NEUROINFLAMMATION AND ITS RESOLUTION: FROM MOLECULAR MECHANISMS TO THERAPEUTIC PERSPECTIVES

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NEUROINFLAMMATION AND ITS RESOLUTION: FROM MOLECULAR MECHANISMS TO THERAPEUTIC PERSPECTIVES

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Editorial: Neuroinflammation and Its Resolution: From Molecular Mechanisms to Therapeutic Perspectives

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Editorial on the Research Topic

Neuroinflammation and Its Resolution: From Molecular Mechanisms to Therapeutic Perspectives

Neuroinflammation, the complex immune response of the central nervous system (CNS), when sustained, is a common denominator in the etiology and course of all major neurological diseases, including neurodevelopmental, neurodegenerative, and psychiatric disorders (e.g., Alzheimer's disease, AD; Parkinson's disease, PD; multiple sclerosis, MS; motor neuron disease; depression; autism spectrum disorder; and schizophrenia). Cellular (microglia and mast cells, two brain-resident immune cells, together with astrocytes) and molecular immune components (e.g., cytokines, complement and pattern-recognition receptors) act as key regulators of neuroinflammation (Skaper et al., 2012). In response to pathological triggers or neuronal damage, immune cells start an innate immune response with the aim to eliminate the initial cause of injury. However, when the cellular activity becomes dysregulated, it results in an inappropriate immune response that can be injurious and affect CNS functions. Thus, limiting neuroinflammation and microglia activity represents a potential strategy to alleviate neuroinflammation-related diseases.

The Research Topic collects 20 manuscripts, divided into five sections, that include both original research articles and reviews of the emerging literature and explore the role of neuroinflammation in various neurological diseases. There is particular attention dedicated to the relevant research exploring the mechanisms and mediators involved in the resolution of neuroinflammation. Our aim was to generate a valuable discussion contributing to identify new therapeutic targets in brain damage and providing new drug development opportunities for the prevention and treatment of CNS diseases involving neuroinflammation.

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PATHOPHYSIOLOGY OF NEUROINFLAMMATION

Guzman-Martinez et al. presented an overview of the various biochemical pathways involved in the neuroinflammatory cascade, focusing on the role of neuroinflammation in the process of protein alteration implicated in various neurodegenerative diseases, such as AD, PD, and Huntington disease. Herradon et al. highlighted the crosstalk between peripheral inflammation and neuroinflammation. The authors described cytokines such as pleiotrophin and midkine as key mediators in modulating both peripheral inflammation and neuroinflammation and they discussed the role of these cytokines in mediating both chronic neuroinflammatory conditions (i.e., neurodegenerative diseases) and peripheral inflammation. Furthermore, the existing therapeutics

for overt neuroinflammation are reviewed and new perspectives suggested for the development of innovative strategies that focus on the modulation of the pleiotrophin-midkine pathway.

Oxidative stress is a critical and common feature of a wide spectrum of CNS pathologies and has been strongly implicated in microglial activation and neuroinflammation. Malko et al. provided an extensive review focusing on the role of the transient receptor potential melastatin-related 2 (TRPM2) channel, an oxidative stress-sensitive calcium-permeable cationic channel, in the activation of microglia and neuroinflammation. The authors emphasized that in CNS diseases microglia-related neuroinflammation is strongly attenuated in TRPM2-KO mice or in animals treated with a TRPM2 inhibitor. These results suggested that microglial TRPM2 represents a prospective novel therapeutic target for neuroinflammatory CNS diseases.

In the attempt to find new antiinflammatory treatments, Joffre et al. described the antineuroinflammatory and neuroprotective effects of n-3 long chain polyunsaturated fatty acids and the derived specialized proresolving mediators, with the aim to identify molecules derived from these fatty acids as possible protagonists of the antiinflammatory effects of polyunsaturated fatty acids.

NEUROINFLAMMATION IN AD AND MEMORY IMPAIRMENT

AD is the most common age-related neurodegenerative disease and is characterized by progressive memory decline and cognitive dysfunction. The impact of chronic inflammation is of relevance in the pathophysiology of AD, considering that subjects affected by inflammatory diseases have an increased risk of developing AD (Heneka et al., 2015). Dysregulated immunoactivity in AD has been widely studied and several papers point to microglia as legitimate drug targets in AD. In this context, Biber et al. provided a concise and informative review that reconciled some of the most recent updates in the understanding of microglia biology with drug discovery, noting that the dynamic properties of microglia and their location in the CNS make targeted small molecule therapies relatively difficult to develop. The authors proposed several microglia target candidates (e.g., CD33, kynurenine pathway, Toll-like receptors, the potassium channel $K_{Ca}3.1$, etc.), with the optimistic hope that one of them could be used in developing drugs useful for AD therapy.

In the attempt to understand the mechanisms underlying the link between neuroinflammation and AD, several authors have studied the effects of some small molecules in animal models of AD. Bellozi et al. evaluated the effects of a 14 days oral treatment with dactolisib, a dual phosphatidylinositol 3 kinase (PI3K)/mammalian target of rapamycin (mTOR) inhibitor, in a transgenic mouse model of AD overexpressing the amyloid precursor protein (APP). Among the different pathways involved in the maintenance and progression of AD, an abnormal and continuous activation of PI3K/protein kinase B (Akt)/mTOR signaling contributes to disease progression, because of the disrupted clearance of amyloid β (Aβ) and tau, synaptic loss, and cognitive decline (O'neill, 2013; Heras-Sandoval et al., 2014). Dactolisib reduced social memory impairment, microglia activation in CA3 region of hippocampus, and the levels of hippocampal interleukin (IL)-10, suggesting that an adequate control of PI3K/Akt/mTOR pathway activation might have the potential to ameliorate AD signs and symptoms. Ano et al. demonstrated interesting effects of a short-term intake of iso-α-acids, which are bitter components in beer, in the reduction of the hippocampal inflammation and neural hyperactivation and memory impairment in a transgenic mouse model of AD.

Considering that chronic psychosocial stress represents a risk factor for AD, being associated with cognitive deficits, microglia priming, and inflammatory responses in adult brain (Piirainen et al, 2017), Wang Y. et al. focused on the relation between social stress and impairment of learning and memory in AD. The authors showed that icariin, a natural flavonoid extracted from *Epimedium brevicornum Maxim* (a traditional Chinese herb), attenuated restraint/isolation stress-induced memory damage, Aβ accumulation, and neuroinflammation by reducing microglia M1 activation and activating peroxisome proliferator-activated receptor γ in APP/PS1 mice.

Alagan et al. showed the neuroprotective and antiinflammatory effects of an ethanol extract of *Phyllanthus amarus* in an *in vivo* rat model of lipopolysaccharide (LPS)-induced memory impairment and neuroinflammation. Lykhmus et al. presented an interesting study on the effects of mesenchymal stem cells (MSCs) in a mouse model of memory impairment induced by LPS. Intravenously injection of MSCs or their conditioned media prevented α7 nAChR decrease, Aβ accumulation, and episodic memory decline induced by a single dose of systemic LPS in mice, suggesting that MSCs could be a potential therapeutic tool to treat neuroinflammatory-related cognitive pathology.

NEUROINFLAMMATION IN PSYCHIATRIC DISORDERS

Increasing evidence indicates that dysregulated or enhanced neuroinflammation is closely linked with the pathogenesis of psychiatric disorders, such as schizophrenia, anxiety and depression (Müller and Bechter, 2013; Weber et al., 2017). In a timely and comprehensive review, Müller considered the role of the intracellular adhesion molecule-1 (ICAM-1) in psychiatric illness. ICAM-1, expressed in microglia, astrocytes, and in endothelial cells of the CNS, is of particular interest from two interrelated reasons: it is a cell bound adhesion molecule, intimately associated with the blood brain barrier, and as a soluble protein it has been measured in blood or cerebrospinal fluid as a biomarker for several mental health disorders.

Depression is a recurrent, common, and potentially life-threatening psychiatric disease related to multiple causes. Hyperactivity of the hypothalamic-pituitary-adrenal axis, involved in the pathophysiology of depression, induces microglia activation and the overproduction of proinflammatory cytokines in the brain (Brites and Fernandes, 2015). Extensive research in recent years has been concentrated on the immuno-inflammatory mechanisms for the treatment of depression. Indeed, many antidepressants are endowed with antiinflammatory effects (Kenis and Maes, 2002). However, current antidepressants have important limitations, due to significant adverse reactions, poor compliance, and a high risk of

relapse following drug withdrawal (Berwian et al., 2017). At present, natural plant products are offering attractive alternatives in antidepressant drug development. In this context, Ke et al. examined the effect of quercetin, a natural polyphenol with antiinflammatory and neuroprotective effects, in a rat model of LPS-induced depression. The authors showed that quercetin attenuated the inflammation induced depression-like behavior and improved learning and memory in LPS-challenged rats. These effects were associated with the reversal effects of quercetin on LPS-induced alteration of expression of brain derived neurotrophic factor, Copine 6, and TREM1/2 in the hippocampus and in the prefrontal cortex. Lee et al. found that myelophil, a 30% ethanol extract of Radix Astragali and Radix Salviae, reversed the depressive behavior in a mouse model of unpredictable chronic mild stress. Myelophil also decreased the over-activation of microglia and the inflammatory response in the hippocampus, and reversed the reduction of serotonergic function in the dorsal raphe nuclei and neurogenesis in the subgranular zone of hippocampus.

NEUROINFLAMMATION IN MS AND EXPERIMENTAL AUTOIMMUNE NEURITIS

MS is a CNS autoimmune and neurodegenerative disease that affects ~2.5 million people worldwide and causes a significant financial burden. Several experimental and clinical studies have revealed that microglia/macrophages actively participate in the course of the disease. In this context, Wang J. et al. provided a comprehensive review that attempted to discuss the role of macrophages and microglia in both healthy CNS and in MS. The authors discussed the possibility of a differential manipulation of CNS resident microglia and infiltrating macrophages to potentially target different mechanisms operating in MS and realize efficient treatments for the disease. Zhang F. et al. demonstrated that scopoletin, a phenolic coumarin found in some plants, improved the severity of the disease and prominently decreased inflammation and demyelination in an experimental autoimmune encephalomyelitis mouse model of MS, *via* nuclear factor-κB signaling.

Xu et al. examined the effect of oridonin, a diterpenoid compound extracted from *Rabdosia rubescens*, in an experimental autoimmune neuritis animal model. Oridonin ameliorated inflammatory disease progression and favored the disease outcome by inducing the switch of macrophages toward the antiinflammatory polarization state. Thus, oridonin could be considered a possible therapeutic candidate of inflammatory neuropathies.

NEUROINFLAMMATION IN CHRONIC PAIN, HYPERAMMONIA, CARDIAC ARREST, AND INTRACEREBRAL HEMORRHAGE

Chronic pain is caused by nerve damage which occurs during nerve compression, diabetes, inflammation, and shingles virus infection (Campbell and Meyer, 2006). Cytokines, chemokines, prostaglandins, and nitric oxide released from activated microglia and astrocytes in the dorsal horn of the spinal cord play important roles in the pathogenesis of chronic pain (Skaper et al., 2018). Therefore, several studies have targeted activated microglia to reduce pain hypersensitivity. In light of this, the manuscript by Song et al. showed the positive effect of GNF-2, a selective allosteric inhibitor of Bcr-Abl, initially developed as an anticancer drug, on neuroinflammation and associated pain pathogenesis, using different animal models of pain.

Malaguarnera et al. studied the effect of bicuculline, a $GABA_A$ receptor antagonist, on hyperammonemic rats. In the hippocampus of these animals, GABAergic tone was increased contributing to some aspects of neuroinflammation. Glutamate transmission was altered and spatial learning and memory as well as anxiety were impaired. As expected, the treatment with bicuculline reduced hippocampal astrocytosis, but failed to control glial activation. Furthermore, the antagonist reduced the GABAergic tone and reversed the expression of the GluA1 and GluA2 subunits of AMPA receptors and of the NR2B subunit of NMDA receptors. The treatment with bicuculline also improved spatial learning, working memory and decreased anxiety in these rats.

The manuscript by Ma et al. explored the capacity of a bioactive annexin A1 short peptide to resolve neuroinflammation in a rat model of exsanguinating cardiac arrest treated by emergency preservation and resuscitation. The attenuation of cortical cell death and reduction of several biomarkers of neuroinflammation induced by the peptide were associated with the increased expression of sirtuin 3 and the upregulation of antioxidant pathways.

Zhang J. et al. indicated that a systemic treatment with simvastatin reduced polymorphonuclear neutrophil infiltration into brain tissue, ameliorated brain edema, and reduced proinflammatory mediator expression in perihematomal area in an animal model of neuroinflammation after intracerebral hemorrhage.

In conclusion, the Research Topic "Neuroinflammation and its resolution: from molecular mechanisms to therapeutic perspectives" presents the current state of knowledge about the involvement of microglia and neuroinflammation in neurological disorders and provides several new perspectives and therapeutic approaches for the development of novel pharmacological agents aimed at counteracting neuroinflammatory diseases.

Finally, it should be noted that in six manuscripts (~30%) of this Research Topic, the antiinflammatory effect of plant-derived products has been investigated. There is a high rate of failure in development of drugs for neurological diseases and for AD, in particular. New treatments are urgently needed, but even if progress is being made, we probably need to define new targets and developing new specific agents. In that regard, natural products could be a valuable source to provide candidates to be used in CNS diseases.

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All the authors have contributed to the composition and the revision of this Editorial Article and approved it for publication.

REFERENCES

Berwian, I. M., Walter, H., Seifritz, E., and Huys, Q. J. (2017). Predicting relapse after antidepressant withdrawal—a systematic review. *Psychol. Med.* 47, 426–437. doi: 10.1017/S0033291716002580

Brites, D., and Fernandes, A. (2015). Neuroinflammation and depression: microglia activation, extracellular microvesicles and microRNA dysregulation. *Front. Cell. Neurosci.* 9, 476. doi: 10.3389/fncel.2015.00476

Campbell, J. N., and Meyer, R. A. (2006). Mechanisms of neuropathic pain. *Neuron* 52, 77–92. doi: 10.1016/j.neuron.2006.09.021

Heneka, M. T., Carson, M. J., El Khoury, J., Landreth, G. E., Brosseron, F., Feinstein, D. L., et al. (2015). Neuroinflammation in Alzheimer's disease. *Lancet Neurol.* 14, 388–405. doi: 10.1016/S1474-4422(15)70016-5

Heras-Sandoval, D., Perez-Rojas, J. M., Hernandez-Damian, J., and Pedraza-Chaverri, J. (2014). The role of PI3K/AKT/mTOR pathway in the modulation of autophagy and the clearance of protein aggregates in neurodegeneration. *Cell Signal.* 26, 2694–2701. doi: 10.1016/j.cellsig.2014.08.019

Kenis, G., and Maes, M. (2002). Effects of antidepressants on the production of cytokines. *Int. J. Neuropsychopharmacol.* 5, 401–412. doi: 10.1017/S1461145702003164

Müller, N., and Bechter, K. (2013). The mild encephalitis concept for psychiatric disorders revisited in the light of current psychoneuroimmunological findings. *Neurol. Psychiatry Brain Res.* 19, 87–101. doi: 10.1016/j.npbr.2013.04.004

O'neill, C. (2013). PI3-kinase/Akt/mTOR signaling: impaired on/off switches in aging, cognitive decline and Alzheimer's disease. *Exp. Gerontol.* 48, 647–653. doi: 10.1016/j.exger.2013.02.025

Piirainen, S., Youssef, A., Song, C., Kalueff, A. V., Landreth, G. E., Malm, T., et al. (2017). Psychosocial stress on neuroinflammation and cognitive dysfunctions in Alzheimer's disease: the emerging role for microglia? *Neurosci. Biobehav. Rev.* 77, 148–164. doi: 10.1016/j.neubiorev.2017.01.046

Skaper, S. D., Giusti, P., and Facci, L. (2012). Microglia and mast cells: two tracks on the road to neuroinflammation. *FASEB J.* 26, 3103–3117. doi: 10.1096/fj.11-197194

Skaper, S. D., Facci, L., Zusso, M., and Giusti, P. (2018). An Inflammation-Centric View of Neurological Disease: Beyond the Neuron. *Front. Cell. Neurosci.* 12, 72. doi: 10.3389/fncel.2018.00072

Weber, M. D., Godbout, J. P., and Sheridan, J. F. (2017). Repeated social defeat, neuroinflammation, and behavior: monocytes carry the signal. *Neuropsychopharmacol.* 42, 46–61. doi: 10.1038/npp.2016.102

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ORIGINAL RESEARCH
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Simvastatin Reduces Neutrophils Infiltration Into Brain Parenchyma After Intracerebral Hemorrhage via Regulating Peripheral Neutrophils Apoptosis

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Statins, known for their lipid-lowering effects, also have immunomodulatory properties. This study aims to examine whether systematic simvastatin administration could decrease polymorphonuclear neutrophils (PMNs) infiltration into brain tissue, as well as alleviate neuroinflammation in a rat model of intracerebral hemorrhage (ICH). The ICH model was induced in adult male Sprague–Dawley rats by an injection of autologous blood. Animals randomly received simvastatin (i.p. 2 mg/kg) or vehicle daily from 5 days before ICH until sacrificed. Routine blood counts, brain water content, neurological scoring, immunofluorescence and RT-PCR were conducted to evaluate the anti-inflammatory effect of simvastatin following ICH. Furthermore, flow cytometric and western blotting analysis were implemented for elucidating the mechanisms involved in simvastatin-induced reduction of neutrophil brain-invading. Elevated PMNs count and neutrophil-to-lymphocyte ratio in circulation were detected in rat model of ICH, which was reversed by using simvastatin. Simvastatin effectively alleviated PMNs infiltration and proinflammatory factors release in perihematomal area, as well as attenuated ICH-induced brain edema and neurological deficits. Simvastatin significantly downregulated the expression of antiapoptotic protein-Mcl-1 while increased the level of proapoptotic protein-Bax and cleaved caspase 3 in PMNs. Simvastatin treatment significantly alleviated PMNs brain-infiltrating and subsequent neuroinflammatory reaction after ICH, in part by accelerating peripheral PMNs apoptosis through disorganized the expression of apoptotic related proteins. Our data provided new evidence for simvastatin application on patients with ICH.

Keywords: intracerebral hemorrhage, statins, polymorphonuclear neutrophils, apoptosis, inflammation

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INTRODUCTION

Intracerebral hemorrhage (ICH) accounts for 10–15% of all strokes, and its mortality and morbidity far exceed ischemic strokes (Qureshi et al., 2009). The lack of a specific therapeutic target in the treatment of ICH has increased the need for new treatment options (Xi et al., 2014; Joseph et al., 2016). More and more studies have provided evidence for supporting the key role of neuroinflammation in secondary brain injury following ICH (Zhou et al., 2014).

After ICH, the microglia around the hematoma takes the lead in response to injury, and then releases a variety of chemokines to recruit peripheral inflammatory cells migrate into lesion area of brain (Wan et al., 2016). Among them, the polymorphonuclear neutrophils (PMNs) first arrived perihematomal region. Both previous clinicopathological findings and recent animal experiments suggested that PMNs began to infiltrate into the brain at 6 h after ICH and reached peak on 1˜3 day (Wang, 2010). Subsequently, a large number of proinflammatory cytokines released by PMNs will activate the adjacent microglia/macrophages, leading to the release of more proinflammatory factors and resulting in the "inflammatory cascade effect," which worse the prognosis of patients with ICH (Iadecola and Anrather, 2011; Behrouz, 2016). Taken together, PMNs is considered as the "fuse" of inflammation in the central nervous system (CNS), which could be a promising therapeutic target for ICH. Many years ago, some investigators had tried to deplete peripheral PMNs in a rat model of ICH (Moxon-Emre and Schlichter, 2011; Sansing et al., 2011). They found that PMNs specific antibody injection effectively diminished PMNs and monocyte infiltration into brain, attenuated BBB breakdown and improved functional outcome. However, to some extent, the proper dosage of PMNs antibody is hard to determine among human beings, and rapid loss of PMNs in circulation may breakdown the immune system homeostasis and lead to infection. Therefore, new therapeutic methods were still needed for suppressing PMNs brain-invading post-ICH.

In the past few years, several clinical studies have pointed out that the elevated PMNs count and neutrophil-to-lymphocyte ratio (NLR) in circulation was closely related to the poor prognosis after ICH (Lattanzi et al., 2016; Wang et al., 2016; Gusdon et al., 2017; Tao et al., 2017). However, the mechanisms responsible for this relationship remain poorly characterized. Recently, a randomized, double-blind clinical study reported that systematic administration of simvastatin, an HMG-CoA reductase inhibitor, before cardiopulmonary bypass significantly increased the apoptotic ratio of peripheral PMNs, and ameliorated post-operative inflammation (Chello et al., 2007). Taken together, we hypothesized that simvastatin could be a safe and effective candidate for attenuating PMNs brain-infiltration and subsequent inflammatory reaction following ICH, by reducing the PMNs count and NLR in circulation through regulating the apoptosis of PMNs. The present study was designed to test whether consecutive simvastatin treatment (before and after ICH) could decline the peripheral PMNs count and NLR, by which diminishing PMNs infiltration into brain in a rat model of ICH.

MATERIALS AND METHODS

Animals and ICH Model

Two hundred and eighty-five adult male Sprague-Dawley rats (250–350 g; the Third Military Medical University) were used. All animals were housed with a 12 h light/dark cycle and water and food provided *ad libitum*. A feedback-controlled heating pad was used to maintain rats at 37.0°C during operation. The animals were kept warm at thermostats and clear respiratory secretions after operation, then return the model rats to cages until its fully awake. Animal use procedures were in compliance with the Guide for the Care and Use of Laboratory Animals and approved by the Laboratory Animal Welfare and Ethics Committee of the Third Military Medical University (SCXK-PLA-20120011). Animals were anesthetized with pentobarbital (40 mg/kg IP), and a feedback-controlled heating pad was used to maintain body temperature at 37.0°C. The rat model of ICH was established according to our previous published work (Chen et al., 2017; Jiang et al., 2017). For the model of ICH, a cranial burr hole (1 mm) was drilled, and a 29-gauge needle was inserted stereotaxically into the right caudate nucleus (coordinates: 0.2 mm anterior, 5.5 mm ventral, and 3.5 mm lateral to the bregma). Subsequently, 100 μl of autologous arterial blood was infused in 10 min using a microinfusion pump. Then, the hole was sealed using bone wax. The sham groups received only needle injection.

Experimental Grouping

This study was divided into 3 parts. First, to examine whether simvastatin could decrease peripheral PMNs count and PMNs brain-infiltrating, ninety rats had an intracaudate injection of 100 μl of blood. The sham control received only needle injection. The animals were randomly assigned to three groups. Group 1 received simvastatin (2 mg/kg/d, i.p.) from 5 days before ICH until sacrificed, and the control group received the same volume of vehicle. Some rats (n = 4 per group, each time point) were sacrificed for RT-PCR analysis of TNF-α, IL-6, CCL2 and ICAM-1 at 6, 12, and 24 h after ICH. Other animals (n = 5 for sham group, n = 8 for ICH+simva. group and ICH+Veh. group each time point) were employed for blood cell count and MPO staining at 1, 3, and 7 days after ICH. In the second part, to evaluate the neuroprotective effect of simvastatin treatment on brain injury following ICH, rats were randomly divided into three groups and treated as part I. Neurological function scoring was conducted in some rats one day before ICH and 1, 3, and 7 days following ICH (n = 6 per group). Other animals (n = 6 per group, each time point) were sacrificed for brain water content measurement at 24 and 72 h after ICH. In the third part, to explore the mechanism underlying simvastatin-mediated against peripheral PMNs brain-invading after ICH, rats were randomly divided into three groups and treated as part I. Flow cytometric analysis of peripheral PMNs apoptosis (n = 6 per group, each time point) and lymphocytes apoptosis (n = 6 per group, each time

point) were conducted at days 1, 3, and 7 after ICH. Then, the level of apoptotic related proteins was detected using Western blotting analysis at 24 and 72 h after ICH (n = 4 per group for each time point).

Drug Administration

Simvastatin (Sigma, United States) was prepared as a 4 mg/ml stock, as previously described (Leung et al., 2003; Jin et al., 2013). Briefly, 4 mg of simvastatin was dissolved in 100 μl of ethanol and 150 μl of 0.1 N NaOH, incubated at 50°C for 2 h, then the pH adjusted to 7.0 with 0.1 M HCl, and added water to 1 ml. This simvastatin stock solution was stored at −80°C and diluted with triple volume of sterile saline immediately before use. Animals were randomized to receive intraperitoneal injection of simvastatin (2 mg/kg/d) from 5 days before ICH until sacrificed. The dose regimen of simvastatin was referred to previous studies in rat model of ICH (Karki et al., 2009; Yang et al., 2013; Chen et al., 2017).

Routine Blood Counts

Routine blood counts were performed as previously described (Welles et al., 2009). First, animals were anesthetized with pentobarbital, then the heart was exposed and about 4 ml fresh blood samples were collected using the EDTA-anticoagulated tube. After fully shake, 200 μl blood sample was transferred to an eppendorf tube and analyzed on the bench-top analyzer (Hemavet 950, Shandong Excellent Science Instrument Co. Ltd., CHN). Before analyzer running, the quality of machine cleaning liquid, hemolysin liquid and protective agent were checked, and then use double steamed water for calibration.

PMNs and Lymphocytes Isolation

PMNs isolation and identification were conducted as previously described (Dyugovskaya et al., 2012). The EDTA-anticoagulated whole blood from rats was collected, and it was then mixed with dextran t-500 (1% v/w) for 30 min at 37°C. The upper leukocyte-rich layer was transferred to a new tube and centrifuged at 2000 g for 20 min at 20°C. Pellets were suspended in 2 ml salt solution balanced by D-Hanks; then, they were loaded on the top of the 2 ml Histopaque1119 and Histopaque1083 density gradient carefully and centrifuged at 700 g for 30 min at 20°C. The cells were collected in the PMN-rich layer between Histopaque1083 and Histopaque1119 and were suspended in D-Hanks. The viability of the cells was determined by Trypan blue dye exclusion. The purity of isolated PMNs was detected by Wright-Giemsa staining. PMNs' viability and purity were more than 95%. Refer to the above methods, we also isolated lymphocytes from circulating blood of ICH rat.

Wright-Giemsa Staining

Wright-Giemsa staining was performed standard protocols (Qin et al., 2015). Briefly, the smear of peripheral blood or isolation was staining with Wright-Giemsa stains A dye 10–15 s and then staining with Wright-Giemsa stains B dye 2 min, flush slides under running water for at least 30 s after staining.

PMNs and Lymphocytes Apoptosis Detection

The apoptotic ratio was measured by flow cytometry as previously described (Jin et al., 2013). The D-Hanks-washed cells were incubated on ice with 5 μl Annexin V-fluorescein isothiocyanate (FITC) solution and 10 μl propidium iodide (PI) solution for 15 min in dark. Then, flow cytometry (BD LSRF Ortessa, United States) was used to analyze the apoptosis.

Brain Water Content

Animals were decapitated under deep anesthesia 24 and 72 h after autologous blood injection for brain water content measurement. Brains were removed quickly, and the frontal poles (4 mm) were cut off. The remaining brains were divided into five parts: cerebellum, ipsilateral cortex and basal ganglia, contralateral cortex and basal ganglia. Brain samples were weighed immediately to record the wet weight and were then dried at 100° for 24 h to record the dry weight. Tissue water content was calculated as (wet weight – dry weight)/wet weight. The brain water content was measured by as previously described (Williamson and Colbourne, 2017).

Assessment of Neurological Abnormalities

Neurological dysfunction of rats was evaluated using a modified Neurological Severity Score (mNSS) method, forelimb placing test and corner test as described previously (Hua et al., 2002; Liew et al., 2012). Briefly, the assessment was performed on day 1 before and on days 1, 3, and 7 after ICH. First, the mNSS is a composite test of motor, sensory, and balance functions. Neurological function was graded on a scale of 0–18 (normal score, 0; maximal deficit score, 18). Then, each rat was tested 10 times for each forelimb, and the percentage of trials in which the rat placed the appropriate forelimb on the edge of the countertop in response to vibrissae stimulation was determined. Next, all rats were allowed to proceed into a corner, whose angle was 30°. To exit the corner, the rat could turn to either the left or the right, and that direction was recorded. The test was repeated 10 times, with at least 30 s between trials, and the percentage of right turns was calculated. Testers were highly experienced and blinded to the condition of the animal. The mean neurological score was evaluated by 2 blinded observers.

Cell Counts

The cell counts were performed at days 1, 3, and 7 after ICH, respectively (Chen et al., 2017). Cell counts analysis was performed as previously described. For quantification of the MPO positive cells in the perihematomal area (0.2 mm anterior to bregma), consecutive slices were made, and two sections per animal (n = 6 per group) with 40 μm space in between were used for cell counts. Three high-power images (40× magnification) were used for cell counting. Cell counts were performed by two researchers in a blinded manner. All measurements were repeated three times, and the mean value was used.

TABLE 1 | Primers used for RT-PCR.

Gene	Primers (5′∼ 3′)	Primer location	Product (bp)	Genbank no.
GAPDH	GACATGCCGCCTGGAGAAAC AGCCCAGGATGCCCTTTAGT	792–883	92	NM_017008.4
IL-6	ACTTCCAGCCAGTTGCCTTCTTG TGGTCTGTTGTGGGTGGTATCCTC	87–109	110	NM_012589.2
TNF-α	CACCACGCTCTTCTGTCTACTGAAC TGGGCTACGGGCTTGTCACTC	276–300	141	NM_012675.3
ICAM-1	CTGTCAAACGGGAGATGAATGG TCTGGCGGTAATAGGTGTAAATGG	1403–1421	189	NM_012967.1
CCL2	GCATCAACCCTAAGGACTTCAGC AAGGCATCACATTCCAAATCACA	391–413	155	NM_031530.1

GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; IL-6, Interleukin-6; TNF-α, Tumor Necrosis Factor-α ; CCL2, C-C motif chemokine ligand 2; ICAM-1, Intercellular Adhesion Molecule 1.

Real-Time PCR

The PCR was performed and analyzed, as previously described (Tan et al., 2017). For RT-PCR of TNF-α, IL-6, CCL2, and ICAM-1 gene expression analysis, rats (4/group) were sacrificed 6, 12, and 24 h by decapitation after ICH. The brains were then dissected 2 mm anteriorly and 2 mm posteriorly to the needle entry site (easily identifiable on the brain surface) and were divided into separate hemispheres along the midline. Next, perihematomal brain tissue was used for RNA extraction. Primers were designed with the Primer3 Output program (**Table 1**). Total RNA was extracted using TRIzol reagent (Invitrogen). A positive standard curve for each primer was obtained using a serially diluted cDNA (complementary DNA) sample mixture. Gene expression was quantified with standard samples and normalized with glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The data are expressed as normalized mRNA expression. All data analyses were performed in a blinded manner by 2 observers.

Western Blot Analysis

Western blot analysis was performed as previously described (Zou et al., 2017). Pre-extracted and washed cells were transferred to a sterile EP tube. Then, add the prepared lysis solution to the test tube by a ratio of 1 ml/10^7 cells. Repeatedly pipetting and mixing until all the cellular proteins precipitation. Next, cells were lysed on ice for 5 min, vortexed, and repeat three times. After a centrifugation at 12,000 rpm for 10 min under 4° condition, the supernatant was collected and stored at −80°C for Western blot analysis. Antibodies were used as follows: Rabbit anti-rat Cleaved Caspase-3 (Cell Signaling Technology, United States 1:1000), Rabbit anti-rat Mcl-1 (Cell Signaling Technology, United States 1:1000), Rabbit anti-rat Bcl-2 (Cell Signaling Technology, United States 1:1000), Mice anti-rat Bax (Abcam, United States 1:500). Immunopositive bands of horseradish peroxidase–conjugated secondary antibodies were detected with an ECL system (GE Healthcare). The relative densities of the bands were analyzed using NIH ImageJ software.

Determination of Myeloperoxidase (MPO) Activity

MPO activity has been used as an index of neutrophil infiltration in brain tissues. In this study, we used MPO activity to indicate neutrophils infiltration in the brain tissue after injection. Immunofluorescence staining of brain tissue was performed on fixed frozen sections as previously described (Feng et al., 2017). Rats were anesthetized with pentobarbital (100 mg/kg intraperitoneal) and perfused with 4% paraformaldehyde in 0.1 mol/L pH 7.4 PBS. The brains were removed and kept in 4% paraformaldehyde for 4–6 h and then immersed in 30% sucrose for 3–4 days at 4°C. The brains were embedded in an optimal cutting temperature compound (SAKURA, United States), and 18 mm thick slices were cut using a cryostat. The slices were stored at −20° before staining. Before being blocked with 10% goat serum for 1 h at room temperature, the sections were washed with PBST (PBS with 0.3% Triton X-100) for 30 min. Then, they were incubated with antibodies against MPO (Abcam, United States 1:100) at 4° overnight. The sections were kept at room temperature for 45 min and then washed with PBS. Then, the sections were incubated with an Alexa Fluor 555-conjugated goat anti-rabbit IgG (H+L) (Beyotime, China 1:300) secondary antibody at 37°C for 3 h. Finally, cell nuclei were stained with 40,6-diamidino-2-phenylindole (DAPI). The stained sections were viewed under identical conditions using a 310 or 320 objective on a confocal microscope (LSM-780; Zeiss).

Statistical Analyses

The values in this study are presented as mean ± SD. The data in this study are given as the mean ± SD. Data were analyzed by one-way analysis of variance, followed by Scheffe's *post hoc* test. Differences were considered statistically significant at a *P*-value of less than 0.05.

RESULTS

Elevated Peripheral PMNs Count and Neutrophil-to-Lymphocyte Ratio (NLR) Were Detected in Rat Model of ICH, Which Was Reversed by Using Simvastatin

Many clinical researches have reported that ICH patients with higher peripheral PMNs count and NLR in acute stage predicted more worse outcome (Lattanzi et al., 2016; Wang et al., 2016; Gusdon et al., 2017; Tao et al., 2017). Thus, first, we examined whether this phenomenon also exits in experimental animal model of ICH. As shown in **Figure 1**, compared with the sham group, the ICH rats presented higher PMNs count and higher NLR at 24 h after blood injection. Then, to test the potential effect of simvastatin on modulating leukocyte change following ICH, we conducted the dynamic blood routine analysis. The

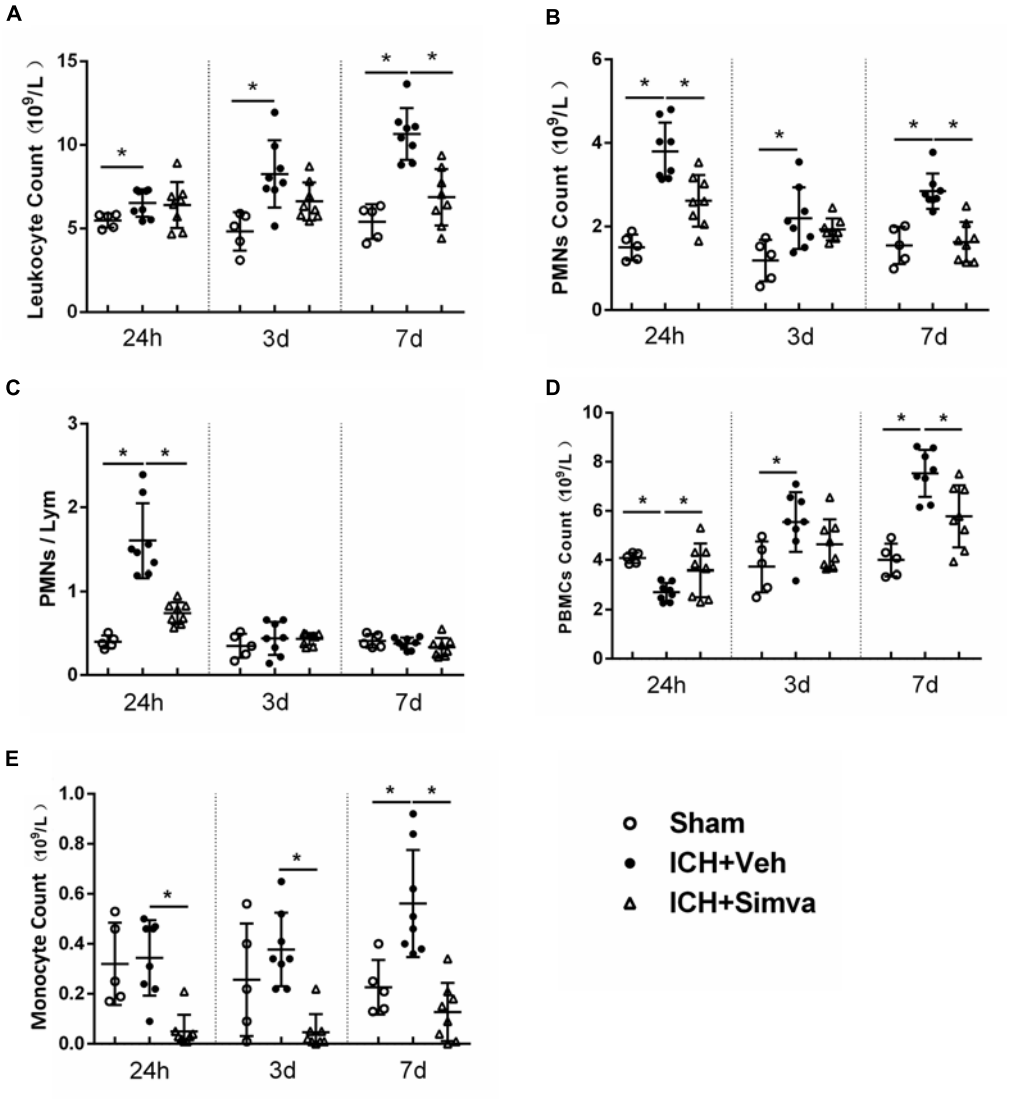

FIGURE 1 | Elevated PMNs count and NLR in circulation were detected in rat model of ICH, which was reversed by using simvastatin. Analysis of peripheral leukocyte count **(A)**, PMNs count **(B)**, NLR **(C)**, PBMCs count **(D),** and monocyte count **(E)** on day1, 3, and 7 days after sham operation (n = 5 each time point), ICH+Vehicle (n = 8 each time point), and ICH+Simvastatin (n = 8 each time point). Values are expressed as mean ± SD; *P < 0.05. ICH, intracerebral hemorrhage; PMNs, polymorphonuclear neutrophils; NLR, neutrophil to lymphocyte ratio; PBMCs, peripheral blood mononuclear cells; Lym, lymphocytes.

total leukocyte count in Veh-group has no difference among the Simva-, Veh- and sham- groups on day 1, but markedly increased on day 3, and reached a peak on day 7 after ICH. However, the total leukocyte count in the Simva-group remains stable on the 3 and 7 days post-ICH (**Figure 1A**). The PMNs count in the Simva-group was significantly lower than the control group on days 1 and 7 after ICH. Of note, 24 h after ICH, the Simva-group displayed dramatically decreased NLR compared to Vehicle controls. These results suggest that elevated PMNs count and NLR also presented in the experimental ICH model in a rat, which was effectively reversed after simvastatin treatment (**Figures 1B,C**). Furthermore, we also analyzed the count of peripheral blood mononuclear cells (PBMCs) and monocyte in this rat model of ICH (**Figures 1D,E**). PBMCs elevates on 72 h after ICH and stay high for up to 7 days. Simvastatin effectively reduced the PBMCs count on day 7 post-ICH. Notably, simvastatin significantly decreased the monocyte count from 24 h to day 7 after ICH.

Simvastatin Effectively Alleviated PMNs Brain-Infiltration and Proinflammatory Mediators Expression in Perihematomal Area

Next, we wondered whether simvastatin also changes the number of PMNs infiltration after ICH. The Laser Confocal Microscope was adopted to trace the infiltrated PMNs around hematoma on diffident time points after ICH. Compared with the Sham-group,

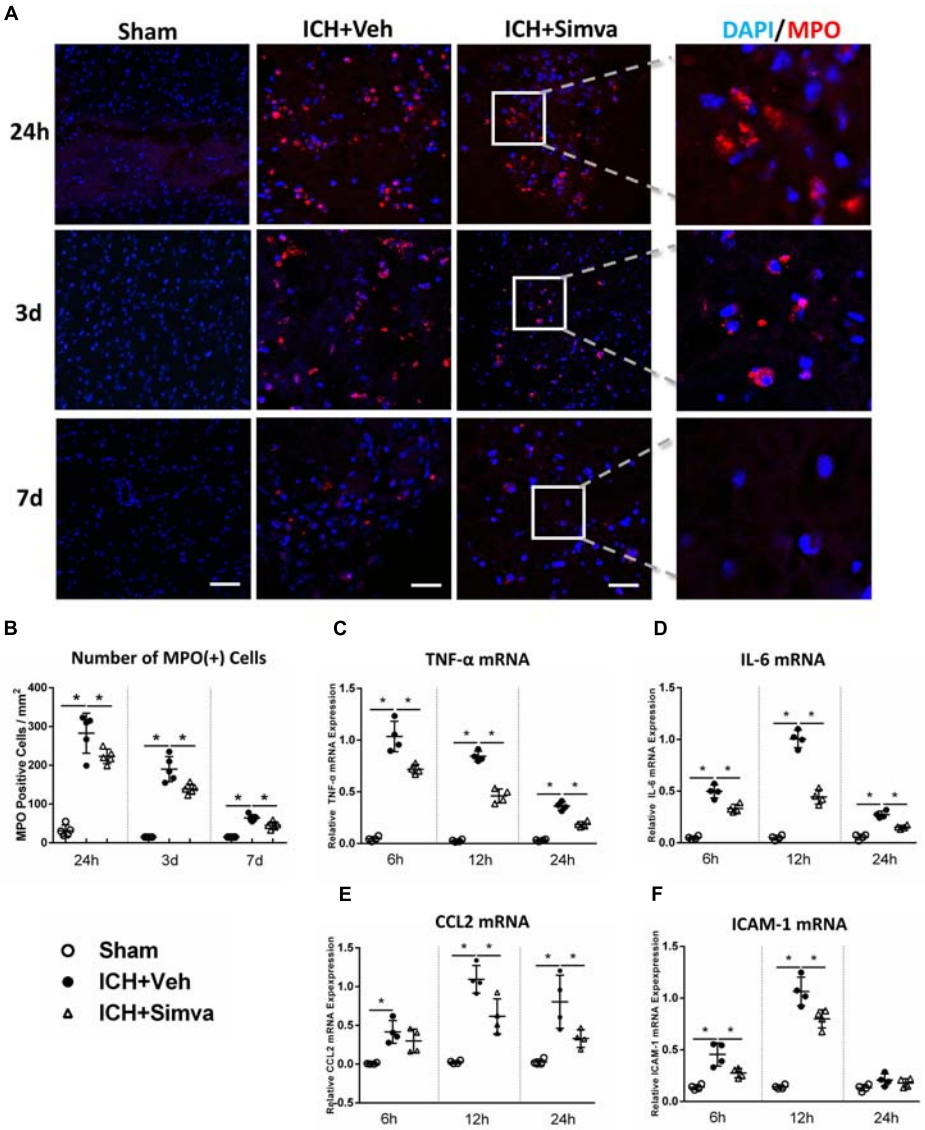

FIGURE 2 | Simvastatin significantly reduced brain-infiltrating PMNs and proinflammatory mediators expression in perihematomal area after ICH. **(A)** Representative images of MPO (+) immunofluorescence around hematoma 1, 3, and 7 days after ICH. **(B)** Perihematomal MPO positive cells count at the indicated time points. Values are expressed as mean ± *SD*, $n = 6$ per group; $^*P < 0.05$ versus sham group. RT-PCR analysis of TNF-α **(C)**, IL-6 **(D)**, CCL2 **(E)**, and ICAM-1 **(F)** mRNA in perihematomal tissue at 6, 12, and 24 h after ICH. Values are expressed as mean ± SD, $n = 4$ per group; $^*P < 0.05$ versus sham group. The scaling bar represents 20 μm.

both Simva-group and Veh-group showed a lot of MPO (+) cells in the area around the hematoma, in which the MPO (+) cells count in the Simva-group was less than the control one on the days 1, 3, and 7 post-ICH (**Figures 2A,B**). This data indicate that simvastatin effectively prevented peripheral PMNs infiltrating into brain parenchyma, which may be attributed by cutting down the number of PMNs in the circulation after ICH. In addition, to assess whether the degressive infiltration impacts on the proinflammatory mediators and adhesion molecule expression around the lesion, we determined the transcriptional level of some factors, like TNF-α, IL-6, CCL2, and ICAM-1 in acute stage of ICH. RT-PCR analysis showed a lower expression level of all above chemokines in the Simva-group than in the control at 6, 12, and 24 h after ICH (**Figures 2C–F**), suggesting simvastatin relieved the early neuroinflammatory response to ICH in some extent.

Simvastatin Significantly Attenuated ICH-Induced Brain Edema and Neurological Deficits

To further investigate the neuroprotective effect of simvastatin on ICH rats, we measured the brain water content of animal models and calculated their neurological scorings on corresponding

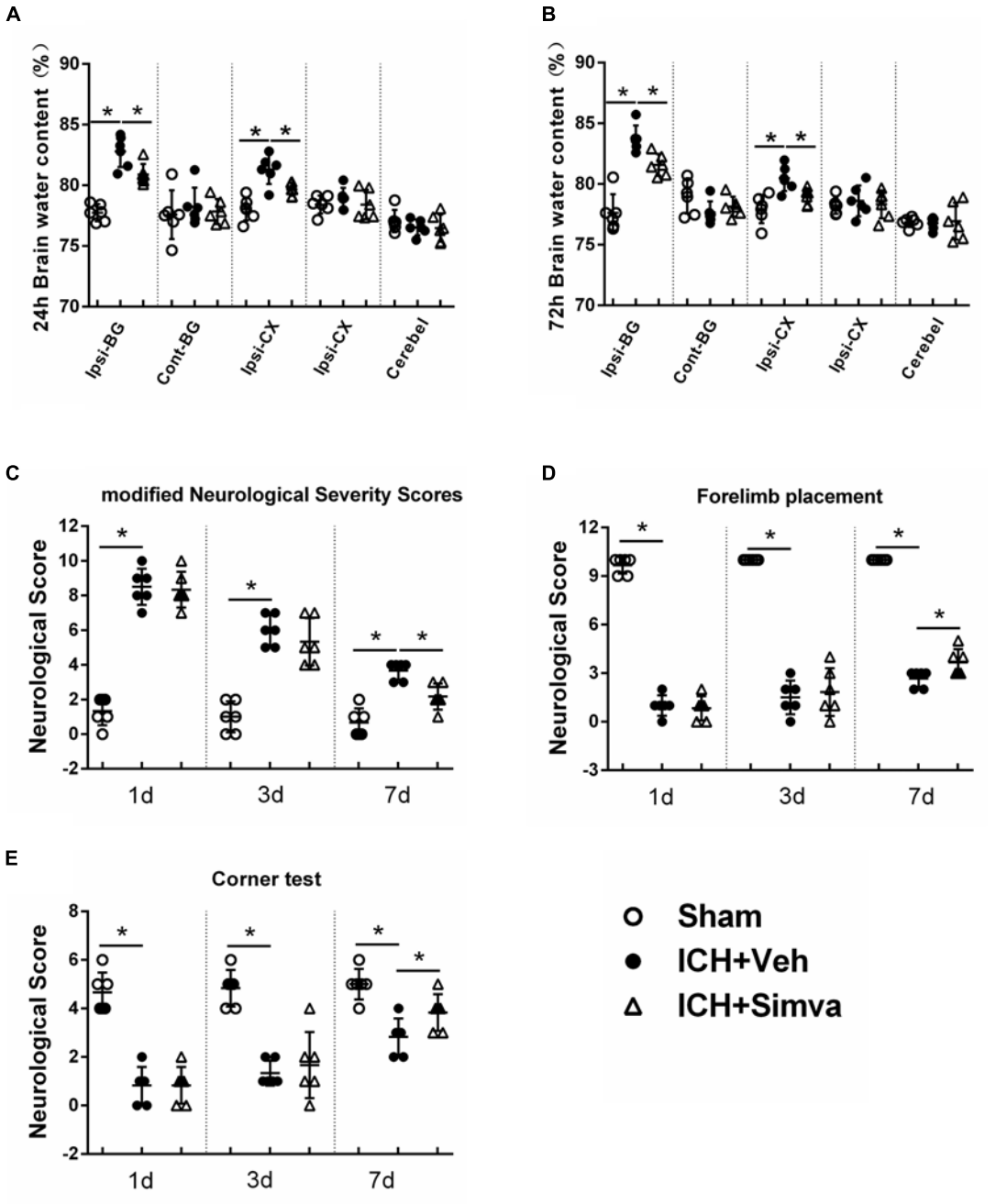

FIGURE 3 | Simvastatin significantly attenuated ICH-induced brain edema and neurological deficits. Measurement of brain water content at 24 h **(A)** and 72 h **(B)** after ICH. Values are expressed as mean ± SD, $n = 6$ per group; $^{*}P < 0.05$ versus sham group; $^{\#}P < 0.05$ versus ICH-Veh group. Ipsi-BG, ipsilateral basal ganglia; Con-BG, contralateral basal ganglia; Ipsi-CX, ipsilateral cerebral cortex; Con-CX, contralateral cerebral cortex; Cerebel, cerebellum. Neurofunctional assessment by using mNSS **(C)**, Forelimb Placement Test **(D)** and Corner Test **(E)** at the indicated time points after ICH. Values are expressed as mean ± SD, $n = 6$ per group; $^{*}P < 0.05$ ICH-Veh group versus Sham group.

time points post-ICH. The brain water contents in both Veh-group and Simva-group showed an obvious increase after blood injection when compared with the Sham-group. Notably, the brain water content of ipsilateral hemisphere (basal ganglia, cortex) in the Veh-group was significantly higher than the treatment group at 24 and 72 h after ICH (**Figures 3A,B**). As shown in **Figure 3C,** both Veh-group and Simva-group presented higher neurological score than the Sham-group at days 1, 3, and 7 after ICH, in which the neurological score of treatment group was significantly lower than controls at day 7. Furthermore, higher neurological scores were observed in the Simva-group than the Veh-group during the forelimb placement test (**Figure 3D**) and

corner test (**Figure 3E**) at day 7 after ICH. Our above data indicate that simvastatin administration distinctly ameliorated brain edema and improved neurological function following ICH.

Simvastatin Broke the Balance Between Apoptotic Related Proteins, Mcl-1/Bax, Which Then Driving PMNs Apoptosis

After isolated from the circulating blood of rat, PMNs were identified by using DAPI and Giemsa staining. As shown in **Figure 4**, at high magnification, PMNs showed the feature typical for PMNs-multilobal nuclei. Compared with the sham control, the other two experimental groups showed higher apoptotic ratio at 24 h after ICH, but obviously declined at days 3 and 7. Thereinto, the simvastatin group presented a more significantly elevated apoptotic ratio at 24 h, whereas a less ratio decreasing than the vehicle control at day 3 (**Figures 5A,C**). Our data indicated that simvastatin markedly enhanced the apoptosis of PMNs in the acute phase of ICH, and maintained PMNs under a higher level of apoptosis than control groups in the subacute stage, which in some extent cut down the lifespan of peripheral PMNs and shortened the time course of neuroinflammatory reaction following ICH. As shown in **Figures 5B,D**, simvastatin also effectively increased the apoptotic ratio of lymphocytes on days 1 and 7 post-ICH, suggesting simvastatin has potential pro-apoptotic effect on various subtypes of leukocyte not just on PMNs. Previous researches have demonstrated that the balance between antiapoptotic proteins and proapoptotic proteins is closely associated with the fate of PMNs (Dzhagalov et al., 2007; Dyugovskaya et al., 2012). Therefore, to further elucidate mechanisms for simvastatin-induced PMNs apoptosis, we traced the level of apoptotic related proteins expression in the peripheral PMNs isolated from ICH rat. As displayed in **Figures 6A,B**, the expression of antiapoptotic proteins, Bcl-2 and Mcl-1, was markedly inhibited at 24 h after ICH, while the proapoptotic proteins, Bax, was significantly upregulated. In comparision with control group, the ICH rats treated with simvastatin showed less antiapoptotic proteins and more proapoptotic proteins expression in peripheral PMNs. In addition, the level of cleaved caspase 3 in Simva-group was higher than the Veh-group at 24 h after ICH. Quantification of these apoptotic related proteins levels by Western blot indicated that simvastatin broke the balance between Mcl-1 and Bax on mitochondria, leading to more caspase 3 release and activation, which then initiated the apoptosis of PMNs after ICH (**Figures 6C,D**). In addition, we also analyzed the protein level of BCL2, MCL1, Bax and Caspase 3 expression on day 3 after ICH. Notably, as displayed in **Figures 6A–D**, simvastatin presented insignificant effect on the expression of apoptotic related proteins.

DISCUSSION

In 2016, a research team from Italy reported that in patients with acute ICH, higher PMNs, lower lymphocytes, and higher neutrophil-to-lymphocyte ratio (NLR) in peripheral circulating blood predicted worse 3 months outcome (Lattanzi et al., 2016). More Recently, a research further presented that higher NLR is independently associated with edema growth following ICH (Gusdon et al., 2017). These clinical trials suggest that peripheral PMNs count and NLR may be closely related to the prognosis of ICH. However, the mechanisms responsible for this relationship remain poorly characterized. Thus, we tested it for the first time in an experimental animal model of ICH. Notably, in this study, we observed the similar trend like in ICH patients, all ICH rats presented higher leukocyte count, PMNs count and NLR when compared with the control groups. Our data indicated that this animal model of ICH successfully reproduced the clinical situation in human beings. As PMNs plays a key role in neuroinflammation after ICH, inhibiting the migration of PMNs into brain could be a promising therapy for ICH. Furthermore, we also found that PBMCs elevates on 72 h after ICH and stay high for up to 7 days. These data partially fit with other literature reports, showing that after acute condition (miocardila infarction), PBMCs elevates within hours and stay high for up to 72 h (Nahrendorf et al., 2010). This discrepancy may be attributed to the difference between "ischemic" and "hemorrhagic" pathological nature here.

In normal conditions, PMNs lifespan was about 8–20 h in circulation. But in pathology situation, inflammatory response will delay the apoptosis of PMNs and extends its lifespan, such as arthritis, diabetes, Soehnlein et al. (2017). On this basis, we thought that is there any safe drug could accelerate PMNs apoptosis and promotes inflammation resolution after ICH. Recently, a randomized, double-blind clinical study reported that simvastatin therapy before cardiopulmonary bypass significantly increased peripheral PMNs apoptotic ratio and reduced the post-operative peak values of interleukin (IL)-6 and IL-8 (Chello et al., 2007). In this study, we observed that animals treated with simvastatin displays higher PMNs apoptotic ratio than control groups, as well as lower PMNs count and NLR in circulation. In addition, fewer PMNs invasion and lower transcriptional level of proinflammatory factors were detected in the perihematomal area after simvastatin administration. According to previous studies, CCL2 and ICAM-1 were closed with PMN invasion to lesion site after injury (Yang et al., 2005; Reichel et al., 2009). Thus, in present study, we detected the mRNA level of CCL2 and ICAM-1 in the perihematomal area at 6, 12, and 24 h after ICH. The data shows that ICH significantly upregulated the mRNA level of these chemokines and vascular adhesion molecule, which is reversed by the treatment with simvastatin. Our data indicated that simvastatin-mediated against PMN infiltration into brain may in part by inhibiting RNA transcription of CCL2 and ICAM-1 following ICH. In view of this, we proposed that simvastatin could be a safe and effective mean for attenuating PMNs brain-infiltration and subsequent inflammatory reaction following ICH.

According to previous literature, neutrophils can promote inflammatory mediators release that help recruit monocytes/macrophages, amplifying the inflammation. When neutrophils adhere to the endothelial surface, the contents of secretory granules are released, including cationic antimicrobial protein of 37 kd (CAP37)/azurocidin and proteinase 3, leading to endothelial cell activation, increased cellular adhesion molecule expression (such as ICAM-1), and increased monocyte

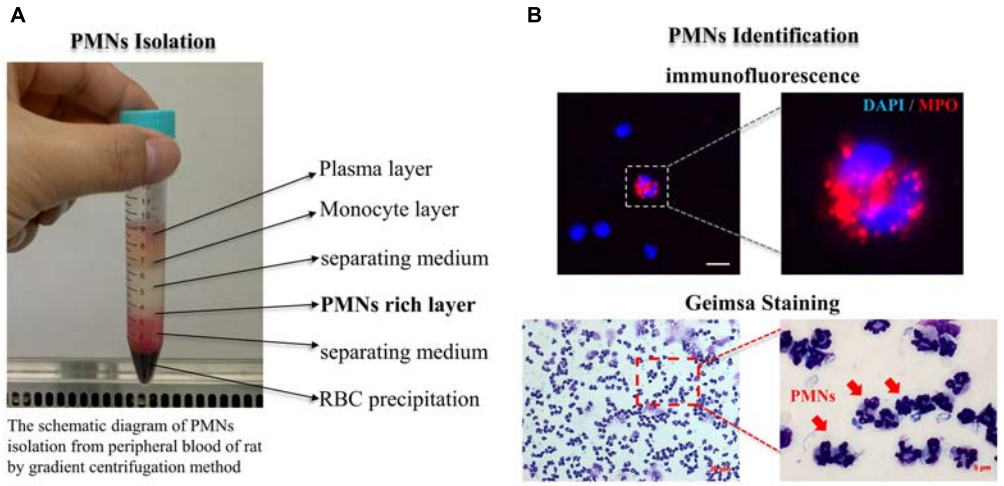

FIGURE 4 | PMNs isolation and identification. **(A)** The schematic diagram of PMNs isolation from peripheral blood of rat by gradient centrifugation method; **(B)** PMNs well identified by using immunofluorescence and Giemsa staining methods. PMNs presented the feature typical for PMNs-multilobal nuclei at high magnification. The scaling bar represents 5 μm in immunofluorescence image.

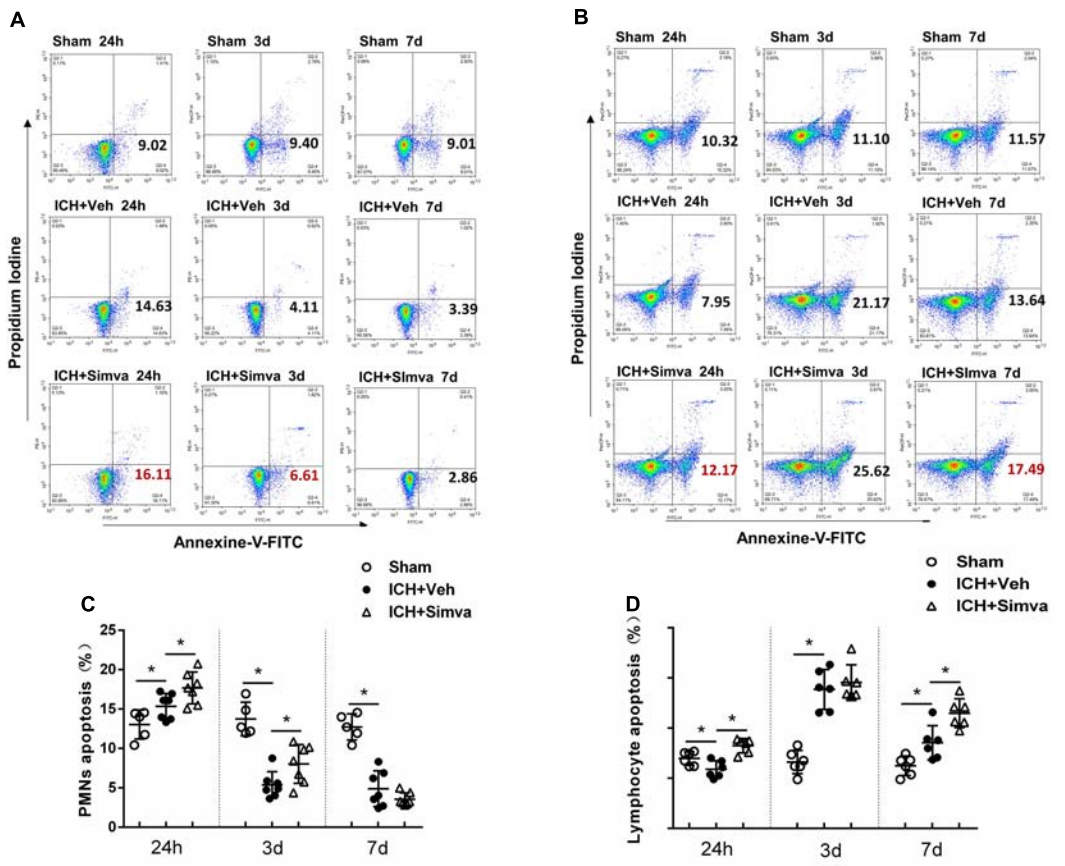

FIGURE 5 | Simvastatin accelerated peripheral PMNs apoptosis after ICH, which in part by broke the balance between antiapoptotic proteins and proapoptotic proteins. Representative flow cytometric dot plots showing circulating PMNs **(A)** and lymphocytes **(B)** apoptosis, which freshly isolated from sham control rat and ICH rat treated with vehicle or simvastatin. Annexin V+ and PI− cells were considered early apoptotic cells (lower right quadrant). Flow cytometric analysis of peripheral PMNs **(C)** and lymphocytes **(D)** in the simvastatin- and vehicle-treated rat days 1, 3, and 7 after ICH. The apoptotic ratio was calculated from the ratio of apoptotic cells to total cells counted. Values are expressed as mean ± SD, $n = 6$ per group; $^*P < 0.05$.

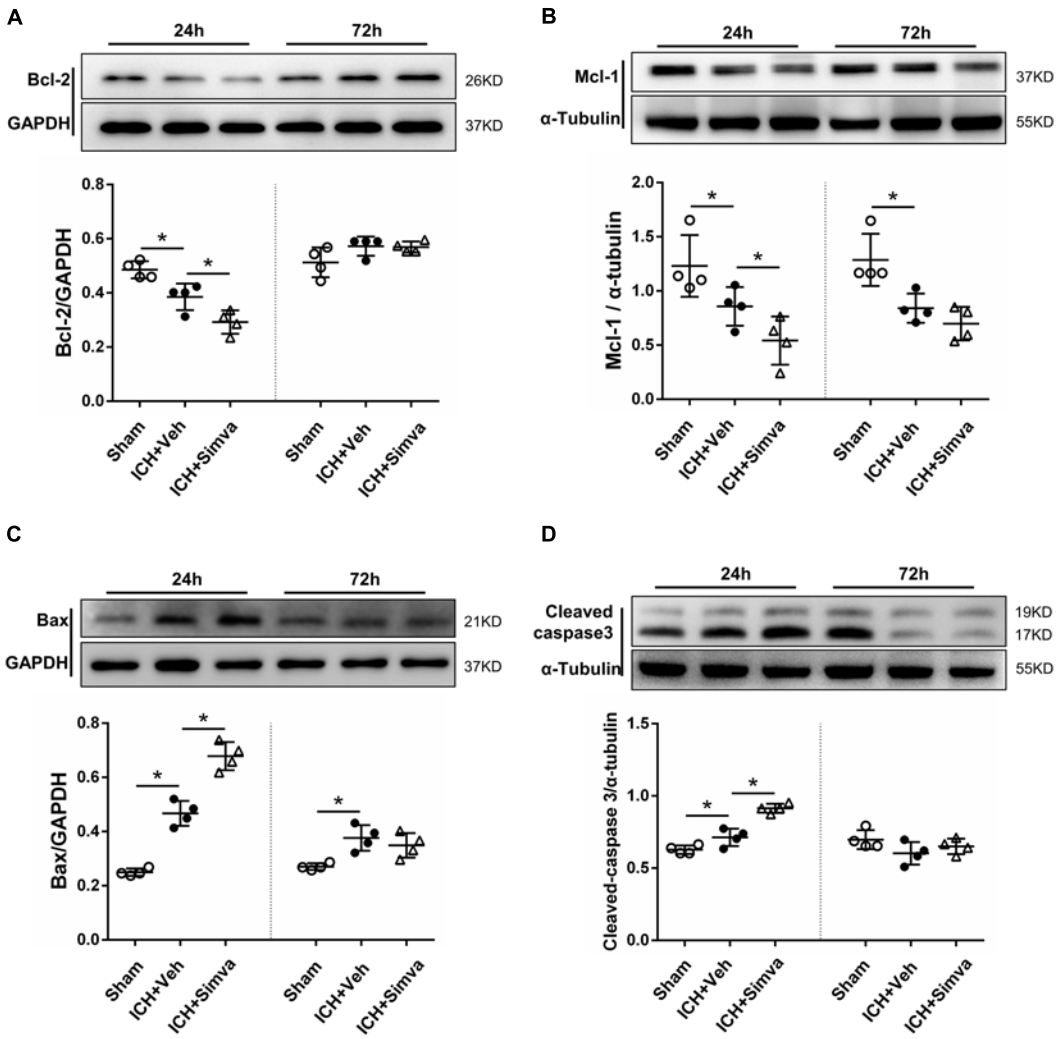

FIGURE 6 | Western blot analysis of the levels of apoptotic related proteins in PMNs after ICH. Representative images and quantitative analysis of Bcl-2 protein **(A)**, Mcl-1 protein **(B)**, Bax protein **(C)**, and caspase 3 protein **(D)** in the isolated peripheral PMNs at 24 h and day 3 after ICH. Values are expressed as mean ± SD, $n = 4$ per group; $^*P < 0.05$ versus sham control.

adhesion (Lee et al., 2003). Neutrophils also contribute to monocyte chemotaxis by releasing LL-37 (De et al., 2000) and cathepsin G (Sun et al., 2004), and enhancing endothelial cell release of monocyte chemoattractant protein-1 (MCP-1) (Taekema-Roelvink et al., 2001). Consistently, in a rat model of ICH, selective depletion of neutrophils resulted in decreased infiltration of monocytes and macrophages into perihematomal area (Moxon-Emre and Schlichter, 2011; Sansing et al., 2011). In our study, neutrophils infiltrated into brain and promoted the mRNA transcription of CCL2 (also referred to as MCP-1), ICAM-1 and pro-inflammatory factors around hematoma. Notably, all of these effects of neutrophils were prevented by simvastatin. Taken together, our data in some extent suggest that neutrophils mobilization play a role in the process of leukocytes activation following ICH.

Previous researches have demonstrated that the balance between Bax/Mcl-1 has a key role in modulating PMNs apoptosis or survival (Dyugovskaya et al., 2012). PMNs constitutively express the proapoptotic members of the Bcl-2 family, including Bax, Bad, Bak, Bid, and Bik. While Mcl-1 is a member of the antiapoptotic proteins of Bcl-2 family, which plays its role by combining with Bax that expression on the outer membrane of mitochondria, forming heteromeric two dimers and stabilizing mitochondrial membrane potential. Then, preventing mitochondrial release of cytochrome C, and ultimately reducing caspase 3 activation to prevent PMN apoptosis. Recent clinical studies have found that the level of Mcl-1 expression is negatively correlated with the severity of PMNs apoptosis (Moulding et al., 1998). Much more, animal study further confirmed that conditional knockout of Mcl-1 gene significantly accelerated the apoptosis of PMN (Dzhagalov et al., 2007). Therefore, to illuminate how simvastatin promoted PMNs apoptosis, we conducted Western blotting analysis of apoptotic related proteins in present study. Our data show that

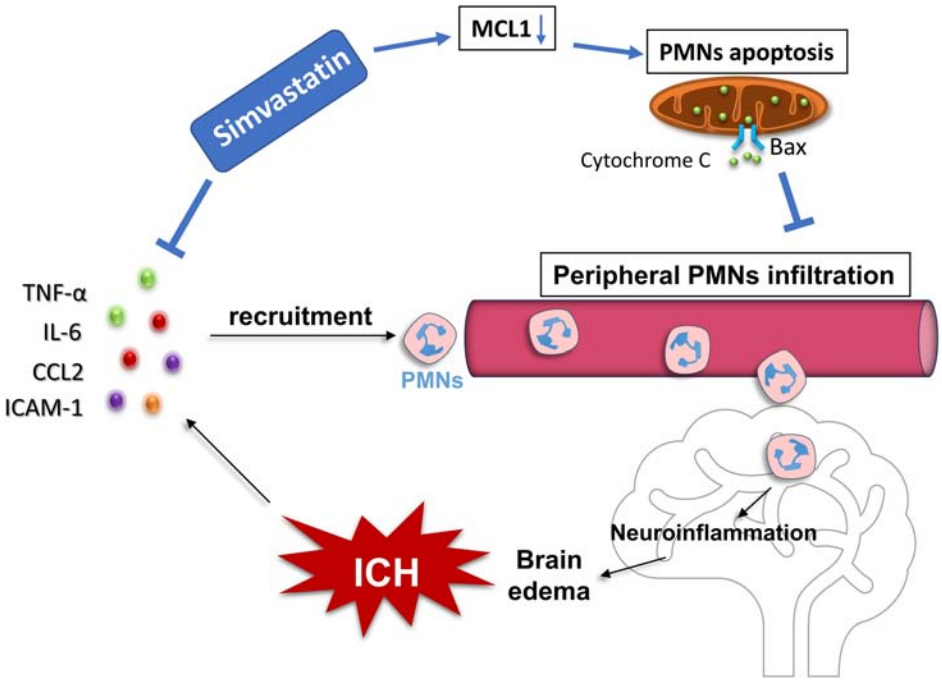

FIGURE 7 | Proposed mechanism underlying simvastatin-mediated against PMNs brain-invading after ICH. It mainly depends on two approaches. On the one hand, simvastatin significantly inhibited the transcription of chemokines and vascular cell adhesion molecule, such as TNF-α, IL-6, CCL2, and ICAM-1, which subsequently reduced the chemotaxis and recruitment of peripheral neutrophil into brain after ICH. On the other hand, through down-regulated the expression of antiapoptotic protein-Mcl-1 while increased the level of proapoptotic protein-Bax expression in PMNs, resulting in more cytochrome C releasing out from mitochondria and caspase 3 activating, which then promoted the apoptosis of PMNs and cut down the number of circulating PMNs.

the level of antiapoptotic protein, Mcl-1, was down-regulated while the expression of proapoptotic protein, Bax, increased after simvastatin used.

HMG-CoA reductase, as a rate-limiting enzyme, catalyzes the cholesterol synthesis pathway in the liver and other tissues. By inhibiting HMG-CoA reductase, statins lower cholesterol levels and might also reduce intracellular levels of isoprenoids, such as farnesyl pyrophosphate and geranylgeranyl pyrophosphate (Weitz-Schmidt, 2002). Isoprenoids are necessary for the post-translational lipid modification (prenylation) of a variety of proteins, thus anchoring them to the cell membrane (Zhang and Casey, 1996). Protein targets include the small guanosine triphosphate (GTP)-binding proteins that have a key role in signal transduction pathways that regulate cell proliferation, cell differentiation, vesicular transport and apoptosis. In addition to their cholesterol-lowering activity, statins also have pleiotropic effects, like anti-inflammation, promoting hematoma absorption, neurogenesis and neuroplasticity in some CNS diseases via the non-cholesterol pathway (Karki et al., 2009; van der Most et al., 2009; Yang et al., 2013; Wang et al., 2018). In the past few years, some retrospective clinical studies on ICH have suggested that statin use may has potential to reduce mortality, improve outcomes, with no risk of inducing rehemorrhage (Flint et al., 2014; Pan et al., 2014; Chen et al., 2015). However, to date, no randomized clinical trials have examined the exact effect. Although preclinical studies from our research team and other centers have made some progress during the past decade, it is still a long way for statin use in patients with ICH. In this study, we observed simvastatin effectively prevented PMNs infiltration, suppressed proinflammatory factors release, and attenuated brain edema following ICH through regulating PMNs apoptosis. Thus, our results may provide another evidence for future statin use in ICH patients, and more related researches were needed to understand the potential mechanism. Interestingly, a recent work reported that systemic depletion of PMNs at 24 h after ICH exacerbated neurological deficits, suggesting that PMNs infiltration into the brain also shows some beneficial function at the subacute stage post-ICH (Zhao et al., 2018). After further investigation, they proposed that PMNs-mediated protective effect in subacute stage may through the two following ways: (1) PMNs can deliver cytoprotective lactoferrin to the ICH-affected brain, neutralizing iron and blocking its toxicity (Zhao et al., 2018). (2) ICH can also transform the phenotype of PMNs that entering brain at the later stages, which then could enhance the beneficial effects like promoted blood detoxification efficacy (Zhao et al., 2017). From the above results, we can read that peripheral PMNs are like a double-edged sword for ICH- it counteracts the iron toxicity as well banes to exacerbate the secondary neuroinflammation at the different stages following ICH. Therefore, correct therapies should be chosen at correct time when targeting circulating blood PMNs after ICH, so as to maximize the natural processes of brain repair. Contrary to traditional belief, increasing evidence support that neutrophils also play a role in chronic

inflammatory disorders, such as obesity and atherosclerosis (Soehnlein et al., 2017). Soehnlein et al. (2017) highlighted some therapeutic strategies to prevent neutrophil-orchestrated chronic inflammation, such as inhibition of neutrophil extracellular trap (NET)-driven inflammation, dampening neutrophil recruitment, promoting neutrophil "reverse migration" from the tissue into the bloodstream, inhibition of cyclin-dependent kinase 9 (CDK9) promotes neutrophil apoptosis.

CONCLUSION

Our data revealed that the PMNs count and NLR in circulation significantly increased in experimental ICH, which in consistent with the clinical situation occurred in ICH patients. Simvastatin effectively reduced the elevated PMNs count and NLR in circulation, and significantly inhibited PMNs brain-invading and ameliorated ICH-induced brain edema and neurological deficits in rats. Moreover, simvastatin-mediated against PMNs brain-infiltration after ICH may in part by accelerating the apoptosis of peripheral PMNs as well as reducing the mRNA level of pro-inflammatory mediators (**Figure 7**). Our results not only provide evidence for simvastatin use on patients with ICH, but also suggest that PMNs apoptosis regulation may be a new therapeutic target for ICH.

ETHICS STATEMENT

All institutional and national guidelines for the care and use of laboratory animals were followed.

AUTHOR CONTRIBUTIONS

JZ, XS, NH, LW, LT, YC, and QC contributed to the implementation of the experiment. QC, HF, ZC, and GZ contributed to the design and paper writing.

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REFERENCES

Behrouz, R. (2016). Re-exploring tumor necrosis factor alpha as a target for therapy in intracerebral hemorrhage. *Transl. Stroke Res.* 7, 93–96. doi: 10.1007/s12975-016-0446-x

Chello, M., Anselmi, A., Spadaccio, C., Patti, G., Goffredo, C., Di Sciascio, G., et al. (2007). Simvastatin increases neutrophil apoptosis and reduces inflammatory reaction after coronary surgery. *Ann. Thorac. Surg.* 83, 1374–1380. doi: 10.1016/j.athoracsur.2006.10.065

Chen, P. S., Cheng, C. L., Chang, Y. C., Kao Yang, Y. H., Yeh, P. S., and Li, Y. H. (2015). Early statin therapy in patients with acute intracerebral hemorrhage without prior statin use. *Eur. J. Neurol.* 22, 773–780. doi: 10.1111/ene.12649

Chen, Q., Shi, X., Tan, Q., Feng, Z., Wang, Y., Yuan, Q., et al. (2017). Simvastatin promotes hematoma absorption and reduces hydrocephalus following intraventricular hemorrhage in part by upregulating CD36. *Transl. Stroke Res.* 8, 362–373. doi: 10.1007/s12975-017-0521-y

De, Y., Chen, Q., Schmidt, A. P., Anderson, G. M., Wang, J. M., Wooters, J., et al. (2000). LL-37, the neutrophil granule- and epithelial cell-derived cathelicidin, utilizes formyl peptide receptor-like 1 (FPRL1) as a receptor to chemoattract human peripheral blood neutrophils, monocytes, and T cells. *J. Exp. Med.* 192, 1069–1074. doi: 10.1084/jem.192.7.1069

Dyugovskaya, L., Polyakov, A., Cohen-Kaplan, V., Lavie, P., and Lavie, L. (2012). Bax/Mcl-1 balance affects neutrophil survival in intermittent hypoxia and obstructive sleep apnea: effects of p38MAPK and ERK1/2 signaling. *J. Transl. Med.* 10:211. doi: 10.1186/1479-5876-10-211

Dzhagalov, I., St John, A., and He, Y. W. (2007). The antiapoptotic protein Mcl-1 is essential for the survival of neutrophils but not macrophages. *Blood* 109, 1620–1626. doi: 10.1182/blood-2006-03-013771

Feng, Z., Tan, Q., Tang, J., Li, L., Tao, Y., Chen, Y., et al. (2017). Intraventricular administration of urokinase as a novel therapeutic approach for communicating hydrocephalus. *Transl. Res. J. Lab. Clin. Med.* 180, 77.e2–90.e2. doi: 10.1016/j.trsl.2016.08.004

Flint, A. C., Conell, C., Rao, V. A., Klingman, J. G., Sidney, S., Johnston, S. C., et al. (2014). Effect of statin use during hospitalization for intracerebral hemorrhage on mortality and discharge disposition. *JAMA Neurol.* 71, 1364–1371. doi: 10.1001/jamaneurol.2014.2124

Gusdon, A. M., Gialdini, G., Kone, G., Baradaran, H., Merkler, A. E., Mangat, H. S., et al. (2017). Neutrophil-Lymphocyte ratio and perihematomal edema growth in intracerebral hemorrhage. *Stroke* 48, 2589–2592. doi: 10.1161/STROKEAHA.117.018120

Hua, Y., Schallert, T., Keep, R. F., Wu, J., Hoff, J. T., and Xi, G. (2002). Behavioral tests after intracerebral hemorrhage in the rat. *Stroke* 33, 2478–2484. doi: 10.1161/01.STR.0000032302.91894.0F

Iadecola, C., and Anrather, J. (2011). The immunology of stroke: from mechanisms to translation. *Nat. Med.* 17, 796–808. doi: 10.1038/nm.2399

Jiang, B., Li, L., Chen, Q., Tao, Y., Yang, L., Zhang, B., et al. (2017). Role of glibenclamide in brain injury after intracerebral hemorrhage. *Transl. Stroke Res.* 8, 183–193. doi: 10.1007/s12975-016-0506-2

Jin, R., Zhu, X., Liu, L., Nanda, A., Granger, D. N., and Li, G. (2013). Simvastatin attenuates stroke-induced splenic atrophy and lung susceptibility to spontaneous bacterial infection in mice. *Stroke* 44, 1135–1143. doi: 10.1161/STROKEAHA.111.000633

Joseph, M. J., Caliaperumal, J., and Schlichter, L. C. (2016). After intracerebral hemorrhage, oligodendrocyte precursors proliferate and differentiate inside white-matter tracts in the rat striatum. *Transl. Stroke Res.* 7, 192–208. doi: 10.1007/s12975-015-0445-3

Karki, K., Knight, R. A., Han, Y., Yang, D., Zhang, J., Ledbetter, K. A., et al. (2009). Simvastatin and atorvastatin improve neurological outcome after experimental intracerebral hemorrhage. *Stroke* 40, 3384–3389. doi: 10.1161/STROKEAHA.108.544395

Lattanzi, S., Cagnetti, C., Provinciali, L., and Silvestrini, M. (2016). Neutrophil-to-Lymphocyte ratio predicts the outcome of acute intracerebral hemorrhage. *Stroke* 47, 1654–1657. doi: 10.1161/STROKEAHA.116.013627

Lee, T. D., Gonzalez, M. L., Kumar, P., Grammas, P., and Pereira, H. A. (2003). CAP37, a neutrophil-derived inflammatory mediator, augments leukocyte adhesion to endothelial monolayers. *Microvasc. Res.* 66, 38–48. doi: 10.1016/S0026-2862(03)00010-4

Leung, B. P., Sattar, N., Crilly, A., Prach, M., McCarey, D. W., Payne, H., et al. (2003). A novel anti-inflammatory role for simvastatin in inflammatory arthritis. *J. Immunol.* 170, 1524–1530. doi: 10.4049/jimmunol.170.3.1524

Liew, H. K., Pang, C. Y., Hsu, C. W., Wang, M. J., Li, T. Y., Peng, H. F., et al. (2012). Systemic administration of urocortin after intracerebral hemorrhage reduces neurological deficits and neuroinflammation in rats. *J. Neuroinflammation* 9:13. doi: 10.1186/1742-2094-9-13

Moulding, D. A., Quayle, J. A., Hart, C. A., and Edwards, S. W. (1998). Mcl-1 expression in human neutrophils: regulation by cytokines and correlation with cell survival. *Blood* 92, 2495–2502.

Moxon-Emre, I., and Schlichter, L. C. (2011). Neutrophil depletion reduces blood-brain barrier breakdown, axon injury, and inflammation after intracerebral hemorrhage. *J. Neuropathol. Exp. Neurol.* 70, 218–235. doi: 10.1097/NEN.0b013e31820d94a5

Nahrendorf, M., Pittet, M. J., and Swirski, F. K. (2010). Monocytes: protagonists of infarct inflammation and repair after myocardial infarction. *Circulation* 121, 2437–2445. doi: 10.1161/CIRCULATIONAHA.109.916346

Pan, Y. S., Jing, J., Wang, Y. L., Zhao, X. Q., Song, B., Wang, W. J., et al. (2014). Use of statin during hospitalization improves the outcome after intracerebral hemorrhage. *CNS Neurosci. Ther.* 20 548–555. doi: 10.1111/cns.12274

Qin, L., Zhao, L., Tan, C., Chen, X. U., Yang, Z., and Mo, W. (2015). A novel method of combining Periodic Acid Schiff staining with Wright-Giemsa staining to identify the pathogens *Penicillium marneffei*, *Histoplasma capsulatum*, Mucor and *Leishmania donovani* in bone marrow smears. *Exp. Ther. Med.* 9, 1950–1954. doi: 10.3892/etm.2015.2357

Qureshi, A. I., Mendelow, A. D., and Hanley, D. F. (2009). Intracerebral haemorrhage. *Lancet* 373 1632–1644. doi: 10.1016/S0140-6736(09)60371-8

Reichel, C. A., Rehberg, M., Lerchenberger, M., Berberich, N., Bihari, P., Khandoga, A. G., et al. (2009). Ccl2 and Ccl3 mediate neutrophil recruitment via induction of protein synthesis and generation of lipid mediators. *Arterioscler. Thromb. Vasc. Biol.* 29 1787–1793. doi: 10.1161/ATVBAHA.109.193268

Sansing, L. H., Harris, T. H., Kasner, S. E., Hunter, C. A., and Kariko, K. (2011). Neutrophil depletion diminishes monocyte infiltration and improves functional outcome after experimental intracerebral hemorrhage. *Acta Neurochir. Suppl.* 111 173–178. doi: 10.1007/978-3-7091-0693-8_29

Soehnlein, O., Steffens, S., Hidalgo, A., and Weber, C. (2017). Neutrophils as protagonists and targets in chronic inflammation. *Nat. Rev. Immunol.* 17, 248–261. doi: 10.1038/nri.2017.10

Sun, R., Iribarren, P., Zhang, N., Zhou, Y., Gong, W., Cho, E. H., et al. (2004). Identification of neutrophil granule protein cathepsin G as a novel chemotactic agonist for the G protein-coupled formyl peptide receptor. *J. Immunol.* 173, 428–436. doi: 10.4049/jimmunol.173.1.428

Taekema-Roelvink, M. E., Kooten, C., Kooij, S. V., Heemskerk, E., and Daha, M. R. (2001). Proteinase 3 enhances endothelial monocyte chemoattractant protein-1 production and induces increased adhesion of neutrophils to endothelial cells by upregulating intercellular cell adhesion molecule-1. *J. Am. Soc. Nephrol.* 12, 932–940.

Tan, Q., Chen, Q., Niu, Y., Feng, Z., Li, L., Tao, Y., et al. (2017). Urokinase, a promising candidate for fibrinolytic therapy for intracerebral hemorrhage. *J. Neurosurg.* 126, 548–557. doi: 10.3171/2016.1.JNS152287

Tao, C., Hu, X., Wang, J., Ma, J., Li, H., and You, C. (2017). Admission neutrophil count and neutrophil to lymphocyte ratio predict 90-day outcome in intracerebral hemorrhage. *Biomark. Med.* 11, 33–42. doi: 10.2217/bmm-2016-0187

van der Most, P. J., Dolga, A. M., Nijholt, I. M., Luiten, P. G., and Eisel, U. L. (2009). Statins: mechanisms of neuroprotection. *Prog. Neurobiol.* 88, 64–75. doi: 10.1016/j.pneurobio.2009.02.002

Wan, S., Cheng, Y., Jin, H., Guo, D., Hua, Y., Keep, R. F., et al. (2016). Microglia activation and polarization after intracerebral hemorrhage in mice: the role of protease-activated receptor-1. *Transl. Stroke Res.* 7, 478–487. doi: 10.1007/s12975-016-0472-8

Wang, F., Hu, S., Ding, Y., Ju, X., Wang, L., Lu, Q., et al. (2016). Neutrophil-to-Lymphocyte Ratio and 30-Day mortality in patients with acute intracerebral hemorrhage. *J. Stroke Cerebrovasc. Dis.* 25, 182–187. doi: 10.1016/j.jstrokecerebrovasdis.2015.09.013

Wang, J. (2010). Preclinical and clinical research on inflammation after intracerebral hemorrhage. *Prog. Neurobiol.* 92 463–477. doi: 10.1016/j.pneurobio.2010.08.001

Wang, Y., Chen, Q., Tan, Q., Feng, Z., He, Z., Tang, J., et al. (2018). Simvastatin accelerates hematoma resolution after intracerebral hemorrhage in a PPARgamma-dependent manner. *Neuropharmacology* 128, 244–254. doi: 10.1016/j.neuropharm.2017.10.021

Weitz-Schmidt, G. (2002). Statins as anti-inflammatory agents. *Trends Pharmacol. Sci.* 23, 482–486. doi: 10.1016/S0165-6147(02)02077-1

Welles, E. G., Hall, A. S., and Carpenter, D. M. (2009). Canine complete blood counts: a comparison of four in-office instruments with the ADVIA 120 and manual differential counts. *Vet. Clin. Pathol.* 38, 20–29. doi: 10.1111/j.1939-165X.2008.00084.x

Williamson, M. R., and Colbourne, F. (2017). Evidence for decreased brain parenchymal volume after large intracerebral hemorrhages: a potential mechanism limiting intracranial pressure rises. *Transl. Stroke Res.* 8, 386–396. doi: 10.1007/s12975-017-0530-x

Xi, G., Strahle, J., Hua, Y., and Keep, R. F. (2014). Progress in translational research on intracerebral hemorrhage: is there an end in sight? *Prog. Neurobiol.* 115, 45–63. doi: 10.1016/j.pneurobio.2013.09.007

Yang, D., Knight, R. A., Han, Y., Karki, K., Zhang, J., Chopp, M., et al. (2013). Statins protect the blood brain barrier acutely after experimental intracerebral hemorrhage. *J. Behav. Brain Sci.* 3, 100–106. doi: 10.4236/jbbs.2013.31010

Yang, L., Froio, R. M., Sciuto, T. E., Dvorak, A. M., Alon, R., and Luscinskas, F. W. (2005). ICAM-1 regulates neutrophil adhesion and transcellular migration of TNF-alpha-activated vascular endothelium under flow. *Blood* 106, 584–592. doi: 10.1182/blood-2004-12-4942

Zhang, F. L., and Casey, P. J. (1996). Protein prenylation: molecular mechanisms and functional consequences. *Annu. Rev. Biochem.* 65, 241–269. doi: 10.1146/annurev.bi.65.070196.001325

Zhao, X., Ting, S. M., Liu, C. H., Sun, G., Kruzel, M., Roy-O'Reilly, M., et al. (2017). Neutrophil polarization by IL-27 as a therapeutic target for intracerebral hemorrhage. *Nat. Commun.* 8:602. doi: 10.1038/s41467-017-00770-7

Zhao, X., Ting, S. M., Sun, G., Roy-O'Reilly, M., Mobley, A. S., Bautista Garrido, J., et al. (2018). Beneficial role of neutrophils through function of lactoferrin after intracerebral hemorrhage. *Stroke* 49, 1241–1247. doi: 10.1161/STROKEAHA.117.020544

Zhou, Y., Wang, Y., Wang, J., Anne Stetler, R., and Yang, Q. W. (2014). Inflammation in intracerebral hemorrhage: from mechanisms to clinical translation. *Prog. Neurobiol.* 115, 25–44. doi: 10.1016/j.pneurobio.2013.11.003

Zou, X., Wu, Z., Zhu, W., Chen, L., Mao, Y., and Zhao, F. (2017). Effectiveness of minocycline in acute white matter injury after intracerebral hemorrhage. *J. Neurosurg.* 126, 1855–1862. doi: 10.3171/2016.5.JNS152670

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Iso-α-Acids, Bitter Components in Beer, Suppress Inflammatory Responses and Attenuate Neural Hyperactivation in the Hippocampus

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Due to the growth in aging populations worldwide, prevention and therapy for age-related cognitive decline and dementia are in great demand. We previously demonstrated that long-term intake of iso-α-acids, which are hop-derived bitter compounds found in beer, prevent Alzheimer's pathology in a rodent model. On the other hand, the effects of iso-α-acids on neural activity in Alzheimer's disease model mice have not been investigated. Here, we demonstrated that short-term intake of iso-α-acids suppresses inflammation in the hippocampus and improves memory impairment even after disease onset. Importantly, we demonstrated that short-term administration of iso-α-acids attenuated the neural hyperactivation in hippocampus. In 6-month-old 5 × FAD mice exhibiting hippocampus inflammation and memory impairment, oral administration of iso-α-acids for 7 days reduced inflammatory cytokines, including MIP-1α and soluble Aβ and improved object memory in the novel object recognition test. In 12-month-old J20 mice, intake of iso-α-acids for 7 days also suppressed inflammatory cytokines and soluble Aβ in the brain. Manganese-enhanced magnetic resonance imaging (MEMRI) of hippocampi of J20 mice showed increased manganese compared with wild type mice, but iso-α-acids canceled this increased MEMRI signal in J20 mice, particularly in the hippocampus CA1 and CA3 region. Taken together, these findings suggest that short-term intake of iso-α-acids can suppress hippocampus inflammation even after disease onset and improve hyper neural activity in Alzheimer's disease model mice.

Keywords: Alzheimer's disease, amyloid β, cognitive decline, hippocampus, inflammation, iso-α-acids, lipopolysaccharide

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INTRODUCTION

The rise in aging populations worldwide is accompanied by increasing rates of dementia and cognitive impairment, which are a burden to national healthcare systems as well as patients and their families. Because of the lack of treatments for dementia, preventive approaches such as diet, exercise, and lifelong learning have received increasing research attention. In etiological studies of lifestyle, low to moderate consumption of alcoholic beverages, such as wine and beer, might

reduce the risk of cognitive decline and the development of dementia. Individuals who consume low to moderate levels of alcohol on a daily basis were shown to have significantly lower risks of neurodegenerative diseases compared with individuals who abstained from alcohol or drank heavily (Matsui et al., 2011; Neafsey and Collins, 2011; Horvat et al., 2015). Apart from the effects of alcohol itself, resveratrol, a polyphenolic compound found in red wine, has been shown to have neuroprotective effects (Vidavalur et al., 2006; Arntzen et al., 2010; Porquet et al., 2014; Witte et al., 2014). Our group previously demonstrated that long-term intake of iso-α-acids for 3 months, which are bitter components in beer, prevented Alzheimer's pathology in a transgenic mouse model. Iso-α-acids are derived from hops, the female inflorescences of the hop plant (*Humulus lupulus* L.), and have been used in beer production since 822. Hops are used as both a preservative and a flavoring agent in the beer-brewing process. Iso-α-acids were shown to activate the peroxisome proliferator-activated receptor-γ (PPAR-γ) and regulate microglial phagocytosis and inflammation (Ano et al., 2017). Our group has previously demonstrated that iso-α-acids prevented dyslipidemia and type 2 diabetes in a diet-induced obese rodent model (Yajima et al., 2004, 2005), and improved glucose metabolism and decreased body fat in a clinical trial (Obara et al., 2009). Long-term administration of iso-α-acids is applicable for the preventive approaches, but the effects for the therapeutic approaches and for neural activity have not been elucidated. It is reported that hyperactivity in hippocampus is associated with cognitive impairment and improvement of the hyperactivity show therapeutic effects on memory impairment (Bakker et al., 2012). In addition, previous study did not conclude that long-term administration of iso-α-acids suppressed the inflammation in the brain directly or as a result of the improvement of amyloid β (Aβ) deposition. To address these research gaps, in the present study, we examined the effects of short-term intake of iso-α-acids on brain inflammation and neural activity in hippocampus using the manganese-enhanced magnetic resonance imaging (MEMRI) in Alzheimer's model mice.

MATERIALS AND METHODS

Animals

Alzheimer's disease model, B6SJL-Tg mice [APPSwFlLon, PSEN1*M146L*L286V[1], (Oakley et al., 2006)], hereafter referred to as 5 × FAD transgenic mice, were purchased from Jackson Laboratory (Sacramento, CA, United States) and maintained by crossing hemizygous transgenic mice with B6SJLF1/J mice at the experimental facility of the University of Tokyo. The 5 × FAD transgenic mice overexpress mutant human APP (695) with the Swedish (K670N, M671L), Florida (I716V), and London (V717I) Familial Alzheimer's Disease (FAD) mutations, along with human PS1 harboring two FAD mutations, namely, M146L and L286V. Non-transgenic wild type (WT) littermates as controls were used in the experiments. All experiments were approved by the Animal Care and Use Committee of the Graduate School of Agricultural and Life Sciences, The University of Tokyo, and conducted in strict accordance with its guidelines. Transgenic (J20) mice express human amyloid precursor protein (hAPP) with the Swedish (K670N, M671L) and Indiana (V717F) mutations under the control of the PDGF β-chain promoter (Mucke et al., 2000). Experiments using J20 mice were approved by the local ethical board and complied with the guidelines for animal experimentation of the National Center for Geriatrics and Gerontology in Japan. Pregnant C57BL/6J mice and 6-week-old CD-1 (ICR) mice were purchased from Charles River Japan (Tokyo, Japan) and maintained at a faculty of Kirin Company Ltd. The experiments were approved by the Animal Experiment Committee of Kirin Company Ltd. and conducted in strict accordance with its guidelines since 2014–2016. All efforts were made to minimize animal suffering. Mice were fed a standard rodent diet (CE-2, CLEA Japan, Tokyo, Japan) and maintained at room temperature (23 ± 1°C) under a constant 12-h light/dark cycle (light period from 8 a.m. to 8 p.m.).

[1]http://jaxmice.jax.org/strain/006554.html

Preparation of Iso-α-Acids

α-Acids predominantly consist of three congeners: cohumulone, humulone, and adhumulone. During the brewing process, they are each isomerized into two epimeric isomers, namely, cis- and trans-iso-α-acids. Purchased isomerized hop extract (IHE) (Hopsteiner, Mainburg, Germany) with 30.5% (w/v) iso-α-acids, comprising trans-isocohumulone (1.74% w/v), cis-isocohumulone (7.61% w/v), trans-isohumulone (3.05% w/v), cis-isohumulone (14.0% w/v), trans-isoadhumulone (0.737% w/v), and cis-isoadhumulone (3.37% w/v) as described previously (Taniguchi et al., 2013).

Primary Microglia Cell Culture

Microglial cells were isolated from brains of newborn C57BL/6J mice (<7 days old) via magnetic cell sorting (MACS) after conjugation with anti-CD11b antibody, as described previously (Ano et al., 2015). Briefly, isolated CD11b-positive cells (>90% pure, evaluated by flow cytometer) were plated into poly-D-lysine (PDL)–coated 96-well plates (BD Biosciences, Billerica, MA, United States) and cultured in a DMEM/F-12 (Gibco, Carlsbad, CA, United States) medium supplemented with 10% fetal calf serum (Gibco) and 100 U/ml penicillium/streptomycin (Sigma-Aldrich, St. Louis, MO, United States). Microglia at a density of 30,000 were treated with each iso-α-acids for 12 h, and then with lipopolysaccharide (LPS, 5 ng/ml, Sigma-Aldrich, St. Louis, MO, United States) and interferon-γ (IFN-γ, 0.5 ng/ml, R&D systems, Minneapolis, MN, United States) for 12 h. After stimulation, the supernatant was used for the TNF-α production assay. Concentrations of cytokines sand chemokines in the supernatant was measured by the Bio-Plex assay system (Bio-Rad, Hercules, CA, United States).

Neuronal Inflammation Induced by Lipopolysaccharide

Six-week-old ICR male mice were orally administered 1 mg/kg of iso-α-acids dissolved in distilled water once a day for

3 days. One hour after the final administration, the mice were deeply anesthetized with sodium pentobarbital (Kyoritsu Seiyaku, Tokyo, Japan) and injected intracerebroventricularly with 0.25 mg/kg of LPS (5 μl, L7895, Sigma) or PBS as controls as previously described (Ano et al., 2015). Briefly, a micro syringe with a 27-gauge stainless steel needle, 2 mm in length, was used for micro injection. The needle was inserted unilaterally 1 mm to the right of the midline at the equal distance between eyes and ears, and perpendicular to the plane of the skull (anteroposterior, −0.22 mm from the bregma; lateral, 1 mm from the bregma). LPS was delivered gradually within 30 s. The needle was withdrawn after waiting 30 s. Twenty-four hour later, the mice were subjected to the Y-maze test for 8 min to evaluate spontaneous alternation. After the test, the hippocampus and cerebral cortex were removed and homogenized in TBS buffer containing a protease inhibitor cocktail (BioVision, CA, United States). To quantify cytokine and chemokine production, the homogenate supernatant was measured by the Bio-Plex assay system (Bio-Rad).

Aβ and Cytokine Measurement in Transgenic Mice

To evaluate the effects of iso-α-acids on Alzheimer's-like disease, 6-month-old transgenic 5 × FAD and WT mice were orally administered daily 1 mg/kg iso-α-acids for 7 days. The mice were subjected to a novel object recognition test after 7 days of administration, and their brains were then removed for quantification of Aβ and cytokines. The hippocampus and cerebral cortex were homogenized in TBS buffer (Wako) with a multibeads shocker (Yasui Kikai, Osaka, Japan). After centrifugation at 50,000 × *g* for 20 min, the supernatant was collected. Pellets were re-homogenized in TBS containing 1% Triton X-100 (Wako) and the supernatant was collected after centrifugation. The total protein concentration of the supernatant was measured with the BCA protein assay kit (ThermoScientific, Yokohama, Japan). The first supernatant was assayed for quantification of soluble Aβ1-42 (Wako) by enzyme-linked immunosorbent assay (ELISA). To quantify cytokines and chemokines, supernatants were evaluated by the Bio-Plex assay system (Bio-Rad). The second supernatant was used for quantification of insoluble Aβ1-42 (Wako) by ELISA.

Spontaneous Alternation Test

The spontaneous alternation test was performed as previously described (Ano et al., 2018). The test used a three-arm Y-maze with equal angles between all arms (25 cm long × 5 cm wide × 20 cm high) and with walls constructed from dark black polyvinyl plastic. Each mouse was initially placed in one arm, and the sequence and number of arm entries were counted for 8 min. The alternation score (%) for each mouse was defined as the ratio of the actual number of alternations to the possible number (defined as the total number of arm entries minus two) multiplied by 100, i.e., % Alternation = [(Number of alternations)/(Total arm entries−2)] × 100.

Novel Object Recognition Test

An object recognition test was performed during the light period in a polyvinyl chloride box (25 cm × 40 cm × 20 cm) without a roof, in accordance with previous work (Ayabe et al., 2018). For the acquisition trial, a pair of wooden triangle poles (4.5 cm × 4.5 cm × 4.5 cm) or wooden pyramids (4.5 cm × 4.5 cm × 4.5 cm) was used; for the retention trial, a pair of poles or pyramids and a golf ball (4.5 cm diameter) were used. In all trials, the objects were placed 7.5 cm from the corner of the box. In the acquisition trial, 1 h after oral administration of the test sample, the mouse was allowed to explore the box containing the two objects for 10 min. Twenty-four hour later, 1 h after administration of the test sample, the mouse was allowed to explore the box containing the novel and familiar objects for 5 min. The discrimination index (DI) was calculated by dividing the difference in time exploring the novel object and familiar object by the total time spent exploring both objects, i.e., (novel object exploration time − familiar object exploration time)/(total exploration time); thus, a DI of 0 indicated equal exploration of both objects.

Manganese-Enhanced MRI

Manganese-enhanced magnetic resonance imaging was performed in accordance with our previous report (Yoshikawa et al., 2018). Mice were administered with $MnCl_2$ (20 mg/kg i.p.) and then returned to their home cages after 30 min, they were exposed to a novel environment (clear Perspex cylinder, 30-cm diameter) for 2 h (cylinder moved every 30 min to prevent habituation) and they were video-recorded. The mice were then returned to their home cage for 90 min before MRI scanning.

Anesthesia was induced with 3.0% isoflurane/air and maintained with 0.5–1.5% isoflurane/air; and deep core body temperature and heart rate were monitored (SA Instruments, Inc., United States) throughout the procedure. Scanning was performed 4 h after $MnCl_2$ injection using a 4.7T AVANCE III PharmaScan (Bruker BioSpin, Germany). Radio frequency transmission and reception were applied with a 23 mm inner diameter birdcage volume coil. Images were acquired with 3D Fast Imaging using a Steady-State Free Precession (FISP) sequence [repetition time (TR) = 8 ms, echo time (TE) = 4 ms, flip angle = 20°, number of acquisition = 7, matrix = 160 × 160 × 160, field of view (FOV) = 20 mm × 20 mm × 20 mm, and voxel size = 0.125 mm × 0.125 mm × 0.125 mm]. The total acquisition time was 31 min. MRI data were analyzed as described previously (Kimura et al., 2007), with the aid of a custom-developed MATLAB function (2012a, MathWorks). Brain slices were aligned with reference to the bregma. MR images were realigned and registered non-rigidly to the mouse brain template constructed by aligning and averaging 10 subject images. All voxel data were smoothed using a 3D Gaussian filter (MATLAB Image Processing Toolbox, version 5.02, MathWorks). Image intensities were normalized to the mean signal in the whole brain of each individual mouse. MR images were visualized with Osirix (version 5.0.2), an open-source software for navigating multidimensional DICOM images.

Statistical Analysis

The data represent the mean and the error bars indicate the SEM. Data were analyzed by Student's *t*-test and one-way analysis of variance (ANOVA) followed by Dunnett's test or the Tukey–Kramer test were performed, as indicated in the Figure legends. All statistical analyses were performed using the Ekuseru-Toukei (2012) software program (Social Survey Research Information, Tokyo, Japan). A p-value <0.05 was considered statistically significant.

RESULTS

Effects of Iso-α-Acids on Microglial Cytokine and Chemokine Productions

To evaluate the effects of iso-α-acids on primary microglia culture, cytokines and chemokines in the supernatant of microglial culture treated with LPS were quantified. The concentrations of IL-1β, TNF-α, IL-6, IL-12p40, MIP-1α, and MCP-1 in supernatant were significantly increased after LPS treatment. The concentrations were, however, significantly reduced by iso-α-acids addition concentration-dependently (**Figures 1A–F**, respectively). These results suggest that iso-α-acids suppress inflammatory cytokines and chemokines.

Effects of Iso-α-Acids on Cytokine Productions and Memory Impairment in Mice Treated With LPS

To evaluate the effects of iso-α-acids on cytokine productions and memory impairment in LPS-injected mice, mice inoculated with LPS were subjected to the Y-maze test. LPS treatment significantly reduced spontaneous alternation, whereas oral administration of iso-α-acids significantly improved the reduction (**Figure 2A**). There were no significant changes in arm entries among each group (**Figure 2B**). Next, we measured the levels of cytokines in the hippocampus. Concentrations of TNF-α, IL-12p40, and MIP-1α in the hippocampus of LPS-injected mice were significantly increased compared to those in untreated mice. The concentrations of TNF-α, IL-12p40 and MIP-1α significantly decreased in mice orally administered with iso-α-acids (**Figures 2D,F,G**). However, the concentrations of IL-1β, IL-6 and MCP-1 were unchanged by LPS injection (**Figures 2C,E,H**, respectively). These results showed that oral administration of iso-α-acids improved memory impairment and suppressed cytokine productions in LPS-injected mice.

Effects of Iso-α-Acids on Inflammation and Memory Impairment in 5 × FAD Mice

To evaluate the effects of iso-α-acids on inflammation and cognitive impairment after disease onset, 5 × FAD mice were administered with 1 mg/kg iso-α-acids for 7 days. In 5 × FAD mice, long-term intake of iso-α-acids for 2.5 months resulted in the reduction of Aβ deposition and in inflammation in the brain (Ano et al., 2017). The amounts of MIP-1α and IL-12p40 in 5 × FAD mice administered with iso-α-acids was significantly lower than those in control 5 × FAD mice, and was significantly increased in control 5 × FAD mice relative to WT mice (**Figures 3B,C**). The amount of TNF-α was also increased in control 5 × FAD mice compared to that in WT mice, while the increase was not observed in 5 × FAD mice treated with iso-α-acids (**Figure 3A**). The amount of TBS-soluble Aβ in the hippocampus of 5 × FAD mice treated with iso-α-acids was significantly reduced compared to that in control 5 × FAD mice (**Figure 3D**). The amounts of TBS insoluble and TBS-T soluble Aβ in the hippocampus of 5 × FAD mice treated with iso-α-acids were unchanged (**Figure 3E**). 5 × FAD mice were also subjected to a novel object recognition test. The mice treated with iso-α-acids explored a novel object for a longer time than the control 5 × FAD mice. The amounts of time spent approaching the novel object and DI of 5 × FAD mice treated with iso-α-acids were significantly higher than that of control 5 × FAD mice (**Figures 3F,G**). These results suggest that short-term treatments with iso-α-acids suppress the levels of inflammatory cytokines and soluble Aβ in the hippocampus, which lead to improve memory impairment even after the onset of Alzheimer's pathology.

Effects of Iso-α-Acids on Inflammation and Neural Activity in J20 Mice

To evaluate the effects of a short-term treatments of iso-α-acids, 12-month-old J20 mice were administered 1 mg/kg iso-α-acids for 7 days and then subjected to MEMRI. The level of MIP-1α in the brain of the mice treated with iso-α-acids was significantly lower than that in control mice, and the level of which was significantly increased compared to that of WT mice (**Figure 4A**). The level of TBS soluble Aβ in the hippocampus of J20 mice treated with iso-α-acids was significantly reduced compared to control J20 mice (**Figure 4B**).

To evaluate the neural activity, mice were injected with $MnCl_2$, allowed to explore a novel space, and then subjected to MRI to detect manganese. Manganese was distributed in the hippocampus and cerebral cortex (**Figure 4C**). The deposition of manganese in some areas of the hippocampus, especially the CA1and CA3 region, of control J20 mice was significantly increased compared to that of WT mice, whereas the increase of deposition was attenuated by iso-α-acids treatment (**Figure 4D**). The normalized intensity of manganese in the whole hippocampus of control J20 mice was significantly increased compared to that of WT mice, whereas an increase in J20 was returned to the level of WT mice by the treatment of iso-α-acids (**Figure 4E**). These results suggest that iso-α-acids suppress inflammation and improve the neural activity in the hippocampus.

DISCUSSION

The present study demonstrated that short-term treatment of iso-α-acids as 7 days suppressed inflammation in the hippocampus and improved memory impairment in Alzheimer's disease model mice, even after disease onset. Notably, short-term administration of iso-α-acids attenuate the neural

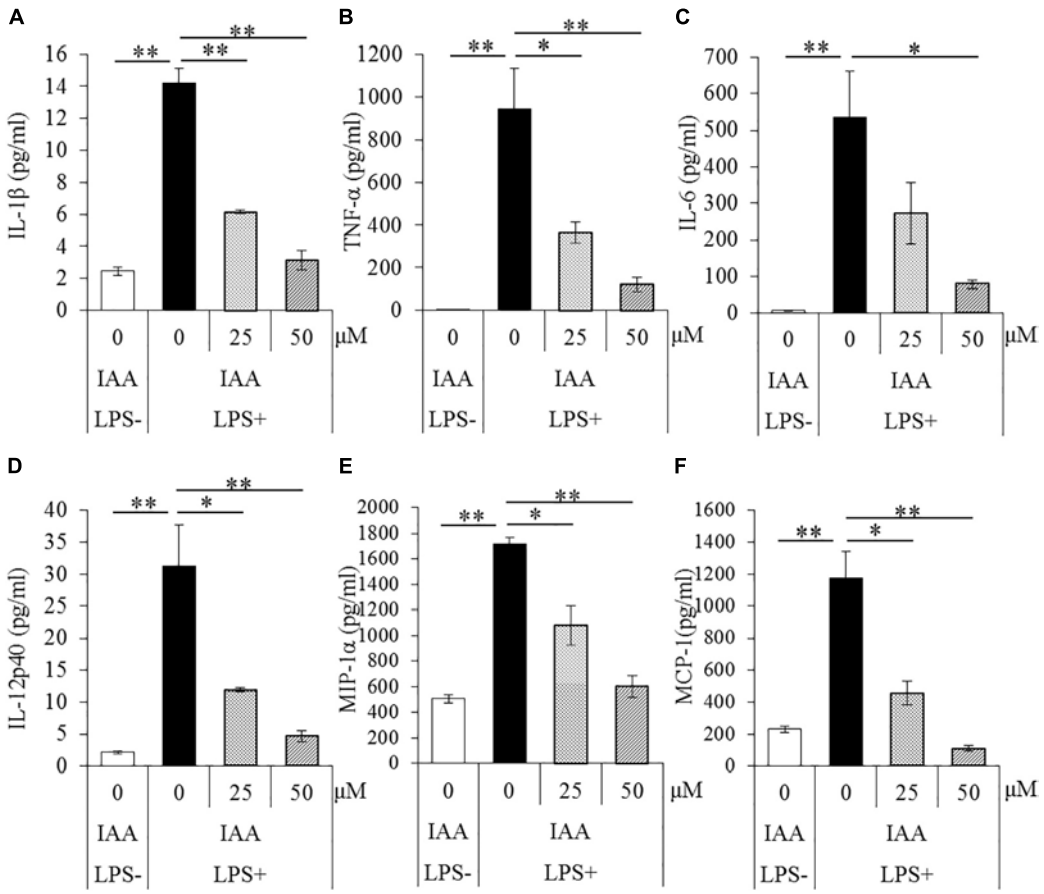

FIGURE 1 | Effects of iso-α-acids on microglial cytokine and chemokine productions. **(A–F)** The amounts of IL-1β, TNF-α, IL-6, IL-12p40, MIP-1α, and MCP-1 in the supernatant of microglial culture pretreated with 0, 25, and 50 μM iso-α-acids and treated with 5 ng/ml LPS and 0.5 ng/ml IFN-γ. IAA: iso-α-acids. Mean ± SE of three wells per sample. The *p*-values shown in the graph were calculated by Student's *t*-test (LPS [–] vs [+] at 0 μM) and one-way ANOVA followed by Dunnett's test. $^{*}p < 0.05$ and $^{**}p < 0.01$.

hyperactivation in hippocampus in Alzheimer's disease model mice detected by MEMRI. Iso-α-acids treatment suppressed the cytokine and chemokine levels in the hippocampus and improved memory impairment induced by LPS injection. The spontaneous alternation test used in this study evaluates spatial working memory, which is a hippocampus-dependent memory function (Lalonde, 2002). We previously reported that iso-α-acids activate the PPAR-γ (Yajima et al., 2004) and that the PPAR-γ activation is involved in the suppression of microglial inflammation (Ano et al., 2017). It has also been reported that PPAR-γ activation changes microglia phenotype to the M2 anti-inflammatory type (Pan et al., 2015; Wen et al., 2018). Pioglitazone, which is a PPAR-γ agonist, also alters microglia to the M2 type *in vivo* (Mandrekar-Colucci et al., 2012). These results suggest that iso-α-acids suppressed the inflammatory response via activation of PPAR-γ and suppression of microglial inflammation in the hippocampus by iso-α-acids may contribute to the prevention of memory impairment. On the other hand, the phenotype of inflammation induced by LPS, which is toll-like receptor agonist, is different from that in the brain of Alzheimer's disease.

Inflammation in the brain has attracted growing attention to a preventive and therapeutic strategy for Alzheimer's disease (Fernandez et al., 2013). Epidemiological investigation has suggested that an intake of non-steroidal anti-inflammatory drugs (NSAIDs) has a preventive effect against Alzheimer's disease (Breitner et al., 1995; Stewart et al., 1997), suggesting the therapeutic potential of pioglitazone. Microglia are known to play a crucial role in inflammation in the brain, and generally remove old synapses and waste products in the brain to maintain the environment (Kettenmann et al., 2013), yet massively activated microglia produce neurotoxic substances, including reactive oxygen species and inflammatory cytokines (Lull and Block, 2010). It has been suggested that the polarization of microglia between the M1 inflammatory and M2 anti-inflammatory types is important for improving neurological pathology and cognitive decline in Alzheimer's disease (Sarlus and Heneka, 2017).

The current study is novel, as we evaluated the effects of iso-α-acids on brain inflammation in Alzheimer's disease after its onset. It was previously demonstrated that a long-term intake of iso-α-acids has a preventive effect against Alzheimer's

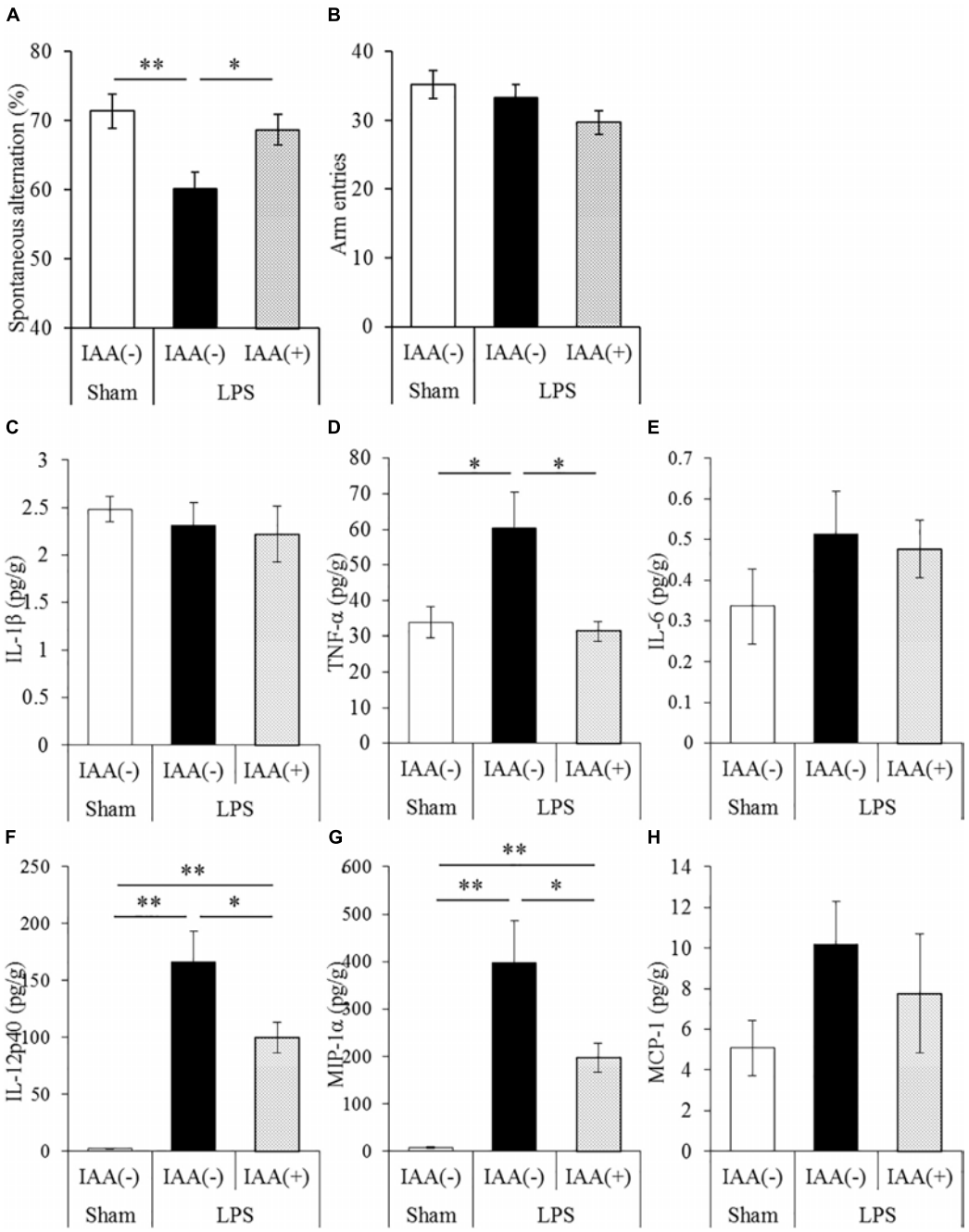

FIGURE 2 | Effects of iso-α-acids on memory impairment induced by LPS. **(A,B)** CD-1 (ICR) mice were orally administered with 0 or 1 mg/kg of iso-α-acids for 3 days and intracerebroventricularly injected with PBS or 0.25 mg/kg LPS 1 h after the last iso-α-acids administration. Mice were subjected to the spontaneous alternation test using the Y-maze 1 day after LPS injection. Spontaneous alternations **(A)** and arm entries **(B)** in the Y-maze test were recorded to evaluate spatial memory. **(C–H)** Concentrations of IL-1β, TNF-α, IL-6, IL-12p40, MIP-1α, and MCP-1 in the hippocampus were measured 24 h after LPS injection, respectively. Data are represented as mean ± SE of 10 mice per group. *p*-Values shown in the graph were calculated by one-way ANOVA followed by the Tukey–Kramer test. $^{*}p < 0.05$ and $^{**}p < 0.01$.

pathology in 5 × FAD mice by suppressing inflammation and Aβ deposition in the brain (Ano et al., 2017). In the study, iso-α-acids were fed as a component (0.05% w/w) of the daily diet, so the daily treatment amount was not strictly controlled. In the present study, we evaluated the therapeutic effects of iso-α-acids on 6-month-old 5 × FAD mice displaying Aβ deposition, inflammation, and cognitive impairment. Treatment with iso-α-acids at 1 mg/kg for 7 days suppressed inflammation in the hippocampus and improved hippocampus-dependent object recognition memory (Cohen and Stackman, 2015). Iso-α-acids

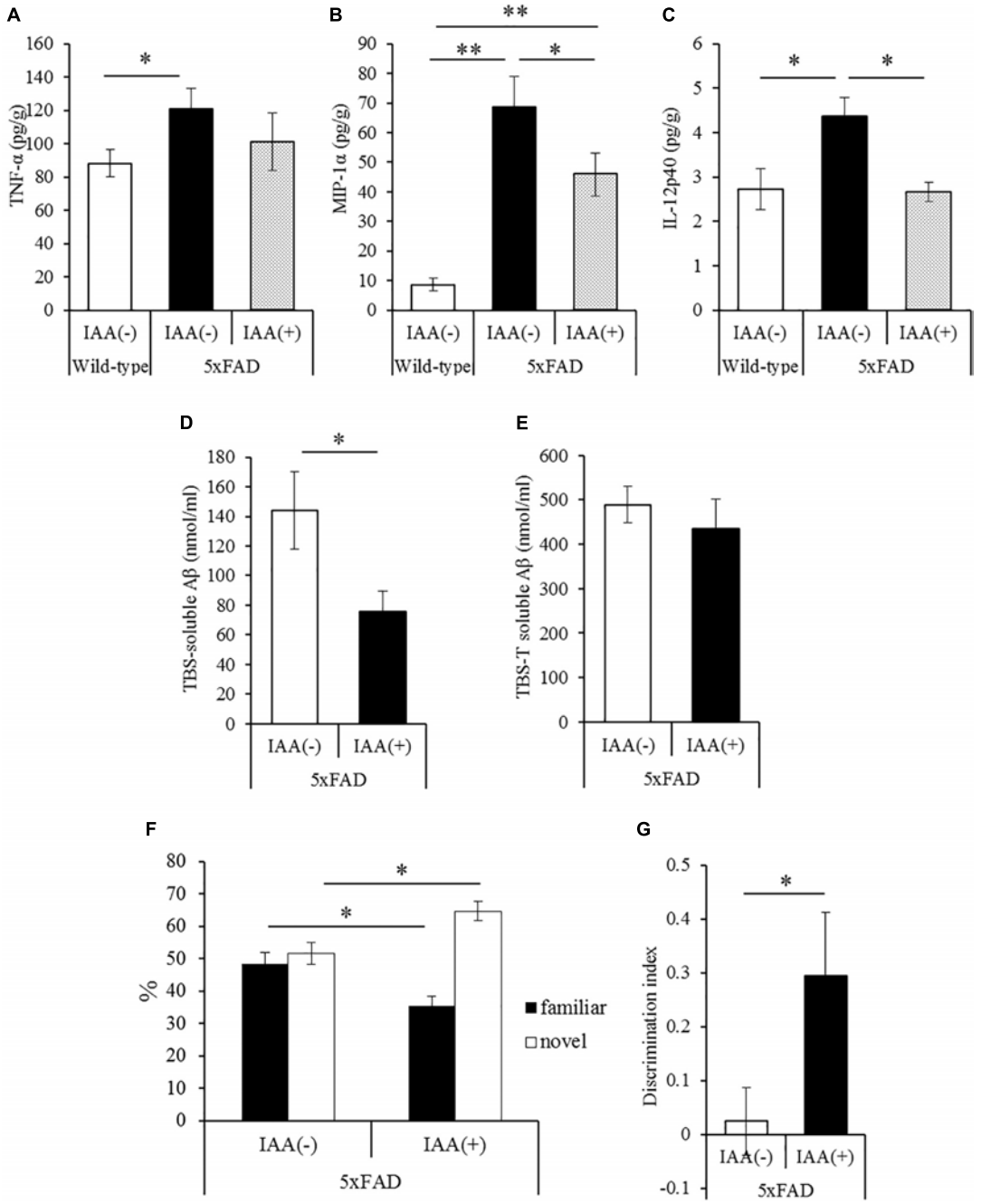

FIGURE 3 | Effects of iso-α-acids on 5 × FAD Alzheimer's disease model mice after disease onset. **(A,C)**, 5 × FAD mice were orally administered with 0 or 1 mg/kg iso-α-acids for 7 days, and the levels of TNF-α **(A)**, MIP-1α **(B)** and IL-12p40 **(C)** in the hippocampus was measured, respectively. **(D,E)** The levels of TBS soluble **(D)** or TBS insoluble and TBS-T soluble **(E)** $A\beta_{1-42}$ in the hippocampus, respectively. **(F,G)** On days 6 and 7 of administration, 5 × FAD mice were subjected to the novel object recognition test. The time spent exploring novel and familiar objects during 5 min of re-exploration was measured **(F)**. The discrimination index = [time spent with object A – time spent with object B]/[total time exploring both objects] is shown **(G)**. Data are mean ± SE of 10 mice (wild type mice) and 8 mice (5 × FAD mice per group). *p*-Values were calculated by Student's *t*-test **(D–G)** and one-way ANOVA followed by the Tukey–Kramer test **(A–C)**. $^{*}p < 0.05$ and $^{**}p < 0.01$.

treatment also reduced the level of soluble $A\beta_{1-42}$ in the hippocampus, which is known to be elevated with inflammation (Lee et al., 2008). Hippocampus-dependent memory is known to decline with inflammation in the hippocampus (Lee et al., 2008), and it has been reported that PPAR-γ activation improves object recognition test performance in 5 × FAD mice (Escribano et al., 2009). These results suggest that suppression of inflammation in the hippocampus by iso-α-acids may contribute to the improvement in memory impairment in Alzheimer's disease model mice.

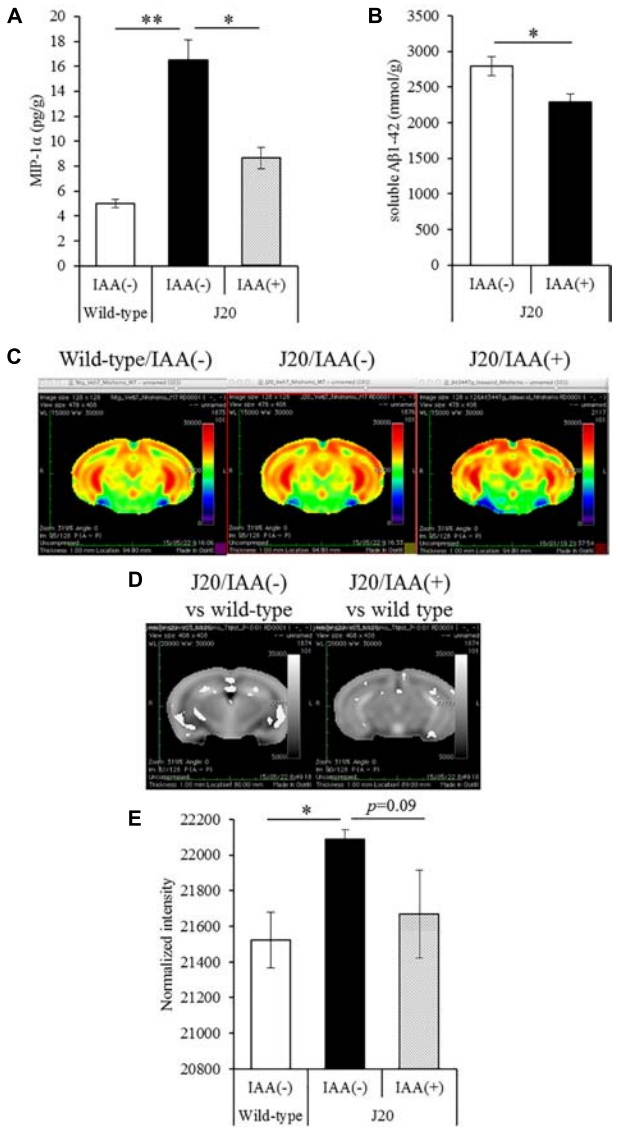

FIGURE 4 | Effects of iso-α-acids on J20 Alzheimer's disease model mice. **(A,B)** J20 mice were orally administered with 0 or 1 mg/kg iso-α-acids for 7 days. The levels of MIP-1α **(A)** and TBS soluble $A\beta_{1-42}$ in the hippocampus was measured, respectively. **(C)** Coronal brain sections from wild type mice (left), J20 mice without iso-α-acids (middle), and J20 mice treated with iso-α-acids (right), all of which were 12 months old. Relative MRI signal intensities, after normalization to the mean signal intensity in the whole brain, are depicted (see color spectrum scale bar). **(D)** Coronal brain sections showing the differences of signal intensities J20 mice between wild type mice and J20 mice without iso-α-acids (left), and between wild type mice and J20 mice with iso-α-acids (right). **(E)** Normal intensities of hippocampus data are mean ± SE of seven mice (wild type mice), eight mice [IAA(–) J20 mice], and five mice [IAA(+) J20 mice]. *p*-Values shown in the graph were calculated by Student's *t*-test **(B)** and one-way ANOVA followed by the Tukey–Kramer test **(A,E)**. $^{*}p < 0.05$ and $^{**}p < 0.01$.

At last, to evaluate the effects of iso-α-acids on neural activity, J20 Alzheimer's disease model mice treated with iso-α-acids at 1 mg/kg for 7 days were subjected to MEMRI analysis after exploration of a novel environment. Our group previously reported that 12-month-old J20 mice displayed hyperactivity detected by MEMRI in hippocampal neurons induced by exploration of a novel environment (Yoshikawa et al., 2018). J20 mice and 5 × FAD mice express human APP with mutations. J20 mice overexpresses human APP with two mutations linked to FAD (the Swedish and Indiana mutations) (Wright et al., 2013), on the other hand, 5 × FAD mice overexpress APP with mutations of Swedish K670N/M671L, Florida I716V and London V717I and PSEN1 with mutation of M146L and L286V (Oakley et al., 2006). 5 × FAD display more severe amyloid pathology including amyloid deposition and inflammation. So in the present study, we evaluate the 6-month-old 5 × FAD and 12-month-old J20 mice. Treatment with iso-α-acids reduced inflammation and soluble $A\beta_{1-42}$ in the hippocampi of J20 mice, which is consistent with the results of the experiments using 5 × FAD mice. Manganese-detected neural activation in the hippocampi of J20 mice was significantly higher than that in age-matched WT mice, whereas the increase was not observed in the hippocampi—especially the CA1 and CA3 region—of J20 mice treated with iso-α-acids. It is reported that hyperactivity in the hippocampal region in individuals with Mild Cognitive Impairment (MCI) is observed by functional MRI (Yassa et al., 2010). It is also reported that hyperactivity in hippocampus lead to cognitive impairment and improvement of the hyperactivity show therapeutic effects on memory impairment (Bakker et al., 2012). It has been also reported that oligomeric Aβ induces neural hyperactivity in hippocampus of Alzheimer's disease model mice (Busche et al., 2012). In hAPP transgenic mice, Aβ-induced dysfunction of inhibitory interneurons induces aberrant synchrony in neural networks (Palop and Mucke, 2010; Busche et al., 2012). Taken together, it is suggested that suppression of inflammation and the level of soluble Aβ by iso-α-acids administration in Alzheimer's disease model mice might contribute to the improvement of neural activity in the hippocampus, and resulting memory improvement, especially for the individuals with MCI. On the other hand, the relationship between suppression of hippocampal inflammation and attenuation of neural hyperactivity by iso-α-acids should be investigated in the further study.

Because iso-α-acids are generated from α-acids in hops, which has been used in brewing for more than 1,000 years, they are considered safe for consumption. The amount of iso-α-acids used in the present study was 1 mg/kg, whereas beer generally contains iso-α-acids at 20–50 μg/ml; thus, the amount of iso-α-acids in approximately 1 l of beer might be equivalent to an effective dosage in humans. However, as beer also contains alcohol, it is difficult to calculate the reduced volume. Given the mounting evidence that iso-α-acids may be beneficial for cognition, a clinical trial is needed to further assess their effects in human populations.

AUTHOR CONTRIBUTIONS

YA conducted most of biochemical analysis and wrote most of the paper. MY, MM, and AT conducted the experiment using MRI

using transgenic mice and analyzed the data, and AT also wrote the manuscript. YT, KU, and HN conducted the experiment using 5 × FAD model mice, and HN also wrote the manuscript.

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REFERENCES

Ano, Y., Ayabe, T., Kutsukake, T., Ohya, R., Takaichi, Y., Uchida, S., et al. (2018). Novel lactopeptides in fermented dairy products improve memory function and cognitive decline. *Neurobiol. Aging* 72, 23–31. doi: 10.1016/j.neurobiolaging.2018.07.016

Ano, Y., Dohata, A., Taniguchi, Y., Hoshi, A., Uchida, K., Takashima, A., et al. (2017). Iso-alpha-acids, bitter components of beer, prevent inflammation and cognitive decline induced in a mouse model of alzheimer's disease. *J. Biol. Chem.* 292, 3720–3728. doi: 10.1074/jbc.M116.763813

Ano, Y., Ozawa, M., Kutsukake, T., Sugiyama, S., Uchida, K., Yoshida, A., et al. (2015). Preventive effects of a fermented dairy product against Alzheimer's disease and identification of a novel oleamide with enhanced microglial phagocytosis and anti-inflammatory activity. *PLoS One* 10:e0118512. doi: 10.1371/journal.pone.0118512

Arntzen, K. A., Schirmer, H., Wilsgaard, T., and Mathiesen, E. B. (2010). Moderate wine consumption is associated with better cognitive test results: a 7 year follow up of 5033 subjects in the tromso study. *Acta Neurol. Scand. Suppl.* 122, 23–29. doi: 10.1111/j.1600-0404.2010.01371.x

Ayabe, T., Ohya, R., Kondo, K., and Ano, Y. (2018). Iso-alpha-acids, bitter components of beer, prevent obesity-induced cognitive decline. *Sci. Rep.* 8:4760. doi: 10.1038/s41598-018-23213-9

Bakker, A., Krauss, G. L., Albert, M. S., Speck, C. L., Jones, L. R., Stark, C. E., et al. (2012). Reduction of hippocampal hyperactivity improves cognition in amnestic mild cognitive impairment. *Neuron* 74, 467–474. doi: 10.1016/j.neuron.2012.03.023

Breitner, J. C., Welsh, K. A., Helms, M. J., Gaskell, P. C., Gau, B. A., Roses, A. D., et al. (1995). Delayed onset of Alzheimer's disease with nonsteroidal anti-inflammatory and histamine H2 blocking drugs. *Neurobiol. Aging* 16, 523–530.

Busche, M. A., Chen, X., Henning, H. A., Reichwald, J., Staufenbiel, M., Sakmann, B., et al. (2012). Critical role of soluble amyloid-beta for early hippocampal hyperactivity in a mouse model of Alzheimer's disease. *Proc. Natl. Acad. Sci. U.S.A.* 109, 8740–8745. doi: 10.1073/pnas.1206171109

Cohen, S. J., and Stackman, R. W. Jr. (2015). Assessing rodent hippocampal involvement in the novel object recognition task. A review. *Behav. Brain Res.* 285, 105–117. doi: 10.1016/j.bbr.2014.08.002

Escribano, L., Simon, A. M., Perez-Mediavilla, A., Salazar-Colocho, P., Del Rio, J., and Frechilla, D. (2009). Rosiglitazone reverses memory decline and hippocampal glucocorticoid receptor down-regulation in an Alzheimer's disease mouse model. *Biochem. Biophys. Res. Commun.* 379, 406–410. doi: 10.1016/j.bbrc.2008.12.071

Fernandez, P. L., Britton, G. B., and Rao, K. S. (2013). Potential immunotargets for Alzheimer's disease treatment strategies. *J. Alzheimers Dis.* 33, 297–312. doi: 10.3233/jad-2012-121222

Horvat, P., Richards, M., Kubinova, R., Pajak, A., Malyutina, S., Shishkin, S., et al. (2015). Alcohol consumption, drinking patterns, and cognitive function in older Eastern European adults. *Neurology* 84, 287–295. doi: 10.1212/wnl.0000000000001164

Kettenmann, H., Kirchhoff, F., and Verkhratsky, A. (2013). Microglia: new roles for the synaptic stripper. *Neuron* 77, 10–18. doi: 10.1016/j.neuron.2012.12.023

Kimura, T., Yamashita, S., Fukuda, T., Park, J. M., Murayama, M., Mizoroki, T., et al. (2007). Hyperphosphorylated tau in parahippocampal cortex impairs place learning in aged mice expressing wild-type human tau. *EMBO J.* 26, 5143–5152. doi: 10.1038/sj.emboj.7601917

Lalonde, R. (2002). The neurobiological basis of spontaneous alternation. *Neurosci. Biobehav. Rev.* 26, 91–104.

Lee, J. W., Lee, Y. K., Yuk, D. Y., Choi, D. Y., Ban, S. B., Oh, K. W., et al. (2008). Neuro-inflammation induced by lipopolysaccharide causes cognitive impairment through enhancement of beta-amyloid generation. *J. Neuroinflamm.* 5:37. doi: 10.1186/1742-2094-5-37

Lull, M. E., and Block, M. L. (2010). Microglial activation and chronic neurodegeneration. *Neurotherapeutics* 7, 354–365. doi: 10.1016/j.nurt.2010.05.014

Mandrekar-Colucci, S., Karlo, J. C., and Landreth, G. E. (2012). Mechanisms underlying the rapid peroxisome proliferator-activated receptor-gamma-mediated amyloid clearance and reversal of cognitive deficits in a murine model of Alzheimer's disease. *J. Neurosci.* 32, 10117–10128. doi: 10.1523/jneurosci.5268-11.2012

Matsui, T., Yoshimura, A., Toyama, T., Matsushita, S., and Higuchi, S. (2011). Preventive effect of moderation in drinking on dementia. *Nihon Rinsho* 69(Suppl. 10, Pt 2), 217–222.

Mucke, L., Masliah, E., Yu, G. Q., Mallory, M., Rockenstein, E. M., Tatsuno, G., et al. (2000). High-level neuronal expression of abeta 1-42 in wild-type human amyloid protein precursor transgenic mice: synaptotoxicity without plaque formation. *J. Neurosci.* 20, 4050–4058.

Neafsey, E. J., and Collins, M. A. (2011). Moderate alcohol consumption and cognitive risk. *Neuropsychiatr. Dis. Treat.* 7, 465–484. doi: 10.2147/ndt.S23159

Oakley, H., Cole, S. L., Logan, S., Maus, E., Shao, P., Craft, J., et al. (2006). Intraneuronal beta-amyloid aggregates, neurodegeneration, and neuron loss in transgenic mice with five familial Alzheimer's disease mutations: potential factors in amyloid plaque formation. *J. Neurosci.* 26, 10129–10140. doi: 10.1523/jneurosci.1202-06.2006

Obara, K., Mizutani, M., Hitomi, Y., Yajima, H., and Kondo, K. (2009). Isohumulones, the bitter component of beer, improve hyperglycemia and decrease body fat in Japanese subjects with prediabetes. *Clin. Nutr.* 28, 278–284. doi: 10.1016/j.clnu.2009.03.012

Palop, J. J., and Mucke, L. (2010). Amyloid-beta-induced neuronal dysfunction in Alzheimer's disease: from synapses toward neural networks. *Nat. Neurosci.* 13, 812–818. doi: 10.1038/nn.2583

Pan, J., Jin, J. L., Ge, H. M., Yin, K. L., Chen, X., Han, L. J., et al. (2015). Malibatol A regulates microglia M1/M2 polarization in experimental stroke in a PPARgamma-dependent manner. *J. Neuroinflamm.* 12:51. doi: 10.1186/s12974-015-0270-3

Porquet, D., Grinan-Ferre, C., Ferrer, I., Camins, A., Sanfeliu, C., Del Valle, J., et al. (2014). Neuroprotective role of trans-resveratrol in a murine model of familial Alzheimer's disease. *J. Alzheimers Dis.* 42, 1209–1220. doi: 10.3233/jad-140444

Sarlus, H., and Heneka, M. T. (2017). Microglia in Alzheimer's disease. *J. Clin. Invest.* 127, 3240–3249. doi: 10.1172/jci90606

Stewart, W. F., Kawas, C., Corrada, M., and Metter, E. J. (1997). Risk of Alzheimer's disease and duration of NSAID use. *Neurology* 48, 626–632.

Taniguchi, Y., Matsukura, Y., Ozaki, H., Nishimura, K., and Shindo, K. (2013). Identification and quantification of the oxidation products derived from alpha-acids and beta-acids during storage of hops (*Humulus lupulus* L.). *J. Agric. Food Chem.* 61, 3121–3130. doi: 10.1021/jf3047187

Vidavalur, R., Otani, H., Singal, P. K., and Maulik, N. (2006). Significance of wine and resveratrol in cardiovascular disease: French paradox revisited. *Exp. Clin. Cardiol.* 11, 217–225.

Wen, L., You, W., Wang, H., Meng, Y., Feng, J., and Yang, X. (2018). Polarization of microglia to the M2 phenotype in a peroxisome proliferator-activated receptor gamma-dependent manner attenuates axonal injury induced by traumatic brain injury in mice. *J. Neurotrauma.* 35, 2330–2340. doi: 10.1089/neu.2017.5540

Witte, A. V., Kerti, L., Margulies, D. S., and Floel, A. (2014). Effects of resveratrol on memory performance, hippocampal functional connectivity, and glucose metabolism in healthy older adults. *J. Neurosci.* 34, 7862–7870. doi: 10.1523/jneurosci.0385-14.2014

Wright, A. L., Zinn, R., Hohensinn, B., Konen, L. M., Beynon, S. B., Tan, R. P., et al. (2013). Neuroinflammation and neuronal loss precede Abeta plaque deposition in the hAPP-J20 mouse model of Alzheimer's disease. *PLoS One* 8:e59586. doi: 10.1371/journal.pone.0059586

Yajima, H., Ikeshima, E., Shiraki, M., Kanaya, T., Fujiwara, D., Odai, H., et al. (2004). Isohumulones, bitter acids derived from hops, activate both peroxisome proliferator-activated receptor alpha and gamma and reduce insulin resistance. *J. Biol. Chem.* 279, 33456–33462. doi: 10.1074/jbc.M403456200

Yajima, H., Noguchi, T., Ikeshima, E., Shiraki, M., Kanaya, T., Tsuboyama-Kasaoka, N., et al. (2005). Prevention of diet-induced obesity by dietary isomerized hop extract containing isohumulones, in rodents. *Int. J. Obes.* 29, 991–997. doi: 10.1038/sj.ijo.0802965

Yassa, M. A., Stark, S. M., Bakker, A., Albert, M. S., Gallagher, M., and Stark, C. E. (2010). High-resolution structural and functional MRI of hippocampal CA3 and dentate gyrus in patients with amnestic mild cognitive impairment. *Neuroimage* 51, 1242–1252. doi: 10.1016/j.neuroimage.2010.03.040

Yoshikawa, M., Soeda, Y., Michikawa, M., Almeida, O. F. X., and Takashima, A. (2018). Tau depletion in APP transgenic mice attenuates task-related hyperactivation of the hippocampus and differentially influences locomotor activity and spatial memory. *Front. Neurosci.* 12:124. doi: 10.3389/fnins.2018.00124

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Bicuculline Reduces Neuroinflammation in Hippocampus and Improves Spatial Learning and Anxiety in Hyperammonemic Rats. Role of Glutamate Receptors

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Patients with liver cirrhosis may develop minimal hepatic encephalopathy (MHE) with mild cognitive impairment. Hyperammonemia is a main contributor to cognitive impairment in MHE, which is mediated by neuroinflammation. GABAergic neurotransmission is altered in hyperammonemic rats. We hypothesized that, in hyperammonemic rats, (a) enhanced GABAergic tone would contribute to induce neuroinflammation, which would be improved by reducing GABAergic tone by chronic bicuculline treatment; (b) this would improve spatial learning and memory impairment; and (c) modulation of glutamatergic neurotransmission would mediate this cognitive improvement. The aim of this work was to assess the above hypotheses. Bicuculline was administrated intraperitoneally once a day for 4 weeks to control and hyperammonemic rats. The effects of bicuculline on microglia and astrocyte activation, IL-1β content, on membrane expression of AMPA and NMDA glutamate receptors subunits in the hippocampus and on spatial learning and memory as well as anxiety were assessed. Treatment with bicuculline reduces astrocyte activation and IL-1β but not microglia activation in the hippocampus of hyperammonemic rats. Bicuculline reverses the changes in membrane expression of AMPA receptor subunits GluA1 and GluA2 and of the NR2B (but not NR1 and NR2A) subunit of NMDA receptors. Bicuculline improves spatial learning and working memory and decreases anxiety in hyperammonemic rats. In hyperammonemia, enhanced activation of $GABA_A$ receptors in the hippocampus contributes to some but not all aspects of neuroinflammation, to altered glutamatergic neurotransmission and to impairment of spatial learning and memory as well as anxiety, all of which are reversed by reducing activation of $GABA_A$ receptors with bicuculline.

Keywords: hepatic encephalopathy, $GABA_A$ receptor, hippocampus, spatial learning and memory, anxiety, glutamate receptors, astrocytes activation, IL-1β

Abbreviations: AMPA, alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; BS3, *Bis*(sulfosuccinimidyl) suberate; cGMP, cyclic guanosine monophosphate; CNS, central nervous system; GABA, gamma-aminobutyric acid; GFAP, glial fibrillary acidic protein; GluA, AMPA receptor subunit; IL, interleukin; IL-1R, interleikin-1 receptor; LPS, lipopolysaccharide; MHE, minimal hepatic encephalopathy; NMDA, *N*-methyl-D-aspartic acid; NR, NMDA receptor subunit; RM, reference memory; ROI, region of interest; TNF, tumor necrosis factor; WM, working memory.

INTRODUCTION

Patients with liver cirrhosis may develop covert or MHE with mild cognitive impairment, attention deficits and psychomotor slowing, which impair quality of life, reduce life span and increase accidents, falls and hospitalizations. MHE affects several million people and is a serious health, social and economic problem (Felipo, 2013).

Hyperammonemia and peripheral inflammation play synergistic roles in inducing the cognitive and motor alterations in MHE (Shawcross et al., 2004; Montoliu et al., 2009; Felipo et al., 2012). These alterations would be mediated by neuroinflammation (Montoliu et al., 2015).

Chronic hyperammonemia *per se* is enough to induce neuroinflammation with activation of microglia and increased inflammatory markers in the brain associated with impaired cognitive function (Rodrigo et al., 2010). Reducing neuroinflammation with ibuprofen restores learning in a Y maze task in hyperammonemic rats (Rodrigo et al., 2010). Hyperammonemic rats also show neuroinflammation in the hippocampus that is associated with altered membrane expression of glutamate receptors and impaired spatial learning and memory (Cabrera-Pastor et al., 2016). These alterations are reversed by treating the rats with sulforaphane, which reduces neuroinflammation in the hippocampus (Hernández-Rabaza et al., 2016).

GABAergic neurotransmission is altered in hyperammonemic rats, which show increased GABAergic tone in the cerebellum. Chronic treatment with bicuculline, a $GABA_A$ receptor antagonist, restores GABAergic tone, the function of the glutamate-nitric oxide-cGMP pathway in the cerebellum and learning of a discrimination task modulated by this pathway (Cauli et al., 2009). The same effects are induced by treatment with pregnenolone sulfate, a negative allosteric modulator of the $GABA_A$ receptor, which also improves motor incoordination caused by increased extracellular GABA in the cerebellum (González-Usano et al., 2013).

Reducing GABAergic tone by treating rats with GR3027, which antagonizes the enhancement of $GABA_A$ receptor activation by neurosteroids, also restores spatial memory modulated mainly in the hippocampus (Johansson et al., 2015).

These reports show that reducing either GABAergic tone or neuroinflammation in the hippocampus of hyperammonemic rats improve spatial learning. This suggests that there would be a cross-talk between GABAergic tone and neuroinflammation in the modulation of the mechanisms involved in spatial learning and maybe also in other functions modulated in the hippocampus such as short-term memory or anxiety.

Recent studies support this cross-talk between GABAergic neurotransmission and neuroinflammation, which seem to modulate each other (reviewed by Crowley et al., 2016). Different pro-inflammatory cytokines, such as TNFα, IL-1β and IL-6, modulate $GABA_A$ receptor function in an area- and dose-dependent manner (Stellwagen et al., 2005; García-Oscos et al., 2012; Pribiag and Stellwagen, 2013). IL-1β suppresses GABA-induced currents in the superficial spinal cord (Kawasaki et al., 2008) in hippocampal slices (Nisticò et al., 2013) and in rat hippocampal neurons (Wang et al., 2000). Contrarily, at a different concentration, IL-1β also increases membrane expression of $GABA_A$ receptor subunits and GABAergic neurotransmission in cultured rat hippocampal neurons (Serantes et al., 2006). Hellstrom et al. (2005) showed that LPS increases GABAergic inhibition in the hippocampus through IL-1β. Additionally, reactive astrocytes release GABA, increasing GABAergic tone in cerebral ischemia (Lin et al., 2018). GABA released from reactive astrocytes impairs learning and memory (Jo et al., 2014). During neuroinflammation, GABAergic tone would increase to reduce excitotoxicity caused by excessive glutamate neurotransmission (Crowley et al., 2016).

In rats with hyperammonemia or hepatic encephalopathy, reducing neuroinflammation reverses the increase of GABAergic tone in the cerebellum and restores impaired motor coordination, suggesting enhancement of GABA neurotransmission by neuroinflammation in the cerebellum in these rats (Rodrigo et al., 2010; Dadsetan et al., 2016a; Hernández-Rabaza et al., 2016; Agusti et al., 2017). These reports show that neuroinflammation modulates GABAergic neurotransmission in different systems, including the cerebellum of hyperammonemic rats.

Gamma-aminobutyric acid neurotransmission also modulates neuroinflammation. Both anti- and pro-inflammatory effects of GABA have been reported. GABA acts as anti-inflammatory in rheumatoid arthritis, downregulating mechanisms that lead to the production of pro-inflammatory agents such as IL-1β (Kelley et al., 2008) and also in neuroinflammation in general (Crowley et al., 2016). GABA acts as anti-inflammatory in microglia through activation of $GABA_A$ receptors (Lee et al., 2011).

Other studies suggest that GABA can induce pro-inflammatory cytokines in pathological conditions. Carmans et al. (2013) showed that exogenous GABA increases IL-6 and TNFα mRNA in the CNS. Sallam et al. (2016) reported that intra-cerebral administration of bicuculline inhibited the increase of IL-6 and TNFα induced by LPS in rats. Increased GABA levels and the subsequent activation of $GABA_A$ receptors induce activation of astrocytes (Runquist and Alonso, 2003).

The mechanisms by which neuroinflammation impairs spatial learning in hyperammonemic rats involve altered membrane expression of AMPA and NMDA receptor subunits. Treatment with sulforaphane reverses changes in membrane expression of the receptors and restores spatial learning (Hernández-Rabaza et al., 2016).

It has not been analyzed whether modulating GABAergic neurotransmission could reduce neuroinflammation in the hippocampus of hyperammonemic rats and restore membrane expression of glutamate receptors and cognitive functions modulated by this area.

We have proposed that there is an interplay between neuroinflammation and GABAergic-glutamatergic neurotransmission in the induction of cognitive and motor alterations in rats with MHE (Agusti et al., 2017). We propose now that a similar interplay in the hippocampus of hyperammonemic rats would induce alterations in spatial learning and is likely in other functions modulated in the hippocampus, such as anxiety or short-term memory.

We hypothesized that, in hyperammonemic rats, (a) enhanced GABAergic tone would contribute to induce neuroinflammation in hippocampus; (b) reducing GABAergic tone by chronic treatment with bicuculline would reduce neuroinflammation; (c) this would be associated with normalization of membrane expression of AMPA receptor subunits and restoration of spatial learning; and (d) bicuculline treatment could also improve other functions modulated in the hippocampus such as short-term-memory and anxiety.

To test these hypotheses, we assessed whether chronic intraperitoneal administration of the $GABA_A$ receptor antagonist bicuculline in hyperammonemic rats modulates neuroinflammation in the hippocampus by analyzing the activation of microglia, astrocytes, and IL-1β content. We also assessed the effects on membrane expression of AMPA and NMDA receptor subunits and on spatial learning and memory, short-term memory and anxiety.

MATERIALS AND METHODS

Study Design, Chronic Hyperammonemia in Rats, and Treatment With Bicuculline

Male Wistar rats (120–140 g, Charles River Laboratories, Barcelona, Spain) were made hyperammonemic by feeding them an ammonium-containing diet as previously described (Felipo et al., 1988). Animals were distributed into four groups: control with vehicle (CV); control treated with bicuculline (CB); hyperammonemic rats (HA); hyperammonemic rats treated with bicuculline (HB). Bicuculline [(+)-Bicuculline, Sigma-Aldrich] was injected intraperitoneally at 0.3 mg/kg once per day. The dose was chosen based on a previous study that shows that hyperammonemia changes GABAergic tone in the CNS (Cauli et al., 2009). This dose is lower than that which induces seizures (>1 mg/kg i.p.) (Meldrum et al., 1987; Giardina, 2000; Martín del Campo et al., 2009). In addition, we did not observe any signs of seizures in any of the injected rats. Bicuculline was dissolved in physiological serum (NaCl 0.9%) with 0.3% DMSO, and this solution was used as vehicle.

The experiment was replicated four times using 36 animals (9 rats per group) each time. A total of 144 rats were used, with 36 rats per group. Not all animals performed behavioral tests because we found that the data obtained with two replicates were enough to reach statistical significance. Concerning the analysis of neuroinflammation and membrane expression of proteins, in each replicate four rats per group were perfused for immunohistochemistry studies while the other five rats per group were used for analysis of membrane surface expression and content of the proteins. The experimental design is summarized in **Figure 1**. The experiments were approved by the Comité de Ética y Bienestar en Experimentación Animal, Prince Felipe Research Center-Consellería de Agricultura, Generalitat Valenciana and carried out in accordance with the Directive of the European Commission (2010/63/EU) for care and management of experimental animals.

Brain Immunohistochemistry

At week 6 of hyperammonemia, the rats were anesthetized with sodium pentobarbital and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were removed and post-fixed in the same fixative solution for 24 h at 4°C. Paraffin-embedded sections (5 μm) were cut and mounted on coated slide glass. The tissue sections were then processed with the Envision Flex+kit (DAKO) blocking endogenous peroxidase activity for 5 min and then incubated with antibodies. Primary antibodies were against Iba-1 (Wako 019-19741; 1:300 for 30 min), GFAP (Dako IR524; ready for use for 20 min) and IL-1β (Abcam AB9722; 1:100 dilution for 30 min). The reaction was visualized by incubation with Envision Flex + horseradish peroxidase for 20 min and finally diaminobenzidine for 10 min. Sections were counterstained with Mayer's hematoxylin for 5 min.

Analysis of Microglia Activation

Analysis of Iba-1-stained microglia was performed in the hippocampus using Image J software. Microglia activation was assessed by measuring the cell perimeter in eight randomly selected areas (0.45 mm^2) per section according to Vinet et al. (2012). The area of interest was selected. Using Auto Local Threshold and Analyze particle functions in ImageJ, the intensity thresholds and size filter were applied. To measure the perimeter of microglia, the Bernsen method was used and a 2000–20,000 size filter was applied. For each rat, at least 30–40 cells were quantified, and the results were converted from pixels to micrometers. The perimeter length for each group is expressed as the percentage of values for control rats.

Analysis of Astrocytes Activation

Astroglial area (μm^2) covered by GFAP was measured using Image J software. Using Auto Local Threshold and Analyze Particles functions, the intensity thresholds and size filter were applied. To measure the total cell size, the Bernsen method (Bernsen, 1986) was used and a 1500–7000 size filter was applied. For each rat, at least 20 cells from three different sections were counted.

Analysis of IL1β Expression

IL1β-positive cells were manually counted by two blinded experimenters and the results (the mean of two blind experimenters) were expressed as a percentage of the total number of cells. For each rat, at least 120–150 cells per section were counted from at least four different sections. The intensity of IL1β in CA1 region was quantified using the ROI manager function in ImageJ. The CA1 region was selected manually. Inverted values of Mean Gray value were recorded and results were expressed as a percentage of control group.

Analysis of Membrane Surface Expression of Receptors

This analysis was performed by cross-linking with B53 as described by Cabrera-Pastor et al. (2016). Hippocampi were dissected and transversal slices (400 μm) were

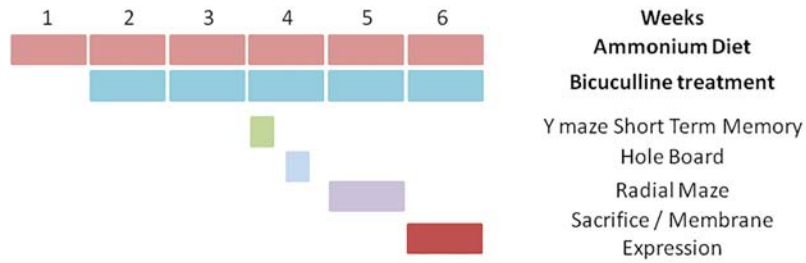

FIGURE 1 | Scheme showing the experimental design.

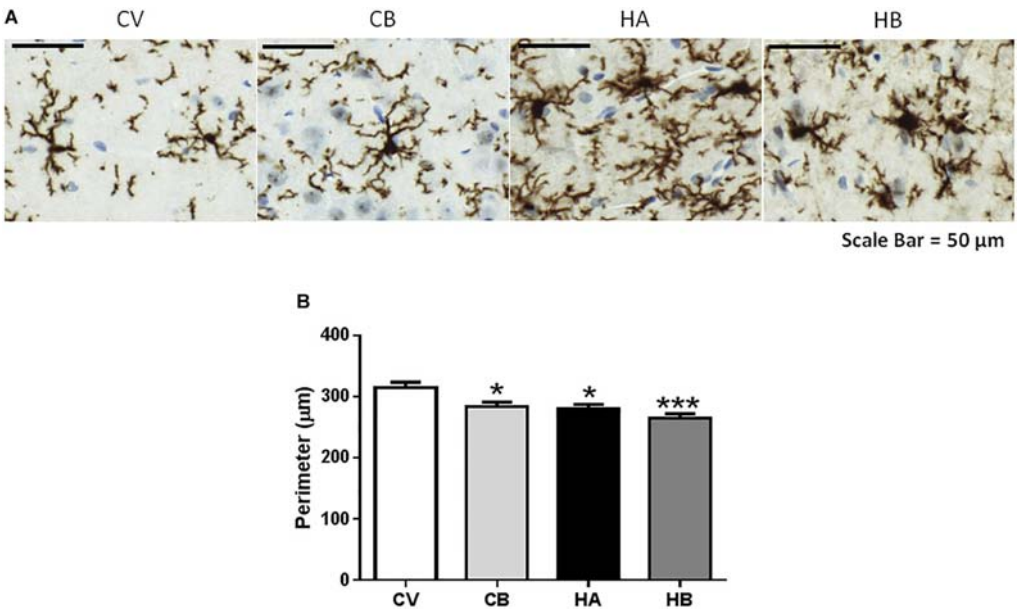

FIGURE 2 | Effect of bicuculline on microglia activation in CA1 region of the hippocampus. Immunohistochemistry was performed as indicated in Section "Materials and Methods" using antibody against Iba1. Representative images of microglial activation **(A)** are shown. Scale bar = 50 um. The perimeter of microglia stained with Iba1 was quantified as described in methods as a measure of microglia activation. **(B)** Data are the mean ± SEM of 10 rats per group. Values significantly different from control rats are indicated by asterisks. *$p \leq 0.05$. CV, control vehicle; CB, control treated with bicuculline; HV, hyperammonemic rats with vehicle; HB, hyperammonemic rats treated with bicuculline. ***$p < 0.001$.

obtained using a chopper. Slices were added to tubes containing ice-cold standard buffer with or without 2 mM BS3 (Pierce, Rockford, IL, United States) and incubated for 30 min at 4°C. Cross-linking was terminated by adding 100 mM glycine (10 min, 4°C). The slices were homogenized by sonicating for 20 s. Samples treated or not with BS3 were analyzed by western blot. The membrane surface expression of each receptor was calculated as the difference between the intensity of the bands without BS3 (total protein) and with BS3 (non-membrane protein) as described by Cabrera-Pastor et al. (2016).

Analysis of Protein Content in Hippocampus by Western Blot

Homogenates of the hippocampus were subjected to immunoblotting according to Felipo et al. (1993). Primary antibodies were against IL-1β 1:500 dilution (AF-510-NA) from R&D SYSTEMS (Minneapolis, MN, United States); GluA1, GluA2, NR2A, and NR2B 1:1000 dilution (cat.# 04-855, AB1768, 04-901 and 06-600, respectively) from Merck Millipore (Darmstadt, Germany); and NR1, 1:1000 dilution (cat.# 556308) from BD Biosciences (San Jose, CA, United States). As a control for protein loading, the same membranes were also incubated with anti-actin (1:5000) from Abcam (Cambridge, MA, United States). Secondary antibodies were anti-rabbit, anti-goat or anti-mouse IgG, 1:4000 dilution (cat.# A8025, A7650, A3562, respectively) conjugated with alkaline phosphatase from Sigma (St. Louis, MO, United States). The images were captured using the ScanJet 5300C (Hewlett-Packard, Amsterdam, Netherlands) and band intensities quantified using the A Imager 2200, version 3.1.2 (AInnotech Corporation, San Francisco, CA, United States).

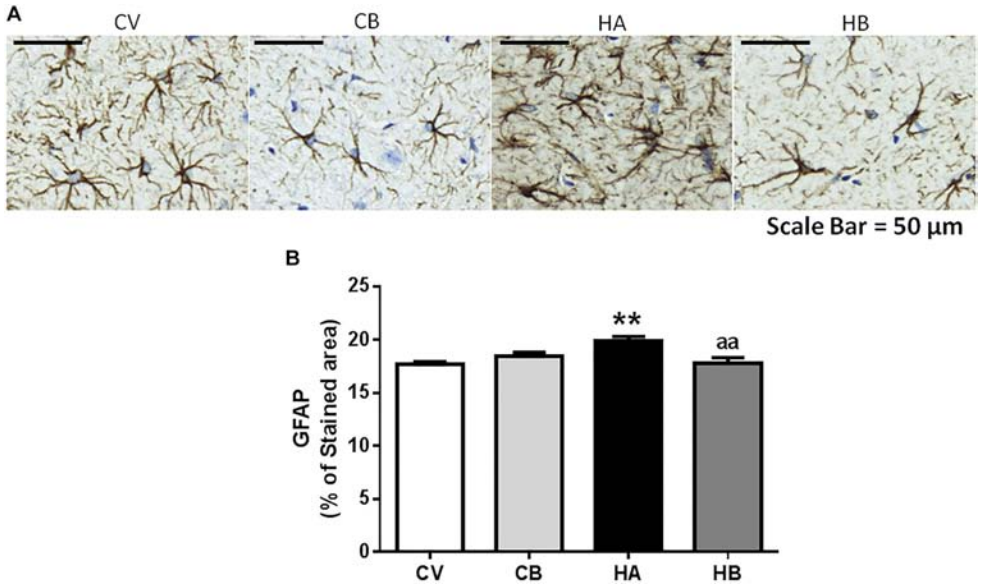

FIGURE 3 | Effect of bicuculline on astrocyte activation in CA1 region of hippocampus. Immunohistochemistry was performed as indicated in Section "Materials and Methods" using antibody against GFAP. Representative images of astrocyte staining **(A)** are shown. Scale bar = 50 um. The percentage of the area stained with GFAP was quantified as described in methods **(B)**. Data are the mean ± SEM of 10 rats per group. Values significantly different from control rats are indicated by asterisks. $^{**}p \leq 0.01$. Values significantly different from hyperammonemic rats are indicated by "aa" $p \leq 0.01$. CV, control vehicle; CB, control treated with bicuculline; HV, hyperammonemic rats with vehicle; HB, hyperammonemic rats treated with bicuculline.

Spatial Learning in the 8-Arms Radial Maze

It was assessed as described in Hernández-Rabaza et al. (2016). Training was performed over 4 days (three trials per day). The task involved locating four pellets, each placed at the end of a different arm according to a random configuration. Configurations were specific for each rat and were kept invariable throughout training. The number of spatial reference errors (reference memory errors, visits to unbaited arms) and WM errors (WM errors, visits to arms already visited in the same trial) were calculated. Learning index is defined as the difference between the number of right choices and reference errors as in Hernández-Rabaza et al. (2016).

Short-Term Spatial Recognition Memory

It was analyzed using a Y-maze consisting of three arms made of black metacrilate joined in the middle to form a "Y" shape. This test is based on the rodents' innate curiosity to explore novel areas and presents no negative or positive reinforcement and very little stress for the rats. The protocol is a modification of the test used by Sarnyai et al. (2000) and Sanderson et al. (2009). The rat was handled for 1 min to reduce stress and anxiety, placed into one of the arms of the maze (start arm) and allowed to explore the maze with one of the arms closed for 2 min (training trial) for three times. After 1 min of inter-trial interval, the rat returned to the Y maze by placing it in the start arm. Then, the rat was allowed to explore freely all three arms of the maze for 2 min (test trial). The number of entries into and the time spent in each arm, the first choice of entry and the discrimination ratio [(Time spent in the novel arm – Time spent in the familiar arm)/Total time passed in the two arms] were registered. Because entry into the novel arm could be altered by anxiety, exploration time was also recorded. No significant differences in exploration time were observed between groups (results not shown).

Analysis of Anxiety Using a Hole-Board Test

This test was performed in a plastic floor with 16 equidistant holes of 4 cm in diameter. The floor was positioned in an open-field activity chamber (43 cm × 43 cm × 30.5 cm) (Med Associates, St Albans, VT, United States), where the animals were positioned. The rat's activity was detected by arrays of infrared motion detection, with two arrays 1 cm above the floor of the chamber and another array 6 cm above the floor, so the upper line of infra-red cells detected the animal movement and the bottom line detected the head-dipping. For the hole-board experiments, each animal was placed in the center of the hole-board and allowed to freely explore the apparatus for 5 min (Kong et al., 2006). Total number of head-dipping and latency to the first head-dipping were recorded by the software Activity Monitor (provided by MED Associates, Inc., St Albans, VT, United States). Anxiety leads to decreased explorative activity that can be quantified by counting the frequency of head-dipping in the holes. The larger is the frequency of head-dipping and the lower is the anxiety of the rat. Novel head dips were also measured to quantify anxiety.

Statistical Analysis

Data are expressed as mean ± SEM. All statistical analyses were performed using the software program GraphPad Prism

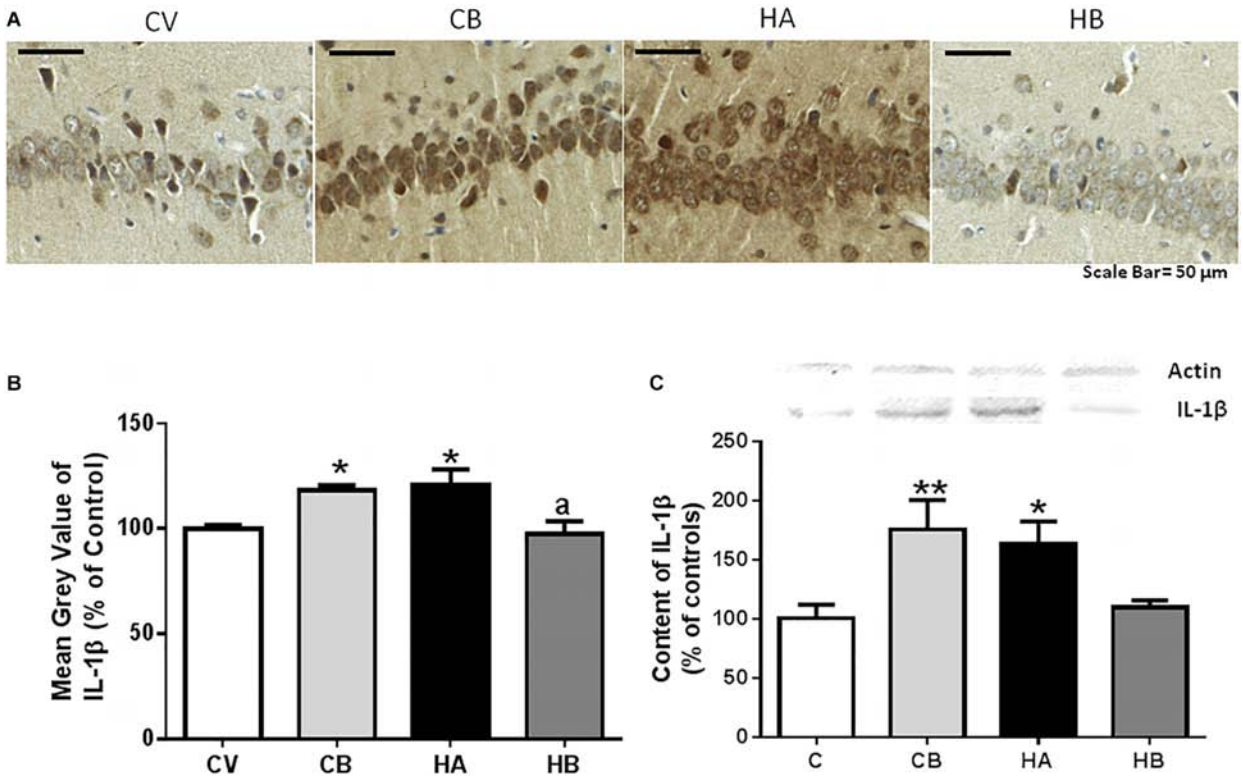

FIGURE 4 | Effect of bicuculline on the content of IL1β in CA1 region of hippocampus. Immunohistochemistry was performed as indicated in Section "Materials and Methods" using antibody against IL1β. Representative images of IL1β staining in CA1 are shown **(A)**. Scale bar = 50 um. The percentage of cells expressing IL1β was quantified as described in methods. Data are the mean ± SEM of 10 rats per group **(B).** The content of IL-1 β was also analyzed by western blot in the total hippocampus **(C)**. Values are mean ± SEM of 12–14 samples per group. Values significantly different from control rats are indicated by asterisks, $*p \leq 0.05$, $**p < 0.01$. Values significantly different from hyperammonemic rats are indicated by "a" $p \leq 0.05$. CV, control vehicle; CB, control treated with bicuculline; HV, hyperammonemic rats with vehicle; HB, hyperammonemic rats treated with bicuculline.

7.0 (GraphPad Prism Software, Inc.). Statistical analysis was carried out using one-way ANOVA or two-way ANOVA with repeated measures, followed by Tukey's *post hoc* test, as indicated in the figure legends. A confidence level of 95% was accepted as significant.

RESULTS

Hyperammonemic rats show activated microglia in the hippocampus, with a less ramified and more ameboid morphology reflected in a reduction ($p < 0.05$) of the perimeter to 280 ± 8 µm compared to 315 ± 9 µm in control rats (**Figure 2**). Activation of microglia is not prevented by bicuculline. The perimeter of microglia in hyperammonemic rats treated with bicuculline was 265 ± 7 µm, similar to untreated hyperammonemic rats. In contrast, bicuculline treatment induced microglia activation in control rats, with reduction of the perimeter to 284 ± 7 µm [$F(3,68) = 12,76$] (**Figure 2**).

Hyperammonemic rats also showed astrocyte activation in the hippocampus, as indicated by the increased area stained by anti-GFAP (19.9 ± 0.4% of the area, $p < 0.01$) compared to control rats (17.7 ± 0.2% of the area). Bicuculline decreased astrocyte activation in hyperammonemic rats and the area stained by anti-GFAP returned to normal values (17.8 ± 0.5% of the area, $p < 0.01$ vs. hyperammonemic rats), indicating de-activation of astrocytes [$F(3,56) = 6,068$] (**Figure 3**).

Neuroinflammation was also reflected in increased IL1β content in hippocampus as analyzed by immunohistochemistry. Anti-IL1β stained mainly neurons of the CA1 region. The number of cells expressing IL1β increased ($p < 0.05$) in hyperammonemic rats to 120 ± 8% of controls. Treatment with bicuculline reversed the increase in IL1β to 97 ± 12% of controls ($p < 0.05$, compared with untreated hyperammonemic rats). In contrast, as occurs for microglial activation, bicuculline increased ($p < 0.05$) the number of cells expressing IL1β in control rats to 118 ± 3% of untreated controls [$F(3,42) = 5,384$] (**Figures 4A,B**).

IL1β was also quantified by western blot, which confirmed an increase of IL1β levels in the hippocampus of hyperammonemic rats to 159 ± 2% of control rats. This increase was reversed by bicuculline treatment, which reduces IL1β levels to 97 ± 12% of controls (**Figure 4C**). Western blot analysis also confirmed the increase ($p < 0.05$) of IL1β in control rats treated with bicuculline, to 189 ± 32% of untreated controls [$F(3,30) = 5,427$] (**Figure 4C**).

We have proposed that neuroinflammation in the hippocampus leads to impairment of spatial learning and

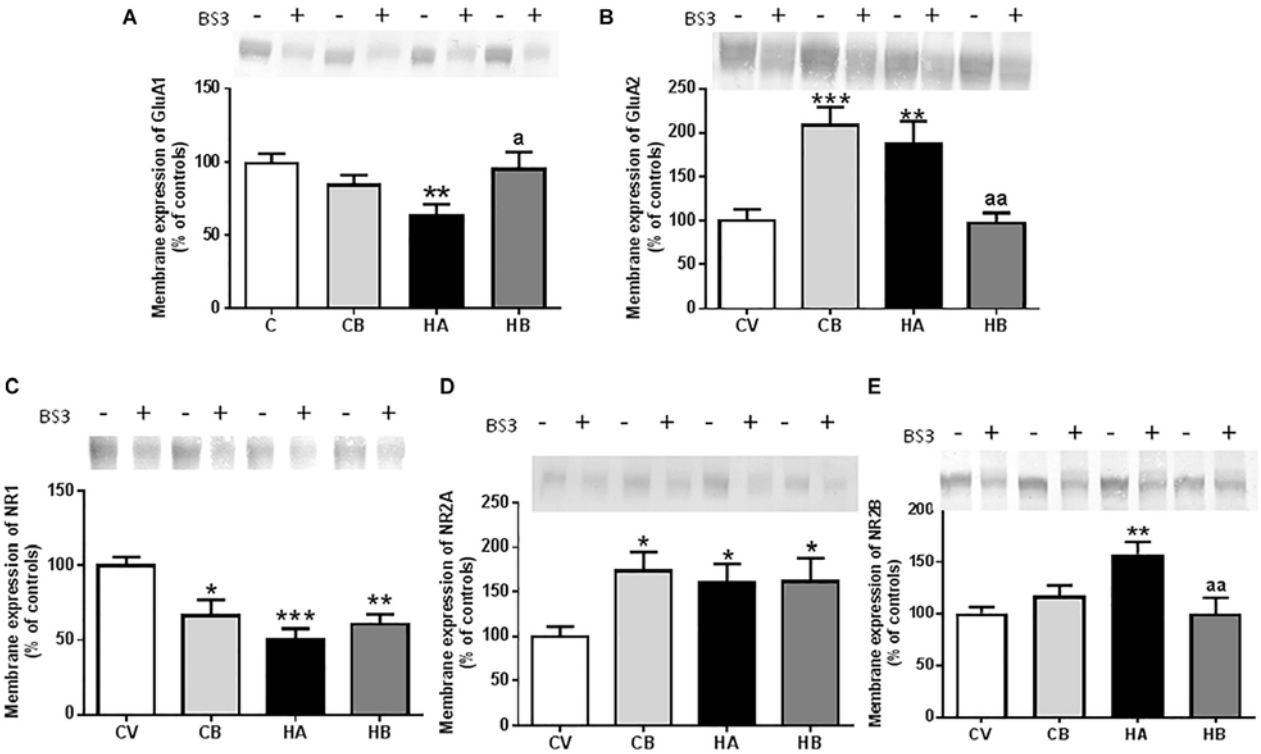

FIGURE 5 | Effect of bicuculline on membrane expression of AMPA and NMDA receptors subunits. Membrane expression of GluA1 **(A)**, GluA2 **(B)**; NR1 **(C)**, NR2A **(D)**, and NR2B **(E)** in the hippocampus was analyzed using the BS3 crosslinker procedure as described in Section "Materials and Methods." Samples incubated in the absence or presence of BS3, were subjected to Western blotting using antibodies for each of the subunits. Representative images are shown. Samples in the absence of BS3 represent the total amount of each protein. Samples in the presence of BS3 represent the non-membrane fraction. The intensities of the bands were quantified and membrane expression was calculated as the difference of intensity between samples without and with BS3. Values are expressed as a percentage of control rats and are the mean ± standard errors of 8–10 rats per group. Values significantly different from control rats are indicated by asterisks and from hyperammonemic rats by "a." *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; a $p < 0.05$; aa $p < 0.01$.

memory by altering membrane expression of GluA1 and GluA2 subunits of AMPA receptors (Cabrera-Pastor et al., 2016). We therefore assessed if treatment with bicuculline normalizes membrane expression of GluA1 and GluA2 subunits of AMPA receptors and spatial learning and memory in hyperammonemic rats.

At 5 weeks of hyperammonemia, membrane expression of GluA1 was significantly reduced ($p < 0.01$) to 65 ± 6% of control rats (**Figure 5A**). Treatment with bicuculline reversed the decrease in membrane expression of GluA1 ($p < 0.01$ compared with hyperammonemic rats), returning to 96 ± 11% of control rats. In control rats, treatment with bicuculline induced a slight reduction (85 ± 6% of control rats) in membrane expression of GluA1 [$F(3,47) = 5,667$] (**Figure 5A**).

Membrane expression of GluA2 was increased ($p < 0.01$) in hyperammonemic rats to 188 ± 25% of control rats (**Figure 5B**). Bicuculline completely reversed the increase of GluA2 ($p < 0.01$, compared with untreated hyperammonemic rats), returning to levels similar to controls (97 ± 12% of controls). In control rats, bicuculline increased membrane expression of GluA2 (208 ± 21% of controls, $p < 0.001$) [$F(3,47) = 10,74$] (**Figure 5B**).

We also assessed the effects on the NR1 and NR2 subunits of NMDA receptors. Membrane expression of NR1 was significantly reduced [$p < 0.001$; $F(3,58) = 7,592$] in hyperammonemic rats to 50 ± 7% of controls (**Figure 5C**). Membrane expression of NR2A was increased [$p < 0.05$; $F(3,49) = 3,752$] to 161 ± 21% of control rats (**Figure 5D**), and that of NR2B to 158 ± 11% of control rats [$p < 0.01$; $F(3,53) = 6,066$] (**Figure 5E**). Treatment with bicuculline in hyperammonemic rats did not reverse the decrease of NR1 nor the increase in NR2A, but completely normalized ($p < 0.01$) the membrane expression of NR2B, returning it to levels similar to control rats (**Figures 5C–E**).

In control rats, bicuculline reduced ($p < 0.05$) membrane expression of NR1 to 66 ± 11% of controls and increased membrane expression of NR2A (173 ± 21% of controls, $p < 0.05$) (**Figures 5C,D**).

Spatial learning and memory were assessed at 4–5 weeks of hyperammonemia in the radial and Y mazes. Hyperammonemia impaired spatial learning in the radial maze. The learning index was lower ($p < 0.05$) than for control rats. Bicuculline reversed the impairment of spatial learning in hyperammonemic rats. The learning index was higher than in untreated hyperammonemic rats [two-way ANOVA Repeated Measure: group by Learning Index. $F(3,44) = 3,684$, $p < 0.01$] and not different from control rats (**Figure 6A**).

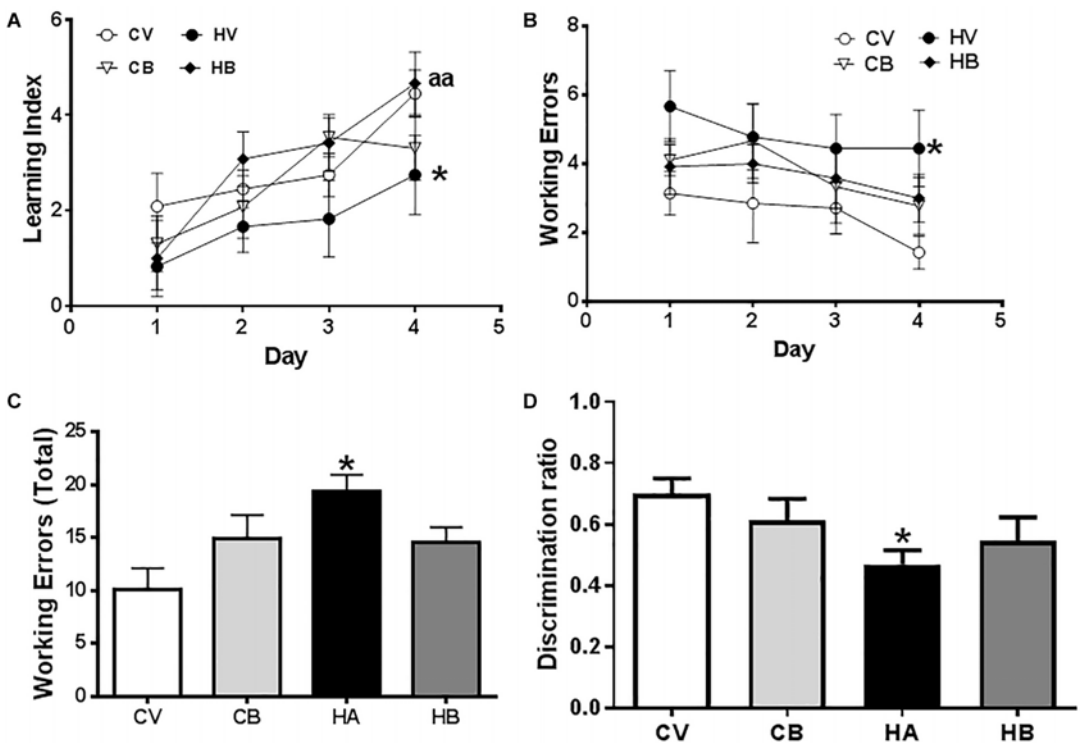

FIGURE 6 | Effects of bicuculline on spatial learning and WM. Spatial learning and memory were assessed in the radial maze. A learning index was calculated as described in Section "Materials and Methods." Learning index increases along the training days **(A)**. **(B)** Working errors at the different days of training. **(C)** Total working errors in the total period of 4 days. Short-term memory in the Y maze was assessed as described in Section "Materials and Methods" and the discrimination index of each experimental group is shown in **(D)**. Values are the mean ± SEM of 12–13 rats per group. Values significantly different from control rats are indicated by asterisks and from hyperammonemic rats by "a." *$p < 0.05$; aa $p < 0.01$.

The number of working errors was also higher in hyperammonemic than in control rats [two-way ANOVA: group by treatment interaction $F(3,33) = 3,706$, $p < 0.05$] (**Figure 6B**). Hyperammonemic rats performed a total of 19 ± 2 working errors [$F(3,33) = 3.7$], which is more ($p < 0.05$) than for control rats (10 ± 2 working errors). Treatment with bicuculline reversed the impairment of WM. Hyperammonemic rats treated with bicuculline performed 15 ± 2 working errors, not significantly different from control rats. In control rats, bicuculline increased the number of working errors (15 ± 2), but the difference with untreated controls was not statistically significant (**Figure 6C**).

We also assessed short-term memory in the Y maze. The discrimination ratio was reduced ($p < 0.05$) in hyperammonemic rats to 0.47 ± 0.05, compared with control rats (0.70 ± 0.05) [$F(3,43) = 2,6$]. Treatment with bicuculline partially restored short-term memory. The discrimination ratio (0.55 ± 0.08) was higher than in untreated hyperammonemic rats (**Figure 6D**).

As anxiety is also modulated by the hippocampus, we also assessed it in hyperammonemic rats, which show a decreased number of head dips [39 ± 5, $p < 0.05$, $F(3,25) = 4,548$] compared to control rats (55 ± 5), indicative of anxiety behavior. Hyperammonemic rats treated with bicuculline showed a tendency to increase the number of head dips, reaching 47 ± 5, which is not significantly different from control rats (**Figure 7A**).

Control rats treated with bicuculline showed a similar number of head dips (37 ± 2, $p < 0.05$) than hyperammonemic rats (**Figure 7A**), which is not surprising since bicuculline has a well-known anxiogenic effect.

We also analyzed the number of novel head dips, i.e., the number of head dips in unexplored holes. This number was reduced ($p < 0.01$) in hyperammonemic rats (13.0 ± 0.5) compared to control rats (15.3 ± 0.2) and was restored by treatment with bicuculline [15.1 ± 0.5; $F(3,25) = 5,482$] (**Figure 7B**). In control rats, treatment with bicuculline reduced the number of novel head dips (14.0 ± 0.3), indicating altered exploratory behavior.

DISCUSSION

We have analyzed the effects of blocking $GABA_A$ receptors with bicuculline on three indicators of neuroinflammation in the hippocampus of hyperammonemic rats: activation of microglia and of astrocytes and content of IL1β. Blocking $GABA_A$ receptors reduces astrocyte activation and IL1β levels, but not microglia activation in the hippocampus of hyperammonemic rats.

$GABA_A$ receptors are expressed in astrocytes (MacVicar et al., 1989; Bureau et al., 1995) and their expression seems to be increased in reactive astrocytes (Hösli et al., 1997).

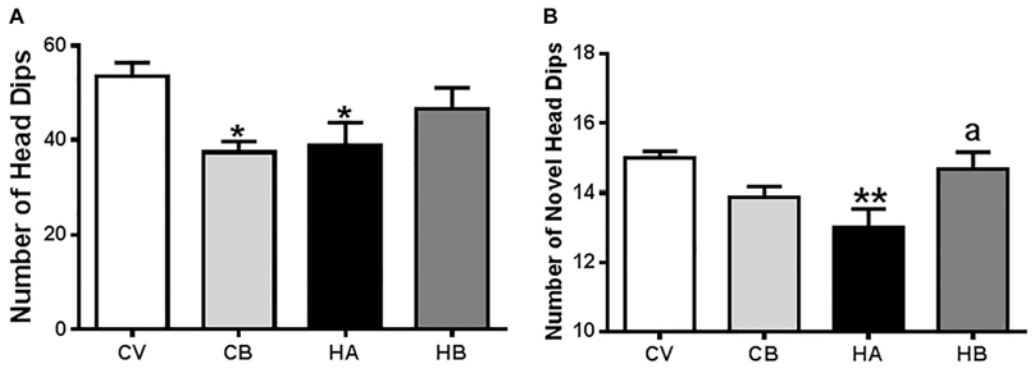

FIGURE 7 | Effects of bicuculline on anxiety. Anxiety was assessed using a hole-board test in an actimeter as indicated in Section "Materials and Methods." Total and novel head dips were quantified by the Activity monitor software and the means ± SEM of eight rats per group are represented in **(A,B)**, respectively. Values significantly different from control rats are indicated by asterisks and from hyperammonemic rats by "a." $*p < 0.05$, $**p < 0.01$; a $p < 0.05$.

Moreover, it has been shown in primary cultures that GABA released by neurons or added exogenously triggers morphological changes in astrocytes through the activation of $GABA_A$ receptors. The effect was blocked by bicuculline (Matsutani and Yamamoto, 1997). A similar effect of GABA on astrocytes morphology through activation of $GABA_A$ receptors has been shown *in vivo* in neonatal rat hypothalamus (Mong et al., 2002).

The increase of GABAergic signaling *in vivo*, by using an inhibitor of GABA transaminase or intracerebral infusion of the GABA-A agonist muscimol, induced activation of astrocytes, with increased branching and GFAP content (Runquist and Alonso, 2003).

The above reports suggest that increasing GABA levels and/or activation of $GABA_A$ receptors is enough to induce activation of astrocytes. As we show that bicuculline treatment reverses activation of astrocytes in the hippocampus of hyperammonemic rats, it is likely that this de-activation of astrocytes would be due directly to the block of $GABA_A$ receptors in astrocytes by bicuculline.

Bicuculline-induced de-activation of astrocytes is associated with a reduction of IL-1β in the hippocampus of hyperammonemic rats. However, IL-1β expression in hyperammonemic rats is increased in neurons of the CA1 region, not in astrocytes, in agreement with previous reports in rats with hyperammonemia and hepatic encephalopathy (Hernandez-Rabaza et al., 2015; Dadsetan et al., 2016b; Balzano et al., 2019). Therefore, the normalization of IL-1β levels by bicuculline would not occur in astrocytes.

It was reported that IL-1β and TNF-a are expressed in hippocampal neurons *in vivo* in response to lesions (Tchélingérian et al., 1996) or to pneumococcal meningitis (Izadpanah et al., 2014). *In situ* hybridization studies show that in murine pneumococcal meningitis IL-1β and TNF-a mRNA were first upregulated in astroglial cells but at 18–24 h were strongly increased in hippocampal neurons (Izadpanah et al., 2014). A similar process occurs in the hippocampus of rats with hepatic encephalopathy, leading to increased expression of IL-1β and TNF-a in neurons (Dadsetan et al., 2016b). It seems therefore that neuronal expression of IL-1β occurs in different pathological situations and would be triggered by previous expression in astrocytes.

These reports suggest therefore that increased expression of IL-1β in CA1 neurons of the hippocampus in hyperammonemic rats could be a consequence of previous activation of astrocytes. Bicuculline would reverse activation of astrocytes and this would lead to a reduced expression of IL-1β in neurons in hyperammonemic rats. Alternatively, other direct effects of blocking $GABA_A$ receptors with bicuculline could be also involved. For example, it has been reported that treatment with bicuculline methiodide, a form of the $GABA_A$ receptor blocker that does not cross the blood–brain barrier, decreases IL-1β in blood in a rat model of sepsis (Hsu and Liu, 2004).

In contrast to astrocyte activation and over-expression of IL-1β, which are reversed by bicuculline in hyperammonemic rats, microglial activation is not reversed. This suggests that hyperammonemic rats show increased GABAergic tone in the hippocampus, which contributes to activation of astrocytes and to over-expression of IL-1β but not to activation of microglia.

It has already been shown that hyperammonemic rats show increased GABAergic tone in the cerebellum but not in the cerebral cortex (Cauli et al., 2009). Increased GABAergic tone in the hippocampus is supported by a report from Johansson et al. (2015) showing that reducing GABAergic tone with GR3027, which antagonizes $GABA_A$ receptor potentiating neurosteroids, restores spatial learning, modulated by the hippocampus, in hyperammonemc rats.

In contrast with the effects of bicuculline in hyperammonemic rats, bicuculline increases neuroinflammation in control rats, inducing microglial activation and increasing IL-1β. It has been proposed that GABA acts as an anti-inflammatory in rheumatoid arthritis downregulating mechanisms that lead to the production of pro-inflammatory agents such as IL-1β (Kelley et al., 2008) and also in neuroinflammation in general (Crowley et al., 2016). GABA acts as an anti-inflammatory in microglia through activation of $GABA_A$ receptors (Lee et al., 2011). In control rats, treatment with bicuculline would prevent this anti-inflammatory effect of GABA resulting in activation of microglia and enhanced production of IL-1β.

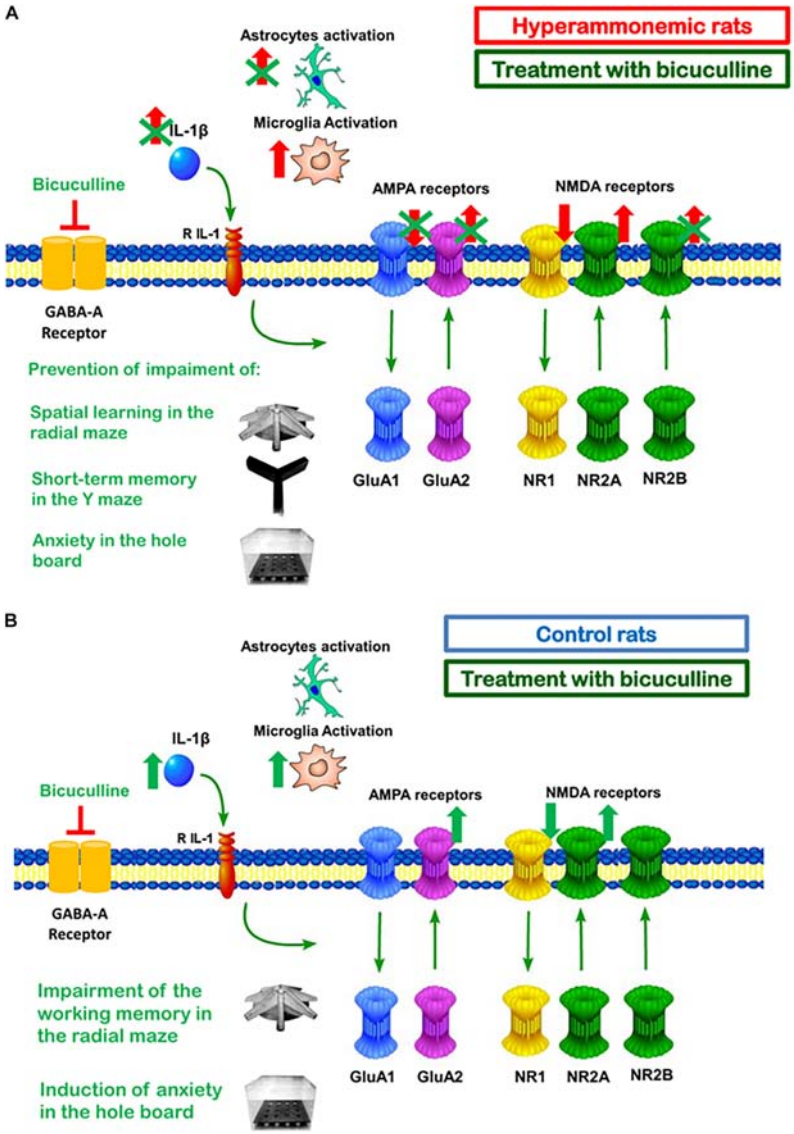

FIGURE 8 | Summary of the effects of bicuculline in hippocampus of hyperammonemic **(A)** and of control **(B)** rats. **(A)** In hyperammonemic rats, treatment with bicuculline reduces astrocytes activation and IL1β expression but not microglia activation. Normalization of IL1βlevels is associated with normalization of membrane expression of the NR2B subunit of the NMDA receptor and of the GluA2 and GluA1 subunits of AMPA receptors, but not of the NR1 and NR2A subunits of the NMDA receptor. This is associated with restoration of spatial learning and decreased anxiety. **(B)** Chronic administration of bicuculline in control rats results in microglia activation and increased IL1β in the hippocampus. This is associated with increased membrane expression of GluA2 and NR2A, a tendency to increase for NR2B and reduced membrane expression of NR1. This is associated with increased anxiety and a tendency to impair WM.

In hyperammonemic rats, normalization of IL-1β levels and astrocyte activation in hippocampus by bicuculline is associated with normalization of membrane expression of the GluA1 and GluA2 subunits of AMPA receptors and of the NR2B subunit of NMDA receptors as well as with improvement of spatial learning. These effects would be a direct consequence of the reduction of IL-1β levels. Taoro-Gonzalez et al. (2018) have shown that in the hippocampus of hyperammonemic rats, increased IL-1β levels over-activate the IL-1 receptor, leading to activation of Src and increased membrane expression of NR2B and GluA2 in addition to reduced membrane expression of GluA1. Blocking IL-1 receptor with the endogenous antagonist IL-1Ra reverses all these changes. This indicates that the effects of bicuculline on GluA1, GluA2, and NR2B are due to the reduction of IL-1β. In contrast, this does not normalize membrane expression of NR1 and NR2A subunits of NMDA receptors, which are not modulated by IL-1β under these conditions (Lai et al., 2006).

It has been reported that altered membrane expression of AMPA receptors in the hippocampus is responsible for impairment of spatial learning and WM in hyperammonemic rats and that reversing these changes by different treatments (sulforaphane, extracellular cGMP) restores spatial learning

(Cabrera-Pastor et al., 2016; Hernández-Rabaza et al., 2016). Normalization of membrane expression of AMPA receptors following normalization of IL-1β by bicuculline treatment would be therefore responsible for improvement of spatial learning and WM in hyperammonemic rats.

Our approach does not allow us to discern if membrane expression of AMPA and NMDA receptors is altered in glial cells or in neurons. However, we can assume that the alterations occur in neurons since, in contrast with other cerebral regions, astrocytes in the hippocampus apparently lack AMPA receptors, and expression of NMDA receptors in glial cells in the hippocampus remains controversial (Rose et al., 2018). Moreover, there is clear evidence of the role of neuronal AMPA and NMDA receptors in hippocampal synaptic plasticity and spatial learning (Morris and Frey, 1997; Shapiro, 2001; Baez et al., 2018).

A direct effect of the blockade of $GABA_A$ receptors by bicuculline on improvement of spatial learning cannot be disregarded. Several studies report beneficial effects on spatial learning and memory of intra-CA1 or intraseptal injection of bicuculline (Roland and Savage, 2009; Torkaman-Boutorabi et al., 2013; Yousefi et al., 2013). Intraperitoneal injection of bicuculline also improves WM in aged ovariectomized rats treated with progesterone (Braden et al., 2015) and in control or chronically stressed rats (Nishimura et al., 2017). On the other hand, bicuculline infusion in the prefrontal cortex impairs working and reference memory (Auger and Floresco, 2017). This, together with increased IL-1β, could be a possible cause of the tendency to increase working errors (not significantly) in control rats treated with bicuculline.

The NR1 subunit of NMDA receptors seems to play a relevant role in short-term memory. The dentate gyrus NR1 knockout mice exhibit a selective impairment in short-term spatial memory (Niewoehner et al., 2007). Treatment with bicuculline did not restore membrane expression of NR1 nor short-term memory in hyperammonemic rats. In both cases, there was a slight tendency to improve that did not reach statistical significance. This suggests that altered membrane expression of NR1 in the hippocampus may contribute to decreased short-term spatial memory in hyperammonemic rats.

Anxiety is common in hyperammonemic cirrhotic patients and decreases quality of life (Nardelli et al., 2013; Malaguarnera et al., 2018). Hyperammonemic rats also show increased anxiety related to neuroinflammation (Luo et al., 2014). Anxiety is modulated by the hippocampus (Nasehi et al., 2011) and by NMDA and GABA receptors (Bina et al., 2014). Injection of NMDA in the ventral hippocampus induces anxiolytic effects, which are prevented by bicuculline, indicating a cross-talk between both neurotransmitter systems in the regulation of anxiety (Bina et al., 2014). In apparent contrast with this report, Barkus et al. (2010) reported that antagonists of NMDA receptors or deletion of the NR1 subunit of NMDA receptors in ventral hippocampus also have an anxiolytic effects.

We found that hyperammonemic rats show increased anxiety and reduced membrane expression of NR1 in the hippocampus. However, treatment with bicuculline reversed anxiety but not membrane expression of NR1, indicating that this would not be directly responsible for anxiety in hyperammonemci rats.

Genetically modified mice lacking the NR2B subunit in hippocampal granule and pyramidal cells in dentate gyrus and CA1, display reduced anxiety (von Engelhardt et al., 2008). As NR2B is increased in membranes in the hippocampus of hyperammonemic rats and treatment with bicuculline reverses this increase, it is possible that the changes in NR2B could contribute to anxiety in hyperammonemic rats and to its reversal by bicuculline.

As summarized in **Figure 8**, this report shows that in hyperammonemic rats, enhanced basal activation of $GABA_A$ receptors in the hippocampus contributes to activation of astrocytes and increased IL-1β, but not to increased activation of microglia. Blocking $GABA_A$ receptor with bicuculline reduces astrocyte activation and IL-1β content in hyperammonemic rats. Normalization of IL-1β levels by bicuculline is associated with reversal of the changes in membrane expression of the GluA1 and GluA2 subunits of AMPA receptors and of the NR2B subunit of NMDA receptors, but not of the NR1 and NR2A subunits of NMDA receptors. Normalization of GluA1, GluA2, and NR2B would be a consequence of normalization of IL-1β and would be responsible for restoration of spatial learning and WM. NR2B would also contribute to reverse the increased anxiety in hyperammonemic rats.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the supplementary files.

AUTHOR CONTRIBUTIONS

MM contributed to most of the experiments including treatment of rats and obtaining samples, immunohistochemical studies and analysis, and membrane expression of receptors, performed the radial maze and anxiety tests, analyzed and interpreted the data, and drafted the manuscript. ML supervised the study, analyzed and interpreted the data, and wrote the manuscript. TB, CA-M, and RM contributed to immunohistochemical studies and analysis. BG-G contributed to treatment of rats, obtaining samples, and radial maze. NM-A contributed to membrane expression of receptors, and immunohistochemical studies and analysis. VF conceived, designed, and supervised the study, obtained funding, analyzed and interpreted the data, and wrote the manuscript.

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REFERENCES

Agusti, A., Hernández-Rabaza, V., Balzano, T., Taoro-Gonzalez, L., Ibañez-Grau, A., Cabrera-Pastor, A., et al. (2017). Sildenafil reduces neuroinflammation in cerebellum, restores GABAergic tone and improves motor in-coordination in rats with hepatic encephalopathy. *CNS Neurosci. Ther.* 23, 386–394. doi: 10.1111/cns.12688

Auger, M. L., and Floresco, S. B. (2017). Prefrontal cortical GABAergic and NMDA glutamatergic regulation of delayed responding. *Neuropharmacology* 113(Pt A), 10–20. doi: 10.1016/j.neuropharm.2016.09.022

Baez, M. V., Cercato, M. C., and Jerusalinsky, D. A. (2018). NMDA receptor subunits change after synaptic plasticity induction and learning and memory acquisition. *Neural Plast.* 2018:5093048. doi: 10.1155/2018/5093048

Balzano, T., Dadsetan, S., Forteza, J., Cabrera-Pastor, A., Taoro-Gonzalez, L., Malaguarnera, M., et al. (2019). Chronic hyperammonemia induces peripheral inflammation that leads to cognitive impairment in rats: reversal by anti-TNFa treatment. *J. Hepatol.* doi: 10.1016/j.jhep.2019.01.008 [Epub ahead of print].

Barkus, C., McHugh, S. B., Sprengel, R., Seeburg, P. H., Rawlins, J. N., and Bannerman, D. M. (2010). Hippocampal NMDA receptors and anxiety: at the interface between cognition and emotion. *Eur. J. Pharmacol.* 626, 49–56. doi: 10.1016/j.ejphar.2009.10.014

Bernsen, J. (1986). "Dynamic thresholding of gray level image," in *Proceedings of the International Conference on Pattern Recognition* (ICPR '86), Berlin, 1251–1255.

Bina, P., Rezvanfard, M., Ahmadi, S., and Zarrindast, M. R. (2014). Anxiolytic-like effects and increase in locomotor activity induced by infusions of NMDA into the ventral hippocampus in rat: interaction with GABAergic system. *Basic Clin. Neurosci.* 5, 267–276.

Braden, B. B., Kingston, M. L., Koenig, E. N., Lavery, C. N., Tsang, C. W., and Bimonte-Nelson, H. A. (2015). The GABAA antagonist bicuculline attenuates progesterone-induced memory impairments in middle-aged ovariectomized rats. *Front. Aging Neurosci.* 7:149. doi: 10.3389/fnagi.2015.00149

Bureau, M., Laschet, J., Bureau-Heeren, M., Hennuy, B., Minet, A., Wins, P., et al. (1995). Astroglial cells express large amounts of GABA A receptor proteins in mature brain. *J. Neurochem.* 65, 2006–2015. doi: 10.1046/j.1471-4159.1995.65052006.x

Cabrera-Pastor, A., Hernandez-Rabaza, V., Taoro-Gonzalez, L., Balzano, T., Llansola, M., and Felipo, V. (2016). In vivo administration of extracellular cGMP normalizes TNF-α and membrane expression of AMPA receptors in hippocampus and spatial reference memory but not IL-1β, NMDA receptors in membrane and working memory in hyperammonemic rats. *Brain Behav. Immun.* 57, 360–370. doi: 10.1016/j.bbi.2016.05.011

Carmans, S., Hendriks, J. J. A., Slaets, H., Thewissen, K., Stinissen, P., Rigo, J. M., et al. (2013). Systemic treatment with the inhibitory neurotransmitter (-aminobutyric acid aggravates experimental autoimmune encephalomyelitis by affecting proinflammatory immune responses. *J. Neuroimmunol.* 255, 45–53. doi: 10.1016/j.jneuroim.2012.11.001

Cauli, O., Mansouri, M. T., Agusti, A., and Felipo, V. (2009). Hyperammonemia increases GABAergic tone in cerebellum but decreases it in rat cortex. *Gastroenterology* 136, 1359–1367. doi: 10.1053/j.gastro.2008.12.057

Crowley, T., Cryan, J. F., Downer, E. J., and O'Leary, O. F. (2016). Inhibiting neuroinflammation: the role and therapeutic potential of GABA in neuro-immune interactions. *Brain Behav. Immun.* 54, 260–277. doi: 10.1016/j.bbi.2016.02.001

Dadsetan, S., Balzano, T., Forteza, J., Agustí, A., Cabrera-Pastor, A., Taoro-González, L., et al. (2016a). Infliximab reduces peripheral inflammation, neuroinflammation, and extracellular GABA in the cerebellum and improves learning and motor coordination in rats with hepatic encephalopathy. *J. Neuroinflamm.* 13:245. doi: 10.1186/s12974-016-0710-8

Dadsetan, S., Balzano, T., Forteza, J., Cabrera-Pastor, A., Taoro-Gonzalez, L., Hernandez-Rabaza, V., et al. (2016b). Reducing peripheral inflammation with infliximab improves neuroinflammation and cognition in rats with hepatic encephalopathy. *Front. Mol. Neurosci.* 9:106. doi: 10.3389/fnmol.2016.00106

Felipo, V. (2013). Hepatic encephalopathy: effects of liver failure on brain function. *Nat. Rev. Neurosci.* 14, 851–858. doi: 10.1038/nrn3587

Felipo, V., Grau, E., Miñana, M. D., and Grisolía, S. (1993). Ammonium injection induces an N methyl D aspartate receptor mediated proteolysis of the microtubule associated protein MAP 2. *J. Neurochem.* 60, 1626–1630. doi: 10.1111/j.1471-4159.1993.tb13384.x

Felipo, V., Miñana, M. D., and Grisolía, S. (1988). Long term ingestion of ammonium increases acetylglutamate and urea levels without affecting the amount of carbamyl phosphate synthase. *Eur. J. Biochem.* 176, 567–571. doi: 10.1111/j.1432-1033.1988.tb14315.x

Felipo, V., Urios, A., Montesinos, E., Molina, I., Garcia-Torres, M. L., Civera, M., et al. (2012). Contribution of hyperammonemia and inflammatory factors to cognitive impairment in minimal hepatic encephalopathy. *Metab. Brain Dis.* 27, 51–58. doi: 10.1007/s11011-011-9269-3

García-Oscos, F., Salgado, H., Hall, S., Thomas, F., Farmer, G. E., Bermeo, J., et al. (2012). The stress-induced cytokine interleukin-6 decreases the inhibition/excitation ratio in the rat temporal cortex via transsignaling. *Biol. Psychiatr.* 71, 574–582. doi: 10.1016/j.biopsych.2011.11.018

Giardina, W. J. (2000). Models of epilepsy: electroshock and chemical induced convulsions in the mouse. *Curr. Prot. Pharmacol.* 45, 5.22.1–5.22.22. doi: 10.1002/0471141755.ph0522s10

González-Usano, A., Cauli, O., Agusti, A., and Felipo, V. (2013). Pregnenolone sulphate restores the glutamate-nitric oxide-cGMP pathway and extracellular GABA in cerebellum and learning and motor coordination in hyperammonemic rats. *ACS Chem. Neurosci.* 5, 100–105. doi: 10.1021/cn400168y

Hellstrom, I. C., Danik, M., Luheshi, G. N., and Williams, S. (2005). Chronic LPS exposure produces changes in intrinsic membrane properties and a sustained IL-beta-dependent increase in GABAergic inhibition in hippocampal CA1 pyramidal neurons. *Hippocampus* 15, 656–664. doi: 10.1002/hipo.20086

Hernandez-Rabaza, V., Agusti, A., Cabrera-Pastor, A., Fustero, S., Delgado, O., Taoro-Gonzalez, L., et al. (2015). Sildenafil reduces neuroinflammation and restores spatial learning in rats with hepatic encephalopathy. underlying mechanisms. *J. Neuroinflamm.* 12:195. doi: 10.1186/s12974-015-0420-7

Hernández-Rabaza, V., Cabrera-Pastor, A., Taoro-González, L., Malaguarnera, M., Agustí, A., Llansola, M., et al. (2016). Hyperammonemia induces glial activation, neuroinflammation and alters neurotransmitter receptors in hippocampus, impairing spatial learning: reversal by sulforaphane. *J. Neuroinflam.* 13:41. doi: 10.1186/s12974-016-0505-y

Hösli, E., Otten, U., and Hösli, L. (1997). Expression of GABA(A) receptors by reactive astrocytes in explant and primary cultures of rat CNS. *Int. J. Dev. Neurosci.* 15, 949–960. doi: 10.1016/S0736-5748(97)00041-5

Hsu, D. Z., and Liu, M. Y. (2004). Bicuculline methiodide attenuates hepatic injury and decreases mortality in septic rats: role of cytokines. *Shock* 22, 347–350. doi: 10.1097/01.shk.0000136705.33995.bd

Izadpanah, K., Freyer, D., Weber, J. R., and Braun, J. S. (2014). Brain parenchymal TNF-α and IL-1β induction in experimental pneumococcal meningitis. *J. Neuroimmunol.* 276, 104–111. doi: 10.1016/j.jneuroim.2014.08.625

Jo, S., Yarishkin, O., Hwang, Y. J., Chun, Y. E., Park, M., Woo, D. H., et al. (2014). GABA from reactive astrocytes impairs memory in mouse models of alzheimer's disease. *Nat. Med.* 20, 886–896. doi: 10.1038/nm.3639

Johansson, M., Agusti, A., Llansola, M., Montoliu, C., Strömberg, J., Malinina, E., et al. (2015). GR3027 antagonizes GABAA receptor-potentiating neurosteroids and restores spatial learning and motor coordination in rats with chronic hyperammonemia and hepatic encephalopathy. *Am. J. Physiol. Gastro. Liver Physiol.* 309, G400–G409. doi: 10.1152/ajpgi.00073.2015

Kawasaki, Y., Zhang, L., Cheng, J. K., and Ji, R. R. (2008). Cytokine mechanisms of central sensitization: distinct and overlapping role of interleukin-1beta, interleukin-6, and tumor necrosis factor-α in regulating synaptic and neuronal activity in the superficial spinal cord. *J. Neurosci.* 28, 5189–5194. doi: 10.1523/JNEUROSCI.3338-07.2008

Kelley, J. M., Hughes, L. B., and Bridges, S. L. Jr. (2008). Does gamma-aminobutyric acid (GABA) influence the development of chronic inflammation in rheumatoid arthritis? *J. Neuroinflam.* 3:1. doi: 10.1186/1742-2094-5-1

Kong, W. X., Chen, S. W., Li, Y. L., Zhang, Y. J., Wang, R., Min, L., et al. (2006). Effects of taurine on rat behaviors in three anxiety models. *Pharmacol. Biochem. Behav.* 83, 271–276. doi: 10.1016/j.pbb.2006.02.007

Lai, A. Y., Swayze, R. D., El-Husseini, A., and Song, C. (2006). Interleukin-1 beta modulates AMPA receptor expression and phosphorylation in hippocampal neurons. *J. Neuroimmunol.* 175, 97–106. doi: 10.1016/j.jneuroim.2006.03.001

Lee, M., Schwab, C., and McGeer, P. L. (2011). Astrocytes are GABAergic cells that modulate microglial activity. *Glia* 59, 152–165. doi: 10.1002/glia.21087

Lin, Y. H., Liang, H. Y., Xu, K., Ni, H. Y., Dong, J., Xiao, H., et al. (2018). Dissociation of nNOS from PSD-95 promotes functional recovery after cerebral ischaemia in mice through reducing excessive tonic GABA release from reactive astrocytes. *J. Pathol.* 244, 176–188. doi: 10.1002/path.4999

Luo, J., Wang, T., Liang, S., Hu, X., Li, W., and Jin, F. (2014). Ingestion of *Lactobacillus* strain reduces anxiety and improves cognitive function in the hyperammonemia rat. *Sci. China Life Sci.* 57, 327–335. doi: 10.1007/s11427-014-4615-4

MacVicar, B. A., Tse, F. W. Y., Crichton, S. A., and Kettenmann, H. (1989). GABA-activated Cl-channels in astrocytes of hippocampal slices. *J. Neurosci.* 9, 3577–3583. doi: 10.1523/JNEUROSCI.09-10-03577.1989

Malaguarnera, G., Pennisi, M., Bertino, G., Motta, M., Borzì, A. M., Vicari, E., et al. (2018). Resveratrol in patients with minimal hepatic encephalopathy. *Nutrients* 10:E329. doi: 10.3390/nu10030329

Martín del Campo, C., Pérez Velázquez, J. L., and Cortez, M. A. (2009). EEG Recording in rodents, with a focus on epilepsy. *Curr. Prot. Neurosci.* 49, 6.24.1–6.24.24. doi: 10.1002/0471142301.ns0624s49

Matsutani, S., and Yamamoto, N. (1997). Neuronal regulation of astrocyte morphology in vitro is mediated by GABAergic signaling. *Glia* 20, 1–9. doi: 10.1002/(SICI)1098-1136(199705)20:1<1::AID-GLIA1>3.0.CO;2-E

Meldrum, B. S., Swan, J. H., Ottersen, O. P., and Storm-Mathisen, J. (1987). Redistribution of transmitter amino acids in rat hippocampus and cerebellum during seizures induced by l-allylglycine and bicuculline: an immunocytochemical study with antisera against conjugated GABA, glutamate and aspartate. *Neuroscience* 22, 17–20. doi: 10.1016/0306-4522(87)90194-1

Mong, J. A., Nuñez, J. L., and McCarthy, M. M. (2002). GABA mediates steroid-induced astrocyte differentiation in the neonatal rat hypothalamus. *J. Neuroendocrinol.* 14, 45–55. doi: 10.1046/j.1365-2826.2002.00737.x

Montoliu, C., Llansola, M., and Felipo, V. (2015). Neuroinflammation and neurological alterations in chronic liver diseases. *Neuroimmunol. Neuroinflam.* 2, 138–144. doi: 10.4103/2347-8659.160845

Montoliu, C., Piedrafita, B., Serra, M. A., del Olmo, J. A., Urios, A., Rodrigo, J. M., et al. (2009). IL-6 and IL-18 in blood may discriminate cirrhotic patients with and without minimal hepatic encephalopathy. *J. Clin. Gastroenterol.* 43, 272–279. doi: 10.1097/MCG.0b013e31815e7f58

Morris, R. G., and Frey, U. (1997). Hippocampal synaptic plasticity: role in spatial learning or the automatic recording of attended experience? *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 352, 1489–1503. doi: 10.1098/rstb.1997.0136

Nardelli, S., Pentassuglio, I., Pasquale, C., Ridola, L., Moscucci, F., Merli, M., et al. (2013). Depression, anxiety and alexithymia symptoms are major determinants of health related quality of life (HRQoL) in cirrhotic patients. *Metab. Brain Dis.* 28, 239–243. doi: 10.1007/s11011-012-9364-0

Nasehi, M., Mafi, F., Oryan, S., Nasri, S., and Zarrindast, M. R. (2011). The effects of dopaminergic drugs in the dorsal hippocampus of mice in the nicotine-induced anxiogenic-like response. *Pharmacol. Biochem. Behav.* 98, 468–473. doi: 10.1016/j.pbb.2011.02.011

Niewoehner, B., Single, F. N., Hvalby, Ø, Jensen, V., Meyer zum Alten Borgloh, S., Seeburg, P. H., et al. (2007). Impaired spatial working memory but spared spatial reference memory following functional loss of NMDA receptors in the dentate gyrus. *Eur. J. Neurosci.* 25, 837–846. doi: 10.1111/j.1460-9568.2007.05312.x

Nishimura, K. J., Ortiz, J. B., and Conrad, C. D. (2017). Antagonizing the GABAA receptor during behavioral training improves spatial memory at different doses in control and chronically stressed rats. *Neurobiol. Learn. Mem.* 145, 114–118. doi: 10.1016/j.nlm.2017.09.002

Nisticò, R., Mango, D., Mandolesi, G., Piccinin, S., Berretta, N., Pignatelli, M., et al. ((2013). Inflammation subverts hippocampal synaptic plasticity in experimental multiple sclerosis. *PLoS One* 8:e54666. doi: 10.1371/journal.pone.0054666

Pribiag, H., and Stellwagen, D. (2013). TNF-downregulates inhibitory neurotransmission through protein phosphatase 1-dependent trafficking of GABA(A) receptors. *J. Neurosci.* 33, 15879–15893. doi: 10.1523/JNEUROSCI.0530-13.2013

Rodrigo, R., Cauli, O., Gomez-Pinedo, U., Agusti, A., Hernandez-Rabaza, V., Garcia-Verdugo, J. M., et al. (2010). Hyperammonemia induces neuroinflammation that contributes to cognitive impairment in rats with hepatic encephalopathy. *Gastroenterology* 139, 675–684. doi: 10.1053/j.gastro.2010.03.040

Roland, J. J., and Savage, L. M. (2009). Blocking GABA-A receptors in the medial septum enhances hippocampal acetylcholine release and behavior in a rat model of diencephalic amnesia. *Pharmacol. Biochem. Behav.* 92, 480–487. doi: 10.1016/j.pbb.2009.01.022

Rose, C. R., Felix, L., Zeug, A., Dietrich, D., Reiner, A., and Henneberger, C. (2018). Astroglial glutamate signaling and uptake in the hippocampus. *Front. Mol. Neurosci.* 10:451. doi: 10.3389/fnmol.2017.00451

Runquist, M., and Alonso, G. (2003). Gabaergic signaling mediates the morphological organization of astrocytes in the adult rat forebrain. *Glia* 15, 137–151. doi: 10.1002/glia.10166

Sallam, M. Y., El-Gowilly, S. M., Abdel-Galil, A. G., and El-Mas, M. M. (2016). Central GABAA receptors are involved in inflammatory and cardiovascular consequences of endotoxemia in conscious rats. *Naunyn Schmiedebergs Arch Pharmacol.* 389, 279–288. doi: 10.1007/s00210-015-1201-7

Sanderson, D. J., Good, M. A., Skelton, K., Sprengel, R., Seeburg, P. H., Rawlins, J. N., et al. (2009). Enhanced long-term and impaired short-term spatial memory in GluA1 AMPA receptor subunit knockout mice: evidence for a dual-process memory model. *Learn. Mem.* 16, 379–386. doi: 10.1101/lm.1339109

Sarnyai, Z., Sibille, E. L., Pavlides, C., Fenster, R. J., McEwen, B. S., and Toth, M. (2000). Impaired hippocampal-dependent learning and functional abnormalities in the hippocampus in mice lacking serotonin(1A) receptors. *Proc. Natl. Acad. Sci. U S A.* 97, 14731–14736. doi: 10.1073/pnas.97.26.14731

Serantes, R., Arnalich, F., Figueroa, M., Salinas, M., Andrés-Mateos, E., Codoceo, R., et al. (2006). Interleukin-1beta enhances GABAA receptor cell-surface expression by a phosphatidylinositol 3-kinase/aktpathway: relevance to sepsis-associated encephalopathy. *J. Biol.Chem.* 281, 14632–14643. doi: 10.1074/jbc.M512489200

Shapiro, M. (2001). Plasticity, hippocampal place cells, and cognitive maps. *Arch. Neurol.* 58, 874–881. doi: 10.1001/archneur.58.6.874

Shawcross, D. L., Davies, N. A., William, R., and Jalan, R. (2004). Systemic inflammatory response exacerbates the neuropsychological effects of induced hyperammonemia in cirrhosis. *J. Hepatol.* 40, 247–254. doi: 10.1016/j.jhep.2003.10.016

Stellwagen, D., Beattie, E. C., Seo, J. Y., and Malenka, R. C. (2005). Differential regulation of AMPA receptor and GABA receptor trafficking by tumor necrosis factor-α. *J. Neurosci.* 25, 3219–3228. doi: 10.1523/JNEUROSCI.4486-04.2005

Taoro-Gonzalez, L., Arenas, Y. M., Cabrera-Pastor, A., and Felipo, V. (2018). Hyperammonemia alters membrane expression of GluA1 and GluA2 subunits of AMPA receptors in hippocampus by enhancing activation of the IL-1 receptor: underlying mechanisms. *J. Neuroinflam.* 15:36. doi: 10.1186/s12974-018-1082-z

Tchélingérian, J. L., Le Saux, F., and Jacque, C. (1996). Identification and topography of neuronal cell populations expressing TNF alpha and IL-1 alpha in response to hippocampal lesion. *J. Neurosci. Res.* 43, 99–106. doi: 10.1002/jnr.490430113

Torkaman-Boutorabi, A., Soltani, S., Oryan, S., Ebrahimi-Ghiri, M., Torabi-Nami, M., and Zarrindast, M. R. (2013). Involvement of the dorsal hippocampal GABA-A receptors in histamine-induced facilitation of memory in the morris water maze. *Pharmacol. Biochem. Behav.* 105, 142–150. doi: 10.1016/j.pbb.2013.02.007

Vinet, J., Weering, H. R., Heinrich, A., Kälin, R. E., Wegner, A., Brouwer, N., et al. (2012). Neuroprotective function for ramified microglia in hippocampal excitotoxicity. *J. Neuroinflam.* 31:27. doi: 10.1186/1742-2094-9-27

von Engelhardt, J., Doganci, B., Jensen, V., Hvalby, Ø, Göngrich, C., Taylor, A., et al. (2008). Contribution of hippocampal and extra-hippocampal NR2B-containing NMDA receptors to performance on spatial learning tasks. *Neuron* 60, 846–860. doi: 10.1016/j.neuron.2008.09.039

Wang, S., Cheng, Q., Malik, S., and Yang, J. (2000). Interleukin-1beta inhibits gamma -aminobutyric acid type a (GABAA) receptor current in cultured hippocampal neurons. *J. Pharmacol. Exp. Ther.* 292, 497–504.

Yousefi, B., Farjad, M., Nasehi, M., and Zarrindast, M. R. (2013). Involvement of the CA1 GABAA receptors in ACPA-induced impairment of spatial and non-spatial novelty detection in mice. *Neurobiol. Learn. Mem.* 100, 32–40. doi: 10.1016/j.nlm.2012.12.001

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REVIEW
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TRPM2 Channel in Microglia as a New Player in Neuroinflammation Associated With a Spectrum of Central Nervous System Pathologies

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Microglial cells in the central nervous system (CNS) are crucial in maintaining a healthy environment for neurons to function properly. However, aberrant microglial cell activation can lead to excessive generation of neurotoxic proinflammatory mediators and neuroinflammation, which represents a contributing factor in a wide spectrum of CNS pathologies, including ischemic stroke, traumatic brain damage, Alzheimer's disease, Parkinson's disease, multiple sclerosis, psychiatric disorders, autism spectrum disorders, and chronic neuropathic pain. Oxidative stress is a salient and common feature of these conditions and has been strongly implicated in microglial cell activation and neuroinflammation. The transient receptor potential melastatin-related 2 (TRPM2) channel, an oxidative stress-sensitive calcium-permeable cationic channel, is highly expressed in microglial cells. In this review, we examine the recent studies that provide evidence to support an important role for the TRPM2 channel, particularly TRPM2-mediated Ca^{2+} signaling, in mediating microglial cell activation, generation of proinflammatory mediators and neuroinflammation, which are of relevance to CNS pathologies. These findings lead to a growing interest in the TRPM2 channel, a new player in neuroinflammation, as a novel therapeutic target for CNS diseases.

Keywords: TRPM2 channel, microglial cell activation, CNS pathologies, neuroinflammation, proinflammatory mediators

INTRODUCTION

The central nervous system (CNS), which is composed of the brain and spinal cord, is a highly integrated and complex network made up principally by neuronal and glial cells. Neuronal cells or neurons as the working unit of the CNS are specialized to transmit information. Glial cells function more in a supportive capacity to surrounding neurons and, nonetheless, as has been

increasingly recognized, also actively participate in many functional aspects of the CNS through bi-directional and dynamic interactions (Jäkel and Dimou, 2017; Allen and Lyons, 2018; Luca et al., 2018). There are several types of glial cells with different embryonic origins (Menassa and Gomez-Nicola, 2018). Astrocytes, oligodendrocytes, and neural-glial antigen 2-positive cells are derived from neuro-ectoderm that also gives rise to neurons, whereas microglial cells are myeloid-lineage cells originated from mesoderm that generates cells of the blood and immune system. Therefore, microglial cells are privileged to be the immune-competent cells of the CNS, like macrophages in the systemic immune system, and thus are often referred to as CNS-resident macrophages. Under healthy or steady-state conditions, microglial cells exhibit a distinctive morphology characteristic of high ramification with an extensive network of fine processes stemming from a small cell body and a resting phenotype (Saijo and Glass, 2011). Microglial cells can secret neurotrophic factors [e.g., brain-derived neurotrophic factor (BDNF)] and, using their phagocytic capability, eliminate excessive or dysfunctional synapses and clear apoptotic developing neurons. In this way, microglial cells support neuronal functions, particularly important processes such as neurogenesis and synaptogenesis during brain development and in the adult brain (Marin-Teva et al., 2004; Sierra et al., 2010; Kettenmann et al., 2013; Yirmiya et al., 2015; Kierdorf and Prinz, 2017; Ising and Heneka, 2018; Luca et al., 2018). In addition, microglial cells act as the sentinel of the CNS and unceasingly patrol the surroundings with their fine processes to monitor environmental changes and provide the first defensive mechanism in response to damage and infection. Microglial cells express a repertoire of the so-called pattern recognition receptors (PRRs), with Toll-like receptors (TLRs), and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) being two example groups. PRRs detect danger-associated molecular patterns (DAMPs) released from host cells due to damage or stress or pathogen-associated molecular patterns (PAMPs) generated by invading pathogens (Brubaker et al., 2015; Jassam et al., 2017). Upon ligation of PRRs by DAMPs and/or PAMPs, microglial cells become activated and, after retracting their processes and taking on a spherical form, adopt an amoeboid morphology, proliferate and migrate to the site of damage or infection, where they remove damaged cells or pathogens via phagocytosis (Hanisch and Kettenmann, 2007). Microglial cells can generate proinflammatory mediators that are instrumental in heightening acute immune responses, including chemokines [e.g., C-X-C motif ligand 2 (CXCL2)], cytokines [e.g., interleukin (IL)-1β, tumor necrosis factor (TNF)-α, IL-6], nitric oxide (NO), and reactive oxygen species (ROS). Activated microglial cells can also assume distinctive and anti-inflammatory phenotypes and produce anti-inflammatory cytokines and neurotrophic factors [e.g., IL-10, tissue growth factor (TGF)-β and BDNF] that are important in resolving inflammation and stimulating tissue repair (Wang et al., 2015; Tay et al., 2017; Luca et al., 2018). It is increasingly clear that microglial cells exhibit a high level of heterogeneity in the developing brain and an increase in varied proinflammatory subtypes in the aged, inflamed or neurodegenerative brain (Hammond et al., 2018; Sousa et al., 2018).

It is known that numerous DAMPs are released by cells in the CNS as a result of aging, traumatic damage, chronic psychological stress or neurodegenerative diseases, with ATP being one such well-documented example (Jassam et al., 2017; Wei et al., 2018). It is also well-known that DAMPs are released from degenerating neurons in the brain, such as misfolded amyloid β-peptides (Aβ), α-synuclein, and superoxide dismutase 1 (Glass et al., 2010). These DAMPs are potent inducers of chronical activation or senescence of microglial cells, leading to elevated generation of pro-inflammatory mediators that alters neuronal functions and induces neurotoxicity, a process often referred to as neuroinflammation (Glass et al., 2010; Heneka et al., 2018; Luca et al., 2018). Studies over the past decade have gathered a large body of evidence to support that microglial cells play a key role in mediating neuroinflammation as a significant contributing factor in the progression of aging and a wide spectrum of CNS conditions, including ischemic stroke, traumatic brain damage, Alzheimer's disease (AD), Parkinson's disease (AD), multiple sclerosis (MS), amyotrophic lateral sclerosis (ALS), neuropsychiatric disorders [e.g., depression, bipolar disorder (BD), and schizophrenia], autism spectrum disorders (ASD), and neuropathic pain (Glass et al., 2010; Yirmiya et al., 2015; Du et al., 2017; Inoue, 2017; Jassam et al., 2017; Maiti et al., 2017; Ramirez et al., 2017; Salter and Stevens, 2017; Alibhai et al., 2018; Bodnar et al., 2018; Ising and Heneka, 2018; Luca et al., 2018; Shetty et al., 2018; Szepesi et al., 2018; Voet et al., 2018).

Oxidative stress, resulting from excessive ROS generation, impaired antioxidant capacity, or both, is a common and salient feature in aging and the aforementioned CNS diseases. The transient receptor potential melastatin-related 2 (TRPM2) channel is a Ca^{2+}-permeable cationic channel with a high sensitivity to oxidative stress or ROS (Hara et al., 2002; Zhang et al., 2003) and is a member of the large transient receptor potential (TRP) superfamily (Clapham, 2003). In the systemic immune system, the TRPM2 channel has been recognized as an important molecular mechanism mediating DAMP/PAMP-induced generation of proinflammatory mediators and innate immune responses (Knowles et al., 2013; Syed Mortadza et al., 2015). Expression of the TRPM2 channel is widely distributed in the CNS with a high level in microglial cells. In this article, we focus on the TRPM2 channel in microglial cells and its role in neuroinflammation. We start with a brief introduction of the TRPM2 channel activation followed by a summary of the evidence supporting TRPM2 channel expression in microglial cells. We proceed to describe the studies that show an important role of the TRPM2 channel in microglial cell activation and generation of proinflammatory mediators in response to various DAMPs and PAMPs, and also the current understanding regarding the molecular mechanisms responsible for DAMP/PAMP-induced TRPM2 channel activation and the downstream TRPM2-dependent signaling pathways engaged in microglial cell activation and generation of proinflammatory mediators. We also discuss

the studies using rodent models that demonstrate the role of the TRPM2 channel in microglial cell activation and neuroinflammation in CNS diseases. Finally, we highlight the gaps in our understanding that require further investigation in order to test whether targeting the TRPM2 channel, a new player in neuroinflammation, could represent a neuroprotective approach to tempering the progression of aging or CNS diseases.

TRPM2 CHANNEL ACTIVATION

Up to now, it has been established both functionally and structurally that the TRPM2 channel is a ligand-gated Ca^{2+}-permeable cationic channel activated by intracellular ADP-ribose (ADPR), and that ADPR-induced TRPM2 channel activation displays strong dependence of intracellular Ca^{2+} (**Figure 1A**) (Perraud et al., 2001; McHugh et al., 2003; Mei et al., 2006; Tong et al., 2006; Xia et al., 2008; Du et al., 2009; Tóth and Csanády, 2010; Huang et al., 2018; Wang et al., 2018; Zhang et al., 2018). Several ADPR analogs, including ADPR-2′-phosphate, 2′-*O*-acetyl-ADPR and 2′-deoxy-ADPR, have been shown to gate the TRPM2 channel (**Figure 1A**) (Grubisha et al., 2006; Toth et al., 2015; Fliegert et al., 2017). Cyclic ADPR (cADPR), nicotinamide adenine dinucleotide (NAD) and other structurally or metabolically ADPR-related compounds were also reported in earlier studies using whole-cell recording to activate the TRPM2 channel (Sano et al., 2001; Kolisek et al., 2005; Beck et al., 2006; Togashi et al., 2006). This notion however has been challenged by more recent studies using the excised inside-out recording to show that application of these compounds to the intracellular face of the TRPM2 channel failed to induce TRPM2 channel activation (Tóth and Csanády, 2010; Toth et al., 2015).

It is also known that warm temperature (≥35°C) induces TRPM2 channel activation alone or in synergy with other TRPM2 channel activators (**Figure 1A**), as shown in pancreatic β-cells and macrophages (Togashi et al., 2006; Kashio et al., 2012; Kashio and Tominaga, 2015). In this aspect, recent studies have revealed an important role for the TRPM2 channel in sensory neurons in the peripheral and central nervous systems in detecting non-noxious warmth and regulating body temperature (Song et al., 2016; Tan and McNaughton, 2016).

As introduced above, TRPM2 channels display high sensitivity to activation under oxidative stress or more specifically exposure to elevated levels of ROS, thus gaining increasing recognition for their role in mediating cellular responses to oxidative stress (Jiang et al., 2010; Miller and Zhang, 2011; Takahashi et al., 2011; Knowles et al., 2013; Ru and Yao, 2014; Li et al., 2015, 2017; Syed Mortadza et al., 2015; Yamamoto and Shimizu, 2016). While some earlier studies suggested that ROS such as H_2O_2 may directly activate the TRPM2 channel, it is now widely accepted that ROS-induced TRPM2 channel activation is indirect and depends on mechanisms that promote an increase in intracellular ADPR level (Jiang et al., 2010). One widely-employed mechanism in many types of mammalian cells is generation of ADPR from NAD by poly(ADPR)-polymerase (PARP), particularly PARP-1, and poly(ADPR)-glycohydrolase (PARG) in the nucleus (**Figure 1B**). Some evidence exists to suggest that ADPR generation from NAD catalyzed by NADase in the mitochondria also contributes in ROS-induced TRPM2 channel activation (Perraud et al., 2005).

TRPM2 CHANNEL EXPRESSION IN MICROGLIAL CELLS

Studies examined TRPM2 channel expression in microglial cells at the mRNA, protein and/or functional levels using reverse transcription-polymerase chain reaction (RT-PCR), immunostaining, western blotting, Ca^{2+} imaging and/or patch-clamp current recording (Kraft et al., 2004; Fonfria et al., 2006; Lee et al., 2010; Jeong et al., 2017; Syed Mortadza et al., 2017). Kraft et al. (2004) were the first to examine TRPM2 channel expression in cultured rat microglial cells. A high level of TRPM2 mRNA expression was detected, and exposure to H_2O_2 induced extracellular Ca^{2+} influx, leading to an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). Furthermore, application of intracellular ADPR opened a cationic conductance with a linear current-voltage (I-V) relationship and a single channel conductance of ∼65 pS (Kraft et al., 2004), the key biophysical characteristics of the TRPM2 channels (Jiang et al., 2010). A recent study shows strong TRPM2 mRNA and protein expression and ADPR-induced cationic currents in cultured mouse microglial cells (Jeong et al., 2017). Consistently, exposure to H_2O_2 (10–300 μM) induced concentration-dependent Ca^{2+} influx and increase in $[Ca^{2+}]_i$ in cultured mouse microglial cells from wild-type (WT) but not TRPM2-knockout (TRPM2-KO) mice (Syed Mortadza et al., 2017). Profiling the TRPM2 mRNA level in numerous human tissues, including the brain and spinal cord, revealed abundant expression and a wide distribution of TRPM2 expression in the CNS (Fonfria et al., 2006). In C13, a human microglial cell line, TRPM2 mRNA transcripts were also readily detected, and exposure to H_2O_2 induced a robust increase in $[Ca^{2+}]_i$. Both the mRNA expression level and H_2O_2-induced Ca^{2+} responses were reduced in C13 cells after treatment with TRPM2-specific antisense oligomers (Fonfria et al., 2006). Furthermore, application of intracellular ADPR or extracellular H_2O_2 elicited cationic currents that exhibited an almost linear I-V relationship and a strong sensitivity to inhibition by flufenamic acid (FFA), a TRPM2 channel inhibitor (**Figure 1A**). In cultured human microglial cells isolated from surgically resected temporal lobe tissues, exposure to H_2O_2 elicited a strong increase in $[Ca^{2+}]_i$ that was inhibited by treatment with clotrimazole (Lee et al., 2010), a TRPM2 channel inhibitor (**Figure 1A**). These studies have gathered compelling evidence to support TRPM2 channel expression in human and rodent microglial cells as a Ca^{2+} influx pathway with a significant role in ROS-induced Ca^{2+} signaling.

Interestingly, an earlier study noted that there were significantly greater H_2O_2-induced Ca^{2+} responses and

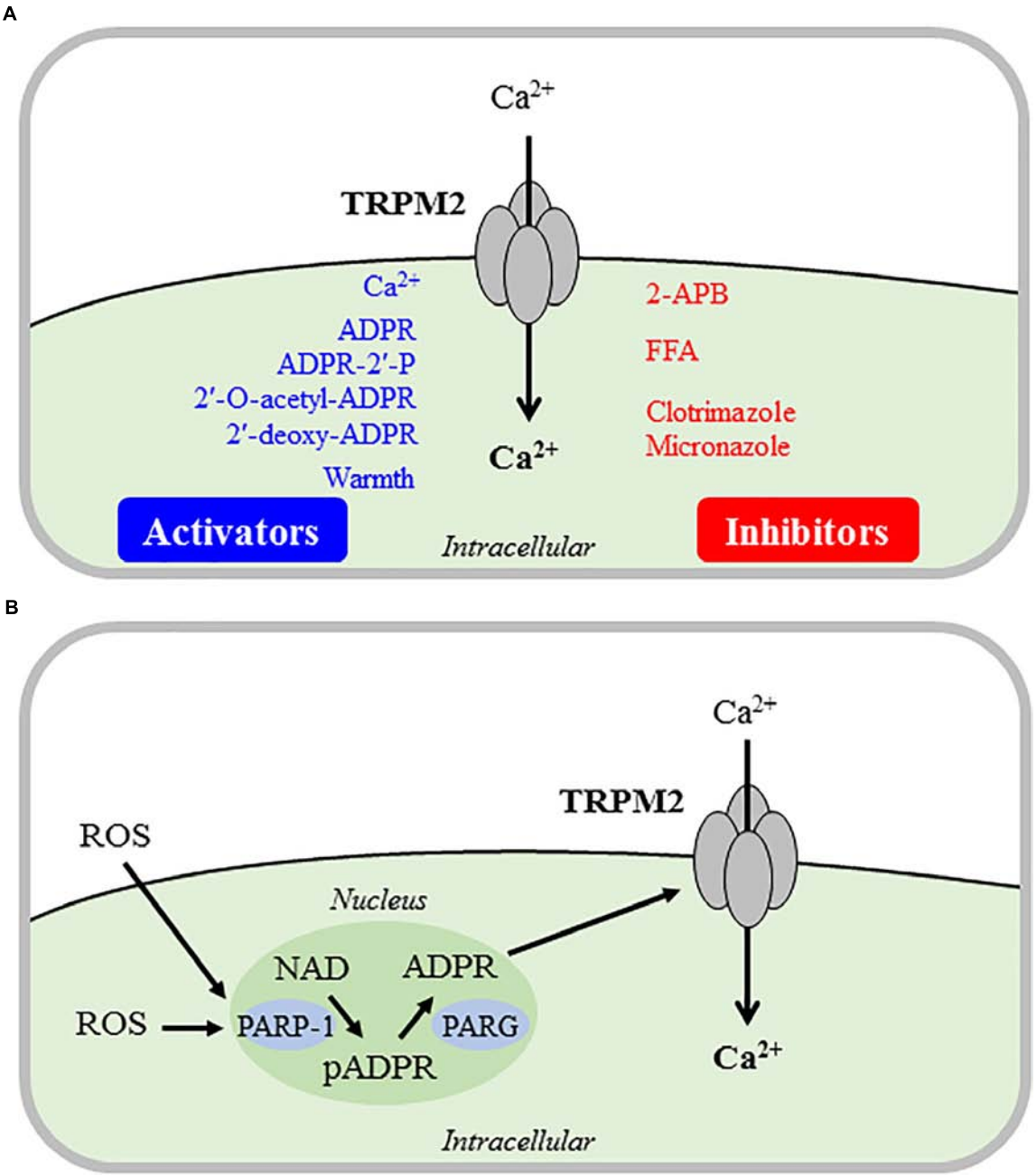

FIGURE 1 | Direct and indirect mechanisms inducing TRPM2 channel activation. Summary of the major mechanisms that are responsible for direct **(A)** or indirect **(B)** activation of the Ca^{2+}-permeable TRPM2 channel on the cell surface that mediates Ca^{2+} influx leading to an increase in intracellular Ca^{2+} concentrations. **(A)** Intracellular Ca^{2+}, ADPR and several ADPR analogs binds to and activate the TRPM2 channel alone or in synergy. Warm temperature can also induce TRPM2 channel activation in a yet not well-defined mechanism. The TRPM2 channel inhibitors used in the studies discussed in this review are shown here, and note that none of these inhibitors are TRPM2-specific. **(B)** ROS can potently but indirectly induce TRPM2 channel activation, mainly via PARP-1/PARG-mediated ADPR generation from NAD in the nucleus. ADPR, ADP-ribose; ADPR-2′-P, ADPR-2′-phosphate; 2-APB, 2-aminoethyl diphenylborinate; FFA, flufenamic acid; ROS, reactive oxygen species; NAD, nicotinamide adenine dinucleotide; pADPR, poly(ADPR); PARP1, poly(ADPR)-polymerase 1; PARG, poly(ADPR)-glycohydrolase.

more readily detectable H_2O_2-induced currents in cultured rat microglial cells after exposure to H_2O_2 or treatment with lipopolysaccharide (LPS), an endotoxin found in the outer membrane of Gram-negative bacteria and a widely-used PAMP to induce immune cell activation via TLR4 activation (Kraft et al., 2004). TRPM2 mRNA expression was up-regulated in C13 cells after treatment with IL-1β (Fonfria et al., 2006). As we discuss below, exposure to diverse pathological stimuli or conditions can increase TRPM2 channel expression in microglial cells.

TRPM2 CHANNEL IN MICROGLIAL CELL ACTIVATION AND GENERATION OF PROINFLAMMATORY MEDIATORS

An increasing number of studies have, mainly using cultured microglial cells, investigated the role of the TRPM2 channel in microglial cell activation and generation of proinflammatory mediators in response to diverse pathological stimuli. Furthermore, as discussed in detail next, efforts have been made to gain considerable insights into the mechanisms by which the TRPM2 channel is activated by such stimuli and the downstream TRPM2-dependent signaling pathways in microglial cell activation and generation of proinflammatory mediators (**Figure 2**).

Generation of TNF-α and IL-6 Resulting From Impaired Antioxidant Capacity

Glutathione (GSH) is present as one of the important reducing agents in most mammalian tissues including the CNS that equip cells with a non-enzymatic antioxidant capacity (Meister and Anderson, 1983). Glutamatecysteine ligase (or γ-glutamylcysteine synthase) is a rate-limiting step in GSH synthesis and thus D,L-buthionine-*S*,*R*-sulfoximine (BSO), an inhibitor of glutamatecysteine ligase, can cause depletion of intracellular GSH and cellular oxidative stress. It has been proposed that a reduction in intracellular GSH with aging increases age-related susceptibility to oxidative stress, which is worsened in many neurodegenerative conditions (Sohal and Weindruch, 1996). A previous study investigated the role of the TRPM2 channel in generating neurotoxic proinflammatory mediators in cultured human microglial cells under BSO-induced oxidative stress (Lee et al., 2010). Exposure to BSO (1–24 h) induced an exposure duration-dependent increase in $[Ca^{2+}]_i$. Exposure to BSO for 2 h was sufficient to activate mitogen-activated protein kinases (MAPK), p38, extracellular signal-regulated kinase (ERK) and Jun-N-terminal kinase (JNK), and furthermore downstream nuclear factor NF-κB. BSO-induced increase in $[Ca^{2+}]_i$ and activation of MAPK and NF-κB signaling pathways were significantly suppressed by supplementation with GSH or treatment with clotrimazole. Exposure to BSO (0.1, 0.5 and 1 mM) also induced concentration-dependent release of TNF-α and IL-6 from microglial cells, which was reduced by treatment with TRPM2-specific small interference RNA (siRNA) (Lee et al., 2010). These results suggest that oxidative stress resulting from GSH depletion activates the TRPM2 channel and TRPM2-mediated Ca^{2+} influx in turn initiates downstream MAPK and NF-kB signaling pathways, leading to generation of TNF-α and IL-6 (**Figure 2A**). Human neuroblastoma SH-SY5Y cells cultured in the medium conditioned by BSO-treated microglial cells exhibited substantial cell death (Lee et al., 2010). Such cell death was significantly attenuated in the conditioned culture medium that was prior depleted of TNF-α and IL-6. Moreover, SH-SY5Y cell death in the conditioned culture medium was strongly suppressed by supplementing microglial cell culture medium with GSH or treating microglial cells with clotrimazole or TRPM2-siRNA (Lee et al., 2010). Collectively, these results suggest that TNF-α and IL-6, generated by microglial cells in a TRPM2-dependent manner, under BSO-induced oxidative stress can induce neurotoxicity.

LPC-Induced Microglial Cell Activation

It is known that lysophosphatidylcholine (LPC), an inflammatory phospholipid endogenously generated under physiological and various pathological conditions, can induce extracellular Ca^{2+} influx in microglial cells and microglial cell activation (Schilling et al., 2004; Sheikh et al., 2009). A recent study has investigated the role of the TRPM2 channel in LPC-induced Ca^{2+}-signaling and microglial cell activation in cultured mouse microglial cells (Jeong et al., 2017). Exposure to LPC induced cationic currents as well as an extracellular Ca^{2+}-dependent increase in $[Ca^{2+}]_i$. LPC exposure also resulted in phosphorylation of p38 (p-p38), an indicator of microglial cell activation. Consistently, intrathecal injection of LPC enhanced expression of ionized calcium binding adapter molecule 1 (Iba1) and CD11 in spinal microglial cells, suggesting microglial cell activation (Jeong et al., 2017). Such LPC-induced *in vitro* or *in vivo* effects in microglial cells were largely prevented by TRPM2-KO (Jeong et al., 2017). These results support a key role for the TRPM2 channel in LPC-induced Ca^{2+} signaling and activation of downstream p38 MAPK signaling pathways, leading to microglial cell activation (Jeong et al., 2017) (**Figure 2B**). It remains unclear regarding the mechanisms by which LPC induces TRPM2 channel activation, and the types of proinflammatory mediators that are generated as a result of LPC-induced microglial cell activation. This study has made an interesting observation that the levels of both total and cell surface TRPM2 protein expression was significantly increased in LPC-treated microglial cells but it is not elucidated how such up-regulation of TRPM2 expression and membrane trafficking occurs.

LPS/IFNγ-Induced Activation of iNOS and Generation of NO

The TRPM2 channel was shown, in an *in vivo* study discussed below, to play a significant role in mediating spinal microglial cell activation and neuropathic pain (Haraguchi et al., 2012). In this study the authors particularly revealed a role for the TRPM2 channel in cultured microglial cells in the activation of inducible NO synthase (iNOS) and generation of NO after exposure to LPS and IFNγ. A subsequent study by the same group investigated the signaling pathways engaged in LPS/IFNγ-induced TRPM2 channel activation and NO generation (Miyake et al., 2014). LPS/IFNγ exposure evoked extracellular Ca^{2+} influx to increase $[Ca^{2+}]_i$, which was prevented by TRPM2-KO or treatment with miconazole, a TRPM2 channel inhibitor (**Figure 1A**). Such Ca^{2+} response was also efficiently inhibited by treatment with diphenylene iodonium (DPI) and ML-171, inhibitors of nicotinamide adenine dinucleotide phosphate (NADPH)-dependent oxidases (NOXs). LPS/IFNγ-induced NO generation was

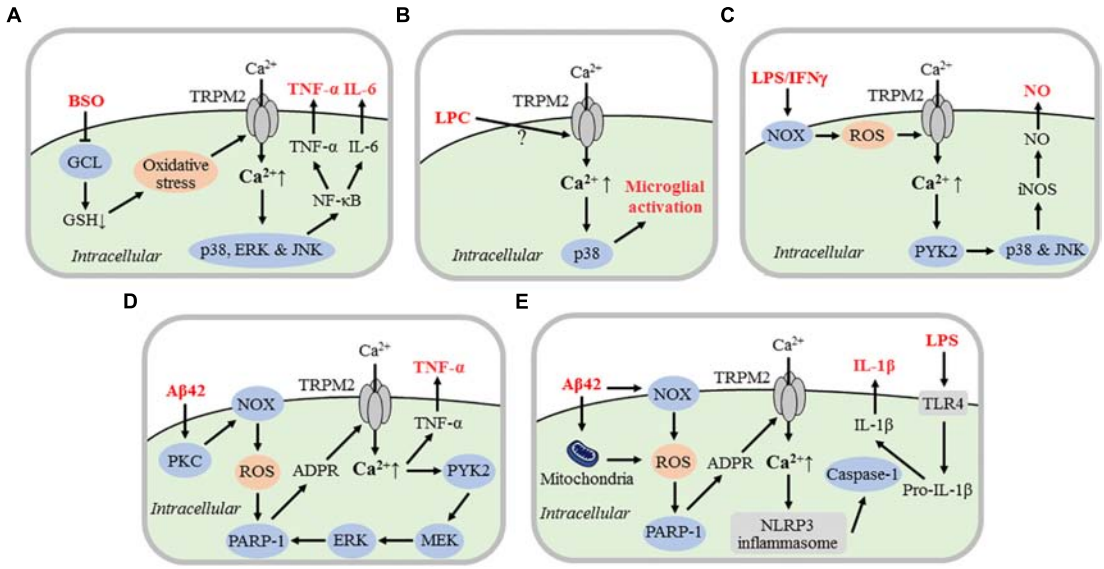

FIGURE 2 | TRPM2 channel mechanisms mediating microglial cell activation and generation of proinflammatory mediators. Schematic illustration of the current knowledge of the signaling mechanisms by which various danger- or pathogen-associated molecular patterns activate the TRPM2 channel in microglial cells, leading to microglial cell activation and generation of proinflammatory mediators. **(A)** BSO-induced GSH depletion via inhibition of GCL-mediated GSH synthesis results in oxidative stress that activates the TRPM2 channel. TRPM2-mediated Ca^{2+} influx induces activation of p38, ERK, and JNK MAPKs and NF-κB pathways that drive expression of TNF-α and IL-6. **(B)** Exposure to LPC induces TRPM2 channel activation via currently unknown mechanisms and ensuring TRPM2-mediated Ca^{2+} influx activates p38, leading to microglial cell activation. **(C)** Exposure to LPS/IFN-γ induces NOX-mediated ROS generation and TRPM2 channel activation. TRPM2-mediated Ca^{2+} influx activates Ca^{2+}-sensitive proline rich tyrosine kinase PYK2 and downstream p38 and JNK, triggering iNOS expression and NO generation. **(D)** Exposure to Aβ42 induces TRPM2 channel activation via PKC/NOX-mediated ROS generation, activation of nuclear PARP-1 and ADPR generation. TRPM2-mediated Ca^{2+} and subsequent activation of PYK2 and MEK/ERK serves as a positive feedback mechanism for further TRPM2 channel activation. TRPM2-mediated Ca^{2+} signaling induces TNF-α expression. **(E)** LPS priming of microglial cells promotes expression of biologically inactive pro-IL-1β via TLR4. Exposure to Aβ42 induces mitochondrial and NOX-mediated ROS generation, activation of nuclear PARP-1, and generation of ADPR which opens the TRPM2 channel. TRPM2-mediated Ca^{2+} influx activates NLRP3 inflammasome and subsequently caspase-1. Caspase-1 converts by cleavage pro-IL-1β into biologically active IL-1β. For the evidence that supports or suggests these TRPM2 channel mechanisms in mediating microglial cell activation and generation of proinflammatory mediators, refer to the studies discussed in detail in the text. BSO, D,L-buthionine-*S*,*R*-sulfoximine; GSH, glutathione; GCL, glutamatecysteine ligase; ERK, extracellular signal-regulated kinase; JNK, Jun-N-terminal kinase; MAPK, mitogen-activated protein kinase; TNF-α, tumor-necrosis factor-α; IL, interleukin; LPC, lysophosphatidylcholine; Aβ42, amyloid-β peptide 42; LPS, lipopolysaccharide; IFNγ, interferon γ; NOX; NADPH oxidases; NO, nitric oxide; iNOS, inducible NO synthase; PKC, protein kinase C; PARP-1, poly(ADPR) polymerase 1; TLR4; Toll-like receptor 4; NLRP3, nucleotide binding domain-containing leucine-rich repeat protein 3.

also significantly reduced by TRPM2-KO, or by inclusion of 1,2-*bis*(*o*-aminophenoxy)ethane-*N,N,N,N*-tetraacetic acid (BAPTA), a Ca^{2+} chelator, to remove extracellular Ca^{2+}. These results support that LPS/IFNγ induce NOX-mediated ROS generation, TRPM2 channel activation and an increase in $[Ca^{2+}]_i$, leading to NO generation (**Figure 2C**). Moreover, LPS/IFNγ-induced NO generation was attenuated by treatment with AG17, an inhibitor for Ca^{2+}-sensitive proline-rich tyrosine kinase 2 (PYK2), SB203580, a p38 inhibitor, or SP600125, a JNK inhibitor. Inhibition of LPS/IFNγ-induced NO generation by BAPTA, AG17, SB203580 or SP600125 was abolished by TRPM2-KO. LPS/IFNγ-induced NO generation in microglial cells from both WT and TRPM2-KO mice was attenuated by treatment with PD98059, a MEK/ERK inhibitor. Likewise, exposure to LPS/IFNγ induced selective activation of p38 in WT but not TRPM2-KO microglial cells, but indiscriminate activation of ERK in both WT and TRPM2-KO microglial cells. Overall, these results suggest that LPS/IFNγ-induced TRPM2-mediated Ca^{2+} signaling initiates activation of PYK2 and downstream p38/JNK MAPK signaling pathways for activation of iNOS and subsequent NO generation (**Figure 2C**).

Aβ42-Induced Microglial Cell Activation and Generation of TNF-α

A recent *in vivo* study using the APP/PS1 mouse model of AD, as discussed further below, has disclosed an important role of the TRPM2 channel in Aβ-induced AD pathologies, including microglial cell activation (Ostapchenko et al., 2015). It is well-established that TNF-α contributes to AD and neurodegenerative diseases via direct interaction with its death receptor on neurons as well as induction of microglial cell activation to generate additional neurotoxic mediators (Alam et al., 2016; Jiang et al., 2018). Our recent study has explored the molecular mechanisms responsible for TRPM2 channel activation and TNF-α generation in cultured mouse microglial cells induced by exposure to Aβ42, one of the amyloid-β peptides of high relevance to AD (Syed Mortadza et al., 2018). Exposure to Aβ42 (30–300 nM) induced a concentration-dependent and extracellular Ca^{2+}-dependent increase in $[Ca^{2+}]_i$. Aβ42-induced

Ca^{2+} response was strongly suppressed by treatment with 2-APB, a TRPM2 channel inhibitor (**Figure 1**), or BAPTA-AM as a membrane-permeable and thus intracellular Ca^{2+} chelator, and furthermore by TRPM2-KO. Exposure to Aβ42 induced cellular ROS generation and activation of nuclear PARP-1. Both Aβ42-induced PARP-1 activation and increase in $[Ca^{2+}]_i$ were suppressed by treatment with PJ34, an inhibitor of PARP enzymes including PARP-1. Furthermore, Aβ42-induced ROS generation, PARP-1 activation and Ca^{2+} responses were inhibited by treatment with chelerythrine, a protein kinase C (PKC) inhibitor, GKT137831, a NOX1/4-seletive inhibitor, or Phox-I2, a NOX2 inhibitor as well as the NOX generic inhibitor DPI. These results indicate that Aβ42 activates the TRPM2 channel by inducing PKC/NOX-mediated ROS generation and subsequent PARP-1 activation and generation of ADPR (**Figure 2D**). Aβ42-induced PARP-1 activation and increase in $[Ca^{2+}]_i$ were also prevented by treatment with PF431396, a PYK2 inhibitor, or U0126, a MEK/ERK inhibitor. Aβ42-induced PARP-1 activation was significantly reduced but incompletely abolished by TRPM2-KO, and the remaining Aβ42-induced PARP-1 activity in TRPM2-KO microglial cells was prevented by treatment with GKT137831 or Phox-I2 and, in striking contrast, not altered by treatment with PF431396 or U0126. Taken together, these results suggest that Aβ42 stimulates PKC/NOX-mediated ROS generation and PARP-1 activation leading to initial TRPM2 channel activation, and that subsequent TRPM2-mediated Ca^{2+} flux and activation of PYK2, MEK/ERK, and PARP-1 serves as a positive feedback mechanism for further TRPM2 channel activation (**Figure 2D**). Moreover, exposure to Aβ42 induced noticeable morphological changes in microglial cells and an increase in the expression and release of TNF-α. Aβ42-induced morphological changes and TNF-α generation were prevented by TRPM2-KO and, moreover, by pharmacological inhibition of the aforementioned signaling pathways responsible for TRPM2 channel activation (Syed Mortadza et al., 2018).

Aβ42-Induced Activation of NLRP3 Inflammasome and Generation of IL-1β

The nucleotide binding domain-containing leucine-rich repeat protein 3 (NLRP3) is a member of the NOD family of PRRs in the cytosol. In response to damage or infection, NLRP3, apoptosis-associated speck-like protein containing a caspase recruitment domain, and procaspase-1, via protein-protein interactions, assemble a multi-protein complex often termed as the NLRP3 inflammasome. NLRP3 inflammasome activation is required to activate caspase-1, which in turn cleaves pro-IL-1β into IL-1β (Tschopp and Schroder, 2010; Brubaker et al., 2015; Jassam et al., 2017; Song et al., 2017; White et al., 2017). It was shown that genetic inactivation of the NLRP3 inflammasome in APP/PS1 mice reduced IL-1β generation by microglial cells, leading to improved spatial memory and attenuation of other AD-related pathological phenotypes (Heneka et al., 2013). In addition, NLRP3 inflammasome inactivation shifted microglial cells toward an anti-inflammatory phenotype that cleared Aβ peptides, thereby resulting in a reduction in amyloid-β deposition (Heneka et al., 2013). Therefore, neuroinflammation resulting from NLPR3 inflammasome activation and IL-1β generation in microglial cells has emerged as an important factor contributing to AD pathogenesis, inciting an interest in targeting the NLRP3 inflammasome as a therapeutic approach to AD (Heneka et al., 2014; White et al., 2017). It is well-known that NLRP3 inflammasome activation and IL-1β generation in immune cells including microglial cells exhibit a striking convergence on ROS generation (Tschopp and Schroder, 2010; Song et al., 2017). A recent pharmacological study has examined the potential role of the TRPM2 channel in Aβ42-induced NLRP3 inflammasome activation and IL-1β generation in LPS-primed microglial cells (Aminzadeh et al., 2018). Exposure to Aβ42 at a relatively high concentration (10 μM) induced mitochondrial ROS generation and also IL-1β generation, both of which were suppressed by treatment with DPI at a high concentration (20 μM) that presumably targets mitochondrial ROS generation. Aβ42-induced IL-1β generation was inhibited by treatment with VAS2870 or (2R,4R)-4-aminopyrrolidine-2,4-dicarboxylate, NOX inhibitors, indicating engagement of NOX-meditated ROS generation. In addition, Aβ42-induced IL-1β generation was reduced by treatment with *N*-acetylcysteine, a ROS scavenger, or DPQ, a PARP-1 inhibitor (Aminzadeh et al., 2018). Exposure to Aβ42 resulted in a Ca^{2+} influx-dependent increase in $[Ca^{2+}]_i$ that was also strongly inhibited by treatment with DPI, VAS2870, DPQ, or BAPTA-AM. Finally, Aβ42-induced caspase-1 activation, as shown by western blotting, was inhibited by treatment with DPQ or BAPTA-AM (Aminzadeh et al., 2018). These results are consistent with the notion that Aβ42 induces NLRP3 inflammasome activation and IL-1β generation via stimulating mitochondrial and NOX-mediated ROS generation, activation of PARP-1 and the TRPM2 channel, and subsequent TRPM2-mediated Ca^{2+} influx (**Figure 2E**). However, more and definitive evidence is required to corroborate the proposed role of the TRPM2 channel.

TRPM2 CHANNEL IN NEUROINFLAMMATION AND CNS PATHOLOGIES

It is clear from the above discussion that studies based on cultured microglial cells support an important role for the TRPM2 channel in microglial cell activation and generation of neurotoxic proinflammatory mediators in response to DAMPs/PAMPs of high relevance to various CNS diseases. As discussed next, there is increasing evidence from *in vivo* studies using rodent models that supports a critical role for the TRPM2 channel in microglial cells in microglial cell activation, generation of proinflammatory mediators and neuroinflammation in the pathogenesis of CNS diseases (**Figure 3**).

Neuropathic Pain

It is well-recognized that microglial cell activation in the spinal cord, as well as peripheral neuroinflammation, plays

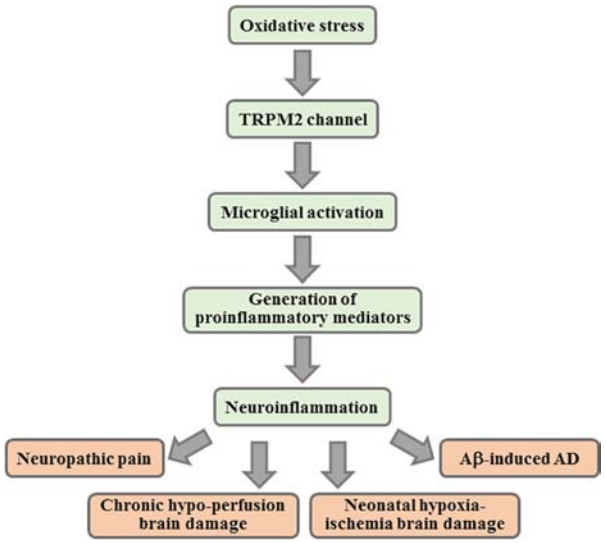

FIGURE 3 | Contribution of TRPM2 channel-mediated neuroinflammation in CNS pathologies. Summary of the key events in TRPM2-mediated neuroinflammation implicated in various CNS pathologies. Activation of the TRPM2 channel in microglial cells mediates microglial cell activation, generation of proinflammatory mediators and/or neuroinflammation that have been shown to contribute to the pathogenesis of neuropathic pain, brain damage by chronic cerebral hypo-perfusion and neonatal hypoxia-ischemia, and Aβ-induced AD. CNS, central nervous system; Aβ, amyloid β peptides; AD, Alzheimer's disease.

a significant role in the development of chronic neuropathic pain (Ji and Suter, 2007; Costigan et al., 2009; Tsuda et al., 2013). The role of the TRPM2 channel in mediating spinal microglial cell activation and neuropathic pain was explored in a previous study using two mouse models of neuropathic pain induced by partial sciatic nerve ligation (SNL) and spinal nerve transection (SNT), respectively (Haraguchi et al., 2012). Both mechanical allodynia and thermal hyperalgesia observed in WT mice during the 2 weeks following SNL were largely absent in TRPM2-KO mice. In the sciatic nerves on the ligation site, the TRPM2 mRNA level was markedly elevated. SNL induced a significant increase in the number of neutrophils and also in generation of CXCL2 in WT mice, both of which were mitigated or completely prevented in TRPM2-KO mice. SNL also resulted in a strong increase in the TRPM2 mRNA expression in dorsal microglial cells and in the intensity of immunoreactivity for both Iba1 and CD11b. Moreover, there was an increase in p-p38 and strong co-localization of p-p38 and CD11b, further indicating microglial cell activation. SNL-induced increase in the intensity of immunoreactivity for Iba1, CD11b nd p-p38, and co-localization of p-p38 and CD11b in spinal microglial cells was largely prevented by TRPM2-KO. Similarly, SNT induced mechanical allodynia, increased intensity of immunoreactivity to CD11b and p-p38 and their co-localization in dorsal microglial cells, all of which were significantly subdued in TRPM2-KO mice. The study further examined the role of the TRPM2 channel in the generation of proinflammatory mediators in cultured microglial cells exposed to LPS/IFNγ. LPS/IFNγ stimulated CXCL2 generation and NO release as well as an increase in the mRNA expression of CXCL2, TNF-α, IL-1β, IL-6, and iNOS. LPS/IFNγ-induced generation of CXCL2 and NO, and increased mRNA expression of CXCL2 and iNOS were significantly lowered by TRPM2-KO (Haraguchi et al., 2012). However, the study revealed no significant effect of TRPM2-KO on the mRNA expression of TNF-α, IL-1β and IL-6, indicating engagement of TRPM2-independent mechanisms. These results support the notion that the TRPM2 channel in spinal microglial cells contributes to neuropathic pain by mediating the generation of proinflammatory mediators to aggravate pro-nociceptive inflammatory responses. As discussed above, LPS/IFNγ-induced NO generation depends on TRPM2-mediated Ca^{2+} signaling and activation of downstream PYK2 and MAPK p38 and JNK signaling pathways (**Figure 2C**).

Alzheimer's Disease

Alzheimer's disease is an age-related neurodegenerative disease with increasing prevalence in a rapidly aging society, representing the most common cause of dementia that afflicts tens of millions of older people worldwide. Aβ accumulation is widely thought to be an early and pathogenic event in AD pathogenesis. Oxidative damage is a conspicuous but mechanistically poorly understood feature of AD. As has been recently reviewed (Jiang et al., 2018), studies have shown wide expression of the TRPM2 channel in the brain and strong evidence for the TRPM2 channel as a nexus from Aβ generation and oxidative damage to AD pathologies via multiple cellular and molecular mechanisms, including microglial cell activation. Microglial cells are known to have a dual role in AD (Boche and Nicoll, 2008). They provide a protective role by phagocytic clearance of Aβ, but such a beneficial capacity declines with aging and is overwhelmed by excessive toxic aggregates, becoming inefficient. As introduced above, Aβ can induce chronic activation and senescence of microglial cells leading to excessive generation of ROS and numerous neurotoxic proinflammatory cytokines, such as TNF-α, IL-1β and IL-6, which constitutes a critical component of AD pathogenesis. APP/PS1 mice co-express a chimeric mouse/human amyloid precursor protein (APP) with the Swedish mutations (K670N and M671L) and human presenilin 1 (PS1) with deletion of exon 9 (Jankowsky et al., 2003). A recent study has examined the role of the TRPM2 channel in Aβ-induced AD pathogenesis using this mouse AD model (Ostapchenko et al., 2015). As has been well-documented, the APP/PS1 mice exhibit excessive Aβ generation, amyloid deposits and synaptic loss in the hippocampus and cortex, microglial cell activation, and severe impairment in age-related spatial memory. Genetic deletion of TRPM2 expression in APP/PS1 mice, while resulting in no alteration in amyloid deposition, essentially reversed Aβ-induced synaptic loss, microglial cell activation, and memory impairment (Ostapchenko et al., 2015). These results provide compelling evidence to support a critical role for the TRPM2 channel in Aβ-induced AD-related pathologies. As already discussed above, recent *in vitro* studies reveal an important role of the TRPM2 channel in mediating Aβ42-induced microglial cell activation and generation of

TNF-α (Syed Mortadza et al., 2018) and possibly IL-1β (Aminzadeh et al., 2018).

Brain Damage by Deficient Cerebral Blood Circulation

The brain is well-known for its vulnerability to damage by deprivation or restriction of oxygen and/or glucose supply that can occur under conditions such as cerebral ischemic stroke, cardiac arrest, chronic cerebral hypo-perfusion, and neonatal hypoxia-ischemia. Oxidative stress, mainly due to increased ROS generation, is a common characteristic of these conditions. An early study demonstrated elevated TRPM2 mRNA expression in rat brains at 1 and 4 weeks after transient middle cerebral artery occlusion (MCAO), a widely-used rodent model of ischemic stroke (Fonfria et al., 2006). A number of recent studies, using various *in vitro* and *in vivo* mouse models of ischemia-reperfusion in conjunction with pharmacological inhibition or genetic deletion of the TRPM2 channel, have supported a critical role of the TRPM2 channel in ischemia-reperfusion brain damage and associated cognitive dysfunction (Jia et al., 2011; Alim et al., 2013; Shimizu et al., 2013, 2016; Gelderblom et al., 2014; Ye et al., 2014). There is also emerging evidence to indicate a role for the TRPM2 channel in mediating brain damage due to hypoxia-ischemia in neonates (Huang et al., 2017) and chronic cerebral hypo-perfusion in adults (Miyanohara et al., 2018).

Of notice, much of the research in this area has so far been devoted to the TRPM2 channel in mediating neuronal death. Nonetheless, there is increasing evidence to suggest a significant contribution of TRPM2-mediated neuroinflammation. For example, selective deletion of the TRPM2 expression in peripheral immune cells substantially protected infarction and cognitive impairment in mice after transient MCAO and reperfusion (Gelderblom et al., 2014). However, the role of the TRPM2 channel in microglial cells in ischemia-reperfusion brain damage largely remains unclear. In the case of neonatal hypoxia-ischemia, a recent study shows that infarction in postnatal day 7 pups, induced by ligating the right common carotid artery and reducing oxygen supply and examined 24 h or 7 days afterward, was considerably attenuated in heterozygous and homozygous TRPM2-KO pups. In addition, WT pups exhibited sensorimotor dysfunction at 7 days post hypoxia-ischemia, and such deficits were less noticeable in heterozygous and homozygous TRPM2-KO pups. In WT pups, the TRPM2 mRNA expression was greater in the damaged hemisphere than the healthy hemisphere. Hypoxia-ischemia induced a massive increase in the number of glial fibrillary acidic protein (GFAP) positive cells and Iba1-positive cells in WT pups, but not in heterozygous and homozygous TRPM2-KO pups. These results suggest that the TRPM2 channel plays an important role in mediating activation of glial cells, including microglial cells, thereby inducing neonatal hypoxic-ischemic brain damage. The role of the TRPM2 channel in microglial cells has been best understood in brain damage by chronic cerebral hypo-perfusion (Miyanohara et al., 2018). Mice manifested significant white matter damage and cognitive dysfunction 28 days after introduction of bilateral common carotid artery stenosis (BCAS), a model of chronic cerebral hypo-perfusion. At this time point, there was also a significant increase in the TRPM2 mRNA expression and in the level of IL-1β, TNF-α and IL-6 in the corpus callosum. Such BCAS-induced effects, namely, white matter damage, cognitive dysfunction and increased generation of IL-1β, TNF-α and IL-6, were prevented by TRPM2-KO. There was an increase in the number of GFAP positive cells and Iba-1 positive cells in the corpus callosum at 14 and 28 days after BCAS, but only the number of Iba-1 positive cells was strongly suppressed by TRPM2-KO. The increase in the number of Iba1-positive cells and cognitive dysfunction in BCAS-operated mice was effectively prevented by administration of minocycline, an inhibitor of microglial cell and macrophage activation. Further analysis, using WT and TRPM2-KO mice with bone marrow (BM)-derived cells replaced by WT GFP-labeled BM-derived cells, indicates that the Iba-1 positive cells in white matter mainly are largely microglial cells. Collectively, these results therefore support a critical role for the TRPM2 channel in mediating microglial cell activation and generation of proinflammatory cytokines, IL-1β, TNF-α and IL-6, in the aggravation of cognitive impairment by chronic cerebral hypo-perfusion.

SUMMARY AND PERSPECTIVES

In summary, the TRPM2 channel is highly expressed in microglial cells and mainly functions as a plasma membrane Ca^{2+}-permeable cationic channel with a key role in mediating ROS-induced Ca^{2+} signaling (**Figure 1B**). In addition, the TRPM2 channel in microglial cells is potently activated by diverse DAMPs and/or PAMPs that induce mitochondrial and/or NOX-mediated ROS generation, activation of PARP-1 and ADPR generation (**Figure 2**). Studies using rodent models in combination with pharmacological and genetic interventions support a significant role for the TRPM2 channel in microglial cell activation and neuroinflammation in the pathogenesis of various CNS conditions. Currently, this includes neuropathic pain, chronic cerebral hypo-perfusion brain damage, neonatal hypoxia-ischemia and Aβ-induced AD (**Figure 3**). As mentioned in the introduction, microglial cell-mediated neuroinflammation is a well-recognized factor in the pathogenesis of many other CNS conditions besides the aforementioned conditions. Research has also implicated TRPM2 channel in PD (Sun et al., 2018; An et al., 2019; Li and Jiang, 2019), MS (Tsutsui et al., 2018), traumatic brain damage (Cook et al., 2010; Yürüker et al., 2015), and neurodevelopmental disorders such as ASD (Higashida et al., 2018) and depression (Xu et al., 2006; Jang et al., 2015; Zhong et al., 2016; Ko et al., 2019) as well as ischemic stroke brain damage. Evidently, further research is required to investigate whether the TRPM2 channel in microglial cells in mediating neuroinflammation plays a significant role in these CNS conditions.

As discussed above, recent studies have gained significant insights into the molecular mechanisms by which DAMPs and/or PAMPs induce activation of the TRPM2 channel and generation of diverse proinflammatory mediators that are of strong relevance

to various CNS diseases. It is clear from the discussion that the current understanding remains fragmented with better insights in some cases than others (**Figure 2**). Further research is required to provide a coherent understanding of how the TRPM2 channel is activated in response to distinctive stimuli or under different conditions, leading to activation of downstream Ca^{2+} signaling pathways, and ultimately how such TRPM2-dependent signaling pathways drive microglial cell activation and generation of proinflammatory mediators.

Given the widespread indication of a significant role for the TRPM2 channel in mediating neuroinflammation and CNS diseases, the TRPM2 channel represents an attractive therapeutic target. The TRPM2 channel also plays important roles in a number of physiological processes, such as insulin release from pancreatic β-cells, regulation of temperature sensation, and peripheral immune responses, which may complicate the concept of targeting TRPM2 as a therapeutic strategy. However, TRPM2 channel expression in the CNS is selectively up-regulated by diverse pathological stimuli or diseased conditions. With continual research into TRPM2 modulation and function in specific cell types, future developments may focus on pharmacological agents that can improve the outcome for patients with CNS diseases while sparing the physiological functions of the channel. Targeting the TRPM2 channel in microglial cells, a newly-emerged player in neuroinflammation, represents an interesting a venue of development of promising therapeutics.

AUTHOR CONTRIBUTIONS

L-HJ and PM wrote the manuscript. All the authors contributed to literature research and analysis, developed the review topic, and approved the manuscript.

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REFERENCES

Alam, Q., Alam, M. Z., Mushtaq, G., Damanhouri, G. A., Rasool, M., Kamal, M. A., et al. (2016). Inflammatory process in Alzheimer's and parkinson's diseases: central role of cytokines. *Curr. Pharm. Des.* 22, 541–548. doi: 10.2174/1381612822666151125000300

Alibhai, J. D., Diack, A. B., and Manson, J. C. (2018). Unravelling the glial response in the pathogenesis of Alzheimer's disease. *FASEB J.* 32, 5766–5777. doi: 10.1096/fj.201801360R

Alim, I., Teves, L., Li, R., Mori, Y., and Tymianski, M. (2013). Modulation of NMDAR subunit expression by TRPM2 channels regulates neuronal vulnerability to ischemic cell death. *J. Neurosci.* 33, 17264–17277. doi: 10.1523/JNEUROSCI.1729-13.2013

Allen, N. J., and Lyons, D. A. (2018). Glia as architects of central nervous system formation and function. *Science* 362, 181–185. doi: 10.1126/science.aat0473

Aminzadeh, M., Roghani, M., Sarfallah, A., and Riazi, G. H. (2018). TRPM2 dependence of ROS-induced NLRP3 activation in Alzheimer's disease. *Int. Immunopharmacol.* 54, 78–85. doi: 10.1016/j.intimp.2017.10.024

An, X., Fu, Z., Mai, C., Wang, W., Wei, L., Li, D., et al. (2019). Increasing the TRPM2 channel expression in human neuroblastoma SH-SY5Y cells augments the susceptibility to ROS-induced cell death. *Cells.* 8:E28. doi: 10.3390/cells8010028

Beck, A., Kolisek, M., Bagley, L. A., Fleig, A., and Penner, R. (2006). Nicotinic acid adenine dinucleotide phosphate and cyclic ADP-ribose regulate TRPM2 channels in T lymphocytes. *FASEB J.* 20, 962–964. doi: 10.1096/fj.05-5538fje

Boche, D., and Nicoll, J. A. (2008). The role of the immune system in clearance of Abeta from the brain. *Brain Pathol.* 18, 267–278. doi: 10.1111/j.1750-3639.2008.00134.x

Bodnar, C. N., Morganti, J. M., and Bachstetter, A. D. (2018). Depression following a traumatic brain injury: uncovering cytokine dysregulation as a pathogenic mechanism. *Neural. Regen. Res.* 13, 1693–1704. doi: 10.4103/1673-5374.238604

Brubaker, S. W., Bonham, K. S., Zanoni, I., and Kagan, J. C. (2015). Innate immune pattern recognition: a cell biological perspective. *Annu. Rev. Immunol.* 33, 257–290. doi: 10.1146/annurev-immunol-032414-112240

Clapham, D. E. (2003). TRP channels as cellular sensors. *Nature* 426, 517–524. doi: 10.1038/nature02196

Cook, N. L., Vink, R., Helps, S. C., Manavis, J., and Van Den Heuvel, C. (2010). Transient receptor potential melastatin 2 expression is increased following experimental traumatic brain injury in rats. *J. Mol. Neurosci.* 42, 192–199. doi: 10.1007/s12031-010-9347-8

Costigan, M., Scholz, J., and Woolf, C. J. (2009). Neuropathic pain: a maladaptive response of the nervous system to damage. *Annu. Rev. Neurosci.* 32, 1–32. doi: 10.1146/annurev.neuro.051508.135531

Du, J., Xie, J., and Yue, L. (2009). Intracellular calcium activates TRPM2 and its alternative spliced isoforms. *Proc. Natl. Acad. Sci. U.S.A.* 106, 7239–7244. doi: 10.1073/pnas.0811725106

Du, L., Zhang, Y., Chen, Y., Zhu, J., Yang, Y., and Zhang, H. L. (2017). Role of microglia in neurological disorders and their potentials as a therapeutic target. *Mol. Neurobiol.* 54, 7567–7584. doi: 10.1007/s12035-016-0245-0

Fliegert, R., Watt, J. M., Schobel, A., Rozewitz, M. D., Moreau, C., Kirchberger, T., et al. (2017). Ligand-induced activation of human TRPM2 requires the terminal ribose of ADPR and involves Arg1433 and Tyr1349. *Biochem. J.* 474, 2159–2175. doi: 10.1042/BCJ20170091

Fonfria, E., Murdock, P. R., Cusdin, F. S., Benham, C. D., Kelsell, R. E., and McNulty, S. (2006). Tissue distribution profiles of the human TRPM cation channel family. *J. Recept. Signal Transduct. Res.* 26, 159–178. doi: 10.1080/10799890600637506

Gelderblom, M., Melzer, N., Schattling, B., Göb, E., Hicking, G., Arunachalam, P., et al. (2014). Transient receptor potential melastatin subfamily member 2 cation channel regulates detrimental immune cell invasion in ischemic stroke. *Stroke* 45, 3395–3402. doi: 10.1161/STROKEAHA.114.005836

Glass, C. K., Saijo, K., Winner, B., Marchetto, M. C., and Gage, F. H. (2010). Mechanisms underlying inflammation in neurodegeneration. *Cell* 140, 918–934. doi: 10.1016/j.cell.2010.02.016

Grubisha, O., Rafty, L. A., Takanishi, C. L., Xu, X., Tong, L., Perraud, A. L., et al. (2006). Metabolite of SIR2 reaction modulates TRPM2 ion channel. *J. Biol. Chem.* 281, 14057–14065. doi: 10.1074/jbc.M513741200

Hammond, T. R., Robinton, D., and Stevens, B. (2018). Microglia and the brain: complementary partners in development and disease. *Annu. Rev. Cell Dev. Biol.* 34, 523–544. doi: 10.1146/annurev-cellbio-100616-060509

Hanisch, U. K., and Kettenmann, H. (2007). Microglia: active sensor and versatile effector cells in the normal and pathologic brain. *Nat. Neurosci.* 10, 1387–1394. doi: 10.1038/nn1997

Hara, Y., Wakamori, M., Ishii, M., Maeno, E., Nishida, M., Yoshida, T., et al. (2002). LTRPC2 Ca^{2+}-permeable channel activated by changes in redox status confers

susceptibility to cell death. *Mol. Cell* 9, 163–173. doi: 10.1016/S1097-2765(01)00438-5

Haraguchi, K., Kawamoto, A., Isami, K., Maeda, S., Kusano, A., Asakura, K., et al. (2012). TRPM2 contributes to inflammatory and neuropathic pain through the aggravation of pronociceptive inflammatory responses in mice. *J. Neurosci.* 32, 3931–3941. doi: 10.1523/JNEUROSCI.4703-11.2012

Heneka, M. T., Kummer, M. P., and Latz, E. (2014). Innate immune activation in neurodegenerative disease. *Nat. Rev. Immunol.* 14, 463–477. doi: 10.1038/nri3705

Heneka, M. T., Kummer, M. P., Stutz, A., Delekate, A., Schwartz, S., Vieira-Saecker, A., et al. (2013). NLRP3 is activated in Alzheimer's disease and contributes to pathology in APP/PS1 mice. *Nature* 493, 674–678. doi: 10.1038/nature11729

Heneka, M. T., Mcmanus, R. M., and Latz, E. (2018). Inflammasome signalling in brain function and neurodegenerative disease. *Nat. Rev. Neurosci.* 19, 610–621. doi: 10.1038/s41583-018-0055-7

Higashida, H., Yuhi, T., Akther, S., Amina, S., Zhong, J., Liang, M., et al. (2018). Oxytocin release via activation of TRPM2 and CD38 in the hypothalamus during hyperthermia in mice: implication for autism spectrum disorder. *Neurochem. Int.* 119, 42–48. doi: 10.1016/j.neuint.2017.07.009

Huang, S., Turlova, E., Li, F., Bao, M. H., Szeto, V., Wong, R., et al. (2017). Transient receptor potential melastatin 2 channels (TRPM2) mediate neonatal hypoxic-ischemic brain injury in mice. *Exp. Neurol.* 296, 32–40. doi: 10.1016/j.expneurol.2017.06.023

Huang, Y., Winkler, P. A., Sun, W., Lu, W., and Du, J. (2018). Architecture of the TRPM2 channel and its activation mechanism by ADP-ribose and calcium. *Nature* 562, 145–149. doi: 10.1038/s41586-018-0558-4

Inoue, K. (2017). Purinergic signaling in microglia in the pathogenesis of neuropathic pain. *Proc. Jpn. Acad. Ser. B. Phys. Biol. Sci.* 93, 174–182. doi: 10.2183/pjab.93.011

Ising, C., and Heneka, M. T. (2018). Functional and structural damage of neurons by innate immune mechanisms during neurodegeneration. *Cell Death Dis.* 9:120. doi: 10.1038/s41419-017-0153-x

Jäkel, S., and Dimou, L. (2017). Glial cells and their function in the adult brain: a journey through the history of their ablation. *Front. Cell Neurosci.* 11:24. doi: 10.3389/fncel.2017.00024

Jang, Y., Lee, S. H., Lee, B., Jung, S., Khalid, A., Uchida, K., et al. (2015). TRPM2, a susceptibility gene for bipolar disorder, regulates glycogen synthase kinase-3 activity in the brain. *J. Neurosci.* 35, 11811–11823. doi: 10.1523/JNEUROSCI.5251-14.2015

Jankowsky, J. L., Xu, G., Fromholt, D., Gonzales, V., and Borchelt, D. R. (2003). Environmental enrichment exacerbates amyloid plaque formation in a transgenic mouse model of Alzheimer disease. *J. Neuropathol. Exp. Neurol.* 62, 1220–1227. doi: 10.1093/jnen/62.12.1220

Jassam, Y. N., Izzy, S., Whalen, M., Mcgavern, D. B., and El Khoury, J. (2017). Neuroimmunology of traumatic brain injury: time for a paradigm shift. *Neuron* 95, 1246–1265. doi: 10.1016/j.neuron.2017.07.010

Jeong, H., Kim, Y. H., Lee, Y., Jung, S. J., and Oh, S. B. (2017). TRPM2 contributes to LPC-induced intracellular Ca2+ influx and microglial cell activation. *Biochem. Biophys. Res. Commun.* 485, 301–306. doi: 10.1016/j.bbrc.2017.02.087

Ji, R. R., and Suter, M. R. (2007). p38 MAPK, microglial signaling, and neuropathic pain. *Mol. Pain* 3:33. doi: 10.1186/1744-8069-3-33

Jia, J., Verma, S., Nakayama, S., Quillinan, N., Grafe, M. R., Hurn, P. D., et al. (2011). Sex differences in neuroprotection provided by inhibition of TRPM2 channels following experimental stroke. *J. Cereb. Blood Flow Metab.* 31, 2160–2168. doi: 10.1038/jcbfm.2011.77

Jiang, L. H., Li, X., Syed Mortadza, S. A., Lovatt, M., and Yang, W. (2018). The TRPM2 channel nexus from oxidative damage to Alzheimer's pathologies: an emerging novel intervention target for age-related dementia. *Ageing Res. Rev.* 47, 67–79. doi: 10.1016/j.arr.2018.07.002

Jiang, L. H., Yang, W., Zou, J., and Beech, D. J. (2010). TRPM2 channel properties, functions and therapeutic potentials. *Expert Opin. Ther. Targets* 14, 973–988. doi: 10.1517/14728222.2010.510135

Kashio, M., Sokabe, T., Shintaku, K., Uematsu, T., Fukuta, N., Kobayashi, N., et al. (2012). Redox signal-mediated sensitization of transient receptor potential melastatin 2 (TRPM2) to temperature affects macrophage functions. *Proc. Natl. Acad. Sci. U.S.A.* 109, 6745–6750. doi: 10.1073/pnas.1114193109

Kashio, M., and Tominaga, M. (2015). Redox signal-mediated enhancement of the temperature sensitivity of transient receptor potential melastatin 2 (TRPM2) elevates glucose-induced insulin secretion from pancreatic islets. *J. Biol. Chem.* 290, 12435–12442. doi: 10.1074/jbc.M115.649913

Kettenmann, H., Kirchhoff, F., and Verkhratsky, A. (2013). Microglia: new roles for the synaptic stripper. *Neuron* 77, 10–18. doi: 10.1016/j.neuron.2012.12.023

Kierdorf, K., and Prinz, M. (2017). Microglia in steady state. *J. Clin. Invest.* 127, 3201–3209. doi: 10.1172/JCI90602

Knowles, H., Li, Y., and Perraud, A. L. (2013). The TRPM2 ion channel, an oxidative stress and metabolic sensor regulating innate immunity and inflammation. *Immunol. Res.* 55, 241–248. doi: 10.1007/s12026-012-8373-8

Ko, S. Y., Wang, S. E., Lee, H. K., Jo, S., Han, J., Lee, S. H., et al. (2019). Transient receptor potential melastatin 2 governs stress-induced depressive-like behaviors. *Proc. Natl. Acad. Sci. U.S.A.* 116, 1770–1775. doi: 10.1073/pnas.1814335116

Kolisek, M., Beck, A., Fleig, A., and Penner, R. (2005). Cyclic ADP-ribose and hydrogen peroxide synergize with ADP-ribose in the activation of TRPM2 channels. *Mol. Cell* 18, 61–69. doi: 10.1016/j.molcel.2005.02.033

Kraft, R., Grimm, C., Grosse, K., Hoffmann, A., Sauerbruch, S., Kettenmann, H., et al. (2004). Hydrogen peroxide and ADP-ribose induce TRPM2-mediated calcium influx and cation currents in microglia. *Am. J. Physiol. Cell Physiol.* 286, C129–C137. doi: 10.1152/ajpcell.00331.2003

Lee, M., Cho, T., Jantaratnotai, N., Wang, Y. T., Mcgeer, E., and Mcgeer, P. L. (2010). Depletion of GSH in glial cells induces neurotoxicity: relevance to aging and degenerative neurological diseases. *FASEB J.* 24, 2533–2545. doi: 10.1096/fj.09-149997

Li, C., Meng, L., Li, X., Li, D., and Jiang, L. H. (2015). Non-NMDAR neuronal Ca2+-permeable channels in delayed neuronal death and as potential therapeutic targets for ischemic brain damage. *Expert Opin. Ther. Targets* 19, 879–892. doi: 10.1517/14728222.2015.1021781

Li, J., Gao, Y., Bao, X., Li, F., Yao, W., Feng, Z., et al. (2017). TRPM2: a potential drug target to retard oxidative stress. *Front. Biosci.* 22:1427–1438.

Li, X., and Jiang, L.-H. (2019). A critical role of the transient receptor potential melastatin 2 channel in a positive feedback mechanism for reactive oxygen species-induced delayed cell death. *J. Cell. Physiol.* 234, 3647–3660. doi: 10.1002/jcp.27134

Luca, A., Calandra, C., and Luca, M. (2018). Molecular bases of Alzheimer's disease and neurodegeneration: the role of neuroglia. *Aging Dis.* 9, 1134–1152. doi: 10.14336/AD.2018.0201

Maiti, P., Manna, J., and Dunbar, G. L. (2017). Current understanding of the molecular mechanisms in Parkinson's disease: targets for potential treatments. *Transl. Neurodegener.* 6:28. doi: 10.1186/s40035-017-0099-z

Marin-Teva, J. L., Dusart, I., Colin, C., Gervais, A., Van Rooijen, N., and Mallat, M. (2004). Microglia promote the death of developing Purkinje cells. *Neuron* 41, 535–547. doi: 10.1016/S0896-6273(04)00069-8

McHugh, D., Flemming, R., Xu, S. Z., Perraud, A. L., and Beech, D. J. (2003). Critical intracellular Ca2+ dependence of transient receptor potential melastatin 2 (TRPM2) cation channel activation. *J. Biol. Chem.* 278, 11002–11006. doi: 10.1074/jbc.M210810200

Mei, Z. Z., Xia, R., Beech, D. J., and Jiang, L. H. (2006). Intracellular coiled-coil domain engaged in subunit interaction and assembly of melastatin-related transient receptor potential channel 2. *J. Biol. Chem.* 281, 38748–38756. doi: 10.1074/jbc.M607591200

Meister, A., and Anderson, M. E. (1983). Glutathione. *Annu. Rev. Biochem.* 52, 711–760. doi: 10.1146/annurev.bi.52.070183.003431

Menassa, D. A., and Gomez-Nicola, D. (2018). Microglial dynamics during human brain development. *Front. Immunol.* 9:1014. doi: 10.3389/fimmu.2018.01014

Miller, B. A., and Zhang, W. (2011). TRP channels as mediators of oxidative stress. *Adv. Exp. Med. Biol.* 704, 531–544. doi: 10.1007/978-94-007-0265-3_29

Miyake, T., Shirakawa, H., Kusano, A., Sakimoto, S., Konno, M., Nakagawa, T., et al. (2014). TRPM2 contributes to LPS/IFNγ-induced production of nitric oxide via the p38/JNK pathway in microglia. *Biochem. Biophys. Res. Commun.* 444, 212–217. doi: 10.1016/j.bbrc.2014.01.022

Miyanohara, J., Kakae, M., Nagayasu, K., Nakagawa, T., Mori, Y., Arai, K., et al. (2018). TRPM2 channel aggravates CNS inflammation and cognitive

impairment via activation of microglia in chronic cerebral hypoperfusion. *J. Neurosci.* 38, 3520–3533. doi: 10.1523/JNEUROSCI.2451-17.2018

Ostapchenko, V. G., Chen, M., Guzman, M. S., Xie, Y. F., Lavine, N., Fan, J., et al. (2015). The transient receptor potential melastatin 2 (TRPM2) channel contributes to β-amyloid oligomer-related neurotoxicity and memory impairment. *J. Neurosci.* 35, 15157–15169. doi: 10.1523/JNEUROSCI.4081-14.2015

Perraud, A. L., Fleig, A., Dunn, C. A., Bagley, L. A., Launay, P., Schmitz, C., et al. (2001). ADP-ribose gating of the calcium-permeable LTRPC2 channel revealed by Nudix motif homology. *Nature* 411, 595–599. doi: 10.1038/35079100

Perraud, A. L., Takanishi, C. L., Shen, B., Kang, S., Smith, M. K., Schmitz, C., et al. (2005). Accumulation of free ADP-ribose from mitochondria mediates oxidative stress-induced gating of TRPM2 cation channels. *J. Biol. Chem.* 280, 6138–6148. doi: 10.1074/jbc.M411446200

Ramirez, A. I., De Hoz, R., Salobrar-Garcia, E., Salazar, J. J., Rojas, B., Ajoy, D., et al. (2017). The role of microglia in retinal neurodegeneration: Alzheimer's Disease, Parkinson, and glaucoma. *Front. Aging Neurosci.* 9:214. doi: 10.3389/fnagi.2017.00214

Ru, X., and Yao, X. (2014). TRPM2: a multifunctional ion channel for oxidative stress sensing. *Sheng Li Xue Bao* 66, 7–15.

Saijo, K., and Glass, C. K. (2011). Microglial cell origin and phenotypes in health and disease. *Nat. Rev. Immunol.* 11, 775–787. doi: 10.1038/nri3086

Salter, M. W., and Stevens, B. (2017). Microglia emerge as central players in brain disease. *Nat. Med.* 23, 1018–1027. doi: 10.1038/nm.4397

Sano, Y., Inamura, K., Miyake, A., Mochizuki, S., Yokoi, H., Matsushime, H., et al. (2001). Immunocyte Ca2+ influx system mediated by LTRPC2. *Science* 293, 1327–1330. doi: 10.1126/science.1062473

Schilling, T., Lehmann, F., Ruckert, B., and Eder, C. (2004). Physiological mechanisms of lysophosphatidylcholine-induced de-ramification of murine microglia. *J. Physiol.* 557, 105–120. doi: 10.1113/jphysiol.2004.060632

Sheikh, A. M., Nagai, A., Ryu, J. K., Mclarnon, J. G., Kim, S. U., and Masuda, J. (2009). Lysophosphatidylcholine induces glial cell activation: role of rho kinase. *Glia* 57, 898–907. doi: 10.1002/glia.20815

Shetty, A. K., Kodali, M., Upadhya, R., and Madhu, L. N. (2018). Emerging anti-aging strategies - scientific basis and efficacy. *Aging Dis.* 9, 1165–1184. doi: 10.14336/AD.2018.1026

Shimizu, T., Dietz, R. M., Cruz-Torres, I., Strnad, F., Garske, A. K., Moreno, M., et al. (2016). Extended therapeutic window of a novel peptide inhibitor of TRPM2 channels following focal cerebral ischemia. *Exp. Neurol.* 283, 151–156. doi: 10.1016/j.expneurol.2016.06.015

Shimizu, T., Macey, T. A., Quillinan, N., Klawitter, J., Perraud, A. L., Traystman, R. J., et al. (2013). Androgen and PARP-1 regulation of TRPM2 channels after ischemic injury. *J. Cereb. Blood Flow Metab.* 33, 1549–1555. doi: 10.1038/jcbfm.2013.105

Sierra, A., Encinas, J. M., Deudero, J. J., Chancey, J. H., Enikolopov, G., Overstreet-Wadiche, L. S., et al. (2010). Microglia shape adult hippocampal neurogenesis through apoptosis-coupled phagocytosis. *Cell Stem Cell* 7, 483–495. doi: 10.1016/j.stem.2010.08.014

Sohal, R. S., and Weindruch, R. (1996). Oxidative stress, caloric restriction, and aging. *Science* 273, 59–63. doi: 10.1126/science.273.5271.59

Song, K., Wang, H., Kamm, G. B., Pohle, J., Reis, F. C., Heppenstall, P., et al. (2016). The TRPM2 channel is a hypothalamic heat sensor that limits fever and can drive hypothermia. *Science* 353, 1393–1398. doi: 10.1126/science.aaf7537

Song, L., Pei, L., Yao, S., Wu, Y., and Shang, Y. (2017). NLRP3 inflammasome in neurological diseases, from functions to therapies. *Front. Cell Neurosci.* 11:63. doi: 10.3389/fncel.2017.00063

Sousa, C., Golebiewska, A., Poovathingal, S. K., Kaoma, T., Pires-Afonso, Y., Martina, S., et al. (2018). Single-cell transcriptomics reveals distinct inflammation-induced microglia signatures. *EMBO Rep.* 19:e46171. doi: 10.15252/embr.201846171

Sun, Y., Sukumaran, P., Selvaraj, S., Cilz, N. I., Schaar, A., Lei, S., et al. (2018). TRPM2 promotes neurotoxin MPP+/MPTP-induced cell death. *Mol. Neurobiol.* 55, 409–420. doi: 10.1007/s12035-016-0338-9

Syed Mortadza, S. A., Sim, J. A., Neubrand, V. E., and Jiang, L. H. (2018). A critical role of TRPM2 channel in Aβ42 -induced microglial cell activation and generation of tumor necrosis factor-α. *Glia* 66, 562–575. doi: 10.1002/glia.23265

Syed Mortadza, S. A., Sim, J. A., Stacey, M., and Jiang, L. H. (2017). Signalling mechanisms mediating Zn2+-induced TRPM2 channel activation and cell death in microglial cells. *Sci. Rep.* 7:45032. doi: 10.1038/srep45032

Syed Mortadza, S. A., Wang, L., Li, D., and Jiang, L. H. (2015). TRPM2 channel-mediated ROS sensitive Ca2+ signaling mechanisms in immune cells. *Front. Immunol.* 6:407. doi: 10.3389/fimmu.2015.00407

Szepesi, Z., Manouchehrian, O., Bachiller, S., and Deierborg, T. (2018). Bidirectional microglia-neuron communication in health and disease. *Front. Cell Neurosci.* 12:323. doi: 10.3389/fncel.2018.00323

Takahashi, N., Kozai, D., Kobayashi, R., Ebert, M., and Mori, Y. (2011). Roles of TRPM2 in oxidative stress. *Cell Calcium* 50, 279–287. doi: 10.1016/j.ceca.2011.04.006

Tan, C. H., and McNaughton, P. A. (2016). The TRPM2 ion channel is required for sensitivity to warmth. *Nature* 536, 460–463. doi: 10.1038/nature19074

Tay, T. L., Savage, J. C., Hui, C. W., Bisht, K., and Tremblay, M. E. (2017). Microglia across the lifespan: from origin to function in brain development, plasticity and cognition. *J. Physiol.* 595, 1929–1945. doi: 10.1113/JP272134

Togashi, K., Hara, Y., Tominaga, T., Higashi, T., Konishi, Y., Mori, Y., et al. (2006). TRPM2 activation by cyclic ADP-ribose at body temperature is involved in insulin secretion. *EMBO J.* 25, 1804–1815. doi: 10.1038/sj.emboj.7601083

Tong, Q., Zhang, W., Conrad, K., Mostoller, K., Cheung, J. Y., Peterson, B. Z., et al. (2006). Regulation of the transient receptor potential channel TRPM2 by the Ca2+ sensor calmodulin. *J. Biol. Chem.* 281, 9076–9085. doi: 10.1074/jbc.M510422200

Tóth, B., and Csanády, L. (2010). Identification of direct and indirect effectors of the transient receptor potential melastatin 2 (TRPM2) cation channel. *J. Biol. Chem.* 285, 30091–30102. doi: 10.1074/jbc.M109.066464

Toth, B., Iordanov, I., and Csanady, L. (2015). Ruling out pyridine dinucleotides as true TRPM2 channel activators reveals novel direct agonist ADP-ribose-2'-phosphate. *J. Gen. Physiol.* 145, 419–430. doi: 10.1085/jgp.201511377

Tschopp, J., and Schroder, K. (2010). NLRP3 inflammasome activation: the convergence of multiple signalling pathways on ROS production? *Nat. Rev. Immunol.* 10, 210–215. doi: 10.1038/nri2725

Tsuda, M., Masuda, T., Tozaki-Saitoh, H., and Inoue, K. (2013). Microglial regulation of neuropathic pain. *J. Pharmacol. Sci.* 121, 89–94. doi: 10.1254/jphs.12R14CP

Tsutsui, M., Hirase, R., Miyamura, S., Nagayasu, K., Nakagawa, T., Mori, Y., et al. (2018). TRPM2 exacerbates central nervous system inflammation in experimental autoimmune encephalomyelitis by increasing production of CXCL2 chemokines. *J. Neurosci.* 38, 8484–8495. doi: 10.1523/JNEUROSCI.2203-17.2018

Voet, S., Prinz, M., and Van Loo, G. (2018). Microglia in central nervous system inflammation and multiple sclerosis pathology. *Trends Mol. Med.* 25, 112–123. doi: 10.1016/j.molmed.2018.11.005

Wang, L., Fu, T. M., Zhou, Y., Xia, S., Greka, A., and Wu, H. (2018). Structures and gating mechanism of human TRPM2. *Science* 362:eaav4809. doi: 10.1126/science.aav4809

Wang, W. Y., Tan, M. S., Yu, J. T., and Tan, L. (2015). Role of pro-inflammatory cytokines released from microglia in Alzheimer's disease. *Ann. Transl. Med.* 3:136. doi: 10.3978/j.issn.2305-5839.2015.03.49

Wei, L., Syed Mortadza, S. A., and Jiang, L. H. (2018). Melastatin-related transient receptor potential 2 channel in Abeta42-induced neuroinflammation: implications to Alzheimer's disease mechanism and development of therapeutics. *Neural Regen. Res.* 13, 419–420. doi: 10.4103/1673-5374.228720

White, C. S., Lawrence, C. B., Brough, D., and Rivers-Auty, J. (2017). Inflammasomes as therapeutic targets for Alzheimer's disease. *Brain Pathol.* 27, 223–234. doi: 10.1111/bpa.12478

Xia, R., Mei, Z. Z., Mao, H. J., Yang, W., Dong, L., Bradley, H., et al. (2008). Identification of pore residues engaged in determining divalent cationic permeation in transient receptor potential melastatin subtype channel 2. *J. Biol. Chem.* 283, 27426–27432. doi: 10.1074/jbc.M801049200

Xu, C., Macciardi, F., Li, P. P., Yoon, I. S., Cooke, R. G., Hughes, B., et al. (2006). Association of the putative susceptibility gene, transient receptor potential protein melastatin type 2, with bipolar disorder. *Am. J. Med. Genet. B Neuropsychiatr. Genet.* 141B, 36–43. doi: 10.1002/ajmg.b.30239

Yamamoto, S., and Shimizu, S. (2016). Targeting TRPM2 in ROS-coupled diseases. *Pharmaceuticals* 9:E57. doi: 10.3390/ph9030057

Ye, M., Yang, W., Ainscough, J. F., Hu, X. P., Li, X., Sedo, A., et al. (2014). TRPM2 channel deficiency prevents delayed cytosolic Zn2+ accumulation and CA1 pyramidal neuronal death after transient global ischemia. *Cell Death Dis.* 5:e1541. doi: 10.1038/cddis.2014.494

Yirmiya, R., Rimmerman, N., and Reshef, R. (2015). Depression as a microglial disease. *Trends Neurosci.* 38, 637–658. doi: 10.1016/j.tins.2015.08.001

Yürüker, V., Naziroðlu, M., and §cenol, N. (2015). Reduction in traumatic brain injury-induced oxidative stress, apoptosis, and calcium entry in rat hippocampus by melatonin: possible involvement of TRPM2 channels. *Metab. Brain Dis.* 30, 223–231. doi: 10.1007/s11011-014-9623-3

Zhang, W., Chu, X., Tong, Q., Cheung, J. Y., Conrad, K., Masker, K., et al. (2003). A novel TRPM2 isoform inhibits calcium influx and susceptibility to cell death. *J. Biol. Chem.* 278, 16222–16229. doi: 10.1074/jbc.M300298200

Zhang, Z., Tóth, B., Szollosi, A., Chen, J., and Csanády, L. (2018). Structure of a TRPM2 channel in complex with Ca2+ explains unique gating regulation. *eLife* 7:e36409. doi: 10.7554/eLife.36409

Zhong, J., Amina, S., Liang, M., Akther, S., Yuhi, T., Nishimura, T., et al. (2016). Cyclic ADP-ribose and heat tegulate oxytocin release via CD38 and TRPM2 in the hypothalamus during social or psychological stress in mice. *Front. Neurosci.* 10:304. doi: 10.3389/fnins.2016.00304

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REVIEW
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Targeting Microglia and Macrophages: A Potential Treatment Strategy for Multiple Sclerosis

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Multiple sclerosis (MS) is a chronic inflammatory neurodegenerative disease of the central nervous system (CNS). The early stage is characterized by relapses and the later stage, by progressive disability. Results from experimental and clinical investigations have demonstrated that microglia and macrophages play a key part in the disease course. These cells actively initiate immune infiltration and the demyelination cascade during the early phase of the disease; however, they promote remyelination and alleviate disease in later stages. This review aims to provide a comprehensive overview of the existing knowledge regarding the neuromodulatory function of macrophages and microglia in the healthy and injured CNS, and it discusses the feasibility of harnessing microglia and macrophage physiology to treat MS. The review encourages further investigations into macrophage-targeted therapy, as well as macrophage-based drug delivery, for realizing efficient treatment strategies for MS.

Keywords: multiple sclerosis, microglia, macrophages, central nervous system, targeted therapy

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INTRODUCTION

Multiple sclerosis, a CNS autoimmune and neurodegenerative disease, affects approximately 2.5 million people worldwide, seriously diminishing their quality of life and causing a significant financial burden (Compston and Coles, 2008; Lemus et al., 2018; Thompson et al., 2018). Studies using EAE, an animal model of MS, have revealed that microglia/macrophages actively participate in the pathogenesis of EAE progression (Jiang et al., 2014). The CNS contains several resident macrophages, such as microglia and non-parenchymal macrophages, in the choroid plexus, perivascular space, and meninges, which function to maintain CNS homeostasis (Prinz et al., 2011). Alterations in CNS homeostasis lead to the recruitment of peripheral blood-derived monocytes (known as monocyte-derived macrophages) into the CNS, the activation of microglia, and the presence of foamy macrophages (Bogie et al., 2014; Kuhlmann et al., 2017; Mrdjen et al., 2018; Zéphir, 2018). This phenomenon appears to be a type of self-protective mechanism designed to eliminate abnormalities and recover a steady state.

Abbreviations: APCs, antigen-presenting cells; BBB, blood–brain barrier; BDNF, brain-derived neurotrophic factor; CCL, C-C motif chemokine ligands; CNS, central nervous system; CSF, cerebrospinal fluid; CXCL, C-X-C motif chemokine ligands; EAE, experimental autoimmune encephalomyelitis; IFN, interferon; IL, interleukin; MHC, major histocompatibility complex; MS, multiple sclerosis; TNF, tumor necrosis factor.

Monocyte-derived macrophages and CNS-resident phagocytic cells can promote neuroinflammation, thereby inducing MS, but they can also play neuroprotective and anti-inflammatory roles, depending on many factors. In this review, we discuss the protective and pathogenic mechanism of microglia and macrophages in the development of MS and EAE, and propose the use of macrophages in MS therapy for repairing CNS damage.

MICROGLIA AND MACROPHAGES IN A HEALTHY CNS

In a healthy CNS, microglia reside in the parenchyma, while non-parenchymal macrophages are present in boundary regions, including perivascular spaces, the meninges, and the choroid plexus. These cells have different developmental origins and serve distinct functions by specific subtypes.

Origin of CNS Macrophages and Microglia

The earlier view that CNS macrophages are derived from blood-borne myeloid cells during adulthood has now been challenged, because studies using novel transgenic mouse models have shown that macrophages other than choroid plexus macrophages originate exclusively from the yolk sac (**Figure 1**; Gomez Perdiguero et al., 2015; Goldmann et al., 2016). The application of tamoxifen to adult *Cx3cr1*CreER:*R26*-YFP mice has further demonstrated that not only microglia but also perivascular and meningeal macrophages retain the yfp label from embryonic day (E)16.0 to 8–9 weeks of age (Goldmann et al., 2016), indicating that non-parenchymal meningeal and perivascular CNS macrophages and microglia arise entirely from embryonic precursor cells. Fate-mapping tools have helped reveal that CNS macrophages arise from the primitive c-kit$^+$ erythromyeloid precursors located in the yolk sac during E7.5∼8.0. Uncommitted erythromyeloid precursors subsequently disappear at E9.0 and develop into CD45$^+$CX3CR1^{hi}F4/80^{hi} (A2) macrophage progenitors via immature CD45$^+$CX3CR1^{lo}F4/80^{lo} (A1) cells. Proliferating A2 cells then become CD11b$^+$F4/80$^+$ microglia and perivascular, meningeal, and choroid plexus macrophages (Bertrand et al., 2005; Kierdorf et al., 2013; Hoeffel et al., 2015; Matcovitch-Natan et al., 2016). Once established in the CNS, microglia and perivascular and meningeal macrophages persist throughout the life of the organism due to their longevity and their capacity of self-proliferation, rather than the infiltration of peripheral myeloid cells (Ajami et al., 2007, 2011). In contrast, choroid plexus macrophages mainly depend on blood-derived immigrating Ly6C^{hi} monocytes after birth (Goldmann et al., 2016). The astonishing finding of such close ontogeny between microglia and the other non-parenchymal macrophages in the CNS prompts the question of whether they can transform into one another under specific physiological conditions.

Functions of Microglia

Microglia are widely scattered throughout the brain and spinal cord, and they come into close contact with neurons, astrocytes, and oligodendrocytes (Ransohoff and Cardona, 2010; Szepesi et al., 2018). Studies have demonstrated that microglia mediate synaptic pruning and trophic-factor production through fractalkine-CX3CR1 signaling in neurodevelopment (Tremblay et al., 2010; Ransohoff and El Khoury, 2014; Schafer and Stevens, 2015; Miyamoto et al., 2016). Fractalkine receptor-deficient mice showed decreased neuron survival in layer V cortical neurons during the first postnatal week because of the reduced secretion of insulin-like growth factor from microglia (Ueno et al., 2013). Furthermore, microglia depletion by the *Cre-Lox* technique led to deficiencies in multiple learning tasks, as well as a significant reduction in learning-related synapse formation (Parkhurst et al., 2014). In addition, microglial-secreted BDNF could increase the activity of neuronal tropomyosin-related kinase receptor B, an important modulator in synaptic plasticity, prompting a crucial role of BDNF signaling in learning and memory-related synapse formation (Parkhurst et al., 2014). Microglia may communicate with neurons; changes in the physiological environment such as chronic stress and light deprivation can cause hyper-ramification of microglia and more frequent neuron–microglia contacts (Torres-Platas et al., 2014; Welberg, 2014; Yirmiya et al., 2015). Moreover, microglia act as scavengers in the adult CNS to remove damaged components, apoptotic cells, and misfolded proteins (Sierra et al., 2010). Once "danger" signals are detected in the surrounding environment, microglia rapidly change into a reactive phenotype, characterized by larger soma, and release various complement factors to facilitate neuron and tissue repair (Hanisch and Kettenmann, 2007; Graeber and Streit, 2010; Kettenmann et al., 2011).

Functions of Non-parenchymal Macrophages

Non-parenchymal brain macrophages have been largely ignored, but recent studies have refocused on their relevant functions in the perivascular space, meninges, and choroid plexus (Herz et al., 2017). Perivascular macrophages are critical in BBB establishment (Mendes-Jorge et al., 2009; He et al., 2016), and blockage of colony stimulating factor 1 receptor signaling leads to a decreased coverage of pericytes in brain vessels (Yamamoto et al., 2017). Perivascular macrophages continuously retract and protract along blood vessels in the adult brain (Daneman, 2012; Goldmann et al., 2016), suggesting that they may play a role in immune surveillance. Moreover, they can protect against bacterial infection by recruiting circulating leukocytes (Polfliet et al., 2001). The study of meningeal macrophages has been limited; however, it was reported that meninges possess a "glymphatic" system, which guides the clearance of waste products such as β-amyloid in the CNS (Raper et al., 2016). However, whether meningeal macrophages instruct lymphangiogenesis in the "glymphatic" system, similar to their role in the peripheral immune system, remains to be ascertained (Gordon et al., 2011; Louveau et al., 2015). In addition, meningeal macrophages function as APCs, detecting cell debris and antigens to maintain immune homeostasis (Kivisakk et al., 2009). Choroid plexus macrophages are located near the microvilli of the choroid plexus (Liddelow, 2015; Marques et al., 2017), and their main function may support CSF release and flux (Goldmann et al., 2016).

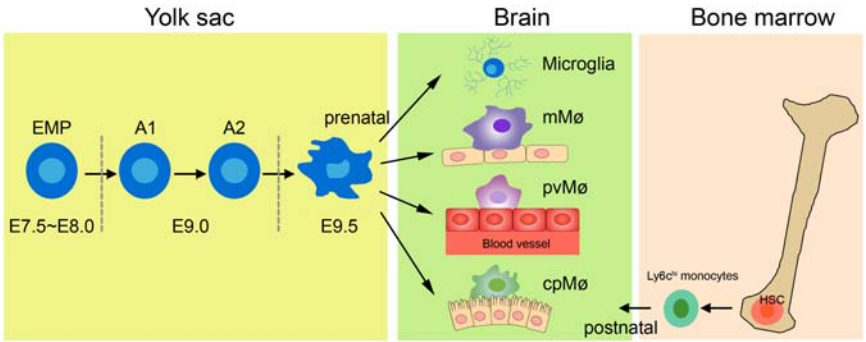

FIGURE 1 | Origin of tissue macrophages in healthy CNS. Microglia and tissue-resident macrophages are derived from prenatal sources. The primitive erythromyeloid precursors (EMPs) located in the yolk sac during E7.5∼8.0 soon develop into A1 cells and finally become A2 cells. The matured A2 cells then differentiate into microglia, meningeal macrophages (mMø), perivascular macrophages (pvMø), and choroid-plexus macrophages (cpMø). However, after birth, cpMΦ originate exclusively from bone Ly6c^{hi} monocytes.

In conclusion, considerable fundamental details about non-parenchymal macrophages remain to be determined.

Polarization of Microglia and Macrophages

Macrophages (including microglia) are often classified into M1 (inflammatory) and M2 (anti-inflammatory) phenotypes (Mikita et al., 2011; Liu et al., 2013). However, this simple classification does not reveal the full spectrum of macrophages under different stimulation conditions owing to their diversity and plasticity (Prinz and Priller, 2014). Transcriptome profiles have revealed at least nine different subtypes of human macrophages (Veremeyko et al., 2018). In this review, M1 and M2 represent the activation states of macrophages rather than specific cell subtypes.

Interferon-γ and lipopolysaccharide treatment can generate M1 cells, while IL-4, IL-10, or IL-13 are activators of M2 cells. M1 macrophages and microglia are characterized by an amoeboid shape, and they release pro-inflammatory cytokines, such as IL-6, IFN-γ, IL-23, TNF-α; inducible nitric oxide synthase; chemokines, including CCL 4, CCL5, and CCL8; and CXCL 2, CXCL4, and CXCL9 (Juhas et al., 2015; Kapellos and Iqbal, 2016). M1 macrophages and microglia are potent APCs, causing an adaptive immune response to clear foreign substances and abnormal proteins (Das et al., 2015). In contrast, M2 macrophages and microglia have smaller cell bodies and a branched structure (Ransohoff and Perry, 2009), and they express a variety of anti-inflammatory molecules, including IL-4, IL-10, IL-13, and transforming growth factor-β (David and Kroner, 2011). These contribute to immunoregulation by inhibiting inflammation, remodeling tissue, promoting angiogenesis, and clearing parasites (Banerjee et al., 2013). M2 microglia promote oligodendrocyte differentiation, and their depletion inhibits remyelination (Kotter et al., 2005; Miron et al., 2013). M2-polarized macrophages and microglia can be further subdivided into M2a, M2b, and M2c cells. These have different activation and functional mechanisms but have some biochemical overlap (**Table 1**; Murray et al., 2014). Increasing evidence suggests that M1 cells prefer an anaerobic environment, whereas oxygen consumption and oxidative phosphorylation benefit M2 macrophages (Shi et al., 2017; Wang et al., 2018).

In conclusion, microglia and non-parenchymal macrophages are independent immune populations in the CNS. Besides different cell origins, they have their own functions in CNS development, homeostasis, and tissue healing (Lopez-Atalaya et al., 2018). Under steady state, microglia are primarily responsible for supporting the development of the healthy brain, they shape and maintain the neuronal synaptic network, interact with neurons and produce beneficial components (Hagemeyer et al., 2017). And non-parenchymal macrophages strictly limits access of peripheral immune cells (T cells, B cells, and monocytes) reach the CNS parenchyma (Polfliet et al., 2001). Upon detecting "danger" signals, both microglia and non-parenchymal macrophages produce large amounts of chemokines and cytokines to promote leukocyte infiltration into the CNS parenchyma and function as APCs to reactivate T cells in the CNS (Galli et al., 2011; Krausgruber et al., 2011). Moreover, microglia phagocytose damaged cell debris, as well as unnecessary neurons and synapses resulting from defective differentiation and/or migration (Paolicelli et al., 2011; Lopez-Atalaya et al., 2018).

MICROGLIA AND MACROPHAGES IN MS

After CNS injury and inflammation, while microglia situated in the parenchyma and macrophages in the choroid plexus, perivascular space, and the meninges are activated, monocytes from the blood cross the damaged BBB and become monocyte-derived macrophages in the CNS (Lassmann et al., 2001). These spatial differences may determine subtype-specific effector functions of these distinctive phagocytes in the diseased CNS. In this section, we compare and elaborate the functions of microglia and macrophages in the progression of MS and EAE.

Distinguishing Microglia From Other Macrophages

A lack of specific markers that distinguish resident microglia from other macrophages in MS lesions hampers the accurate

evaluation of their contributions to human brain pathology. Originally, the expression profiles of CD11b and CD45 allowed the discrimination of $CD11b^{+}CD45^{med}$ microglia from $CD11b^{+}CD45^{hi}$ monocyte-derived macrophages (Martin et al., 2017). However, such a classification remains controversial (Koeniger and Kuerten, 2017). Studies have suggested TMEM119 (Bennett et al., 2016; Satoh et al., 2016; Li et al., 2019), Sall1 (Buttgereit et al., 2016), Siglec-H (Konishi et al., 2017), and P2Y12 (Mildner et al., 2017) as more valuable markers for distinguishing microglia from other macrophages. For instance, TMEM119 is expressed exclusively on $Iba1^{+}CD68^{+}$ microglia and not on infiltrated $Iba1^{+}CD68^{+}$ macrophages within demyelinating lesions of MS (Satoh et al., 2016). However, TMEM119 expression is absent in immature microglia (Satoh et al., 2016). Sall1 expression is abundant in neuronal and glial progenitor cells during CNS development (Harrison et al., 2012; Buttgereit et al., 2016), and P2Y12 shows decreased expression in activated microglia (Amadio et al., 2014; Mildner et al., 2017). Almost all microglia in the CNS parenchyma expressed Siglec-H, from developmental to mature stages, and the expression was maintained in activated microglia after CNS injuries (Koso et al., 2018). In contrast, Siglec-H expression was largely absent from other myeloid cells in the CNS (Koso et al., 2018). Overall, these findings suggest that Siglec-H is a promising specific marker for the specific identification of microglia from CNS-associated macrophages. However, these results are all from mice, and human Siglec-L2 is only ∼42% homologous with Siglec-H (Zhang et al., 2006). Whether Siglec-L2 might be a marker for microglia warrants further investigation.

Microglia

Pronounced microglial activation with high expression levels of pro-inflammatory genes can be found in lesion areas and normal-appearing white matter of MS patients (Lucchinetti et al., 2000; Singh et al., 2013; Zrzavy et al., 2017). These clusters of activated microglia are regarded as "pre-active lesions," with an absence of leukocyte infiltration and demyelination; however, they may eventually develop into active demyelinating MS lesions (Singh et al., 2013). In contrast to healthy controls, the number of $P2Y12^{+}$ homeostatic microglia is significantly reduced in the white matter of MS patients, and $TMEM119^{+}$ microglia predominantly govern the edge of active lesions in MS (Zrzavy et al., 2017), indicating that microglial activation is related to disease development.

TSPO tracers have demonstrated inflammatory processes with microglia involvement in MS (Vas et al., 2008; Oh et al., 2011). These microglia are enriched with iron, express pro-inflammatory cytokines, and cause persistent tissue damage (Wisnieff et al., 2015). Although microglia are important in synaptic plasticity, this function is destroyed under pathological insults, which causes synaptic loss in MS and eventually, cognitive decline (Michailidou et al., 2015; Hong et al., 2016; Salter and Stevens, 2017; Di Filippo et al., 2018). Activated microglia in EAE mice may produce large amounts of TNF-α and thus induce excessive glutamate, leading to spontaneous and miniature excitatory post-synaptic currents (Centonze et al., 2009, 2010). Furthermore, astroglial ATP amplifies microglial signals and subsequently promotes glutamate release from astroglia, which directly inhibits synaptic transmission (Kettenmann et al., 2013). Classically activated neuroinflammatory microglia secrete TNF-α, and C1q, inducing A1 astrocyte dysfunction, thereby resulting in the dysfunction of both oligodendrocytes and neurons (Liddelow et al., 2017; Rothhammer et al., 2018). Importantly, mitochondrial disturbances have been demonstrated in modulating MS lesions, reactive oxygen species and reactive nitrogen species generated by the activated microglia also lead to intra-axonal mitochondrial injury (Balaban et al., 2005; Haile et al., 2017). Kinetics studies from EAE mice indicate that microglia are the first cell line to take up myelin antigens (Sosa et al., 2013), subsequently restimulating encephalitogenic T cells in the CNS through major histocompatibility complex (MHC) molecules and costimulatory molecules (Perry, 1998). Consistent with this, time-course studies in EAE mice showed that the appearance of inflammatory T cells in the CNS is consistent with the activation of $CD11b^{+}$ microglia (Murphy et al., 2010). However, specific deletion of MHCII in microglia does not affect disease progression, suggesting that microglia are not important in reactivating efficient T cells in the CNS (Wolf et al., 2018). Microglia also secrete a series of pro-inflammatory cytokines such as IL-18, IL-6, and IL-1b and chemokines like CCL2 and CCL5 to aggravate both MS and EAE (Merson et al., 2010; Jiang et al., 2014). Moreover, activated microglia in white matter lesions may lead to greater brain atrophy in secondary progressive disease (Datta et al., 2017). In conclusion, microglia exert detrimental functions in MS and strongly damage myelin.

Microglial activation may also be beneficial for remyelination in MS due to the phagocytotic ability of microglia, as well as their secretion of neuroprotective molecules and anti-inflammatory cytokines (Du et al., 2017). Microglia-specific deletion of A20, the crucial protein in regulating microglial activation, makes microglia lose their ability to regulate neuronal synaptic function, thus exacerbating MS-like disease (Voet et al., 2018). Several receptors, including TREM2, complement receptor 3, and signal regulatory protein-α, invoke microglia to phagocytose myelin debris in lesions (Brendecke and Prinz, 2015). Microglia-specific ablation of TREM2 cannot upregulate genes associated with phagocytosis (Poliani et al., 2015), and the blockage of TREM2 results in increased demyelination in EAE (Piccio et al., 2007). Moreover, microglia secrete

TABLE 1 | Polarization of macrophages and microglia (Murray et al., 2014).

Phenotype	Stimulation	Marker	Function
M1	IFN-γ, lipopolysaccharide	CD86, CD40, MHCII	T cell priming
M2a	IL-4, IL-13	CD206, FIZZ1, ARG1, YM1	Immunity against parasites, tissue repair, collagen formation
M2b	Immune complexes	MHC-II, CD86	Recruitment of regulatory T cells
M2c	IL-10, TGF-β1, glucocorticoids	CD163	Wound healing

trypsinogen, insulin-like growth factor, and fibroblast growth factor during activation to promote neurogenesis (Pérez-Martín et al., 2010; Voss et al., 2012). Anti-inflammatory molecules from microglia also benefit remyelination in EAE, for example, IL-4 enhances oligodendrogenesis (Butovsky et al., 2006), and activin A drives oligodendrocyte differentiation (Miron et al., 2013). Interestingly, pro-inflammatory cytokine TNF has neuroprotective functions in EAE (Wolf et al., 2017), and deletion of microglial TNFR2 causing early disease onset, with increased leukocyte infiltration, demyelination and T cell activation in CNS (Gao et al., 2017). However, soluble TNF repair remyelination by suppressing clearance of myelin debris (Karamita et al., 2017). Other evidence indicated that microglia can prompt remyelination through P2X4R signaling, and that the blockade of P2X4R results in microglial activation to a pro-inflammatory phenotype and exacerbates the clinical symptoms of EAE mice (Zabala et al., 2018).

Monocyte-Derived Macrophages

During the effector stage of EAE, Ly6C^{hi} monocytes rapidly infiltrate into the inflamed CNS with the help of matrix metalloproteinases (Ajami et al., 2011; Yamasaki et al., 2014). Monocytes then differentiate into macrophages, express MHC II and costimulatory molecules, and produce pro-inflammatory factors that actively contribute to the demyelination process (Jiang et al., 2014; Yamasaki et al., 2014). Consistently, the abundance of CCR1^{+}/CCR5^{+} hematogenous monocytes has been found to appear in demyelination zones compared with the periplaque white matter of MS patients (Trapp et al., 1998; Trebst et al., 2001), and Kim1p staining shows abundant subcortical macrophage infiltration (Tobin et al., 2017).

The activated monocyte-derived macrophages is generally considered to be harmful in MS (Yamasaki et al., 2014). Infiltrating monocytes accumulate at the nodes of Ranvier during early stages of EAE and actively initiate the demyelination of axons (Yamasaki et al., 2014), similar to microglia, but they do not affect CNS-resident microglia (Ajami et al., 2011). Bone marrow chimeric mice show that TRPM2 expressed by CNS-infiltrating macrophages prompts the production of CXCL2, thus increasing neutrophil infiltration and contributing to the progression of EAE (Tsutsui et al., 2018). High F4/80^{+}CD45^{hi} macrophage/T-cell ratios were found in EAE mice (McMahon et al., 2005), indicating that recruited macrophages participate in T-cell proliferation after myelin antigen stimulation. Macrophages also secrete large amounts of pro-inflammatory cytokines, such as IL-6, IL-12, TNF-α, IL-1, and IL-23, to aggravate the inflammatory response (Valentin-Torres et al., 2016). IL-1β expression has been found to be pivotal in the polarization of Th17 cells and therefore for EAE induction (Sutton et al., 2006). IL-6 has also been demonstrated to be critical for CNS autoimmunity, as IL-6-deficient mice demonstrated alleviated EAE (Samoilova et al., 1998). Macrophages also express high levels of CCR4 during the disease, and CCR4 deletion leading to reduced accumulation of macrophages in CNS and attenuated EAE symptoms (Forde et al., 2011). These findings suggest that macrophages are responsible for demyelination, but their direct functions on axons remain to be determined.

Other studies have also defined the neuron-supportive activities of Ly6C^{hi} monocytes in EAE. The activation of invariant natural killer T cells converts inflammatory Ly6C^{hi} monocytes to M2 macrophages in the CNS, with associated improvement in neurological impairment (Denney et al., 2012; Jiang et al., 2017). Recent studies have showed that TG2 mRNA levels are increased in monocytes derived from MS patients and correlates with anti-inflammatory cytokine expression, proposing a more anti-inflammatory monocytes status in MS (Sestito et al., 2017). Monocyte-derived macrophages can also promote CNS repair in the injured spinal cord by clearing myelin debris through scavenger receptors (GrandPré et al., 2000; Kotter et al., 2006). In contrast to microglia, macrophages express TREM-1 rather than TREM-2, and although this isoform plays a role in inflammatory responses (Colonna and Facchetti, 2003), the functions of TREM-1 still need to be further studied in a CNS autoimmunity context. In addition, monocyte-derived macrophages secrete trophic factors and anti-inflammatory factors to alleviate sympathetic neuron dysfunction (Hikawa and Takenaka, 1996; Miron et al., 2013). The transcription factor Nr4a1 represses the autocrine production of norepinephrine in macrophages, and myeloid cells lacking Nr4a1 lead to more leukocyte infiltration within the CNS and more severe EAE clinical symptoms (Shaked et al., 2015). Consistently, expression levels of Nr4a were down-regulated in peripheral blood mononuclear cells from MS patients, and Fingolimod treatment could recovery MS from Nr4a2 deficit (Montarolo et al., 2018). Besides Ly6C^{hi} monocytes, a small number of LyC6^{lo} monocytes also recruited in the injured CNS (Saederup et al., 2010). Although studies have pointed toward a reparative role of infiltrated Ly6C^{lo} monocytes in a spinal cord injury model (Arnold et al., 2007; Nahrendorf et al., 2007); however, more research is needed to determine whether Ly6C^{lo} monocytes do promote repair processes in the pathogenesis of EAE.

Non-parenchymal Macrophages

Perivascular macrophages, typically CD163^{+}HLADR^{+}, are markedly increased in MS patients and EAE-affected animals (Zhang et al., 2011; Mammana et al., 2018). Substantial evidence indicates that perivascular macrophages have both supportive and neuroprotective roles in MS. Selective elimination of both perivascular and meningeal macrophages by treatment with clodronate-loaded liposomes reduces the progression of EAE in rats (Polfliet et al., 2002), suggesting that perivascular macrophages may act as competent APCs in EAE. EAE-related perivascular macrophages upregulate the expression of MHC II and can present peptides to myelin-specific T cells in the CNS, thereby accelerating disease progression (Perry, 1998; Fabriek et al., 2005; Greter et al., 2005; Vogel et al., 2013). Furthermore, perivascular macrophages express high levels of adhesion molecules such as VCAM-1 and ICAM-1 and chemokines like CCL2 and CCL3 (Hofmann et al., 2002; Vercellino et al., 2017), which may mediated by chondroitin sulfate proteoglycans (Stephenson et al., 2018), to promote leukocyte infiltration into the CNS. However, perivascular macrophages during the early stage of EAE are characterized as mannose receptor-positive M2 macrophages, produce leukemia

inhibitory factors and neuroprotective factors, and secrete anti-inflammatory IL-6 and IL-1ra to protect glial and neuronal cells (Boven et al., 2006; Vanderlocht et al., 2006). Moreover, M2 perivascular macrophages can promote the differentiation of Th2 and Treg cells due to their anti-inflammatory characteristic (Weber et al., 2007).

An extensive number of meningeal immune cells in the CNS leads to a more severe clinical course of MS (Howell et al., 2011; Choi et al., 2012; Kilsdonk et al., 2017; Kolber et al., 2017; Bevan et al., 2018). In concordance, meningeal macrophages are equipped to present peptide antigens to T cells, triggering neuroinflammatory events (Bartholomäus et al., 2009; Kivisakk et al., 2009; Ransohoff and Cardona, 2010). In addition, meningeal macrophages can release inflammatory and toxic mediators or even invade the CNS parenchyma to sustain MS pathology (Maxwell et al., 1990; Garabedian et al., 2000; Reuter et al., 2001). In contrast, meningeal macrophages produce neurotrophic and anti-inflammatory factors to inhibit leukocyte activity and their infiltration into the CNS at particular disease stages (Bogie et al., 2014). Leptomeningeal macrophages increase the expression of transforming growth factor-β in glial cells and cortical neurons by releasing prostaglandin E2 during inflammation (Wu et al., 2007). However, other studies demonstrated that prostaglandin E2 could also exacerbate EAE (Lima et al., 2012), depending on the disease stage and microenvironment.

The choroid plexus, the site of CSF production, forms the interface between blood and the CNS (Wolburg and Paulus, 2010; Kaur et al., 2016). It is thus an important element in the communication between the vascular compartment and the CNS during inflammation (Breuer et al., 2018). The severity of MS coincides with enhanced levels of infiltrated leukocytes in the choroid plexus (Engelhardt et al., 2001; Vercellino et al., 2008; Mitchell et al., 2009), strongly indicating that inflammation at the choroid plexus contributes to the disease. Chemokines like CXCL9 and CXCL10 accumulate in large quantities in the CSF during MS progression (Edwards et al., 2013; Puthenparampil et al., 2017), but whether they are secreted by macrophages needs to be confirmed. Choroid plexus macrophages may release neurotoxic mediators and pro-inflammatory factors into the CSF during disease to impact perivascular and meningeal inflammation, as well as the activation and integrity of neurons and glial cells (Garabedian et al., 2000; Bragg et al., 2002a,b). However, remote brain choroid plexus can also recruit M2-like macrophages to an injured spinal cord, which might benefit myelin repair (Shechter et al., 2013).

Foamy Macrophages

Ample evidence indicates that abundant foamy macrophages are present in MS lesions (Grajchen et al., 2018). After disease initiation, both resident microglia and recruited monocytes ingest and accumulate vast amounts of myelin-derived lipids and thereby acquire their distinctive morphology (Li et al., 1996; Bogie et al., 2017), but how foamy macrophages function in the process of MS is still poorly understood. MHC II and costimulatory molecules expressed on foamy macrophages may present myelin antigens to autoreactive T cells (de Vos et al., 2002; Boven et al., 2006; van Zwam et al., 2011), thereby driving epitope spread and MS progression or even initiation (Stys et al., 2012). In agreement with this, numerous studies have found M1-like foamy macrophages present within MS lesions (Bogie et al., 2014). Interestingly, yet another study showed that microglia show the M1 phenotype after the first 6 h of myelin uptake, and they soon convert to the M2 phenotype after prolonged exposure (Liu et al., 2006), indicating that the polarization of foamy macrophages can be reversed. Both *in vivo* studies and *in vitro* cultures demonstrated that foamy macrophages prefer to express anti-inflammatory molecules rather than secrete pro-inflammatory cytokines (van Zwam et al., 2011), indicating that myelin macrophages may resolve the inflammation in MS demyelination lesions. Consistent with this, researchers found that mouse myelin macrophages could inhibit IFN-γ release by Th1 cells and suppress EAE severity (Bogie et al., 2014). Collectively, these studies imply that besides being aggressors in MS pathogenesis, foamy macrophages can also destroy T cell-induced autoimmunity. However, more research is warranted to confirm the specific role of foamy macrophages in MS progression.

In fact, among numerous phagocytic cells, only microglia and infiltrating macrophages play a crucial role in MS progression. Both of them participate in demyelination and remyelination through antigen presentation, phagocytosis, and inflammatory mediator secretion (Shechter et al., 2009; Ruckh et al., 2012; Li and Barres, 2018); however, microglia alone could affect neuron cells directly through fractalkine-CX3CR1 signaling (Eyo et al., 2016). Although microglia and infiltrating macrophages are potent APCs in EAE, the expression levels of MHC II and costimulatory molecules on microglia are lower (Brendecke and Prinz, 2015), indicating that inflammation in the CNS is mainly activated by infiltrating macrophages. The roles of perivascular macrophages, as well as macrophages, in the meninges and choroid plexus in MS are not yet completely understood. In conclusion, whether these macrophages are unique phagocyte subsets in MS, and if so, the underlying mechanism thereof, needs to be determined.

THERAPEUTIC PERSPECTIVES

As discussed in the previous sections, any change in the physiological functions of macrophages and microglia can impact neuroinflammatory and neurodegenerative events. Therefore, selectively promoting macrophage polarization to the anti-inflammatory subtype is an attractive therapeutic option (**Figure 2**).

MS Therapy by Regulating Macrophages

Although the crucial role of macrophages and microglia in neurodegenerative events has been proven, therapeutics solely targeting these phagocytes are still lacking. Currently, the clinical treatment of MS includes recombinant IFNβ-1a, glatiramer acetate, and natalizumab to mainly regulate T and B cells (Cheng et al., 2017). Studies have found that the neuroprotective effects of glatiramer acetate are mediated by activated M2

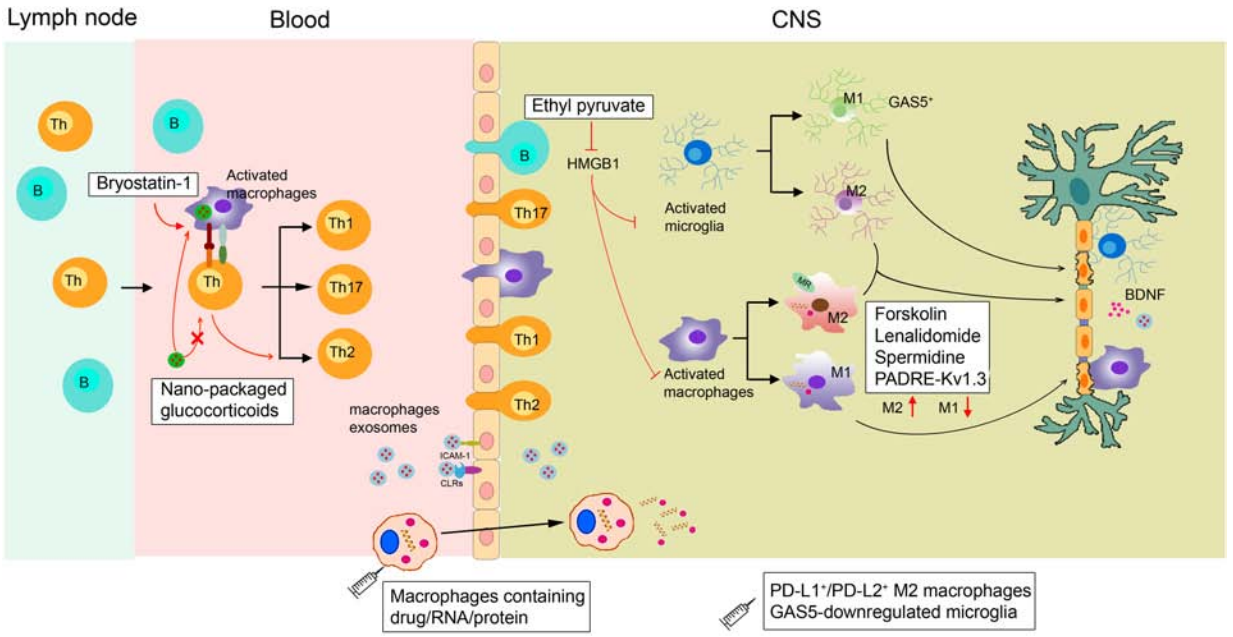

FIGURE 2 | Proposed actions of disease-modifying multiple sclerosis therapies targeting macrophages. In the periphery, bryostatin-1 promotes the differentiation of lymphocytes into anti-inflammatory Th2 cells by acting on macrophages. Ethyl pyruvate prevents the activation of macrophages/microglia within the CNS. Lenalidomide, spermidine, forskolin, and the novel PADRE-Kv1.3 vaccine can promote macrophage/microglia M2 polarization. Transplanting GAS5-downregulated microglia into the lateral ventricles of EAE mice could promote remyelination. Exogenous supplementation of M2 macrophages such as PD-L1$^+$/PD-L2$^+$ M2 cells can also alleviate demyelination. Micro-based drug delivery systems for delivering GC to targeted macrophages have been widely used in EAE. As macrophages can easily migrate into the brain during MS, they can be used as cellular vehicles for the delivery of small drugs, RNAs, and proteins to the brain parenchyma. Macrophage exosomes could cross the BBB and deliver BDNF to recover injury. HMGB1, high-mobility group box 1; GC, glucocorticoids; MR, mineralocorticoid receptors; CLRs, C-type lectin receptors.

microglia (Ratchford et al., 2012). Furthermore, diverse new compounds that influence macrophage and microglial functions are undergoing non-clinical testing. These agents include ethyl pyruvate (Djedović et al., 2017), spermidine (Yang et al., 2016), bryostatin-1 (Kornberg et al., 2018), forskolin (Veremeyko et al., 2018), and lenalidomide (Weng et al., 2018). Ethyl pyruvate can reduce high-mobility group box 1 expression in activated ED1$^+$ macrophages and Iba1$^+$ microglia, thus inhibiting the activation of macrophages/microglia within the CNS to protect against EAE (Djedović et al., 2017). In addition, forskolin has been found to alleviate EAE by suppressing the expression of CD86 while enhancing the expression of ARG1 in macrophages (Veremeyko et al., 2018). Bryostatin-1 provides marked benefits in mice with EAE by acting on APCs, including macrophages, to promote the differentiation of lymphocytes into Th2 cells (Kornberg et al., 2018).

The novel PADRE-Kv1.3 vaccine has been applied to treat EAE. After vaccination, infiltrated microglia/macrophages significantly shift toward the M2 subtype in the CNS (Fan et al., 2018). Mineralocorticoid receptors expressed on macrophages promote their polarization toward M2 cells, and mice selectively lacking mineralocorticoid receptors in their myeloid cells show diminished clinical symptoms of EAE (Montes-Cobos et al., 2017b). Transferring M2 macrophages to eliminate EAE has been widely applied. The long non-coding RNA GAS5, a suppresser of microglial M2 polarization, is expressed at a high level within microglia in MS patients. Transplanting GAS5-downregulated microglia into the lateral ventricles of EAE mice could promote remyelination, similar to a lysolecithin-induced demyelination model (Sun et al., 2017). In addition, Ly6C$^+$ monocytes could give rise to PD-L1$^+$/PD-L2$^+$ M2 cells, and transferring PD-L1$^+$/PD-L2$^+$ M2 macrophages might reduce the incidence of EAE (Terrazas et al., 2017).

Macrophage-Targeted Therapeutics via Micro-Based Drug Delivery Systems in Neurodegenerative Diseases

Because most drugs do not effectively reach macrophages at therapeutic levels, micro-based drug delivery systems for targeting macrophages have been widely used in treating CNS diseases. For example, glucocorticoids are used extensively to treat acute relapses in MS patients (Pato-Pato et al., 2003; Cohen et al., 2009), but their therapeutic effect is accompanied by serious side effects due to their broad spectrum of immunosuppressive actions (Baschant and Tuckermann, 2010). Interestingly, *in vivo*, glucocorticoids packaged with specific nanoparticles vs. free glucocorticoids are preferentially taken up by phagocytic cells, including macrophages, rather than T cells. This approach allows their therapeutic efficacy to be retained in EAE mice (Montes-Cobos et al., 2017a). Other research showed that infiltrated CD163$^+$ macrophages in brains

from a Parkinson's disease model modulate local microglia to promote neuroprotection. Specifically designed liposomes can load dexamethasone solely to $CD163^{+}$ macrophages. This modification leads to decreased dopaminergic cell death and better motor performance (Tentillier et al., 2016). However, we cannot ignore that several nanoparticles themselves also induce macrophage polarization toward various phenotypes and modulate macrophage reprogramming (Miao et al., 2017).

Macrophages as Drug Carriers for MS Therapy

Many CNS diseases are thought to be untreatable because drugs rarely pass through the BBB at therapeutic levels (Fischbach et al., 2013). Chemokine gradients secreted from the CNS parenchyma can induce macrophage migration to the brain, making macrophages potential carriers for drug/nano formulations across the BBB to reach target sites (Ye et al., 2018). Macrophages transporting superparamagnetic iron oxide nanoparticles and exogenous genes were injected intravenously into a lipopolysaccharide-induced acute neuroinflammatory mouse model. The differentiated macrophages demonstrated an excellent ability to spread into the brain, and the number of transported macrophages positively correlated with the number of intravenous cells (Tong et al., 2016). Moreover, macrophage exosomes can interact with brain microvessel endothelial cells that comprise the BBB by binding to carbohydrate-binding C-type lectin receptors. BBB cells can absorb more macrophage exosomes when inflammation occurs due to the upregulated secretion of ICAM-1. After intravenous administration, macrophage exosomes have been shown to cross the BBB and deliver a cargo protein, BDNF, in the presence of CNS inflammation (Yuan et al., 2017). Overall, these results support the concept of using monocytes/macrophages or macrophage exosomes as cellular vehicles for the delivery of small drugs, RNAs, and proteins to the brain parenchyma.

PERSPECTIVE

It is becoming increasingly clear that microglia and macrophages play critical roles in healthy, inflamed, injured, and recovering CNS due to their dual natures. We have summarized the origin, subtypes, and functions of these phagocytic cells in MS. Therapeutic interventions that block the pro-inflammatory effects of macrophages/microglia during disease progression, while preserving their anti-inflammatory functions on EAE, have achieved great success. However, as infiltrating inflammatory monocytes and microglia contribute differentially to EAE pathophysiology, strategies that allow the differential manipulation of CNS-resident microglia and infiltrating macrophages to optimally target different mechanisms operating in MS need to be developed. Moreover, extensive additional research is needed before utilizing the physiology of microglia and macrophage subsets for MS therapy.

AUTHOR CONTRIBUTIONS

QW and QH conceived the review article and made the corrections in the manuscript. BY provided some critical comments. JyW wrote the manuscript. JjW and JcW collected the related research articles.

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REFERENCES

Ajami, B., Bennett, J. L., Krieger, C., McNagny, K. M., and Rossi, F. M. (2011). Infiltrating monocytes trigger EAE progression, but do not contribute to the resident microglia pool. *Nat. Neurosci.* 14, 1142–1150. doi: 10.1038/nn.2887

Ajami, B., Bennett, J. L., Krieger, C., Tetzlaff, W., and Rossi, F. M. (2007). Local self-renewal can sustain CNS microglia maintenance and function throughout adult life. *Nat. Neurosci.* 10, 1538–1543. doi: 10.1038/nn2014

Amadio, S., Parisi, C., Montilli, C., Carrubba, A. S., Apolloni, S., and Volonté, C. (2014). P2Y(12) receptor on the verge of a neuroinflammatory breakdown. *Mediators Inflamm.* 2014:975849. doi: 10.1155/2014/975849

Arnold, L., Henry, A., Poron, F., Baba-Amer, Y., van Rooijen, N., Plonquet, A., et al. (2007). Inflammatory monocytes recruited after skeletal muscle injury switch into antiinflammatory macrophages to support myogenesis. *J. Exp. Med.* 204, 1057–1069. doi: 10.1084/jem.20070075

Balaban, R. S., Nemoto, S., and Finkel, T. (2005). Mitochondria, oxidants, and aging. *Cell* 120, 483–495. doi: 10.1016/j.cell.2005.02.001

Banerjee, S., Cui, H., Xie, N., Tan, Z., Yang, S., Icyuz, M., et al. (2013). MiR-125a-5p regulates differential activation of macrophages and inflammation. *J. Biol. Chem.* 288, 35428–35436. doi: 10.1074/jbc.M112.426866

Bartholomäus, I., Kawakami, N., Odoardi, F., Schläger, C., Miljkovic, D., Ellwart, J. W., et al. (2009). Effector T cell interactions with meningeal vascular structures in nascent autoimmune CNS lesions. *Nature* 462, 94–98. doi: 10.1038/nature08478

Baschant, U., and Tuckermann, J. (2010). The role of the glucocorticoid receptor in inflammation and immunity. *J. Steroid Biochem. Mol. Biol.* 120, 69–75. doi: 10.1016/j.jsbmb.2010.03.058

Bennett, M. L., Bennett, F. C., Liddelow, S. A., Ajami, B., Zamanian, J. L., Fernhoff, N. B., et al. (2016). New tools for studying microglia in the mouse and human CNS. *Proc. Natl. Acad. Sci. U.S.A.* 113, E1738–E1746. doi: 10.1073/pnas.1525528113

Bertrand, J. Y., Jalil, A., Klaine, M., Jung, S., Cumano, A., and Godin, I. (2005). Three pathways to mature macrophages in the early mouse yolk sac. *Blood* 106, 3004–3011. doi: 10.1182/blood-2005-02-0461

Bevan, R. J., Evans, R., Griffiths, L., Watkins, L. M., Rees, M. I., Magliozzi, R., et al. (2018). Meningeal inflammation and cortical demyelination in acute multiple sclerosis. *Ann. Neurol.* 84, 829–842. doi: 10.1002/ana.25365

Bogie, J. F., Mailleux, J., Wouters, E., Jorissen, W., Grajchen, E., Vanmol, J., et al. (2017). Scavenger receptor collectin placenta 1 is a novel receptor involved in the uptake of myelin by phagocytes. *Sci. Rep.* 7:44794. doi: 10.1038/srep44794

Bogie, J. F., Stinissen, P., and Hendriks, J. J. (2014). Macrophage subsets and microglia in multiple sclerosis. *Acta Neuropathol.* 128, 191–213. doi: 10.1007/s00401-014-1310-2

Boven, L. A., Van Meurs, M., Van Zwam, M., Wierenga-Wolf, A., Hintzen, R. Q., Boot, R. G., et al. (2006). Myelin-laden macrophages are anti-inflammatory, consistent with foam cells in multiple sclerosis. *Brain* 129, 517–526. doi: 10.1093/brain/awh707

Bragg, D. C., Boles, J. C., and Meeker, R. B. (2002a). Destabilization of neuronal calcium homeostasis by factors secreted from choroid plexus macrophage cultures in response to feline immunodeficiency virus. *Neurobiol. Dis.* 9, 173–186. doi: 10.1006/nbdi.2001.0459

Bragg, D. C., Hudson, L. C., Liang, Y. H., Tompkins, M. B., Fernandes, A., and Meeker, R. B. (2002b). Choroid plexus macrophages proliferate and release toxic factors in response to feline immunodeficiency virus. *J. Neurovirol.* 8, 225–239. doi: 10.1080/13550280290049679

Brendecke, S. M., and Prinz, M. (2015). Do not judge a cell by its cover—diversity of CNS resident, adjoining and infiltrating myeloid cells in inflammation. *Semin. Immunopathol.* 37, 591–605. doi: 10.1007/s00281-015-0520-6

Breuer, J., Korpos, E., Hannocks, M. J., Schneider-Hohendorf, T., Song, J., Zondler, L., et al. (2018). Blockade of MCAM/CD146 impedes CNS infiltration of T cells over the choroid plexus. *J. Neuroinflammation* 15, 236. doi: 10.1186/s12974-018-1276-4

Butovsky, O., Landa, G., Kunis, G., Ziv, Y., Avidan, H., Greenberg, N., et al. (2006). Induction and blockage of oligodendrogenesis by differently activated microglia in an animal model of multiple sclerosis. *J. Clin. Invest.* 116, 905–915. doi: 10.1172/JCI26836

Buttgereit, A., Lelios, I., Yu, X., Vrohlings, M., Krakoski, N. R., Gautier, E. L., et al. (2016). Sall1 is a transcriptional regulator defining microglia identity and function. *Nat. Immunol.* 17, 1397–1406. doi: 10.1038/ni.3585

Centonze, D., Muzio, L., Rossi, S., Cavasinni, F., De Chiara, V., Bergami, A., et al. (2009). Inflammation triggers synaptic alteration and degeneration in experimental autoimmune encephalomyelitis. *J. Neurosci.* 29, 3442–3452. doi: 10.1523/JNEUROSCI.5804-08.2009

Centonze, D., Muzio, L., Rossi, S., Furlan, R., Bernardi, G., and Martino, G. (2010). The link between inflammation, synaptic transmission and neurodegeneration in multiple sclerosis. *Cell Death Differ.* 17, 1083–1091. doi: 10.1038/cdd.2009.179

Cheng, Y., Sun, L., Xie, Z., Fan, X., Cao, Q., Han, J., et al. (2017). Diversity of immune cell types in multiple sclerosis and its animal model: pathological and therapeutic implications. *J. Neurosci. Res.* 95, 1973–1983. doi: 10.1002/jnr.24023

Choi, S. R., Howell, O. W., Carassiti, D., Magliozzi, R., Gveric, D., Muraro, P. A., et al. (2012). Meningeal inflammation plays a role in the pathology of primary progressive multiple sclerosis. *Brain* 135, 2925–2937. doi: 10.1093/brain/aws189

Cohen, J. A., Imrey, P. B., Calabresi, P. A., Edwards, K. R., Eickenhorst, T., Felton, W. L., et al. (2009). Results of the avonex combination trial (ACT) in relapsing-remitting MS. *Neurology* 72, 535–541. doi: 10.1212/01.wnl.0000341934.12142.74

Colonna, M., and Facchetti, F. (2003). TREM-1 (triggering receptor expressed on myeloid cells): a new player in acute inflammatory responses. *J. Infect. Dis.* 187, S397–S401. doi: 10.1086/374754

Compston, A., and Coles, A. (2008). Multiple sclerosis. *Lancet* 372, 1502–1517. doi: 10.1016/S0140-6736(08)61620-7

Daneman, R. (2012). The blood-brain barrier in health and disease. *Ann. Neurol.* 72, 648–672. doi: 10.1002/ana.23648

Das, A., Sinha, M., Datta, S., Abas, M., Chaffee, S., Sen, C. K., et al. (2015). Monocyte and macrophage plasticity in tissue repair and regeneration. *Am. J. Pathol.* 185, 2596–2606. doi: 10.1016/j.ajpath.2015.06.001

Datta, G., Colasanti, A., Rabiner, E. A., Gunn, R. N., Malik, O., Ciccarelli, O., et al. (2017). Neuroinflammation and its relationship to changes in brain volume and white matter lesions in multiple sclerosis. *Brain* 140, 2927–2938. doi: 10.1093/brain/awx228

David, S., and Kroner, A. (2011). Repertoire of microglial and macrophage responses after spinal cord injury. *Nat. Rev. Neurosci.* 12, 388–399. doi: 10.1038/nrn3053

de Vos, A. F., van Meurs, M., Brok, H. P., Boven, L. A., Hintzen, R. Q., van der Valk, P., et al. (2002). Transfer of central nervous system autoantigens and presentation in secondary lymphoid organs. *J. Immunol.* 169, 5415–5423. doi: 10.4049/jimmunol.169.10.5415

Denney, L., Kok, W. L., Cole, S. L., Sanderson, S., McMichael, A. J., and Ho, L. P. (2012). Activation of invariant NKT cells in early phase of experimental autoimmune encephalomyelitis results in differentiation of Ly6Chi inflammatory monocyte to M2 macrophages and improved outcome. *J. Immunol.* 189, 551–557. doi: 10.4049/jimmunol.1103608

Di Filippo, M., Portaccio, E., Mancini, A., and Calabresi, P. (2018). Multiple sclerosis and cognition: synaptic failure and network dysfunction. *Nat. Rev. Neurosci.* 19, 599–609. doi: 10.1038/s41583-018-0053-9

Djedović, N., Stanisavljevic, S., Jevtić, B., Momčilović, M., Lavrnja, I., and Miljković, D. (2017). Anti-encephalitogenic effects of ethyl pyruvate are reflected in the central nervous system and the gut. *Biomed. Pharmacother.* 96, 78–85. doi: 10.1016/j.biopha.2017.09.110

Du, L., Zhang, Y., Chen, Y., Zhu, J., Yang, Y., and Zhang, H. L. (2017). Role of microglia in neurological disorders and their potentials as a therapeutic target. *Mol. Neurobiol.* 54, 7567–7584. doi: 10.1007/s12035-016-0245-0

Edwards, K. R., Goya, J., Plavina, T., Czerkowicz, J., Goelz, S., Ranger, A., et al. (2013). Feasibility of the use of combinatorial chemokine arrays to study blood and CSF in multiple sclerosis. *PLoS One* 8:e81007. doi: 10.1371/journal.pone.0081007

Engelhardt, B., Wolburg-Buchholz, K., and Wolburg, H. (2001). Involvement of the choroid plexus in central nervous system inflammation. *Microsc. Res. Tech.* 52, 112–129. doi: 10.1002/1097-0029(20010101)52:1<112::AID-JEMT13>3.0.CO;2-5

Eyo, U. B., Peng, J., Murugan, M., Mo, M., Lalani, A., Xie, P., et al. (2016). Regulation of physical microglia–neuron interactions by fractalkine signaling after status epilepticus. *eNeuro* 3:ENEURO.209–ENEURO.216. doi: 10.1523/ENEURO.0209-16.2016

Fabriek, B. O., Van Haastert, E. S., Galea, I., Polfliet, M. M., Döpp, E. D., Van Den Heuvel, M. M., et al. (2005). CD163-positive perivascular macrophages in the human CNS express molecules for antigen recognition and presentation. *Glia* 51, 297–305. doi: 10.1002/glia.20208

Fan, C., Long, R., You, Y., Wang, J., Yang, X., Huang, S., et al. (2018). A novel PADRE-Kv1.3 vaccine effectively induces therapeutic antibodies and ameliorates experimental autoimmune encephalomyelitis in rats. *Clin. Immunol.* 193, 98–109. doi: 10.1016/j.clim.2018.02.012

Fischbach, M. A., Bluestone, J. A., and Lim, W. A. (2013). Cell-based therapeutics: the next pillar of medicine. *Sci. Transl. Med.* 5:179s7. doi: 10.1126/scitranslmed.3005568

Forde, E. A., Dogan, R. N. E., and Karpus, W. J. (2011). CCR4 contributes to the pathogenesis of experimental autoimmune encephalomyelitis by regulating inflammatory macrophage function. *J. Neuroimmunol.* 236, 17–26. doi: 10.1016/j.jneuroim.2011.04.008

Galli, S. J., Borregaard, N., and Wynn, T. A. (2011). Phenotypic and functional plasticity of cells of innate immunity: macrophages, mast cells and neutrophils. *Nat. Immunol.* 12, 1035–1044. doi: 10.1038/ni.2109

Gao, H., Danzi, M. C., Choi, C. S., Taherian, M., Dalby-Hansen, C., Ellman, D. G., et al. (2017). Opposing functions of microglial and macrophagic TNFR2 in the pathogenesis of experimental autoimmune encephalomyelitis. *Cell Rep.* 18, 198–212. doi: 10.1016/j.celrep.2016.11.083

Garabedian, B. V., Lemaigre-Dubreuil, Y., and Mariani, J. (2000). Central origin of IL-1β produced during peripheral inflammation: role of meninges. *Brain Res. Mol. Brain Res.* 75, 259–263. doi: 10.1016/S0169-328X(99)00320-4

Goldmann, T., Wieghofer, P., Jordão, M. J., Prutek, F., Hagemeyer, N., Frenzel, K., et al. (2016). Origin, fate and dynamics of macrophages at CNS interfaces. *Nat. Immunol.* 17, 797–805. doi: 10.1038/ni.3423

Gomez Perdiguero, E., Klapproth, K., Schulz, C., Busch, K., Azzoni, E., Crozet, L., et al. (2015). Tissue-resident macrophages originate from yolk-sac-derived erythro-myeloid progenitors. *Nature* 518, 547–551. doi: 10.1038/nature13989

Gordon, E. J., Rao, S., Pollard, J. W., Nutt, S. L., Lang, R. A., and Harvey, N. L. (2011). Macrophages define dermal lymphatic vessel calibre during development by regulating lymphatic endothelial cell proliferation. *Development* 137, 3899–3910. doi: 10.1242/dev.050021

Graeber, M. B., and Streit, W. J. (2010). Microglia: biology and pathology. *Acta Neuropathol.* 119, 89–105. doi: 10.1007/s00401-009-0622-0

Grajchen, E., Hendriks, J. J. A., and Bogie, J. F. J. (2018). The physiology of foamy phagocytes in multiple sclerosis. *Acta Neuropathol. Commun.* 6:124. doi: 10.1186/s40478-018-0628-8

GrandPré, T., Nakamura, F., Vartanlan, T., and Strittmatter, S. M. (2000). Identification of the Nogo inhibitor of axon regeneration as a Reticulon protein. *Nature* 403, 439–444. doi: 10.1038/35000226

Greter, M., Heppner, F. L., Lemos, M. P., Odermatt, B. M., Goebels, N., Laufer, T., et al. (2005). Dendritic cells permit immune invasion of the CNS in an animal model of multiple sclerosis. *Nat. Med.* 11, 328–334. doi: 10.1038/nm1197

Hagemeyer, N., Hanft, K. M., Akriditou, M. A., Unger, N., Park, E. S., Stanley, E. R., et al. (2017). Microglia contribute to normal myelinogenesis and to oligodendrocyte progenitor maintenance during adulthood. *Acta Neuropathol.* 134, 441–458. doi: 10.1007/s00401-017-1747-1

Haile, Y., Deng, X., Ortiz-Sandoval, C., Tahbaz, N., Janowicz, A., Lu, J. Q., et al. (2017). Rab32 connects ER stress to mitochondrial defects in multiple sclerosis. *J. Neuroinflammation* 14:19. doi: 10.1186/s12974-016-0788-z

Hanisch, U. K., and Kettenmann, H. (2007). Microglia: active sensor and versatile effector cells in the normal and pathologic brain. *Nat. Neurosci.* 10, 1387–1394. doi: 10.1038/nn1997

Harrison, S. J., Nishinakamura, R., Jones, K. R., and Monaghan, A. P. (2012). Sall1 regulates cortical neurogenesis and laminar fate specification in mice: implications for neural abnormalities in Townes-Brocks syndrome. *Dis. Model. Mech.* 5, 351–365. doi: 10.1242/dmm.002873

He, H., Mack, J. J., Güç, E., Warren, C. M., Squadrito, M. L., Kilarski, W. W., et al. (2016). Perivascular macrophages limit permeability. *Arterioscler. Thromb. Vasc. Biol.* 36, 2203–2212. doi: 10.1161/ATVBAHA.116.307592

Herz, J., Filiano, A. J., Smith, A., Yogev, N., and Kipnis, J. (2017). Myeloid cells in the central nervous system. *Immunity* 46, 943–956. doi: 10.1016/j.immuni.2017.06.007

Hikawa, N., and Takenaka, T. (1996). Myelin-stimulated macrophages release neurotrophic factors for adult dorsal root ganglion neurons in culture. *Cell. Mol. Neurobiol.* 16, 517–528. doi: 10.1007/BF02150231

Hoeffel, G., Chen, J., Lavin, Y., Low, D., Almeida, F. F., See, P., et al. (2015). C-Myb(+) erythro-myeloid progenitor-derived fetal monocytes give rise to adult tissue-resident macrophages. *Immunity* 42, 665–678. doi: 10.1016/j.immuni.2015.03.011

Hofmann, N., Lachnit, N., Streppel, M., Witter, B., Neiss, W. F., Guntinas-Lichius, O., et al. (2002). Increased expression of ICAM-1, VCAM-1, MCP-1, and MIP-1α by spinal perivascular macrophages during experimental allergic encephalomyelitis in rats. *BMC Immunol.* 3:11. doi: 10.1186/1471-2172-3-11

Hong, S., Beja-Glasser, V. F., Nfonoyim, B. M., Frouin, A., Li, S., Ramakrishnan, S., et al. (2016). Complement and microglia mediate early synapse loss in Alzheimer mouse models. *Science* 352, 712–716. doi: 10.1126/science.aad8373

Howell, O. W., Reeves, C. A., Nicholas, R., Carassiti, D., Radotra, B., Gentleman, S. M., et al. (2011). Meningeal inflammation is widespread and linked to cortical pathology in multiple sclerosis. *Brain* 134, 2755–2771. doi: 10.1093/brain/awr182

Jiang, W., Li, D., Han, R., Zhang, C., Jin, W. N., Wood, K., et al. (2017). Acetylcholine-producing NK cells attenuate CNS inflammation via modulation of infiltrating monocytes/macrophages. *Proc. Natl. Acad. Sci. U.S.A.* 114, E6202–E6211. doi: 10.1073/pnas.1705491114

Jiang, Z., Jiang, J. X., and Zhang, G. X. (2014). Macrophages: a double-edged sword in experimental autoimmune encephalomyelitis. *Immunol. Lett.* 160, 17–22. doi: 10.1016/j.imlet.2014.03.006

Juhas, U., Ryba-Stanisławowska, M., Szargiej, P., and Myśliwska, J. (2015). Different pathways of macrophage activation and polarization. *Postepy Hig. Med. Dosw.* 69, 496–502. doi: 10.5604/17322693.1150133

Kapellos, T. S., and Iqbal, A. J. (2016). Epigenetic control of macrophage polarisation and soluble mediator gene expression during inflammation. *Mediators Inflamm.* 2016:6591703. doi: 10.1155/2016/6591703

Karamita, M., Barnum, C., Möbius, W., Tansey, M. G., Szymkowski, D. E., Lassmann, H., et al. (2017). Therapeutic inhibition of soluble brain TNF promotes remyelination by increasing myelin phagocytosis by microglia. *JCI Insight* 2:87455. doi: 10.1172/jci.insight.87455

Kaur, C., Rathnasamy, G., and Ling, E. A. (2016). The choroid plexus in healthy and diseased brain. *J. Neuropathol. Exp. Neurol.* 75, 198–213. doi: 10.1093/jnen/nlv030

Kettenmann, H., Hanisch, U. K., Noda, M., and Verkhratsky, A. (2011). Physiology of microglia. *Physiol. Rev.* 91, 461–553. doi: 10.1152/physrev.00011.2010

Kettenmann, H., Kirchhoff, F., and Verkhratsky, A. (2013). Microglia: new roles for the synaptic stripper. *Neuron.* 77, 10–18. doi: 10.1016/j.neuron.2012.12.023

Kierdorf, K., Erny, D., Goldmann, T., Sander, V., Schulz, C., Perdiguero, E. G., et al. (2013). Microglia emerge from erythromyeloid precursors via Pu.1-and Irf8-dependent pathways. *Nat. Neurosci.* 16, 273–280. doi: 10.1038/nn.3318

Kilsdonk, I. D., Schoonheim, M., and Wattjes, M. P. (2017). In-vivo imaging of meningeal inflammation in multiple sclerosis: presence of evidence or evidence of presence? *Mult. Scler.* 23, 1169–1171. doi: 10.1177/1352458517704924

Kivisakk, P., Imitola, J., Rasmussen, S., Elyaman, W., Zhu, B., Ransohoff, R. M., et al. (2009). Localizing central nervous system immune surveillance: meningeal antigen-presenting cells activate T cells during experimental autoimmune encephalomyelitis. *Ann. Neurol.* 65, 457–469. doi: 10.1002/ana.21379

Koeniger, T., and Kuerten, S. (2017). Splitting the "unsplittable": dissecting resident and infiltrating macrophages in experimental autoimmune encephalomyelitis. *Int. J. Mol. Sci.* 18:E2072. doi: 10.3390/ijms18102072

Kolber, P., Droby, A., Roebroeck, A., Goebel, R., Fleischer, V., Groppa, S., et al. (2017). A "kissing lesion": in-vivo 7T evidence of meningeal inflammation in early multiple sclerosis. *Mult. Scler.* 23, 1167–1169. doi: 10.1177/1352458516683267

Konishi, H., Kobayashi, M., Kunisawa, T., Imai, K., Sayo, A., Malissen, B., et al. (2017). Siglec-H is a microglia-specific marker that discriminates microglia from CNS-associated macrophages and CNS-infiltrating monocytes. *Glia* 65, 1927–1943. doi: 10.1002/glia.23204

Kornberg, M. D., Smith, M. D., Shirazi, H. A., Calabresi, P. A., Snyder, S. H., and Kim, P. M. (2018). Bryostatin-1 alleviates experimental multiple sclerosis. *Proc. Natl. Acad. Sci. U.S.A.* 115, 2186–2191. doi: 10.1073/pnas.1719902115

Koso, H., Nishinakamura, R., and Watanabe, S. (2018). Sall1 regulates microglial morphology cell autonomously in the developing retina. *Adv. Exp. Med. Biol.* 1074, 209–215. doi: 10.1007/978-3-319-75402-4_26

Kotter, M. R., Li, W. W., Zhao, C., and Franklin, R. J. (2006). Myelin impairs CNS remyelination by inhibiting oligodendrocyte precursor cell differentiation. *J. Neurosci.* 26, 328–332. doi: 10.1523/JNEUROSCI.2615-05.2006

Kotter, M. R., Zhao, C., Van Rooijen, N., and Franklin, R. J. (2005). Macrophage-depletion induced impairment of experimental CNS remyelination is associated with a reduced oligodendrocyte progenitor cell response and altered growth factor expression. *Neurobiol. Dis.* 18, 166–175. doi: 10.1016/j.nbd.2004.09.019

Krausgruber, T., Blazek, K., Smallie, T., Alzabin, S., Lockstone, H., Sahgal, N., et al. (2011). IRF5 promotes inflammatory macrophage polarization and TH1-TH17 responses. *Nat. Immunol.* 12, 231–238. doi: 10.1038/ni.1990

Kuhlmann, T., Ludwin, S., Prat, A., Antel, J., Brück, W., and Lassmann, H. (2017). An updated histological classification system for multiple sclerosis lesions. *Acta Neuropathol.* 133, 13–24. doi: 10.1007/s00401-016-1653-y

Lassmann, H., Brück, W., and Lucchinetti, C. (2001). Heterogeneity of multiple sclerosis pathogenesis: implications for diagnosis and therapy. *Trends Mol. Med.* 7, 115–121. doi: 10.1016/S1471-4914(00)01909-2

Lemus, H. N., Warrington, A. E., and Rodriguez, M. (2018). Multiple sclerosis: mechanisms of disease and strategies for myelin and axonal repair. *Neurol. Clin.* 36, 1–11. doi: 10.1016/j.ncl.2017.08.002

Li, H., Cuzner, M. L., and Newcombe, J. (1996). Microglia-derived macrophages in early multiple sclerosis plaques. *Neuropathol. Appl. Neurobiol.* 22, 207–215. doi: 10.1111/j.1365-2990.1996.tb00896.x

Li, Q., and Barres, B. A. (2018). Microglia and macrophages in brain homeostasis and disease. *Nat. Rev. Immunol.* 18, 225–242. doi: 10.1038/nri.2017.125

Li, Q., Lan, X., Han, X., and Wang, J. (2019). Expression of Tmem119/Sall1 and Ccr2/CD69 in FACS-sorted microglia- and monocyte/macrophage-enriched cell populations after intracerebral hemorrhage. *Front. Cell. Neurosci.* 12:520. doi: 10.3389/fncel.2018.00520

Liddelow, S. A. (2015). Development of the choroid plexus and blood-CSF barrier. *Front. Neurosci.* 9:32. doi: 10.3389/fnins.2015.00032

Liddelow, S. A., Guttenplan, K. A., Clarke, L. E., Bennett, F. C., Bohlen, C. J., Schirmer, L., et al. (2017). Neurotoxic reactive astrocytes are induced by activated microglia. *Nature* 541, 481–487. doi: 10.1038/nature21029

Lima, I. V., Bastos, L. F., Limborço-Filho, M., Fiebich, B. L., and de Oliveira, A. C. (2012). Role of prostaglandins in neuroinflammatory and neurodegenerative diseases. *Mediators Inflamm.* 2012:946813. doi: 10.1155/2012/946813

Liu, C., Li, Y., Yu, J., Feng, L., Hou, S., Liu, Y., et al. (2013). Targeting the shift from M1 to M2 macrophages in experimental autoimmune encephalomyelitis mice treated with fasudil. *PLoS One* 8:e54841. doi: 10.1371/journal.pone.0054841

Liu, Y., Hao, W., Letiembre, M., Walter, S., Kulanga, M., Neumann, H., et al. (2006). Suppression of microglial inflammatory activity by myelin phagocytosis: role

of p47-PHOX-mediated generation of reactive oxygen species. *J. Neurosci.* 26, 12904–12913. doi: 10.1523/JNEUROSCI.2531-06.2006

Lopez-Atalaya, J. P., Askew, K. E., Sierra, A., and Gomez-Nicola, D. (2018). Development and maintenance of the brain's immune toolkit: microglia and non-parenchymal brain macrophages. *Dev. Neurobiol.* 78, 561–579. doi: 10.1002/dneu.22545

Louveau, A., Smirnov, I., Keyes, T. J., Eccles, J. D., Rouhani, S. J., Peske, J. D., et al. (2015). Structural and functional features of central nervous system lymphatic vessels. *Nature* 523, 337–341. doi: 10.1038/nature14432

Lucchinetti, C., Brück, W., Parisi, J., Scheithauer, B., Rodriguez, M., and Lassmann, H. (2000). Heterogeneity of multiple sclerosis lesions: implications for the pathogenesis of demyelination. *Ann. Neurol.* 47, 707–717. doi: 10.1002/1531-8249(200006)47:6<707::AID-ANA3>3.0.CO;2-Q

Mammana, S., Fagone, P., Cavalli, E., Basile, M. S., Petralia, M. C., Nicoletti, F., et al. (2018). The role of macrophages in neuroinflammatory and neurodegenerative pathways of Alzheimer's disease, amyotrophic lateral sclerosis, and multiple sclerosis: pathogenetic cellular effectors and potential therapeutic targets. *Int. J. Mol. Sci.* 19:E831. doi: 10.3390/ijms19030831

Marques, F., Sousa, J. C., Brito, M. A., Pahnke, J., Santos, C., Correia-Neves, M., et al. (2017). The choroid plexus in health and in disease: dialogues into and out of the brain. *Neurobiol. Dis.* 107, 32–40. doi: 10.1016/j.nbd.2016.08.011

Martin, E., El-Behi, M., Fontaine, B., and Delarasse, C. (2017). Analysis of microglia and monocyte-derived macrophages from the central nervous system by flow cytometry. *J. Vis. Exp.* 124:e55781. doi: 10.3791/55781

Matcovitch-Natan, O., Winter, D. R., Giladi, A., Vargas Aguilar, S., Spinrad, A., Sarrazin, S., et al. (2016). Microglia development follows a stepwise program to regulate brain homeostasis. *Science* 353:aad8670. doi: 10.1126/science.aad8670

Maxwell, W. L., Follows, R., Ashhurst, D. E., and Berry, M. (1990). The response of the cerebral hemisphere of the rat to injury. II. The neonatal rat. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 328, 501–513. doi: 10.1098/rstb.1990.0122

McMahon, E. J., Bailey, S. L., Castenada, C. V., Waldner, H., and Miller, S. D. (2005). Epitope spreading initiates in the CNS in two mouse models of multiple sclerosis. *Nat. Med.* 11, 335–339. doi: 10.1038/nm1202

Mendes-Jorge, L., Ramos, D., Luppo, M., Llombart, C., Alexandre-Pires, G., Nacher, V., et al. (2009). Scavenger function of resident autofluorescent perivascular macrophages and their contribution to the maintenance of the blood-retinal barrier. *Invest. Ophthalmol. Vis. Sci.* 50, 5997–6005. doi: 10.1167/iovs.09-3515

Merson, T. D., Binder, M. D., and Kilpatrick, T. J. (2010). Role of cytokines as mediators and regulators of microglial activity in inflammatory demyelination of the CNS. *Neuromolecular Med.* 12, 99–132. doi: 10.1007/s12017-010-8112-z

Miao, X., Leng, X., and Zhang, Q. (2017). The current state of nanoparticle-induced macrophage polarization and reprogramming research. *Int. J. Mol. Sci.* 18:E336. doi: 10.3390/ijms18020336

Michailidou, I., Willems, J. G., Kooi, E. J., Van Eden, C., Gold, S. M., Geurts, J. J., et al. (2015). Complement C1q-C3-associated synaptic changes in multiple sclerosis hippocampus. *Ann. Neurol.* 77, 1007–1026. doi: 10.1002/ana.24398

Mikita, J., Dubourdieu-Cassagno, N., Deloire, M. S., Vekris, A., Biran, M., Raffard, G., et al. (2011). Altered M1/M2 activation patterns of monocytes in severe relapsing experimental rat model of multiple sclerosis. Amelioration of clinical status by M2 activated monocyte administration. *Mult. Scler.* 17, 2–15. doi: 10.1177/1352458510379243

Mildner, A., Huang, H., Radke, J., Stenzel, W., and Priller, J. (2017). P2Y12 receptor is expressed on human microglia under physiological conditions throughout development and is sensitive to neuroinflammatory diseases. *Glia* 65, 375–387. doi: 10.1002/glia.23097

Miron, V. E., Boyd, A., Zhao, J. W., Yuen, T. J., Ruckh, J. M., Shadrach, J. L., et al. (2013). M2 microglia and macrophages drive oligodendrocyte differentiation during CNS remyelination. *Nat. Neurosci.* 16, 1211–1218. doi: 10.1038/nn.3469

Mitchell, K., Yang, H. Y., Berk, J. D., Tran, J. H., and Iadarola, M. J. (2009). Monocyte chemoattractant protein-1 in the choroid plexus: a potential link between vascular pro-inflammatory mediators and the CNS during peripheral tissue inflammation. *Neuroscience* 158, 885–895. doi: 10.1016/j.neuroscience.2008.10.047

Miyamoto, A., Wake, H., Ishikawa, A. W., Eto, K., Shibata, K., Murakoshi, H., et al. (2016). Microglia contact induces synapse formation in developing somatosensory cortex. *Nat. Commun.* 7:12540. doi: 10.1038/ncomms12540

Montarolo, F., Perga, S., Martire, S., Brescia, F., Caldano, M., Lo Re, M., et al. (2018). Study of the NR4A family gene expression in patients with multiple sclerosis treated with Fingolimod. *Eur. J. Neurol.* 26, 667–672. doi: 10.1111/ene.13875

Montes-Cobos, E., Ring, S., Fischer, H. J., Heck, J., Strauß, J., Schwaninger, M., et al. (2017a). Targeted delivery of glucocorticoids to macrophages in a mouse model of multiple sclerosis using inorganic-organic hybrid nanoparticles. *J. Control. Release* 245, 157–169. doi: 10.1016/j.jconrel.2016.12.003

Montes-Cobos, E., Schweingruber, N., Li, X., Fischer, H. J., Reichardt, H. M., and Lühder, F. (2017b). Deletion of the mineralocorticoid receptor in myeloid cells attenuates central nervous system autoimmunity. *Front. Immunol.* 8:1319. doi: 10.3389/fimmu.2017.01319

Mrdjen, D., Pavlovic, A., Hartmann, F. J., Schreiner, B., Utz, S. G., Leung, B. P., et al. (2018). High-dimensional single-cell mapping of central nervous system immune cells reveals distinct myeloid subsets in health, aging, and disease. *Immunity* 48, 380–395.e6. doi: 10.1016/j.immuni.2018.01.011

Murphy, A. C., Lalor, S. J., Lynch, M. A., and Mills, K. H. (2010). Infiltration of Th1 and Th17 cells and activation of microglia in the CNS during the course of experimental autoimmune encephalomyelitis. *Brain. Behav. Immun.* 24, 641–651. doi: 10.1016/j.bbi.2010.01.014

Murray, P. J., Allen, J. E., Biswas, S. K., Fisher, E. A., Gilroy, D. W., Goerdt, S., et al. (2014). Macrophage activation and polarization: nomenclature and experimental guidelines. *Immunity* 41, 14–20. doi: 10.1016/j.immuni.2014.06.008

Nahrendorf, M., Swirski, F. K., Aikawa, E., Stangenberg, L., Wurdinger, T., Figueiredo, J. L., et al. (2007). The healing myocardium sequentially mobilizes two monocyte subsets with divergent and complementary functions. *J. Exp. Med.* 204, 3037–3047. doi: 10.1084/jem.20070885

Oh, U., Fujita, M., Ikonomidou, V. N., Evangelou, I. E., Matsuura, E., Harberts, E., et al. (2011). Translocator protein PET imaging for glial activation in multiple sclerosis. *J. Neuroimmune Pharmacol.* 6, 354–361. doi: 10.1007/s11481-010-9243-6

Paolicelli, R. C., Bolasco, G., Pagani, F., Maggi, L., Scianni, M., Panzanelli, P., et al. (2011). Synaptic pruning by microglia is necessary for normal brain development. *Science* 333, 1456–1458.

Parkhurst, C. N., Yang, G., Ninan, I., Savas, J. N., Yates, J. R., Lafaille, J. J., et al. (2014). Microglia promote learning-dependent synapse formation through BDNF. *Cell* 155, 1596–1609. doi: 10.1016/j.cell.2013.11.030

Pato-Pato, A., Prieto, J. M., Lema, M., Dapena-Bolaño, D., Abella-Corral, J., and Pumar, J. M. (2003). [Cerebral atrophy in multiple sclerosis patients treated periodically with boluses of methylprednisolone]. *Rev. Neurol.* 37, 501–506.

Pérez-Martín, M., Cifuentes, M., Grondona, J. M., López-Avalos, M. D., Gómez-Pinedo, U., García-Verdugo, J. M., et al. (2010). IGF-I stimulates neurogenesis in the hypothalamus of adult rats. *Eur. J. Neurosci.* 31, 1533–1548. doi: 10.1111/j.1460-9568.2010.07220.x

Perry, V. H. (1998). A revised view of the central nervous system microenvironment and major histocompatibility complex class II antigen presentation. *J. Neuroimmunol.* 90, 113–121. doi: 10.1016/S0165-5728(98)00145-3

Piccio, L., Buonsanti, C., Mariani, M., Cella, M., Gilfillan, S., Cross, A. H., et al. (2007). Blockade of TREM-2 exacerbates experimental autoimmune encephalomyelitis. *Eur. J. Immunol.* 37, 1290–1301. doi: 10.1002/eji.200636837

Polfliet, M. M., van de Veerdonk, F., Döpp, E. A., van Kesteren-Hendrikx, E. M., van Rooijen, N., Dijkstra, C. D., et al. (2002). The role of perivascular and meningeal macrophages in experimental allergic encephalomyelitis. *J. Neuroimmunol.* 122, 1–8. doi: 10.1016/S0165-5728(01)00445-3

Polfliet, M. M., Zwijnenburg, P. J., van Furth, A. M., van der Poll, T., Döpp, E. A., Renardel de Lavalette, C., et al. (2001). Meningeal and perivascular macrophages of the central nervous system play a protective role during bacterial meningitis. *J. Immunol.* 167, 4644–4650. doi: 10.4049/jimmunol.167.8.4644

Poliani, P. L., Wang, Y., Fontana, E., Robinette, M. L., Yamanishi, Y., Gilfillan, S., et al. (2015). TREM2 sustains microglial expansion during aging and response to demyelination. *J. Clin. Invest.* 125, 2161–2170. doi: 10.1172/JCI77983

Prinz, M., and Priller, J. (2014). Microglia and brain macrophages in the molecular age: from origin to neuropsychiatric disease. *Nat. Rev. Neurosci.* 15, 300–312. doi: 10.1038/nrn3722

Prinz, M., Priller, J., Sisodia, S. S., and Ransohoff, R. M. (2011). Heterogeneity of CNS myeloid cells and their roles in neurodegeneration. *Nat. Neurosci.* 14, 1227–1235. doi: 10.1038/nn.2923

Puthenparampil, M., Federle, L., Miante, S., Zito, A., Toffanin, E., Ruggero, S., et al. (2017). BAFF Index and CXCL13 levels in the cerebrospinal fluid associate respectively with intrathecal IgG synthesis and cortical atrophy in multiple sclerosis at clinical onset. *J. Neuroinflammation* 14:11. doi: 10.1186/s12974-016-0785-2

Ransohoff, R. M., and Cardona, A. E. (2010). The myeloid cells of the central nervous system parenchyma. *Nature* 468, 253–262. doi: 10.1038/nature09615

Ransohoff, R. M., and El Khoury, J. (2014). Microglia in health and disease. *Cold Spring Harb. Perspect. Biol.* 8:a020560. doi: 10.1101/cshperspect.a020560

Ransohoff, R. M., and Perry, V. H. (2009). Microglial physiology: unique stimuli, specialized responses. *Annu. Rev. Immunol.* 27, 119–145. doi: 10.1146/annurev.immunol.021908.132528

Raper, D., Louveau, A., and Kipnis, J. (2016). How do meningeal lymphatic vessels drain the CNS? *Trends Neurosci.* 39, 581–586. doi: 10.1016/j.tins.2016.07.001

Ratchford, J. N., Endres, C. J., Hammoud, D. A., Pomper, M. G., Shiee, N., McGready, J., et al. (2012). Decreased microglial activation in MS patients treated with glatiramer acetate. *J. Neurol.* 259, 1199–1205. doi: 10.1007/s00415-011-6337-x

Reuter, U., Bolay, H., Jansen-Olesen, I., Chiarugi, A., Sanchez del Rio, M., Letourneau, R., et al. (2001). Delayed inflammation in rat meninges: implications for migraine pathophysiology. *Brain* 124, 2490–2502. doi: 10.1093/brain/124.12.2490

Rothhammer, V., Borucki, D. M., Tjon, E. C., Takenaka, M. C., Chao, C. C., Ardura-Fabregat, A., et al. (2018). Microglial control of astrocytes in response to microbial metabolites. *Nature* 557, 724–728. doi: 10.1038/s41586-018-0119-x

Ruckh, J. M., Zhao, J. W., Shadrach, J. L., van wijngaarden, P., Rao, T. N., Wagers, A. J., et al. (2012). Rejuvenation of regeneration in the aging central nervous system. *Cell Stem Cell* 10, 96–103. doi: 10.1016/j.stem.2011.11.019

Saederup, N., Cardona, A. E., Croft, K., Mizutani, M., Cotleur, A. C., Tsou, C. L., et al. (2010). Selective chemokine receptor usage by central nervous system myeloid cells in CCR2-red fluorescent protein knock-in mice. *PLoS One* 5:e13693. doi: 10.1371/journal.pone.0013693

Salter, M. W., and Stevens, B. (2017). Microglia emerge as central players in brain disease. *Nat. Med.* 23, 1018–1027. doi: 10.1038/nm.4397

Samoilova, E. B., Horton, J. L., Liu, T. S., and Chen, Y. (1998). IL-6-deficient mice are resistant to experimental autoimmune encephalomyelitis: roles of IL-6 in the activation and differentiation of autoreactive T cells. *J. Immunol.* 161, 6480–6486.

Satoh, J., Kino, Y., Asahina, N., Takitani, M., Miyoshi, J., Ishida, T., et al. (2016). TMEM119 marks a subset of microglia in the human brain. *Neuropathology* 36, 39–49. doi: 10.1111/neup.12235

Schafer, D. P., and Stevens, B. (2015). Microglia function in central nervous system development and plasticity. *Cold Spring Harb. Perspect. Biol.* 7:a020545. doi: 10.1101/cshperspect.a020545

Sestito, C., Brevé, J. J. P., van Eggermond, M. C. J. A., Killestein, J., Teunissen, C. E., van Rossum, J., et al. (2017). Monocyte-derived tissue transglutaminase in multiple sclerosis patients: reflecting an anti-inflammatory status and function of the cells? *J. Neuroinflammation* 14, 257. doi: 10.1186/s12974-017-1035-y

Shaked, I., Hanna, R. N., Shaked, H., Chodaczek, G., Nowyhed, H. N., Tweet, G., et al. (2015). Transcription factor Nr4a1 couples sympathetic and inflammatory cues in CNS-recruited macrophages to limit neuroinflammation. *Nat. Immunol.* 16, 1228–1234. doi: 10.1038/ni.3321

Shechter, R., London, A., varol, C., Raposo, C., Cusimano, M., Yovel, G., et al. (2009). Infiltrating blood-derived macrophages are vital cells playing an anti-inflammatory role in recovery from spinal cord injury in mice. *PLoS Med.* 6:e1000113. doi: 10.1371/journal.pmed.1000113

Shechter, R., Miller, O., Yovel, G., Rosenzweig, N., London, A., Ruckh, J., et al. (2013). Recruitment of beneficial M2 macrophages to injured spinal cord is orchestrated by remote brain choroid plexus. *Immunity* 38, 555–569. doi: 10.1016/j.immuni.2013.02.012

Shi, Y., Pan, C., Auckloo, B. N., Chen, X., Chen, C. T. A., Wang, K., et al. (2017). Stress-driven discovery of a cryptic antibiotic produced by *Streptomyces* sp. WU20 from Kueishantao hydrothermal vent with an integrated metabolomics strategy. *Appl. Microbiol. Biotechnol.* 101, 1395–1408. doi: 10.1007/s00253-016-7823-y

Sierra, A., Encinas, J. M., Deudero, J. J., Chancey, J. H., Enikolopov, G., Overstreet-Wadiche, L. S., et al. (2010). Microglia shape adult hippocampal neurogenesis through apoptosis-coupled phagocytosis. *Cell Stem Cell* 7, 483–495. doi: 10.1016/j.stem.2010.08.014

Singh, S., Metz, I., Amor, S., van der Valk, P., Stadelmann, C., and Brück, W. (2013). Microglial nodules in early multiple sclerosis white matter are associated with degenerating axons. *Acta Neuropathol.* 125, 595–608. doi: 10.1007/s00401-013-1082-0

Sosa, R. A., Murphey, C., Ji, N., Cardona, A. E., and Forsthuber, T. G. (2013). The kinetics of myelin antigen uptake by myeloid cells in the central nervous system during experimental autoimmune encephalomyelitis. *J. Immunol.* 191, 5848–5857. doi: 10.4049/jimmunol.1300771

Stephenson, E. L., Mishra, M. K., Moussienko, D., Laflamme, N., Rivest, S., Ling, C. C., et al. (2018). Chondroitin sulfate proteoglycans as novel drivers of leucocyte infiltration in multiple sclerosis. *Brain* 141, 1094–1110. doi: 10.1093/brain/awy033

Stys, P. K., Zamponi, G. W., Van Minnen, J., and Geurts, J. J. G. (2012). Will the real multiple sclerosis please stand up? *Nat. Rev. Neurosci.* 13, 507–514. doi: 10.1038/nrn3275

Sun, D., Yu, Z., Fang, X., Liu, M., Pu, Y., Shao, Q., et al. (2017). LncRNA GAS5 inhibits microglial M2 polarization and exacerbates demyelination. *EMBO Rep.* 18, 1801–1816. doi: 10.15252/embr.201643668

Sutton, C., Brereton, C., Keogh, B., Mills, K. H., and Lavelle, E. C. (2006). A crucial role for interleukin (IL)-1 in the induction of IL-17–producing T cells that mediate autoimmune encephalomyelitis. *J. Exp. Med.* 203, 1685–1691. doi: 10.1084/jem.20060285

Szepesi, Z., Manouchehrian, O., Bachiller, S., and Deierborg, T. (2018). Bidirectional microglia–neuron communication in health and disease. *Front. Cell. Neurosci.* 12:323. doi: 10.3389/fncel.2018.00323

Tentillier, N., Etzerodt, A., Olesen, M. N., Rizalar, F. S., Jacobsen, J., Bender, D., et al. (2016). Anti-inflammatory modulation of microglia via CD163-targeted glucocorticoids protects dopaminergic neurons in the 6-OHDA Parkinson's disease model. *J. Neurosci.* 36, 9375–9390. doi: 10.1523/JNEUROSCI.1636-16.2016

Terrazas, C., de Dios Ruiz-Rosado, J., Amici, S. A., Jablonski, K. A., Martinez-Saucedo, D., Webb, L. M., et al. (2017). Helminth-induced Ly6Chi monocyte-derived alternatively activated macrophages suppress experimental autoimmune encephalomyelitis. *Sci. Rep.* 7:40814. doi: 10.1038/srep40814

Thompson, A. J., Baranzini, S. E., Geurts, J., Hemmer, B., and Ciccarelli, O. (2018). Multiple sclerosis. *Lancet* 391, 1622–1636. doi: 10.1016/S0140-6736(18)30481-1

Tobin, W. O., Costanzi, C., Guo, Y., Parisi, J. E., Weigand, S. D., and Lucchinetti, C. F. (2017). Clinical-radiological-pathological spectrum of central nervous system-idiopathic inflammatory demyelinating disease in the elderly. *Mult. Scler.* 23, 1204–1213. doi: 10.1177/1352458516675748

Tong, H. I., Kang, W., Shi, Y., Zhou, G., and Lu, Y. (2016). Physiological function and inflamed-brain migration of mouse monocyte-derived macrophages following cellular uptake of superparamagnetic iron oxide nanoparticles-Implication of macrophage-based drug delivery into the central nervous system. *Int. J. Pharm.* 505, 271–282. doi: 10.1016/j.ijpharm.2016.03.028

Torres-Platas, S. G., Cruceanu, C., Chen, G. G., Turecki, G., and Mechawar, N. (2014). Evidence for increased microglial priming and macrophage recruitment in the dorsal anterior cingulate white matter of depressed suicides. *Brain Behav. Immun.* 42, 50–59. doi: 10.1016/j.bbi.2014.05.007

Trapp, B. D., Peterson, J., Ransohoff, R. M., Rudick, R., Mörk, S., and Bö, L. (1998). Axonal transection in the lesions of multiple sclerosis. *N. Engl. J. Med.* 338, 278–285. doi: 10.1056/NEJM199801293380502

Trebst, C., Sørensen, T. L., Kivisäkk, P., Cathcart, M. K., Hesselgesser, J., Horuk, R., et al. (2001). CCR1+/CCR5+ mononuclear phagocytes accumulate in the central nervous system of patients with multiple sclerosis. *Am. J. Pathol.* 159, 1701–1710. doi: 10.1016/S0002-9440(10)63017-9

Tremblay, M. Ė, Lowery, R. L., and Majewska, A. K. (2010). Microglial interactions with synapses are modulated by visual experience. *PLoS Biol.* 8:e1000527. doi: 10.1371/journal.pbio.1000527

Tsutsui, M., Hirase, R., Miyamura, S., Nagayasu, K., Nakagawa, T., Mori, Y., et al. (2018). TRPM2 exacerbates central nervous system inflammation in experimental autoimmune encephalomyelitis by increasing production of

CXCL2 chemokines. *J. Neurosci.* 38, 8484–8495. doi: 10.1523/JNEUROSCI.2203-17.2018

Ueno, M., Fujita, Y., Tanaka, T., Nakamura, Y., Kikuta, J., Ishii, M., et al. (2013). Layer v cortical neurons require microglial support for survival during postnatal development. *Nat. Neurosci.* 16, 543–551. doi: 10.1038/nn.3358

Valentin-Torres, A., Savarin, C., Hinton, D. R., Phares, T. W., Bergmann, C. C., and Stohlman, S. A. (2016). Sustained TNF production by central nervous system infiltrating macrophages promotes progressive autoimmune encephalomyelitis. *J. Neuroinflammation* 13:46. doi: 10.1186/s12974-016-0513-y

van Zwam, M., Samsom, J. N., Nieuwenhuis, E. E., Melief, M.-J., Wierenga-Wolf, A. F., Dijke, I. E., et al. (2011). Myelin ingestion alters macrophage antigen-presenting function in vitro and in vivo. *J. Leukoc. Biol.* 90, 123–132. doi: 10.1189/jlb.1209813

Vanderlocht, J., Hellings, N., Hendriks, J. J., Vandenabeele, F., Moreels, M., Buntinx, M., et al. (2006). Leukemia inhibitory factor is produced by myelin-reactive T cells from multiple sclerosis patients and protects against tumor necrosis factor-alpha-induced oligodendrocyte apoptosis. *J. Neurosci. Res.* 83, 763–774. doi: 10.1002/jnr.20781

Vas, A., Shchukin, Y., Karrenbauer, V. D., Cselényi, Z., Kostulas, K., Hillert, J., et al. (2008). Functional neuroimaging in multiple sclerosis with radiolabelled glia markers: preliminary comparative PET studies with [11C]vinpocetine and [11C]PK11195 in patients. *J. Neurol. Sci.* 264, 9–17. doi: 10.1016/j.jns.2007.07.018

Vercellino, M., Trebini, C., Capello, E., Mancardi, G. L., Giordana, M. T., and Cavalla, P. (2017). Inflammatory responses in Multiple Sclerosis normal-appearing white matter and in non-immune mediated neurological conditions with wallerian axonal degeneration: a comparative study. *J. Neuroimmunol.* 312, 49–58. doi: 10.1016/j.jneuroim.2017.09.004

Vercellino, M., Votta, B., Condello, C., Piacentino, C., Romagnolo, A., Merola, A., et al. (2008). Involvement of the choroid plexus in multiple sclerosis autoimmune inflammation: a neuropathological study. *J. Neuroimmunol.* 199, 133–141. doi: 10.1016/j.jneuroim.2008.04.035

Veremeyko, T., Yung, A. W. Y., Dukhinova, M., Kuznetsova, I. S., Pomytkin, I., Lyundup, A., et al. (2018). Cyclic AMP pathway suppress autoimmune neuroinflammation by inhibiting functions of encephalitogenic CD4 T cells and enhancing M2 macrophage polarization at the site of inflammation. *Front. Immunol.* 9:50. doi: 10.3389/fimmu.2018.00050

Voet, S., Prinz, M., and van Loo, G. (2018). Microglia in central nervous system inflammation and multiple sclerosis pathology. *Trends Mol. Med.* 25, 112–123. doi: 10.1016/j.molmed.2018.11.005

Vogel, D. Y., Vereyken, E. J., Glim, J. E., Heijnen, P. D., Moeton, M., van der Valk, P., et al. (2013). Macrophages in inflammatory multiple sclerosis lesions have an intermediate activation status. *J. Neuroinflammation* 10, 35. doi: 10.1186/1742-2094-10-35

Voss, E. V., Škuljec, J., Gudi, V., Skripuletz, T., Pul, R., Trebst, C., et al. (2012). Characterisation of microglia during de- and remyelination: can they create a repair promoting environment? *Neurobiol. Dis.* 45, 519–528. doi: 10.1016/j.nbd.2011.09.008

Wang, Q., He, Z., Huang, M., Liu, T., Wang, Y., Xu, H., et al. (2018). Vascular niche IL-6 induces alternative macrophage activation in glioblastoma through HIF-2α. *Nat. Commun.* 9:559. doi: 10.1038/s41467-018-03050-0

Weber, M. S., Prod'homme, T., Youssef, S., Dunn, S. E., Rundle, C. D., Lee, L., et al. (2007). Type II monocytes modulate T cell-mediated central nervous system autoimmune disease. *Nat. Med.* 13, 935–943. doi: 10.1038/nm1620

Welberg, L. (2014). A synaptic role for microglia. *Nat. Rev. Neurosci.* 15, 68–69. doi: 10.1038/nrn3678

Weng, Q., Wang, J., Wang, J., Wang, J., Sattar, F., Zhang, Z., et al. (2018). Lenalidomide regulates CNS autoimmunity by promoting M2 macrophages polarization article. *Cell Death Dis.* 9:251. doi: 10.1038/s41419-018-0290-x

Wisnieff, C., Ramanan, S., Olesik, J., Gauthier, S., Wang, Y., and Pitt, D. (2015). Quantitative susceptibility mapping (QSM) of white matter multiple sclerosis lesions: interpreting positive susceptibility and the presence of iron. *Magn. Reson. Med.* 74, 564–570. doi: 10.1002/mrm.25420

Wolburg, H., and Paulus, W. (2010). Choroid plexus: biology and pathology. *Acta Neuropathol.* 119, 75–88. doi: 10.1007/s00401-009-0627-8

Wolf, Y., Shemer, A., Levy-Efrati, L., Gross, M., Kim, J. S., Engel, A., et al. (2018). Microglial MHC class II is dispensable for experimental autoimmune encephalomyelitis and cuprizone-induced demyelination. *Eur. J. Immunol.* 48, 1308–1318. doi: 10.1002/eji.201847540

Wolf, Y., Shemer, A., Polonsky, M., Gross, M., Mildner, A., Yona, S., et al. (2017). Autonomous TNF is critical for in vivo monocyte survival in steady state and inflammation. *J. Exp. Med.* 214, 905–917. doi: 10.1084/jem.20160499

Wu, Z., Hayashi, Y., Zhang, J., and Nakanishi, H. (2007). Involvement of prostaglandin E2 released from leptomeningeal cells in increased expression of transforming growth factor-beta in glial cells and cortical neurons during systemic inflammation. *J. Neurosci. Res.* 85, 184–192. doi: 10.1002/jnr.21100

Yamamoto, S., Muramatsu, M., Azuma, E., Ikutani, M., Nagai, Y., Sagara, H., et al. (2017). A subset of cerebrovascular pericytes originates from mature macrophages in the very early phase of vascular development in CNS. *Sci. Rep.* 7:3855. doi: 10.1038/s41598-017-03994-1

Yamasaki, R., Lu, H., Butovsky, O., Ohno, N., Rietsch, A. M., Cialic, R., et al. (2014). Differential roles of microglia and monocytes in the inflamed central nervous system. *J. Exp. Med.* 211, 1533–1549. doi: 10.1084/jem.20132477

Yang, Q., Zheng, C., Cao, J., Cao, G., Shou, P., Lin, L., et al. (2016). Spermidine alleviates experimental autoimmune encephalomyelitis through inducing inhibitory macrophages. *Cell Death Differ.* 23, 1850–1861. doi: 10.1038/cdd.2016.71

Ye, Z. P., Ai, X. L., Faramand, A. M., and Fang, F. (2018). Macrophages as nanocarriers for drug delivery: novel therapeutics for central nervous system diseases. *J. Nanosci. Nanotechnol.* 18, 471–485. doi: 10.1166/jnn.2018.15218

Yirmiya, R., Rimmerman, N., and Reshef, R. (2015). Depression as a microglial disease. *Trends Neurosci.* 38, 637–658. doi: 10.1016/j.tins.2015.08.001

Yuan, D., Zhao, Y., Banks, W. A., Bullock, K. M., Haney, M., Batrakova, E., et al. (2017). Macrophage exosomes as natural nanocarriers for protein delivery to inflamed brain. *Biomaterials* 142, 1–12. doi: 10.1016/j.biomaterials.2017.07.011

Zabala, A., Vazquez-Villoldo, N., Rissiek, B., Gejo, J., Martin, A., Palomino, A., et al. (2018). P2X4 receptor controls microglia activation and favors remyelination in autoimmune encephalitis. *EMBO Mol. Med.* 10:e8743. doi: 10.15252/emmm.201708743

Zéphir, H. (2018). Progress in understanding the pathophysiology of multiple sclerosis. *Rev. Neurol.* 174, 358–363. doi: 10.1016/j.neurol.2018.03.006

Zhang, J., Raper, A., Sugita, N., Hingorani, R., Salio, M., Palmowski, M. J., et al. (2006). Characterization of Siglec-H as a novel endocytic receptor expressed on murine plasmacytoid dendritic cell precursors. *Blood* 107, 3600–3608. doi: 10.1182/blood-2005-09-3842

Zhang, Z., Zhang, Z. Y., Schittenhelm, J., Wu, Y., Meyermann, R., and Schluesener, H. J. (2011). Parenchymal accumulation of CD163+ macrophages/microglia in multiple sclerosis brains. *J. Neuroimmunol.* 237, 73–79. doi: 10.1016/j.jneuroim.2011.06.006

Zrzavy, T., Hametner, S., Wimmer, I., Butovsky, O., Weiner, H. L., and Lassmann, H. (2017). Loss of "homeostatic" microglia and patterns of their activation in active multiple sclerosis. *Brain* 140, 1900–1913. doi: 10.1093/brain/awx113

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Icariin Attenuates M1 Activation of Microglia and Aβ Plaque Accumulation in the Hippocampus and Prefrontal Cortex by Up-Regulating PPARγ in Restraint/Isolation-Stressed APP/PS1 Mice

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Background: Studies have shown that psychosocial stress is involved in Alzheimer's disease (AD) pathogenesis; it induces M1 microglia polarization and production of pro-inflammatory cytokines, leading to neurotoxic outcomes and decreased β-amyloid (Aβ) clearance. Icariin has been proven to be an effective anti-inflammatory agent and to activate peroxisome proliferator-activated receptors gamma (PPARγ) which induces the M2 phenotype in the microglia. However, whether restraint/isolation stress reduces the clearance ability of microglia by priming and polarizing microglia to the M1 phenotype, and the effects of icariin in attenuating the inflammatory response and relieving the pathological changes of AD are still unclear.

Methods: APP/PS1 mice (male, aged 3 months) were randomly divided into a control group, a restraint/isolation stress group, and a restraint/isolation stress + icariin group. The restraint/isolation stress group was subjected to a paradigm to build a depressive animal model. Sucrose preference, open field, elevated plus maze, and Y maze test were used to assess the stress paradigm. The Morris water maze test was performed to evaluate spatial reference learning and memory. Enzyme-linked immunosorbent assay and immunohistochemistry were used to identify the microglia phenotype and Aβ accumulation. Western blotting was used to detect the expression of PPARγ in the hippocampus and prefrontal cortex (PFC).

Results: Restraint/isolation stress induced significant depressive-like behaviors in APP/PS1 mice at 4 months of age and memory impairment at 10 months of age,

while 6 months of icariin administration relieved the memory damage. Restraint/isolation stressed mice had elevated pro-inflammatory cytokines, decreased anti-inflammatory cytokines, increased Aβ plaque accumulation and more M1 phenotype microglia in the hippocampus and PFC at 10 months of age, while 6 months of icariin administration relieved these changes. Moreover, restraint/isolation stressed mice had down-regulated PPARγ expression in the hippocampus and PFC at 10 months of age, while 6 months of icariin administration reversed the alteration, especially in the hippocampus.

Conclusion: Restraint/isolation stress induced depressive-like behaviors and spatial memory damage, over-expression of M1 microglia markers and more severe Aβ accumulation by suppressing PPARγ in APP/PS1 mice. Icariin can be considered a new treatment option as it induces the switch of the microglia phenotype by activating PPARγ.

Keywords: stress, Alzheimer's disease, microglia, icariin, PPARγ, cytokine, hippocampus, prefrontal cortex

INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by progressive cognitive impairment (Selkoe and Schenk, 2003). Prominent neuropathological features of AD are β-amyloid (Aβ) plaques and neurofibrillary tauopathy consisting of threads and tangles (NFT), which are observed throughout the brain, including the areas critically involved in memory formation and emotional regulation (Braak and Braak, 1996).

Mounting evidence shows that psychosocial stress is involved in AD pathogenesis, as patients with posttraumatic stress disorder (PTSD) or depression often develop dementia and even clinical AD (Green et al., 2003; Wilson et al., 2005; Qureshi et al., 2010; Yaffe et al., 2010; Gracia-Garcia et al., 2015; Sacuiu et al., 2016). The elevated level of cortisol, a stress hormone, found in AD patients plays a crucial role in contribution to the comorbidity (Hartmann et al., 1997; Elgh et al., 2006). Studies in animal models further demonstrated the association of hypothalamic–pituitary–adrenal axis activation with AD pathogenesis in the frontal cortex and hippocampus (Green et al., 2006; Sotiropoulos et al., 2008; Joshi et al., 2012; Justice et al., 2015). Chronic unpredictable stress (CUS) significantly increased both serum corticosterone levels (Liu et al., 2010; Yang et al., 2012) and amyloid precursor protein (APP) fragments. Aβ infusion triggered APP misprocessing in the hippocampus and prefrontal cortex (PFC) of rats, which was further exacerbated by stress (Catania et al., 2009). Furthermore, chronic stress including restraint/isolation stress in particularly elevated Aβ40 and Aβ42 levels of brain, accelerated amyloid plaque formation and impaired learning and memory in mice (Jeong et al., 2006; Carroll et al., 2011; Lee and Han, 2013). More importantly, a recent study demonstrated that prenatal stress induced a constant neuroinflammatory response which contributes to a more vulnerable profile; the latter can subsequently lead to an aberrant response to accumulating Aβ peptides, and ultimately modify the extent of Aβ neuropathology. The study concluded that early-life stress aggravated plaque pathology in 10-month-old APP/PS1 mice, and was accompanied by reduced microglial accumulation and increased level of pro-inflammatory tumor necrosis factor-α (TNF-α) (Hoeijmakers et al., 2016).

Microglial activation is often classified into classical (M1) and alternative (M2). M1 microglia may contribute to dysfunction of the neurotrophic system by expressing pro-inflammatory cytokines, such as TNF-α, interleukin-1β (IL-1β) and IL-6 (Michelucci et al., 2009). In the M1 phenotype of the microglia, the activation of nuclear factor κB (NF-κB) may play a critical role in the production of pro-inflammatory cytokines, leading to neurotoxic outcomes (Park et al., 2015). The M2 phenotype, also known as the neuroprotective microglial phenotype, releases different mediators including IL-4, IL-10, and transforming growth factor-β (TGF-β) (Michelucci et al., 2009) to antagonize inflammation-induced damage in the central nervous system (CNS) (Kobayashi et al., 2013; Zhao et al., 2015), and is associated with enhanced phagocytosis of deposited amyloid (Mandrekar-Colucci et al., 2012; Heneka et al., 2013; He et al., 2016; Huang et al., 2017). Stress paradigms, including CUS and chronic social defeat stress, are among the factors that could induce M1 microglia polarization (Zhao et al., 2016; Tang et al., 2018).

Peroxisome proliferator-activated receptors (PPARs) are a group of nuclear receptor proteins regulating gene expression as ligand-activated transcription factors (Michalik et al., 2006). Three closely related PPAR isoforms have been identified (alpha, delta, and gamma), transcribed from different genes and characterized by different tissue distribution, ligand specificity, and physiological roles (Berger and Moller, 2002; Breidert et al., 2002; Chang et al., 2007; Tontonoz and Spiegelman, 2008). Among the three isoforms, PPARγ has the highest expression in the CNS, where it has been identified in neurons, astrocytes, and glial cells (Moreno et al., 2004). Specifically, PPARγ appears to be linked to stress modulation (Garcia-Bueno et al., 2005) and to mediate the conversion between microglia phenotypes (Yamanaka et al., 2012; Saijo et al., 2013). PPARγ induced the M2 phenotype microglia under Aβ toxicity via adiponectin treatment through its effects on the anti-inflammatory response (Song et al., 2017). Moreover, pioglitazone which was used to treat depressive-like behaviors in a chronic mild stress mouse model is

associated with PPARγ-mediated M2 activation of the microglia (Zhao et al., 2016).

Icariin, a natural flavonoid compound extracted from Epimedium brevicornum Maxim (a traditional Chinese herb), has been proven to have a wide range of effects including anti-tumor, anti-oxidant, anti-bacterial, and anti-inflammatory properties (Luo et al., 2007; Zhou et al., 2011). Previous studies investigated the pharmacological properties of icariin in the CNS and showed that it can markedly attenuate cognitive deficits in several models of AD (Li et al., 2010; Nie et al., 2010; Jin et al., 2014) and alleviate the neuronal injury induced by ischemia (Li et al., 2005). It was also reported that icariin can attenuate the inflammatory response through PPARγ activation in rats (Xiong et al., 2016).

Previous studies also showed that various stress can activate M1 microglia (Zhao et al., 2016; Tang et al., 2018), which is associated with decreased Aβ clearance. In the present study, we first hypothesized that restraint/isolation stress might prime and polarize microglia to the M1 phenotype and reduce its clearing ability. Second, we aimed to probe the effects of icariin in attenuating the inflammatory response and relieving the pathological changes of AD by targeting PPARγ activation in the hippocampus and PFC of APP/PS1 double-transgenic mice.

MATERIALS AND METHODS

Animals and Experimental Procedure

Thirty male APP/PS1 mice, aged 3 months and weighing 24–30 g, obtained from Beijing HFK Bioscience Co., Ltd. (Beijing, China), were housed (five per cage) and maintained under a 12-h light-dark cycle, at 20–24°C with free access to food and water. After 7 days of adaptation, the mice were randomly divided into three groups (10 mice per group): a control group, a restraint/isolation stress group (RIS), and a restraint/isolation stress + icariin group (RIS+ICA). Mice in both the RIS and RIS+ICA groups were subjected to 28 days of restraint/isolation stress from 3 to 4 months of age. The mice in the RIS+ICA group received daily administration of icariin (60 mg/kg) for 6 months from 4 to 10 months of age. The mice weight was recorded at baseline, every week during the stress procedure, and every month during drug administration. Behavioral tests after stress included the sucrose preference test (SPT), open field test (OFT), elevated plus maze test and Y maze test. The Morris water maze (MWM) test was used to measure learning and memory after drug administration at 10 months of age. Enzyme-linked immunosorbent assay (ELISA) and immunohistochemistry were used to detect Aβ plaque accumulation and microglia phenotypes, whereas western blotting was used to detect the expression of PPARγ in the hippocampus and the PFC. The experimental paradigm is presented in **Figure 1**.

Restraint/Isolation Stress and Drug Treatments

Restraint/isolation stress is a paradigm used to build depressive animal models (Carroll et al., 2011). Each mouse was individually restrained in restraint tubes for 6 h per day, and the mice were deprived of water and food during the restraint/isolation stress.

Icariin (Cat no. I8760, Beijing Solarbio Science & Technology Co., Ltd.) was orally administered daily to the mice in the RIS+ICA group (60 mg/kg, freshly suspended in ddH_2O) from 4 to 10 months of age (Wang et al., 2013; Li et al., 2015; Hoeijmakers et al., 2017). The mice in the control group and restraint/isolation stress group were given ddH_2O (vehicle) in the same volume to balance the systematic error.

Sucrose Preference Test (SPT)

The SPT test was carried out at the end of the exposure to restraint/isolation stress. A decreased sucrose preference is considered to be homologous to anhedonia, the inability to experience pleasure, and thus simulates the defining symptom of major depression. Mice were individually housed during the SPT. Before the test, the mice were trained to adapt to sucrose solution (1%, w/v): Two bottles of sucrose solution were placed in each cage for 24 h, then one bottle of sucrose solution was replaced with pure water for 24 h. After adaptation, mice were deprived of water and food for 24 h. Then, each mouse was given free access to two bottles for 1 h, one with 200 ml of 1% (w/v) sucrose solution and the other with 200 ml of pure water. The drinking bottles were weighed to calculate fluid consumption 1 h later. The sucrose preference percentage was evaluated as the amount of sucrose solution consumed during consumption of all fluids.

Open Field Test (OFT)

The OFT was used to test the motivation of mice to explore. The open field apparatus is a white wooden box (90 cm in diameter and 45 cm in wall height). The bottom of the apparatus is divided into 25 squares. The mice were individually placed at the center of the apparatus and left free to explore the arena for 5 min. The following indices were recorded: number of grid crossings (horizontal movement), defined as crossing into the nearby grids with more than three paws or half of the body; and number of rears (vertical movement), defined as both forelimbs raised at least 1 cm above the ground. The box was thoroughly cleaned between tests. Horizontal and vertical movements were recorded using a camera linked with a computer fitted with a SMART video tracking system (SMART v3.0, Panlab, Spain).

Elevated Plus Maze (EPM)

The experimental apparatus consisted of a central part (5 cm× 5 cm), two opposing open arms (30 cm × 5 cm) and two opposing closed arms (30 cm × 5 cm) with non-transparent walls of 15 cm height. The maze was elevated 50 cm above the floor. The mice were individually placed in the center of the maze facing an open arm and allowed free exploration for 5 min. The number of entries into the open and closed arms and the total time spent in the open and closed arms were recorded and measured by the SMART video tracking system. The time and entry ratios were used to obtain the anxiety score. The time ratio was defined as the ratio of total time spent in the open arms of the maze to the total time spent in any arm. The entry ratio was defined as the ratio of the number of entries into the open arms of the maze to the total number of entries into any arm of the maze. An anxiety score

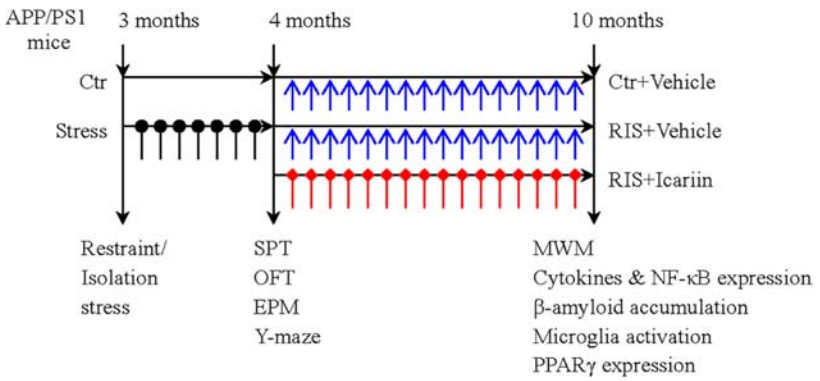

FIGURE 1 | The experimental paradigm. Thirty male APP/PS1 mice were randomly divided into three groups (10 mice per group): control group (Ctr), restraint/isolation stress group (RIS), and restraint/isolation stress + icariin group (RIS+ICA). Mice in both the RIS and RIS+ICA groups were subjected to 28 days of restraint/isolation stress procedure, from 3 to 4 months of age. Mice in the RIS+ICA group were administered icariin (60 mg/kg) daily for 6 months, from 4 to 10 months of age. The weight of the mice was recorded at baseline, every week during the stress procedure and every month during the period of drug administration. The behavioral tests performed after stress included the sucrose preference test (SPT), open field test (OFT), elevated plus maze (EPM) and Y maze test in this order. After drug administration, the Morris water maze (MWM) test was performed. Enzyme-linked immunosorbent assay and immunohistochemistry were used to assess the microglia phenotype and western blotting was used to measure the expression of PPARγ in the hippocampus and PFC.

was calculated as 1 − (time ratio + entry ratio/2). Anxious mice were more likely to stay in the closed arms, so that a smaller time ratio or entry ratio indicates more anxious behavior. The maze platforms and walls were thoroughly cleaned with 75% ethanol between test sessions and allowed to dry.

Y Maze Test

The Y maze test was used to assess various parameters related to spatial memory. The apparatus consisted of three arms (50 cm × 16 cm × 32 cm) made of black non-transparent plastic to form a "Y" shape. Visual cues made of colored paper were placed on the walls of the arms. The floor of the maze was covered with padding. The mice were placed into one of the arms of the maze (start arm) and allowed to explore the maze with one of the arms closed for 15 min (training trial). One hour later, the mice were returned to the start arm, and allowed to explore all three arms of the maze freely for 5 min (test trial). At the end of the training trial, the mice were returned to their home cage and the padding inside the maze was mixed to reduce the possibility of odor interference. The number of entries, the time spent and the distance traveled in each arm were measured by the SMART video tracking system. Ratio time was defined as the total time spent in the novel arm divided by the total time spent in any arm of the maze. Ratio entry was defined as the number of entries into the novel arm divided by the total number of entries into any arm of the maze. Ratio distance was defined as the total distance covered in the novel arm divided by the total distance covered in any arm of the maze. Due to their natural curiosity, mice are more likely to explore the novel arm, so that these parameters reflect spatial recognition memory. More entries or exploration of the novel arm indicate better spatial memory.

Morris Water Maze (MWM)

The MWM test was performed to evaluate spatial reference learning and memory of APP/PS1 mice at 10 months old. The maze was a blue pool (0.8 m in diameter) filled with water (0.3 m deep, at 25 ± 1°C). Geometric pictures pasted on the surrounding walls were used by the mice for spatial orientation. The maze was divided into four equal quadrants corresponding to four directions: I, II, III, and IV. The mice movements were captured by a CCD camera connected with a computer. All mice were allowed to swim freely for 60 s within 24 h before the formal training. During the following 5 consecutive days, mice were trained to find a platform (12 cm in diameter) hidden under the water surface in quadrant IV four times per day. If a mouse failed to find the platform within 60 s, it was manually guided to the platform and allowed to remain there for 10 s, and the escape latency was scored as 60 s. On the sixth day, all mice were released into the maze, without the hidden platform, from an identical point of quadrant I and allowed to swim freely for 60 s. The escape latency of training days as well as the percentage of time spent in and the number of entries into quadrant IV on the sixth day were analyzed with the SMART video tracking system.

Protein Isolation

Mice (six per group) were sacrificed under deep anesthesia, and both sides of the frontal and parietal bones were removed to obtain the whole brain from the cranial cavity. The PFC and the hippocampus were collected and immediately immersed in liquid nitrogen, and stored at −80°C for later protein isolation. The tissue was dissociated using an ultrasonic cell disruptor and lysed in a cold lysis buffer containing 10 mM Tris-HCl, pH 8.0, 240 mM NaCl, 5 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethanesulfonyl fluoride, 1% Triton X-100, 1 mM sodium vanadate and 1 g/ml of leupeptin, pepstatin and aprotinin. Tissue lysates were incubated at 4°C for 20 min. The samples were centrifuged at 12,000 rpm for 10 min at 4°C, then for each sample the supernatant was collected and protein content was determined using BCA protein assay reagents (Beyotime Institute of Biotechnology, China).

Enzyme-Linked Immunosorbent Assay (ELISA)

The levels of IL-1β, IL-6, TNF-α, IL-4, IL-10, TGF-β1, NF-κB component p65, and Aβ 1-42 were measured using commercially available ELISA kits according to the manufacturer's instructions (Beijing Andy Huatai Technology Co., Ltd., China). Briefly, serial dilutions of protein standards and samples were added to 96-well ELISA plates followed by HRP labeled antibodies for IL-1β, IL-6, TNF-α, IL-4, IL-10, TGF-β1, NF-κB p65, and Aβ 1-42 to form antibody-antigen-enzyme labeled antibody complexes. After complete washing with wash solution, TMB substrate solution was added, which under the catalysis of HRP was converted to blue. The reaction was stopped using the stop solution. Optical density at 450 nm was detected using the iMark Microplate Absorbance Reader (Bio-Rad, United States). The concentration of each sample was calculated from the linear equation derived from the standard curve of known concentrations of NF-κB p65, Aβ 1-42, and the cytokines.

Western Blotting

Brain protein samples containing the same amount of total protein were mixed with a 5x Laemmli loading buffer (protein volume: loading buffer = 4: 1). The mixed protein sample was heated at 99°C for 5 min to achieve protein denaturation, then 15 μg of protein sample was separated on 10% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel and electro-transferred to polyvinylidene difluoride (PVDF) membranes (Servicebio, China). The membrane was blocked with 5% skim milk in TBS containing 0.1% Tween-20 (TBST) for 1.5 h and incubated in a refrigerator with primary antibodies against PPARγ (1:1000, Abcam, United States) or GAPDH (1:10000, Biogot Technology Co., Ltd., China) at 4°C overnight. The following day, after washing with TBST three times for 5 min, the PVDF membrane was incubated for 1 h with the secondary antibody (ZSGB-BIO, China). Then, the PVDF membrane was washed again with TBST three times for 15 min; the western blots were visualized after being incubated with ECL solution (Millipore Corp., United States) for 1 min and exposed onto photographic films (Eastman Kodak Company, United States) for 10–90 s. Signal intensities were quantified using the ImageJ 14.0 software, and the intensity value of the protein band of interest was normalized according to that of the GAPDH band of the same sample.

Immunohistochemistry

The mice (four per group) were anesthetized with pentobarbital and perfused with 50 ml of 0.1 M PBS, followed by 100 ml of ice-cold 4% paraformaldehyde (PFA). Paraffin-embedded PFC and hippocampus tissues were cut into 5 μm thick sections with a microtome. Coronal sections of the hippocampus were selected between bregma −1.22 mm and bregma −3.64 mm, and sections of the PFC were selected between anterior +4.7 mm to bregma posterior +3.7 mm to bregma. After rehydration, the sections were heated at 95–98°C in 0.01 M citrate buffer (pH 6.0) for 15 min, then cooled to room temperature for 30 min and washed with PBS. Thereafter, the sections were incubated with fresh 3% H_2O_2 for 25 min at room temperature to block endogenous peroxidase activity. After washing with PBS, the sections were blocked with 3% BSA for 30 min, incubated with mouse anti-Aβ 1-42 antibody (1:1000, BioLegend, United States), rabbit anti-ionized calcium-binding adapter molecule 1 (Iba-1) antibody (1:2000, Servicebio, China) or rabbit anti-inducible nitric oxide synthase (iNOS) antibody (1:100, Abcam, United States) at 4°C overnight. After washing with PBS, the sections were incubated with HRP labeled anti-mouse or anti-rabbit antibodies at room temperature for 50 min, rewashed with PBS and incubated in diaminobenzidine (DAB) (Servicebio, China) for chromogen development under a microscope. Finally, the sections were dehydrated, cleared and mounted. After completing the staining, each section was viewed under 400× magnification. The expression of Aβ and the positive reactive cells of Iba-1 and iNOS were observed. Sections from corresponding locations were selected in each group, and five different visual fields (50 μm × 50 μm) of expression areas in the hippocampus [CA1, CA3, and dentate gyrus (DG)] and PFC were selected in each section under a high power lens to count Iba-1- and iNOS-positive cells.

Statistics

Data are presented as mean ± standard error of mean (SEM). Statistical analysis was carried out by one-way analysis of variance (ANOVA) with Student–Newman–Keuls (SNK) *post hoc* test. Differences were considered statistically significant if the p-value was less than 0.05.

RESULTS

Effect of RIS on Behavioral Changes in SPT, OFT, EPM, and Y Maze Test

Sucrose Preference Test

As shown in **Figure 2A**, stress exposure significantly reduced the percentage of sucrose consumption in stressed APP/PS1 mice in both the RIS and the RIS+ICA groups compared with the control animals [$F(2,27) = 5.664$, $p = 0.009$; *post hoc* $p < 0.05$ for both comparisons]. The results indicated that stress decreased sucrose preference, considered a major symptom of depression, in APP/PS1 mice.

Open Field Test

As shown in **Figure 2B**, the APP/PS1 mice in the two stress groups showed decreased horizontal and vertical movements compared with the control group [$F(2,27) = 10.093$, $p = 0.001$; *post hoc* $p < 0.01$ for both comparisons]. The results indicated that stress induced decreased interest in a new environment and behavioral suppression in APP/PS1 mice.

Elevated Plus Maze

As shown in **Figure 2C**, 4 weeks of exposure to RI stress did not significantly change the anxiety score of mice in the two stress groups [$F(2,27) = 0.183$, $p = 0.834$], indicating that the level of stress was too mild to induce anxiety-like behaviors.

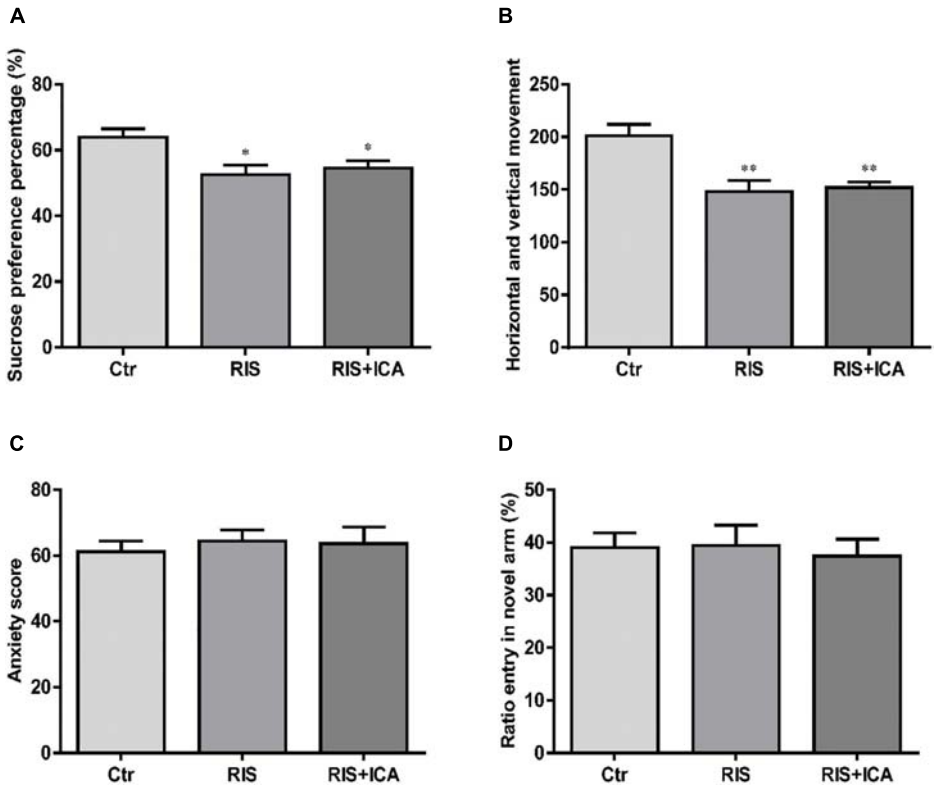

FIGURE 2 | Effect of RIS on behavioral changes in SPT **(A)**, OFT **(B)**, EPM **(C)**, and Y-maze test **(D)**. **(A)** Stress exposure significantly reduced the percentage of sucrose consumption in stressed APP/PS1 mice, in both the RIS and the RIS+ICA groups, compared with the control animals. **(B)** APP/PS1 mice in the two stress groups showed decreased horizontal and vertical movements compared with the control group. **(C)** Four weeks of RI stress exposure did not significantly change the anxiety score of mice in the two stress groups. **(D)** Four weeks of RI stress exposure did not significantly reduce the ratio entry. The results are expressed as the mean ± SEM, $n = 10$. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. the control group.

Y Maze Test

As shown in **Figure 2D**, 4 weeks of exposure to RI stress did not significantly reduce the Ratio entry [$F(2,27) = 0.101$, $p = 0.904$], indicating the absence of memory impairment in stressed animals.

Effect of Icariin on Learning and Memory in MWM

As shown in **Figure 3A**, mice in the RIS group had longer escape latency on the first, second, and third learning day, but there were no significant differences in escape latency at any day among the three groups [$F(2,27) = 1.210$, $p = 0.314$ (first day); $F(2,27) = 0.902$, $p = 0.418$ (second day); $F(2,27) = 0.728$, $p = 0.492$ (third day); $F(2,27) = 0.419$, $p = 0.662$ (fourth day) and $F(2,27) = 0.146$, $p = 0.865$ (fifth day)]. However, as shown in **Figures 3B–D**, mice in the RIS group spent less time in the target quadrant in the MWM test than the control group did [$F(2,27) = 6.849$, $p = 0.004$; *post hoc* $p < 0.01$]. Moreover, RIS mice showed decreased entry ratio in the target quadrant in comparison with the control mice [$F(2,27) = 8.148$, $p = 0.002$; *post hoc* $p < 0.01$]. Icariin administration significantly relieved these behavioral alterations in the RIS+ICA group as compared with the RIS group ($p < 0.01$, $p < 0.05$, respectively). The results indicated that long-term icariin administration effectively relieved the memory impairment of restraint/isolation-stressed mice.

Effect of Icariin on Cytokines and NF-κB in the Hippocampus and PFC

Cytokines and NF-κB Expression in the Hippocampus

Cytokine expression in the hippocampus is shown in **Figures 4A–F**. **Figure 4A** shows that IL-1β levels increased in the RIS group compared with the control group [$F(2,15) = 32.507$, $p < 0.001$; *post hoc* $p < 0.01$]; however, icariin relieved the alteration ($p < 0.01$). **Figure 4B** shows that RIS significantly increased IL-6 levels [$F(2,15) = 69.126$, $p < 0.001$; *post hoc* $p < 0.01$] compared with controls, while icariin decreased them ($p < 0.01$). **Figure 4C** shows that stress increased TNF-α in APP/PS1 mice [$F(2,15) = 10.718$, $p = 0.001$, *post hoc* $p < 0.01$], while icariin could reverse this change ($p < 0.01$).

Figure 4D shows that IL-4 levels decreased in the RIS group compared with controls [$F(2,15) = 74.971$, $p < 0.001$; *post hoc* $p < 0.01$], and icariin relieved such alteration ($p < 0.01$). Similarly, **Figure 4E** shows that RIS significantly decreased IL-10 levels compared with controls [$F(2,15) = 119.031$, $p < 0.001$;

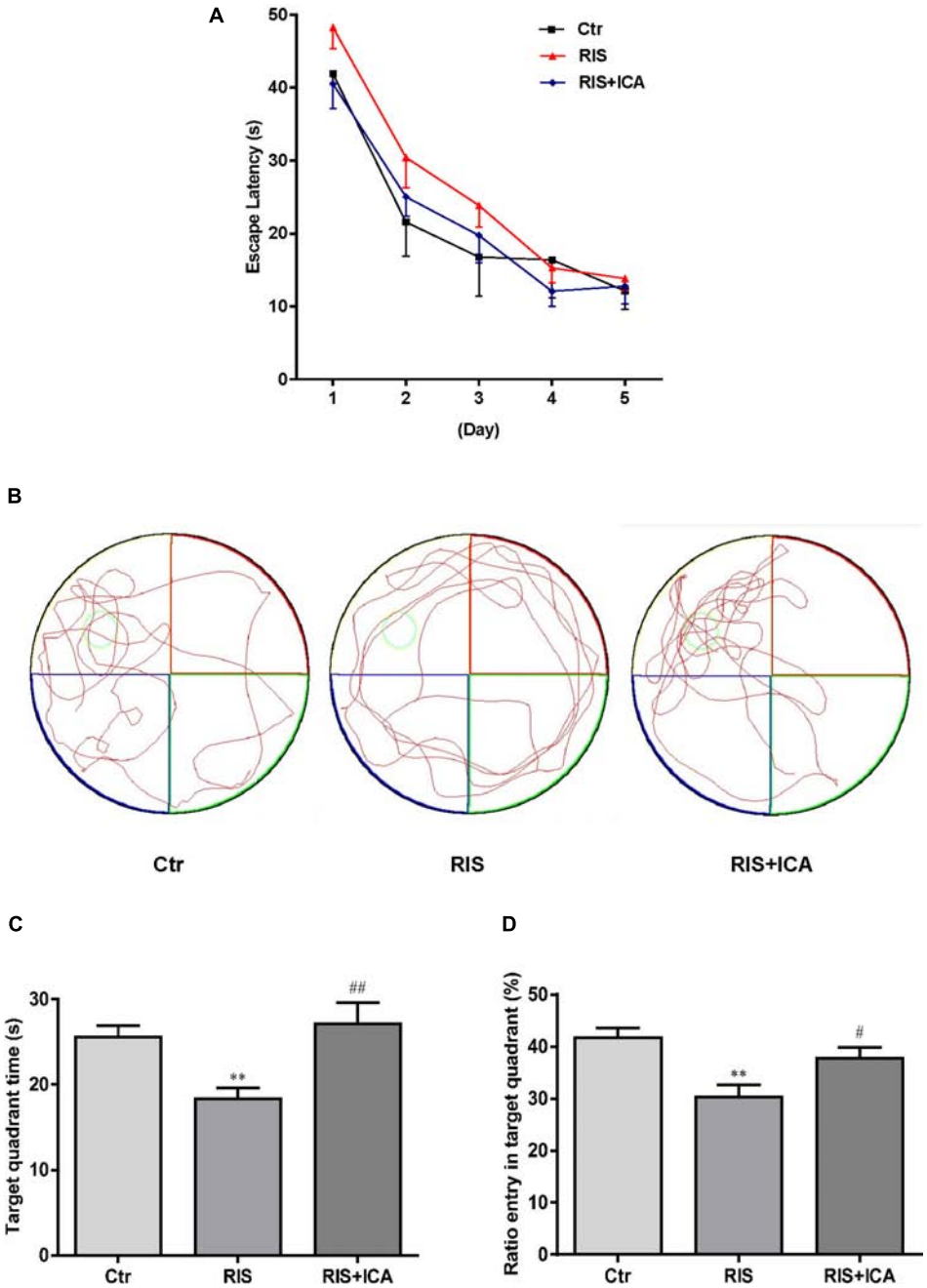

FIGURE 3 | Effect of icariin on learning and memory in MWM. **(A)** The escape latency of training days. **(B)** The tracks in the MWM test. **(C)** Target quadrant time. **(D)** Ratio entry in the target quadrant. Mice in RIS group spent less time in the target quadrant and showed decreased entry ratio in the target quadrant in the MWM test, in comparison with the control group ($p < 0.01$). Icariin administration significantly relieved the behavioral alterations of the mice in the RIS+ICA group, compared with the RIS group. The results are expressed as the mean ± SEM, $n = 10$. $^{**}p < 0.01$ vs. control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. RIS group.

post hoc $p < 0.01$], while icariin increased them ($p < 0.01$). **Figure 4F** shows that stress decreased TGF-β1 expression in APP/PS1 mice compared with controls [$F(2,15) = 82.481$, $p < 0.001$; *post hoc* $p < 0.01$], while icariin could reverse such change ($p < 0.01$).

Figure 4G shows that NF-κB levels increased in the RIS group compared with controls [$F(2,15) = 16.709$, $p < 0.001$: *post hoc* $p < 0.01$]; however, icariin relieved the alteration ($p < 0.01$).

Cytokines and NF-κB Expression in the PFC

Cytokine expression in the PFC is shown in **Figures 5A–F**. **Figure 5A** shows that IL-1β levels increased in the RIS group compared with controls [$F(2,15) = 17.565$, $p < 0.001$; *post hoc*

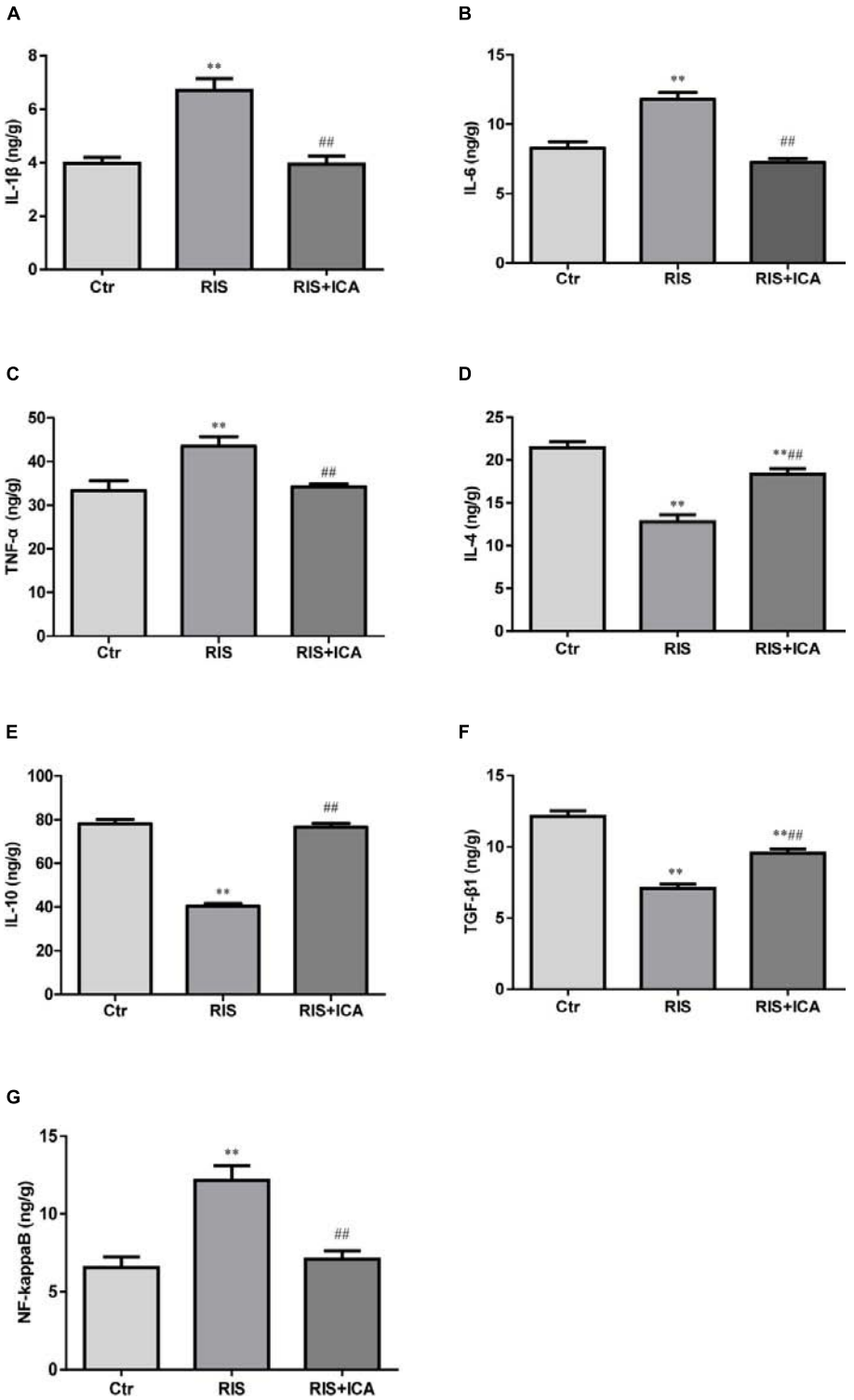

FIGURE 4 | Effect of icariin on cytokines and NF-κB in the hippocampus. **(A–C)** The level of IL-1β **(A)**, IL-6 **(B)**, and TNF-α **(C)** increased in the RIS group, and icariin relieved the alteration. **(D–F)** The level of IL-4 **(D)**, IL-10 **(E)**, and TGF-β1 **(F)** decreased in the RIS group, and icariin relieved the alteration. **(G)** The level of NF-κB increased in the RIS group, and icariin relieved the alteration. The results are expressed as the mean ± SEM, $n = 6$. $^{**}p < 0.01$ vs. control group; $^{\#\#}p < 0.01$ vs. RIS group.

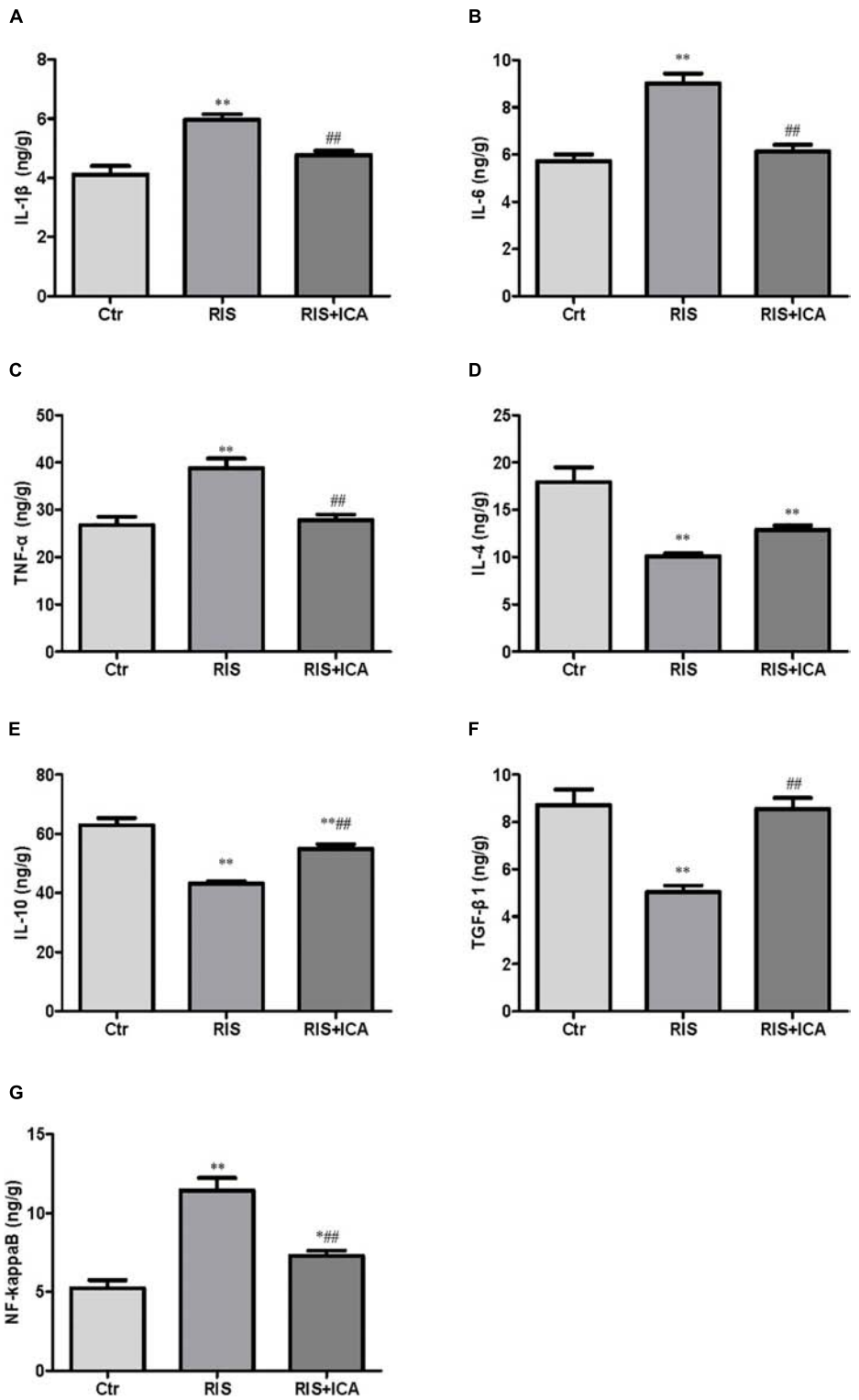

FIGURE 5 | Effect of icariin on cytokines and NF-κB in the PFC. **(A–C)** The level of IL-1β **(A)**, IL-6 **(B)**, and TNF-α **(C)** increased in the RIS group, and icariin relieved the alteration. **(D)** The levels of IL-4 decreased in the RIS group, but icariin failed to relieve the alteration. **(E,F)** RIS significantly decreased the level of IL-10 **(E)** and TGF-β1 **(F)** compared with controls, and icariin could reverse the change. **(G)** NF-κB levels increased in the RIS group, and icariin relieved the alteration. The results are expressed as the mean ± SEM, $n = 6$. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control group; $^{\#\#}p < 0.01$ vs. RIS group.

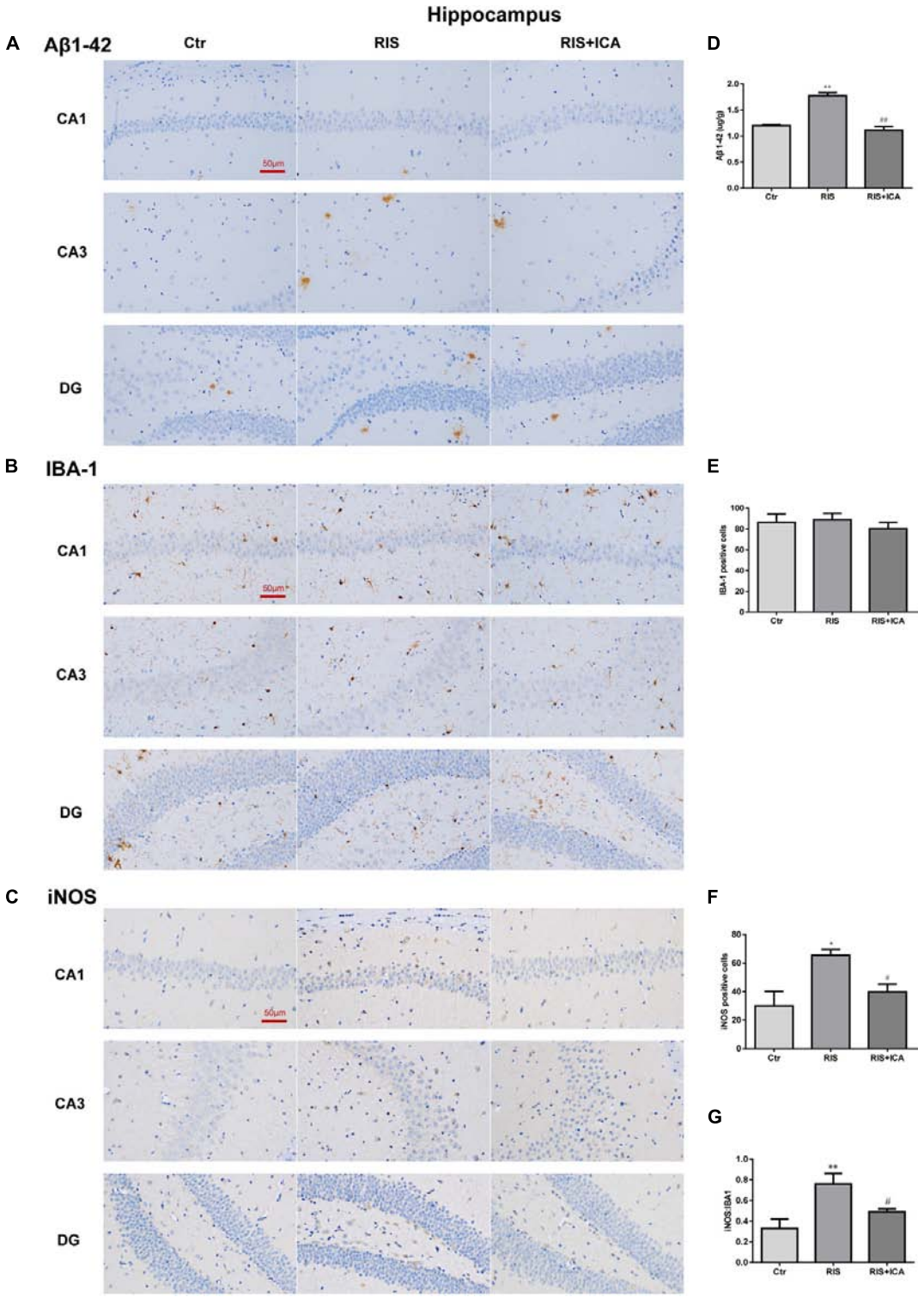

FIGURE 6 | Effect of icariin on Aβ plaques and microglia phenotype in the hippocampus. **(A–C)** The immunohistochemical staining results of Aβ, Iba-1, and iNOS (400×). **(D)** RIS significantly increased the levels of Aβ in the RIS group compared with the controls, and administration of icariin decreased its levels in the RIS+ICA group. **(E)** The number of Iba-1+ microglia positive cells was not significantly different among the three groups. **(F)** The number of iNOS+ microglia positive cells in the hippocampus of stressed mice was higher than that of control mice, and icariin administration reversed the change in the RIS+ICA group. **(G)** The RIS group showed higher ratio of iNOS+ to Iba-1+ microglia, while the RIS+ICA group showed a lower ratio. The results of Aβ are expressed as the mean ± SEM, $n = 6$; the results of Iba-1 and iNOS are expressed as the mean ± SEM, $n = 4$. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. RIS group.

$p < 0.01$]; however, icariin relieved the alteration ($p < 0.01$). **Figure 5B** shows that RIS significantly increased the level of IL-6 [$F(2,15) = 51.672$, $p < 0.001$; *post hoc* $p < 0.01$], which was reversed by treatment of icariin ($p < 0.01$). **Figure 5C** shows that stress increased TNF-α expression compared with controls [$F(2,15) = 16.475$, $p < 0.001$; *post hoc* $p < 0.01$], while icariin could reverse this change ($p < 0.01$).

Figure 5D shows that IL-4 levels decreased in the RIS group compared with controls [$F(2,15) = 25.263$, $p < 0.001$; *post hoc* $p < 0.01$], and icariin failed to relieved this alteration. **Figure 5E** shows that RIS also significantly decreased the level of IL-10 [$F(2,15) = 41.906$, $p < 0.001$; *post hoc* $p < 0.01$], which was enhanced by the administration of icariin ($p < 0.01$). **Figure 5F** shows that stress decreased TGF-β1 compared with controls [$F(2,15) = 23.709$, $p < 0.001$; *post hoc* $p < 0.01$], while icariin could reverse this change ($p < 0.01$).

Figure 5G shows that the level of NF-κB increased in the RIS group compared with controls [$F(2,15) = 37.217$, $p < 0.001$; *post hoc* $p < 0.01$]; however, icariin relieved the alteration ($p < 0.01$).

Effect of Icariin on Aβ Plaques and Microglia Phenotype in the Hippocampus and PFC

Aβ Plaques and Microglia Phenotype in the Hippocampus

As shown in **Figure 6A**, the amount and size of Aβ plaques in the hippocampus of the RIS APP/PS1 mice were larger than those in the mice of the control group, while icariin relieved the alteration in the RIS+ICA group. To accurately evaluate the changes in Aβ, we measured the Aβ level in the hippocampus using ELISA, as shown in **Figure 6D**. RIS significantly increased Aβ levels in the RIS group compared with the control group [$F(2,15) = 74.140$, $p < 0.001$; *post hoc* $p < 0.01$], while administration of icariin decreased the levels of Aβ in the RIS+ICA group ($p < 0.01$). The counting of Iba-1+ microglia positive cells did not reveal significant differences among the three groups as shown in **Figures 6B,E** [$F(2,9) = 0.395$, $p = 0.685$]. However, the number of iNOS+ microglia positive cells in the hippocampus of the stressed mice was higher than that in control mice [$F(2,9) = 6.640$, $p = 0.017$; *post hoc* $p < 0.05$], and icariin administration reversed the change in the RIS+ICA group ($p < 0.05$, **Figures 6C,F**). The higher ratio of iNOS+ microglia to Iba-1+ microglia in the stressed group and the lower ratio in the RIS+ICA group further support the promotion by icariin of a microglia phenotype switch in the hippocampus of RIS APP/PS1 mice [$F(2,9) = 7.837$, $p = 0.011$; *post hoc* $p < 0.01$, control vs. RIS; $p < 0.05$, RIS vs. RIS+ICA] (**Figure 6G**).

Aβ Plaques and Microglia Phenotype in the PFC

As shown in **Figure 7A**, the amount and size of Aβ plaques in the PFC of RIS APP/PS1 mice were larger than that in the PFC of control mice, while icariin relieved such alterations. In order to accurately evaluate the change of Aβ, Aβ levels were also measured in the PFC using ELISA as shown in **Figure 7B**. Compared with controls, RIS significantly increased Aβ levels [$F(2,15) = 4.368$, $p = 0.032$; *post hoc* $p < 0.05$], while icariin decreased them ($p < 0.05$). The counting of Iba-1+ and iNOS+ microglia cells showed no significant differences among the three groups, as shown in **Figures 7C,D** [$F(2,9) = 0.922$, $p = 0.432$; $F(2,9) = 1.502$, $p = 0.274$, respectively]. As a result, the ratio of iNOS+ to Iba-1+ microglia also was not significantly different among the three groups [$F(2,9) = 0.229$, $p = 0.800$] (**Figure 7E**).

Effect of Icariin on PPARγ in the Hippocampus and PFC

Figure 8A shows PPARγ expression in the hippocampus as assessed using western blotting. The RIS procedure induced a marked decrease in PPARγ levels in APP/PS1 mice compared with controls [$F(2,9) = 7.917$, $p = 0.010$; *post hoc* $p < 0.05$], and icariin could reverse such alteration ($p < 0.05$).

Figure 8B shows PPARγ expression in the PFC as assessed using western blotting. The RIS procedure induced a marked decrease in PPARγ levels in APP/PS1 mice compared with controls [$F(2,12) = 3.916$, $p = 0.049$; *post hoc* $p < 0.05$], and icariin could partially reverse the alteration ($p > 0.05$, control group vs. RIS+ICA).

DISCUSSION

Mounting evidence has shown that chronic psychosocial stress is a major risk factor for late-onset AD, and is associated with cognitive deficits (Piirainen et al., 2017). Chronic stress could prime the microglia and induce inflammatory responses in the adult brain, thereby compromising the synapse-supportive roles of microglia and contributing to age-related cognitive deterioration. In order to examine the effects of chronic stress on neuroinflammation and neurodegeneration in AD, we used restraint/isolation stress to build a stressed AD model (Jeong et al., 2006; Carroll et al., 2011; Lee and Han, 2013). Consistent with previous studies, 1 month of restraint/isolation stress induced lower sucrose consumption and lower horizontal and vertical movements in the OFT in APP/PS1 mice aged 4 months, compared with unstressed APP/PS1 mice, indicating that stressed animals showed anhedonia and were less interested in a new environment, thus suggesting the validity of the stressed AD model.

The role of chronic neuroinflammation in AD-related dementia has recently gained attention, implicating an early involvement of inflammation in the onset and progression of the disease (Querfurth and LaFerla, 2010). Microglia are the first line of defense against invading pathogens in the CNS (Piirainen et al., 2017). Studies have confirmed that stress could stimulate the microglia and prime them toward the M1 phenotype, which is responsible for neuroinflammation (Zhao et al., 2016; Tang et al., 2018). In the present study, M1 phenotype markers including IL-1β, IL-6, TNF-α, and NF-κB, and M2 markers such as IL-4, IL-10, and TGF-β1 in the hippocampus and the PFC were measured in 10-month-old mice in the control, RIS, and RIS+ICA groups by ELISA (Michelucci et al., 2009; Park et al., 2015). The results showed that stressed APP/PS1 mice had higher levels of M1 markers and lower levels of M2 markers in both

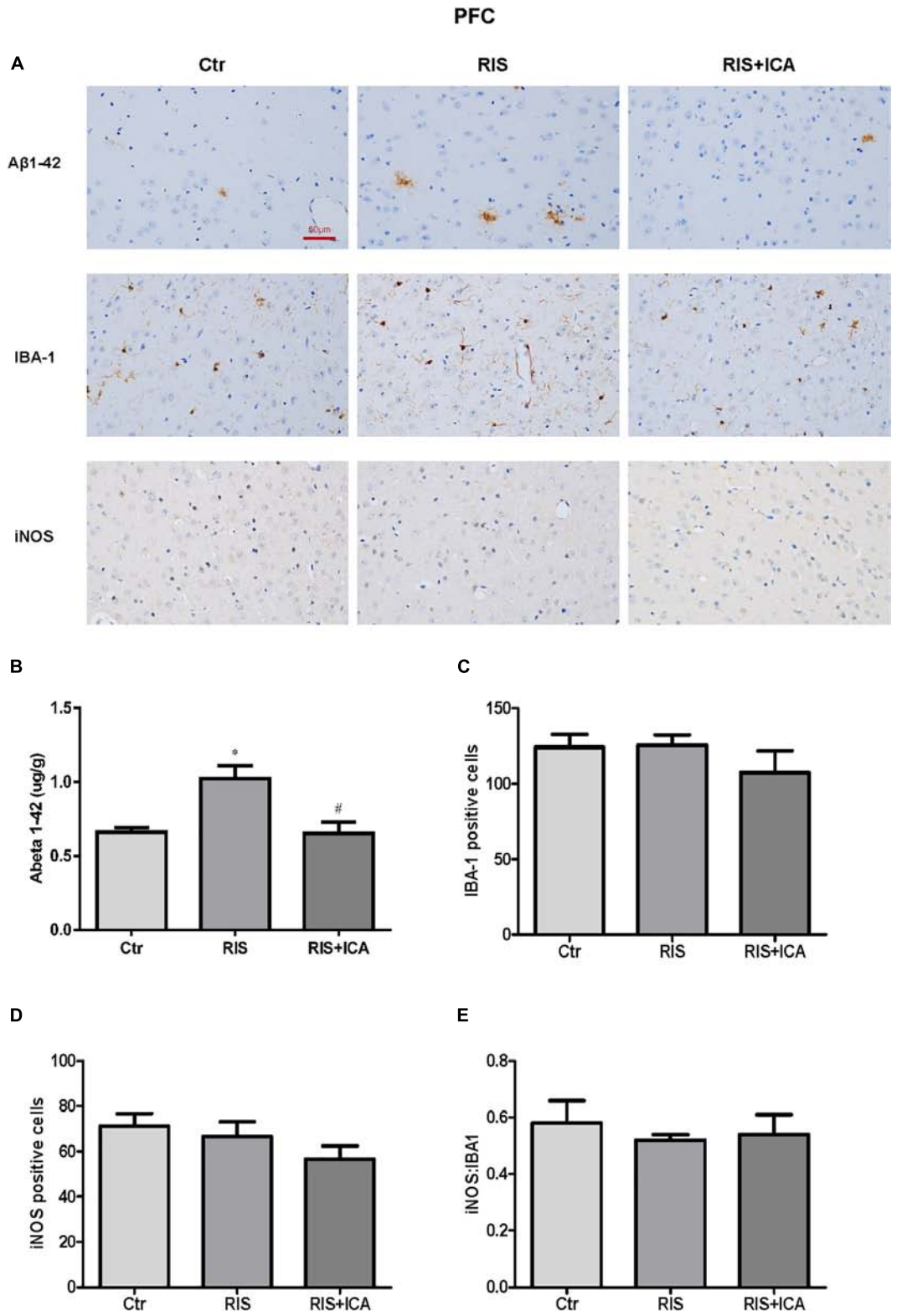

FIGURE 7 | Effect of icariin on Aβ plaques and microglia phenotype in the PFC. **(A)** Results of immunohistochemical staining (400×). **(B)** RIS significantly increased the level of Aβ compared with controls, and icariin decreased it. **(C–E)** The number of Iba-1+ **(C)** and iNOS+ **(D)** microglia cells was not significantly different among the three groups. As a result, the ratio of iNOS+ to Iba-1+ microglia **(E)** was not significantly different either among the three groups. The results of Aβ are expressed as the mean ± SEM, $n = 6$; the results of Iba-1 and iNOS are expressed as the mean ± SEM, $n = 4$. $^{*}p < 0.05$ vs. control group; $^{\#}p < 0.05$ vs. RIS group.

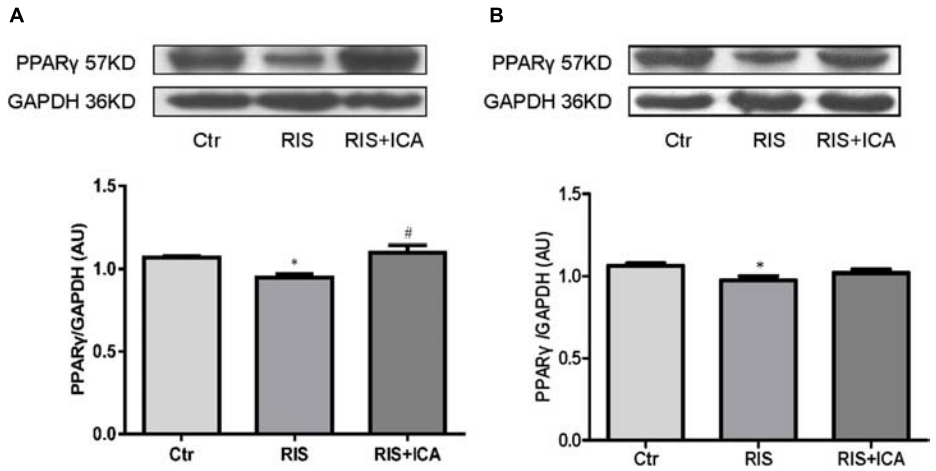

FIGURE 8 | Effect of icariin on PPARγ in the hippocampus and PFC. **(A)** PPARγ expression in the hippocampus using western blotting. The RIS procedure induced a marked decrease in PPARγ levels in APP/PS1 mice compared with controls, and icariin could reverse the alteration. **(B)** PPARγ expression in the PFC using western blotting. The RIS procedure induced a marked decrease in PPARγ level in APP/PS1 mice compared with controls, and icariin could partially reverse the alteration. The results of hippocampus are expressed as the mean ± SEM, $n = 4$; the results of PFC are expressed as the mean ± SEM, $n = 5$. $^{*}p < 0.05$ vs. control group; $^{\#}p < 0.05$ vs. RIS group.

the hippocampus and the PFC, indicating that stress induced microglia M1 activation in APP/PS1 mice. We also assessed the levels of the M1 phenotype marker iNOS and its ratio to the microglia marker Iba-1. We found that the fraction of M1 microglia was higher in the hippocampus, but not in the PFC of stressed mice than in control mice. A possible explanation is that the hippocampus is the area most sensitive to stress, because it shows the highest expression of corticosterone receptors among brain regions (Anacker et al., 2011; Zhang et al., 2018). Indeed the stress-induced activation of inflammatory pathways in the brain was shown to be region-specific (Ogundele et al., 2017). Given that the microglia M2 activation state was associated with the suppression of inflammation, promotion of phagocytosis and tissue repair (Chawla, 2010; Chinetti-Gbaguidi et al., 2011), as well as characterized by increased Aβ clearance and enhanced tissue remodeling (Heneka et al., 2013), increased microglia M1 activation in stressed APP/PS1 mice resulted in lower clearance ability and higher neurotoxicity of the microglia (Park et al., 2015). Moreover, microglia M1 activation and the release of inflammatory cytokines increased the neuroinflammation of brain, which could result in AD progression (Mandrekar-Colucci et al., 2012; Huang et al., 2017). In the present study, we observed that RIS mice had worse memory impairment in the MWM, together with higher levels of M1 phenotype markers and lower level of M2 phenotype markers in both hippocampus and PFC at 10 months of age, while icariin could attenuate these alterations. The results are consistent with the conclusion that the imbalance of M1/M2 microglia activation is involved in AD pathogenesis (Mandrekar-Colucci et al., 2012; Huang et al., 2017).

Previous studies confirmed that the failure of microglia to clear abnormally accumulating Aβ peptide led to neuroinflammation and neurodegeneration in AD models in which RIS elevated Aβ40 and Aβ42 levels in the brain, accelerated amyloid plaque formation, and impaired learning and memory (Jeong et al., 2006; Carroll et al., 2011; Lee and Han, 2013). In our study, the stressed APP/PS1 mice had higher level of Aβ accumulation in both the hippocampus and the PFC compared with unstressed mice. More importantly, the stressed APP/PS1 mice showed more severe memory impairment in the MWM test at 10 months of age compared with unstressed mice. These findings support the previous conclusion that psychological stress induces lower ability to clear accumulating Aβ peptide, resulting in impaired cognition (Jeong et al., 2006; Hoeijmakers et al., 2017).

The PPARs are a group of nuclear receptor proteins regulating gene expression as ligand-activated transcription factors (Michalik et al., 2006). Studies demonstrated that PPARγ can mediate the conversion of the microglia into the M2 phenotype (Yamanaka et al., 2012; Saijo et al., 2013). Chronic stress induced lower PPARγ expression in the adipose tissue and PPARγ knockout mice displayed more anxiety-like behaviors (Domi et al., 2016; Guo et al., 2017). Moreover, studies have suggested that activation of PPARγ-mediated anti-inflammatory signaling might be a potential therapeutic strategy for AD (Vallee et al., 2017). Our results showed that PPARγ expression levels in the hippocampus and the PFC were significantly lower in stressed than in unstressed mice, which indicates that stress induced microglia M1 activation is via suppression of PPARγ expression.

Epimedium (family Berberidaceae), commonly known as horny goat weed in the West, is known as Yin Yang Huo in Chinese medicine. Icariin is a highly potent active ingredient of this herb, believed to be the source of its many potential health benefits (Lee et al., 1995; Lin et al., 2004). Icariin was proven to possess anti-bacterial and anti-inflammatory efficacy (Luo et al., 2007; Zhou et al., 2011). A previous study indicated that it improved spatial learning and memory abilities in lipopolysaccharide (LPS)-induced rat brain dysfunction through the inhibition of hippocampus IL-1β and cyclooxygenase-2

(COX-2) expression (Guo et al., 2010). Accumulating evidence indicates that, in addition to inhibiting the activation of innate immune cells (including microglia) producing TNF-α and IL-1β, icariin led to stable suppression of NF-κB signaling activation in the hippocampus (Liu et al., 2015). Thus, icariin plays a counter-regulatory role resulting in neuroinflammation suppression. Studies have investigated the pharmacological properties of icariin in the CNS and showed that it can markedly attenuate cognitive deficits in several models of AD (Li et al., 2010; Nie et al., 2010; Jin et al., 2014), and also alleviate neuronal injury induced by ischemia (Li et al., 2005). It was also reported that icariin can attenuate inflammatory response through PPARγ activation in rats (Xiong et al., 2016). However, the role of icariin in attenuating PPARγ alterations in the AD brain has not yet been clarified. The present study compared the expression of PPARγ in the hippocampus and the PFC between controls, RIS, and RIS+ICA mice and found that stress induced lower PPARγ expression in the RIS mice, while icariin relieved such alteration in the RIS+ICA mice. Moreover, the results about the microglia activation phenotype and Aβ accumulation demonstrate that icariin administration normalized the Aβ clearance ability by up-regulating PPARγ in the RIS+ICA mice. As a result, the memory impairment of RIS+ICA mice was milder than that of untreated stressed mice.

CONCLUSION

Our results demonstrate that RIS induced depressive-like behaviors, spatial memory impairment, over-expression of M1 microglia markers and increased Aβ accumulation by suppressing PPARγ in APP/PS1 mice. Icariin, which induces a microglia phenotype switch through the activation of PPARγ, is a promising candidate for new therapeutic strategies. In conclusion, our study may provide novel insights into the role of chronic psychosocial stress in the pathogenesis and progression of AD and the development of new therapeutic approaches. Icariin affecting the microglia phenotype and cytokine release through other pathways should be further studied both *in vitro* and *in vivo*.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the supplementary files.

ETHICS STATEMENT

All procedures used in the study were reviewed and approved by the Ethics Committee of School of Medicine, Shandong University, and comply with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals (NIH publication no. 85-23, revised 1985). In the handling and care of all animals, we followed the international guiding principles for animal research, as stipulated by the World Health Organization (WHO) Chronicle (World Health Organization, 1985), as adopted by the Laboratory Animal Center, Shandong University.

AUTHOR CONTRIBUTIONS

XL was involved in study design and data interpretation. YW performed the majority of the laboratory work and contributed to the analysis of data and the writing of the manuscript. TZ, MW, FZ, GZ, JZ, and YZ were responsible for the animal model and the behavioral tests. EW revised the manuscript. All authors approved the final version to be submitted.

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REFERENCES

Anacker, C., Zunszain, P. A., Carvalho, L. A., and Pariante, C. M. (2011). The glucocorticoid receptor: pivot of depression and of antidepressant treatment? *Psychoneuroendocrinology* 36, 415–425. doi: 10.1016/j.psyneuen.2010.03.007

Berger, J., and Moller, D. E. (2002). The mechanisms of action of PPARs. *Annu. Rev. Med.* 53, 409–435. doi: 10.1146/annurev.med.53.082901.104018

Braak, H., and Braak, E. (1996). Evolution of the neuropathology of Alzheimer's disease. *Acta Neurol. Scand. Suppl.* 165, 3–12. doi: 10.1111/j.1600-0404.1996.tb05866.x

Breidert, T., Callebert, J., Heneka, M. T., Landreth, G., Launay, J. M., and Hirsch, E. C. (2002). Protective action of the peroxisome proliferator-activated receptor-gamma agonist pioglitazone in a mouse model of Parkinson's disease. *J. Neurochem.* 82, 615–624. doi: 10.1046/j.1471-4159.2002.00990.x

Carroll, J. C., Iba, M., Bangasser, D. A., Valentino, R. J., James, M. J., Brunden, K. R., et al. (2011). Chronic stress exacerbates tau pathology, neurodegeneration, and cognitive performance through a corticotropin-releasing factor receptor-dependent mechanism in a transgenic mouse model of tauopathy. *J. Neurosci.* 31, 14436–14449. doi: 10.1523/JNEUROSCI.3836-11.2011

Catania, C., Sotiropoulos, I., Silva, R., Onofri, C., Breen, K. C., Sousa, N., et al. (2009). The amyloidogenic potential and behavioral correlates of stress. *Mol. Psychiatry* 14, 95–105. doi: 10.1038/sj.mp.4002101

Chang, F., Jaber, L. A., Berlie, H. D., and O'Connell, M. B. (2007). Evolution of peroxisome proliferator-activated receptor agonists. *Ann. Pharmacother.* 41, 973–983. doi: 10.1345/aph.1K013

Chawla, A. (2010). Control of macrophage activation and function by PPARs. *Circ. Res.* 106, 1559–1569. doi: 10.1161/CIRCRESAHA.110.216523

Chinetti-Gbaguidi, G., Baron, M., Bouhlel, M. A., Vanhoutte, J., Copin, C., Sebti, Y., et al. (2011). Human atherosclerotic plaque alternative macrophages display low cholesterol handling but high phagocytosis because of distinct activities of the PPARgamma and LXRalpha pathways. *Circ. Res.* 108, 985–995. doi: 10.1161/CIRCRESAHA.110.233775

Domi, E., Uhrig, S., Soverchia, L., Spanagel, R., Hansson, A. C., Barbier, E., et al. (2016). Genetic deletion of neuronal PPARgamma enhances the emotional

response to acute stress and exacerbates anxiety: an effect reversed by rescue of amygdala PPARgamma function. *J. Neurosci.* 36, 12611–12623. doi: 10.1523/JNEUROSCI.4127-15.2016

Elgh, E., Lindqvist Astot, A., Fagerlund, M., Eriksson, S., Olsson, T., and Nasman, B. (2006). Cognitive dysfunction, hippocampal atrophy and glucocorticoid feedback in Alzheimer's disease. *Biol. Psychiatry* 59, 155–161. doi: 10.1016/j.biopsych.2005.06.017

Garcia-Bueno, B., Madrigal, J. L., Lizasoain, I., Moro, M. A., Lorenzo, P., and Leza, J. C. (2005). Peroxisome proliferator-activated receptor gamma activation decreases neuroinflammation in brain after stress in rats. *Biol. Psychiatry* 57, 885–894. doi: 10.1016/j.biopsych.2005.01.007

Gracia-Garcia, P., de-la-Camara, C., Santabarbara, J., Lopez-Anton, R., Quintanilla, M. A., Ventura, T., et al. (2015). Depression and incident Alzheimer disease: the impact of disease severity. *Am. J. Geriatr. Psychiatry* 23, 119–129. doi: 10.1016/j.jagp.2013.02.011

Green, K. N., Billings, L. M., Roozendaal, B., McGaugh, J. L., and LaFerla, F. M. (2006). Glucocorticoids increase amyloid-beta and tau pathology in a mouse model of Alzheimer's disease. *J. Neurosci.* 26, 9047–9056. doi: 10.1523/JNEUROSCI.2797-06.2006

Green, R. C., Cupples, L. A., Kurz, A., Auerbach, S., Go, R., Sadovnick, D., et al. (2003). Depression as a risk factor for Alzheimer disease: the MIRAGE Study. *Arch. Neurol.* 60, 753–759. doi: 10.1001/archneur.60.5.753

Guo, J., Li, F., Wu, Q., Gong, Q., Lu, Y., and Shi, J. (2010). Protective effects of icariin on brain dysfunction induced by lipopolysaccharide in rats. *Phytomedicine* 17, 950–955. doi: 10.1016/j.phymed.2010.03.007

Guo, M., Li, C., Lei, Y., Xu, S., Zhao, D., and Lu, X. Y. (2017). Role of the adipose PPARgamma-adiponectin axis in susceptibility to stress and depression/anxiety-related behaviors. *Mol. Psychiatry* 22, 1056–1068. doi: 10.1038/mp.2016.225

Hartmann, A., Veldhuis, J. D., Deuschle, M., Standhardt, H., and Heuser, I. (1997). Twenty-four hour cortisol release profiles in patients with Alzheimer's and Parkinson's disease compared to normal controls: ultradian secretory pulsatility and diurnal variation. *Neurobiol. Aging* 18, 285–289. doi: 10.1016/S0197-4580(97)80309-0

He, Y., Li, H., Zhang, F., Zhang, G., Tang, X., Zhu, T., et al. (2016). Immunotherapeutic effects of lymphocytes co-cultured with human cord blood-derived multipotent stem cells transplantation on APP/PS1 mice. *Behav. Brain Res.* 315, 94–102. doi: 10.1016/j.bbr.2016.08.025

Heneka, M. T., Kummer, M. P., Stutz, A., Delekate, A., Schwartz, S., Vieira-Saecker, A., et al. (2013). NLRP3 is activated in Alzheimer's disease and contributes to pathology in APP/PS1 mice. *Nature* 493, 674–678. doi: 10.1038/nature11729

Hoeijmakers, L., Ruigrok, S. R., Amelianchik, A., Ivan, D., Dam, A. V., Lucassen, P. J., et al. (2016). Early-life stress lastingly alters the neuroinflammatory response to amyloid pathology in an Alzheimer's disease mouse model. *Brain Behav. Immun.* 63, 160–175. doi: 10.1016/j.bbi.2016.12.023

Hoeijmakers, L., Ruigrok, S. R., Amelianchik, A., Ivan, D., van Dam, A. M., Lucassen, P. J., et al. (2017). Early-life stress lastingly alters the neuroinflammatory response to amyloid pathology in an Alzheimer's disease mouse model. *Brain Behav. Immun.* 63, 160–175. doi: 10.1016/j.bbi.2016.12.023

Huang, C., Dong, D., Jiao, Q., Pan, H., Ma, L., and Wang, R. (2017). Sarsasapogenin-AA13 ameliorates Abeta-induced cognitive deficits via improving neuroglial capacity on Abeta clearance and antiinflammation. *CNS Neurosci. Ther.* 23, 498–509. doi: 10.1111/cns.12697

Jeong, Y. H., Park, C. H., Yoo, J., Shin, K. Y., Ahn, S. M., Kim, H. S., et al. (2006). Chronic stress accelerates learning and memory impairments and increases amyloid deposition in APPV717I-CT100 transgenic mice, an Alzheimer's disease model. *FASEB J.* 20, 729–731. doi: 10.1096/fj.05-4265fje

Jin, F., Gong, Q. H., Xu, Y. S., Wang, L. N., Jin, H., Li, F., et al. (2014). Icariin, a phosphodiesterase-5 inhibitor, improves learning and memory in APP/PS1 transgenic mice by stimulation of NO/cGMP signalling. *Int. J. Neuropsychopharmacol.* 17, 871–881. doi: 10.1017/S1461145713001533

Joshi, Y. B., Chu, J., and Pratico, D. (2012). Stress hormone leads to memory deficits and altered tau phosphorylation in a model of Alzheimer's disease. *J. Alzheimers Dis.* 31, 167–176. doi: 10.3233/JAD-2012-120328

Justice, N. J., Huang, L., Tian, J. B., Cole, A., Pruski, M., Hunt, A. J., et al. (2015). Posttraumatic stress disorder-like induction elevates beta-amyloid levels, which directly activates corticotropin-releasing factor neurons to exacerbate stress responses. *J. Neurosci.* 35, 2612–2623. doi: 10.1523/JNEUROSCI.3333-14.2015

Kobayashi, K., Imagama, S., Ohgomori, T., Hirano, K., Uchimura, K., Sakamoto, K., et al. (2013). Minocycline selectively inhibits M1 polarization of microglia. *Cell Death Dis.* 4:e525. doi: 10.1038/cddis.2013.54

Lee, J. E., and Han, P. L. (2013). An update of animal models of Alzheimer disease with a reevaluation of plaque depositions. *Exp. Neurobiol.* 22, 84–95. doi: 10.5607/en.2013.22.2.84

Lee, M. K., Choi, Y. J., Sung, S. H., Shin, D. I., Kim, J. W., and Kim, Y. C. (1995). Antihepatotoxic activity of icariin, a major constituent of *Epimedium koreanum*. *Planta Med.* 61, 523–526. doi: 10.1055/s-2006-959362

Li, F., Dong, H. X., Gong, Q. H., Wu, Q., Jin, F., and Shi, J. S. (2015). Icariin decreases both APP and Abeta levels and increases neurogenesis in the brain of Tg2576 mice. *Neuroscience* 304, 29–35. doi: 10.1016/j.neuroscience.2015.06.010

Li, F., Gong, Q. H., Wu, Q., Lu, Y. F., and Shi, J. S. (2010). Icariin isolated from *Epimedium brevicornum* Maxim attenuates learning and memory deficits induced by d-galactose in rats. *Pharmacol. Biochem. Behav.* 96, 301–305. doi: 10.1016/j.pbb.2010.05.021

Li, L., Zhou, Q. X., and Shi, J. S. (2005). Protective effects of icariin on neurons injured by cerebral ischemia/reperfusion. *Chin. Med. J.* 118, 1637–1643.

Lin, C. C., Ng, L. T., Hsu, F. F., Shieh, D. E., and Chiang, L. C. (2004). Cytotoxic effects of *Coptis chinensis* and *Epimedium sagittatum* extracts and their major constituents (berberine, coptisine and icariin) on hepatoma and leukaemia cell growth. *Clin. Exp. Pharmacol. Physiol.* 31, 65–69. doi: 10.1111/j.1440-1681.2004.03951.x

Liu, B., Xu, C., Wu, X., Liu, F., Du, Y., Sun, J., et al. (2015). Icariin exerts an antidepressant effect in an unpredictable chronic mild stress model of depression in rats and is associated with the regulation of hippocampal neuroinflammation. *Neuroscience* 294, 193–205. doi: 10.1016/j.neuroscience.2015.02.053

Liu, X., Yang le, J., Fan, S. J., Jiang, H., and Pan, F. (2010). Swimming exercise effects on the expression of HSP70 and iNOS in hippocampus and prefrontal cortex in combined stress. *Neurosci. Lett.* 476, 99–103. doi: 10.1016/j.neulet.2010.04.011

Luo, Y., Nie, J., Gong, Q. H., Lu, Y. F., Wu, Q., and Shi, J. S. (2007). Protective effects of icariin against learning and memory deficits induced by aluminium in rats. *Clin. Exp. Pharmacol. Physiol.* 34, 792–795. doi: 10.1111/j.1440-1681.2007.04647.x

Mandrekar-Colucci, S., Karlo, J. C., and Landreth, G. E. (2012). Mechanisms underlying the rapid peroxisome proliferator-activated receptor-gamma-mediated amyloid clearance and reversal of cognitive deficits in a murine model of Alzheimer's disease. *J. Neurosci.* 32, 10117–10128. doi: 10.1523/JNEUROSCI.5268-11.2012

Michalik, L., Auwerx, J., Berger, J. P., Chatterjee, V. K., Glass, C. K., Gonzalez, F. J., et al. (2006). International Union of Pharmacology. LXI. Peroxisome proliferator-activated receptors. *Pharmacol. Rev.* 58, 726–741. doi: 10.1124/pr.58.4.5

Michelucci, A., Heurtaux, T., Grandbarbe, L., Morga, E., and Heuschling, P. (2009). Characterization of the microglial phenotype under specific pro-inflammatory and anti-inflammatory conditions: effects of oligomeric and fibrillar amyloid-beta. *J Neuroimmunol* 210, 3–12. doi: 10.1016/j.jneuroim.2009.02.003

Moreno, S., Farioli-Vecchioli, S., and Ceru, M. P. (2004). Immunolocalization of peroxisome proliferator-activated receptors and retinoid X receptors in the adult rat CNS. *Neuroscience* 123, 131–145. doi: 10.1016/j.neuroscience.2003.08.064

Nie, J., Luo, Y., Huang, X. N., Gong, Q. H., Wu, Q., and Shi, J. S. (2010). Icariin inhibits beta-amyloid peptide segment 25-35 induced expression of beta-secretase in rat hippocampus. *Eur. J. Pharmacol.* 626, 213–218. doi: 10.1016/j.ejphar.2009.09.039

Ogundele, O. M., Ebenezer, P. J., Lee, C. C., and Francis, J. (2017). Stress-altered synaptic plasticity and DAMP signaling in the hippocampus-PFC axis; elucidating the significance of IGF-1/IGF-1R/CaMKIIalpha expression in neural changes associated with a prolonged exposure therapy. *Neuroscience* 353, 147–165. doi: 10.1016/j.neuroscience.2017.04.008

Park, J., Min, J. S., Kim, B., Chae, U. B., Yun, J. W., Choi, M. S., et al. (2015). Mitochondrial ROS govern the LPS-induced pro-inflammatory response in microglia cells by regulating MAPK and NF-kappaB pathways. *Neurosci. Lett.* 584, 191–196. doi: 10.1016/j.neulet.2014.10.016

Piirainen, S., Youssef, A., Song, C., Kalueff, A. V., Landreth, G. E., Malm, T., et al. (2017). Psychosocial stress on neuroinflammation and cognitive dysfunctions in Alzheimer's disease: the emerging role for microglia? *Neurosci. Biobehav. Rev.* 77, 148–164. doi: 10.1016/j.neubiorev.2017.01.046

Querfurth, H. W., and LaFerla, F. M. (2010). Alzheimer's disease. *N. Engl. J. Med.* 362, 329–344. doi: 10.1056/NEJMra0909142

Qureshi, S. U., Kimbrell, T., Pyne, J. M., Magruder, K. M., Hudson, T. J., Petersen, N. J., et al. (2010). Greater prevalence and incidence of dementia in older veterans with posttraumatic stress disorder. *J. Am. Geriatr. Soc.* 58, 1627–1633. doi: 10.1111/j.1532-5415.2010.02977.x

Sacuiu, S., Insel, P. S., Mueller, S., Tosun, D., Mattsson, N., Jack, C. R., et al. (2016). Chronic depressive symptomatology in mild cognitive impairment is associated with frontal atrophy rate which hastens conversion to Alzheimer Dementia. *Am. J. Geriatr. Psychiatry* 24, 126–135. doi: 10.1016/j.jagp.2015.03.006

Saijo, K., Crotti, A., and Glass, C. K. (2013). Regulation of microglia activation and deactivation by nuclear receptors. *Glia* 61, 104–111. doi: 10.1002/glia.22423

Selkoe, D. J., and Schenk, D. (2003). Alzheimer's disease: molecular understanding predicts amyloid-based therapeutics. *Annu. Rev. Pharmacol. Toxicol.* 43, 545–584. doi: 10.1146/annurev.pharmtox.43.100901.140248

Song, J., Choi, S. M., and Kim, B. C. (2017). Adiponectin regulates the polarization and function of microglia via PPAR-gamma signaling under amyloid beta toxicity. *Front. Cell. Neurosci.* 11:64. doi: 10.3389/fncel.2017.00064

Sotiropoulos, I., Catania, C., Riedemann, T., Fry, J. P., Breen, K. C., Michaelidis, T. M., et al. (2008). Glucocorticoids trigger Alzheimer disease-like pathobiochemistry in rat neuronal cells expressing human tau. *J. Neurochem.* 107, 385–397. doi: 10.1111/j.1471-4159.2008.05613.x

Tang, J., Yu, W., Chen, S., Gao, Z., and Xiao, B. (2018). Microglia polarization and endoplasmic reticulum stress in chronic social defeat stress induced depression mouse. *Neurochem. Res.* 43, 985–994. doi: 10.1007/s11064-018-2504-0

Tontonoz, P., and Spiegelman, B. M. (2008). Fat and beyond: the diverse biology of PPARgamma. *Annu. Rev. Biochem.* 77, 289–312. doi: 10.1146/annurev.biochem.77.061307.091829

Vallee, A., Lecarpentier, Y., Guillevin, R., and Vallee, J. N. (2017). Effects of cannabidiol interactions with Wnt/beta-catenin pathway and PPARgamma on oxidative stress and neuroinflammation in Alzheimer's disease. *Acta Biochim. Biophys. Sin.* 49, 853–866. doi: 10.1093/abbs/gmx073

Wang, X., Li, J., Qian, L., Zang, X. F., Zhang, S. Y., Wang, X. Y., et al. (2013). Icariin promotes histone acetylation and attenuates post-stroke cognitive impairment in the central cholinergic circuits of mice. *Neuroscience* 236, 281–288. doi: 10.1016/j.neuroscience.2012.12.074

Wilson, R. S., Barnes, L. L., Bennett, D. A., Li, Y., Bienias, J. L., Mendes de Leon, C. F., et al. (2005). Proneness to psychological distress and risk of Alzheimer disease in a biracial community. *Neurology* 64, 380–382. doi: 10.1212/01.WNL.0000149525.53525.E7

World Health Organization (1985). International guiding principles for animal research. *Chronicle* 39, 52–56.

Xiong, D., Deng, Y., Huang, B., Yin, C., Liu, B., Shi, J., et al. (2016). Icariin attenuates cerebral ischemia-reperfusion injury through inhibition of inflammatory response mediated by NF-kappaB, PPARalpha and PPARgamma in rats. *Int. Immunopharmacol.* 30, 157–162. doi: 10.1016/j.intimp.2015.11.035

Yaffe, K., Vittinghoff, E., Lindquist, K., Barnes, D., Covinsky, K. E., Neylan, T., et al. (2010). Posttraumatic stress disorder and risk of dementia among US veterans. *Arch. Gen. Psychiatry* 67, 608–613. doi: 10.1001/archgenpsychiatry.2010.61

Yamanaka, M., Ishikawa, T., Griep, A., Axt, D., Kummer, M. P., and Heneka, M. T. (2012). PPARgamma/RXRalpha-induced and CD36-mediated microglial amyloid-beta phagocytosis results in cognitive improvement in amyloid precursor protein/presenilin 1 mice. *J. Neurosci.* 32, 17321–17331. doi: 10.1523/JNEUROSCI.1569-12.2012

Yang, L. J., Liu, X., Liu, D. X., Jiang, H., Mao, X. Q., Wang, C., et al. (2012). Effects of different adrenergic blockades on the stress resistance of Wistar rats. *Neurosci. Lett.* 511, 95–100. doi: 10.1016/j.neulet.2012.01.046

Zhang, L., Zhang, J., and You, Z. (2018). Switching of the microglial activation phenotype is a possible treatment for depression disorder. *Front. Cell. Neurosci.* 12:306. doi: 10.3389/fncel.2018.00306

Zhao, Q., Wu, X., Yan, S., Xie, X., Fan, Y., Zhang, J., et al. (2016). The antidepressant-like effects of pioglitazone in a chronic mild stress mouse model are associated with PPARgamma-mediated alteration of microglial activation phenotypes. *J. Neuroinflammation* 13:259. doi: 10.1186/s12974-016-0728-y

Zhao, Q., Xie, X., Fan, Y., Zhang, J., Jiang, W., Wu, X., et al. (2015). Phenotypic dysregulation of microglial activation in young offspring rats with maternal sleep deprivation-induced cognitive impairment. *Sci. Rep.* 5:9513. doi: 10.1038/srep09513

Zhou, J., Wu, J., Chen, X., Fortenbery, N., Eksioglu, E., Kodumudi, K. N., et al. (2011). Icariin and its derivative, ICT, exert anti-inflammatory, anti-tumor effects, and modulate myeloid derived suppressive cells (MDSCs) functions. *Int. Immunopharmacol.* 11, 890–898. doi: 10.1016/j.intimp.2011.01.007

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Natural Diterpenoid Oridonin Ameliorates Experimental Autoimmune Neuritis by Promoting Anti-inflammatory Macrophages Through Blocking Notch Pathway

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The diterpenoid compound, Oridonin, extracted from the Chinese herb, *Rabdosia rubescens*, possesses multiple biological activities and properties. Oridonin exhibited efficient anti-inflammatory activity by inducing a switch in macrophage polarization to the anti-inflammatory phenotype through inhibition of the Notch pathway in our *in vitro* study; therefore, its potential therapeutic effects were further investigated in the animal model of human Guillain-Barré syndrome (GBS) and other polyneuropathies – experimental autoimmune neuritis (EAN). Either preventive or therapeutic treatments with Oridonin greatly attenuated disease peak severity, suppressed paraparesis, shortened disease duration, and even delayed EAN onset. Progression of neuropathic pain, demyelination, inflammatory cellular accumulations, and inflammatory cytokines in peripheral nerves were significantly attenuated. Meanwhile, accumulation of immune cells in the spinal roots and microglial activation in the lumbar spinal cord were also reduced. Interestingly, Oridonin treatment significantly increased the proportion of anti-inflammatory macrophages and made them locally dominant among all infiltrated macrophages in the peripheral nerves. The down-regulation of local Notch pathway proteins, together with our *in vitro* results indicated their possible involvement. Taken together, our results demonstrated that Oridonin effectively suppressed EAN by attenuating local inflammatory reaction and increasing the proportion of immune regulating macrophages in the peripheral nerves, possibly through blockage of the Notch pathway, which suggests Oridonin as a potential therapeutic candidate for human GBS and neuropathies.

Keywords: Guillain-Barré syndrome, experimental autoimmune neuritis, Oridonin, anti-inflammatory macrophage, Notch pathway

Abbreviations: CFA, complete Freund's adjuvant; CMC, carboxymethylcellulose; EAN, experimental autoimmune neuritis; FBS, fetal bovine serum; GBS, Guillain-Barré syndrome; GFAP, glial fibrillary acidic protein; HWT, hind-paw withdrawal threshold; IL-1β, interleukin-1β; iNOS, inducible nitric oxide synthase; IR, immunoreactivity; LFB, Luxol fast blue; LPS, lipopolysaccharide; MNCs, mononuclear cells; PNS, peripheral nervous system; SEM, standard errors of means; TBS, Tris-buffered saline; TNF, tumor necrosis factor.

INTRODUCTION

Guillain-Barré syndrome (GBS) is an immune-mediated polyneuropathy; the world's leading cause of acute autoimmune neuromuscular paralysis, caused by an autoimmune attack on the peripheral nervous system (PNS) (Willison, 2005; Kuwabara and Yuki, 2013). Existing treatments, including supportive managements, like advanced intensive care and assisted respiration, as well as some active treatments such as plasma exchange and intravenous immunoglobulin, are not satisfactory (Hughes, 2002). Not all GBS patients respond to these treatments, and even if they do, they still do not recover fully; and continuing weakness, motor units deprivation, and the problem of fatigue continues to be detected (Winer, 2001; Kieseier et al., 2004). Therefore, more effective, and convenient therapeutic approaches for the inflammatory peripheral demyelinating disease are needed. Experimental autoimmune neuritis (EAN), an autoantigen-specific, T cell-mediated animal model for demyelinating inflammatory diseases of the PNS, is broadly accepted and used to study pathogenesis, pathological changes, and potential therapeutic approaches for human polyneuropathies like GBS, by mirroring many of their neurological, electrophysiological, immunological, and pathological features (Hughes and Cornblath, 2005).

Experimental autoimmune neuritis is actively induced by immunization with autoantigen-like purified myelin or P2 peptides. Following the breakdown of the blood-nerve barrier, reactivated T cells and macrophages are abundantly infiltrated and accumulated in the PNS, which results in a robust local inflammation, demyelination, and axon degeneration, and all of these are crucial for the progression of EAN (Kieseier et al., 2004; Zhang et al., 2008a). Modulating or controlling the activation of these immune cells is, thus, considered an important therapeutic strategy for EAN and human neuropathies. As the main infiltrating cell population and the major effector cell present during EAN progression, macrophages are responsible for most neuropathological changes due to their antigen-presenting property and direct phagocytosis of axon myelin (Kiefer et al., 2001; Maurer et al., 2002). Infiltrating macrophages also cause tissue damage by secretion of inflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor (TNF)-α, and these are responsible for demyelination and axon loss as well (Kiefer et al., 2001). Inhibition of macrophage activity or their depletion was proved to prevent EAN, as observed through all the clinical, electrophysiological, and histological changes (Craggs et al., 1984). However, recruited macrophages can be broadly divided into two particular phenotypes: classically activated macrophages that promote inflammatory reaction and tissue damage and alternatively activated macrophages with anti-inflammatory activity and tissue-repairing effects (Mosser and Edwards, 2008; Ricardo et al., 2008). Pro-inflammatory macrophages execute host-defense against infection, but also cause inflammatory tissue damage and lead to the high expression of pro-inflammatory cytokines such as IL-12, IL-23, and TNF-α and inducible nitric oxide synthase (iNOS). Anti-inflammatory macrophages are considered to control inflammatory reactions and are responsible for tissue repair; they are characterized by the high expression of anti-inflammatory molecules such as IL-10 and arginase-I (Mosser and Edwards, 2008; Ricardo et al., 2008). It has been previously reported that a phenotypic switch of macrophage polarization to the anti-inflammatory state can attenuate pathological progression and even determine the outcome of EAN (Zhang Z. Y. et al., 2010; Han et al., 2016a,b).

Plant-derived compounds have gained increased attention as nutraceutical treatments for various diseases, including cardiovascular diseases, mental disorders, cancers, and inflammatory diseases (Kumar, 2006). Oridonin, a natural diterpenoid compound extracted from a Chinese herb *Rabdosia rubescens* (Zhang Z. et al., 2010), exhibits numerous biological activities and effects, including oxygen free-radical clearing, anti-mutagenic, and anti-microbial activities, and is recently being studied intensively for promising anti-neoplastic activity (Ji et al., 2011, Jia et al., 2012). Moreover, its anti-inflammatory activities (He et al., 2018; Zhou et al., 2018), especially anti-neuroinflammatory and neuroprotective effects, have been reported recently in different *in vitro* and *in vivo* studies (Liu et al., 2007; Xu et al., 2009; Chen et al., 2012), which indicates its therapeutic potential in treating inflammatory disorders of the nervous system. As a widely used Traditional Chinese Medicine (TCM) and a main ingredient of many commonly available over-the-counter (OTC) herbal medicines in China, *R. rubescens* and its aqueous extract have shown excellent tolerance and oral bioavailability; therefore, the proven safety and reliability of the herb further ascertains its promising potential in clinical applications (Chen et al., 2009; Ma et al., 2011; Cheng et al., 2017).

Notch signaling is initiated by the Notch receptor-ligand binding (Mailhos et al., 2001). Typical signaling proteins such as Notch1, Jagged-2, and Hes-1 are usually tested for the activation or inhibition of Notch signaling, and were also investigated in our study. After Oridonin showed efficient anti-inflammatory activity and induced a phenotypic switch of macrophage polarization toward the anti-inflammatory state in our cell culture, the potential therapeutic effects of Oridonin were further investigated in the rat model of EAN, as we hypothesized that the possible effects might be connected to the regulation of immune cells, especially macrophages polarization.

MATERIALS AND METHODS

Animals

Male Lewis rats (10–12 weeks, 230–250 g, Charles River, Sulzfeld, Germany) were housed under a 12 h light-12 h dark cycle with free access to food and water. The guideline EU Directive 2010/63/EU for animal experiments was followed and all animal procedures were approved by the local Administration District Official Committee. All efforts were made to minimize the number of animals and their suffering.

Isolation of Primary Peritoneal Macrophages and Cell Culture

The rats were treated with 4 ml thioglycollate medium intraperitoneally. Seven days later, peritoneal cells were obtained

by peritoneal lavage with 12 ml of ice-cold PBS (phosphate-buffered saline) containing 2% FBS (fetal bovine serum) (Invitrogen, Karlsruhe, Germany). FITC-conjugated anti-CD68 antibodies (Serotec, Oxford, United Kingdom) were used to confirm the purity of the macrophages and more than 90% of the peritoneal cells were macrophages.

Primary peritoneal macrophages and a common murine macrophage cell line, RAW 264.7, (Sigma-Aldrich, Munich, Germany) were both used to evaluate the effects of Oridonin on the inflammatory reaction and polarization of macrophages *in vitro*. Primary macrophages and RAW cells were maintained in complete RPMI 1640 media, from Gibco (Invitrogen, Karlsruhe, Germany), containing 10% FBS (Invitrogen, Karlsruhe, Germany), penicillin (100 U/ml), and streptomycin (100 U/ml). 10^5 cells were seeded into 12-well plates. After 24 h of culture, lipopolysaccharide (LPS) (Sigma-Aldrich, Munich, Germany) was added in some wells and co-cultured for another 24 h; some groups were treated with Oridonin (0.25, 1, or 4 μM). A previous pharmacokinetic study (Xu et al., 2006) reported that the plasma concentrations of Oridonin after oral dosing of 20 mg/kg or 40 mg/kg in rats were between 0.1 and 0.35 μg/ml for over 10 h, which were equivalent to 0.25 to 0.95 μM. Our *in vitro* study, therefore, used working concentrations of 0.25, 1, and 4 μM. Further, some groups of cells were incubated for 48 h with 30 μM DAPT (Sigma-Aldrich, Munich, Germany), an inhibitor of the Notch signaling pathway. Thereafter, supernatants from different wells were collected and a standard Griess assay (Sigma-Aldrich, Munich, Germany) was performed to analyze the production of nitric oxide (NO). Total RNA was extracted from cells with the RNeasy Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. QuantiTect Reverse Transcription Kit (QIAGEN, Hilden, Germany) was utilized to reverse transcribe RNA (1 μg) into cDNA. Real-time PCR analysis was used to measure the mRNA expression. ELISA kits for IL-1β (Thermo Scientific, Waltham, MA, United States), TNF-α, and IL-10 (Bio legend Inc., San Diego, CA, United States) were used to detect the concentrations of the cytokines in macrophage culture supernatants.

Transient Transfection

The Notch1-NICD (Notch intracellular domain) was generated using the following primers: sense 5′-CGCGGATCCATGCACCTGGATGCCGCTGACCTG-3′ and antisense 5′-ACGTCTAGACTYGAAGGCCTCCGGAATGCG-3′. The pair of primers were designed for Notch1-NICD gene sequences from the GenBank database, and the fragment of Notch1-NICD was cloned into the pcDNA3.1 vector (Invitrogen, Karlsruhe, Germany). Lipofectamine 2000 (Invitrogen, Karlsruhe, Germany) was used to conduct transfection based on the manufacturer's protocols. The Real-time-PCR was conducted to monitor the transfection efficiency. RAW cells from the other comparable groups, including the control group, were all transiently transfected with a plasmid vehicle.

EAN Induction and Oridonin Treatment

After dissolving in PBS at a concentration of 2 mg/ml, the synthetic neurogenic peptide, P2 57-81 (GeneScript Corporation, Scotch Plains, NJ, United States) was emulsified with 2 mg/ml *Mycobacterium tuberculosis* mixed within Complete Freund's Adjuvant (CFA) (Sigma-Aldrich; Munich, Germany), and the final concentration of peptide in the emulsion was 1 mg/ml. Each rat was injected subcutaneously at the base of the tail with 100 μl emulsion containing 100 μg peptide.

Once a day, neurological signs of EAN were recorded and graded as follows: normal (score = 0), reduced tonus of tail (score = 1), impaired righting, limp tail (score = 2), absent righting (score = 3), gait ataxia (score = 4), mild paresis of the hind limbs (score = 5), moderate paraparesis (score = 6), severe paraparesis or paraplegia of the hind limbs (score = 7), tetraparesis (score = 8), moribund (score = 9), and death (score = 10).

Four rounds of Oridonin treatments were performed: two each of therapeutic and preventive treatments. EAN rats received daily gavages of Oridonin from Day 0 to Day 14 as the preventive treatment or from Day 9 to Day 14 as the therapeutic treatment (six rats/group); rats were prepared for tissue analysis on Day 15. In addition, another three groups of rats administered identical treatments as described were observed until they fully recovered to cover the entire spectrum of disease development and recovery. Oridonin, suspended in 1% carboxymethylcellulose (CMC) (Blanose®, Hercules-Aqualon, Düsseldorf, Germany), was fed by gavage once a day at doses of 7 or 20 mg/kg of body weight.

It was reported that 175 mg of Oridonin administered orally to humans, which is a common dosage prescribed for inflammatory conditions in TCM, was comparable to the dose of 13.5 mg/kg administered to rats (Xu et al., 2006). The oral dosing of 20 mg/kg was comparable to the treatments of lupus-like symptoms (Zhou et al., 2013) and neuroinflammation of cerebral amyloidosis (Zhang et al., 2013) in mice. A daily dose of 10 mg/kg of body weight administered by the intraperitoneal (i.p.) route was reported previously to treat animal models of rats (Li et al., 2018). A previous pharmacokinetic study of Oridonin indicated that the oral absolute bioavailability of Oridonin was rather low and it was approximately 1/2–1/3 that of i.p. treatments (Xu et al., 2006). The same pharmacokinetic study further reported that the plasma concentrations of Oridonin after oral dosing of 20 mg/kg or 40 mg/kg in rats were between 0.25 and 0.95 μM, which is comparable to the working concentrations used in our *in vitro* study. All these reports indicated that the oral dose of 20 mg/kg to EAN rats is comparable to the dosage reported in most published studies and for human treatments in TCM with the herb, *R. rubescens*. Additionally, we tested the dose of 7 mg/kg (1/3 of the 20 mg/kg dose) in the preventive treatment round to observe the dose-dependent effect.

Mechanical Allodynia

Mechanical allodynia was defined by the reduction of the hind-paw withdrawal threshold (HWT), which was measured using a mechanical plantar test apparatus, namely, an automatic von Frey system (Ugo Basile, Milan, Italy). All rats were trained every day, starting six days before the first HWT measurement. HWT was examined between 10:00 and 14:00 every day from two days prior to immunization to 14 days post-immunization. Briefly, rats were

habituated for 10 min and acclimated for another 10 min. The mechanical force (from 0 to 50 *g* over a period of 15 s) was exerted onto the middle of the hind-paws using a fine metal filament. Left and right hind-paws were measured 10 times each, one after the other. The forces under which the rats actively lifted their paws on their own initiative were recorded and mean values were calculated. All EAN rats for mechanical allodynia assays were re-grouped before immunization according to the measurement results of the training sessions to stratify the experimental groups.

Immunohistochemistry

On Day 15, after deep anesthesia and intracardial perfusion with 4% paraformaldehyde at 4°C, the rats were prepared for analysis. To detect the infiltration of inflammatory cells and the levels of pathological changes in the PNS, sciatic nerves from both the legs and the lumbar spinal cord were removed quickly and fixed overnight in 4% paraformaldehyde at 4°C. Each specimen was cut into two equal long segments, embedded in paraffin, serially sectioned (3 μm), and mounted on slides.

Sections were dewaxed and boiled in a microwave oven (600 W) in citrate buffer (sodium citrate 2.1 g/L, pH 6) for 15 min. Methanol containing 1% H_2O_2 was used to inhibit endogenous peroxidase for 15 min. After blockage with 10% pig serum (Biochrom, Berlin, Germany), cross sections were incubated with monoclonal antibodies as follows: CD3 (1:50; Serotec, Oxford, United Kingdom) for T cells, OX22 (1:200; Serotec, Oxford, United Kingdom) for B lymphocytes, glial fibrillary acidic protein (GFAP) (1:500; Chemicon International, Temecula, CA, United States) for astrocytes. Activated macrophages/microglia and anti-inflammatory macrophages were detected by ED1 (a lysosomal membrane protein which recognizes CD68) (1:100; Serotec, Oxford, United Kingdom) and ED2 (1:100; Serotec, Oxford, United Kingdom), respectively. Biotinylated IgG F(ab)2 (DAKO, Hamburg, Germany) was used as secondary antibody fragment to visualize the antibodies binding to sections. A horseradish peroxidase-conjugated streptavidin complex (DAKO, Hamburg, Germany) and diaminobenzidine (DAB) substrate (Fluka, Neu-Ulm, Germany) was subsequently added to sections. Tissues were counterstained with Maier's Hemalum in the end.

The ratio of immunoreactivity (IR) areas to sciatic nerve cross-section areas were calculated as described previously (Zhang et al., 2008a). Briefly, micro-images of staining were captured with a Nikon Cool-scope (Nikon, Düsseldorf, Germany); MetaMorph Offline 7.1 (Molecular Devices, Toronto, ON, Canada) was applied on the micro images to outline and analyze the cross sections of the sciatic nerves. In regions of interest, the percentages of specific IR were selected by color threshold segmentation and analyzed. For each staining, all parameters were fixed for all the images and no automatic adjustments were used. Results are shown as the arithmetic means of percentages of IR area to interest areas on cross-sections and standard errors of means (SEM).

Luxol Fast Blue (LFB) (Sigma-Aldrich; Munich, Germany) staining was used to visualize myelin. For quantification of the demyelination of impacted nerves, the software MetaMorph Offline 7.1 (Molecular Devices, Toronto, ON, Canada) was used to analyze the relative optical density of the nerves from all groups. Raw images of nerve cross sections were acquired at the same exposure level and converted to 8-bit gray scale files. For removing the image background, the mean density calculated from the threshold pixels.

Furthermore, between Oridonin-treated and control EAN rats, histological alterations were analyzed by a semi-quantitative method established previously (Zhang et al., 2008a). In short, cross-sections of middle levels of the left and right sciatic nerves from EAN rats were evaluated. All cross-sections were collected and perivascular areas were evaluated by two pathologists. The levels of pathological changes were semi-quantitatively graded as: normal perivascular area (0); mild inflammatory cellular infiltration in immediate proximity to the blood vessels (1); cellular infiltration and demyelination adjacent to the blood vessels (2); and cellular infiltration plus demyelination throughout the cross-sections (3). Results are shown as mean histological scores (Hartung et al., 1988).

Tissue Preparation, RNA Isolation, Reverse Transcription, and Semi-Quantitative PCR

Experimental autoimmune neuritis rats were deeply anesthetized and intracardially perfused with 4°C PBS. The sciatic nerves, lumbar spinal cords, livers and inguinal lymph nodes were removed quickly and stored in liquid nitrogen immediately for RNA isolation. Trizol LS Reagent (Invitrogen, Karlsruhe, Germany) was applied to isolate total RNA and QuantiTect Reverse Transcription Kit (QIAGEN, Hilden, Germany) was used to reverse transcribe RNA to cDNA, The resulting cDNA was used to measure semi-quantitatively the expression of genes using SYBR green qPCR master mix according to the manufacturer's protocol (BioRad, Hercules, CA, United States). Real-time measurements of gene expression were performed with an iCycler thermocycler system and iQ5 optical system software (BioRad, Hercules, CA, United States). All the results are presented as mRNA expression levels relative to the housekeeping gene β-actin.

Flow Cytometric Analysis

Single-cell suspensions were prepared from the spleens taken from the rats on Day 15. RBCs were lysed and monocytes were suspended in 100 μL PBS containing 1% FBS. All cells were stained, according to the manufacturer's protocol, with FITC-conjugated antibody against CD11b and anti-CD206-PE (eBioscience, San Diego, CA, United States), which is a specific anti-inflammatory marker. Data were analyzed using FlowJo Flow Cytometry Analysis Software[1].

Western Blot Analysis

On Day 15, total proteins was extracted from the sciatic nerves of various groups of EAN rats. All samples were adjusted to an equal volume and content and separated electrophoretically on 12% SDS-PAGE gels. Then, proteins were transferred to PVDF membranes (Millipore, Billerica, MA, United States). After being blocked in Tris-buffered saline solution (TBS) containing

[1] flowjo.com

5% BSA for 2 h, membranes were incubated at 4°C overnight with primary antibodies: Notch1 (1:1000), Jagged-2 (1:500) (Cell Signaling Technology, Beverly, MA, United States), Hes-1 (1:500) (Millipore, Single Oak Drive, Temecula, CA, United States), and β-actin (1:500) (Sigma, St. Louis, MO, United States). The proteins were visualized by secondary antibodies with enhanced chemiluminescence. The signals of specific proteins were detected with a Gel Doc imager (Serial No. 721BR08844; Bio-Rad, Hercules, CA, United States) and reported as the fraction of control β-actin.

Evaluation and Statistical Analysis

Differences between the neurologic scores and histological scores were evaluated with the Mann–Whitney *U* test. For paired and multiple group comparisons, Student's *t*-test and one-way analysis of variance (ANOVA) were applied, respectively. The results are represented as means ± SEM. Statistical significance was defined as p-values < 0.05. Statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA, United States). For the *in vitro* studies with cell cultures, graphs show mean ± SEM of triplicate wells and represent data from three independent experiments.

RESULTS

Oridonin Inhibited Inflammatory Response and Induced Phenotypic Switch of Macrophage Polarization in Cell Culture

As the anti-inflammatory phenotype of macrophages can attenuate the inflammatory response and promote tissue repair, we first investigated whether Oridonin could inhibit the inflammatory response of the macrophages cell line, RAW, and the primary peritoneal macrophages by favoring a switch of macrophages from classically activated inflammatory into the anti-inflammatory phenotype *in vitro*.

Upon LPS induction, increased NO production (**Figure 1A**) and mRNA expression of characteristic inflammatory cytokines IL-1β, IL-6, and iNOS (**Figure 1B**) indicated a classically pro-inflammatory phenotype in both the RAW cells and the primary macrophages. Oridonin treatments significantly reduced the NO production (**Figure 1A**) and reduced mRNA levels of these inflammatory cytokines in dose-dependent patterns, suggesting a suppressed inflammatory reaction (**Figures 1B,C**). Further, mRNA levels of anti-inflammatory phenotype markers IL-10 and CD206 were significantly increased, also in dose-dependent patterns, indicating a switch in the phenotype of the macrophages to the anti-inflammatory phenotype (**Figures 1B,C**). Additional MTT assays excluded possible differences in cell viability among the different treatments (data not shown).

The expression levels of IL-1β, TNF-α, and IL-10 from the primary macrophages were detected with the ELISA assay. The productions of IL-1β and TNF-α were significantly reduced by Oridonin. Further, the macrophages treated with Oridonin released much more IL-10 than LPS induced cells (**Figure 1D**). Therefore, Oridonin showed efficient anti-inflammatory activity and induced a switch of macrophage polarization toward the anti-inflammatory phenotype, which suggested its further application in the inflammatory animal model of EAN.

Oridonin Induced Phenotypic Switch in Macrophage Polarization by Blocking the Notch Signaling

We further investigated the possible mechanism behind the anti-inflammatory effects of Oridonin. The Notch pathway is a direct target of Oridonin, and Notch inhibition was reported to enhance monocyte differentiation into the anti-inflammatory (M2) phenotype (Dong et al., 2014; Singla et al., 2014, 2017; Xia et al., 2017). We, therefore, investigated whether Oridonin exerts these effects by affecting Notch signaling. In LPS-stimulated RAW cells, Oridonin treatment significantly reduced the mRNA levels of Jagged-2, Notch1, and Hes-1, all of which are involved in the Notch pathway (**Figure 1E**). Further, the effects of Oridonin were abolished by the Notch1 transient transfection. Cells from the other comparable groups, especially the control group, were all transient transfected with the plasmid vehicle. Simultaneously, the Notch inhibitor DAPT exhibited anti-inflammatory activity similar to that of Oridonin, the mRNA levels of pro-inflammatory cytokines, including IL-1β, IL-6, and iNOS were decreased, and the anti-inflammatory phenotype markers IL-10 and CD206 were significantly increased (**Figure 1F**). Taken together, our results suggest that Oridonin induced the phenotypic switch of LPS-stimulated macrophages from the pro-inflammatory phenotype to the anti-inflammatory phenotype by blocking the Notch signaling.

Oridonin Treatment Suppressed EAN Neurological Progression and Accompanying Mechanical Allodynia

Experimental autoimmune neuritis was induced by subcutaneous immunization of Lewis rats with 100 μg of synthetic neurogenic P2 peptide. Oridonin or CMC (the control group) was given by daily gastric gavage starting at Day 0 as a preventive or on Day 9 (when the first neurological sign occurred) as a therapeutic treatment. In the control group, the first neurologic signs were established on Day 9 (0.33 ± 0.17); the neurologic severity represented by the scores peaked on Day 15 (6.3 ± 0.8), receded thereafter, and disappeared by Day 21 (0 ± 0). In comparison, preventive treatment of Oridonin at the dose of 20 mg/kg, initiated since Day 0, effectively delayed EAN onset (the first neurological sign occurred on Day 11), significantly reduced the severity at disease peak (2.7 ± 0.3, $p < 0.05$, compared to the control group), and shortened disease duration (until Day 18). Moreover, therapeutic treatment with the dose of 20 mg/kg, started after the first neurological sign was seen, also significantly attenuated the disease peak (4.5 ± 0.4, $p < 0.05$, compared to the control group) and lead to an earlier recovery from EAN (until Day 20) (**Figure 2A**). Both the therapeutic and preventive treatments with Oridonin significantly ameliorated the reduction of body weight, which is also a characteristic sign

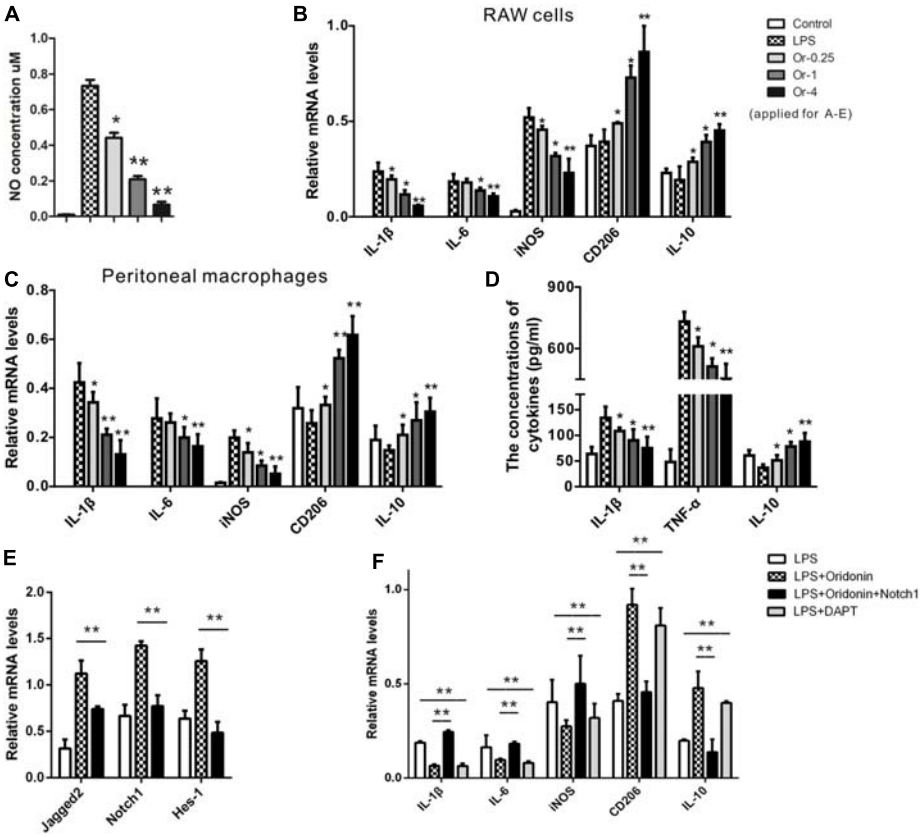

FIGURE 1 | Effects of Oridonin on phenotypic switch of macrophage polarization and Notch pathway *in vitro*. *In vitro* cultured macrophage cells were stimulated by LPS, transfected with Notch1 or vehicle plasmid, and treated with Oridonin or DAPT. NO concentrations, proportions of PCR products relative to the house keeping β-actin and cytokine concentrations are presented as bar graphs. **(A)** Upon the induction of LPS, NO production in RAW cell culture was increased compared to the control. Further, NO concentrations were significantly reduced by Oridonin treatment, in a clear dose-dependent pattern. **(B,C)** In both RAW cells and primary macrophages, the RNA levels of IL-1β, IL-6 and iNOS, the markers of inflammatory macrophages, were significantly decreased by Oridonin treatment. On the contrary, CD206 and IL-10 mRNA levels, the markers of anti-inflammatory phenotype, were significantly increased. All these results also presented clear dose-dependent patterns. **(D)** ELISA assays showed that the concentrations of IL-1β and TNF-α were reduced by Oridonin treatment and the level of IL-10 was increased in primary macrophages, in a dose-dependent pattern as well. **(E)** mRNA levels of Notch pathway involved molecules Jagged-2, Notch1 and Hes-1 were remarkably inhibited by Oridonin treatment in RAW cells. **(F)** In RAW cells, increased mRNA levels of IL-1β, IL-6 and iNOS were induced by LPS. Their levels were decreased by Oridonin or DAPT, whereas the levels of CD206 and IL-10 were reversed. But the Oridonin's effects were abolished by the transient transfection of Notch1. Cells from the other groups were all transiently transfected with vehicle plasmid. (Or-0.25, -1, -4 = Oridonin concentrations of 0.25, 1, and 4 μM. $^{*}p < 0.05$, $^{**}p < 0.01$ compared to their respective controls). Graphs show mean ± SEM of triplicate wells and represent data from three independent experiments.

of EAN (**Figure 2B**). Additionally, a lower dose of 7 mg/kg also showed therapeutic, but significantly reduced, effects in the preventive treatment. Further, repeated ANOVA assays indicated a significant difference between all the four groups, both in the neurological scores and body weight time courses.

Mechanical allodynia is a characteristic neurological sign of both human neuropathies and animal EAN. For the EAN rats, it can be recorded as a significant reduction of the hind-paw withdrawal threshold (HWT) in comparison to individual baselines (average HWT of the first four days after immunization). Mechanical allodynia was established on Day 7 except in the group treated with Oridonin at the dose of 20 mg/kg from Day 0 (**Figure 2C**). In the control group, HWT decreased persistently and gradually, presenting a steady progression of mechanical allodynia. Meanwhile, in the therapeutic treatment group (since Day 9), reduction of HWT was steady, like in the control group until Day 9, but was significantly slower after that. As shown in **Figure 2C**, HWT values detected between the therapeutic treatment and control groups were significantly different since Day 10, one day after the first treatment, and the differences could be seen till Day 13, the end of the observation period (when the EAN rats in the control groups could not lift their paws anymore). In the group treated prophylactically with Oridonin (20 mg/kg) since Day 0, a significant reduction could not be seen until Day 10; the HWT decreased slowly after that, but the reduction was always a little less than in the other two groups. The additional group administered the preventive treatment with the lower dose of 7 mg/kg also showed protective, but significantly reduced, effects in this test. Our results indicate that Oridonin treatment significantly suppressed and slowed the progression of mechanical allodynia in the EAN rats.

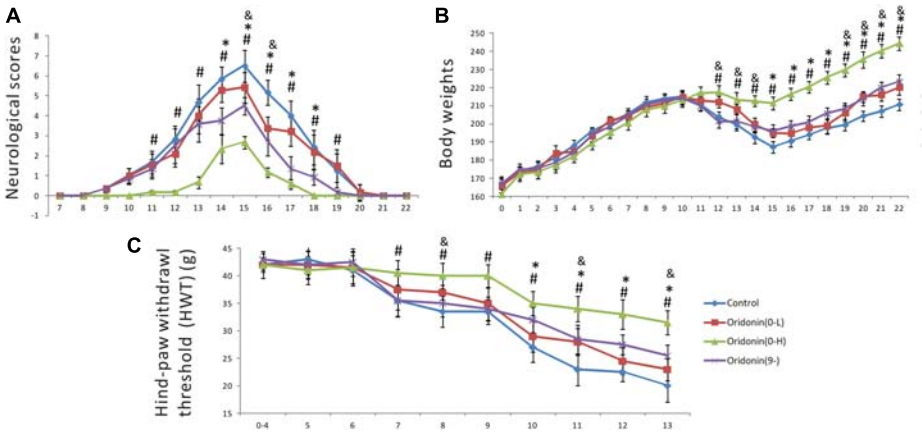

FIGURE 2 | Preventive and therapeutic Oridonin treatments attenuated neurological disease severity, shortened disease duration and suppressed mechanical allodynia in EAN rats. EAN rats (n = 6) were orally administrated daily with Oridonin (7 or 20 mg/kg in CMC) or CMC alone (same volume), since Day 0 as preventive or since Day 9 (first neurological sign occurred) as therapeutic treatment. Neurological scores and body weights were recorded every day post-immunization. **(A)** Both preventive and therapeutic Oridonin treatments decreased neurological severity of EAN and shortened EAN duration. **(B)** Meanwhile, the reduction of body weights, which is also a characteristic sign of EAN, was significantly ameliorated by both therapeutic and preventive treatments with Oridonin. **(C)** The another characteristic neurological sign of EAN, mechanical allodynia, defined as reduction of HWT, was observed as early as Day 7 up to the end of the experiment (Day 13) in the control group. Either therapeutic or preventive treatment with Oridonin at high or low doses significantly restored the reductions. Significant differences of HWT among control, Oridonin therapeutic and preventive groups were confirmed. Finally, repeated ANOVA assays indicated significant difference between all the three groups, in neurological scores, body weight time courses and HWT. $^{*}p < 0.05$ comparison of the preventive treatment group to the control group, $^{\&}p < 0.05$ comparison of the preventive treatment group at 7 mg/kg to the control group, $^{\#}p < 0.05$ comparison of the therapeutic treatment group to the control group, n = 6.

Oridonin Treatment Ameliorated Pathological Changes of EAN by Reducing Infiltration of Inflammatory Cells and Inflammation-Related Cytokines

Following therapeutic or preventive treatments with CMC or Oridonin, EAN rats were prepared for analysis at the peak of the EAN progression, namely on Day 15, and sciatic nerves were collected for further histological analysis (n = 6). LFB staining and quantification were first performed to investigate nerve demyelination and local infiltration of immune cells. Severe perivascular demyelination and massive infiltration of inflammatory immune cells were observed in the control group. Oridonin treatment significantly attenuated the degree of demyelination and the severity of cellular infiltration (**Figures 3A,E,I**). These pathological changes were evaluated, scored, and compared between treatment and control groups. As shown in the **Figure 3J**, in comparison to the control group (2.23 ± 0.16), both Oridonin preventive- (Oridonin 0) and therapeutic treatments (Oridonin 9) significantly decreased the mean histological scores of the sciatic nerves (1.14 ± 0.08 and 1.68 ± 0.13, respectively, $p < 0.05$).

Further immunohistochemical analysis showed significant reduction in the local aggregation of various infiltrated cellular populations following the Oridonin treatments. In the control group, massive infiltration of macrophages (ED1^{+}), pan-T cells (W3/13^{+}), and B cells (OX22^{+}) were observed and the macrophages were dominant in the cross-sections (**Figures 3B–D**). These infiltrated cells were not uniformly distributed in the cross-sections but were more concentrated around vessels and in the perineurium; some of them were also seen in the endoneurium. Both preventive and therapeutic Oridonin treatments significantly attenuated infiltration of all these cell types ($p < 0.05$) but did not alter their distribution pattern (**Figures 3F–H,K**).

In addition, mRNA levels of inflammatory cytokines in sciatic nerves, including those of IL-1β, iNOS, and IL-6, which are crucial for inflammatory progression in EAN, were further analyzed by real-time PCR. As shown in **Figure 3L**, the mRNA levels were significantly lower in the Oridonin-treated group than in the control group. However, the IL-10 mRNA level was not significantly changed, probably due to the dramatically reduced macrophage number in the local region.

Oridonin Treatment Decreased Inflammatory Cell Accumulation in Spinal Roots and Microglial Activation in the Spinal Cord

In EAN rats, significant accumulation of ED1^{+} and W3/13^{+} cells were observed in the dorsal and ventral roots. Like the sciatic nerves, they mainly accumulated around vessels in the endoneurium and perineurium. In comparison, macrophages and T cells were rarely seen in the dorsal and ventral roots of the spinal cord in naive animals and with a lower cell density than in sciatic nerves, as reported in our previous studies (Zhang et al., 2009). Following the preventive Oridonin treatment, their accumulations were significantly reduced, for ED1: 1.9 ± 0.3% in the

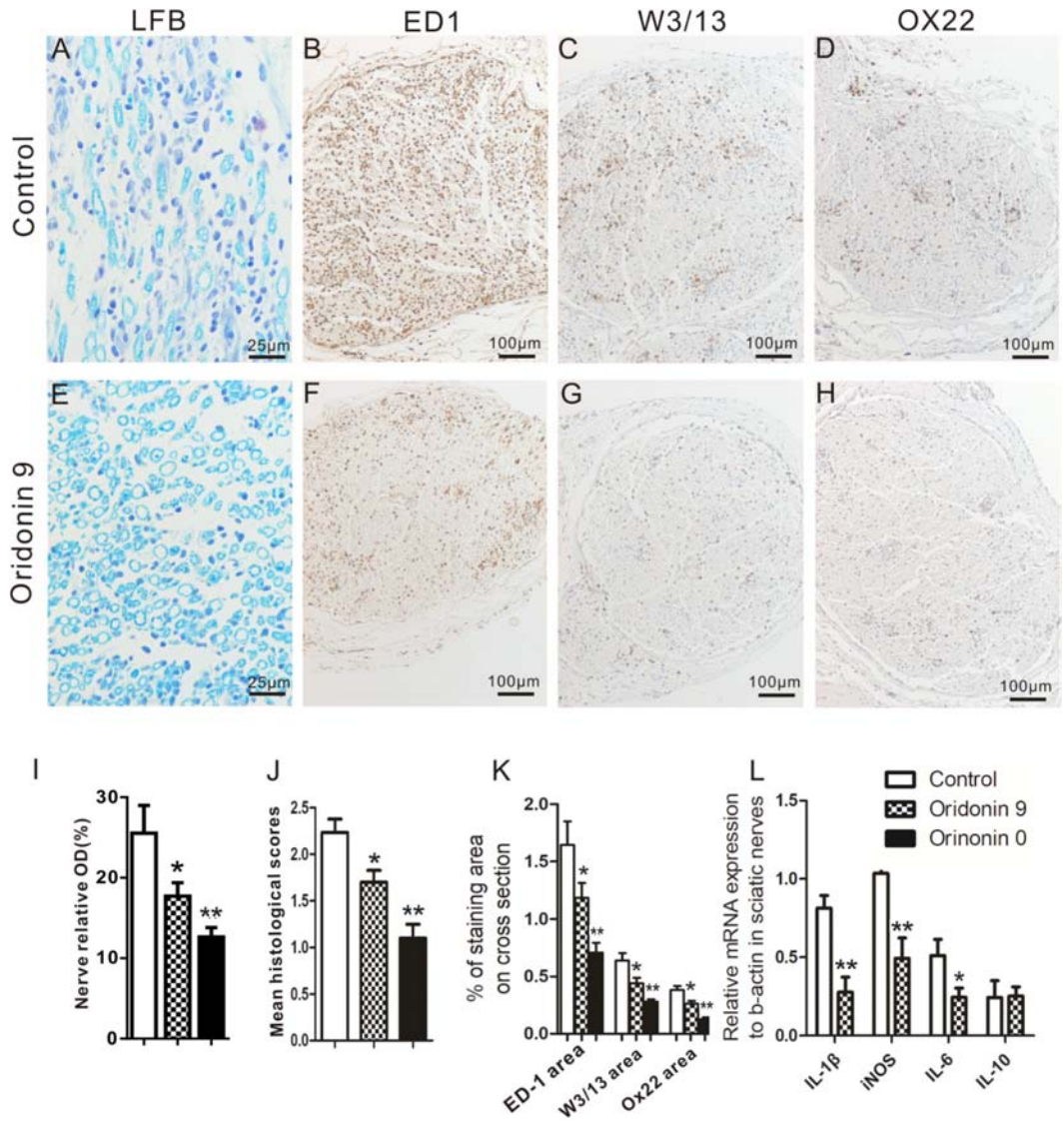

FIGURE 3 | Oridonin treatment attenuated local demyelination, inflammatory cell infiltration and inflammatory cytokines in sciatic nerves of EAN rats. Following preventive or therapeutic treatments, rats were sacrificed on Day 15 and sciatic nerves were taken for LFB and immunohistochemical staining or semi-quantitative PCR. Representative microphotos of LFB and IHC staining for control **(A–D)** or Oridonin treated **(E–H)** EAN rats are shown. Quantification of demyelination, mean histological scores and percentages of immune cells marker IR on cross sections were calculated as described in Materials and Methods. Preventive or therapeutic Oridonin treatments significantly reduced the demyelination levels; mean histological scores; percentages of ED1 (macrophages), W3/13 (pan-T cells) and OX22 (B cells) IR in sciatic nerves, compared to the control EAN rats **(I–K)**. **(L)** mRNA levels of IL-1β, IL-6, iNOS and IL-10 in sciatic nerves of these EAN rats was analyzed by real-time PCR. Therapeutic Oridonin treatment significantly reduced mRNA levels of IL-1β, IL-6 and iNOS in sciatic nerves, but did not change the mRNA level of IL-10 significantly. $^{*}p < 0.05$, $^{**}p < 0.01$ compared to the control group, $n = 6$.

control group and 0.6 ± 0.1% in the treatment group ($p < 0.05$) (**Figures 4A,D,G**); for W3/13: 0.8 ± 0.1% in the control group and 0.3 ± 0.1% in the treatment group ($p < 0.05$) (**Figures 4B,E,H**).

ED1 is also considered the microglial activation marker in CNS and its expression in the spinal cord was further analyzed. According to our previously published data (Zhang et al., 2009), only a few microglial cells could be observed in the spinal cord of naive rats. In our EAN rats, however, obvious ED1 IR could be seen, mostly in the gray matter of the lumbar spinal cord, especially in the superficial layers of the dorsal horns. They accumulated near large neurons, particularly in the control group. They exhibited an activated hypertrophic morphology, which was characterized by thicker, shorter, and less branched processes; and enlarged, darkened soma. In the Oridonin group, however, fewer ED1 IR and $ED1^{+}$ cells with relatively smaller soma were observed (**Figures 4C,F**). The density of ED1 IR was then analyzed in the dorsal horns, where $ED1^{+}$ cells were relatively more abundant. The quantity of ED1 IR was significantly lower in

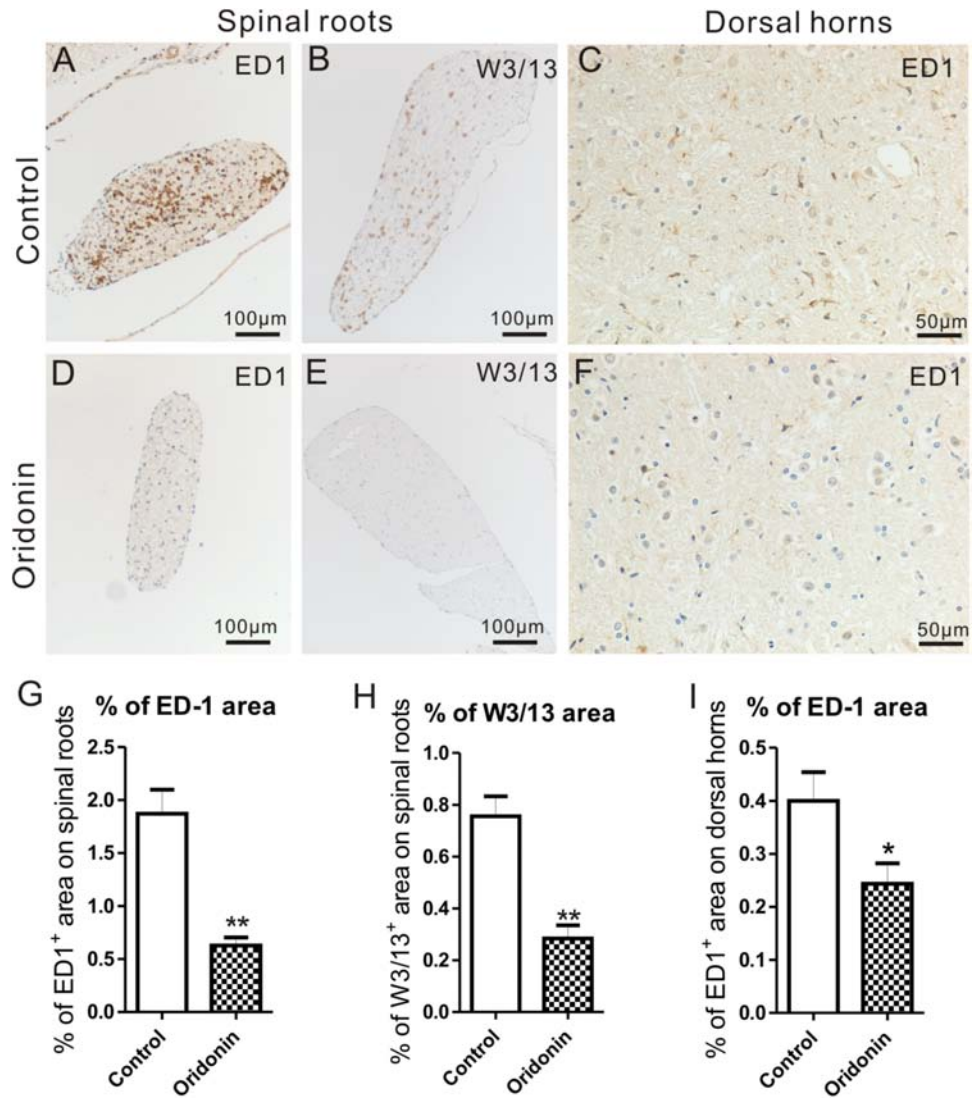

FIGURE 4 | Oridonin treatments attenuated cellular infiltration in spinal roots and inflammatory microglial activation in lumbar spinal cords of EAN rats. EAN Rats were preventively treated with Oridonin and sacrificed on Day 15, spinal cords were taken for immunohistochemical staining and analyzed, especially focusing on lumbar sections. Significant accumulation of ED1$^+$ and W3/13$^+$ cells was mainly observed in dorsal roots of our EAN rats **(A,B)**. Similar to sciatic nerves, they were mainly detected to concentrate around vessels in perineurium and endoneurium. Following the Oridonin treatment, their accumulations were significantly reduced **(D,E,G,H)**.The expression of ED1 in lumbar spinal cords was further analyzed. In lumbar spinal cords of EAN rats, significantly increased ED1 expression could be observed in the control group **(C)**. ED1 IR was mainly detected in gray matter, particularly in the superficial layers of dorsal horns. The density of ED1 IR in dorsal horns was then investigated, and it was significantly decreased by Oridonin treatment **(F)**, compared to the control group **(I)**. $^*p < 0.05$, $^{**}p < 0.01$ compared to the control group, $n = 6$.

the Oridonin preventive treatment group than in the control group (0.40 ± 0.04% for control, 0.24 ± 0.03% for treatment, $p < 0.05$) (**Figure 4I**).

Oridonin Increased the Proportion of Anti-inflammatory Macrophages in EAN Rats

A significantly increased expression of CD163 is characteristically observed on an anti-inflammatory activated phenotype of macrophages (Moestrup and Moller, 2004; Badylak et al., 2008). ED2 antibody against CD163 was therefore used to detect these macrophages in the sciatic nerves of our EAN rats (**Figures 5A,B**). Even though the total numbers of accumulated macrophages were dramatically reduced (ED1$^+$ cells, **Figures 3B,F**), CD163/ED2 IR (0.36 ± 0.04%) in the sciatic nerves of the Oridonin group was slightly increased (0.33 ± 0.03%, $p > 0.05$, compared to the control group, **Figure 5C**). Moreover, the proportion of CD163 IR among total macrophages (ED1$^+$ cells) was significantly increased following the treatment (36% for the control and 57% for the Oridonin group, **Figure 5D**), indicating a switch of

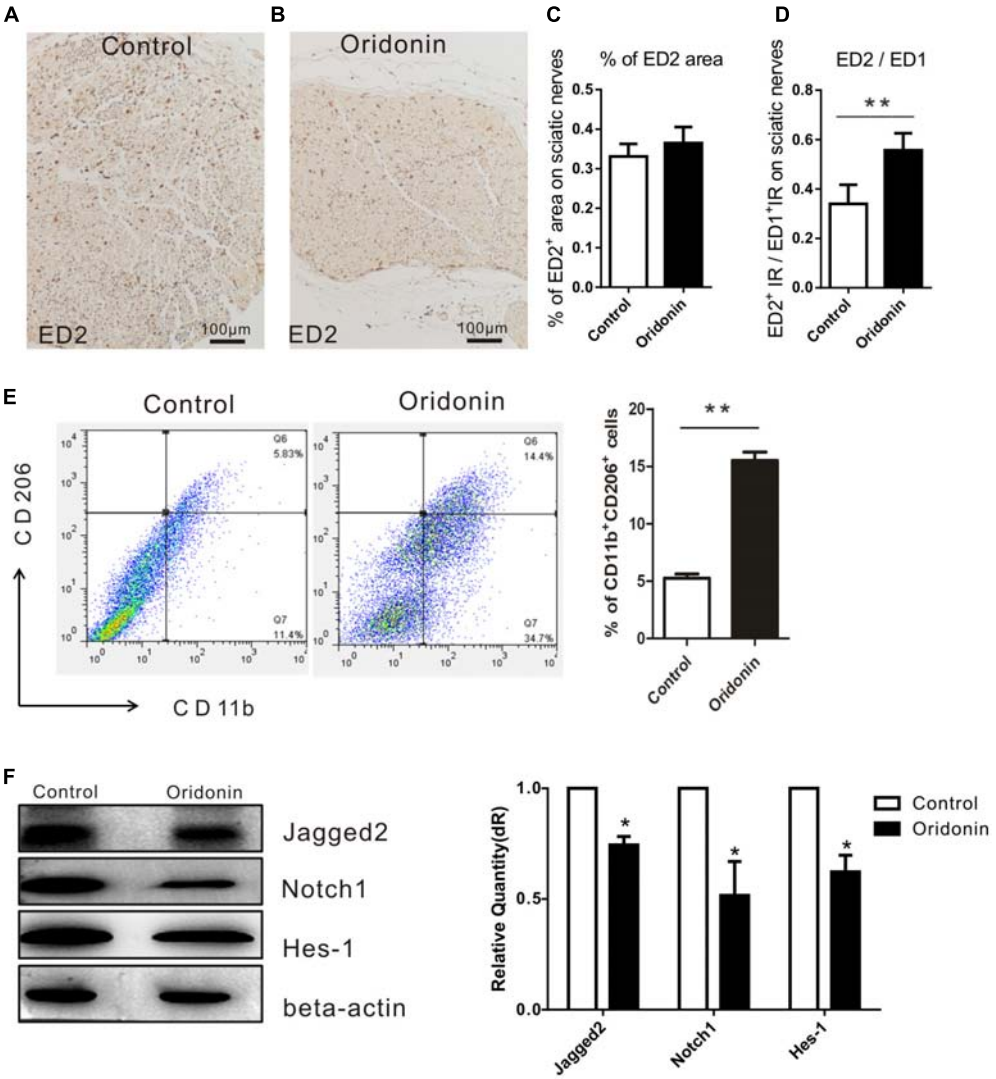

FIGURE 5 | Oridonin increased proportion of anti-inflammatory macrophages in EAN rats through suppression of Notch pathway. Rats were treated therapeutically with Oridonin and sacrificed on Day 15; sciatic nerves were taken for immunohistochemical staining. By staining, anti-inflammatory macrophages were identified by expression of CD163, which is detected by ED2 antibody. Representative microphotos show $ED2^+$ cells in sciatic nerves of EAN rats treated by Oridonin **(B)** in comparison to the control **(A)**. In the control group, much more infiltrates could be observed, but CD163 expression was not significantly different from the treatment group, where much less accumulated immune cells could be seen. **(C,D)**: The bar graphs show no difference in CD163 IR levels between control and Oridonin groups, but the proportions of $ED2^+$ over $ED1^+$ IR areas in sciatic nerves were significantly increased in comparison to controls. Further, the frequency of $CD206^+CD11b^+$ cells from spleen were tested by flow cytometry. **(E)** Spleen mononuclear cells (MNCs) were isolated from the control and Oridonin treated EAN rats at day 15 post-immunization and examined by flow cytometry. Percentage of anti-inflammatory macrophages ($CD206^+CD11b^+$ cells) in spleen MNCs was significantly increased in Oridonin treated rats compared to the control group. **(F)** On day 15, sciatic nerves of each rat were harvested for Western blotting. Relative expression of Jagged-2, Notch1 and Hes-1 in Oridonin-treated groups was significantly lower compared to the control group. $^*p < 0.05$, $^{**}p < 0.01$ compared to the control group, $n = 6$.

the local macrophage population from classically activated inflammatory into the anti-inflammatory phenotype, as well as the dominance of the anti-inflammatory phenotype among all infiltrated macrophages.

Further, the effect of Oridonin on the phenotypic polarization of macrophages in the entire body was studied. Mononuclear cells (MNCs) derived from the spleens of EAN rats were determined with flow cytometry and results demonstrated a higher proportion of anti-inflammatory macrophages in spleen MNCs from the Oridonin treated EAN rats than in those of the control group rats (**Figure 5E**).

Oridonin Inhibits Notch Signaling Protein Expressions in Sciatic Nerves of EAN Rats

As proved by the results from our *in vitro* study, Oridonin is a potent suppressor of the Notch signaling pathway and may

thereby induce the phenotypic switch in macrophage polarization and suppress the inflammatory reactions. We therefore explored the expressions of the major products of the Notch pathway, including Jagged-2, Notch1, and Hes1 in the EAN sciatic nerves from both groups. Results from Western blotting analysis show potent local suppression of Jagged-2, Notch1, and Hes-1 by Oridonin treatment (**Figure 5F**). The involvement of the Notch pathway has been thereby approved in our EAN rats and all the representative molecules were modified following Oridonin treatments, in accordance with previous reports; therefore, no further investigations were performed to explore the pathway in detail as this has been previously reported in other studies.

DISCUSSION

As the prime animal model of human GBS and polyneuropathies, EAN is widely accepted and applied in disease mechanism investigation and development of novel therapeutic approaches. After we proved efficient anti-inflammatory activity of Oridonin and ability of inducing an anti-inflammatory phenotypic switch of macrophage polarization in cell culture, potential therapeutic values of Oridonin were further investigated in our animal model of human polyneuropathies and GBS. Our results show that either therapeutic or preventive treatments with Oridonin significantly shortened the disease duration, attenuated the peak severity, and paraparesis, or even delayed EAN onset. Oridonin treatments also significantly attenuated neuropathic pain, inflammatory infiltration of activated macrophages and other immune cells in the peripheral nerves and reduced microglial activation in the lumbar spinal cord. Interestingly, Oridonin treatment increased the systematic and local proportions of anti-inflammatory macrophages over the pro-inflammatory phenotypic population and resulted in the dominance of the anti-inflammatory phenotype among all infiltrated macrophages, which may be crucial for the anti-inflammatory activity, and ameliorated EAN progression. Our results from both *in vitro* and *in vivo* experiments suggest that the macrophage phenotypic switching effect of Oridonin may be associated with the inhibition of the Notch pathway.

As an effective diterpenoid isolated from *R. rubescens*, Oridonin has a variety of physiological and pharmacological properties/activities including anti-bacterial, anti-tumor, and anti-inflammatory. *R. rubescens* has a long history in TCM for the treatment of tumor and inflammatory diseases (Dong et al., 2014). The herb or its aqueous extract has been successfully applied in TCM for treatment of human inflammation such as gingivitis (Chen et al., 2009) and pharyngitis (Ma et al., 2011) for a long time. Several studies reported that Oridonin inhibited the expressions of COX-2 and iNOS by blocking NF-κB activity (Ikezoe et al., 2005; Leung et al., 2005); inhibited further pro-inflammatory molecules including IL-6, IL-2, IL-12, TNF-α, and IFN-γ; induced apoptosis of immune cells; as well as affected the anti-inflammatory target HO-1 (Hu et al., 2008); indicating its immunosuppressive and anti-inflammatory properties. However, a significantly modified expression of NF-κB was not observed in our EAN sciatic nerves, which indicated that other mechanisms which could be involved needed further investigation. Moreover, Oridonin has neuro-protective and anti-neuroinflammatory effects by modulating multiple microglial functions (Xu et al., 2009), suggesting its potential therapeutic application against inflammatory disorders of the nervous system.

In this present study, both preventive and therapeutic treatments with Oridonin significantly suppressed disease progression and eventually improved EAN outcome, by attenuating immune cell accumulation and reducing expressions of inflammatory cytokines in peripheral nerves, including the sciatic nerves and spinal roots. The infiltration of reactive leukocytes, mainly macrophages and T cells, into the PNS is the characteristic pathological change of EAN (Schabet et al., 1991); especially the activated macrophages triggered nerve demyelination by secretion of inflammatory mediators and direct phagocytic attack (Kiefer et al., 2001; Maurer et al., 2002). In EAN peripheral nerves, inflammatory cytokines are expressed and secreted by different immune cell types and modulate/regulate inflammatory responses. Pro-inflammatory molecules like IL-1β, IL-6, and iNOS are believed to promote EAN disease progression. IL-1β is considered a participant in the initiation of the autoimmune response of human neuropathies and the animal model EAN (Bettelli et al., 2008), IL-6 is believed to amplify local inflammation and be crucial for the EAN progression (Zhang et al., 2008b), iNOS produces pro-inflammatory NO (Abramson et al., 2001), and iNOS up-regulation in EAN was proved to contribute to the demyelination and even axonal damage in PNS (Conti et al., 2004). Therefore, Oridonin attenuated local inflammatory reactions and demyelination in the PNS and finally favored EAN outcome, by reducing local infiltration of reactivated immune cells and expression of inflammatory cytokines/molecules. In addition, the working concentrations used in our macrophage cell culture were comparable to the oral doses used in a previous study and presented significant dose-dependent anti-inflammatory patterns. The dosage was also comparable to orally administered Oridonin and the standard treatment followed in TCM (Xu et al., 2006).

Besides macrophages, we also observed a microglial accumulation in the lumbar spinal cord, together with pain hypersensitivity. While the EAN pathological changes were mainly studied and observed in the PNS, pathological changes in EAN spinal cord have drawn increased attention, especially focusing on spinal microglia (Piehl and Lidman, 2001; Nakamura, 2002), as they were considered to play a key role in inducing neuropathic pain in a variety of models (Moalem and Tracey, 2006). In the spinal cord of EAN animals, the activation of microglia is seen (Beiter et al., 2005), which is considered crucial in the induction of neuropathic pain (Raghavendra et al., 2003). Microglia are very sensitive to micro-environmental changes and spinal microglia are stimulated by various factors ranging from peripheral injury to central nervous system (CNS) inflammation. Reactive microglia in the spinal cord express various receptors and release inflammatory molecules acting either directly on pain transmitting dorsal horn neurons (nociceptive neurons) or on primary afferents, inducing raised sensitivity of the nociceptive neurons and contributing indirectly to central sensitization of neuropathic pain (Beggs et al., 2012; Ji et al., 2016). Therefore,

activation of spinal microglia is essential and sufficient to initiate neuropathic pain. Suppression of spinal microglia can significantly relieve or even block the process of neuropathic pain in various animal models (Ji and Strichartz, 2004). Our study showed significantly reduced microglial accumulation, which might be due to attenuated stimuli from the periphery or direct inhibiting effects on spinal microglia. Our results indicate that Oridonin could suppress neuropathic pain, exert direct or indirect neuro-protective effects in the animal model, and possibly even in human patients, which makes the therapeutic and preventive effects of Oridonin more comprehensive.

As the major effector cells responsible for the main EAN pathological changes, macrophages' activation states/status and phenotype may determine the disease progression. Based on our *in vitro* and *in vivo* results, the potent anti-inflammatory activity of Oridonin during EAN progression is, at least partially, due to the phenotypic switch toward anti-inflammatory macrophages and eventually their local dominance in peripheral nerves. This is also in accordance with previous studies demonstrating that anti-inflammatory activated macrophages played a protective and immune-modulatory role in EAN progression; as well as a switch of macrophage polarization from the pro-inflammatory phenotype toward the anti-inflammatory could resolve inflammation and thereby favor the outcome of different inflammatory disorders (Li et al., 2016; Klinkert et al., 2017); including animal models of EAN (Zhang Z. Y. et al., 2010; Han et al., 2016b). In our EAN rats, Oridonin raised the proportion of anti-inflammatory macrophages ($ED2^+$ cells) substantially over the pro-inflammatory phenotype population not only locally in the peripheral nerves but also systemically as determined in the spleen, the most important storage organ of the peripheral immune cells. The latter may also suggest an improved environmental milieu in immune organs. Moreover, Oridonin efficiently reduced local expression of pro-inflammatory representative cytokines/molecules. Although the anti-inflammatory representative cytokine IL-10 was not significantly changed, considering the dramatically reduced number of total immune cells, a slight increase of IL-10 could also support the dominance of anti-inflammatory over pro-inflammatory cells.

Although the phenotypic switch of macrophage polarization has not been associated directly with Oridonin treatment, particularly in an inflammatory condition, previous data suggest possible involvement of the Notch pathway: the Notch pathway is a direct target of Oridonin and Notch inhibition could promote monocyte differentiation into anti-inflammatory macrophages (Singla et al., 2017; Xia et al., 2017). Dong et al. (2014) demonstrated that Oridonin could suppress the Notch activity, down-regulate Notch pathway proteins such as Jagged-2, Notch-1, and their downstream molecules, in the same manner as a Notch inhibitor could. Singla et al. reported that suppression of the Notch-1 pathway or of several further Notch signaling proteins decreased the inflammatory macrophages and expression of their cytokines, enhanced

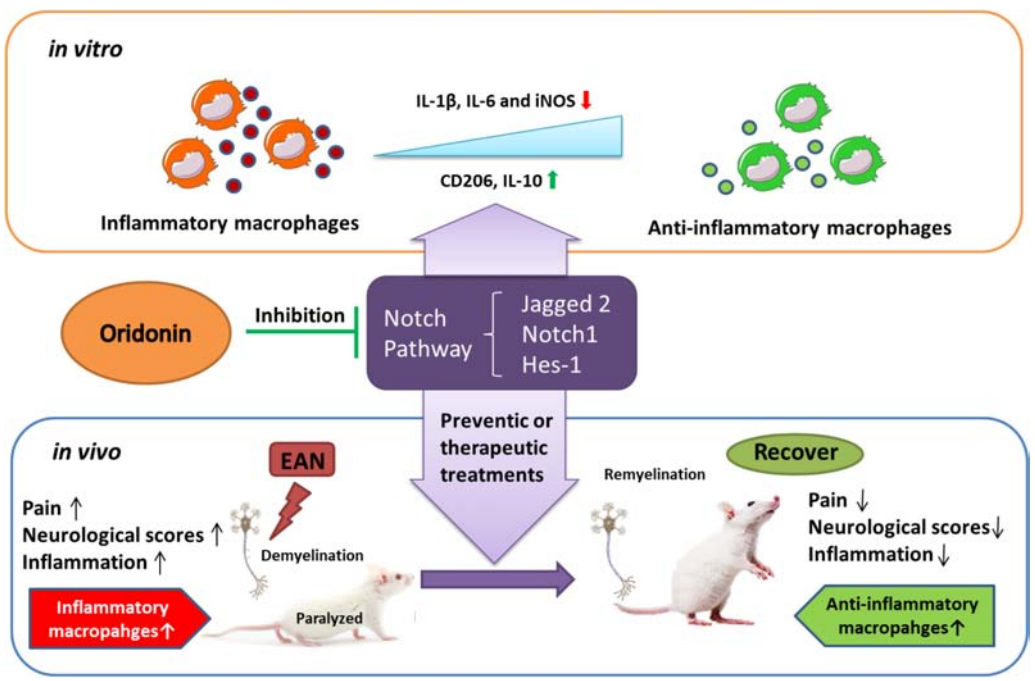

FIGURE 6 | Schematic diagram showing how Oridonin ameliorates experimental autoimmune neuritis by promoting anti-inflammatory macrophages through blocking Notch pathway. Oridonin exhibits efficient anti-inflammatory activity and induced a switch in macrophage polarization to the anti-inflammatory phenotype through inhibition of the Notch pathway in our *in vitro* study. Further in the EAN rats, Oridonin significantly reduced neuropathic pain, demyelination, inflammatory cellular accumulations and inflammatory cytokines in peripheral nerves, effectively suppressed disease progression by attenuating local inflammatory reaction and increasing the proportion of immune regulating macrophages, possibly through blockage of the Notch pathway.

secretion of anti-inflammatory molecules, and subsequently promoted anti-inflammatory macrophage polarization (Singla et al., 2014), even for human primary monocytes (Singla et al., 2017). In the present study, we demonstrated that the anti-inflammatory switch of macrophage polarization in LPS-induced cell culture was promoted by Oridonin or the Notch inhibitor DAPT, but the transfection of Notch-1 could abolish this effect by Oridonin. This proves the effects of Oridonin on inhibition of the Notch-1 pathway. Further, expression levels of the Notch pathway proteins were also decreased locally in EAN sciatic nerves following Oridonin treatment, meanwhile proportions of anti-inflammatory macrophages were increased locally or systemically in the spleen. All these suggest that inhibition of the Notch pathway by Oridonin may be, at least partially, involved in the anti-inflammatory phenotypic switch of macrophage polarization.

CONCLUSION

Oridonin ameliorated inflammatory EAN progression and finally favored the disease outcome through inducing the phenotypic switch of macrophage polarization toward the anti-inflammatory state, probably by the blockage of the Notch pathway (**Figure 6**). Therefore, with its proven safety and biocompatibility, Oridonin could be considered a potential anti-inflammatory therapeutic candidate of human GBS and other inflammatory neuropathies.

AUTHOR CONTRIBUTIONS

HS and Z-YZ designed the experiments and obtained resources and funding acquisition. LX and Z-YZ conducted the experiments with assistance from LL and C-YZ. LX, LL, C-YZ, and Z-YZ collected the data and contributed to the statistical analysis. LX, Z-YZ, and HS analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

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REFERENCES

Abramson, S. B., Amin, A. R., Clancy, R. M., and Attur, M. (2001). The role of nitric oxide in tissue destruction. *Best Pract. Res. Clin. Rheumatol.* 15, 831–845. doi: 10.1053/berh.2001.0196

Badylak, S. F., Valentin, J. E., Ravindra, A. K., McCabe, G. P., and Stewart-Akers, A. M. (2008). Macrophage phenotype as a determinant of biologic scaffold remodeling. *Tissue Eng. Part A* 14, 1835–1842. doi: 10.1089/ten.tea.2007.0264

Beggs, S., Trang, T., and Salter, M. W. (2012). P2X4R+ microglia drive neuropathic pain. *Nat. Neurosci.* 15, 1068–1073. doi: 10.1038/nn.3155

Beiter, T., Artelt, M. R., Trautmann, K., and Schluesener, H. J. (2005). Experimental autoimmune neuritis induces differential microglia activation in the rat spinal cord. *J. Neuroimmunol.* 160, 25–31. doi: 10.1016/j.jneuroim.2004.10.027

Bettelli, E., Korn, T., Oukka, M., and Kuchroo, V. K. (2008). Induction and effector functions of T(H)17 cells. *Nature* 453, 1051–1057. doi: 10.1038/nature07036

Chen, S., Liu, J., and Zhang, H. (2009). Efficacy of rabdosia rubescens in the treatment of gingivitis. *J. Huazhong Univ. Sci. Technol. Med. Sci.* 29, 659–663. doi: 10.1007/s11596-009-0525-2

Chen, S. S., Michael, A., and Butler-Manuel, S. A. (2012). Advances in the treatment of ovarian cancer: a potential role of antiinflammatory phytochemicals. *Discov. Med.* 13, 7–17.

Cheng, W., Huang, C., Ma, W., Tian, X., and Zhang, X. (2017). Recent development of oridonin derivatives with diverse pharmacological activities. *Mini Rev. Med. Chem.* 19, 114–124. doi: 10.2174/1389557517666170417170609

Conti, G., Rostami, A., Scarpini, E., Baron, P., Galimberti, D., Bresolin, N., et al. (2004). Inducible nitric oxide synthase (iNOS) in immune-mediated demyelination and Wallerian degeneration of the rat peripheral nervous system. *Exp. Neurol.* 187, 350–358. doi: 10.1016/j.expneurol.2004.01.026

Craggs, R. I., King, R. H., and Thomas, P. K. (1984). The effect of suppression of macrophage activity on the development of experimental allergic neuritis. *Acta Neuropathol.* 62, 316–323. doi: 10.1007/BF00687614

Dong, Y., Zhang, T., Li, J., Deng, H., Song, Y., Zhai, D., et al. (2014). Oridonin inhibits tumor growth and metastasis through anti-angiogenesis by blocking the Notch signaling. *PLoS One* 9:e113830. doi: 10.1371/journal.pone.0113830

Han, R., Gao, J., Zhai, H., Xiao, J., Ding, Y., and Hao, J. (2016a). RAD001 (everolimus) attenuates experimental autoimmune neuritis by inhibiting the mTOR pathway, elevating Akt activity and polarizing M2 macrophages. *Exp. Neurol.* 280, 106–114. doi: 10.1016/j.expneurol.2016.04.005

Han, R., Xiao, J., Zhai, H., and Hao, J. (2016b). Dimethyl fumarate attenuates experimental autoimmune neuritis through the nuclear factor erythroid-derived 2-related factor 2/hemoxygenase-1 pathway by altering the balance of M1/M2 macrophages. *J. Neuroinflammation* 13:97. doi: 10.1186/s12974-016-0559-x

Hartung, H. P., Schafer, B., Heininger, K., Stoll, G., and Toyka, K. V. (1988). The role of macrophages and eicosanoids in the pathogenesis of experimental allergic neuritis. Serial clinical, electrophysiological, biochemical and morphological observations. *Brain* 111(Pt 5), 1039–1059. doi: 10.1093/brain/111.5.1039

He, H., Jiang, H., Chen, Y., Ye, J., Wang, A., Wang, C., et al. (2018). Oridonin is a covalent NLRP3 inhibitor with strong anti-inflammasome activity. *Nat. Commun.* 9:2550. doi: 10.1038/s41467-018-04947-6

Hu, A. P., Du, J. M., Li, J. Y., and Liu, J. W. (2008). Oridonin promotes CD4+/CD25+ Treg differentiation, modulates Th1/Th2 balance and induces HO-1 in rat splenic lymphocytes. *Inflamm. Res.* 57, 163–170. doi: 10.1007/s00011-007-7193-0

Hughes, R. A. (2002). Systematic reviews of treatment for inflammatory demyelinating neuropathy. *J. Anat.* 200, 331–339. doi: 10.1046/j.1469-7580.2002.00041.x

Hughes, R. A., and Cornblath, D. R. (2005). Guillain-Barre syndrome. *Lancet* 366, 1653–1666. doi: 10.1016/s0140-6736(05)67665-9

Ikezoe, T., Yang, Y., Bandobashi, K., Saito, T., Takemoto, S., Machida, H., et al. (2005). Oridonin, a diterpenoid purified from Rabdosia rubescens, inhibits the proliferation of cells from lymphoid malignancies in association with blockade of the NF-kappa B signal pathways. *Mol. Cancer Ther.* 4, 578–586. doi: 10.1158/1535-7163.MCT-04-0277

Ji, R. R., Chamessian, A., and Zhang, Y. Q. (2016). Pain regulation by non-neuronal cells and inflammation. *Science* 354, 572–577. doi: 10.1126/science.aaf8924

Ji, R. R., and Strichartz, G. (2004). Cell signaling and the genesis of neuropathic pain. *Sci. STKE* 2004:reE14. doi: 10.1126/stke.2522004re14

Ji, Z., Tang, Q., Zhang, J., Yang, Y., Liu, Y., and Pan, Y. (2011). Oridonin-induced apoptosis in SW620 human colorectal adenocarcinoma cells. *Oncol. Lett.* 2, 1303–1307. doi: 10.3892/ol.2011.408

Jia, L., Shen, J., Zhang, D., Duan, C., Liu, G., Zheng, D., et al. (2012). In vitro and in vivo evaluation of oridonin-loaded long circulating nanostructured lipid carriers. *Int. J. Biol. Macromol.* 50, 523–529. doi: 10.1016/j.ijbiomac.2012.01.024

Kiefer, R., Kieseier, B. C., Stoll, G., and Hartung, H. P. (2001). The role of macrophages in immune-mediated damage to the peripheral nervous system. *Prog. Neurobiol.* 64, 109–127. doi: 10.1016/S0301-0082(00)00060-5

Kieseier, B. C., Kiefer, R., Gold, R., Hemmer, B., Willison, H. J., and Hartung, H. P. (2004). Advances in understanding and treatment of immune-mediated disorders of the peripheral nervous system. *Muscle Nerve* 30, 131–156. doi: 10.1002/mus.20076

Klinkert, K., Whelan, D., Clover, A. J. P., Leblond, A. L., Kumar, A. H. S., and Caplice, N. M. (2017). Selective M2 macrophage depletion leads to prolonged inflammation in surgical wounds. *Eur. Surg. Res.* 58, 109–120. doi: 10.1159/000451078

Kumar, V. (2006). Potential medicinal plants for CNS disorders: an overview. *Phytother. Res.* 20, 1023–1035. doi: 10.1002/ptr.1970

Kuwabara, S., and Yuki, N. (2013). Axonal Guillain-Barre syndrome: concepts and controversies. *Lancet Neurol.* 12, 1180–1188. doi: 10.1016/s1474-4422(13)70215-1

Leung, C. H., Grill, S. P., Lam, W., Han, Q. B., Sun, H. D., and Cheng, Y. C. (2005). Novel mechanism of inhibition of nuclear factor-kappa B DNA-binding activity by diterpenoids isolated from Isodon rubescens. *Mol. Pharmacol.* 68, 286–297. doi: 10.1124/mol.105.012765

Li, D., Wang, C., Yao, Y., Chen, L., Liu, G., Zhang, R., et al. (2016). mTORC1 pathway disruption ameliorates brain inflammation following stroke via a shift in microglia phenotype from M1 type to M2 type. *FASEB J.* 30, 3388–3399. doi: 10.1096/fj.201600495R

Li, J., Bao, L., Zha, D., Zhang, L., Gao, P., Zhang, J., et al. (2018). Oridonin protects against the inflammatory response in diabetic nephropathy by inhibiting the TLR4/p38-MAPK and TLR4/NF-κB signaling pathways. *Int. Immunopharmacol.* 55, 9–19. doi: 10.1016/j.intimp.2017.11.040

Liu, J., Yang, F., Zhang, Y., and Li, J. (2007). Studies on the cell-immunosuppressive mechanism of Oridonin from *Isodon serra*. *Int. Immunopharmacol.* 7, 945–954. doi: 10.1016/j.intimp.2007.03.001

Ma, Z., Hu, C., and Zhang, Y. (2011). Therapeutic effect of Rabdosia rubescens aqueous extract on chronic pharyngitis and its safety. *Zhong Nan Da Xue Xue Bao Yi Xue Ban* 36, 170–173. doi: 10.3969/j.issn.1672-7347.2011.02.014

Mailhos, C., Modlich, U., Lewis, J., Harris, A., Bicknell, R., and Ish-Horowicz, D. (2001). Delta4, an endothelial specific notch ligand expressed at sites of physiological and tumor angiogenesis. *Differentiation* 69, 135–144. doi: 10.1046/j.1432-0436.2001.690207.x

Maurer, M., Toyka, K. V., and Gold, R. (2002). Cellular immunity in inflammatory autoimmune neuropathies. *Rev. Neurol.* 158(12 Pt 2), S7–S15.

Moalem, G., and Tracey, D. J. (2006). Immune and inflammatory mechanisms in neuropathic pain. *Brain Res. Rev.* 51, 240–264. doi: 10.1016/j.brainresrev.2005.11.004

Moestrup, S. K., and Moller, H. J. (2004). CD163: a regulated hemoglobin scavenger receptor with a role in the anti-inflammatory response. *Ann. Med.* 36, 347–354. doi: 10.1080/07853890410033171

Mosser, D. M., and Edwards, J. P. (2008). Exploring the full spectrum of macrophage activation. *Nat. Rev. Immunol.* 8, 958–969. doi: 10.1038/nri2448

Nakamura, Y. (2002). Regulating factors for microglial activation. *Biol. Pharm. Bull.* 25, 945–953. doi: 10.1248/bpb.25.945

Piehl, F., and Lidman, O. (2001). Neuroinflammation in the rat–CNS cells and their role in the regulation of immune reactions. *Immunol. Rev.* 184, 212–225. doi: 10.1034/j.1600-065x.2001.1840119.x

Raghavendra, V., Tanga, F., Rutkowski, M. D., and DeLeo, J. A. (2003). Anti-hyperalgesic and morphine-sparing actions of propentofylline following peripheral nerve injury in rats: mechanistic implications of spinal glia and proinflammatory cytokines. *Pain* 104, 655–664. doi: 10.1016/S0304-3959(03)00138-6

Ricardo, S. D., van Goor, H., and Eddy, A. A. (2008). Macrophage diversity in renal injury and repair. *J. Clin. Invest.* 118, 3522–3530. doi: 10.1172/jci36150

Schabet, M., Whitaker, J. N., Schott, K., Stevens, A., Zurn, A., Buhler, R., et al. (1991). The use of protease inhibitors in experimental allergic neuritis. *J. Neuroimmunol.* 31, 265–272. doi: 10.1016/0165-5728(91)90048-C

Singla, D. K., Wang, J., and Singla, R. (2017). Primary human monocytes differentiate into M2 macrophages and involve Notch-1 pathway. *Can. J. Physiol. Pharmacol.* 95, 288–294. doi: 10.1139/cjpp-2016-0319

Singla, R. D., Wang, J., and Singla, D. K. (2014). Regulation of Notch 1 signaling in THP-1 cells enhances M2 macrophage differentiation. *Am. J. Physiol. Heart Circ. Physiol.* 307, H1634–H1642. doi: 10.1152/ajpheart.00896.2013

Willison, H. J. (2005). The immunobiology of Guillain-Barre syndromes. *J. Peripher. Nerv. Syst.* 10, 94–112. doi: 10.1111/j.1085-9489.2005.0010202.x

Winer, J. B. (2001). Guillain Barre syndrome. *Mol. Pathol.* 54, 381–385.

Xia, S., Zhang, X., Li, C., and Guan, H. (2017). Oridonin inhibits breast cancer growth and metastasis through blocking the Notch signaling. *Saudi Pharm. J.* 25, 638–643. doi: 10.1016/j.jsps.2017.04.037

Xu, W., Sun, J., Zhang, T. T., Ma, B., Cui, S. M., Chen, D. W., et al. (2006). Pharmacokinetic behaviors and oral bioavailability of oridonin in rat plasma. *Acta Pharmacol. Sin.* 27, 1642–1646. doi: 10.1111/j.1745-7254.2006.00440.x

Xu, Y., Xue, Y., Wang, Y., Feng, D., Lin, S., and Xu, L. (2009). Multiple-modulation effects of Oridonin on the production of proinflammatory cytokines and neurotrophic factors in LPS-activated microglia. *Int. Immunopharmacol.* 9, 360–365. doi: 10.1016/j.intimp.2009.01.002

Zhang, Z. Y., Zhang, Z., and Schluesener, H. J. (2010). MS-275, an histone deacetylase inhibitor, reduces the inflammatory reaction in rat experimental autoimmune neuritis. *Neuroscience* 169, 370–377. doi: 10.1016/j.neuroscience.2010.04.074

Zhang, Z., Zhang, X., Xue, W., Yangyang, Y., Xu, D., Zhao, Y., et al. (2010). Effects of oridonin nanosuspension on cell proliferation and apoptosis of human prostatic carcinoma PC-3 cell line. *Int. J. Nanomed.* 5, 735–742. doi: 10.2147/ijn.s13537

Zhang, Z., Zhang, Z. Y., Fauser, U., and Schluesener, H. J. (2008a). FTY720 ameliorates experimental autoimmune neuritis by inhibition of lymphocyte and monocyte infiltration into peripheral nerves. *Exp. Neurol.* 210, 681–690. doi: 10.1016/j.expneurol.2007.12.025

Zhang, Z., Zhang, Z. Y., Fauser, U., and Schluesener, H. J. (2008b). Valproic acid attenuates inflammation in experimental autoimmune neuritis. *Cell. Mol. Life Sci.* 65, 4055–4065. doi: 10.1007/s00018-008-8521-4

Zhang, Z., Zhang, Z. Y., and Schluesener, H. J. (2009). Compound A, a plant origin ligand of glucocorticoid receptors, increases regulatory T cells and M2 macrophages to attenuate experimental autoimmune neuritis with reduced side effects. *J. Immunol.* 183, 3081–3091. doi: 10.4049/jimmunol.0901088

Zhang, Z. Y., Daniels, R., and Schluesener, H. J. (2013). Oridonin ameliorates neuropathological changes and behavioural deficits in a mouse model of cerebral amyloidosis. *J. Cell. Mol. Med.* 17, 1566–1576. doi: 10.1111/jcmm.12124

Zhou, L., Sun, L., Wu, H., Zhang, L., Chen, M., Liu, J., et al. (2013). Oridonin ameliorates lupus-like symptoms of MRL(lpr/lpr) mice by inhibition of B-cell activating factor (BAFF). *Eur. J. Pharmacol.* 715, 230–237. doi: 10.1016/j.ejphar.2013.05.016

Zhou, M., Yi, Y., and Hong, L. (2018). Oridonin ameliorates lipopolysaccharide-induced endometritis in mice via inhibition of the TLR-4/NF-kappaBpathway. *Inflammation* 42, 81–90. doi: 10.1007/s10753-018-0874-8

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REVIEW
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Connecting Metainflammation and Neuroinflammation Through the PTN-MK-RPTPβ/ζ Axis: Relevance in Therapeutic Development

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Inflammation is a common factor of pathologies such as obesity, type 2 diabetes or neurodegenerative diseases. Chronic inflammation is considered part of the pathogenic mechanisms of different disorders associated with aging. Interestingly, peripheral inflammation and the associated metabolic alterations not only facilitate insulin resistance and diabetes but also neurodegenerative disorders. Therefore, the identification of novel pathways, common to the development of these diseases, which modulate the immune response and signaling is key. It will provide highly relevant information to advance our knowledge of the multifactorial process of aging, and to establish new biomarkers and/or therapeutic targets to counteract the underlying chronic inflammatory processes. One novel pathway that regulates peripheral and central immune responses is triggered by the cytokines pleiotrophin (PTN) and midkine (MK), which bind its receptor, Receptor Protein Tyrosine Phosphatase (RPTP) β/ζ, and inactivate its phosphatase activity. In this review, we compile a growing body of knowledge suggesting that PTN and MK modulate the immune response and/or inflammation in different pathologies characterized by peripheral inflammation associated with insulin resistance, such as aging, and in central disorders characterized by overt neuroinflammation, such as neurodegenerative diseases and endotoxemia. Evidence strongly suggests that regulation of the PTN and MK signaling pathways may provide new therapeutic opportunities particularly in those neurological disorders characterized by increased PTN and/or MK cerebral levels and neuroinflammation. Importantly, we discuss existing therapeutics, and others being developed, that modulate these signaling pathways, and their potential use in pathologies characterized by overt neuroinflammation.

Keywords: pleiotrophin, midkine, PTPRZ, neuroinflammation, inflammation, aging, obesity, neurodegeneration

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NEUROINFLAMMATION AND CNS DISORDERS

Activation of the innate immunity in the Central Nervous System (CNS) is a critical step in the healing process after brain injury. However, cumulative evidence points to deleterious effects of chronic neuroinflammation (Szepesi et al., 2018). Activation of astrocytes and microglia is a hallmark of neuroinflammation. Activated glial cells release free radicals, cytokines and other

pro-inflammatory factors. Activated microglia can be prophylactic and protect the brain from a wide variety of events including traumatic injury, stroke or neurodegenerative diseases, through modulation of neuronal synapses, by promoting neurogenesis, clearing debris, and suppressing inflammation (Chen and Trapp, 2016). However, exacerbated or persistent neuroinflammation can potentially exert negative effects on CNS integrity and function accompanying a wide variety of CNS diseases (Kielian, 2016). Sustained neuroinflammation is implicated in the progressive nature of neurodegenerative diseases (Faden et al., 2016; Gasiorowski et al., 2017). In addition to the peripheral stimuli that can cause chronic neuroinflammation, inflammatory processes in the CNS are also observed in response to different central events such as contact with neurotoxins or traumatic brain injury (TBI) (Spielman et al., 2014) and have been related to vascular injury and angiogenesis (Popp et al., 2017). The perpetuation of these inflammatory processes can subsequently cause acute secondary injury, leading to chronic neurodegenerative diseases (Simon et al., 2017). Supporting this role of neuroinflammation in neurodegenerative diseases, genome-wide association studies have shown associations with neurodegenerative disorders susceptibility, such as Parkinson's disease (PD), in the human leukocyte antigen region and other polymorphisms related to the immune system including inflammatory cytokines and their receptors (Nalls et al., 2014). In addition, chronic neuroinflammation has been shown to contribute to neuronal loss by apoptosis in different pathologies, including multiple sclerosis (Mori F. et al., 2014; Mandolesi et al., 2017), drug addiction and alcoholism, and psychiatric disorders such as major depression (Montesinos et al., 2016; Neupane, 2016). In the light of this dual role of neuroinflammation and microglial activation in health and disease, the identification of the mechanisms responsible for maintenance of microglial activation and associated continuous neuron damage is critical to understand the etiology and pathology of diseases characterized by overt neuroinflammation.

Pleiotrophin and Midkine: Novel Modulators of Neuroinflammation

In neuroinflammatory processes, the activation of innate immunity, contribution of Toll-like receptors (TLRs) and induction of expression of inflammation-related molecules such as cytokines, have been described in detail. Although the discovery of new modulators of neuroinflammation can potentially translate in novel targets and biomarkers for different diseases, the genetic bases of neuroinflammation are still far from being fully understood. Recently, we identified Pleiotrophin (PTN) and Midkine (MK) as two novel neurotrophic factors that modulate neuroinflammation in different contexts (**Figure 1**; Vicente-Rodriguez et al., 2016a,b; Fernandez-Calle et al., 2017). Pleiotrophin and MK are the only cytokines that constitute the *Ptn/Mk* developmental gene family (Kadomatsu et al., 1988; Milner et al., 1989). The pattern of expression of these cytokines in developing and adult nervous system has been extensively described in different species and led to study the importance

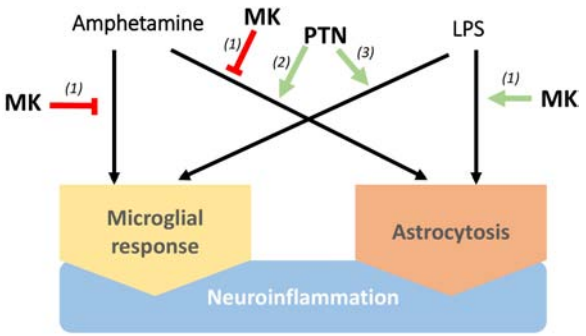

FIGURE 1 | Differential regulation of neuroinflammation by Pleiotrophin (PTN) and Midkine (MK). MK lessens microglia responses and astrocytosis induced by amphetamine in the striatum, although lipopolysaccharide (LPS)-induced neuroinflammation seems to be potentiated by MK. On the other hand, PTN enhances neuroinflammation induced by different stimuli, including microglial activation, increase of pro-inflammatory cytokines in the brain and astrocytosis. (1) Vicente-Rodriguez et al. (2016a), (2) Vicente-Rodriguez et al. (2016b), (3) Fernandez-Calle et al. (2017).

of these cytokines in neural-glial interactions (Vanderwinden et al., 1992; Silos-Santiago et al., 1996; Xu et al., 2014). They are widely expressed during development but, in adults, their pattern of expression is restricted to a few cell types in different organs, including the brain, where they are mainly expressed in neurons. Importantly, the levels of expression of MK and PTN are highly upregulated after injury in different cells including microglia and inflammatory macrophages (Jin et al., 2009; Martin et al., 2011; Muramatsu, 2011; Gonzalez-Castillo et al., 2014). As suggested by their similar pattern of expression, PTN and MK overlap many functions including wound repair and survival of neurons (Gramage and Herradon, 2011; Herradon and Perez-Garcia, 2014). Accordingly, PTN and MK are upregulated in senile plaques and sera of patients with Alzheimer's disease (AD) and in the substantia nigra of patients with PD among other neurodegenerative disorders (Herradon and Perez-Garcia, 2014). Midkine is also found in glial cytoplasmic inclusions of multiple system atrophy brains (Kato et al., 2000). In addition, both cytokines are also upregulated in the brain in pathological conditions characterized by neuroinflammation such as different types of brain injury, including ischemia, and after administration of drugs of abuse like amphetamine, alcohol and opioids (**Table 1**; Herradon and Perez-Garcia, 2014). It has been demonstrated that $Ptn^{-/-}$ mice exhibit exacerbated amphetamine-induced dopaminergic injury in the nigrostriatal pathway (Gramage et al., 2010), which correlated with changes in the striatal phosphoproteome similar to those found in PD (Gramage et al., 2013). Other authors have demonstrated that *Ptn* overexpression in the brain exerts neurotrophic effects in rodent models of PD (Gombash et al., 2012). Taken together, these findings suggest that PTN protects against dopaminergic neurodegeneration in different pathological contexts.

Midkine exerts relevant functions in peripheral inflammatory processes in different pathological conditions (Muramatsu, 2014; Weckbach et al., 2014), facilitating the migration of macrophages

TABLE 1 | Pleiotrophin and Midkine expression is upregulated in different pathologies with a neuroinflammatory component.

Neuroinflammatory conditions	Pleiotrophin upregulation	Midkine upregulation
Amphetamine	Le Greves, 2005	–
Alcohol	Vicente-Rodriguez et al., 2014	Flatscher-Bader and Wilce, 2008; He et al., 2015
Opioid	Garcia-Perez et al., 2015	Ezquerra et al., 2007; Garcia-Perez et al., 2015
Neuropathic pain	Ezquerra et al., 2008	–
Parkinson's disease	Marchionini et al., 2007	–
Alzheimer's disease	Skillback et al., 2017	Yasuhara et al., 1993; Xiong et al., 2019
Brain injury	Yeh et al., 1998; Poulsen et al., 2000; Iseki et al., 2002	Yoshida et al., 1995; Mochizuki et al., 1998; Wada et al., 2002; Otsuka et al., 2016
Other neurodegenerative disorders	–	Yasuhara et al., 1996; Kato et al., 2000

The upregulation of these cytokines in the CNS has been described after different stimuli or in pathologies related to neuroinflammatory processes.

and neutrophils (Takada et al., 1997; Horiba et al., 2000; Sato et al., 2001) and preventing differentiation of regulatory T-cells (Wang et al., 2008; Sonobe et al., 2012). In the CNS, we found that amphetamine-induced microglial response and astrocytosis are enhanced in the striatum, but not in the hippocampus, of $Mk^{-/-}$ mice. In contrast, lipopolysaccharide (LPS)-induced striatal astrogliosis was blocked by genetic inactivation of *Mk*, suggesting a differential regulation of astrocytosis by MK depending on the inflammatory stimulus (**Figure 1**; Vicente-Rodriguez et al., 2016a). On the other hand, *Ptn* overexpression in the brain (*Ptn*-Tg mice) potentiates the striatal astrocytosis induced by acute administrations of amphetamine (Vicente-Rodriguez et al., 2016b). Pleiotrophin overexpression also potentiates microglial activation after an acute systemic administration of LPS (Fernandez-Calle et al., 2017). Importantly, LPS-induced increases of pro-inflammatory cytokines in the brain, including TNF-α and IL-1β, were significantly enhanced in *Ptn*-Tg mice (Fernandez-Calle et al., 2017). The data suggest that PTN potentiates the acute neuroimmune response induced by different stimuli, including microglial activation (**Figure 1**), which is necessary and critical for host defense (Chen and Trapp, 2016) and could contribute to the known neurotrophic effects of this cytokine. However, although upregulation of PTN levels in the brain has been described in different chronic diseases such as AD and PD (Herradon and Perez-Garcia, 2014), in which long-term neuroinflammation is a pathological hallmark, the possible modulatory role of PTN in persistent neuroinflammation remains to be elucidated. If chronic upregulation of PTN levels in these diseases contributes to prolonged neuroinflammation, PTN would parallel the dual role of glial activation in health and disease depending on the duration of its actions. Although further studies are needed to better understand the roles of PTN and MK in the regulation of neuroinflammation in chronic CNS disorders, evidence strongly suggests that pharmacological modulation of the actions of PTN and MK is a novel strategy to treat disorders characterized by neuroinflammation.

It has to be noted that aging-associated neuroinflammatory conditions (e.g., AD) have been related with vascular injury and angiogenesis (Popp et al., 2017) and PTN expression levels are associated strongly with aging in human cerebrospinal fluid (Wyss-Coray, 2016). Both PTN and MK induce tumor growth mainly by promoting angiogenesis (Perez-Pinera et al., 2008; Weckbach et al., 2012; Papadimitriou et al., 2016). Pleiotrophin exerts mitogenic activity in fibroblasts (Milner et al., 1989), brain capillary endothelial cells (Courty et al., 1991), and in adrenal carcinoma cells (Fang et al., 1992). Importantly, *Ptn* is upregulated in vascular endothelial cells in sites of neovascularization after focal cerebral ischemia (Yeh et al., 1998). The angiogenic potential of PTN has been shown in studies in ischemic myocardium in rats, in which it was demonstrated that PTN stimulates significant increases in normal appearing new capillaries, arterioles, and newly formed blood vessels that interconnect with the existent coronary vascular system (Christman et al., 2005a). On the other hand, MK promotes endothelial cell proliferation and the recruitment of inflammatory cells to lesions (Kadomatsu et al., 2014). As in the case of PTN, an important pro-angiogenic role under hypoxia conditions has been described for MK (Weckbach et al., 2012). Thus, both cytokines have been proposed as important factors for inflammation, tissue repair and angiogenesis. Accordingly, it seems reasonable to propose the modulation of PTN/MK signaling pathways for the treatment of the above mentioned disorders characterized by neuroinflammation and vascular injury.

The Mechanism of Action of Pleiotrophin and Midkine in Relation to Neuroinflammation

Midkine and PTN bind different receptors such as Receptor Protein Tyrosine Phosphatase (RPTP) β/ζ (Maeda et al., 1996, 1999; Meng et al., 2000), syndecan-3 (Rauvala et al., 2000), and anaplastic lymphoma kinase (ALK) (Stoica et al., 2001). Midkine also binds other proteoglycans (Muramatsu, 2011; Kadomatsu et al., 2013), including Neuroglycan C, through which MK has been shown to promote neurites in oligodendrocyte precursor-like cells (Ichihara-Tanaka et al., 2006). Also, MK has been shown to bind low density lipoprotein receptor-related protein (LRP), integrin α4β1 and integrin α6β1 in the brain (Muramatsu et al., 2000, 2004).

RPTPβ/ζ (a.k.a. RPTPβ, PTPRZ, and PTPζ) (Krueger and Saito, 1992), is abundantly expressed in the CNS as a chondroitin sulfate proteoglycan (Krueger and Saito, 1992; Maeda et al., 1994). Pleiotrophin and RPTPβ/ζ are highly expressed in human white matter oligodendroglial precursor cells (OPCs), and PTN-PTPRZ signaling promotes postnatal OPC differentiation during developmental myelination and remyelination after injury (Harroch et al., 2002; Sim et al., 2006; Kuboyama et al., 2015; Tanga et al., 2019). RPTPβ/ζ is composed of an N-terminal carbonic anhydrase-like domain, a fibronectin type III domain, a serine, glycine-rich domain that is thought to be chondroitin

sulfate attachment region, a transmembrane segment, and two tyrosine phosphatase domains (Krueger and Saito, 1992). There are four splice variants of this molecule including a full-length (PTPζ-A), a short form (PTPζ-B), the secreted form (PTPζ-S or 6B4 proteoglycan/phosphacan), which corresponds to the extracellular region of PTPζ-A (Maeda et al., 1994; Maurel et al., 1994) and the PSI isoform, expressed only in neurons (Heck et al., 2005). Midkine binds mostly to the chondroitin sulfate portion of RPTPβ/ζ (Maeda et al., 1999) which has been proven to be essential for MK-induced neuron survival effects. The interaction of RPTPβ/ζ with PTN inactivates the intrinsic tyrosine phosphatase activity of RPTPβ/ζ, presumably by enforcing a conformational change in RPTPβ/ζ that denies substrates access to its active site in the D1 domain (Meng et al., 2000).

These signaling pathways triggered by PTN and MK through RPTPβ/ζ support important roles of these cytokines in neuroinflammation (**Figure 1**) since they modulate the tyrosine phosphorylation of substrates of RPTPβ/ζ that are known regulators of neuroinflammation such as TrkA (Shintani and Noda, 2008) and Fyn kinase (Pariser et al., 2005; Panicker et al., 2015). After LPS administration, Fyn is activated in microglia (Panicker et al., 2015). Activated Fyn phosphorylates PKCδ at Y311, contributing to an increase in PKCδ kinase activity and activation of the NFκB pathway (Panicker et al., 2015), suggesting that the PTN/RPTPβ/ζ is a major regulatory pathway of this pro-inflammatory signaling cascade. These signaling events are also observed in animal models of PD (Panicker et al., 2015).

The wide pattern of expression of *Ptn* in developing and adult tissues, including nervous system and peripheral organs, has been described in detail (Vanderwinden et al., 1992; Xu et al., 2014). The sites of expression of RPTPβ/ζ in peripheral organs are highly relevant for its potential role in inflammation. RPTPβ/ζ is expressed in the intestine, mononuclear cells, monocytes, macrophages (Zwicker et al., 2016), in hematopoietic stem cells (HSC) and B cells, in which RPTPβ/ζ promotes HSC maintenance and B cell survival (Sorrelle et al., 2017). RPTPβ/ζ expression is increased in inflammatory processes associated with kidney injury, in which interleukin 34 (IL-34) increases its phosphatase activity and promotes monocyte and macrophage infiltration in the kidney, contributing to chronic renal damage (Sanchez-Nino et al., 2016). As in the case of PTN and MK, these findings point to an important role of RPTPβ/ζ in the regulation of central and peripheral inflammatory processes. However, the relevance of the changes in the phosphatase activity of RPTPβ/ζ may differ in acute and chronic inflammatory processes and has not been studied before, which is due, in part, to the lack of proper ligands of this receptor. Recently, Fujikawa et al. (2016) identified SCB4380 as a potent inhibitor of RPTPβ/ζ in a rat allograft model of glioblastoma. Unfortunately, the low lipophilicity of this inhibitor, which requires liposome carriers for intracellular delivery *in vitro*, is a critical limitation for its use in studies in the CNS. To fill this gap, we recently developed BBB permeable inhibitors of RPTPβ/ζ, selecting the lead compound (MY10) after *in vitro* and *in vivo* validation (Fernandez-Calle et al., 2018; Pastor et al., 2018).

METAINFLAMMATION AND NEUROINFLAMMATION

Inflammatory responses in the brain in response to metabolic stress have been observed in both mice and humans, but little is known about the mechanisms that activate them (Thaler et al., 2012). For instance, low level inflammation in response to an excess of nutrients or energy is not necessarily a pathological process but reflects on a series of potentially harmful alterations in metabolic homeostasis, which has been called metainflammation (Gregor and Hotamisligil, 2011). Chronic overfeeding is associated with excess peripheral pro-inflammatory mediators that contribute to neuroinflammation, which subsequently exacerbates neurodegeneration (Spielman et al., 2014). Accordingly, epidemiological studies indicate that AD and PD risk positively correlate with pro-inflammatory conditions such as diabetes mellitus or metabolic syndrome (Spielman et al., 2014). Thus, the discovery of new pharmacological targets involved in the preservation of a healthy basal metabolism would potentially translate in novel therapeutic interventions not only in metabolic diseases but in neuroinflammatory CNS disorders. In this context, we will now summarize the evidence demonstrating that PTN is essential to preserve the appropriate basal metabolism.

Pleiotrophin: A Novel Modulator of Metainflammation, Insulin Resistance, and Adipose Tissue Plasticity

Although *Ptn* is expressed in adults (Vanderwinden et al., 1992), its role in adipose tissue is not fully understood. *Ptn* expression is found in human adipose tissue, being higher in visceral white adipose tissue (WAT) than in subcutaneous WAT (Hoggard et al., 2012). In addition, the expression of *Ptn* in mice also depends on the type of visceral deposit, being about 30 times higher in the mesenteric than in the perigonadal fat. Pleiotrophin deletion is associated with a lipodystrophic phenotype (Sevillano et al., 2012), altered energy metabolism and insulin resistance (Sevillano et al., 2019). These results, together with previous reports from our group showing that PTN is increased in the visceral WAT of obese pregnant women, point to a novel role of PTN in metabolic homeostasis and metainflammation.

The association of insulin resistance with inflammation has been studied in depth during the last few decades (Shoelson et al., 2006; Olefsky and Glass, 2010; Hotamisligil, 2017), including the role of macrophages (Olefsky and Glass, 2010). It has been shown that inflammatory cytokines activate kinases that promote Ser phosphorylation of insulin receptor substrate 1 (pSerIRS1) (Bluher et al., 2009; Copps and White, 2012), which is considered a marker of insulin resistance in adipose tissue, not only in pathological (Schmitz-Peiffer and Whitehead, 2003) but also physiological situations, as shown previously (Sevillano et al., 2005, 2007; de Castro et al., 2011). An additional factor is the heterogeneity of macrophages; M1 are pro-inflammatory while M2 are anti-inflammatory and participate in homeostasis and tissue remodeling (Stienstra et al., 2008). Peroxisome proliferator-activated receptor gamma (PPAR-γ) is key in

metainflammation, since it inhibits the inflammatory response in macrophages (Welch et al., 2003). Thus, activators of PPAR-γ, such as thiazolidinediones (TZD), improve the insulin response of adipose tissue by inhibiting the production of inflammatory mediators, including TNF-α (Sharma and Staels, 2007), and by increasing the levels of adiponectin (Combs et al., 2002), an anti-inflammatory adipokine that is downregulated in states of insulin resistance such as obesity or type 2 diabetes (T2D) (Statnick et al., 2000). In the same manner, treatment with TZDs reverses the polarization toward the pro-inflammatory phenotype M1 in diet-induced obesity models (Stienstra et al., 2008; Fujisaka et al., 2009). Precisely, we have proposed very recently that defective PPAR-γ activation may underlie insulin resistance of *Ptn* deficient mice by promoting an inflammatory condition that impairs lipid and glucose homeostasis (Sevillano et al., 2019).

The great plasticity of WAT enables this tissue to contract or expand in response to alterations in the energy balance. It has been proposed that WAT plasticity is a key factor in metabolic alterations such as obesity (Carobbio et al., 2017). Expansion of adipose tissue can be caused by hypertrophy, due to accumulation of triacylglycerides and by hyperplasia characterized by adipogenesis. Numerous molecules participate in this process, such as WNT and its downstream effectors, transcription factors, such as PPAR-γ (Spiegelman, 1998), or oxidative stress (Lowe et al., 2011). Interestingly, it has been suggested that PTN could inhibit the expression of PPAR-γ, which would block white adipocyte differentiation (Gu et al., 2007). The role of PTN in this context, however, remains unclear, since rPTN has been found to decrease Pparγ2 expression *in vitro*, but the same study shows *in vivo* that injection of a PTN-neutralizing antibody slightly decreased Pparγ2 in adipose tissue (Wong et al., 2016). Beige adipocytes can develop in WAT, a process known as browning, in response to certain stimuli, such as treatment with TZDs or cold exposure (Wang and Seale, 2016). WAT browning and brown adipose tissue (BAT) (Bartelt and Heeren, 2014; Lee et al., 2014) may represent important therapeutic targets because they facilitate the oxidation of excess lipids (Kajimura et al., 2015), thus preventing hyperlipidaemia and potentially also lipotoxicity. In this context, it has been recently demonstrated that PTN impairs brown adipocyte differentiation (Sevillano et al., 2019). In fact, the lipodystrophic phenotype of $Ptn^{-/-}$ mice is related to an enhanced thermogenesis in BAT (Sevillano et al., 2019). The molecular events responsible for these newly discovered functions of PTN seems to involve the extracellular matrix (ECM).

The plasticity of WAT also requires vascular remodeling and ECM (Pellegrinelli et al., 2016). Obesity is usually accompanied by infiltration of macrophages in the tissue, and it has been proposed that the matrix metalloproteinases (MMPs) secreted by resident macrophages in WAT participate in the degradation of ECM proteins and promote adipocyte-induced secretion of MMPs (Mariman and Wang, 2010), thus supporting WAT remodeling. In this context, it is important the phosphatase ADAMTS1, an MMP that contributes to the degradation of highly expressed collagens in WAT, such as Col1α1 (Mori S. et al., 2014). It has been proposed that ADAMTS1 plays a modulatory role in ECM and adipogenesis by inhibiting the differentiation of mesenchymal cells to preadipocytes. In fact, in a diet-induced obesity mouse model, expression of Adamts1 is decreased in the perigonadal WAT, which is consequently expanded by an activated adipogenesis. Accordingly, an inverse correlation between Adamts1 expression and body mass index (BMI) has been found in humans (Chen et al., 2016). It is important to note that ADAMTS1 induces *Ptn* expression through the WNT/β-catenin signaling pathway, suggesting a role of this cytokine in the effects of MMPs on adipocyte differentiation. This is supported by studies in humans with diet-induced weight gain that show concomitant increases in the levels of expression of ADAMTS1, PTN and different mediators of WNT signaling (Wong et al., 2016). Interestingly, preliminary results from our group showed that, when brown adipocytes differentiate, Adamts1 expression is blunted, which correlates with a decreased expression of *Ptn* (Sevillano et al., 2019).

The Metainflammation-Neuroinflammation Connection: Role of Pleiotrophin

Although metainflammation was first described in WAT, it may also affect other tissues such as the liver, pancreas, or hypothalamus. In fact, to maintain organism homeostasis, the brain must control the energy state of the periphery, as the CNS depends on the continuous supply of nutrients from the general circulation. Adipose tissue is innervated by the sympathetic endings of the autonomic nervous system, with BAT being much more innervated than WAT (**Figure 2**). It has been shown that CNS neurons are involved in the multisynaptic pathways to both liver and WAT, which allows a coordinated control of peripheral metabolism (Stanley et al., 2010). The alteration of any of the key components of this system, as it occurs in inflammation, or the failure of its integration could be involved in the etiopathogenesis of metabolic disorders such as obesity and diabetes, but also in CNS disorders such as neurodegenerative diseases (**Figure 2**; Santiago and Potashkin, 2014). As mentioned before, peripheral inflammatory diseases such as T2D and obesity confers an increased risk of developing AD and PD (Spielman et al., 2014), presumably through their capacity to cause prolonged low-grade neuroinflammation. It is conceivable, therefore, that bidirectional communication and cross-talk between the CNS and the periphery may be relevant to these pathological conditions (**Figure 2**). In fact, the CNS seems to be involved in the control of proliferation and differentiation of white adipocytes. Accordingly, *in vivo* surgical or pharmacological denervation of WAT triggers an increase in the number of white pre-adipocytes and adipocytes (Cousin et al., 1993; Bowers et al., 2004), whereas noradrenaline inhibits the proliferation of adipocyte precursor cells *in vitro* (Jones et al., 1992). On the other hand, when BBB homeostasis is altered, as it occurs in some metabolic disorders, the development of central pathological events such as neurodegeneration are facilitated (Mauro et al., 2014), probably by increasing brain sensitivity to peripheral stimuli that eventually end up causing chronic neuroinflammation. For instance, systemic

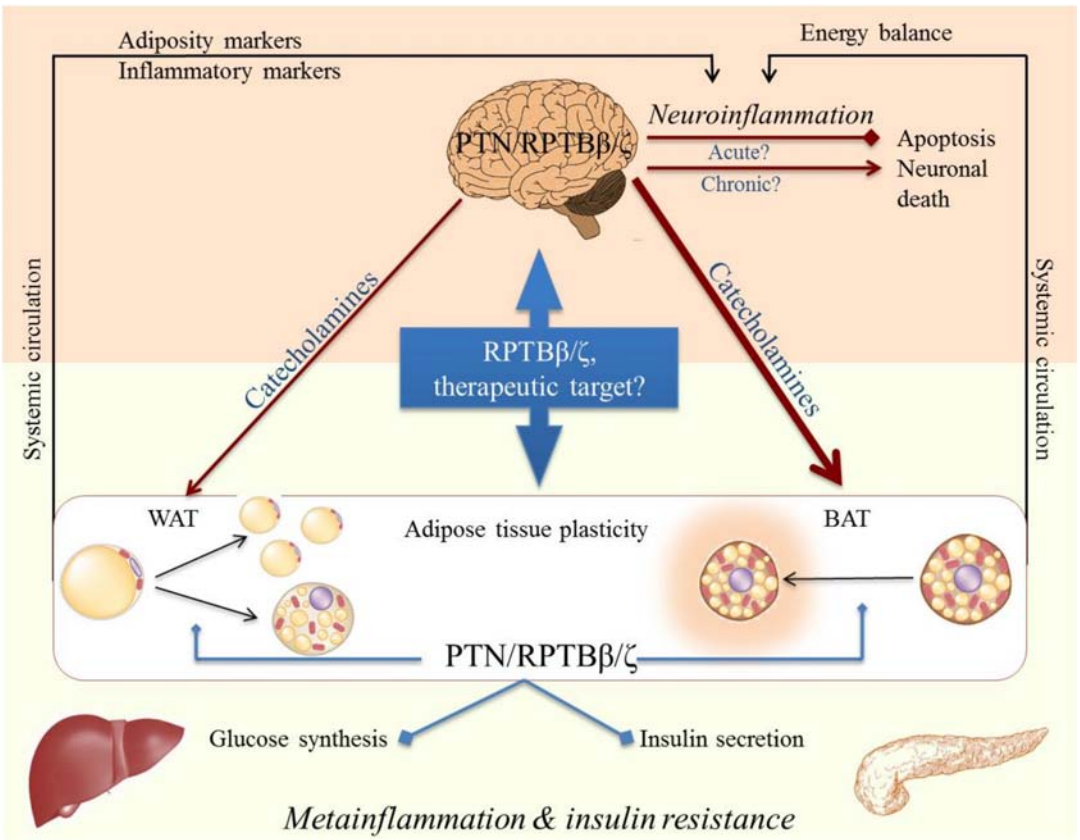

FIGURE 2 | Cross-talk CNS-periphery in inflammation and its possible modulation by RPTBβ/ζ and its ligand Pleiotrophin (PTN).

administration of LPS is an animal model of peripherally-induced neuroinflammation and neurodegeneration (Qin et al., 2007, 2013), and there is evidence that peripheral infections accompanied by inflammation represent major risk factors for the development of sporadic AD and PD (Holmes, 2013; Su and Federoff, 2014). Other alterations of BBB such as pericyte dysfunction at the neurovascular unit have been linked with aberrant angiogenesis (Sweeney et al., 2016). As PTN is known to modulate the astrocytic response in different contexts and to play a role in vascular formation (Martin et al., 2013; Zhang et al., 2014; Wang et al., 2017; Fernandez-Calle et al., 2018), a dual function of this cytokine in both angiogenesis and neuroinflammation in these conditions should not be ruled out.

In addition, it has to be noted that PPAR-γ is known to be involved in degenerative processes in the brain and peripheral inflammation in pathologies such as DM or obesity (Villapol, 2018). Importantly, PPAR-γ is also associated with the control of angiogenesis in the brain (Arany et al., 2008). As explained before, PTN is involved in the regulation of PPAR-γ expression (Gu et al., 2007), suggesting the interesting possibility that this mechanism may underlie the common role of this cytokine in diabetes, neurodegeneration and angiogenesis.

It is also important to identify modulatory factors regarding the communication between the CNS and the immune system involved in the control of immune responses both centrally and peripherally. Extracellular vesicles (ExV), including exosomes, are released by all cells, including those of adipose tissue and nervous system. Their capacity to deliver a variety of bioactive molecules (e.g., protein, mRNA, miRNA, DNA, and lipids) to both nearby and distal cells suggests that exosomes and specific miRNAs play a role in cancer, diabetes, obesity, cardiovascular disease (Trajkovski et al., 2011) and CNS disorders, including neuroinflammation and PD (Leggio et al., 2017). In this context, it has to be noted that PTN is secreted by murine and human neural precursor cells (NPCs) (Qin et al., 2017), which is consistent with reports identifying PTN in the secretomes of various neural stem cell populations (Furuta et al., 2004; Lee et al., 2012).

Since adipocytes secrete large proportions of exosomes, it has been proposed that exosomes and miRNAs derived from fat play a role in pathologies related with insulin resistance (Thomou et al., 2017). Furthermore, there is evidence that exosomes can penetrate the BBB, moving from the peripheral circulation to the CNS (Matsumoto et al., 2017). It is also interesting to note that brain endothelial exosomes may be used as serological biomarkers for BBB status during neuroinflammation (Ramirez et al., 2018), pointing to a key role of exosomes in CNS-periphery cross-talk. The possible involvement of exosomes in PTN-mediated effects needs to be elucidated.

THERAPEUTIC PERSPECTIVES

Pleiotrophin and MK are novel modulators of neuroinflammation that play different roles depending on the inflammatory stimulus and the duration of the neuroinflammatory processes. Further studies are needed to fully understand the role of these cytokines on neuroinflammation, particularly in chronic neuroinflammation. However, in the light of evidence summarized here, it is reasonable to hypothesize that both potentiation and inhibition of the PTN/MK signaling pathways are novel therapeutic strategies to modulate neuroinflammation in acute and chronic pathological states. For instance, potentiation of the acute neuroinflammatory actions of these cytokines may exert therapeutic effects in different types of brain injury through limitation of the damage and improvement of brain repair. In terms of metainflammation, potentiation of PTN/MK signaling pathways may contribute to control the inflammatory condition associated to metabolic disorders. On the contrary, inhibiting the actions of these cytokines may be beneficial in chronic neuroinflammatory states that promotes exacerbation of neurodegeneration.

The administration of rMK and rPTN has been proposed in a number of CNS and peripheral injuries, rending already significant effects in preclinical models, particularly in the case of myocardial ischemia (Christman et al., 2005b; Fukui et al., 2008) and bone repair (Lamprou et al., 2014; Liedert et al., 2014). Gene therapy or stem cells overexpressing these genes have also been proposed as delivery systems of these cytokines in different animal models of peripheral and central disorders and have been reviewed elsewhere (Muramatsu, 2011; Herradon and Perez-Garcia, 2014; Kadomatsu et al., 2014).

The potential of therapeutic inhibition of MK and PTN has been studied in depth for the treatment of malignant diseases in different experimental models, including siRNA, shRNA, antibodies and RNA aptamers (Muramatsu, 2011; Kishida and Kadomatsu, 2014; Shi et al., 2017). Interestingly, an aptamer to MK has already been used to treat experimental autoimmune encephalitis (EAE) in mice (Wang et al., 2008), which is an animal model of multiple sclerosis.

Although promising, the above mentioned strategies to potentiate or inhibit the actions of PTN and MK in neuroinflammation in CNS disorders are commonly restricted by the limitation of the route of administration (e.g., intracranial). Thus, these effective therapeutic strategies in preclinical models of different diseases are not likely to progress through clinical development in the midterm. We will now summarize the existing modulators of PTN/MK signaling with approved therapeutic uses in humans and other promising strategies at the preclinical level that are more likely to progress through clinical development in the midterm.

Potentiation of MK/PTN Actions

RPTPβ/ζ Inhibitors

Recently, Fujikawa et al. (2016) described SCB4380 as a potent inhibitor of PTPRZ (RPTPβ/ζ) as a potential new drug for glioma therapy. Unfortunately, its physicochemical properties make very difficult for this molecule to go through biological barriers. Thus, SCB4380 required liposome carriers for intracellular delivery (Fujikawa et al., 2016). To overcome this limitation in the RPTPβ/ζ inhibition strategy, novel BBB permeable selective small-molecule inhibitors of RPTPβ/ζ were recently designed and synthesized through rational drug design (Pastor et al., 2018). The lead compound, MY10, interacts with the intracellular domain PD1 of RPTPβ/ζ and inhibits its tyrosine phosphatase activity. MY10 has been validated *in vitro* and *in vivo*. Interestingly, systemic administration of MY10 reduces alcohol consumption and blocks the rewarding effects of alcohol in mice (Fernandez-Calle et al., 2018), replicating the effects obtained before with the transgenic mouse model (*Ptn*-Tg) overexpressing in the brain *Ptn*, the endogenous inhibitor of RPTPβ/ζ (Vicente-Rodriguez et al., 2014). Using these *Ptn*-Tg mice, it was demonstrated that PTN overexpression in the brain potentiates LPS-induced microglial activation and neuroinflammation (Fernandez-Calle et al., 2017), suggesting that acute treatment with MY10 would cause similar effects and could be used in brain repair after injury by promoting acute neuroimmune responses. However, persistent and/or over-activation of microglia is deleterious. Thus, more knowledge regarding the role of PTN in chronic neuroinflammation is needed to substantiate the pharmacological use of RPTPβ/ζ inhibitors as a potential therapeutic strategy in CNS disorders related with chronic neuroinflammation.

Inhibition of MK/PTN Actions

ALK Inhibitors

ALK has been identified as a receptor for PTN (Stoica et al., 2001), but is also a substrate of RPTPβ/ζ (Perez-Pinera et al., 2007). It has been shown to mediate many of the central actions of PTN and MK (Herradon and Perez-Garcia, 2014; Dutton et al., 2017) and has been recently identified as a potent regulator of NLRP3 inflammasome activation in macrophages through its capacity to activate NF-kB (Zhang et al., 2018). Interestingly, it has been recently demonstrated that PTN activates AKT signaling through ALK to promote the morphological maturation and synaptic integration of newborn neurons (Tang et al., 2019). Pleiotrophin or MK-induced inhibition of RPTPβ/ζ causes an increase in tyrosine phosphorylation in ALK and subsequent activation of its tyrosine kinase activity (Perez-Pinera et al., 2007; Herradon et al., 2009).

Three generations of ALK inhibitors have been developed for the treatment of non–small cell lung cancer with certain mutations in Alk (Fan et al., 2018; Rothenstein and Chooback, 2018). Interestingly, these inhibitors are also effective in preclinical models of non-malignant completely unrelated pathological conditions. For instance, inhibition of ALK with alectinib, a second generation ALK inhibitor, significantly reduces alcohol binge drinking in rodents (Dutton et al., 2017). Inhibition of ALK with ceritinib and lorlatinib blocks NLRP3 inflammasome activation in macrophages (Zhang et al., 2018). Taking together, evidence suggests that the effects of the PTN-MK-RPTPβ/ζ axis on neuroinflammation could be

modulated through pharmacologic regulation of one of its main effectors: ALK.

Fyn Inhibitors

Fyn kinase is a substrate of RPTPβ/ζ (Pariser et al., 2005). Fyn has been found activated in microglia in animal models of PD and after treatment with LPS and triggers the activation of the pro-inflammatory NFκB pathway in these models (Panicker et al., 2015). These data suggest that Fyn is a mediator of the actions of the PTN-MK-RPTPβ/ζ axis on neuroinflammation and, thus, pharmacologic inhibition of Fyn could be considered as a potential strategy to modulate the effects of this axis in diseases characterized by neuroinflammatory processes. In this context, it is interesting to note that Poli et al. (2018) have recently developed through rational drug design new derivatives of previous hit compounds Fyn inhibitors with considerable potency (IC_{50} = 0.76 μM).

MK Inhibitors

Small-molecule midkine inhibitors have been investigated mainly in the field of cancer therapy (Matsui et al., 2010). A promising compound (iMDK) has been recently proven to be effective in preclinical models of non–small cell lung cancer (Hao et al., 2013), oral squamous cell carcinoma (Masui et al., 2016) and prostate cancer (Erdogan et al., 2017). By inhibiting the actions of MK on RPTPβ/ζ, iMDK would leave unchecked the intrinsic phosphatase activity of this receptor. As a result, decreased phosphorylation and activation of its substrates (e.g., Fyn, ALK) would contribute to decreased neuroimmune responses.

CONCLUSION

The expression of the components of the PTN-MK-RPTPβ/ζ axis in immune cells and in inflammatory diseases suggests important roles for this axis in inflammation. Pleiotrophin has been recently identified as a limiting factor of metainflammation, a chronic pathological state that contributes to neuroinflammation and neurodegeneration. Pleiotrophin also seems to potentiate acute neuroinflammation independently of the inflammatory stimulus while MK seems to play different -even opposite- roles in acute neuroinflammation depending on the stimulus. Which are the functions of MK and PTN in chronic neuroinflammation is still a question of great biologic interest.

For its pattern of expression and its known signaling cascades involving important regulators of inflammation as Fyn kinase and ALK, RPTPβ/ζ is a target receptor for PTN and MK in neuroinflammation. Pharmacologic modulation of the PTN, MK, RPTPβ/ζ and/or its downstream effectors, Fyn and ALK, is a novel therapeutic strategy to modulate neuroinflammation, from central or peripheral origin, in different pathological contexts.

AUTHOR CONTRIBUTIONS

GH and EG wrote the manuscript and built **Figure 1** and **Table 1**. MPR-A wrote the manuscript and built **Figure 2**.

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REFERENCES

Arany, Z., Foo, S. Y., Ma, Y., Ruas, J. L., Bommi-Reddy, A., Girnun, G., et al. (2008). HIF-independent regulation of VEGF and angiogenesis by the transcriptional coactivator PGC-1alpha. *Nature* 451, 1008–1012. doi: 10.1038/nature06613

Bartelt, A., and Heeren, J. (2014). Adipose tissue browning and metabolic health. *Nat. Rev. Endocrinol.* 10, 24–36. doi: 10.1038/nrendo.2013.204

Bluher, M., Bashan, N., Shai, I., Harman-Boehm, I., Tarnovscki, T., Avinaoch, E., et al. (2009). Activated Ask1-MKK4-p38MAPK/JNK stress signaling pathway in human omental fat tissue may link macrophage infiltration to whole-body Insulin sensitivity. *J. Clin. Endocrinol. Metab.* 94, 2507–2515. doi: 10.1210/jc.2009-0002

Bowers, R. R., Festuccia, W. T., Song, C. K., Shi, H., Migliorini, R. H., and Bartness, T. J. (2004). Sympathetic innervation of white adipose tissue and its regulation of fat cell number. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 286, R1167–R1175. doi: 10.1152/ajpregu.00558.2003

Carobbio, S., Pellegrinelli, V., and Vidal-Puig, A. (2017). Adipose tissue function and expandability as determinants of lipotoxicity and the metabolic syndrome. *Adv. Exp. Med. Biol.* 960, 161–196. doi: 10.1007/978-3-319-48382-5_7

Chen, S. Z., Ning, L. F., Xu, X., Jiang, W. Y., Xing, C., Jia, W. P., et al. (2016). The miR-181d-regulated metalloproteinase Adamts1 enzymatically impairs adipogenesis via ECM remodeling. *Cell Death Differ.* 23, 1778–1791. doi: 10.1038/cdd.2016.66

Chen, Z., and Trapp, B. D. (2016). Microglia and neuroprotection. *J. Neurochem.* 136(Suppl. 1), 10–17. doi: 10.1111/jnc.13062

Christman, K. L., Fang, Q., Kim, A. J., Sievers, R. E., Fok, H. H., Candia, A. F., et al. (2005a). Pleiotrophin induces formation of functional neovasculature in vivo. *Biochem. Biophys. Res. Commun.* 332, 1146–1152. doi: 10.1016/j.bbrc.2005.04.174

Christman, K. L., Fang, Q., Yee, M. S., Johnson, K. R., Sievers, R. E., and Lee, R. J. (2005b). Enhanced neovasculature formation in ischemic myocardium following delivery of pleiotrophin plasmid in a biopolymer. *Biomaterials* 26, 1139–1144. doi: 10.1016/j.biomaterials.2004.04.025

Combs, T. P., Wagner, J. A., Berger, J., Doebber, T., Wang, W. J., Zhang, B. B., et al. (2002). Induction of adipocyte complement-related protein of 30 kilodaltons by PPARgamma agonists: a potential mechanism of insulin sensitization. *Endocrinology* 143, 998–1007. doi: 10.1210/endo.143.3.8662

Copps, K. D., and White, M. F. (2012). Regulation of insulin sensitivity by serine/threonine phosphorylation of insulin receptor substrate proteins IRS1 and IRS2. *Diabetologia* 55, 2565–2582. doi: 10.1007/s00125-012-2644-8

Courty, J., Dauchel, M. C., Caruelle, D., Perderiset, M., and Barritault, D. (1991). Mitogenic properties of a new endothelial cell growth factor related to pleiotrophin. *Biochem. Biophys. Res. Commun.* 180, 145–151. doi: 10.1016/S0006-291X(05)81267-7

Cousin, B., Casteilla, L., Lafontan, M., Ambid, L., Langin, D., Berthault, M. F., et al. (1993). Local sympathetic denervation of white adipose tissue in rats induces preadipocyte proliferation without noticeable changes in metabolism. *Endocrinology* 133, 2255–2262. doi: 10.1210/endo.133.5.8404678

de Castro, J., Sevillano, J., Marciniak, J., Rodriguez, R., Gonzalez-Martin, C., Viana, M., et al. (2011). Implication of low level inflammation in the insulin

resistance of adipose tissue at late pregnancy. *Endocrinology* 152, 4094–4105. doi: 10.1210/en.2011-0068

Dutton, J. W. III, Chen, H., You, C., Brodie, M. S., and Lasek, A. W. (2017). Anaplastic lymphoma kinase regulates binge-like drinking and dopamine receptor sensitivity in the ventral tegmental area. *Addict. Biol.* 22, 665–678. doi: 10.1111/adb.12358

Erdogan, S., Doganlar, Z. B., Doganlar, O., Turkekul, K., and Serttas, R. (2017). Inhibition of midkine suppresses prostate cancer CD133(+) Stem cell growth and migration. *Am. J. Med. Sci.* 354, 299–309. doi: 10.1016/j.amjms.2017.04.019

Ezquerra, L., Alguacil, L. F., Nguyen, T., Deuel, T. F., Silos-Santiago, I., and Herradon, G. (2008). Different pattern of pleiotrophin and midkine expression in neuropathic pain: correlation between changes in pleiotrophin gene expression and rat strain differences in neuropathic pain. *Growth Factors* 26, 44–48. doi: 10.1080/08977190801987711

Ezquerra, L., Pérez-García, C., Garrido, E., Díez-Fernández, C., Deuel, T. F., Alguacil, L. F., et al. (2007). Morphine and yohimbine regulate midkine gene expression in the rat hippocampus. *Eur. J. Pharmacol.* 557, 147–150. doi: 10.1016/j.ejphar.2006.11.024

Faden, A. I., Wu, J., Stoica, B. A., and Loane, D. J. (2016). Progressive inflammation-mediated neurodegeneration after traumatic brain or spinal cord injury. *Br. J. Pharmacol.* 173, 681–691. doi: 10.1111/bph.13179

Fan, J., Fong, T., Xia, Z., Zhang, J., and Luo, P. (2018). The efficacy and safety of ALK inhibitors in the treatment of ALK-positive non-small cell lung cancer: a network meta-analysis. *Cancer Med.* 7, 4993–5005. doi: 10.1002/cam4.1768

Fang, W., Hartmann, N., Chow, D. T., Riegel, A. T., and Wellstein, A. (1992). Pleiotrophin stimulates fibroblasts and endothelial and epithelial cells and is expressed in human cancer. *J. Biol. Chem.* 267, 25889–25897.

Fernandez-Calle, R., Vicente-Rodriguez, M., Gramage, E., Pita, J., Perez-Garcia, C., Ferrer-Alcon, M., et al. (2017). Pleiotrophin regulates microglia-mediated neuroinflammation. *J. Neuroinflammation* 14:46. doi: 10.1186/s12974-017-0823-8

Fernandez-Calle, R., Vicente-Rodriguez, M., Pastor, M., Gramage, E., Di Geronimo, B., Zapico, J. M., et al. (2018). Pharmacological inhibition of Receptor Protein Tyrosine Phosphatase beta/zeta (PTPRZ1) modulates behavioral responses to ethanol. *Neuropharmacology* 137, 86–95. doi: 10.1016/j.neuropharm.2018.04.027

Flatscher-Bader, T., and Wilce, P. A. (2008). Impact of alcohol abuse on protein expression of midkine and excitatory amino acid transporter 1 in the human prefrontal cortex. *Alcohol. Clin. Exp. Res.* 32, 1849–1858. doi: 10.1111/j.1530-0277.2008.00754.x

Fujikawa, A., Nagahira, A., Sugawara, H., Ishii, K., Imajo, S., Matsumoto, M., et al. (2016). Small-molecule inhibition of PTPRZ reduces tumor growth in a rat model of glioblastoma. *Sci. Rep.* 6:20473. doi: 10.1038/srep20473

Fujisaka, S., Usui, I., Bukhari, A., Ikutani, M., Oya, T., Kanatani, Y., et al. (2009). Regulatory mechanisms for adipose tissue M1 and M2 macrophages in diet-induced obese mice. *Diabetes* 58, 2574–2582. doi: 10.2337/db08-1475

Fukui, S., Kitagawa-Sakakida, S., Kawamata, S., Matsumiya, G., Kawaguchi, N., Matsuura, N., et al. (2008). Therapeutic effect of midkine on cardiac remodeling in infarcted rat hearts. *Ann. Thorac. Surg.* 85, 562–570. doi: 10.1016/j.athoracsur.2007.06.002

Furuta, M., Shiraishi, T., Okamoto, H., Mineta, T., Tabuchi, K., and Shiwa, M. (2004). Identification of pleiotrophin in conditioned medium secreted from neural stem cells by SELDI-TOF and SELDI-tandem mass spectrometry. *Brain Res. Dev. Brain Res.* 152, 189–197. doi: 10.1016/j.devbrainres.2004.06.014

Garcia-Perez, D., Laorden, M. L., and Milanes, M. V. (2015). Regulation of pleiotrophin, midkine, receptor protein tyrosine phosphatase beta/zeta, and their intracellular signaling cascades in the nucleus accumbens during opiate administration. *Int. J. Neuropsychopharmacol.* 19:pyv077. doi: 10.1093/ijnp/pyv077

Gasiorowski, K., Brokos, B., Leszek, J., Tarasov, V. V., Ashraf, G. M., and Aliev, G. (2017). Insulin Resistance in Alzheimer Disease: p53 and MicroRNAs as Important Players. *Curr. Top. Med. Chem.* 17, 1429–1437. doi: 10.2174/1568026617666170103161233

Gombash, S. E., Lipton, J. W., Collier, T. J., Madhavan, L., Steece-Collier, K., Cole-Strauss, A., et al. (2012). Striatal pleiotrophin overexpression provides functional and morphological neuroprotection in the 6-hydroxydopamine model. *Mol. Ther.* 20, 544–554. doi: 10.1038/mt.2011.216

Gonzalez-Castillo, C., Ortuno-Sahagun, D., Guzman-Brambila, C., Pallas, M., and Rojas-Mayorquin, A. E. (2014). Pleiotrophin as a central nervous system neuromodulator, evidences from the hippocampus. *Front. Cell Neurosci.* 8:443. doi: 10.3389/fncel.2014.00443

Gramage, E., and Herradon, G. (2011). Connecting Parkinson's disease and drug addiction: common players reveal unexpected disease connections and novel therapeutic approaches. *Curr. Pharm. Des.* 17, 449–461. doi: 10.2174/138161211795164103

Gramage, E., Herradon, G., Martin, Y. B., Vicente-Rodriguez, M., Rojo, L., Gnekow, H., et al. (2013). Differential phosphoproteome of the striatum from pleiotrophin knockout and midkine knockout mice treated with amphetamine: correlations with amphetamine-induced neurotoxicity. *Toxicology* 306, 147–156. doi: 10.1016/j.tox.2013.02.013

Gramage, E., Rossi, L., Granado, N., Moratalla, R., and Herradon, G. (2010). Genetic inactivation of pleiotrophin triggers amphetamine-induced cell loss in the substantia nigra and enhances amphetamine neurotoxicity in the striatum. *Neuroscience* 170, 308–316. doi: 10.1016/j.neuroscience.2010.06.078

Gregor, M. F., and Hotamisligil, G. S. (2011). Inflammatory mechanisms in obesity. *Annu. Rev. Immunol.* 29, 415–445. doi: 10.1146/annurev-immunol-031210-101322

Gu, D., Yu, B., Zhao, C., Ye, W., Lv, Q., Hua, Z., et al. (2007). The effect of pleiotrophin signaling on adipogenesis. *FEBS Lett.* 581, 382–388. doi: 10.1016/j.febslet.2006.12.043

Hao, H., Maeda, Y., Fukazawa, T., Yamatsuji, T., Takaoka, M., Bao, X. H., et al. (2013). Inhibition of the growth factor MDK/midkine by a novel small molecule compound to treat non-small cell lung cancer. *PLoS One* 8:e71093. doi: 10.1371/journal.pone.0071093

Harroch, S., Furtado, G. C., Brueck, W., Rosenbluth, J., Lafaille, J., Chao, M., et al. (2002). A critical role for the protein tyrosine phosphatase receptor type Z in functional recovery from demyelinating lesions. *Nat. Genet.* 32, 411–414. doi: 10.1038/ng1004

He, D., Chen, H., Muramatsu, H., and Lasek, A. W. (2015). Ethanol activates midkine and anaplastic lymphoma kinase signaling in neuroblastoma cells and in the brain. *J. Neurochem.* 135, 508–521. doi: 10.1111/jnc.13252

Heck, N., Klausmeyer, A., Faissner, A., and Garwood, J. (2005). Cortical neurons express PSI, a novel isoform of phosphacan/RPTPbeta. *Cell Tissue Res.* 321, 323–333. doi: 10.1007/s00441-005-1135-3

Herradon, G., Ezquerra, L., Gramage, E., and Alguacil, L. F. (2009). Targeting the pleiotrophin/receptor protein tyrosine phosphatase beta/zeta signaling pathway to limit neurotoxicity induced by drug abuse. *Mini Rev. Med. Chem.* 9, 440–447. doi: 10.2174/138955709787847895

Herradon, G., and Perez-Garcia, C. (2014). Targeting midkine and pleiotrophin signalling pathways in addiction and neurodegenerative disorders: recent progress and perspectives. *Br. J. Pharmacol.* 171, 837–848. doi: 10.1111/bph.12312

Hoggard, N., Cruickshank, M., Moar, K. M., Bashir, S., and Mayer, C. D. (2012). Using gene expression to predict differences in the secretome of human omental vs. subcutaneous adipose tissue. *Obesity* 20, 1158–1167. doi: 10.1038/oby.2012.14

Holmes, C. (2013). Review: systemic inflammation and Alzheimer's disease. *Neuropathol. Appl. Neurobiol.* 39, 51–68. doi: 10.1111/j.1365-2990.2012.01307.x

Horiba, M., Kadomatsu, K., Nakamura, E., Muramatsu, H., Ikematsu, S., Sakuma, S., et al. (2000). Neointima formation in a restenosis model is suppressed in midkine-deficient mice. *J. Clin. Invest.* 105, 489–495. doi: 10.1172/JCI7208

Hotamisligil, G. S. (2017). Inflammation, metaflammation and immunometabolic disorders. *Nature* 542, 177–185. doi: 10.1038/nature21363

Ichihara-Tanaka, K., Oohira, A., Rumsby, M., and Muramatsu, T. (2006). Neuroglycan C is a novel midkine receptor involved in process elongation of oligodendroglial precursor-like cells. *J. Biol. Chem.* 281, 30857–30864. doi: 10.1074/jbc.M602228200

Iseki, K., Hagino, S., Mori, T., Zhang, Y., Yokoya, S., Takaki, H., et al. (2002). Increased syndecan expression by pleiotrophin and FGF receptor-expressing astrocytes in injured brain tissue. *Glia* 39, 1–9. doi: 10.1002/glia.10078

Jin, L., Jianghai, C., Juan, L., and Hao, K. (2009). Pleiotrophin and peripheral nerve injury. *Neurosurg. Rev.* 32, 387–393. doi: 10.1007/s10143-009-0202-8

Jones, D. D., Ramsay, T. G., Hausman, G. J., and Martin, R. J. (1992). Norepinephrine inhibits rat pre-adipocyte proliferation. *Int. J. Obes. Relat. Metab. Disord.* 16, 349–354.

Kadomatsu, K., Bencsik, P., Gorbe, A., Csonka, C., Sakamoto, K., Kishida, S., et al. (2014). Therapeutic potential of midkine in cardiovascular disease. *Br. J. Pharmacol.* 171, 936–944. doi: 10.1111/bph.12537

Kadomatsu, K., Kishida, S., and Tsubota, S. (2013). The heparin-binding growth factor midkine: the biological activities and candidate receptors. *J. Biochem.* 153, 511–521. doi: 10.1093/jb/mvt035

Kadomatsu, K., Tomomura, M., and Muramatsu, T. (1988). cDNA cloning and sequencing of a new gene intensely expressed in early differentiation stages of embryonal carcinoma cells and in mid-gestation period of mouse embryogenesis. *Biochem. Biophys. Res. Commun.* 151, 1312–1318. doi: 10.1016/S0006-291X(88)80505-9

Kajimura, S., Spiegelman, B. M., and Seale, P. (2015). Brown and beige fat: physiological roles beyond heat generation. *Cell Metab.* 22, 546–559. doi: 10.1016/j.cmet.2015.09.007

Kato, S., Shinozawa, T., Takikawa, M., Kato, M., Hirano, A., Awaya, A., et al. (2000). Midkine, a new neurotrophic factor, is present in glial cytoplasmic inclusions of multiple system atrophy brains. *Acta Neuropathol.* 100, 481–489. doi: 10.1007/s004010000214

Kielian, T. (2016). Multifaceted roles of neuroinflammation: the need to consider both sides of the coin. *J. Neurochem.* 136(Suppl. 1), 5–9. doi: 10.1111/jnc.13530

Kishida, S., and Kadomatsu, K. (2014). Involvement of midkine in neuroblastoma tumourigenesis. *Br. J. Pharmacol.* 171, 896–904. doi: 10.1111/bph.12442

Krueger, N. X., and Saito, H. (1992). A human transmembrane protein-tyrosine-phosphatase, PTP zeta, is expressed in brain and has an N-terminal receptor domain homologous to carbonic anhydrases. *Proc. Natl. Acad. Sci. U.S.A.* 89, 7417–7421. doi: 10.1073/pnas.89.16.7417

Kuboyama, K., Fujikawa, A., Suzuki, R., and Noda, M. (2015). Inactivation of protein tyrosine phosphatase receptor type Z by pleiotrophin promotes remyelination through activation of differentiation of oligodendrocyte precursor cells. *J. Neurosci.* 35, 12162–12171. doi: 10.1523/JNEUROSCI.2127-15.2015

Lamprou, M., Kaspiris, A., Panagiotopoulos, E., Giannoudis, P. V., and Papadimitriou, E. (2014). The role of pleiotrophin in bone repair. *Injury* 45, 1816–1823. doi: 10.1016/j.injury.2014.10.013

Le Greves, P. (2005). Pleiotrophin gene transcription in the rat nucleus accumbens is stimulated by an acute dose of amphetamine. *Brain Res. Bull.* 65, 529–532. doi: 10.1016/j.brainresbull.2005.03.010

Lee, J. Y., Jeong, W., Lim, W., Kim, J., Bazer, F. W., Han, J. Y., et al. (2012). Chicken pleiotrophin: regulation of tissue specific expression by estrogen in the oviduct and distinct expression pattern in the ovarian carcinomas. *PLoS One* 7:e34215. doi: 10.1371/journal.pone.0034215

Lee, Y. H., Mottillo, E. P., and Granneman, J. G. (2014). Adipose tissue plasticity from WAT to BAT and in between. *Biochim. Biophys. Acta* 1842, 358–369. doi: 10.1016/j.bbadis.2013.05.011

Leggio, L., Vivarelli, S., L'Episcopo, F., Tirolo, C., Caniglia, S., Testa, N., et al. (2017). MicroRNAs in Parkinson's Disease: from pathogenesis to novel diagnostic and therapeutic approaches. *Int. J. Mol. Sci.* 18:E2698. doi: 10.3390/ijms18122698

Liedert, A., Schinke, T., Ignatius, A., and Amling, M. (2014). The role of midkine in skeletal remodelling. *Br. J. Pharmacol.* 171, 870–878. doi: 10.1111/bph.12412

Lowe, C. E., O'Rahilly, S., and Rochford, J. J. (2011). Adipogenesis at a glance. *J. Cell Sci.* 124(Pt 16), 2681–2686. doi: 10.1242/jcs.079699

Maeda, N., Hamanaka, H., Shintani, T., Nishiwaki, T., and Noda, M. (1994). Multiple receptor-like protein tyrosine phosphatases in the form of chondroitin sulfate proteoglycan. *FEBS Lett.* 354, 67–70. doi: 10.1016/0014-5793(94)01093-5

Maeda, N., Ichihara-Tanaka, K., Kimura, T., Kadomatsu, K., Muramatsu, T., and Noda, M. (1999). A receptor-like protein-tyrosine phosphatase PTPzeta/RPTPbeta binds a heparin-binding growth factor midkine. Involvement of arginine 78 of midkine in the high affinity binding to PTPzeta. *J. Biol. Chem.* 274, 12474–12479. doi: 10.1074/jbc.274.18.12474

Maeda, N., Nishiwaki, T., Shintani, T., Hamanaka, H., and Noda, M. (1996). 6B4 proteoglycan/phosphacan, an extracellular variant of receptor-like protein-tyrosine phosphatase zeta/RPTPbeta, binds pleiotrophin/heparin-binding growth-associated molecule (HB-GAM). *J. Biol. Chem.* 271, 21446–21452. doi: 10.1074/jbc.271.35.21446

Mandolesi, G., De Vito, F., Musella, A., Gentile, A., Bullitta, S., Fresegna, D., et al. (2017). miR-142-3p Is a Key Regulator of IL-1beta-Dependent Synaptopathy in Neuroinflammation. *J. Neurosci.* 37, 546–561. doi: 10.1523/JNEUROSCI.0851-16.2016

Marchionini, D. M., Lehrmann, E., Chu, Y., He, B., Sortwell, C. E., Becker, K. G., et al. (2007). Role of heparin binding growth factors in nigrostriatal dopamine system development and Parkinson's disease. *Brain Res.* 1147, 77–88. doi: 10.1016/j.brainres.2007.02.028

Mariman, E. C., and Wang, P. (2010). Adipocyte extracellular matrix composition, dynamics and role in obesity. *Cell Mol. Life Sci.* 67, 1277–1292. doi: 10.1007/s00018-010-0263-4

Martin, Y. B., Gramage, E., and Herradon, G. (2013). Maintenance of amphetamine-induced place preference does not correlate with astrocytosis. *Eur. J. Pharmacol.* 699, 258–263. doi: 10.1016/j.ejphar.2012.11.011

Martin, Y. B., Herradon, G., and Ezquerra, L. (2011). Uncovering new pharmacological targets to treat neuropathic pain by understanding how the organism reacts to nerve injury. *Curr. Pharm. Des.* 17, 434–448. doi: 10.2174/138161211795164130

Masui, M., Okui, T., Shimo, T., Takabatake, K., Fukazawa, T., Matsumoto, K., et al. (2016). Novel Midkine inhibitor iMDK inhibits tumor growth and angiogenesis in oral squamous cell Carcinoma. *Anticancer Res.* 36, 2775–2781.

Matsui, T., Ichihara-Tanaka, K., Lan, C., Muramatsu, H., Kondou, T., Hirose, C., et al. (2010). Midkine inhibitors: application of a simple assay procedure to screening of inhibitory compounds. *Int. Arch. Med.* 3:12. doi: 10.1186/1755-7682-3-12

Matsumoto, J., Stewart, T., Banks, W. A., and Zhang, J. (2017). The transport mechanism of extracellular vesicles at the blood-brain barrier. *Curr. Pharm. Des.* 23, 6206–6214. doi: 10.2174/1381612823666170913164738

Maurel, P., Rauch, U., Flad, M., Margolis, R. K., and Margolis, R. U. (1994). Phosphacan, a chondroitin sulfate proteoglycan of brain that interacts with neurons and neural cell-adhesion molecules, is an extracellular variant of a receptor-type protein tyrosine phosphatase. *Proc. Natl. Acad. Sci. U.S.A.* 91, 2512–2516. doi: 10.1073/pnas.91.7.2512

Mauro, C., De Rosa, V., Marelli-Berg, F., and Solito, E. (2014). Metabolic syndrome and the immunological affair with the blood-brain barrier. *Front. Immunol.* 5:677. doi: 10.3389/fimmu.2014.00677

Meng, K., Rodriguez-Pena, A., Dimitrov, T., Chen, W., Yamin, M., Noda, M., et al. (2000). Pleiotrophin signals increased tyrosine phosphorylation of beta beta-catenin through inactivation of the intrinsic catalytic activity of the receptor-type protein tyrosine phosphatase beta/zeta. *Proc. Natl. Acad. Sci. U.S.A.* 97, 2603–2608. doi: 10.1073/pnas.020487997

Milner, P. G., Li, Y. S., Hoffman, R. M., Kodner, C. M., Siegel, N. R., and Deuel, T. F. (1989). A novel 17 kD heparin-binding growth factor (HBGF-8) in bovine uterus: purification and N-terminal amino acid sequence. *Biochem. Biophys. Res. Commun.* 165, 1096–1103. doi: 10.1016/0006-291X(89)92715-0

Mochizuki, R., Takeda, A., Sato, N., Kimpara, T., Onodera, H., Itoyama, Y., et al. (1998). Induction of midkine expression in reactive astrocytes following rat transient forebrain ischemia. *Exp. Neurol.* 149, 73–78. doi: 10.1006/exnr.1997.6687

Montesinos, J., Alfonso-Loeches, S., and Guerri, C. (2016). Impact of the innate immune response in the actions of ethanol on the central nervous system. *Alcohol. Clin. Exp. Res.* 40, 2260–2270. doi: 10.1111/acer.13208

Mori, F., Nistico, R., Mandolesi, G., Piccinin, S., Mango, D., Kusayanagi, H., et al. (2014). Interleukin-1beta promotes long-term potentiation in patients with multiple sclerosis. *Neuromolecular Med.* 16, 38–51. doi: 10.1007/s12017-013-8249-7

Mori, S., Kiuchi, S., Ouchi, A., Hase, T., and Murase, T. (2014). Characteristic expression of extracellular matrix in subcutaneous adipose tissue development and adipogenesis; comparison with visceral adipose tissue. *Int. J. Biol. Sci.* 10, 825–833. doi: 10.7150/ijbs.8672

Muramatsu, H., Zou, K., Sakaguchi, N., Ikematsu, S., Sakuma, S., and Muramatsu, T. (2000). LDL receptor-related protein as a component of the midkine receptor. *Biochem. Biophys. Res. Commun.* 270, 936–941. doi: 10.1006/bbrc.2000.2549

Muramatsu, H., Zou, P., Suzuki, H., Oda, Y., Chen, G. Y., Sakaguchi, N., et al. (2004). alpha4beta1- and alpha6beta1-integrins are functional receptors for midkine, a heparin-binding growth factor. *J. Cell Sci.* 117(Pt 22), 5405–5415. doi: 10.1242/jcs.01423

Muramatsu, T. (2011). Midkine: a promising molecule for drug development to treat diseases of the central nervous system. *Curr. Pharm. Des.* 17, 410–423. doi: 10.2174/138161211795164167

Muramatsu, T. (2014). Structure and function of midkine as the basis of its pharmacological effects. *Br. J. Pharmacol.* 171, 814–826. doi: 10.1111/bph.12353

Nalls, M. A., Pankratz, N., Lill, C. M., Do, C. B., Hernandez, D. G., Saad, M., et al. (2014). Large-scale meta-analysis of genome-wide association data identifies six new risk loci for Parkinson's disease. *Nat. Genet* 46, 989–993. doi: 10.1038/ng.3043

Neupane, S. P. (2016). Neuroimmune interface in the comorbidity between alcohol use disorder and major depression. *Front. Immunol.* 7:655. doi: 10.3389/fimmu.2016.00655

Olefsky, J. M., and Glass, C. K. (2010). Macrophages, inflammation, and insulin resistance. *Annu. Rev. Physiol.* 72, 219–246. doi: 10.1146/annurev-physiol-021909-135846

Otsuka, S., Sakakima, H., Sumizono, M., Takada, S., Terashi, T., and Yoshida, Y. (2016). The neuroprotective effects of preconditioning exercise on brain damage and neurotrophic factors after focal brain ischemia in rats. *Behav. Brain Res.* 303, 9–18. doi: 10.1016/j.bbr.2016.01.049

Panicker, N., Saminathan, H., Jin, H., Neal, M., Harischandra, D. S., Gordon, R., et al. (2015). Fyn kinase regulates microglial neuroinflammatory responses in cell culture and animal models of Parkinson's Disease. *J. Neurosci.* 35, 10058–10077. doi: 10.1523/JNEUROSCI.0302-15.2015

Papadimitriou, E., Pantazaka, E., Castana, P., Tsalios, T., Polyzos, A., and Beis, D. (2016). Pleiotrophin and its receptor protein tyrosine phosphatase beta/zeta as regulators of angiogenesis and cancer. *Biochim. Biophys. Acta* 1866, 252–265. doi: 10.1016/j.bbcan.2016.09.007

Pariser, H., Ezquerra, L., Herradon, G., Perez-Pinera, P., and Deuel, T. F. (2005). Fyn is a downstream target of the pleiotrophin/receptor protein tyrosine phosphatase beta/zeta-signaling pathway: regulation of tyrosine phosphorylation of Fyn by pleiotrophin. *Biochem. Biophys. Res. Commun.* 332, 664–669. doi: 10.1016/j.bbrc.2005.05.007

Pastor, M., Fernandez-Calle, R., Di Geronimo, B., Vicente-Rodriguez, M., Zapico, J. M., Gramage, E., et al. (2018). Development of inhibitors of receptor protein tyrosine phosphatase beta/zeta (PTPRZ1) as candidates for CNS disorders. *Eur. J. Med. Chem.* 144, 318–329. doi: 10.1016/j.ejmech.2017.11.080

Pellegrinelli, V., Carobbio, S., and Vidal-Puig, A. (2016). Adipose tissue plasticity: how fat depots respond differently to pathophysiological cues. *Diabetologia* 59, 1075–1088. doi: 10.1007/s00125-016-3933-4

Perez-Pinera, P., Berenson, J. R., and Deuel, T. F. (2008). Pleiotrophin, a multifunctional angiogenic factor: mechanisms and pathways in normal and pathological angiogenesis. *Curr. Opin. Hematol.* 15, 210–214. doi: 10.1097/MOH.0b013e3282fdc69e

Perez-Pinera, P., Zhang, W., Chang, Y., Vega, J. A., and Deuel, T. F. (2007). Anaplastic lymphoma kinase is activated through the pleiotrophin/receptor protein-tyrosine phosphatase beta/zeta signaling pathway: an alternative mechanism of receptor tyrosine kinase activation. *J. Biol. Chem.* 282, 28683–28690. doi: 10.1074/jbc.M704505200

Poli, G., Lapillo, M., Granchi, C., Caciolla, J., Mouawad, N., Caligiuri, I., et al. (2018). Binding investigation and preliminary optimisation of the 3-amino-1,2,4-triazin-5(2H)-one core for the development of new Fyn inhibitors. *J. Enzyme Inhib. Med. Chem.* 33, 956–961. doi: 10.1080/14756366.2018.1469017

Popp, J., Oikonomidi, A., Tautvydaite, D., Dayon, L., Bacher, M., Migliavacca, E., et al. (2017). Markers of neuroinflammation associated with Alzheimer's disease pathology in older adults. *Brain Behav. Immun.* 62, 203–211. doi: 10.1016/j.bbi.2017.01.020

Poulsen, F. R., Lagord, C., Courty, J., Pedersen, E. B., Barritault, D., and Finsen, B. (2000). Increased synthesis of heparin affin regulatory peptide in the perforant path lesioned mouse hippocampal formation. *Exp. Brain Res.* 135, 319–330. doi: 10.1007/s002210000536

Qin, E. Y., Cooper, D. D., Abbott, K. L., Lennon, J., Nagaraja, S., Mackay, A., et al. (2017). Neural precursor-derived pleiotrophin mediates subventricular zone invasion by glioma. *Cell* 170, 845–859.e19. doi: 10.1016/j.cell.2017.07.016

Qin, L., Liu, Y., Hong, J. S., and Crews, F. T. (2013). NADPH oxidase and aging drive microglial activation, oxidative stress, and dopaminergic neurodegeneration following systemic LPS administration. *Glia* 61, 855–868. doi: 10.1002/glia.22479

Qin, L., Wu, X., Block, M. L., Liu, Y., Breese, G. R., Hong, J. S., et al. (2007). Systemic LPS causes chronic neuroinflammation and progressive neurodegeneration. *Glia* 55, 453–462. doi: 10.1002/glia.20467

Ramirez, S. H., Andrews, A. M., Paul, D., and Pachter, J. S. (2018). Extracellular vesicles: mediators and biomarkers of pathology along CNS barriers. *Fluids Barriers CNS* 15:19. doi: 10.1186/s12987-018-0104-7

Rauvala, H., Huttunen, H. J., Fages, C., Kaksonen, M., Kinnunen, T., Imai, S., et al. (2000). Heparin-binding proteins HB-GAM (pleiotrophin) and amphoterin in the regulation of cell motility. *Matrix Biol.* 19, 377–387. doi: 10.1016/S0945-053X(00)00084-6

Rothenstein, J. M., and Chooback, N. (2018). ALK inhibitors, resistance development, clinical trials. *Curr. Oncol.* 25(Suppl. 1), S59–S67. doi: 10.3747/co.25.3760

Sanchez-Nino, M. D., Sanz, A. B., and Ortiz, A. (2016). Chronicity following ischaemia-reperfusion injury depends on tubular-macrophage crosstalk involving two tubular cell-derived CSF-1R activators: CSF-1 and IL-34. *Nephrol. Dial Transplant.* 31, 1409–1416. doi: 10.1093/ndt/gfw026

Santiago, J. A., and Potashkin, J. A. (2014). System-based approaches to decode the molecular links in Parkinson's disease and diabetes. *Neurobiol. Dis.* 72(Pt A), 84–91. doi: 10.1016/j.nbd.2014.03.019

Sato, W., Kadomatsu, K., Yuzawa, Y., Muramatsu, H., Hotta, N., Matsuo, S., et al. (2001). Midkine is involved in neutrophil infiltration into the tubulointerstitium in ischemic renal injury. *J. Immunol.* 167, 3463–3469. doi: 10.4049/jimmunol.167.6.3463

Schmitz-Peiffer, C., and Whitehead, J. P. (2003). IRS-1 regulation in health and disease. *IUBMB Life* 55, 367–374. doi: 10.1080/1521654031000138569

Sevillano, J., de Castro, J., Bocos, C., Herrera, E., and Ramos, M. P. (2007). Role of insulin receptor substrate-1 serine 307 phosphorylation and adiponectin in adipose tissue insulin resistance in late pregnancy. *Endocrinology* 148, 5933–5942. doi: 10.1210/en.2007-0352

Sevillano, J., Lopez-Perez, I. C., Herrera, E., Del Pilar Ramos, M., and Bocos, C. (2005). Englitazone administration to late pregnant rats produces delayed body growth and insulin resistance in their fetuses and neonates. *Biochem. J.* 389(Pt 3), 913–918. doi: 10.1042/BJ20041837

Sevillano, J., Sanchez-Alonso, M. G., Gramage, E., Limones, M., Alcala, M., Viana, M., et al. (2012). Effect of pleiotrophin in tissular lipid accumulation. *IUBMB FEBS Congr. FEBS J.* 279, 52–57.

Sevillano, J., Sanchez-Alonso, M. G., Zapateria, B., Calderon, M., Alcala, M., Limones, M., et al. (2019). Pleiotrophin deletion alters glucose homeostasis, energy metabolism and brown fat thermogenic function in mice. *Diabetologia* 62, 123–135. doi: 10.1007/s00125-018-4746-4

Sharma, A. M., and Staels, B. (2007). Review: peroxisome proliferator-activated receptor gamma and adipose tissue–understanding obesity-related changes in regulation of lipid and glucose metabolism. *J. Clin. Endocrinol. Metab.* 92, 386–395. doi: 10.1210/jc.2006-1268

Shi, Y., Ping, Y. F., Zhou, W., He, Z. C., Chen, C., Bian, B. S., et al. (2017). Tumour-associated macrophages secrete pleiotrophin to promote PTPRZ1 signalling in glioblastoma stem cells for tumour growth. *Nat. Commun.* 8:15080. doi: 10.1038/ncomms15080

Shintani, T., and Noda, M. (2008). Protein tyrosine phosphatase receptor type Z dephosphorylates TrkA receptors and attenuates NGF-dependent neurite outgrowth of PC12 cells. *J. Biochem.* 144, 259–266. doi: 10.1093/jb/mvn064

Shoelson, S. E., Lee, J., and Goldfine, A. B. (2006). Inflammation and insulin resistance. *J. Clin. Invest* 116, 1793–1801. doi: 10.1172/JCI29069

Silos-Santiago, I., Yeh, H. J., Gurrieri, M. A., Guillerman, R. P., Li, Y. S., Wolf, J., et al. (1996). Localization of pleiotrophin and its mRNA in subpopulations of neurons and their corresponding axonal tracts suggests important roles in neural-glial interactions during development and in maturity. *J. Neurobiol.* 31, 283–296. doi: 10.1002/(SICI)1097-4695(199611)31:3<283::AID-NEU2>3.0.CO;2-6

Sim, F. J., Lang, J. K., Waldau, B., Roy, N. S., Schwartz, T. E., Pilcher, W. H., et al. (2006). Complementary patterns of gene expression by human oligodendrocyte progenitors and their environment predict determinants of progenitor maintenance and differentiation. *Ann. Neurol.* 59, 763–779. doi: 10.1002/ana.20812

Simon, D. W., McGeachy, M. J., Bayir, H., Clark, R. S., Loane, D. J., and Kochanek, P. M. (2017). The far-reaching scope of neuroinflammation after traumatic brain injury. *Nat. Rev. Neurol.* 13, 171–191. doi: 10.1038/nrneurol.2017.13

Skillback, T., Mattsson, N., Hansson, K., Mirgorodskaya, E., Dahlen, R., van der Flier, W., et al. (2017). A novel quantification-driven proteomic strategy identifies an endogenous peptide of pleiotrophin as a new biomarker of Alzheimer's disease. *Sci. Rep.* 7:13333. doi: 10.1038/s41598-017-13831-0

Sonobe, Y., Li, H., Jin, S., Kishida, S., Kadomatsu, K., Takeuchi, H., et al. (2012). Midkine inhibits inducible regulatory T cell differentiation by suppressing the development of tolerogenic dendritic cells. *J. Immunol.* 188, 2602–2611. doi: 10.4049/jimmunol.1102346

Sorrelle, N., Dominguez, A. T. A., and Brekken, R. A. (2017). From top to bottom: midkine and pleiotrophin as emerging players in immune regulation. *J. Leukoc. Biol.* 102, 277–286. doi: 10.1189/jlb.3MR1116-475R

Spiegelman, B. M. (1998). PPAR-gamma: adipogenic regulator and thiazolidinedione receptor. *Diabetes* 47, 507–514. doi: 10.2337/diabetes.47.4.507

Spielman, L. J., Little, J. P., and Klegeris, A. (2014). Inflammation and insulin/IGF-1 resistance as the possible link between obesity and neurodegeneration. *J. Neuroimmunol.* 273, 8–21. doi: 10.1016/j.jneuroim.2014.06.004

Stanley, S., Pinto, S., Segal, J., Perez, C. A., Viale, A., DeFalco, J., et al. (2010). Identification of neuronal subpopulations that project from hypothalamus to both liver and adipose tissue polysynaptically. *Proc. Natl. Acad. Sci. U.S.A.* 107, 7024–7029. doi: 10.1073/pnas.1002790107

Statnick, M. A., Beavers, L. S., Conner, L. J., Corominola, H., Johnson, D., Hammond, C. D., et al. (2000). Decreased expression of apM1 in omental and subcutaneous adipose tissue of humans with type 2 diabetes. *Int. J. Exp. Diabetes Res.* 1, 81–88. doi: 10.1155/EDR.2000.81

Stienstra, R., Duval, C., Keshtkar, S., van der Laak, J., Kersten, S., and Muller, M. (2008). Peroxisome proliferator-activated receptor gamma activation promotes infiltration of alternatively activated macrophages into adipose tissue. *J. Biol. Chem.* 283, 22620–22627. doi: 10.1074/jbc.M710314200

Stoica, G. E., Kuo, A., Aigner, A., Sunitha, I., Souttou, B., Malerczyk, C., et al. (2001). Identification of anaplastic lymphoma kinase as a receptor for the growth factor pleiotrophin. *J. Biol. Chem.* 276, 16772–16779. doi: 10.1074/jbc.M010660200

Su, X., and Federoff, H. J. (2014). Immune responses in Parkinson's disease: interplay between central and peripheral immune systems. *Biomed. Res. Int.* 2014:275178. doi: 10.1155/2014/275178

Sweeney, M. D., Ayyadurai, S., and Zlokovic, B. V. (2016). Pericytes of the neurovascular unit: key functions and signaling pathways. *Nat. Neurosci.* 19, 771–783. doi: 10.1038/nn.4288

Szepesi, Z., Manouchehrian, O., Bachiller, S., and Deierborg, T. (2018). Bidirectional microglia-neuron communication in health and disease. *Front. Cell Neurosci.* 12:323. doi: 10.3389/fncel.2018.00323

Takada, T., Toriyama, K., Muramatsu, H., Song, X. J., Torii, S., and Muramatsu, T. (1997). Midkine, a retinoic acid-inducible heparin-binding cytokine in inflammatory responses: chemotactic activity to neutrophils and association with inflammatory synovitis. *J. Biochem.* 122, 453–458. doi: 10.1093/oxfordjournals.jbchem.a021773

Tang, C., Wang, M., Wang, P., Wang, L., Wu, Q., and Guo, W. (2019). Neural stem cells behave as a functional niche for the maturation of newborn neurons through the secretion of PTN. *Neuron* 101, 32–44.e6. doi: 10.1016/j.neuron.2018.10.051

Tanga, N., Kuboyama, K., Kishimoto, A., Kiyonari, H., Shiraishi, A., Suzuki, R., et al. (2019). The PTN-PTPRZ signal activates the AFAP1L2-dependent PI3K-AKT pathway for oligodendrocyte differentiation: targeted inactivation of PTPRZ activity in mice. *Glia* 67, 967–984. doi: 10.1002/glia.23583

Thaler, J. P., Yi, C. X., Schur, E. A., Guyenet, S. J., Hwang, B. H., Dietrich, M. O., et al. (2012). Obesity is associated with hypothalamic injury in rodents and humans. *J. Clin. Invest.* 122, 153–162. doi: 10.1172/JCI59660

Thomou, T., Mori, M. A., Dreyfuss, J. M., Konishi, M., Sakaguchi, M., Wolfrum, C., et al. (2017). Adipose-derived circulating miRNAs regulate gene expression in other tissues. *Nature* 542, 450–455. doi: 10.1038/nature21365

Trajkovski, M., Hausser, J., Soutschek, J., Bhat, B., Akin, A., Zavolan, M., et al. (2011). MicroRNAs 103 and 107 regulate insulin sensitivity. *Nature* 474, 649–653. doi: 10.1038/nature10112

Vanderwinden, J. M., Mailleux, P., Schiffmann, S. N., and Vanderhaeghen, J. J. (1992). Cellular distribution of the new growth factor pleiotrophin (HB-GAM) mRNA in developing and adult rat tissues. *Anat. Embryol.* 186, 387–406. doi: 10.1007/BF00185989

Vicente-Rodriguez, M., Fernandez-Calle, R., Gramage, E., Perez-Garcia, C., Ramos, M. P., and Herradon, G. (2016a). Midkine is a novel regulator of amphetamine-induced striatal gliosis and cognitive impairment: evidence for a stimulus-dependent regulation of neuroinflammation by midkine. *Mediators Inflamm.* 2016:9894504. doi: 10.1155/2016/9894504

Vicente-Rodriguez, M., Perez-Garcia, C., Ferrer-Alcon, M., Uribarri, M., Sanchez-Alonso, M. G., Ramos, M. P., et al. (2014). Pleiotrophin differentially regulates the rewarding and sedative effects of ethanol. *J. Neurochem.* 131, 688–695. doi: 10.1111/jnc.12841

Vicente-Rodriguez, M., Rojo Gonzalez, L., Gramage, E., Fernandez-Calle, R., Chen, Y., Perez-Garcia, C., et al. (2016b). Pleiotrophin overexpression regulates amphetamine-induced reward and striatal dopaminergic denervation without changing the expression of dopamine D1 and D2 receptors: implications for neuroinflammation. *Eur. Neuropsychopharmacol.* 26, 1794–1805. doi: 10.1016/j.euroneuro.2016.09.002

Villapol, S. (2018). Roles of peroxisome proliferator-activated receptor gamma on brain and peripheral inflammation. *Cell Mol. Neurobiol.* 38, 121–132. doi: 10.1007/s10571-017-0554-5

Wada, M., Kamata, M., Aizu, Y., Morita, T., Hu, J., and Oyanagi, K. (2002). Alteration of midkine expression in the ischemic brain of humans. *J. Neurol. Sci.* 200, 67–73. doi: 10.1016/S0022-510X(02)00134-X

Wang, J., Takeuchi, H., Sonobe, Y., Jin, S., Mizuno, T., Miyakawa, S., et al. (2008). Inhibition of midkine alleviates experimental autoimmune encephalomyelitis through the expansion of regulatory T cell population. *Proc. Natl. Acad. Sci. U.S.A.* 105, 3915–3920. doi: 10.1073/pnas.0709592105

Wang, W., LeBlanc, M. E., Chen, X., Chen, P., Ji, Y., Brewer, M., et al. (2017). Pathogenic role and therapeutic potential of pleiotrophin in mouse models of ocular vascular disease. *Angiogenesis* 20, 479–492. doi: 10.1007/s10456-017-9557-6

Wang, W., and Seale, P. (2016). Control of brown and beige fat development. *Nat. Rev. Mol. Cell Biol.* 17, 691–702. doi: 10.1038/nrm.2016.96

Weckbach, L. T., Gola, A., Winkelmann, M., Jakob, S. M., Groesser, L., Borgolte, J., et al. (2014). The cytokine midkine supports neutrophil trafficking during acute inflammation by promoting adhesion via beta2 integrins (CD11/CD18). *Blood* 123, 1887–1896. doi: 10.1182/blood-2013-06-510875

Weckbach, L. T., Groesser, L., Borgolte, J., Pagel, J. I., Pogoda, F., Schymeinsky, J., et al. (2012). Midkine acts as proangiogenic cytokine in hypoxia-induced angiogenesis. *Am. J. Physiol. Heart Circ. Physiol.* 303, H429–H438. doi: 10.1152/ajpheart.00934.2011

Welch, J. S., Ricote, M., Akiyama, T. E., Gonzalez, F. J., and Glass, C. K. (2003). PPARgamma and PPARdelta negatively regulate specific subsets of lipopolysaccharide and IFN-gamma target genes in macrophages. *Proc. Natl. Acad. Sci. U.S.A.* 100, 6712–6717. doi: 10.1073/pnas.1031789100

Wong, J. C., Krueger, K. C., Costa, M. J., Aggarwal, A., Du, H., McLaughlin, T. L., et al. (2016). A glucocorticoid- and diet-responsive pathway toggles adipocyte precursor cell activity in vivo. *Sci. Signal.* 9:ra103. doi: 10.1126/scisignal.aag0487

Wyss-Coray, T. (2016). Ageing, neurodegeneration and brain rejuvenation. *Nature* 539, 180–186. doi: 10.1038/nature20411

Xiong, F., Ge, W., and Ma, C. (2019). Quantitative proteomics reveals distinct composition of amyloid plaques in Alzheimer's disease. *Alzheimers Dement.* 15, 429–440. doi: 10.1016/j.jalz.2018.10.006

Xu, C., Zhu, S., Wu, M., Han, W., and Yu, Y. (2014). Functional receptors and intracellular signal pathways of midkine (MK) and pleiotrophin (PTN). *Biol. Pharm. Bull.* 37, 511–520. doi: 10.1248/bpb.b13-00845

Yasuhara, O., Muramatsu, H., Kim, S. U., Muramatsu, T., Maruta, H., and McGeer, P. L. (1993). Midkine, a novel neurotrophic factor, is present in senile plaques of Alzheimer disease. *Biochem. Biophys. Res. Commun.* 192, 246–251. doi: 10.1006/bbrc.1993.1406

Yasuhara, O., Schwab, C., Matsuo, A., Kim, S. U., Steele, J. C., Akiguchi, I., et al. (1996). Midkine-like immunoreactivity in extracellular neurofibrillary tangles in brains of patients with parkinsonism-dementia complex of Guam. *Neurosci. Lett.* 205, 107–110. doi: 10.1016/0304-3940(96)12385-5

Yeh, H. J., He, Y. Y., Xu, J., Hsu, C. Y., and Deuel, T. F. (1998). Upregulation of pleiotrophin gene expression in developing microvasculature, macrophages, and astrocytes after acute ischemic brain injury. *J. Neurosci.* 18, 3699–3707. doi: 10.1523/JNEUROSCI.18-10-03699.1998

Yoshida, Y., Goto, M., Tsutsui, J., Ozawa, M., Sato, E., Osame, M., et al. (1995). Midkine is present in the early stage of cerebral infarct. *Brain Res. Dev. Brain Res.* 85, 25–30. doi: 10.1016/0165-3806(94)00183-Z

Zhang, B., Wei, W., and Qiu, J. (2018). ALK is required for NLRP3 inflammasome activation in macrophages. *Biochem. Biophys. Res. Commun.* 501, 246–252. doi: 10.1016/j.bbrc.2018.04.226

Zhang, S., Liang, F., Wang, B., Le, Y., and Wang, H. (2014). Elevated expression of pleiotrophin in pilocarpine-induced seizures of immature rats and in pentylenetetrazole-induced hippocampal astrocytes in vitro. *Acta Histochem.* 116, 415–420. doi: 10.1016/j.acthis.2013.09.003

Zwicker, S., Bureik, D., Bosma, M., Martinez, G. L., Almer, S., and Bostrom, E. A. (2016). Receptor-type protein-tyrosine phosphatase zeta and colony stimulating factor-1 receptor in the intestine: cellular expression and cytokine- and chemokine responses by interleukin-34 and colony stimulating factor-1. *PLoS One* 11:e0167324. doi: 10.1371/journal.pone.0167324

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Intravenously Injected Mesenchymal Stem Cells Penetrate the Brain and Treat Inflammation-Induced Brain Damage and Memory Impairment in Mice

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Neuroinflammation is regarded as one of the pathogenic factors of Alzheimer disease (AD). Previously, we showed that mice regularly injected with bacterial lipopolysaccharide (LPS) possessed the AD-like symptoms like episodic memory decline, elevated amounts of amyloid beta (Aβ) peptide (1–42), and decreased levels of nicotinic acetylcholine receptors (nAChRs) in the brain. The use of mesenchymal stem cells (MSCs), which can differentiate into multiple cell types, including neurons, is an attractive idea of regenerative medicine, in particular, for neurodegenerative disorders like AD. In the present study, we aimed to investigate whether pathogenic effect of LPS on the brain and behavior of mice can be prevented or treated by injection of MSCs or MSC-produced soluble factors. Fluorescently-labeled MSCs, injected intravenously, were found in the brain blood vessels of LPS-treated mice. Mice co-injected with LPS and MSCs did not demonstrate episodic memory impairment, Aβ (1–42) accumulation, and nAChR decrease in the brain and brain mitochondria. Their mitochondria released less cytochrome *c* under the effect of Ca^{2+} compared to mitochondria of LPS-only-treated mice. Moreover, MSCs could reverse the pathogenic symptoms developed 3 weeks after LPS injection. Cultured MSCs produced IL-6 in response to LPS and MSCs effect *in vivo* was accompanied by additional stimulation of both micro- and macroglia. Xenogeneic (human) MSCs were almost as efficient as allogeneic (mouse) ones and regular injections of human MSC-conditioned medium also produced positive effect. These data allow suggesting MSCs as a potential therapeutic tool to cure neuroinflammation-related cognitive pathology.

Keywords: neuroinflammation, mesenchymal stem cells, nicotinic acetylcholine receptor, episodic memory, mitochondria, Alzheimer disease

INTRODUCTION

Alzheimer disease (AD) is an age-dependent neurodegenerative disorder resulting in impairment of memory, speech, and practical habits. The brains of AD patients are characterized by cholinergic deficiency and accumulation of extracellular senile plaques formed by oligomerized amyloid beta (Aβ) peptides. AD is accompanied by inflammatory reactions; moreover, neuroinflammation often precedes the development of cognitive symptoms and may be regarded as one of the pathogenic factors causing neurodegeneration (reviewed in Skok and Lykhmus, 2016).

Cholinergic deficiency is manifested as the decrease of acetylcholine content in the brain due to decreased activity of choline acetyltransferase and the loss of nicotinic acetylcholine receptors (nAChRs) in the cognitively important brain areas leading to degeneration of cholinergic neurons (Keverne and Ray, 2005). Consequently, the use of either acetylcholine esterase (AChE) inhibitors or selective agonists of certain nAChR subtypes aimed to enhance cholinergic signaling has been suggested as a current medication for symptomatic treatment of AD patients (Russo et al., 2014).

The nAChRs are ligand-gated ion channels composed of various combinations of α (α1–α10) and β (β1–β4) subunits (reviewed in Changeux, 2012). The two main nAChR subtypes found in the brain are α7 and α4β2 (Champtiaux and Changeux, 2002; Dineley et al., 2015) and both of them are related to AD pathogenesis (Wevers et al., 1999; Guan et al., 2000; Gotti et al., 2006; Posadas et al., 2013). The α4β2 nAChR signaling underlies the pro-cognitive effects of nicotine (Picciotto et al., 2001), the absence of α4β2 nAChRs in knockout mice favors neurodegeneration upon ageing (Zoli et al., 1999), and the density of α4β2 nAChRs is decreased in people with neurodegenerative diseases including the AD (Wevers et al., 1999; Guan et al., 2000). The α7 nAChRs directly interact with Aβ to affect its proper metabolism (Wang et al., 2000; Parri and Dineley, 2010). This nAChR subtype is expressed in both the plasma membrane and mitochondria of the brain cells to support their viability (Parada et al., 2010; Gergalova et al., 2012; Lykhmus et al., 2014). In addition, the α7 nAChRs expressed in the glial cells regulate inflammatory reactions in the brain (Suzuki et al., 2006; Tyagi et al., 2010; Thomsen and Mikkelsen, 2012).

In our previous studies, we showed that mice regularly injected with bacterial lipopolysaccharide (LPS) possessed decreased levels of α7 and α4β2 nAChRs, elevated amounts of Aβ (1–42) in the brain, and demonstrated episodic memory decline (Lykhmus et al., 2015b, 2017). The LPS treatment stimulated astrocytosis in the cortex and striatum and evidently decreased the number of cells in the hippocampus and striatum. The brain mitochondria of LPS-treated mice also contained less α7 nAChRs, more Aβ peptides (1–40) and (1–42) and released more cytochrome *c* (cyt *c*) in response to apoptogenic doses of Ca^{2+}. These data allowed us to suggest that neuroinflammation caused by external inflammatory stimuli led to α7 nAChR down-regulation, accumulation of Aβ (1–42), and mitochondria impairment resulting in memory decline and finally neurodegeneration.

Mesenchymal stem cells (MSCs) are self-renewing multipotent cells able to differentiate into multiple cell types including neurons (NIH Stem Cell Information Home Page, 2016). In addition, MSCs produce numerous trophic and growth factors affecting neurogenesis, synaptogenesis, astrocytosis and cell survival (Konala et al., 2016). The efficiency of regenerative MSC therapy has been studied in many experimental models (Kariminekoo et al., 2016) including transgenic mice bearing AD-related mutations (Yang et al., 2013; Chang et al., 2014; Shin et al., 2014) and their role in modulating inflammation (Klinker and Wei, 2015; Zachar et al., 2016) have been demonstrated. In addition, the regenerative potential of MSC-secreted factors vs. the cells *per se* is being widely discussed (Konala et al., 2016).

In the present study, we put an aim to investigate whether pathogenic effect of LPS on the brain and behavior of mice can be prevented or reversed by MSCs, and if yes, whether the effect can be reproduced by MSC-produced soluble factors.

MATERIALS AND METHODS

Materials

All reagents were of chemical grade and purchased from Sigma-Aldrich (Saint Louis, USA), unless specially indicated. Antibodies against α3(181–192), α4(181–192), α7(179–190), α9(11–23), β2(190–200) or β4(190–200) nAChR fragments and rabbit cyt *c*-specific antibodies were generated using methods previously developed in our lab (Skok et al., 1999; Koval et al., 2004, 2011; Lykhmus et al., 2010; Gergalova et al., 2014). The antibodies were biotinylated according to standard procedures (Harlow and Lane, 1988). Antibodies against Aβ (1–42), ionized calcium binding adaptor molecule 1 [(Iba-1) or allograft inflammatory factor (AIF-1)] and neutravidin-peroxidase conjugate were purchased from ALT Ukraine Ltd (representing Thermo Fisher Scientific in Ukraine). Rabbit polyclonal antibody against glial fibrillary acidic protein (GFAP) was from Dako (Agilent Technologies); goat anti-rabbit IgG Alexa 488-labeled and IL-6-specific antibody pair were from Invitrogen.

Animals

As MSCs recipients, we used C57BL/6 J female mice 3–5 months of age. Placental MSCs were obtained from 6 week-old female FVB wild-type pregnant mice, and GFP-labeled MSCs were obtained from FVB-Cg-Tg (GFPU) 5Nagy/J mice, transgenic by green fluorescent protein (GFP) gene. Mice were kept in the animal facilities of either Palladin Institute of Biochemistry NAS of Ukraine or Dmitry F. Chebotarev Institute of Gerontology NAMS of Ukraine in Kyiv. Mice were housed in quiet, temperature-controlled rooms, and provided with water and food pellets *ad libitum*. Before removing the brains, mice were

Abbreviations: AD, Alzheimer disease; cyt *c*, cytochrome *c*; GFP, green fluorescence protein; LPS, lipopolysaccharide; MSCs, mesenchymal stem cells; nAChR, nicotinic acetylcholine receptor.

sacrificed by cervical dislocation. All procedures complied with the ARRIVE guidelines, were carried out in accordance with the Directive 2010/63/EU for animal experiments and were approved by the Animal Care and Use Committee of Palladin Institute of Biochemistry.

Mesenchymal Stem Cells Isolation, Propagation, and Characterization

Human MSCs (hMSCs) were obtained from Wharton jelly (WJ) using the explant method (Maslova et al., 2013; Shuvalova and Kordium, 2016). Umbilical cords were collected from healthy donors (39–40 weeks of gestation) after their consent. The umbilical cord fragment (5–10 cm) was washed with PBS, the vessels were mechanically removed. WJ was cut into 0.4–0.5 mm pieces that were placed in the 75 cm^2 cultural flasks containing complete growth medium α-MEM (BioWest, Austria) supplemented with 10% fetal bovine serum (Invitogen), penicillin 100 U/ml (Arterium, Ukraine), and streptomycin 100 μg/ml (Arterium, Ukraine). Cultivation was performed under conditions of humidified air with 5% CO_2 at 37°C. The medium was changed every 4–5 days. The first attached cells were visible on the 7–10th day. After 14 days the clones reached the size and confluence (70–80%) sufficient for passing, which was performed by standard method with the use of trypsin-EDTA mixture (Shuvalova et al., 2013). The surface marker proteins CD34, CD45, CD90, CD73, CD105 expression was determined at the second passage by flow cytometry (BD FACS Aria) with FITC- and PE- conjugated antibodies (UsBiological, USA) according to minimal criteria for defining multipotent mesenchymal stromal cells (Dominici et al., 2006). The cells of the second passage were used for both *in vitro* assays and transplantation into LPS-treated mice.

Murine placental multipotent mesenchymal stem cells (mMMSCs, further mMSCs) were obtained from FVB-Cg-Tg (GFPU) 5Nagy/J mice 19th day of pregnancy. Under sterile conditions, placentae were transferred into a Petri dish with cold PBS, containing antibiotics. Fetal membranes were minced and incubated with 0.1% collagenase type I (Sigma-Aldrich, USA) for 90 min at 37°C. Cell pellet obtained after digestion and filtration was washed and seeded in 75 cm^2 flasks containing culture medium DMEM-LG (Low Glucose, 1 g/L) supplemented with 10% fetal bovine serum, penicillin 100 U/ml, streptomycin 100 μg/ml and 1:100 nonessential amino acids (Sigma-Aldrich, USA). Cultivation was carried out in CO_2-incubator under conditions of humidified air with 5% CO_2 at 37°C. The medium was changed every 3–4 days. After approximately 14 days, the cells were rinsed with Dulbecco's Phosphate Buffered Saline (Sigma-Aldrich, USA), and then exposed to pre-warmed trypsin–EDTA (0.25% trypsin, 4 mM EDTA, Invitrogen) for 2 min. The resulting detached cells were resuspended in serum-supplemented medium, counted and seeded as first passage cultures at 4,000 cells per cm^2. Subcultivation was performed at 80% confluence of the monolayer. Cells of the second passage were used in the experiment (Fhilho and Oliveira, 2012).

Phenotyping of cells for markers CD34, CD44, CD45, CD73, CD90, CD105 was performed using fluorochrome-labeled monoclonal antibodies to mouse membrane antigens by flow cytometry. Obtained cell cultures satisfied criteria of MMSCs by phenotype and ability to directed multilinear differentiation.

In vitro Assays

Mouse MSCs (4×10^4 cells per well) were seeded in 96-well tissue culture plates containing complete growth medium DMEM/F12 supplemented with 10% fetal bovine serum, penicillin 100 U/ml, streptomycin 100 μg/ml (all–Sigma-Aldrich, USA) and were cultured in the presence of different doses of LPS at 37°C and 5% CO_2 during 72 h. Then, the cell supernatant was collected and the cells' quantity/viability was studied in MTT test (Carmichael et al., 1987). The supernatants were tested for the presence of IL-6 using the Murine IL-6 ELI-Pair kit from Diaclone (Gen-Probe, France), according to manufacturer's instructions.

Animal Treatment and Brain Preparations

In the first set of experiments, three groups of C57Bl/6 mice, eight animals per group, were intraperitoneally injected with 2 mg kg^{-1} LPS (*E. coli* strain 055:B5) in 0.1 ml of saline. Two of these groups, in addition, obtained intravenously, in the tail vein, 10^6 mMSCs or hMSCs in 0.1 ml of incubation medium. Three weeks thereafter, mice were examined in behavioral novel object recognition test, sacrificed and their brains were removed for examination.

In the second set of experiments, three groups of mice, five animals in each, were injected with LPS as described above. After 3 weeks, the mice were examined in behavioral test and one group obtained hMSCs (10^6 in the tail vein), while another group was injected intraperitoneally with 0.3 ml of hMSC-conditioned medium obtained after 2 days of cells incubation in serum-free medium. Injections of conditioned medium were repeated every 7 days for 3 weeks more and mice were examined in behavioral test every week. In a month, a week after the last conditioned medium injection, mice were sacrificed and their brains were removed for examination.

To study if intravenously introduced MSC penetrate into the brain parenchyma, GFP-labeled mMSCs (10^6 per animal) were injected into two mice pre-injected with LPS a day before. Mice were sacrificed 24 and 72 h thereafter and their brains were removed for examination.

For sandwich ELISA experiments and mitochondria examination, the mouse brains were homogenized in a glass homogenizer. The homogenate was fractionated into mitochondria and non-mitochondria by standard procedure of differential centrifugation (Gergalova et al., 2012; Lykhmus et al., 2015a). The purity of fractions obtained was evaluated by ELISA using the antibodies against different cellular compartments, as described previously (Uspenska et al., 2017). Live mitochondria were further examined in functional test of cytochrome c (cyt *c*) release (see below), while the pellets of both mitochondria and non-mitochondria fractions were used to prepare the detergent lysates, as described previously (Lykhmus et al., 2015b). Protein concentration was measured with the BCA kit (Thermo Scientific, France).

For immunohistochemistry studies, the brains were fixed in 4% paraformaldehyde for 48 h, washed in PBS, dehydrated

with increasing concentrations of alcohol and embedded in ParaplastX-TRA (McCormick Scientific LLC). The paraplast-embedded specimens were cut into serial frontal 5 μM sections with rotational microtome (HM 325, MICROM International GmbH). The sections were placed onto adhesive microscopic slides to be further examined by immunohistochemistry.

To study MSC penetration into the brain, the brains were fixed in 4% paraformaldehyde and cut by vibratome into coronal 40 μm sections. The floated sections were placed onto microscopic slides to be examined by confocal microscopy.

ELISA Assays

To determine the level of various nAChR subunits within the brain or mitochondria preparations, the immunoplates (Nunc, Maxisorp) were coated with rabbit α7(1–208)-specific antibody (20 μg/ml), blocked with 1% BSA, and the detergent lysates of brain tissue or mitochondria were applied into the wells (1 μg of protein per 0.05 ml per well) for 2 h at 37°C. The plates were washed with water and the second biotinylated α3(181–192)-, α4(181–192)-, α7(179–190)-, α9(11–23)-, β2(190–200)- or β4(190–200)-specific antibody was applied for additional 2 h being revealed with Neutravidin-peroxidase conjugate and *o*-phenylendiamine-containing substrate solution.

To determine the level of Aβ (1–42) bound to α7 nAChR, the plates were coated with α7(1-208)-specific antibody, and the α7-Aβ complex from the brain or mitochondria preparation applied as described above was revealed with biotinylated Aβ (1–42)-specific antibody, Neutravidin-peroxidase conjugate and *o*-phenylendiamine-containing substrate solution. The optical density was read at 490 nm using Stat-Fax 2000 ELISA Reader (Awareness Technologies, USA).

Immunohistochemistry and Confocal Microscopy

Before immunohistochemical staining, the brain sections were de-paraffinated by standard procedure; the nonspecific binding was blocked with 1% BSA in PBS (30 min, RT). For staining the Aβ (1–42), the slides were incubated with biotinylated mouse Aβ (1–42)-specific antibody (1:200) overnight at room temperature, washed with PBS and incubated with Extravidin-Cy3 (1,200) in 1% BSA-containing PBS for 1 h at RT. Nonspecific binding was blocked with goat anti-mouse IgG.

For staining the astrocytes, the slides were incubated with rabbit anti-GFAP antibody (1:100) followed by goat anti-rabbit-Alexa 488.

For staining the microglia, the slides were incubated with biotinylated anti-Iba-1 antibody (1:100) followed by Streptavidin-Cy3.

As negative controls, the incubations without primary antibody were performed in both control and experimental sections.

All slides with paraplast sections were embedded in MOWIOL-DABCO, while slides with floated brain sections were embedded in Vectashield and examined under Zeiss LSM 510 Meta confocal lasers canning microscope (Zeiss, Germany). The brain regions were identified according to Paxinos and Franklin, 2001.

Mitochondria Functional Assay

The purified live mitochondria (120 μg of protein per ml) were incubated with either 0.1 μM $CaCl_2$ or 0.9 μM $CaCl_2$ for 5 min at room temperature and were immediately pelleted by centrifugation (10 min, 7,000 g) at 4°C. The incubation medium contained 10 mM HEPES, 125 mM KCl, 25 mM NaCl, 5 mM sodium succinate and 0.1 mM Pi(K), pH 7.4. The mitochondria supernatants were collected and tested for the presence of cyt *c* by sandwich assay as described previously (Gergalova et al., 2012, 2014).

Behavioral Experiments

Mice of all groups were tested in the "Novel Object Recognition" (NOR) behavioral test (Antunes and Biala, 2012; Lykhmus et al., 2015a,b) prior and post-treatments. Briefly, the animals were individually placed in a rectangular novel open field containing two identical objects with distinctive features (shape and texture). The animals were subjected to a 10-min session of exploration of the objects followed by 10 min in a waiting cage. During the second 10-min session, one of the objects was replaced by a novel one, and we scored the time spent in contact with each object. It is widely acknowledged that rodents spontaneously explore novel objects by touching the objects with their nose and prefer novel objects to familiar ones that reflect their episodic memory (Save et al., 1992; Thinus-Blanc, 1996). Therefore, the time spent in contact with each object reflects the time of exploration for the object. The results of NOR test are presented as discrimination index (DI) calculated as the difference in the number of "novel" and "famous" object explorations divided by the total number of explorations of two identical objects.

Statistical Analysis

ELISA experiments have been performed in triplicates and mean values for individual mice were used for statistical analysis assessed using one-way ANOVA test. Behavioral tests were also performed in triplicate for each mouse and mean values for individual mice were taken for statistical analysis. The data are presented as mean ± SD; $^{*}p < 0.05$; $^{**}\ p < 0.005$; $^{***}\ p < 0.0005$.

RESULTS

Both Mouse and Human MSCs Prevent Pathogenic Effect of LPS on Mouse Brains and Behavior

We used MSCs from two sources: either human umbilical cord (hMSCs) or mouse placenta (mMSCs). Both types of cells satisfied minimal criteria for defining multipotent mesenchymal stromal cells according to CD34, CD44, CD45, CD90, CD73, CD105 surface markers expression (Dominici et al., 2006) and were used for transplantation after two passages *in vitro*. Morphology of mMSCs in primary culture and at the second passage is shown in **Figures 1A,B**. Both mMSCs and hMSCs were shown to proliferate and to produce IL-6 in response to LPS stimulation (data for mMSCs are shown in **Figures 1C,D**).

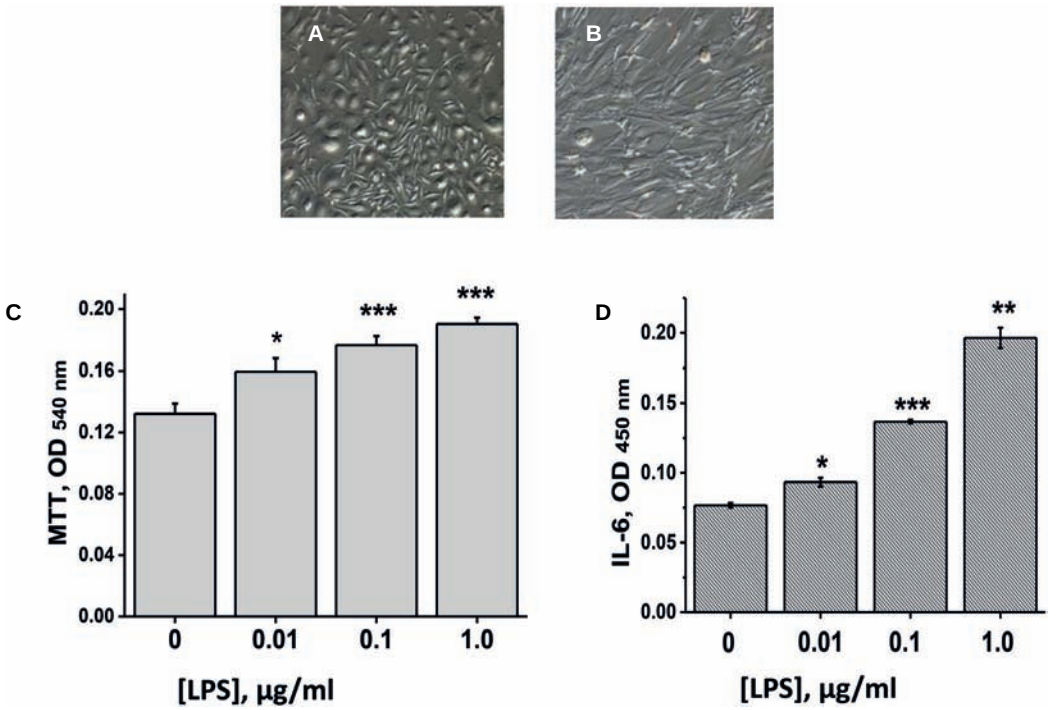

FIGURE 1 | Placental mMSCs cultured *in vitro*. **(A,B)** – microscopic images of primary **(A)** and passage 2 cells **(B)**; their proliferation (MTT test) **(C)**, and IL-6 production **(D)** under the effect of LPS. Each column in C and D corresponds to M ± SD of triplicate measurements; $*p < 0.05$; $**p < 0.005$; $***p < 0.0005$ compared to Ctrl (no LPS).

In the first set of experiments, mice were injected intraperitoneally with LPS and intravenously with either hMSCs or mMSCs (10^6 cells per mouse) at the same time. Three weeks later, they were examined in memory test and their brains and brain mitochondria were studied for the levels of α7 nAChR, Aβ (1–42) and cyt *c* released upon Ca^{2+} stimulation.

It was found that MSCs injection prevented α7 nAChR decrease, Aβ (1–42) accumulation and increased cyt *c* release from mitochondria, as well as episodic memory decline caused by LPS. Human MSCs were almost as efficient as mouse ones in a majority of tests (**Figure 2**).

The use of GFP-labeled mouse MSCs demonstrated that green fluorescent signal could be found along/around the brain blood vessels 3 days after intravenous injection (**Figure 3**).

Both Human MSCs and Their Conditioned Media Reverse the Pathogenic Effects Developed 3 Weeks After LPS Injection

The second set of experiments was undertaken to find out whether the MSCs effect is due to cellular or humoral influence and whether it is only prophylactic or can also be therapeutic, i.e., cure the already developed pathogenic symptoms caused by LPS.

For this purpose, mice were injected intravenously with xenogeneic (human) MSCs 3 weeks after LPS injection when memory decline was already observed. Another group of LPS-treated mice was injected once per week intraperitoneally with the conditioned culture medium in which hMSCs were grown to 80% confluency and were maintained for 2 days without serum. Mice were examined in memory test every week thereafter, then sacrificed and their brains and brain mitochondria were studied as in the previous set of experiments. In this case, we studied a broader range of nAChR subunits in both the mitochondrial and nonmitochondrial brain fractions. It was found that either hMSCs or their supernatants up-regulated α4, α9 and β2 nAChR subunits in their brains (**Figure 4A**) and brain mitochondria (**Figure 4C**); MSCs additionally up-regulated β4 subunits and decreased the level of Aβ (1–42) (**Figures 4B,D**). Either hMSCs or their supernatants also restored memory of LPS-treated mice and significantly improved mitochondria sustainability to Ca^{2+} (**Figures 5A,B**). In contrast to MSCs, which supported memory of LPS-treated mice for at least 3 weeks, the effect of a single injection of conditioned medium was transient and disappeared after 2 weeks (**Figure 5A**).

The visible decrease of Aβ (1–42) accumulated in the frontal cortex, striatum and hippocampus of LPS injected mice under the effect of either MSC or their conditioned medium was found by means of immunohistochemistry (**Figure 6**).

Finally, MSC visibly increased the green signal for GFAP (**Figure 7**), while their conditioned medium increased Iba-1-specific staining (**Figure 8**) in the brains of LPS-treated mice. Although no quantitative analysis has been performed in this experiment, the data allow suggesting that the treatments increased the number of activated astrocytes (GFAP) or microglial cells (Iba-1).

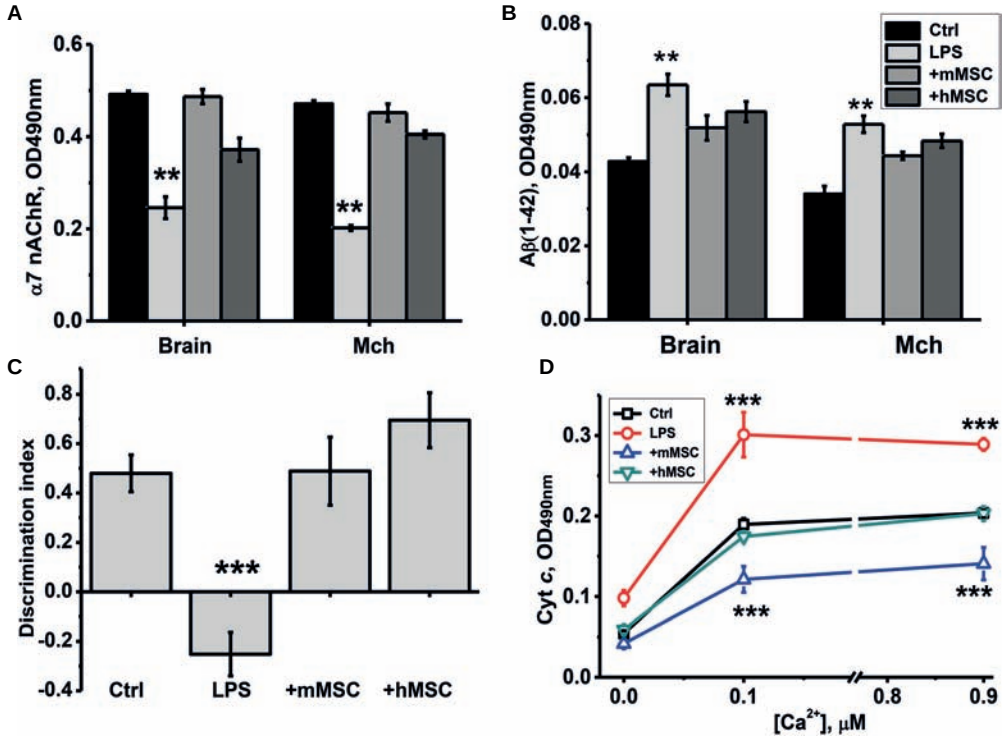

FIGURE 2 | The effect of either hMSCs or mMSCs on α7 nAChR **(A)** or Aβ (1–42) **(B)** levels in the brain, memory impairment **(C)** and cyt *c* release from the brain mitochondria (Mch) under the effect of Ca^{2+} **(D)**. Ctrl – samples of non-treated mice. Each column **(A–C)** or point **(D)** corresponds to M ± SD of data for separate mice in each group (n = 8). $^{**}p < 0.005$; $^{***}p < 0.0005$ compared to Ctrl. Designations of columns are similar in **(A)** and **(B)**.

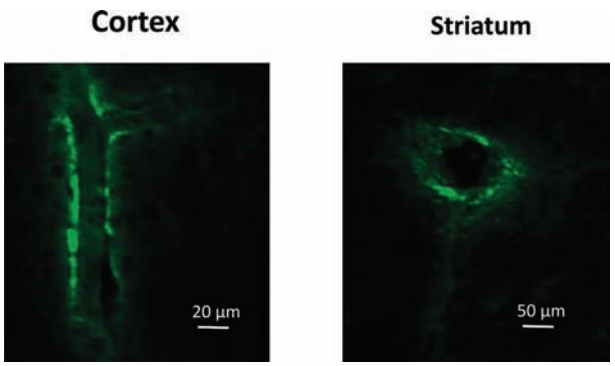

FIGURE 3 | Confocal microscopy images of the blood vessels in the *Cortex* (lengthwise projection) and *Striatum* (cross-cut projection) of LPS-treated mice prepared 72 h after intravenous injection of GFP-labeled mMSCs.

DISCUSSION

The data obtained in the first set of experiments indicated that MSCs injected intravenously prevent the pathogenic effect of LPS on the brain, brain mitochondria and behavior of LPS-treated mice. In particular, they prevented the α7 nAChR decrease, Aβ (1–42) accumulation, mitochondria and episodic memory impairment caused by LPS injection. Xenogeneic (human) MSCs were almost as efficient as allogeneic (mouse) ones suggesting that their effect was mostly due to soluble growth/trophic factors produced. This suggestion was approved in the second set of experiments where regular injections of hMSC-conditioned medium appeared to be almost as efficient as MSC themselves. In addition, it was found that MSCs can not only prevent the pathogenic effect of LPS, but also reverse the already developed nAChR decrease, Aβ (1–42) accumulation, mitochondria, and memory impairment. The anti-LPS effect of MSCs was accompanied by additional stimulation of both micro- and macroglia suggesting that these cells are the targets of MSC-produced soluble factors. We can speculate that positive effect of MSCs is, at least partly, due to activation of trophic functions of glial cells.

The use of multipotent stem cells is an attractive idea of regenerative medicine, in particular, for neurodegenerative disorders like AD. The therapeutic effect of MSCs has been already studied in several AD models. The first studies were performed with the bone-marrow-derived MSCs transplanted intracerebrally (Lee et al., 2009, 2010a,b, 2012; Zhang et al., 2012; Bae et al., 2013) in mice either pre-injected with Aβ (Lee et al., 2009, 2010a) or transgenic for APP/PS1 (Lee et al., 2010b, 2012; Bae et al., 2013). A big piece of studies was performed with human MSCs from adipose tissue, a good source of autologous MSCs (Ma et al., 2013; Chang et al., 2014; Yan et al., 2014). Human umbilical cord MSCs were also transplanted intracerebrally in APP/PS1 mice (Lee et al., 2012; Yang et al., 2013). In all cases, the improvement of

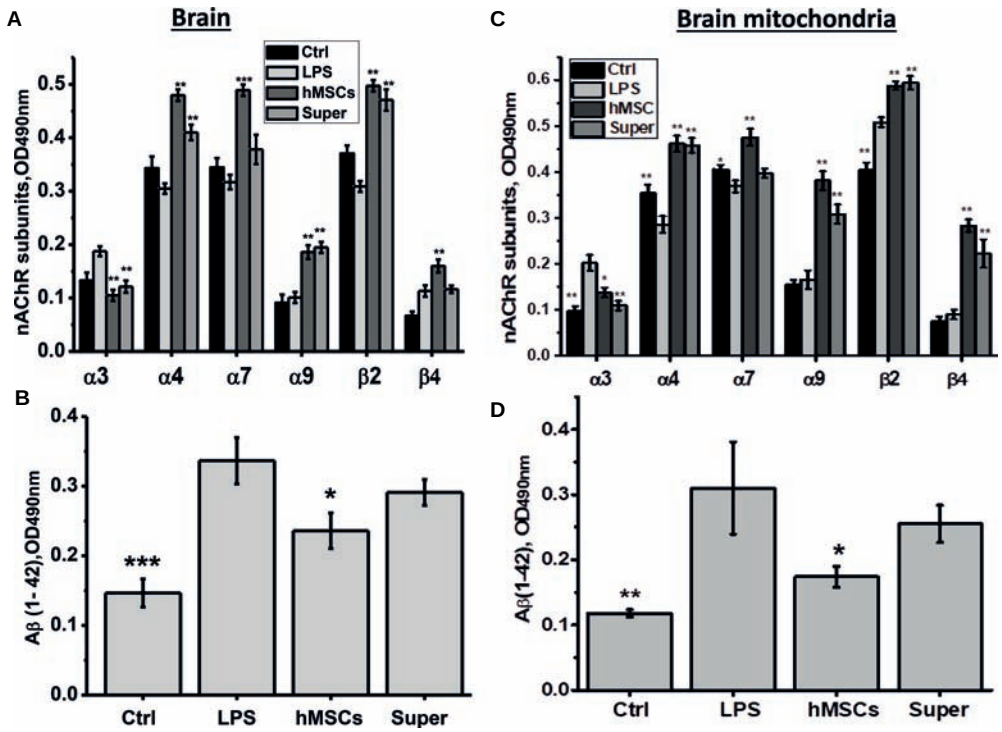

FIGURE 4 | The effects of either hMSCs or their conditioned medium (Super) on the level of nAChR subunits **(A,C)** or α7-bound Aβ (1–42) **(B,D)** in the brain and brain mitochondria of mice pre-injected with LPS 3 weeks before. Each column corresponds to M ± SD of data for separate mice in each group (n = 5) *p < 0.05; **p < 0.005; ***p < 0.0005 compared to LPS-treated samples/mice.

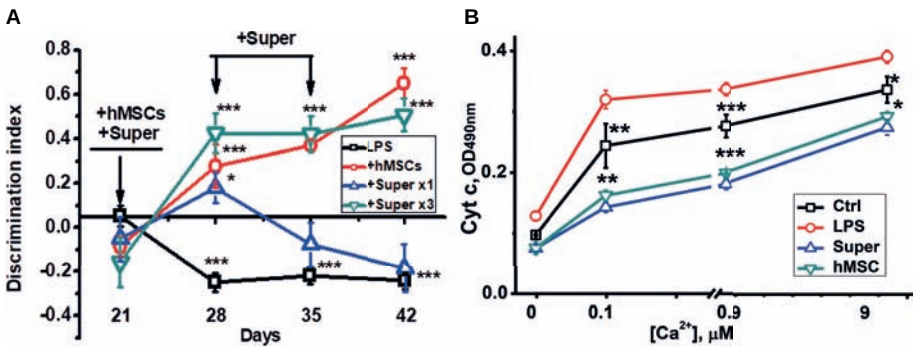

FIGURE 5 | The effects of either hMSCs or their conditioned medium (Super) on episodic memory **(A)** and cyt *c* release from the brain mitochondria under the effect of Ca^{2+} **(B)** in mice pre-injected with LPS 3 weeks before. Arrows in **(A)** indicate the time points of LPS, MSCs or conditioned medium injections. Each point corresponds to M ± SD of data for separate mice in each group (n = 5) *p < 0.05; **p < 0.005; ***p < 0.0005 compared to LPS-treated samples/mice.

cognitive (memory) and synaptic functions, as well as reduced Aβ deposition has been observed that is in accord with our data. Advantage of our model is that the Aβ accumulation in the brain occurred in a natural way in genetically non-modified mice. The wild-type mice do not form senile plaques; however, significant accumulation of soluble Aβ could be observed and it was significantly decreased upon MSC injection. Moreover, we show that MSCs can not only prevent the pathogenic effect of LPS, but cure the already developed pathogenic symptoms including memory impairment.

In contrast to the studies cited above, we injected MSCs intravenously. The brain is protected from penetration of peripheral cells by the blood-brain barrier. However, the integrity of the cerebral vasculature is compromised following inflammation (Zlokovic, 2008). In particular, a diminished function of the blood-brain barrier is an early event in multiple sclerosis when inflammation facilitates the massive influx of leukocytes into the brain parenchyma inducing demyelination, tissue damage and axonal disfunction (Kamphuis et al., 2015). Previously, we reported that the blood-brain barrier in

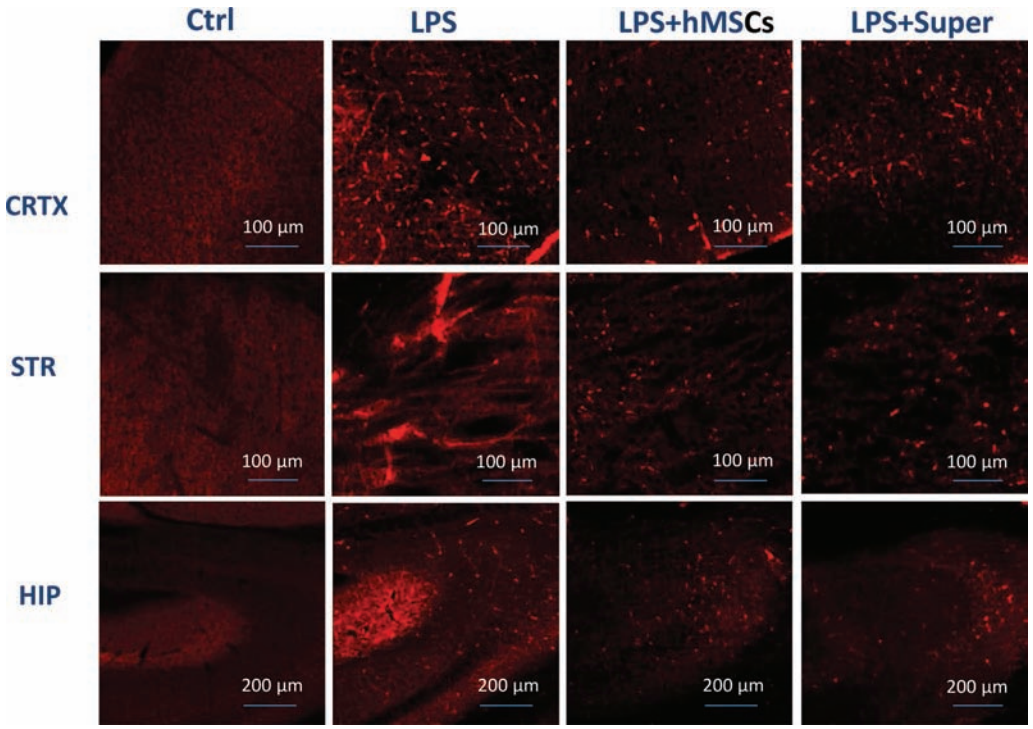

FIGURE 6 | Confocal microscopy images of the brain slices obtained from non-treated (Ctrl), LPS-treated, LPS + hMSCs-treated or LPS + Sup-treated mice and stained with Aβ (1–42)-specific antibody (red). CRTX, cortex; STR, striatum; HIP, hippocampus.

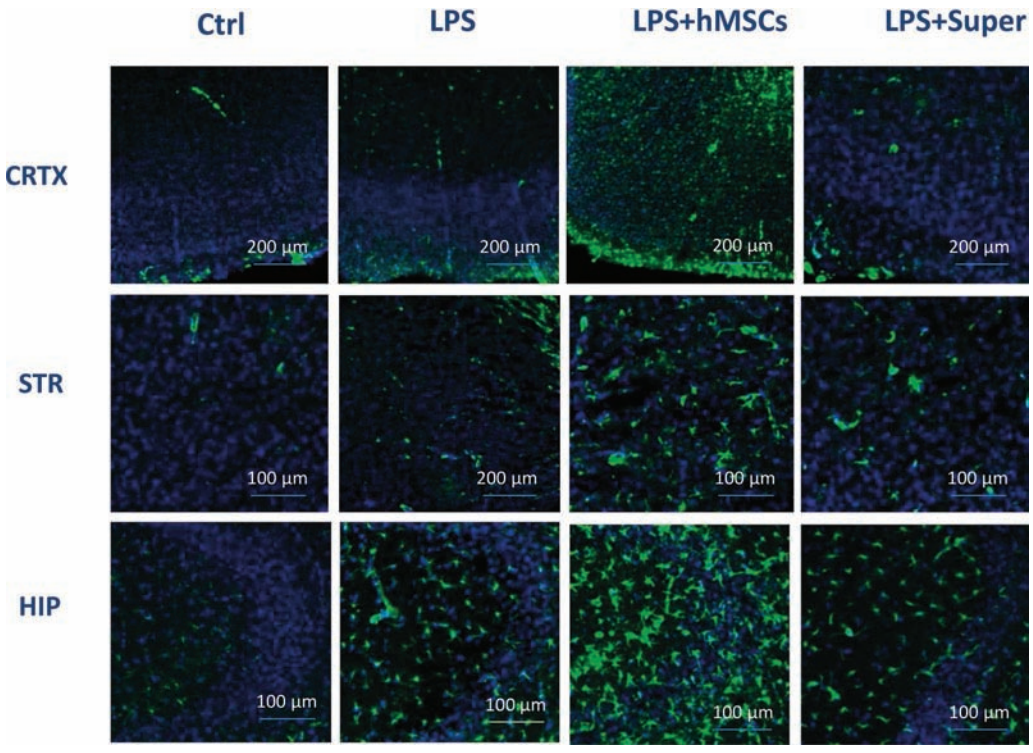

FIGURE 7 | Confocal microscopy images of the brain slices obtained from non-treated (Ctrl), LPS-treated, LPS + hMSCs-treated or LPS + Sup-treated mice and stained with GFAP-specific antibody (green) and DAPI (blue). CRTX, cortex; STR, striatum; HIP, hippocampus.

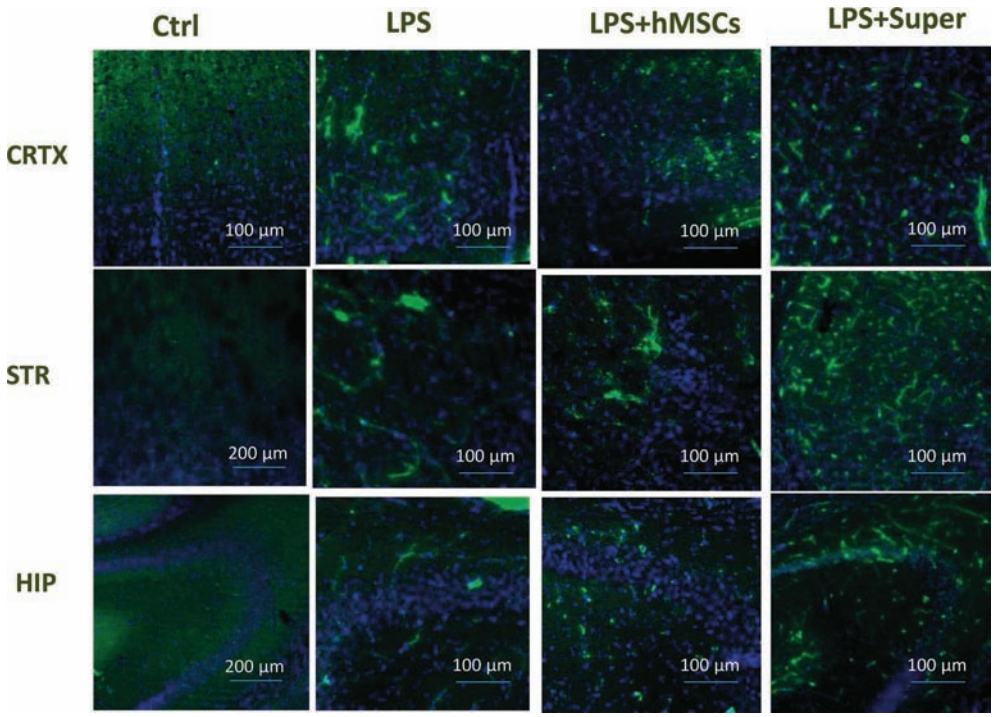

FIGURE 8 | Confocal microscopy images of the brain slices obtained from non-treated (Ctrl), LPS-treated, LPS + hMSCs-treated or LPS + Sup-treated mice and stained with Iba-1-specific antibody (green) and DAPI (blue). CRTX, cortex; STR, striatum; HIP, hippocampus.

LPS-treated mice had been disturbed to allow the nAChR-specific antibody penetration (Lykhmus et al., 2015b). Here, we show that MSCs can be found along the brain vessels of LPS-pre-treated mice 3 days after intravenous injection. This is in accord with the data of Lee et al., who found intraarterially injected MSCs in the brain vasculature (Lee et al., 2016). It was also shown that, at least *in vitro*, MSCs possess leukocyte-like molecular mechanisms enabling interaction with vascular endothelial cells (Rüster et al., 2006). Therefore, it is reasonable to expect that MSCs can penetrate the brain parenchyma *via* transmigration. Indeed, intravenously injected mouse bone marrow cells or human adipose-derived MSCs did cross the blood-brain barrier and migrated into the brain in a rat (Salem et al., 2014) or mouse models for AD (Kim et al., 2012). The AD pathology in humans is accompanied by elevated pro-inflammatory cytokines, which can affect the integrity of the blood-brain barrier (Zenaro et al., 2017; Toropova et al., 2018). However, whether its damage is comparable to that found upon multiple sclerosis and other autoimmune pathologies of the central nervous system and whether it is sufficient to allow intravenously injected MSC to penetrate the brain parenchyma is a reasonable question which needs to be addressed.

The important fact, not examined in previous studies, is that MSC injection restores/elevates the level of nAChRs in the brain and brain mitochondria decreased as a result of LPS treatment. The MSCs up-regulated α4, α7, α9, β2 and β4 subunits in the brain and brain mitochondria, while the conditioned medium did not affect α7 subunits decreased by LPS. Both α7 and α4β2 nAChRs expressed in the brain are involved in regulating cognition and memory (Gotti et al., 2006) and mitochondria-expressed α7β2, α4β2, and α9 nAChRs are involved in the anti-apoptotic pathways (Gergalova et al., 2012, 2014; Lykhmus et al., 2014; Uspenska et al., 2018); therefore, their increased levels make mitochondria more resistant to apoptogenic influence and support the viability of brain cells. In one of the published papers, MSCs were introduced together with galantamine-containing nanoparticles; that was expected to inhibit AChE and increase cholinergic signaling (Misra et al., 2016). Our data indicate that MSCs themselves contribute to cholinergic signaling in the brain by increasing the level of nAChRs.

Another important question arising from positive effects of MSC in AD models is whether they are due to direct involvement of differentiated MSC into neuronal networks in the brain (the true regenerative medicine) or are mediated by numerous trophic and growth factors produced by MSCs to stimulate the host cells. The latter possibility is therapeutically important because it allows avoiding cell transplantation by substituting it with the use of MSC-produced substances. Previous studies demonstrated that human MSCs stimulated neurogenesis both *in vitro* (Park et al., 2016) and *in vivo* (Yan et al., 2014; Kim et al., 2015; Oh et al., 2015) by producing soluble factors like activin A, growth differentiation factor-15 and activating Wnt signaling pathway in neuronal progenitor cells. Moreover, it was found that MSCs produce extracellular vesicles (exosomes), which contain neprilysin, enzyme involved in Aβ degradation (Katsuda et al., 2013), and these exosomes can be suggested for AD therapy (Katsuda et al., 2015).

We show here that supernatants of hMSC culture, applied intraperitoneally, improved memory of LPS-treated mice and affected their brains almost similarly to MSCs. However, in contrast to MSCs, which improved the state of mice for at least 3 weeks after a single injection, the effect of MSC supernatant was transient and regular injections were required to maintain the improvement achieved. Additional studies are required to establish whether MSC-conditioned medium can provide a stable therapeutic effect.

Finally, it is not definitely clear which cells in the brain are the targets for MSC-produced factors. The effects of MSC co-culture with neuronal progenitor cells suggested the direct influence on the brain neurons (Oh et al., 2015; Park et al., 2016). However, other studies demonstrated that intracerebrally transplanted human adipose MSCs activate microglia around senile plaques in the brain of APP/PS1 transgenic mice (Ma et al., 2013). We observed a visible activation of both microglia and astrocytes in the brains of MSC-injected mice, additional to that induced by LPS, and showed that MSCs produce IL-6 in response to LPS stimulation *in vitro*. Therefore, injecting MSCs simultaneously with LPS could stimulate MSCs for IL-6 production. IL-6 is a pro-inflammatory cytokine, but is also known as a neurotrophic factor (Hirota et al., 1996; Wagner, 1996). Recently, it was reported that mesenchymal progenitor cells derived from induced pluripotent stem cells enhance neuritogenesis *via* neurotrophin and cytokine (including IL-6) production (Brick et al., 2018). Therefore, IL-6 may be one of neurotrophic soluble factors produced by MSCs penetrating the brain that can affect both neurons and glial cells.

The data obtained put a wider question on what happens in the mouse brain under the effect of injected MSCs. The ability of MSCs to prevent LPS pathogenic effect (simultaneous injection of LPS and MSCs) indicates that MSCs do not allow such effect to develop. However, positive MSCs effect when pathological symptoms have already developed (MSCs injection 3 weeks after LPS) allows suggesting that MSCs, or their soluble factors, directly or indirectly (by activating glial cells) restore the activity of damaged brain neurons. Further experiments are needed to reveal whether the recovery observed under the MSCs effect is long-lasting or just temporal.

CONCLUSIONS

1. Intravenously injected MSCs penetrate the brain of LPS-treated mice.
2. Either allogenic (mouse) or xenogeneic (human) MSCs prevent and reverse the pathogenic effect of LPS on the brain nAChRs, Aβ accumulation, mitochondria and memory impairment.
3. The MSCs therapeutic action is largely due to their humoral factors and is accompanied by activation of micro- and macroglia.

ETHICS STATEMENT

This study was carried out in accordance with the recommendation of the guidelines of the Animal Care and Use Committee of Palladin Institute of Biochemistry, Kiev. The protocol was approved by the IACUC of Palladin Institute of Biochemistry.

AUTHOR CONTRIBUTIONS

MS, OD, SK, OL, and VKo made substantial contributions to the conception or design of the work. LK, OL, LV, KU, MS, NS, and AU contributed to acquisition, analysis, and interpretation of data for the work. MS drafted the work. OD, VKy, and AU revised it critically for important intellectual content. LK, OL, LV, KU, SK, VKo, OD, NS, VKy, and AU finally approved the version to be published. LK, OL, LV, KU, SK, VKo, OD, NS, VKy, and AU agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

REFERENCES

Antunes, M., and Biala, G. (2012). The novel object recognition memory: neurobiology, test procedure, and its modifications. *Cogn. Process.* 13, 93–110. doi: 10.1007/s10339-011-0430-z

Bae, J. S., Jin, H. K., Lee, J. K., Richardson, J. C., and Carter, J. E. (2013). Bone marrow-derived mesenchymal stem cells contribute to the reduction of amyloid-β deposits and the improvement of synaptic transmission in a mouse model of pre-dementia Alzheimer's disease. *Curr. Alzheimer Res.* 10, 524–531. doi: 10.2174/15672050113109990027

Brick, R. M., Sun, A. X., and Tuan, R. S. (2018). Neurotrophically induced mesenchymal progenitor cells derived from induced pluripotent stem cells enhance neuritogenesis via neurotrophin and cytokine production. *Stem Cells Transl. Med.* 7, 45–58. doi: 10.1002/sctm.17-0108

Carmichael, J., DeGraff, W. G., Gazdar, A. F., Minna, J. D., and Mitchell, J. B. (1987). Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of radiosensitivity. *Cancer Res.* 47, 943–946.

Champtiaux, N., and Changeux, J. P. (2002). Knock-out and knock-in mice to investigate the role of nicotinic receptors in the central nervous system. *Curr. Drug Targets CNS Neurol. Disord.* 1, 319–330.

Chang, K. A., Kim, H. J., Joo, Y., Ha, S., and Suh, Y. H. (2014). The therapeutic effects of human adipose-derived stemcells in Alzheimer's disease mouse models. *Neurodegener. Dis.* 13, 99–102. doi: 10.1159/000355261

Changeux, J. P. (2012). Thenicotinic acetylcholine receptor: the founding father of the pentameric ligand-gated ion channel superfamily. *J. Biol. Chem.* 287, 40207–40215. doi: 10.1074/jbc.R112.407668

Dineley, K. T., Pandya, A. A., and Yakel, J. L. (2015). Nicotinic ACh receptors as therapeutic targets in CNS disorders. *Trends Pharmacol. Sci.* 36, 96–108. doi: 10.1016/j.tips.2014.12.002

Dominici, M., Le Blanc, K., Mueller, I., Slaper-Cortenbach, I., Marini, F., Krause, D., et al. (2006). Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 8, 315–317. doi: 10.1080/14653240600855905

Fhilho, J. B. B., and Oliveira, M. S. (2012). *Placental structure and biological aspects of fetal membranes cultured in vitro*. Open access chapter from "Biomedical Tissue Culture". eds. L. Ceccherini-Nelli, and B. Matteoli. doi: 10.5772/51274

Gergalova, G., Lykhmus, O., Komisarenko, S., and Skok, M. (2014). α7 Nicotinic acetylcholine receptors control cytochrome c release from isolated mitochondria

through kinase-mediated pathways. *Int. J. Biochem. Cell Biol.* 49, 26–31. doi: 10.1016/j.biocel.2014.01.001

Gergalova, G. L., Lykhmus, O. Y., Kalashnyk, O. M., Koval, L. M., Chernyshov, V. I., Kryukova, E., et al. (2012). Mitochondria express α7 nicotinic acetylcholine receptors to regulate Ca^{2+} accumulation and cytochrome *c* release: study on isolated mitochondria. *PLoS One* 7:e31361. doi: 10.1371/journal.pone.0031361

Gotti, C., Zoli, M., and Clementi, F. (2006). Brain nicotinic acetylcholine receptors: native subtypes and their relevance. *Trends Pharmacol. Sci.* 27, 482–491. doi: 10.1016/j.tips.2006.07.004

Guan, Z., Zhang, X., Ravid, R., and Nordberg, A. (2000). Decreased protein levels of nicotinic receptor subunits in the hippocampus and temporal cortex of patients with Alzheimer's disease. *J. Neurochem.* 74, 237–243. doi: 10.1046/j.1471-4159.2000.0740237.x

Harlow, E., and Lane, D. (1988). *Antibodies. A laboratory manual*. (New York: Cold Spring Harbor Laboratory), 341–342.

Hirota, H., Kiyama, H., Kishimoto, T., and Taga, T. (1996). Accelerated nerve regeneration in mice by upregulated expression of interleukin (IL) 6 and IL-6 receptor after trauma. *J. Exp. Med.* 183, 2627–2634. doi: 10.1084/jem.183.6.2627

Kamphuis, W. W., Derada Troletti, C., Reijerkerk, A., Romero, I. A., and de Vries, H. E. (2015). The blood-brain barrier in multiple sclerosis: micro RNAs as key regulators. *CNS Neurol. Disord. Drug Targets* 14, 157–167. doi: 10.2174/1871527314666150116125246

Kariminekoo, S., Movassaghpour, A., Rahimzadeh, A., Talebi, M., Shamsasenjan, K., and Akbarzadeh, A. (2016). Implications of mesenchymal stem cells in regenerative medicine. *Artif. Cells Nanomed. Biotechnol.* 44, 749–757. doi: 10.3109/21691401.2015.1129620

Katsuda, T., Oki, K., and Ochiya, T. (2015). Potential application of extracellular vesicles of human adipose tissue-derived mesenchymal stem cells in Alzheimer's disease therapeutics. *Methods Mol. Biol.* 1212, 171–181. doi: 10.1007/7651_2014_98

Katsuda, T., Tsuchiya, R., Kosaka, N., Yoshioka, Y., Takagaki, K., Oki, K., et al. (2013). Human adipose tissue-derived mesenchymal stem cells secrete functional neprilysin-bound exosomes. *Sci. Rep.* 3:e53131. doi: 10.1371/journal.pone.0053131

Keverne, J., and Ray, M. (2005). Neurochemistry of Alzheimer's disease. *Psychiatry* 4, 40–42. doi: 10.1383/psyt.4.1.40.58307

Kim, D. H., Lee, D., Chang, E. H., Kim, J. H., Hwang, J. W., Kim, J. Y., et al. (2015). GDF-15 secreted from human umbilical cord blood mesenchymal stem cells delivered through the cerebrospinal fluid promotes hippocampal neurogenesis and synaptic activity in an Alzheimer's disease model. *Stem Cells Dev.* 24, 2378–2390. doi: 10.1089/scd.2014.0487

Kim, S., Kim, D. H., Lee, D., Chang, E. H., Kim, J. H., Hwang, J. W., et al. (2012). The preventive and therapeutic effects of intravenous human adipose-derived stem cells in Alzheimer's disease mice. *PLoS One* 7:e45757. doi: 10.1371/journal.pone.0045757

Klinker, M. W., and Wei, C. H. (2015). Mesenchymal stem cells in the treatment of inflammatory and autoimmune diseases in experimental animal models. *World J. Stem Cells* 7, 556–567. doi: 10.4252/wjsc.v7.i3.556

Konala, V. B., Mamidi, M. K., Bhonde, R., Das, A. K., Pochampally, R., and Pal, R. (2016). The current landscape of the mesenchymal stromal cell secretome: a new paradigm for cell-free regeneration. *Cytotherapy* 18, 13–24. doi: 10.1016/j.jcyt.2015.10.008

Koval, L., Lykhmus, O., Zhmak, M., Khruschov, A., Tsetlin, V., Magrini, E., et al. (2011). Differential involvement of α4β2, α7 and α9α10 nicotinic acetylcholine receptors in B lymphocyte activation in vitro. *Int. J. Biochem. Cell Biol.* 43, 516–524. doi: 10.1016/j.biocel.2010.12.003

Koval, O. M., Voitenko, L. P., Skok, M. V., Lykhmus, E. Y., Tsetlin, V. I., Zhmak, M. N., et al. (2004). The β-subunit composition of nicotinic acetylcholine receptors in the neurons of the guinea pig inferior mesenteric ganglion. *Neurosci. Lett.* 365, 143–146. doi: 10.1016/j.neulet.2004.04.071

Lee, J. K., Jin, H. K., and Bae, J. S. (2009). Bone marrow-derived mesenchymal stem cells reduce brain amyloid-beta deposition and accelerate the activation of microglia in an acutely induced Alzheimer's disease mouse model. *Neurosci. Lett.* 450, 136–141. doi: 10.1016/j.neulet.2008.11.059

Lee, J. K., Jin, H. K., and Bae, J. S. (2010a). Bone marrow-derived mesenchymal stem cells attenuate amyloid β-induced memory impairment and apoptosis by inhibiting neuronal cell death. *Curr. Alzheimer Res.* 7, 540–548.

Lee, J. K., Jin, H. K., Endo, S., Schuchman, E. H., Carter, J. E., and Bae, J. S. (2010b). Intracerebral transplantation of bone marrow-derived mesenchymal stem cells reduces amyloid-beta deposition and rescues memory deficits in Alzheimer's disease mice by modulation of immune responses. *Stem Cells* 28, 329–343. doi: 10.1002/stem.277

Lee, H. J., Lee, J. K., Lee, H., Carter, J. E., Chang, J. W., Oh, W., et al. (2012). Human umbilical cord blood-derived mesenchymal stem cells improve neuropathology and cognitive impairment in an Alzheimer's disease mouse model through modulation of neuroinflammation. *Neurobiol. Aging* 33, 588–602. doi: 10.1016/j.neurobiolaging.2010.03.024

Lee, N. K., Yang, J., Chang, E. H., Park, S. E., Lee, J., Choi, S. J., et al. (2016). Intra-arterially delivered mesenchymal stem cells are not detected in the brain parenchyma in an Alzheimer's disease mouse model. *PLoS One* 11:e0155912. doi: 10.1371/journal.pone.0155912

Lykhmus, O., Gergalova, G., Koval, L., Zhmak, M., Komisarenko, S., and Skok, M. (2014). Mitochondria express several nicotinic acetylcholine receptor subtypes to control various pathways of apoptosis induction. *Int. J. Biochem. Cell Biol.* 53, 246–252. doi: 10.1016/j.biocel.2014.05.030

Lykhmus, O., Gergalova, G., Zouridakis, M., Tzartos, S., Komisarenko, S., and Skok, M. (2015a). Inflammation decreases the level of alpha 7 nicotinic acetylcholine receptors in the brain mitochondria and makes them more susceptible to apoptosis induction. *Int. Immunopharmacol.* 29, 148–151. doi: 10.1016/j.intimp.2015.04.007

Lykhmus, O., Koval, L., Pavlovych, S., Zouridakis, M., Zisimopoulou, P., Tzartos, S., et al. (2010). Functional effects of antibodies against non-neuronal nicotinic acetylcholine receptors. *Immunol. Lett.* 128, 68–73. doi: 10.1016/j.imlet.2009.11.006

Lykhmus, O., Uspenska, K., Koval, L., Lytovchenko, D., Voytenko, L., Horid'ko, T., et al. (2017). N-stearoylethanolamine protects the brain and improves memory of mice treated with lipopolysaccharide or immunized with the extracellular domain of α7 nicotinic acetylcholine receptor. *Int. Immunopharmacol.* 52, 290–296. doi: 10.1016/j.intimp.2017.09.023

Lykhmus, O., Voytenko, L., Koval, , Mykhalskiy, S., Kholin, V., Peschana, K., et al. (2015b). α7 Nicotinic acetylcholine receptor-specific antibody induces inflammation and amyloid β42 accumulation in the mouse brain to impair memory. *PLoS One* 10:e0122706. doi: 10.1371/journal.pone.0122706

Ma, T., Gong, K., Ao, Q., Yan, Y., Song, B., Huang, H., et al. (2013). Intracerebral transplantation of adipose-derived mesenchymal stem cells alternatively activates microglia and ameliorates neuropathological deficits in Alzheimer's disease mice. *Cell Transplant.* 22(Suppl. 1), 113–126. doi: 10.3727/096368913x672181

Maslova, O. O., Shuvalova, N. S., Sukhorada, O. M., Zhukova, S. M., Deryabina, O. G., Makarenko, M. V., et al. (2013). Heterogeneity of umbilical cords as a source for mesenchymal stem cells. *Dataset Pap. Biol.* 1–4. doi: 10.7167/2013/370103

Misra, S., Chopra, K., Saikia, U. N., Sinha, V. R., Sehgal, R., Modi, M., et al. (2016). Effect of mesenchymal stem cells and galantamine nanoparticles in rat model of Alzheimer's disease. *Regen. Med.* 11, 629–646. doi: 10.2217/rme-2016-0032

NIH Stem Cell Information Home Page (2016). *Stem cell information* [World Wide Website]. Bethesda, MD: National Institutes of Health, U.S. Department of Health and Human Services. Available at: stemcells.nih.gov/info/basics/1.htm

Oh, S. H., Kim, H. N., Park, H. J., Shin, J. Y., and Lee, P. H. (2015). Mesenchymal stem cells increase hippocampal neurogenesis and neuronal differentiation by enhancing the Wnt signaling pathway in an Alzheimer's disease model. *Cell Transplant.* 24, 1097–10109. doi: 10.3727/096368914X679237

Parada, E., Egea, J., Romero, A., del Barrio, L., García, A. G., and López, M. G. (2010). Poststress treatment with PNU282987 can rescue SH-SY5Y cells undergoing apoptosis via α7 nicotinic receptors linked to a Jak2/Akt/HO-1 signaling pathway. *Free Radic. Biol. Med.* 49, 1815–1821. doi: 10.1016/j.freeradbiomed.2010.09.017

Park, S. E., Lee, J., Chang, E. H., Kim, J. H., Sung, J. H., Na, D. L., et al. (2016). Activin A secreted by human mesenchymal stem cells induces neuronal development and neurite outgrowth in an in vitro model of Alzheimer's disease: neurogenesis induced by MSCs via activin A. *Arch. Pharm. Res.* 39, 1171–1179. doi: 10.1007/s12272-016-0799-4

Parri, H. R., and Dineley, K. T. (2010). Nicotinic acetylcholine receptor interaction with β-amyloid: molecular, cellular, and physiological consequences. *Curr. Alzheimer Res.* 7, 27–39. doi: 10.2174/156720510790274464

Paxinos, G., and Franklin, K. B. J. (2001). *The mouse brain in stereotaxic coordinates*. (New York: Academic Press).

Picciotto, M. R., Caldarone, B. J., Brunzell, D. H., Zachariou, V., Stevens, T. R., and King, S. L. (2001). Neuronal nicotinic acetylcholine receptor subunit knockout mice: physiological and behavioral phenotypes and possible clinical implications. *Pharmacol. Ther.* 92, 89–108. doi: 10.1016/S0163-7258(01)00161-9

Posadas, I., López-Hernández, B., and Ceña, V. (2013). Nicotinic receptors in neurodegeneration. *Curr. Neuropharmacol.* 11, 298–314. doi: 10.2174/1570159X11311030005

Russo, P., Del Bufalo, A., Frustaci, A., Fini, M., and Cesario, A. (2014). Beyond acetylcholinesterase inhibitors for treating Alzheimer's disease: α7-nAChR agonists in human clinical trials. *Curr. Pharm. Res.* 20, 6014–6021.

Rüster, B. R., Göttig, S., Ludwig, R. J., Bistrian, R., Müller, S., and Seifried, E. (2006). Mesenchymal stem cells display coordinated rolling and adhesion behavior on endothelial cells. *Blood* 108, 3938–3944. doi: 10.1182/blood-2006-05-025098

Salem, A. M., Ahmed, H. H., Atta, H. M., Ghazy, M. A., and Aglan, H. A. (2014). Potential of bone marrow mesenchymal stem cells in management of Alzheimer's disease in female rats. *Cell Biol. Int.* 38, 1367–1383. doi: 10.1002/cbin.10331

Save, E., Poucet, B., Foreman, N., and Buhot, M.-C. (1992). Object exploration and reactions to spatial and nonspatial changes in hooded rats following damage to parietal cortex or hippocampal formation. *Behav. Neurosci.* 106, 447–456. doi: 10.1037/0735-7044.106.3.447

Shin, J. Y., Park, H. J., Kim, H. N., Oh, S. H., Bae, J. S., Ha, H. J., et al. (2014). Mesenchymal stem cells enhance autophagy and increase β-amyloid clearance in Alzheimer disease models. *Autophagy* 10, 32–44. doi: 10.4161/auto.26508

Shuvalova, N. S., and Kordium, V. A. (2016). Morphological characteristics of mesenchymal stem cells from Wharton jelly, cultivated under physiological oxygen tensions, in various gas mixtures. *Biopolym. Cell* 32, 262–270. doi: 10.7124/bc.000928

Shuvalova, N. S., Maslova, O. A., Sukhorada, O. M., Deryabina, O. G., and Kordium, V. A. (2013). Maintenance of mesenchymal stem cells culture due to the cells with reduced attachment rate. *Biopolym. Cell* 29, 75–78. doi: 10.7124/bc000809

Skok, M., and Lykhmus, O. (2016). The role of α7 nicotinic acetylcholine receptors and α7-specific antibodies in neuroinflammation related to Alzheimer disease. *Curr. Pharm. Des.* 22, 2035–2049.

Skok, M. V., Voitenko, L. P., Voitenko, S. V., Lykhmus, E. Y., Kalashnik, E. N., Litvin, T. I., et al. (1999). Alpha subunit composition of nicotinic acetylcholine receptors in the rat autonomic ganglia neurons as determined with subunit-specific anti-alpha(181-192) peptide antibodies. *Neuroscience* 93, 1427–1436. doi: 10.1016/S0306-4522(99)00160-8

Suzuki, T., Hide, I., Matsubara, A., Hama, C., Harada, K., Miyano, K., et al. (2006). Microglial alpha7 nicotinic acetylcholine receptors drive a phospholipase C/IP3 pathway and modulate the cell activation toward a neuroprotective role. *J. Neurosci. Res.* 83, 1461–1470. doi: 10.1002/jnr20850

Thinus-Blanc, C. (1996). *Animal spatial cognition. Behavioral and neural approaches*. (Singapore, New Jersey, London, Hong Kong: World Scientific Publishing Co).

Thomsen, M. S., and Mikkelsen, J. D. (2012). Theα7 nicotinic acetylcholine receptor ligands methyllycaconitine, NS6740 and GTS-21 reduce lipopolysaccharide-induced TNF-α release from microglia. *J. Neuroimmunol.* 251, 65–72. doi: 10.1016/jneuroim.2012.07.006

Toropova, A. P., Toropov, A. A., Begum, S., and Achary, P. G. R. (2018). Blood brain barrier and Alzheimer's disease: similarity and dissimilarity of molecular alerts. *Curr. Neuropharmacol.* 16, 769–785. doi: 10.2174/1570159X15666171016163951

Tyagi, E., Agrawal, R., Nath, C., and Shukla, R. (2010). Cholinergic protection via alpha7 nicotinic acetylcholine receptors and PI3K-Akt pathway in LPS-induced neuroinflammation. *Neurochem. Int.* 56, 135–142. doi: 10.1016/j.neuint.2009.09.011

Uspenska, K., Lykhmus, O., Gergalova, G., Chernyshov, V., Arias, H. R., Komisarenko, S., et al. (2017). Nicotine facilitates nicotinic acetylcholine receptor targeting to mitochondria but makes them less susceptible to selective ligands. *Neurosci. Lett.* 656, 43–50. doi: 10.1016/j.neulet.2017.07.009

Uspenska, K., Lykhmus, O., Obolenskaya, M., Pons, S., Maskos, U., Komisarenko, S., et al. (2018). Mitochondrial nicotinic acetylcholine receptors support liver cells viability after partial hepatectomy. *Front. Pharmacol.* 9:626. doi: 10.3389/fphar.2018.00626

Wagner, J. A. (1996). Is IL-6 both a cytokine and a neurotrophic factor? *J. Exp. Med.* 183, 2417–2419. doi: 10.1084/jem.183.6.2417

Wang, H. Y., Lee, D. H., D'Andrea, M. R., Peterson, P. A., Shank, R. P., and Reitz, A. B. (2000). β-Amyloid 1–42 binds to α7 nicotinic acetylcholine receptor with high affinity. Implications for Alzheimer's disease pathology. *J. Biol. Chem.* 275, 5626–5632. doi: 10.1074/jbc.275.8.5626

Wevers, A., Monteggia, L., Nowacki, S., Bloch, W., Schütz, U., Lindstrom, J., et al. (1999). Expression of nicotinic acetylcholine receptor subunits in the cerebral cortex in Alzheimer's disease: histotopographical correlation with amyloid plaques and hyperphosphorylated-tau protein. *Eur. J. Neurosci.* 11, 2551–2565. doi: 10.1046/j.1460-9568.1999.00676.x

Yan, Y., Ma, T., Gong, K., Ao, Q., Zhang, X., and Gong, Y. (2014). Adipose-derived mesenchymalstemcell transplantation promotes adult neurogenesis in the brains of Alzheimer's disease mice. *Neural Regen. Res.* 9, 798–805. doi: 10.4103/1673-5374.131596

Yang, H., Xie, Z., Wei, L., Yang, H., Yang, S., Zhu, Z., et al. (2013). Human umbilical cord mesenchymalstemcell-derived neuron-like cells rescue memory deficits and reduce amyloid-beta deposition in an AβPP/PS1 transgenic mouse model. *Stem Cell Res. Ther.* 4:76. doi: 10.1186/scrt227

Zachar, L., Bačenková, D., and Rosocha, J. (2016). Activation, homing, and role of the mesenchymal stem cells in the inflammatory environment. *J. Inflamm. Res.* 9, 231–240. doi: 10.2147/JIR.S121994

Zenaro, E., Piacentino, G., and Constantin, G. (2017). The blood-brain barrier in Alzheimer's disease. *Neurobiol. Dis.* 107, 1–56. doi: 10.3389/fimmu.2017.00211

Zhang, P., Zhao, G., Kang, X., and Su, L. (2012). Effects of lateral ventricular transplantation of bone marrow-derived mesenchymal stem cells modified with brain-derived neurotrophic factor gene on cognition in a rat model of Alzheimer's disease. *Neural Regen. Res.* 7, 245–250. doi: 10.3969/j.issn.1673-5374.2012.04.001

Zlokovic, B. V. (2008). The blood-brain barrier in health and chronic neurodegenerative disorders. *Neuron* 57, 178–201. doi: 10.1016/j.neuron.2008.01.003

Zoli, M., Picciotto, M. R., Ferrari, R., Cocchi, D., and Changeux, J. P. (1999). Increased neurodegeneration during ageing in mice lacking high-affinity nicotine receptors. *EMBO J.* 18, 1235–1244. doi: 10.1093/emboj/18.5.1235

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A Bcr-Abl Inhibitor GNF-2 Attenuates Inflammatory Activation of Glia and Chronic Pain

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GNF-2 is an allosteric inhibitor of Bcr-Abl. It was developed as a new class of anti-cancer drug to treat resistant chronic myelogenous leukemia. Recent studies suggest that c-Abl inhibition would provide a neuroprotective effect in animal models of Parkinson's disease as well as in clinical trials. However, the role of c-Abl and effects of GNF-2 in glia-mediated neuroinflammation or pain hypersensitivity has not been investigated. Thus, in the present study, we tested the hypothesis that c-Abl inhibition by GNF-2 may attenuate the inflammatory activation of glia and the ensuing pain behaviors in animal models. Our results show that GNF-2 reduced lipopolysaccharide (LPS)-induced nitric oxide and pro-inflammatory cytokine production in cultured glial cells in a c-Abl-dependent manner. The small interfering ribonucleic acid (siRNA)-mediated knockdown of c-Abl attenuated LPS-induced nuclear factor kappa light chain enhancer of activated B cell (NF-κB) activation and the production of pro-inflammatory mediators in glial cell cultures. Moreover, GNF-2 administration significantly attenuated mechanical and thermal hypersensitivities in experimental models of diabetic and inflammatory pain. Together, our findings suggest the involvement of c-Abl in neuroinflammation and pain pathogenesis and that GNF-2 can be used for the management of chronic pain.

Keywords: GNF-2, c-Abl, glia, neuroinflammation, pain

INTRODUCTION

Neuroinflammation is highly associated with several neurodegenerative diseases including Alzheimer's disease (AD), Parkinson's disease (PD), and chronic pain (Katsulov and Mazneikova, 1987; Mosley et al., 2006; Tansey et al., 2007; Calsolaro and Edison, 2016; Chen et al., 2018). The chronic pain pathophysiology is complex and includes peripheral and central neuronal alterations and neuroinflammation. The neuroinflammatory process is characterized by the activation of microglia and astrocytes, macrophage infiltration, release of diverse proinflammatory mediators

[e.g., nitric oxide (NO), cytokines, and chemokines]. This process leads to neuronal death or neurodegeneration (Frank-Cannon et al., 2009; Song and Suk, 2017). In this regard, important clues to the molecular mechanisms of neuropathic pain may be found by closely examining the microglial inflammatory activation and neuroinflammation (Carniglia et al., 2017; Kiguchi et al., 2017; Chen et al., 2018). Therefore, studies which target inflammatory mediators may provide novel therapeutic approaches for chronic pain management.

Non-receptor tyrosine kinase c-Abl activation is associated with AD and PD pathogenesis in human and animal models. c-Abl phosphorylation is robustly increased in brain samples from AD and PD patients as well as in animal models of AD, PD, and synucleinopathies (Ko et al., 2010; Imam et al., 2011; Vargas et al., 2018). Furthermore, imatinib or nilotinib, which are FDA-approved c-Abl inhibitors, showed neuroprotective effects when administered in animal models of PD and AD (Cancino et al., 2008; Hebron et al., 2013). More recently, activated c-Abl was observed in the spinal cord of G93A-SOD1 transgenic mice, a widely-used model of amyotrophic lateral sclerosis (ALS). This study revealed that the administration of dasatinib (a c-Abl inhibitor) improved the innervation status of neuromuscular junctions (Katsumata et al., 2012). It is quite well known that oxidative stress-induced c-Abl activation leads to nuclear factor kappa light chain enhancer of activated B cell (NF-κB) activation and neuronal death (Xiao et al., 2011). However, most studies have focused on the neuroprotective effects of c-Abl inhibitors and related molecular mechanisms in neurons. Recent findings on pain pathogenesis demonstrate that glial cells, particularly microglia and astrocytes, are an important source of inflammatory mediators fundamentally involved in the pathogenesis of inflammatory and neuropathic pain (Carniglia et al., 2017; Chen et al., 2018). Therefore, it is necessary to study the function of glial c-Abl in the pathogenesis of both inflammatory and neuropathic pain.

GNF-2 is a selective allosteric inhibitor of Bcr-Abl (the oncogenic fusion protein of Bcr and c-Abl caused by reciprocal chromosomal translocations), which was developed as an anti-cancer drug (Zhang et al., 2010; Rossari et al., 2018). GNF-2 binds to the myristate-binding site of c-Abl, leading to improved pharmacokinetic properties (Fabbro et al., 2010; Zhang et al., 2010). GNF-2 is a very selective non-ATP competitive inhibitor of Bcr-Abl and c-Abl. Unlike other inhibitors, it does not show activity against many other kinases such as fms-like tyrosine kinase 3, platelet-derived growth factor receptor, Janus kinase-1, tyrosine-protein kinase Met. Therefore, in this study, we used GNF-2 to assess the effect of c-Abl on neuroinflammation and associated pain pathogenesis using multiple pain models. It has been reported that reactive microglia release a various array of toxic molecules including pro-inflammatory cytokines, NO, and superoxide, which have been shown to play a complex role in the pathogenesis of neuropathic pain. However, the effects of c-Abl inhibition by GNF-2 on neuroinflammation and associated chronic pain pathogenesis remain elusive. Thus, in the present study, we investigated the role of c-Abl in the inflammatory activation of glia and their contribution to the pathogenesis of inflammatory and neuropathic pain by the *in vitro* and *in vivo* application of the Bcr-Abl inhibitor GNF-2.

MATERIALS AND METHODS

Materials

GNF-2 and methylated GNF-2 compounds were prepared as described previously (Adrian et al., 2006). Lipopolysaccharide (LPS) was purchased from Sigma-Aldrich. It was obtained from *Escherichia coli* 0111:B4 prepared by phenolic extraction and gel filtration chromatography. Recombinant mouse interferon-γ (IFN-γ) protein was purchased from R&D Systems. The c-Abl siRNA (1:1 mix of siRNA #2 and #3) and control siRNA were purchased from Genolution Pharmaceuticals (Seoul, South Korea); siCont- 5′-CCUCGUGCCGUUCCAUCAGG UAGUU-3′, siAbl-#2, 5′-GCAACAAGCCCACUAUCUAUU-3′, siAbl-#3, 5′-UGAUGAAGGAGAUCAAACAUU-3′.

Cell Culture

BV-2 immortalized murine microglial cell line was maintained in Dulbecco's modified Eagle's medium (DMEM) containing 5% heat-inactivated fetal bovine serum (FBS) and 50 mg/ml gentamicin at 37°C. For mouse primary mixed glial cells (MGCs) culture, the brains of 3-day old C57BL6 mice were isolated and homogenized and mechanically disrupted by a nylon mesh. The MGCs were seeded in poly-L-lysine-coated culture flasks with DMEM containing 10% FBS, 100 U/ml of penicillin, and 100 μg/ml of streptomycin (Gibco, Grand Island, NY, United States) and allowed to grow at 37°C in a humidified atmosphere with 5% CO_2. Culture medium was changed initially after 5 days and then changed every 3 days. After 14 days of culture, MGCs (mixed microglial and astrocytes) were prepared by trypsinization, as previously described (Song et al., 2016). The collected cells were further plated using the same media condition and used for experiments.

Nitric Oxide Production

The BV-2 cells (4×10^4 cells/well in 96-well plates) were treated with 100 ng/ml of LPS and the level of NO production was assessed by measuring the amount of nitrite as previously described (Lee et al., 2009). Briefly, After 24-h of incubation, 50 μl of the cell culture media was mixed with an equal volume of a Griess reagent (0.1% naphthylethylenediamine dihydrochloride and 1% sulfanilamide in 5% phosphoric acid) in a 96-well microtiter plate. Absorbance at 540 nm was measured on a microplate reader. Sodium nitrite was used as the standard curve to calculate NO concentration.

Assessment of Cell Viability

Both BV-2 microglia and primary MGCs (4×10^4 cells/well in 96-well plates) were used to measure cell viability using 3-(4, 5 dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) assay, as previously described (Song et al., 2016). After 24 h of LPS treatment, the culture media was removed and MTT (0.5 mg/ml in PBS) was added to the cells, which were then incubated at 37°C for 2 h in a 5% CO_2 incubator. The insoluble

formazan crystals were completely dissolved in DMSO. The absorbance at 570 nm was measured using a microplate reader.

Enzyme-Linked Immunosorbent Assay (ELISA) for TNF-α

The BV-2 cells or primary cells were treated with LPS either in the presence or absence of GNF-2 for 24 h. The concentration of TNF-α protein in the culture media was assessed using a rat monoclonal anti-mouse TNF-α antibody (capture antibody), and a goat biotinylated polyclonal anti-mouse TNF-α antibody (detection antibody), as described in the product manual (ELISA development reagent; R&D systems, Minneapolis, MN, United States). The recombinant TNF-α protein was used as a standard.

Small Interfering Ribonucleic Acid (siRNA)-Mediated Knockdown of the c-Abl Gene

Cells were transfected with siRNAs using Lipofectamine™ iMAX (Invitrogen, Carlsbad, CA, United States), based on the manufacturer's instructions. The cells were used after 48 h of transfection.

Traditional and Real-Time Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total ribonucleic acid (RNA) was extracted from the treated cells or tissues (spinal cord and brain) using TRIZOL reagent (Invitrogen, Carlsbad, CA, United States). Reverse transcription (RT) was conducted using the Superscript II reverse transcriptase (Invitrogen) and an oligo (dT) primer. Traditional PCR amplification was done using specific primer sets at 55–60°C as annealing temperature and 25–32 cycles in a C1000 Touch Thermal Cycler (Bio-Rad, Richmond, CA, United States). PCR products with ethidium bromide were electrophoresed on a 1% agarose gel, and bands were observed under ultraviolet light for analysis. Real-time PCR was performed using One Step SYBR PrimeScript RT-PCR Kit (Takara Bio, Otsu, Shiga, Japan), according to the manufacturer's instructions, followed by detection using the ABI Prism 7000 Sequence Detection System (Applied Biosystems, California, CA, United States). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. The primer sequences were designed based on published complementary deoxyribonucleic acid (cDNA) sequences (**Table 1**).

TABLE 1 | DNA sequences of the primers used for RT-PCR.

Target genes	Forward primer (5′–>3′)	Reverse primer (5′–>3′)
c-Abl	GAGCCTGGCCTACAACAAGT	TGTCCAGTGCATCGCTTTCT
TNF-α	CATCTTCTCAAAATTCGAGTGACAA	ACTTGGGCAGATTGACCTCAG
IL-1β	GCAACTGTTCCTGAACTC	CTCGGAGCCTGTAGTGCA
IL-6	AGTTGCCTTCTTGGGACTGA	TCCACGATTTCCCAGAGAAC
GAPDH	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA

Western Blotting Analysis

Cells or brain tissues were lysed in 300 μl of lysis buffer [150 mM sodium chloride, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS), 50 mM Tris-HCl (pH 7.5), 2 mM EDTA] containing mixture of Halt™ protease and phosphatase inhibitors (1×) (Thermo Fisher Scientific). The brain tissues were individually homogenized and then centrifuged at 13,400× *g* at 4°C for 15 min. Protein concentration was determined using the Pierce BCA protein assay kit (Thermo Fisher Scientific). Bovine serum albumin was used as the standard. Proteins (20–30 μg) for each sample were separated using 12% sodium dodecyl sulfate-PAGE and transferred to polyvinylidene fluoride filter membranes (Bio-Rad) by the semi-dry electroblotting method. The membranes were blocked with 5% skim milk and incubated sequentially with the following primary antibodies against either c-Abl (rabbit monoclonal antibody, 1:1000; Santa Cruz), p-p65, p-65, p-IκB, IκB (rabbit monoclonal antibody, 1:1000; Cell Signaling), TNF-α (rat anti-mouse monoclonal antibody, 1:500; Millipore) or α-tubulin (mouse monoclonal antibody, 1:2000; Sigma-Aldrich) and horseradish peroxidase-conjugated secondary antibodies (anti-rabbit or mouse IgG antibody; Cell Signaling), followed by chemiluminescence detection (Thermo Fisher Scientific).

Animals and Maintenance

All experiments were conducted in accordance with approved animal protocols and guidelines established by the Animal Care Committee of Kyungpook National University. All efforts were made to reduce the number of animals and their sufferings. Age-matched male C57BL/6 mice (8–10 weeks old) were supplied by Samtako Bio (South Korea). Mice were housed in the groups of three to five per cage under standard condition using a 12-h light/dark cycle (lights on 07:00–19:00) at a constant ambient temperature of 23 ± 2°C. Each individual animal was used for a single experiment.

Neuroinflammation Model Based on Intraperitoneal LPS Injection

Lipopolysaccharide was administered to evoke neuroinflammation in mice as described previously (Jo et al., 2017). Mice were injected a single dose of vehicle or LPS (5 mg/kg) intraperitoneally. Phosphate buffered saline (PBS) was used as vehicle and administered the same volume. Mice were sacrificed 48 h after injection and brain tissues collected for further analysis.

The Complete Freund's Adjuvant (CFA)-Induced Chronic Inflammatory Pain Model

Chronic inflammation in mice was induced by a single dose of CFA injection, as described previously (Jha et al., 2015). Briefly, mice were gently anesthetized with 5% of isoflurane for induction and 2% for maintenance. They received CFA (30 μl, 0.5 mg/ml; Sigma-Aldrich) unilaterally in their left hind paws (ipsilateral paws) by intraplantar injections. Mice in the control group received an equal amount of saline in their left hind

paws. Pain behaviors were assessed before and up to 5 days post-CFA administration.

Measurement of CFA-Induced Paw Edema

Complete Freund's adjuvant-induced paw edema was assessed by the measurement of paw thickness. One experimenter, who was blinded to the treatment conditions, handled and tested all the animals. The dorsoventral thickness of the middle portions of the hind paws were measured using a caliper, as described previously (Jha et al., 2015).

Streptozotocin (STZ)-Induced Diabetes Model

The mouse model of diabetes was generated as described previously (Rahman et al., 2016). Briefly, type-1 diabetes was induced by an intraperitoneal administration of STZ (150 mg/kg body weight; Sigma-Aldrich), prepared in 0.1 M citrate buffer (pH 4.5). An equal amount of citrate buffer was injected into control animals. Glycaemia level was tested in blood samples collected from the tail vein 3 days post-STZ injection by using an SD CodeFree^{TM} glucometer (SD Biosensor Inc., Suwon-si, South Korea). Mice with fasting blood glucose levels over 260 mg/dl were considered diabetic and used for further study.

Behavioral Test for Pain

Before the actual test, mice were allowed to familiarize the experimenter, testing room, and equipment for at least 1 week. Paw withdrawal thresholds (PWTs) in response to mechanical stimulations were measured at different time points following CFA and STZ injection. The mechanical sensitivity was examined using calibrated Von Frey filaments (Bioseb^{TM}, Chaville, France), as described previously (Rahman et al., 2016). PWT was calculated from five consecutive withdrawal responses using Dixon's up-down method. Thermal hyperalgesia is defined as a decrease in paw withdrawal latencies (PWL) in response to a noxious thermal stimulus. The thermal sensitivity was tested using the Hargreaves' Plantar Test Analgesy-Meter (Ugo Basile), as previously described (Jha et al., 2015). To obtain the mean PWL values, tests were repeated at least three times and averaged with 5 min intervals between tests to avoid heat-induced sensitization. One experimenter, who was unaware of the experimental conditions, handled and examined all the animals.

Immunohistochemistry and Histopathology

Mice were deeply anesthetized and then subjected to intracardiac perfusion-fixation through the aorta with 0.1 M PBS followed by 4% paraformaldehyde dissolved in 0.1 M PBS. The tissues were further post-fixed in the same paraformaldehyde overnight. Tissues were washed with 0.1 M PBS and cryoprotected in 30% sucrose in 0.1 M PBS overnight at 4°C. Tissues were embedded in frozen section compound (FSC 22 Clear; Leica), and a cryostat was used to prepare 20 μm-thick cross-sections for the spinal cord tissues and 30 μm-thick coronal sections for the brain tissues. Tissue sections or fixed cells were then blocked with 1% bovine serum albumin or normal serum in 0.3% Triton X-100 for 60 min at room temperature. For immunofluorescence staining, tissue sections were incubated with the following primary antibodies against c-Abl (rabbit, 1:100; Santa Cruz), Iba-1 (goat, 1:200; Novus Biologicals, Littleton, CO, United States), GFAP (mouse, 1:500; BD Biosciences), inducible nitric oxide synthase (iNOS) antibody (mouse, 1:200 dilution; BD Transduction Laboratories) or NF-κB p65 (rabbit, 1:500) overnight at 4°C, and then incubated with Cy3- or FITC-conjugated secondary antibodies (1:200; Jackson ImmunoResearch, West Grove, PA, United States). Slides were washed three times with 0.1 M PBS in 0.3% Triton X-100, and mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA, United States) using glass cover-slips, and visualized under a fluorescence microscope (Leica Microsystems, DM2500, Wetzlar, Germany).

Quantification and Statistical Analysis

Statistical analysis was performed using either a Student's *t* test or a one/two-way ANOVA with Dunnett's multiple-comparisons test using GraphPad Prism (version 5.01). Differences with *p*-values <0.05 were considered to be statistically significant. For the immunohistochemical analysis, 5–6 microscopic images were chosen randomly for statistical analysis. For the measurement of either immunofluorescence or western blot band intensities, the area of the whole image or each band was selected, and the mean intensity was measured using the ImageJ software (National Institutes of Health, Bethesda, MD, United States). The background intensity of the band was also measured and deducted from the values obtained.

RESULTS

GNF-2 Inhibits LPS-Induced Inflammatory Activation of Glial Cells in Culture

c-Abl is activated by oxidative stress and its activation in neurons increases NF-κB activation leading to neuronal death (Xiao et al., 2011). In this study, we examined whether c-Abl is involved in the process of inflammatory microglial activation. To investigate the role of c-Abl in inflammatory microglial activation, BV-2 immortalized mouse microglial cell line was stimulated with LPS after GNF-2 pre-treatment (**Figure 1A**). GNF-2 significantly inhibited LPS-induced NO (**Figure 1B**) and TNF-α production (**Figure 1D**) in a dose-dependent manner and GNF-2 did not show any apparent cytotoxicity in the microglia (**Figure 1C**). Similarly, exposure of BV2 microglia to LPS significantly increased the expression of *IL-1*β mRNA, whereas GNF-2 treatment markedly attenuated LPS-induced upregulation of *IL-1*β (**Figure 1E**). GNF-2 treatment significantly reduced LPS-induced NF-κB activation in BV-2 microglial cells. Notably, LPS-induced NF-κB activation (phosphorylation of IκB and p65) was decreased in microglia following GNF-2 treatment (**Figures 1F–I**). NF-κB activation is associated with nuclear translocation of the p65, a component of the NF-κB complex (Tanaka and Iino, 2016). LPS-induced nuclear translocation

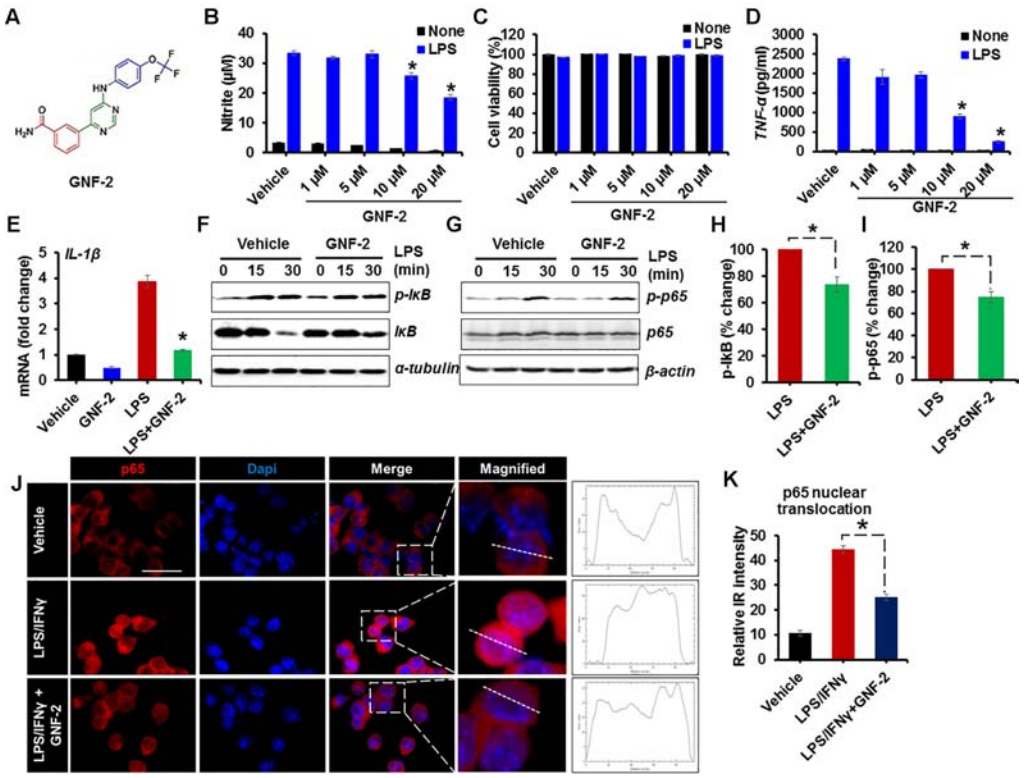

FIGURE 1 | GNF-2 inhibits LPS-induced inflammatory activation of microglia. **(A)** Chemical structure of GNF-2. **(B)** The dose-dependent effect of GNF-2 on BV-2 cells in either the presence or absence of LPS (100 ng/ml) stimulation. **(C)** The cytotoxicity was measured using the MTT assay 24 h after treatment with LPS and the indicated compound concentration. **(D)** TNF-α release was measured by ELISA in BV-2 cells treated with LPS and GNF-2 for 24 h. **(E)** Real-time PCR for IL-1β mRNA expression in BV-2 cells treated with LPS or GNF-2 (10 μM) for 24 h. **(F,G)** Western blot analysis for the phosphorylation of IκB (F) or NF-kB p65 **(G)** in BV-2 cells treated with LPS for 15 and 30 min following 1 h of GNF-2 (10 μM) pre-treatment. IκB degradation was measured by anti-IκB blotting. α-tubulin and β-actin were used as loading control. **(H)** Quantification for the relative % change in p-IkB at 30 min after LPS or GNF-2 treatment. **(I)** Quantification for the relative intensity for p-p65 western blot bands at 30 min after LPS or GNF-2 treatment. **(J)** Immunocytochemistry for p65 in BV-2 cells in either presence or absence of GNF-2 with LPS treatment. Fluorescence intensity profile for p65 across a transverse section of one cell is presented adjacent to the magnified images. The dotted line shows the cross section of the single cell for the fluorescence intensity profile. **(K)** Quantification for the relative IR intensity for the p65 nuclear translocation is presented in the adjacent graph. Nuclear translocation of p65 IF intensity was measured for 10 randomly selected cells from each group with ImageJ. Data are presented as mean ± SEM. *$p < 0.05$ vs. LPS from ANOVA and unpaired two-tailed Student's *t* test; $n = 3$ for each group. Scale bar, 50 μm.

of p65 was measured in BV-2 cells after pretreatment with GNF-2. GNF-2 significantly reduced nuclear p65 expression (**Figures 1J,K**). These findings suggest that GNF-2 attenuates inflammatory activation of microglia induced by LPS through inhibition of c-Abl activity.

Knockdown of c-Abl Expression Inhibits LPS-Induced Glial Activation

It has been documented that the expression of active c-Abl in adult mouse forebrain neurons induces severe and progressive neurodegeneration in the Cornu Ammonis 1 (CA1) region of the hippocampus and reactive gliosis (Schlatterer et al., 2011). However, the function of c-Abl in microglia upon inflammatory stimulation has not been studied yet. To examine the role of c-Abl expression in microglia following inflammatory stimulation, BV-2 cells were transfected with siRNA for c-Abl knockdown and the inflammatory activation of microglia was examined (**Figures 2A,B**). The knockdown of the c-Abl gene expression (more than 50%) in BV-2 cells significantly attenuated LPS-induced nitric oxide production (**Figure 2C**) as well as the expression of pro-inflammatory *TNF*-α mRNA (**Figure 2D**).

Verification of the Anti-inflammatory Effect of GNF-2 in Primary Microglia and Astrocytes

To determine whether the anti-inflammatory effect of GNF-2 is also observed in primary microglia and astrocytes, primary MGCs were treated with various inflammatory stimuli including LPS, TNF-α, or combination of LPS and interferon-gamma (IFN-γ). As in the BV-2 cells, GNF-2 significantly inhibited LPS-induced NO release. In addition, the anti-inflammatory effect of GNF-2 in MGC upon stimulation with recombinant TNF-α protein, an alternative potent inflammatory stimulus, was also assessed. Similarly, GNF-2 significantly inhibited TNF-α-induced NO production in primary glial cells (**Figure 3A**). Further, these findings led us to investigate whether GNF-2 can

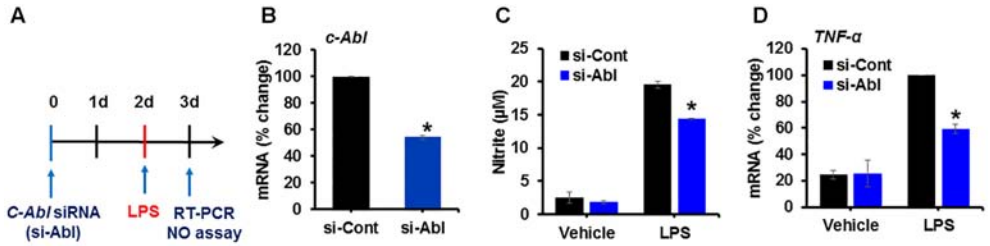

FIGURE 2 | Knockdown of c-Abl expression inhibits LPS-induced inflammatory activation of microglia. **(A)** Experimental scheme. BV-2 cells were transfected with siRNA for the c-Abl gene and then treated with LPS (100 ng/ml) on day 2. Real-time PCR was performed on BV-2 cells on day 3 after transfection. **(B)** c-Abl gene expression was significantly reduced after siRNA transfection. **(C)** Nitric oxide (NO) production was measured in cells treated with LPS for 24 h after siRNA transfection. **(D)** TNF-α mRNA measured by real-time RT-PCR. si-Cont, control siRNA, si-Abl, siRNA for c-Abl gene knockdown. $*p < 0.05$ vs. si-Cont from ANOVA.

inhibit LPS and IFN-γ-induced production of pro-inflammatory mediators in primary glial cells or not. We found that GNF-2 pre-treatment dramatically suppressed such upregulation of *IL-1*β mRNA expression (**Figure 3B**). However, pre-treatment with a methylated GNF-2 analog, mGNF-2 (methylation of the aniline nitrogen at the C4 position of the pyrimidine) (Choi et al., 2009), did not suppress LPS-induced NO production. This is because methylation of GNF-2 abolished the binding specificity of GNF-2 for c-Abl (Choi et al., 2009), suggesting that the effect of GNF-2 is highly specific for c-Abl (**Figure 3C**). Subsequently, the effects of GNF-2 on NF-κB activation after LPS and IFN-γ stimulation was examined in MGCs pre-treated with GNF-2. The pre-exposure of MGCs to GNF-2 strongly inhibited LPS and IFN-γ–induced NF-κB activation. This was assessed by western blot analyses of phosphorylated-p65 and -IκB protein (**Figures 3D–F**). Furthermore, knocking down the c-Abl gene by siRNA also significantly inhibited LPS/IFN-γ-induced NO production (**Figure 3G**), *TNF*-α mRNA expression (**Figures 3H,I**), and NF-κB activation (**Figures 3J–L**) in the primary MGC. Taken together, these findings suggest that GNF-2 may have a potent anti-inflammatory role in glia-mediated neuroinflammation.

Anti-inflammatory Effects of GNF-2 in a Neuroinflammation Model

To investigate the role of c-Abl in neuroinflammation, a mouse model of neuroinflammation induced by the intraperitoneal (IP) administration of LPS was used. First, the expression of c-Abl at the level of the mRNA and protein was examined by using RT-PCR and immunostaining in brain tissues isolated from LPS-injected mice. RT-PCR analysis revealed enhanced expression of *c-Abl* mRNA after 2 days post-LPS injection (**Figures 4A,B**). Similarly, immunofluorescence analysis showed a substantial upregulation of c-Abl protein in the brain cortex following LPS administration (**Figure 4C**). Co-immunostaining analysis revealed that c-Abl is found to be expressed in Iba-1-positive microglial cells, particularly in hyperactivated microglia with amoeboid shape (indicated by arrows in **Figure 4C**). The effect of GNF-2 on microglial activation was further tested *in vivo* using a mouse model of LPS-induced neuroinflammation. As shown in **Figure 5A**, GNF-2 treatment in the pre-treatment group started 24 h before LPS injection. It was administered daily for three more days. The mice were then sacrificed and examined for neuroinflammation. To evaluate the anti-inflammatory effect of GNF-2 *in vivo*, the expression of pro-inflammatory cytokines was measured in brain tissues at the levels of both mRNA and protein. The expression levels of *TNF*-α and *IL-1*β mRNA were significantly diminished following GNF-2 pre-treatment (**Figures 5B,G**). Similarly, GNF-2 pre-treatment significantly reduced the expression of TNF-α protein induced by LPS (**Figures 5C,H**). Our *in vitro* data showed a potent inhibitory effect of GNF-2 on NO production by glial cells upon inflammatory stimulation. To confirm whether GNF-2 can reduce the expression level of iNOS in the mouse brain following LPS administration, immunostaining of brain tissue sections isolated from mice treated with LPS and GNF-2 was performed. The immunostaining analyses revealed an upregulation of iNOS immunoreactivity in the brain cortex after 48 h of LPS injection when compared with vehicle-injected control animals; the immunoreactivity was significantly attenuated in GNF-2-injected mice (**Figures 5D,I**). Further, the levels of Iba-1 and GFAP immunoreactivity were assessed, since they are molecular markers of microglia and astrocyte activation (Rahman et al., 2016). Upon LPS injection, a significant increase in the number of Iba-1-positive microglial cells was observed in the cortex of mice brain, where microglia displayed enhanced Iba-1 immunoreactivity with short and thick processes when compared to control mice (**Figures 5E,J**). These morphological features of the microglia and the increased Iba-1 immunoreactivity in the cortex were attenuated in mice treated with GNF-2. Similarly, the GFAP-positive astrocytes in the cortex of LPS-injected mice showed enhanced immunoreactivity and hypertrophic morphology in comparison to that in the cortex of the vehicle-injected control animals; the immunoreactivity and hypertrophic morphology were both significantly attenuated in GNF-2-treated mice (**Figures 5F,K**). These findings suggest that the intraperitoneal administration of GNF-2 has a potent anti-inflammatory effect on LPS-induced neuroinflammation. The potential brain uptake mechanism of small-molecule compounds is based on physiochemical and molecular properties (Mikitsh and Chacko, 2014). In line with these parameters, GNF-2 is a small-molecule compound with comparatively low molecular weight (374.323) and has a high lipophilicity (Adrian et al., 2006),

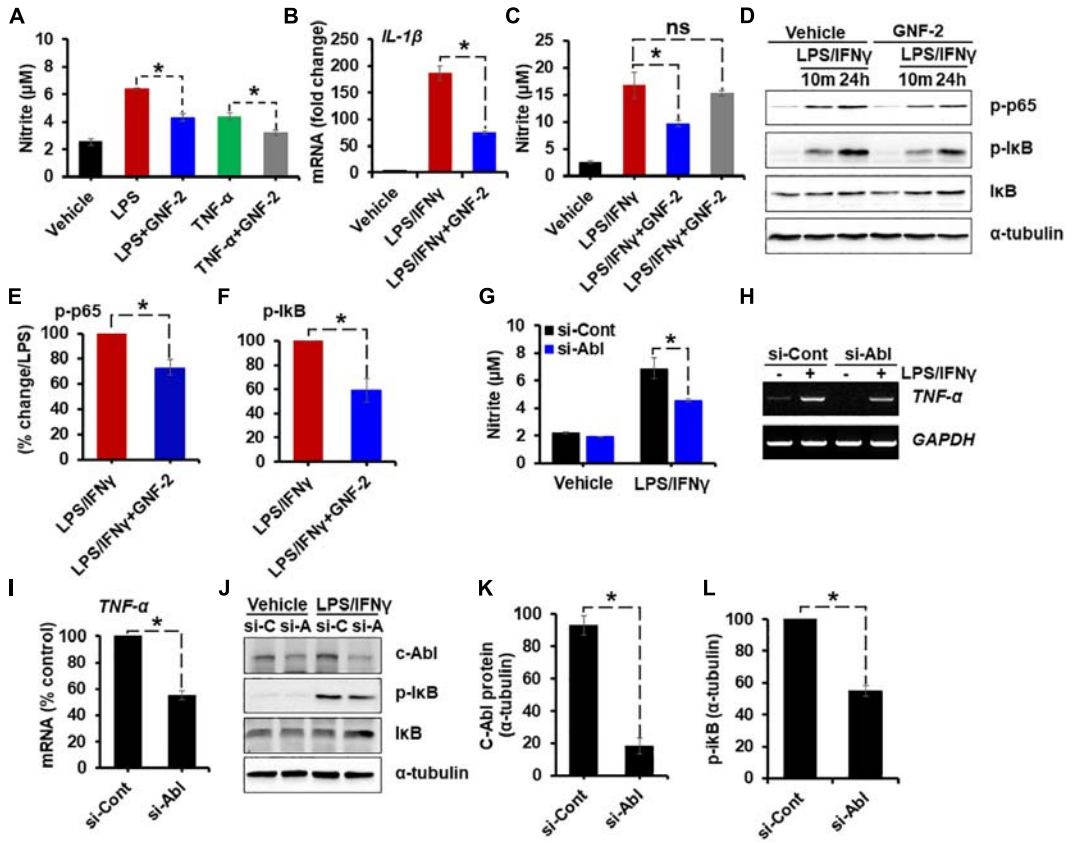

FIGURE 3 | Verification of the anti-inflammatory effect of GNF-2 in primary microglia and astrocytes. Mouse mixed glial cells (MGC) were plated on 96-well plates. **(A)** Nitric oxide (NO) production induced by LPS (1 μg/ml) or TNF-α (10 μg/ml) was significantly reduced by GNF-2 treatment. **(B)** IL-1β mRNA expression induced by LPS (1 μg/ml) and IFN-γ (50 U/ml) was significantly reduced by GNF-2 treatment. **(C)** NO production induced by LPS (1 μg/ml) and IFN-γ (50 U/ml) was significantly reduced by GNF-2 treatment but not by mGNF-2, the methylated version of GNF-2. **(D)** NF-κB activation was reduced by GNF-2 pre-treatment for 1 h. Western blotting was performed to measure phosphorylation of p65 and IκB in MGC after treatment with the indicated reagents. **(E,F)** Quantification for the relative % change of p-p65 **(E)** and p-IκB **(F)** at 24 h after LPS/IFN-γ or GNF-2 treatment. **(G–L)** MGC were transfected with siRNA for the c-Abl gene and incubated for 2 days. Cells were then treated with LPS and IFN-γ for 24 h. siRNA-mediated knockdown of the c-Abl gene significantly reduced LPS/IFN-γ-induced NO production **(G)**, TNF-α mRNA expression **(H)**. **(I)** Quantification for the relative % change of TNF-α mRNA following LPS/IFN-γ treatment presented in the adjacent graph. **(J)** c-Abl expression and phosphorylation of IκB, measured by Western blot analysis. **(K)** Quantification for the relative protein expression of c-Abl after c-Abl gene knockdown following LPS/IFN-γ treatment. **(L)** Quantification for the relative protein expression for p-IκB after c-Abl gene knockdown following LPS/IFN-γ treatment. si-C, control siRNA; si-A, siRNA for c-Abl gene knockdown. Alpha-tubulin was used as the protein loading control. Data are presented as mean ± SEM. $^*p < 0.05$ from ANOVA and unpaired two-tailed Student's *t* test; $n = 3$ for each group. ns, not significant.

which are important criteria for BBB permeability (Mikitsh and Chacko, 2014). In addition, several studies have demonstrated that mice injected with LPS intraperitoneally show blood-brain barrier (BBB) disruption and increased permeability (Jangula and Murphy, 2013; Banks et al., 2015; Varatharaj and Galea, 2017). Thus, BBB disruption and increased permeability in the LPS model may facilitate GNF-2 transportation from circulation to the central nervous system (CNS), thereby enabling the anti-inflammatory effects of peripherally administered GNF-2 on CNS events.

GNF-2 Ameliorates Inflammatory Pain Hypersensitivity

Our *in vitro* and *in vivo* studies strongly indicated that treatment with GNF-2 suppresses neuroinflammation. Based on these observations, the pharmacological efficacy of GNF-2 was validated in a mouse model of CFA-induced chronic inflammatory pain. This model recapitulates several key inflammatory phenotypes including paw edema and pro-inflammatory cytokine release in the hind paw and spinal cord tissues, which has been suggested as a mechanistic consequence of peripheral and CNS pathology of CFA-induced pain hypersensitivity (Jha et al., 2014, 2016). To investigate whether intraperitoneally administering GNF-2 in this mouse model could inhibit CFA-induced pain hypersensitivity (**Figure 6A**), a single injection of GNF-2 (10 mg/kg body weight) was administered 30 min before CFA-administration. GNF-2-injected mice showed significantly diminished CFA (10 mg/kg)-induced paw edema formation (**Figure 6B**), the development of thermal hyperalgesia (**Figure 6C**), and mechanical allodynia (**Figure 6D**). However, the analgesic effect of 1 mg/kg GNF-2

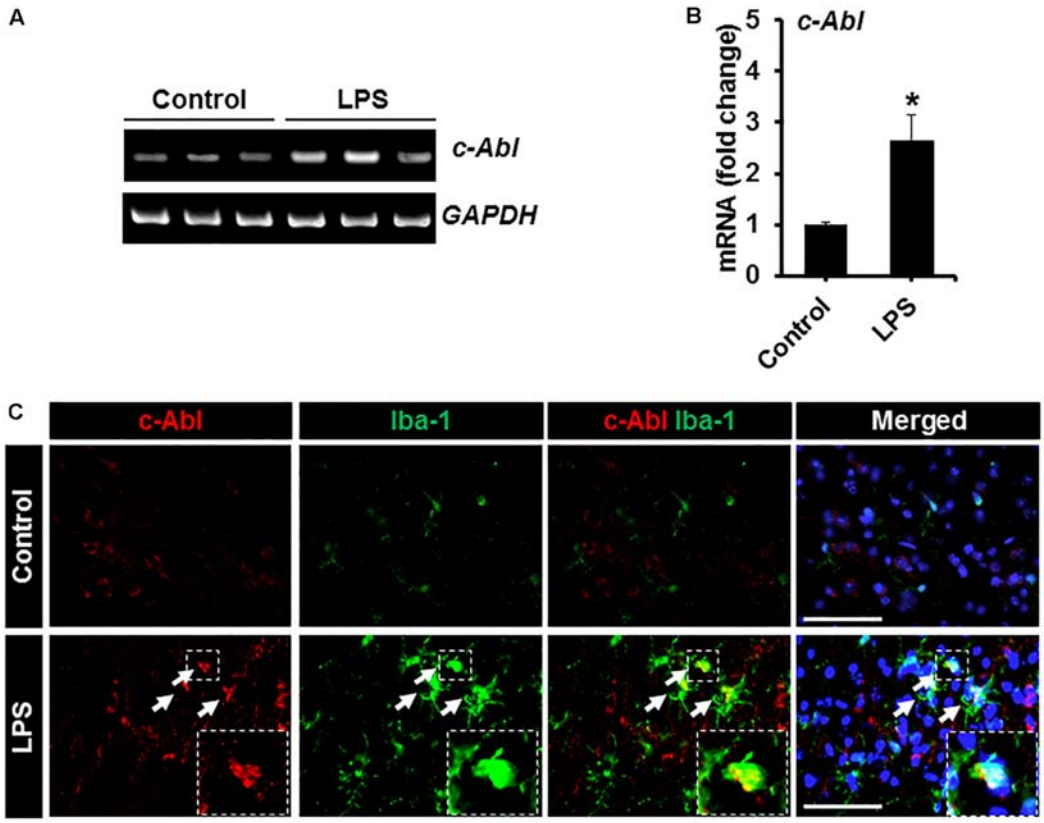

FIGURE 4 | Expression of c-Abl in brain tissues after LPS injection. **(A)** The expression of c-Abl mRNA in brain tissues 48 h after the intraperitoneal injection of LPS was assessed by conventional RT-PCR. **(B)** Quantification for the c-Abl mRNA expression normalized to GAPDH. **(C)** Double immunostaining showed that c-Abl (red) expression co-localized with Iba-1 (green)-positive microglia in the cortex area of mouse brain 48 h post-LPS injection. Arrows indicate the double-labeled cells. The enlarged amoeboid shape of microglia is magnified as indicated in the dotted area. The nuclei were stained with DAPI (blue). $^*p < 0.05$ vs. the vehicle-treated control animals; unpaired two-tailed Student's *t* test; $n = 3$ for each group; data are presented as mean ± SEM. Scale bar, 100 μm.

was partial (**Figures 6C,D**). The withdrawal latency in response to thermal stimuli and the withdrawal threshold in response to mechanical stimuli were unchanged in the contralateral hind paws following CFA and GNF-2 treatment (data not shown). These results demonstrate the crucial role of c-Abl in chronic inflammatory pain.

GNF-2 Attenuates Diabetes-Induced Neuroinflammation and Pain Hypersensitivity

To investigate the role of GNF-2 in neuroinflammation and pain hypersensitivity, an STZ-induced painful diabetic mouse model was also used. The mRNA and protein levels of c-Abl were first examined in spinal cord tissues isolated from mice 2 weeks after STZ injection using RT-PCR and immunostaining (**Figure 7A**). RT-PCR analysis revealed that the induction of diabetes significantly increases the expression of c-Abl mRNA in the spinal cord (**Figures 7B,C**). Similarly, immunofluorescence analysis showed a substantial upregulation of the c-Abl protein in the dorsal horn of the lumbar segment of the spinal cord (**Figure 7D**). In addition, c-Abl was expressed in GFAP-positive astrocytes, particularly those located in the lamina I region of the diabetic spinal cord. Subsequently, the expression levels of pro-inflammatory cytokines and glial activation were examined in the spinal cord of diabetic mice. The expression of *TNF*-α and *IL-1*β mRNAs in the lumbar segment of the spinal cord of mice with diabetes was significantly increased 2 weeks after STZ injection (**Figures 8A,B**). The intraperitoneal administration of GNF-2 significantly decreased the diabetes-induced increase in the expression of pro-inflammatory cytokines such as *TNF*-α and *IL-1*β mRNAs in the spinal cord tissues (**Figure 8B**). To evaluate the effects of GNF-2 on neuroinflammation, we also assessed diabetes-induced changes in glial activation and proliferation in the spinal cord using immunostaining. In diabetic mice, there was a significant increase in the number of Iba-1-positive microglial cells in the dorsal horn of the spinal cord tissues isolated from the lumbar segment, where the microglia displayed enhanced Iba-1 immunoreactivity with reactive morphological changes (**Figure 8C**). Similarly, the number of GFAP-positive astrocytes was markedly increased in the spinal cord dorsal horn of STZ-induced diabetic mice. This increase was accompanied by an increase in GFAP immunoreactivity and hypertrophic morphology with

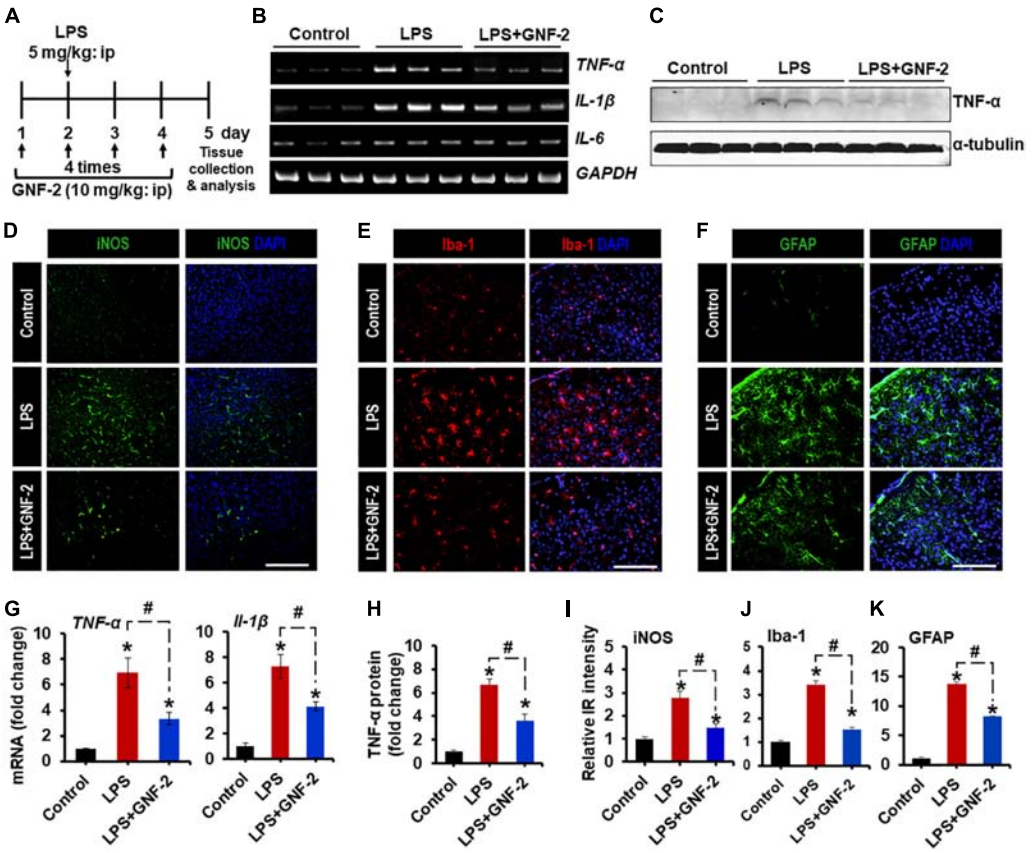

FIGURE 5 | Effects of GNF-2 administration on LPS-induced neuroinflammation *in vivo*. **(A)** To determine the role of c-Abl in neuroinflammation, GNF-2, and LPS were administered intraperitoneally at the indicated time points as shown in the experimental outline. **(B)** The expression of TNF-α and IL-1β mRNAs in the brain tissues after GNF-2 and LPS injection was evaluated by conventional RT-PCR. **(C)** The western blot detection of TNF-α protein in the brain tissue after GNF-2 and LPS injection. **(D–F)** The immunoreactivity (IR) of iNOS, Iba-1, and GFAP was increased in the brain of LPS-injected mice, whereas GNF-2 administration significantly attenuated this increase in IR. The nuclei were stained with DAPI. **(G)** Quantification for the TNF-α and IL-1β mRNA expression is displayed as the fold change of gene expression normalized to GAPDH. **(H)** Quantification for the TNF-α protein from the western blot. **(I–K)** Quantification for the relative intensities of iNOS, Iba-1, and GFAP IR is presented in the graph. $^{*}p < 0.05$ vs. vehicle-treated control animals; $^{\#}p < 0.05$ between the indicated groups; unpaired two-tailed Student's *t* test; $n = 3$ for each group; data are presented as mean ± SEM. Scale bar 400 μm **(D)**, 200 μm **(E,F)**.

thick processes (**Figure 8D**). Notably, GNF-2 administration significantly downregulated Iba-1 and GFAP immunoreactivity in the spinal cord of STZ-injected mice (**Figures 8E,F**). These results obtained through the pharmacological inhibition of c-Abl demonstrate that c-Abl plays a crucial role in diabetes-associated neuroinflammation.

Furthermore, the contribution of c-Abl activation to the pathogenesis of diabetic pain was assessed through the pharmacological inhibition of c-Abl. A single intraperitoneal injection of mice with GNF-2 (10 mg/kg) significantly attenuated diabetes-induced thermal hyperalgesia (**Figure 9A**) as well as mechanical allodynia (**Figure 9B**). However, the vehicle alone did not alter the withdrawal latency in response to thermal stimuli or withdrawal threshold in response to mechanical stimuli. These findings suggest that c-Abl plays a critical role in diabetes-induced neuroinflammation and associated pain hypersensitivity. Thus, GNF-2 might be a potent therapeutic agent for the treatment of chronic pain which results from diabetic peripheral neuropathy.

DISCUSSION

In this study, we show that the upregulation of c-Abl expression promotes the classical pro-inflammatory activation of microglia and that GNF-2, a potent c-Abl inhibitor, attenuates neuroinflammation and pain hypersensitivities in CFA- and STZ- induced pain models. Our findings demonstrate that c-Abl contributes to the pathogenesis of chronic pain by regulating microglial activation and neuroinflammation.

Chronic pain is caused by nerve damage which occurs during nerve compression, diabetes, inflammation, and shingles virus infection (Campbell and Meyer, 2006). Specifically, cytokines, chemokines, prostaglandins, and NO released from activated microglia and astrocytes in the dorsal horn of the spinal cord are known to play important roles in the pathogenesis of chronic pain (Skaper et al., 2012; Vega-Avelaira et al., 2013). Therefore, several studies have targeted activated microglia in order to reduce pain hypersensitivity (Hsieh et al., 2018). For example, treatment with minocycline, a microglial inhibitor,

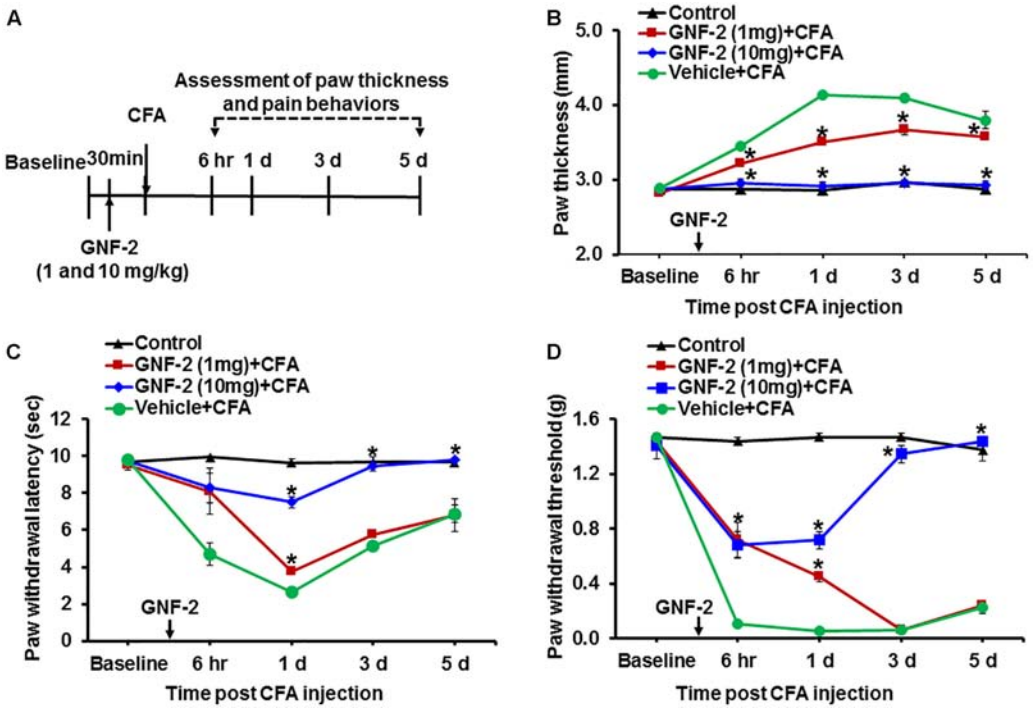

FIGURE 6 | Effects of GNF-2 on CFA-induced paw edema and inflammatory pain behaviors. **(A)** To investigate the role of c-Abl in CFA-induced inflammatory pain phenotypes, GNF-2 were administered into hind paw 30 min before the intraplantar injection of CFA at the indicated time points as shown in the experimental outline. Paw edema and pain responses were measured at 6 h, 1, 3, and 5 d post-CFA injection. In the ipsilateral sides, CFA injection increased paw thickness **(B)** and reduced PWL to heat **(C)** as well as PWT to force **(D)** when compared with vehicle-injected control animals. The CFA-induced paw edema and pain hypersensitivity were attenuated in the GNF-2-injected mice (1 and 10 mg/kg) in a dose-dependent manner. No significant change in paw edema or pain-related behavior was observed in the contralateral sides and vehicle-injected animals. $^{*}p < 0.05$ vs. vehicle + CFA injected animals Student's *t* test; $n = 3$ for each group; data are represented as mean ± SEM. d, day (s).

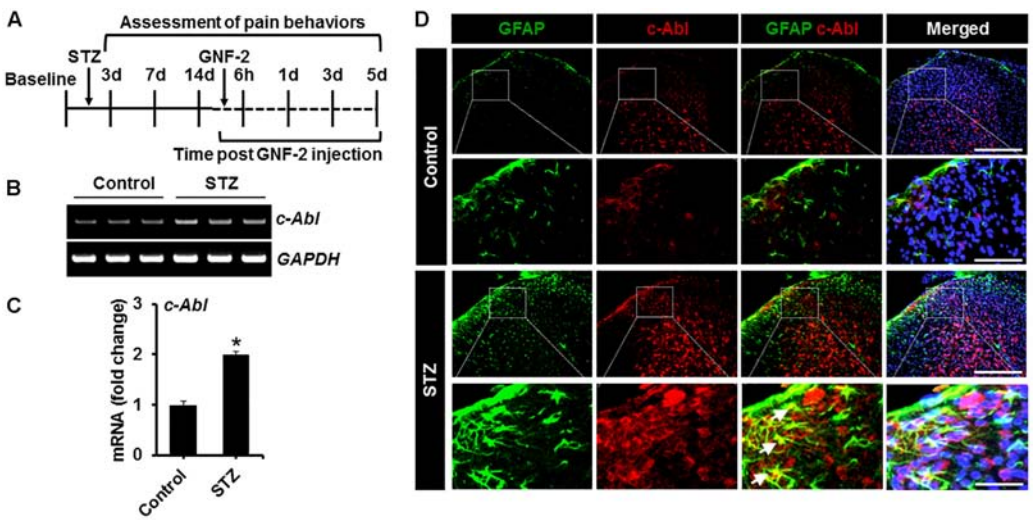

FIGURE 7 | c-Abl expression in the STZ-induced neuropathic pain model. **(A)** STZ and GNF-2 were administered intraperitoneally as shown in the experimental outline. **(B)** The expression of c-Abl mRNA in the spinal cord tissues after STZ injection was evaluated by conventional RT-PCR. **(C)** Quantification for the mRNA expression is displayed as fold change of gene expression normalized to GAPDH. **(D)** Glial expression of c-Abl (arrows) was confirmed by co-staining with GFAP, a marker of astrocyte GFAP (an astrocyte marker). $^{*}p < 0.05$ vs. vehicle-treated control animals; $^{\#}p < 0.05$ between the indicated groups; unpaired two-tailed Student's *t* test; $n = 3$ for each group; data are represented as mean ± SEM. Scale bar, 100 and 200 μm.

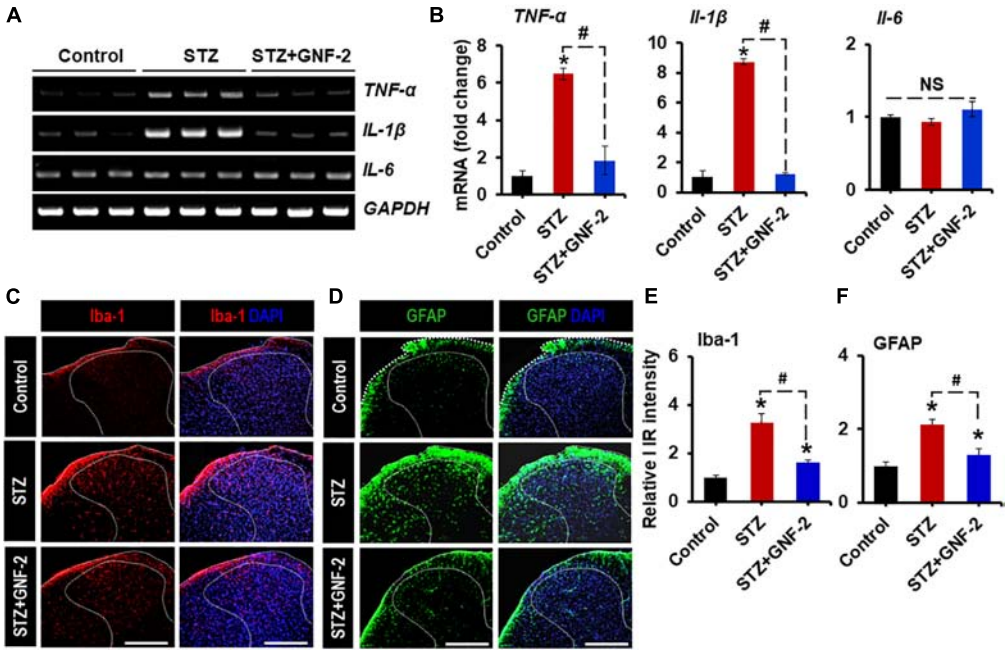

FIGURE 8 | GNF-2 attenuates STZ-induced neuroinflammation. **(A)** The expression of TNF-α, IL-1β, and IL-6 mRNAs in the spinal cord tissues after GNF-2 and STZ injection was evaluated by conventional RT-PCR. **(B)** Quantification for mRNA expression is displayed as the fold increase of gene expression normalized to Gapdh. **(C,D)** Immunoreactivity (IR) of Iba-1 and GFAP was increased in the spinal cord (L4-6) of LPS-injected mice, whereas GNF-2 administration significantly attenuated this increase in IR. **(E,F)** Quantification for the relative intensity of Iba-1 and GFAP IR is presented adjacent to the microscopic images. $^{*}p < 0.05$ vs. vehicle-treated control animals; $^{\#}p < 0.05$ between the indicated groups; unpaired two-tailed Student's *t* test; $n = 3$ for each group; data are represented as mean ± SEM. Scale bar, 100 μm.

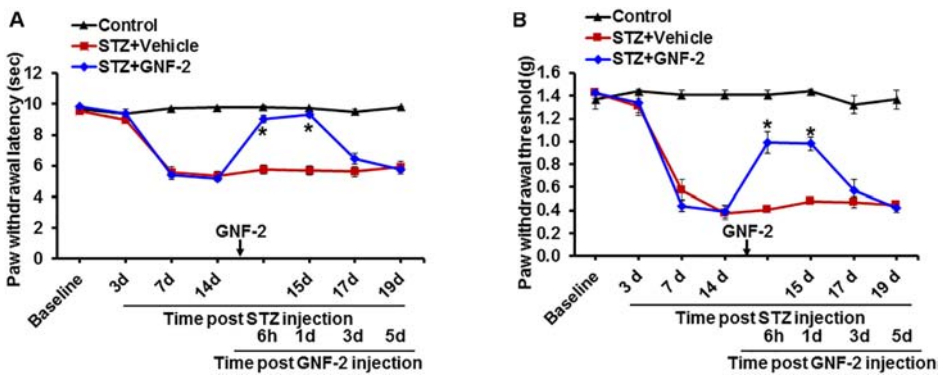

FIGURE 9 | Effects of GNF-2 on STZ-induced diabetic pain. To investigate the role of c-Abl in STZ-induced pain phenotypes, GNF-2 was administered intraperitoneally at the indicated time point. STZ injection reduced PWL to heat **(A)** as well as PWT to force **(B)** when compared with vehicle-injected control animals. The STZ-induced pain hypersensitivity was attenuated in the GNF-2-injected mice (10 mg/kg). No significant change in pain-related behavior was observed in the contralateral sides and vehicle injected animals. $^{*}p < 0.05$ vs. vehicle + STZ-injected animals Student's *t* test; $n = 3$ for each group; data are represented as mean ± SEM. d, day (s).

prevents pain hypersensitivity caused by systemic LPS exposure in neonates, and its protective effect may be related to its ability to attenuate LPS-induced microglial activation, pro-inflammatory cytokine IL-1β, and pain mediator PGE_2. In addition, microglial activation is pivotal to the development and maintenance of allodynia after spinal cord injury through mechanisms involving both TNF-α and IL-1β and in chronic states, IL-6 (Detloff et al., 2008). Previous studies have shown that inflammation and oxidative stress are associated with the overexpression and activation of c-Abl (Schlatterer et al., 2011; Lawana et al., 2017). In fact, in our study, an increase in c-Abl expression was observed in the inflammatory pain models and glial activation and pain hypersensitivity were reduced by the c-Abl inhibitor.

The activation of c-Abl has been reported to play a role in neurodegenerative diseases. For example, in AD,

beta-amyloid (Aβ) activates c-Abl in hippocampal neurons (Alvarez et al., 2004) and c-Abl levels are increased in pre-tangle neurons in AD (Derkinderen et al., 2005). The inhibition of c-Abl activity by Imatinib (STI-571) protects hippocampal neurons from Aβ-induced apoptosis and the reduction of c-Abl mRNA levels protects neuronal cells from Aβ-induced toxicity (Alvarez et al., 2004). Recently, it has been reported that the tyrosine phosphorylation of parkin by the oxidative stress-induced c-Abl is part of a regulatory mechanism in parkin function (Imam et al., 2011). Tyrosine phosphorylation of parkin results in impaired E3-ubiquitin ligase activity and auto-ubiquitination of parkin. c-Abl activation also plays a key role in α-synuclein neurodegeneration. c-Abl overexpression in mice leads to dopaminergic neuron degeneration and α-synuclein pathologies, while c-Abl deletion reduces α-synuclein pathologies. Activation of c-Abl leads to tyrosine 39 phosphorylation of α-synuclein, which strongly correlates with disease progression in hA53Tα-syn transgenic mice, suggesting a critical role of c-Abl in neuronal function and survival (Brahmachari et al., 2016).

The use of c-Abl inhibitors such as imatinib and nilotinib have been proposed for the treatment of AD and PD. Indeed, there have been a few reports which indicated that c-Abl inhibition might be beneficial in PD and α-synucleinopathies (Ko et al., 2010; Hebron et al., 2013). The c-Abl inhibitor, STI-571 (Imatinib), restores the E3 ligase activity of parkin and reduces the accumulation of parkin substrates, thereby protecting against 1-methyl-4-phenylpyridinium (MPP+)-induced neurotoxicity *in vitro* (Ko et al., 2010; Imam et al., 2011). However, in the aforementioned studies, it was difficult to conclude whether c-Abl inhibition could be an effective neuroprotective strategy because of the lack of selectivity of the c-Abl inhibitors they used. Imatinib and nilotinib are potent inhibitors of tyrosine kinases which include c-Abl, Src families, c-Kit, and PDGFR. However, GNF-2, a third-generation c-Abl inhibitor, had no detectable inhibitory effect on the Src family kinases Hck, Lyn, Lck, and c-Src (Choi et al., 2009). Therefore, in the current study, we used GNF-2, which is a more specific c-Abl inhibitor, and siRNA to knockdown c-Abl gene expression in order to investigate the role of c-Abl in neuroinflammation and related pathology.

CONCLUSION

In conclusion, GNF-2 significantly inhibits NF-κB activation and LPS-induced pro-inflammatory molecules including TNF-α and NO in microglia and *in vivo* models of chronic inflammatory and neuropathic pain. Furthermore, we show that GNF-2 very efficiently prevents inflammatory and diabetic pain in animal models. Our results buttress the role of c-Abl in the pathogenesis of neuroinflammatory diseases. These findings indicate that c-Abl can be therapeutically targeted for both the prevention and reversal of chronic pathological pain.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of Animal Care Committee of Kyungpook National University.

AUTHOR CONTRIBUTIONS

KS and GS: conceptualization. GS, MR, MJ, DG, SP, and J-HK: data acquisition and methodology. S-HL, TS, I-KL, YB, W-HL, GS, and KS: investigation. KS: project administration, resources, and supervision. GS and KS: validation. All authors wrote and edited the manuscript.

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REFERENCES

Adrian, F. J., Ding, Q., Sim, T., Velentza, A., Sloan, C., Liu, Y., et al. (2006). Allosteric inhibitors of Bcr-abl-dependent cell proliferation. *Nat. Chem. Biol.* 2, 95–102. doi: 10.1038/nchembio760

Alvarez, A. R., Sandoval, P. C., Leal, N. R., Castro, P. U., and Kosik, K. S. (2004). Activation of the neuronal c-Abl tyrosine kinase by amyloid-beta-peptide and reactive oxygen species. *Neurobiol. Dis.* 17, 326–336. doi: 10.1016/j.nbd.2004.06.007

Banks, W. A., Gray, A. M., Erickson, M. A., Salameh, T. S., Damodarasamy, M., Sheibani, N., et al. (2015). Lipopolysaccharide-induced blood-brain barrier disruption: roles of cyclooxygenase, oxidative stress, neuroinflammation, and elements of the neurovascular unit. *J. Neuroinflammation* 12:223. doi: 10.1186/s12974-015-0434-431

Brahmachari, S., Ge, P., Lee, S. H., Kim, D., Karuppagounder, S. S., Kumar, M., et al. (2016). Activation of tyrosine kinase c-Abl contributes to alpha-synuclein-induced neurodegeneration. *J. Clin. Invest.* 126, 2970–2988. doi: 10.1172/JCI85456

Calsolaro, V., and Edison, P. (2016). Neuroinflammation in Alzheimer's disease: current evidence and future directions. *Alzheimers Dement.* 12, 719–732. doi: 10.1016/j.jalz.2016.02.010

Campbell, J. N., and Meyer, R. A. (2006). Mechanisms of neuropathic pain. *Neuron* 52, 77–92. doi: 10.1016/j.neuron.2006.09.021

Cancino, G. I., Toledo, E. M., Leal, N. R., Hernandez, D. E., Yevenes, L. F., Inestrosa, N. C., et al. (2008). STI571 prevents apoptosis, tau phosphorylation and behavioural impairments induced by Alzheimer's beta-amyloid deposits. *Brain* 131(Pt 9), 2425–2442. doi: 10.1093/brain/awn125

Carniglia, L., Ramirez, D., Durand, D., Saba, J., Turati, J., Caruso, C., et al. (2017). Neuropeptides and microglial activation in inflammation, pain, and neurodegenerative diseases. *Mediators Inflamm.* 2017:5048616. doi: 10.1155/2017/5048616

Chen, G., Zhang, Y. Q., Qadri, Y. J., Serhan, C. N., and Ji, R. R. (2018). Microglia in pain: detrimental and protective roles in pathogenesis and resolution of pain. *Neuron* 100, 1292–1311. doi: 10.1016/j.neuron.2018.11.009

Choi, Y., Seeliger, M. A., Panjarian, S. B., Kim, H., Deng, X., Sim, T., et al. (2009). N-myristoylated c-Abl tyrosine kinase localizes to the endoplasmic reticulum upon binding to an allosteric inhibitor. *J. Biol. Chem.* 284, 29005–29014. doi: 10.1074/jbc.M109.026633

Derkinderen, P., Scales, T. M., Hanger, D. P., Leung, K. Y., Byers, H. L., Ward, M. A., et al. (2005). Tyrosine 394 is phosphorylated in Alzheimer's paired helical filament tau and in fetal tau with c-Abl as the candidate tyrosine kinase. *J. Neurosci.* 25, 6584–6593. doi: 10.1523/JNEUROSCI.1487-05.2005

Detloff, M. R., Fisher, L. C., McGaughy, V., Longbrake, E. E., Popovich, P. G., and Basso, D. M. (2008). Remote activation of microglia and pro-inflammatory cytokines predict the onset and severity of below-level neuropathic pain after spinal cord injury in rats. *Exp. Neurol.* 212, 337–347. doi: 10.1016/j.expneurol.2008.04.009

Fabbro, D., Manley, P. W., Jahnke, W., Liebetanz, J., Szyttenholm, A., Fendrich, G., et al. (2010). Inhibitors of the Abl kinase directed at either the ATP- or myristate-binding site. *Biochim. Biophys. Acta* 1804, 454–462. doi: 10.1016/j.bbapap.2009.12.009

Frank-Cannon, T. C., Alto, L. T., McAlpine, F. E., and Tansey, M. G. (2009). Does neuroinflammation fan the flame in neurodegenerative diseases? *Mol. Neurodegener.* 4:47. doi: 10.1186/1750-1326-4-47

Hebron, M. L., Lonskaya, I., and Moussa, C. E. (2013). Nilotinib reverses loss of dopamine neurons and improves motor behavior via autophagic degradation of alpha-synuclein in Parkinson's disease models. *Hum. Mol. Genet.* 22, 3315–3328. doi: 10.1093/hmg/ddt192

Hsieh, C. T., Lee, Y. J., Dai, X., Ojeda, N. B., Lee, H. J., Tien, L. T., et al. (2018). Systemic lipopolysaccharide-induced pain sensitivity and spinal inflammation were reduced by minocycline in neonatal Rats. *Int. J. Mol. Sci.* 19:2947. doi: 10.3390/ijms19102947

Imam, S. Z., Zhou, Q., Yamamoto, A., Valente, A. J., Ali, S. F., Bains, M., et al. (2011). Novel regulation of parkin function through c-Abl-mediated tyrosine phosphorylation: implications for Parkinson's disease. *J. Neurosci.* 31, 157–163. doi: 10.1523/JNEUROSCI.1833-10.2011

Jangula, A., and Murphy, E. J. (2013). Lipopolysaccharide-induced blood brain barrier permeability is enhanced by alpha-synuclein expression. *Neurosci. Lett.* 551, 23–27. doi: 10.1016/j.neulet.2013.06.058

Jha, M. K., Jeon, S., Jin, M., Ock, J., Kim, J. H., Lee, W. H., et al. (2014). The pivotal role played by lipocalin-2 in chronic inflammatory pain. *Exp. Neurol.* 254, 41–53. doi: 10.1016/j.expneurol.2014.01.009

Jha, M. K., Rahman, M. H., Park, D. H., Kook, H., Lee, I. K., Lee, W. H., et al. (2016). Pyruvate dehydrogenase kinase 2 and 4 gene deficiency attenuates nociceptive behaviors in a mouse model of acute inflammatory pain. *J. Neurosci. Res.* 94, 837–849. doi: 10.1002/jnr.23727

Jha, M. K., Song, G. J., Lee, M. G., Jeoung, N. H., Go, Y., Harris, R. A., et al. (2015). Metabolic connection of inflammatory pain: pivotal role of a pyruvate dehydrogenase kinase-pyruvate dehydrogenase-lactic acid axis. *J. Neurosci.* 35, 14353–14369. doi: 10.1523/JNEUROSCI.1910-15.2015

Jo, M., Kim, J. H., Song, G. J., Seo, M., Hwang, E. M., and Suk, K. (2017). Astrocytic orosomucoid-2 modulates microglial activation and neuroinflammation. *J. Neurosci.* 37, 2878–2894. doi: 10.1523/JNEUROSCI.2534-16.2017

Katsulov, A., and Mazneikova, V. (1987). Treatment of eclamptic convulsions with the so-called lytic cocktail. *Akush. Ginekol.* 26, 71–74.

Katsumata, R., Ishigaki, S., Katsuno, M., Kawai, K., Sone, J., Huang, Z., et al. (2012). c-Abl inhibition delays motor neuron degeneration in the G93A mouse, an animal model of amyotrophic lateral sclerosis. *PLoS One* 7:e46185. doi: 10.1371/journal.pone.0046185

Kiguchi, N., Kobayashi, D., Saika, F., Matsuzaki, S., and Kishioka, S. (2017). Pharmacological regulation of neuropathic pain driven by inflammatory macrophages. *Int. J. Mol. Sci.* 18:2296. doi: 10.3390/ijms18112296

Ko, H. S., Lee, Y., Shin, J. H., Karuppagounder, S. S., Gadad, B. S., Koleske, A. J., et al. (2010). Phosphorylation by the c-Abl protein tyrosine kinase inhibits parkin's ubiquitination and protective function. *Proc. Natl. Acad. Sci. U.S.A.* 107, 16691–16696. doi: 10.1073/pnas.1006083107

Lawana, V., Singh, N., Sarkar, S., Charli, A., Jin, H., Anantharam, V., et al. (2017). Involvement of c-Abl kinase in microglial activation of NLRP3 inflammasome and impairment in autolysosomal system. *J. Neuroimmune Pharmacol.* 12, 624–660. doi: 10.1007/s11481-017-9746-9745

Lee, S., Park, J. Y., Lee, W. H., Kim, H., Park, H. C., Mori, K., et al. (2009). Lipocalin-2 is an autocrine mediator of reactive astrocytosis. *J. Neurosci.* 29, 234–249. doi: 10.1523/JNEUROSCI.5273-08.2009

Mikitsh, J. L., and Chacko, A. M. (2014). Pathways for small molecule delivery to the central nervous system across the blood-brain barrier. *Perspect. Medicin. Chem.* 6, 11–24. doi: 10.4137/PMC.S13384

Mosley, R. L., Benner, E. J., Kadiu, I., Thomas, M., Boska, M. D., Hasan, K., et al. (2006). Neuroinflammation, oxidative stress and the pathogenesis of Parkinson's disease. *Clin. Neurosci. Res.* 6, 261–281. doi: 10.1016/j.cnr.2006.09.006

Rahman, M. H., Jha, M. K., Kim, J. H., Nam, Y., Lee, M. G., Go, Y., et al. (2016). Pyruvate dehydrogenase kinase-mediated glycolytic metabolic shift in the dorsal root ganglion drives painful diabetic neuropathy. *J. Biol. Chem.* 291, 6011–6025. doi: 10.1074/jbc.M115.699215

Rossari, F., Minutolo, F., and Orciuolo, E. (2018). Past, present, and future of Bcr-Abl inhibitors: from chemical development to clinical efficacy. *J. Hematol. Oncol.* 11:84. doi: 10.1186/s13045-018-0624-622

Schlatterer, S. D., Tremblay, M. A., Acker, C. M., and Davies, P. (2011). Neuronal c-Abl overexpression leads to neuronal loss and neuroinflammation in the mouse forebrain. *J. Alzheimers Dis.* 25, 119–133. doi: 10.3233/JAD-2011-102025

Skaper, S. D., Giusti, P., and Facci, L. (2012). Microglia and mast cells: two tracks on the road to neuroinflammation. *FASEB J.* 26, 3103–3117. doi: 10.1096/fj.11-197194

Song, G. J., Nam, Y., Jo, M., Jung, M., Koo, J. Y., Cho, W., et al. (2016). A novel small-molecule agonist of PPAR-gamma potentiates an anti-inflammatory M2 glial phenotype. *Neuropharmacology* 109, 159–169. doi: 10.1016/j.neuropharm.2016.06.009

Song, G. J., and Suk, K. (2017). Pharmacological modulation of functional phenotypes of microglia in neurodegenerative diseases. *Front. Aging Neurosci.* 9:139. doi: 10.3389/fnagi.2017.00139

Tanaka, T., and Iino, M. (2016). Nuclear translocation of p65 is controlled by sec6 via the degradation of ikappaBalpha. *J. Cell. Physiol.* 231, 719–730. doi: 10.1002/jcp.25122

Tansey, M. G., McCoy, M. K., and Frank-Cannon, T. C. (2007). Neuroinflammatory mechanisms in Parkinson's disease: potential environmental triggers, pathways, and targets for early therapeutic intervention. *Exp. Neurol.* 208, 1–25. doi: 10.1016/j.expneurol.2007.07.004

Varatharaj, A., and Galea, I. (2017). The blood-brain barrier in systemic inflammation. *Brain Behav. Immun.* 60, 1–12. doi: 10.1016/j.bbi.2016.03.010

Vargas, L. M., Cerpa, W., Munoz, F. J., Zanlungo, S., and Alvarez, A. R. (2018). Amyloid-beta oligomers synaptotoxicity: the emerging role of EphA4/c-Abl signaling in Alzheimer's disease. *Biochim. Biophys. Acta Mol. Basis Dis.* 1864(4 Pt A), 1148–1159. doi: 10.1016/j.bbadis.2018.01.023

Vega-Avelaira, D., Ballesteros, J. J., and Lopez-Garcia, J. A. (2013). Inflammation-induced hyperalgesia and spinal microglia reactivity in neonatal rats. *Eur. J. Pain* 17, 1180–1188. doi: 10.1002/j.1532-2149.2013.00308.x

Xiao, L., Chen, D., Hu, P., Wu, J., Liu, W., Zhao, Y., et al. (2011). The c-Abl-MST1 signaling pathway mediates oxidative stress-induced neuronal cell death. *J. Neurosci.* 31, 9611–9619. doi: 10.1523/JNEUROSCI.0035-11.2011

Zhang, J., Adrian, F. J., Jahnke, W., Cowan-Jacob, S. W., Li, A. G., Iacob, R. E., et al. (2010). Targeting Bcr-Abl by combining allosteric with ATP-binding-site inhibitors. *Nature* 463, 501–506. doi: 10.1038/nature08675

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Protective Effects of *Phyllanthus amarus* Against Lipopolysaccharide-Induced Neuroinflammation and Cognitive Impairment in Rats

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Background: *Phyllanthus amarus* (PA) is widely studied for its hepatoprotective properties but has recently received increasing attention due to its diverse anti-inflammatory effects. However, the effects of PA in modulating immune responses in the central nervous system leading to protection against functional changes remain unexplored. Therefore, we sought to examine the protective effects of 80% v/v ethanol extract of PA on lipopolysaccharide (LPS)-induced non-spatial memory impairment and neuroinflammation.

Methods: Selected major phytoconstituents of PA extract were identified and quantified using high-performance liquid chromatography. Subchronic neurotoxicity was performed in male Wistar rats given daily oral administration of 100, 200, and 400 mg/kg of the PA extract. Their neurobehavioral activities (functional observation battery and locomotor activity) were scored, and the extracted brains were examined for neuropathological changes. Rats were treated orally with vehicle (5% Tween 20), PA extract (100, 200, and 400 mg/kg), or ibuprofen (IBF; 40 mg/kg) for 14 and 28 days before being subjected to novel object discrimination test. All groups were challenged with LPS (1 mg/kg) given intraperitoneally a day prior to the behavioral tests except for the negative control group. At the end of the behavioral tests, the levels of tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, nitric oxide (NO), inducible nitric oxide synthase (iNOS), CD11b/c integrin expression, and synaptophysin immunoreactivity were determined in the brain tissues.

Results: Gallic acid, ellagic acid, corilagin, geraniin, niranthin, phyllanthin, hypophyllanthin, phyltetralin, and isonirtetralin were identified in the PA extract. Subchronic administration of PA extract (100, 200, and 400 mg/kg) showed no abnormalities in neurobehavior and brain histology. PA extract administered at 200 and 400 mg/kg for 14 and 28 days effectively protected the rodents from LPS-induced memory impairment. Similar doses significantly ($p < 0.05$) decreased the release of proteins like TNF-α, IL-1β, and iNOS in the brain tissue. NO levels, CD11b/c integrin expression, and synaptophysin immunoreactivity were also reduced as compared with those in the LPS-challenged group.

Conclusion: Pre-treatment with PA extract for 14 and 28 days was comparable with pre-treatment with IBF in prevention of memory impairment and alleviation of neuroinflammatory responses induced by LPS. Further studies are essential to identify the bioactive phytochemicals and the precise underlying mechanisms.

Keywords: *Phyllanthus amarus*, neuroinflammation, neuroprotection, non-spatial memory, pro-inflammatory markers

INTRODUCTION

Phyllanthus amarus Schumah & Thonn. (PA) belongs to the Euphorbiaceae family and is traditionally used for kidney ailments, diabetes, pain, jaundice, gonorrhea, chronic dysentery, skin ulcer, and hepatitis B. Recently, the plant has received increasing attention and has been studied for various pharmacological properties such as immunomodulatory, antinociceptive, anti-inflammatory, antioxidant, antibacterial, anticancer, antiulcer, gastroprotective, antifungal, antiplasmodic, antiviral, aphrodisiac, contraceptive, hepatoprotective, antihyperglycemic, antilipidemic, nephroprotective, and anti-amnesic activities (Joshi and Parle, 2007; Patel et al., 2011). Although it demonstrates a wide spectrum of pharmacological actions, the unifying features of all these actions are directed towards the anti-inflammatory and antioxidant properties of the plant. PA contains various phytoconstituents such as lignans, alkaloids, phenolics, terpenes, tannins, flavonoids, sterols, and volatile oils (Patel et al., 2011). Of all these phytochemicals, phyllanthin, hypophyllanthin, corilagin, and geraniin are found in abundance and potentially responsible for the reported anti-inflammatory actions of PA (Patel et al., 2011; Jantan et al., 2014). Most of the anti-inflammatory studies were performed in models of inflammation either *in vitro* or *in vivo*. However, limited available data to substantiate the effects of PA in neuroinflammation have warranted a study to explore the anti-inflammatory actions of PA in the central nervous system (CNS).

Neuroinflammation has been implicated in the pathogenesis of neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), and multiple sclerosis (MS) and of neuropsychiatric disorders such as major depressive disorder, schizophrenia, and bipolar disorder (Radtke et al., 2017). Unlike other systemic immune responses, microglia are the resident macrophage in the brain. Microglial activation forms the basis of many immune-mediated responses of the CNS, which enables it to cope with pathogens, toxins, trauma, and degeneration. However, it is the hyperactivation of microglial cells that initiates excessive production of inflammatory mediators leading to destructive inflammatory responses in the brain (Kempuraj et al., 2016). Additionally, the brain is also exposed to constitutive defense responses, such as systemic inflammation. The systemic inflammation may also lead to the initiation of circulating cytokines, thereby impacting CNS and triggering neuroinflammation. Over the decades, it was evident that the immune system plays a central role in learning, memory modulation, and neural plasticity. Under normal quiescent conditions, immune mechanisms positively regulate the neural circuits remodeling, promotion of memory functions, and neurogenesis (Thomson and Sutherland, 2005). In conditions under which the immune system is strongly activated by infection, injury, or severe or chronic stressful conditions, the inflammatory mediators disrupt the delicate balance needed for neurophysiological actions and produces detrimental effects on memory, neural plasticity, and neurogenesis (Okun et al., 2012).

Bacterial infections initiate innate immune responses including activation of toll-like receptor 4 (TLR4) and the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) in microglia and macrophages, which subsequently provoke the expression of cytokines and generation of nitric oxide (NO) (Parajuli et al., 2012). These pathways play a basic role in the degradation of bacteria by immune cells, which unfavorably influence neuronal death. Lipopolysaccharide (LPS) is a cell wall component of gram-negative bacteria and a ligand of TLR4 that initiates immune responses to infections. As compared with other glial cells, microglial cells are markedly responsive to LPS leading to learning and memory impairment. Intraperitoneal injection of LPS induces secretion of pro-inflammatory cytokines resulting in neuroinflammation, hippocampal apoptosis, cognitive impairment, learning deficits, and even beta-amyloid plaques generation in the hippocampus (Rock et al., 2004; Zarifkar et al., 2010).

Previous studies in our laboratory identified the presence of phyllanthin, hypophyllanthin, gallic acid, geraniin, corilagin, ellagic acid, and niranthin in 80% ethanol extract of PA (Jantan et al., 2014). It was also found that *P. amarus* at doses of 100 to 500 mg/kg for 14 days revealed non-toxic effect with no abnormalities in general behavior and physiology of rats (Ilangkovan et al., 2015). Additionally, a single or daily repeated doses administration of PA for 28 days revealed no morphological changes in histopathological observation of the kidney, liver, and pancreas (Lawson-Evi et al., 2008; Kushwaha et al., 2013). Lack of study for assessment of neurotoxicity of PA has led us to examine the effects of this plant extract on neurobehavior and brain histopathological changes in rats.

Although the anti-inflammatory activities of PA have been documented (Ilangkovan et al., 2015; Harikrishnan et al., 2018), there is a lack of evidence to substantiate similar effects in the CNS. Treatment with PA extract and phyllanthin was found to improve memory impairment and exhibited anticholinesterase activity in young and older mice (Joshi and Parle, 2006; Joshi and Parle, 2007). These are important early findings that demonstrated the plant activity in the brain suggestive of its potential value in

Abbreviations: PA, *Phyllanthus amarus*; LPS, lipopolysaccharide; IBF, ibuprofen; i.p., intraperitoneal injection; CNS, central nervous system; AD, Alzheimer's disease; PD, Parkinson's disease; MS, multiple sclerosis; TLR4, toll-like receptor 4; NF-κB, nuclear transcription factor; HPLC, high-performance liquid chromatography; LOD, limit of detection; LOQ, limit of quantification; NOD, novel object discrimination test, TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; NO, nitric oxide; iNOS, inducible nitric oxide synthase; CD11b/c, cluster differentiation 11b/c.

the prevention and treatment of neurodegenerative diseases. Similarly, other *Phyllanthus* species such as *Phyllanthus niruri* (Ambali et al., 2012) and *Phyllanthus emblica* (Ashwlayan and Singh, 2011) have also been reported to reverse memory deficits induced by scopolamine, sodium nitrite, or chlorpyrifos in different animal models of cognitive behavior, which further support a notion of their neuroprotective role. Therefore, the present study sought to examine the neuroprotective effects of PA extract as compared with IBF, a widely studied nonsteroidal anti-inflammatory drug, for its neuroprotective effects against LPS-induced memory impairment and inflammation in rodents.

MATERIALS AND METHODS

Animals

Adult male Wistar rats weighing 190–200 g (5 weeks old) were obtained from the Laboratory Animal Resource Unit (LARU), Universiti Kebangsaan Malaysia (UKM), Malaysia. The rats were housed in a temperature-controlled room (22–25°C) and exposed to 12 h dark/light cycles. Experiments were carried out on the basis of procedures approved by UKM Animal Ethics Committee. Animals were allowed to acclimatize for 7 days before the initiation of treatment. The animal laboratory was maintained under standard conditions. The studies were performed according to procedures for the use of animals in research as approved by the UKM Animal Ethics Committee with the approval number FF/2017/NORAZRINA/24-MAY/850-JUNE-2017-JULY-2018 for the toxicity assessment in rats and FF/2015/NORAZRINA/20-MAY/683-MAY-2015-MAY-2016 for the efficacy and molecular study done in rats.

Chemicals

LPS from *Escherichia coli* (055:B5) and ibuprofen (IBF; >98% purity) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Radioimmunoprecipitation assay (RIPA) buffer, enzyme-linked immunosorbent assay (ELISA) kits for tumor necrosis factor-α (TNF-α), and interleukin (IL)-1β were obtained from R&D Technology (Minneapolis, MN, USA). Inducible nitric oxide synthase (iNOS), anti-CD11b/c, and synaptophysin were from Abcam (Cambridge, MA, USA). β-Actin was from Cell Signaling Technology (Beverly, MA, USA). Methanol and acetonitrile [high-performance liquid chromatography (HPLC) grade] were brought from Fisher Scientific (Loughborough, UK). Phyllanthin, hypophyllanthin, gallic acid, geraniin, corilagin, ellagic acid, niranthin, phyltetralin, and isonirtetralin were purchased from ChromaDex (CA, USA) with purity > 98%.

Preparation of Extract

The whole plant of PA Schumah & Thonn. was obtained from Marang, Kuala Terengganu, Malaysia, in the month of February 2016. Dr. Abdul Latif Mohamad from the Faculty of Science and Technology, Universiti Kebangsaan Malaysia (UKM), identified the plant, and a voucher specimen (*P. amarus* UKMB 30078) was deposited at the Herbarium of UKM, Bangi, Malaysia. After collection, the plant was allowed to dry for a week and then ground to form the coarse powder. A total of 1 kg of the coarse powder was soaked in 80% ethanol for 9 days. The extract was filtered, and the solvent was changed every 72 h. The solvent was removed using a rotary evaporator, and the extract was subjected to freeze-drying and stored in −20°C.

High-Performance Liquid Chromatography Analysis of 80% Ethanol Extract of *Phyllanthus amarus*

Qualitative and quantitative HPLC analysis was performed according to the method of Jantan and colleagues, with a slight modification (Jantan et al., 2014). Twenty milligrams of PA extract and 1 mg of reference standards (gallic acid, ellagic acid, corilagin, geraniin, niranthin, phyllanthin, hypophyllanthin, phyltetralin, and isonirtetralin) each in 1 mL of methanol were ultra-sonicated for 10 min. Then they were filtered through 0.45-μm Millipore Millex polytetrafluoroethylene membrane (Maidstone, Kent, UK). The diluted extract and the reference standards were analyzed using the following conditions: reversed-phase column, C-18 (100 mm × 4.6 mm inner diameter, 5 μm, XBridge, Waters, Ireland); detector, photodiode array (Waters 2998); wavelength, 220 nm; and flow rate, 1.0 mL/min. Phyllanthin and hypophyllanthin were eluted isocratically (mobile phase A, acetonitrile; B, acidified water with 0.1% orthophosphoric acid) with 5% B, increased to 95% over 20 min, and then followed by 95% for 15 min. In addition to that, gallic acid, ellagic acid, corilagin, geraniin, niranthin, phyltetralin, and isonirtetralin were eluted by gradient method (mobile phase A, acetonitrile; B, acidified water with 0.2% orthophosphoric acid) with 5–70% A (0 to 15 min) and 70% to 95% A (15 to 30 min) and then followed by 95% A for 20 min. Compound identification was done by comparing the retention time and spectra peaks with the reference standards. A calibration curve was plotted with three different concentrations (250, 500, and 1,000 μg/mL) for each standard versus the area under the peaks. The standard curve equations obtained from each bioactive compound were used to quantify the compounds in the extract (Jantan et al., 2014; Yuandani et al., 2016).

High-Performance Liquid Chromatography Validation

Validation was performed by determining the linearity, precision, limit of detection (LOD), and limit of quantification (LOQ). Linear calibration analysis was evaluated to determine the linearity by calculating the correlation coefficient (r^2) from the calibration curve. A calibration curve was plotted with three different concentrations (250, 500, and 1,000 μg/mL) for each standard. The method precision was determined by intra-day and inter-day assays, and they are performed by injecting the extract (20 mg/mL) and standards (1 mg/mL) separately three times in a day and on three different days. The mean values of the area under the peak and retention time obtained from the intra-day and inter-day assay were used to confirm the reproducibility of the results. LOD and LOQ were calculated using the formula LOD = 3.3 × (σ/*S*) and LOQ = 10 × (σ/*S*), where σ = standard deviation of the response and *S* = slope.

Subchronic Neurotoxicity Study

Toxicity tests were conducted according to Organisation for Economic Co-operation and Development 423 guidelines with modified procedure (Organisation for Economic Co-operation and Development, 2002). Twenty adult male Wistar rats were divided into four groups randomly. Group 1 was considered as vehicle control with 5% Tween 20; groups 2, 3, and 4 were administered orally with 100, 200, and 400 mg/kg of PA extract for 28 days. The general behaviors of rats were observed by functional observational battery (FOB) test and followed by histopathological evaluation.

Functional Observational Battery

Neurobehavioral changes were evaluated by FOB, a widely used screening method to identify potential neurotoxicity of new and existing chemicals. The procedure was based on the McDaniel and Moser (1993) protocol with slight modifications. Home-cage movement and hand-held observations were performed in the FOB. The number of line crossings, rearing against a wall, center square entry, grooming, defecation, and urination were observed in the open field activity test for 28 days (McDaniel and Moser, 1993; Brown et al., 1999).

Histopathological Evaluation

After behavioral analysis, rats were sacrificed, and their brains were dissected and weighed. They were immersed in 10% neutral buffered formalin and stored until further analysis. Tissues were processed, embedded in paraffin, and sectioned at 5-µm thickness. The slides were stained with hematoxylin and eosin (H&E) and viewed under a light microscope for semiquantitative histological evaluation (Clausen et al., 2009) and morphological observation of the neurons and pyramidal cells. Responses to insult were evaluated as edema of the brain parenchyma and infiltration of the cortical lesion and hippocampus ipsilaterally by macrophages, neutrophils, and lymphocytes as the signs of inflammation. Evaluation of morphological and pathological changes was performed by an investigator (MAM) blinded to the treatment status of each animal.

Experimental Design for 14 and 28 Days of *Phyllanthus amarus* Extract Treatment

The animals were divided into six groups of eight and received daily oral administration of 5% Tween 20 (negative and vehicle control groups), IBF 40 mg/kg (positive control), and PA extract at different doses (100, 200, and 400 mg/kg). The treatment was scheduled for 14 and 28 days. A day prior to the cognitive behavioral tests, all groups were challenged with 1 mg/kg of LPS given intraperitoneally except the negative control group, which received an intraperitoneal injection of the vehicle. The animals were decapitated at the end of the experiment, and their brains were collected for further analysis.

Cognitive Behavioral Studies

Novel Object Discrimination Task

Novel object discrimination test (NOD) is utilized to assess the non-spatial memory in rodents. This test examines the time spent in exploring the novel object and a familiar object. The method was adopted from previous studies with minor modifications (Ennaceur, 2010; Azmi et al., 2011). NOD was performed in an open box (width × length × height = 40 cm × 40 cm × 40 cm) made of Perspex. The lighting setup was fixed above the ceiling inside the behavior room with constant illumination. The object was a glass bottle made with a distinct color. The floor was divided into nine equal squares by black lines for scoring of locomotor activity. The procedure consisted of three phases: habituation, familiarization, and test phases. Each rat was pre-habituated for 1 h per day before the experiment. On the experiment day, animals were allowed to explore the empty arena for 3 min individually, followed by the familiarization phase, where an animal was allowed to explore two identical objects (A1 and A2) for another 3 min. During the test phase, animals were allowed to explore one identical object that has been explored before (A3) and one novel object (B) inside the arena for 3 min. In between phases, the open field and objects were cleaned with 70% v/v ethanol to eliminate olfactory cues while the rat was returned to its home cage. The location and combination of objects used were alternated between rats to decrease bias. Scoring of exploration was carried out when the rat nose was directed towards the object at a distance of ≤2 cm or touching the object. Turning around, sitting, or climbing on the object was not scored as an exploratory behavior. Their exploratory behavior was scored during a playback of the closed-circuit television (CCTV) footage. The non-spatial memory was determined using the values of discrimination index (DI). DI was calculated for every rat using the formula DI = (B − A3)/(B + A3).

Locomotor Activity

The locomotor activity was measured for each rat by scoring the number of line crossings inside the arena during familiarization and test phases. The activity was scored as the frequency of line crossings with all four paws across the line during a playback of the CCTV footage.

Brain Sample Collection

After the behavioral study, animals were decapitated and their brains dissected into two hemispheres to be stored in −80°C until further analysis. The left and right hemispheres were collected alternately from each group and homogenized in an equal amount of RIPA (R&D Technology, Minneapolis, MN, USA) buffer with protease inhibitor followed by centrifugation at 12,000 rpm for 20 min at 4°C. The supernatant was collected and molecular analysis performed.

Measurement of Cytokines

The concentrations of pro-inflammatory cytokines TNF-α and IL-1β were estimated using ELISA kits (R&D Systems, Minneapolis, MN, USA) as per manufacturer protocol. Cytokine levels were expressed as percentage of inhibition (Achoui et al., 2010; Harikrishnan et al., 2018). The percentage inhibition was calculated as

$$\%\,Inhibition = 100 \times \left\{ \frac{[(cytokine)_{control} - (cytokine)_{sample}]}{(cytokine)_{control}} \right\}$$

Griess Assay

Nitrite levels were measured in the brain tissue using the Griess assay. The Griess reagent consisted of 0.1% *N*-(1-naphthyl) ethylenediamine dihydrochloride, 1% sulfanilamide, and 2.5% phosphoric acid. Equal volume of Griess reagent and brain sample was mixed and incubated at 37°C for 10 min. The absorbance was read at 542-nm wavelength spectrophotometrically in ELISA plate reader. Nitrite levels were expressed as percentage of inhibition (Green et al., 1982; Achoui et al., 2010). The percentage inhibition was calculated as

$$\%\,Inhibition = 100 \times \left\{ \frac{[(NO_2)_{control} - (NO_2)_{sample}]}{(NO_2)_{control}} \right\}$$

Western Blot

Total protein concentrations in the left and right hemispheres were quantified using NanoQuant. Equal amount of protein (60 μg) was separated by 8% and 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membrane. Blots were blocked for 2 h at room temperature with 1% bovine serum albumin (BSA) in tris-buffered saline with Tween 20 (TBST) and washed. The blots were incubated with primary antibody iNOS (1:1,000, Abcam, Cambridge, MA, USA) and synaptophysin (1:500, Abcam, Cambridge, MA, USA) overnight. After being washed, blots were incubated with horseradish peroxidase-conjugated secondary antibody for 2 h. The membrane was then developed for visualizing the protein band in gel doc image analyzer using enhanced chemiluminescence kit (R&D Systems) (Zhu et al., 2016; Harikrishnan et al., 2018).

Immunohistochemistry of Brain Slices

After the behavioral study, two rats from each group were anesthetized with a combination of ketamine, xylazine, tiletamine, and zolazepam. Transcardiac perfusion with normal saline (0.9%) and buffered 4% paraformaldehyde was performed to fix the brain. The whole brain was collected and cryoprotected with 30% sucrose. The hippocampus area was sectioned coronally in the cryostat (30 μm in thickness) and stored in anti-freezing solution. Then the sections were washed twice with 0.1M phosphate-buffered saline (PBS). The sections were blocked with 3% BSA, 0.75% Triton X, and 0.1M PBS for 1 h in 60 rpm at room temperature. They were incubated overnight with mouse monoclonal anti-CD11b/c (1:500, Abcam, Cambridge, MA, USA). The next day, the sections were washed twice and kept for 1-h incubation with Alexa Fluor 488 secondary antibody. Finally, the sections were mounted on slides after being washed and viewed under a confocal microscope (Nikon Microscope ECLIPSE TE 2000-E) with a 20× objective. The immunostained area in the hippocampus for CD11b/c was measured using ImageJ software referred to a particular area (4×10^4 μm^2) (Hernangomez et al., 2016; Shen et al., 2016).

Statistical Analysis

Data obtained were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's *post hoc* test using GraphPad Prism 5. All experimental data were expressed as mean ± standard error of mean (SEM), where "*n*" denotes the number of rats involved in the test. $p < 0.05$ value was set as statistically significant.

RESULTS

Qualitative and Quantitative Analysis of 80% Ethanolic Extract of *Phyllanthus amarus*

Identification of major phytochemicals in 80% ethanol extract of PA was carried out using HPLC. Separation of phyllanthin and hypophyllanthin was identified at 25.423 and 25.617 min, respectively (**Figure 1A**). Gallic acid, geraniin, corilagin, ellagic acid, niranthin, phyltetralin, and isonirtetralin appeared at 11.156, 14.204, 15.273, 16.283, 23.933, 32.157, and 33.628 min, respectively (**Figure 1B**). Compound identification was accomplished by comparing retention time with respective standard compounds (**Table 1**). The LOD and LOQ of the identified compounds are listed in **Table 1**. Among the identified compounds in PA extract, ellagic acid (218.06 μg/mL) content was found to be the highest followed by phyllanthin (162.69 μg/mL), corilagin (158.68 μg/mL), phyltetralin (145.83 μg/mL), niranthin (102.97 μg/mL), hypophyllanthin (95.37 μg/mL), isonirtetralin (71.80 μg/mL), geraniin (67.47 μg/mL), and gallic acid (18.32 μg/mL).

Functional Observational Battery

Rats treated with PA extract for 28 days did not show any abnormal behavioral changes while handling and home-cage movement. No significant changes were observed in body weight, and there was no mortality rate. In locomotor activity, PA extract administered rats did not show any significant changes in the frequency of line crossing, rearing, central square entry, defecation, and urination (**Supplementary Figure S1**).

Histopathological Evaluation of *Phyllanthus amarus*-Treated Brain Tissues

The cerebral cortex and hippocampus were sectioned and stained to identify whether PA extract has shown any morphological changes in rats. The vehicle control group and PA extract-treated group at three different doses did not show any edema or inflammatory changes in the cerebral cortex and hippocampus. In addition, no neuronal morphological changes were observed in the neurons and pyramidal cells (**Supplementary Figure S2**).

Effects of *Phyllanthus amarus* Extract on Cognitive Behavioral Studies

The DI was calculated as mentioned earlier and shown in **Figure 2**. **Figure 2A**,**B** reveals that the group that received LPS alone showed negative DI values, indicating non-spatial memory impairment where the rats spent more time exploring the familiar object than the novel object. Pre-treatment of PA extract (100, 200, and 400 mg/kg) for 14 days demonstrated positive DI,

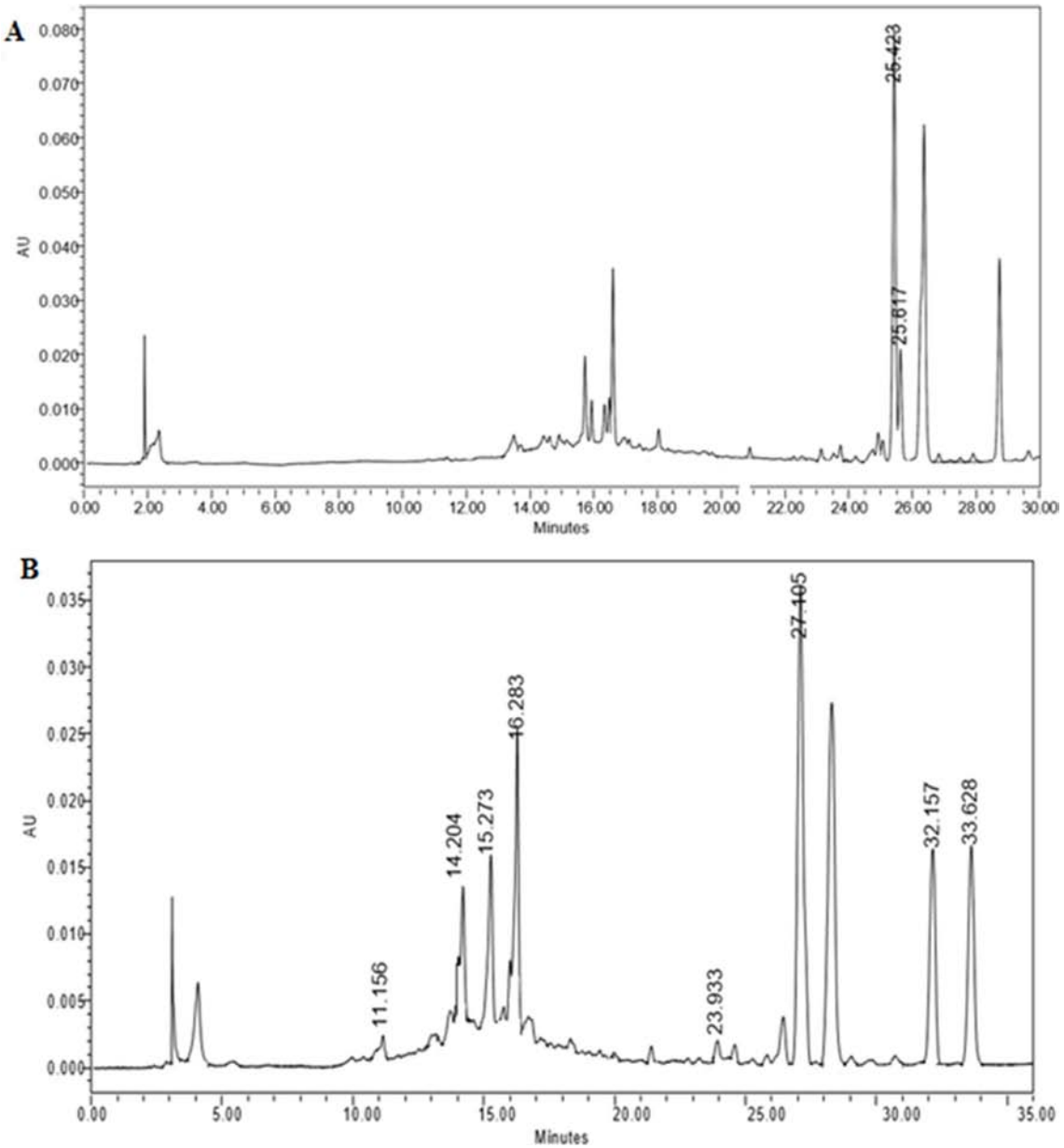

FIGURE 1 | (A) High-performance liquid chromatography (HPLC) chromatogram profiling of *Phyllanthus amarus* (PA) extract with identified retention time of phyllanthin (25.423 min) and hypophyllanthin (25.617 min). **(B)** Retention time of gallic acid (11.156 min), geraniin (14.204 min), corilagin (15.273 min), ellagic acid (16.283 min), niranthin (23.933 min), phyltetralin (32.157 min), and isonirtetralin (33.628 min) in the extract.

indicating that the rats were able to discriminate the novel from familiar objects significantly (*$p < 0.05$, **$p < 0.01$). However, after 28 days of treatment, only groups treated with 200 (*$p < 0.05$) and 400 mg/kg of PA extract showed a positive DI. Taken together, it was evident that PA extract at 200 and 400 mg/kg protected against LPS-induced memory impairment. Similarly, the positive control (**$p < 0.01$) group also demonstrated a positive DI value. Locomotor activity was evaluated by the number of line crossings in the test arena. **Figure 2C**,**D** indicates that all groups demonstrated significantly higher locomotor activity (***$p < 0.001$, **$p < 0.01$) than did the group that received LPS alone.

Effects of *Phyllanthus amarus* Extract on Lipopolysaccharide-Induced Pro-Inflammatory Cytokines

The concentrations of TNF-α and IL-1β proteins were measured in rat brains. Elevation of pro-inflammatory cytokines in the LPS-induced group was observed as an evidence for

TABLE 1 | Limit of detection (LOD) and limit of quantification (LOQ) of the identified compounds.

Standards	RT	Intra-day (n = 3)		Inter-day (n = 3)	
		RT (RSD) (%)	Peak area (RSD) (%)	RT (RSD) (%)	Peak area (RSD) (%)
Gallic acid	11.15	1.297	6.638	1.014	4.742
Geraniin	14.20	0.500	3.501	0.285	4.096
Corilagin	15.27	0.277	1.083	0.294	1.066
Ellagic acid	16.28	2.149	4.507	0.421	4.715
Niranthin	23.93	0.216	0.303	0.164	7.567
Phyllanthin	25.42	0.087	3.792	0.017	5.364
Hypophyllanthin	25.61	0.146	2.104	0.056	4.964
Phyltetralin	32.15	0.714	6.380	0.627	8.230
Isonirtetralin	33.62	0.714	6.380	0.017	7606

RT, retention time; RSD, relative standard deviation.

neuroinflammation through systemic inflammation. PA extract-treated groups showed a significant decrease of TNF-α (***p < 0.001, **p < 0.01, respectively) and IL-1β (*p < 0.05, **p < 0.01, ***p < 0.001, respectively) levels, than did vehicle control in 14- and 28-day treatments. The percentage of inhibition of TNF-α and IL-1β is shown in **Table 2**.

Determination of Nitric Oxide Levels

Administration of PA extract for 14 and 28 days at three different doses showed significant reduction of NO production (**p < 0.01, ***p < 0.001). NO level in the LPS-induced group was greatly increased than in other groups (**Table 3**).

Effects of *Phyllanthus amarus* Extract in Lipopolysaccharide-Induced Changes in Pro-Inflammatory Enzymes and Synaptic Marker

Figure 3 shows the expression of iNOS protein (A and B) and synaptophysin immunoreactivity (C and D) following 14 and 28 days' treatment of PA extract in the brain tissue homogenate. The doses of 100 and 200 mg/kg of PA extract significantly decreased the LPS-induced iNOS protein expression (*p < 0.05, **p < 0.01, ***p < 0.001), respectively, after 14 and 28 days of pre-treatment. The levels of presynaptic marker synaptophysin

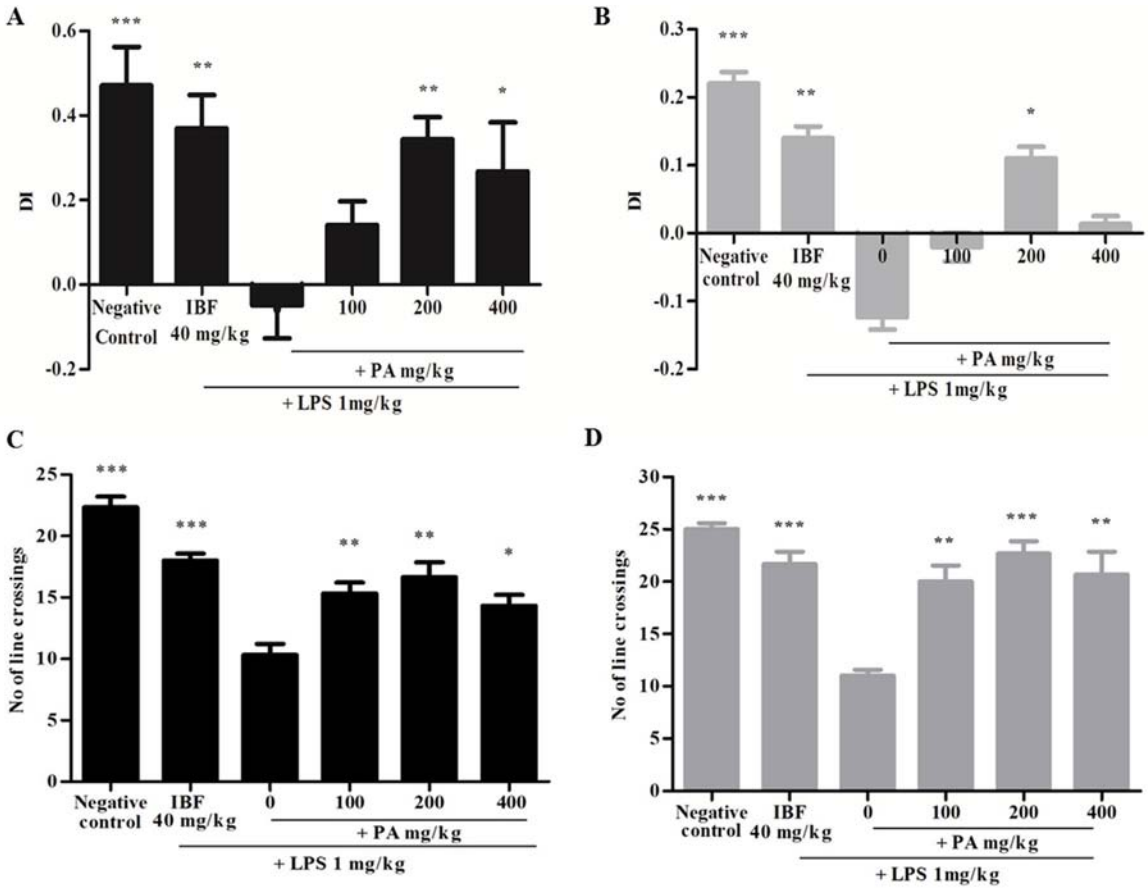

FIGURE 2 | Discrimination index of PA extract treatment after 14 **(A)** and 28 **(B)** days. Data expressed as mean time in seconds ± standard error of the mean [*p < 0.05, **p < 0.01, ***p < 0.001 vs. lipopolysaccharide (LPS), respectively]. Locomotor activity after 14 **(C)** and 28 **(D)** days' treatment of PA extract. Data expressed as the mean number of line crossings ± standard error of the mean (*p < 0.05, **p < 0.01, ***p < 0.001 vs. LPS, respectively). LPS, lipopolysaccharide; IBF, ibuprofen (positive control); and PA, *Phyllanthus amarus* extract.

TABLE 2 | Percentage inhibition of necrosis factor-α (TNF-α) and interleukin (IL)-1β production.

Treatment	Percentage of inhibition (%)			
	14 days' treatment		28 days' treatment	
	TNF-α	IL-1β	TNF-α	IL-1β
Negative control	100***	100***	100***	100***
IBF 40 mg/kg + LPS	78.82 ± 0.43**	76.92 ± 0.51***	78.19 ± 0.57***	69.25 ± 0.58**
Vehicle control (LPS)	0 ± 0.43	0 ± 0.51	0 ± 0.67	0 ± 0.61
PA 100 mg/kg + LPS	77.11 ± 0.52**	72.1 ± 0.74***	69.53 ± 0.81**	55.68 ± 0.46*
PA 200 mg/kg + LPS	87.15 ± 0.32***	79.51 ± 0.43***	80.09 ± 0.72***	75.80 ± 0.61**
PA 400 mg/kg + LPS	81.96 ± 0.63**	75.82 ± 0.41***	74.3 ± 0.71***	72.66 ± 0.52**

*TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1-beta; IBF, ibuprofen; PA, Phyllanthus amarus; LPS, lipopolysaccharide. Data expressed as mean ± standard error of the mean (n = 3); ***p < 0.001 and **p < 0.01 compared with vehicle control (vehicle + LPS).*

TABLE 3 | Percentage inhibition of nitric oxide (NO) production.

Treatment	Percentage inhibition of NO production (%)	
	14 days' treatment	28 days' treatment
Negative control	100***	100***
IBF 40 mg/kg + LPS	69.89 ± 0.15**	68.33 ± 0.22***
Vehicle control (LPS)	0 ± 0.423	0 ± 1.37
PA 100 mg/kg + LPS	67.01 ± 0.11**	59.27 ± 0.08**
PA 200 mg/kg + LPS	74.11 ± 0.07***	73.32 ± 1.55***
PA 400 mg/kg + LPS	67.8 ± 0.31**	64.19 ± 0.66***

*TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1-beta; IBF, ibuprofen; PA, Phyllanthus amarus; LP, lipopolysaccharide. Data expressed as mean ± standard error of the mean (n = 3); ***p < 0.001 and **p < 0.01 compared with vehicle control (vehicle + LPS).*

were significantly decreased by LPS (***$p < 0.001$), whereas in the PA-treated groups, the level of synaptophysin significantly increased (***$p < 0.001$).

Protective Effects of *Phyllanthus amarus* Extract Against Glial Cell Activation

The CD11b/c integrin is expressed in activated microglial as a surface marker. It is an effective marker to recognize microglial activation at the time of neurodegeneration. **Figure 4A**,**B** shows the immunohistochemistry staining and intensity of CD11b/c integrin expression in the hippocampal region of the rat brain after 14 and 28 days' administration of PA extract. The LPS-treated group, as compared with other treated groups, showed small and numerous slender cell morphological features, which clearly explains the presence of activated microglia. Pre-treatment of PA extract of 200 and 400 mg/kg in 14 (*$p < 0.05$) and 28 days (**$p < 0.01$) showed a significant reduction of CD11b/c integrin expression comparable with that of the positive control.

DISCUSSION

PA has been widely studied for its anti-inflammatory activity in *in vitro* and *in vivo* models. A previous study demonstrated that PA prevented memory impairment and possessed anticholinergic and anti-inflammatory properties (Joshi and Parle, 2006). These findings suggested that PA is neuroactive and can alter the brain functions. However, to the best of our knowledge, no studies have been done to determine the effects of PA on neuroinflammation. Therefore, the present study sought to investigate the protective effects of PA extract against memory impairment and immune responses in LPS-induced neuroinflammation in rodents. The biological activities of medicinal plants are usually due to the presence of bioactive phytoconstituents. Similar to other studies, our results showed the presence of major phytoconstituents such as gallic acid, geraniin, corilagin, ellagic acid, niranthin, phyllanthin, hypophyllanthin, phyltetralin, and isonirtetralin in PA. The present finding is in accordance with previous studies reported for the presence of lignans and polyphenols in this species (Jantan et al., 2014). We have also demonstrated that subchronic treatment of PA extract did not show any signs of toxicity in the animal behavior. In addition, no pathological changes were detected in pyramidal cells of the hippocampus and cerebral cortex. A similar study in our laboratory showed that 80% ethanol extract of PA at doses ranging from 100 to 500 mg/kg given orally did not cause any abnormal behavior and mortality in rats (Ilangkovan et al., 2015). Taken together, it indicates that the concentrations of the extract used for this study were within the safe limit.

Novel object discrimination task was performed to assess the rodents' working memory and preferences towards novelty (Ennaceur, 2010). After 14 days' administration of PA extract (100, 200, and 400 mg/kg), rats showed positive preference towards the novel object than did the vehicle control group. However, 28 days' administration of PA extract at 200 mg/kg showed a significant positive preference for the novel object but not with the other doses. It is suggested that pre-treatment with PA protected against LPS-induced impairment in non-spatial memory at an optimum dose of 200 mg/kg and prevented hypolocomotion induced by LPS (Custódio et al., 2013; Tortorelli et al., 2015), which were comparable with the effects of IBF. Cognitive studies done with other *Phyllanthus* species, namely, *P. emblica*, *P. niruri*, and *P. reticulates* have also demonstrated reversal of memory impairment possibly due to the anti-cholinesterase activity of this genus (Ambali et al., 2012; Malve et al., 2014; Uddin et al., 2016).

Cognitive impairment is noticeable in Alzheimer's disease (AD) and also in other disorders where neuroinflammation is proposed to play a prominent role (Ownby, 2010). Initiation of TLR4, which is expressed in astrocytes, microglia, and neuron can antagonistically

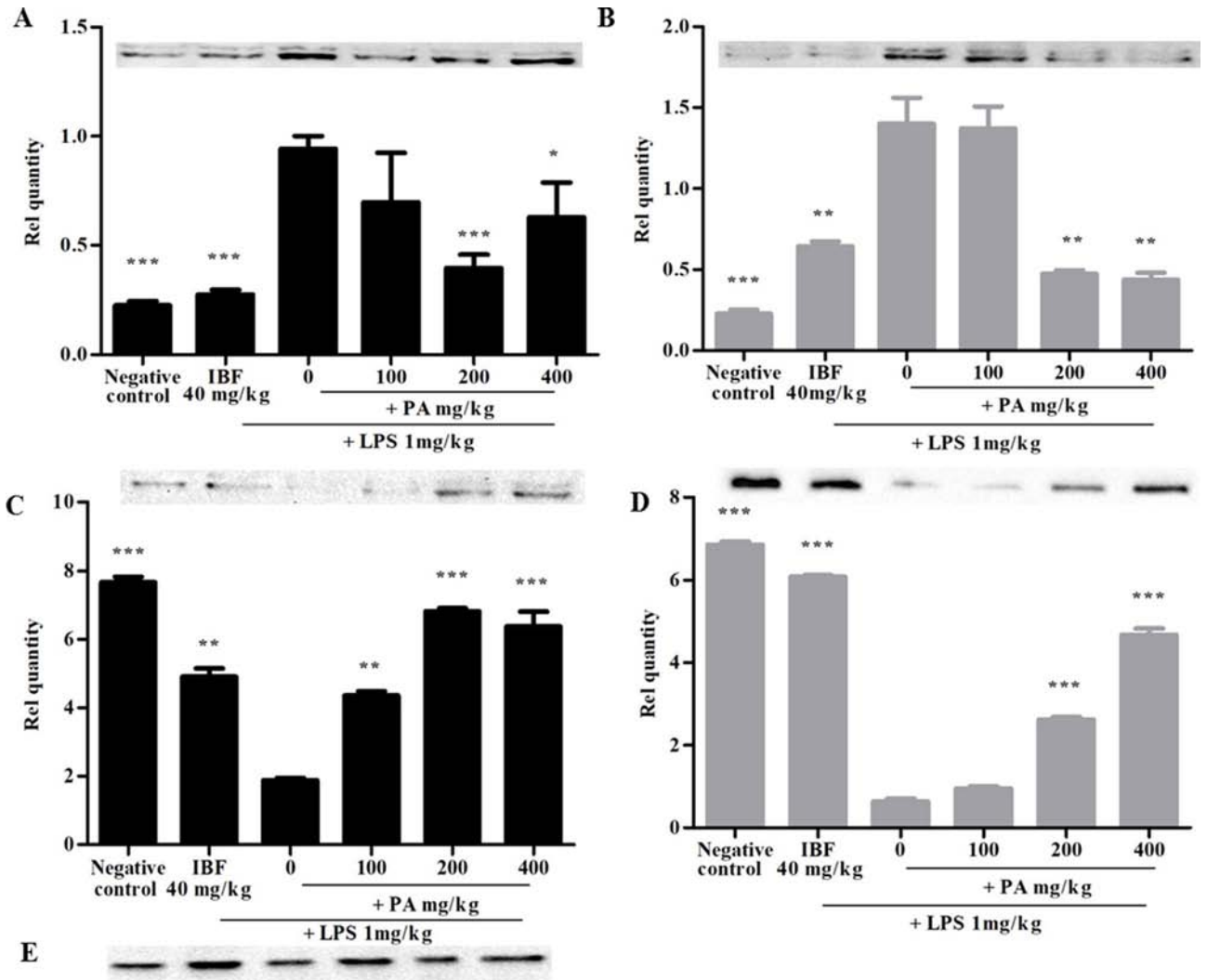

FIGURE 3 | Expression of inducible nitric oxide synthase (iNOS) **(A, B)** and synaptophysin **(C, D)** after 14 **(A, C)** and 28 days' **(B, D)** treatment of PA extract. **E** refers to β-actin (control used for **A**, **B**, **C**, and **D**). Data expressed as mean ± standard error of the mean (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. LPS, respectively). LPS, lipopolysaccharide; IBF, ibuprofen; and PA, *Phyllanthus amarus* extract.

influence neuronal plasticity and survival in brain injury (Okun et al., 2012). Additionally, TLR4 signaling reduces neurogenesis and results in cognitive impairment. As a consequence, microglial cells are activated and release inflammatory mediators like TNF-α, IL-1β, IL-6, iNOS, NO, reactive nitrogen species, and reactive oxygen species which mediates oxidative stress (Zhang et al., 2018). Indeed, an inflammatory response is a common observation in the brain tissue of patients with dementia (Zhao et al., 2018). Likewise, our present study showed that LPS increased the levels of pro-inflammatory cytokines (TNF-α and IL-1β), which act as a central part in the onset and maintenance of inflammation. Related proteins such as iNOS and NO were significantly increased, and a decrease in the level of synaptic marker was noted after exposure to LPS. Indeed, it is known that high concentrations of NO but not the lower concentrations may advance excitotoxicity and result in cognitive impairment. It was suggested that LPS might stimulate the inflammatory cytokines levels in the brain *via* TLR4 activation and induces neuroinflammation (Zhang et al., 2018).

The immune-mediated changes seen with LPS were effectively prevented by pre-treatment of PA extract at 200 and 400 mg/kg for 14 and 28 days. In fact, previous studies have reported that corilagin from PA, ellagic acid, and geraniin were found to decrease the release of TNF-α and IL-β in the brain (Farbood et al., 2015; Tong et al., 2016). Additionally, there is a correlation between pro-inflammatory cytokine release with the decreased level of synaptophysin, which affects memory status (Strużynska et al., 2006). In the present study, we demonstrated that 200 and 400 mg/kg of PA extract given for 14 and 28 days attenuated the LPS-induced pro-inflammatory cytokine release with resulting synaptic loss and memory impairment suggestive of a protective effect against LPS-induced neuroinflammation. Neuroinflammatory conditions, for example, traumatic brain damage, AD, Down syndrome, and

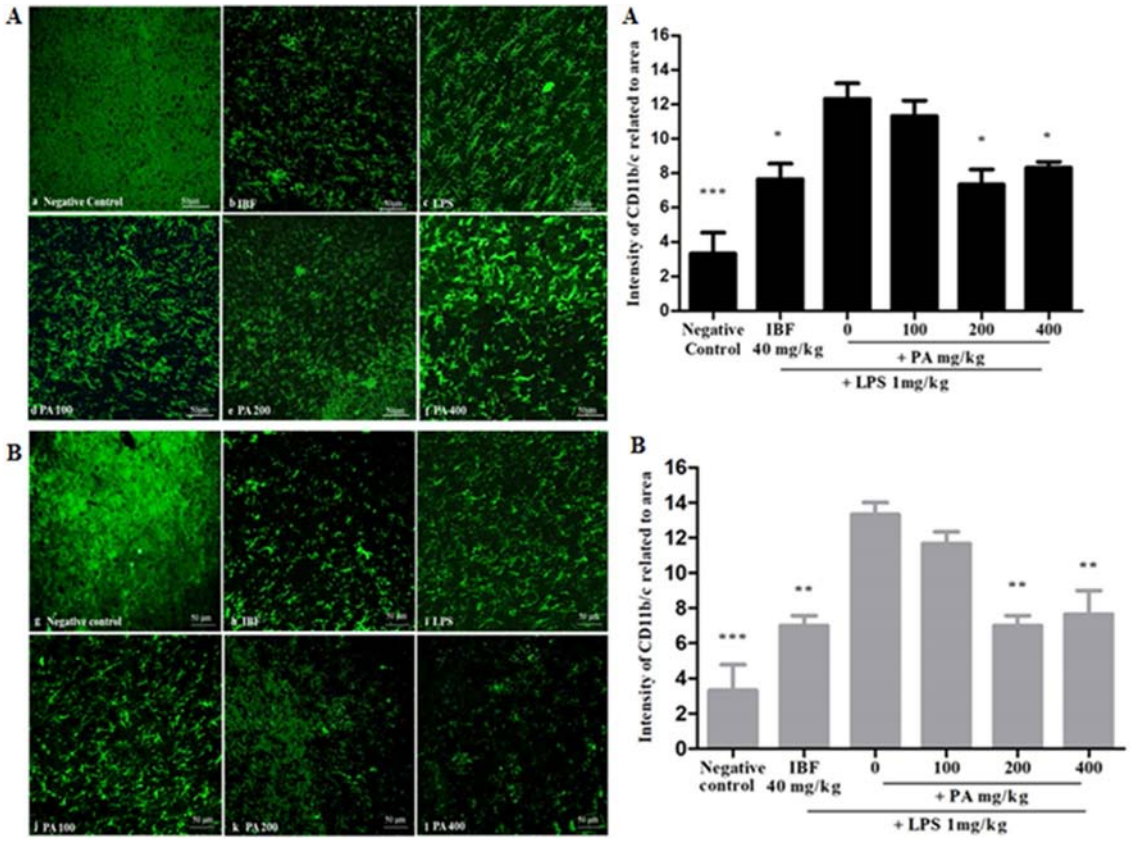

FIGURE 4 | Immunohistochemistry staining of CD11b/c in the hippocampal region of the rat brain (20×): the photomicrographs of brain section presenting the microglial activation in hippocampus after 14 **(A)** and 28 **(B)** days' treatment. Graphical representation **(A, B)** refers to CD11b/c immunoreactivity area in rats' hippocampus. Data expressed as mean ± standard error of the mean (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. LPS, respectively). LPS, lipopolysaccharide; IBF, ibuprofen (positive control); and PA, *Phyllanthus amarus* extract.

aging are often presented with memory impairment. A relationship between cytokine expression in the brain and memory deficits has also been established. For example, it has been demonstrated that the release of TNF-α and IL-1β is a sign of neuroinflammatory-induced memory impairment. Indeed, acute inflammation induced by LPS or IL-1β infusion led to memory impairment (Shaftel et al., 2007). Similarly, previous findings demonstrated that hippocampal IL-1β overexpression hinders contextual and spatial long-term memory (Thomson and Sutherland, 2005). It becomes increasingly evident that prevention of memory impairment observed in the PA-treated groups could be related to immunological changes in the brain.

Astrocytes, microglia, and neurons respond to the acute or chronic stimulation and can aggressively influence neuronal plasticity and surface marker (Okun et al., 2012) resulting in neuroinflammation. CD11b/c is considered as an active integral marker of the innate immune response in microglial cells. Researches revealed that activation of microglia is often noted by expressing the surface marker CD11b/c in either the administration of endotoxins (for example LPS) or injury (Liu et al., 2016). It is believed that microglial activation expresses various proteins and surface markers. Among them, CD11b/c is more sensitive and an intense marker, which plays a role as a binding protein for intracellular adhesion molecule-1 and complement receptor type 3. Therefore, CD11b/c was examined in the hippocampal region of the brain tissue. The outcome of the present study showed that PA extract and IBF attenuated CD11b/c integrin expression in LPS-induced rat brain, which points to a notion that PA inhibits microglial activation similar to IBF. Studies have shown that increased microglial marker CD11b/c are influenced by NO production. However, their complete signaling mechanism is uncertain to researchers (Nillert et al., 2017).

Most of the changes in cognitive function and immunological markers were optimum at 200 mg/kg of PA extract, and increasing the dose by two-fold did not cause any further increase in the effects. It is highly unlikely that the lack of dose–response effects between 200 and 400 mg/kg in many of the parameters measured was due to toxicity. This is evident from our toxicity study that did not demonstrate any neurotoxicity at the highest dose of 400 mg/kg. The lack of dose–response effects could also be due to saturation of receptors at higher concentrations where a similar observation has been reported in other studies (Lima et al., 2016; Manalo et al., 2017). In the present study, it is unclear as to whether the observed effects were produced by the major phytoconstituents of the plant or as a result of a combined effects of various phytochemicals present in the extract. Indeed, this study sought to determine the efficacy

of PA crude extract in modulating inflammatory responses in the brain by measuring only selected markers of neuroinflammation. Although limited, the present findings provide an early indication of the protective actions of PA against non-spatial memory impairment, which may result from inflammatory processes in the brain. It is also unclear if PA alleviated inflammation in the brain by inhibiting TLR4 activation induced by LPS. A different group from our laboratory has similarly demonstrated an anti-inflammatory action of PA through inhibition of LPS-induced responses in human macrophages (Harikrishnan et al., 2018). The study revealed that PA targeted the NF-κB, mitogen-activated protein kinase, and phosphatidylinositol 3-kinase/protein kinase B signaling pathways to exert its anti-inflammatory effects. Therefore, on the basis of our present findings, we suggest that PA exhibited anti-inflammatory actions *via* LPS-induced signaling pathway.

CONCLUSION

Pre-treatment with PA extract for 14 and 28 days was comparable with that of IBF in the prevention of non-spatial memory impairment and alleviation of neuroinflammatory responses induced by LPS. However, further investigations are warranted to support this notion and to better understand the exact protective mechanisms of PA in altering immune and inflammatory responses in the brain. Identifying the bioactive compounds in the plant that are responsible for these effects is also essential in future studies.

ETHICS STATEMENT

The studies were performed according to procedures for the use of animals in research as approved by the UKM Animal Ethics Committee with the approval number FF/2017/NORAZRINA/24-MAY/850-JUNE-2017-JULY-2018 for the toxicity assessment in rats and FF/2015/NORAZRINA/20-MAY/683-MAY-2015-MAY-2016 for the efficacy and molecular study done in rats.

AUTHOR CONTRIBUTIONS

NA, IJ, and EK were involved in the study design, analysis, and interpretation of the data. AA performed the experiments and data analysis. SO participated in the immunohistochemistry work, and MA analyzed the brain histology. The manuscript draft was written by AA and finalized by NA, IJ, EK, SO, and MA.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2019.00632/full#supplementary-material

REFERENCES

Achoui, M., Appleton, D., Abdulla, M. A., Awang, K., Mohd, M. A., and Mustafa, M. R. (2010). *In vitro* and *in vivo* anti-inflammatory activity of 17-*O*-acetylacuminolide through the inhibition of cytokines, NF-κB translocation and IKKβ activity. *PLoS One* 5, e15105. doi: 10.1371/journal.pone.0015105

Ambali, S. F., Makinde, A. O., Shittu, M., Adeniyi, S. A., and Mowuogwu, F. O. (2012). Alleviating effect of *Phyllanthus niruri* on sensorimotor and cognitive changes induced by subacute chlorpyrifos exposure in Wistar rats. *Am. J. Med. Med. Sci.* 2, 50–58. doi: 10.5923/j.ajmms.20120203.05

Ashwlayan, V. D., and Singh, R. (2011). Reversal effect of *Phyllanthus emblica* (Euphorbiaceae) Rasayana on memory deficits in mice. *Int. J. App. Pharm.* 3, 10–15.

Azmi, N., Loh, W. T., Omar, S. S., Jalil, J., and Adam, A. (2011). Effects of aqueous extract of *Prismatomeris glabra* root on non-spatial memory in rats using object discrimination test. *Sains Malays* 40, 1097–1103.

Brown, R. E., Corey, S. C., and Moore, A. K. (1999). Differences in measures of exploration and fear in MHC-congenic C57BL/6J and B6-H-2K mice. *Behav. Genet.* 29 (4), 263–271. doi: 10.1023/A:1021694307672

Clausen, F., Hanell, A., Bjork, M., Hillered, L., Mir, A. K., Gram, H., et al. (2009). Neutralization of interleukin-1β modifies the inflammatory response and improves histological and cognitive outcome following traumatic brain injury in mice. *Eur. J. Neurosci.* 30 (3), 385–396. doi: 10.1111/j.1460-9568.2009.06820.x

Custódio, C. S., Mello, B. S., Cordeiro, R. C., de Araújo, F. Y., Chaves, J. H., Vasconcelos, S. M., et al. (2013). Time course of the effects of lipopolysaccharide on prepulse inhibition and brain nitrite content in mice. *Eur. J. Pharmacol.* 713 (1–3), 31–38. doi: 10.1016/j.ejphar.2013.04.040

Ennaceur, A. (2010). One-trial object recognition in rats and mice: methodological and theoretical issues. *Behav. Brain Res.* 215, 244–254. doi: 10.1016/j.bbr.2009.12.036

Farbood, Y., Sarkaki, A., Dianat, M., Khodadadi, A., Haddad, M. K., and Mashhadizadeh, S. (2015). Ellagic acid prevents cognitive and hippocampal long-term potentiation deficits and brain inflammation in rat with traumatic brain injury. *Life Sci.* 124, 120–127. doi: 10.1016/j.lfs.2015.01.013

Green, L. C., Wagner, D. A., Glogowski, J., Skipper, P. L., Wishnok, J. S., and Tannenbaum, S. R. (1982). Analysis of nitrate, nitrite, and [15N] nitrate in biological fluids. *Anal. Biochem.* 126, 131–138. doi: 10.1016/0003-2697(82)90118-X

Harikrishnan, H., Jantan, I., Haque, M. A., and Kumolosasi, E. (2018). Phyllanthin from *Phyllanthus amarus* inhibits LPS-induced proinflammatory responses in U937 macrophages *via* downregulation of NF-κB/MAPK/PI3K-Akt signaling pathways. *Phytother. Res.* 32, 2510–2519. doi: 10.1002/ptr.6190

Hernangomez, M., Klusakova, I., Joukal, M., Hradilova-Svizenska, I., Guaza, C., and Dubovy, P. (2016). CD200R1 agonist attenuates glial activation, inflammatory reactions, and hypersensitivity immediately after its intrathecal application in a rat neuropathic pain model. *J. Neuroinflammation* 13, 43. doi: 10.1186/s12974-016-0508-8

Ilangkovan, M., Jantan, I., Mesaik, M. A., and Bukhari, S. N. A. (2015). Immunosuppressive effects of the standardized extract of *Phyllanthus amarus* on cellular immune responses in Wistar–Kyoto rats. *Drug Des. Devel. Ther.* 9, 4917. doi: 10.2147/DDDT.S88189

Jantan, I., Ilangkovan, M., and Mohamad, H. F. (2014). Correlation between the major components of *Phyllanthus amarus* and *Phyllanthus urinaria* and their inhibitory effects on phagocytic activity of human neutrophils. *BMC Complement. Altern. Med.* 14, 429. doi: 10.1186/1472-6882-14-429

Joshi, H., and Parle, M. (2006). Evaluation of antiamnestic potentials of [6]-gingerol and phyllanthin in mice. *Nat. Prod.* 2, 109–117.

Joshi, H., and Parle, M. (2007). Pharmacological evidences for antiamnesic potentials of *Phyllanthus amarus* in mice. *Afr. J. Biomed. Res.* 10. doi: 10.4314/ajbr.v10i2.50622

Kempuraj, D., Thangavel, R., Natteru, P. A., Selvakumar, G. P., Saeed, D., Zahoor, H., et al. (2016). Neuroinflammation induces neurodegeneration. *J. Neurol. Neurosurg. Spine* 1, 1003.

Kushwaha, S. K., Dashora, A., Dashora, N., Patel, J. R., and Kori, M. L. (2013). Acute oral toxicity studies of the standardized methanolic extract of *Phyllanthus amarus* Schum & Thonn. *J. Pharm. Res.* 67, 720–724. doi: 10.1016/j.jopr.2013.04.020

Lawson-Evi, P., Eklu-Gadegbeku, K., Agbonon, A., Aklikokou, K., Moukha, S., Creppy, E. E., et al. (2008). Toxicological assessment on extracts of *Phyllanthus amarus* Schum and Thonn. *Sci. Res. Essays* 39, 410–415.

Lima, A. L., Alves, A. F., Xavier, A. L., Mozzini-Monteiro, T., Oliveira, T. R., Leite, F. C., et al. (2016). Anti-inflammatory activity and acute toxicity studies of hydroalcoholic extract of *Herissantia tiubae*. *Rev. Bras. Farmacogn.* 26, 225–232. doi: 10.1016/j.bjp.2015.11.001

Liu, G., Hu, Y., Xiao, J., Li, X., Li, Y., Tan, H., et al. (2016). 99mTc-labelled anti-CD11b SPECT/CT imaging allows detection of plaque destabilization tightly linked to inflammation. *Sci. Rep.* 6, 20900. doi: 10.1038/srep20900

Malve, H. O., Raut, S. B., Marathe, P. A., and Rege, N. N. (2014). Effect of combination of *Phyllanthus emblica*, *Tinospora cordifolia*, and *Ocimum sanctum* on spatial learning and memory in rats. *J. Ayurveda Integr. Med.* 5, 209. doi: 10.4103/0975-9476.146564

Manalo, R. V., Silvestre, M. A., Barbosa, A. L. A., and Medina, P. M. (2017). Coconut (*Cocos nucifera*) ethanolic leaf extract reduces amyloid-β (1-42) aggregation and paralysis prevalence in transgenic *Caenorhabditis elegans* independently of free radical scavenging and acetylcholinesterase inhibition. *Biomedicines* 5, 17. doi: 10.3390/biomedicines5020017

McDaniel, K. L., and Moser, V. C. (1993). Utility of a neurobehavioral screening battery for differentiating the effects of two pyrethroids, permethrin and cypermethrin. *Neurotoxicol. Teratol.* 15 (2), 71–83. doi: 10.1016/0892-0362(93)90065-V

Nillert, N., Pannangrong, W., Welbat, J. U., Chaijaroonkhanarak, W., Sripanidkulchai, K., and Sripanidkulchai, B. (2017). Neuroprotective effects of aged garlic extract on cognitive dysfunction and neuroinflammation induced by β-amyloid in rats. *Nutrients* 9, 24. doi: 10.3390/nu9010024

Organisation for Economic Co-operation and Development (2002). *Test no. 423: acute oral toxicity—acute toxic class method*. Paris, France: OECD Publishing. doi: 10.1787/9789264071001-en

Okun, E., Barak, B., Saada-Madar, R., Rothman, S. M., Griffioen, K. J., Roberts, N., et al. (2012). Evidence for a developmental role for TLR4 in learning and memory. *PLoS One* 7, e47522. doi: 10.1371/journal.pone.0047522

Ownby, R. L. (2010). Neuroinflammation and cognitive aging. *Curr. Psychiatry Rep.* 12 (1), 39–45. doi: 10.1007/s11920-009-0082-1

Parajuli, B., Sonobe, Y., Kawanokuchi, J., Doi, Y., Noda, M., Takeuchi, H., et al. (2012). GM-CSF increases LPS-induced production of proinflammatory mediators *via* upregulation of TLR4 and CD14 in murine microglia. *J. Neuroinflammation* 9 (1), 268. doi: 10.1186/1742-2094-9-268

Patel, J. R., Tripathi, P., Sharma, V., Chauhan, N. S., and Dixit, V. K. (2011). *Phyllanthus amarus*: ethnomedicinal uses, phytochemistry and pharmacology: a review. *J. Ethnopharmacol.* 138, 286–313. doi: 10.1016/j.jep.2011.09.040

Radtke, F. A., Chapman, G., Hall, J., and Syed, Y. A. (2017). Modulating neuroinflammation to treat neuropsychiatric disorders. *BioMed Res. Int.* 2017, 5071786. doi: 10.1155/2017/5071786

Rock, R. B., Gekker, G., Hu, S., Sheng, W. S., Cheeran, M., Lokensgard, J. R., et al. (2004). Role of microglia in central nervous system infections. *Clin. Microbiol. Rev.* 17 (4), 942–964. doi: 10.1128/CMR.17.4.942-964.2004

Shaftel, S. S., Carlson, T. J., Olschowka, J. A., Kyrkanides, S., Matousek, S. B., and O'Banion, M. K. (2007). Chronic interleukin-1β expression in mouse brain leads to leukocyte infiltration and neutrophil-independent blood–brain barrier permeability without overt neurodegeneration. *J. Neurosci.* 27, 9301–9309. doi: 10.1523/JNEUROSCI.1418-07.2007

Shen, Y., McMackin, M. Z., Shan, Y., Raetz, A., David, S., and Cortopassi, G. (2016). Frataxin deficiency promotes excess microglial DNA damage and inflammation that is rescued by PJ34. *PLoS One* 11, e0151026. doi: 10.1371/journal.pone.0151026

Strużynska, L., Dąbrowska-Bouta, B., Koza, K., and Sulkowski, G. (2006). Inflammation-like glial response in lead-exposed immature rat brain. *Toxicol. Sci.* 95, 156–162. doi: 10.1093/toxsci/kfl134

Thomson, L. M., and Sutherland, R. J. (2005). Systemic administration of lipopolysaccharide and interleukin-1β have different effects on memory consolidation. *Brain Res. Bull.* 67, 24–29. doi: 10.1016/j.brainresbull.2005.05.024

Tong, F., Zhang, J., Liu, L., Gao, X., Cai, Q., Wei, C., et al. (2016). Corilagin attenuates radiation-induced brain injury in mice. *Mol. Neurobiol.* 53, 6982–6996. doi: 10.1007/s12035-015-9591-6

Tortorelli, L. S., Engelke, D. S., Lunardi, P., Mello e Souza, T., Santos-Junior, J. G., and Gonçalves, C. A. (2015). Cocaine counteracts LPS-induced hypolocomotion and triggers locomotor sensitization expression. *Behav. Brain Res.* 287, 226–229. doi: 10.1016/j.bbr.2015.03.054

Uddin, M. S., Mamun, A. A., Iqbal, M. A., Islam, A., Hossain, M. F., Khanum, S., et al. (2016). Analyzing nootropic effect of *Phyllanthus reticulatus* Poir. on cognitive functions, brain antioxidant enzymes and acetylcholinesterase activity against aluminium-induced Alzheimer's model in rats: applicable for controlling the risk factors of Alzheimer's disease. *Adv. Alzheimer. Dis.* 5, 87–102. doi: 10.4236/aad.2016.53007

Yuandani, I. J., Ilangkovan, M., Husain, K., and Chan, K. M. (2016). Inhibitory effects of compounds from *Phyllanthus amarus* on nitric oxide production, lymphocyte proliferation, and cytokine release from phagocytes. *Drug Des. Devel. Ther.* 10, 1935. doi: 10.2147/DDDT.S105651

Zarifkar, A., Choopani, S., Ghasemi, R., Naghdi, N., Maghsoudi, A. H., Maghsoudi, N., et al. (2010). Agmatine prevents LPS-induced spatial memory impairment and hippocampal apoptosis. *Eur. J. Pharmacol.* 634, 1–3, 84–88. doi: 10.1016/j.ejphar.2010.02.029

Zhang, Y., Xu, T., Pan, Z., Ge, X., Sun, C., Lu, C., et al. (2018). Shikonin inhibits myeloid differentiation protein 2 to prevent LPS-induced acute lung injury. *Br. J. Pharmacol.* 175, 840–854. doi: 10.1111/bph.14129

Zhao, X., Liao, Y., Morgan, S., Mathur, R., Feustel, P., Mazurkiewicz, J., et al. (2018). Noninflammatory changes of microglia are sufficient to cause epilepsy. *Cell Rep.* 22, 2080–2093. doi: 10.1016/j.celrep.2018.02.004

Zhu, L., Nang, C., Luo, F., Pan, H., Zhang, K., Liu, J., et al. (2016). Esculetin attenuates lipopolysaccharide (LPS)-induced neuroinflammatory processes and depressive-like behavior in mice. *Physiol. Behav.* 163, 184–192. doi: 10.1016/j.physbeh.2016.04.051

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frontiers in Pharmacology

ORIGINAL RESEARCH
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Antidepressant-Like Activity of Myelophil *via* Attenuation of Microglial-Mediated Neuroinflammation in Mice Undergoing Unpredictable Chronic Mild Stress

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Myelophil, a 30% ethanol extract that has an equal rate in both *Astragali Radix* and *Salviae Radix*, is a remedy for the treatment of fatigue-linked disorders in traditional Oriental medicine. The majority of patients with chronic fatigue have a risk of comorbidity with depression symptoms. To evaluate the anti-depressant activity of Myelophil, mice were subjected to unpredictable chronic mild stress (UCMS, eight different stresses) for 3 weeks with daily administration of distilled water, Myelophil (25, 50, or 100 mg/kg), or *n*-acetyl-*l*-cysteine (NAC) (100 mg/kg). After the final stress exposure, three behavioral tests, including the open field test (OFT), forced swimming test (FST), and tail suspension test (TST), and stress-derived alterations of the serotonergic signal and inflammatory response in the hippocampus were measured. UCMS notably induced depressive behaviors, whereas these behavioral alterations were significantly reversed by the administration of Myelophil in regard to the OFT, FST, and TST results. Myelophil also significantly attenuated the over-activation of microglial cells and the inflammatory response in the hippocampal region (TNF-α, tumor necrosis factor-alpha; IL-1β, interleukin-1beta; and caspase-1). Furthermore, Myelophil significantly restored the distortions of serotonergic function in the dorsal raphe nuclei and neurogenesis in the subgranular zone of the hippocampus. These results support the clinical relevance of the anti-depressant activity of Myelophil, specifically by modulating serotonergic function and the neuroinflammatory response.

Keywords: myelophil, depression, anxiety, antidepressants, microglia, serotonin

INTRODUCTION

Depression, a pervasive emotional disorder, is the single largest contributor to the global burden of disease (WHO, 2017). The number of patients who suffer from depressive disorder is estimated to be 322 million worldwide (WHO, 2017). Its major symptoms, such as low mood, a feeling of sadness,

and a loss of interest in things, lead to low socio-economic activity (Nestler et al., 2002). Annually, 0.8 million patients with depressive disorder commit suicide (Klonsky et al., 2016). However, the pathophysiological mechanisms have been unclear to date, and no curative therapeutics exists yet.

As a credible etiology, the hypothalamic–pituitary–adrenal (HPA) axis hypothesis is suggested (Pariante and Lightman, 2008). Accumulating evidence suggests that HPA axis hyperactivity induces the overproduction of brain pro-inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α) and interleukin-1beta (IL-1β), through microglial activation (Brites and Fernandes, 2015; Yirmiya et al., 2015), and this phenomenon has consistently been observed in subjects with depressive disorders (Zou et al., 2018). Recently, the role of Nucleotide-binding oligomerization domain (NACHT), Leucine-rich repeat (LRR), and Pyrin domation (PYD) domains-containing protein 3 (NLRP3) inflammasome-dependent IL-1β has emerged as a novel contributor to depressive disorders (Alcocer-Gomez and Cordero, 2014; Kaufmann et al., 2017). These molecular alterations eventually led to impaired serotonergic synthesis and neurotransmission, which are characteristics of depression (Velasquez and Rappaport, 2016). Therefore, maintenance of serotonergic homeostasis is considered as a main strategy for psychiatric disorders (Nordquist and Oreland, 2010).

Antidepressants have been developed on the basis of serotoninergic signal modulation, while selective serotonin reuptake inhibitors (SSRIs) and serotonin–norepinephrine reuptake inhibitors (SNRIs) represent the majority of the antidepressant market (Artigas, 2013). The global antidepressant market was estimated to be 11.6 billion dollars in 2017 (Mordor-Intelligence, 2017), and serotoninergic modulators account for 90% of market (Artigas, 2013). Nevertheless, current antidepressants have critical limitations, such as extensive adverse effects, poor treatment compliance and remission rate, and a high risk of relapse following drug withdrawal, for example, 61.8% in the case of fluoxetine (Gaynes et al., 2009; Andrews et al., 2012; Berwian et al., 2017).

Medicinal herbs have been attractive as candidates in antidepressant drug development (Lee and Bae, 2017). Myelophil, a 30% ethanol extract of *Astragali Radix* and *Salviae Radix*, is used to treat fatigue-associated disorders, including idiopathic chronic fatigue and chronic fatigue syndrome, in clinics of traditional Korean medicine. We previously presented that Myelophil exerted anti-fatigue in both clinical and experimental studies (Cho et al., 2009; Lee et al., 2015). Besides, our previous findings showed the neuropharmacological actions of Myelophil against hippocampal memory dysfunction, brain oxidative damage, and endocrine abnormality of HPA axis (Kim et al., 2013; Kim et al., 2014; Lee et al., 2014). However, there is no scientific evidence for the pharmacological property of Myelophil in depressive disorder. We hypothesized that Myelophil might have antidepressant-like effects because of the high relevance and comorbidity between chronic fatigue and depression (Demyttenaere et al., 2005; Penner and Paul, 2017).

The present study aimed to investigate the antidepressant-like properties of Myelophil and its underlying mechanisms using an unpredictable chronic mild stress (UCMS)-induced depression mouse model. In order to compare the relative pharmacological potential, NAC was adapted as a positive control.

MATERIALS AND METHODS

Myelophil Preparation and Standardization

Myelophil is composed of 30% ethanol extract in equal amounts of *Astragali Radix* (*Astragalus membranaceus*) and *Salviae Radix* (*Salvia miltiorrhizae*). Myelophil was manufactured by Kyung-Bang Pharmacy (Incheon, Republic of Korea, lot. no. KB-Myelo-1801) according to the approved good manufacturing practice (GMP) guidelines of the Korean Ministry of Food and Drug Safety (MFDS). Fingerprinting analysis of Myelophil was performed to confirm the reproducibility as previously described (Lee et al., 2018). Briefly, ultra-high-performance liquid chromatography (UHPLC, Thermo Scientific, San Jose, CA, USA) coupled with a high-resolution LTQ Orbitrap mass spectrometry (MS) system (Thermo Scientific Co., San Jose, CA, USA) was used, and identifying analysis was conducted with each relative reference compound (rosmarinic acid; salvianolic acid A, B, C, and D; and formononetin).

Animals and Stress Procedure

Forty-eight specific pathogen-free BALB/c male mice (8 weeks old, 22–24 g) were purchased from Dae Han Biolink Co., Ltd. (Eumseong, Republic of Korea). They were housed in plastic cages maintained at 23 ± 1°C with a 12-h light–dark cycle and freely fed food pellets (Cargill Agri Purina, Gyeonggido, Republic of Korea) and water. After acclimation for 1 week, the mice were randomly divided into six groups (n = 8): vehicle, UCMS, Myelophil (25, 50, or 100 mg/kg), and NAC (100 mg/kg, as a positive control) groups.

Animal care and experiments were conducted in accordance with the guidelines issued by the Institutional Animal Care and Use Committee of Daejeon University (Daejeon, Republic of Korea; Approval No. DJUARB 2017-017) and the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health.

The UCMS procedure was conducted as previously described (Nollet et al., 2013) with slight modifications. Briefly, except vehicle group, mice were subjected to a stress paradigm once per day over a period of 3 weeks: continuous illumination during the dark cycle, wet bedding for 24 h, isolation stress for 24 h, 45° tilting for 12 h, food and/or water deprivation for 12 h, restraint stress for 3 h, 4°C cold stress for 1 h, and swimming in cold water for 15 min. Detailed information for stress procedure is indicated in **Supplementary Figure 2**. After the final day of stress, the mice were sequentially subjected to behavioral tests to assess the depression/anxiety-like behavior [open field test (OFT) on day 22, a forced swimming test (FST) on day 23, and a tail suspension test (TST) on day 24]. During the entire experiment period, the mice were orally administered with distilled water (vehicle and UCMS), Myelophil, or NAC, respectively, at 11:00 am every day. The experimental scheme is summarized in **Supplementary Figure 2**.

Open Field Test, Forced Swimming Test, and Tail Suspension Test

The OFT was performed as previously described (Ieraci and Herrera, 2006) with slight modification. The plastic enclosure box for the open field apparatus was contained in the black square side (40 × 40 × 30 cm), and the center of the field was

distinguishable in the recording software. To evaluate the depressive and anxious conditions, each mouse was placed in the center of the field, and their spent time in the center zone and the total distance were subsequently recorded for 5 min at 25-lux illumination using a video camera connected to the corresponding software (Smart Junior).

The FST was performed as previously described (Porsolt et al., 1977; Chatterjee et al., 2012) with slight modification. The apparatus was contained in the plastic cylinder (30 × 30 × 50 cm), and it was filled with tap water at 25 ± 1°C up to 23 cm in height. Individual mouse was allowed to swim for 1 min (pre-test), and the immobility time (passive floating with no additional activity) and global activity (swimming duration) were recorded for 5 min at 30-lux illumination. The behavior between immobility and activity was judged by corresponding software (Smart Junior).

The TST was conducted as previously described (Steru et al., 1985; Chatterjee et al., 2012) with slight modification. The apparatus for the test consisted of a rectangular box (30 × 30 × 50 cm) with a rack on the top. Each mouse was individually suspended for 1 min for pre-test, and behaviors such as immobility and activity were recorded for 5 min at 25-lux illumination by software (Smart Junior).

Animal behavior tracking software (Smart Junior, Panlab SL; Barcelona, Spain) was used for recording distances, speeds, trajectories, and global activity (activity duration). Immobility and activity duration were judged by designated threshold (immobility, 0 to 120; low and high activities, 121 to 300). Behavioral tests were performed by researchers as a blind manner to experimental conditions.

Sample Preparation

After the final behavioral tests, the mice were sacrificed under CO_2 anesthesia on day 25. The serum was collected by centrifugation at 3,000 × *g* for 15 min. The brains of the five mice for each group were immediately removed and dissected to isolate the hippocampal tissue. The sera and hippocampi were stored at −80°C or RNAlater (Ambion, TX, USA) until use. The hippocampal tissue was homogenized in a radioimmunoprecipitation assay (RIPA) buffer, which was used for the biochemical analysis. For the immunohistological analysis, the remaining three mice for each group were subjected to transcardial perfusion with heparin (10 units/mL) and paraformaldehyde (PFA) solution, and the brains were maintained in 4% PFA solution. The total protein concentrations were measured using a bicinchoninic acid protein assay kit (Sigma). The absorbance at 560 nm was measured using a UV spectrophotometer (Molecular Devices Corp., Sunnyvale, CA, USA).

Immunohistological Analysis of DCX, Iba-1, and 5-HT

The brains were gradually cryoprotected in 10%, 20%, and 30% sucrose for each 24-h interval and were subsequently embedded in tissue-freezing medium (Leica Microsystems, Bensheim, Germany) with liquid nitrogen. They were cut into frozen coronal sections (35 μm) using a Leica CM3050 cryostat. The sections were stored in free-floating buffer. For the immunohistological analysis, after washing with ice-cold PBS, the sections were blocked in 5% normal chicken serum (which contained 0.3% Triton X-100 in PBS) for 1 h. After being washed, the sections were incubated with primary antibodies against doublecortin (DCX, 1:200, sc-8066, Santa Cruz Biotechnology), ionized calcium binding adaptor molecule 1 (Iba-1, 1:200, 019-19741, Wako), or 5-hydroxytryptamine (5-HT, 1:200, ab66047, Abcam) overnight at 4°C. The sections were incubated with donkey anti-goat IgG H&L (1:400, Alexa Fluor® 488, ab150129, Abcam) or goat anti-rabbit IgG Horseradish peroxidase (HRP) (1:400, ab6722, Abcam) secondary antibodies for 2 h at room temperature. For the Iba-1-positive signal, the sections were subsequently exposed to an avidin–biotin peroxidase complex (Vectastain ABC kit, Vector Laboratories) for 2 h. The peroxidase activity was visualized using a stable diaminobenzidine solution. For the DCX- and 5-HT-positive signal, the sections were subsequently exposed to 4', 6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) (1:1,000, D9542, Sigma) to stain cell nuclei. Immunoreactions were observed under an Axio-phot microscope (Carl Zeiss, Germany), and the signals were quantified using ImageJ 1.46 software (NIH, Bethesda, MD, USA).

Determination of Corticosterone and Pro-Inflammatory Cytokines

The serum corticosterone level was determined using a DetectX® corticosterone Arbor assay kit (Ann Arbor, MI, USA). The absorbance at 450 nm was measured using a UV spectrophotometer. The levels of pro-inflammatory cytokines in the hippocampal homogenates were determined using commercially available enzyme immunoassay (EIA) kits for TNF-α (BD Biosciences, San Diego, CA, USA) and IL-1β (R&D Systems Inc., Minneapolis, MN, USA), and the absorbance was read within 10 min at 450 and 570 nm using a UV spectrophotometer (Molecular Devices).

Determination of Caspase-1 Activity

The caspase-1 activity in the hippocampal homogenates was measured using a mouse caspase-1 enzyme-linked immunosorbent assay kit (Novus Biologicals, Littleton, CO, USA). The absorbance at 450 nm was measured using a UV spectrophotometer (Molecular Devices).

Western Blotting Analysis

The protein expressions of NACHT, LRR, and PYD Domains-Containing Protein 3 (NLRP3), apoptosis-associated Speck-like protein containing a CARD (ASC), pro-IL-1β and mature IL-1β, and β-actin in the hippocampal homogenates were evaluated using a western blotting method. The protein concentration of the homogenates was equalized, and the samples were separated by 10% polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride (PVDF) membranes. To minimize the non-specific binding, the membrane was blocked in 5% bovine serum albumin for 1 h. The membranes were incubated with primary antibodies, such as NLRP3 (1:500, ab214185, Abcam), ASC (1:500, sc-271054, Santa Cruz), pro-IL-1β and mature IL-1β (1:1,000, ab9722, Abcam), or β-actin (1:2500, PA1-183, Thermo-Fisher Scientific), overnight at 4°C. After being washed, the

membranes were incubated with an HRP-conjugated anti-rabbit or anti-mouse antibody (GeneTex, Inc., Irvine, CA) for 1 h. The western blotting results were visualized with an enhanced chemiluminescence (ECL) advanced kit. The intensity was analyzed with ImageJ version 1.46 (NIH, Bethesda, MD, USA).

Determination of Nitric Oxide in BV2 Microglial Cells

Mouse microglia cells (BV2 cell line) were cultured in DMEM supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin–streptomycin. BV2 cells were incubated at 37°C under 5% CO_2. The BV2 cells were seeded at 2×10^4 cells/well into 96-well microplates. After incubation for 12 h, the BV2 cells were pre-treated with Myelophil (5, 10, or 20 μg/mL) or NAC (100 μM) for 2 h. After exposure to the gram-negative lipopolysaccharide bacteria (LPS, 1 μg/mL) for 24 h, the nitric oxide levels of the cell supernatants were determined using the previously described method (Green et al., 1982). Briefly, the cell supernatant was responded by the Griess reagent [1% sulfanilamide, 0.1% *N*-(1-naphthyl) ethylenediamine hydrochloride, and 2.5% H_3PO_4] at 37°C for 20 min, and the resulting purple azo dye product was subsequently measured at 540 nm using a UV spectrophotometer (Molecular Devices).

Statistical Analysis

Data are expressed as the mean ± standard error of the mean (SEM). The statistically significant differences between the groups were evaluated by one-way analysis of variance (ANOVA) followed by *post hoc* multiple comparisons with Tukey's honestly significant difference (HSD) test using IBM SPSS statistics software, ver. 25.0 (SPSS Inc., Chicago, IL, USA). Differences at $p < 0.05$ indicate statistical significance.

RESULTS

Compounds Present in Myelophil

The major peaks and their retention times, formulae, and molecular weight were consistent with previous analysis (Lee et al., 2018). Six major peaks were detected at 7.47, 8.14, 8.27, 8.71, 9.38, and 12.65 min of retention time under the UV wavelength of 254 nm. Their mass to charge ratios (*m*/*z*) were analyzed in 419.0969 (salvianolic acid D), 361.0917 (rosmarinic acid), 493.1125 (salvianolic acid C), 719.1604 (salvianolic acid B), 495.1276 (salvianolic acid A), and 269.0806 (formononetin) *m*/*z* as displayed in **Supplementary Figure 1**.

Effects on Depressive and Anxious-Like Behaviors

As an anxiety-based test, the OFT was conducted, and the UCMS significantly decreased the total distance [$F(5, 42) = 12.68$; $p < 0.001$] ($p < 0.01$) and time spent in the center zone [$F(5, 42) = 5.69$; $p < 0.001$] ($p < 0.05$) compared with those in the vehicle-treated group. These reductions were completely reversed by the treatment of Myelophil, particularly at the dose of 100 mg/kg, compared with those in the UCMS group ($p < 0.05$ and $p < 0.01$, respectively, **Figure 1A** and **B**).

To evaluate depressive-like behaviors, the forced swimming test (FST) and tail suspension test (TST) were performed. Regarding the FST results, the UCMS significantly reduced the global activity [$F(5, 42) = 18.59$; $p < 0.001$] and elevated immobility time [$F(5, 42) = 19.96$; $p < 0.001$] compared with those in the vehicle-treated group ($p < 0.01$ for both parameters). Myelophil treatment, however, showed noteworthy antidepressant-like activity particularly for the 100 mg/kg dose ($p < 0.01$, **Figure 1C** and **D**).

A low global activity [$F(5, 42) = 42.55$; $p < 0.001$] and prolonged immobility time [$F(5, 42) = 55.84$; $p < 0.001$] in the TST were induced by the UCMS, and they were significantly different compared with those in the vehicle-treated group ($p < 0.01$ for both parameters). Myelophil treatment (100 mg/kg) significantly attenuated these behavioral alterations compared with those in the UCMS group ($p < 0.01$, **Figure 1E** and **F**). NAC also showed positive effects in the depressive and anxious-like behaviors compared with those in the vehicle-treated group; however, these effects were not significant in all tests.

Effects on Microglial Activation in Hippocampus

The UCMS remarkably increased the microglial activation in cornus ammonis (CA)1 [$F(5, 12) = 20.52$; $p < 0.001$], dentate gyrus [$F(5, 12) = 23.88$; $p < 0.001$], and CA3 region [$F(5, 12) = 14.28$; $p < 0.001$] of the hippocampus ($p < 0.01$ for all regions). Hyper-activation of microglia cell was significantly attenuated by the Myelophil treatment especially in doses of 50 and 100 mg/kg ($p < 0.05$ or $p < 0.01$, **Figure 2A** and **B**). NAC also showed similar effects against microglial activation.

Effects on Serum Corticosterone

The UCMS significantly increased the serum corticosterone (2.9-fold) compared with that in the vehicle-treated group [$F(5, 24) = 4.69$; $p = 0.004$] ($p < 0.01$), while the Myelophil-treated group showed significant reductions for the high corticosterone level ($p < 0.05$ for both 50 and 100 mg/kg, **Figure 3A**). Treatment with NAC also showed similar effects as Myelophil.

Effects on Nitric Oxide in BV2 Microglia Cells

The LPS induced an inflammatory response in BV2 cells, as evidenced by the increase of nitric oxide [$F(5, 24) = 99.67$; $p < 0.001$] (4.3-fold, $p < 0.01$) in the cell supernatant. Pretreatment with Myelophil inhibited the highly increased nitric oxide level than did the LPS-treated cells ($p < 0.01$ for all doses, **Figure 3B**). Nitric oxide scavenging activity was also present in NAC-pretreated cells similar to Myelophil.

Effects on Pro-Inflammatory Cytokines in Hippocampus

The UCMS-subjected group showed significant elevations of TNF-α [$F(5, 24) = 9.29$; $p < 0.001$] (1.9-fold) and IL-1β

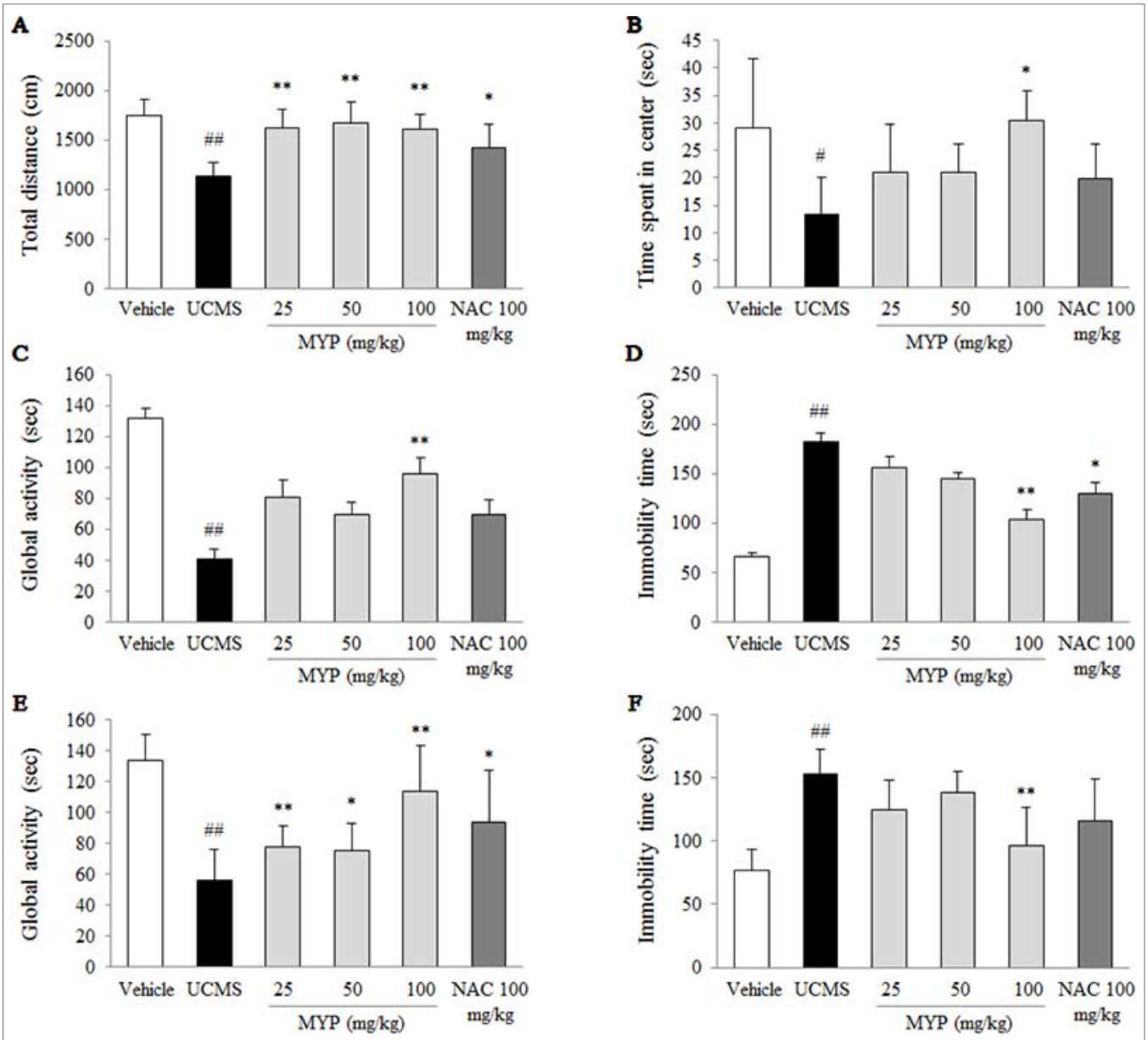

FIGURE 1 | Depressive and anxiety behavioral tests. After unpredictable chronic mild stress (UCMS) with/without oral administration of Myelophil (MYP) or *n*-acetyl-*l*-cysteine (NAC) for 21 days, the total distance **(A)** and spent time in the center zone **(B)** in the open field test (on the 22nd day), the global activity **(C)** and immobility time **(D)** in the forced swimming test (on the 23rd day), and the global activity **(E)** and immobility time **(F)** in the tail suspension test (on the 24th day) were assessed. The data are expressed as the mean ± standard error of the mean (SEM) (n = 8). Significant differences were evaluated by one-way analysis of variance (ANOVA) with Tukey's honestly significant difference (HSD) *post hoc* test. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared with the vehicle-treated group; $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with the UCMS-subjected group.

[$F(5, 24) = 2.67$; $p = 0.047$] (1.8-fold) in the hippocampal tissue than did the vehicle-treated group ($p < 0.05$ and $p < 0.01$, respectively). These elevations were significantly attenuated by Myelophil treatment compared with the UCMS group: TNF-α level ($p < 0.05$ for 25 and 100 mg/kg, $p < 0.01$ for 50 mg/kg) and IL-1β level ($p < 0.05$ for 50 and 100 mg/kg; **Figure 3C** and **D**). The anti-inflammatory effects of NAC were only present in the TNF-α result.

Effects on Hippocampal NLRP3 Inflammasome

The UCMS significantly activated the NLRP3 inflammasome, as evidenced by NLRP3 [$F(5, 24) = 143.56$; $p < 0.001$] (2-fold, $p < 0.01$), ASC [$F(5, 24) = 113.80$; $p < 0.001$] (1.5-fold, $p < 0.01$), pro-IL-1β [$F(5, 24) = 50.25$; $p < 0.001$] ($p < 0.05$), and mature IL-1β [$F(5, 24) = 220.91$; $p < 0.001$] (1.9-fold, $p < 0.01$) compared with those in the vehicle-treated group. Myelophil treatment

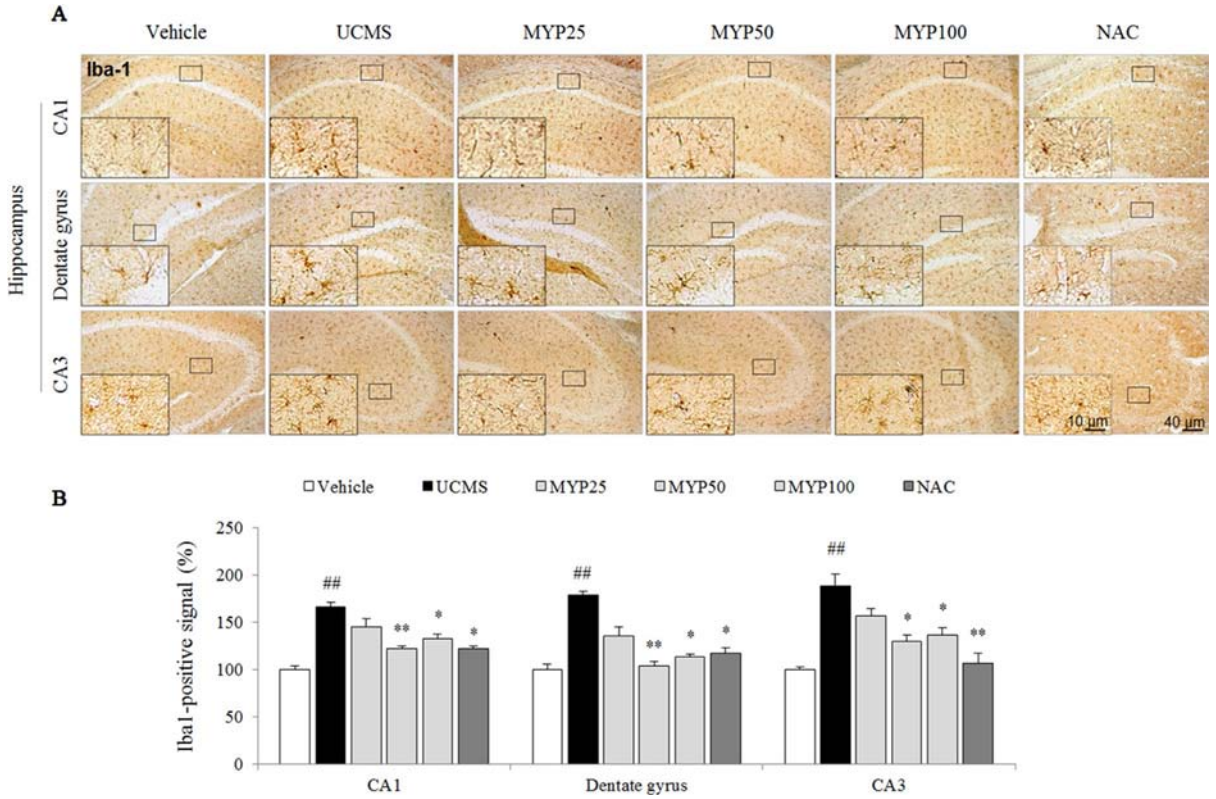

FIGURE 2 | Microglial activation in CA1, dentate gyrus, and CA3 of hippocampus. After behavioral tests, mice were sacrificed on the 25th day. Microglial activation was evaluated with Iba-1 immunofluorescence analysis in the hippocampus CA1, dentate gyrus, and CA3 region **(A)**, and its signal was semi-quantified **(B)**. The data are expressed as the mean ± SEM (n = 3). Significant differences were evaluated by one-way ANOVA with Tukey's HSD *post hoc* test. $^{\#\#}p < 0.01$ compared with the vehicle-treated group; $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with the UCMS-subjected group.

notably inhibited against NLRP3 inflammasome activation; the differences in NLRP3, ASC, pro-IL-1β, and mature IL-1β were statistically significant compared with those in the UCMS group ($p < 0.05$ or $p < 0.01$ for 100 mg/kg, **Figure 4A** and **C**).

Effects on Caspase-1 Activity in Hippocampus

The caspase-1 activity in the hippocampal tissue of the UCMS-subjected group was higher than that in the vehicle-treated group [$F(5, 24) = 5.17$; $p = 0.002$] (approximately 2.1-fold, $p < 0.01$), whereas the hyper-activity of caspase-1 was significantly inhibited by Myelophil treatment compared with the UCMS group ($p < 0.05$ for 100 mg/kg, **Figure 4B**). NAC treatment also had a similar effect on the caspase-1 hyper-activation.

Effects on Hippocampal Neurogenesis and Serotonergic Function in Dorsal Raphe Nuclei

The UCMS exerted a predominant reduction of the 5-HT activity in the region of the dorsal raphe nuclei compared with those in the vehicle-treated group [$F(5, 12) = 33.42$; $p < 0.001$] ($p < 0.01$), whereas treatment with Myelophil notably recovered the low 5-HT activity ($p < 0.01$ for both 50 and 100 mg/kg). The DCX-positive signal in the subgranular zone of the hippocampus was markedly decreased by UCMS compared with that in the vehicle-treated group [$F(5, 12) = 14.13$; $p < 0.001$] ($p < 0.01$). Moreover, an increase of DCX-positive neuronal dendrites was observed in the Myelophil-treated group ($p < 0.01$ for 50 mg/kg, $p < 0.05$ for 100 mg/kg). NAC also showed similar effects as Myelophil. The immunofluorescence staining results were quantified, and there was a significant difference between the UCMS-subjected and Myelophil-treated groups ($p < 0.01$, **Figures 5** and **6**).

DISCUSSION

In present study, we found that Myelophil could alleviate the depressive-like behaviors *via* modulation of microglial-mediated neuroinflammation. Our findings are the first evidence of the antidepressant-like effects of Myelophil, which indicates the therapeutic possibilities on the mood disorders.

To verify the hypothesis that Myelophil exerts antidepressant-like effect, we chose the BALB/c mouse strain to induce depressive behaviors because it is known to be more susceptible to UCMS relative to other rodent strains (Farley et al., 2012), and the UCMS method has been commonly used as a depressive and anxious animal model (Nollet et al., 2013). The reliability of the

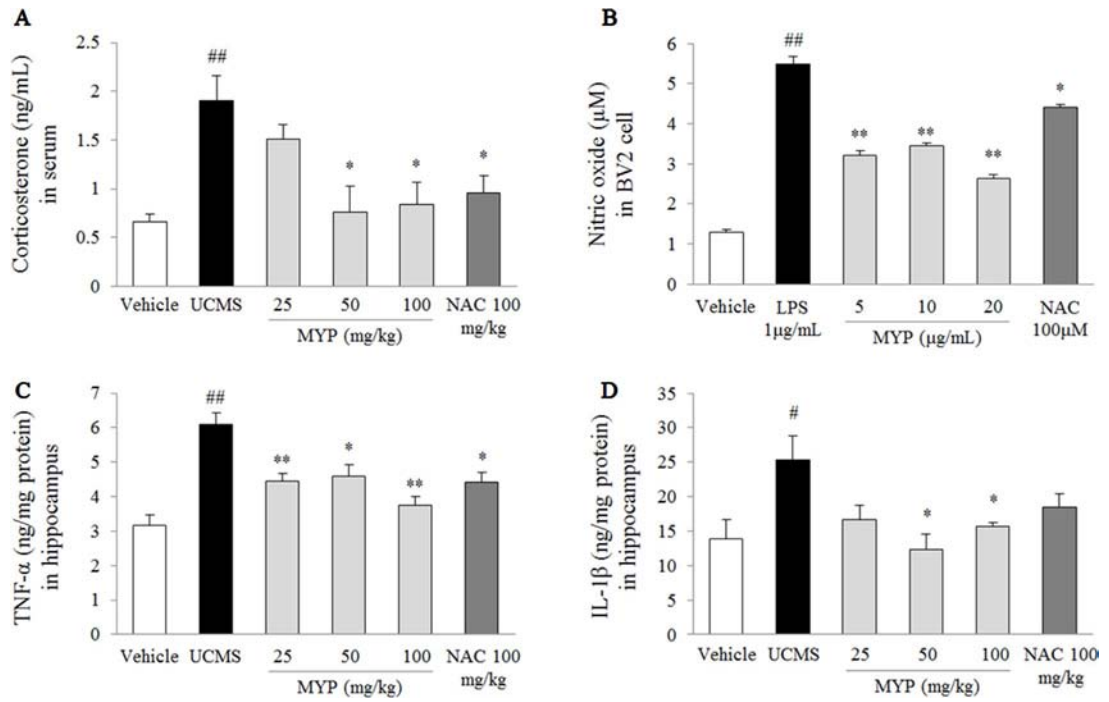

FIGURE 3 | Serum corticosterone and nitric oxide in BV2 cells and pro-inflammatory cytokines in hippocampus. After behavioral tests, mice were sacrificed on the 25th day. Serum corticosterone was evaluated *via* enzyme immunoassay (EIA) method **(A)**, nitric oxide in BV2 microglial cells was evaluated by Griess method **(B)**, and hippocampal tumor necrosis factor (TNF)-α **(C)** and interleukin (IL)-1β **(D)** were measured by EIA method. The data are expressed as the mean ± SEM ($n = 5$). Significant differences were evaluated by one-way ANOVA with Tukey's HSD *post hoc* test. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared with the vehicle-treated group or cells; $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with the UCMS-subjected group or lipopolysaccharide-treated cells.

UCMS animal model for inducing depression has been validated by numerous studies, and the unpredictable stress model can mimic the human psychopathology (Willner, 2017). We also adopted the FST, TST, and OFT as the behavioral tests, which are representative assessments for evaluating antidepressant interventions (Cryan et al., 2005; Slattery and Cryan, 2012; Daniel et al., 2017). As expected, chronic exposure to unpredictable stress led to predominant behavioral alterations in the FST, TST, and OFT, while these depression-related behaviors were significantly ameliorated by administration of Myelophil (**Figure 1A** to **F**). These Myelophil-derived results were similar with those of another study using hesperidin (a kind of citrus bioflavonoid) under UCMS-induced depressive behavioral tests (Fu et al., 2019).

To explain the underlying mechanisms of Myelophil, we subsequently examined the stress-responsive system, particularly focusing on the HPA axis hyper-activation. The HPA axis abnormality has been implicated in the pathophysiology of major depressive disorder. Hypercortisolemia is commonly observed in 40% to 60% of depressed patients (Murphy, 1991). The glucocorticoid receptor-mediated negative regulation of cortisol release is impaired in conditions of psychiatric pathology (Pariante and Lightman, 2008). In a previous animal study, chronically dexamethasone-injected mice have shown depressive-like behaviors and a decrease of hippocampal gene expression for glucocorticoid receptor (Skupio et al., 2015). Consistently with previous reports, we verified the increase of serum corticosterone by UCMS. However, this over-release of corticosterone was significantly attenuated by Myelophil administration, which proposed the antidepressant-like activity of Myelophil *via* balancing the endogenous glucocorticoid system (**Figure 3A**).

High glucocorticoids lead to morphological and functional changes of microglial cells in the brain, for example, from resting state into reactive phenotype as a neuroinflammatory response (Nair and Bonneau, 2006). Microglia, resident immune cells in the central nervous system, plays a pivotal role in the pathogenesis of depression (Yirmiya et al., 2015). Antidepressants, such as imipramine, suppressed the M1 phenotype microglia in the hippocampus of mice exposed to chronic mild stress (Zhao et al., 2016). As expected, the administration of Myelophil predominantly attenuated the over-activation of microglia in the CA1, dentate gyrus, and CA3 of the hippocampus (**Figure 2A** and **B**). Microglial activation in the hippocampal granule cell layer, hilus, CA1, and CA3 regions was known to trigger the onset of depression (Iwata et al., 2016). We further confirmed the inhibitory effects of Myelophil against the LPS-induced production of nitric oxide in the BV2 murine microglia cell line (**Figure 3B**). Researchers in pharmacology are attempting to identify the antidepressant agents that involve the regulation of microglia-derived neuroinflammation (Chen et al., 2017). In our study, the hippocampal pro-inflammatory cytokines, including TNF-α and IL-1β, were also completely normalized by Myelophil treatment (**Figure 3C** and **D**).

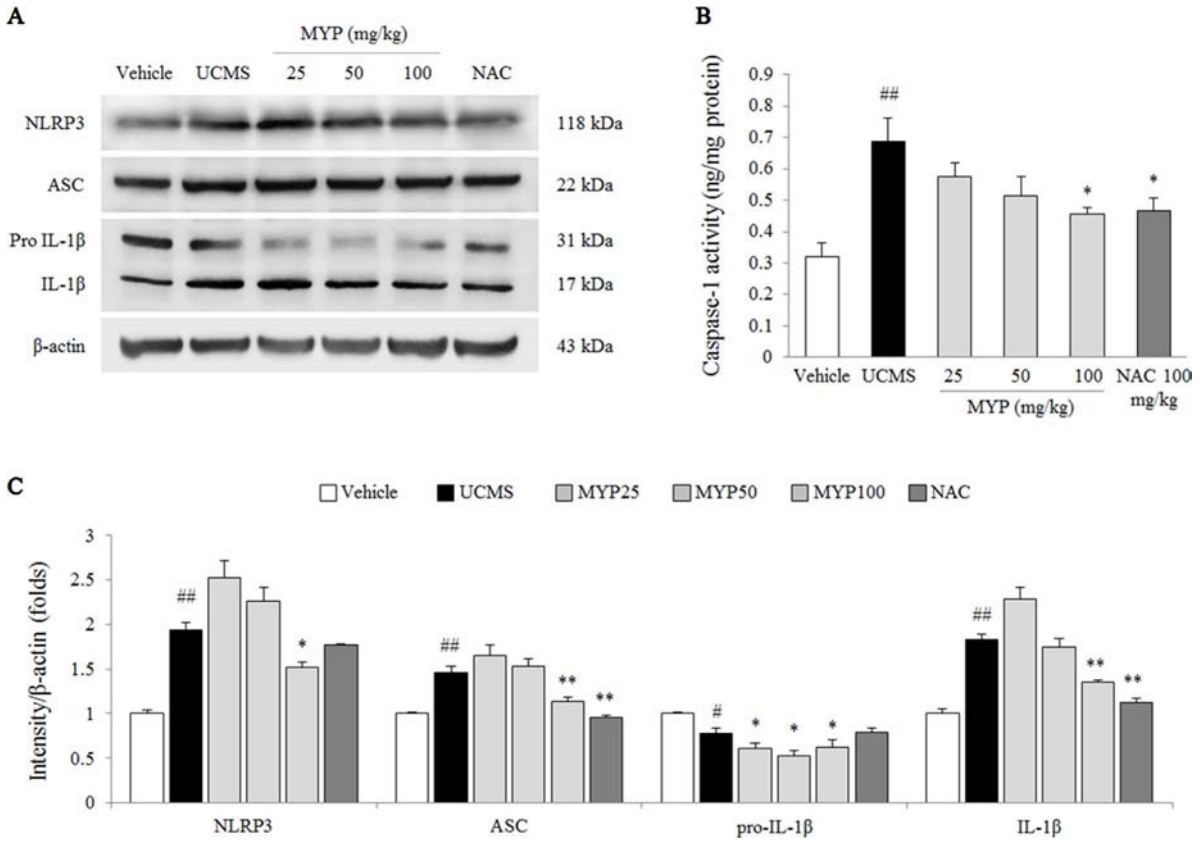

FIGURE 4 | NLRP3 inflammasome in hippocampus. After behavioral tests, mice were sacrificed on the 25th day. Protein levels of NACHT, LRR, and PYD domains-containing protein (NLRP) 3, apoptosis-associated Speck-like protein containing a CARD (ASC), and pro-IL-1β and mature IL-1β **(A)** were determined by western blotting method. Caspase-1 activity in the hippocampus was measured by EIA method. Each protein expression was semi-quantified **(C)**. The data are expressed as the mean ± SEM (n = 5). Significant differences were evaluated by one-way ANOVA with Tukey's HSD *post hoc* test. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared with the vehicle-treated group; $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with the UCMS-subjected group.

Emerging evidence reinforces the importance of NLRP3 inflammasome in neuropsychiatric disorders, particularly in depression and anxiety (Velasquez and Rappaport, 2016; Song et al., 2017). One preclinical study showed that 4 weeks of UCMS in mice resulted in depressive-like behaviors, and its contributing factor was NLRP3 inflammasome in the hippocampal region (Zhang et al., 2015). As expected, we found that Myelophil (100 mg/kg) significantly inhibited the hippocampal protein expressions of NLRP3, ASC, and caspase-1 activity (**Figure 4A–C**). These results implied the possibility of an NLRP3-dependent antidepressant-like action of Myelophil. In a case–control study, 20 patients with major depressive disorder exhibited high anxiety score and increased levels of caspase-1 and NLRP3 expression in peripheral blood mononuclear cells than did the healthy group (Alcocer-Gomez et al., 2016). The glucocorticoid-induced NLRP3 inflammasome formation was observed in a primary microglia cell isolated from mouse hippocampus (Frank et al., 2014). Furthermore, one clinical study identified a high correlation between the glial activation level and depressive score in patients with CFS using positron emission tomography (PET) (Nakatomi et al., 2014). These facts support that antidepressant-like effects of Myelophil are linked to anti-fatigue activity.

Excessive microglial activation causes an impairment of the hippocampal neurogenesis under conditions of stress and inflammation (Sierra et al., 2014). In addition, the reduced hippocampal neurogenesis is closely involved in the pathophysiology of major depression (Snyder et al., 2011). Our data also showed a notable suppression of the hippocampal neurogenesis, which was significantly ameliorated by Myelophil treatment (**Figure 5**). It is known that chronic antidepressant treatment (serotonergic modulator) increases adult hippocampal neurogenesis in both non-human primates and humans (Perera et al., 2011; Boldrini et al., 2012). Serotonin, 5-HT, as a monoamine neurotransmitter, is the most important target molecule in depressive disorder. Therefore, serotonin-target medications, such as SSRIs and SNRIs, are prescribed most frequently in approximately 90% of cases (Artigas, 2013). It is particularly interesting that dysregulation of the HPA axis attenuates 5-HT neurotransmission (Mahar et al., 2014). In our results, the

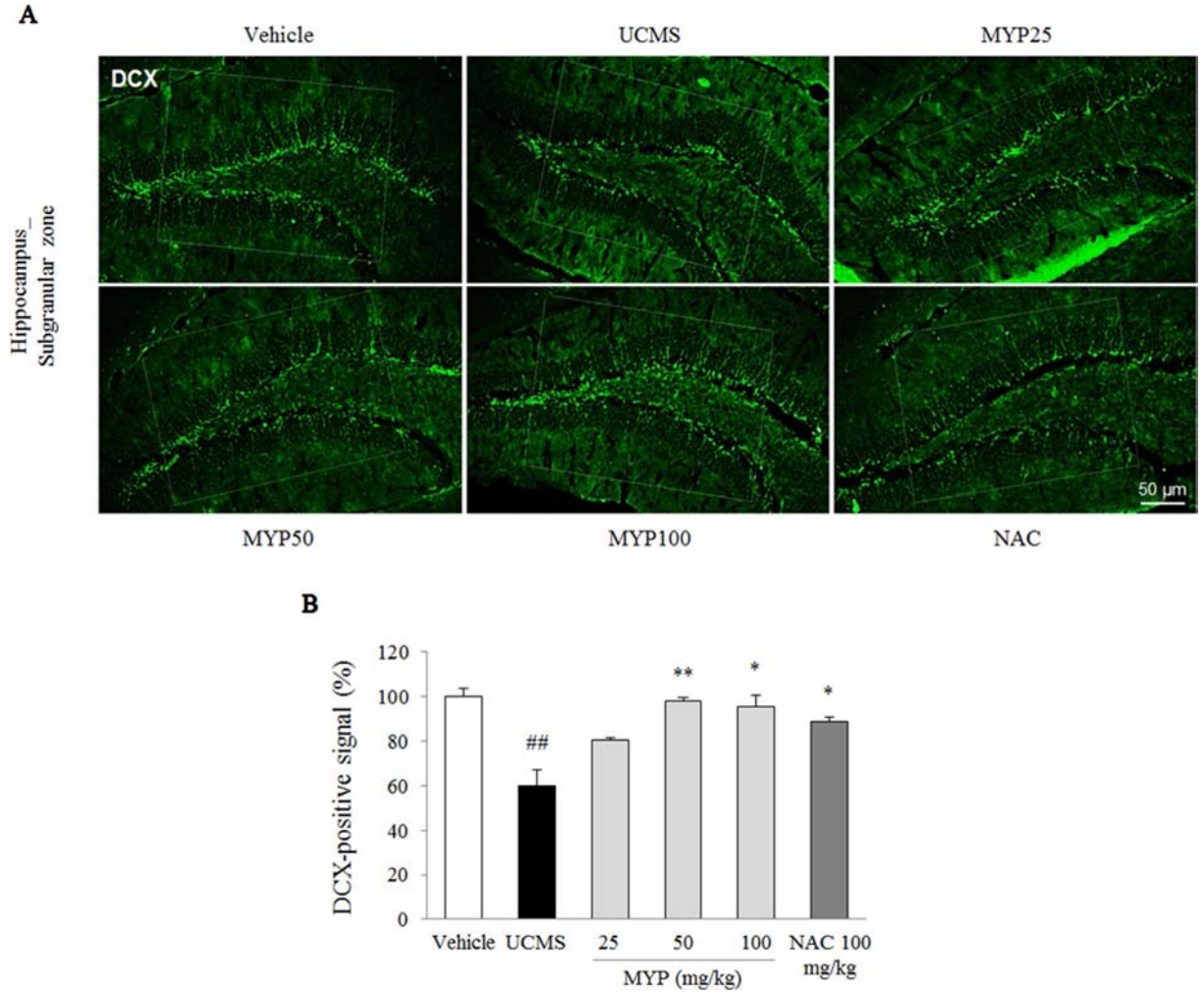

FIGURE 5 | Hippocampal neurogenesis. After behavioral tests, mice were sacrificed on the 25th day. Hippocampal neurogenesis was confirmed by doublecortin (DCX) immunofluorescence analysis in the hippocampus dentate gyrus regions **(A)**, and its signal was semi-quantified **(B)**. The data are expressed as the mean ± SEM ($n = 3$). Significant differences were evaluated by one-way ANOVA with Tukey's HSD *post hoc* test. $^{\#\#}p < 0.01$ compared with the vehicle-treated group; $^{*}p < 0.05$ compared with the UCMS-subjected group.

altered 5-HT signals in the dorsal raphe nuclei were restored by Myelophil treatment (**Figure 6A** and **B**).

Myelophil was developed for the treatment of fatigue-related disorders on the basis of the theory of traditional Chinese/Korean medicine. *Astragali Radix* and *Salviae miltiorrhizae Radix* are the main materials in Myelophil, and these two herbal plants are commonly used to maintain qi and bloodstream homeostasis in the human body (Cho et al., 2009). From our previous studies, Myelophil showed pharmacological actions against fatigue-related pathology such as brain oxidative stress and memory deficit (Lee et al., 2012; Lee et al., 2015). Our present data supported the applicability of Myelophil to depressive symptoms, which clearly accompany chronic fatigue disorders. However, the present study has a limitation of unknown information regarding the active compounds that correspond to the anti-depressive property. A study found that salvianolic acid B, a major compound in *Salviae Radix*, exhibits anti-inflammatory effects by modulating NLRP3 inflammasome (Jiang et al., 2017). Salvianolic acid B promotes microglial M2-polarization against UCMS-induced M1 phenotype (Zhang et al., 2017). Astragaloside IV, a main *Astragali Radix* compound, inhibits depressive-like behaviors by regulating the nuclear factor-κB/NLRP3 axis (Song et al., 2018). Further studies are required to identify the major active compound in the future. We used NAC as a positive control because the antidepressant-like effects of NAC have been explored in several studies (Ferreira et al., 2008; Costa-Campos et al., 2013). One study group is planning to evaluate the inflammation-inhibiting NAC effects on depressive disorder in a randomized placebo-controlled trial (Yang et al., 2018).

Taken together, our findings comprise the first evidence for the antidepressant-like effects of Myelophil, and its underlying mechanism may involve the regulation of NLRP3-dependent

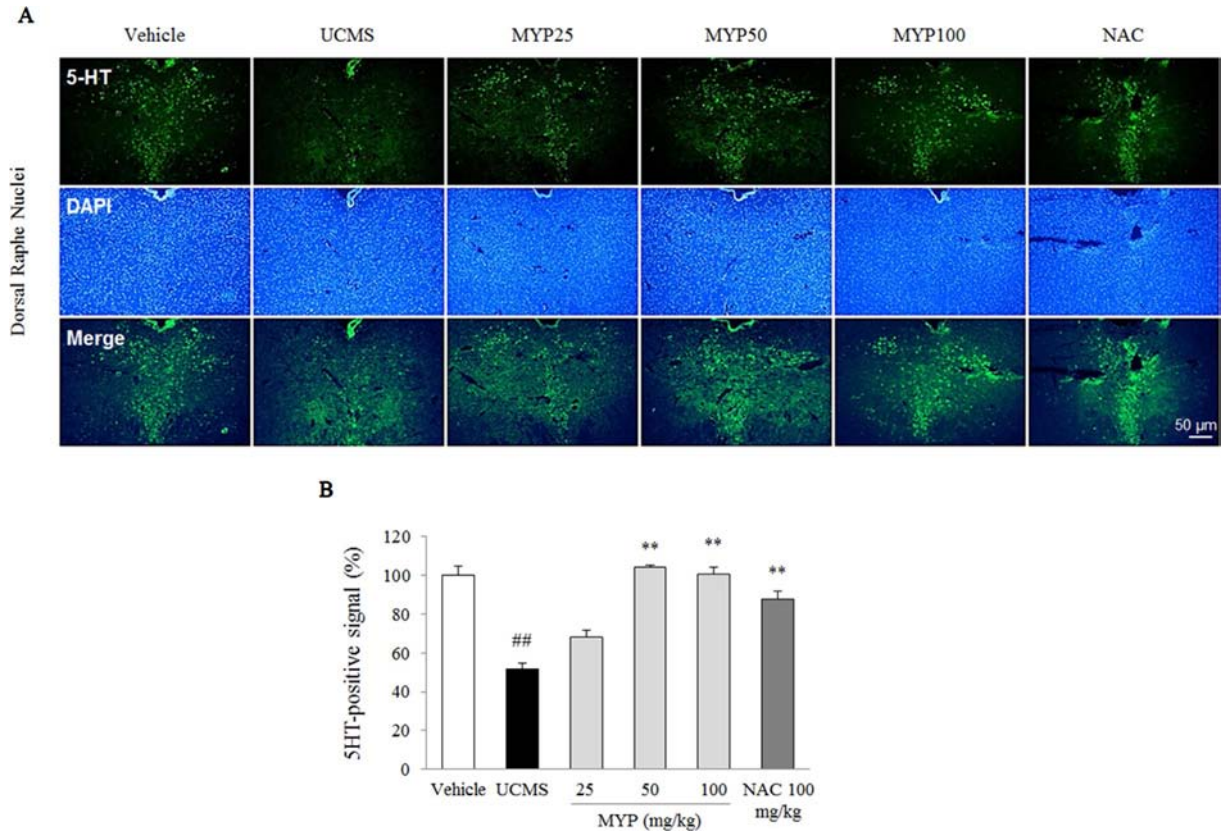

FIGURE 6 | Serotonergic signal in dorsal raphe nuclei. After behavioral tests, mice were sacrificed on the 25th day. Serotonergic signal was confirmed by 5-hydroxytryptamine (5-HT) immunofluorescence analysis in the dorsal raphe nuclei **(A)**, and its signal was semi-quantified **(B)**. The data are expressed as the mean ± SEM (n = 3). Significant differences were evaluated by one-way ANOVA with Tukey's HSD *post hoc* test. $^{\#\#}p < 0.01$ compared with the vehicle-treated group; $^{**}p < 0.01$ compared with the UCMS-subjected group.

neuroinflammation and the serotonergic signal. This study would support the pharmacological applicability of Myelophil in chronic fatigue, as well as neuropsychiatric disorders.

ETHICS STATEMENT

Animal care and experiments were conducted in accordance with the guidelines issued by the Institutional Animal Care and Use Committee of Daejeon University (Daejeon, Republic of Korea; Approval No. DJUARB 2017-017) and the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health.

AUTHOR CONTRIBUTIONS

JL wrote the main manuscript text and conducted the experiments. WK supported the behavioral test for depression and anxiety. YJ prepared **Figure 2** and other immunofluorescence staining results. SL performed a statistical analysis. DL advised on the mechanism of NLRP3 inflammasome activation. CS supervised the preparation of manuscript and directed the final version of all contents. All authors reviewed and approved this manuscript.

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SUPPLEMENTARY MATERIAL

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REFERENCES

Alcocer-Gomez, E., and Cordero, M. D. (2014). NLRP3 inflammasome: a new target in major depressive disorder. *CNS Neurosci. Ther.* 20, 294–295. doi: 10.1111/cns.12230

Alcocer-Gomez, E., Ulecia-Moron, C., Marin-Aguilar, F., Rybkina, T., Casas-Barquero, N., Ruiz-Cabello, J., et al. (2016). Stress-induced depressive behaviors require a functional NLRP3 inflammasome. *Mol. Neurobiol.* 53, 4874–4882. doi: 10.1007/s12035-015-9408-7

Andrews, P. W., Thomson, J. A., Jr., Amstadter, A., and Neale, M. C. (2012). Primum non nocere: an evolutionary analysis of whether antidepressants do more harm than good. *Front Psychol.* 3, 117. doi: 10.3389/fpsyg.2012.00117

Artigas, F. (2013). Serotonin receptors involved in antidepressant effects. *Pharmacol. Ther.* 137, 119–131. doi: 10.1016/j.pharmthera.2012.09.006

Berwian, I. M., Walter, H., Seifritz, E., and Huys, Q. J. (2017). Predicting relapse after antidepressant withdrawal—a systematic review. *Psychol. Med.* 47, 426–437. doi: 10.1017/S0033291716002580

Boldrini, M., Hen, R., Underwood, M. D., Rosoklija, G. B., Dwork, A. J., Mann, J. J., et al. (2012). Hippocampal angiogenesis and progenitor cell proliferation are increased with antidepressant use in major depression. *Biol Psychiatry* 72, 562–571. doi: 10.1016/j.biopsych.2012.04.024

Brites, D., and Fernandes, A. (2015). Neuroinflammation and depression: microglia activation, extracellular microvesicles and microRNA dysregulation. *Front Cell Neurosci.* 9, 476. doi: 10.3389/fncel.2015.00476

Chatterjee, M., Jaiswal, M., and Palit, G. (2012). Comparative evaluation of forced swim test and tail suspension test as models of negative symptom of schizophrenia in rodents. *ISRN Psychiatry* 2012 595141. doi: 10.5402/2012/595141

Chen, Q., Feng, G., Liu, L., Wang, X., Wan, Y. Q., Li, M., et al. (2017). The effect of ketamine on microglia and proinflammatory cytokines in the hippocampus of depression-like rat. *Neuropsychiatry (London)* 7, 77–85. doi: 10.4172/Neuropsychiatry.1000183

Cho, J. H., Cho, C. K., Shin, J. W., Son, J. Y., Kang, W., and Son, C. G. (2009). Myelophil, an extract mix of Astragali Radix and Salviae Radix, ameliorates chronic fatigue: a randomised, double-blind, controlled pilot study. *Complement. Ther. Med.* 17, 141–146. doi: 10.1016/j.ctim.2008.11.003

Costa-Campos, L., Herrmann, A. P., Pilz, L. K., Michels, M., Noetzold, G., and Elisabetsky, E. (2013). Interactive effects of N-acetylcysteine and antidepressants. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 44, 125–130. doi: 10.1016/j.pnpbp.2013.02.008

Cryan, J. F., Mombereau, C., and Vassout, A. (2005). The tail suspension test as a model for assessing antidepressant activity: review of pharmacological and genetic studies in mice. *Neurosci. Biobehav. Rev.* 29, 571–625. doi: 10.1016/j.neubiorev.2005.03.009

Daniel, P., Andrea, S., Griffin, Ignasi, B., and Daniel, S. (2017). Revisiting the open-field test: what does it really tell us about animal personality? *Anim. Behav.* 123, 69–79. doi: 10.1016/j.anbehav.2016.10.006

Demyttenaere, K., De Fruyt, J., and Stahl, S. M. (2005). The many faces of fatigue in major depressive disorder. *Int. J. Neuropsychopharmacol.* 8, 93–105. doi: 10.1017/S1461145704004729

Farley, S., Dumas, S., El Mestikawy, S., and Giros, B. (2012). Increased expression of the vesicular glutamate transporter-1 (VGLUT1) in the prefrontal cortex correlates with differential vulnerability to chronic stress in various mouse strains: effects of fluoxetine and MK-801. *Neuropharmacology* 62, 503–517. doi: 10.1016/j.neuropharm.2011.09.010

Ferreira, F. R., Biojone, C., Joca, S. R., and Guimaraes, F. S. (2008). Antidepressant-like effects of N-acetyl-L-cysteine in rats. *Behav. Pharmacol.* 19, 747–750. doi: 10.1097/FBP.0b013e3283123c98

Frank, M. G., Hershman, S. A., Weber, M. D., Watkins, L. R., and Maier, S. F. (2014). Chronic exposure to exogenous glucocorticoids primes microglia to pro-inflammatory stimuli and induces NLRP3 mRNA in the hippocampus. *Psychoneuroendocrinology* 40, 191–200. doi: 10.1016/j.psyneuen.2013.11.006

Fu, H., Liu, L., Tong, Y., Li, Y., Zhang, X., Gao, X., et al. (2019). The antidepressant effects of hesperidin on chronic unpredictable mild stress-induced mice. *Eur. J. Pharmacol.* 853, 236–246. doi: 10.1016/j.ejphar.2019.03.035

Gaynes, B. N., Warden, D., Trivedi, M. H., Wisniewski, S. R., Fava, M., and Rush, A. J. (2009). What did STAR*D teach us? Results from a large-scale, practical, clinical trial for patients with depression. *Psychiatr. Serv.* 60, 1439–1445. doi: 10.1176/ps.2009.60.11.1439

Green, L. C., Wagner, D. A., Glogowski, J., Skipper, P. L., Wishnok, J. S., and Tannenbaum, S. R. (1982). Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. *Anal Biochem.* 126, 131–138. doi: 10.1016/0003-2697(82)90118-X

Ieraci, A., and Herrera, D. G. (2006). Nicotinamide protects against ethanol-induced apoptotic neurodegeneration in the developing mouse brain. *PLoS Med.* 3, e101. doi: 10.1371/journal.pmed.0030101

Iwata, M., Ishida, H., Kaneko, K., and Shirayama, Y. (2016). Learned helplessness activates hippocampal microglia in rats: a potential target for the antidepressant imipramine. *Pharmacol. Biochem. Behav.* 150, 138–146. doi: 10.1016/j.pbb.2016.10.005

Jiang, P., Guo, Y., Dang, R., Yang, M., Liao, D., Li, H., et al. (2017). Salvianolic acid B protects against lipopolysaccharide-induced behavioral deficits and neuroinflammatory response: involvement of autophagy and NLRP3 inflammasome. *J. Neuroinflammation* 14, 239. doi: 10.1186/s12974-017-1013-4

Kaufmann, F. N., Costa, A. P., Ghisleni, G., Diaz, A. P., Rodrigues, A. L. S., Peluffo, H., et al. (2017). NLRP3 inflammasome-driven pathways in depression: clinical and preclinical findings. *Brain Behav. Immun.* 64, 367–383. doi: 10.1016/j.bbi.2017.03.002

Kim, H. G., Lee, J. S., Han, J. M., Lee, J. S., Choi, M. K., Son, S. W., et al. (2013). Myelophil attenuates brain oxidative damage by modulating the hypothalamus–pituitary–adrenal (HPA) axis in a chronic cold-stress mouse model. *J. Ethnopharmacol.* 148, 505–514. doi: 10.1016/j.jep.2013.04.046

Kim, H. G., Lee, J. S., Choi, M. K., Han, J. M., and Son, C. G. (2014). Ethanolic extract of Astragali Radix and Salviae Radix prohibits oxidative brain injury by psycho-emotional stress in whisker removal rat model. *PLoS One* 9, e98329. doi: 10.1371/journal.pone.0098329

Klonsky, E. D., May, A. M., and Saffer, B. Y. (2016). Suicide, suicide attempts, and suicidal ideation. *Annu. Rev. Clin. Psychol.* 12, 307–330. doi: 10.1146/annurev-clinpsy-021815-093204

Lee, G., and Bae, H. (2017). Therapeutic Effects of phytochemicals and medicinal herbs on depression. *Biomed. Res. Int.* 2017, 6596241. doi: 10.1155/2017/6596241

Lee, J. S., Cho, J. H., Lee, D. S., and Son, C. G. (2018). Genotoxicity evaluation of an ethanol extract mixture of Astragali Radix and Salviae miltiorrhizae Radix. *Evid. Based Complement. Alternat. Med.* 2018, 5684805. doi: 10.1155/2018/5684805

Lee, J. S., Kim, H. G., Han, J. M., Kim, D. W., Yi, M. H., Son, S. W., et al. (2014). Ethanol extract of Astragali Radix and Salviae miltiorrhizae Radix, Myelophil, exerts anti-amnesic effect in a mouse model of scopolamine-induced memory deficits. *J. Ethnopharmacol.* 153, 782–792. doi: 10.1016/j.jep.2014.03.048

Lee, J. S., Kim, H. G., Han, J. M., Kim, Y. A., and Son, C. G. (2015). Anti-fatigue effect of Myelophil in a chronic forced exercise mouse model. *Eur. J. Pharmacol.* 764, 100–108. doi: 10.1016/j.ejphar.2015.06.055

Lee, J. S., Kim, H. G., Han, J. M., Lee, J. S., Son, S. W., Ahn, Y. C., et al. (2012). Myelophil ameliorates brain oxidative stress in mice subjected to restraint stress. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 39, 339–347. doi: 10.1016/j.pnpbp.2012.07.006

Mahar, I., Bambico, F. R., Mechawar, N., and Nobrega, J. N. (2014). Stress, serotonin, and hippocampal neurogenesis in relation to depression and antidepressant effects. *Neurosci. Biobehav. Rev.* 38, 173–192. doi: 10.1016/j.neubiorev.2013.11.009

Mordor-Intelligence (2017) Global antidepressants market—segmented by antidepressant therapies, depressive disorders, and geography 2017–2022. *Mordor Intell.* 4388394, 205.

Murphy, B. E. (1991). Steroids and depression. *J. Steroid Biochem. Mol. Biol.* 38, 537–559. doi: 10.1016/0960-0760(91)90312-S

Nair, A., and Bonneau, R. H. (2006). Stress-induced elevation of glucocorticoids increases microglia proliferation through NMDA receptor activation. *J. Neuroimmunol.* 171, 72–85. doi: 10.1016/j.jneuroim.2005.09.012

Nakatomi, Y., Mizuno, K., Ishii, A., Wada, Y., Tanaka, M., Tazawa, S., et al. (2014). Neuroinflammation in patients with chronic fatigue syndrome/myalgic encephalomyelitis: an (1)(1)C-(R)-PK11195 PET study. *J. Nucl. Med.* 55, 945–950. doi: 10.2967/jnumed.113.131045

Nestler, E. J., Barrot, M., Dileone, R. J., Eisch, A. J., Gold, S. J., and Monteggia, L. M. (2002). Neurobiology of depression. *Neuron* 34, 13–25. doi: 10.1016/S0896-6273(02)00653-0

Nollet, M., Le Guisquet, A. M., and Belzung, C. (2013). Models of depression: unpredictable chronic mild stress in mice. *Curr. Protoc. Pharmacol. Chapter 5 Unit* 5, 65. doi: 10.1002/0471141755.ph0565s61

Nordquist, N., and Oreland, L. (2010). Serotonin, genetic variability, behaviour, and psychiatric disorders—a review. *Ups. J. Med. Sci.* 115, 2–10. doi: 10.3109/03009730903573246

Pariante, C. M., and Lightman, S. L. (2008). The HPA axis in major depression: classical theories and new developments. *Trends Neurosci.* 31, 464–468. doi: 10.1016/j.tins.2008.06.006

Penner, I. K., and Paul, F. (2017). Fatigue as a symptom or comorbidity of neurological diseases. *Nat. Rev. Neurol.* 13, 662–675. doi: 10.1038/nrneurol.2017.117

Perera, T. D., Dwork, A. J., Keegan, K. A., Thirumangalakudi, L., Lipira, C. M., Joyce, N., et al. (2011). Necessity of hippocampal neurogenesis for the therapeutic action of antidepressants in adult nonhuman primates. *PLoS One* 6, e17600. doi: 10.1371/journal.pone.0017600

Porsolt, R. D., Bertin, A., and Jalfre, M. (1977). Behavioral despair in mice: a primary screening test for antidepressants. *Arch Int. Pharmacodyn. Ther.* 229, 327–336.

Sierra, A., Beccari, S., Diaz-Aparicio, I., Encinas, J. M., Comeau, S., and Tremblay, M. E. (2014). Surveillance, phagocytosis, and inflammation: how never-resting microglia influence adult hippocampal neurogenesis. *Neural Plast.* 2014, 610343. doi: 10.1155/2014/610343

Skupio, U., Tertil, M., Sikora, M., Golda, S., Wawrzczak-Bargiela, A., and Przewlocki, R. (2015). Behavioral and molecular alterations in mice resulting from chronic treatment with dexamethasone: relevance to depression. *Neuroscience* 286, 141–150. doi: 10.1016/j.neuroscience.2014.11.035

Slattery, D. A., and Cryan, J. F. (2012). Using the rat forced swim test to assess antidepressant-like activity in rodents. *Nat. Protoc.* 7, 1009–1014. doi: 10.1038/nprot.2012.044

Snyder, J. S., Soumier, A., Brewer, M., Pickel, J., and Cameron, H. A. (2011). Adult hippocampal neurogenesis buffers stress responses and depressive behaviour. *Nature* 476, 458–461. doi: 10.1038/nature10287

Song, L., Pei, L., Yao, S., Wu, Y., and Shang, Y. (2017). NLRP3 inflammasome in neurological diseases, from functions to therapies. *Front Cell Neurosci.* 11, 63. doi: 10.3389/fncel.2017.00063

Song, M. T., Ruan, J., Zhang, R. Y., Deng, J., Ma, Z. Q., and Ma, S. P. (2018). Astragaloside IV ameliorates neuroinflammation-induced depressive-like behaviors in mice via the PPARgamma/NF-kappaB/NLRP3 inflammasome axis. *Acta Pharmacol. Sin.* 39, 1559–1570. doi: 10.1038/aps.2017.208

Steru, L., Chermat, R., Thierry, B., and Simon, P. (1985). The tail suspension test: a new method for screening antidepressants in mice. *Psychopharmacology (Berl)* 85, 367–370. doi: 10.1007/BF00428203

Velasquez, S., and Rappaport, J. (2016). Inflammasome activation in major depressive disorder: a pivotal linkage between psychological stress, purinergic signaling, and the kynurenine pathway. *Biol Psychiatry* 80, 4–5. doi: 10.1016/j.biopsych.2016.04.019

WHO (2017) *Depression and other common mental disorders*. World Health Organization.

Willner, P. (2017). Reliability of the chronic mild stress model of depression: a user survey. *Neurobiol. Stress.* 6, 68–77. doi: 10.1016/j.ynstr.2016.08.001

Yang, C., Bosker, F. J., Li, J., and Schoevers, R. A. (2018). N-Acetylcysteine as add-on to antidepressant medication in therapy refractory major depressive disorder patients with increased inflammatory activity: study protocol of a double-blind randomized placebo-controlled trial. *BMC Psychiatry* 18, 279. doi: 10.1186/s12888-018-1845-1

Yirmiya, R., Rimmerman, N., and Reshef, R. (2015). Depression as a microglial disease. *Trends Neurosci.* 38, 637–658. doi: 10.1016/j.tins.2015.08.001

Zhang, J., Xie, X., Tang, M., Zhang, J., Zhang, B., Zhao, Q., et al. (2017). Salvianolic acid B promotes microglial M2-polarization and rescues neurogenesis in stress-exposed mice. *Brain Behav. Immun.* 66, 111–124. doi: 10.1016/j.bbi.2017.07.012

Zhang, Y., Liu, L., Liu, Y. Z., Shen, X. L., Wu, T. Y., Zhang, T., et al. (2015). NLRP3 inflammasome mediates chronic mild stress-induced depression in mice via neuroinflammation. *Int. J. Neuropsychopharmacol.* 18, 1–8. doi: 10.1093/ijnp/pyv006

Zhao, Q., Wu, X., Yan, S., Xie, X., Fan, Y., Zhang, J., et al. (2016). The antidepressant-like effects of pioglitazone in a chronic mild stress mouse model are associated with PPARgamma-mediated alteration of microglial activation phenotypes. *J. Neuroinflammation* 13, 259. doi: 10.1186/s12974-016-0728-y

Zou, W., Feng, R., and Yang, Y. (2018). Changes in the serum levels of inflammatory cytokines in antidepressant drug-naive patients with major depression. *PLoS One* 13, e0197267. doi: 10.1371/journal.pone.0197267.

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frontiers in Neuroscience

ORIGINAL RESEARCH
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Annexin A1 Bioactive Peptide Promotes Resolution of Neuroinflammation in a Rat Model of Exsanguinating Cardiac Arrest Treated by Emergency Preservation and Resuscitation

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Neuroinflammation initiated by damage-associated molecular patterns, including high mobility group box 1 protein (HMGB1), has been implicated in adverse neurological outcomes following lethal hemorrhagic shock and polytrauma. Emergency preservation and resuscitation (EPR) is a novel method of resuscitation for victims of exsanguinating cardiac arrest, shown in preclinical studies to improve survival with acceptable neurological recovery. Sirtuin 3 (SIRT3), the primary mitochondrial deacetylase, has emerged as a key regulator of metabolic and energy stress response pathways in the brain and a pharmacological target to induce a neuronal pro-survival phenotype. This study aims to examine whether systemic administration of an Annexin-A1 bioactive peptide (ANXA1sp) could resolve neuroinflammation and induce sirtuin-3 regulated cytoprotective pathways in a novel rat model of exsanguinating cardiac arrest and EPR. Adult male rats underwent hemorrhagic shock and ventricular fibrillation, induction of profound hypothermia, followed by resuscitation and rewarming using cardiopulmonary bypass (EPR). Animals randomly received ANXA1sp (3 mg/kg, in divided doses) or vehicle. Neuroinflammation (HMGB1, TNFα, IL-6, and IL-10 levels), cerebral cell death (TUNEL, caspase-3, pro and antiapoptotic protein levels), and neurologic scores were assessed to evaluate the inflammation resolving effects of ANXA1sp following EPR. Furthermore, western blot analysis and immunohistochemistry were used to interrogate the mechanisms involved. Compared to vehicle controls, ANXA1sp effectively reduced expression of cerebral HMGB1, IL-6, and TNFα and increased IL-10 expression, which were associated with improved neurological scores.

ANXA1sp reversed EPR-induced increases in expression of proapoptotic protein Bax and reduction in antiapoptotic protein Bcl-2, with a corresponding decrease in cerebral levels of cleaved caspase-3. Furthermore, ANXA1sp induced autophagic flux (increased LC3II and reduced p62 expression) in the brain. Mechanistically, these findings were accompanied by upregulation of the mitochondrial protein deacetylase Sirtuin-3, and its downstream targets FOXO3a and MnSOD in ANXA1sp-treated animals. Our data provide new evidence that engaging pro-resolving pharmacological strategies such as Annexin-A1 biomimetic peptides can effectively attenuate neuroinflammation and enhance the neuroprotective effects of EPR after exsanguinating cardiac arrest.

Keywords: sirtuins, autophagy, apoptosis, neuroprotection, HMGB1

INTRODUCTION

Exsanguinating hemorrhage, leading to cardiac arrest and multiple organ failure, remains the most common cause of death among trauma patients without traumatic brain injury, and neurologic outcomes in survivors are poor. An new therapeutic paradigm – termed EPR – involves rapidly cooling victims of exsanguinating cardiac arrest to deep or profound hypothermia levels ($\leq$20°C), in an effort to extend ischemic time, maintain organ viability during severe shock, allow operative repair of injuries and resuscitation, and ultimately improve survival and preserve neurological function (Tisherman et al., 2017). Following initial characterization of EPR, a growing body of proof-of-concept large animal studies have investigated the optimal depth and rate of cooling, duration of EPR, and physiological conditions prior to induction of EPR (Rhee et al., 2000; Alam et al., 2002, 2004, 2005, 2006a,b; Wu et al., 2006). Rapid cooling and subsequent controlled resuscitation and rewarming is accomplished through the use of CPB (Sailhamer et al., 2007; Alam et al., 2008). The development of rodent models of HS and EPR enabled characterization of key cellular and molecular changes involved in exsanguination cardiac arrest (Drabek et al., 2007a,b) – chief among those are neuroinflammation and apoptosis – which constitute targets for adjunctive therapies (Han et al., 2008; Alam et al., 2010).

Neuroinflammation is a complex immune response commonly observed following acute brain insults such as HS and associated warm ischemia, ischemia-reperfusion injury (I/RI), therapeutic hypothermia, exposure to CPB, and post-cardiac arrest syndrome. Multiple rodent models of surgical trauma, cardiac arrest and resuscitation have identified upregulation of pro-inflammatory cytokines and inflammatory mediators in both peripheral tissues and the central nervous system (CNS) (Terrando et al., 2010a, 2011). Acute neuroinflammation is characterized by increased cytokines, such as interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), HMGB1 and their cognate receptors ultimately leading to neuron-glia dysfunction and blood–brain barrier impairments (Cao et al., 2010; Terrando et al., 2010b, 2011; He et al., 2012; Liu and McCullough, 2013; Wohleb et al., 2014; Xiang et al., 2016; Skvarc et al., 2018). Oxidative stress and mitochondrial dysfunction converge with the neuroinflammatory pathway, creating a positive feedback loop (Netto et al., 2018). Collectively, these mechanisms actively contribute to neuronal death and cognitive impairment. There is evidence that the neuroinflammatory *milieu* persists following surgical trauma, HS, I/RI, and resuscitation from cardiac arrest because is not efficiently controlled by endogenous anti-inflammatory mechanisms, thereby contributing to secondary injury, CNS dysfunction, and neuronal network hyperexcitability (Tahsili-Fahadan et al., 2018). Despite improvements in resuscitation science for victims of HS cardiac arrest, including EPR, total body cooling is technically and logistically challenging and the development of effective therapies to decrease morbidity and improve long-term neurologic outcomes following exsanguinating cardiac arrest remains a critical need.

Dysfunction of inflammation-resolving pathways have been described in experimental rodent trauma models, leading to exaggerated post-injury cognitive decline (Su et al., 2013). In recent years, a number of lipid mediators such as resolvins, lipoxins, and maresins have begun to receive attention as possible resolvers of neuroinflammation (Terrando et al., 2013; Vacas et al., 2013). Resolvins act to block neutrophil and monocyte migration and reduce the oxidative burst of neutrophils (Serhan et al., 2007; Perretti et al., 2015). Similarly, the glucocorticoid-regulated protein Annexin A1 and its peptide mimetics display pro-resolution effects, and have been shown to mitigate cerebral I/RI by attenuating neuroinflammation in many experimental models (Ansari et al., 2018) including cardiac surgery with deep hypothermic circulatory arrest (Zhang et al., 2017). The aim of this study was to determine the neuroprotective efficacy of a bioactive Annexin A1 short peptide (ANXA1sp) in a small animal model of exsanguination cardiac arrest and EPR. We hypothesized that ANXA1sp treatment would attenuate neuroinflammation and neuronal cell death, in part through upregulation of the mitochondrial protein deacetylase SIRT3 and downstream cytoprotective pathways.

Abbreviations: ANXA1sp, Annexin A1 short peptide; CPB, cardiopulmonary bypass; EPR, emergency preservation and resuscitation; HMGB1, high mobility group box 1; HS, hemorrhagic shock; LC3-II, microtubule-associated protein 1A/1B-light chain 3-phosphatidylethanolamine conjugate; MnSOD, manganese superoxide dismutase; p62/SQSTM1, sequestosome-1; ROS, reactive oxygen species; SIRT3, sirtuin-3.

MATERIALS AND METHODS

Animals

The experimental protocol was approved by the Duke University Animal Care and Use Committee. All procedures met the guidelines of the National Institutes of Health for animal care (Guide for the Care and Use of Laboratory Animals, Health and Human Services, National Institute of Health Publication No. 86-23, revised 1996). Adult male Sprague–Dawley rats (age 13–15 weeks; weight 400–450 g; Charles River Laboratories, Wilmington, MA, United States) were housed (two animals per cage) in a 12-h light-dark cycle environment with free access to food and water. Rats were acclimated for at least 1 week before starting the experiment.

Surgical Preparation for Experimental EPR

Experimental procedures are summarized in **Figure 1**. Fasted rats were anesthetized with isoflurane, intubated, and mechanically ventilated to maintain the arterial $PaCO_2$ between 35 and 45 mmHg. Anesthesia was provided with isoflurane (1.5–2%) with an oxygen and air mixture at a FiO_2 of 0.5. Bupivacaine 0.25% was injected at the operative sites to achieve local anesthesia. Routine physiologic parameters, and rectal and pericranial [hypodermic needle probe (Omega®, Stamford, CT, United States) placed beneath the temporalis muscle] temperatures were monitored. The right caudal epigastric artery was cannulated with a polyethylene catheter (PE10, Clay Adams, Sparks, MD, United States) and used for systemic mean arterial pressure (MAP) monitoring. The tail artery was cannulated with a 20-gauge catheter, which served as blood sampling and inflow CPB cannula. The right internal jugular vein was cannulated with a customized 4.5-F multi-orifice catheter advanced into the right atrium and used for withdrawing blood and venous out-flow CPB cannula. The external carotid artery was cannulated with a polyethylene catheter (PE50; Clay Adams, Sparks, MD, United States) advanced into the aortic arch and used for induction of profound hypothermia by flushing ice-cold normal saline. During surgical preparation, normothermia was maintained using a heat lamp.

Induction of Hemorrhagic Shock and Ventricular Fibrillation

After a 30-min equilibration period, rapid exsanguination (50% blood volume, 0.034 ml/g body weight, 12–15 ml of blood) over 10 min was performed via the jugular venous cannula and MAP was allowed to drop below 20 mmHg. For the next 35 min (simulating pre-hospital transport time), the MAP remained <20 mm Hg without any resuscitative efforts HS. The shed blood was salvaged in a 20 ml syringe prefilled with 2 ml citrate phosphate dextrose solution (Fenwal Inc., Lake Zurich, IL, United States) and stored at 4°C for later transfusion/resuscitation. At the end of HS, ventricular fibrillation (VF) was induced by transesophageal burst pacing at 50 V (S48 stimulator, RI, United States) for 90 s, and confirmed electrocardiographically and echocardiographically (Phillips Sonos7500 system, Andover, Mass 01810). During HS, anesthesia was maintained using 0.4% isoflurane. No neuromuscular blocker was administered until just prior to VF. A dose of vecuronium bromide (0.1 mg/kg) was given intravenously to prevent muscle contraction during VF and cooling.

Induction of Profound Hypothermia

After the induction of VF, the animals were rapidly cooled to a core temperature of 10–15°C for 45 min by flushing ice-cold solution [total volume 45 ml = 40 ml normal saline + 2 ml 5% human plasma protein fraction (Plasmanate®, Grifols Therapeutics, Inc., NC, United States) + 3 ml 8.5% Sodium Bicarbonate] into the ascending aorta/aortic arch and returning from right jugular vein using a peristaltic pump (Masterflex, model 770201-60, Cole-Parmer, Vernon Hills, IL, United States) at rate of 20 ml/kg/min (T208 transonic volume flow meter, Transonic Systems Inc. Ithaca, NY, United States). A water bath with ice water as well as topical cooling with ice bags was also applied.

Resuscitation and Rewarming With Cardiopulmonary Bypass

Following 45 min of profound hypothermia, rats were rewarmed and resuscitated using CPB. The CPB circuit consisted of a peristaltic pump (MasterflexC, Cole-Parmer, Vernon Hills, IL, United States), a custom-designed oxygenator, and a venous reservoir, as previously described (Bartels et al., 2014; Shim et al., 2014; Zhang et al., 2017). The reservoir was primed with 3 ml of 6% hydroxyethylstarch 130/0.4 and 2 ml of previously shed and salvaged blood. Heparin (200 IU) and vecuronium bromide (0.1 mg/kg) were added to the venous reservoir. CPB was initiated at a flow rate of 20–30 ml/min, which was gradually increased to 50–60 ml/min upon reaching 33–34°C. The gradient between the water bath and core body temperatures was not allowed to exceed 10°C. Once core temperature increased from 10 to 34°C, all salvaged blood was gradually re-transfused to keep up with the increased oxygen demand. The animals were rewarmed for 80 min until core temperatures of 34°C were achieved; subsequently, CPB was terminated. Rewarming rate was maintained at approximately 0.4°C/min. Acid-base abnormalities were corrected as needed. During rewarming, MAP was kept above 50 mmHg once core temperature reached >30°C using intermittent administration of epinephrine.

After decannulation, animals were kept ventilated for 2 h under anesthesia with 0.5–1% isoflurane (core temperature 36–37°C). To increase the hematocrit value to greater than 30%, the remaining autologous blood in the CPB circuit was collected, concentrated by centrifugation (3000 rpm for 5 min), and re-transfused. Heparin-induced anticoagulation was not reversed and allowed to dissipate spontaneously. Routine blood gas analysis was conducted serially (GEM Premier 3000 analyzer, Instrumentation Laboratory, Bedford, MA, United States). After spontaneous ventilation had resumed, animals were extubated and allowed to recover in an oxygen-enriched and humidified environment for 24 h, with free access to water and food.

To harvest the brain, 24 h after EPR rats were re-anesthetized, intubated, and mechanically ventilated. One sample of brain

tissue was immediately fixed in 10% buffered formalin and paraffin-embedded for immunostaining. The remaining brain tissue was frozen in liquid nitrogen and stored at −80°C until further use. Blood samples were also collected before and after induction of HS and at 0–120 min and 24 h after CPB and stored at −80°C until analysis. Naïve rats were sacrificed under 5% isoflurane.

Drug Treatments

Annexin A1 biomimetic tripeptide (ANXA1sp or Ac-QAW, Ac = acetyl, MW = 445.47 Da) was synthesized and purified (>98% purity) by GenScript (Piscataway, NJ, United States). The peptide was suspended in 100% DMSO. For experiments, this stock solution was diluted in saline to a final dose of 1 mg/kg ANXA1sp and a concentration of 1% DMSO in saline as vehicle (control). ANXA1sp treatment solutions were freshly prepared immediately before use.

Rats were randomly assigned to three groups (*EPR + ANXA1sp*, $n = 14$; *EPR+ vehicle*, $n = 15$; *naïve controls*, $n = 3$) and terminated for histologic and biochemical analyses at 24 h after EPR. Rats received ANXA1sp (1 mg/kg iv) or vehicle (1% DMSO iv) in 1 ml saline immediately after induction of VF, then again at 1 h after CPB. Additionally, blood salvaged during exsanguination HS was also treated *ex vivo* with ANXA1sp 1 mg/kg or 1% DMSO and re-transfused during CPB rewarming and resuscitation (**Figure 1**). All treatments were administered in a blinded manner.

Cell Death Assessment

Apoptosis was determined by terminal deoxynucleotidyl nick-end labeling (TUNEL) per assay manufacturer's protocol

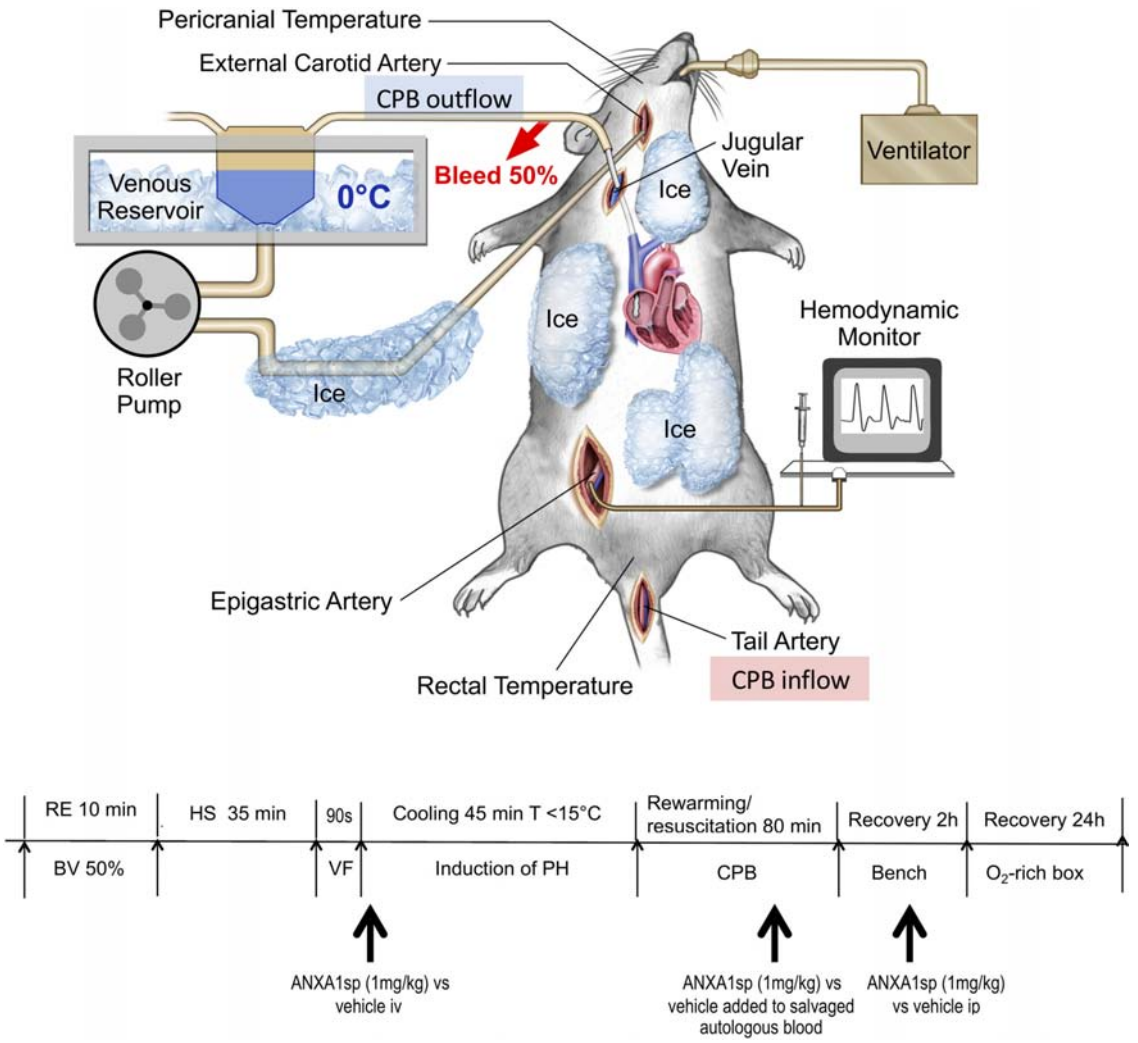

FIGURE 1 | Experimental model of exsanguination cardiac arrest including rapid hemorrhagic shock followed by 90 s of fibrillatory arrest, induction of profound hypothermia (<15°C), resuscitation and rewarming on CPB (emergency preservation and resuscitation, EPR) – diagram of surgical model and experimental timeline. RE, rapid exsanguination; BV, blood volume; HS, hemorrhagic shock; VF, ventricular fibrillation; PH, profound hypothermia; CPB, cardiopulmonary bypass; ANXA1sp, Annexin A1 short peptide.

(Roche Diagnostics, Indianapolis, IN, United States). Briefly, sections of paraffin-embedded brain tissue samples (5 μm thick) were deparaffinized using xylene and descending grades of ethanol, and pretreated with microwave radiation (350 W, in 200 mL of 0.1 M Citrate buffer, pH 6.0) for 5 min. Tissue sections were then incubated with terminal deoxynucleotidyl transferase (TdT) for 1.5 h at 37°C and then rinsed with PBS. Slides of five representative areas of the retrosplenial and posterior parietal cortex and CA1 area of the hippocampus were mounted using UltraCruz™ Mounting Medium with DAPI (Santa Cruz Biotechnology, Santa Cruz, CA, United States). Negative controls were incubated in label solution without TdT. A separate set of brain tissue sections was stained with acid fuchsin-celestine blue to identify possible necrotic cells. Cell counting was performed in a blinded manner across five representative areas of the cerebral cortex and CA1 areas using fluorescence microscopy (Leica DM IRB, Germany) with a 20×/0.4 PH objective at 1.5-fold magnification. Data obtained in every field were added together to make a final data count for each slide and expressed as percentage of total cell number within the relevant fields.

Western Blots

Frozen brain samples were homogenized and protein quantified by BCA assay (Thermo Fisher Scientific). Western blotting was performed using SDS–PAGE 4–20 and 8–16% gels gradient gels (Bio-Rad) with the following antibodies: rabbit anti-HMGB1 (Bioss Antibodies Inc., Woburn, MA, United States), rabbit polyclonal antibodies against SIRT3 (28-kDa isoform, Cell Signaling and Abcam), FOXO3a (Cell Signaling), Mn-SOD (Santa Cruz), cleaved caspase-3 (Cell Signaling), Bax and Bcl-2 (Santa Cruz), rabbit anti-LC3B (cell signaling), mouse anti-p62/SQSTM1 (R&D Systems, Minneapolis, MN, United States), and rabbit monoclonal antibody against GAPDH (Cell Signaling Technology, Boston, MA, United States). The bands were detected by Super-signal West Dura Extended Duration Substrate (Thermo Scientific Fisher, Rockford, IL, United States). Band intensities of HMGB1, SIRT3, FOXO3a, Mn-SOD, cleaved caspase-3, Bax, Bcl-2, LC3II, p62/SQSTM1 were all normalized with a GAPDH loading control.

Cytokine Measurements

Concentrations of IL-10, IL-6, and TNFα in brain homogenates were measured using rat-specific ELISA kits per manufacturer's protocol (Thermo Fisher Scientific, Grand Island, NY, United States). Brain homogenates were separated by centrifugation at 14,000 *g* for 10 min at 4°C to remove cellular debris. In addition, left ventricular myocardial concentrations of IL-6 and TNFα were analyzed by ELISA. Change in absorbance in every well was detected at 450 nm on a microplate reader. All measurements were performed in triplicate.

Confocal and Fluorescence Microscopy

After deparaffinization, brain tissue sections were treated with 10 mM citrate buffer (pH 6.0) for antigen retrieval. After blocking with 10% normal goat serum at RT for 1 h, the sections were incubated with rabbit anti-SIRT3 antibody (1:300) and mouse anti-COXIV (1:500, Santa Cruz Biotechnology, Santa Cruz, CA, United States), or rabbit anti-LC3B (1:400) and mouse anti-p62/SQSTM1 (1:400), respectively, at 4°C overnight. The sections were then incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:500; Invitrogen, Carlsbad, CA, United States) and Alexa Fluor 550-conjugated goat anti-mouse IgG (1:500; Invitrogen, Carlsbad, CA, United States) at RT for 1 h. After washing with PBS, slides were prepared and mounted using UltraCruz™ Mounting Medium with DAPI (Santa Cruz Biotechnology, Santa Cruz, CA, United States) to detect nuclei. Images were captured on a Leica SP5 confocal microscope (Leica Microsystems, Germany) using a 40×/1.25–0.75 Plan APO oil objective, and the images were analyzed by NIH ImageJ software (version 1.51).

To assess biodistribution of systemically administered ANXA1sp, rats were anesthetized, intubated and mechanically ventilated, and the tail vein cannulated as described above. Fluorescein isothiocyanate (FITC)-conjugated ANXA1sp (FITC-QAW, MW = 905.48, >98% purity) was synthesized and purified by GenScript (Piscataway, NJ, United States) and injected via the tail vein (0.5 mg/kg). Brain tissue was harvested at 1 h after injection, fixed with 10% neutrally buffered formalin and embedded in paraffin. The 5 μm sections were deparaffinized and evaluated by fluorescence microscopy as above.

Neurologic Evaluation

On postoperative day 1, rats underwent standardized functional neurologic testing by an observer blinded to group assignment, using an established neurologic scoring system that evaluates motor deficit (Homi et al., 2010). Briefly, rats were first placed on a 35 × 31 cm screen (grid size 0.6 × 0.6 cm) that could be rotated from horizontal (0°) to vertical (90°). The length of time that the rat could hold onto the screen after being rotated from 0 to 90° was recorded to a maximum of 15 s (0–3). Rats were then tested for balance on a horizontal wooden rod, and the time lapse before falling off the rod was recorded to a maximum of 30 s (0–3). Finally, rats underwent a prehensile traction test, and the length of time that the rat could cling to a horizontal rope was recorded to a maximum of 5 s (0–3). Animals received a score for each of the three tests. The final score was the sum of the individual test scores, with 0 the best score, and 9 the worst score.

Statistical Analysis

Statistical analysis was performed using Prism 8 (GraphPad Software, San Diego, CA, United States). Results are expressed as mean ± standard deviation (SD). Parametric values, including physiologic values, data from western blots, ELISA were compared between groups using one-way analysis of variance (ANOVA) with *post hoc* Tukey's multiple comparison test, or a Student's *t*-test (equal variance not assumed), according to the characteristics of each experiment. Statistical significance was defined as $p < 0.05$.

RESULTS

The 24 h survival rates were 75.0% (9/12) and 61.5% (8/13) for the ANXA1sp and vehicle treated groups, respectively. One rat

in vehicle group and three in ANXAsp1 group died from acute cardiac failure during the early postoperative phase. Additionally, three rats died of severe spinal cord injury and one of severe brain injury, all in the vehicle treated group. Two rats in each group died of technical failures and were excluded from analyses.

Baseline and intraoperative physiological parameters were similar between groups and are shown in **Figures 2A,B** and **Table 1**. Profound systemic hypotension (MAP < 20 mmHg) occurred during rapid exsanguination and cardiac arrest (**Figure 2A**) and was associated with severe metabolic acidosis (**Table 1**). All animals underwent the same cooling and rewarming protocol (**Figure 2B**). Rats exhibited spontaneous hypothermia (from 37°C to 32°C) before the onset of systemic cooling (shock-induced hypothermia). Following induction of hypothermia, pericranial temperature rapidly dropped to 23°C at 5 min (rate of cooling 1.8°C/min) and to 15°C at 20 min (rate of cooling 0.85°C/min). After 45 min of profound hypothermia (10–15°C), animals were resuscitated and slowly rewarmed using CPB to 34–35°C over 80 min (rate of rewarming 0.4°C/min).

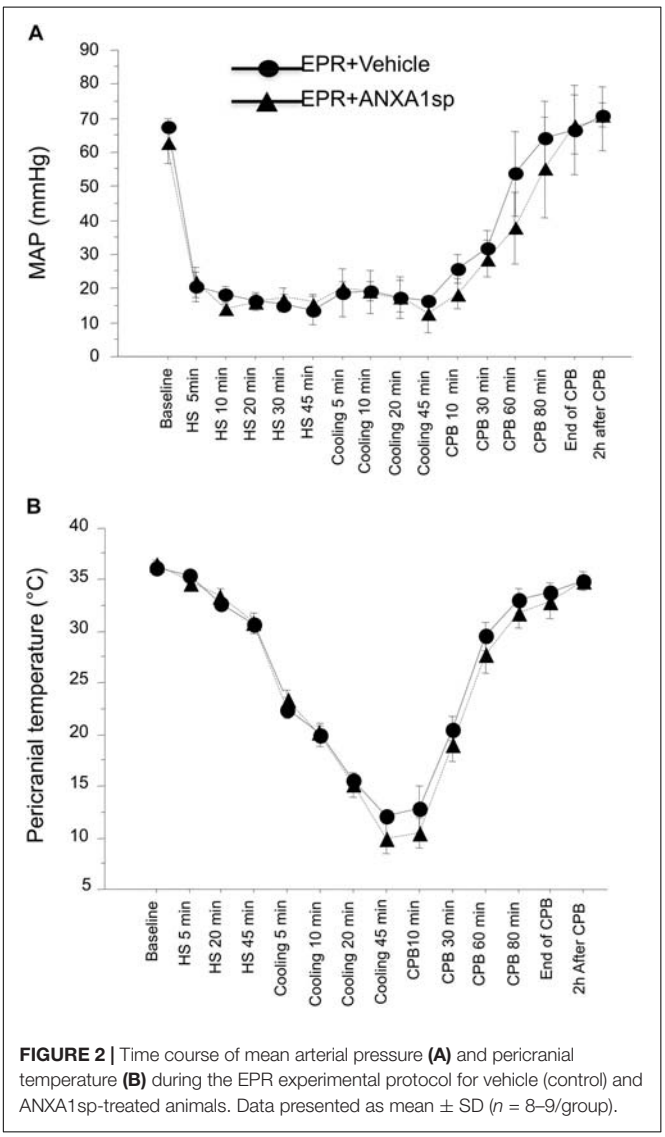

FIGURE 2 | Time course of mean arterial pressure **(A)** and pericranial temperature **(B)** during the EPR experimental protocol for vehicle (control) and ANXA1sp-treated animals. Data presented as mean ± SD (n = 8–9/group).

Systemically Administered ANXA1sp Traverses the Blood-Brain Barrier and Attenuates Neuroinflammation After EPR

Relative abundance of HMBG1, a key initiator of neuroinflammation, was assessed by Western analysis in brain homogenates. We found a 10% ($p < 0.05$) attenuation in cerebral expression of HMGB1 in ANXA1sp compared to HS-EPR vehicle treated animals (**Figures 3A,B**). Moreover, brain levels of the pro-inflammatory cytokines IL-6 and TNFα were reduced by 55% ($p < 0.01$) and 27% ($p = 0.059$), respectively, with ANXA1sp treatment (**Figures 3C,D**). Conversely, brain levels of the anti-inflammatory cytokine IL-10 were increased by 25% ($p < 0.05$) in ANXA1sp-treated compared to vehicle controls at 24 h after EPR (**Figure 3E**).

Fluorescence-conjugated peptide (FITC-QAW) was detectable in all areas of brain parenchyma at 1 h following intravenous injection (**Supplementary Figure 1**).

Regulation of Cell Death by ANXA1sp After EPR

Acid fuchsin-celestine blue staining revealed acidophilic neurons and possible necrosis in the cortex at 24 h after EPR in vehicle-treated animals, which were reduced by 72% ($p < 0.05$) following ANXA1sp treatment (**Figures 4A,B**). ANXA1sp treatment was also associated with a 62% ($p < 0.05$) reduction in TUNEL-positive cells in the cerebral cortex, but not in the hippocampus, at 24 h after EPR (**Figures 4C,D**). This was corroborated by a 56% ($p < 0.05$) reduction in cerebral expression of cleaved caspase-3 in ANXA1sp-treated animals (**Figures 4E,F**).

We used Western analysis of brain homogenates to assess changes in ratios of death and survival factors. At 24 h, a modest but significant (10%, $p < 0.05$) increase in the relative abundance of the anti-cell death protein Bcl-2 was observed in ANXA1sp compared to vehicle-treated animals after HS-EPR (**Figures 5A,B**). Conversely, a marked 5-fold ($p < 0.01$) increase in expression of the pro-cell death protein Bax was seen after HS-EPR in vehicle-treated animals, with a significant 33% ($p < 0.05$) reduction following ANXA1sp treatment (**Figures 5A,C**). Consequently, the near 5-fold surge in Bax:Bcl-2 ratio detected after HS-EPR in vehicle-treated compared to naïve animals ($p < 0.001$) was attenuated by 38% ($p < 0.01$) with ANXA1sp treatment (**Figure 5D**).

Modulation of SIRT3 Expression and SIRT3 Pathway by ANXA1sp

Western blot analysis in brain homogenates revealed a 33% ($p < 0.01$) reduction in relative abundance of the primary mitochondrial deacetylase SIRT3 (28 kDa isoform) in vehicle-treated HS-EPR compared to naïve animals, which was partially restored with ANXA1sp treatment (increased by 25%, $p < 0.05$) (**Figures 6A,B**). Increased SIRT3 expression with ANXA1sp treatment in both cortical and hippocampal

TABLE 1 | Physiologic data.

Parameter	Group	Baseline	End of HS	End of cooling	CPB 30 min	CPB 80 min	End of CPB	2 h after CPB
pH	Vehicle	7.42 ± 0.06	7.19 ± 0.08	7.34 ± 0.09	7.39 ± 0.04	7.45 ± 0.09	7.29 ± 0.08	7.48 ± 0.07
	ANXA1sp	7.47 ± 0.04	7.17 ± 0.04	7.27 ± 0.05	7.35 ± 0.06	7.40 ± 0.1	7.32 ± 0.06	7.47 ± 0.05
PaO_2 (mmHg)	Vehicle	202 ± 46	26 ± 8	46 ± 11*	291 ± 47	285 ± 89	240 ± 102	249 ± 77
	ANXA1sp	193 ± 26	25 ± 4	31 ± 3	313 ± 40	268 ± 65	253 ± 73	277 ± 131
$PaCO_2$ (mmHg)	Vehicle	37.5 ± 6.9	54.8 ± 9.	52.2 ± 11.1	35.6 ± 6.6	34.0 ± 7.8	56.3 ± 13.4	42.2 ± 6.6
	ANXA1sp	32.9 ± 4.9	54.4 ± 4.7	57.2 ± 6.1	39.4 ± 5.0	39.3 ± 11.8	50.8 ± 5.1	41.6 ± 4.7
Hct (%)	Vehicle	38.00 ± 3.8	33.75 ± 2.4		18.63 ± 1.9	22.29 ± 2.4	23.57 ± 2.9	26.70 ± 4.4
	ANXA1sp	38.22 ± 5.5	35.22 ± 2.5		19.00 ± 1.7	23.78 ± 2.0	26.11 ± 5.9	32.50 ± 3.1
BE (mmol/L)	Vehicle	0.08 ± 3.6	−7.30 ± 2.9	1.26 ± 4.1	−3.03 ± 3.5	−0.94 ± 2.3	0.16 ± 2.6	6.86 ± 4.5
	ANXA1sp	0.47 ± 3.8	−8.57 ± 2.1	−1.69 ± 2.4	−3.46 ± 2.4	−1.31 ± 2.3	−0.51 ± 2.8	6.06 ± 1.8

*Results are shown as Mean ± SD; *$p < 0.05$ between groups. ANXA1sp, Annexin A1 short peptide; Hct, hematocrit; BE, base excess; End of HS, at the end of 45 min of hemorrhagic shock; End of cooling, at the end of 45 min cooling; End of CPB, at the end of cardiopulmonary bypass.*

cells was further confirmed by confocal microscopy, with SIRT3 immunoreactivity colocalizing with the mitochondrial protein COXIV (**Figure 6E**). We further measured expression of FOXO3a and MnSOD, two downstream targets of SIRT3. EPR resulted in an 87% ($p < 0.01$) reduction in relative abundance of the transcription factor FOXO3a and a 42% ($p < 0.001$) reduction its regulated antioxidant enzyme MnSOD compared to naïve animals, and were both partially restored with ANXA1sp treatment (a 4-fold increase in FOXO3a expression, $p < 0.05$; and a 18% increase in MnSOD expression, $p < 0.05$) (**Figures 6A,C,D**).

ANXA1sp Promotes Autophagy Following EPR

Western blot was performed to detect the expression of autophagy associated proteins in brain homogenates. Compared to vehicle, ANXA1sp treatment was associated with a 35% ($p < 0.01$) increased expression of LC3II and a 25% ($p < 0.05$) reduced expression of p62/SQSTM1 (**Figures 7A,B,C**). In addition, we detected expression of LC3B by immunohistochemistry, with LC3B and p62 colocalizing in cortical cells (**Figure 7D**).

Neurologic Outcome After EPR and ANXA1sp Treatment

Finally, we evaluated neurologic changes at 24 h after EPR and ANXA1sp treatment. Compared to vehicle controls, ANXA1sp-treated animals had significantly lower neurologic severity scores, showing improved sensory-motor functions (including processing involving retrosplenial and posterior parietal cortex, **Figure 4G**).

Systemically Administered ANXA1sp Also Attenuates Myocardial Inflammation

To evaluate the effects of ANXA1sp in organs that are not shielded by the blood-brain barrier, we assessed pro-inflammatory cytokines in left ventricular myocardial homogenates between groups. At 24 h after EPR, heart levels of IL-6 and TNFα were reduced in ANXA1sp-treated animals by 10% ($p < 0.05$) and 17% ($p = 0.054$), respectively (**Supplementary Figure 2**).

DISCUSSION

In this study, we used a novel experimental model of acute neuroinflammation triggered by exsanguinating cardiac arrest and treated by emergent preservation and resuscitation (EPR) to test the neuroprotective effects and mechanisms of ANXA1sp administration. Together with our previous findings that ANXA1sp exerts pro-resolving effects in a rat model of CPB with global ischemia-reperfusion via deep hypothermic circulatory arrest (Zhang et al., 2017), we continue to illustrate how this small peptide impacts neuroinflammation. Here, we show that ANXA1sp treatment is superior to induction of profound hypothermia (EPR) alone by increasing cortical cell viability, promoting a favorable expression of pro-survival (Bcl-2) versus pro-death (Bax) factors in the brain, reducing caspase-3 activation, and overall improving neurological performance at 24 h after EPR.

High mobility group box 1 is a key endogenous danger associated molecular pattern which acts as a mediator of neuroinflammation resulting from a variety of conditions such as cerebral ischemia-reperfusion (Kim et al., 2006; Yang et al., 2011), septic shock, and traumatic brain injury (Parker et al., 2017). HMGB1 is actively released by neurons and glial cells upon inflammasome activation, and in turn activates two pattern recognition receptors on target cells (TLR4 and RAGE) (Gao et al., 2012), leading to NF-κB mediated production of pro-inflammatory cytokines (Paudel et al., 2018). HMGB1 also plays a pivotal role in BBB disruption (He et al., 2012; Festoff et al., 2016; Yang et al., 2018), either directly via cytokine mediated activation of metalloproteinase or via disruption of tight junctions (Gloor et al., 2001; Utech et al., 2010), although the precise mechanism remains elusive. Nonetheless, altered BBB permeability is known to then amplify neuroinflammation and neuronal excitation (Frank et al., 2015, 2016). We provide primary evidence that HS and exsanguinating cardiac arrest treated with induction of profound hypothermia and EPR

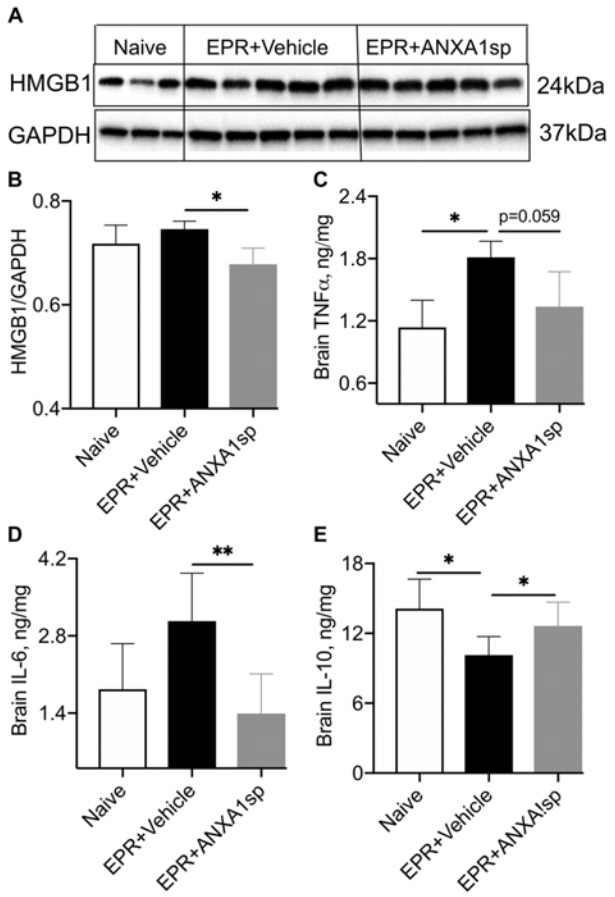

FIGURE 3 | Modulation of indices of neuroinflammation after EPR by experimental group. **(A,B)** High mobility group protein B1 (HMGB1) expression in brain homogenates from ANXA1sp-treated animals were significantly reduced compared to vehicle (controls). **(C,D)** Cerebral levels of tumor necrosis alpha (TNFα) and interleukin 6 (IL-6) were increased in the vehicle controls, and reduced with systemic administration of ANXA1sp. **(E)** Cerebral interleukin 10 (IL-10) was significantly reduced after induction of profound hypothermia (EPR + vehicle) and restored with administration of ANXA1sp. Data presented as mean ± SD (n = 3–7/group), $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to vehicle controls or naïve animals, analyzed by one-way ANOVA with Tukey's multiple comparisons test. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

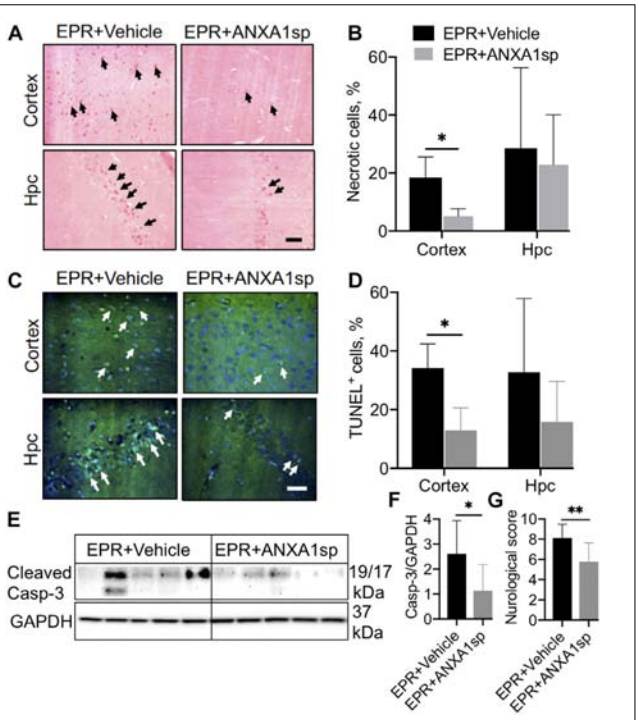

FIGURE 4 | Necrosis and apoptosis in the CNS after exsanguination cardiac arrest/EPR and ANXA1sp or vehicle treatment. **(A,B)** Necrosis, detected by acid fuchsin-celestine blue, was reduced in the cerebral cortex of ANXA1sp treated rats. Arrowheads in panel A identify acidophilic neurons in the cortex and hippocampus (Hpc). **(C,D)** Terminal deoxynucleotidyl nick-end labeling (TUNEL) staining and quantification of apoptotic cells. Apoptosis was significantly reduced in the cerebral cortex of ANXA1sp-treated rats on postoperative day 1. Arrowheads in panel **(C)** identify pyknotic positive cells. Scale bar: 20 μm. **(E,F)** Western blot analysis of cleaved caspase-3 revealed significantly reduced levels in brain homogenates from ANXA1sp-treated animals compared to vehicle (controls). **(G)** Neurologic score (sensory-motor function) was significantly improved at postoperative day 1 in ANXA1sp rats. Data presented as mean ± SD (n = 5–7/group), $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to vehicle (controls) analyzed by unpaired t-test **(B,D,F)** or Mann–Whitney U-test **(G)**.

elicit neuroinflammation. Further, we show ANXA1sp treatment to favorably modulate the neuroinflammatory response, by attenuating the HS-EPR induced increase in cerebral levels of the proinflammatory alarmin HMGB1 and altering the balance of pro-inflammatory (IL-6 and TNFα) and anti-inflammatory cytokines (IL-10) (**Figures 3A–E**). This is consistent with the pleiotropic immunomodulatory functions of IL-10, to polarize the inflammatory system toward an anti-inflammatory phenotype (Ip et al., 2017), aiding in the resolution of neuroinflammation (Garcia et al., 2017). The pro-resolving effects were accompanied by improved cell survival (**Figures 4A–E**) and neurological function (**Figure 4G**). This study builds on our previous findings demonstrating beneficial effects of ANXA1sp on neuroinflammation, microglial activation, NF-κB activation and postoperative neurocognitive performance following global I/RI associated with deep hypothermic circulatory arrest (Zhang et al., 2017). While our experimental model is complex, sequentially involving HS, warm cardiac arrest, induction of profound hypothermia followed by rewarming and resuscitation, the protective effects of ANXA1sp recapitulate those seen in other experimental models of cerebral I/RI (Relton et al., 1991; Gavins et al., 2007; Smith et al., 2015; Vital et al., 2016).

Systemically administered ANXA1sp was also associated with anti-inflammatory effects in the heart (reduced levels of IL-6 and TNFα) following HS-EPR. Consistent with previous reports in experimental models of regional myocardial I/RI (La et al., 2001; Qin et al., 2015), these results support the broader therapeutic roles attributed to ANXA1 and its peptide mimetics in reducing systemic inflammation and conferring organ protection from a variety of insults involving IR/I.

To better understand the added neuroprotective effects and mechanisms of action of ANXA1sp in the setting of HS-EPR with

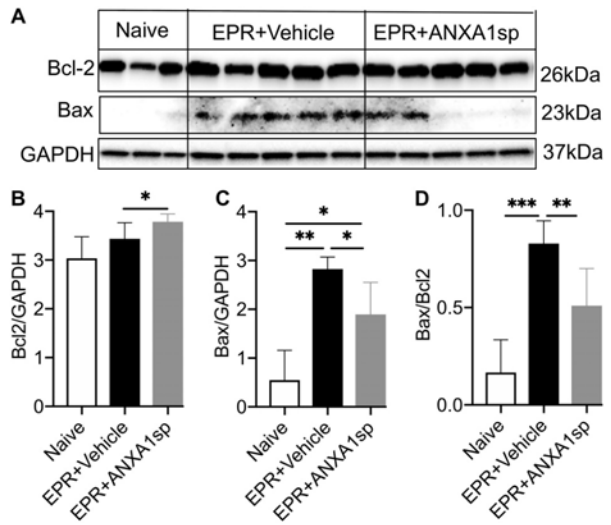

FIGURE 5 | Western blot analysis of cerebral Bcl-2 and Bax levels after exsanguination cardiac arrest/EPR and ANXA1sp or vehicle treatment. Intensities of protein bands corresponding to Bcl-2 (26 kDa) **(A,B)** and Bax (23 kDa) **(A,C)** were measured using computer assisted densitometric analysis, normalized to the intensity of GAPDH and compared between experimental groups. ANXA1sp treatment resulted in significantly increased expression of the anti-apoptotic protein Bcl-2 **(B)**, reduced expression of the pro-apoptotic protein Bax **(C)**, with a corresponding reduction in the Bcl-2:Bax ratio **(D)**. Data presented as mean ± SD (n = 3–8/group), $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared to either naïve or vehicle controls analyzed by one-way ANOVA with Tukey's multiple comparisons test.

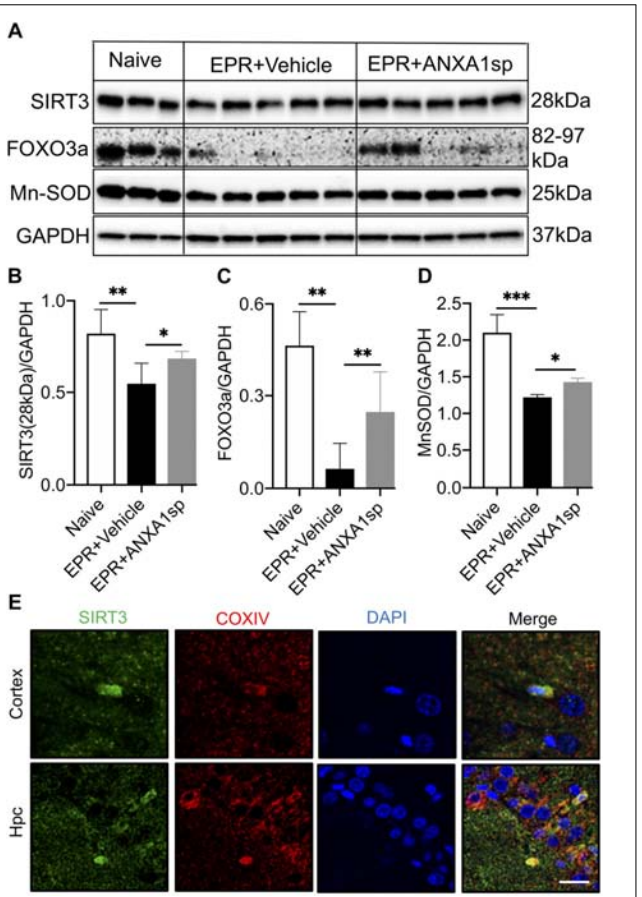

FIGURE 6 | Cerebral expression of Sirtuin-3 (SIRT3) and downstream regulated proteins after exsanguination cardiac arrest/EPR and ANXA1sp or vehicle treatment. **(A,B)** A significant reduction in SIRT3 (28 kDa short, processed, active form) was seen after EPR + vehicle treatment compared to naïve animals, and was rescued in ANXA1sp-treated animals. This was accompanied by similar expression changes for the forkhead box O3a (FOXO3a) transcription factor **(C)**, known to transactivate the antioxidant enzyme manganese superoxide dismutase (MnSOD) **(D)**. GAPDH was used as loading control **(E)**. At 24 h postoperatively, colocalization of sirtuin-3 (SIRT3) with the mitochondrial protein cytochrome c oxidase complex IV (COXIV) in cortical and hippocampal slides from ANXA1sp-treated animals was visualized via double immunofluorescence staining and confocal microscopy. Data presented as mean ± SD (n = 3–7/group), $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared to either naïve or vehicle controls analyzed by one-way ANOVA with Tukey's multiple comparisons test.

profound hypothermia, we focused on SIRT3, a mitochondrial protein deacetylase known to be widely and abundantly expressed in most cell types in the CNS (Kim et al., 2011) and differentially regulated in brain regions and across stages of development in the rat (Sidorova-Darmos et al., 2014). SIRT3 is part of the silent information regulator of transcription (SIRT) family of NAD^{+}-dependent protein deacetylases, which have emerged as key regulators of metabolic and energy stress-response pathways including neuroinflammation (Someya et al., 2010; Lee et al., 2013; Hattori et al., 2015; Liu L. et al., 2015; Yuan et al., 2016), but also systemic inflammation (Preyat and Leo, 2013; Vachharajani et al., 2014; Liu T. F. et al., 2015; Traba et al., 2015). Notably, SIRT3 exerts neuroprotective effects. For instance, SIRT3 overexpression rescues mutant SOD1-induced neuronal cell death (Song et al., 2013), increases neuronal lifespan under mitochondrial oxidative stress (Weir et al., 2012), protects against excitotoxic injury (Kim et al., 2011), mediates adaptive neuronal responses (resistance to oxidative stress, apoptotic cell death) to *in vitro* bioenergetic, oxidative and excitatory stress (Cheng et al., 2016), and protects mice against noise-induced hearing loss *in vivo* (Someya et al., 2010). Specifically, SIRT3 has been shown to have a major involvement in CNS ROS metabolism by regulating FOXO3a transcription factor and contributing to transactivation of key antioxidant enzymes including MnSOD and catalase (Rangarajan et al., 2015). Together, even though results from rodent studies are not completely consistent (Novgorodov et al., 2016), most data suggest that SIRT3 expression is vital for neuronal survival and suppression of neuroinflammation following physical stressors. In this study, we noted that relative abundance of SIRT3 – the 28 kDa processed active isoform, known to possess deacetylase activity (Schwer et al., 2002; Sundaresan et al., 2008) – as well as FOXO3a and MnSOD, were all reduced in brain homogenates following HS-EPR, consistent with previous reports in experimental models of cerebral I/R (Zhao et al., 2018), but were rescued with systemic administration of ANXA1sp (**Figure 6**). HS, ischemia-reperfusion, and EPR incite neuroinflammation, and SIRT3-mediated upregulation of FOXO3a-dependent antioxidant gene expression could be

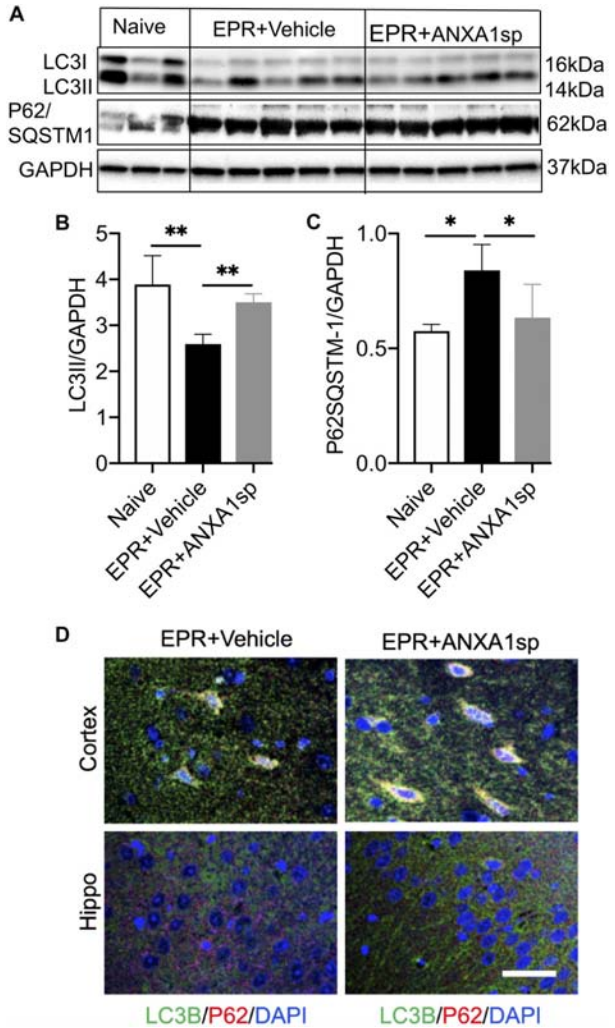

FIGURE 7 | ANXA1sp promotes cerebral autophagy after exsanguinating cardiac arrest/EPR. The expression of LC3II and p62/SQSTM1 (sequestosome 1) was assessed by western blot and normalized to GAPDH **(A–C)**. **(D)** Colocalization of LC3B with p62 in cortical and hippocampal slides from animals treated with ANXA1sp and vehicle (controls) was visualized by double immunofluorescence staining and confocal microscopy. Increased number of LC3B positive/p62 positive puncta appears in the ANXA1sp-treated group. $^{**}p < 0.01$; $^{*}p < 0.05$.

implicated in the attenuation of ROS neurotoxicity associated with acute neuroinflammation and activation of microglia. This may translate into attenuation of secondary brain injury and decreased cerebral edema. Along with downregulation of pro-apoptotic proteins, these findings suggest that ANXA1sp treatment offers an element of neuroprotection.

Modulation of protein acetylation is emerging as a therapeutic strategy to create a pro-survival and anti-inflammatory phenotype in various shock states, including lethal HS (Li and Alam, 2011). Intriguingly, promoting histone acetylation via administration of the histone deacetylase (HDAC) inhibitor valproic acid has been shown to decrease neuronal apoptosis, neuroinflammation, and brain lesion size while improving neural plasticity and resulting in faster neurocognitive and neurologic recovery in pre-clinical models of traumatic brain injury, HS, and polytrauma (Nikolian et al., 2017, 2018; Chang et al., 2019). An imbalance in histone acetyltransferase/deacetylase activity has been reported in both HS (Lin et al., 2006) and neurodegeneration (Rouaux et al., 2003). Valproic acid administration results in epigenetic and posttranslational modifications that induce differential gene expression (Dekker et al., 2014; Bambakidis et al., 2016), metabolic changes (Hwabejire et al., 2013) and alterations of the proteome. It appears that promoting acetylation of histones and other target transcription factors through the use of HDAC inhibitors (like valproic acid), as well as promoting mitochondrial protein deacetylation via induction of SIRT3 (in this study using ANXA1sp) can both exert neuroprotective effects in the setting of lethal HS. The precise mechanisms by which modulation of protein acetylation results in attenuation or resolution of neuroinflammation remain to be elucidated.

We found that ANXA1sp treatment promoted cerebral autophagy following EPR. This was accompanied by increased SIRT3 protein expression, reduced cell apoptosis and improved neuro severity scores. Importantly, upregulation of SIRT3 in response to ANXA1sp treatment was associated with increased expression of LC3II and a reduced level of p62/SQSTM1, indicating increased autophagic flux (**Figure 7**). These results are consistent with previous reports implicating SIRT3 as an activator of autophagy in neuronal cells (Dai et al., 2017; Yan et al., 2018) and macrophages (Liu et al., 2018) under various stress conditions *in vitro*, and *in vivo* studies showing sirtuin activation to be protective in spinal cord injury, brain trauma, and cerebral ischemia (He et al., 2017; Zhao et al., 2017). A schematic representation of the relationships between ANXA1sp, the SIRT3 pathway and neuroinflammation in the context of cerebral I/RI is shown in **Figure 8**.

Although the therapeutic effects of hypothermia in HS in general, and those of EPR for exsanguinating cardiac arrest in particular, have been previously reported in large animal experiments (Alam et al., 2002, 2004, 2005, 2006a,b; Chen et al., 2005; Wu et al., 2006), benefits of the clinically realistic rat model described in this study include lower costs to test efficacy of selective pharmacological neuroprotective interventions (such as the pro-resolving ANXA1sp) in enhancing clinical outcomes after EPR, as well as availability of molecular biology tools to understand the mechanisms at play. To increase clinical relevance, we modified previously described rodent models of EPR (Drabek et al., 2007a,b) in several ways: first, we introduced a prolonged period of HS followed by induction of fibrillatory cardiac arrest; second, we rapidly induced profound hypothermia via aortic flushing of a relatively low volume (1.5–2 fold the estimated blood volume) of ice-cold saline-based solution using a light weight cycling system at 20 ml/min; finally, we re-transfused salvaged autologous blood during resuscitation, thereby avoiding additional confounding effects from exposure to allogeneic blood transfusions. In this severe model, administration of a pro-resolving ANXA1 bioactive peptide during EPR was superior to EPR alone in attenuating neuroinflammation, increasing pro-survival protein expression and autophagic flux.

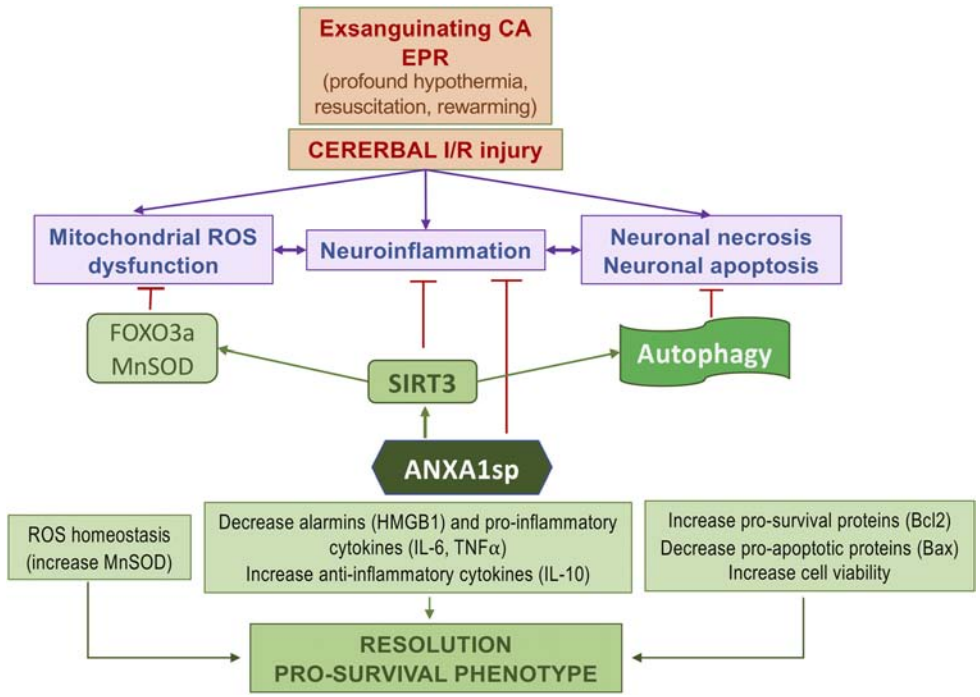

FIGURE 8 | Simplified diagram of the protective effects of ANXA1sp following cerebral ischemia-reperfusion injury associated with experimental exsanguinating cardiac arrest (CA) and emergent preservation and resuscitation (EPR).

Several limitations of this study follow. HS was achieved by closely controlled exsanguination of blood, which was salvaged and re-transfused. Although we designed the model to closely mimic the clinical scenario, HS inevitably requires surgical bleeding control and transfusion of allogeneic blood products, which would significantly amplify the inflammatory response. Rats serve as imperfect surrogates for human subjects, and only male young adult animals were used. An injured but un-resuscitated control group was not used, as the survival without resuscitation is extremely poor. Instead, we used a naïve uninjured control group, to allow assessing whether ANXA1sp effects are restorative or *de novo*. We have not measured directly markers of resolution of inflammation (neutrophil apoptosis/efferocytosis, functional macrophage switches), or microglial activation. However, microglial activation was attenuated by ANXA1sp treatment in our previously reported rat deep hypothermic circulatory arrest study (Zhang et al., 2017). This study focuses on ANXA1sp-induced upregulation of the SIRT3 pathway, and thus we have not explored the traditional mechanisms implicated in the pro-resolving effects of Annexin A1 and its bioactive peptides (via the FPR2/ALX receptor pathway). The neuroinflammatory response to hypothermic cardiac arrest is not uniform, displaying important spatio-temporal differences between brain regions (Drabek et al., 2015). Our investigation focused on the cortex and hippocampus as two vulnerable brain regions, but other areas displaying robust early neuroinflammatory responses (e.g., the striatum) were not studied. Temporally, our observation period was limited to 24 h, and longer-term evaluations are required to fully assess ANXA1sp's protective effects, including neurocognitive tests. Although previous reports from experimental focal cerebral I/RI in the rat suggest neutrophil accumulation in the infarct area (which correlates closely with cortical lesion size) to be maximal at 24 h post-reperfusion (Zhang et al., 1994), it should be noted that our results may represent a delay rather than elimination of neuronal death in the selectively vulnerable brain regions studied.

CONCLUSION

Using a clinically relevant rodent model of lethal HS and EPR-induced acute neuroinflammation, we show that administration of a pro-resolving ANXA1 peptide mimetic results in a pro-survival phenotype with attenuated cortical cell death, reduction of several common functional biomarkers of neuroinflammation (HMGB1, IL-6, and TNFα), and improved early neurological outcomes, compared to induction of profound hypothermia (EPR) alone. The protective effects were associated with increased expression of SIRT3 and corresponding upregulation of the FOXO3a-MnSOD antioxidant pathway. In addition, we note increased cerebral autophagy in ANXA1sp treated animals. Further studies need to refine the mechanisms involved and evaluate the use of SIRT3-activating drugs to resolve acute neuroinflammation and attenuate the uncontrolled production of ROS by activated microglia, as well as the long-term benefits of the proposed therapeutics.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the **Supplementary Files**.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the National Institutes of Health for animal care (Guide for the Care and Use of Laboratory Animals, Health and Human Services, National Institute of Health Publication No. 86-23, revised 1996). The protocol was approved by the Duke University Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

ZZ, QM, J-KS, and MP designed and performed the research. TV, CL, JM, and NT contributed new reagents and analytic tools. ZZ, QM, QQ, and MP analyzed the data. QM, ZZ, and MP wrote the manuscript. QQ, JM, and NT provided the critical edits to the manuscript. All authors read and approved the final draft of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2019.00608/full#supplementary-material

REFERENCES

Alam, H. B., Bowyer, M. W., Koustova, E., Gushchin, V., Anderson, D., Stanton, K., et al. (2002). Learning and memory is preserved after induced asanguineous hyperkalemic hypothermic arrest in a swine model of traumatic exsanguination. *Surgery* 132, 278–288. doi: 10.1067/msy.2002.125787

Alam, H. B., Chen, Z., Ahuja, N., Chen, H., Conran, R., Ayuste, E. C., et al. (2005). Profound hypothermia protects neurons and astrocytes, and preserves cognitive functions in a swine model of lethal hemorrhage. *J. Surg. Res.* 126, 172–181. doi: 10.1016/j.jss.2005.01.019

Alam, H. B., Chen, Z., Honma, K., Koustova, E., Querol, R. I., Jaskille, A., et al. (2004). The rate of induction of hypothermic arrest determines the outcome in a Swine model of lethal hemorrhage. *J. Trauma* 57, 961–969. doi: 10.1097/01.ta.0000149549.72389.3f

Alam, H. B., Chen, Z., Li, Y., Velmahos, G., DeMoya, M., Keller, C. E., et al. (2006a). Profound hypothermia is superior to ultraprofound hypothermia in improving survival in a swine model of lethal injuries. *Surgery* 140, 307–314. doi: 10.1016/j.surg.2006.03.015

Alam, H. B., Rhee, P., Honma, K., Chen, H., Ayuste, E. C., Lin, T., et al. (2006b). Does the rate of rewarming from profound hypothermic arrest influence the outcome in a swine model of lethal hemorrhage. *J. Trauma* 60, 134–146. doi: 10.1097/01.ta.0000198469.95292.ec

Alam, H. B., Duggan, M., Li, Y., Spaniolas, K., Liu, B., Tabbara, M., et al. (2008). Putting life on hold-for how long? Profound hypothermic cardiopulmonary bypass in a Swine model of complex vascular injuries. *J. Trauma* 64, 912–922. doi: 10.1097/TA.0b013e3181659e7f

Alam, H. B., Hashmi, S., Finkelstein, R. A., Shuja, F., Fukudome, E. Y., Li, Y., et al. (2010). Alterations in gene expression after induction of profound hypothermia for the treatment of lethal hemorrhage. *J. Trauma* 68, 1084–1098. doi: 10.1097/TA.0b013e3181d76bd1

Ansari, J., Kaur, G., and Gavins, F. N. E. (2018). Therapeutic potential of annexin A1 in ischemia reperfusion injury. *Int. J. Mol. Sci.* 19:E1211. doi: 10.3390/ijms19041211

Bambakidis, T., Dekker, S. E., Sillesen, M., Liu, B., Johnson, C. N., Jin, G., et al. (2016). Resuscitation with valproic acid alters inflammatory genes in a porcine model of combined traumatic brain injury and hemorrhagic shock. *J. Neurotrauma* 33, 1514–1521. doi: 10.1089/neu.2015.4163

Bartels, K., Ma, Q., Venkatraman, T. N., Campos, C. R., Smith, L., Cannon, R. E., et al. (2014). Effects of deep hypothermic circulatory arrest on the blood brain barrier in a cardiopulmonary bypass model–a pilot study. *Heart Lung. Circ.* 23, 981–984. doi: 10.1016/j.hlc.2014.04.131

Cao, X. Z., Ma, H., Wang, J. K., Liu, F., Wu, B. Y., Tian, A. Y., et al. (2010). Postoperative cognitive deficits and neuroinflammation in the hippocampus triggered by surgical trauma are exacerbated in aged rats. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 34, 1426–1432. doi: 10.1016/j.pnpbp.2010.07.027

Chang, P., Williams, A. M., Bhatti, U. F., Biesterveld, B. E., Liu, B., Nikolian, V. C., et al. (2019). Valproic acid attenuates neural apoptosis, inflammation, and degeneration 30 days after traumatic brain injury, hemorrhagic shock, and polytrauma in a swine model. *J. Am. Coll. Surg.* 228, 265–275. doi: 10.1016/j.jamcollsurg.2018.12.026

Chen, Z., Chen, H., Rhee, P., Koustova, E., Ayuste, E. C., Honma, K., et al. (2005). Induction of profound hypothermia modulates the immune/inflammatory response in a swine model of lethal hemorrhage. *Resuscitation* 66, 209–216. doi: 10.1016/j.resuscitation.2005.01.021

Cheng, A., Yang, Y., Zhou, Y., Maharana, C., Lu, D., Peng, W., et al. (2016). Mitochondrial SIRT3 mediates adaptive responses of neurons to exercise and metabolic and excitatory challenges. *Cell Metab.* 23, 128–142. doi: 10.1016/j.cmet.2015.10.013

Dai, S. H., Chen, T., Li, X., Yue, K. Y., Luo, P., Yang, L. K., et al. (2017). Sirt3 confers protection against neuronal ischemia by inducing autophagy: involvement of the AMPK-mTOR pathway. *Free Radic. Biol. Med.* 108, 345–353. doi: 10.1016/j.freeradbiomed.2017.04.005

Dekker, S. E., Bambakidis, T., Sillesen, M., Liu, B., Johnson, C. N., Jin, G., et al. (2014). Effect of pharmacologic resuscitation on the brain gene expression profiles in a swine model of traumatic brain injury and hemorrhage. *J. Trauma Acute Care Surg.* 77, 906–12;discussion 912. doi: 10.1097/TA.0000000000000345

Drabek, T., Stezoski, J., Garman, R. H., Han, F., Henchir, J., Tisherman, S. A., et al. (2007a). Exsanguination cardiac arrest in rats treated by 60 min, but not 75 min, emergency preservation and delayed resuscitation is associated with intact outcome. *Resuscitation* 75, 114–123. doi: 10.1016/j.resuscitation.2007.03.012

Drabek, T., Stezoski, J., Garman, R. H., Wu, X., Tisherman, S. A., Stezoski, S. W., et al. (2007b). Emergency preservation and delayed resuscitation allows normal recovery after exsanguination cardiac arrest in rats: a feasibility trial. *Crit. Care Med.* 35, 532–537. doi: 10.1097/01.CCM.0000253398.61666.0D

Drabek, T., Wilson, C. D., Janata, A., Stezoski, J. P., Janesko-Feldman, K., Garman, R. H., et al. (2015). Unique brain region-dependent cytokine signatures after prolonged hypothermic cardiac arrest in rats. *Ther. Hypothermia Temp. Manag.* 5, 26–39. doi: 10.1089/ther.2014.0013

Festoff, B. W., Sajja, R. K., van Dreden, P., and Cucullo, L. (2016). HMGB1 and thrombin mediate the blood-brain barrier dysfunction acting as biomarkers of neuroinflammation and progression to neurodegeneration in Alzheimer's disease. *J. Neuroinflammation.* 13:194. doi: 10.1186/s12974-016-0670-z

Frank, M. G., Weber, M. D., Fonken, L. K., Hershman, S. A., Watkins, L. R., and Maier, S. F. (2016). The redox state of the alarmin HMGB1 is a pivotal factor in neuroinflammatory and microglial priming: a role for the NLRP3 inflammasome. *Brain Behav. Immun.* 55, 215–224. doi: 10.1016/j.bbi.2015.10.009

Frank, M. G., Weber, M. D., Watkins, L. R., and Maier, S. F. (2015). Stress sounds the alarmin: the role of the danger-associated molecular pattern HMGB1 in stress-induced neuroinflammatory priming. *Brain Behav. Immun.* 48, 1–7. doi: 10.1016/j.bbi.2015.03.010

Gao, T. L., Yuan, X. T., Yang, D., Dai, H. L., Wang, W. J., Peng, X., et al. (2012). Expression of HMGB1 and RAGE in rat and human brains after traumatic brain injury. *J. Trauma Acute Care Surg.* 72, 643–649. doi: 10.1097/TA.0b013e31823c54a6

Garcia, J. M., Stillings, S. A., Leclerc, J. L., Phillips, H., Edwards, N. J., Robicsek, S. A., et al. (2017). Role of interleukin-10 in acute brain injuries. *Front. Neurol.* 8:244. doi: 10.3389/fneur.2017.00244

Gavins, F. N., Dalli, J., Flower, R. J., Granger, D. N., and Perretti, M. (2007). Activation of the annexin 1 counter-regulatory circuit affords protection in the mouse brain microcirculation. *FASEB J.* 21, 1751–1758. doi: 10.1096/fj.06-7842com

Gloor, S. M., Wachtel, M., Bolliger, M. F., Ishihara, H., Landmann, R., and Frei, K. (2001). Molecular and cellular permeability control at the blood-brain barrier. *Brain Res. Brain Res. Rev.* 36, 258–264. doi: 10.1016/s0165-0173(01)00102-3

Han, F., Drabek, T., Stezoski, J., Janesko-Feldman, K., Stezoski, S. W., Clark, R. S., et al. (2008). Protein nitration and poly-ADP-ribosylation in brain after rapid exsanguination cardiac arrest in a rat model of emergency preservation and resuscitation. *Resuscitation* 79, 301–310. doi: 10.1016/j.resuscitation.2008.06.004

Hattori, Y., Okamoto, Y., Nagatsuka, K., Takahashi, R., Kalaria, R. N., Kinoshita, M., et al. (2015). SIRT1 attenuates severe ischemic damage by preserving cerebral blood flow. *Neuroreport* 26, 113–117. doi: 10.1097/WNR.0000000000000308

He, H. J., Wang, Y., Le, Y., Duan, K. M., Yan, X. B., Liao, Q., et al. (2012). Surgery upregulates high mobility group box-1 and disrupts the blood-brain barrier causing cognitive dysfunction in aged rats. *CNS Neurosci. Ther.* 18, 994–1002. doi: 10.1111/cns.12018

He, Q., Li, Z., Wang, Y., Hou, Y., Li, L., and Zhao, J. (2017). Resveratrol alleviates cerebral ischemia/reperfusion injury in rats by inhibiting NLRP3 inflammasome activation through Sirt1-dependent autophagy induction. *Int. Immunopharmacol.* 50, 208–215. doi: 10.1016/j.intimp.2017.06.029

Homi, H. M., Calvi, C. L., Lynch, J., and Grocott, H. P. (2010). Longitudinal assessment of neurocognitive function in rats after cardiopulmonary bypass: evidence for long-term deficits. *J. Cardiothorac. Vasc. Anesth.* 24, 293–299. doi: 10.1053/j.jvca.2009.07.020

Hwabejire, J. O., Jin, G., Imam, A. M., Duggan, M., Sillesen, M., Deperalta, D., et al. (2013). Pharmacologic modulation of cerebral metabolic derangement and excitotoxicity in a porcine model of traumatic brain injury and hemorrhagic shock. *Surgery* 154, 234–243. doi: 10.1016/j.surg.2013.04.008

Ip, W. K. E., Hoshi, N., Shouval, D. S., Snapper, S., and Medzhitov, R. (2017). Anti-inflammatory effect of IL-10 mediated by metabolic reprogramming of macrophages. *Science* 356, 513–519. doi: 10.1126/science.aal3535

Kim, J. B., Sig Choi, J., Yu, Y. M., Nam, K., Piao, C. S., Kim, S. W., et al. (2006). HMGB1, a novel cytokine-like mediator linking acute neuronal death and delayed neuroinflammation in the postischemic brain. *J. Neurosci.* 26, 6413–6421. doi: 10.1523/JNEUROSCI.3815-05.2006

Kim, S. H., Lu, H. F., and Alano, C. C. (2011). Neuronal Sirt3 protects against excitotoxic injury in mouse cortical neuron culture. *PLoS One* 6:e14731. doi: 10.1371/journal.pone.0014731

La, M., D'Amico, M., Bandiera, S., Di Filippo, C., Oliani, S. M., Gavins, F. N., et al. (2001). Annexin 1 peptides protect against experimental myocardial ischemia-reperfusion: analysis of their mechanism of action. *FASEB J.* 15, 2247–2256. doi: 10.1096/fj.01-0196com

Lee, O. H., Kim, J., Kim, J. M., Lee, H., Kim, E. H., Bae, S. K., et al. (2013). Decreased expression of sirtuin 6 is associated with release of high mobility group box-1 after cerebral ischemia. *Biochem. Biophys. Res. Commun.* 438, 388–394. doi: 10.1016/j.bbrc.2013.07.085

Li, Y., and Alam, H. B. (2011). Modulation of acetylation: creating a pro-survival and anti-inflammatory phenotype in lethal hemorrhagic and septic shock. *J. Biomed. Biotechnol.* 2011:523481. doi: 10.1155/2011/523481

Lin, T., Alam, H. B., Chen, H., Britten-Webb, J., Rhee, P., Kirkpatrick, J., et al. (2006). Cardiac histones are substrates of histone deacetylase activity in hemorrhagic shock and resuscitation. *Surgery* 139, 365–376. doi: 10.1016/j.surg.2005.08.022

Liu, F., and McCullough, L. D. (2013). Inflammatory responses in hypoxic ischemic encephalopathy. *Acta Pharmacol. Sin.* 34, 1121–1130. doi: 10.1038/aps.2013.89

Liu, L., Peritore, C., Ginsberg, J., Kayhan, M., and Donmez, G. (2015). SIRT3 attenuates MPTP-induced nigrostriatal degeneration via enhancing mitochondrial antioxidant capacity. *Neurochem. Res.* 40, 600–608. doi: 10.1007/s11064-014-1507-8

Liu, T. F., Vachharajani, V., Millet, P., Bharadwaj, M. S., Molina, A. J., and McCall, C. E. (2015). Sequential actions of SIRT1-RELB-SIRT3 coordinate nuclear-mitochondrial communication during immunometabolic adaptation to acute inflammation and sepsis. *J. Biol. Chem.* 290, 396–408. doi: 10.1074/jbc.M114.566349

Liu, P., Huang, G., Wei, T., Gao, J., Huang, C., Sun, M., et al. (2018). Sirtuin 3-induced macrophage autophagy in regulating NLRP3 inflammasome activation. *Biochim. Biophys. Acta Mol. Basis Dis.* 1864, 764–777. doi: 10.1016/j.bbadis.2017.12.027

Netto, M. B., de Oliveira Junior, A. N., Goldim, M., Mathias, K., Fileti, M. E., da Rosa, N., et al. (2018). Oxidative stress and mitochondrial dysfunction contributes to postoperative cognitive dysfunction in elderly rats. *Brain Behav. Immun.* 73, 661–669. doi: 10.1016/j.bbi.2018.07.016

Nikolian, V. C., Dennahy, I. S., Higgins, G. A., Williams, A. M., Weykamp, M., Georgoff, P. E., et al. (2018). Transcriptomic changes following valproic acid treatment promote neurogenesis and minimize secondary brain injury. *J. Trauma Acute Care Surg.* 84, 459–465. doi: 10.1097/TA.0000000000001765

Nikolian, V. C., Georgoff, P. E., Pai, M. P., Dennahy, I. S., Chtraklin, K., Eidy, H., et al. (2017). Valproic acid decreases brain lesion size and improves neurologic recovery in swine subjected to traumatic brain injury, hemorrhagic shock, and polytrauma. *J. Trauma Acute Care Surg.* 83, 1066–1073. doi: 10.1097/TA.0000000000001612

Novgorodov, S. A., Riley, C. L., Keffler, J. A., Yu, J., Kindy, M. S., Macklin, W. B., et al. (2016). SIRT3 deacetylates ceramide synthases: implications for mitochondrial dysfunction and brain injury. *J. Biol. Chem.* 291, 1957–1973. doi: 10.1074/jbc.M115.668228

Parker, T. M., Nguyen, A. H., Rabang, J. R., Patil, A. A., and Agrawal, D. K. (2017). The danger zone: systematic review of the role of HMGB1 danger signalling in traumatic brain injury. *Brain Inj.* 31, 2–8. doi: 10.1080/02699052.2016.1217045

Paudel, Y. N., Shaikh, M. F., Chakraborti, A., Kumari, Y., Aledo-Serrano, Á., Aleksovska, K., et al. (2018). HMGB1: a common biomarker and potential target for TBI, neuroinflammation, epilepsy, and cognitive dysfunction. *Front. Neurosci.* 12:628. doi: 10.3389/fnins.2018.00628

Perretti, M., Leroy, X., Bland, E. J., and Montero-Melendez, T. (2015). Resolution pharmacology: opportunities for therapeutic innovation in inflammation. *Trends Pharmacol. Sci.* 36, 737–755. doi: 10.1016/j.tips.2015.07.007

Preyat, N., and Leo, O. (2013). Sirtuin deacylases: a molecular link between metabolism and immunity. *J. Leukoc. Biol.* 93, 669–680. doi: 10.1189/jlb.1112557

Qin, C., Yang, Y. H., May, L., Gao, X., Stewart, A. G., Tu, Y., et al. (2015). Cardioprotective potential of annexin-A1 mimetics in myocardial infarction. *Pharmacol. Ther.* 148, 47–65. doi: 10.1016/j.pharmthera.2014.11.012

Rangarajan, P., Karthikeyan, A., Lu, J., Ling, E. A., and Dheen, S. T. (2015). Sirtuin 3 regulates Foxo3a-mediated antioxidant pathway in microglia. *Neuroscience* 311, 398–414. doi: 10.1016/j.neuroscience.2015.10.048

Relton, J. K., Strijbos, P. J., O'Shaughnessy, C. T., Carey, F., Forder, R. A., Tilders, F. J., et al. (1991). Lipocortin-1 is an endogenous inhibitor of ischemic damage in the rat brain. *J. Exp. Med.* 174, 305–310. doi: 10.1084/jem.174.2.305

Rhee, P., Talon, E., Eifert, S., Anderson, D., Stanton, K., Koustova, E., et al. (2000). Induced hypothermia during emergency department thoracotomy: an animal model. *J. Trauma* 48, 439–447;discussion 447.

Rouaux, C., Jokic, N., Mbebi, C., Boutillier, S., Loeffler, J. P., and Boutillier, A. L. (2003). Critical loss of CBP/p300 histone acetylase activity by caspase-6 during neurodegeneration. *EMBO J.* 22, 6537–6549. doi: 10.1093/emboj/cdg615

Sailhamer, E. A., Chen, Z., Ahuja, N., Velmahos, G. C., de Moya, M., Rhee, P., et al. (2007). Profound hypothermic cardiopulmonary bypass facilitates survival without a high complication rate in a swine model of complex vascular, splenic, and colon injuries. *J. Am. Coll. Surg.* 204, 642–653. doi: 10.1016/j.jamcollsurg.2007.01.017

Schwer, B., North, B. J., Frye, R. A., Ott, M., and Verdin, E. (2002). The human silent information regulator (Sir)2 homologue hSIRT3 is a mitochondrial nicotinamide adenine dinucleotide-dependent deacetylase. *J. Cell Biol.* 158, 647–657. doi: 10.1083/jcb.200205057

Serhan, C. N., Brain, S. D., Buckley, C. D., Gilroy, D. W., Haslett, C., O'Neill, L. A., et al. (2007). Resolution of inflammation: state of the art, definitions and terms. *FASEB J.* 21, 325–332. doi: 10.1096/fj.06-7227rev

Shim, J. K., Ma, Q., Zhang, Z., Podgoreanu, M. V., and Mackensen, G. B. (2014). Effect of pregabalin on cerebral outcome after cardiopulmonary bypass with deep hypothermic circulatory arrest in rats. *J. Thorac. Cardiovasc. Surg.* 148, 298–303. doi: 10.1016/j.jtcvs.2014.02.076

Sidorova-Darmos, E., Wither, R. G., Shulyakova, N., Fisher, C., Ratnam, M., Aarts, M., et al. (2014). Differential expression of sirtuin family members in the developing, adult, and aged rat brain. *Front. Aging Neurosci.* 6:333. doi: 10.3389/fnagi.2014.00333

Skvarc, D. R., Berk, M., Byrne, L. K., Dean, O. M., Dodd, S., Lewis, M., et al. (2018). Post-operative cognitive dysfunction: an exploration of the inflammatory hypothesis and novel therapies. *Neurosci. Biobehav. Rev.* 84, 116–133. doi: 10.1016/j.neubiorev.2017.11.011

Smith, H. K., Gil, C. D., Oliani, S. M., and Gavins, F. N. (2015). Targeting formyl peptide receptor 2 reduces leukocyte-endothelial interactions in a murine model of stroke. *FASEB J.* 29, 2161–2171. doi: 10.1096/fj.14-263160

Someya, S., Yu, W., Hallows, W. C., Xu, J., Vann, J. M., Leeuwenburgh, C., et al. (2010). Sirt3 mediates reduction of oxidative damage and prevention of age-related hearing loss under caloric restriction. *Cell* 143, 802–812. doi: 10.1016/j.cell.2010.10.002

Song, W., Song, Y., Kincaid, B., Bossy, B., and Bossy-Wetzel, E. (2013). Mutant SOD1G93A triggers mitochondrial fragmentation in spinal cord motor neurons: neuroprotection by SIRT3 and PGC-1α. *Neurobiol. Dis.* 51, 72–81. doi: 10.1016/j.nbd.2012.07.004

Su, X., Feng, X., Terrando, N., Yan, Y., Chawla, A., Koch, L. G., et al. (2013). Dysfunction of inflammation-resolving pathways is associated with exaggerated postoperative cognitive decline in a rat model of the metabolic syndrome. *Mol. Med.* 18, 1481–1490. doi: 10.2119/molmed.2012.00351

Sundaresan, N. R., Samant, S. A., Pillai, V. B., Rajamohan, S. B., and Gupta, M. P. (2008). SIRT3 is a stress-responsive deacetylase in cardiomyocytes that protects cells from stress-mediated cell death by deacetylation of Ku70. *Mol. Cell Biol.* 28, 6384–6401. doi: 10.1128/MCB.00426-08

Tahsili-Fahadan, P., Farrokh, S., and Geocadin, R. G. (2018). Hypothermia and brain inflammation after cardiac arrest. *Brain Circ.* 4, 1–13. doi: 10.4103/bc.bc_4_18

Terrando, N., Eriksson, L. I., Ryu, J. K., Yang, T., Monaco, C., Feldmann, M., et al. (2011). Resolving postoperative neuroinflammation and cognitive decline. *Ann. Neurol.* 70, 986–995. doi: 10.1002/ana.22664

Terrando, N., Gómez-Galán, M., Yang, T., Carlström, M., Gustavsson, D., Harding, R. E., et al. (2013). Aspirin-triggered resolvin D1 prevents surgery-induced cognitive decline. *FASEB J.* 27, 3564–3571. doi: 10.1096/fj.13-230276

Terrando, N., Monaco, C., Ma, D., Foxwell, B. M., Feldmann, M., and Maze, M. (2010a). Tumor necrosis factor-alpha triggers a cytokine cascade yielding postoperative cognitive decline. *Proc. Natl. Acad. Sci. U.S.A.* 107, 20518–20522. doi: 10.1073/pnas.1014557107

Terrando, N., Rei Fidalgo, A., Vizcaychipi, M., Cibelli, M., Ma, D., Monaco, C., et al. (2010b). The impact of IL-1 modulation on the development of lipopolysaccharide-induced cognitive dysfunction. *Crit. Care.* 14:R88. doi: 10.1186/cc9019

Tisherman, S. A., Alam, H. B., Rhee, P. M., Scalea, T. M., Drabek, T., Forsythe, R. M., et al. (2017). Development of the emergency preservation and resuscitation for cardiac arrest from trauma clinical trial. *J. Trauma Acute Care Surg.* 83, 803–809. doi: 10.1097/TA.0000000000001585

Traba, J., Kwarteng-Siaw, M., Okoli, T. C., Li, J., Huffstutler, R. D., Bray, A., et al. (2015). Fasting and refeeding differentially regulate NLRP3 inflammasome activation in human subjects. *J. Clin. Invest.* 125, 4592–4600. doi: 10.1172/JCI83260

Utech, M., Mennigen, R., and Bruewer, M. (2010). Endocytosis and recycling of tight junction proteins in inflammation. *J. Biomed. Biotechnol.* 2010:484987. doi: 10.1155/2010/484987

Vacas, S., Degos, V., Feng, X., and Maze, M. (2013). The neuroinflammatory response of postoperative cognitive decline. *Br. Med. Bull.* 106, 161–178. doi: 10.1093/bmb/ldt006

Vachharajani, V., Liu, T., and McCall, C. E. (2014). Epigenetic coordination of acute systemic inflammation: potential therapeutic targets. *Expert Rev. Clin. Immunol.* 10, 1141–1150. doi: 10.1586/1744666X.2014.943192

Vital, S. A., Becker, F., Holloway, P. M., Russell, J., Perretti, M., Granger, D. N., et al. (2016). Formyl-peptide receptor 2/3/lipoxin A4 receptor regulates neutrophil-platelet aggregation and attenuates cerebral inflammation: impact for therapy in cardiovascular disease. *Circulation* 133, 2169–2179. doi: 10.1161/CIRCULATIONAHA.115.020633

Weir, H. J., Murray, T. K., Kehoe, P. G., Love, S., Verdin, E. M., O'Neill, M. J., et al. (2012). CNS SIRT3 expression is altered by reactive oxygen species and in Alzheimer's disease. *PLoS One* 7:e48225. doi: 10.1371/journal.pone.0048225

Wohleb, E. S., McKim, D. B., Sheridan, J. F., and Godbout, J. P. (2014). Monocyte trafficking to the brain with stress and inflammation: a novel axis of immune-to-brain communication that influences mood and behavior. *Front. Neurosci.* 8:447. doi: 10.3389/fnins.2014.00447

Wu, X., Drabek, T., Kochanek, P. M., Henchir, J., Stezoski, S. W., Stezoski, J., et al. (2006). Induction of profound hypothermia for emergency preservation and resuscitation allows intact survival after cardiac arrest resulting from prolonged lethal hemorrhage and trauma in dogs. *Circulation* 113, 1974–1982. doi: 10.1161/CIRCULATIONAHA.105.587204

Xiang, Y., Zhao, H., Wang, J., Zhang, L., Liu, A., and Chen, Y. (2016). Inflammatory mechanisms involved in brain injury following cardiac arrest and cardiopulmonary resuscitation. *Biomed. Rep.* 5, 11–17. doi: 10.3892/br.2016.677

Yan, W. J., Liu, R. B., Wang, L. K., Ma, Y. B., Ding, S. L., Deng, F., et al. (2018). Sirt3-mediated autophagy contributes to resveratrol-induced protection against ER stress in HT22 cells. *Front. Neurosci.* 12:116. doi: 10.3389/fnins.2018.00116

Yang, L., Wang, F., Yang, L., Yuan, Y., Chen, Y., Zhang, G., et al. (2018). HMGB1 a-box reverses brain edema and deterioration of neurological function in a traumatic brain injury mouse model. *Cell Physiol. Biochem.* 46, 2532–2542. doi: 10.1159/000489659

Yang, Q. W., Lu, F. L., Zhou, Y., Wang, L., Zhong, Q., Lin, S., et al. (2011). HMBG1 mediates ischemia-reperfusion injury by TRIF-adaptor independent Toll-like receptor 4 signaling. *J. Cereb. Blood Flow Metab.* 31, 593–605. doi: 10.1038/jcbfm.2010.129

Yuan, F., Xu, Z. M., Lu, L. Y., Nie, H., Ding, J., Ying, W. H., et al. (2016). SIRT2 inhibition exacerbates neuroinflammation and blood-brain barrier disruption in experimental traumatic brain injury by enhancing NF-κB p65 acetylation and activation. *J. Neurochem.* 136, 581–593. doi: 10.1111/jnc.13423

Zhang, R. L., Chopp, M., Chen, H., and Garcia, J. H. (1994). Temporal profile of ischemic tissue damage, neutrophil response, and vascular plugging following permanent and transient (2H) middle cerebral artery occlusion in the rat. *J. Neurol. Sci.* 125, 3–10. doi: 10.1016/0022-510x(94)90234-8

Zhang, Z., Ma, Q., Shah, B., Mackensen, G. B., Lo, D. C., Mathew, J. P., et al. (2017). Neuroprotective effects of annexin A1 tripeptide after deep hypothermic circulatory arrest in rats. *Front. Immunol.* 8:1050. doi: 10.3389/fimmu.2017.01050

Zhao, H., Chen, S., Gao, K., Zhou, Z., Wang, C., Shen, Z., et al. (2017). Resveratrol protects against spinal cord injury by activating autophagy and inhibiting apoptosis mediated by the SIRT1/AMPK signaling

pathway. *Neuroscience* 348, 241–251. doi: 10.1016/j.neuroscience.2017.02.027

Zhao, H., Luo, Y., Chen, L., Zhang, Z., Shen, C., Li, Y., et al. (2018). Sirt3 inhibits cerebral ischemia-reperfusion injury through normalizing Wnt/β-catenin pathway and blocking mitochondrial fission. *Cell Stress Chaperones* 23, 1079–1092. doi: 10.1007/s12192-018-0917-y

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Scopoletin Suppresses Activation of Dendritic Cells and Pathogenesis of Experimental Autoimmune Encephalomyelitis by Inhibiting NF-κB Signaling

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Scopoletin, a phenolic coumarin derived from many medical or edible plants, is involved in various pharmacological functions. In the present study, we showed that Scopoletin effectively ameliorated experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis (MS), through novel regulatory mechanisms involving inhibition of NF-κB activity in dendritic cells (DCs). Scopoletin treatment significantly improved the severity of the disease and prominently decreased inflammation and demyelination of central nervous system (CNS) in EAE mice. Disease alleviation correlated with the downregulation of major histocompatibility complex (MHC) class II, CD80 and CD86, expressed on DCs of CNS or spleens, and the infiltration and polarization of encephalitogenic Th1/Th17 cells. Consistent with the *in vivo* data, Scopoletin-treated, bone marrow-derived dendritic cells (BM-DCs) exhibited reduced expression of MHC class II and costimulatory molecules (e.g., CD80 and CD86) and reduced NF-κB phosphorylation. These findings, for the first time, demonstrated the ability of Scopoletin to impair DC activation, downregulating pathogenic Th1/Th17 inflammatory cell responses and, eventually, reducing EAE severity. Our study demonstrates new evidence that natural products derived from medical or edible plants, such as Scopoletin, will be valuable in developing a novel therapeutic agent for MS in the future.

Keywords: scopoletin, experimental autoimmune encephalomyelitis, multiple sclerosis, dendritic cells, NF-κB signaling

INTRODUCTION

Multiple sclerosis (MS) is a T-cell-mediated chronic autoimmune disease featured by neuroinflammation and demyelination in the central nervous system (CNS), affecting 2.5 million people worldwide, and the incidence continues to increase (Hemmer et al., 2015). Experimental autoimmune encephalomyelitis (EAE) is a well-known animal model of MS that is used to study the underlying mechanism and that provides the theoretical basis for developing new therapies of MS (Procaccini et al., 2015; Lassmann and Bradl, 2016).

Dendritic cells (DCs) are antigen-presenting cells that connect the innate and adaptive immune response. Depending on the variety activation status, DCs can either activate or modulate naive T lymphocytes. When DCs are activated, costimulatory molecules are upregulated and produce cytokines that drive T-cell priming and effector differentiation (Amodio and Gregori, 2012). In the absence of activation, antigen presentation by steady-state DCs can result to T-cell unresponsiveness and tolerance (Besusso et al., 2015). Therefore, modulation of DCs has been shown to be a promising area for the treatment of autoimmune disease. For instance, it was proved that DCs treated with natural compounds or synthetic drugs blocked the inflammatory signal and halted disease development (Ginwala et al., 2016; Kornberg et al., 2018). DC-induced suppression is mainly dependent on inhibiting Th17 cell differentiation or promoting Treg cell generation (Thome et al., 2014a; Thome et al., 2018). It is known that naive $CD4^+$ T cells can develop into various effector subsets containing Th1, Th2, Th17, and Treg cells, etc.; among these, Th17 cells are crucially related to the pathogenesis of MS/EAE (He et al., 2017; Malik et al., 2017). Suppression of the cellular response, Th17 cells in particular, can therefore act as a therapeutic target for controlling autoimmune and inflammatory diseases.

NF-κB is a broad transcription factor which can regulate various biological functions containing inflammation, immunity, cell growth, and survival, and its signaling pathway plays a role in DC activation and maturation (Ardeshna et al., 2000). Phosphorylation of NF-κB in innate immune cells induces inflammation reaction, cytokine secretion, and tissue damage, thus promoting to the progress of autoimmune diseases including EAE (Lu et al., 2013). In addition, immature DCs respond to danger signals or antigen and, when mature, produce abundance of proinflammatory cytokines, such as type I interferons (IFN). Subsequently, mature DCs present antigens to T lymphocytes to stimulate a sustained immune response, given the interferon regulatory factor (IRF) family's important role in DC development and maturation. For example, IRF-7 is necessary for producing IFN-α and IFN-β, and it serves as the prime regulator of type-I interferon-dependent immune responses (Honda et al., 2005; Gabriele and Ozato, 2007; Yasuda et al., 2007).

Scopoletin is a phenolic coumarin extracted from many medical plants, including *Erycibe obtusifolia*, *Aster tataricus*, *Foeniculum vulgare*, and *Artemisia iwayomogi*, as well as some edible plants, such as *Lycium barbarum* and *Morinda citrifolia* (Dou et al., 2013; Lee et al., 2014; Forino et al., 2016; Shalan et al., 2016). Scopoletin has been shown to possess anti-inflammatory, antioxidant, antidepressant, hypouricemic, and neuroprotective effects (Ding et al., 2005; Basu et al., 2016). Moreover, a recent report suggests that Scopoletin is effective in an adjuvant-induced arthritis rat model, increasing the possibility of its application as a therapeutic agent for autoimmune diseases (Pan et al., 2010). However, little is known about the regulatory properties and underlying mechanism of Scopoletin relative to innate immune cells such as DCs and its effect on the pathogenic progression of MS and EAE. In light of the pharmacological profile of Scopoletin and its reported immunoregulatory properties, we hypothesized that Scopoletin might have a therapeutic effect on the animal model of MS and EAE. The purpose of this study was, therefore, to test the therapeutic activity of Scopoletin on EAE and to elucidate the underlying therapeutic mechanisms of its action, as part of our ongoing search for development of immunomodulation medicine extracted from medical plants or edible plants (Zhao et al., 2018).

MATERIALS AND METHODS

Reagents and Animals

Scopoletin was purchased from Sigma (Sigma-Aldrich, St. Louis, MO). C57BL/6 mice, female, 8 weeks old, were obtained from the Fourth Military University (Xi'an, China). Studies were carried out by a protocol approved by the Animal Ethics Experimental Committee at of Shaanxi Normal University, and all experiments were operated strictly according to approved institutional guidelines and regulations.

EAE Induction and Treatment

EAE was induced by subcutaneously injecting an emulsion containing 200 μg Myelin Oligodendrocyte $Glycoprotein_{35–55}$ ($MOG_{35–55}$) (Genescript, Piscataway, NJ), complete Freund's adjuvant (CFA) (Sigma-Aldrich), and 5 mg/ml *Mycobacterium tuberculosis* H37Ra (Difco Laboratories, Lawrence, KS), and intraperitoneally (i.p.) injected 200 ng pertussis toxin (Sigma-Aldrich) in PBS on day 0 and 2 postimmunization (p.i.) (Zhang et al., 2015). Disease progression of EAE mouse was evaluated daily in a blind way by two researchers, scoring standard for EAE disease progression as follows: 0, no symptoms; 0.5, stiff tail; 1, limp tail; 1.5, limp tail and waddle; 2, paralysis of one limb; 2.5, paralysis of one limb and weakness of one other limb; 3, complete paralysis of two hind limbs; 4, moribund; and 5, death. (Li et al., 2016). Scopoletin was prepared in dimethylsulfoxide (DMSO) at 100 mg/ml for stock, which was further diluted 20 times with PBS as a working solution. DMSO (5%) was dissolved in PBS as vehicle. Vehicle or Scopoletin (50 mg/kg) was injected i.p. daily from the day 0 p.i.

Histopathology

Mice were killed at day 20 postimmunization (p.i.). To assess CNS histopathological, mice were perfused through the heart with cold PBS; spinal cord was then fixed with 4% paraformaldehyde, cut into 7-μm sections, and stained with hematoxylin and eosin (H&E) or Luxol fast blue (LFB) for evaluation of inflammation or demyelination, respectively. Slides were evaluated in a blinded fashion for inflammation using a 0–3 scale as description, and two investigators selected 10 areas in the white matter of the spinal cord and scored for slides, evaluation methods, and criteria as description followed our previous studies (Li et al., 2016). The white matter area was manually outlined, and the Image-Pro Plus software was used to calculated areas (%) of demyelination for evaluation of demyelination (Li et al., 2017). Demyelination area percentage was quantified as the area without LFB staining (white) in white matter divided by the area white matter of spinal cord slices. Fixed spinal cords were embedded in optimum cutting temperature (OCT) solution (Tissue-Tek, Sakura Finetek, Japan) and then sectioned coronally cut into 12 μm. Finally,

sections stained with primary and secondary antibodies. Results were acquired by Nikon Eclipse E600 fluorescent microscopy (Melville, NY).

Mononuclear Cells (MNCs) and BMDCs Preparation

Spleens were mechanically grinded and filtrated with a 100-μm cell strainer (Falcon, Tewksbury, MA) to harvest splenic cells, subsequently incubated with red blood cell lysis buffer (Biolegend, San Diego, CA) for 60 s. After washing cells with cold PBS, cells were stimulated with MOG_{35-55} *in vitro*. To acquire CNS infiltrating cells, the protocol was followed as previously described (Li et al., 2017). To acquire the bone-marrow-derived dendritic cells (BM-DCs), femurs and tibias were separated from the naive adult C57BL/6 mice, and the cells were blown out of the bone marrow with a syringe full of the precooled PBS. After filtering with 100-μm cell strainer, the harvested cells were subsequently cultured with PRMI1640 containing granulocyte colony-stimulating factor (GM-CSF) cytokine (10 ng/ml) until the ninth day to obtain mature BM-DCs. Dendritic cells are cultured in medium without GM-CSF cytokine after ninth day.

Drug Preparation for Cell Experiments

Stock solution of Scopoletin was dissolved into the concentration of 30 mM for cell experiments with DMSO.

T-cell Proliferation Assay

Splenic CD4+ T cells were isolated from the naive adult C57BL/6 mice using a CD4+ T Cell Isolation Kit (Miltenyi Biotec) and labeled with CFSE. Both BM-DCs and T cells were cocultured at 1:10 ratio in the presence of 10 μg/ml of MOG_{35-55} (Genescript, Piscataway, NJ) and 100 ng/ml of lipopolysaccharide (LPS) (Sigma-Aldrich). T-cell proliferation was measured by analyzing CFSE intensity by flow cytometry after 72 h.

ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

DCs culture medium or supernatants from splenocytes were harvested at 18 or 72 h, and sandwich ELISA was performed to determine the concentrations of cytokine production by ELISA kits (R&D Systems, Minneapolis, MN). according to the manufacturer's instructions, including IFN-γ, IL-17, GM-CSF, IL-1β, IL-6, and IL-23. Briefly, the capture antibody was used to precoat overnight. Then, the plate was washed with wash buffer (R&D Systems, Minneapolis, MN). for three times. Blocking with 1% BSA in PBS for 1 h, gradient standard, and samples were added to the plate for 2 h incubation. Next, the samples and standard were removed clearly, and the detection antibody was incubated for 2 h. The wells were again washed, a TMB substrate solution was added to the wells, and color developed in proportion to the amounts of cytokines. The Stop Solution changed color from blue to yellow, and the intensity of the color was measured at 450 nm. A standard curve was run for each microwell plate. According to the standard curve, the concentration of cytokine was determined.

Flow Cytometry Analysis

For surface-marker staining, cells were washed with PBS and incubated 30 min at 4°C with anti-CD4, anti-CD8, anti-CD11b, anti-CD11c, anti-CD80, and anti-CD86 (BD Biosciences, San Jose, CA) or isotype control Abs according to the manufacturer's instructions to dilute. With 25 or 10 μg/ml MOG_{35-55} to stimulate splenocytes or infiltrating MNCs in CNS for 72 h or overnight to analyze Th1 and Th17 response to MOG_{35-55}. For intracellular staining, cells were stimulated with 50 ng/ml PMA, 500 ng/ml ionomycin, and GolgiPlug for 5 h. For blocking the nonspecific staining, 1 μg Fc Block reagent for the FcγII/III receptor (BD Biosciences, San Jose, CA) was addded to 1×10^6 cells in 100 μl staining buffer (BD Biosciences) and incubated for 15 min at 4°C. The cells are then washed. For surface staining, cells were incubated with anti-CD4, anti-CD8, anti-CD11b, anti-CD11c, anti-MHC class II, anti-CD80, and anti-CD86. Then, cells were fixed and permeabilized by Fix & Perm Medium (Invitrogen, Waltham, MA), using Abs anti-IL-17, anti-IFN-γ, or anti-GM-CSF (BD Biosciences, San Jose, CA) and were incubated overnight at 4°C to stain intracellular cytokines. FACS Aria was used for flow cytometric analysis (BD Biosciences, San Jose, CA). The results were evaluated by FlowJo (Treestar, Ashland, OR).

Western Blot

Mature BM-DCs were placed in six-well plates with a density of 1.5×10^6 cells/ml and stimulated with 100 ng/ml LPS to induce DC activation. Meanwhile, 100 μM Scopoletin was added to DCs. After 18 h, proteins were extracted from BM-DCs as described. The total protein contents were measured by Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, MA). DC proteins were equally loaded onto SDS-PAGE, electrophoresed, transferred onto PVDF membranes, and after blocking with 5% nonfat milk in TBS, the PVDF membranes were incubated with primary antibody over 12 h at 4°C (Li et al., 2018), including JNK, p-JNK, NF-κB, p-NF-κB, p38, p-p38 (cell signal technology), IRF-7, ERK1/2, p-ERK1/2 (Abcam, Cambridge, UK), and p-IRF-7 (Signalway Antibody, College Park, MD). After washing, the membranes were incubated with 0.5% horseradish peroxidase-labeled IgG. Temembrane-bound antibodies were detected with an Immolilon™ Western chemiluminescent HRP Substrate (Millipore, Billerica, MA) and analyzed with an ImageJ (ChemiDoc XRS System; Bio-Rad).

Quantitative RT-PCR

Total RNA was extracted by RNeasy Mini Kit (Qiagen, Valencia, CA). cDNA was subsequently synthesized by QuantiTect® Reverse Transcription Kit (QIAGEN). Quantitative RT-PCR was performed with the QuantiFast™ SYBR® Green PCR Kit (QIAGEN) under standard thermocycler conditions (Applied Biosystems, Foster City, CA). Quantification was performed by normalization with housekeeping genes glyceraldehyde

3-phosphate dehydrogenase (GAPDH) and relative expression level as fold changes *via* the $2^{-\Delta\Delta Ct}$ method. Primers are summarized in **Supplementary Table 1.**

Statistical Analysis

Results are described as mean ± SD. Statistical differences between two or multiple groups were implemented using Student's *t*-test or the ANOVA. $P < 0.05$ was considered statistically significant. All statistical analyses were performed by GraphPad Prism 6 (GraphPad, La Jolla, CA).

RESULTS

Scopoletin Suppressed the Development of EAE

Previous studies have shown that Scopoletin is safe at the dosage of 10–100 mg/kg daily with no other side effects in *in vivo* experiment (Jamuna et al., 2015; Basu et al., 2016; Zeng et al., 2017). To assess the effect of Scopoletin on the disease course of EAE, EAE mice were i.p. injected Scopoletin (50 mg/kg) from day 0 p.i. Vehicle-treated EAE mice demonstrated a progressive increase in disease severity after day 10 p.i., while the Scopoletin-treated group showed a marked decrease in mean disease course from day 14 to 20 p.i. (**Figure 1A**). Consistent with the disease development, mice treated with vehicle demonstrated abundant inflammatory cell infiltrated in the white matter compared with the Scopoletin-treated EAE mice (**Figures 1B, C**). At the same time, there was a dramatic decrease in demyelination areas in the spinal cord of Scopoletin-treated EAE mice compared to control (**Figures 1D, E**). In the therapeutic regimen, Scopoletin administration starting from disease onset (day 11 p.i.) effectively ameliorated EAE progression (**Supplementary Figure 1**). Together, these results indicate that Scopoletin effectively suppressed EAE pathogenesis.

Scopoletin Treatment Inhibited DC Activation *In Vivo*

To assess the impact of Scopoletin on peripheral immune responses in EAE, splenocytes were harvested from EAE mice treated with Scopoletin or vehicle at day 20 p.i. and cultured in the presence of MOG_{35-55} (25 μg/ml) for 72 h. Expression of costimulatory molecules on $CD11c^+$ DCs was examined by flow cytometry, which showed significantly decreased levels of MHC class II, CD80 and CD86 in Scopoletin-treated EAE mice compared to the vehicle group (**Figures 2A–D**). These results indicate that Scopoletin inhibited DC activation during the development of EAE.

Consistent with the observed effect of Scopoletin in EAE and DC activation, there was a remarkable diminishment in the percentages of $CD4^+$ and $CD8^+$ T cells in the periphery (**Figures 2E, F**). The percentages of Th1 and Th17 cells were also determined by intracellular staining and showed an obvious inhibition under Scopoletin treatment (**Figures 2G–I**). When stimulated with MOG_{35-55} *ex vivo*, splenocytes of Scopoletin-treated EAE mice secreted significantly lower levels of MOG-induced IFN-γ, IL-17, and GM-CSF (**Figure 2J**). When EAE symptoms is at its peak, spleen cells were harvested at 15 days p.i. and stimulated with MOG_{35-55} for 3 days. The concentration of IFN-γ, IL-17, and GM-CSF in cells culture supernatants were significantly decreased in Scopoletin-treated group (**Supplementary Figure 3A**). Furthermore, the inflammatory cytokine expression levels from sera were also detected by ELISA and RT-PCR. Although we did not detected protein expression by ELISA due to limited samples from sera, RT-PCR results showed that IL-17A, GM-CSF, and IL-1β expression were significantly decreased after Scopoletin treatment (**Supplementary Figure 3B**). These results indicated that Scopoletin alleviated the disease development of EAE by altering expression of costimulatory molecules and reduced activation of DCs, inhibiting Th1 and Th17 cells development, as well as suppressing proinflammatory cytokine secretion.

Scopoletin Treatment Alleviated CNS Inflammation

In the CNS, effector T cells produce a number of molecules that recruit inflammatory leukocytes, ultimately leading to demyelination (Thome et al., 2014b). To evaluate the effect of Scopoletin on EAE-related CNS pathology, thoracic spinal cords were harvested from Scopoletin- or vehicle-treated EAE mice. Immunofluorescence evaluation of CNS tissues showed that Scopoletin markedly decreased infiltration of $CD45^+$ cells compared with control mice (**Figures 3A, B**). The number of MNCs in the CNS of Scopoletin-treated mice was dramatically decreased compared to the vehicle group (**Figure 3C**). Furthermore, flow cytometry was used to analyze $CD45^+$ (leukocyte), $CD11c^+$ (DC), and $CD11b^+$ (macrophage/microglia) cell populations. Results showed that the percentages and absolute numbers of these cells were significantly reduced in the CNS of Scopoletin-treated mice compared with vehicle-treated EAE mice. In addition, Scopoletin-treated mice have lower percentages and absolute numbers of $CD4^+$ and $CD8^+$ T cells in the CNS (**Figures 3D–F**).

Emerging data suggest that both interferon-γ-producing (Th1) and interlukin-17-producing Th17 (Th17) cells contribute to CNS autoimmunity and mediate disease pathogenesis in EAE (El-Behi et al., 2010). In addition, although various cell types produce GM-CSF, myelin-specific CD4+ T cells are essential to EAE development (Rasouli et al., 2015). To explore potential mechanisms underlying the therapeutic effect of Scopoletin in EAE, we measured the numbers of Th1, Th17, and pathogenic $CD4^+GM\text{-}CSF^+$ (Rasouli et al., 2015) cells in the CNS of EAE mice. As shown in **Figures 4A–C**, the percentages and absolute numbers of Th17 cells were reduced in the Scopoletin-treated group in comparison to the vehicle-treated mice. While the percentages of $CD4^+IFN\text{-}\gamma^+$ and $CD4^+IL\text{-}17^+$ T cells were not significantly changed, the absolute numbers of these cells were decreased in the Scopoletin-treated group (**Figures 4A–C**) due to the reduced total numbers of $CD4^+$ T cells in the CNS after Scopoletin treatment

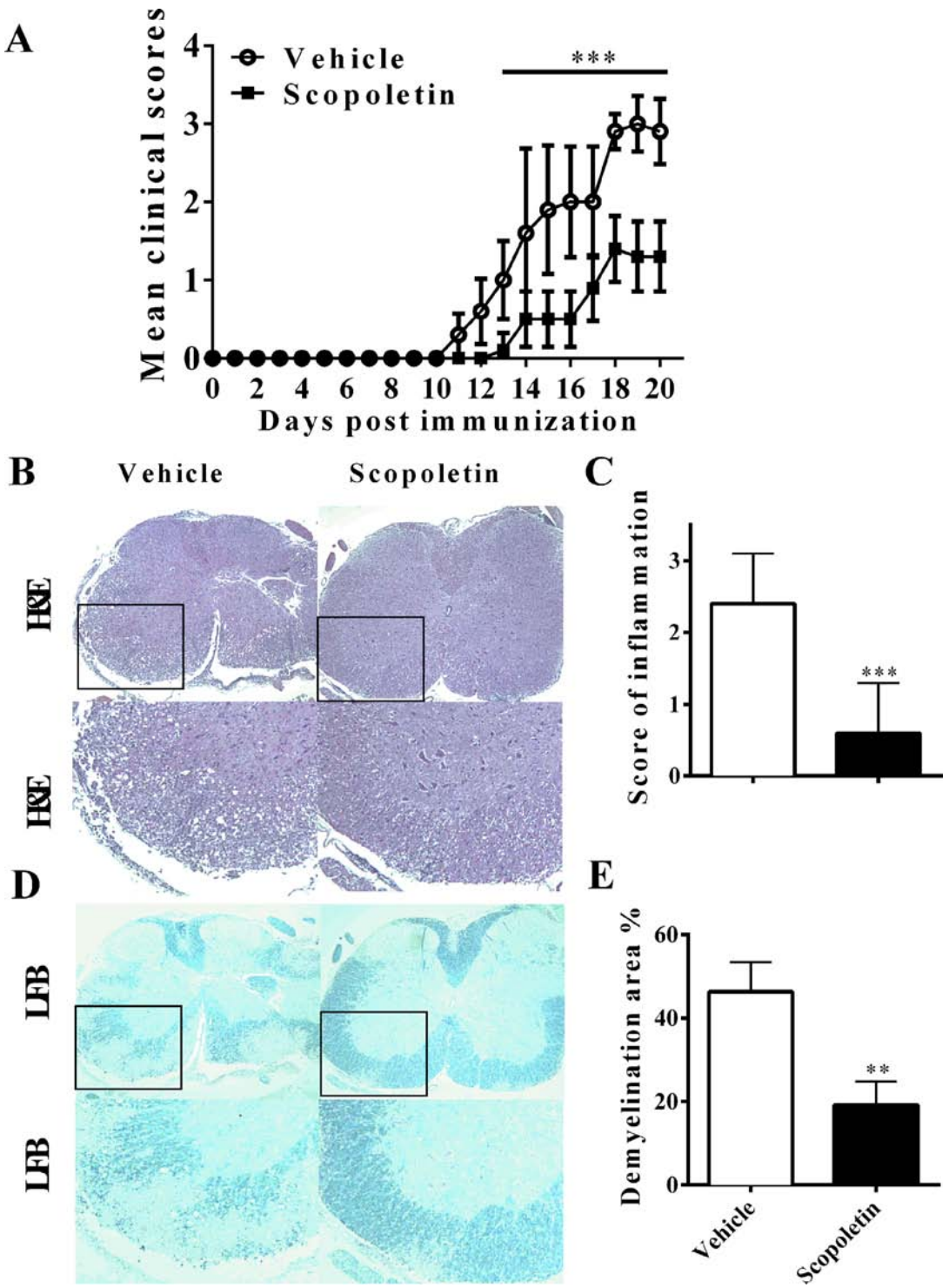

FIGURE 1 | Scopoletin suppressed the development of experimental autoimmune encephalomyelitis (EAE). **(A)** Mice were injected i.p. with vehicle or Scopoletin (50 mg/kg) daily from day 0 p.i. to day 20 p.i. experimental autoimmune encephalomyelitis (EAE) development were evaluated and recorded daily following a 0–5 scale. **(B)** Hematoxylin and eosin (H&E), below is high magnification of H&E analysis of the spinal cord sections, and **(D)** Luxol fast blue staining, below is high magnification of Luxol fast blue (LFB) analysis of the spinal cord sections. **(C)** Mean score of inflammation in H&E staining. **(E)** Quantification of demyelination area was analyzed by Image-Pro Plus software. Scale bar = 100 μm. $n = 8$ mice each group. Symbols represent mean ± SD, ** $P < 0.01$ and *** $P < 0.001$, determined by two-way ANOVA **(A)**, or Student's *t*-test **(B, D, F)**. Data are combined from three independent experiments.

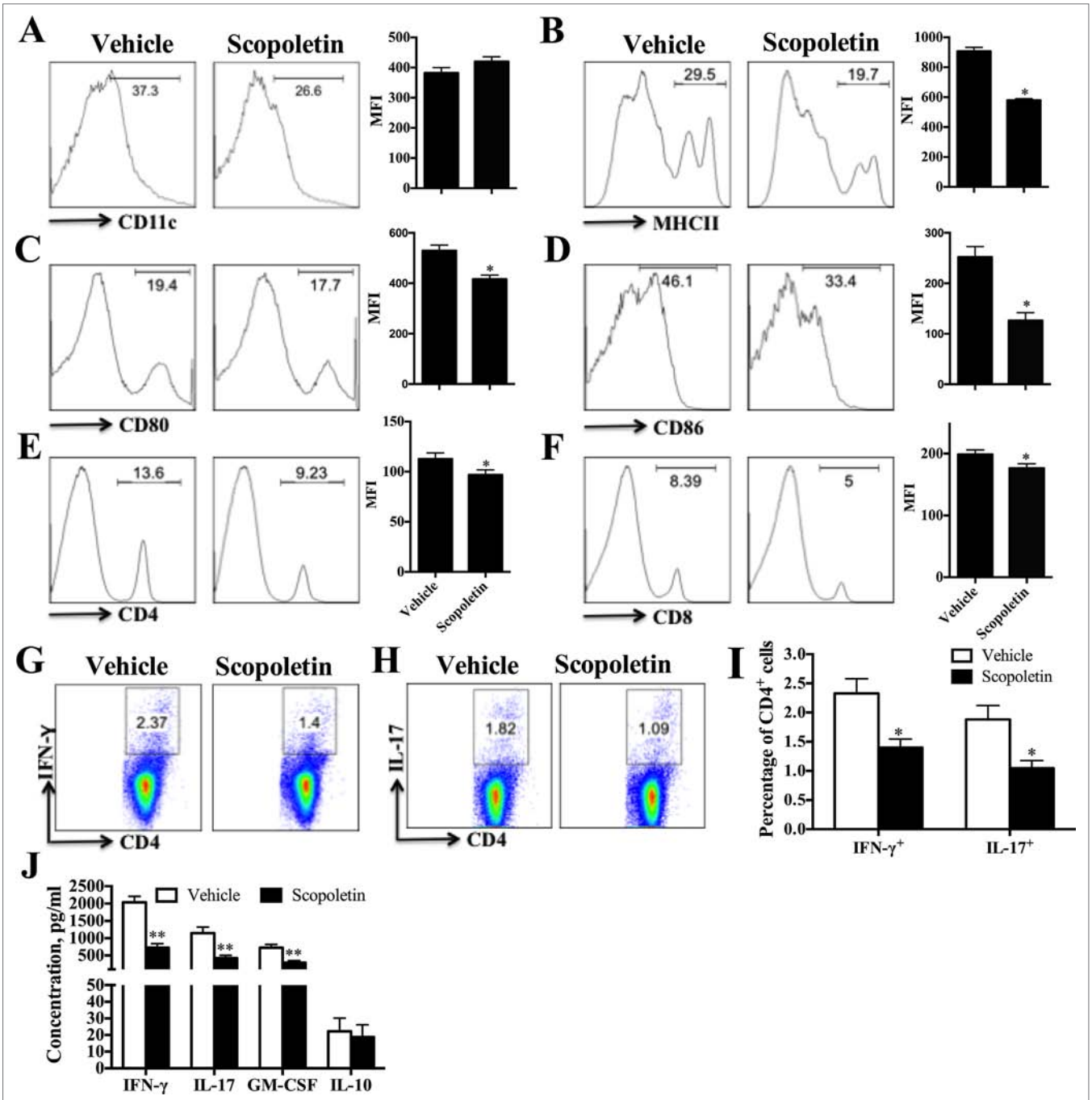

FIGURE 2 | Scopoletin treatment reduced periphery inflammatory responses. Scopoletin- or vehicle-treated EAE mice were killed at day 20 p.i. and spleens were harvested. **(A–F)** Splenocytes were cultured in the presence of MOG_{35-55} (25 μg/ml) for 72 h and analyzed for the median fluorescence intensity (MFI) of **(A)** $CD11c^+$, **(B)** MHC II^+, **(C)** $CD80^+$, **(D)** $CD86^+$ (the positive cells of CD80 and CD86 are gated below the CD11c positive cells), **(E)** $CD4^+$, and **(F)** $CD8^+$. **(G, H)** Frequencies of Th1 and Th17 cells were assessed by flow cytometry. **(I)** Percentages of Th1 and Th17 cells were analyzed. **(J)** Supernatants derived from splenocyte cultures were analyzed for the level of indicated cytokines. Figures **(A–H)** are one representative of three independent experiments. Data **(A–F, I, J)** were determined by Student's *t*-test and symbols represent mean ± SD, $^*P < 0.05$ and $^{**}P < 0.01$.

(**Figures 3D–F**). $CD4^+GM\text{-}CSF^+$ T cells infiltration in the CNS was found in these groups. Our data therefore suggest that the effect of Scopoletin in alleviating EAE development may be due to an alteration in the differentiation of Th1, Th17, and $CD4^+GM\text{-}CSF^+$ T cells (**Figures 4A–C**).

Scopoletin Inhibited the Activation of Murine BM-DCs *In Vitro*

We have shown the inhibitory effect of Scopoletin on DC activation *in vivo* in EAE mice (**Figures 2A–D**); here, we evaluated its direct effects on DCs *in vitro*. To that end, BM-DCs were generated. Upon

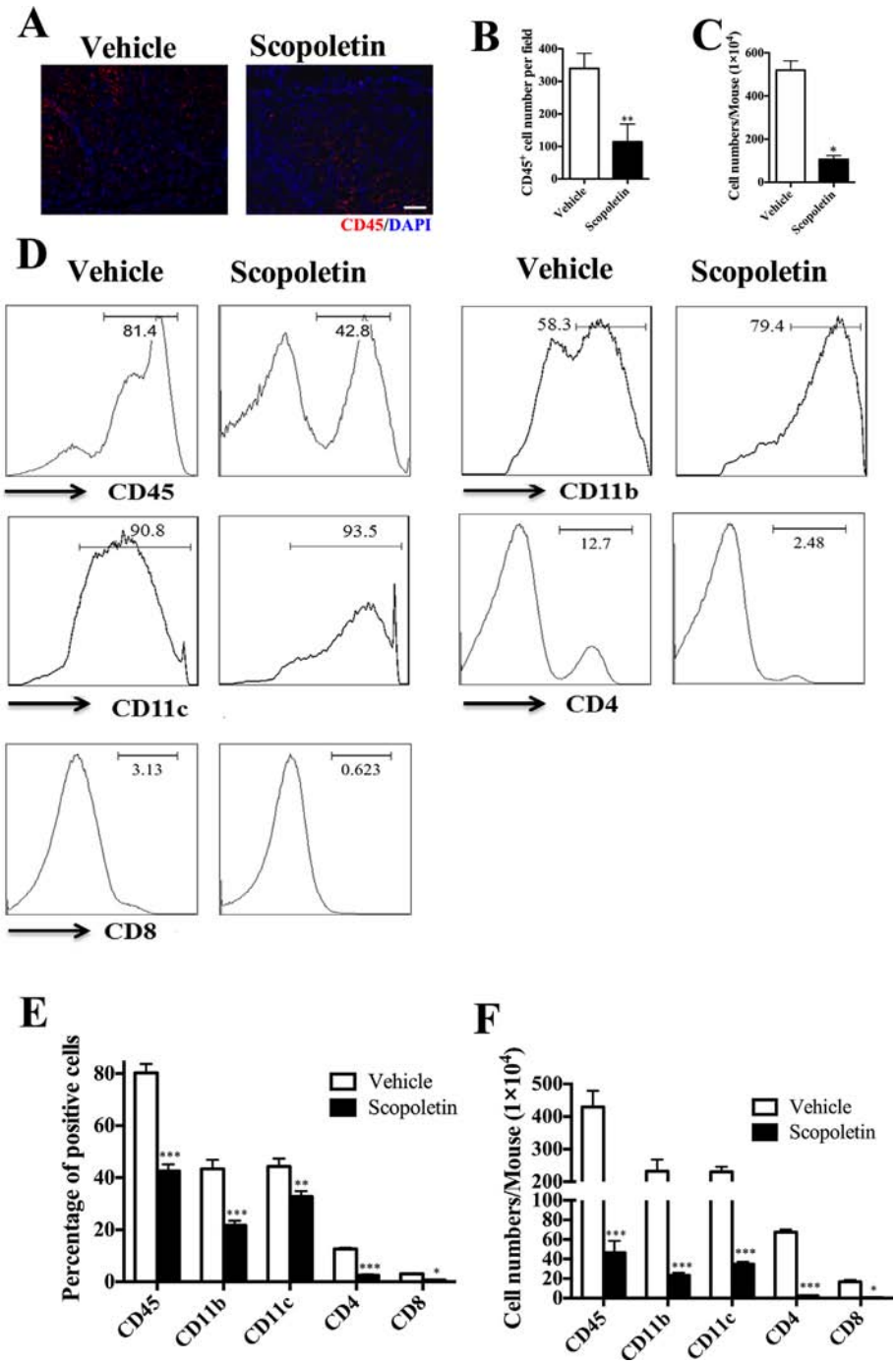

FIGURE 3 | Scopoletin treatment alleviated central nervous system (CNS) inflammation. Scopoletin- or vehicle-treated EAE mice were killed at day 20 p.i., mononuclear cells (MNCs) were isolated from spinal cords and brains. **(A, B)** Spinal cords were subjected to immunofluorescent staining analysis. Representative sections of thoracic spinal cord from vehicle-treated mice or Scopoletin-treated mice were stained with CD45 to evaluate inflammatory infiltration. Scale bar = 100 μm. **(C)** Total MNCs numbers in CNS were counted under light microscopy. **(D, E)** The percentages of $CD45^+$ leukocytes, $CD11b^+$ microglia/macrophage cells, $CD11c^+$ DCs, $CD4^+$, and $CD8^+$ T cells were measured by flow cytometry. **(F)** Absolute numbers of different subtypes of CNS infiltrates were calculated by multiplying the percentages of these cells with total numbers of MNCs in each spinal cord and brain tissue. Figures **(A, D)** are one representative of three independent experiments. Data were determined by Student's *t*-test, and symbols represent mean ± SD, $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

LPS stimulation, DCs expressed relatively high levels of MHC class II, CD80, and CD86, the markers of DCs activation, and their expression was significantly inhibited by Scopoletin treatment (**Figures 5A–F**). Scopoletin, thus, showed a potential for restricting DCs activation and inhibiting expression of costimulatory molecules in BM-DCs cultures.

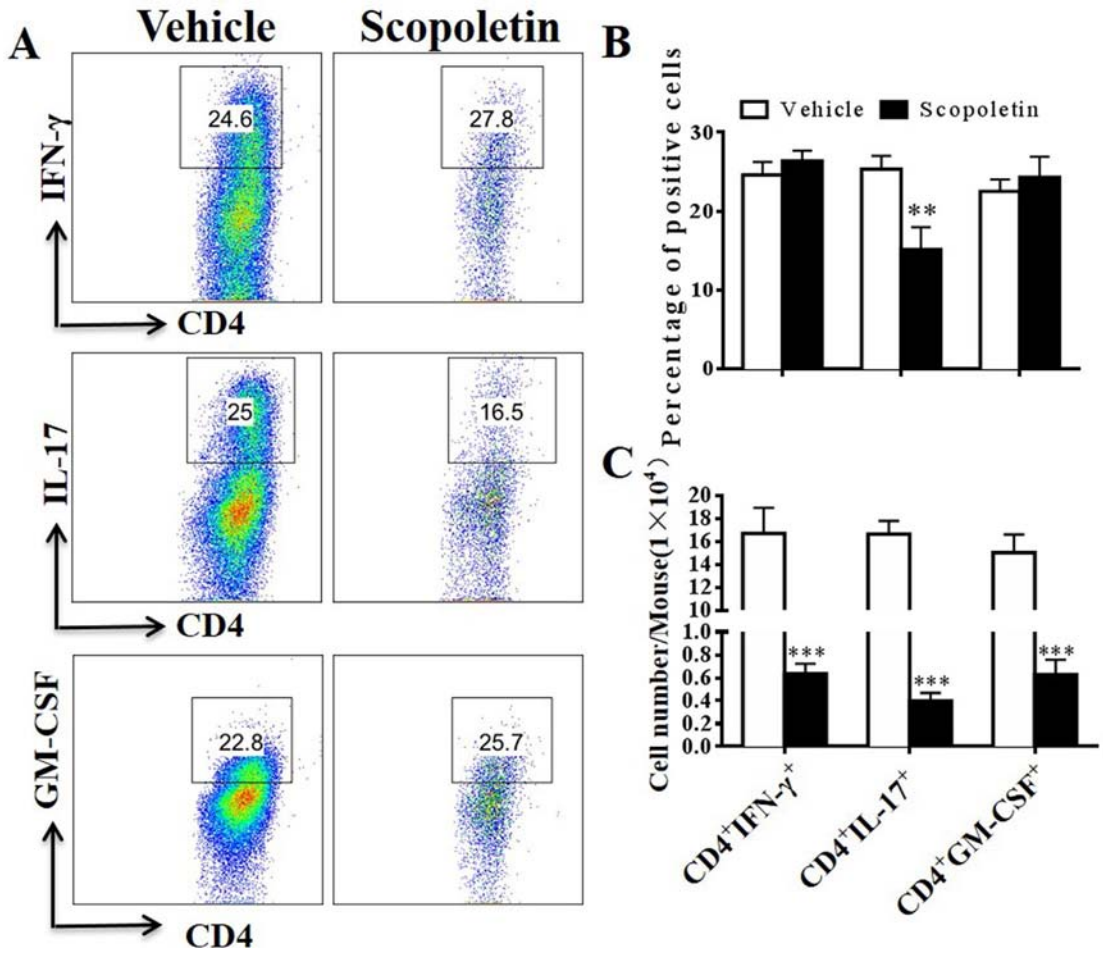

FIGURE 4 | Scopoletin treatment reduced numbers of IFN-γ, IL-17-, and GM-CSF-producing CD4⁺ T cells in the CNS. Scopoletin- or vehicle-treated EAE mice were killed at day 20 p.i., spinal cords and brains were harvested, and MNCs were isolated. **(A, B)** Percentages of IFN-γ-, IL-17-, and GM-CSF-producing CD4⁺ T cells were assessed by flow cytometry. **(C)** Absolute numbers of these cells were calculated by multiplying their percentages of cytokine-producing cells and total CD4⁺ cell numbers in each CNS. Data are representative of three independent experiments. Symbols represent mean ± SD (n = 5 each group), $^{**}P < 0.01$ and $^{***}P < 0.001$, determined by Student's t-test.

To further examine the inhibitory effect of Scopoletin on BM-DCs activation, we then determined the mRNA levels of multiple inflammatory-associated genes expressed by BM-DCs, such as IL-6, IL-12p35, IL-12/IL23p40, IL-23p19, IL-1β, and TNF-α. Previous studies have shown that IL-12 is probably related to Th1 cells differentiation, and IL-23 and IL-6 are crucial to the Th17 cells differentiation (Luckheeram et al., 2012). Importantly, Scopoletin treatment caused a remarkably inhibition in the expression of these genes except for IL-1β (**Figure 6A**). ELISA analysis indicated the suppressive effects of Scopoletin on the secretion of IL-6, IL-23, and TNF-α by BM-DCs (**Figure 6B**). These findings suggest that the effect of Scopoletin treatment may be through inhibiting production of proinflammatory cytokines and expression of costimulatory molecules in DCs.

To test the effects of Scopoletin in DC-induced T-cell proliferation assays, we performed the DC and T-cell coculture and quantified the T-cell proliferation with CFSE; the results showed that Scopoletin reduced the T-cell proliferation *via* DCs induction (**Supplementary Figure 3C**).

Scopoletin Inhibited NF-κB Signaling During LPS-Stimulated DC Activation *In Vitro*

It is well known that NF-κB and MAPK signaling pathways play a central role in the coordinated regulation of gene expression during DC activation (Lu et al., 2014; Baratin et al., 2015). Thus, expression of the total and phosphorylation forms of IRF-7, p38, JNK, and NF-κB in BM-DCs was determined by Western blot. The level of phosphorylation of NF-κB was significantly lower in Scopoletin-treated BM-DCs when compared to DCs stimulated with LPS alone. In contrast, expression of IRF-7, p38, and JNK were not markedly affected by Scopoletin treatment (**Figures 7A**, **B**). In addition, BM-DCs were pretreated with SN50 (20 μM) for 1 h, a specific NF-κB peptide inhibitor (Lin et al., 1995), before LPS (100 ng/ml) stimulation and Scopoletin (100 μM) treatment, after 4 h cells were collected, and then, expression level of cytokines were determined by quantitative real time PCR (qRT-PCR). The expression of NF-κB downstream genes such as IL-1β and TNF-α was significantly decreased by Scopoletin and SN50 treatment. When combined with SN-50 and Scopoletin, TNF-α expression

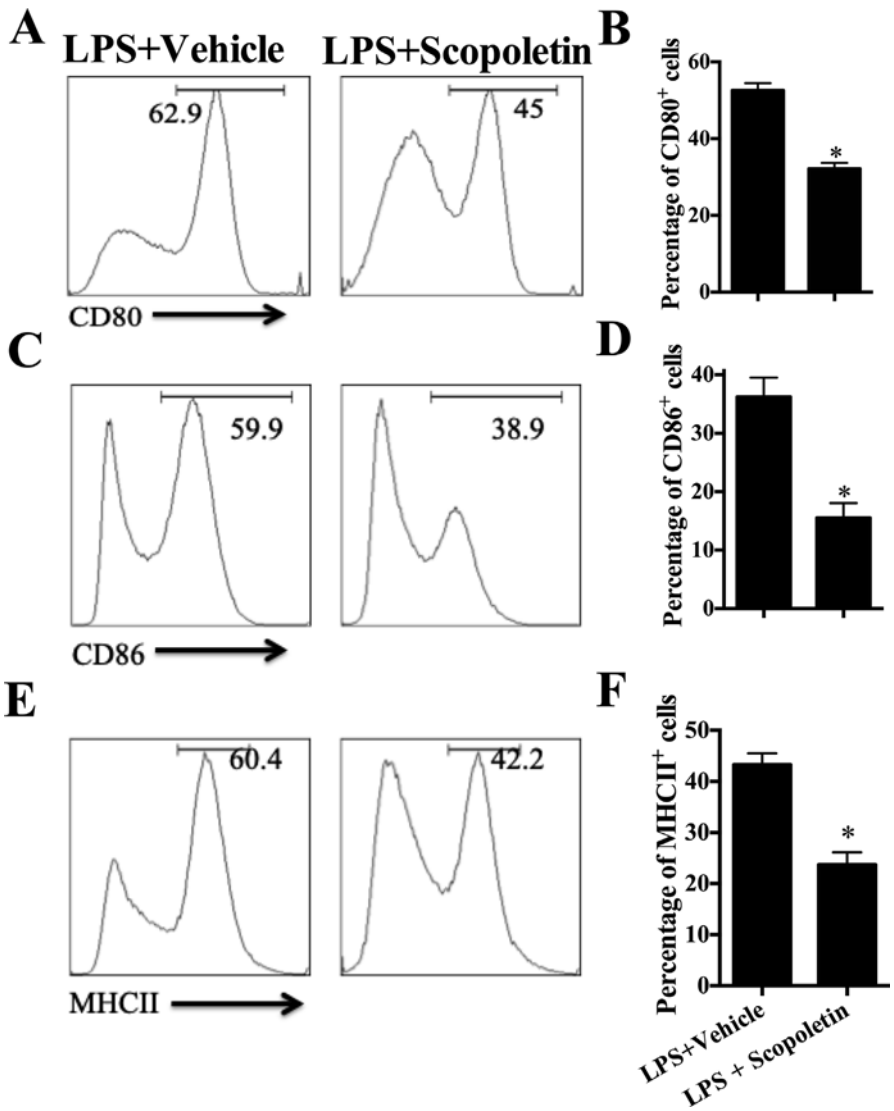

FIGURE 5 | Scopoletin inhibited the activation of murine bone marrow-derived dendritic cells (BM-DCs) by inhibiting NF-κB signaling *in vitro*. BM-DCs were generated and stimulated with 100 ng/μl LPS, and cultured with Scopoletin at a dose of 100 μM. After 18 h, expression of **(A)** CD80, **(C)** CD86, **(E)** MHC II (the positive cells of CD80, CD86, and MHC II are gated below the CD11c positive cells) was measured by flow cytometry following overnight incubation and treatment with or without Scopoletin. **(B, D, F)** Percentages of each molecule were counted. Figures **(A, C, E**) are one representative of three independent experiments. Statistical data are expressed as mean ± SD of three independent experiments. *$P < 0.05$ by Student's *t*-test.

level was significantly enhanced compared with Scopoletin only (**Supplementary Figure 3D**). These data suggest that Scopoletin may exert its action and ameliorate the progression of EAE specifically through inhibiting NF-κB signaling.

DISCUSSION

In this study, we provide evidence, for the first time, of the anti-inflammatory activities and protective effects of Scopoletin in the mouse model of MS, EAE. Our data present a promising bioactive drug, derived from a natural source, for the treatment of MS and possibly other autoimmune diseases.

In addition, Scopoletin is also the main coumarin constituent occurring in the stems of *Erycibe obtusifolia*, a classical medicinal plant that has demonstrated various biological activities mainly used for rheumatoid arthritis with a long history (Pan et al., 2010). Scopoletin has been reported to have anti-inflammatory properties, e.g., inhibition of lymphocyte proliferation and reduction of cytokine production (Arcos et al., 2006). *In vitro* studies showed that Scopoletin inhibited proinflammatory cytokine secretion from RAW 246.7 and HMC-1 cell lines (Moon et al., 2007; Connell et al., 2017). Scopoletin also exhibited anti-inflammatory, antioxidant, and antiacetylcholinesterase potential against Alzheimer's disease (Garcia-Morales et al., 2015). However, its role is unknown in the regulation of APC functions in an autoimmune disease such as MS.

In autoimmune diseases, DCs as professional APCs have a potent ability to trigger naive T-cell reaction and activate an

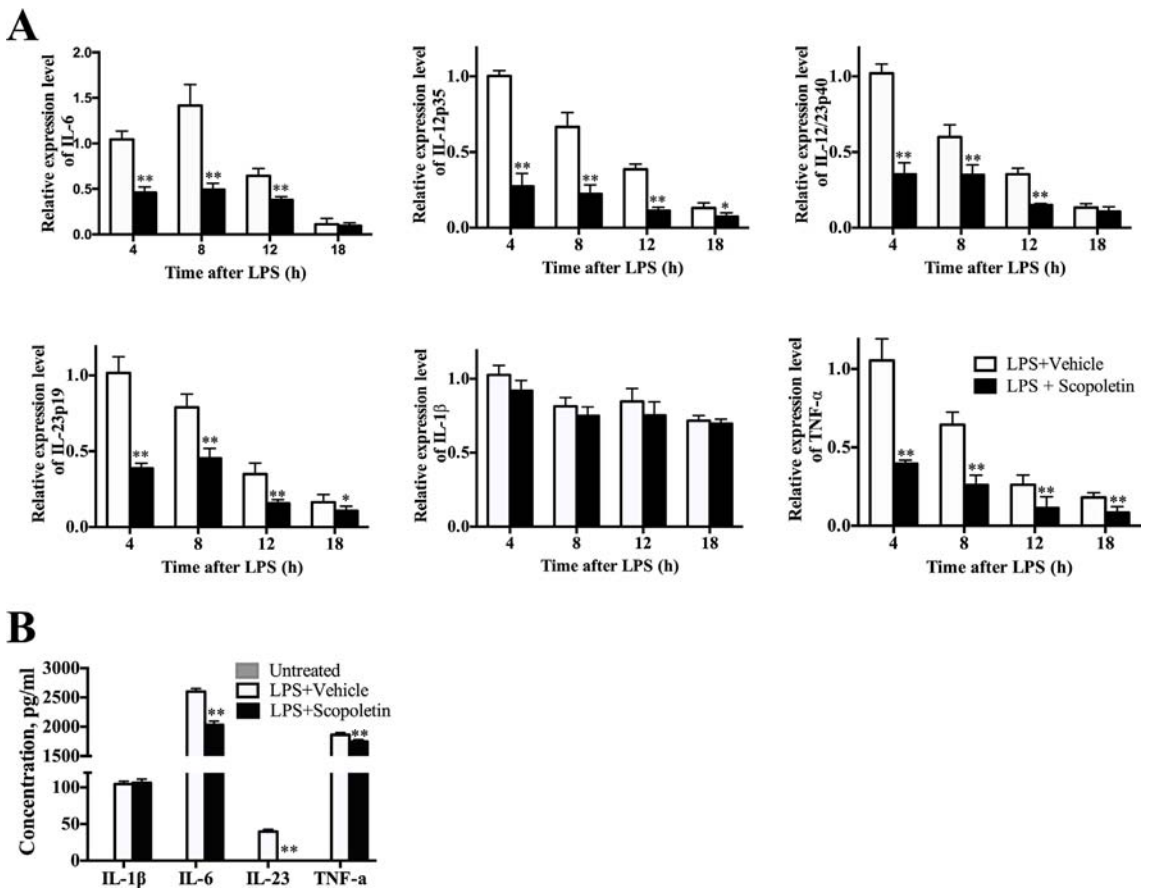

FIGURE 6 | Scopoletin treatment decreased expression of inflammation-related genes and production of proinflammatory cytokines of BM-DCs. **(A)** BM-DCs were generated and activated with 100 ng/μl LPS. mRNA levels of inflammatory-related genes from BM-DCs were determined at 4, 8, 12, and 18 h by qRT-PCR after Scopoletin treatment. **(B)** Supernatants were assayed by ELISA for production of IL-1β, IL-6, IL-23, and TNF-α. Data are mean ± SD of three independent experiments. * $P < 0.05$ and ** $P < 0.01$ by two-way ANOVA.

autoreactive response (Thompson et al., 2018), and decreasing CD80/86 prevented T-cell activation and Th-cell differentiation by inhibiting the costimulatory second signal (Grimbert et al., 2011; Yamamoto et al., 2012). Our results showed that the therapeutic effects of Scopoletin were largely dependent on the suppression of DC activation. Scopoletin reduced the expression of costimulatory molecules CD80 and CD86 as well as MHC class II *in vivo* and *in vitro.* Furthermore, in MS patients and EAE mice, there is a marked increase in the levels of IFN-γ and IL-17, signature cytokines for Th1 and Th17 cells, respectively (Arellano et al., 2017). As drivers of autoimmune response, pathogenic Th1 and Th17 cells are therefore considered to be the main culprits in MS and EAE (Grigoriadis et al., 2015). Our data demonstrated that Scopoletin treatment remarkably decreased the percentages of Th1 and Th17 cells. On the other hand, GM-CSF, which can be produced by both Th1 and Th17 cells, is required for encephalitogenicity of these pathogenic T-cell subsets (Codarri et al., 2011; El-Behi et al., 2011). Reduced numbers of $CD4^+GM\text{-}CSF^+$ T cells in the spleen and CNS following Scopoletin treatment would, therefore, be an important mechanism underlying the effect of Scopoletin on EAE. When the direct effect of Scopoletin on Th1 and Th17 cell differentiation was further investigated *in vitro*, our results showed that differentiation of Th1 and Th17 was not significantly altered by Scopoletin treatment (**Supplementary Figure 2**). Furthermore, the inhibitory effect of Scopoletin treatment on Th1/Th17 cells *in vivo* in EAE mice must therefore be through an indirect mechanism, i.e., by inhibition of DC activation during EAE progression.

It has been well documented that IL-6 plays a key role in determination of Th17 polarization, and knockout of these cytokines completely inhibits EAE development (Giralt et al., 2013). In addition, IL-23 is important for expanding and maintaining the developing Th17 population (Smith and Colbert, 2014) and is required for differentiation of Th17 cells and the pathogenic role of these T cells (El-Behi et al., 2011). Given that DCs are major producers of these cytokines, differentiation of naive T cells into Th17 phenotype is dependent on activated DCs (Agalioti et al., 2018). IL-6 signaling plays a key role in the differentiation of CD4 + T cells, in combination with TGF-β, IL-1β, IL-23, and IL-6, promotes the differentiation of Th17 cells by activating transcription factors containing signal transducers and activators of transcription 3 (STAT3), retinoic acid-related orphan receptors gamma t (RORγt) (Yao et al., 2014). Here, we found that Scopoletin treatment significantly decreased expression of activation and costimulation markers of DCs and their IL-6 and IL-23 production, further

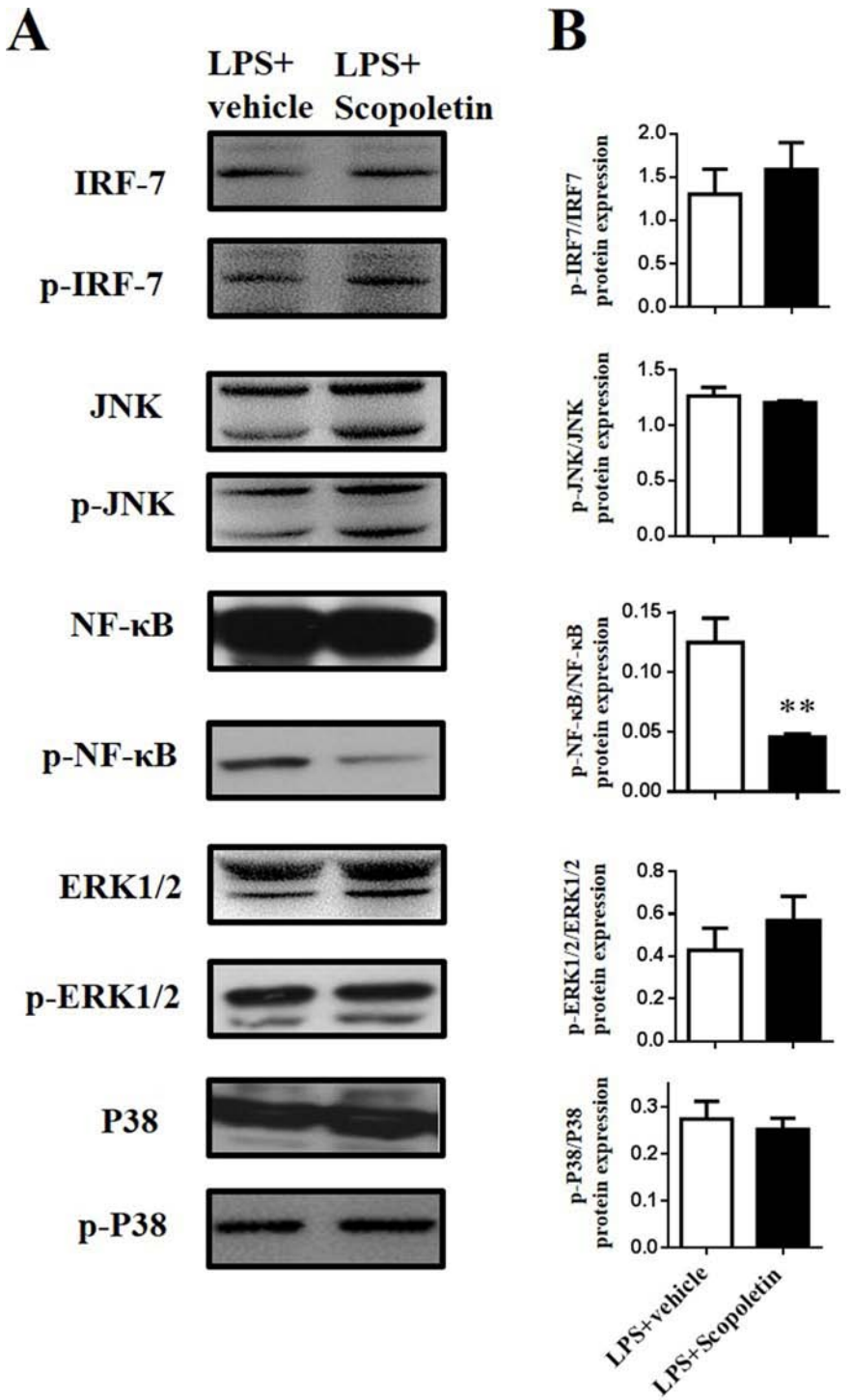

FIGURE 7 | Scopoletin inhibited NF-κB signaling in LPS-stimulated BM-DCs *in vitro*. BM-DCs were activated with LPS and cultured with Scopoletin at a dose of 100 μM. After 18 h, cells were harvested and homogenized for Western blot. **(A)** Protein expression levels of nuclear transcription factors and protein kinase were measured by Western blot. **(B)** Densitometry was determined by ImageJ, and data were analyzed by GraphPad Prism 6. Data are expressed as mean ± SD of three biological replicates. $^{**}P < 0.01$ by Student's *t*-test. Figure A is one representative of three independent experiments.

indicating that Scopoletin blocked DC secretion of Th17-inducing cytokines, thus resulting in inhibited Th17 phenotype formation.

When antigen presentation occurs, epitope-major histocompatibility complexes identified by T-cell receptors and costimulatory signals are sent by interaction of CD80, CD86 of DCs and CD28 expressing on T cells, as well as the cytokine milieu formed by DCs and other inflammatory cells in the microenvironment (Thome et al., 2014b). These signals working

together results in T cell polarization into Th1, Th17, and other effector $CD4^+$ T cells. Studies have shown that the activation of DCs is possibly regulated by three intracellular signaling pathways, including NF-κB, MAPKs, and PI3K (Chesi et al., 2016; Rescigno et al., 1998). To further explore the mechanism of Scopoletin-induced amelioration of EAE, we investigated the effects of Scopoletin on BM-DCs *in vitro,* which indicated decreased expression of a series of inflammation-related genes. While the activation of MAPKs containing ERK, p38, and JNK was not prominently influenced in LPS-stimulated DCs, Scopoletin treatment exerted a profound inhibitory effect on NF-κB activity during DC activation. It was shown that Scopoletin markedly suppressed the phosphorylation of NF-κB, which plays a significant role in the activation and maturation of DCs. Previous studies showed that IL-6 gene expression was reduced through downregulating of NF-κB signaling (Chiang et al., 2014), and the promoter activity of IL-23 was modulated by the NF-κB pathway. Our results indicated that the therapeutic effect of Scopoletin may be mediated by inhibiting the NF-κB signaling pathway, which is vitally involved in producing proinflammatory cytokines.

In summary, we reported, for the first time, that natural compound Scopoletin ameliorated the severity of EAE by regulating DC activation and reducing CNS inflammation *via* suppression of NF-κB signaling. Scopoletin treatment could therefore be a potential candidate for the modulation of inflammatory conditions of autoimmune diseases.

CONCLUSION

Our study demonstrates that natural products Scopoletin derived from medical or edible plants will be valuable in developing a novel therapeutic agent for MS in the future.

DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of 'institutional guidelines and regulations approved by the Animal Ethics Experimental Committee at of Shaanxi Normal University', 'the Animal Ethics Experimental Committee at of Shaanxi Normal University'. The protocol was approved by the 'Animal Ethics Experimental Committee at of Shaanxi Normal University'.

AUTHOR CONTRIBUTIONS

FZ, YZ, TY, and XL conceived and designed the experiments. FZ, Z-QY, TY, H-RF, and J-JH carried out the experiments. FZ, YZ, TY, JT, and XL analyzed data and wrote the manuscript. Z-ZW and XL cosupervised the study and revised the paper. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2019.00863/full#supplementary-material

REFERENCES

Agalioti, T., Villablanca, E. J., Huber, S., and Gagliani, N. (2018). TH17cell plasticity: the role of dendritic cells and molecular mechanisms. *J. Autoimmun.* 87, 50–60. doi: 10.1016/j.jaut.2017.12.003

Amodio, G., and Gregori, S. (2012). Dendritic cells a double-edge sword in autoimmune responses. *Front. Immunol.* 3, 233. doi: 10.3389/fimmu.2012.00233

Arcos, M. L. B., Cremaschi, G., Werner, S., Coussio, J., Ferraro, G., and Anesini, C. (2006). *Tilia cordata* Mill. extracts and scopoletin (isolated compound): differential cell growth effects on lymphocytes. *Phytother. Res.* 20, 34–40. doi: 10.1002/ptr.1798

Ardeshna, K. M., Pizzey, A. R., and Devereux, S. a. K. A. (2000). The PI3 kinase, p38 SAP kinase, and NF-κB signal transduction pathways are involved in the survival and maturation of lipopolysaccharide-stimulated human monocyte-derived dendritic cells. *Blood* 96, 1039-1046.

Arellano, G., Acuna, E., Reyes, L. I., Ottum, P. A., De Sarno, P., Villarroel, L., et al. (2017). Th1 and Th17 cells and associated cytokines discriminate among clinically isolated syndrome and multiple sclerosis phenotypes. *Front. Immunol.* 8, 753. doi: 10.3389/fimmu.2017.00753

Baratin, M., Foray, C., Demaria, O., Habbeddine, M., Pollet, E., Maurizio, J., et al. (2015). Homeostatic NF-kappaB signaling in steady-state migratory dendritic cells regulates immune homeostasis and tolerance. *Immunity* 42, 627–639. doi: 10.1016/j.immuni.2015.03.003

Basu, M., Mayana, K., Xavier, S., Balachandran, S., and Mishra, N. (2016). Effect of scopoletin on monoamine oxidases and brain amines. *Neurochem. Int.* 93, 113–117. doi: 10.1016/j.neuint.2016.01.001

Besusso, D., Saul, L., Leech, M. D., O'connor, R. A., Macdonald, A. S., Anderton, S. M., et al. (2015). 1,25-Dihydroxyvitamin D3-conditioned CD11c+ dendritic cells are effective initiators of CNS autoimmune disease. *Front. Immunol.* 6, 575. doi: 10.3389/fimmu.2015.00575

Chesi, M., Mirza, N. N., Garbitt, V. M., Sharik, M. E., Dueck, A. C., Asmann, Y. W., et al. (2016). IAP antagonists induce anti-tumor immunity in multiple myeloma 22. *Nat. Med.* 1411–1420. doi: 10.1038/nm.4229

Chiang, K. C., Tsui, K. H., Chung, L. C., Yeh, C. N., Chen, W. T., Chang, P. L., et al. (2014). Celastrol blocks interleukin-6 gene expression *via* downregulation of NF-kappaB in prostate carcinoma cells. *PLoS One* 9, e93151. doi: 10.1371/journal.pone.0093151

Codarri, L., Gyulveszi, G., Tosevski, V., Hesske, L., Fontana, A., Magnenat, L., et al. (2011). RORgammat drives production of the cytokine GM-CSF in helper T cells, which is essential for the effector phase of autoimmune neuroinflammation. *Nat. Immunol.* 12, 560–567. doi: 10.1038/ni.2027

Connell, B. J., Saleh, M. C., Rajagopal, D., and Saleh, T. M. (2017). UPEI-400, a conjugate of lipoic acid and scopoletin, mediates neuroprotection in a rat model of ischemia/reperfusion. *Food Chem. Toxicol.* 100, 175–182. doi: 10.1016/j.fct.2016.12.026

Ding, Z., Dai, Y., and Wang, Z. (2005). Hypouricemic action of scopoletin arising from xanthine oxidase inhibition and uricosuric activity. *Planta Medica* 71, 183–185. doi: 10.1055/s-2005-837789

Dou, Y., Tong, B., Wei, Z., Li, Y., Xia, Y., and Dai, Y. (2013). Scopoletin suppresses IL-6 production from fibroblast-like synoviocytes of adjuvant arthritis rats induced by IL-1beta stimulation. *Int. Immunopharmacol.* 17, 1037–1043. doi: 10.1016/j.intimp.2013.10.011

El-Behi, M., Ciric, B., Dai, H., Yan, Y., Cullimore, M., Safavi, F., et al. (2011). The encephalitogenicity of T(H)17 cells is dependent on IL-1- and IL-23-induced production of the cytokine GM-CSF. *Nat. Immunol.* 12, 568–575. doi: 10.1038/ni.2031

El-Behi, M., Rostami, A., and Ciric, B. (2010). Current views on the roles of Th1 and Th17 cells in experimental autoimmune encephalomyelitis. *J. Neuroimmune Pharmacol.* 5, 189–197. doi: 10.1007/s11481-009-9188-9

Forino, M., Tartaglione, L., Dell'aversano, C., and Ciminiello, P. (2016). NMR-based identification of the phenolic profile of fruits of Lycium barbarum (goji berries). Isolation and structural determination of a novel N-feruloyl tyramine dimer as the most abundant antioxidant polyphenol of goji berries. *Food Chem.* 194, 1254–1259. doi: 10.1016/j.foodchem.2015.08.129

Gabriele, L., and Ozato, K. (2007). The role of the interferon regulatory factor (IRF) family in dendritic cell development and function. *Cytokine Growth Factor Rev.* 18, 503–510. doi: 10.1016/j.cytogfr.2007.06.008

Garcia-Morales, G., Huerta-Reyes, M., Gonzalez-Cortazar, M., Zamilpa, A., Jimenez-Ferrer, E., Silva-Garcia, R., et al. (2015). Anti-inflammatory, antioxidant and anti-acetylcholinesterase activities of *Bouvardia ternifolia*: potential implications in Alzheimer's disease. *Arch. Pharm. Res.* 38, 1369–1379. doi: 10.1007/s12272-015-0587-6

Ginwala, R., Mctish, E., Raman, C., Singh, N., Nagarkatti, M., Nagarkatti, P., et al. (2016). Apigenin, a natural flavonoid, attenuates EAE severity through the modulation of dendritic cell and other immune cell functions. *J. Neuroimmune Pharmacol.* 11, 36–47. doi: 10.1007/s11481-015-9617-x

Giralt, M., Ramos, R., Quintana, A., Ferrer, B., Erta, M., Castro-Freire, M., et al. (2013). Induction of atypical EAE mediated by transgenic production of IL-6 in astrocytes in the absence of systemic IL-6. *Glia* 61, 587–600. doi: 10.1002/glia.22457

Grigoriadis, N., Van Pesch, V., and Paradig, M. S. G. (2015). A basic overview of multiple sclerosis immunopathology. *Eur. J. Neurol.* 22 (Suppl 2), 3–13. doi: 10.1111/ene.12798

Grimbert, P., Audard, V., Diet, C., Matignon, M., Plonquet, A., Mansour, H., et al. (2011). T-cell phenotype in protocol renal biopsy from transplant recipients treated with belatacept-mediated co-stimulatory blockade. *Nephrol. Dial. Transplant* 26, 1087–1093. doi: 10.1093/ndt/gfq453

He, Z., Ma, J., Wang, R., Zhang, J., Huang, Z., Wang, F., et al. (2017). A two-amino-acid substitution in the transcription factor RORgammat disrupts its function in TH17 differentiation but not in thymocyte development. *Nat. Immunol.* 18, 1128–1138. doi: 10.1038/ni.3832

Hemmer, B., Kerschensteiner, M., and Korn, T. (2015). Role of the innate and adaptive immune responses in the course of multiple sclerosis. *Lancet Neurol.* 14, 406–419. doi: 10.1016/S1474-4422(14)70305-9

Honda, K., Yanai, H., Negishi, H., Asagiri, M., Sato, M., Mizutani, T., et al. (2005). IRF-7 is the master regulator of type-I interferon-dependent immune responses. *Nature* 434, 772–777. doi: 10.1038/nature03464

Jamuna, S., Karthika, K., Paulsamy, S., Thenmozhi, K., Kathiravan, S., and Venkatesh, R. (2015). Confertin and scopoletin from leaf and root extracts of *Hypochaeris radicata* have anti-inflammatory and antioxidant activities. *Ind. Crops Prod.* 70, 221–230. doi: 10.1016/j.indcrop.2015.03.039

Kornberg, M. D., Smith, M. D., Shirazi, H. A., Calabresi, P. A., Snyder, S. H., and Kim, P. M. (2018). Bryostatin-1 alleviates experimental multiple sclerosis. *Proc. Nat. Acad. Sci. U. S. A.* 115, 2186–2191. doi: 10.1073/pnas.1719902115

Lassmann, H., and Bradl, M. (2016). Multiple sclerosis: experimental models and reality. *Acta Neuropathol.* 133, 223-244. doi: 10.1007/s00401-016-1631-4

Lee, H. I., Yun, K. W., Seo, K. I., Kim, M. J., and Lee, M. K. (2014). Scopoletin prevents alcohol-induced hepatic lipid accumulation by modulating the AMPK-SREBP pathway in diet-induced obese mice. *Metabolism* 63, 593–601. doi: 10.1016/j.metabol.2014.01.003

Li, G., Yamasaki, R., Fang, M., Masaki, K., Ochi, H., Matsushita, T., et al. (2018). Novel disease-modifying anti-rheumatic drug iguratimod suppresses chronic experimental autoimmune encephalomyelitis by down-regulating activation of macrophages/microglia through an NF-kappaB pathway. *Sci. Rep.* 8, 1933. doi: 10.1038/s41598-018-20390-5

Li, X., Zhang, Y., Yan, Y., Ciric, B., Ma, C. G., Chin, J., et al. (2017). LINGO-1-Fc-transduced neural stem cells are effective therapy for chronic stage experimental autoimmune encephalomyelitis. *Mol. Neurobiol.* 54, 4365–4378. doi: 10.1007/s12035-016-9994-z

Li, X., Zhang, Y., Yan, Y., Ciric, B., Ma, C. G., Gran, B., et al. (2016). Neural stem cells engineered to express three therapeutic factors mediate recovery from chronic stage CNS autoimmunity. *Mol. Ther.* 24, 1456–1469. doi: 10.1038/mt.2016.104

Lin, Y. Z., Yao, S. Y., Veach, R. A., Torgerson, T. R., and Hawiger, J. (1995). Inhibition of nuclear translocation of transcription factor NF-kappa B by a synthetic peptide containing a cell membrane-permeable motif and nuclear localization sequence. *J. Biol. Chem.* 270, 14255–14258. doi: 10.1074/jbc.270.24.14255

Lu, Y., Chen, B., Song, J. H., Zhen, T., Wang, B. Y., Li, X., et al. (2013). Eriocalyxin B ameliorates experimental autoimmune encephalomyelitis by suppressing Th1 and Th17 cells. *PNAS* 110 (116), 2258–2263. doi: 10.1073/pnas.1222426110

Lu, Y., Zhang, M., Wang, S., Hong, B., Wang, Z., Li, H., et al. (2014). p38 MAPK-inhibited dendritic cells induce superior antitumour immune responses and overcome regulatory T-cell-mediated immunosuppression. *Nat. Commun.* 5, 4229. doi: 10.1038/ncomms5229

Luckheeram, R. V., Zhou, R., Verma, A. D., and Xia, B. (2012). CD4+T Cells: Differentiation and functions. *Clin. Dev. Immunol.* 2012, 1–12. doi: 10.1155/2012/925135

Malik, S., Sadhu, S., Elesela, S., Pandey, R. P., Chawla, A. S., Sharma, D., et al. (2017). Transcription factor Foxo1 is essential for IL-9 induction in T helper cells. *Nat. Commun.* 8, 815. doi: 10.1038/s41467-017-00674-6

Moon, P. D., Lee, B. H., Jeong, H. J., An, H. J., Park, S. J., Kim, H. R., et al. (2007). Use of scopoletin to inhibit the production of inflammatory cytokines through inhibition of the IkappaB/NF-kappaB signal cascade in the human mast cell line HMC-1. *Eur. J. Pharmacol.* 555, 218–225. doi: 10.1016/j.ejphar.2006.10.021

Pan, R., Gao, X. H., Li, Y., Xia, Y. F., and Dai, Y. (2010). Anti-arthritic effect of scopoletin, a coumarin compound occurring in *Erycibe obtusifolia* Benth stems, is associated with decreased angiogenesis in synovium. *Fundam. Clin. Pharmacol.* 24, 477–490. doi: 10.1111/j.1472-8206.2009.00784.x

Procaccini, C., De Rosa, V., Pucino, V., Formisano, L., and Matarese, G. (2015). Animal models of multiple sclerosis. *Eur. J. Pharmacol.* 759, 182–191. doi: 10.1016/j.ejphar.2015.03.042

Rasouli, J., Ciric, B., Imitola, J., Gonnella, P., Hwang, D., Mahajan, K., et al. (2015). Expression of GM-CSF in T cells is increased in multiple sclerosis and suppressed by IFN-beta therapy. *J. Immunol.* 194, 5085–5093. doi: 10.4049/jimmunol.1403243

Rescigno, M., Martino, M., Sutherland, C. L., Gold, M. R., and Ricciardi, C. P. (1998). Dendritic cell survival and maturation are regulated by different signaling pathways. *J. Exp. Med.* 188, 2175–2180. doi: 10.1084/jem.188.11.2175

Shalan, N.a.M., Mustapha, N. M., and Mohamed, S. (2016). *Morinda citrifolia* leaf enhanced performance by improving angiogenesis, mitochondrial biogenesis, antioxidant, anti-inflammatory & stress responses. *Food Chem.* 212, 443–452. doi: 10.1016/j.foodchem.2016.05.179

Smith, J. A., and Colbert, R. A. (2014). Review: The interleukin-23/interleukin-17 axis in spondyloarthritis pathogenesis: Th17 and beyond. *Arthritis Rheumatol.* 66, 231-241. doi: 10.1002/art.38291

Thome, R., Bonfanti, A. P., Rasouli, J., Mari, E. R., Zhang, G. X., Rostami, A., et al. (2018). Chloroquine-treated dendritic cells require STAT1 signaling for their tolerogenic activity. *Eur. J. Immunol.* 48, 1228–1234. doi: 10.1002/eji.201747362

Thome, R., Issayama, L. K., Alves Da Costa, T., Gangi, R. D., Ferreira, I. T., Raposo, C., et al. (2014a). Dendritic cells treated with crude *Plasmodium berghei* extracts acquire immune-modulatory properties and suppress the development of autoimmune neuroinflammation. *Immunology* 143, 164–173. doi: 10.1111/imm.12298

Thome, R., Issayama, L. K., Digangi, R., Bombeiro, A. L., Da Costa, T. A., Ferreira, I. T., et al. (2014b). Dendritic cells treated with chloroquine modulate

experimental autoimmune encephalomyelitis. *Immunol. Cell Biol.* 92, 124–132. doi: 10.1038/icb.2013.73

Thompson, A. J., Baranzini, S. E., Geurts, J., Hemmer, B., and Ciccarelli, O. (2018). Multiple sclerosis. *Lancet* 391, 1622–1636. doi: 10.1016/S0140-6736(18)30481-1

Yamamoto, S., Nava, R. G., Zhu, J., Huang, H. J., Ibrahim, M., Mohanakumar, T., et al. (2012). Cutting edge: *Pseudomonas aeruginosa* abolishes established lung transplant tolerance by stimulating B7 expression on neutrophils. *J. Immunol.* 189, 4221–4225. doi: 10.4049/jimmunol.1201683

Yao, X., Huang, J., Zhong, H., Shen, N., Faggioni, R., Fung, M., et al. (2014). Targeting interleukin-6 in inflammatory autoimmune diseases and cancers. *Pharmacol. Ther.* 141, 125–139. doi: 10.1016/j.pharmthera.2013.09.004

Yasuda, K., Richez, C., Maciaszek, J. W., Agrawal, N., Akira, S., Marshak-Rothstein, A., et al. (2007). Murine dendritic cell type I IFN production induced by human IgG-RNA immune complexes is IFN regulatory factor (IRF)5 and IRF7 dependent and is required for IL-6 production. *J. Immunol.* 178, 6876–6885. doi: 10.4049/jimmunol.178.11.6876

Zeng, Y. C., Li, S., Liu, C., Gong, T., Sun, X., Fu, Y., et al. (2017). Soluplus micelles for improving the oral bioavailability of scopoletin and their hypouricemic effect *in vivo*. *Acta Pharmacol. Sin.* 38, 424–433. doi: 10.1038/aps.2016.126

Zhang, Y., Li, X., Ciric, B., Ma, C. G., Gran, B., Rostami, A., et al. (2015). Therapeutic effect of baicalin on experimental autoimmune encephalomyelitis is mediated by SOCS3 regulatory pathway. *Sci. Rep.* 5, 17407. doi: 10.1038/srep17407

Zhao, L., Li, X., Ye, Z. Q., Zhang, F., Han, J. J., Yang, T., et al. (2018). Nutshell extracts of *Xanthoceras sorbifolia*: a new potential source of bioactive phenolic compounds as a natural antioxidant and immunomodulator. *J. Agric. Food Chem.* 66, 3783–3792. doi: 10.1021/acs.jafc.7b05590

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Corrigendum: Scopoletin Suppresses Activation of Dendritic Cells and Pathogenesis of Experimental Autoimmune Encephalomyelitis by Inhibiting NF-κB Signaling

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In the published article, author Zhe-Zhi Wang's identity was incorrect. Instead of "co-author," it should be "co-corresponding author." The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

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REVIEW
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Microglial Drug Targets in AD: Opportunities and Challenges in Drug Discovery and Development

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Alzheimer's disease (AD) is a large and increasing unmet medical need with no disease-modifying treatment currently available. Genetic evidence from genome-wide association studies (GWASs) and gene network analysis has clearly revealed a key role of the innate immune system in the brain, of which microglia are the most important element. Single-nucleotide polymorphisms (SNPs) in genes predominantly expressed in microglia have been associated with altered risk of developing AD. Furthermore, microglia-specific pathways are affected on the messenger RNA (mRNA) expression level in post-mortem AD tissue and in mouse models of AD. Together these findings have increased the interest in microglia biology, and numerous scientific reports have proposed microglial molecules and pathways as drug targets for AD. Target identification and validation are generally the first steps in drug discovery. Both target validation and drug lead identification for central nervous system (CNS) targets and diseases entail additional significant obstacles compared to peripheral targets and diseases. This makes CNS drug discovery, even with well-validated targets, challenging. In this article, we will illustrate the special challenges of AD drug discovery by discussing the viability/practicality of possible microglia drug targets including cluster of differentiation 33 (CD33), $K_{Ca}3.1$, kynurenines, ionotropic P2 receptor 7 (P2X7), programmed death-1 (PD-1), Toll-like receptors (TLRs), and triggering receptor expressed in myeloid cells 2 (TREM2).

Keywords: drug target, microglia, target identification, target validation, screening cascade

HIGHLIGHTS:

- Glial cell–based targets receive increased attention in drug development.
- What makes a good drug target?
- Steps and pitfalls for preclinical drug development.
- Specific consideration for putative microglial drug targets in Alzheimer's disease.

INTRODUCTION

Microglial reactivity has long been recognized as a pathological hallmark of a wide variety of neurological diseases. While morphological changes of microglia were initially interpreted as a reactive response to neuronal damage, growing evidence suggests that these changes are not merely "reactive" but indicate that glial pathology contributes to disease progression (Raj et al., 2014a; Verkhratsky et al., 2014; Ben Haim et al., 2015; Bergles and Richardson, 2015; Biber et al., 2015; Heneka et al., 2015; Heppner et al., 2015; Jain et al., 2015; Ransohoff and El Khoury, 2015; Robel and Sontheimer, 2015; Zuchero and Barres, 2015; Osborn et al., 2016). Especially with regard to Alzheimer's disease (AD), numerous reviews cover various aspects of microglial biology in the context of disease (see for example: (Guedes et al., 2018; Hansen et al., 2018; Kinney et al., 2018; Shi and Holtzman, 2018; Thei et al., 2018; Ulland and Colonna, 2018; Henstridge et al., 2019). Using the search term "microglia Alzheimer," PubMed lists close to 100 reviews that were published in the last 2 years (Jan 2017–Jan 2019). It is not the purpose of the current review to discuss the potential role of microglia in AD, but to focus on the challenges and practical aspects of microglial proteins or pathways as potential drug targets.

A considerable set of genetic risk factors for AD are predominantly expressed in microglia (Wes et al., 2016; Henstridge et al., 2019). Thus, it is very clear from human genetics that regardless of being "reactive," "active," "dystrophic," "senescent," "dysfunctional," or "disease associated," microglial involvement in AD offers a hitherto unexplored intervention point. Many publications concerning microglia in AD include conclusions like: "The presented data here suggest that protein 'XYZ' can be considered as a drug target for AD." As there are some common misunderstandings between colleagues focused on biology vs. drug discovery regarding the definition of a target, we first aim to clarify what it takes to consider a microglial molecule suitable for a drug discovery program. We will then review some commonly suggested microglial targets and pathways, i.e., cluster of differentiation 33 (CD33), $K_{Ca}3.1$, kynurenines (KYN), ionotropic P2 receptor 7 (P2X7), programmed death-1 (PD-1), Toll-like receptors (TLRs), and triggering receptor expressed in myeloid cells 2 (TREM2), discussing their potential role in AD and their respective challenges in regard to drug discovery.

TARGET IDENTIFICATION

In general, preclinical drug discovery can be staged as follows: target identification, in which the validity of a target for modulating a disease process and the initial chemical matter along with the means of characterizing them are established; lead optimization, in which target validity is further strengthened while identifying candidate molecules with the full range of necessary drug-like properties; and finally, preclinical testing for safety and efficacy. Apart from target identification, we will not discuss the other stages of preclinical drug development (**Figure 1**). The interested reader is referred to (Möller and Boddeke, 2016) for a detailed description of the whole process.

For potential targets, it is of utmost importance to demonstrate relevancy to the disease and that modulating the target would result in therapeutic benefit with an acceptable safety margin. Evidence for a role of a candidate target in disease might include i) altered expression in disease, ii) integral relationship to disease pathophysiology, iii) genetic association with disease, iv) having a mechanistic link to disease etiology, and v) demonstration of a beneficial effect when modulated in a disease-relevant *in vitro* or (preferably) *in vivo* model.

Unfortunately, generating expression data for the human central nervous system (CNS) is extremely difficult. While for peripheral diseases biopsies, or blood draws are routine, for the CNS, most parameters can only be derived from post-mortem brain tissue that at best represents end-stage disease, or by limited imaging methods, making it difficult to observe changes in earlier and therapeutically more relevant disease states. Non-human models can also provide insight, but with significant translational uncertainties.

A strong genetic association of a microglial molecule with AD, for example, CD33, complement receptor 1 (CR1), phospholipase C gamma 2 (PLCγ2), and TREM2 (Wes et al., 2016; Henstridge et al., 2019), also suggests promise as a drug target. If such evidence can further be strengthened by a mechanistic link to a disease model by *in vitro* or *in vivo* genetic or pharmacologic intervention studies [e.g., knocking out CD33 (Bradshaw et al., 2013)], that would serve to increase confidence in the target.

WHAT MAKES A GOOD (MICROGLIAL) TARGET?

Drugability

Besides linking a target to disease, there are other more pragmatic considerations for selecting a target. Admittedly, "drugable" is not a well-defined term; it usually includes chemical tractability with a ligand (small molecule or antibody) but can also refer more broadly to the properties of such ligands, e.g., pharmacokinetics, distribution, metabolism (and the pharmacologic properties of

FIGURE 1 | Schematic representation of the preclinical drug discovery process. The preclinical drug discovery process can generally be divided into six unique steps from target identification to preclinical testing.

significant metabolites), pharmaceutical properties including chemical stability, and manufacturability.

Traditionally, a target would be considered chemically tractable if a small molecule or antibody can bind with high affinity and specificity and induce the desired biological effect. For example, G-protein coupled receptors (GPCRs) are generally considered chemically tractable; more than 30% of currently marketed drugs target GPCRs (Ma and Zemmel, 2002; Hauser et al., 2017). Ion channels or enzymes are also considered tractable as those targets often include structural features that permit potent and specific ligand binding, with consequent direct inhibition of conductance or catalytic activity. Blocking protein–protein interactions is generally more difficult; however, recent advances have challenged this view, and modulation of protein–protein interactions by small molecules is now an area of active research and will increase the number of targets considered drugable (Higueruelo et al., 2013). Furthermore, blocking protein–protein interactions can often be accomplished with therapeutic antibodies, and several innovative approaches have been devised to improve antibody penetration into the brain (Watts and Dennis, 2013; Salameh and Banks, 2014; Pardridge, 2015). Despite these recent advancements, developing therapeutics for the CNS remains challenging, and while there are many biologically interesting molecules related to microglia, only a small subset of them might be considered drugable.

Specificity

It is not merely enough to generate a potent pharmacological agent against a drug target; it is also of utmost importance that the molecule is selectively modulating only this one target to avoid off-target effects that may hamper the interpretation of experimental data. Unfortunately, many so-called "reference compounds," such as commercially available inhibitors, are not as specific as advertised by the suppliers (Frye, 2010). A group of 50 scientists from different life science disciplines have covered this topic in detail in Commentary in *Nature Chemical Biology* (Arrowsmith et al., 2015). This lack of specificity makes interpretation of results gained with many compounds virtually impossible. A prominent example in microglia biology (although not directly related to a single target) is minocycline, which often is referred to as a "microglia inhibitor" (Möller et al., 2016).

Safety

Arguably the most important criterion for target selection, however, is safety. During clinical development, many drugs fail, not due to the lack of efficacy but due to adverse safety events inadequately predicted by preclinical studies. Toxicity might not only arise from modulating the target itself, meaning that the therapeutic window is small, but that there could be off-target effects of a drug, or that toxicity may also arise from a metabolite of the drug that itself is toxic. It is therefore important to select targets with the highest likelihood of being safe as early as possible. A common question is how broadly a target is expressed. Localized expression preferentially in the target cell and/or tissue is considered advantageous as there is less likelihood for adverse effects unrelated to the desired mechanism of action. Unfortunately, there are very few potential targets restricted to microglia as most potential targets are also expressed in peripheral myeloid cells, so effects on the peripheral immune system are a persistent concern. Safety concerns, whether based on side effects of a development compound, a target knockout phenotype, or suspected essential physiological roles of the target, need to be addressed as early as possible by experiments.

Taken together, there is a whole range of drugability considerations that must be met to make a target practical for drug discovery. There are few targets in microglia that are often discussed in the literature to be of value in AD. Here we will discuss the potential and challenges around these targets when it comes down to drugability aspects.

CURRENTLY PROPOSED MICROGLIAL DRUG TARGETS

Proposed Role of CD33 in AD

In 2011, large-scale genome-wide association studies (GWASs) identified CD33, a member of the sialic acid–binding immunoglobulin-like lectins (siglecs) family, as a genetic locus associated with the risk to develop AD (Hollingworth et al., 2011; Naj et al., 2011). This association with AD risk has been confirmed in a variety of subsequent genetic studies in different ethnic groups, with odds ratios between 0.7 and 0.9; thus, the CD33 rs3865444A allele protects from the development of AD [for recent meta-analysis, see (Jiang et al., 2018)]. It was furthermore described that the protective single-nucleotide polymorphism (SNP) rs3865444A is in linkage disequilibrium with rs12459419T, which causes increased splicing of exon2, and therefore deletes the sialic acid–binding site of CD33. The protective SNP therefore leads to increased expression of a truncated, non-functional version of the receptor, called d2-CD33 or CD33m (Bradshaw et al., 2013; Malik et al., 2013; Raj et al., 2014b). A reduction in full-length CD33 expression was observed in myeloid cells of CD33 rs3865444A allele carriers, resulting in an overall reduced CD33 function (Bradshaw et al., 2013; Raj et al., 2014b).

CD33 signaling in humans depends on two intracellular inhibitory domains, one immunoreceptor tyrosine-based inhibitory motif (ITIM), and one ITIM-like domain. CD33 is therefore considered to be an inhibitory immune receptor. Indeed, when activated CD33 becomes tyrosine-phosphorylated by Src kinases, which subsequently recruit Src homology-2 domain (SH2)–containing tyrosine phosphatases (SHP1/2), this results in the dephosphorylation and inhibition of signaling cascades (see for review Macauley et al., 2014). CD33 is expressed in myeloid cells, and its signaling negatively regulates the function of these cells. Accordingly, knockdown of CD33 in human monocytes leads to spontaneous release of interleukin 1 beta (IL-1β), Tumor necrosis factor alpha (TNFα), and IL-8 (Lajaunias et al., 2005). In monocytes from carriers of the CD33 rs3865444A allele (decreased CD33 function), an increased uptake capacity of amyloid beta(1–42) ($A\beta_{1-42}$) was described (Bradshaw et al., 2013).

In the brain, CD33 is specifically expressed in microglia (Bradshaw et al., 2013; Griciuc et al., 2013; Malik et al., 2013; Walker et al., 2015), where an inverse relationship between CD33 expression and Aβ uptake capacity was observed

(Griciuc et al., 2013). Accordingly, in the cortex of AD patients, a positive correlation of microglial CD33 expression and amyloid pathology was described, indicating that increased CD33 expression in microglia promotes plaque pathology (Griciuc et al., 2013). CD33-deficient Amyloid precursor protein (APP)/ Presenilin 1 (PS1) mice showed reduced amyloid pathology compared to age-matched wildtype (WT) controls, supporting the idea that reduced microglial CD33 function is beneficial with respect to amyloid pathology (Griciuc et al., 2013). Given this variety of data concerning the role of CD33 as regulator of microglial Aβ uptake capacity, this siglec receptor is currently considered as drug target in AD. It is considered that inhibition of CD33 function in microglia would increase their amyloid uptake capacity, which in turn would be beneficial in AD **Figure 2** (Heneka et al., 2015).

Target Challenges Around CD33

There are many challenges with respect to CD33 as a drug target in AD. In general, finding ligands for siglecs is difficult, as glycan binding often is of relatively low affinity and relies on multivalency to achieve adequate affinity (Varki, 2009). There is evidence that CD33 binds α2.3 sialic acid and α2.6 sialic acid; the corresponding glycosylated proteins that are decorated with these sialic acids, however, have not been identified (Linnartz-Gerlach et al., 2014; Macauley et al., 2014). As CD33 is the shortest of all known siglecs, it most likely is activated in a cis-manner (O'Reilly and Paulson, 2009; Linnartz-Gerlach et al., 2014; Macauley et al., 2014),

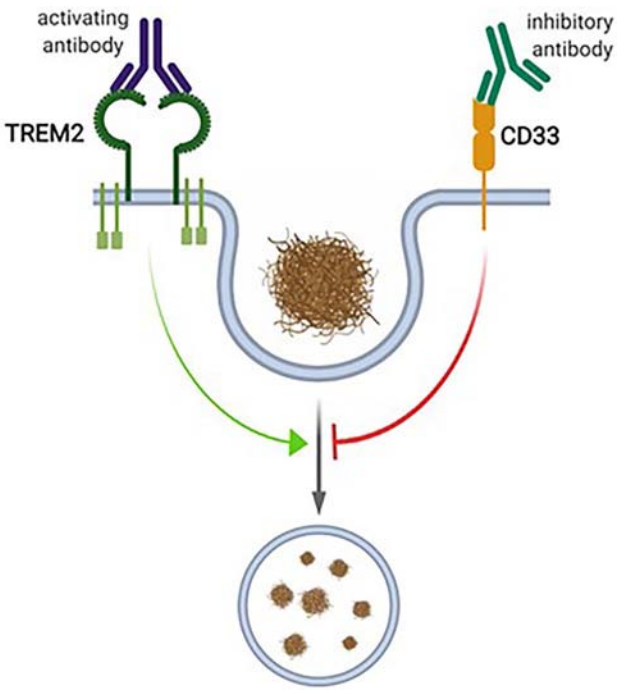

FIGURE 2 | The proposed interplay between CD33 and TREM2 in microglial phagocytosis. CD33 and TREM2 have been proposed to be inhibitors or activators of microglial phagocytosis, respectively. For TREM2, activating antibodies, and for CD33, inhibitory antibodies, have been proposed as therapeutic interventions. Figure created with Biorender.com

meaning that the sialylated glycoproteins that activate CD33 would also be expressed in microglia. One report provides data that soluble sialyllactosamine in the mM range might activate CD33 in de-sialylated monocytes (Lajaunias et al., 2005). This finding awaits independent confirmation. Apart from our very limited understanding around natural ligands for CD33, functional tool compounds for CD33 have not yet been described. The only published molecule displaying a reasonable binding at CD33 is cpd22, which displaces bead-coupled α2.6 sialic acid with an half maximal effective concentration (EC_{50}) of about 10 μM (Rillahan et al., 2014). It therefore is suggested that cpd22 binds CD33 at the sialic acid–binding domain; whether this binding affects CD33 signaling is not understood. Thus, it is not clear whether cpd22 might function as an agonist or antagonist for CD33. The lack of ligands or tool compounds seriously hampers the development of functional analysis of CD33 in cellular assays, making the development of functional inhibitors for CD33 difficult.

Therapeutic inhibitors of CD33 are hypothesized to increase phagocytic properties in microglia; from a drug development perspective, they might also be envisaged to inhibit the CD33 signaling cascade. It is not yet understood how recruitment of SHP1 leads to inhibition of cellular phagocytosis in CD33-expressing myeloid cells (Linnartz-Gerlach et al., 2014).

Another challenge concerns the large species difference of CD33. Mouse and human CD33 differ not only in the extracellular domain but also in the transmembrane domain as well as intracellularly. In contrast to human CD33, mouse CD33 only has one ITIM-like domain but lacks the ITIM domain (Cao and Crocker, 2011). Moreover, in the transmembrane region of mouse CD33, a positively charged residue is present, which might enable mouse CD33 to recruit immunoreceptor tyrosine-based activation motif (ITAM)-motif–containing adapter proteins like DNAX activating protein of 12 kDa (DAP12) (Linnartz-Gerlach et al., 2014). These data suggest that mouse CD33 can act as an activating receptor instead of being inhibitory, a hypothesis that awaits experimental evidence. In cultured microglia from CD33KO mice, it was shown that CD33 negatively couples to phagocytosis (Griciuc et al., 2013). Whether this is due to ITIM-like signaling or recruitment of ITAM-motif–containing adapter proteins is currently an open question. Potential drugs that inhibit CD33 function, however, need to be tested in animal models. Whether or not the mouse would be an adequate model system to do so is questionable given the species differences and the limited knowledge on CD33 function in mouse cells.

Taken together, despite the genetic association between CD33 and AD development and the straightforward hypothesis of how reduced CD33 function might protect from AD, CD33 remains a target with its unique set of challenges.

Proposed Role of $K_{Ca}3.1$ in AD

$K_{Ca}3.1$ is an intermediate-conductance potassium channel that is activated when intracellular calcium is increased. The Ca^{2+} binds to calmodulin, which is constitutively bound to the calmodulin binding domain of the channel, resulting in the opening of the K^+ channel (K_d ~ 300 nM) (Xia et al., 1998; Fanger et al., 1999; Bouhy et al., 2011). The $K_{Ca}3.1$-mediated K^+ efflux augments the driving force

for further Ca^{2+} influx *via* Ca^{2+} release-activated calcium (CRAC) channels and subsequent refilling of intracellular calcium stores. Thus, one of the primary functions of $K_{Ca}3.1$ is the augmentation of Ca^{2+} signaling (Feske et al., 2012). Expression of $K_{Ca}3.1$ in the CNS and its controversies have been reviewed (Dale et al., 2016). Here we will focus on microglia, as the expression of $K_{Ca}3.1$ was established using well-validated antibodies and demonstration of voltage-independent $K_{Ca}3.1$-like Ca^{2+}-activated K^+ currents.

Voltage-independent $K_{Ca}3.1$-like Ca^{2+}-activated K^+ currents in cultured murine microglia were first reported by Eder et al. (Eder et al., 1997). Khanna et al. subsequently demonstrated the presence of $K_{Ca}3.1$ mRNA in cultured rat and mouse microglia (Khanna et al., 2001). The conclusion that microglia express $K_{Ca}3.1$ was supported by pharmacological studies that demonstrated that charybdotoxin and clotrimazole could inhibit the voltage-independent $K_{Ca}3.1$-like Ca^{2+}-activated K^+ currents (Schilling et al., 2002; Schilling and Eder, 2004). There is further evidence that charybdotoxin and clotrimazole prevent the induced microglial oxidative burst (Khanna et al., 2001) and microglial migration (Schilling and Eder, 2004). The $K_{Ca}3.1$ inhibitor TRAM-34 reduced lipopolysaccharide (LPS)-induced microglial neurotoxicity (Kaushal et al., 2007), and inhibition of $K_{Ca}3.1$ using senicapoc (see below) blocked the LPS-induced IL-1β and Nitric oxide (NO) production by microglia (Staal et al., 2017a).

Early *in vivo* studies using antibodies validated in $K_{Ca}3.1$ WT and knock out (KO) mice did not provide any evidence of $K_{Ca}3.1$ expression on any cells in the mouse or human brain (including juvenile, adult, and aged mice) (Schilling and Eder, 2004; Schilling and Eder, 2007). However, studies using antibodies validated in this manner demonstrated $K_{Ca}3.1$ expression on CD68-positive cells (microglia or macrophages) upon injury to the CNS as induced by ischemic injury (Chen et al., 2011), suggesting that $K_{Ca}3.1$ is expressed by microglia only in response to injury. The study by Chen et al. also demonstrated the therapeutic efficacy of inhibiting $K_{Ca}3.1$ (using TRAM-34) in ischemic injury, a finding they validated using $K_{Ca}3.1$-null mice (Chen et al., 2011; Chen et al., 2015). Many other studies have since demonstrated the therapeutic efficacy of $K_{Ca}3.1$ inhibition in other models of neurological injury and disease in which microglia are believed to play a significant role, including experimental autoimmune encephalomyelitis, traumatic brain injury, spinal cord injury, optic nerve transection, glioblastoma multiforme, neuropathic pain, and AD (Dale et al., 2016; Staal et al., 2017b).

Taken together, there is convincing evidence for the expression of $K_{Ca}3.1$ in cultured rodent microglia. While the details of regulation of $K_{Ca}3.1$ await confirmation with more specific tool compounds, the available data suggest a role for $K_{Ca}3.1$ at the very least in microglia reactive oxygen species (ROS) production and signaling.

Challenges in $K_{Ca}3.1$ Inhibition as Therapeutic Intervention in AD

The presence of "activated" microglia identified by expression of markers of microglial activation [major histocompatibility complex II (MHCII), Iba1] or an activated morphology (enlarged cell bodies and shorter, thicker processes) in brains of AD is well established. The observation that microglial "activation" can occur prior to plaque deposition and neurofibrillary tangle formation, and correlates with cognitive deficits, is consistent with the theory that microglial activation occurs at the earliest stages of disease (perhaps even before disease onset) and is somehow tied to its progression (Okello et al., 2009a; Okello et al., 2009b; Sastre et al., 2011). Maezawa et al. demonstrated that TRAM-34 blocks Aβ-induced proliferation of microglia, p38 Mitogen-activated protein kinase (MAPK) phosphorylation, nuclear factor kappa b (NF-κb) activation, and NO generation in primary microglia cultures (Maezawa et al., 2012). Furthermore, TRAM-34 inhibited neurotoxic effects of Aβ oligomers in mixed microglia–neuron cultures and in organotypic hippocampal slices by decreasing microglial activation and partially preventing synaptic loss (Maezawa et al., 2011).

Senicapoc is a potent and selective inhibitor of $K_{Ca}3.1$ developed for the treatment of sickle cell anemia [half maximal inhibitory concentration (IC_{50}) = 11 nM; screening of 57 targets yielded no hits below 1 μM other than $K_{Ca}3.1$] (Ataga et al., 2006; Ataga et al., 2008; Ataga and Stocker, 2009; Dale et al., 2016; Staal et al., 2017a). In a recent and comprehensive study, Maezawa and colleagues demonstrated that $K_{Ca}3.1$ is functionally upregulated in microglia in brains from 5xFAD mice as well as AD patients (Jin et al., In Press). The authors demonstrate that Aβ oligomers impair long-term potentiation (LTP) and that this effect is reversed by the $K_{Ca}3.1$ inhibitor TRAM-34 as well as senicapoc in hippocampal slices or *in vivo*. It was furthermore demonstrated in this paper that senicapoc can reduce the pro-inflammatory effects as well as rescue the hippocampal LTP affected by Aβ oligomer (Jin et al., In Press). Senicapoc was then tested in the 5xFAD mice, starting at 6 months of age and continuing for three months. Results show that treatment with senicapoc reduced the amyloid load as well as neuroinflammation while enhancing neuronal plasticity compared to vehicle-treated mice (Jin et al., In Press). Thus, the study provides a strong rationale for repurposing senicapoc for the treatment of AD.

In fact, the Alzheimer's Drug Discovery Foundation has provided funding to manufacture clinical-grade senicapoc and conduct the required stability testing in support of an investigational new drug (IND) filing. Upon approval of the IND filing, senicapoc will be tested in a phase II clinical trial in prodromal and mild AD (https://www.alzdiscovery.org/news-room/announcements/1.8-million-in-new-funding-supports-clinical-stage-treatments).

Senicapoc has been through extensive preclinical safety testing as well as phase I, II, and III clinical trials with few significant side effects noted. Given our knowledge of the pharmacokinetics of senicapoc, including its CNS penetrance (Staal et al., 2017b), it is likely that a dose similar to that used in the previous clinical trials will be used. AD patients, however, are very different from patients with sickle cell disease. It is likely that the AD patients will be older and have more comorbidities than those with sickle cell anemia. The age and comorbidities could reveal underlying susceptibilities to $K_{Ca}3.1$ inhibition that may not have been problematic in younger patients with sickle cell disease (e.g., susceptibility to infections). On the other hand, many diseases or conditions afflicting the elderly have inflammatory components, and a mild immunosuppressant such as senicapoc may benefit those conditions as well (e.g., stroke, chronic and neuropathic pain, atherosclerosis).

Yet, the current AD study is still incredibly important to test the hypothesis that targeting neuroinflammation, specifically $K_{Ca}3.1$, can slow or halt the progression of AD. If the trial results demonstrated significant and convincing slowing of disease progression, pharmaceutical companies could initiate drug discovery projects to develop improved CNS penetrant $K_{Ca}3.1$ inhibitors.

Targeting the Kynurenine Pathway to Treat AD

Development of drugs that target products of tryptophan metabolism has transformed the treatment of psychiatric disorders, particularly with the advent of serotonin (5-HT) reuptake inhibitors (Wong et al., 1995). However, it is noteworthy that metabolism to 5-HT represents only a small fraction of the fate of free tryptophan, and in fact, the great majority is converted to KYN (Leklem, 1971) and its subsequent products by an array of enzymes located throughout the body and brain. Yet, surprisingly, there are no marketed medications that directly target metabolism of the KYN pathway.

Entry into the KYN pathway begins with metabolism of tryptophan into N-formylkynurenine by indole-2,3-dioxygenase (IDO) or tryptophan-2,3-dioxygenase (TDO), which is then rapidly converted to L-kynurenine (Takikawa et al., 1986; Zhang et al., 2007). Metabolism of KYN produces a host of neuro-active products in the brain that may contribute to CNS diseases (Török et al., 2016; Lovelace et al., 2017; Schwarcz and Stone, 2017). The KYN pathway bifurcates into neurotoxic and neuroprotective metabolic routes. As an example, astrocytes largely express kynurenine aminotransferases (KATs) and so are the primary source of the neuroprotective metabolite, kynurenic acid (KYNA), in the brain (Guidetti et al., 2007). Alternatively, microglia express kynurenine 3-monooxygenase (KMO), which converts KYN to neurotoxic metabolites such as 3-hyroxykynurenine (3-HK) and subsequently quinolinic acid (Quin) (Heyes et al., 1996; Guillemin et al., 2003a) (**Figure 3**). While the topic of this discussion is targeting the disruption in microglial KYN metabolism, one should understand that the various branches of KYN metabolism do not act in isolation, and so a change in one may cause a change in the others by altering the availability of substrate.

Inflammation triggers activation of microglia and dysregulation of KYN metabolism, leading to production of neurotoxic metabolites from the KMO branch, a process that is predicted to be involved in the development of AD pathology (Campbell et al., 2014; Zádori et al., 2018). However, direct evidence of microglial KYN metabolism from human AD subjects suggests that this effect may be limited to localized pathological events rather than global changes in CNS KYN metabolism. As an example, levels of KYN and 3-HK were both surprisingly reduced in CSF of AD patients with dementia (Tohgi et al., 1992). However, tryptophan, 5-HT, and melatonin were all also significantly reduced in the same study. In addition, the same group reported that KYN was reduced in a second cohort of patients with AD dementia but not in ones with vascular dementia (Tohgi et al., 1995). Furthermore, Quin (Mourdian et al., 1989; Heyes et al., 1992) and KYNA (Wennström et al., 2014) were reportedly unchanged in recent studies, though earlier evidence indicated that KYNA could also be decreased (Heyes et al., 1992; Hartai et al., 2007; Gulaj et al., 2010) in AD patients. Overall, there is limited evidence that CSF tryptophan/KYN metabolites are reproducibly altered in AD, though where it has been assessed, most studies point to either no consistent changes in metabolites or a reduction in KYN metabolism (**Table 1**).

In post-mortem AD brain tissue, early studies reported a lack of change in KYN metabolism. Indeed, Quin levels were shown to be unchanged (Moroni et al., 1986; Mourdian et al., 1989; Sofic et al., 1989; Pearson and Reynolds, 1992), as were KYNA concentrations (Flint Beal et al., 1992). However, more recent analysis revealed

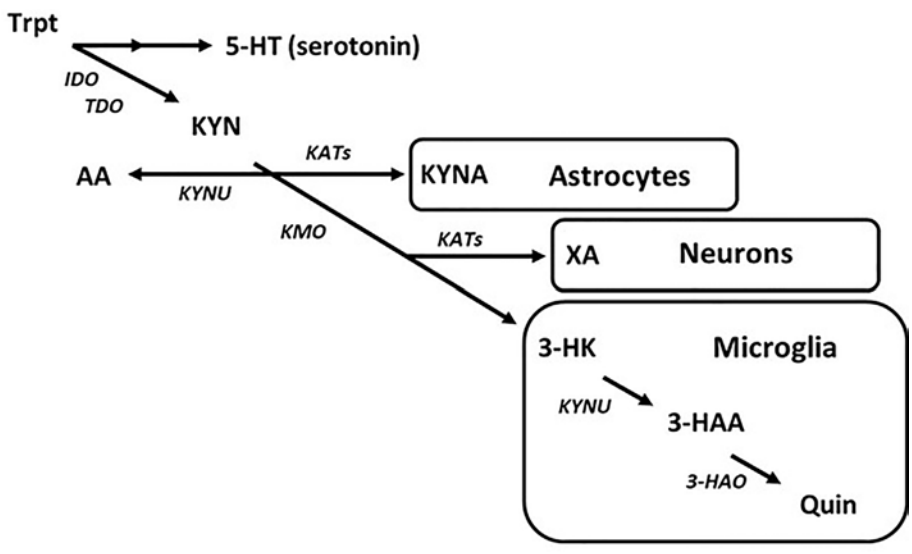

FIGURE 3 | The kynurenine pathway of tryptophan metabolism in the brain. Simplified diagram depicting the tryptophan–kynurenine pathway. Metabolism of tryptophan along the kynurenine pathway in the brain is regulated by a variety of enzymes that are largely segregated by cell type.

TABLE 1 | Kynurenine metabolite profile from patients with Alzheimer's Disease.

Biosample	Metabolite	Effect	Reference
Serum	Trpt	Decreased	Widner et al., 1999;
		No Change	Widner et al., 2000 Schwarz et al., 2013; Oxenkrug et al., 2017
	KYN	Increased	Widner et al., 1999;
		No Change	Widner et al., 2000; Atukeren et al., 2018 Oxenkrug et al., 2017
	AA	Decreased	Oxenkrug et al., 2017
	XA	ND	
	KYNA	No Change	Schwarz et al., 2013; Oxenkrug et al., 2017
	3-HK	Increased	Schwarz et al., 2013; Oxenkrug et al., 2017
	Quin	No Change	Schwarz et al., 2013
Plasma	Trpt	Decreased	Greilberger et al., 2010; Giil et al., 2017
	KYN	No Change	Greilberger et al., 2010
	AA	Increased	Chouraki et al., 2017
	XA	Decreased	Giil et al., 2017
	KYNA	Decreased	Heyes et al., 1992; Hartai et al., 2007; Gulaj et al., 2010
	3-HK	Increased	Gulaj et al., 2010
	Quin	Increased	Gulaj et al., 2010
		Decreased	Giil et al., 2017
CSF	Trpt	Decreased	Tohgi et al., 1992; Muguruma et al., 2018
	KYN	Decreased	Tohgi et al., 1992; Tohgi et al., 1995; Muguruma et al., 2018
	AA	Decreased	Muguruma et al., 2018
	XA	ND	
	KYNA	No Change	Wennström et al., 2014
		Decreased	Heyes et al., 1992; Hartai et al., 2007; Gulaj et al., 2010
	3-HK	Decreased	Tohgi et al., 1992; Muguruma et al., 2018
	Quin	No Change	Mourdian et al., 1989; Heyes et al., 1992
Brain (post mortem)	KYN	No Change (regional)	Baran et al., 1999
	AA	ND	
	XA	ND	
	KYNA	No Change	Beal et al., 1992
		Increase (caudate/ putamen)	Baran et al., 1999
	3-HK	No Change (regional)	Baran et al., 1999
	Quin	No Change (regional)	Moroni et al., 1986; Mourdian et al., 1989;
		Increased (plaques/tangles)	Sofic et al., 1989; Pearson and Reynolds, 1992; Guillemin et al., 2005; Wu et al., 2013

regional changes that indicated greater production of KYN as well as shunting of KYN metabolism toward production of neurotoxic microglial metabolites. Immunohistochemical analysis showed that both TDO and IDO were elevated in the hippocampus of AD patients (Guillemin et al., 2005; Wu et al., 2013). Importantly, the increase in IDO expression was found in microglia, astrocytes, and neurons, with the primary product found in proximity to senile plaques and neurofibrillary tangles in post-mortem AD brain being Quin, the microglial product (Guillemin et al., 2005; Bonda et al., 2010). These data are in line with reports that treatment of microglia with $A\beta_{1-42}$ induces IDO expression (Guillemin et al., 2003a; Guillemin et al., 2003b) and that Quin may seed α-synuclein aggregation (Tavassoly et al., 2018) and induce tau hyperphosphorylation (Rahman et al., 2009). Given the well-documented evidence that tryptophan and KYN metabolism is sensitive to inflammatory stimuli (Campbell et al., 2014; Strasser et al., 2017), it is plausible to predict that neuroinflammation centered around plaques and tangles in the brains of AD patients produces localized pro-inflammatory activation of microglia, resulting in dysregulation of KYN metabolism, which favors production of neurotoxic metabolites such as 3-HK and Quin. A deleterious impact resulting in local brain circuit disruption and longer-term neurodegeneration is hypothesized, but further analysis in longitudinal studies is needed to confirm or disprove this.

Challenges of Developing Kynurenine Pathway Drugs for AD

The main challenge for developing KYN metabolite inhibitors for AD is a clear understanding of the role of KYN metabolic products in the development and/or progression of AD, as well as the availability of agents to test the efficacy of CNS inhibition of neurotoxic KYN enzymes, specifically KMO. Peripheral measures of KYN metabolism in serum and plasma support the hypothesis that the KYN pathway is induced in AD, leading to increased production of KYN and products associated with the microglial metabolic branch. However, it is unclear whether this peripheral pattern of metabolism reflects CNS KYN metabolism or contributes to neural pathology. Indeed, distribution of metabolites in the CNS (brain, CSF) suggest a more nuanced pattern of regulation that also supports a shunting of tryptophan metabolism toward neurotoxic KYN metabolites, but only in proximity to plaques and tangles, indicating a need for brain-penetrable KMO inhibitors to act locally at the site of pathology. As discussed above, there is also a lack of longitudinal data on KYN metabolism in AD patients and an analysis of how these data correlate with disease progression or severity state. The limited correlation between blood and CSF metabolite profiles, as well as with post-mortem brain patterns, highlights the need for methodologies that correlate disease progression with direct measurements of brain KYN metabolites and/or enzymes in living AD patients. A concerted effort to develop positron-emission tomography (PET) ligands and/or other imaging agents targeting the KYN pathway would be a great benefit toward understanding the role of this pathway in AD and improving the rationale for the substantial investment required to develop brain-penetrant drugs.

While KMO has been targeted by the pharmaceutical industry for at least 3 decades, there are no published agents with good CNS drug exposure. Importantly, targeting KMO in the periphery creates a substantial increase in substrate, KYN, which is actively transported into the brain, where it is converted to substances such as KYNA, which could be neuroprotective, but also into 3-HK and Quin (Beaumont et al., 2016), which may increase

oxidative stress and neuronal damage. This could be especially problematic in neurodegenerative disorders such as AD where neuroinflammation associated with plaques and tangles is predicted to increase KMO expression, thus more readily converting KYN to 3-HK and Quin (Guillemin et al., 2003a; Guillemin et al., 2005; Bonda et al., 2010). Therefore, it will be necessary to focus drug discovery efforts on identifying KMO inhibitor chemotypes with a high degree of CNS penetrance to test their therapeutic benefit in AD. Indeed, it may even be advantageous to attempt developing non-competitive KMO inhibitors along the lines of recent advances in KAT II inhibitors (Dounay et al., 2012) since they would not compete with increased KYN pumped into the brain from the periphery that could otherwise limit their efficacy. Finally, while the paucity of brain-penetrant drugs targeting the KMO branch of the KYN pathway is discouraging, advances have been made in developing agents that stimulate the neuroprotective KAT branch, such as 4-Chlorokynurenine (4CL-KYN; AV-101). Peripheral administration of 4Cl-KYN is transported into the brain, where it is converted to 7-chloro-KYNA, thereby acting as a locally synthesized KYNA mimetic (Hokari et al., 1996), where it appears to have promising neuroprotective effects (Wu et al., 2000; Wu et al., 2002). These data are encouraging and support the hypothesis that development of centrally acting KMO inhibitors could be neuroprotective in AD since blocking microglial KYN metabolism would not only prevent production of neurotoxic metabolites such as 3-HK and Quin but also indirectly increase endogenously produced KYNA. Thus, a greater understanding of the impact of rebalancing the neurotoxic and neuroprotective branches of the KYN pathway in AD may provide additional mechanistic evidence to support development of a new generation of neuroprotective brain-penetrant KMO inhibitors.

The P2X7–NLRP3 Axis in Alzheimer's Disease

The P2X7–NLRP3 (nucleotide-binding oligomerization domain, leucine-rich repeat, and pyrin domain–containing inflammasome complex 3) pathway has received extensive attention, with several publications pointing to its potential role in neuroinflammatory disorders, from psychiatry to neurodegenerative diseases. Microglia express several cell-surface and cytoplasmic sensors of danger signals that react to invading "dangers" and cause neuroinflammation. The ATP-gated ion channel P2X7 is expressed abundantly in microglia and responds to extracellular ATP, which often occurs during tissue distress or damage and can lead to activation of the NLRP3 inflammasome complex, producing pro-inflammatory cytokines, IL-1β and IL-18 (Bhattacharya and Biber, 2016). As such, the P2X7–NLRP3 pathway has been the focus of intense drug discovery efforts: while brain-penetrant P2X7 antagonists have made progress and are in the clinic (**Table 2**), brain-penetrant, drug-like NLRP3 inhibitors are the focus of medicinal chemistry discovery programs.

P2X7 belongs to the P2X family of trimeric ligand–gated cation channels. Its activation by ATP allows for the influx of several cations, including Ca^{2+}, Na^{+}, and K^{+} (Bartlett et al., 2014). Even though P2X7 expression is abundant in microglia (and in peripheral immune cells), it is "silent" under normal physiology, where ATP concentrations do not reach the high micromolar levels required to activate its ion channel (Bhattacharya and Biber, 2016). As such, P2X7 is an attractive drug target as antagonism of a silent channel by true neutral antagonists would not cause any serious target mediated (adverse) effects: antagonism will only be evident when the channel is activated by high ATP concentrations, which is believed to occur during neuroinflammatory disorders of the CNS. NLRP3, on the other hand, is a convergent point of inflammasome activation, both dependent and independent of P2X7 activation (Bhattacharya and Jones, 2018), and as such is an attractive drug target. The advantages and disadvantages of targeting P2X7 vs. NLRP3 in neuroinflammatory disorders remain to be determined.

The P2X7–NLRP3 pathway is a case in point where microglia-driven neuroinflammation may play a role in AD causality (Heneka et al., 2013), and there has been growing interest in probing the role of pathogenic Aβ in neuroinflammation, although the exact mechanism of its involvement is still unknown (Illes et al., 2019; Martin et al., 2019; Thawkar and Kaur, 2019). P2X7 activation and consequent release of IL-1β is one such mechanism of interest in the AD field in the quest to understand the role of neuroinflammation in AD (Sanz et al., 2009). P2X7 upregulation has been reported in post-mortem AD brains and in animal models of tauopathy and amyloid deposition, mostly around the areas of pathology (Parvathenani et al., 2003; McLarnon et al., 2006; Lopez-Gonzalez et al., 2015). For example, P2X7 upregulation was seen in cortical samples from AD patients compared to healthy volunteers (Martin et al., 2019). With the recent discoveries of clinically available P2X7 PET ligands (Bhattacharya, 2018), the detection of P2X7 upregulation in humans becomes possible. In a mouse model of AD/frontotemporal lobar degeneration (FTLD) (P301S), enhanced uptake of P2X7 PET signal was detected in brain regions of tauopathy (Jin et al., 2018). P2X7 activation has also been linked to neuronal damage and synaptotoxicity in transgenic AD models (Lee et al., 2011). There are signals of an active P2X7–NLRP3 axis in animal models of AD. For example, in the J20 mouse model of AD, which overexpresses the human APP gene with two familial AD-linked mutations, the authors noted that P2X7 activation prevents APP processing by α-secretase, facilitating the formation of toxic Aβ *via* P2X7-mediated activation of glycogen synthase kinase 3 (GSK-3) (Diaz-Hernandez et al., 2012). Supporting these data, inhibition of P2X7 with Brilliant Blue G (BBG) in the same mouse model increased α-secretase activity and reduced the formation of Aβ plaques, supporting a beneficial role of P2X7 antagonism in AD (Chen et al., 2014). Recently, it was reported that mice lacking P2X7 have reduced Aβ load and cognitive impairment in another AD model overexpressing both human mutated APP and mutated presenilin-1 (APP–PSEN1) (Martin et al., 2018). In addition to P2X7, the downstream intracellular NLRP3 inflammasome has been receiving special attention through several publications, and there is a growing interest to bring forward NLRP3 inhibitors for clinical testing in AD (Heneka, 2017; Heneka et al., 2018). For example, MCC950 (NLRP3 inhibitor) has been reported to prevent cognitive decline in APP/PS1 mice (Dempsey et al., 2017). A comprehensive study by Venegas et al. demonstrated mechanistically that ASC [adapter

protein apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (CARD)] specks (involved in NLRP3 activation) contributed to the spreading of Aβ plaques in APP/PSEN1 double-mutant mice (Venegas et al., 2017). The study by Martin et al. points to the unique role of P2X7, outside the NLRP3–IL-1β signaling cascade. While support for the NLRP3 pathway being involved in disease is gaining momentum, there are subtleties of targeting P2X7 vs. NLRP3 inflammasome with therapeutics. P2X7 antagonism may bring additional benefits as highlighted in the study by Martin et al. (2018). While NLRP3 is downstream of P2X7 and may be a critical mediator of IL-1β release through the inflammasome, P2X7 antagonism may provide additional benefits such as modulation of synaptic plasticity, modulation of chemokine release (e.g., CCL3), and consequent effects on $CD8^+$ T cell recruitment to focal regions of the diseased brain.

Challenges Around P2X7/NLRP3

While the scientific community continues to generate data supporting the role of P2X7–NLRP3 in models of AD, there must be a healthy balance of caution and opportunistic investments in clinical trials to test these new mechanisms. The biggest challenge of NLRP3 for AD is a lack thus far of a suitable clinical compound with acceptable brain penetration. For P2X7, several brain-penetrant antagonists have been disclosed, some of which are in clinical development (**Table 2**). With the availability of brain-penetrant P2X7 antagonists and P2X7 PET ligands, these clinical tools could be tested in a patient population, perhaps with the help of PET and associated biomarkers (CSF, blood, genomics) that would enrich AD patients with an active P2X7–NLRP3 axis. In addition to using P2X7 PET ligands as imaging tools to enrich patient clusters for proof-of-concept testing, one must also demonstrate target engagement at clinically achievable doses. Target engagement in patients receiving a clinical candidate for proof-of-concept testing is critical to the evaluation of primary end points. Emerging data supporting the role of microglia-driven processes (neuroinflammation, phagocytosis), and description of different phenotypes of microglia in normal and disease states has prompted renewed thinking regarding their role in AD. To that end, it is of some comfort to witness that the role of microglia and microglia-driven neuroinflammation in AD has been the subject of some high-profile publications in the last few years, including significant progress with clinical compounds. While brain-penetrant P2X7 compounds are clinically available (**Table 2**), NLRP3 inhibitors that adequately demonstrate central target engagement (with NLRP3 PET ligands) are currently lacking. Demonstration of central target engagement is fundamental to drug development; once a dose-occupancy is established, moving to AD patients is the next step, with the challenge of selecting patients who can be intervened in early on in the disease process.

TABLE 2 | Clinical strategies used to dampen IL-1β signaling.

P2X7 Antagonists	Indication(s)	Comments
CE-224,535	Rheumatoid arthritis	Failed efficacy study (Ph-II)
(Pfizer)	Not in development	Not brain penetrant
		Blocked ex-vivo human IL-1β
AZD-9056	Rheumatoid arthritis	+ve signal in Chron's
(AstraZeneca)	Crohn's	+ve & -ve signals in RA
	Not in development	Blocked ex vivo human IL-1β
		Not brain penetrant
GSK-1482160	Pain (intended)	Phase-I safety study
(GlaxoSmithKline)	Not in development	Blocked ex-vivo human IL-1β
		Brain penetrant
SGM-1019	NASH (intended)	Phase-I safety study
(Second Genome)	In active development	No information on compound
JNJ-54175446	CNS	Phase-I safety study
(Janssen)	In active development	Blocked ex-vivo human IL-1β
		Brain penetrant
JNJ-55308942	CNS	Phase-I safety study
(Janssen)	In active development	Brain penetrant
NLRP3 Inhibitors	**Indication(s)**	**Comments**
OLT-1177	Inflammatory disorders	Phase-II (CAPS)
(Olatec)	In active development	No ex-vivo human IL-1β data
		In-vitro blockade of IL-1β
		No brain penetration data
Caspase-1 inhibitors	**Indications**	**Comments**
VX-765	Epilepsy	Brain penetrant
(Vertex)	Psoriasis	
	Not in development	
Vx-740	Rheumatoid arthritis	Pro-drug
(Vertex)	Psoriasis	
	Not in development	
IL-1β biologics	**Indications**	**Comments**
Anakinra	Rheumatoid arthritis	IL-1r antibody
		Marketed product (injection)
Rilonacept	Inflammatory disorders	IL-1β and IL-1α antibody
	CAPS, FCAS MWS	Marketed product (injection)
Canakinumab	Atherosclerosis	IL-1β antibody
(Novartis)	Lung cancer	Phase-III

Proposed Role of PD-1 Immune Checkpoint Blockade in AD

PD-1 (or CD279) is a member of the CD28 family of T cell regulators (Riley and June, 2005). It is weakly expressed on naive T cells but can be induced upon activation in several types of immune cells, including B cells, T cells, natural killer cells, dendritic cells, and monocytes. PD-1 plays an important role in immune responses related to B cell activity and/or T cell activity, such as antibody production, immune tolerance, and autoimmunity (Okazaki et al., 2013). PD-1 function is important to preserve immune tolerance. Taken together, PD-1 signaling ameliorates immune overreaction.

Recent studies have indicated that boosting the immune response in the brain might have therapeutic potential. It was shown that inhibition of forkhead box protein P3 (FOXP3)-positive regulatory T cells increased interferon-γ (IFNγ)–dependent leukocyte trafficking to the brain, promoting clearance of Aβ and improving cognitive function in a mouse model of amyloidosis (Baruch et al., 2015). As PD-1 checkpoint blockade was previously reported to stimulate IFNγ-dependent immune responses in cancer immunotherapy, the therapeutic potential of PD-1 blockade in AD models was explored (Baruch et al., 2016).

In their studies, the authors treated the 5XFAD mouse model of amyloidosis at 10 months of age (an age with advanced pathology) with two intraperitoneal injections of a PD-1–specific antibody with 3-day intervals. One week after the first treatment, the mice were reported to exhibit a systemic IFNγ immune response. Further analysis of the myeloid cell populations in the brain upon treatment documented IFNγ-dependent recruitment of monocyte-derived macrophages as characterized by high expression of lymphocyte antigen 6c and expression of the chemokine receptor CCR2. Intriguingly, treatment also resulted in reduced cognitive deficits. In detail, spatial learning and memory in the radial-arm water-maze task was improved 1 month after treatment. Furthermore, 2 months after initial treatment, 5XFAD mice that had received 2 rounds of the anti–PD-1 treatment at a 1-month interval performed indistinguishably from non-transgenic mice. In comparison, mice with a single round of PD-1 blockade that were examined 2 months later were not different from placebo control and hardly showed improved memory. These results indicated that repeated treatment was needed to maintain improved cognition. Treatment with the PD-1–specific antibodies also caused a reduction in plaque load in the hippocampus and in the cortex. This treatment effect was more pronounced after two rounds of anti–PD-1 treatment. In addition, astrogliosis was reduced in the hippocampus of 5XFAD mice with either one or two rounds of anti–PD-1 treatment. The beneficial effects described in 5xFAD mice after PD-1 blockade were largely confirmed in a second mouse model of amyloidosis (APP/PS1APPswe, PSEN1dE9), in which treatment reduced plaque deposition in hippocampus.

Given the fact that various antibodies or small molecules targeting PD-1/PD-1L are already marketed or under development (Kobold et al., 2018; Shaabani et al., 2018), the findings by Baruch et al. triggered considerable interest in immune checkpoint blockade as a novel therapeutic strategy for AD. Accordingly, this study stimulated evaluation of the PD-1 immune checkpoint blockade for AD by three independent pharmaceutical companies.

Challenges of PD-1 Immune Checkpoint Blockade as Therapeutic Intervention in AD

In subsequent studies jointly published in *Glia* (Latta-Mahieu et al., 2018) by Sanofi, Janssen, and Eli Lilly, the consequences of PD-1 immunotherapy in a range of different APP transgenic models (ThyAPP/PS1M146L, ThyAPP/PS1A246E, PD-APPAPPV717F) with the same anti–PD-1 antibody were documented. In addition to the antibody used by Baruch et al., two mouse chimeric variants and corresponding Immunglobulin G (IgG) controls were tested. In accordance with Baruch et al. (2016), PD-1 immunotherapy caused a systemic IFNγ-dependent immune response (Latta-Mahieu et al., 2018). However, in contrast to Baruch et al. (2016) there was no effect of anti–PD-1 treatment either on amyloid pathology or on monocyte-derived macrophage infiltration into the brain. This result point is consistent with data from another laboratory that explored the consequences of PD-1 deficiency in a murine prion disease model of neurodegeneration (ME7). In this study, there was no detectable infiltration of peripheral myeloid cells into the brain (Obst et al., 2018).

Latta-Mahieu and coworkers used, in addition to the rat mouse antibody (mAb) used by Baruch et al., two mouse chimeric variants with mouse IgG1 and IgG2a Fc domains as additional controls to evaluate whether observed effects were directly related to PD-1 target engagement. The *in vivo* studies were designed to ensure high statistical power (n = 9/group at Sanofi, n = 20/group at Janssen, and n = 25/group at Eli Lilly) and carried out in a blinded fashion. Furthermore, additional cohorts of animals for baseline measurement of pathology at the start of the anti–PD-1 treatment have been included. This way, age-dependent increases in amyloid pathology in each model of amyloidosis could be compared to anti–PD-1 treatment effects at the end of the study (Latta-Mahieu et al., 2018).

The reason for the different findings between Baruch et al. and Latta-Mathieu et al. is unclear, and more research is needed to understand the potential of PD-1 as a target for AD. It should be noted that in the study by Baruch et al., a xenogeneic rat antibody was used that potentially made the interpretation of the results difficult. Thus, it remained uncertain whether PD-1 target engagement or stimulation of the immune system, independent of PD-1, was causing the reduction in pathology and the functional improvements.

The Proposed Role of Toll-Like Receptors in AD

The innate immune system is the first line of defense against pathogens and tissue damage and elicits a rapid and robust response. Within the CNS, the innate immune system performs a similar role that is mediated by microglial and astroglial cells (Ransohoff and Brown, 2012). In the case of CNS infection or injury, microglial cells detect pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs) *via* specialized cell-surface pattern recognition receptors (PRRs). PRRs transmit information about the CNS microenvironment to the glial cells, which then orchestrate an appropriate immune response (Kigerl et al., 2014). Glia direct the neuroimmune response by migrating toward lesions or pathology, producing pro- and anti-inflammatory cytokines and chemokines, releasing growth factors and up regulating phagocytic mechanisms (Hansen et al., 2018). After a period, there is a resolution signal, which returns glia to their homeostatic state (Chavan et al., 2017). However, in AD, where Aβ, hyperphosphorylated tau, and neuronal damage persist for decades, there is no resolution, resulting in chronic activation of the innate immune system. This persistent alteration of glial cell phenotype is believed to contribute to AD progression and severity, and therefore, the identification of drugable molecular targets within the innate immune system of the CNS is of paramount interest.

TLRs are a major class of PRRs characterized by an extracellular leucine-rich repeat domain and an intracellular TIR domain (Kielian, 2006). Ten mammalian TLRs have been identified, each with a particular ligand specificity. Complicating the picture, TLRs can hetero-dimerize with additional co-receptors, resulting in an expansion of their ligand repertoire (Kielian, 2006). TLRs are expressed in microglia (Laflamme and Rivest, 2001; Laflamme

et al., 2001; Bsibsi et al., 2002; Dalpke et al., 2002; Olson and Miller, 2004; Zhang et al., 2012), and several have been shown to have increased expression both in the brains of AD transgenic animals and in human AD patients (Liu et al., 2005; Walter et al., 2007; Frank et al., 2009; Letiembre et al., 2009; Wirths et al., 2010; Rangasamy et al., 2018). The primary consensus among researchers is that persistent TLR signaling in AD models is detrimental; however, conflicting evidence also suggests beneficial roles of TLR signaling. The most extensively studied TLRs, in the context of AD, are TLR2 and TLR4 along with their co-receptor, CD14.

TLR2 and its co-receptor CD14 are upregulated in AD transgenic mouse models and in human AD brain sections (Letiembre et al., 2009). Functional characterization of TLR2 in primary mouse microglia and the mouse BV-2 microglia cell line demonstrated that fibrillar preparations of Aβ peptide can increase expression of NO synthase, pro-inflammatory cytokines, and integrin markers in a TLR2-dependent manner (Jana et al., 2008). Consistent with this, primary microglia and bone marrow–derived macrophages from TLR2 knockout mice show a reduced pro-inflammatory reaction and an enhanced phagocytic response when stimulated with aggregated $A\beta_{1-42}$ peptide (Liu et al., 2012). Anti-TLR2 antibodies, administered over seven months, improved spatial learning, reduced gliosis [as assessed by immunoreactivity of CD68 and glial fibrillary acidic protein (GFAP)], and decreased Aβ plaque burden (McDonald et al., 2016). Anti-TLR2 antibodies can prevent a metabolic switch in microglia toward glycolysis, which results in an increase in phagocytosis (Rubio-Araiz et al., 2018). A peptide corresponding to the TLR2-interacting domain of myeloid differentiation primary response 88 (MyD88) (TIDM) disrupted the interaction of TLR2 with MyD88, resulting in a blockade of TLR2 signaling (Rangasamy et al., 2018). When administered intranasally to 5xFAD transgenic mice, the peptide could be detected in the hippocampus, where it reduced glial activation and Aβ plaque burden (Rangasamy et al., 2018). In contrast, several studies have postulated that TLR2 can promote beneficial responses in AD models. TLR2 deficiency accelerated spatial and contextual memory deficits while increasing levels of $A\beta_{1-42}$ in the brains of PS1/APP transgenic mice (Richard et al., 2008). In addition, the TLR2 agonist peptidoglycan (PGN) enhanced uptake of $A\beta_{1-42}$ in primary mouse microglia (Chen et al., 2006).

Like TLR2, TLR4 is expressed in microglial cells and can mediate binding and internalization of $A\beta_{1-42}$ *in vitro* (Tahara et al., 2006). TLR4 was originally identified as an endotoxin receptor, mediating immune response to stimuli such as LPSs (Janssens and Beyaert, 2003). Studies using LPS as a tool to activate TLR4 signaling in AD transgenic mice have produced conflicting results. Intra-hippocampal administration of LPS to Tg2576 or PS1/APP mice activated microglia (as assessed by CD45 and CR3 or MHCII immunoreactivity) and reduced diffuse Aβ staining but not compact plaque (DiCarlo et al., 2001; Herber et al., 2007). Multiple doses of peripheral LPS improved cognitive function and reduced tau pathology in the P301S model (Qin et al., 2016). In contrast, repeated peripheral administration of LPS to WT mice was shown to increase Aβ levels, activate microglia, and induce cognitive impairment (Lee et al., 2008; Lee et al., 2012). Studies with in APPswe PS1dE9 and PS1/APP TLR4 knockout mice confirm an increase in plaque pathology (Tahara et al., 2006; Song et al., 2011), resulting in reduced glial activation and improved cognitive function (Song et al., 2011). Therapeutic blockade of TLR4 signaling using the cyanobacterial product CyP (Macagno et al., 2006) injected intracerebroventricularly (ICV) was able to reduce memory impairment and glial cell activation induced by ICV administration of Aβ oligomers (Balducci et al., 2017).

Challenges Targeting TLRs

Over the last two decades, considerable attention has been paid to understanding the role of TLRs in innate immunity, but their role in AD initiation and progression remains elusive. As highlighted above, the transgenic models used, the timing of the intervention, and the end points measured can impact the conclusions made. AD is a complex disease, and the effects of modulating TLRs may differ depending on the stage of the disease during intervention. For instance, memory impairment was reduced after ICV injection of Aβ oligomers by pre-treatment with TLR2 and TLR4 agonists (Pourbadie et al., 2018). In contrast co-administration of TLR4 antagonist produced similar results (Balducci et al., 2017). Additional complexities in targeting TLRs arise from their ability to downregulate the immune response following repeated exposures to TLR agonists like LPS. This "endotoxin tolerance" has been described in animals and a variety of human diseases (Lopez-Collazo and del Fresno, 2013): this might confound interpretation of experimental data using TLR agonists. This is highlighted by recent work that demonstrated that the microglia response to LPS increases in aged WT animals compared to young WT mice. However, in the PS1/APP model, the microglia response to LPS is decreased in aged animals compared to WT (Go et al., 2016). This altered TLR signaling (i.e., tolerance) could affect the efficacy of TLR agonists or antagonists especially where chronic administration of the drug will be needed, such as in AD.

TLRs are expressed on a wide variety of cells, including peripheral immune cells and non-immune cells within the CNS such a neurons (Kielian, 2009). With traditional small molecule approaches, selectively modulating CNS and not peripheral TLRs is not feasible. Therefore, a suitable therapeutic window will need to be established for any TLR therapeutic developed. This will require a delicate balance between achieving the brain exposures needed to drive a therapeutically beneficial effect and minimizing peripheral exposures to reduce the risk for potential toxicities. The typically lower brain exposure compared to the periphery that is observed for many drugs adds to this challenge.

Several small molecule TLR agonists and antagonists have been described (Zhu et al., 2018). Discovery of TLR8 inhibitors was greatly facilitated by structure-based design (Hu et al., 2018). Similar approaches may be useful in designing small molecules for additional TLRs. Rather than drugging TLRs directly, others have sought to interfere with key protein–protein interactions required for TLR signaling. The TIDM peptide serves as a MyD88 decoy, essentially capping TLR2,

preventing assembly of the myddosome and subsequent TLR2 signaling (Rangasamy et al., 2018). TAK-242 is a small molecule that interacts with cysteine-747 in TLR4, preventing interaction with TIR domain–containing adaptor protein (TIRAP) or TRAM resulting in blockade of TLR4 signaling (Matsunaga et al., 2011). Degrader approaches may also be useful to selectively shut down TLR signaling, but the use of such technology has not been demonstrated on TLRs. The therapeutic potential of TLRs in AD remains compelling, and the recent development of selective TLR ligands will support investigations of the role of TLRs in AD while helping to frame a robust therapeutic hypothesis.

Proposed Role of TREM2 in AD

TREM2 is an immunoreceptor of partially understood function, expressed in myeloid cells in the periphery including dendritic cells, tissue macrophages and osteoclasts, and brain microglia (Colonna and Wang, 2016). TREM2 is a prominent AD risk gene (Pimenova et al., 2018) and is remarkable in the context of AD for at least two reasons: 1) its selective brain expression in microglia clearly links microglia specifically to neurodegenerative disease, and 2) there is the possibility of pharmacologic modulation of TREM2 to treat neurodegenerative disease.

In 2013, two landmark papers (Guerreiro et al., 2013; Jonsson et al., 2013) identified, by exome sequencing, rare mutants in TREM2 associated with an AD risk similar to that of a single allele of the strongest known risk factor, the E4 isoform of Apolipoprotein E (ApoE). Specifically, they showed that the mutation R47H, present in about 0.2% of the human population, elevated the AD odds ratio to at least 3.0. Several subsequent studies found slightly lower odds ratios but have confirmed the TREM2 R47H observations and identified additional TREM2 risk alleles and variants of unknown consequence (Bellenguez et al., 2017; Sims et al., 2017) that, in total, are present in about 1.5% of the human population (http://exac.broadinstitute.org/). Many other genes of modest AD risk have been identified before and since (Pimenova et al., 2018). But TREM2 is the strongest risk factor expressed in the brain only in microglia, unambiguously linking innate immune mechanisms in general, and microglial biology in particular, to AD. This observation has focused a great deal of attention on the role of microglia in AD and encouraged exploration of novel microglia-specific approaches to AD therapy (Hansen et al., 2018; Song and Colonna, 2018).

Functions of TREM2 in health and disease are summarized in several excellent reviews (for example, Hansen et al., 2018). AD-associated TREM2 mutants confer at least partially reduced ligand affinity, signaling, phagocytosis, and microgliosis (Ulrich et al., 2017). Significant evidence suggests that TREM2 is a direct phagocytic receptor (Takahashi et al., 2005; N'Diaye et al., 2009), which implies that failure to phagocytose aggregated proteins is a key driver of AD and related diseases. Note, however, that TREM2 is but one of many phagocytic receptors in microglia (Sierra et al., 2013), and it is unclear why the contribution of TREM2 in particular to phagocytosis would so strongly affect AD risk. Perhaps TREM2 has a prominent role in the phagocytosis of substrates specific to AD, e.g., Aβ or tau, or perhaps compromised TREM2 function has other pathological consequences (**Figure 4**). TREM2 also seems necessary for microglial plaque association in mouse models and in human disease (Wang et al., 2016; Yuan et al., 2016). Functional studies have suggested that TREM2 is a lipid receptor, and that the binding of some but not all lipids is compromised by AD-associated mutations (Wang et al., 2015). Evidence for other ligands including ApoE (Atagi et al., 2015; Bailey et al., 2015; Yeh et al., 2016) and Aβ peptide (Lessard et al., 2018; Zhao et al., 2018; Zhong et al., 2018) has been presented. These results should be interpreted cautiously. Aβ peptide is markedly hydrophobic, making specific interactions difficult to interpret. Also, depending on the presence and nature of bound lipids, ApoE is likely to have different interaction properties. The key ligand(s) for TREM2 in AD may not yet be known, or TREM2 may simply be a promiscuous receptor (Kober and Brett, 2017). The phenotype of mouse ApoE knockouts mimics TREM2 knockouts with respect to microglial plaque association (Shi and Holtzman, 2018; Ulrich et al., 2018), supporting ApoE as a TREM2 ligand and suggesting a model in which TREM2-mediated effects on plaque may require or be enhanced by a ternary complex with ApoE. Structural studies provide some insight into the specific effects of AD risk of TREM2 mutations. R47, for example, is a surface residue that may constitute part of the TREM2 ligand binding surface (Kober et al., 2016). In mutant form (R47H), histidine substantially alters the conformation of a surface loop, which may change the energetics of ligand binding (Sudom et al., 2018). It should be noted that some TREM2 mutants, including ones that eliminate expression by chain termination, are associated with a different neurologic condition called Nasu–Hakola disease (NHD). AD-associated mutations change amino acids on the outer surface of the protein and are predicted to have subtle structural effects. In contrast, NHD sequence variants are buried in the core of the protein and are predicted to have profound effects on protein structure and expression (Kober et al., 2016). Thus, relatively conservative mutations increase the risk of AD, while protein elimination is associated with NHD.

Some challenges in the TREM2 field have slowed progress in our understanding of its role in AD. Many studies are in cells other than microglia—often, peripheral macrophages. Many studies are done with TREM2 knockouts. As noted above, such knockouts are associated with NHD rather than AD, and the mechanistic relationships between the two are essentially not known. Some studies rely on overexpression of TREM2 (e.g., Jiang et al., 2016; Lee et al., 2018), which risks overwhelming TREM2 signaling partners in these cells, with uncertain consequences. Much of this is understandable. A major problem is that the phenotype of the AD risk sequence variants is quite subtle, making differential readouts and clear conclusions difficult. Some investigators use heterozygous as well as homozygous knockouts to reveal trends that are complex to interpret. Likewise, the relationship between a heterozygous knockout and, for example, the R47H sequence variant is not

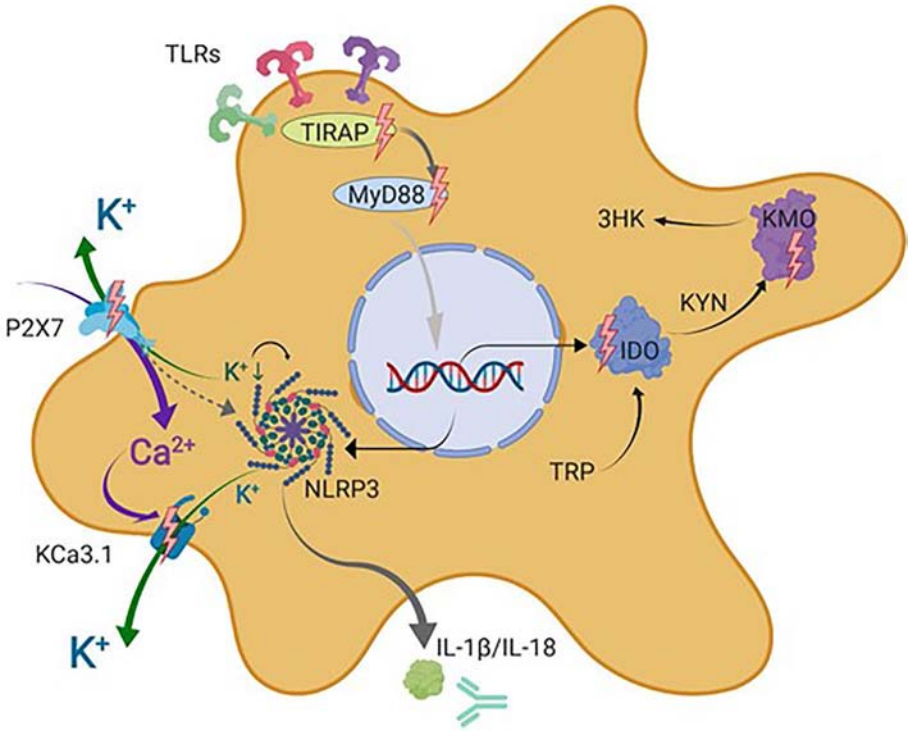

FIGURE 4 | Schematic representation of microglial drug targets discussed in this review. This simplified schematic does not contain all signal transduction molecules known to be involved in the described signaling cascades but focuses on the microglial drug targets discussed in this review indicated by lighting bolts and antibody symbol. IDO, indolamine-2,3-dioxygenase; IL-1β, interleukin 1 beta; IL-18, interleukin 18; $K_{Ca}3.1$, intermediate-conductance calcium-activated potassium channel 3.1; KYN, kynurenine; AA, anthranilic acid; 3-HK, 3-hydroxykynurenine; KATs, kynurenine aminotransferases; KMO, kynurenine 3-monooxygenase (kynurenine 3-hydroxylase); NLRP3, nucleotide-binding oligomerization domain, leucine-rich repeat, and pyrin domain–containing inflammasome complex 3; P2X7, ionotropic P2 receptor 7; TIRAP, TIR domain–containing adaptor protein; MyD88, myeloid differentiation primary response protein 88; TDO, tryptophan-2,3-dioxygenase; TRP, tryptophan. Figure created with Biorender.com

clear, and the latter is the preferable experimental target (for example, Cheng et al., 2018; Song et al., 2018). Compounding the TREM2 phenotype problem are difficulties common to all studies of microglia; these cells are extremely sensitive to their environment, rapidly changing their gene expression profile *ex vivo* (Bohlen et al., 2017). Human primary cells are available, of course, only from cadavers, and even then, only after several hours of hypoxia, with uncertain impact. Inducible pluripotent stem cell (iPSC)-derived "microglia-like" cells have, at best, an uncertain relationship with human microglia in their natural setting. Finally, due largely to the lack of reagents, intervention studies with pharmacologic modulators are rare: one such study using an antibody reported to activate TREM2 signaling caused significant increases in microglial survival and migration (Cheng et al., 2018). More studies with better-characterized reagents are needed.

TREM2 as Drug Target: Challenges

Besides directing attention to microglia as an approach to AD drug discovery, TREM2 itself may have promise as a target if the appropriate pharmacologic modulation can be identified. As mutants that increase AD risk seem to have a partial loss of TREM2 function, and considering that overexpression studies with TREM2 seem to enhance its function in processes associated with AD (Takahashi et al., 2005; Lee et al., 2018), one hypothesis is that augmenting the amount or function of TREM2 might oppose AD progression. However, increasing the function of a protein is challenging. Antibodies stabilizing TREM2 on the cell surface might be another possibility, including those that increase cell-surface TREM2 by blocking its proteolytic cleavage and release (Schlepckow et al., 2017; Thornton et al., 2017). Small molecule binders that stabilize TREM2 by blocking proteolytic cleavage or other mechanisms are conceivable but likely quite difficult to find. More promising, but further off, would be modulation of TREM2 regulatory factors. Such signaling or regulatory mechanisms specific enough to TREM2 to expect avoidance of undesired pleiotropic effects have not been reported. These may be revealed by ongoing research, including phenotypic screens for genes or compounds that boost TREM2 function. But as a caveat to this whole strategy, recall that TREM2 mutants that increase AD risk are quite rare. The vast majority of AD patients carry the common variant sequence of TREM2. There is no direct evidence in humans, and only limited evidence in model systems, that boosting the function of "normal" TREM2 would have beneficial effects for most AD patients. There is much to be learned about TREM2 and its promise as a drug target.

CONCLUSIONS

To date, no disease-modifying treatment for AD has achieved Food and Drug Administration (FDA) approval despite many studies showing their efficacy in mouse models. Aβ and tau are key features of AD, and either could be a "disease-modifying" target in principle. Multiple failures to stop AD by facilitating Aβ clearance or inhibition of production have considerably increased the interest in other modalities to target this devastating disease, such as neuroinflammation.

Microglia were first recognized as key players in neurodegenerative diseases more than two decades ago. More recently, unambiguous genetic evidence clearly links microglia function to AD pathogenesis. A large percentage of disease-associated SNPs are present in genes that are specifically expressed in microglia, a finding that has boosted the interest in understanding how microglia could be targeted to treat AD. Accordingly, numerous microglia proteins or pathways have been suggested to be drug targets. Here we reviewed several target candidates and their drugability challenges. Ideal drug targets should have a strong biological case, ideally combining genetic evidence with a well-understood mechanism of action that is supported by *in vitro* and *in vivo* data. However, even targets which fullfill this criteria need to be evaluated on whether they can be drugged safely. For the targets that have been briefly discussed in this review, these high hurdles have not yet been overcome. We remain optimistic that microglia-driven mechanisms will eventually bear clinical success and that one of the many discussed targets in this article will find a drug in the market helping AD patients.

AUTHOR CONTRIBUTIONS

KB, AB, BC, JP, MR, RS, RT, TM wrote the manuscript. KB, RT, and TM edited the manuscript.

REFERENCES

Arrowsmith, C. H., Audia, J. E., Austin, C., Baell, J., Bennett, J., Blagg, J., et al. (2015). The promise and peril of chemical probes. *Nat. Chem. Biol.* 11 (8), 536–541. doi: 10.1038/nchembio.1867

Ataga, K. I., Orringer, E. P., Styles, L., Vichinsky, E. P., Swerdlow, P., Davis, G. A., et al. (2006). Dose-escalation study of ICA-17043 in patients with sickle cell disease. *Pharmacotherapy* 26 (11), 1557–1564. doi: 10.1592/phco.26.11.1557

Ataga, K. I., Smith, W. R., De Castro, L. M., Swerdlow, P., Saunthararajah, Y., Castro, O., et al. (2008). Efficacy and safety of the Gardos channel blocker, senicapoc (ICA-17043), in patients with sickle cell anemia. *Blood* 111 (8), 3991–3997. doi: 10.1182/blood-2007-08-110098

Ataga, K. I., and Stocker, J. (2009). Senicapoc (ICA-17043): a potential therapy for the prevention and treatment of hemolysis-associated complications in sickle cell anemia. *Expert Opin. Investig. Drugs* 18 (2), 231–239. doi: 10.1517/13543780802708011

Atagi, Y., Liu, C.-C., Painter, M. M., Chen, X.-F., Verbeeck, C., Zheng, H., et al. (2015). Apolipoprotein E is a ligand for triggering receptor expressed on myeloid cells 2 (TREM2). *J. Biol. Chem.* 290 (43), 26043–26050. doi: 10.1074/jbc.M115.679043

Atukeren, P., Cengiz, M., Yavuzer, H., Gelisgen, R., Altunoglu, E., Oner, S., et al. (2018). The efficacy of donepezil administration on acetylcholinesterase activity and altered redox homeostasis in Alzheimer's disease. *Biomed. Pharmacother.* 90, 786–795. doi: 10.1016/j.biopha.2017.03.101

Bailey, C. C., DeVaux, L. B., and Farzan, M. (2015). The triggering receptor expressed on myeloid cells 2 binds apolipoprotein E. *J. Biol. Chem.* 290 (43), 26033–26042. doi: 10.1074/jbc.M115.677286

Balducci, C., Frasca, A., Zotti, M., La Vitola, P., Mhillaj, E., Grigoli, E., et al. (2017). Toll-like receptor 4–dependent glial cell activation mediates the impairment in memory establishment induced by beta-amyloid oligomers in an acute mouse model of Alzheimer's disease. *Brain Behav. Immun.* 60, 188–197. doi: 10.1016/j.bbi.2016.10.012

Baran, H., Jellinger, K., and Deecke, L. (1999). Kynurenine metabolism in Alzheimer's disease. *J. Neural. Transm.* (Vienna). 106 (2), 165–181. doi: 10.1007/s007020050149

Bartlett, R., Stokes, L., and Sluyter, R. (2014). The P2X7 receptor channel: recent developments and the use of P2X7 antagonists in models of disease. *Pharmacol. Rev.* 66 (3), 638–675. doi: 10.1124/pr.113.008003

Baruch, K., Deczkowska, A., Rosenzweig, N., Tsitsou-Kampeli, A., Sharif, A. M., Matcovitch-Natan, O., et al. (2016). PD-1 immune checkpoint blockade reduces pathology and improves memory in mouse models of Alzheimer's disease. *Nat. Med.* 22 (2), 135–137. doi: 10.1038/nm.4022

Baruch, K., Rosenzweig, N., Kertser, A., Deczkowska, A., Sharif, A. M., Spinrad, A., et al. (2015). Breaking immune tolerance by targeting Foxp3(+) regulatory T cells mitigates Alzheimer's disease pathology. *Nat. Commun.* 6, 7967. doi: 10.1038/ncomms8967

Beal, M. F., Matson, W. R., Storey, E., Milbury, P., Ryan, E. A., Ogawa, T., et al. (1992). Kynurenic acid concentrations are reduced in Huntington's disease cerebral cortex. *J. Neurol. Sci.* 108 (1), 80-87. doi: 10.1016/0022-510x(92)90191-m

Beaumont, V., Mrzljak, L., Dijkman, U., Freije, R., Heins, M., Rassoulpour, A., et al. (2016). The novel KMO inhibitor CHDI-340246 leads to a restoration of electrophysiological alterations in mouse models of Huntington's disease. *Exp. Neurol.* 282, 99–118. doi: 10.1016/j.expneurol.2016.05.005

Bellenguez, C., Charbonnier, C., Grenier-Boley, B., Quenez, O., Le Guennec, K., Nicolas, G., et al. (2017). Contribution to Alzheimer's disease risk of rare variants in TREM2, SORL1, and ABCA7 in 1779 cases and 1273 controls. *Neurobiol. Aging.* 59, 220.e1-220.e9. doi: 10.1016/j.neurobiolaging.2017.07.001

Ben Haim, L., Carrillo-de Sauvage, M. A., Ceyzeriat, K., and Escartin, C. (2015). Elusive roles for reactive astrocytes in neurodegenerative diseases. *Front. Cell. Neurosci.* 9, 278. doi: 10.3389/fncel.2015.00278

Bergles, D. E., and Richardson, W. D. (2015). Oligodendrocyte development and plasticity. *Cold Spring Harb.* 8 (2), a020453. doi: 10.1101/cshperspect.a020453

Bhattacharya, A. (2018). Recent advances in CNS P2X7 physiology and pharmacology: focus on neuropsychiatric disorders. *Front. Pharmacol.* 9, 30. doi: 10.3389/fphar.2018.00030

Bhattacharya, A., and Biber, K. (2016). The microglial ATP-gated ion channel P2X7 as a CNS drug target. *Glia* 64 (10), 1772–1787. doi: 10.1002/glia.23001

Bhattacharya, A., and Jones, D. N. C. (2018). Emerging role of the P2X7–NLRP3–IL1beta pathway in mood disorders. *Psychoneuroendocrinology* 98, 95–100. doi: 10.1016/j.psyneuen.2018.08.015

Biber, K., Moller, T., Boddeke, E., and Prinz, M. (2015). Central nervous system myeloid cells as drug targets: current status and translational challenges. *Nat. Rev. Drug Discov.* 15 (2), 110–124. doi: 10.1038/nrd.2015.14

Bohlen, C. J., Bennett, F. C., Tucker, A. F., Collins, H. Y., Mulinyawe, S. B., and Barres, B. A. (2017). Diverse requirements for microglial survival, specification, and function revealed by defined-medium cultures. *Neuron* 94 (4), 759–773 e758. doi: 10.1016/j.neuron.2017.04.043

Bonda, D. J., Mailankot, M., Stone, J. G., Garrett, M. R., Staniszewska, M., Castellani, R. J., et al. (2010). Indoleamine 2,3-dioxygenase and 3-hydroxykynurenine modifications are found in the neuropathology of Alzheimer's disease. *Redox Rep.* 15 (4), 161–168. doi: 10.1179/174329210X12650506623645

Bouhy, D., Ghasemlou, N., Lively, S., Redensek, A., Rathore, K. I., Schlichter, L. C., et al. (2011). Inhibition of the Ca(2)(+)-dependent K(+) channel, KCNN4/KCa3.1, improves tissue protection and locomotor recovery after spinal cord injury. *J. Neurosci.* 31 (45), 16298–16308. doi: 10.1523/JNEUROSCI.0047-11.2011

Bradshaw, E. M., Chibnik, L. B., Keenan, B. T., Ottoboni, L., Raj, T., Tang, A., et al. (2013). CD33 Alzheimer's disease locus: altered monocyte function and amyloid biology. *Nat. Neurosci.* 16 (7), 848–850. doi: 10.1038/nn.3435

Bsibsi, M., Ravid, R., Gveric, D., and van Noort, J. M. (2002). Broad expression of Toll-like receptors in the human central nervous system. *J. Neuropathol. Exp. Neurol.* 61 (11), 1013–1021. doi: 10.1093/jnen/61.11.1013

Campbell, B. M., Charych, E., Lee, A. W., and Möller, T. (2014). Kynurenines in CNS disease: regulation by inflammatory cytokines. *Front. Neurosci.* 8, 12. doi: 10.3389/fnins.2014.00012

Cao, H., and Crocker, P. R. (2011). Evolution of CD33-related siglecs: regulating host immune functions and escaping pathogen exploitation? *Immunology* 132 (1), 18–26. doi: 10.1111/j.1365-2567.2010.03368.x

Chavan, S. S., Pavlov, V. A., and Tracey, K. J. (2017). Mechanisms and therapeutic relevance of neuro-immune communication. *Immunity* 46 (6), 927–942. doi: 10.1016/j.immuni.2017.06.008

Chen, K., Iribarren, P., Hu, J., Chen, J., Gong, W., Cho, E. H., et al. (2006). Activation of Toll-like receptor 2 on microglia promotes cell uptake of Alzheimer disease–associated amyloid beta peptide. *J. Biol. Chem.* 281 (6), 3651–3659. doi: 10.1074/jbc.M508125200

Chen, X., Hu, J., Jiang, L., Xu, S., Zheng, B., Wang, C., et al. (2014). Brilliant Blue G improves cognition in an animal model of Alzheimer's disease and inhibits amyloid-beta–induced loss of filopodia and dendrite spines in hippocampal neurons. *Neuroscience* 279, 94–101. doi: 10.1016/j.neuroscience.2014.08.036

Chen, Y. J., Raman, G., Bodendiek, S., O'Donnell, M. E., and Wulff, H. (2011). The KCa3.1 blocker TRAM-34 reduces infarction and neurological deficit in a rat model of ischemia/reperfusion stroke. *J. Cereb. Blood Flow Metab.* 31 (12), 2363–2374. doi: 10.1038/jcbfm.2011.101

Chen, Y. J., Wallace, B. K., Yuen, N., Jenkins, D. P., Wulff, H., and O'Donnell, M. E. (2015). Blood–brain barrier KCa3.1 channels: evidence for a role in brain Na uptake and edema in ischemic stroke. *Stroke* 46 (1), 237–244. doi: 10.1161/STROKEAHA.114.007445

Cheng, Q., Danao, J., Talreja, S., Wen, P., Yin, J., Sun, N., et al. (2018). TREM2-activating antibodies abrogate the negative pleiotropic effects of the Alzheimer's disease variant TREM2R47H on murine myeloid cell function. *J. Biol. Chem.* 293 (32), 12620–12633. doi: 10.1074/jbc.RA118.001848

Chouraki, V., Preis, S. R., Yang, Q., Beiser, A., Li, S., Larson, M. G., et al. (2017). Association of amine biomarkers with incident dementia and Alzheimer's disease in the Framingham Study. *Alzheimers Dement.* 13 (12), 1327–1336. doi: 10.1016/j.jalz.2017.04.009

Colonna, M., and Wang, Y. (2016). TREM2 variants: new keys to decipher Alzheimer disease pathogenesis. *Nat. Rev. Neurosci.* 17 (4), 201–207. doi: 10.1038/nrn.2016.7

Dale, E., Staal, R. G., Eder, C., and Moller, T. (2016). KCa 3.1—a microglial target ready for drug repurposing? *Glia* 64 (10), 1733–1741. doi: 10.1002/glia.22992

Dalpke, A. H., Schafer, M. K., Frey, M., Zimmermann, S., Tebbe, J., Weihe, E., et al. (2002). Immunostimulatory CpG-DNA activates murine microglia. *J. Immunol.* 168 (10), 4854–4863. doi: 10.4049/jimmunol.168.10.4854

Dempsey, C., Rubio Araiz, A., Bryson, K. J., Finucane, O., Larkin, C., Mills, E. L., et al. (2017). Inhibiting the NLRP3 inflammasome with MCC950 promotes non-phlogistic clearance of amyloid-beta and cognitive function in APP/PS1 mice. *Brain Behav. Immun.* 61, 306–316. doi: 10.1016/j.bbi.2016.12.014

Diaz-Hernandez, J. I., Gomez-Villafuertes, R., Leon-Otegui, M., Hontecillas-Prieto, L., Del Puerto, A., Trejo, J. L., et al. (2012). *In vivo* P2X7 inhibition reduces amyloid plaques in Alzheimer's disease through GSK3beta and secretases. *Neurobiol. Aging* 33 (8), 1816–1828. doi: 10.1016/j.neurobiolaging.2011.09.040

DiCarlo, G., Wilcock, D., Henderson, D., Gordon, M., and Morgan, D. (2001). Intrahippocampal LPS injections reduce Abeta load in APP+PS1 transgenic mice. *Neurobiol. Aging* 22 (6), 1007–1012. doi: 10.1016/S0197-4580(01)00292-5

Dounay, A. B., Anderson, M., Bechle, B. M., Campbell, B. M., Claffey, M. M., Evdokimov, A., et al. (2012). Discovery of brain-penetrant, irreversible kynurenine aminotransferase II inhibitors for schizophrenia. *ACS Med. Chem. Lett.* 3 (3), 187–192. doi: 10.1021/ml200204m

Eder, C., Klee, R., and Heinemann, U. (1997). Pharmacological properties of Ca2+-activated K+ currents of ramified murine brain macrophages. *Naunyn Schmiedebergs Arch. Pharmacol.* 356 (2), 233–239. doi: 10.1007/PL00005046

Fanger, C. M., Ghanshani, S., Logsdon, N. J., Rauer, H., Kalman, K., Zhou, J., et al. (1999). Calmodulin mediates calcium-dependent activation of the intermediate conductance KCa channel, IKCa1. *J. Biol. Chem.* 274 (9), 5746–5754. doi: 10.1074/jbc.274.9.5746

Feske, S., Skolnik, E. Y., and Prakriya, M. (2012). Ion channels and transporters in lymphocyte function and immunity. *Nat. Rev. Immunol.* 12 (7), 532–547. doi: 10.1038/nri3233

Flint Beal, M., Matson, W. R., Storey, E., Milbury, P., Ryan, E. A., Ogawa, T., et al. (1992). Kynurenic acid concentrations are reduced in Huntington's disease cerebral cortex. *J. Neurol. Sci.* 108 (1), 80–87. doi: 10.1016/0022-510X(92)90191-M

Frank, S., Copanaki, E., Burbach, G. J., Muller, U. C., and Deller, T. (2009). Differential regulation of toll-like receptor mRNAs in amyloid plaque–associated brain tissue of aged APP23 transgenic mice. *Neurosci. Lett.* 453 (1), 41–44. doi: 10.1016/j.neulet.2009.01.075

Frye, S. V. (2010). The art of the chemical probe. *Nat. Chem. Biol.* 6 (3), 159–161. doi: 10.1038/nchembio.296

Giil, L. M., Midttun, Ø., Refsum, H., Ulvik, A., Advani, R., Smith, A. D., et al. (2017). Kynurenine Pathway Metabolites in Alzheimer's Disease. *J. Alzheimers Dis.* 60 (2), 495–504. doi: 10.3233/JAD-170485

Go, M., Kou, J., Lim, J. E., Yang, J., and Fukuchi, K. I. (2016). Microglial response to LPS increases in wild-type mice during aging but diminishes in an Alzheimer's mouse model: implication of TLR4 signaling in disease progression. *Biochem. Biophys. Res. Commun.* 479 (2), 331–337. doi: 10.1016/j.bbrc.2016.09.073

Greilberger, J., Fuchs, D., Leblhuber, F., Greilberger, M., Wintersteiger, R., and Tafeit, E. (2010). Carbonyl proteins as a clinical marker in Alzheimer's disease and its relation to tryptophan degradation and immune activation. *Clin. Lab.* 56 (9–10), 441–448.

Griciuc, A., Serrano-Pozo, A., Parrado, A. R., Lesinski, A. N., Asselin, C. N., Mullin, K., et al. (2013). Alzheimer's disease risk gene CD33 inhibits microglial uptake of amyloid beta. *Neuron* 78 (4), 631–643. doi: 10.1016/j.neuron.2013.04.014

Guedes, J. R., Lao, T., Cardoso, A. L., and El Khoury, J. (2018). Roles of microglial and monocyte chemokines and their receptors in regulating Alzheimer's disease–associated amyloid-beta and tau pathologies. *Front. Neurol.* 9, 549. doi: 10.3389/fneur.2018.00549

Guerreiro, R., Wojtas, A., Bras, J., Carrasquillo, M., Rogaeva, E., Majounie, E., et al. (2013). TREM2 variants in Alzheimer's disease. *N. Engl. J. Med.* 368 (2), 117–127. doi: 10.1056/NEJMoa1211851

Guidetti, P., Hoffman, G. E., Melendez-Ferro, M., Albuquerque, E. X., and Schwarcz, R. (2007). Astrocytic localization of kynurenine aminotransferase II in the rat brain visualized by immunocytochemistry. *Glia* 55 (1), 78–92. doi: 10.1002/glia.20432

Guillemin, G. J., Brew, B. J., Noonan, C. E., Takikawa, O., and Cullen, K. M. (2005). Indoleamine 2,3 dioxygenase and quinolinic acid immunoreactivity in Alzheimer's disease hippocampus. *Neuropathol. Appl. Neurobiol.* 31 (4), 395–404. doi: 10.1111/j.1365-2990.2005.00655.x

Guillemin, G. J., Smith, D. G., Smythe, G. A., Armati, P. J., and Brew, B. J. (2003a). Expression of the kynurenine pathway enzymes in human microglia and macrophages. *Adv. Exp. Med. Biol.* 527, 105–112. doi: 10.1007/978-1-4615-0135-0_12

Guillemin, G. J., Smythe, G. A., Veas, L. A., Takikawa, O., and Brew, B. J. (2003b). Aβ1–42 induces production of quinolinic acid by human macrophages and microglia. *Neuroreport* 14 (18), 2311–2315. doi: 10.1097/00001756-200312190-00005

Gulaj, E., Pawlak, K., Bien, B., and Pawlak, D. (2010). Kynurenine and its metabolites in Alzheimer's disease patients. *Adv. Med. Sci.* 55 (2), 204–211. doi: 10.2478/v10039-010-0023-6

Hansen, D. V., Hanson, J. E., and Sheng, M. (2018). Microglia in Alzheimer's disease. *J. Cell Biol.* 217 (2), 459–472. doi: 10.1083/jcb.201709069

Hartai, Z., Juhász, A., Rimanóczy, A., Janáky, T., Donkó, T., Dux, L., et al. (2007). Decreased serum and red blood cell kynurenic acid levels in Alzheimer's disease. *Neurochem. Int.* 50 (2), 308–313. doi: 10.1016/j.neuint.2006.08.012

Hauser, A. S., Attwood, M. M., Rask-Andersen, M., Schioth, H. B., and Gloriam, D. E. (2017). Trends in GPCR drug discovery: new agents, targets and indications. *Nat. Rev. Drug Discov.* 16 (12), 829–842. doi: 10.1038/nrd.2017.178

Heneka, M. T. (2017). Inflammasome activation and innate immunity in Alzheimer's disease. *Brain Pathol.* 27 (2), 220–222. doi: 10.1111/bpa.12483

Heneka, M. T., Carson, M. J., El Khoury, J., Landreth, G. E., Brosseron, F., Feinstein, D. L., et al. (2015). Neuroinflammation in Alzheimer's disease. *Lancet Neurol.* 14 (4), 388–405. doi: 10.1016/S1474-4422(15)70016-5

Heneka, M. T., Kummer, M. P., Stutz, A., Delekate, A., Schwartz, S., Vieira-Saecker, A., et al. (2013). NLRP3 is activated in Alzheimer's disease and contributes to pathology in APP/PS1 mice. *Nature* 493 (7434), 674–678. doi: 10.1038/nature11729

Heneka, M. T., McManus, R. M., and Latz, E. (2018). Inflammasome signalling in brain function and neurodegenerative disease. *Nat. Rev. Neurosci.* 19 (10), 610–621. doi: 10.1038/s41583-018-0055-7

Henstridge, C. M., Hyman, B. T., and Spires-Jones, T. L. (2019). Beyond the neuron-cellular interactions early in Alzheimer disease pathogenesis. *Nat. Rev. Neurosci.* 20 (2), 94–108. doi: 10.1038/s41583-018-0113-1

Heppner, F. L., Ransohoff, R. M., and Becher, B. (2015). Immune attack: the role of inflammation in Alzheimer disease. *Nat. Rev. Neurosci.* 16 (6), 358–372. doi: 10.1038/nrn3880

Herber, D. L., Mercer, M., Roth, L. M., Symmonds, K., Maloney, J., Wilson, N., et al. (2007). Microglial activation is required for Abeta clearance after intracranial injection of lipopolysaccharide in APP transgenic mice. *J. Neuroimmune Pharmacol.* 2 (2), 222–231. doi: 10.1007/s11481-007-9069-z

Heyes, M. P., Achim, C. L., Wiley, C. A., Major, E. O., Saito, K., and Markey, S. P. (1996). Human microglia convert l-tryptophan into the neurotoxin quinolinic acid. *Biochem. J.* 320 (2), 595–597. doi: 10.1042/bj3200595

Heyes, M. P., Saito, K., Crowley, J. S., Davis, L. E., Demitrack, M. A., Der, M., et al. (1992). Quinolinic acid and kynurenine pathway metabolism in inflammatory and non-inflammatory neurological disease. *Brain* 115 (5), 1249–1273. doi: 10.1093/brain/115.5.1249

Higueruelo, A. P., Jubb, H., and Blundell, T. L. (2013). Protein–protein interactions as druggable targets: recent technological advances. *Curr. Opin. Pharmacol.* 13 (5), 791–796. doi: 10.1016/j.coph.2013.05.009

Hokari, M., Wu, H.-Q., Schwarcz, R., and Smith, Q. R. (1996). Facilitated brain uptake of 4-chlorokynurenine and conversion to 7-chlorokynurenic acid. *Neuroreport* 8 (1), 15–18. doi: 10.1097/00001756-199612200-00004

Hollingworth, P., Harold, D., Sims, R., Gerrish, A., Lambert, J. C., Carrasquillo, M. M., et al. (2011). Common variants at ABCA7, MS4A6A/MS4A4E, EPHA1, CD33 and CD2AP are associated with Alzheimer's disease. *Nat. Genet.* 43 (5), 429–435. doi: 10.1038/ng.803

Hu, Z., Tanji, H., Jiang, S., Zhang, S., Koo, K., Chan, J., et al. (2018). Small-molecule TLR8 antagonists *via* structure-based rational design. *Cell Chem. Biol.* 25 (10), 1286–1291 e1283. doi: 10.1016/j.chembiol.2018.07.004

Illes, P., Rubini, P., Huang, L., and Tang, Y. (2019). The P2X7 receptor: a new therapeutic target in Alzheimer's disease. *Expert Opin. Ther. Targets*. 23 (3), 165–176. doi: 10.1080/14728222.2019.1575811

Jain, P., Wadhwa, P. K., and Jadhav, H. R. (2015). Reactive astrogliosis: role in Alzheimer's disease. *CNS Neurol. Dis. Drug Targets* 14 (7), 872–879. doi: 10.2174/1871527314666150713104738

Jana, M., Palencia, C. A., and Pahan, K. (2008). Fibrillar amyloid-beta peptides activate microglia *via* TLR2: implications for Alzheimer's disease. *J. Immunol.* 181 (10), 7254–7262. doi: 10.4049/jimmunol.181.10.7254

Janssens, S., and Beyaert, R. (2003). Role of Toll-like receptors in pathogen recognition. *Clin. Microbiol. Rev.* 16 (4), 637–646. doi: 10.1128/CMR.16.4.637-646.2003

Jiang, T., Zhang, Y. D., Chen, Q., Gao, Q., Zhu, X. C., Zhou, J. S., et al. (2016). TREM2 modifies microglial phenotype and provides neuroprotection in P301S tau transgenic mice. *Neuropharmacology* 105, 196–206. doi: 10.1016/j.neuropharm.2016.01.028

Jiang, Y. T., Li, H. Y., Cao, X. P., and Tan, L. (2018). Meta-analysis of the association between CD33 and Alzheimer's disease. *Ann. Transl. Med.* 6 (10), 169. doi: 10.21037/atm.2018.04.21

Jin, H., Han, J., Resing, D., Liu, H., Yue, X., Miller, R. L., et al. (2018). Synthesis and *in vitro* characterization of a P2X7 radioligand [(123)I]TZ6019 and its response to neuroinflammation in a mouse model of Alzheimer disease. *Eur. J. Pharmacol.* 820, 8–17. doi: 10.1016/j.ejphar.2017.12.006

Jin, L. D. L., J, Nguyen, H., Singh, V., Sing, L., Chavez, M., et al. (In Press). Repurposing the KCa3.1 inhibitor senicapoc as a microglia-targeted therapeutic candidate for Alzheimer's disease. *Brain*.

Jonsson, T., Stefansson, H., Steinberg, S., Jonsdottir, I., Jonsson, P. V., Snaedal, J., et al. (2013). variant of TREM2 associated with the risk of Alzheimer's disease. *N. Engl. J. Med.* 368 (2), 107–116. doi: 10.1056/NEJMoa1211103

Kaushal, V., Koeberle, P. D., Wang, Y., and Schlichter, L. C. (2007). The Ca2+-activated K+ channel KCNN4/KCa3.1 contributes to microglia activation and nitric oxide–dependent neurodegeneration. *J. Neurosci.* 27 (1), 234–244. doi: 10.1523/JNEUROSCI.3593-06.2007

Khanna, R., Roy, L., Zhu, X., and Schlichter, L. C. (2001). K+ channels and the microglial respiratory burst. *Am. J. Physiol. Cell Physiol.* 280 (4), C796–C806. doi: 10.1152/ajpcell.2001.280.4.C796

Kielian, T. (2006). Toll-like receptors in central nervous system glial inflammation and homeostasis. *J. Neurosci. Res.* 83 (5), 711–730. doi: 10.1002/jnr.20767

Kielian, T. (2009). Overview of toll-like receptors in the CNS. *Curr. Top. Microbiol. Immunol.* 336, 1–14. doi: 10.1007/978-3-642-00549-7_1

Kigerl, K. A., de Rivero Vaccari, J. P., Dietrich, W. D., Popovich, P. G., and Keane, R. W. (2014). Pattern recognition receptors and central nervous system repair. *Exp. Neurol.* 258, 5–16. doi: 10.1016/j.expneurol.2014.01.001

Kinney, J. W., Bemiller, S. M., Murtishaw, A. S., Leisgang, A. M., Salazar, A. M., and Lamb, B. T. (2018). Inflammation as a central mechanism in Alzheimer's disease. *Alzheimers Dement. (N. Y.)* 4, 575–590. doi: 10.1016/j.trci.2018.06.014

Kober, D. L., and Brett, T. J. (2017). TREM2–ligand interactions in health and disease. *J. Mol. Biol.* 429 (11), 1607–1629. doi: 10.1016/j.jmb.2017.04.004

Kober, D. L., Alexander-Brett, J. M., Karch, C. M., Cruchaga, C., Colonna, M., Holtzman, M. J., et al. (2016). Neurodegenerative disease mutations in TREM2 reveal a functional surface and distinct loss-of-function mechanisms. *Elife* 5, e20391. doi: 10.7554/eLife.20391

Kobold, S., Pantelyushin, S., Rataj, F., and Vom Berg, J. (2018). Rationale for combining bispecific T cell activating antibodies with checkpoint blockade for cancer therapy. *Front. Oncol.* 8, 285. doi: 10.3389/fonc.2018.00285

Laflamme, N., and Rivest, S. (2001). Toll-like receptor 4: the missing link of the cerebral innate immune response triggered by circulating gram-negative bacterial cell wall components. *FASEB J.* 15 (1), 155–163. doi: 10.1096/fj.00-0339com

Laflamme, N., Soucy, G., and Rivest, S. (2001). Circulating cell wall components derived from gram-negative, not gram-positive, bacteria cause a profound induction of the gene-encoding Toll-like receptor 2 in the CNS. *J. Neurochem.* 79 (3), 648–657. doi: 10.1046/j.1471-4159.2001.00603.x

Lajaunias, F., Dayer, J. M., and Chizzolini, C. (2005). Constitutive repressor activity of CD33 on human monocytes requires sialic acid recognition and phosphoinositide 3-kinase–mediated intracellular signaling. *Eur. J. Immunol.* 35 (1), 243–251. doi: 10.1002/eji.200425273

Latta-Mahieu, M., Elmer, B., Bretteville, A., Wang, Y., Lopez-Grancha, M., Goniot, P., et al. (2018). Systemic immune-checkpoint blockade with anti-PD1 antibodies does not alter cerebral amyloid-β burden in several amyloid transgenic mouse models. *Glia* 66 (3), 492–504. doi: 10.1002/glia.23260

Lee, C. Y. D., Daggett, A., Gu, X., Jiang, L. L., Langfelder, P., Li, X., et al. (2018). Elevated TREM2 gene dosage reprograms microglia responsivity and ameliorates pathological phenotypes in Alzheimer's disease models. *Neuron* 97 (5), 1032–1048 e1035. doi: 10.1016/j.neuron.2018.02.002

Lee, H. G., Won, S. M., Gwag, B. J., and Lee, Y. B. (2011). Microglial $P2X_7$ receptor expression is accompanied by neuronal damage in the cerebral cortex of the APPswe/PS1dE9 mouse model of Alzheimer's disease. *Exp. Mol. Med.* 43 (1), 7–14. doi: 10.3858/emm.2011.43.1.001

Lee, J. W., Lee, Y. K., Yuk, D. Y., Choi, D. Y., Ban, S. B., Oh, K. W., et al. (2008). Neuro-inflammation induced by lipopolysaccharide causes cognitive impairment through enhancement of beta-amyloid generation. *J. Neuroinflammation* 5, 37. doi: 10.1186/1742-2094-5-37

Lee, Y. J., Choi, D. Y., Choi, I. S., Kim, K. H., Kim, Y. H., Kim, H. M., et al. (2012). Inhibitory effect of 4-O-methylhonokiol on lipopolysaccharide-induced neuroinflammation, amyloidogenesis and memory impairment *via* inhibition of nuclear factor-kappaB *in vitro* and *in vivo* models. *J. Neuroinflammation* 9, 35. doi: 10.1186/1742-2094-9-35

Leklem, J. E. (1971). Quantitative aspects of tryptophan metabolism in humans and other species: a review. *Am. J. Clin. Nutr.* 24 (6), 659–672. doi: 10.1093/ajcn/24.6.659

Lessard, C. B., Malnik, S. L., Zhou, Y., Ladd, T. B., Cruz, P. E., Ran, Y., et al. (2018). High affinity interactions and signal transduction between Aβ oligomers and TREM2. *EMBO Mol. Med.* 10 (11), e9027. doi: 10.15252/emmm.201809027

Letiembre, M., Liu, Y., Walter, S., Hao, W., Pfander, T., Wrede, A., et al. (2009). Screening of innate immune receptors in neurodegenerative diseases: a similar pattern. *Neurobiol. Aging* 30 (5), 759–768. doi: 10.1016/j.neurobiolaging.2007.08.018

Linnartz-Gerlach, B., Kopatz, J., and Neumann, H. (2014). Siglec functions of microglia. *Glycobiology* 24 (9), 794–799. doi: 10.1093/glycob/cwu044

Liu, S., Liu, Y., Hao, W., Wolf, L., Kiliaan, A. J., Penke, B., et al. (2012). TLR2 is a primary receptor for Alzheimer's amyloid beta peptide to trigger neuroinflammatory activation. *J. Immunol.* 188 (3), 1098–1107. doi: 10.4049/jimmunol.1101121

Liu, Y., Walter, S., Stagi, M., Cherny, D., Letiembre, M., Schulz-Schaeffer, W., et al. (2005). LPS receptor (CD14): a receptor for phagocytosis of Alzheimer's amyloid peptide. *Brain* 128 (Pt 8), 1778–1789. doi: 10.1093/brain/awh531

Lopez-Collazo, E., and del Fresno, C. (2013). Pathophysiology of endotoxin tolerance: mechanisms and clinical consequences. *Crit. Care* 17 (6), 242. doi: 10.1186/cc13110

Lopez-Gonzalez, I., Schluter, A., Aso, E., Garcia-Esparcia, P., Ansoleaga, B., Llorens F., et al. (2015). Neuroinflammatory signals in Alzheimer disease and APP/PS1 transgenic mice: correlations with plaques, tangles, and oligomeric species. *J. Neuropathol. Exp. Neurol.* 74 (4), 319–344. doi: 10.1097/NEN.0000000000000176

Lovelace, M. D., Varney, B., Sundaram, G., Lennon, M. J., Lim, C. K., Jacobs, K., et al. (2017). Recent evidence for an expanded role of the kynurenine pathway of tryptophan metabolism in neurological diseases. *Neuropharmacology* 112, 373–388. doi: 10.1016/j.neuropharm.2016.03.024

Ma, P., and Zemmel, R. (2002). Value of novelty? *Nat. Rev. Drug Discov.* 1 (8), 571–572. doi: 10.1038/nrd884

Macagno, A., Molteni, M., Rinaldi, A., Bertoni, F., Lanzavecchia, A., Rossetti, C., et al. (2006). A cyanobacterial LPS antagonist prevents endotoxin shock and blocks sustained TLR4 stimulation required for cytokine expression. *J. Exp. Med.* 203 (6), 1481–1492. doi: 10.1084/jem.20060136

Macauley, M. S., Crocker, P. R., and Paulson, J. C. (2014). Siglec-mediated regulation of immune cell function in disease. *Nat. Rev. Immunol.* 14 (10), 653–666. doi: 10.1038/nri3737

Maezawa, I., Jenkins, D. P., Jin, B. E., and Wulff, H. (2012). Microglial KCa3.1 Channels as a potential therapeutic target for Alzheimer's disease. *Int. J. Alzheimers Dis.* 2012, 868972. doi: 10.1155/2012/868972

Maezawa, I., Zimin, P. I., Wulff, H., and Jin, L. W. (2011). Amyloid-beta protein oligomer at low nanomolar concentrations activates microglia and induces microglial neurotoxicity. *J. Biol. Chem.* 286 (5), 3693–3706. doi: 10.1074/jbc.M110.135244

Malik, M., Simpson, J. F., Parikh, I., Wilfred, B. R., Fardo, D. W., Nelson, P. T., et al. (2013). CD33 Alzheimer's risk–altering polymorphism, CD33 expression, and exon 2 splicing. *J. Neurosci.* 33 (33), 13320–13325. doi: 10.1523/JNEUROSCI.1224-13.2013

Martin, E., Amar, M., Dalle, C., Youssef, I., Boucher, C., Le Duigou, C., et al. (2018). New role of P2X7 receptor in an Alzheimer's disease mouse model. *Mol. Psychiatry*. 24 (1), 108–125. doi: 10.1038/s41380-018-0108-3

Martin, E., Amar, M., Dalle, C., Youssef, I., Boucher, C., Le Duigou, C., et al. (2019). New role of P2X7 receptor in an Alzheimer's disease mouse model. *Mol. Psychiatry* 24 (1), 108–125. doi: 10.1038/s41380-018-0108-3

Matsunaga, N., Tsuchimori, N., Matsumoto, T., and Ii, M. (2011). TAK-242 (resatorvid), a small-molecule inhibitor of Toll-like receptor (TLR) 4 signaling, binds selectively to TLR4 and interferes with interactions between TLR4 and its adaptor molecules. *Mol. Pharmacol.* 79 (1), 34–41. doi: 10.1124/mol.110.068064

McDonald, C. L., Hennessy, E., Rubio-Araiz, A., Keogh, B., McCormack, W., McGuirk, P., et al. (2016). Inhibiting TLR2 activation attenuates amyloid accumulation and glial activation in a mouse model of Alzheimer's disease. *Brain Behav. Immun.* 58, 191–200. doi: 10.1016/j.bbi.2016.07.143

McLarnon, J. G., Ryu, J. K., Walker, D. G., and Choi, H. B. (2006). Upregulated expression of purinergic P2X(7) receptor in Alzheimer disease and amyloid-beta peptide-treated microglia and in peptide-injected rat hippocampus. *J. Neuropathol. Exp. Neurol.* 65 (11), 1090–1097. doi: 10.1097/01.jnen.0000240470.97295.d3

Möller, T., and Boddeke, H. W. (2016). Glial cells as drug targets: what does it take? *Glia* 64 (10), 1742–1754. doi: 10.1002/glia.22993

Möller, T., Bard, F., Bhattacharya, A., Biber, K., Campbell, B., Dale, E., et al. (2016). Critical data-based re-evaluation of minocycline as a putative specific microglia inhibitor. *Glia* 64 (10), 1788–1794. doi: 10.1002/glia.23007

Moroni, F., Lombardi, G., Robitaille, Y., and Etienne, P. (1986). Senile dementia and Alzheimer's disease: lack of changes of the cortical content of quinolinic acid. *Neurobiol. Aging* 7 (4), 249–253. doi: 10.1016/0197-4580(86)90003-5

Mourdian, M. M., Heyes, M. P., Pan, J. B., Heuser, I. J. E., Markey, S. P., and Chase, T. N. (1989). No changes in central quinolinic acid levels in Alzheimer's disease. *Neurosci. Lett.* 105 (1–2), 233–238. doi: 10.1016/0304-3940(89)90043-8

Muguruma, Y., Tsutsui, H., Noda, T., Akatsu, H., and Inoue, K. (2018). Widely targeted metabolomics of Alzheimer's disease postmortem cerebrospinal fluid based on 9-fluorenylmethyl chloroformate derivatized ultra-high performance liquid chromatography tandem mass spectrometry. *J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci.* 1091, 53–66. doi: 10.1016/j.jchromb.2018.05.031

N'Diaye, E.-N., Branda, C. S., Branda, S. S., Nevarez, L., Colonna, M., Lowell, C., et al. (2009). TREM-2 (triggering receptor expressed on myeloid cells 2) is a phagocytic receptor for bacteria. *J. Cell Biol.* 184 (2), 215–223. doi: 10.1083/jcb.200808080

Naj, A. C., Jun, G., Beecham, G. W., Wang, L. S., Vardarajan, B. N., Buros, J., et al. (2011). Common variants at MS4A4/MS4A6E, CD2AP, CD33 and EPHA1 are associated with late-onset Alzheimer's disease. *Nat. Genet.* 43 (5), 436–441. doi: 10.1038/ng.801

O'Reilly, M. K., and Paulson, J. C. (2009). Siglecs as targets for therapy in immune-cell–mediated disease. *Trends Pharmacol. Sci.* 30 (5), 240–248. doi: 10.1016/j.tips.2009.02.005

Obst, J., Mancuso, R., Simon, E., and Gomez-Nicola, D. (2018). PD-1 deficiency is not sufficient to induce myeloid mobilization to the brain or alter the inflammatory profile during chronic neurodegeneration. *Brain Behav. Immun.* 73, 708–716. doi: 10.1016/j.bbi.2018.08.006

Okazaki, T., Chikuma, S., Iwai, Y., Fagarasan, S., and Honjo, T. (2013). A rheostat for immune responses: the unique properties of PD-1 and their advantages for clinical application. *Nat. Immunol.* 14 (12), 1212–1218. doi: 10.1038/ni.2762

Okello, A., Edison, P., Archer, H. A., Turkheimer, F. E., Kennedy, J., Bullock, R., et al. (2009a). Microglial activation and amyloid deposition in mild cognitive impairment: a PET study. *Neurology* 72 (1), 56–62. doi: 10.1212/01.wnl.0000338622.27876.0d

Okello, A., Koivunen, J., Edison, P., Archer, H. A., Turkheimer, F. E., Nagren, K., et al. (2009b). Conversion of amyloid positive and negative MCI to AD over 3 years: an 11C-PIB PET study. *Neurology* 73 (10), 754–760. doi: 10.1212/WNL.0b013e3181b23564

Olson, J. K., and Miller, S. D. (2004). Microglia initiate central nervous system innate and adaptive immune responses through multiple TLRs. *J. Immunol.* 173 (6), 3916–3924. doi: 10.4049/jimmunol.173.6.3916

Osborn, L. M., Kamphuis, W., Wadman, W. J., and Hol, E. M. (2016). Astrogliosis: an integral player in the pathogenesis of Alzheimer's disease. *Prog. Neurobiol.* 144, 121–141. doi: 10.1016/j.pneurobio.2016.01.001

Oxenkrug, G., van der Hart, M., Roeser, J., and Summergrad, P. (2017). Peripheral Tryptophan - Kynurenine Metabolism Associated with Metabolic Syndrome is Different in Parkinson's and Alzheimer's Diseases. *Endocrinol. Diabetes Metab. J.* 1 (4).

Pardridge, W. M. (2015). Targeted delivery of protein and gene medicines through the blood–brain barrier. *Clin. Pharmacol. Ther.* 97 (4), 347–361. doi: 10.1002/cpt.18

Parvathenani, L. K., Tertyshnikova, S., Greco, C. R., Roberts, S. B., Robertson, B., and Posmantur, R. (2003). P2X7 mediates superoxide production in primary microglia and is up-regulated in a transgenic mouse model of Alzheimer's disease. *J. Biol. Chem.* 278 (15), 13309–13317. doi: 10.1074/jbc.M209478200

Pearson, S. J., and Reynolds, G. P. (1992). Increased brain concentrations of a neurotoxin, 3-hydroxykynurenine, in Huntington's disease. *Neurosci. Lett.* 144 (1–2), 199–201. doi: 10.1016/0304-3940(92)90749-W

Pimenova, A. A., Raj, T., and Goate, A. M. (2018). Untangling genetic risk for Alzheimer's disease. *Biol. Psychiatry* 83 (4), 300–310. doi: 10.1016/j.biopsych.2017.05.014

Pourbadie, H. G., Sayyah, M., Khoshkholgh-Sima, B., Choopani, S., Nategh, M., Motamedi, F., et al. (2018). Early minor stimulation of microglial TLR2 and TLR4 receptors attenuates Alzheimer's disease–related cognitive deficit in rats: behavioral, molecular, and electrophysiological evidence. *Neurobiol. Aging* 70, 203–216. doi: 10.1016/j.neurobiolaging.2018.06.020

Qin, Y., Liu, Y., Hao, W., Decker, Y., Tomic, I., Menger, M. D., et al. (2016). Stimulation of TLR4 attenuates Alzheimer's disease–related symptoms and pathology in tau-transgenic mice. *J. Immunol.* 197 (8), 3281–3292. doi: 10.4049/jimmunol.1600873

Rahman, A., Ting, K., Cullen, K. M., Braidy, N., Brew, B. J., and Guillemin, G. J. (2009). The excitotoxin quinolinic acid induces tau phosphorylation in human neurons. *PLoS One* 4 (7), e6344. doi: 10.1371/journal.pone.0006344

Raj, D. D., Jaarsma, D., Holtman, I. R., Olah, M., Ferreira, F. M., Schaafsma, W., et al. (2014a). Priming of microglia in a DNA-repair deficient model of accelerated aging. *Neurobiol. Aging* 35 (9), 2147–2160. doi: 10.1016/j.neurobiolaging.2014.03.025

Raj, T., Ryan, K. J., Replogle, J. M., Chibnik, L. B., Rosenkrantz, L., Tang, A., et al. (2014b). CD33: increased inclusion of exon 2 implicates the Ig V-set domain in Alzheimer's disease susceptibility. *Hum. Mol. Genet.* 23 (10), 2729–2736. doi: 10.1093/hmg/ddt666

Rangasamy, S. B., Jana, M., Roy, A., Corbett, G. T., Kundu, M., Chandra, S., et al. (2018). Selective disruption of TLR2-MyD88 interaction inhibits inflammation and attenuates Alzheimer's pathology. *J. Clin. Invest.* 128 (10), 4297–4312. doi: 10.1172/JCI96209

Ransohoff, R. M., and Brown, M. A. (2012). Innate immunity in the central nervous system. *J. Clin. Invest.* 122 (4), 1164–1171. doi: 10.1172/JCI58644

Ransohoff, R. M., and El Khoury, J. (2015). Microglia in health and disease. *Cold Spring Harb. Perspect. Biol.* 8 (1), a020560. doi: 10.1101/cshperspect.a020560

Richard, K. L., Filali, M., Prefontaine, P., and Rivest, S. (2008). Toll-like receptor 2 acts as a natural innate immune receptor to clear amyloid beta 1–42 and delay the cognitive decline in a mouse model of Alzheimer's disease. *J. Neurosci.* 28 (22), 5784–5793. doi: 10.1523/JNEUROSCI.1146-08.2008

Riley, J. L., and June, C. H. (2005). The CD28 family: a T-cell rheostat for therapeutic control of T-cell activation. *Blood* 105 (1), 13–21. doi: 10.1182/blood-2004-04-1596

Rillahan, C. D., Macauley, M. S., Schwartz, E., He, Y., McBride, R., Arlian, B. M., et al. (2014). Disubstituted sialic acid ligands targeting siglecs CD33 and CD22 associated with myeloid leukaemias and B cell lymphomas. *Chem. Sci.* 5 (6), 2398–2406. doi: 10.1039/c4sc00451e

Robel, S., and Sontheimer, H. (2015). Glia as drivers of abnormal neuronal activity. *Nat. Neurosci.* 19 (1), 28–33. doi: 10.1038/nn.4184

Rubio-Araiz, A., Finucane, O. M., Keogh, S., and Lynch, M. A. (2018). Anti-TLR2 antibody triggers oxidative phosphorylation in microglia and increases phagocytosis of beta-amyloid. *J. Neuroinflammation* 15 (1), 247. doi: 10.1186/s12974-018-1281-7

Salameh, T. S., and Banks, W. A. (2014). Delivery of therapeutic peptides and proteins to the CNS. *Adv. Pharmacol.* 71, 277–299. doi: 10.1016/bs.apha.2014.06.004

Sanz, J. M., Chiozzi, P., Ferrari, D., Colaianna, M., Idzko, M., Falzoni, S., et al. (2009). Activation of microglia by amyloid {beta} requires P2X7 receptor expression. *J. Immunol.* 182 (7), 4378–4385. doi: 10.4049/jimmunol.0803612

Sastre, M., Richardson, J. C., Gentleman, S. M., and Brooks, D. J. (2011). Inflammatory risk factors and pathologies associated with Alzheimer's disease. *Curr. Alzheimer Res.* 8 (2), 132–141. doi: 10.2174/156720511795256062

Schilling, T., and Eder, C. (2004). A novel physiological mechanism of glycine-induced immunomodulation: Na+-coupled amino acid transporter currents in cultured brain macrophages. *J. Physiol.* 559 (Pt 1), 35–40. doi: 10.1113/jphysiol.2004.070763

Schilling, T., and Eder, C. (2007). TRAM-34 inhibits nonselective cation channels. *Pflugers Arch.* 454 (4), 559–563. doi: 10.1007/s00424-007-0232-4

Schilling, T., Repp, H., Richter, H., Koschinski, A., Heinemann, U., Dreyer, F., et al. (2002). Lysophospholipids induce membrane hyperpolarization in microglia by activation of IKCa1 Ca(2+)-dependent K(+) channels. *Neuroscience* 109 (4), 827–835. doi: 10.1016/S0306-4522(01)00534-6

Schlepckow, K., Kleinberger, G., Fukumori, A., Feederle, R., Lichtenthaler, S. F., Steiner, H., et al. (2017). An Alzheimer-associated TREM2 variant occurs at the ADAM cleavage site and affects shedding and phagocytic function. *EMBO Mol. Med.* 9 (10), 1356–1365. doi: 10.15252/emmm.201707672

Schwarz, M. J., Guillemin, G. J., Teipel, S. J., Buerger, K., and Hampel, H. (2013). Increased 3-hydroxykynurenine serum concentrations differentiate Alzheimer's disease patients from controls. *Eur. Arch. Psychiatry Clin. Neurosci.* 263 (4), 345–352. doi: 10.1007/s00406-012-0384-x

Schwarcz, R., and Stone, T. W. (2017). The kynurenine pathway and the brain: challenges, controversies and promises. *Neuropharmacology* 112, 237–247. doi: 10.1016/j.neuropharm.2016.08.003

Shaabani, S., Huizinga, H. P. S., Butera, R., Kouchi, A., Guzik, K., Magiera-Mularz, K., et al. (2018). A patent review on PD-1/PD-L1 antagonists: small molecules, peptides, and macrocycles (2015–2018). *Expert Opin. Ther. Pat.* 28 (9), 665–678. doi: 10.1080/13543776.2018.1512706

Shi, Y., and Holtzman, D. M. (2018). Interplay between innate immunity and Alzheimer disease: APOE and TREM2 in the spotlight. *Nat. Rev. Immunol.* 18 (12), 759–772. doi: 10.1038/s41577-018-0051-1

Sierra, A., Abiega, O., Shahraz, A., and Neumann, H. (2013). Janus-faced microglia: beneficial and detrimental consequences of microglial phagocytosis. *Front. Cell. Neurosci.* 7, 6. doi: 10.3389/fncel.2013.00006

Sims, R., van der Lee, S. J., Naj, A. C., Bellenguez, C., Badarinarayan, N., Jakobsdottir, J., et al. (2017). Rare coding variants in PLCG2, ABI3, and TREM2 implicate microglial-mediated innate immunity in Alzheimer's disease. *J. Prev. Alzheimers Dis.* 4 (4), 247–254. doi: 10.1038/ng.3916

Sofic, E., Halket, J., Przyborowska, A., Riederer, P., Beckmann, H., Sandler, M., et al. (1989). Brain quinolinic acid in Alzheimer's dementia. *Eur. Arch. Psychiatry Clin. Neurosci.* 239 (3), 177–179. doi: 10.1007/BF01739651

Song, W. M., and Colonna, M. (2018). The microglial response to neurodegenerative disease. *Adv. Immunol.* 139, 1–50. doi: 10.1016/bs.ai.2018.04.002

Song, M., Jin, J., Lim, J. E., Kou, J., Pattanayak, A., Rehman, J. A., et al. (2011). TLR4 mutation reduces microglial activation, increases Abeta deposits and exacerbates cognitive deficits in a mouse model of Alzheimer's disease. *J. Neuroinflammation* 8, 92. doi: 10.1186/1742-2094-8-92

Song, W. M., Joshita, S., Zhou, Y., Ulland, T. K., Gilfillan, S., and Colonna, M. (2018). Humanized TREM2 mice reveal microglia-intrinsic and -extrinsic effects of R47H polymorphism. *J. Exp. Med.* 215 (3), 745–760. doi: 10.1084/jem.20171529

Staal, R. G. W., Khayrullina, T., Zhang, H., Davis, S., Fallon, S. M., Cajina, M., et al. (2017a). Inhibition of the potassium channel KCa3.1 by senicapoc reverses tactile allodynia in rats with peripheral nerve injury. *Eur. J. Pharmacol.* 795, 1–7. doi: 10.1016/j.ejphar.2016.11.031

Staal, R. G. W., Weinstein, J. R., Nattini, M., Cajina, M., Chandresana, G., and Moller, T. (2017b). Senicapoc: repurposing a drug to target microglia KCa3.1 in stroke. *Neurochem. Res.* 42 (9), 2639–2645. doi: 10.1007/s11064-017-2223-y

Strasser, B., Becker, K., Fuchs, D., and Gostner, J. M., (2017). Kynurenine pathway metabolism and immune activation: peripheral measurements in psychiatric and co-morbid conditions. *Neuropharmacology* 112, Pt B, 286–296. doi: 10.1016/j.neuropharm.2016.02.030

Sudom, A., Talreja, S., Danao, J., Bragg, E., Kegel, R., Min, X., et al. (2018). Molecular basis for the loss-of-function effects of the Alzheimer's disease–associated R47H variant of the immune receptor TREM2. *J. Biol. Chem.* 293 (32), 12634–12646. doi: 10.1074/jbc.RA118.002352

Tahara, K., Kim, H. D., Jin, J. J., Maxwell, J. A., Li, L., and Fukuchi, K. (2006). Role of toll-like receptor signalling in Abeta uptake and clearance. *Brain* 129 (Pt 11), 3006–3019. doi: 10.1093/brain/awl249

Takahashi, K., Rochford, C. D. P., and Neumann, H. (2005). Clearance of apoptotic neurons without inflammation by microglial triggering receptor expressed on myeloid cells-2. *J. Exp. Med.* 201 (4), 647–657. doi: 10.1084/jem.20041611

Takikawa, O., Yoshida, R., Kido, R., and Hayaishi, O. (1986). Tryptophan degradation in mice initiated by indoleamine 2,3-dioxygenase. *J. Biol. Chem.* 261 (8), 3648–3653.

Tavassoly, O., Sade, D., Bera, S., Shaham-Niv, S., Vocadlo, D. J., and Gazit, E. (2018). Quinolinic acid amyloid-like fibrillar assemblies seed α-synuclein aggregation. *J. Mol. Biol.* 430 (20), 3847–3862. doi: 10.1016/j.jmb.2018.08.002

Thawkar, B. S., and Kaur, G. (2019). Inhibitors of NF-kappaB and P2X7/NLRP3/caspase 1 pathway in microglia: novel therapeutic opportunities in neuroinflammation induced early-stage Alzheimer's disease. *J. Neuroimmunol.* 326, 62–74. doi: 10.1016/j.jneuroim.2018.11.010

Thei, L., Imm, J., Kaisis, E., Dallas, M. L., and Kerrigan, T. L. (2018). Microglia in Alzheimer's disease: a role for ion channels. *Front. Neurosci.* 12, 676. doi: 10.3389/fnins.2018.00676

Thornton, P., Sevalle, J., Deery, M. J., Fraser, G., Zhou, Y., Stahl, S., et al. (2017). TREM2 shedding by cleavage at the H157–S158 bond is accelerated for the Alzheimer's disease–associated H157Y variant. *EMBO Mol. Med.* 9 (10), 1366–1378. doi: 10.15252/emmm.201707673

Tohgi, H., Abe, T., Takahashi, S., Kimura, M., Takahashi, J., and Kikuchi, T. (1992). Concentrations of serotonin and its related substances in the cerebrospinal fluid in patients with Alzheimer type dementia. *Neurosci. Lett.* 141 (1), 9–12. doi: 10.1016/0304-3940(92)90322-X

Tohgi, H., Abe, T., Takahashi, S., Saheki, M., and Kimura, M. (1995). Indoleamine concentrations in cerebrospinal fluid from patients with Alzheimer type and Binswanger type dementias before and after administration of citalopram, a

synthetic serotonin uptake inhibitor. *J. Neural Transm. Park. Dis. Dement. Sect.* 9 (2-3), 121–131. doi: 10.1007/BF02259654

Török, N., Majláth, Z., Fülöp, F., Toldi, J., and Vécsei, L. (2016). Brain aging and disorders of the central nervous system: kynurenines and drug metabolism. *Curr. Drug Metab.* 17 (5), 412–429. doi: 10.2174/1389200217666151222155043

Ulland, T. K., and Colonna, M. (2018). TREM2—a key player in microglial biology and Alzheimer disease. *Nat. Rev. Neurol.* 14 (11), 667–675. doi: 10.1038/s41582-018-0072-1

Ulrich, J. D., Ulland, T. K., Colonna, M., and Holtzman, D. M. (2017). Elucidating the role of TREM2 in Alzheimer's disease. *Neuron* 94 (2), 237–248. doi: 10.1016/j.neuron.2017.02.042

Ulrich, J. D., Ulland, T. K., Mahan, T. E., Nystrom, S., Nilsson, K. P., Song, W. M., et al. (2018). ApoE facilitates the microglial response to amyloid plaque pathology. *J. Exp. Med.* 215 (4), 1047–1058. doi: 10.1084/jem.20171265

Varki, A. (2009). Natural ligands for CD33-related siglecs? *Glycobiology* 19 (8), 810–812. doi: 10.1093/glycob/cwp063

Venegas, C., Kumar, S., Franklin, B. S., Dierkes, T., Brinkschulte, R., Tejera, D., et al. (2017). Microglia-derived ASC specks cross-seed amyloid-beta in Alzheimer's disease. *Nature* 552 (7685), 355–361. doi: 10.1038/nature25158

Verkhratsky, A., Parpura, V., Pekna, M., Pekny, M., and Sofroniew, M. (2014). Glia in the pathogenesis of neurodegenerative diseases. *Biochem. Soc. Trans.* 42 (5), 1291–1301. doi: 10.1042/BST20140107

Walker, D. G., Whetzel, A. M., Serrano, G., Sue, L. I., Beach, T. G., and Lue, L. F. (2015). Association of CD33 polymorphism rs3865444 with Alzheimer's disease pathology and CD33 expression in human cerebral cortex. *Neurobiol. Aging* 36 (2), 571–582. doi: 10.1016/j.neurobiolaging.2014.09.023

Walter, S., Letiembre, M., Liu, Y., Heine, H., Penke, B., Hao, W., et al. (2007). Role of the toll-like receptor 4 in neuroinflammation in Alzheimer's disease. *Cell. Physiol. Biochem.* 20 (6), 947–956. doi: 10.1159/000110455

Wang, Y., Cella, M., Mallinson, K., Ulrich, Jason, D., Young, et al. (2015). TREM2 lipid sensing sustains the microglial response in an Alzheimer's disease model. *Cell* 160 (6), 1061–1071. doi: 10.1016/j.cell.2015.01.049

Wang, Y., Ulland, T. K., Ulrich, J. D., Song, W., Tzaferis, J. A., Hole, J. T., et al. (2016). TREM2-mediated early microglial response limits diffusion and toxicity of amyloid plaques. *J. Exp. Med.* 213 (5), 667–675. doi: 10.1084/jem.20151948

Watts, R. J., and Dennis, M. S. (2013). Bispecific antibodies for delivery into the brain. *Curr. Opin. Chem. Biol.* 17 (3), 393–399. doi: 10.1016/j.cbpa.2013.03.023

Wennström, M., Nielsen, H. M., Orhan, F., Londos, E., Minthon, L., and Erhardt, S. (2014). Kynurenic acid levels in cerebrospinal fluid from patients with Alzheimer's disease or dementia with Lewy bodies. *Int. J. Tryptophan. Res.* 7, 1–7. doi: 10.4137/IJTR.S13958

Wes, P. D., Holtman, I. R., Boddeke, E. W., Möller, T., and Eggen, B. J. (2016). Next generation transcriptomics and genomics elucidate biological complexity of microglia in health and disease. *Glia* 64 (2), 197–213. doi: 10.1002/glia.22866

Widner, B., Leblhuber, F., Walli, J., Tilz, G. P., Demel, U., Fuchs, D. (1999). Degradation of tryptophan in neurodegenerative disorders. *Adv. Exp. Med. Biol.* 467, 133–138. doi: 10.1007/978-1-4615-4709-9_19

Widner, B., Leblhuber, F., Walli, J., Tilz, G. P., Demel, U., and Fuchs, D. (2000). Tryptophan degradation and immune activation in Alzheimer's disease. *J. Neural. Transm.* (Vienna). 107 (3), 343–353. doi: 10.1007/s007020050029

Wirths, O., Breyhan, H., Marcello, A., Cotel, M. C., Bruck, W., and Bayer, T. A. (2010). Inflammatory changes are tightly associated with neurodegeneration in the brain and spinal cord of the APP/PS1KI mouse model of Alzheimer's disease. *Neurobiol. Aging* 31 (5), 747–757. doi: 10.1016/j.neurobiolaging.2008.06.011

Wong, D. T., Bymaster, F. P., and Engleman, E. A. (1995). Prozac (fluoxetine, Lilly 110140), the first selective serotonin uptake inhibitor and an antidepressant drug: twenty years since its first publication. *Life Sci.* 57 (5), 411–441. doi: 10.1016/0024-3205(95)00209-O

Wu, H.-Q., Lee, S.-C., Scharfman, H. E., and Schwarcz, R. (2002). L-4-chlorokynurenine attenuates kainate-induced seizures and lesions in the rat. *Exp. Neurol.* 177 (1), 222–232. doi: 10.1006/exnr.2002.7971

Wu, H. Q., Lee, S. C., and Schwarcz, R. (2000). Systemic administration of 4-chlorokynurenine prevents quinolinate neurotoxicity in the rat hippocampus. *Eur. J. Pharmacol.* 390 (3), 267–274. doi: 10.1016/S0014-2999(00)00024-8

Wu, W., Nicolazzo, J. A., Wen, L., Chung, R., Stankovic, R., Bao, S. S., et al. (2013). Expression of tryptophan 2,3-dioxygenase and production of kynurenine pathway metabolites in triple transgenic mice and human Alzheimer's disease brain. *PLoS One* 8 (4), e59749. doi: 10.1371/journal.pone.0059749

Xia, X. M., Fakler, B., Rivard, A., Wayman, G., Johnson-Pais, T., Keen, J. E., et al. (1998). Mechanism of calcium gating in small-conductance calcium-activated potassium channels. *Nature* 395 (6701), 503–507. doi: 10.1038/26758

Yeh, F., Wang, Y., Tom, I., Gonzalez, L., and Sheng, M. (2016). TREM2 binds to apolipoproteins, including APOE and CLU/APOJ, and thereby facilitates uptake of amyloid-beta by microglia. *Neuron* 91 (2), 328–340. doi: 10.1016/j.neuron.2016.06.015

Yuan, P., Condello, C., Keene, C. D., Wang, Y., Bird, T. D., Paul, S. M., et al. (2016). TREM2 haplodeficiency in mice and humans impairs the microglia barrier function leading to decreased amyloid compaction and severe axonal dystrophy. *Neuron* 90 (4), 724–739. doi: 10.1016/j.neuron.2016.05.003

Zádori, D., Veres, G., Szalárdy, L., Klivényi, P., and Vécsei, L. (2018). Alzheimer's disease: recent concepts on the relation of mitochondrial disturbances, excitotoxicity, neuroinflammation, and kynurenines. *J. Alzheimers Dis.* 62 (2), 523–547. doi: 10.3233/JAD-170929

Zhang, W., Wang, L. Z., Yu, J. T., Chi, Z. F., and Tan, L. (2012). Increased expressions of TLR2 and TLR4 on peripheral blood mononuclear cells from patients with Alzheimer's disease. *J. Neurol. Sci.* 315 (1-2), 67–71. doi: 10.1016/j.jns.2011.11.032

Zhang, Y., Kang, S. A., Mukherjee, T., Bale, S., Crane, B. R., Begley, T. P., et al. (2007). Crystal structure and mechanism of tryptophan 2,3-dioxygenase, a heme enzyme involved in tryptophan catabolism and in quinolinate biosynthesis. *Biochemistry* 46 (1), 145–155. doi: 10.1021/bi0620095

Zhao, Y., Wu, X., Li, X., Jiang, L. L., Gui, X., Liu, Y., et al. (2018). TREM2 is a receptor for beta-amyloid that mediates microglial function. *Neuron* 97 (5), 1023–1031 e1027. doi: 10.1016/j.neuron.2018.01.031

Zhong, L., Wang, Z., Wang, D., Wang, Z., Martens, Y. A., Wu, L., et al. (2018). Amyloid-beta modulates microglial responses by binding to the triggering receptor expressed on myeloid cells 2 (TREM2). *Mol. Neurodegener.* 13 (1), 15. doi: 10.1186/s13024-018-0247-7

Zhu, G., Xu, Y., Cen, X., Nandakumar, K. S., Liu, S., and Cheng, K. (2018). Targeting pattern-recognition receptors to discover new small molecule immune modulators. *Eur. J. Med. Chem.* 144, 82–92. doi: 10.1016/j.ejmech.2017.12.026

Zuchero, J. B., and Barres, B. A. (2015). Glia in mammalian development and disease. *Development* 142 (22), 3805–3809. doi: 10.1242/dev.129304

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REVIEW
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Neuroinflammation as a Common Feature of Neurodegenerative Disorders

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Neurodegenerative diseases share the fact that they derive from altered proteins that undergo an unfolding process followed by formation of β-structures and a pathological tendency to self-aggregate in neuronal cells. This is a characteristic of tau protein in Alzheimer's disease and several tauopathies associated with tau unfolding, α-synuclein in Parkinson's disease, and huntingtin in Huntington disease. Usually, the self-aggregation products are toxic to these cells, and toxicity spreads all over different brain areas. We have postulated that these protein unfolding events are the molecular alterations that trigger several neurodegenerative disorders. Most interestingly, these events occur as a result of neuroinflammatory cascades involving alterations in the cross-talks between glial cells and neurons as a consequence of the activation of microglia and astrocytes. The model we have hypothesized for Alzheimer's disease involves damage signals that promote glial activation, followed by nuclear factor NF-kβ activation, synthesis, and release of proinflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1, IL-6, and IL-12 that affect neuronal receptors with an overactivation of protein kinases. These patterns of pathological events can be applied to several neurodegenerative disorders. In this context, the involvement of innate immunity seems to be a major paradigm in the pathogenesis of these diseases. This is an important element for the search for potential therapeutic approaches for all these brain disorders.

Keywords: Alzheimer's disease, Parkinson's disease, tauopathies, neuroinflammation, microglia, astrocytes, proinflammatory cytokines

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CROSS-TALKS BETWEEN GLIAL CELLS AND NEURONS AND ORIGINS OF ALZHEIMER'S DISEASE

The German physician Alois Alzheimer discovered, in the beginning of the past century, a neuropsychiatric disorder, with clinical features of a dementia, called Alzheimer's disease (AD) after him. He analyzed the *postmortem* brain of an elderly woman with cognitive impairment and found anomalous structures which correspond to the intracellular neurofibrillary tangles (NFTs) formed by aggregates of hyperphosphorylated tau protein. These along with the oligomers of β-amyloid (Aβ) peptide became the major hallmarks of this disease. Along with these hallmarks, during many years of research, several factors have been elucidated, neuroinflammation being a key element in the development of the disease. In dementia, one of the most frequent is AD that affects mainly people

over 65 years old. Because of the expansion of life expectancy, AD has become a major health problem, with an estimated 50 million people all over the world having it (Bettens et al., 2010). According to the World Health Organization (WHO), AD progressively affects learning and memory as well as mood and behavior, displaying a constantly increasing prevalence and impact (Maccioni, 2012; Guzman-Martinez et al., 2013).

A major constituent of NFTs is a hyperphosphorylated form of the axonal protein tau, whereas a major constituent of senile plaques (SPs) is Aβ protein. SPs are extracellular deposits and correspond to deposition of Aβ peptides, derived from the amyloid precursor protein (AβPP) (Chapman et al., 2002). Aβ is generated by a sequential processing of the AβPP by two proteases and usually exported from the brain to the cerebrospinal fluid (CSF) and local degradation by microglia, the major constituent of the brain's innate immune system. In principle, microglia can engulf Aβ by phagocytosis (Heneka et al., 2015).

Hyperphosphorylated tau protein originally forms oligomeric structures called paired helical filaments (PHFs); then it turns into NFTs. The deposition of these structures causes loss of synaptic function and finally neuronal death (Giannakopoulos et al., 2003). Evidence supports the toxicity of tau aggregates when they are exported into the extracellular environment, along with being spread all over the brain (Neumann et al., 2011; Andrade et al., 2017). Studies of cell morphology and organelle distribution under tau overexpression show alterations in transport through the axis by motor axonal microtubule-associated proteins (MAPs) (Cambiazo et al., 1995).

On the other hand, in AD pathophysiology, a key event is neuroinflammation in the central nervous system (CNS). Thus, in this review, we will focus on how neuroinflammatory processes are directly related to cognitive impairment and to the neurodegenerative processes, describing yet the implications of the involvement of both astrocytes and microglia in the inflammatory and neuro-immunomodulatory processes (Fernandez et al., 2008; Morales et al., 2010; Maccioni, 2011; Neumann et al., 2011). The microglial cells regulate the innate immune functions of astrocytes, under both physiological and pathological conditions; the inflammatory factors released by activated microglia can induce transduction of intracellular signals in astrocytes. On the other hand, the reactive astrocytes release factors that favor changes in the permeability of the blood–brain barrier (BBB), resulting in the recruitment of immune cells in the brain parenchyma. This leads to an amplification of the initial innate immune response. In turn, these reactive astrocytes secrete a wide range of factors, such as neurotrophic factors, growth factors, and cytokines, promoting neuronal survival, neurite growth, and neurogenesis. Both the microglia and the astrocytes release various signaling molecules, establishing an autocrine feedback. The feedback between both types of glial cells generates a close reciprocal modulation for various lesions in the CNS (Jha et al., 2019).

There are several neuroinflammatory factors that are involved in both the onset and the progression of AD. This process depends on the innate immune system which includes microglia and astrocytes (Maccioni et al., 2009). Residues from bacteria, viruses, fungi, abnormal endogenous proteins, iron overload, complement factors, antibodies, cytokines, and chemokines, including toll-like receptors (TLRs) and receptor for advanced glycation end products (RAGE), comprise a large number of damage signals, which represent a danger for homeostasis of the CNS, and participate in microglial action and its activation (Shastri et al., 2013). Under these conditions, microglial cells regulate the expression of different surface markers, such as the major histocompatibility complex II (MHC-II) molecular pattern recognition receptors (PPRs), which produce cytokine proinflammatory drugs such as interleukin (IL) 1 beta (IL-1β), IL-6, IL-12, interferon (IFN) gamma (IFN-γ), and tumor necrosis factor (TNF) alpha (TNF-α). They also synthesize and release short-lived cytotoxic factors, such as superoxide radicals (O_2^-), nitric oxide (NO), and reactive oxygen species (ROS) (Meda et al., 2001; Colton and Wilcock, 2010). Therefore, and in relation to the above, microglial cells have an important role in innate immunity and are the main source of proinflammatory factors in the human brain. The microglial activation process depends on phenotypic characteristics and is functionally diverse, because the response depends on the type, intensity, and context of the stimulus that generates it. The factors that affect microglia can also generate neuroprotection. Under pathological conditions, neurotoxicity will be expressed, due to the breakdown of the delicate balance between neurotoxic and neuroprotective effects.

Microglial cells exhibit ramified processes having high motility and allowing a dynamic and continual survey of the healthy brain as observed by using *in vivo* two-photon imaging (Nimmerjahn et al., 2005). They sample, detect, and eliminate debris or apoptotic neurons by phagocytosis, but this ability is considerably decreased in a proinflammatory context (Koenigsknecht-Talboo and Landreth, 2005). Microglia is involved in multiple processes such as neurogenesis, synapse elimination in a complement-dependent manner, or synapse plasticity (Paolicelli et al., 2014). The involvement of microglia in AD pathogenesis was studied in the light of the Aβ (Guillot-Sestier et al., 2015; Heneka et al., 2015) and also in the context of tau oligomerization (Maccioni et al., 2009; Morales et al., 2010; Maccioni, 2012; Morales et al., 2014).

Another key factor is the accumulation of monocytes and microglia around blood vessels, due to the CCL2 chemoattractant protein and its affinity receptor CCR2. Studies showed that removal of the receptor increases the microglia accumulation phenomena, possibly through recruitment of mononuclear phagocytes and bone marrow, which promotes the deposition of perivascular Aβ (Ransohoff, 2016b). Care should be taken, since most experiments of circulating monocytes include conditions in which the BBB is open by irradiation procedures in AD, leading to controversy. Interestingly, the reduction of monocyte infiltration following ccr2 deficiency has been involved in tau hyperphosphorylation in traumatic brain injury (TBI) (Ransohoff, 2016b).

It is known that Aβ oligomers induce the activation of microglia through oligomers-surface receptors such as TLRs, being part of a physiological duty to eliminate them *via* phagocytosis (Walter et al., 2007). Certain receptors are associated with the reduction of microglial Aβ phagocytic capacity like triggering receptors expressed in myeloid cells 2 (*TREM2*), whose specific missense mutations increase the risk of AD (Jonsson et al., 2013;

Parhizkar et al., 2019). Deficiencies in receptor CX3CR1, a chemokine CX3CL1 microglial receptor which partly mediates the infiltration of monocytes, induce overexpression or activation of microglia and tau hyperphosphorylation (Maphis et al., 2015), increased CD33 with a specific single-nucleotide polymorphism (SNP) in the promoter which inhibits immune response promoting $A\beta_{1-42}$ accumulation (Griciuc et al., 2013), B3 domain-containing transcription factor ABI3 (*ABI3*) (Sims et al., 2017), and several other factors.

On the other hand, some variants in phospholipase C gamma 2 (*PLCG2*) have a positive outcome for AD, reducing the late onset of the disease (Sims et al., 2017). Friedman et al. (2018) determined the gene expression profile associated with neurodegeneration, where 75% of these genes are linked with gene ontology (GO) related to plasmatic membrane. Altogether, this information and the effect of mutations in several receptors and other plasma membrane proteins suggest that changes depend on the interaction with the environment. The paper of Keren-Shaul et al. (2017) describes a new kind of microglia, the disease-associated microglia (DAM) that only gets expressed in AD. The study determined several gene modules. In DAM, a neurodegeneration gene core is expressed. Other modules include the IFN gene. In AD animal models, there are abundant cells that express the IFN module. The DAM gene expression changes as follows: there is downregulation of homeostatic genes like CX3CR1 and upregulation of genes associated with the disease like Apoε and phagocytic genes for plaque clearance. Every microglia has promoters and enhancers associated with DAMs, indicating that these stage changes might pass through an epigenomic change. They also discovered the three stages of microglia: homeostatic, intermediated, and finally DAM stage through an unknown Trem2-independent mechanism. In microglia gene expression modules, lipopolysaccharide (LPS) and neutrophil/monocyte are exacerbated, suggesting that inflammation and infiltration elements are involved in the neurodegenerative disease (ND).

Along with microglia, astrocytes are involved in the neuroinflammation process. Astrocytes have roles in metabolic regulation, neuronal scaffold, and synaptogenesis. In addition, there is a close contact with microglia and blood vessels in BBB (Morales et al., 2014). It also participates in the clearance of Aβ, by enzyme secretion (Mulder et al., 2012), and APOε from the ε2 allele is considered a protective factor. (Koistinaho et al., 2004). Like microglia, astrocytes also surround Aβ plaques (Medeiros and LaFerla, 2013), turning into an activated phase. Calcium deregulation, expression of the APOε4 allele, gives rise to APOε4 activity, which does not affect the synthesis of Aβ but does increase the deposition of the same, meaning a defect in the Aβ clearance (Holtzman et al., 2000). The astrocytes can be activated through a pathway involving NF-κB, to release a C3 complement which binds to the C3aR receptor, inducing neuronal damage (Lian et al., 2015), along with soluble CD40, which binds to microglia and induces the release of TNF-α and other proinflammatory cytokines (Frankola et al., 2011). In neuroinflammation, astrocytes also contribute to NO toxicity, by expressing inducible NO synthase (iNOS) (Phillips et al., 2014). Besides, there is overexpression of the glial fibrillary acidic protein (GFAP), a protein essential in the astrocyte cytoskeleton, related to astrocyte activation (Hol and Pekny, 2015). In the tauopathy context, Aβ can bind to the calcium sensing receptor (CaSR) in astrocytes, which triggers signaling pathways involved in the production and release of phosphorylated tau (Chiarini et al., 2017).

NEUROINFLAMMATION IN AD

Neuroinflammation is a process related with the onset of several neurodegenerative disorders and it is an important contributor to AD pathogenesis and progression. Several damage signals appear to induce neuroinflammation, such as trauma, infection, oxidative agents, redox iron, oligomers of tau, and Aβ. In effect, neuroinflammation is responsible for an abnormal secretion of proinflammatory cytokines that trigger signaling pathways that activate brain tau hyperphosphorylation in residues that are not modified under normal physiological conditions. Indeed, evidence exists that AD pathogenesis is not restricted to the neuronal compartment but includes strong interactions with immunological cells in the brain such as astrocytes, microglia, and infiltrating immune cells from the periphery, which could contribute to the modification of the process of neuroinflammation and neurodegeneration in AD brains. In this context, this is where our theory of neuroimmunomodulation plays an important role and focuses on the link between neuronal damage and brain inflammatory process, mediated by the progressive activation of astrocytes and microglial cells with the consequent overproduction of proinflammatory agents (Maccioni et al., 2009). Despite clinical and pathological differences, increasing experimental evidence indicates that neuroinflammatory events lead to tau protein misfolding (Cortes et al., 2018).

The participation of the innate immune system in disease progression has shown a harmful bidirectional connection with regard to tau pathology. It is known that the tau protein belongs to the family of MAPs and is expressed mainly by neurons with preferential axonal localization. It has been observed that tau *in vitro* promotes the polymerization of tubulin and decreases the transition rate between the phases of growth and contraction, generating a stable but dynamic state in microtubules (Weingarten et al., 1975; Drechsel et al., 1992).

Tau is found mainly in axons, but a small amount is distributed physiologically in dendrites. The postsynaptic function of tau is not yet well defined, but it may be involved in synaptic plasticity. On the other hand, in addition to axons and dendrites, a nuclear function of tau (Citron, 2010) has been discovered, which could be regulating transcriptional activity and maintaining DNA/RNA integrity under physiological and stress conditions (Weingarten et al., 1975; Violet et al., 2014).

The tau structure corresponds to a hierarchical phosphorylation process in which different sites modulate the conformation of the protein, promoting the action of secondary kinases. In AD, different sites are phosphorylated earlier than others, leading to the creation of new epitopes. This sequential process has been studied by the use of antibodies such us AT100, whose epitopes in PHFs only appear after successive phosphorylation of residues Thr212 and Ser214, by glycogen synthase kinase (GSK)-3β and protein kinase A (PKA) along with Ser199, Ser202, Ser208, and Thr205 (Bussiere et al., 1999; Malia et al., 2016).

It was also shown that the expression of tau by microglial cells promotes its activation (Wang et al., 2013). Overall, the exact pathway leading to phosphorylation of tau remains poorly defined, but subsequent structural changes induce its detachment from the microtubules and produce higher levels of soluble free tau. Before the formation of NFTs, the hyperphosphorylation of tau favors a dynamic and progressive self-assembly of tau in oligomeric forms and insoluble materials such as PHFs throughout the disease with different degrees of neurotoxicity (Braak and Braak, 1991).

NEUROINFLAMMATION IN SEVERAL TAUOPATHIES

Neuroinflammation in the Context of Tau and Tauopathies

These neurodegenerative disorders (tauopathies) do not have a defined clinical, biochemical, and morphological characteristic, like other diseases. Neurodegenerative disorders are distinguished by accumulation of misfolded proteins, such as α-synuclein (α-syn) protein in Parkinson's disease (PD) and tau protein in AD (Cortes et al., 2018). An important group of degenerative diseases are the so-called tauopathies, which consist in the pathological accumulation of tau protein in intracellular fibrillary aggregates. The spectrum of tauopathies covers a large number of disorders such as progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), frontotemporal dementia (FTD), FTD and parkinsonism linked to chromosome 17, chronic traumatic encephalopathy, and argyrophilic grain disease (Spillantini and Goedert, 2013; Arendt et al., 2016). On the other hand, this last pathology is identified by atrophy of the ambient gyrus and presence of argyrophilic and 4R-tau immunoreactive grains in medial temporal-lobe structures (Tolnay and Clavaguera, 2004). On the other hand, AD is considered a secondary tauopathy because it also presents aggregates of Aβ (SPs) (Musiek and Holtzman, 2015). On the other hand, the pathological aggregates of tau protein, cells of the cerebral immune system, such as activated astrocytes and microglia, are other common pathological features of tauopathies (Wyss-Coray and Mucke, 2002; Ransohoff, 2016a). The existence of neuroinflammatory processes exacerbated in various tau pathologies, known as the *theory of neuroimmunomodulation*, was initially described in AD by Dr. Maccioni's group, in which the bases of the molecular cascades associated with these events were laid (Maccioni et al., 2009). Recent studies have discovered multiple mechanisms by which an overstimulation of glial cells causes a harmful neuroinflammation that would influence the tau pathology and accelerate the neurodegenerative processes. The chronic activation of glial cells alters the physiological function of the tau protein, inducing the activation of enzymes that phosphorylate tau, such as the enzymes CDK5 and GSK-3β, giving way to the formation of NFTs, thus decreasing the neuronal capacity (Wyss-Coray and Mucke, 2002; Lull and Block, 2010; Ransohoff, 2016a). In addition, glial cells can also contribute physically to the spread of tau pathology (Asai et al., 2015). In turn, the glial cells are also positively fed back by the tau pathology, since the degenerating neurons and their axons and dendrites release aggregated and toxic tau species, generating a constant neuroinflammatory cycle (Morales et al., 2010; Morales et al., 2013; Cortes et al., 2018).

As indicated, activated microglia release proinflammatory cytokines to their cell environment, among which we can highlight IL-1β, IL-6, IL-12, IFN-γ, and TNF-α. In turn, they can also produce ROS and NO, among others that may be characteristic of neurodegenerative disorders (Wang et al., 2015). On the other hand, the astrocytes are the main and most numerous of the glial cells; they are fundamental to supporting the function and health of the neuronal cells. In turn, astrogliosis can also be considered as an important factor in chronic neuroinflammation, affecting considerably the neuron and its integrity (Sofroniew and Vinters, 2010). As microglia, astrocytes also synthesize and secrete proinflammatory cytokines. In addition, experimental evidence has indicated that ILs such as IL-1β, TNF-α, IL-6, and C1q (secreted by the microglia) coactivate the astrocytes, resulting in neuronal dysfunction and ultimately death (Jacobs and Tavitian, 2012). In contrast, in PSP, only IL-1β increased significantly in the *substantia nigra* and in the *subthalamic nucleus* (Fernandez-Botran et al., 2011; Lopez Gonzalez et al., 2016).

Currently, there is an increase in the evidence that the pathological activation of both microglia and astrocytes causes chronic neuroinflammation in patients with tauopathies, negatively affecting the progression of the disease, although the first signs of neuroinflammation considered reactive gliosis in tauopathies and other NDs (Leyns and Holtzman, 2017).

Frontotemporal Dementia

More than a new pathological entity is the redefinition of the classic Pick's disease. The term FTD is used to mean all those primary degenerative processes of the anterior portion of the brain, characterized by their clinical manifestations, neuroimaging findings, and histopathological elements, which are of particular importance for psychiatry, due to the tendency of patients to present behavioural disorders, being a frequent cause of dementia (Neary et al., 1988). FTD is a heterogeneous syndrome that involves several disorders that originate mainly in the frontal lobe and temporal areas of the human brain. These alterations can cause problems in language and motor and behavioural disorders (Neary et al., 1998; Olney et al., 2017). In terms of prevalence, after AD, FTD is considered the second most important type of neurodegenerative disorder (Knopman and Roberts, 2011; Hughes et al., 2015). The onset of FTD is between 45 and 65 years of age, with a survival range of between 2 and 20 years, averaging 8 years. In turn, demographic data indicate that the distribution of this disease is similar among men and women (Hodges et al., 2003; Onyike and Diehl-Schmid, 2013; Coyle-Gilchrist et al., 2016), with a prevalence between 15 and 22 people per 100,000 inhabitants, varying according to the age of onset (Irwin et al., 2015). On the other hand, FTD associated with pathological tau represents 36–50% of all cases of FTD (Bang et al., 2015). There are different subtypes of FTD, which are directly related to an existing clinical classification, establishing the following main forms of FTD: the behavior variant of FTD (bvFTD), the non-fluid variant (nfFTD), and the semantic variant (svFTD).

Both nfFTD and svFTD are classified as primary progressive aphasia, since they mainly affect language functions, according

to the criteria of clinical diagnosis. The type of FTD that presents the greatest insight is bvFTD, which covers about 60% of cases (Rascovsky et al., 2011; Onyike and Diehl-Schmid, 2013; Olney et al., 2017). Likewise, other alterations in the FTD category are related to motor neuron disorder (MNFTD), PSP-FTD, and corticobasal syndrome (CBS) (Wang and Mandelkow, 2015; Olney et al., 2017). In the same context, frontotemporal lobar degeneration (FTLD) is based on the neuropathological alterations that occur in the frontal and temporal lobes. Different types of FTD are determined by protein aggregates. In this way, the protein aggregates associated with FTD are the following: (i) tau protein (FTLD-tau); (ii) ubiquitin proteasome system (FTLD-UPS); (iii) transactive response of the DNA binding protein (FTLD-TDP); and (iv) fused in sarcoma, Ewing sarcoma, and TAF15 protein family (FTLD-FET) (Mackenzie et al., 2010; Irwin et al., 2015; Olney et al., 2017). For the purpose of this review, we focus on FTDs caused by pathological inclusions of tau, which correspond to approximately half of FTDs, whose histopathological hallmark corresponds to aggregates of tau protein in both neurons and glial cells (Sieben et al., 2012). These include Pick's disease (PiD-FTD), CB, PSP-FTD, and other rare FTDs, described as globular glial tauopathies and argyrophilic grain disease, which are included in former classifications (Mackenzie et al., 2010; Ghetti et al., 2015; Olney et al., 2017). The pathologies described previously are principally characterized by repetitions of 4R-tau sequences; on the other hand, PiD-FTD are related to 3R-tau aggregates (Ghetti et al., 2015).

Previously, a study reported an increase in the levels of cytokines associated to inflammation, specifically TNF-α and transforming growth factor (TGF)-β in subjects affected by an unspecified form of FTD as compared to healthy people, suggesting a likely interference of inflammatory proteins in the pathogenesis of FTD that was immediately verified by Bellucci et al. (2004); this group reported a considerable rise of cytokines as IL-1 and cyclooxygenase-2 (COX2), both being involved in proinflammatory response; also, studies in transgenic mice with mutation of tau show activation of microglia cells with tau inclusions in the brainstem and spinal cord. Moreover, studies conducted in transgenic mouse with mutation of human tau associated with FTD model reported microgliosis and synaptic disruption, prior to formation of NFT in the hippocampus, suggesting that inflammatory response can carry over to the formation of NFT in FTD (Yoshiyama et al., 2007), in accordance with our theory of neuroimmunomodulation (Fernandez et al., 2008; Rojo et al., 2008; Maccioni et al., 2009). In turn, several trans-models with the FTD tau mutation model have also reported a microglial activation with the subsequent inflammatory process, which further emphasizes that these alterations directly depended on the expression of tau (Wes et al., 2014). In this way, the neuroinflammatory process has been proposed as possible diagnostic tools, through *in vivo* uptake of the microglia, using positron emission tomography (PET) images with the translocator protein (TSPO) ligand [^{11}C]-PK11195 in the FTD and other tauopathies (Cagnin et al., 2004; Venneti et al., 2009; Zhang, 2015).

Progressive Supranuclear Palsy

It is a rare neurodegenerative disorder that is increasing over time. It affects movement, walking, balance, speech, swallowing, vision, mood, behavior, and thinking. One of the classic signs of the disease is the inability to focus and move the eyes correctly, which people can manifest as blurred vision. The prevalence of PSP is 5.8 to 6.5 per 100,000 (Ling, 2016). Like CBD, PSP presents hyperphosphorylated 4R-tau in neurons and glial cells. PSP is defined primarily by tau-positive NFTs, coiled bodies, threads, and tufted astrocytes, in contrast to the ballooned neurons, pre-tangles, threads, and astrocytic plaques that are characteristic of CBD (Yoshida, 2014).

Imaging studies used conventional magnetic resonance; atrophy can be seen at the level of the midbrain and superior cerebellar peduncle. In turn, when using diffusion tensor, the white matter of degeneration can be appreciated, especially in the superior cerebellar peduncles and the superior longitudinal fasciculus in the case of Richardson's syndrome; dopamine transporter single-photon emission computed tomography imaging shows reduced tracer uptake in the striatum; finally, fluorodeoxyglucose PET may identify focal midbrain hypometabolism (Yoshida, 2014; Ling, 2016; Kovacs, 2017).

The NFTs of tau in subcortical structures are a characteristic sign of PSP. These pathological aggregates of tau are located especially in the subthalamic nucleus, the basal ganglia, and the brainstem. The subcortical NFTs are associated in a variable but characteristic way, with astrocytes in tufts and spiral oligodendroglial bodies, as well as with threads, which present immunoreactivity for the isoform of tau 4 repetitions (4R-tau). Studies conducted by Williams et al. (2007) identified that certain brain regions are affected by these pathological tau aggregates. In this context, the pallido-luyso-nigral system is affected early, followed by the basal ganglia, the pontine nuclei, and the dentate nucleus; then the frontal and parietal lobes; and finally other neocortical areas and cerebellar structures (Williams et al., 2007; Kovacs, 2017). When doing comparative studies between CBD and PSP, a greater amount of pathological tau is observed in neurons in the anterior brain in CBD, whereas in PSP the structures of the posterior brain are mainly affected (Dickson, 1999). In addition, the different astroglial pathology associated with the pathological tau protein, associated with the presence of subcortical NFTs in the PSP, facilitates its neuropathological differentiation (Clavaguera et al., 2013).

Corticobasal Degeneration

This disease, a rare and progressive neurodegenerative disorder that affects about 4.9 to 7.3 per 100,000 of the population (Mahapatra et al., 2004), is characterized pathophysiologically by neuronal loss, asymmetric frontoparietal cortical atrophy, gliosis, and swollen achromatic neuronal cell bodies (Rebeiz et al., 1968; Gibb et al., 1989). However, the exact cause of CBD still remains unknown, and although CBD is considered a sporadic disease, there have been some reports of families with pathology similar to CBD (Uchihara and Nakayama, 2006) or mutations in tau protein, a gene linked to pathological findings similar to CBD (Mirra et al., 1999; Spillantini et al., 2000). The average age at which the symptoms begin to manifest in the affected subjects, due to this disease, is close to 62 ± 7 years. The average survival can vary from 2 to 13 years (Rinne et al., 1994c;

Schneider et al., 1997; Wenning et al., 1998; Josephs et al., 2006a). The youngest case, according to the pathological confirmation, began at age 45 (Wenning et al., 1998). Demographic studies indicate a higher incidence of this disease in women compared to men of the same age (Rinne et al., 1994c).

CBD can be characterized by the following signs and symptoms: action and postural tremor, resting tremor, bradykinesia, myoclonus, ideomotor apraxia, exotic extremity phenomena, extremity dystonia, gait deterioration, dysarthria, aphasia, speech apraxia, and/or dementia; these symptoms can be presented, in turn, in combination (Wenning et al., 1998). In this disease, cognitive deterioration is more affected in the area of speech and language (Josephs, 2010) and, in a lesser extent, visuospatial and perceptual deficits (Tang-Wai et al., 2003a; Bak et al., 2006). The CBD is molecularly characterized by deposits of hyperphosphorylated 4R-tau protein; this makes it possible to differentiate it from other tauopathies, which consist mainly of 3R-tau or a mixture of 3R- and 4R-tau (Trojanowski and Dickson, 2001). Brain neurons with CBD react positively with antibodies generated against ubiquitin, hyperphosphorylated tau, phosphorylated neurofilament protein, alpha-B crystal, and synaptophysin. In addition, aggregates of Aβ have been found in some cases of CBD, which are similar to those found in AD (Armstrong, 2015).

Neuropathological CBD alters the anatomical pathways associated with movement control: (i) striatum and (ii) *substantia nigra*, which usually presents with loss of pigmented neurons. In turn, it also presents at the neuronal level cytoplasmic inclusions and neuronal bodies with greater volume, compared with normal neuronal cells, also presenting glial pathology that affects both oligodendroglia and astrocytes (Armstrong et al., 2000). These damages are especially complex in the posterior frontal area anterior to the precentral gyrus, but they are lesser in the primary motor area (Tsuchiya et al., 1997). These neuronal hallmarks of CBD are found in several brain regions, including the superior temporal gyrus, the frontal cortex, the motor cortex, the brainstem tegmentum, the basal ganglia, the thalamus, and the amygdala (Halliday et al., 1995; Matsumoto et al., 1996).

The experimental evidence shows that the dysregulation of proinflammatory cytokines is one of the most harmful factors in tauopathies. Several investigations have been carried out, using neuroimaging technologies by means of PET, in order to examine more deeply the neuroinflammatory events in the neurodegenerative process. These studies have been used with markers that bind to the TSPO, which is expressed by glial cells (microglia and astrocytes) and other cells of the immune system infiltrating the brain. These findings have shown that the TSPO signal increases proportionally with the activation of microglia in various tauopathies, which include AD, PSP, FTD, and FTDP-17 (Maeda et al., 2011; Zhang, 2015), as well as other NDs, and in models of brain injuries such as FTD, PD, stroke, and traumatic brain injuries (Wu et al., 2013).

The overexpression of IL-1β, TNF-α, and IL-6 has one positive feedback, which generates a cascade that leads to an increase in the hyperphosphorylation of the tau protein, reducing the markers of synapses and finally leading to degeneration and neuronal death (Morales et al., 2014). Currently, less information is available about the levels of cytokine transcription in other less common tauopathies; However, the histological characteristics of activated glial cells are a common characteristic of tau protein aggregates (Morales et al., 2010; Leyns and Holtzman, 2017; Cortes et al., 2018).

In summary, tauopathies including AD involve the neuroinflammatory cascade that leads to tau modifications and subsequent oligomerization. However, in PD, the neuroinflammatory cascade seems to trigger α-syn oligomerization, an event that is critical in PD (**Figure 1**).

FUTURE DIRECTIONS: SEARCH FOR THERAPEUTIC TARGETS BASED ON THE NEUROIMMUNOLOGICAL MODEL

Alzheimer Disease

According to the information collected, the microglial cells become one of the points of convergence in the development of the neuroinflammatory process, since not only did the activation process and the changes that occur determine this condition, but it was also shown that a permanent activation can increase the permeability of the BBB and promote an increase in macrophage infiltration peripherals These alterations can complicate the vicinity of the damaged area and contribute to neuronal dysfunction, thus accelerating the neurodegenerative process (Zarrouk et al., 2018).

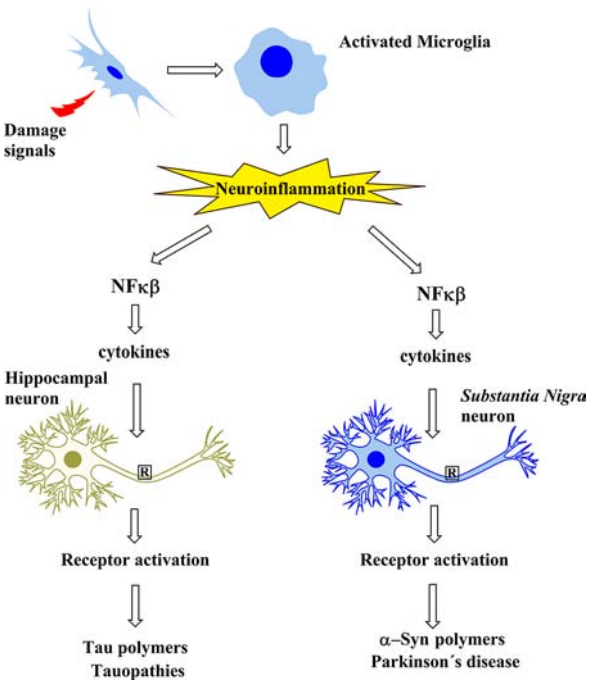

FIGURE 1 | Schematic representation showing that the protein aggregates involved in Alzheimer's disease and in tauopathies, as well as those of Parkinson's disease, share a common cascade of molecular signaling as a result of the activation of microglia by signals of damage.

Within the various factors that modulate the phenotypes activated by the glia, the APO immunomodulatory genotype and the recently identified AD genes can be included. Another important factor to mention is normal aging, which is also associated with the chronic activation of the glia (Zverova et al., 2018) and the factors induced by lesions depending on the focal stage. Context-dependent responses must be expected for nonsteroidal anti-inflammatory drugs (NSAIDs), which act as inhibitors of cyclooxygenase by reducing the concentrations of prostaglandin products, in particular PGE2, by tapping PTGER1–4 receptors and producing very different results. One can highlight the activation of PTGER2, which is predominantly dedicated to proinflammatory neurotoxic pathways, whereas the ligands of PTGER4 have been found to produce anti-inflammatory and neuroprotective effects (Salmon et al., 2015; Alzheimer's Association, 2018). Thus, NSAIDs that inhibit conventional cyclooxygenase could cause a blockage of the incipient pathogenesis of AD driven by inflammation in the early stages. In addition, these NSAIDs may have adverse effects in advanced disease, mainly by restriction of resolution and interference with phagocytic clearance of Aβ and extracellular tau aggregates (McKee et al., 2008).

Recent findings suggest for anti-Aβ immunotherapy that stage-dependent efficacy also stimulates microglial phagocytosis of Aβ, and the potential benefits could be seen only with early intervention. A remarkable argument is that aging or inflammation induced by Aβ or by lesions initiates tauopathy, leading to neurodegeneration and subsequent clinical decline. Therefore, possible explanations for the failure of immunotherapy to treat established dementia include an inability to stop the spread of fully established and planted tauopathy and to rescue deficits caused by the loss of neurons. Whatever the explanations for the failures of previous NSAID trials, based on new and convincing genetic evidence of the causal role of innate immunity in AD risk, new trials with longer and earlier interventions and alternative approaches to treatment are warranted.

There is evidence that neuroinflammation could drive the pathogenic process in AD. In this context, it should be noted that the brain can no longer be seen as an organ with immune privileges, and advances in immunology must be integrated into the known pathological pathways of various neurodegenerative disorders. Ligand–receptor interactions in the CNS microenvironment, which keep the microglia under strict control in the healthy brain, may be disturbed in chronic ND, although it is not yet clear when and how this occurs in AD. While it is true that the simple idea of activated microglia has been useful, it has undoubtedly prevented the understanding and recognition of the diversity of microglial phenotypes and the extraordinary plasticity of these cells. An important approach that future studies should have should be to better understand the individual contributions of microglia and other cell types to the neuroinflammatory response during the course of AD (Maccioni, 2012; Guzman-Martinez et al., 2013; Morales et al., 2013; Morales et al., 2014).

Innate immune cells of the brain can respond rapidly to systemic events, and these responses are further accentuated in the stages of aging and the diseased brain. In future studies, the effect of systemic comorbidities of AD (such as diabetes and hypertension), associated systemic inflammation, and aging as an important risk factor for AD should be considered in order to understand and exploit the immunological processes associated with AD. The recognition that the modification of the immune system contributes to the pathogenesis of chronic NDs could open several pathways in potential treatments to delay its onset and progression (Morales et al., 2014; Andrade et al., 2017; Cortes et al., 2018).

On the other hand, to date, treatments for AD have been have established based on the nature of the symptomatic AD and, in most cases, are used to counteract the disturbance produced in the level of the neurotransmitters involved in this disease. Therapies based on tau protein suggest that this protein is an interesting target, because the formation of PHFs constitute a critical event in the neurodegenerative process.

An important research suggests that the anti-inflammatory activity may be controlled with a natural formula which contains the Andean compound, a natural product endemic to the north of Chile, and vitamins of the B complex (i.e., B6, B9, and B12), called BrainUp-10®. Interestingly, according to studies done by Cornejo et al. (2011), the active principal of this compound, fulvic acid, is able to block auto-aggregation of tau *in vitro* that inhibits the length and morphology of the PHFs generated. Indeed, this compound can disassemble the preformed PHFs and oligomers and tau species released into the extracellular environment. This natural compound is a potent anti-inflammatory substance and a biologically safe nutraceutical. In a clinical trial pilot, patients with AD who underwent treatment with this compound showed less tendency towards cognitive deterioration, in addition to a reduction in neuropsychological symptoms and less neuropsychiatric stress for caregivers of patients. The Andean compound is a complex mixture of humic substances generated by decomposition of plant material through thousands of years (Carrasco-Gallardo et al., 2012). Currently, there are therapeutic approaches based on the use of antioxidant and anti-inflammatory nutraceutical compounds, a multi-target therapy which appears to have benefits as compared with the mono-target approach using drugs, thus contributing to the health and quality of life of AD patients. Studies based on this type of natural compounds have been growing, as well as the search for natural antioxidant compounds with a strong anti-inflammatory activity and the ability to cross the BBB. Therefore, an important strategy to prevent brain damage is based on changes in lifestyles, diet, and science-based nutraceuticals, among other multiple factors. However, there is a need for further investigations, in order to medically validate this natural approach (Andrade et al., 2017; Cortes et al., 2018).

Treatments for Tauopathies

Drugs such as cholinesterase inhibitors and *N*-methyl-D-aspartate receptor antagonist are being used to treat cognitive problems. On the other hand, speech and physical therapies have shown positive effects on patients with aphasia syndromes and alterations in motor function, respectively. Serotonin reuptake inhibitors (SSRIs) have been used as a therapy in cases of apathy and depression (Halliday et al., 1995). In addition, specifically,

medicaments such as sertraline, paroxetine, and fluvoxamine, which may improve behavioral and psychiatric symptoms but not cognitive impairment, have been selected to treat FTD due to the serotonergic deficit involved (Yoshida, 2014). Citalopram has been reported to lead to an improvement in behavioral symptoms after treatment (Irwin et al., 2015). Acetylcholinesterase inhibitors (AChEIs) are well-established treatments of AD, but until now, there is no report of their efficacy in patients affected by FTD (Kempster et al., 2007). Memantine, which has some effects in advanced AD and neuroprotective characteristics (Ling, 2016), could improve behavioral disorders and enhance metabolic rate in certain brain regions (Williams et al., 2007).

Treatment of cognitive and other symptoms of PSP based on serotonergic drugs has shown to be ineffective, despite its positive effect on depression (Dickson, 1999). Rivastigmine trials in a group of PSP patients reported a mild improvement in cognitive disorders (Garcia-Reitboeck et al., 2013) and had also reported improvement in neuropsychiatric symptoms, but they did not show an effect in cognition (Goedert et al., 2013). Levodopa trials have shown only 20–40% of response in patients affected by PSP, and studies of CBD patients have reported its ineffectiveness because of unresponsiveness (Harper et al., 2008). Actually, some therapeutic strategies are linked to inhibition of tau posttranslational modifications, proteolytic activity, and self-aggregation. The decrease of tau mRNA with antisense oligonucleotides has been reported to decrease harmful tau aggregates, disrupt neuronal loss, and increase life duration of transgenic mice expressing human tau harboring the disease-associated P301S mutation (PS19 mouse model). Furthermore, a study of monkeys has reported a drastic decrease of tau mRNA and protein in the CNS (King et al., 2013). Also, the Food and Drug Administration (FDA) has approved an antisense oligonucleotide directed against mutant survival motor neuron gene 1 (*SMN1*) for treatment of spinal muscular atrophy (Bezard et al., 2013), characterized by alteration of lower motor neurons. Studies of brain injury in mice treated with an antibody directed against phospho-tau (p-tau) reported that it halted formation of tau oligomers and complexes, prevented expansion of harmful tau in neighbor cells, decreased brain atrophy, and regained long-term potentiation (Arendt et al., 2016).

According to the existing relation among tauopathies and insulin resistance, therapies previously utilized for treatment of diabetes approved by the FDA are being tested in preclinical and clinical trials. Insulin supplied intranasally in subjects presenting amnestic mild cognitive disorder or AD has been reported to have cognitive benefits (Jochum et al., 2004), (Musiek and Holtzman, 2015), and thus, this opened the way to a new clinical trial: the Study of Nasal Insulin in the Fight Against Forgetfulness (SNIFF, NCT01767909). This trial is studying the effects of insulin supplied intranasally on cognition and brain atrophy; it is not actually certain how insulin administered by this way affects tau protein (Orr et al., 2017). Liraglutide, a drug which stimulates insulin production, has been shown to prevent and decrease the phosphorylation of tau in a mouse model of type II diabetes (Wyss-Coray and Mucke, 2002) and to decrease phosphorylated tau and improve motor function in a mouse model of tauopathy (hTauP301L); also, there is a report about improvement in motor function on this (Ransohoff, 2016a). Studies on 3xTg-AD mice treated with linagliptin has shown that there is a decrease of tau phosphorylation and improvement in cognition (Asai et al., 2015). Metformin, a suppressor of hepatic glucose production, tested in a neuronal cell model of insulin resistance has been reported to prevent tau phosphorylation (Sarazin et al., 2003). A decrease in pathological tau phosphorylation has been reported in trials with tau transgenic mice, along with an increase in tau cleavage, aggregation, synaptic disruption, and hind limb atrophy (Maccioni et al., 2009).

Immunotherapy-based antibodies against tau and active immunizations targeting pathogenic tau oligomers are actually in clinical trials (Riederer et al., 2003). These would prevent intercellular tau spread. For instance, tau vaccine (AADvac1) has been reported to have favorable safety and immunogenicity outcomes (Bussiere et al., 2003), and antibodies against tau have been shown to be assimilated by neurons in *ex vivo* cultured brain slices *in vivo* (Grant et al., 1997; Kovari et al., 2003; Josephs et al., 2006b; Yu et al., 2016), and tau linked to an antibody may be removed by a pathway implying lysosome (Krishnamurthy et al., 2011; Collin et al., 2014). Rapamycin, a mammalian target of rapamycin (mTOR) inhibitor, upregulates autophagy, thus decreasing aberrant tau and improving cognition in multiple mouse models (Laihinen et al., 1994; Lee et al., 1994; Rinne et al., 1994a; Rinne et al., 1994c), and prevents neuronal death in tau transgenic *Drosophila* (Rinne et al., 1994b). Studies conducted by Salonen et al (1994) with transgenic mice of human tau P301S, observed that rapamycin decreases both phosphorylation of tau and its aggregate state when it is delivered at different stages of the disease. In turn, in trials with 3xTg-AD mice, the intake of prophylactic rapamycin notably prevents the formation of aberrant tau deposits (Salonen et al., 1994). Analogs of rapamycin have been reported to increase autophagy, decrease p-tau and NFTs, and improve cognition in studies with tau transgenic mice (Tang-Wai et al., 2003b; Bak et al., 2006). The natural compound wogonin that inhibits mTOR demonstrated a reduction of p-tau in cultured cells (Tang-Wai et al., 2003a). In *Drosophila*, a decrease of tau-induced neurotoxicity has been achieved by multiple strategies such as heterochromatin loosening, genetic reversal of filamentous actin, nuclear envelope disorder, and decline of oxidative stress (Schmidt et al., 2001; Trojanowski and Dickson, 2001; Fulga et al., 2007; Kandimalla et al., 2014; Armstrong, 2015).

THE NEUROIMMUNE CONTEXT OF PD

Neuroimmunology of PD

After AD, the second most common neurodegenerative disorder is PD. This pathology affects the normal movement of the subject, as a consequence of multifactorial factors, such as environmental and genetic factors. The molecular basis is still unclear. The triggering causes have not yet been determined, but it is known that factors such as oligomerization of α-syn, mitochondrial dysfunction, oxidative stress, inflammation, and aging have pathogenic roles in the disease Its main neuropathological marker is the degeneration of neurons which contain neuromelanin in *substantia nigra pars compacta*; this results in loss of dopamine and cytoplasmic protein aggregates, called Lewy bodies (LBs),

mainly composed by α-syn filaments (Forno, 1996). α-Syn has 140 amino acids and three regions, a carboxyl end which is negatively charged, an amino terminal end which is charged positively, and a hydrophobic segment at the center, between residues 61 and 90 (considered as the non-amyloid component or NAC). The protein has four tyrosine residues, Tyr39 next to the amino region and Tyr125, Tyr133, and Tyr136 close to the C-terminus. It is also able to bind lipids (Uversky, 2007).

Among people over 50 years, ~2.0% is affected with PD (Maarouf et al., 2012). The clinical signature consists of motor, cognitive (dementia), neuropsychiatric (depression and anxiety), and autonomic dysfunctions (hypotension and constipation). The motor affections usually present (i) rest tremor, (ii) bradykinesia (impairment in the normal movement, mainly of complex voluntary movements), (iii) postural instability, and (iv) rigidity (Thomas and Beal, 2007). There are a diminished number of dopaminergic neurons in the *substantia nigra*, which consequently ameliorates dopamine in the striatum, promoting dysregulation in the basal ganglia. The previous effects trigger the motor symptoms observed. Briefly, this pathology is considered a ND characterized among the synucleinopathies, which also considers PD with dementia (PDD), LB dementia (DLB), and multiple-system atrophy (MSA). DLB is also able to present parkinsonism, but also hallucinations (mainly visual) and dementia (McKeith et al., 2005). These symptoms make the diagnosis for this disease difficult (McKeith et al., 2005).

Relationships Between Tauopathies and Synucleinopathies

α-Syn hyperphosphorylation promotes misfolding and oligomerization. α-Syn deposits are ubiquitous in the CNS, commonly at presynaptic neuron terminals. These molecular effects are categorized among synucleinopathies (Golde and Miller, 2009; Uversky, 2009). Synucleinopathies usually share their occurrence with tauopathies and different diseases commonly associated with protein misfolding. Although the etiology of most of the processes involved in these pathologies suggest the effect of misfolded proteins in pathways, also affecting them, they are still unknown (Jellinger, 2010a; Jellinger, 2010b; Kovacs et al., 2010). It seems there is an overlap in both diseases, but they also present different genetic, clinical and pathological characteristics. It is common to find NFTs and LB presence in the brain or in a cell (Arai et al., 2001; Iseki et al., 2003). Their co-occurrence has been previously reported several times by (i) Schneider et al. (2006), who found NFT in the substantia nigra of PD patients with displacement damage, and (ii) Joachim et al. (1987), who found them in the same region in AD patients, Down syndrome, and PD. Furthermore, the presence of p-tau has been noticed in dopaminergic neurons of PD and PDD subjects (Wills et al., 2010). p-Tau has also been seen in striatal neurons of a transgenic model that overexpress human α-syn (Haggerty et al., 2011). Moreover, it was noticed that the phosphorylation of the GSK-3β protein does not happen if the expression of α-syn is silenced (Duka et al., 2006). Finally, these mechanisms considering tau and α-syn can be discussed by this manner: the promotion of α-syn expression leads to its accumulation in the brain, so on, GSK-3β gets phosphorylated, which in turn phosphorylate tau (Duka et al., 2006). NFT start forming because of the increase in p-tau. It is also interesting that almost 60% of AD subjects present LB, in familial and sporadic cases (Arai et al., 2001; Jellinger, 2011). We can consider an aggressive progression of these pathologies and an accelerated cognitive dysfunction because of the presence of synucleinopathies and tauopathies at the same time (Langlais et al., 1993; Olichney et al., 1998; Kraybill et al., 2005). It is suggested that the synergistic interaction between tau, Aβ, α-syn and the activate form of GSK-3β could trigger their misfolding, oligomerization and accumulation (Giasson et al., 2003; Lee et al., 2004).

There has been studies that shown the tau/α-syn binding *in vitro*, which in turn, promote their phosphorylations (Jensen et al., 1999). Tau fibril generation could be induced by α-syn, but also they induce pathological filaments formation between each other when they are co-incubated (Giasson et al., 2003). The tau and α-syn interaction have also been observed *in vivo*, using mice that overexpress Ala53Thr α-syn (A53T SNCA), evidencing aggregation in both proteins (Giasson et al., 2003). Besides, p-tau in Ser396 and Ser202, 396/404, were found in PD cortex synapses (Muntane et al., 2008) and brainstem samples, respectively, in mice models overexpressing A-309P α-syn (Frasier et al., 2005). Moreover, the direct relation of tau and α-syn in these diseases is supported by: (i) hyperphosphorylation of tau as consequence of α-syn effect in the mice model for PD, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Duka et al., 2006), (ii) α-syn and p-tau presence in NFT and LBs (Shao et al., 2006); (iii) the proteasome promotes the oxidation of α-syn, which in turn triggers recruitment of tau inside oligodendroglial cells in synucleinopathies (Riedel et al., 2009). In addition, it was also observed tau phosphorylation by α-syn in an *in vitro* study, in the residues Ser262 and Ser356 by the PKA (Jensen et al., 1999). As an interesting fact, GSK-3β does not phosphorylate tau in Ser262, meanwhile PKA does not in residues Ser396 and Ser404, suggesting their synergic role in the development of tauopathies mediated by α-syn.

It was reported that α-syn could promote activation of GSK-3β, hyperphosphorylation tau in Thr181, Ser396, and Ser404 (Duka et al., 2006; Duka et al., 2009; Kawakami et al., 2011; Ciaccioli et al., 2013). It seems that this is a consequence of an augmented activity in GSK-3β (Duka et al., 2009; Wills et al., 2011) but also of the generation of a tau, α-syn, and GSK-3β complexes. Although there are more kinases binding to hyperphosphorylated tau and α-syn. Actually, c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK), which also phosphorylate tau in Ser404 and Ser396, were shown by fluorescence intensity distribution analysis (FIDA) to have correlation with the p-tau presence in mice models of PD (α-syn overexpression) (Frasier et al., 2005; Kaul et al., 2011; Oaks et al., 2013). It was also showed that in the presence of inductors for cationic aggregation (Fe^{3+}, Al^{3+}, or DMSO) co-oligomers of α-syn and tau, and their co-aggregated forms are present at nanomolar concentrations (Nubling et al., 2012). Nonetheless, tau phosphorylation by GSK-3β strongly promotes mixed oligomers appearance (Nubling et al., 2012). Finally, we are demonstrating that α-syn polymerization is promoted by tau and that α-syn induce the same to tau, through the NAC hydrophobic region. In line with this, the main difference of both, is that α-syn aggregates itself, meanwhile

tau cannot and needs an inductor (Goedert et al., 1996). Considering all the previous data, we are able to integrate the molecular mechanisms discussed into the neuroimmunomodulation theory (Maccioni et al., 2009) described above, since there are common effectors with the capacity to trigger neuronal damage and death by inflammatory processes.

Neuroinflammation and Parkinsonism

Following the inflammatory cascade triggered by damage signals, there is synapse impair by several molecular mechanisms. The previous process also generates a positive feedback loop promoting even more damage, mainly mediated by microglial cells (Rojo et al., 2008; Andrade et al., 2017).

Increased permeability of the BBB and neurovascular dysfunction have been associated with severe conditions in PD. This effect could be linked to infiltration of inflammation molecules to the middle brain, microglia activation and dopaminergic neurons death (Collins et al., 2012). The systemic inflammatory response in PD seems to be promoted by peripheral lymphocytes activation and augmented levels of serum cytokines, such as IL-2, IL-6 and TNF-α in PD patients (Collins et al., 2012). However, there is not a general confirmation on the release of proinflammatory cytokines associated with PD. The adaptive immune response could be explained by elevated levels of MHC II in astrocytes of the ventral midbrain and microglia in a mice PD model, after inflammatory processes induction (Martin et al., 2016). Among the different mechanisms which direct microorganisms or non-cerebral immune cells into the brain, one of the recently described are direct vascular channels. They connect the skull bone marrow to the brain surface through the meninges, enabling other cells to travel into this region, which is commonly considered as "aseptic" (Kandimalla et al., 2011b). Leukocytes derived from the bone marrow can trigger inflammatory processes in the tissue they exert their protective function. In this context, it is well established that several brain pathologies, which involved neurodegeneration, commonly present neuroimmune dynamics triggering neuroinflammation, among their paramount action mechanisms (Rojo et al., 2008; Andrade et al., 2017). Since the immune response is also triggered by pathogens, inflammatory processes are elicited as well. This could explain part of the onset and progression of not just PD, but also, several neuronal illness conditions, after their invasion in the brain.

As widely discussed, microglia activation can be triggered by different damage signals including pathogens, toxins, endogenous proteins, products generated by dying neurons, and other toxic agents. The constitutive expression of proinflammatory cytokines, such as IL-2, IL-6, IL-1β, TNF-α, and IFN-γ, the presence of ROS and eicosanoids, were also noticed in *postmortem* patients with PD by cerebral analysis. Moreover, it was seen in serum and CSF *in vivo*, and in animal models for PD. The microglial activation product of neuronal death may lead to a vicious circle of neuroinflammation and neurodegeneration (Collins et al., 2012). Some of these substances released upon degeneration of neurons include aggregates of α-syn, neuromelanin, adenosine triphosphate, and metalloproteinase-3 (MMP-3) (Collins et al., 2012). The activation of microglial cells by the pathological forms of α-syn in PD, DLB and multisystemic atrophy, results in a balance between the elimination of α-syn-mediated phagocytosis through neuronal dysfunction, TLR4 microglia and degeneration of neurons because of proinflammatory cytokines and ROS presence (Bruck et al., 2016). Recently, astrocytes have been also implied in brain degradation of α-syn fibrils, instead of spreading. This mechanism is triggered by the transfer of α-syn to neighbor cells, which is more efficient into astrocytes, which then localize inside lysosomes, where they seem to be degraded (Kandimalla et al., 2011a). Later, since lysosomal dysfunction is a common hallmark of NDs, it was shown that α-syn fibrils are transferred through tunneling nanotubes (TNTs) between neurons inside lysosomes and induce the misfolding/aggregation of the normal soluble protein (Wani et al., 2011). Finally, the stressed lysosomes that function as the vehicle of transfer α-syn fibrils inside TNTs allows, after fibrils escape, the seeding of cytosolic protein, thus explaining the progression of the pathology and highlighting an unsuspected role of lysosomes in this process. (Kandimalla et al., 2013). Thus, and considering also the presence p-tau and NFT in regions affected in PD as discussed before, we are again, able to consider the neuroimmunomodulation theory in the context of inflammation for PD.

The new insights are the gut-brain axis regulation, and its role in the pathogenesis of PD. Gut disorders present another way to induce an immune response and inflammatory processes which could contribute to PD pathology. The most common early manifestations in PD that don't affect movement or motor skills, are olfactory impairment and constipation. This is shared with the Braak staging system: the enteric nervous system (ENS), α-syn in the dorsal motor nucleus of the vagus nerve, the olfactory bulb and the submandibular gland, since all of them are a gateway to the environment. The neuropathological process leading to the pathology seems to start in the ENS or the olfactory bulb, spreading by the rostrocranial transmission to the *substantia Nigra* and finally to the CNS, suggesting that the environment could be part of the disease onset and development.

For instance, it was shown that the pesticide rotenone can almost completely reproduce the common clinical and pathogenic features of PD after intragastric administration (Kandimalla et al., 2011b). It has been reported that there is an altered intestinal microbiome in patients with PD and that there is an influence of gut microbiota in enteric neuron activity (Chang et al., 2017; Maldonado-Lasuncion et al., 2018; Wei et al., 2018). Alselmi and co-workers evaluated that the gastric coadministration of subthreshold doses of lectins and paraquat reproduces the disease symptoms in rats and its behavioral affections. They used a solution containing paraquat + lectin for administration *via* gastric gavage and then evaluated behavior in the context of PD and gastric dysmotility. Pathological α-syn in the dorsal motor nucleus of the vagus (DMV) and in the *substantia nigra pars compacta* of SNpc neurons was also observed. Besides, nigrovagally evoked gastric motility was affected in the rats which underwent the treatment. This was seen before the onset of PD manifestations, which were improved by L-dopa treatment. They also made a vagotomy, preventing the progression of PD effects and constraining the appearance of pathological α-syn only to enteric neurons. These reports demonstrated that coadministration of these molecules induces

a progressive, L-dopa-responsive PD which is preceded by gastric dysmotility (Anselmi et al., 2018). Finally, it is important to notice the role of the microbiota in the CNS, where the host is constantly controlling the maturation and function of the microglial cells (Cosin-Tomas et al., 2018), a reason to consider their effects and imbalances in the subject, which, in the future, could be key in the control, prevention, or even treatment of PD.

Another factor for evaluation is sleep disorders that are common in these diseases, the most common being called rapid eye movement (REM) sleep behavior disorder (RBD), representing between 30% and 46% of the cases for PD (Gagnon et al., 2002) and 50–80% of the cases for DLB (Boeve et al., 2007). Even Donaghy and McKeith (2014) suggest that this symptom could be used as a previous diagnosis for DLB. This is interesting to consider, since previous research has pointed out that REM sleep disturbances could be associated with the formation of aggregated forms of the proteins already discussed, promoting the mechanisms underlying the inflammatory context (Hebert et al., 2008; Leidinger et al., 2013; Nagaraj et al., 2017). As a conclusion, the new pharmacotherapies proposed and their further research for these pathologies, and possible sleep-disruptive effects in PD understanding, are crucial in order to improve the quality of life of the patients.

Novel Therapies for Parkinson

The pharmacological approaches in PD are the usual modality to treat the pathology. Oral levodopa and a dopamine decarboxylase inhibitor, such as carbidopa, are considered as the best therapeutic agents (Geekiyanage and Chan, 2011). In the immunomodulatory context, several approaches target to diminish the inflammatory response. Recently, the focus has been mainly aimed to the immune signaling from the periphery of the CNS. Williams et al. proposed to target the chemokine receptor type 2 (CCR2) and the MHC OO (MHCII), since this has been previously demonstrated as neuroprotective in rodent models of PD (Martinez and Peplow, 2019). The group evaluated the genetic knockout and RNA silencing of the class II transactivator (CIITA), which coactivates transcriptionally MHCII. Their results provided evidence that CIITA is needed for the induction and infiltration of MHCII in peripheral immune cells by α-syn, in a mice model for PD, presenting it as a novel promising therapeutic target (Benussi et al., 2017). In this context, it is also important in a preventive manner, since it has also been reported that peripheral immune cell recruitment occurs prior to neurodegeneration and microglia; monocytes and macrophages all contribute to MHCII expression in PD (Du et al., 2018). Harms et al. also demonstrate that extravasation of proinflammatory peripheral monocytes into the CNS has a paramount role in neurodegeneration. Using a PD synucleinopathy model, they ended up proposing that peripheral monocytes are targets for PD, as a neuroprotective therapy. They observed that the expression of the full-length human α-syn *in vivo* promotes the infiltration of proinflammatory CCR2+ peripheral monocytes into the *substantia nigra*. Moreover, they found that α-syn-induced monocyte entry can be prevented by the genetic deletion of CCR2, attenuating MHCII expression and ending the degeneration of dopaminergic neurons (Sjodin et al., 2017).

Lots of immunotherapies for the treatment of PD use vaccines with AS or antibodies against it. A variety of procedures for vaccination have evidenced that the induction of regulatory T cells in the periphery protects the animal in PD models. In this context, the formulation glatiramer acetate (Copaxone®), which is commonly used for treatment of multiple sclerosis, is presented as a possible candidate because of its capacity to increase the number and the action as suppressors of regulatory T cells (Wani et al., 2014). Several reviewers also considered other approaches in order to diminish the inflammatory context; for instance, transcription factors were proposed in order to address this issue. There are alternatives like this promoting inflammation by effectors such as STAT 3, AP1, NF-κB, and TLRs which are constitutively upregulated in PD, while pathways considered as neuroprotective such as TGF-β, YY1, and mTOR are significantly downregulated in the microglia of patients with PD. Finally, it seems that their regulation could contribute to novel agent generation in order to treat PD, improve patient condition, or prevent the development of the pathology (Pal et al., 2016).

AUTHOR CONTRIBUTIONS

LG-M and NR-E prepared the section on tauopathies and their therapy; LN and MP wrote the Alzheimer's disease section; VA prepared the Parkinson's disease section; and RM organized the strategy and goals of the paper and coordinated the different sections in the final writing of the text.

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REFERENCES

Andrade, V., Guzmán-Martínez, L., Pulgar, K. V., and Maccioni, R. B. (2017). "Neuroimmune dynamics in Alzheimer's disease progression," in *Mechanisms of neuroinflammation*. Ed. G. E. A. Abreu (Rijeka: InTech). doi: 10.5772/intechopen.68941

Anselmi, L., Bove, C., Coleman, F. H., Le, K., Subramanian, M. P., Venkiteswaran, K., et al. (2018). Ingestion of subthreshold doses of environmental toxins induces ascending Parkinsonism in the rat. *NPJ Parkinsons Dis.* 4, 30. doi: 10.1038/s41531-018-0066-0

Arai, Y., Yamazaki, M., Mori, O., Muramatsu, H., Asano, G., and Katayama, Y. (2001). Alpha-synuclein-positive structures in cases with sporadic Alzheimer's

disease: morphology and its relationship to tau aggregation. *Brain Res.* 888 (2), 287–296. doi: 10.1016/S0006-8993(00)03082-1

Arendt, T., Stieler, J. T., and Holzer, M. (2016). Tau and tauopathies. *Brain Res. Bull.* 126 (Pt 3), 238–292. doi: 10.1016/j.brainresbull.2016.08.018

Armstrong, R. A. (2015). "Corticobasal degeneration and dementia," in *Diet and nutrition in dementia and cognitive decline*. Eds. C. R. Martin and V. R. Preedy (San Diego: Academic Press), 35–43. doi: 10.1016/B978-0-12-407824-6.00004-5

Armstrong, R. A., Cairns, N. J., and Lantos, P. L. (2000). A quantitative study of the pathological lesions in the neocortex and hippocampus of twelve patients with corticobasal degeneration. *Exp. Neurol.* 163 (2), 348–356. doi: 10.1006/exnr.2000.7392

Asai, H., Ikezu, S., Tsunoda, S., Medalla, M., Luebke, J., Haydar, T., et al. (2015). Depletion of microglia and inhibition of exosome synthesis halt tau propagation. *Nat. Neurosci.* 18 (11), 1584–1593. doi: 10.1038/nn.4132

Alzheimer's Association (2018). 2018 Alzheimer's disease facts and figures. *Alzheimers Dement.* 14 (3), 367–429. doi: 10.1016/j.jalz.2018.02.001

Bak, T. H., Caine, D., Hearn, V. C., and Hodges, J. R. (2006). Visuospatial functions in atypical parkinsonian syndromes. *J. Neurol. Neurosurg. Psychiatry* 77 (4), 454–456. doi: 10.1136/jnnp.2005.068239

Bang, J., Spina, S., and Miller, B. L. (2015). Frontotemporal dementia. *Lancet* 386 (10004), 1672–1682. doi: 10.1016/S0140-6736(15)00461-4

Bellucci, A., Westwood, A. J., Ingram, E., Casamenti, F., Goedert, M., and Spillantini, M. G. (2004). Induction of inflammatory mediators and microglial activation in mice transgenic for mutant human P301S tau protein. *Am J Pathol* 165, 1643–1652

Benussi, L., Binetti, G., and Ghidoni, R. (2017). Loss of neuroprotective factors in neurodegenerative dementias: the end or the starting point? *Front. Neurosci.* 11, 672. doi: 10.3389/fnins.2017.00672

Bettens, K., Sleegers, K., and Van Broeckhoven, C. (2010). Current status on Alzheimer disease molecular genetics: from past, to present, to future. *Hum. Mol. Genet.* 19 (R1), R4–R11. doi: 10.1093/hmg/ddq142

Bezard, E., Yue, Z., Kirik, D., and Spillantini, M. G. (2013). Animal models of Parkinson's disease: limits and relevance to neuroprotection studies. *Mov. Disord.* 28 (1), 61–70. doi: 10.1002/mds.25108

Boeve, B. F., Silber, M. H., Saper, C. B., Ferman, T. J., Dickson, D. W., Parisi, J. E., et al. (2007). Pathophysiology of REM sleep behaviour disorder and relevance to neurodegenerative disease. *Brain* 130 (Pt 11), 2770–2788. doi: 10.1093/brain/awm056

Braak, H., and Braak, E. (1991). Neuropathological stageing of Alzheimer-related changes. *Acta Neuropathol.* 82 (4), 239–259. doi: 10.1007/BF00308809

Bruck, D., Wenning, G. K., Stefanova, N., and Fellner, L. (2016). Glia and alpha-synuclein in neurodegeneration: a complex interaction. *Neurobiol. Dis.* 85, 262–274. doi: 10.1016/j.nbd.2015.03.003

Bussiere, T., Gold, G., Kovari, E., Giannakopoulos, P., Bouras, C., Perl, D. P., et al. (2003). Stereologic analysis of neurofibrillary tangle formation in prefrontal cortex area 9 in aging and Alzheimer's disease. *Neuroscience* 117 (3), 577–592. doi: 10.1016/S0306-4522(02)00942-9

Bussiere, T., Hof, P. R., Mailliot, C., Brown, C. D., Caillet-Boudin, M. L., Perl, D. P., et al. (1999). Phosphorylated serine422 on tau proteins is a pathological epitope found in several diseases with neurofibrillary degeneration. *Acta Neuropathol.* 97 (3), 221–230. doi: 10.1007/s004010050978

Cagnin, A., Rossor, M., Sampson, E. L., Mackinnon, T., and Banati, R. B. (2004). *In vivo* detection of microglial activation in frontotemporal dementia. *Ann. Neurol.* 56 (6), 894–897. doi: 10.1002/ana.20332

Cambiazo, V., Gonzalez, M., and Maccioni, R. B. (1995). DMAP-85: a tau-like protein from *Drosophila melanogaster* larvae. *J. Neurochem.* 64 (3), 1288–1297. doi: 10.1046/j.1471-4159.1995.64031288.x

Carrasco-Gallardo, C., Farias, G. A., Fuentes, P., Crespo, F., and Maccioni, R. B. (2012). Can nutraceuticals prevent Alzheimer's disease? Potential therapeutic role of a formulation containing shilajit and complex B vitamins. *Arch. Med. Res.* 43 (8), 699–704. doi: 10.1016/j.arcmed.2012.10.010

Chang, W. S., Wang, Y. H., Zhu, X. T., and Wu, C. J. (2017). Genome-wide profiling of miRNA and mRNA expression in Alzheimer's disease. *Med. Sci. Monit.* 23, 2721–2731. doi: 10.12659/MSM.905064

Chapman, M. R., Robinson, L. S., Pinkner, J. S., Roth, R., Heuser, J., Hammar, M., et al. (2002). Role of *Escherichia coli* curli operons in directing amyloid fiber formation. *Science* 295 (5556), 851–855. doi: 10.1126/science.1067484

Chiarini, A., Armato, U., Gardenal, E., Gui, L., and Dal Prà, I. (2017). Amyloid β-exposed human astrocytes overproduce phospho-tau and overrelease it within exosomes, effects suppressed by calcilytic NPS 2143—further implications for Alzheimer's therapy. *Front. Neurosci.* 11, 217. doi: 10.3389/fnins.2017.00217

Ciaccioli, G., Martins, A., Rodrigues, C., Vieira, H., and Calado, P. (2013). A powerful yeast model to investigate the synergistic interaction of α-synuclein and tau in neurodegeneration. *PLoS One* 8 (2), e55848. doi: 10.1371/journal.pone.0055848

Citron, M. (2010). Alzheimer's disease: strategies for disease modification. *Nat. Rev. Drug Discov.* 9 (5), 387–398. doi: 10.1038/nrd2896

Clavaguera, F., Akatsu, H., Fraser, G., Crowther, R. A., Frank, S., Hench, J., et al. (2013). Brain homogenates from human tauopathies induce tau inclusions in mouse brain. *Proc. Natl. Acad. Sci.* 110 (23), 9535–9540. doi: 10.1073/pnas.1301175110

Collin, L., Bohrmann, B., Gopfert, U., Oroszlan-Szovik, K., Ozmen, L., and Gruninger, F. (2014). Neuronal uptake of tau/pS422 antibody and reduced progression of tau pathology in a mouse model of Alzheimer's disease. *Brain* 137 (10), 2834–2846. doi: 10.1093/brain/awu213.

Collins, L. M., Toulouse, A., Connor, T. J., and Nolan, Y. M. (2012). Contributions of central and systemic inflammation to the pathophysiology of Parkinson's disease. *Neuropharmacology* 62 (7), 2154–2168. doi: 10.1016/j.neuropharm.2012.01.028

Colton, C., and Wilcock, D. M. (2010). Assessing activation states in microglia. *CNS Neurol. Disord. Drug Targets* 9 (2), 174–191. doi: 10.2174/187152710791012053

Cornejo, A., Jimenez, J. M., Caballero, L., Melo, F., and Maccioni, R. B. (2011). Fulvic acid inhibits aggregation and promotes disassembly of tau fibrils associated with Alzheimer's disease. *J. Alzheimers Dis.* 27 (1), 143–153. doi: 10.3233/JAD-2011-110623

Cortes, N., Andrade, V., Guzman-Martinez, L., Estrella, M., and Maccioni, R. B. (2018). Neuroimmune tau mechanisms: their role in the progression of neuronal degeneration. *Int. J. Mol. Sci.* 19 (4), 956. doi: 10.3390/ijms19040956

Cosin-Tomas, M., Alvarez-Lopez, M. J., Companys-Alemany, J., Kaliman, P., Gonzalez-Castillo, C., Ortuno-Sahagun, D., et al. (2018). Temporal integrative analysis of mRNA and microRNAs expression profiles and epigenetic alterations in female SAMP8, a model of age-related cognitive decline. *Front. Genet.* 9, 596. doi: 10.3389/fgene.2018.00596

Coyle-Gilchrist, I. T., Dick, K. M., Patterson, K., Vazquez Rodriquez, P., Wehmann, E., Wilcox, A., et al. (2016). Prevalence, characteristics, and survival of frontotemporal lobar degeneration syndromes. *Neurology* 86 (18), 1736–1743. doi: 10.1212/WNL.0000000000002638

Dickson, D. W. (1999). Neuropathologic differentiation of progressive supranuclear palsy and corticobasal degeneration. *J. Neurol.* 246 (2), II6–II15. doi: 10.1007/BF03161076

Donaghy, P. C., and McKeith, I. G. (2014). The clinical characteristics of dementia with Lewy bodies and a consideration of prodromal diagnosis. *Alzheimers Res. Ther.* 6 (4), 46. doi: 10.1186/alzrt274

Drechsel, D. N., Hyman, A. A., Cobb, M. H., and Kirschner, M. W. (1992). Modulation of the dynamic instability of tubulin assembly by the microtubule-associated protein tau. *Mol. Biol. Cell* 3 (10), 1141–1154. doi: 10.1091/mbc.3.10.1141

Du, Y., Wu, H. T., Qin, X. Y., Cao, C., Liu, Y., Cao, Z. Z., et al. (2018). Postmortem brain, cerebrospinal fluid, and blood neurotrophic factor levels in Alzheimer's disease: a systematic review and meta-Analysis. *J. Mol. Neurosci.* 65 (3), 289–300. doi: 10.1007/s12031-018-1100-8

Duka, T., Duka, V., Joyce, J. N., and Sidhu, A. (2009). α-Synuclein contributes to GSK-3β-catalyzed tau phosphorylation in Parkinson's disease models. *FASEB J.* 23 (9), 2820–2830. doi: 10.1096/fj.08-120410

Duka, T., Rusnak, M., Drolet, R. E., Duka, V., Wersinger, C., Goudreau, J. L., et al. (2006). Alpha-synuclein induces hyperphosphorylation of tau in the MPTP model of parkinsonism. *FASEB J.* 20 (13), 2302–2312. doi: 10.1096/fj.06-6092com

Fernandez-Botran, R., Ahmed, Z., Crespo, F. A., Gatenbee, C., Gonzalez, J., Dickson, D. W., et al. (2011). Cytokine expression and microglial activation in progressive supranuclear palsy. *Parkinsonism Relat. Disord.* 17 (9), 683–688. doi: 10.1016/j.parkreldis.2011.06.007

Fernandez, J. A., Rojo, L., Kuljis, R. O., and Maccioni, R. B. (2008). The damage signals hypothesis of Alzheimer's disease pathogenesis. *J. Alzheimers Dis.* 14 (3), 329–333. doi: 10.3233/JAD-2008-14307

Forno, L. S. (1996). Neuropathology of Parkinson's disease. *J. Neuropathol. Exp. Neurol.* 55 (3), 259–272. doi: 10.1097/00005072-199603000-00001

Frankola, K. A., Greig, N.H., Luo, W., and Tweedie, D. (2011). Targeting TNF-alpha to elucidate and ameliorate neuroinflammation in neurodegenerative diseases. *CNS Neurol. Disord. Drug Targets* 10 (3), 391–403. doi: 10.2174/187152711794653751

Frasier, M., Walzer, M., McCarthy, L., Magnuson, D., Lee, J. M., Haas, C., et al. (2005). Tau phosphorylation increases in symptomatic mice overexpressing A30P α-synuclein. *Exp. Neurol.* 192 (2), 274–287. doi: 10.1016/j.expneurol.2004.07.016

Friedman, B. A., Srinivasan, K., Ayalon, G., Meilandt, W. J., Lin, H., Huntley, M. A., et al. (2018). Diverse brain myeloid expression profiles reveal distinct microglial activation states and aspects of Alzheimer's disease not evident in mouse models. *Cell Rep.* 22 (3), 832–847. doi: 10.1016/j.celrep.2017.12.066

Fulga, T. A., Elson-Schwab, I., Khurana, V., Steinhilb, M. L., Spires, T. L., Hyman,B. T., et al. (2007). Abnormal bundling and accumulation of F-actin mediates tau-induced neuronal degeneration *in vivo. Nat. Cell Biol.* 9 (2), 139–148. doi: 10.1038/ncb1528

Gagnon, J. F., Bedard, M. A., Fantini, M. L., Petit, D., Panisset, M., Rompre, S., et al. (2002). REM sleep behavior disorder and REM sleep without atonia in Parkinson's disease. *Neurology* 59 (4), 585–589. doi: 10.1212/WNL.59.4.585

Garcia-Reitboeck, P., Anichtchik, O., Dalley, J. W., Ninkina, N., Tofaris, G. K., Buchman, V. L., et al. (2013). Endogenous alpha-synuclein influences the number of dopaminergic neurons in mouse substantia nigra. *Exp. Neurol.* 248, 541–545. doi: 10.1016/j.expneurol.2013.07.015

Geekiyanage, H., and Chan, C. (2011). MicroRNA-137/181c regulates serine palmitoyltransferase and in turn amyloid beta, novel targets in sporadic Alzheimer's disease. *J. Neurosci.* 31 (41), 14820–14830. doi: 10.1523/JNEUROSCI.3883-11.2011

Ghetti, B., Oblak, A. L., Boeve, B. F., Johnson, K. A., Dickerson, B. C., and Goedert, M. (2015). Invited review: frontotemporal dementia caused by microtubule-associated protein tau gene (MAPT) mutations: a chameleon for neuropathology and neuroimaging. *Neuropathol. Appl. Neurobiol.* 41 (1), 24–46. doi: 10.1111/nan.12213

Giannakopoulos, P., Herrmann, F. R., Bussiere, T., Bouras, C., Kovari, E., Perl, D. P., et al. (2003). Tangle and neuron numbers, but not amyloid load, predict cognitive status in Alzheimer's disease. *Neurology* 60 (9), 1495–1500. doi: 10.1212/01.WNL.0000063311.58879.01

Giasson, B. I., Forman, M. S., Higuchi, M., Golbe, L. I., Graves, C. L., Kotzbauer, P. T., et al. (2003). Initiation and synergistic fibrillization of tau and alpha-synuclein. *Science* 300 (5619), 636–640. doi: 10.1126/science.1082324

Gibb, W. R., Luthert, P. J., and Marsden, C. D. (1989). Corticobasal degeneration. *Brain* 112 (Pt 5), 1171–1192. doi: 10.1093/brain/112.5.1171

Goedert, M., Jakes, R., Spillantini, M. G., Hasegawa, M., Smith, M. J., and Crowther, R. A. (1996). Assembly of microtubule-associated protein tau into Alzheimer-like filaments induced by sulphated glycosaminoglycans. *Nature* 383 (6600), 550–553. doi: 10.1038/383550a0

Goedert, M., Spillantini, M. G., Del Tredici, K., and Braak, H. (2013). 100 years of Lewy pathology. *Nat. Rev. Neurol.* 9 (1), 13–24. doi: 10.1038/nrneurol.2012.242

Golde, T. E., and Miller, V. M. (2009). Proteinopathy-induced neuronal senescence: a hypothesis for brain failure in Alzheimer's and other neurodegenerative diseases. *Alzheimers Res. Ther.* 1 (2), 5. doi: 10.1186/alzrt5

Grant, R. E., Schneider, J. A., Ferguson, E. J., and Cummings, P. B. (1997). Total hip reconstruction in a woman with Cornelia de Lange syndrome: a case report. *J. Natl. Med. Assoc.* 89 (8), 530–532.

Griciuc, A., Serrano-Pozo, A., Parrado, A. R., Lesinski, A. N., Asselin, C. N., Mullin, K., et al. (2013). Alzheimer's disease risk gene CD33 inhibits microglial uptake of amyloid beta. *Neuron* 78 (4), 631–643. doi: 10.1016/j.neuron.2013.04.014

Guillot-Sestier, M. V., Doty, K. R., and Town, T. (2015). Innate immunity fights Alzheimer's disease. *Trends Neurosci.* 38 (11), 674–681. doi: 10.1016/j.tins.2015.08.008

Guzman-Martinez, L., Farias, G. A., and Maccioni, R. B. (2013). Tau oligomers as potential targets for Alzheimer's diagnosis and novel drugs. *Front. Neurol.* 4, 167. doi: 10.3389/fneur.2013.00167

Haggerty, T., Credle, J., Rodriguez, O., Wills, J., Oaks, A. W., Masliah, E., et al. (2011). Hyperphosphorylated tau in an α-synuclein-overexpressing transgenic model of Parkinson's disease. *Eur. J. Neurosci.* 33 (9), 1598–1610. doi: 10.1111/j.1460-9568.2011.07660.x

Halliday, G. M., Davies, L., McRitchie, D. A., Cartwright, H., Pamphlett, R., and Morris, J. G. L. (1995). Ubiquitin-positive achromatic neurons in corticobasal degeneration. *Acta Neuropathol.* 90 (1), 68–75. doi: 10.1007/BF00294461

Harper, D. G., Stopa, E. G., Kuo-Leblanc, V., McKee, A. C., Asayama, K., Volicer, L., et al. (2008). Dorsomedial SCN neuronal subpopulations subserve different functions in human dementia. *Brain* 131 (Pt 6), 1609–1617. doi: 10.1093/brain/awn049

Hebert, S. S., Horre, K., Nicolai, L., Papadopoulou, A. S., Mandemakers, W., Silahtaroglu, A. N., et al. (2008). Loss of microRNA cluster miR-29a/b-1 in sporadic Alzheimer's disease correlates with increased BACE1/beta-secretase expression. *Proc. Natl. Acad. Sci. U.S.A.* 105 (17), 6415–6420. doi: 10.1073/pnas.0710263105

Heneka, M. T., Golenbock, D. T., and Latz, E. (2015). Innate immunity in Alzheimer's disease. *Nat. Immunol.* 16 (3), 229–236. doi: 10.1038/ni.3102

Hodges, J. R., Davies, R., Xuereb, J., Kril, J., and Halliday, G. (2003). Survival in frontotemporal dementia. *Neurology* 61 (3), 349–354. doi: 10.1212/01.WNL.0000078928.20107.52

Hol, E. M., and Pekny, M. (2015). Glial fibrillary acidic protein (GFAP) and the astrocyte intermediate filament system in diseases of the central nervous system. *Curr. Opin. Cell Biol.* 32, 121–130. doi: 10.1016/j.ceb.2015.02.004

Holtzman, D. M., Bales, K. R., Tenkova, T., Fagan, A. M., Parsadanian, M., Sartorius, L. J., et al. (2000). Apolipoprotein E isoform-dependent amyloid deposition and neuritic degeneration in a mouse model of Alzheimer's disease. *Proc. Natl. Acad. Sci.* 97 (6), 2892–2897. doi: 10.1073/pnas.050004797

Hughes, L. E., Rittman, T., Regenthal, R., Robbins, T. W., and Rowe, J. B. (2015). Improving response inhibition systems in frontotemporal dementia with citalopram. *Brain* 138 (Pt 7), 1961–1975. doi: 10.1093/brain/awv133

Irwin, D. J., Cairns, N. J., Grossman, M., McMillan, C. T., Lee, E. B., Van Deerlin, V. M., et al. (2015). Frontotemporal lobar degeneration: defining phenotypic diversity through personalized medicine. *Acta Neuropathol.* 129 (4), 469–491. doi: 10.1007/s00401-014-1380-1

Iseki, E., Togo, T., Suzuki, K., Katsuse, O., Marui, W., de Silva, R., et al. (2003). Dementia with Lewy bodies from the perspective of tauopathy. *Acta Neuropathol.* 105 (3), 265–270. doi: 10.1007/s00401-002-0644-3

Jacobs, A. H., and Tavitian, B. (2012). Noninvasive molecular imaging of neuroinflammation. *J. Cereb. Blood Flow Metab.* 32 (7), 1393–1415. doi: 10.1038/jcbfm.2012.53

Jellinger, K. A. (2010a). Basic mechanisms of neurodegeneration: a critical update. *J. Cell. Mol. Med.* 14 (3), 457–487. doi: 10.1111/j.1582-4934.2010.01010.x

Jellinger, K. A. (2010b). The neuropathologic substrate of Parkinson disease dementia. *Acta Neuropathol.* 119 (1), 151–153. doi: 10.1007/s00401-009-0613-1

Jellinger, K. A. (2011). Interaction between α-synuclein and tau in Parkinson's disease: comment on Wills et al.: elevated tauopathy and α-synuclein pathology in postmortem Parkinson's disease brains with and without dementia. Exp Neurol 2010; 225: 210-218. *Exp. Neurol.* 227 (1), 13–18. doi: 10.1016/j.expneurol.2010.10.006

Jensen, P. H., Hager, H., Nielsen, M. S., Hojrup, P., Gliemann, J., and Jakes, R. (1999). α-Synuclein binds to tau and stimulates the protein kinase A-catalyzed tau phosphorylation of serine residues 262 and 356. *J. Biol. Chem.* 274 (36), 25481–25489. doi: 10.1074/jbc.274.36.25481

Jha, M. K., Jo, M., Kim, J. H., and Suk, K. (2019). Microglia–astrocyte crosstalk: an intimate molecular conversation. *Neuroscientist* 25 (3), 227–240. doi: 10.1177/1073858418783959

Joachim, C. L., Morris, J. H., Kosik, K. S., and Selkoe, D. J. (1987). Tau antisera recognize neurofibrillary tangles in a range of neurodegenerative disorders. *Ann. Neurol.* 22 (4), 514–520. doi: 10.1002/ana.410220411

Jochum, W., Hanggi, D., Bruder, E., Jeck, T., Novotny, H., Probst, A., et al. (2004). Inflammatory myofibroblastic tumour of the sella turcica. *Neuropathol. Appl. Neurobiol.* 30 (6), 692–695. doi: 10.1111/j.1365-2990.2004.00611.x

Jonsson, T., Stefansson, H., Steinberg, S., Jonsdottir, I., Jonsson, P. V., Snaedal, J., et al. (2013). Variant of TREM2 associated with the risk of Alzheimer's disease. *N. Engl. J. Med.* 368 (2), 107–116. doi: 10.1056/NEJMoa1211103

Josephs, K. A. (2010). "Corticobasal ganglionic degeneration," in *Blue books of neurology*. Eds. A. H. V. Schapira, A. E. T. Lang, and S. Fahn (Butterworth-Heinemann), 375–396. doi: 10.1016/B978-1-4160-6641-5.00022-2

Josephs, K. A., Petersen, R. C., Knopman, D. S., Boeve, B. F., Whitwell, J. L., Duffy, J. R., et al. (2006a). Clinicopathologic analysis of frontotemporal and

corticobasal degenerations and PSP. *Neurology* 66 (1), 41–48. doi: 10.1212/01.wnl.0000191307.69661.c3

Josephs, K. A., Whitwell, J. L., Boeve, B. F., Knopman, D. S., Tang-Wai, D. F., Drubach, D. A., et al. (2006b). Visual hallucinations in posterior cortical atrophy. *Arch. Neurol.* 63 (10), 1427–1432. doi: 10.1001/archneur.63.10.1427

Kandimalla, R. J., Anand, R., Veeramanikandan, R., Wani, W. Y., Prabhakar, S., Grover, V. K., et al. (2014). CSF ubiquitin as a specific biomarker in Alzheimer's disease. *Curr. Alzheimer Res.* 11 (4), 340–348. doi: 10.2174/1567205011666140331161027

Kandimalla, R. J., Prabhakar, S., Binukumar, B. K., Wani, W. Y., Gupta, N., Sharma, D. R., et al. (2011a). Apo-epsilon4 allele in conjunction with Abeta42 and tau in CSF: biomarker for Alzheimer's disease. *Curr. Alzheimer Res.* 8 (2), 187–196. doi: 10.2174/156720511795256071

Kandimalla, R. J., Prabhakar, S., Binukumar, B. K., Wani, W. Y., Sharma, D. R., Grover, V. K., et al. (2011b). Cerebrospinal fluid profile of amyloid beta42 (Abeta42), hTau and ubiquitin in North Indian Alzheimer's disease patients. *Neurosci Lett* 487 (2), 134–138. doi: 10.1016/j.neulet.2010.06.075

Kandimalla, R. J., Prabhakar, S., Wani, W. Y., Kaushal, A., Gupta, N., Sharma, D. R., et al. (2013). CSF p-tau levels in the prediction of Alzheimer's disease. *Biol. Open* 2 (11), 1119–1124. doi: 10.1242/bio.20135447

Kaul, T., Credle, J., Haggerty, T., Oaks, A. W., Masliah, E., and Sidhu, A. (2011). Region-specific tauopathy and synucleinopathy in brain of the alpha-synuclein overexpressing mouse model of Parkinson's disease. *BMC Neurosci.* 12, 79. doi: 10.1186/1471-2202-12-79

Kawakami, F., Suzuki, M., Shimada, N., Kagiya, G., Ohta, E., Tamura, K., et al. (2011). Stimulatory effect of alpha-synuclein on the tau-phosphorylation by GSK-3beta. *FEBS J.* 278 (24), 4895–4904. doi: 10.1111/j.1742-4658.2011.08389.x

Kempster, P. A., Williams, D. R., Selikhova, M., Holton, J., Revesz, T., and Lees, A. J. (2007). Patterns of levodopa response in Parkinson's disease: a clinico-pathological study. *Brain* 130 (Pt 8), 2123–2128. doi: 10.1093/brain/awm142

Keren-Shaul, H., Spinrad, A., Weiner, A., Matcovitch-Natan, O., Dvir-Szternfeld, R., Ulland, T. K., et al. (2017). A unique microglia type associated with restricting development of Alzheimer's disease. *Cell* 169 (7), 1276–1290, e1217. doi: 10.1016/j.cell.2017.05.018

King, A., Al-Sarraj, S., Troakes, C., Smith, B. N., Maekawa, S., Iovino, M., et al. (2013). Mixed tau, TDP-43 and p62 pathology in FTLD associated with a C9ORF72 repeat expansion and p.Ala239Thr MAPT (tau) variant. *Acta Neuropathol.* 125 (2), 303–310. doi: 10.1007/s00401-012-1050-0

Knopman, D. S., and Roberts, R. O. (2011). Estimating the number of persons with frontotemporal lobar degeneration in the US population. *J. Mol. Neurosci.* 45 (3), 330–335. doi: 10.1007/s12031-011-9538-y

Koenigsknecht-Talboo, J., and Landreth, G. E. (2005). Microglial phagocytosis induced by fibrillar beta-amyloid and IgGs are differentially regulated by proinflammatory cytokines. *J. Neurosci.* 25 (36), 8240–8249. doi: 10.1523/JNEUROSCI.1808-05.2005

Koistinaho, M., Lin, S., Wu, X., Esterman, M., Koger, D., Hanson, J., et al. (2004). Apolipoprotein E promotes astrocyte colocalization and degradation of deposited amyloid-β peptides. *Nat. Med.* 10 (7), 719. doi: 10.1038/nm1058

Kovacs, G. G. (2017). Tauopathies. *Handb. Clin. Neurol.* 145, 355–368. doi: 10.1016/B978-0-12-802395-2.00025-0

Kovacs, G. G., Botond, G., and Budka, H. (2010). Protein coding of neurodegenerative dementias: the neuropathological basis of biomarker diagnostics. *Acta Neuropathol.* 119 (4), 389–408. doi: 10.1007/s00401-010-0658-1

Kovari, E., Gold, G., Herrmann, F. R., Canuto, A., Hof, P. R., Bouras, C., et al. (2003). Lewy body densities in the entorhinal and anterior cingulate cortex predict cognitive deficits in Parkinson's disease. *Acta Neuropathol.* 106 (1), 83–88. doi: 10.1007/s00401-003-0705-2

Kraybill, M. L., Larson, E. B., Tsuang, D. W., Teri, L., McCormick, W. C., Bowen, J. D., et al. (2005). Cognitive differences in dementia patients with autopsy-verified AD, Lewy body pathology, or both. *Neurology* 64 (12), 2069–2073. doi: 10.1212/01.WNL.0000165987.89198.65

Krishnamurthy, P. K., Deng, Y., and Sigurdsson, E. M. (2011). Mechanistic studies of antibody-mediated clearance of tau aggregates using an *ex vivo* brain slice model. *Front. Psychiatry* 21, 2:59. doi: 10.3389/fpsyt.2011.00059. eCollection 2011.

Laihinen, A. O., Rinne, J. O., Ruottinen, H. M., Nagren, K. A., Lehikoinen, P. K., Oikonen, V. J., et al. (1994). PET studies on dopamine D1 receptors in the human brain with carbon-11-SCH 39166 and carbon-11-NNC 756. *J. Nucl. Med.* 35 (12), 1916–1920.

Langlais, P. J., Thal, L., Hansen, L., Galasko, D., Alford, M., and Masliah, E. (1993). Neurotransmitters in basal ganglia and cortex of Alzheimer's disease with and without Lewy bodies. *Neurology* 43 (10), 1927–1934. doi: 10.1212/WNL.43.10.1927

Lee, V. M., Giasson, B. I., and Trojanowski, J. Q. (2004). More than just two peas in a pod: common amyloidogenic properties of tau and alpha-synuclein in neurodegenerative diseases. *Trends Neurosci.* 27 (3), 129–134. doi: 10.1016/j.tins.2004.01.007

Lee, M. S., Rinne, J. O., Ceballos-Baumann, A., Thompson, P. D., and Marsden, C. D. (1994). Dystonia after head trauma. *Neurology* 44 (8), 1374–1378. doi: 10.1212/WNL.44.8.1374

Leidinger, P., Backes, C., Deutscher, S., Schmitt, K., Mueller, S. C., Frese, K., et al. (2013). A blood based 12-miRNA signature of Alzheimer disease patients. *Genome Biol.* 14 (7), R78. doi: 10.1186/gb-2013-14-7-r78

Leyns, C. E. G., and Holtzman, D. M. (2017). Glial contributions to neurodegeneration in tauopathies. *Mol. Neurodegener.* 12 (1), 50. doi: 10.1186/s13024-017-0192-x

Lian, H., Yang, L., Cole, A., Sun, L., Chiang, A. C.-A., Fowler, S. W., et al. (2015). NFκB-activated astroglial release of complement C3 compromises neuronal morphology and function associated with Alzheimer's disease. *Neuron* 85 (1), 101–115. doi: 10.1016/j.neuron.2014.11.018

Ling, H. (2016). Clinical approach to progressive supranuclear palsy. *JMD* 9 (1), 3–13. doi: 10.14802/jmd.15060

Lopez Gonzalez, I., Garcia-Esparcia, P., Llorens, F., and Ferrer, I. (2016). Genetic and transcriptomic profiles of inflammation in neurodegenerative diseases: Alzheimer, Parkinson, Creutzfeldt-Jakob and tauopathies. *Int. J. Mol. Sci.* 17 (2), 206. doi: 10.3390/ijms17020206

Lull, M. E., and Block, M. L. (2010). Microglial activation and chronic neurodegeneration. *Neurotherapeutics* 7 (4), 354–365. doi: 10.1016/j.nurt.2010.05.014

Maarouf, C. L., Beach, T. G., Adler, C. H., Shill, H. A., Sabbagh, M. N., Wu, T., et al. (2012). Cerebrospinal fluid biomarkers of neuropathologically diagnosed Parkinson's disease subjects. *Neurol. Res.* 34 (7), 669–676. doi: 10.1179/1743132812Y.0000000063

Maccioni, R. B. (2011). Tau protein in Alzheimer's disease. *Curr. Alzheimer Res.* 8 (6), 607. doi: 10.2174/156720511796717159

Maccioni, R. B. (2012). Introductory remarks. Molecular, biological and clinical aspects of Alzheimer's disease. *Arch. Med. Res.* 43 (8), 593–594. doi: 10.1016/j.arcmed.2012.11.001

Maccioni, R. B., Rojo, L. E., Fernandez, J. A., and Kuljis, R. O. (2009). The role of neuroimmunomodulation in Alzheimer's disease. *Ann. N. Y. Acad. Sci.* 1153, 240–246. doi: 10.1111/j.1749-6632.2008.03972.x

Mackenzie, I. R., Neumann, M., Bigio, E. H., Cairns, N. J., Alafuzoff, I., Kril, J., et al. (2010). Nomenclature and nosology for neuropathologic subtypes of frontotemporal lobar degeneration: an update. *Acta Neuropathol.* 119 (1), 1–4. doi: 10.1007/s00401-009-0612-2

Maeda, J., Zhang, M. R., Okauchi, T., Ji, B., Ono, M., Hattori, S., et al. (2011). *In vivo* positron emission tomographic imaging of glial responses to amyloid-beta and tau pathologies in mouse models of Alzheimer's disease and related disorders. *J. Neurosci.* 31 (12), 4720–4730. doi: 10.1523/JNEUROSCI.3076-10.2011

Mahapatra, R. K., Edwards, M. J., Schott, J. M., and Bhatia, K. P. (2004). Corticobasal degeneration. *Lancet Neurol.* 3 (12), 736–743. doi: 10.1016/S1474-4422(04)00936-6

Maldonado-Lasuncion, I., Atienza, M., Sanchez-Espinosa, M. P., and Cantero, J. L. (2018). Aging-related changes in cognition and cortical integrity are associated with serum expression of candidate microRNAs for Alzheimer disease. *Cereb. Cortex*. 2018, 1–12. doi: 10.1093/cercor/bhy323

Malia, T. J., Teplyakov, A., Ernst, R., Wu, S. J., Lacy, E. R., Liu, X., et al. (2016). Epitope mapping and structural basis for the recognition of phosphorylated tau by the anti-tau antibody AT8. *Proteins* 84 (4), 427–434. doi: 10.1002/prot.24988

Maphis, N., Xu, G., Kokiko-Cochran, O. N., Jiang, S., Cardona, A., Ransohoff, R. M., et al. (2015). Reactive microglia drive tau pathology and contribute to the spreading of pathological tau in the brain. *Brain* 138 (6), 1738–1755. doi: 10.1093/brain/awv081

Martin, H. L., Santoro, M., Mustafa, S., Riedel, G., Forrester, J. V., and Teismann, P. (2016). Evidence for a role of adaptive immune response in the disease

pathogenesis of the MPTP mouse model of Parkinson's disease. *Glia* 64 (3), 386–395. doi: 10.1002/glia.22935

Martinez, B., and Peplow, P. V. (2019). MicroRNAs as diagnostic and therapeutic tools for Alzheimer's disease: advances and limitations. *Neural Regen. Res.* 14 (2), 242–255. doi: 10.4103/1673-5374.244784

Matsumoto, S., Udaka, F., Kameyama, M., Kusaka, H., Ito, H., and Imai, T. (1996). Subcortical neurofibrillary tangles, neuropil threads, and argentophilic glial inclusions in corticobasal degeneration. *Clin. Neuropathol.* 15 (4), 209–214.

McKee, A. C., Carreras, I., Hossain, L., Ryu, H., Klein, W. L., Oddo, S., et al. (2008). Ibuprofen reduces Abeta, hyperphosphorylated tau and memory deficits in Alzheimer mice. *Brain Res.* 1207, 225–236. doi: 10.1016/j.brainres.2008.01.095

McKeith, I. G., Dickson, D. W., Lowe, J., Emre, M., O'Brien, J. T., Feldman, H., et al. (2005). Diagnosis and management of dementia with Lewy bodies: third report of the DLB Consortium. *Neurology* 65 (12), 1863–1872. doi: 10.1212/01.wnl.0000187889.17253.b1

Meda, L., Baron, P., and Scarlato, G. (2001). Glial activation in Alzheimer's disease: the role of Abeta and its associated proteins. *Neurobiol. Aging* 22 (6), 885–893. doi: 10.1016/S0197-4580(01)00307-4

Medeiros, R., and LaFerla, F. M. (2013). Astrocytes: conductors of the Alzheimer disease neuroinflammatory symphony. *Exp. Neurol.* 239, 133–138. doi: 10.1016/j.expneurol.2012.10.007

Mirra, S. S., Murrell, J. R., Gearing, M., Spillantini, M. G., Goedert, M., Crowther, R. A., et al. (1999). Tau pathology in a family with dementia and a P301L mutation in tau. *J. Neuropathol. Exp. Neurol.* 58 (4), 335–345. doi: 10.1097/00005072-199904000-00004

Morales, I., Farias, G., and Maccioni, R. B. (2010). Neuroimmunomodulation in the pathogenesis of Alzheimer's disease. *Neuroimmunomodulation* 17 (3), 202–204. doi: 10.1159/000258724

Morales, I., Guzman-Martinez, L., Cerda-Troncoso, C., Farias, G. A., and Maccioni, R. B. (2014). Neuroinflammation in the pathogenesis of Alzheimer's disease. A rational framework for the search of novel therapeutic approaches. *Front. Cell. Neurosci.* 8, 112. doi: 10.3389/fncel.2014.00112

Morales, I., Jimenez, J. M., Mancilla, M., and Maccioni, R. B. (2013). Tau oligomers and fibrils induce activation of microglial cells. *J. Alzheimers Dis.* 37 (4), 849–856. doi: 10.3233/JAD-131843

Mulder, S. D., Veerhuis, R., Blankenstein, M. A., and Nielsen, H. M. (2012). The effect of amyloid associated proteins on the expression of genes involved in amyloid-β clearance by adult human astrocytes. *Exp. Neurol.* 233 (1), 373–379. doi: 10.1016/j.expneurol.2011.11.001

Muntane, G., Dalfó, E., Martinez, A., and Ferrer, I. (2008). Phosphorylation of tau and alpha-synuclein in synaptic-enriched fractions of the frontal cortex in Alzheimer's disease, and in Parkinson's disease and related alpha-synucleinopathies. *Neuroscience* 152 (4), 913–923. doi: 10.1016/j.neuroscience.2008.01.030

Musiek, E. S., and Holtzman, D. M. (2015). Three dimensions of the amyloid hypothesis: time, space and 'wingmen'. *Nat. Neurosci.* 18 (6), 800–806. doi: 10.1038/nn.4018

Nagaraj, S., Laskowska-Kaszub, K., Debski, K. J., Wojsiat, J., Dabrowski, M., Gabryelewicz, T., et al. (2017). Profile of 6 microRNA in blood plasma distinguish early stage Alzheimer's disease patients from non-demented subjects. *Oncotarget* 8 (10), 16122–16143. doi: 10.18632/oncotarget.15109

Neary, D., Snowden, J. S., Gustafson, L., Passant, U., Stuss, D., Black, S., et al. (1998). Frontotemporal lobar degeneration: a consensus on clinical diagnostic criteria. *Neurology* 51 (6), 1546–1554. doi: 10.1212/WNL.51.6.1546

Neary, D., Snowden, J. S., Northen, B., and Goulding, P. (1988). Dementia of frontal-lobe type. *J. Neurol. Neurosurg. Psychiatry* 51 (3), 353–361. doi: 10.1136/jnnp.51.3.353

Neumann, K., Farias, G., Slachevsky, A., Perez, P., and Maccioni, R. B. (2011). Human platelets tau: a potential peripheral marker for Alzheimer's disease. *J. Alzheimers Dis.* 25 (1), 103–109. doi: 10.3233/JAD-2011-101641

Nimmerjahn, A., Kirchhoff, F., and Helmchen, F. (2005). Resting microglial cells are highly dynamic surveillants of brain parenchyma *in vivo*. *Science* 308 (5726), 1314–1318. doi: 10.1126/science.1110647

Nubling, G., Bader, B., Levin, J., Hildebrandt, J., Kretzschmar, H., and Giese, A. (2012). Synergistic influence of phosphorylation and metal ions on tau oligomer formation and coaggregation with alpha-synuclein at the single molecule level. *Mol. Neurodegener.* 7, 35. doi: 10.1186/1750-1326-7-35

Oaks, A. W., Frankfurt, M., Finkelstein, D. I., and Sidhu, A. (2013). Age-dependent effects of A53T alpha-synuclein on behavior and dopaminergic function. *PLoS One* 8 (4), e60378. doi: 10.1371/journal.pone.0060378

Olichney, J. M., Galasko, D., Salmon, D. P., Hofstetter, C. R., Hansen, L. A., Katzman, R., et al. (1998). Cognitive decline is faster in Lewy body variant than in Alzheimer's disease. *Neurology* 51 (2), 351–357. doi: 10.1212/WNL.51.2.351

Olney, N. T., Spina, S., and Miller, B. L. (2017). Frontotemporal dementia. *Neurol. Clin.* 35 (2), 339–374. doi: 10.1016/j.ncl.2017.01.008

Onyike, C. U., and Diehl-Schmid, J. (2013). The epidemiology of frontotemporal dementia. *Int. Rev. Psychiatry* 25 (2), 130–137. doi: 10.3109/09540261.2013.776523

Orr, M. E., Sullivan, A. C., and Frost, B. (2017). A brief overview of tauopathy: causes, consequences, and therapeutic strategies. *Trends Pharmacol. Sci.* 38, 637–648. doi. 10.1016/j.tips.2017.03.011

Pal, R., Tiwari, P. C., Nath, R., and Pant, K. K. (2016). Role of neuroinflammation and latent transcription factors in pathogenesis of Parkinson's disease. *Neurol. Res.* 38 (12), 1111–1122. doi: 10.1080/01616412.2016.1249997

Paolicelli, R. C., Bisht, K., and Tremblay, M. E. (2014). Fractalkine regulation of microglial physiology and consequences on the brain and behavior. *Front. Cell. Neurosci.* 8, 129. doi: 10.3389/fncel.2014.00129

Parhizkar, S., Arzberger, T., Brendel, M., Kleinberger, G., Deussing, M., Focke, C., et al. (2019). Loss of TREM2 function increases amyloid seeding but reduces plaque-associated ApoE. *Nat. Neurosci.* 22, 191–204. doi: 10.1038/s41593-018-0296-9

Phillips, E. C., Croft, C. L., Kurbatskaya, K., O'Neill, M. J., Hutton, M. L., Hanger, D. P. et al. (2014). Astrocytes and neuroinflammation in Alzheimer's disease. *Biochem. Soc. Trans.* 42 (5), 1321–1325. doi: 10.1042/BST20140155

Ransohoff, R. M. (2016a). How neuroinflammation contributes to neurodegeneration. *Science* 353 (6301), 777–783. doi: 10.1126/science.aag2590

Ransohoff, R. M. (2016b). A polarizing question: do M1 and M2 microglia exist? *Nat. Neurosci.* 19 (8), 987. doi: 10.1038/nn.4338

Rascovsky, K., Hodges, J. R., Knopman, D., Mendez, M. F., Kramer, J. H., Neuhaus, J., et al. (2011). Sensitivity of revised diagnostic criteria for the behavioural variant of frontotemporal dementia. *Brain* 134 (Pt 9), 2456–2477. doi: 10.1093/brain/awr179

Rebeiz, J. J., Kolodny, E. H., and Richardson, E. P., Jr. (1968). Corticodentatonigral degeneration with neuronal achromasia. *Arch. Neurol.* 18 (1), 20–33. doi: 10.1001/archneur.1968.00470310034003

Riedel, M., Goldbaum, O., and Richter-Landsberg, C. (2009). α-Synuclein promotes the recruitment of tau to protein inclusions in oligodendroglial cells: effects of oxidative and proteolytic stress. *J. Mol. Neurosci.* 39 (1-2), 226–234. doi: 10.1007/s12031-009-9190-y

Riederer, I. M., Pfulg, C., Bouras, C., Giannakopoulos, P., and Riederer, B. M. (2003). Human immunoglobulins and Fc fragments promote microtubule assembly *via* tau proteins and induce conformational changes of neuronal microtubules *in vitro*. *Neuroreport* 14 (1), 117–121. doi: 10.1097/00001756-200301200-00022

Rinne, J. O., Daniel, S. E., Scaravilli, F., Harding, A. E., and Marsden, C. D. (1994a). Nigral degeneration in neuroacanthocytosis. *Neurology* 44 (9), 1629–1632. doi: 10.1212/WNL.44.9.1629

Rinne, J. O., Daniel, S. E., Scaravilli, F., Pires, M., Harding, A. E., and Marsden, C. D. (1994b). The neuropathological features of neuroacanthocytosis. *Mov. Disord.* 9 (3), 297–304. doi: 10.1002/mds.870090303

Rinne, J. O., Lee, M. S., Thompson, P. D., and Marsden, C. D. (1994c). Corticobasal degeneration. A clinical study of 36 cases. *Brain* 117 (Pt 5), 1183–1196. doi: 10.1093/brain/117.5.1183

Rojo, L. E., Fernandez, J. A., Maccioni, A. A., Jimenez, J. M., and Maccioni, R. B. (2008). Neuroinflammation: implications for the pathogenesis and molecular diagnosis of Alzheimer's disease. *Arch. Med. Res.* 39 (1), 1–16. doi: 10.1016/j.arcmed.2007.10.001

Salmon, E., Bernard Ir, C., and Hustinx, R. (2015). Pitfalls and limitations of PET/CT in brain imaging. *Semin. Nucl. Med.* 45 (6), 541–551. doi: 10.1053/j.semnuclmed.2015.03.008

Salonen, R., Rinne, J. O., Halonen, P., Puusa, A., Marttila, R., and Viljanen, M. K. (1994). Lyme borreliosis associated with complete flaccid paraplegia. *J. Infect.* 28 (2), 181–184. doi: 10.1016/S0163-4453(94)95660-X

Sarazin, M., Michon, A., Pillon, B., Samson, Y., Canuto, A., Gold, G., et al. (2003). Metabolic correlates of behavioral and affective disturbances in frontal lobe pathologies. *J. Neurol.* 250 (7), 827–833. doi: 10.1007/s00415-003-1087-z

Schmidt, M. L., Zhukareva, V., Perl, D. P., Sheridan, S. K., Schuck, T., Lee, V. M., et al. (2001). Spinal cord neurofibrillary pathology in Alzheimer disease and Guam parkinsonism–dementia complex. *J. Neuropathol. Exp. Neurol.* 60 (11), 1075–1086. doi: 10.1093/jnen/60.11.1075

Schneider, J. A., Li, J. L., Li, Y., Wilson, R. S., Kordower, J. H., and Bennett, D. A. (2006). Substantia nigra tangles are related to gait impairment in older persons. *Ann. Neurol.* 59 (1), 166–173. doi: 10.1002/ana.20723

Schneider, J. A., Watts, R. L., Gearing, M., Brewer, R. P., and Mirra, S. S. (1997). Corticobasal degeneration: neuropathologic and clinical heterogeneity. *Neurology* 48 (4), 959–969. doi: 10.1212/WNL.48.4.959

Shao, C. Y., Crary, J. F., Rao, C., Sacktor, T. C., and Mirra, S. S. (2006). Atypical protein kinase C in neurodegenerative disease II: PKCiota/lambda in tauopathies and alpha-synucleinopathies. *J. Neuropathol. Exp. Neurol.* 65 (4), 327–335. doi: 10.1097/01.jnen.0000218441.00040.82

Shastri, A., Bonifati, D. M., and Kishore, U. (2013). Innate immunity and neuroinflammation. *Mediators Inflamm.* 2013, 342931. doi: 10.1155/2013/342931

Sieben, A., Van Langenhove, T., Engelborghs, S., Martin, J. J., Boon, P., Cras, P., et al. (2012). The genetics and neuropathology of frontotemporal lobar degeneration. *Acta Neuropathol.* 124 (3), 353–372. doi: 10.1007/s00401-012-1029-x

Sims, R., Van Der Lee, S. J., Naj, A. C., Bellenguez, C., Badarinarayan, N., Jakobsdottir, J., et al. (2017). Rare coding variants in PLCG2, ABI3, and TREM2 implicate microglial-mediated innate immunity in Alzheimer's disease. *Nat. Genet.* 49 (9), 1373. doi: 10.1038/ng.3916

Sjodin, S., Hansson, O., Ohrfelt, A., Brinkmalm, G., Zetterberg, H., Brinkmalm, A., et al. (2017). Mass spectrometric analysis of cerebrospinal fluid ubiquitin in Alzheimer's disease and Parkinsonian disorders. *Proteomics Clin. Appl.* 11, 1–9, 1700100. doi: 10.1002/prca.201700100

Sofroniew, M. V., and Vinters, H. V. (2010). Astrocytes: biology and pathology. *Acta Neuropathol.* 119 (1), 7–35. doi: 10.1007/s00401-009-0619-8

Spillantini, M. G., and Goedert, M. (2013). Tau pathology and neurodegeneration. *Lancet Neurol.* 12 (6), 609–622. doi: 10.1016/S1474-4422(13)70090-5

Spillantini, M. G., Yoshida, H., Rizzini, C., Lantos, P. L., Khan, N., Rossor, M. N., et al. (2000). A novel tau mutation (N296N) in familial dementia with swollen achromatic neurons and corticobasal inclusion bodies. *Ann. Neurol.* 48 (6), 939–943. doi: 10.1002/1531-8249(200012)48:6<939::AID-ANA17>3.0.CO;2-1

Tang-Wai, D. F., Josephs, K. A., Boeve, B. F., Dickson, D. W., Parisi, J. E., and Petersen, R. C. (2003a). Pathologically confirmed corticobasal degeneration presenting with visuospatial dysfunction. *Neurology* 61 (8), 1134–1135. doi: 10.1212/01.WNL.0000086814.35352.B3

Tang-Wai, D. F., Knopman, D. S., Geda, Y. E., Edland, S. D., Smith, G. E., Ivnik, R. J., et al. (2003b). Comparison of the short test of mental status and the mini-mental state examination in mild cognitive impairment. *Arch. Neurol.* 60 (12), 1777–1781. doi: 10.1001/archneur.60.12.1777

Thomas, B., and Beal, M. F. (2007). Parkinson's disease. *Hum. Mol. Genet.* 16 Spec No2, R183–R194. doi: 10.1093/hmg/ddm159

Tolnay, M., and Clavaguera, F. (2004). Argyrophilic grain disease: a late-onset dementia with distinctive features among tauopathies. *Neuropathology* 24 (4), 269–283. doi: 10.1111/j.1440-1789.2004.00591.x

Trojanowski, J. Q., and Dickson, D. (2001). Update on the neuropathological diagnosis of frontotemporal dementias. *J. Neuropathol. Exp. Neurol.* 60 (12), 1123–1126. doi: 10.1093/jnen/60.12.1123

Tsuchiya, K., Ikeda, K., Uchihara, T., Oda, T., and Shimada, H. (1997). Distribution of cerebral cortical lesions in corticobasal degeneration: a clinicopathological study of five autopsy cases in Japan. *Acta Neuropathol.* 94 (5), 416–424. doi: 10.1007/s004010050728

Uchihara, T., and Nakayama, H. (2006). Familial tauopathy mimicking corticobasal degeneration an autopsy study on three siblings. *J. Neurol. Sci.* 246 (1-2), 45–51. doi: 10.1016/j.jns.2006.02.005

Uversky, V. N. (2007). Neuropathology, biochemistry, and biophysics of alpha-synuclein aggregation. *J. Neurochem.* 103 (1), 17–37. doi: 10.1111/j.1471-4159.2007.04764.x

Uversky, V. N. (2009). Intrinsically disordered proteins and their environment: effects of strong denaturants, temperature, pH, counter ions, membranes, binding partners, osmolytes, and macromolecular crowding. *Protein J.* 28 (7-8), 305–325. doi: 10.1007/s10930-009-9201-4

Venneti, S., Wiley, C. A., and Kofler, J. (2009). Imaging microglial activation during neuroinflammation and Alzheimer's disease. *J. Neuroimmune Pharmacol.* 4 (2), 227–243. doi: 10.1007/s11481-008-9142-2

Violet, M., Delattre, L., Tardivel, M., Sultan, A., Chauderlier, A., Caillierez, R., et al. (2014). A major role for tau in neuronal DNA and RNA protection *in vivo* under physiological and hyperthermic conditions. *Front. Cell. Neurosci.* 8, 84. doi: 10.3389/fncel.2014.00084

Walter, S., Letiembre, M., Liu, Y., Heine, H., Penke, B., Hao, W., et al. (2007). Role of the toll-like receptor 4 in neuroinflammation in Alzheimer's disease. *Cell. Physiol. Biochem.* 20 (6), 947–956. doi: 10.1159/000110455

Wang, Y., and Mandelkow, E. (2015). Tau in physiology and pathology. *Nat. Rev. Neurosci.* 17 (1), 5–21. doi: 10.1038/nrn.2015.1

Wang, L., Jiang, Q., Chu, J., Lin, L., Li, X. G., Chai, G. S., et al. (2013). Expression of Tau40 induces activation of cultured rat microglial cells. *PLoS One* 8 (10), e76057. doi: 10.1371/journal.pone.0076057

Wang, W. Y., Tan, M. S., Yu, J. T., and Tan, L. (2015). Role of pro-inflammatory cytokines released from microglia in Alzheimer's disease. *Ann. Transl. Med.* 3 (10), 136. doi: 10.3978/j.issn.2305-5839.2015.03.49

Wani, W. Y., Gudup, S., Sunkaria, A., Bal, A., Singh, P. P., Kandimalla, R. J., et al. (2011). Protective efficacy of mitochondrial targeted antioxidant MitoQ against dichlorvos induced oxidative stress and cell death in rat brain. *Neuropharmacology* 61 (8), 1193–1201. doi: 10.1016/j.neuropharm.2011.07.008

Wani, W. Y., Sunkaria, A., Sharma, D. R., Kandimalla, R. J., Kaushal, A., Gerace, E., et al. (2014). Caspase inhibition augments dichlorvos-induced dopaminergic neuronal cell death by increasing ROS production and PARP1 activation. *Neuroscience* 258, 1–15. doi: 10.1016/j.neuroscience.2013.11.004

Wei, H., Zhu, X., and Li, Y. (2018). Application value of serum biomarkers for choosing memantine therapy for moderate AD. *J. Neurol.* 265 (8), 1844–1849. doi: 10.1007/s00415-018-8926-4

Weingarten, M. D., Lockwood, A. H., Hwo, S. Y., and Kirschner, M. W. (1975). A protein factor essential for microtubule assembly. *Proc. Natl. Acad. Sci. U.S.A.* 72 (5), 1858–1862. doi: 10.1073/pnas.72.5.1858

Wenning, G. K., Litvan, I., Jankovic, J., Granata, R., Mangone, C. A., McKee, A., et al. (1998). Natural history and survival of 14 patients with corticobasal degeneration confirmed at postmortem examination. *J. Neurol. Neurosurg. Psychiatry* 64 (2), 184–189. doi: 10.1136/jnnp.64.2.184

Wes, P. D., Easton, A., Corradi, J., Barten, D. M., Devidze, N., DeCarr, L. B., et al. (2014). Tau overexpression impacts a neuroinflammation gene expression network perturbed in Alzheimer's disease. *PLoS One* 9 (8), e106050. doi: 10.1371/journal.pone.0106050

Williams, D. R., Holton, J. L., Strand, C., Pittman, A., de Silva, R., Lees, A. J., et al. (2007). Pathological tau burden and distribution distinguishes progressive supranuclear palsy-parkinsonism from Richardson's syndrome. *Brain* 130 (Pt 6), 1566–1576. doi: 10.1093/brain/awm104

Wills, J., Credle, J., Haggerty, T., Lee, J. H., Oaks, A. W., and Sidhu, A. (2011). Tauopathic changes in the striatum of A53T alpha-synuclein mutant mouse model of Parkinson's disease. *PLoS One* 6 (3), e17953. doi: 10.1371/journal.pone.0017953

Wills, J., Jones, J., Haggerty, T., Duka, V., Joyce, J. N., and Sidhu, A. (2010). Elevated tauopathy and alpha-synuclein pathology in postmortem Parkinson's disease brains with and without dementia. *Exp. Neurol.* 225 (1), 210–218. doi: 10.1016/j.expneurol.2010.06.017

Wu, C., Ma, G., Li, J., Zheng, K., Dang, Y., Shi, X., et al. (2013). *In vivo* cell tracking *via* 18F-fluorodeoxyglucose labeling: a review of the preclinical and clinical applications in cell-based diagnosis and therapy. *Clin. Imaging* 37 (1), 28–36. doi: 10.1016/j.clinimag.2012.02.023

Wyss-Coray, T., and Mucke, L. (2002). Inflammation in neurodegenerative disease—a double-edged sword. *Neuron* 35 (3), 419–432. doi: 10.1016/S0896-6273(02)00794-8

Yoshida, M. (2014). Astrocytic inclusions in progressive supranuclear palsy and corticobasal degeneration. *Neuropathology* 34 (6), 555–570. doi: 10.1111/neup.12143

Yoshiyama, Y., Higuchi, M., Zhang, B., Huang, S. M., Iwata, N., Saido, T. C., et al. (2007). Synapse loss and microglial activation precede tangles in a P301S tauopathy mouse model. *Neuron* 53 (3), 337–351. doi: 10.1016/j.neuron.2007.01.010

Yu, D. M., Zhao, L. Y., Yang, Z. Y., Chang, S. Y., Yu, W. T., Fang, H. Y., et al. (2016). Comparison of undernutrition prevalence of children under 5 years in China

between 2002 and 2013. *Biomed. Environ. Sci.* 29 (3), 165–176. doi: 10.3967/bes2016.021

Zarrouk, A., Debbabi, M., Bezine, M., Karym, E. M., Badreddine, A., Rouaud, O., et al. (2018). Lipid biomarkers in Alzheimer's disease. *Curr. Alzheimer Res.* 15 (4), 303–312. doi: 10.2174/1567205014666170505101426

Zhang, J. (2015). Mapping neuroinflammation in frontotemporal dementia with molecular PET imaging. *J. Neuroinflammation* 12, 108. doi: 10.1186/s12974-015-0236-5

Zverova, M., Kitzlerova, E., Fisar, Z., Jirak, R., Hroudova, J., Benakova, H., et al. (2018). Interplay between the APOE genotype and possible plasma biomarkers in Alzheimer's disease. *Curr. Alzheimer Res.* 15 (10), 938–950. doi: 10.2174/1567205015666180601090533

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REVIEW
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N-3 Polyunsaturated Fatty Acids and the Resolution of Neuroinflammation

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In the past few decades, as a result of their anti-inflammatory properties, n-3 long chain polyunsaturated fatty acids (n-3 LC-PUFAs), have gained greater importance in the regulation of inflammation, especially in the central nervous system (in this case known as neuroinflammation). If sustained, neuroinflammation is a common denominator of neurological disorders, including Alzheimer's disease and major depression, and of aging. Hence, limiting neuroinflammation is a real strategy for neuroinflammatory disease therapy and treatment. Recent data show that n-3 LC-PUFAs exert anti-inflammatory properties in part through the synthesis of specialized pro-resolving mediators (SPMs) such as resolvins, maresins and protectins. These SPMs are crucially involved in the resolution of inflammation. They could be good candidates to resolve brain inflammation and to contribute to neuroprotective functions and could lead to novel therapeutics for brain inflammatory diseases. This review presents an overview 1) of brain n-3 LC-PUFAs as precursors of SPMs with an emphasis on the effect of n-3 PUFAs on neuroinflammation, 2) of the formation and action of SPMs in the brain and their biological roles, and the possible regulation of their synthesis by environmental factors such as inflammation and nutrition and, in particular, PUFA consumption.

Keywords: n-3 long-chain PUFAs, docosahexaenoic acid, eicosapentaenoic acid, specialized pro-resolving mediators, nutrition, neuroinflammation, resolvins

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INTRODUCTION

Inflammation is a critical process in host defense, facilitating tissue repair, regeneration and maintenance of homeostasis. However, if uncontrolled, it becomes a chronic low-grade inflammation that is characterized by the production of pro-inflammatory cytokines and adipokines leading to tissue damage and loss of function (López-Vicario et al., 2016). This chronic low-grade inflammation is observed in many chronic pathologies including neurodegenerative diseases and also in aging. Hence it becomes a significant public health concern and constitutes a financial burden that impacts millions worldwide (Serhan, 2017b). It is thus of importance to find treatments that ensure resolution of inflammation in a specific time-limited manner. Nutrition has gained importance in recent years since nutriments such as n-3 polyunsaturated fatty acids (PUFAs) have anti-inflammatory properties (Calder, 2016; Calder et al., 2017). They exert their effect in part through their conversion into bioactive lipid mediators called specialized pro-resolving mediators (SPMs) that underlie most of the beneficial effects attributed to their precursors (Serhan, 2014; López-Vicario et al., 2016). Recently, attention has been paid to these derivatives in the regulation of neuroinflammation. In this review, we present an overview of brain n-3 PUFAs and their effect on neuroinflammation and of the formation and mechanisms of the action of SPMs in the brain.

BRAIN N-3 PUFAS AS PRECURSORS OF SPMs

Brain n-3 PUFAs

The brain contains high levels of PUFAs (25–30%) that are mainly docosahexaenoic acid (DHA, n-3 PUFA) (12–14% of total fatty acids) and arachidonic acid (AA, n-6 PUFA) (8–10% of total fatty acids) (Carrie et al., 2000; Xiao et al., 2005; McNamara and Carlson, 2006; Little et al., 2007; Chung et al., 2008; Joffre et al., 2016). There are regional differences: the hippocampus and prefrontal cortex contain the highest DHA content whereas the hypothalamus has the lowest (Carrie et al., 2000; Xiao et al., 2005; Joffre et al., 2016). There are also cellular differences: astrocytes, oligodendrocytes, and microglial cells (representing respectively 70%, 10–15% and 10–15% of brain glial cells; Renaud et al., 2015) contain DHA in different proportions. DHA represents 10–12% in astrocytes, 5% in the oligodendrocytes and up to 2% in microglial cells (Bourre et al., 1984; Rey et al., 2018). Neurons cannot synthesize long-chain PUFAs (LC-PUFAs) but can incorporate them in their membranes: DHA represents 8.3% of the total fatty acids in neurons (Bourre et al., 1984).

Numerous studies have discussed the transport of DHA through the blood–brain barrier (BBB). DHA enters the brain as unesterified DHA that is the major pool supplying the brain with DHA. However, the precise mechanisms of entry are still not fully described. Some transporters facilitate the uptake of DHA into the brain: fatty acid transport proteins (FATPs), fatty acid translocase (CD36) and major facilitator superfamily domain containing 2A (MFSD2A) (Chouinard-Watkins and Bazinet, 2018). Fernandez et al. (2018) recently reported that a member of the long-chain acyl-CoA synthetase (ACSL) family, ACSL6, is also involved in brain DHA uptake but its role has yet to be determined (Fernandez et al., 2018).

This brain fatty acid composition can be affected by environmental factors such as nutrition, something to which individuals are continuously exposed. Indeed, the PUFA content in all brain structures is strongly impacted by the PUFAs present in the diet (Alashmali et al., 2016; Joffre et al., 2016). A diet rich in n-3 PUFAs (DHA found in fish or its precursor, alpha-linolenic acid, found in vegetable oil) increases brain DHA in rodents (Hiratsuka et al., 2009; de Theije et al., 2015; Skorve et al., 2015; Kitson et al., 2016). However, DHA supplementation is more effective than alpha-linolenic supplementation in increasing the DHA content in the brain (Lacombe et al., 2017; Rey et al., 2019). In rodents, DHA supplementation from 16 weeks to 16 months or from 20 to 22 months of age compensates a DHA decrease due to aging (Little et al., 2007; Labrousse et al., 2012; Bascoul- Colombo et al., 2016). On the other hand, a diet deficient in n-3 PUFAs decreases brain DHA in all brain structures, with the hippocampus, containing most DHA, being the most affected and the hypothalamus the least affected (Delpech et al., 2015b; Joffre et al., 2016; Manduca et al., 2017).

Studies from Broadhurst and Crawford suggest that the amount of DHA incorporated into the brain depends on the complexity of the brain structure and on behavior development (Crawford et al., 1999; Broadhurst et al., 2002). A decrease in brain DHA induced by a deficient diet during gestation and lactation can be reversed by 2-month DHA supplementation at weaning (Orr et al., 2013). The organism also adapts its metabolism to an n-3 PUFA deficiency condition: the half-life of DHA increases in the brain to reduce its loss (Rapoport et al., 2007) and the activity of the enzymes responsible for DHA conversion, the Δ6 desaturase and elongase, is increased in the liver (Cho et al., 1999; Wang et al., 2005; Igarashi et al., 2007). Brain cells are also impacted by dietary PUFA supply. An n-3 PUFA deficient diet decreases DHA in astrocytes, neurons and oligodendrocytes (Bourre et al., 1984) whereas n-3 PUFA supplementation increases DHA levels in glial cells (Bowen and Clandinin, 2005; Destaillats et al., 2010). Moreover, we recently showed that the fatty acid composition of the microglial cells is also modulated by n-3 PUFA dietary intake during the gestation/lactation period. Maternal n-3 LC-PUFA dietary supplementation during gestation and lactation increases the DHA level in the offspring's microglia at P21 as compared with a maternal diet that contains equilibrated levels of n-6 and n-3 PUFA precursors (Rey et al., 2018). Interestingly, maternal n-3 PUFA deficiency does not impact the DHA level suggesting that microglial cells are protected from n-3 PUFA deficiency (Rey et al., 2018).

n-3 PUFAs as Regulators of Neuroinflammation

n-3 PUFAs have powerful anti-inflammatory properties (Calder, 2005). They play an important role in the regulation of the synthesis and release of pro-inflammatory mediators (Delpech et al., 2015b; Hanisch and Kettenmann, 2007; Cunningham and Sanderson, 2008; Yirmiya and Goshen, 2011; Pascual et al., 2012). Pro-inflammatory factors include interleukin-1 beta (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α) and play a role in neuronal plasticity (Delpech et al., 2015b; Yirmiya and Goshen, 2011). If sustained, uncontrolled inflammation can lead to neuronal damage that is involved in many neuronal pathologies (Blais and Rivest, 2003; Layé, 2010; Solito and Sastre, 2012). Hence, limiting the inflammation and enhancing the resolution of inflammation is of great interest.

Evidence in Humans

In humans, the anti-inflammatory properties of n-3 PUFAs were first identified in epidemiological studies in Eskimos that consume a lot of n-3 LC-PUFAs from eating fish (Dyerberg and Bang, 1979; Kromann and Green, 1980; Simopoulos, 2008). Clinical studies have highlighted the beneficial effect of n-3 LC-PUFAs in chronic inflammatory and autoimmune diseases. Indeed, fish oil supplementation decreases pro-inflammatory cytokine expression, such as IL-1β in blood monocytes, and improves the symptoms of patients suffering from rheumatoid arthritis (Kremer et al., 1990; James et al., 1997; Kremer, 2000) or multiple sclerosis (Stewart and Bowling, 2005; Weinstock-Guttman et al., 2005). Moreover, DHA supply significantly decreases the circulating inflammatory markers and the oxidative stress (Freund-Levi et al., 2006; Kiecolt-Glaser et al., 2012). DHA supply for several months also improves the working

and long-term memories in patients with moderate cognitive alterations (Freund-Levi et al., 2006; Lee et al., 2013).

Evidence in Animals

In animals, numerous studies have demonstrated the anti-inflammatory properties of n-3 PUFAs in the brain. In lipopolysaccharide (LPS)-, or IL-1β-, induced inflammation models, dietary n-3 LC-PUFA supplementation in adulthood prevents LPS-induced hippocampal increase of pro-inflammatory cytokines IL-1β and TNF-α in rats and mice (Orr et al., 2013; Dehkordi et al., 2015; Rey et al., 2019). A dietary supply in eicosapentaenoic acid (EPA) decreases the production of LPS-induced pro-inflammatory cytokines IL-1β, IL-6 and TNF-α in the hippocampus and increases the production of anti-inflammatory cytokines IL-10 and IL-4 in both rats (Kavanagh et al., 2004; Lonergan et al., 2004; Dong et al., 2017) and mice (Shi et al., 2016). This modification is associated with a decrease in the phosphorylation of c-Jun and c-Jun N-terminal kinase proteins and in nuclear factor-kappa B (NFκB) that regulate inflammation (Lonergan et al., 2004; Shi et al., 2016). EPA dietary supply attenuates the activation of microglial cells and astrocytes triggered by an intracerebral IL-1β administration and increases the production of IL-10 in the hippocampus in rats (Song and Horrobin, 2004; Song et al., 2008; Dong et al., 2017). Following dietary DHA supplementation, there is an increase of DHA in the phospholipid and free fatty acid fractions. However, only the unesterified DHA is necessary to attenuate neuroinflammation. Nevertheless, the DHA phospholipid pool is an important source of unesterified DHA (Orr et al., 2013). On the contrary, in mice, an n-3 PUFA deficiency from the first day of gestation to weaning increases the expression of pro-inflammatory markers in the hippocampus and an alteration in the motility and the phenotype of microglial cells and alters synaptic plasticity (Lafourcade et al., 2011; Madore et al., 2014; Thomazeau et al., 2017). Using the model of intraperitoneal (ip) injection of LPS, we showed that IL-6 expression is strongly induced in n-3 PUFA-deficient mice whereas sickness behavior is down-regulated *via* the impairment of IL-6 signaling in the brain (Mingam et al., 2008). Moreover, when the deficiency continues in adulthood, it alters GABAergic, dopaminergic and cholinergic neurotransmission (Zimmer et al., 2000; Aid et al., 2003; Chalon, 2006; Larrieu et al., 2014) and anxiety-related, depressive-like behaviors that can be compared with autistic behavior in adults (Mingam et al., 2008; Larrieu et al., 2012; Larrieu et al., 2014; Hughes et al., 2016; Manduca et al., 2017). Following ip injection of LPS, n-3 PUFA-deficient mice display altered hippocampal synaptic plasticity that likely contributes to spatial memory impairment and higher glucocorticoid levels (Delpech et al., 2015b). On the contrary, an n-3 PUFA dietary supply improves emotional behavior alteration and memory deficit in rats (Song and Horrobin, 2004; Song et al., 2008; Dong et al., 2017), restores social and memory performance altered in autism models and improves depressive symptoms in both mice (Pietropaolo et al., 2014; Madore et al., 2016; Weiser et al., 2016) and rats (Bove et al., 2018; Morgese et al., 2018). Of note, n-3 LC-PUFA dietary supplementation attenuates depressive symptoms in depressed patients presenting inflammation (McNamara, 2015; Rapaport et al., 2016; Larrieu and Layé, 2018; Layé et al., 2018). This protective effect seems to be linked to EPA which could target microglia to reduce neuroinflammation (Bazinet et al., 2019). n-3 LC-PUFAs could also modulate neuroinflammation through their effect on the HPA axis (Larrieu et al., 2014; Hughes et al., 2016; Larrieu and Layé, 2018). With Alzheimer's disease, β-amyloid triggers depressive symptoms through neuroinflammatory processes that could be improved by dietary n-3 PUFAs (Bove et al., 2018; Morgese et al., 2018). Altogether these data reinforce the importance of n-3 PUFAs as regulators of inflammation and associated depressive symptoms.

Another way to modulate neuroinflammation is to administer n-3 PUFAs directly into the brain or peripherally. Indeed, a 24-hour intracerebroventricular (icv) DHA brain infusion attenuates hippocampal neuroinflammation initiated by icv LPS in mice (Orr et al., 2013). In a mouse model of cerebral ischemia, DHA icv administration inhibits NFκB activation and cyclooxygenase-2 (COX-2) expression in the hippocampus (Marcheselli et al., 2003). Moreover, intrathecal injection of DHA decreases microglial activation, mitogen-activated protein kinase (MAPK) phosphorylation and the production of pro-inflammatory cytokines in the spinal cord of mice (Lu et al., 2013). In addition, in a model of traumatic brain injury in rats, n-3 PUFA ip injection attenuates microglial-induced inflammation by inhibiting the NFκB pathway (Chen et al., 2017).

Dietary n-3 LC-PUFA supplementation requires the use of fish oil. However, fish oil may provide confusing factors such as vitamins, for example. Thus, the use of Fat-1 transgenic mice that convert n-6 to n-3 PUFAs through a desaturase from *C. elegans* is a model that enables the elimination of confusing factors provided by the diet (Kang et al., 2004). Such mice have higher DHA levels in the hippocampus and cortex (Delpech et al., 2015a; Boudrault et al., 2010; Orr et al., 2013). They express less COX-2 (involved in the production of lipid mediators) in the cortex than wild type mice (Boudrault et al., 2010). They are protected against cognitive deficits induced by ip LPS injection through a decrease in neuroinflammation (Orr et al., 2013; Delpech et al., 2015a). This is associated with a decrease in microglial activation (Orr et al., 2013).

Evidence in *In Vitro* Studies

In vitro studies have shown that n-3 PUFAs have an inhibitory effect on the production of pro-inflammatory cytokines in microglial cells. Indeed, in the microglial cell line or primary culture microglial cells, DHA prevents LPS-induced NKκB activation and then cytokine production by inhibiting LPS receptor presentation and decreases the oxidative stress, nitric oxide (NO) production and inducible nitric oxide synthase (iNOS) expression (De Smedt-Peyrusse et al., 2008; Lu et al., 2010; Antonietta Ajmone-Cat et al., 2012; Pettit et al., 2013; Chen et al., 2014; Corsi et al., 2015; Fourrier et al., 2017; Inoue et al., 2017;). Moreover, DHA prevents LPS-induced MAPK phosphorylation (MAPK pathway playing essential roles in the expression of inflammatory molecules) and induces peroxisome proliferator-activated receptor (PPAR)-γ nuclear translocation that exerts anti-inflammatory effects (Antonietta Ajmone-Cat et al., 2012). Of interest, EPA also affects the production of TNF-α, IL-6 and

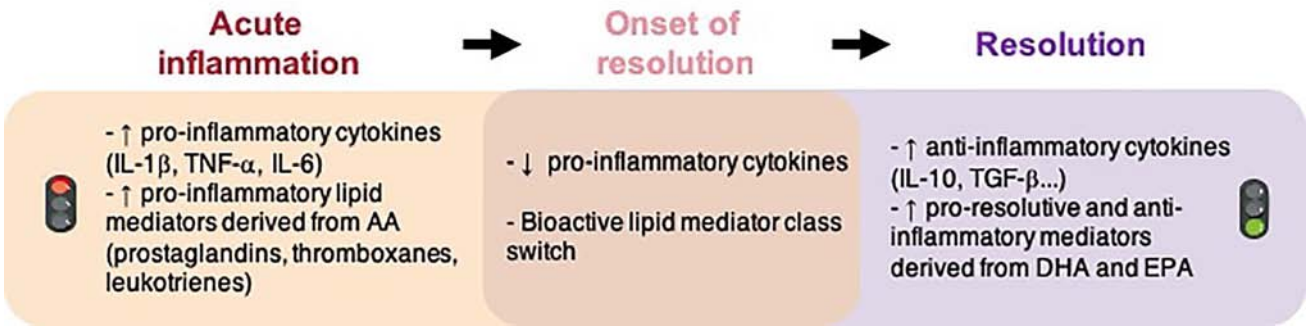

FIGURE 1 | Molecular events implicated in inflammation and the resolution of inflammation. AA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; IL, interleukin; TGF, transforming growth factor; TNF, tumor necrosis factor.

NO by inhibiting NFκB phosphorylation *via* sirtuin-1 (SIRT-1) (Moon et al., 2007; Chen et al., 2014; Inoue et al., 2017). It is likely that DHA attenuates the inflammatory response in LPS-activated microglia by remodeling lipid bodies that are dynamic organelles in which DHA is incorporated and by altering their interplay with mitochondria and other associated organelles (Tremblay et al., 2016). In addition, DHA and EPA are able to enhance myelin or amyloid β (Aβ) peptide phagocytosis that is associated with a shift in microglial polarization toward the beneficial M2 phenotype and to a decrease in pro-inflammatory cytokine production (Hjorth et al., 2013; Chen et al., 2014).

This modulation of neuroinflammation induced by n-3 LC-PUFA supply is attributed, in part, to SPM synthesis (Barden et al., 2016). In the various phases of inflammatory response, prostaglandins, leukotrienes and thromboxane are synthesized first (**Figure 1**). They permit the propagation of inflammation. They also stimulate the synthesis of SPMs with pro-resolutive properties (Levy et al., 2001; Serhan and Savill, 2005).

n-3 PUFA-Derived SPMs and Their Synthesis Pathway

Many of the n-3 PUFA-derived immunomodulators that orchestrate the inflammatory response are lipids (Doyle et al., 2018). Some of them are involved in the regulation of inflammation (prostaglandins, thromboxanes, leukotrienes, etc.) and others are implicated in the resolution of inflammation (resolvins, protectins and maresins). There is a temporal shift in the lipid mediator synthesis from the initiation of inflammation to the resolution that allows the formation of different lipid mediators at different times (**Figure 1**). Here we have focused on SPMs, which are of great interest since they permit a return to homeostasis (Serhan et al., 2015; Sugimoto et al., 2016). Charles N. Serhan first identified these SPMs at the periphery and characterized their anti-inflammatory and pro-resolutive properties (Serhan et al., 2000). SPMs actively orchestrate and finely tune the inflammatory response. They decrease pro-inflammatory cytokines and increase anti-inflammatory cytokines, accelerate the phagocytosis of cellular debris and dead cells without immune suppression. A failure in the resolution of inflammation is detrimental for tissue and contributes to a chronic inflammatory response.

BIOSYNTHESIS AND BIOLOGICAL ROLES OF n-3 PUFA-DERIVED SPMs

Biosynthesis of n-3 PUFA-Derived SPMs

Free (unesterified) n-3 LC-PUFAs are released from membrane phospholipids through the action of phospholipases A2 (PLA2) in response to stimulation. DHA is hydrolyzed by calcium independent PLA2 (iPLA2) from phospholipids and plasmenylethanolamine-PLA2 from plasmalogens (Farooqui and Horrocks, 2006). After this step, n-3 LC-PUFAs undergo an enzymatic conversion to generate SPMs (**Figures 2** and **3**).

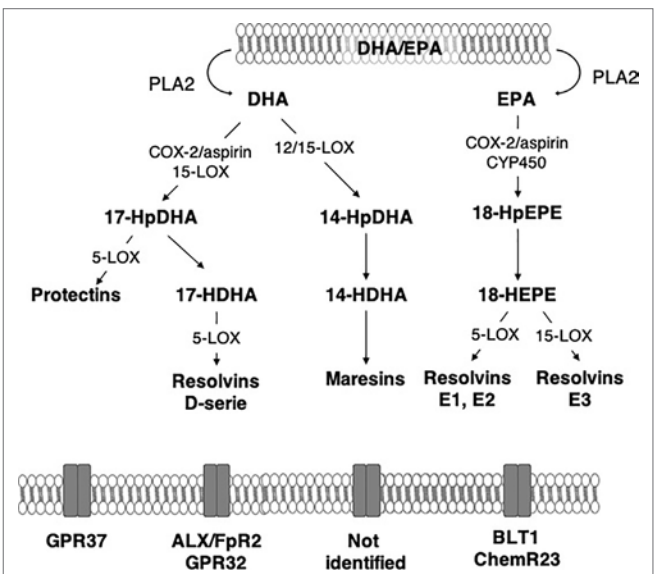

FIGURE 2 | Main synthesis pathway of n-3 long-chain PUFA-derived lipid mediators. ALX/FpR2, lipoxin A4 receptor/formyl peptide receptor 2; BLT1, Leukotriene B4 receptor 1; ChemR23, chemokine-like receptor 1; COX-2, cyclooxygenase-2; CYP450, monoxygenases cytochrome P450; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; GPR, G protein-coupled receptor; HDHA, hydroxy-docosahexaenoic acid; HEPE, hydroxy-eicosapentaenoic acid; HpDHA, hydroperoxyl-docosahexaenoic acid; HpEPE, hydroperoxy-eicosapentaenoic acid; LOX, lipoxygenases; PLA2, phospholipase A2.

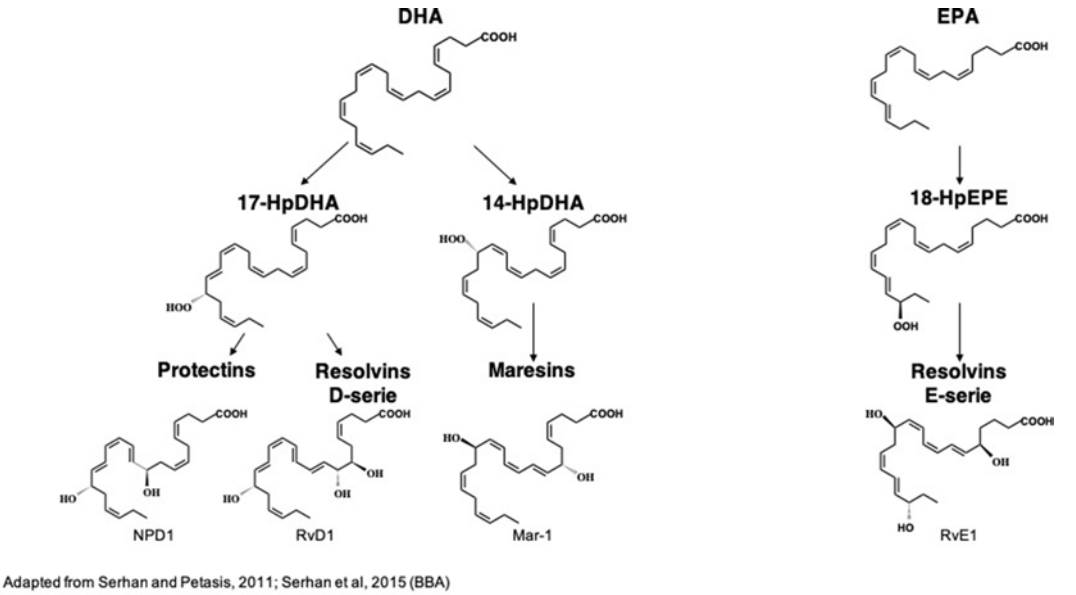

FIGURE 3 | Biochemical structures of the main n-3 long-chain PUFA-derived lipid mediators. DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; HpDHA, hydroperoxyl-docosahexaenoic acid; HpEPE, hydroperoxy-eicosapentaenoic acid; Mar1, maresin 1; NPD1, neuroproetectin D1; RvD1, resolvin D1; RvE1, resolvin E1. (Serhan and Petasis, 2011; Serhan et al., 2015)

n-3 PUFA-derived SPMs are synthesized mainly from DHA and EPA *via* COX-2, lipoxygenases (LOX) and CYP450 monoxygenases (CYP450). In the brain, 15-LOX, 12/15-LOX and 5-LOX are the most abundant LOX and are widely distributed, suggesting the potential production of SPMs (Shalini et al., 2017). They are expressed in the second step of inflammation in the hippocampus (Czapski et al., 2010; Birnie et al., 2013). 15-LOX is involved in neurodegeneration and neurotoxicity due to the increased oxidative stress it generates in models of Alzheimer's disease (Pratico et al., 2004; Wang et al., 2015) and brain ischemia (Yigitkanli et al., 2017). However, it is also implicated in neuroprotection (Sun et al., 2015). Indeed, it increases the production of 12-HETE and 15-HETE that promote the activation of PPARγ that is neuroprotective through its anti-inflammatory properties. The inhibition of 15-LOX induces hippocampus-dependent cognitive alterations (Shalini et al., 2017). Indeed, it prevented long term potentiation, a major molecular mechanism that describes the strengthening of synapses and underlies learning and memory. Moreover, 15-LOX deletion drastically decreases SPM production in the brain while SPMs also modulate synaptic plasticity (Park et al., 2011; Shalini et al., 2017). In the brain, the inducible COX-2 is activated *via* an NFκB pathway (Nadjar et al., 2005). COX-2 catalyzes the first step of the synthesis of prostaglandins and thromboxanes derived from n-6 PUFAs that contribute to the initiation of inflammation (Davidson et al., 2001; Salinas et al., 2007; Engstrom et al., 2012). COX-2 also produces hydroxyl fatty acids from DHA in both an aspirin-dependent and aspirin-independent manner (Serhan et al., 2002; Oh et al., 2011; Gabbs et al., 2015; Nebert, 2017). In response to LPS, COX-2 is rapidly expressed in the hippocampus (Czapski et al., 2010; Rey et al., 2019). Inhibition of COX-2 delays resolution of acute inflammation (Schwab et al., 2007). CYP450s play a complex role in inflammation by producing n-6 PUFA-derived bioactive mediators such as epoxides that are anti-inflammatory in monocytes and macrophages (Bystrom et al., 2011; Fleming, 2011; Nebert et al., 2013; Gilroy et al., 2016).

DHA is the precursor of resolvins D1-6 (RvD1-6), neuroprotectin D1 (NPD1) and maresins 1–2 (Mar1-2) which all have pro-resolutive and anti-inflammatory properties (Spite and Serhan, 2010; Halade et al., 2018). RvD1-6 are synthesized from DHA but RvD1 is the most studied because it has powerful anti-inflammatory and pro-resolutive properties. DHA is converted into monohydroxy DHA, 17-hydroxy docosahexaenoic acid (17-HDHA) by acetylated COX-2, CYP450 and 15-LOX (Barden et al., 2016; Halade et al., 2018) and then into RvD1 by 5-LOX (Sun et al., 2007; Recchiuti, 2013). DHA is also converted into di-hydroxy-DHA, termed protectin D1 (PD1) or neuroprotectin D1 (NPD1) when produced in the central nervous system by 5- and 15-LOX (Hong et al., 2003; Aursnes et al., 2014; Kuda, 2017; Doyle et al., 2018). Acetylated COX-2 permits the synthesis of aspirin-triggered PD1 (AT-PD1) which has powerful protective effects (Bazan et al., 2012). DHA is transformed into Mar 1-2 by 12/15-LOX *via* the synthesis of 14-HDHA (Serhan et al., 2009; Barden et al., 2016; Halade et al., 2018).

EPA is the precursor of resolvins E1 (RvE1), E2 and E3 that have many biological roles (Serhan et al., 2000; Rey et al., 2016; Halade et al., 2018). It is converted by aspirin-triggered acetylated COX-2 or CYP450 into 18R-hydroxyeicosapentaenoic acid (18R-HEPE), that is transformed into RvE1 or E2 by 5-LOX (Ohira et al., 2010; Barden et al., 2016) or into RvE3 by 15-LOX (Isobe et al., 2012).

Little is known concerning the pharmacokinetics and dynamics of oxylipins. They are synthesized *in situ*, reinforcing the importance of the fatty acid composition of the brain. They are not stored, but produced on demand and they are unstable, being rapidly metabolized.

The structure of all derivatives is highly preserved in the evolution from fish to humans suggesting their great bioactive role in all organ systems. Dysfunction of SPM production can be due to insufficient EPA and DHA supply leading to inadequate production of SPMs or to the polymorphism of the enzymes involved in their synthesis or to a defect in the binding of SPMs to their receptors (Sugimoto et al., 2016). In humans, reduced SPM production is associated with chronicity and the magnitude of persistent inflammation. SPMs have multiple biological roles in the maintenance of homeostasis.

Biological Roles of DHA-Derived SPMs

In human serum, the DHA-derivatives represent 30.7% of the identified SPMs (Colas et al., 2014; Serhan et al., 2018). RvD1, PD1 and Mar1 were detected at 30.9 pg/mL, 5.6 pg/mL and 21.2 pg/mL, respectively. They act in the nanomolar or picomolar range as compared with DHA and EPA acting in the micromolar range (López-Vicario et al., 2016; Clària et al., 2017; Serhan, 2017a; Wang and Colgan, 2017; Rosenthal et al., 2018).

Resolvins

RvDs have been identified in mice peritoneal exudates (Serhan et al., 2002). They have many properties at the periphery: protection against bacterial infection, prevention of leucocyte infiltration, inhibition of the production of cytokines, etc. (Spite et al., 2009; Serhan and Petasis, 2011; Chiang et al., 2012; Dalli et al., 2013a; Winkler et al., 2016). RvD1 and its precursor metabolites have been detected in the brain.

In vitro, 17-HDHA has been found in glial cells after DHA and inflammatory stimulus (Hong et al., 2003). *In vivo*, RvD1 has been identified in mouse brain following cerebral ischemia (Marcheselli et al., 2003). Intravenous (iv) DHA injection increases RvD1 levels in rats suggesting the conversion of DHA into RvD1 (Mulik et al., 2016). RvD1 acts through the lipoxin A4 receptor/formyl peptide receptor 2 (ALX/Fpr2) in rodents and the G protein coupling receptor 32 (GPR32) in humans (Recchiuti, 2013) in the picomolar range but induces biological effects in the nanomolar range (Sun et al., 2007; Krishnamoorthy et al., 2010). An overexpression of ALX/Fpr2 or GPR32 increases in phagocytosis by macrophages whereas their deletion decreases the phagocytosis response (Perretti et al., 2002; Krishnamoorthy et al., 2010; Cooray et al., 2013). RvD1 regulates the expression of specific micro-RNA (miR) to control the intensity and the length of inflammation *via* the regulation of target genes such as inflammatory cytokine genes (Fredman and Serhan, 2011; Recchiuti et al., 2011; Rey et al., 2016; Bisicchia et al., 2018). RvD1 increases the expression of miR-21, miR-146b and miR-219 and decreases the expression of miR-208a in macrophages and peritoneal exudates in mice (Recchiuti et al., 2011; Krishnamoorthy et al., 2012). RvD1 modulates miR-155, miR-146, miR-21 and miR-219 in microglial cells (Rey et al., 2016). These miRs have different biological roles: miR-21 is essential to the production of anti-inflammatory cytokine IL-10, miR-146 regulates the transcription of cytokines, chemokines and their receptor, miR-219 decreases the transcription of TNF-α and miR-208a regulates the activation of NFkB (Recchiuti et al., 2011).

RvD1 controls the inflammatory response in many animal models *via* its anti-inflammatory and pro-resolutive properties. In rats, endogenous RvD1 levels decrease at the beginning of inflammation and then increase during the resolution phase (Sun et al., 2014). Recent studies describe the anti-inflammatory properties of RvD1 in microglia and astrocyte cell cultures (Abdelmoaty et al., 2013; Li et al., 2014; Rey et al., 2016). Indeed, in BV2 microglia cell culture, RvD1 promotes the IL-4-induced M2 phenotype (Li et al., 2014) and inhibits LPS-induced pro-inflammatory cytokines (Rey et al., 2016). In astrocyte cell culture, RvD1 attenuates LPS-induced TNF-α (Abdelmoaty et al., 2013). *In vivo*, in a model of remote damage, Bisicchia et al. recently show in rats that RvD1 promotes functional recovery and reduces neuroinflammation *via* miRs (Bisicchia et al., 2018).

Biological Roles of RvD1 in Humans

The effect of RvD1 has been studied in patients suffering from Alzheimer's disease. This pathology is characterized by an increase in microglial activation and in pro-inflammatory cytokine production in the brain (Griffin et al., 1989; Cagnin et al., 2001). Interestingly, RvD1 levels in the cerebrospinal fluid are positively correlated with the enhancement of cognitive functions of patients with dementia (Wang et al., 2015). Indeed, RvD1 may be involved in Aβ phagocytosis. This has been shown *in vitro* in macrophages isolated from Alzheimer's patients (Mizwicki et al., 2013; Famenini et al., 2017). Thus the decrease in RvD1 levels in Alzheimer patients' brains could contribute to the evolution of the disease.

Biological Roles of RvD1 in Rodents

RvD1 attenuates the pro-inflammatory status in the central nervous system. Indeed, an intrathecal injection of 17R-HDHA decreases TNF-α release in the spinal cord in rats (Abdelmoaty et al., 2013). Orr et al. show that icv injection of 17S-HDHA into mice decreases the expression of hippocampal pro-inflammatory cytokines IL-1β and TNF-α induced by LPS acute icv injection (Orr et al., 2013). However, these authors detected NPD1 but did not detect RvD1, suggesting that the effect of 17S-HDHA was rather due to the conversion of 17S-HDHA into NPD1. RvD1 is also able to stimulate phagocytosis in macrophages (Rossi et al., 2015). Indeed, DHA and RvD1 induce the polarization of macrophages toward an M2 phagocytic phenotype in mice (Titos et al., 2011).

Studies have highlighted the protective role of RvD1 in the occurrence of cognitive deficits. Terrando et al. showed that an ip injection of 17-HDHA restores transmission and synaptic plasticity and prevents astrogliosis and cognitive decline in a systemic inflammation model in mice (Terrando et al., 2013). Conversely, an inhibition of 15-LOX, associated with a decrease in RvD1, alters synaptic plasticity and working memory as demonstrated in rats (Shalini et al., 2017). RvD1 plays also a role during the recovery phase following cerebral

ischemia. Indeed, the precursor of 17-HDHA, 17-hydroperoxy docosahexaenoic acid (17-HpDHA), accumulates in the hippocampus of mice (Marcheselli et al., 2003). An ip chronic administration of 17R-HDHA synthesized by acetylated COX-2, prevents cognitive deficits and attenuates motor disorders but doesn't ameliorate microglial activation and sleep quality in mice (Harrison et al., 2015). Moreover, Fat-1 mice that have more brain n-3 LC-PUFAs, have higher hippocampus RvD1 levels, associated with less cognitive deficits, a better neuronal survival, a decrease in astrocyte and microglial activation and a reduction in pro-inflammatory status following brain ischemia (Luo et al., 2014; Delpech et al., 2015a).

Studies have also highlighted the protective role of resolvins in the depressive-like behavior in rodents. Some of them have been recently reported by Furuyashiki et al. (Furuyashiki et al., 2019). An icv injection of RvD1, D2, E1, E2, or E3 significantly decreases LPS-induced depressive-like behavior in mice (Deyama et al., 2017; Deyama et al., 2018a; Deyama et al., 2018b). Moreover, the occurrence of depressive-like behavior associated with pain can also be prevented by an intrathecal injection of 17R-HDHA that is associated with the decrease of pain perception and a restoration of dopamine and glutamate levels in the brain both in rats (Abdelmoaty et al., 2013) and mice (Klein et al., 2014). RvD1 and D2 have also positive effects in chronic mild stress-induced depression and in post-myocardial infarct depression in rats (Gilbert et al., 2014) and mice (Ishikawa et al., 2017).

Biological Roles of RvD1 in In Vitro Models

The effects of RvD1 were tested on different brain cells. In microglial cells, RvD1 potentiates the effect of the anti-inflammatory cytokine IL-4 in the activation of M2 phenotype of microglia (Li et al., 2014). Moreover, we showed that RvD1 decreases LPS-induced pro-inflammatory cytokines (TNF-α, IL-6 and IL-1β) gene expression in microglial BV2 cells *via* the modulation of miRs (Rey et al., 2016). This suggests its pro-resolutive activity in microglia. In astrocytes, RvD1 decreases TNF-α release induced by LPS injection (Abdelmoaty et al., 2013). In neurons from spinal nods, RvD1 increases neurite outgrowth (Shevalye et al., 2015). All these studies suggest that RvD1 can play a central role in the regulation of neuroinflammatory pathologies.

Other D resolvins have been identified in rodent brain: RvD2, RvD4 and RvD5 (Orr et al., 2013; Hashimoto et al., 2015; Winkler et al., 2016). RvD2 limits the activation of microglial cells and inhibits the TLR4/NFκB pathway (Tian et al., 2015). Moreover, chronic intrathecal RvD2 injection prevents the behavioral alterations induced by LPS central injection (Tian et al., 2015). It also prevents depressive-like behavior induced by ip LPS injection by regulating mammalian target of rapamycin complex 1 (mTORC1) complex (Deyama et al., 2017). RvD5 level is decreased in the brain of Alzheimer's disease patients but its role has not yet been identified (Zhu et al., 2016).

Neuroprotectin

Di-hydroxy-DHA termed protection D1 (PD1) has been identified in blood, peritoneal neutrophils and neuroprotectin D1 (NPD1) in the brain in response to zymosan in mice (Hong et al., 2003). Marcheselli et al. have measured NPD1 production in mice hippocampus following brain ischemia (Marcheselli et al., 2003). One receptor for NPD1 has recently been identified as GPR37 (Qu and Caterina, 2018). NPD1 inhibits the oxidative stress in retinal epithelial cells and stimulates their proliferation (Bazan, 2009; Calandria and Bazan, 2010). NPD1 is also able to inhibit neovascularization *via* microglial cell ramification in mice (Sheets et al., 2013). In the central nervous system of mice, the NPD1 level greatly increases in the hippocampus following brain ischemia or acute central LPS injection (Marcheselli et al., 2003; Orr et al., 2013). Hence, NPD1 limits neutrophil infiltration, inhibits NFκB and then decreases pro-inflammatory gene expression (Marcheselli et al., 2003; Bazan et al., 2012; Yao et al., 2013). NPD1 levels decrease in the hippocampus of Alzheimer's disease patients (Lukiw et al., 2005). It plays a role in cellular survival *via* anti-apoptotic protein induction and attenuates pro-inflammatory responses following Aβ exposure *via* NFκB regulation (Lukiw et al., 2005; Bazan, 2008; Asatryan and Bazan, 2017).

Maresins

Mar1 has been identified in mice peritoneal macrophages (Dalli et al., 2013b; Serhan et al., 2009). Its receptor has not been identified yet (Zhu et al., 2016). Mar1 is involved in the resolution of inflammation, prevents neutrophil infiltration, and increases the phagocytosis of apoptotic neutrophils by macrophages in a peritonitis murine model (Serhan et al., 2009). In a murine model of colitis, Mar1 decreases the expression of pro-inflammatory cytokines by inhibiting the NFκB pathway and activating the M2 phenotype in macrophages (Marcon et al., 2013). Mar1 and its precursor 14-HDHA have recently been identified in the hippocampus of mice (Orr et al., 2013). In *post-mortem* brain of Alzheimer's disease patients, the Mar1 level is decreased (Zhu et al., 2016). In this pathology, its role is to stimulate Aβ plaque phagocytosis by microglial cells and to decrease inflammatory marker levels. Hence, Mar1 may play an important role in the pathogenesis of Alzheimer's disease (Zhu et al., 2016). In cerebral ischemia in mice, Mar1 icv injection decreases inflammation and mitochondrial damage and also reduces neurological deficits *via* activation of SIRT-1 signaling (Xian et al., 2016; Xian et al., 2019). After spinal cord injury in mice, iv injection of Mar1 promotes resolution of inflammation (reducing pro-inflammatory cytokines, silencing pro-inflammatory signaling cascades and enhancing the M2 repair macrophage phenotype) and functional recovery (Francos-Quijorna et al., 2017). Mar1 also decreases *in vitro* neuronal death (Zhu et al., 2016).

Biological Roles of EPA-Derived SPMs

RvE1, and its precursor 18-HEPE, have been detected in the hippocampus of rats (Chen et al., 2011) and mice (Orr et al., 2013; Siegert et al., 2017). In human serum, the EPA-derivatives represent 25.9% of the identified SPMs (Colas et al., 2014; Serhan et al., 2018).

RvE1 has been initially identified in mouse exudates (Serhan et al., 2000). RvE1 directly binds to its receptor ChemR23 or

CMKLR1 (chemokine-like receptor 1) (Ohira et al., 2010). It is also a partial agonist of LTB4 receptor (BLT1) (Arita et al., 2007). In the central nervous system, ChemR23 has been identified in the prefrontal cortex, hippocampus and brainstem (Guo et al., 2012), in microglial cells (Graham et al., 2009; Rey et al., 2016) and in neurons (Xu et al., 2010). It is highly expressed in neurons, in microglial cells and in astrocytes in the *post-mortem* hippocampus of Alzheimer's patient brain (Wang et al., 2015). This increase could be due to a compensatory mechanism to counter-balance the decrease in RvE1 in such patients (Wang et al., 2015). *In vitro*, RvE1 plays also a direct role in microglial cells by inhibiting microglial activation and pro-inflammatory cytokine release (Xu et al., 2013; Rey et al., 2016). RvE1 and its precursor, 18R-HEPE, exert anti-inflammatory and anti-apoptotic properties (Hecker et al., 2018). Indeed, they restore mitochondrial dysfunction induced by inflammation in mononuclear blood cells. *In vivo*, RvE1 modulates the inflammatory profile and microglial activation in mice (Xu et al., 2013; Harrison et al., 2015). Intraperitoneal injection of RvE1 also modulates inflammation (by reducing Il-1β, Il-6 and IL-10 levels in the prefrontal cortex) and decreases the measures of Aβ pathology in a murine model of Alzheimer's disease (Kantarci et al., 2018). RvE1 and RvE2 centrally administered also reduce the LPS-induced depressive-like behavior through ChemR23 in mice (Deyama et al., 2018b). RvE1, a total agonist of ChemR23, is more effective than RvE2, which is only a partial agonist of this receptor (Serhan and Chiang, 2013).

REGULATION OF THE n-3 PUFA-DERIVED SPMs

Resolution of inflammation is an active process involving the regulation of the synthesis of numerous mediators in a tightly coordinated manner. The balance between n-3 PUFA-derived SPMs and the pro-inflammatory mediators determines the duration of the inflammatory response and the timing of resolution (Fredman et al., 2017).

Regulation of the n-3 PUFA-Derived SPMs by PUFA Consumption

We have previously shown that PUFA consumption leads to modifications in PUFA levels in the brain. The PUFA derivative levels and their biosynthetic enzyme expression also depend on dietary PUFAs.

SPM levels in peripheral organs and the brain are modulated by different dietary supplies of PUFAs (**Figure 4**). In humans supplemented with 2.4g/day of n-3 LC-PUFAs for 3 weeks (EPA+DHA), SPM levels are generally found to be between 20 and 200 pg/mL (Mas et al., 2012). A shorter supplementation period (5 days) also increased DHA- and EPA- derived SPMs in plasma, especially 18-HEPE, RvE1, 17- and 14-HDHA (Barden et al., 2014). In another study, supplementing patients with a higher fish oil supplement (17.6g/day EPA+DHA) for 24h, only PD1 is detected in the plasma and this increases from 1.0–1.2 pg/mL to 2.7–4.1 pg/mL (Skarke et al., 2015; Barden et al., 2016). These differences in SPM levels in human plasma could be due to differences in study design, dose, duration of n-3 fatty acid supplementation and patient characteristics, etc. (Barden et al., 2016; Chiang and Serhan, 2017). Moreover, dietary supplementation in DHA (1.7g/j) and EPA (0.6g/j) for 6 months increased the RvD1 released by blood mononuclear cells in Alzheimer's disease patients as compared with controls (Wang et al., 2015). Results from intervention studies show that EPA and DHA dietary supplementation increases EPA- and DHA-derived oxylipins although with high inter-individual variances (Ostermann et al., 2017).

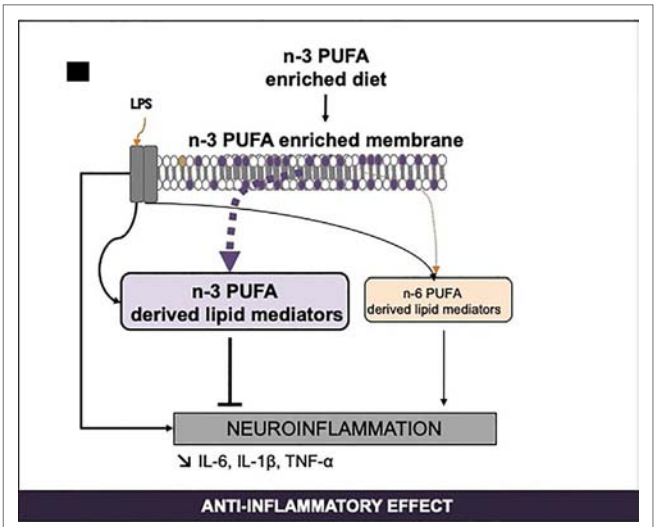

FIGURE 4 | Regulation of n-3 long-chain PUFA-derived SPMs by PUFA consumption and inflammation. IL, interleukine; LPS, lipopolysaccharides; PUFA, polyunsaturated fatty acids; SPM, specialized pro-resolving mediators; TNF, tumor necrosis factor.

In animals, a 3-week fish oil supplementation in arthritic mice increases synthesis of n-3 PUFA-derived SPMs associated with a diminished production of pro-inflammatory mediators (Norling et al., 2016). n-3 PUFA dietary supplementation (140 mg EPA+110 mg DHA/kg animal) in aged rats for 10 weeks enhances the production of lipid mediators derived from DHA and EPA in the cerebral cortex (Hashimoto et al., 2015). DHA and EPA supply also decreases the n-6 PUFA-derived metabolites (which are mainly pro-inflammatory) and increases the n-3 PUFA-derived metabolites in the plasma and at the periphery both in mice (Balvers et al., 2012; Lazic et al., 2014) and rats (Hashimoto et al., 2015). A diet enriched with fish oil (containing 9% EPA + 9% DHA) for 8 weeks in mice increases plasmatic RvD1 (Shevalye et al., 2015). In Fat-1 mice, brain EPA increase is accompanied by an increase in EPA-derived HEPEs (Siegert et al., 2017). Conversely, an n-6 PUFA dietary supply from weaning to 15 weeks of age increases the n-6 PUFA metabolites and decreases the EPA-derived metabolites in the cortex and the plasma of rats (Taha et al., 2016). Recently, we showed that, in mice, n-3 LC-PUFA supplementation from weaning for 2 months induces n-3 LC-PUFA enrichment in the hippocampus and subsequently an increase in n-3 PUFA-derived SPMs and a decrease in n-6

PUFA-derived mediators (Rey et al., 2019). Moreover, in response to LPS, n-3 LC-PUFA-deficient mice present a pro-inflammatory lipid mediator profile whereas n-3 LC-PUFA-supplemented mice display an anti-inflammatory profile in the hippocampus (Rey et al., 2019). Indeed, the consumption of n-3 LC-PUFAs increases the EPA and DHA derivatives. Hashimoto et al. found the same in the cortex of aged rats (Hashimoto et al., 2015). These results differ from those of Trépanier et al. who found that, in a model of Fat-1 mice, a greater increase in brain DHA induced by dietary supply has no effect on the resolution of inflammation after an icv LPS injection (Trépanier et al., 2018).

SPM production is also finely tuned by the regulation of the enzymes in their biosynthesis pathway. A 15-week n-3 PUFA-deficient diet increases the COX-2 expression in the prefrontal cortex of rats, suggesting an increase in AA-derived pro-inflammatory mediator levels (Rao et al., 2007). Conversely, a 15-week n-6 PUFA-deficient diet decreases brain COX-2 expression in rats (Kim et al., 2011). Interestingly, Taha et al. showed that rats fed on an n-6 PUFA-deficient diet for 15 weeks are protected from an increase in COX-2 observed in LPS-treated n-3 PUFA-deficient animals (Taha et al., 2017). However, we showed that 2 months' n-3 LC-PUFA dietary supplementation was not sufficient to modulate COX-2 expression in the brain of mice (Rey et al., 2019). An n-3 LC-PUFA diet increases 15-LOX expression in the hippocampus without changes in the 5-LOX expression (Gabbs et al., 2015). The increase in 15-LOX in the brain has also been described following a 15-week n-6 PUFA-deficient diet in rats (Kim et al., 2011). 15-LOX is responsible for many n-6 and n-3 PUFA derivatives. Those synthesized from AA drastically decrease, and those produced from DHA increase, following a diet enriched in n-3 PUFAs in both mice and rats (Ostermann et al., 2017; Shalini et al., 2017; Rey et al., 2019).

Results on 15-LOX functions on inflammation regulation are conflicting. 15-LOX was initially described as deleterious in neurodegenerative pathologies because it increased the oxidative stress and neuronal degeneration (Pratico et al., 2004; Chinnici et al., 2007; Di Meco et al., 2014; Wang et al., 2015; Yigitkanli et al., 2017). It alters the mitochondrial function of neurons and then induces neuronal death (Pallast et al., 2009). However, recent studies demonstrate the protective role of 15-LOX, particularly in cognition. An increase in its expression is associated with a better working memory (Sun et al., 2015; Shalini et al., 2017). Moreover, an increase in 15-LOX expression during cerebral ischemia is essential for the recovery of neurological functions after the ischemic event (Wang et al., 2017). These results suggest that 15-LOX has a beneficial role in acute inflammation (ischemia, LPS) and is deleterious in chronic inflammation (neurodegenerative pathologies). SPM production is also partly regulated by the availability of free PUFAs that are their precursors. Indeed, PLA2 activity also depends on PUFA dietary supply. An n-6 PUFA-deficient diet increases the iPLA2 that is responsible for the release of DHA from the membranes and a decrease in calcium sensitive cytosolic PLA2 (cPLA2) associated with the hydrolysis of AA from the membranes (Kim et al., 2011).

Lipid nutrition, to which people are exposed throughout their lives, seems to play a major role in the synthesis of bioactive SPMs.

Regulation of the n-3 PUFA-Derived SPMs by Inflammation

Numerous studies have highlighted that inflammation modulates lipid mediator synthesis at the periphery and in the brain (**Figure 4**).

In humans, Wang et al. showed that the RvD1 level in the cerebrospinal fluid (CSF) of Alzheimer's disease patients is positively correlated with cognitive function (Wang et al., 2015). Moreover, 15-LOX was decreased in the CSF suggesting an alteration of the resolution of inflammation in Alzheimer's patients.

In rats, brain ischemia increases the production of 5 of mono-, di- and tri-hydroxy-DHA derivatives (Farias et al., 2008; Hennebelle et al., 2017). However, LPS icv injection does not impact the RvD1 level (Rosenberger et al., 2004; Taha et al., 2017). We have recently shown that, in mice, ip LPS injection modifies the n-6 PUFA derivative profile but not n-3 LC-PUFA derivatives (Rey et al., 2019).

In vitro, most studies have been performed on peripheral immune cells. The lipid mediator profile in rodent neutrophils and macrophages changes with macrophage phenotype M1 or M2, or with the inflammatory stimulus in neutrophils (Dieter et al., 2002). Indeed, M2 macrophages produce more D resolvins, protectins and maresins and less AA-derived pro-inflammatory mediators than M1 macrophages (Dalli and Serhan, 2012).

Inflammation more drastically alters the expression of biosynthetic enzymes than an n-3 LC-PUFA dietary supply. In rats, traumatic brain injury increases the COX-2, 5-LOX, 15-LOX and CYP450 expression in the hippocampus and cortex, suggesting an alteration of all lipid mediator biosynthesis pathways (Birnie et al., 2013). LPS also regulates the expression of these enzymes. An icv LPS injection increases COX-2 expression and activity in the brain in mice (Rey et al., 2019) and rats. Moreover, in *in vitro* human monocytes and dendritic cells, anti-inflammatory cytokines IL-4 or IL-13 increase the production of 15-LOX and decrease the production of 5-LOX (Nassar et al., 1994; Spanbroek et al., 2001).

Neuroinflammation also modifies SPM receptor expression. Indeed, we show that LPS increases significantly the expression of RvD1 and RvE1 receptors (ALX/Fpr2 and ChemR23, respectively) in BV-2 microglial cells (Rey et al., 2016). The increase in ALX/Fpr2 expression was also detected in monocytes, in the hippocampus and in the cortex and in microglial cells in response to an inflammatory stimulus (Krishnamoorthy et al., 2010; Wang et al., 2011; Wang et al., 2015; Guo et al., 2016). Then, inflammation activates the SPM signaling pathway in the brain to regulate the inflammatory response.

HUMAN TRANSLATION

Several possibilities can be considered to translate the findings described above and then attenuate the inflammatory tone, amplitude and duration of inflammation. The first one is to increase the local production of n-3 LC-PUFA-derived SPMs. We see that dietary means is a good way to modulate the level of the fatty acids from which they are synthesized and then to modify their synthesis. The SPM profile synthesized by

each individual could be responsible for the differences in the effects of n-3 PUFA obtained in humans. SPM profiles should be established in patients with different acute and chronic inflammatory pathologies, and in mice under the same conditions to find markers of neuroinflammation in the plasma that can be transposed to humans. Thanks to new technologies in liquid chromatography mass spectrometry (LC-MS/MS), specific mediators produced during physiological and non-physiological conditions should be identified, allowing patient stratification according to disease severity. It could be interesting to determine an individual metabolomic profile to define personalized nutrition (n-3 PUFAs and doses) allowing an increase of n-3 PUFA-derived SPMs in the target tissue. Indeed, there are individual differences in diets and in n-3 PUFA supplementation and also in nutrient metabolism and biological responses to food/nutrients. The aim of personalized nutrition is to increase health using nutrition by delivering specific personalized intervention suited to each individual based on the individual's nutritional phenotype, metabolic profile, and environmental factors in order to prevent and treat chronic disease. Personalized nutrition can also be applied to healthy people. It is nowadays accessible because of a better understanding of the mechanisms of the effect of nutrition on health and also because of the progress in technologies enabling the identification of specific markers. Personalized nutrition has already shown its efficacy, especially in the Food4Me study involving >1600 participants from 7 European countries and in a systematic review and meta-analysis showing a greater efficacy of personalized nutrition in changing diet than a conventional approach (Celis-Morales et al., 2018).

The second possibility for taking advantage of research on the resolution of inflammation is to administer exogenous SPMs. Serhan defines a new concept of resolutive pharmacology targeting the development of SPM analogs, resistant to local inactivation, to stimulate natural circuits of resolution (Chiang and Serhan, 2017). The objective of this new therapeutic pathway is to administer these analogs in association with classical therapy in order to decrease the doses, thus limiting the secondary effects. A clinical trial has reported for the first time the efficacy of an RvE1 analog in patients with dry eye symptoms (Resolvyx Pharmaceuticals). These encouraging results should be extended to the use of such molecules to treat other inflammatory diseases.

CONCLUSION

In the investigation of new anti-inflammatory treatments without the secondary effects of traditional therapy, SPMs are promising therapeutic compounds: they are of natural origin and are active at low concentrations (nM) as compared with their precursor (μM) (Ariel and Serhan, 2007; Bannenberg and Serhan, 2010). SPMs are detectable in the brain and their level can be modulated by dietary supplementation. They have potent anti-inflammatory and pro-resolutive properties and we confirm the main role of nutrition as an environmental factor that greatly influences the inflammatory response. It is important to determine if inflammatory pathologies are due to unresolved inflammation, attributed to a decrease in n-3 LC-PUFA dietary intake leading to a decrease in SPM levels, to an enzyme or receptor polymorphism, to a de-regulation of SPM receptors or to a decrease in their expression. We should also determine if these mechanisms could be restored by n-3 LC-PUFA or SPM analog administration.

AUTHOR CONTRIBUTIONS

All authors (CJ, CR, SL) contributed to the writing of the manuscript.

REFERENCES

Abdelmoaty, S., Wigerblad, G., Bas, D. B., Codeluppi, S., Fernandez-Zafra, T., El-Awady el, S., et al. (2013). Spinal actions of lipoxin A4 and 17(R)-resolvin D1 attenuate inflammation-induced mechanical hypersensitivity and spinal TNF release. *PLoS One* 8, e75543. doi: 10.1371/journal.pone.0075543

Aid, S., Vancassel, S., Poumes-Ballihaut, C., Chalon, S., Guesnet, P., and Lavialle, M. (2003). Effect of a diet-induced n-3 PUFA depletion on cholinergic parameters in the rat hippocampus. *J. Lipid Res.* 44, 1545–1551. doi: 10.1194/jlr.M300079-JLR200

Alashmali, S. M., Hopperton, K. E., and Bazinet, R. P. (2016). Lowering dietary n-6 polyunsaturated fatty acids: interaction with brain arachidonic and docosahexaenoic acids. *Curr. Opin. Lipidol.* 27, 54–66. doi: 10.1097/MOL.0000000000000255

Antonietta Ajmone-Cat, M., Lavinia Salvatori, M., De Simone, R., Mancini, M., Biagioni, S., Bernardo, A., et al. (2012). Docosahexaenoic acid modulates inflammatory and antineurogenic functions of activated microglial cells. *J. Neurosci. Res.* 90, 575–587. doi: 10.1002/jnr.22783

Ariel, A., and Serhan, C. N. (2007). Resolvins and protectins in the termination program of acute inflammation. *Trends Immunol.* 28, 176–183. doi: 10.1016/j.it.2007.02.007

Arita, M., Ohira, T., Sun, Y. P., Elangovan, S., Chiang, N., and Serhan, C. N. (2007). Resolvin E1 selectively interacts with leukotriene B4 receptor BLT1 and ChemR23 to regulate inflammation. *J. Immunol.* 178, 3912–3917. doi: 10.4049/jimmunol.178.6.3912

Asatryan, A., and Bazan, N. G. (2017). Molecular mechanisms of signaling via the docosanoid neuroprotectin D1 for cellular homeostasis and neuroprotection. *J. Biol. Chem.* 292, 12390–12397. doi: 10.1074/jbc.R117.783076

Aursnes, M., Tungen, J. E., Vik, A., Colas, R., Cheng, C.-Y. C., Dalli, J., et al. (2014). Total synthesis of the lipid mediator PD1n-3 DPA: configurational assignments and anti-inflammatory and pro-resolving actions. *J. Nat. Prod.* 77, 910–916. doi: 10.1021/np4009865

Balvers, M. G., Verhoeckx, K. C., Bijlsma, S., Rubingh, C. M., Meijerink, J., Wortelboer, H. M., et al. (2012). Fish oil and inflammatory status alter the n-3 to n-6 balance of the endocannabinoid and oxylipin metabolomes in mouse plasma and tissues. *Metabolomics* 8, 1130–1147. doi: 10.1007/s11306-012-0421-9

Bannenberg, G., and Serhan, C. N. (2010). Specialized pro-resolving lipid mediators in the inflammatory response: an update. *Biochim. Biophys. Acta* 1801, 1260–1273. doi: 10.1016/j.bbalip.2010.08.002

Barden, A. E., Mas, E., and Mori, T. A. (2016). n-3 Fatty acid supplementation and proresolving mediators of inflammation. *Curr. Opin. Lipidol.* 27, 26–32. doi: 10.1097/MOL.0000000000000262

Barden, A., Mas, E., Croft, K. D., Phillips, M., and Mori, T. A. (2014). Short-term n-3 fatty acid supplementation but not aspirin increases plasma proresolving mediators of inflammation. *J. Lipid Res.* 55, 2401–2407. doi: 10.1194/jlr.M045583

Bascoul-Colombo, C., Guschina, I. A., Maskrey, B. H., Good, M., O'Donnell, V. B., and Harwood, J. L. (2016). Dietary DHA supplementation causes selective changes in phospholipids from different brain regions in both wild type mice and the Tg2576 mouse model of Alzheimer's disease. *Biochim. Biophys. Acta* 1861, 524–537. doi: 10.1016/j.bbalip.2016.03.005

Bazan, N. G. (2008). Neurotrophins induce neuroprotective signaling in the retinal pigment epithelial cell by activating the synthesis of the anti-inflammatory and anti-apoptotic neuroprotectin D1. *Adv. Exp. Med. Biol.* 613, 39–44. doi: 10.1007/978-0-387-74904-4_3

Bazan, N. G. (2009). Cellular and molecular events mediated by docosahexaenoic acid-derived neuroprotectin D1 signaling in photoreceptor cell survival and brain protection. *Prostaglandins Leukot. Essent. Fatty Acids* 81, 205–211. doi: 10.1016/j.plefa.2009.05.024

Bazan, N. G., Eady, T. N., Khoutorova, L., Atkins, K. D., Hong, S., Lu, Y., et al. (2012). Novel aspirin-triggered neuroprotectin D1 attenuates cerebral ischemic injury after experimental stroke. *Exp. Neurol.* 236, 122–130. doi: 10.1016/j.expneurol.2012.04.007

Bazinet, R. P., Metherel, A. H., Chen, C. T., Raza Shaikh, S., Nadjar, A., Joffre, C., et al. (2019). Brain eicosapentaenoic acid metabolism as a lead for novel therapeutics in major depression. *Brain Behav. Immun.* doi: 10.1016/j.bbi.2019.07.001

Birnie, M., Morrison, R., Camara, R., and Strauss, K. I. (2013). Temporal changes of cytochrome P450 (Cyp) and eicosanoid-related gene expression in the rat brain after traumatic brain injury. *BMC Genomics* 14, 303. doi: 10.1186/1471-2164-14-303

Bisicchia, E., Sasso, V., Catanzaro, G., Leuti, A., Besharat, Z. M., Chiacchiarini, M., et al. (2018). Resolvin D1 halts remote neuroinflammation and improves functional recovery after focal brain damage via ALX/FPR2 receptor-regulated microRNAs. *Mol. Neurobiol.* 55, 6894–6905. doi: 10.1007/s12035-018-0889-z

Blais, V., and Rivest, S. (2003). [Role of the innate immune response in the brain]. *Med. Sci. (Paris)* 19, 981–987. doi: 10.1051/medsci/20031910981

Boudrault, C., Bazinet, R. P., Kang, J. X., and Ma, D. W. (2010). Cyclooxygenase-2 and n-6 PUFA are lower and DHA is higher in the cortex of fat-1 mice. *Neurochem. Int.* 56, 585–589. doi: 10.1016/j.neuint.2009.12.022

Bourre, J. M., Pascal, G., Durand, G., Masson, M., Dumont, O., and Piciotti, M. (1984). Alterations in the fatty acid composition of rat brain cells (neurons, astrocytes, and oligodendrocytes) and of subcellular fractions (myelin and synaptosomes) induced by a diet devoid of n-3 fatty acids. *J. Neurochem.* 43, 342–348. doi: 10.1111/j.1471-4159.1984.tb00906.x

Bove, M., Mhillaj, E., Tucci, P., Giardino, I., Schiavone, S., Morgese, M. G., et al. (2018). Effects of n-3 PUFA enriched and n-3 PUFA deficient diets in naïve and Aβ-treated female rats. *Biochem. Pharmacol.* 155, 326–335. doi: 10.1016/j.bcp.2018.07.017

Bowen, R. A., and Clandinin, M. T. (2005). Maternal dietary 22 : 6n-3 is more effective than 18 : 3n-3 in increasing the 22 : 6n-3 content in phospholipids of glial cells from neonatal rat brain. *Br. J. Nutr.* 93, 601–611. doi: 10.1079/BJN20041390

Broadhurst, C. L., Wang, Y., Crawford, M. A., Cunnane, S. C., Parkington, J. E., and Schmidt, W. F. (2002). Brain-specific lipids from marine, lacustrine, or terrestrial food resources: potential impact on early African Homo sapiens. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* 131, 653–673. doi: 10.1016/S1096-4959(02)00002-7

Bystrom, J., Wray, J. A., Sugden, M. C., Holness, M. J., Swales, K. E., Warner, T. D., et al. (2011). Endogenous epoxygenases are modulators of monocyte/macrophage activity. *PLoS One* 6, e26591. doi: 10.1371/journal.pone.0026591

Cagnin, A., Brooks, D. J., Kennedy, A. M., Gunn, R. N., Myers, R., Turkheimer, F. E., et al. (2001). In-vivo measurement of activated microglia in dementia. *Lancet* 358, 461–467. doi: 10.1016/S0140-6736(01)05625-2

Calandria, J. M., and Bazan, N. G. (2010). Neuroprotectin D1 modulates the induction of pro-inflammatory signaling and promotes retinal pigment epithelial cell survival during oxidative stress. *Adv. Exp. Med. Biol.* 664, 663–670. doi: 10.1007/978-1-4419-1399-9_76

Calder, P. C. (2005). Polyunsaturated fatty acids and inflammation. *Biochem. Soc. Trans.* 33, 423–427. doi: 10.1042/BST0330423

Calder, P. C. (2016). Docosahexaenoic Acid. *Ann. Nutr. Metab.* 69 Suppl 1, 7–21. doi: 10.1159/000448262

Calder, P. C., Bosco, N., Bourdet-Sicard, R., Capuron, L., Delzenne, N., Doré, J., et al. (2017). Health relevance of the modification of low grade inflammation in ageing (inflammageing) and the role of nutrition. *Ageing Res. Rev.* 40, 95–119. doi: 10.1016/j.arr.2017.09.001

Carrie, I., Clement, M., de Javel, D., Frances, H., and Bourre, J. M. (2000). Specific phospholipid fatty acid composition of brain regions in mice. Effects of n-3 polyunsaturated fatty acid deficiency and phospholipid supplementation. *J. Lipid Res.* 41, 465–472.

Celis-Morales, C., Livingstone, K. M., Affleck, A., Navas-Carretero, S., San-Cristobal, R., Martinez, J. A., et al. (2018). Correlates of overall and central obesity in adults from seven European countries: findings from the Food4Me Study. *Eur. J. Clin. Nutr.* 72, 207–219. doi: 10.1038/s41430-017-0004-y

Chalon, S. (2006). Omega-3 fatty acids and monoamine neurotransmission. *Prostaglandins Leukot. Essent. Fatty Acids* 75, 259–269. doi: 10.1016/j.plefa.2006.07.005

Chen, C. T., Liu, Z., and Bazinet, R. P. (2011). Rapid de-esterification and loss of eicosapentaenoic acid from rat brain phospholipids: an intracerebroventricular study. *J. Neurochem.* 116, 363–373. doi: 10.1111/j.1471-4159.2010.07116.x

Chen, S., Zhang, H., Pu, H., Wang, G., Li, W., Leak, R. K., et al. (2014). n-3 PUFA supplementation benefits microglial responses to myelin pathology. *Sci. Rep.* 4, 7458. doi: 10.1038/srep07458

Chen, X., Wu, S., Chen, C., Xie, B., Fang, Z., Hu, W., et al. (2017). Omega-3 polyunsaturated fatty acid supplementation attenuates microglial-induced inflammation by inhibiting the HMGB1/TLR4/NF-kappaB pathway following experimental traumatic brain injury. *J. Neuroinflamm.* 14, 143. doi: 10.1186/s12974-017-0917-3

Chiang, N., Fredman, G., Backhed, F., Oh, S. F., Vickery, T., Schmidt, B. A., et al. (2012). Infection regulates pro-resolving mediators that lower antibiotic requirements. *Nature* 484, 524–528. doi: 10.1038/nature11042

Chiang, N., and Serhan, C. N. (2017). Structural elucidation and physiologic functions of specialized pro-resolving mediators and their receptors. *Mol. Aspects Med.* 58, 114–129. doi: 10.1016/j.mam.2017.03.005

Chinnici, C. M., Yao, Y., and Pratico, D. (2007). The 5-lipoxygenase enzymatic pathway in the mouse brain: young versus old. *Neurobiol. Aging* 28, 1457–1462. doi: 10.1016/j.neurobiolaging.2006.06.007

Cho, H. P., Nakamura, M. T., and Clarke, S. D. (1999). Cloning, expression, and nutritional regulation of the mammalian Delta-6 desaturase. *J. Biol. Chem.* 274, 471–477. doi: 10.1074/jbc.274.1.471

Chouinard-Watkins, R., and Bazinet, R. P. (2018). ACSL6 is critical for maintaining brain DHA levels. *Proc. Natl. Acad. Sci. U.S.A.* 115, 12343–12345. doi: 10.1073/pnas.1817557115

Chung, W. L., Chen, J. J., and Su, H. M. (2008). Fish oil supplementation of control and (n-3) fatty acid-deficient male rats enhances reference and working memory performance and increases brain regional docosahexaenoic acid levels. *J. Nutr.* 138, 1165–1171. doi: 10.1093/jn/138.6.1165

Clària, J., López-Vicario, C., Rius, B., and Titos, E. (2017). Pro-resolving actions of SPM in adipose tissue biology. *Mol. Aspects Med.* 58, 83–92. doi: 10.1016/j.mam.2017.03.004

Colas, R. A., Shinohara, M., Dalli, J., Chiang, N., and Serhan, C. N. (2014). Identification and signature profiles for pro-resolving and inflammatory lipid mediators in human tissue. *Am. J. Physiol. Cell. Physiol.* 307, C39–C54. doi: 10.1152/ajpcell.00024.2014

Cooray, S. N., Gobbetti, T., Montero-Melendez, T., McArthur, S., Thompson, D., Clark, A. J., et al. (2013). Ligand-specific conformational change of the G-protein-coupled receptor ALX/FPR2 determines proresolving functional responses. *Proc. Natl. Acad. Sci. U. S. A.* 110, 18232–18237. doi: 10.1073/pnas.1308253110

Corsi, L., Dongmo, B. M., and Avallone, R. (2015). Supplementation of omega 3 fatty acids improves oxidative stress in activated BV2 microglial cell line. *Int. J. Food Sci. Nutr.* 66, 293–299. doi: 10.3109/09637486.2014.986073

Crawford, M. A., Bloom, M., Broadhurst, C. L., Schmidt, W. F., Cunnane, S. C., Galli, C., et al. (1999). Evidence for the unique function of docosahexaenoic acid during the evolution of the modern hominid brain. *Lipids* 34 Suppl, S39–S47. doi: 10.1007/BF02562227

Cunningham, C., and Sanderson, D. J. (2008). Malaise in the water maze: untangling the effects of LPS and IL-1beta on learning and memory. *Brain Behav. Immun.* 22, 1117–1127. doi: 10.1016/j.bbi.2008.05.007

Czapski, G. A., Gajkowska, B., and Strosznajder, J. B. (2010). Systemic administration of lipopolysaccharide induces molecular and morphological alterations in the hippocampus. *Brain Res.* 1356, 85–94. doi: 10.1016/j.brainres.2010.07.096

Dalli, J., and Serhan, C. N. (2012). Specific lipid mediator signatures of human phagocytes: microparticles stimulate macrophage efferocytosis and pro-resolving mediators. *Blood* 120, e60–e72. doi: 10.1182/blood-2012-04-423525

Dalli, J., Winkler, J. W., Colas, R. A., Arnardottir, H., Cheng, C. Y., Chiang, N., et al. (2013a). Resolvin D3 and aspirin-triggered resolvin D3 are potent immunoresolvents. *Chem. Biol.* 20, 188–201. doi: 10.1016/j.chembiol.2012.11.010

Dalli, J., Zhu, M., Vlasenko, N. A., Deng, B., Haeggstrom, J. Z., Petasis, N. A., et al. (2013b). The novel 13S,14S-epoxy-maresin is converted by human macrophages to maresin 1 (MaR1), inhibits leukotriene A4 hydrolase (LTA4H), and shifts macrophage phenotype. *FASEB J.* 27, 2573–2583. doi: 10.1096/fj.13-227728

Davidson, J., Abul, H. T., Milton, A. S., and Rotondo, D. (2001). Cytokines and cytokine inducers stimulate prostaglandin E2 entry into the brain. *Pflugers Arch.* 442, 526–533. doi: 10.1007/s004240100572

De Smedt-Peyrusse, V., Sargueil, F., Moranis, A., Harizi, H., Mongrand, S., and Layé, S. (2008). Docosahexaenoic acid prevents lipopolysaccharide-induced cytokine production in microglial cells by inhibiting lipopolysaccharide receptor presentation but not its membrane subdomain localization. *J. Neurochem.* 105, 296–307. doi: 10.1111/j.1471-4159.2007.05129.x

de Theije, C. G., van den Elsen, L. W., Willemsen, L. E., Milosevic, V., Korte-Bouws, G. A., Lopes da Silva, S., et al. (2015). Dietary long chain n-3 polyunsaturated fatty acids prevent impaired social behaviour and normalize brain dopamine levels in food allergic mice. *Neuropharmacology* 90, 15–22. doi: 10.1016/j.neuropharm.2014.11.001

Dehkordi, N. G., Noorbakhshnia, M., Ghaedi, K., Esmaeili, A., and Dabaghi, M. (2015). Omega-3 fatty acids prevent LPS-induced passive avoidance learning and memory and CaMKII-alpha gene expression impairments in hippocampus of rat. *Pharmacol. Rep.* 67, 370–375. doi: 10.1016/j.pharep.2014.10.014

Delpech, J.-C., Madore, C., Joffre, C., Aubert, A., Kang, J. X., Nadjar, A., et al. (2015a). Transgenic increase in n-3/n-6 fatty acid ratio protects against cognitive deficits induced by an immune challenge through decrease of neuroinflammation. *Neuropsychopharmacology* 40, 525–536. doi: 10.1038/npp.2014.196

Delpech, J.-C., Thomazeau, A., Madore, C., Bosch-Bouju, C., Larrieu, T., Lacabanne, C., et al. (2015b). Dietary n-3 PUFAs deficiency increases vulnerability to inflammation—induced spatial memory impairment. *Neuropsychopharmacology* 40, 2774–2787. doi: 10.1038/npp.2015.127

Destaillats, F., Joffre, C., Acar, N., Joffre, F., Bezelgues, J. B., Pasquis, B., et al. (2010). Differential effect of maternal diet supplementation with alpha-Linolenic adcid or n-3 long-chain polyunsaturated fatty acids on glial cell phosphatidylethanolamine and phosphatidylserine fatty acid profile in neonate rat brains. *Nutr. Metab. (Lond.)* 7, 2. doi: 10.1186/1743-7075-7-2

Deyama, S., Ishikawa, Y., Yoshikawa, K., Shimoda, K., Ide, S., Satoh, M., et al. (2017). Resolvin D1 and D2 reverse lipopolysaccharide-induced depression-like behaviors through the mTORC1 signaling pathway. *Int. J. Neuropsychopharmacol.* 20, 575–584. doi: 10.1093/ijnp/pyx023

Deyama, S., Shimoda, K., Ikeda, H., Fukuda, H., Shuto, S., and Minami, M. (2018a). Resolvin E3 attenuates lipopolysaccharide-induced depression-like behavior in mice. *J. Pharmacol. Sci.* 138, 86–88. doi: 10.1016/j.jphs.2018.09.006

Deyama, S., Shimoda, K., Suzuki, H., Ishikawa, Y., Ishimura, K., Fukuda, H., et al. (2018b). Resolvin E1/E2 ameliorate lipopolysaccharide-induced depression-like behaviors via ChemR23. *Psychopharmacol. (Berl.)* 235, 329–336. doi: 10.1007/s00213-017-4774-7

Di Meco, A., Lauretti, E., Vagnozzi, A. N., and Pratico, D. (2014). Zileuton restores memory impairments and reverses amyloid and tau pathology in aged Alzheimer's disease mice. *Neurobiol. Aging* 35, 2458–2464. doi: 10.1016/j.neurobiolaging.2014.05.016

Dieter, P., Scheibe, R., Kamionka, S., and Kolada, A. (2002). LPS-induced synthesis and release of PGE2 in liver macrophages: regulation by CPLA2, COX-1, COX-2, and PGE2 synthase. *Adv. Exp. Med. Biol.* 507, 457–462. doi: 10.1007/978-1-4615-0193-0_71

Dong, Y., Xu, M., Kalueff, A. V., and Song, C. (2017). Dietary eicosapentaenoic acid normalizes hippocampal omega-3 and 6 polyunsaturated fatty acid profile, attenuates glial activation and regulates BDNF function in a rodent model of neuroinflammation induced by central interleukin-1beta administration. *Eur. J. Nutr.* doi: 10.1007/s00394-017-1462-7

Doyle, R., Sadlier, D. M., and Godson, C. (2018). Pro-resolving lipid mediators: agents of anti-ageing? *Semin. Immunol.* 40, 36–48. doi: 10.1016/j.smim.2018.09.002

Dyerberg, J., and Bang, H. O. (1979). Haemostatic function and platelet polyunsaturated fatty acids in Eskimos. *Lancet* 2, 433–435. doi: 10.1016/S0140-6736(79)91490-9

Engstrom, L., Ruud, J., Eskilsson, A., Larsson, A., Mackerlova, L., Kugelberg, U., et al. (2012). Lipopolysaccharide-induced fever depends on prostaglandin E2 production specifically in brain endothelial cells. *Endocrinology* 153, 4849–4861. doi: 10.1210/en.2012-1375

Famenini, S., Rigali, E. A., Olivera-Perez, H. M., Dang, J., Chang, M. T., Halder, R., et al. (2017). Increased intermediate M1-M2 macrophage polarization and improved cognition in mild cognitive impairment patients on omega-3 supplementation. *FASEB J.* 31, 148–160. doi: 10.1096/fj.201600677RR

Farias, S. E., Basselin, M., Chang, L., Heidenreich, K. A., Rapoport, S. I., and Murphy, R. C. (2008). Formation of eicosanoids, E2/D2 isoprostanes, and docosanoids following decapitation-induced ischemia, measured in high-energy-microwaved rat brain. *J. Lipid Res.* 49, 1990–2000. doi: 10.1194/jlr.M800200-JLR200

Farooqui, A. A., and Horrocks, L. A. (2006). Phospholipase A2-generated lipid mediators in the brain: the good, the bad, and the ugly. *Neuroscientist* 12, 245–260. doi: 10.1177/1073858405285923

Fernandez, R. F., Kim, S. Q., Zhao, Y., Foguth, R. M., Weera, M. M., Counihan, J. L., et al. (2018). Acyl-CoA synthetase 6 enriches the neuroprotective omega-3 fatty acid DHA in the brain. *Proc. Natl. Acad. Sci. U. S. A.* 115, 12525–12530. doi: 10.1073/pnas.1807958115

Fleming, I. (2011). Cytochrome P450-dependent eicosanoid production and crosstalk. *Curr. Opin. Lipidol.* 22, 403–409. doi: 10.1097/MOL.0b013e32834a9790

Fourrier, C., Remus-Borel, J., Greenhalgh, A. D., Guichardant, M., Bernoud-Hubac, N., Lagarde, M., et al. (2017). Docosahexaenoic acid-containing choline phospholipid modulates LPS-induced neuroinflammation in vivo and in microglia in vitro. *J. Neuroinflamm.* 14, 170. doi: 10.1186/s12974-017-0939-x

Francos-Quijorna, I., Santos-Nogueira, E., Gronert, K., Sullivan, A. B., Kopp, M. A., Brommer, B., et al. (2017). Maresin 1 promotes inflammatory resolution, neuroprotection, and functional neurological recovery after spinal cord injury. *J. Neurosci.* 37, 11731–11743. doi: 10.1523/JNEUROSCI.1395-17.2017

Fredman, G., Sadhu, S., and Rymut, N. (2017). Fine-tuning inflammation-resolution programs: focus on atherosclerosis. *Curr. Opin. Clin. Nutr. Metab. Care* 20, 117–123. doi: 10.1097/MCO.0000000000000351

Fredman, G., and Serhan, C. N. (2011). Specialized proresolving mediator targets for RvE1 and RvD1 in peripheral blood and mechanisms of resolution. *Biochem. J.* 437, 185–197. doi: 10.1042/BJ20110327

Freund-Levi, Y., Eriksdotter-Jonhagen, M., Cederholm, T., Basun, H., Faxen-Irving, G., Garlind, A., et al. (2006). Omega-3 fatty acid treatment in 174 patients with mild to moderate Alzheimer disease: OmegAD study: a randomized double-blind trial. *Arch. Neurol.* 63, 1402–1408. doi: 10.1001/archneur.63.10.1402

Furuyashiki, T., Akiyama, S., and Kitaoka, S. (2019). Roles of multiple lipid mediators in stress and depression. *Int. Immunol.* 1781–1791. doi: 10.1093/intimm/dxz023

Gabbs, M., Leng, S., Devassy, J. G., Monirujjaman, M., and Aukema, H. M. (2015). Advances in our understanding of oxylipins derived from dietary PUFAs. *Adv. Nutr.* 6, 513–540. doi: 10.3945/an.114.007732

Gilbert, K., Bernier, J., Godbout, R., and Rousseau, G. (2014). Resolvin D1, a metabolite of omega-3 polyunsaturated fatty acid, decreases post-myocardial infarct depression. *Mar. Drugs* 12, 5396–5407. doi: 10.3390/md12115396

Gilroy, D. W., Edin, M. L., De Maeyer, R. P., Bystrom, J., Newson, J., Lih, F. B., et al. (2016). CYP450-derived oxylipins mediate inflammatory resolution. *Proc. Natl. Acad. Sci. U. S. A.* 113, E3240–E3249. doi: 10.1073/pnas.1521453113

Graham, K. L., Zabel, B. A., Loghavi, S., Zuniga, L. A., Ho, P. P., Sobel, R. A., et al. (2009). Chemokine-like receptor-1 expression by central nervous system-infiltrating leukocytes and involvement in a model of autoimmune demyelinating disease. *J. Immunol.* 183, 6717–6723. doi: 10.4049/jimmunol.0803435

Griffin, W. S., Stanley, L. C., Ling, C., White, L., MacLeod, V., Perrot, L. J., et al. (1989). Brain interleukin 1 and S-100 immunoreactivity are elevated in Down syndrome and Alzheimer disease. *Proc. Natl. Acad. Sci. U. S. A.* 86, 7611–7615. doi: 10.1073/pnas.86.19.7611

Guo, X., Fu, Y., Xu, Y., Weng, S., Liu, D., Cui, D., et al. (2012). Chronic mild restraint stress rats decreased CMKLR1 expression in distinct brain region. *Neurosci. Lett.* 524, 25–29. doi: 10.1016/j.neulet.2012.06.075

Guo, Z., Hu, Q., Xu, L., Guo, Z. N., Ou, Y., He, Y., et al. (2016). Lipoxin A4 reduces inflammation through formyl peptide receptor 2/p38 MAPK signaling pathway in subarachnoid hemorrhage rats. *Stroke* 47, 490–497. doi: 10.1161/STROKEAHA.115.011223

Halade, G. V., Black, L. M., and Verma, M. K. (2018). Paradigm shift — metabolic transformation of docosahexaenoic and eicosapentaenoic acids to bioactives exemplify the promise of fatty acid drug discovery. *Biotechnol. Adv.* 36, 935– 953. doi: 10.1016/j.biotechadv.2018.02.014

Hanisch, U. K., and Kettenmann, H. (2007). Microglia: active sensor and versatile effector cells in the normal and pathologic brain. *Nat. Neurosci.* 10, 1387–1394. doi: 10.1038/nn1997

Harrison, J. L., Rowe, R. K., Ellis, T. W., Yee, N. S., O'Hara, B. F., Adelson, P. D., et al. (2015). Resolvins AT-D1 and E1 differentially impact functional outcome, post-traumatic sleep, and microglial activation following diffuse brain injury in the mouse. *Brain Behav. Immun.* 47, 131–140. doi: 10.1016/j.bbi.2015.01.001

Hashimoto, M., Katakura, M., Tanabe, Y., Al Mamun, A., Inoue, T., Hossain, S., et al. (2015). n-3 fatty acids effectively improve the reference memory-related learning ability associated with increased brain docosahexaenoic acid-derived docosanoids in aged rats. *Biochim. Biophys. Acta* 1851, 203–209. doi: 10.1016/j.bbalip.2014.10.009

Hecker, M., Sommer, N., Foch, S., Hecker, A., Hackstein, H., Witzenrath, M., et al. (2018). Resolvin E1 and its precursor 18R-HEPE restore mitochondrial function in inflammation. *Biochim. Biophys. Acta Mol. Cell. Biol. Lipids* 1863, 1016–1028. doi: 10.1016/j.bbalip.2018.06.011

Hennebelle, M., Zhang, Z., Metherel, A. H., Kitson, A. P., Otoki, Y., Richardson, C. E., et al. (2017). Linoleic acid participates in the response to ischemic brain injury through oxidized metabolites that regulate neurotransmission. *Sci. Rep.* 7, 4342. doi: 10.1038/s41598-017-02914-7

Hiratsuka, S., Koizumi, K., Ooba, T., and Yokogoshi, H. (2009). Effects of dietary docosahexaenoic acid connecting phospholipids on the learning ability and fatty acid composition of the brain. *J. Nutr. Sci. Vitaminol. (Tokyo)* 55, 374–380. doi: 10.3177/jnsv.55.374

Hjorth, E., Zhu, M., Toro, V. C., Vedin, I., Palmblad, J., Cederholm, T., et al. (2013). Omega-3 fatty acids enhance phagocytosis of Alzheimer's disease-related amyloid-beta42 by human microglia and decrease inflammatory markers. *J. Alzheimers Dis.* 35, 697–713. doi: 10.3233/JAD-130131

Hong, S., Gronert, K., Devchand, P. R., Moussignac, R. L., and Serhan, C. N. (2003). Novel docosatrienes and 17S-resolvins generated from docosahexaenoic acid in murine brain, human blood, and glial cells. Autacoids in anti-inflammation. *J. Biol. Chem.* 278, 14677–14687. doi: 10.1074/jbc.M300218200

Hughes, M. M., Connor, T. J., and Harkin, A. (2016). Stress-related immune markers in depression: implications for treatment. *Int. J. Neuropsychopharmacol.* 1–19. doi: 10.1093/ijnp/pyw001

Igarashi, M., Ma, K., Chang, L., Bell, J. M., and Rapoport, S. I. (2007). Dietary n-3 PUFA deprivation for 15 weeks upregulates elongase and desaturase expression in rat liver but not brain. *J. Lipid Res.* 48, 2463–2470. doi: 10.1194/jlr.M700315-JLR200

Inoue, T., Tanaka, M., Masuda, S., Ohue-Kitano, R., Yamakage, H., Muranaka, K., et al. (2017). Omega-3 polyunsaturated fatty acids suppress the inflammatory responses of lipopolysaccharide-stimulated mouse microglia by activating SIRT1 pathways. *Biochim. Biophys. Acta* 1862, 552–560. doi: 10.1016/j.bbalip.2017.02.010

Ishikawa, Y., Deyama, S., Shimoda, K., Yoshikawa, K., Ide, S., Satoh, M., et al. (2017). Rapid and sustained antidepressant effects of resolvin D1 and D2 in a chronic unpredictable stress model. *Behav. Brain Res.* 332, 233–236. doi: 10.1016/j.bbr.2017.06.010

Isobe, Y., Arita, M., Matsueda, S., Iwamoto, R., Fujihara, T., Nakanishi, H., et al. (2012). Identification and structure determination of novel anti-inflammatory mediator resolvin E3, 17,18-dihydroxyeicosapentaenoic acid. *J. Biol. Chem.* 287, 10525–10534. doi: 10.1074/jbc.M112.340612

James, S. Y., Williams, M. A., Kelsey, S. M., Newland, A. C., and Colston, K. W. (1997). The role of vitamin D derivatives and retinoids in the differentiation of human leukaemia cells. *Biochem. Pharmacol.* 54, 625–634. doi: 10.1016/S0006-2952(97)00195-0

Joffre, C., Grégoire, S., De Smedt, V., Acar, N., Bretillon, L., Nadjar, A., et al. (2016). Modulation of brain PUFA content in different experimental models of mice. *Prostaglandins Leukot. Essent. Fatty Acids* 114, 1–10. doi: 10.1016/j.plefa.2016.09.003

Kang, J. X., Wang, J., Wu, L., and Kang, Z. B. (2004). Transgenic mice: fat-1 mice convert n-6 to n-3 fatty acids. *Nature* 427, 504. doi: 10.1038/427504a

Kantarci, A., Aytan, N., Palaska, I., Stephens, D., Crabtree, L., Benincasa, C., et al. (2018). Combined administration of resolvin E1 and lipoxin A4 resolves inflammation in a murine model of Alzheimer's disease. *Exp. Neurol.* 300, 111–120. doi: 10.1016/j.expneurol.2017.11.005

Kavanagh, T., Lonergan, P. E., and Lynch, M. A. (2004). Eicosapentaenoic acid and gamma-linolenic acid increase hippocampal concentrations of IL-4 and IL-10 and abrogate lipopolysaccharide-induced inhibition of long-term potentiation. *Prostaglandins Leukot. Essent. Fatty Acids* 70, 391–397. doi: 10.1016/j.plefa.2003.12.014

Kiecolt-Glaser, J. K., Belury, M. A., Andridge, R., Malarkey, W. B., Hwang, B. S., and Glaser, R. (2012). Omega-3 supplementation lowers inflammation in healthy middle-aged and older adults: a randomized controlled trial. *Brain Behav. Immun.* 26, 988–995. doi: 10.1016/j.bbi.2012.05.011

Kim, H. W., Rao, J. S., Rapoport, S. I., and Igarashi, M. (2011). Dietary n-6 PUFA deprivation downregulates arachidonate but upregulates docosahexaenoate metabolizing enzymes in rat brain. *Biochim. Biophys. Acta* 1811, 111–117. doi: 10.1016/j.bbalip.2010.10.005

Kitson, A. P., Metherel, A. H., Chen, C. T., Domenichiello, A. F., Trepanier, M. O., Berger, A., et al. (2016). Effect of dietary docosahexaenoic acid (DHA) in phospholipids or triglycerides on brain DHA uptake and accretion. *J. Nutr. Biochem.* 33, 91–102. doi: 10.1016/j.jnutbio.2016.02.009

Klein, C. P., Sperotto, N. D., Maciel, I. S., Leite, C. E., Souza, A. H., and Campos, M. M. (2014). Effects of D-series resolvins on behavioral and neurochemical changes in a fibromyalgia-like model in mice. *Neuropharmacology* 86, 57–66. doi: 10.1016/j.neuropharm.2014.05.043

Kremer, J. M. (2000). n-3 fatty acid supplements in rheumatoid arthritis. *Am. J. Clin. Nutr.* 71, 349S–351S. doi: 10.1093/ajcn/71.1.349s

Kremer, J. M., Lawrence, D. A., Jubiz, W., DiGiacomo, R., Rynes, R., Bartholomew, L. E., et al. (1990). Dietary fish oil and olive oil supplementation in patients with rheumatoid arthritis. Clinical and immunologic effects. *Arthritis Rheum.* 33, 810–820. doi: 10.1002/art.1780330607

Krishnamoorthy, S., Recchiuti, A., Chiang, N., Fredman, G., and Serhan, C. N. (2012). Resolvin D1 receptor stereoselectivity and regulation of inflammation and proresolving microRNAs. *Am. J. Pathol.* 180, 2018–2027. doi: 10.1016/j.ajpath.2012.01.028

Krishnamoorthy, S., Recchiuti, A., Chiang, N., Yacoubian, S., Lee, C. H., Yang, R., et al. (2010). Resolvin D1 binds human phagocytes with evidence for proresolving receptors. *Proc. Natl. Acad. Sci. U. S. A.* 107, 1660–1665. doi: 10.1073/pnas.0907342107

Kromann, N., and Green, A. (1980). Epidemiological studies in the Upernavik district, Greenland. Incidence of some chronic diseases 1950-1974. *Acta Med. Scand.* 208, 401–406. doi: 10.1111/j.0954-6820.1980.tb01221.x

Kuda, O. (2017). Bioactive metabolites of docosahexaenoic acid. *Biochimie* 136, 12–20. doi: 10.1016/j.biochi.2017.01.002

Labrousse, V. F., Nadjar, A., Joffre, C., Costes, L., Aubert, A., Grégoire, S., et al. (2012). Short-term long chain omega3 diet protects from neuroinflammatory processes and memory impairment in aged mice. *PLoS One* 7, e36861. doi: 10.1371/journal.pone.0036861

Lacombe, R. J. S., Giuliano, V., Colombo, S. M., Arts, M. T., and Bazinet, R. P. (2017). Compound-specific isotope analysis resolves the dietary origin of docosahexaenoic acid in the mouse brain. *J. Lipid Res.* 58, 2071–2081. doi: 10.1194/jlr.D077990

Lafourcade, M., Larrieu, T., Mato, S., Duffaud, A., Sepers, M., Matias, I., et al. (2011). Nutritional omega-3 deficiency abolishes endocannabinoid-mediated neuronal functions. *Nat. Neurosci.* 14, 345–350. doi: 10.1038/nn.2736

Larrieu, T., Hilal, M. L., Hilal, L. M., Fourrier, C., De Smedt-Peyrusse, V., Sans, N., et al. (2014). Nutritional omega-3 modulates neuronal morphology in the prefrontal cortex along with depression-related behaviour through corticosterone secretion. *Transl. Psychiatry* 4, e437. doi: 10.1038/tp.2014.77

Larrieu, T., and Layé, S. (2018). Food for mood: relevance of nutritional omega-3 fatty acids for depression and anxiety. *Front. Physiol.* 9, 1047. doi: 10.3389/fphys.2018.01047

Larrieu, T., Madore, C., Joffre, C., and Layé, S. (2012). Nutritional n-3 polyunsaturated fatty acids deficiency alters cannabinoid receptor signaling pathway in the brain and associated anxiety-like behavior in mice. *J. Physiol. Biochem.* 68, 671–681. doi: 10.1007/s13105-012-0179-6

Layé, S. (2010). Polyunsaturated fatty acids, neuroinflammation and well being. *Prostaglandins Leukot. Essent. Fatty Acids* 82, 295–303. doi: 10.1016/j.plefa.2010.02.006

Layé, S., Nadjar, A., Joffre, C., and Bazinet, R. P. (2018). Anti-inflammatory effects of omega-3 fatty acids in the brain: physiological mechanisms and relevance to pharmacology. *Pharmacol. Rev.* 70, 12–38. doi: 10.1124/pr.117.014092

Lazic, M., Inzaugarat, M. E., Povero, D., Zhao, I. C., Chen, M., Nalbandian, M., et al. (2014). Reduced dietary omega-6 to omega-3 fatty acid ratio and 12/15-lipoxygenase deficiency are protective against chronic high fat diet-induced steatohepatitis. *PLoS One* 9, e107658. doi: 10.1371/journal.pone.0107658

Lee, L. K., Shahar, S., Chin, A. V., and Yusoff, N. A. (2013). Docosahexaenoic acid-concentrated fish oil supplementation in subjects with mild cognitive impairment (MCI): a 12-month randomised, double-blind, placebo-controlled trial. *Psychopharmacol. (Berl.)* 225, 605–612. doi: 10.1007/s00213-012-2848-0

Levy, B. D., Clish, C. B., Schmidt, B., Gronert, K., and Serhan, C. N. (2001). Lipid mediator class switching during acute inflammation: signals in resolution. *Nat. Immunol.* 2, 612–619. doi: 10.1038/89759

Li, L., Wu, Y., Wang, Y., Wu, J., Song, L., Xian, W., et al. (2014). Resolvin D1 promotes the interleukin-4-induced alternative activation in BV-2 microglial cells. *J. Neuroinflamm.* 11, 72. doi: 10.1186/1742-2094-11-72

Little, S. J., Lynch, M. A., Manku, M., and Nicolaou, A. (2007). Docosahexaenoic acid-induced changes in phospholipids in cortex of young and aged rats: a lipidomic analysis. *Prostaglandins Leukot. Essent. Fatty Acids* 77, 155–162. doi: 10.1016/j.plefa.2007.08.009

Lonergan, P. E., Martin, D. S., Horrobin, D. F., and Lynch, M. A. (2004). Neuroprotective actions of eicosapentaenoic acid on lipopolysaccharide-induced dysfunction in rat hippocampus. *J. Neurochem.* 91, 20–29. doi: 10.1111/j.1471-4159.2004.02689.x

López-Vicario, C., Rius, B., Alcaraz-Quiles, J., García-Alonso, V., Lopategi, A., Titos, E., et al. (2016). Pro-resolving mediators produced from EPA and DHA: Overview of the pathways involved and their mechanisms in metabolic syndrome and related liver diseases. *Eur. J. Pharmacol.* 785, 133–143. doi: 10.1016/j.ejphar.2015.03.092

Lu, D. Y., Tsao, Y. Y., Leung, Y. M., and Su, K. P. (2010). Docosahexaenoic acid suppresses neuroinflammatory responses and induces heme oxygenase-1 expression in BV-2 microglia: implications of antidepressant effects for omega-3 fatty acids. *Neuropsychopharmacology* 35, 2238–2248. doi: 10.1038/npp.2010.98

Lu, Y., Zhao, L. X., Cao, D. L., and Gao, Y. J. (2013). Spinal injection of docosahexaenoic acid attenuates carrageenan-induced inflammatory pain through inhibition of microglia-mediated neuroinflammation in the spinal cord. *Neuroscience* 241, 22–31. doi: 10.1016/j.neuroscience.2013.03.003

Lukiw, W. J., Cui, J. G., Marcheselli, V. L., Bodker, M., Botkjaer, A., Gotlinger, K., et al. (2005). A role for docosahexaenoic acid-derived neuroprotectin D1 in neural cell survival and Alzheimer disease. *J. Clin. Invest.* 115, 2774–2783. doi: 10.1172/JCI25420

Luo, C., Ren, H., Wan, J. B., Yao, X., Zhang, X., He, C., et al. (2014). Enriched endogenous omega-3 fatty acids in mice protect against global ischemia injury. *J. Lipid Res.* 55, 1288–1297. doi: 10.1194/jlr.M046466

Madore, C., Leyrolle, Q., Lacabanne, C., Benmamar-Badel, A., Joffre, C., Nadjar, A., et al. (2016). Neuroinflammation in autism: plausible role of maternal inflammation, dietary omega 3, and microbiota. *Neural. Plast.* 2016, 3597209. doi: 10.1155/2016/3597209

Madore, C., Nadjar, A., Delpech, J.-C., Sere, A., Aubert, A., Portal, C., et al. (2014). Nutritional n-3 PUFAs deficiency during perinatal periods alters brain innate immune system and neuronal plasticity-associated genes. *Brain Behav. Immun.* 41, 22–31. doi: 10.1016/j.bbi.2014.03.021

Manduca, A., Bara, A., Larrieu, T., Lassalle, O., Joffre, C., Layé, S., et al. (2017). Amplification of mGlu5-endocannabinoid signaling rescues behavioral and synaptic deficits in a mouse model of adolescent and adult dietary polyunsaturated fatty acid imbalance. *J. Neurosci.* 37, 6851–6868. doi: 10.1523/JNEUROSCI.3516-16.2017

Marcheselli, V. L., Hong, S., Lukiw, W. J., Tian, X. H., Gronert, K., Musto, A., et al. (2003). Novel docosanoids inhibit brain ischemia-reperfusion-mediated leukocyte infiltration and pro-inflammatory gene expression. *J. Biol. Chem.* 278, 43807–43817. doi: 10.1074/jbc.M305841200

Marcon, R., Bento, A. F., Dutra, R. C., Bicca, M. A., Leite, D. F., and Calixto, J. B. (2013). Maresin 1, a proresolving lipid mediator derived from omega-3 polyunsaturated fatty acids, exerts protective actions in murine models of colitis. *J. Immunol.* 191, 4288–4298. doi: 10.4049/jimmunol.1202743

Mas, E., Croft, K. D., Zahra, P., Barden, A., and Mori, T. A. (2012). Resolvins D1, D2, and other mediators of self-limited resolution of inflammation in human blood following n-3 fatty acid supplementation. *Clin. Chem.* 58, 1476–1484. doi: 10.1373/clinchem.2012.190199

McNamara, R. K. (2015). Mitigation of inflammation-induced mood dysregulation by long-chain omega-3 fatty acids. *J. Am. Coll. Nutr.* 34 Suppl 1, 48–55. doi: 10.1080/07315724.2015.1080527

McNamara, R. K., and Carlson, S. E. (2006). Role of omega-3 fatty acids in brain development and function: potential implications for the pathogenesis and prevention of psychopathology. *Prostaglandins Leukot. Essent. Fatty Acids* 75, 329–349. doi: 10.1016/j.plefa.2006.07.010

Mingam, R., Moranis, A., Bluthé, R.-M., De Smedt-Peyrusse, V., Kelley, K. W., Guesnet, P., et al. (2008). Uncoupling of interleukin-6 from its signalling pathway by dietary n-3-polyunsaturated fatty acid deprivation alters sickness behaviour in mice. *Eur. J. Neurosci.* 28, 1877–1886. doi: 10.1111/j.1460-9568.2008.06470.x

Mizwicki, M. T., Liu, G., Fiala, M., Magpantay, L., Sayre, J., Siani, A., et al. (2013). 1alpha,25-dihydroxyvitamin D3 and resolvin D1 retune the balance between amyloid-beta phagocytosis and inflammation in Alzheimer's disease patients. *J. Alzheimers Dis.* 34, 155–170. doi: 10.3233/JAD-121735

Moon, D. O., Kim, K. C., Jin, C. Y., Han, M. H., Park, C., Lee, K. J., et al. (2007). Inhibitory effects of eicosapentaenoic acid on lipopolysaccharide-induced activation in BV2 microglia. *Int. Immunopharmacol.* 7, 222–229. doi: 10.1016/j.intimp.2006.10.001

Morgese, M. G., Schiavone, S., Mhillaj, E., Bove, M., Tucci, P., and Trabace, L. (2018). N-3 PUFA diet enrichment prevents amyloid beta-induced depressive-like phenotype. *Pharmacol. Res.* 129, 526–534. doi: 10.1016/j.phrs.2017.11.034

Mulik, R. S., Bing, C., Ladouceur-Wodzak, M., Munaweera, I., Chopra, R., and Corbin, I. R. (2016). Localized delivery of low-density lipoprotein docosahexaenoic acid nanoparticles to the rat brain using focused ultrasound. *Biomaterials* 83, 257–268. doi: 10.1016/j.biomaterials.2016.01.021

Nadjar, A., Tridon, V., May, M. J., Ghosh, S., Dantzer, R., Amedee, T., et al. (2005). NFkappaB activates in vivo the synthesis of inducible Cox-2 in the brain. *J. Cereb. Blood Flow Metab.* 25, 1047–1059. doi: 10.1038/sj.jcbfm.9600106

Nassar, G. M., Morrow, J. D., Roberts, L. J., Lakkis, F. G., and Badr, K. F. (1994). Induction of 15-lipoxygenase by interleukin-13 in human blood monocytes. *J. Biol. Chem.* 269, 27631–27634.

Nebert, D. W. (2017). Aryl hydrocarbon receptor (AHR): "pioneer member" of the basic-helix/loop/helix per-Arnt-sim (bHLH/PAS) family of "sensors" of foreign and endogenous signals. *Prog. Lipid Res.* 67, 38–57. doi: 10.1016/j.plipres.2017.06.001

Nebert, D. W., Wikvall, K., and Miller, W. L. (2013). Human cytochromes P450 in health and disease. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 368, 20120431. doi: 10.1098/rstb.2012.0431

Norling, L. V., Headland, S. E., Dalli, J., Arnardottir, H. H., Haworth, O., Jones, H. R., et al. (2016). Proresolving and cartilage-protective actions of resolvin D1 in inflammatory arthritis. *JCI Insight* 1, e85922. doi: 10.1172/jci.insight.85922

Oh, S. F., Pillai, P. S., Recchiuti, A., Yang, R., and Serhan, C. N. (2011). Pro-resolving actions and stereoselective biosynthesis of 18S E-series resolvins in human leukocytes and murine inflammation. *J. Clin. Invest.* 121, 569–581. doi: 10.1172/JCI42545

Ohira, T., Arita, M., Omori, K., Recchiuti, A., Van Dyke, T. E., and Serhan, C. N. (2010). Resolvin E1 receptor activation signals phosphorylation and phagocytosis. *J. Biol. Chem.* 285, 3451–3461. doi: 10.1074/jbc.M109.044131

Orr, S. K., Palumbo, S., Bosetti, F., Mount, H. T., Kang, J. X., Greenwood, C. E., et al. (2013). Unesterified docosahexaenoic acid is protective in neuroinflammation. *J. Neurochem.* 378–393. doi: 10.1111/jnc.12392

Ostermann, A. I., Reutzel, M., Hartung, N., Franke, N., Kutzner, L., Schoenfeld, K., et al. (2017). A diet rich in omega-3 fatty acids enhances expression of soluble epoxide hydrolase in murine brain. *Prostaglandins Other Lipid Mediat.* 79–87. doi: 10.1016/j.prostaglandins.2017.06.001

Pallast, S., Arai, K., Wang, X., Lo, E. H., and van Leyen, K. (2009). 12/15-Lipoxygenase targets neuronal mitochondria under oxidative stress. *J. Neurochem.* 111, 882–889. doi: 10.1111/j.1471-4159.2009.06379.x

Park, C.-K., Xu, Z.-Z., Liu, T., Lü, N., Serhan, C. N., and Ji, R.-R. (2011). Resolvin D2 is a potent endogenous inhibitor for transient receptor potential subtype

V1/A1, inflammatory pain, and spinal cord synaptic plasticity in mice: distinct roles of resolvin D1, D2, and E1. *J. Neurosci.* 31, 18433–18438. doi: 10.1523/JNEUROSCI.4192-11.2011

Pascual, O., Ben Achour, S., Rostaing, P., Triller, A., and Bessis, A. (2012). Microglia activation triggers astrocyte-mediated modulation of excitatory neurotransmission. *Proc. Natl. Acad. Sci. U. S. A.* 109, E197–E205. doi: 10.1073/pnas.1111098109

Perretti, M., Chiang, N., La, M., Fierro, I. M., Marullo, S., Getting, S. J., et al. (2002). Endogenous lipid- and peptide-derived anti-inflammatory pathways generated with glucocorticoid and aspirin treatment activate the lipoxin A4 receptor. *Nat. Med.* 8, 1296–1302. doi: 10.1038/nm786

Pettit, L. K., Varsanyi, C., Tadros, J., and Vassiliou, E. (2013). Modulating the inflammatory properties of activated microglia with Docosahexaenoic acid and Aspirin. *Lipids Health Dis.* 12, 16. doi: 10.1186/1476-511X-12-16

Pietropaolo, S., Goubran, M. G., Joffre, C., Aubert, A., Lemaire-Mayo, V., Crusio, W. E., et al. (2014). Dietary supplementation of omega-3 fatty acids rescues fragile X phenotypes in Fmr1-Ko mice. *Psychoneuroendocrinology* 49, 119–129. doi: 10.1016/j.psyneuen.2014.07.002

Pratico, D., Zhukareva, V., Yao, Y., Uryu, K., Funk, C. D., Lawson, J. A., et al. (2004). 12/15-lipoxygenase is increased in Alzheimer's disease: possible involvement in brain oxidative stress. *Am. J. Pathol.* 164, 1655–1662. doi: 10.1016/S0002-9440(10)63724-8

Qu, L., and Caterina, M. J. (2018). Accelerating the reversal of inflammatory pain with NPD1 and its receptor GPR37. *J. Clin. Invest.* 128, 3246–3249. doi: 10.1172/JCI122203

Rao, J. S., Ertley, R. N., DeMar, J. C., Jr., Rapoport, S. I., Bazinet, R. P., and Lee, H. J. (2007). Dietary n-3 PUFA deprivation alters expression of enzymes of the arachidonic and docosahexaenoic acid cascades in rat frontal cortex. *Mol. Psychiatry* 12, 151–157. doi: 10.1038/sj.mp.4001887

Rapaport, M. H., Nierenberg, A. A., Schettler, P. J., Kinkead, B., Cardoos, A., Walker, R., et al. (2016). Inflammation as a predictive biomarker for response to omega-3 fatty acids in major depressive disorder: a proof-of-concept study. *Mol. Psychiatry* 21, 71–79. doi: 10.1038/mp.2015.22

Rapoport, S. I., Rao, J. S., and Igarashi, M. (2007). Brain metabolism of nutritionally essential polyunsaturated fatty acids depends on both the diet and the liver. *Prostaglandins Leukot. Essent. Fatty Acids* 77, 251–261. doi: 10.1016/j.plefa.2007.10.023

Recchiuti, A. (2013). Resolvin D1 and its GPCRs in resolution circuits of inflammation. *Prostaglandins Other Lipid Mediat.* 107, 64–76. doi: 10.1016/j.prostaglandins.2013.02.004

Recchiuti, A., Krishnamoorthy, S., Fredman, G., Chiang, N., and Serhan, C. N. (2011). MicroRNAs in resolution of acute inflammation: identification of novel resolvin D1-miRNA circuits. *Faseb J.* 25, 544–560. doi: 10.1096/fj.10-169599

Renaud, J., Therien, H. M., Plouffe, M., and Martinoli, M. G. (2015). [Neuroinflammation: Dr Jekyll or Mr Hyde?]. *Med. Sci. (Paris)* 31, 979–988. doi: 10.1051/medsci/20153111012

Rey, C., Delpech, J. C., Madore, C., Nadjar, A., Greenhalgh, A. D., Amadieu, C., et al. (2019). Dietary n-3 long chain PUFA supplementation promotes a pro-resolving oxylipin profile in the brain. *Brain Behav. Immun.* 76, 17–27. doi: 10.1016/j.bbi.2018.07.025

Rey, C., Nadjar, A., Buaud, B., Vaysse, C., Aubert, A., Pallet, V., et al. (2016). Resolvin D1 and E1 promote resolution of inflammation in microglial cells in vitro. *Brain Behav. Immun.* 55, 249–259. doi: 10.1016/j.bbi.2015.12.013

Rey, C., Nadjar, A., Joffre, F., Amadieu, C., Aubert, A., Vaysse, C., et al. (2018). Maternal n-3 polyunsaturated fatty acid dietary supply modulates microglia lipid content in the offspring. *Prostaglandins Leukot. Essent. Fatty Acids* 133, 1–7. doi: 10.1016/j.plefa.2018.04.003

Rosenberger, T. A., Villacreses, N. E., Hovda, J. T., Bosetti, F., Weerasinghe, G., Wine, R. N., et al. (2004). Rat brain arachidonic acid metabolism is increased by a 6-day intracerebral ventricular infusion of bacterial lipopolysaccharide. *J. Neurochem.* 88, 1168–1178. doi: 10.1046/j.1471-4159.2003.02246.x

Rosenthal, M. D., Patel, J., Staton, K., Martindale, R. G., Moore, F. A., and Upchurch, G. R. (2018). Can specialized pro-resolving mediators deliver benefit originally expected from fish oil? *Curr. Gastroenterol. Rep.* 20, 40. doi: 10.1007/s11894-018-0647-4

Rossi, S., Di Filippo, C., Gesualdo, C., Potenza, N., Russo, A., Trotta, M. C., et al. (2015). Protection from endotoxic uveitis by intravitreal Resolvin D1: involvement of lymphocytes, miRNAs, ubiquitin-proteasome, and M1/M2 macrophages. *Mediators Inflamm.* 2015, 149381. doi: 10.1155/2015/149381

Salinas, G., Rangasetty, U. C., Uretsky, B. F., and Birnbaum, Y. (2007). The cycloxygenase 2 (COX-2) story: it's time to explain, not inflame. *J. Cardiovasc. Pharmacol. Ther.* 12, 98–111. doi: 10.1177/1074248407301172

Schwab, J. M., Chiang, N., Arita, M., and Serhan, C. N. (2007). Resolvin E1 and protectin D1 activate inflammation-resolution programmes. *Nature* 447, 869–874. doi: 10.1038/nature05877

Serhan, C. N. (2014). Pro-resolving lipid mediators are leads for resolution physiology. *Nature* 510, 92–101. doi: 10.1038/nature13479

Serhan, C. N. (2017a). Discovery of specialized pro-resolving mediators marks the dawn of resolution physiology and pharmacology. *Mol. Aspects Med.* 58, 1–11. doi: 10.1016/j.mam.2017.03.001

Serhan, C. N. (2017b). Treating inflammation and infection in the 21st century: new hints from decoding resolution mediators and mechanisms. *FASEB J.* 31, 1273–1288. doi: 10.1096/fj.201601222R

Serhan, C. N., and Chiang, N. (2013). Resolution phase lipid mediators of inflammation: agonists of resolution. *Curr. Opin. Pharmacol.* 13, 632–640. doi: 10.1016/j.coph.2013.05.012

Serhan, C. N., Chiang, N., and Dalli, J. (2015). The resolution code of acute inflammation: novel pro-resolving lipid mediators in resolution. *Semin. Immunol.* 27, 200–215. doi: 10.1016/j.smim.2015.03.004

Serhan, C. N., Chiang, N., and Dalli, J. (2018). New pro-resolving n-3 mediators bridge resolution of infectious inflammation to tissue regeneration. *Mol. Aspects Med.* 64, 1–17. doi: 10.1016/j.mam.2017.08.002

Serhan, C. N., Clish, C. B., Brannon, J., Colgan, S. P., Chiang, N., and Gronert, K. (2000). Novel functional sets of lipid-derived mediators with antiinflammatory actions generated from omega-3 fatty acids via cyclooxygenase 2-nonsteroidal antiinflammatory drugs and transcellular processing. *J. Exp. Med.* 192, 1197–1204. doi: 10.1084/jem.192.8.1197

Serhan, C. N., Hong, S., Gronert, K., Colgan, S. P., Devchand, P. R., Mirick, G., et al. (2002). Resolvins: a family of bioactive products of omega-3 fatty acid transformation circuits initiated by aspirin treatment that counter proinflammation signals. *J. Exp. Med.* 196, 1025–1037. doi: 10.1084/jem.20020760

Serhan, C. N., and Petasis, N. A. (2011). Resolvins and protectins in inflammation resolution. *Chem. Rev.* 111, 5922–5943. doi: 10.1021/cr100396c

Serhan, C. N., and Savill, J. (2005). Resolution of inflammation: the beginning programs the end. *Nat. Immunol.* 6, 1191–1197. doi: 10.1038/ni1276

Serhan, C. N., Yang, R., Martinod, K., Kasuga, K., Pillai, P. S., Porter, T. F., et al. (2009). Maresins: novel macrophage mediators with potent antiinflammatory and proresolving actions. *J. Exp. Med.* 206, 15–23. doi: 10.1084/jem.20081880

Shalini, S. M., Ho, C. F., Ng, Y. K., Tong, J. X., Ong, E. S., Herr, D. R., et al. (2017). Distribution of Alox15 in the rat brain and its role in prefrontal cortical resolvin D1 formation and spatial working memory. *Mol. Neurobiol.* 1537–1550. doi: 10.1007/s12035-017-0413-x

Sheets, K. G., Jun, B., Zhou, Y., Zhu, M., Petasis, N. A., Gordon, W. C., et al. (2013). Microglial ramification and redistribution concomitant with the attenuation of choroidal neovascularization by neuroprotectin D1. *Mol. Vis.* 19, 1747–1759.

Shevalye, H., Yorek, M. S., Coppey, L. J., Holmes, A., Harper, M. M., Kardon, R. H., et al. (2015). Effect of enriching the diet with menhaden oil or daily treatment with resolvin D1 on neuropathy in a mouse model of type 2 diabetes. *J. Neurophysiol.* 114, 199–208. doi: 10.1152/jn.00224.2015

Shi, Z., Ren, H., Huang, Z., Peng, Y., He, B., Yao, X., et al. (2016). Fish oil prevents lipopolysaccharide-induced depressive-like behavior by inhibiting neuroinflammation. *Mol. Neurobiol.* 7327–7334. doi: 10.1007/s12035-016-0212-9

Siegert, E., Paul, F., Rothe, M., and Weylandt, K. H. (2017). The effect of omega-3 fatty acids on central nervous system remyelination in fat-1 mice. *BMC Neurosci.* 18, 19. doi: 10.1186/s12868-016-0312-5

Simopoulos, A. P. (2008). The importance of the omega-6/omega-3 fatty acid ratio in cardiovascular disease and other chronic diseases. *Exp. Biol. Med. (Maywood)* 233, 674–688. doi: 10.3181/0711-MR-311

Skarke, C., Alamuddin, N., Lawson, J. A., Li, X., Ferguson, J. F., Reilly, M. P., et al. (2015). Bioactive products formed in humans from fish oils. *J. Lipid Res.* 56, 1808–1820. doi: 10.1194/jlr.M060392

Skorve, J., Hilvo, M., Vihervaara, T., Burri, L., Bohov, P., Tillander, V., et al. (2015). Fish oil and krill oil differentially modify the liver and brain lipidome when fed to mice. *Lipids Health Dis.* 14, 88. doi: 10.1186/s12944-015-0086-2

Solito, E., and Sastre, M. (2012). Microglia function in Alzheimer's disease. *Front. Pharmacol.* 3, 14. doi: 10.3389/fphar.2012.00014

Song, C., and Horrobin, D. (2004). Omega-3 fatty acid ethyl-eicosapentaenoate, but not soybean oil, attenuates memory impairment induced by central IL-1beta administration. *J. Lipid Res.* 45, 1112–1121. doi: 10.1194/jlr.M300526-JLR200

Song, C., Manku, M. S., and Horrobin, D. F. (2008). Long-chain polyunsaturated fatty acids modulate interleukin-1beta-induced changes in behavior, monoaminergic neurotransmitters, and brain inflammation in rats. *J. Nutr.* 138, 954–963. doi: 10.1093/jn/138.5.954

Spanbroek, R., Hildner, M., Kohler, A., Muller, A., Zintl, F., Kuhn, H., et al. (2001). IL-4 determines eicosanoid formation in dendritic cells by down-regulation of 5-lipoxygenase and up-regulation of 15-lipoxygenase 1 expression. *Proc. Natl. Acad. Sci. U. S. A.* 98, 5152–5157. doi: 10.1073/pnas.091076998

Spite, M., Norling, L. V., Summers, L., Yang, R., Cooper, D., Petasis, N. A., et al. (2009). Resolvin D2 is a potent regulator of leukocytes and controls microbial sepsis. *Nature* 461, 1287–1291. doi: 10.1038/nature08541

Spite, M., and Serhan, C. N. (2010). Novel lipid mediators promote resolution of acute inflammation: impact of aspirin and statins. *Circ. Res.* 107, 1170–1184. doi: 10.1161/CIRCRESAHA.110.223883

Stewart, T. M., and Bowling, A. C. (2005). Polyunsaturated fatty acid supplementation in MS. *Int. MS. J.* 12, 88–93.

Sugimoto, M. A., Sousa, L. P., Pinho, V., Perretti, M., and Teixeira, M. M. (2016). Resolution of inflammation: what controls its onset? *Front. Immunol.* 7, 160. doi: 10.3389/fimmu.2016.00160

Sun, L., Xu, Y. W., Han, J., Liang, H., Wang, N., and Cheng, Y. (2015). 12/15-Lipoxygenase metabolites of arachidonic acid activate PPARgamma: a possible neuroprotective effect in ischemic brain. *J. Lipid Res.* 56, 502–514. doi: 10.1194/jlr.M053058

Sun, W., Wang, Z. P., Gui, P., Xia, W., Xia, Z., Zhang, X. C., et al. (2014). Endogenous expression pattern of resolvin D1 in a rat model of self-resolution of lipopolysaccharide-induced acute respiratory distress syndrome and inflammation. *Int. Immunopharmacol.* 23, 247–253. doi: 10.1016/j.intimp.2014.09.001

Sun, Y. P., Oh, S. F., Uddin, J., Yang, R., Gotlinger, K., Campbell, E., et al. (2007). Resolvin D1 and its aspirin-triggered 17R epimer. Stereochemical assignments, anti-inflammatory properties, and enzymatic inactivation. *J. Biol. Chem.* 282, 9323–9334. doi: 10.1074/jbc.M609212200

Taha, A. Y., Blanchard, H. C., Cheon, Y., Ramadan, E., Chen, M., Chang, L., et al. (2017). Dietary linoleic acid lowering reduces lipopolysaccharide-induced increase in brain arachidonic acid metabolism. *Mol. Neurobiol.* 54, 4303–4315. doi: 10.1007/s12035-016-9968-1

Taha, A. Y., Hennebelle, M., Yang, J., Zamora, D., Rapoport, S. I., Hammock, B. D., et al. (2016). Regulation of rat plasma and cerebral cortex oxylipin concentrations with increasing levels of dietary linoleic acid. *Prostaglandins Leukot. Essent. Fatty Acids*. doi: 10.1016/j.plefa.2016.05.004

Terrando, N., Gomez-Galan, M., Yang, T., Carlstrom, M., Gustavsson, D., Harding, R. E., et al. (2013). Aspirin-triggered resolvin D1 prevents surgery-induced cognitive decline. *FASEB J.* 27, 3564–3571. doi: 10.1096/fj.13-230276

Thomazeau, A., Bosch-Bouju, C., Manzoni, O., and Layé, S. (2017). Nutritional n-3 PUFA deficiency abolishes endocannabinoid gating of hippocampal long-term potentiation. *Cereb. Cortex* 27, 2571–2579. doi: 10.1093/cercor/bhw052

Tian, Y., Zhang, Y., Zhang, R., Qiao, S., and Fan, J. (2015). Resolvin D2 recovers neural injury by suppressing inflammatory mediators expression in lipopolysaccharide-induced Parkinson's disease rat model. *Biochem. Biophys. Res. Commun.* 460, 799–805. doi: 10.1016/j.bbrc.2015.03.109

Titos, E., Rius, B., Gonzalez-Periz, A., Lopez-Vicario, C., Moran-Salvador, E., Martinez-Clemente, M., et al. (2011). Resolvin D1 and its precursor docosahexaenoic acid promote resolution of adipose tissue inflammation by eliciting macrophage polarization toward an M2-like phenotype. *J. Immunol.* 187, 5408–5418. doi: 10.4049/jimmunol.1100225

Tremblay, M. E., Zhang, I., Bisht, K., Savage, J. C., Lecours, C., Parent, M., et al. (2016). Remodeling of lipid bodies by docosahexaenoic acid in activated microglial cells. *J. Neuroinflamm.* 13, 116. doi: 10.1186/s12974-016-0580-0

Trépanier, M.-O., Hopperton, K. E., Giuliano, V., Masoodi, M., and Bazinet, R. P. (2018). Increased brain docosahexaenoic acid has no effect on the resolution of neuroinflammation following intracerebroventricular lipopolysaccharide injection. *Neurochem. Int.* 118, 115–126. doi: 10.1016/j.neuint.2018.05.010

Wang, D., Liu, Y., Chen, L., Li, P., Qu, Y., and Zhu, Y. (2017). Key role of 15-LO/15-HETE in angiogenesis and functional recovery in later stages of post-stroke mice. *Sci. Rep.* 7, 46698. doi: 10.1038/srep46698

Wang, R. X., and Colgan, S. P. (2017). Special pro-resolving mediator (SPM) actions in regulating gastro-intestinal inflammation and gut mucosal immune responses. *Mol. Aspects Med.* 58, 93–101. doi: 10.1016/j.mam.2017.02.002

Wang, X., Zhu, M., Hjorth, E., Cortes-Toro, V., Eyjolfsdottir, H., Graff, C., et al. (2015). Resolution of inflammation is altered in Alzheimer's disease. *Alzheimers Dement.* 11, 40-50 e1–2. doi: 10.1016/j.jalz.2013.12.024

Wang, Y., Botolin, D., Christian, B., Busik, J., Xu, J., and Jump, D. B. (2005). Tissue-specific, nutritional, and developmental regulation of rat fatty acid elongases. *J. Lipid Res.* 46, 706–715. doi: 10.1194/jlr.M400335-JLR200

Wang, Y. P., Wu, Y., Li, L. Y., Zheng, J., Liu, R. G., Zhou, J. P., et al. (2011). Aspirin-triggered lipoxin A4 attenuates LPS-induced pro-inflammatory responses by inhibiting activation of NF-kappaB and MAPKs in BV-2 microglial cells. *J. Neuroinflamm.* 8, 95. doi: 10.1186/1742-2094-8-95

Weinstock-Guttman, B., Baier, M., Park, Y., Feichter, J., Lee-Kwen, P., Gallagher, E., et al. (2005). Low fat dietary intervention with omega-3 fatty acid supplementation in multiple sclerosis patients. *Prostaglandins Leukot. Essent. Fatty Acids* 73, 397–404. doi: 10.1016/j.plefa.2005.05.024

Weiser, M. J., Mucha, B., Denheyer, H., Atkinson, D., Schanz, N., Vassiliou, E., et al. (2016). Dietary docosahexaenoic acid alleviates autistic-like behaviors resulting from maternal immune activation in mice. *Prostaglandins Leukot. Essent. Fatty Acids* 106, 27–37. doi: 10.1016/j.plefa.2015.10.005

Winkler, J. W., Orr, S. K., Dalli, J., Cheng, C. Y., Sanger, J. M., Chiang, N., et al. (2016). Resolvin D4 stereoassignment and its novel actions in host protection and bacterial clearance. *Sci. Rep.* 6, 18972. doi: 10.1038/srep18972

Xian, W., Li, T., Li, L., Hu, L., and Cao, J. (2019). Maresin 1 attenuates the inflammatory response and mitochondrial damage in mice with cerebral ischemia/reperfusion in a SIRT1-dependent manner. *Brain Res.* 83–90. doi: 10.1016/j.brainres.2019.01.013

Xian, W., Wu, Y., Xiong, W., Li, L., Li, T., Pan, S., et al. (2016). The pro-resolving lipid mediator Maresin 1 protects against cerebral ischemia/reperfusion injury by attenuating the pro-inflammatory response. *Biochem. Biophys. Res. Commun.* 472, 175–181. doi: 10.1016/j.bbrc.2016.02.090

Xiao, Y., Huang, Y., and Chen, Z. Y. (2005). Distribution, depletion and recovery of docosahexaenoic acid are region-specific in rat brain. *Br. J. Nutr.* 94, 544–550. doi: 10.1079/BJN20051539

Xu, Z. Z., Berta, T., and Ji, R. R. (2013). Resolvin E1 inhibits neuropathic pain and spinal cord microglial activation following peripheral nerve injury. *J. Neuroimmune Pharmacol.* 8, 37–41. doi: 10.1007/s11481-012-9394-8

Xu, Z. Z., Zhang, L., Liu, T., Park, J. Y., Berta, T., Yang, R., et al. (2010). Resolvins RvE1 and RvD1 attenuate inflammatory pain via central and peripheral actions. *Nat. Med.* 16, 592–7, 1p following 597. doi: 10.1038/nm.2123

Yao, C., Zhang, J., Chen, F., and Lin, Y. (2013). Neuroprotectin D1 attenuates brain damage induced by transient middle cerebral artery occlusion in rats through TRPC6/CREB pathways. *Mol. Med. Rep.* 8, 543–550. doi: 10.3892/mmr.2013.1543

Yigitkanli, K., Zheng, Y., Pekcec, A., Lo, E. H., and van Leyen, K. (2017). Increased 12/15-lipoxygenase leads to widespread brain injury following global cerebral ischemia. *Transl. Stroke Res.* 8, 194–202. doi: 10.1007/s12975-016-0509-z

Yirmiya, R., and Goshen, I. (2011). Immune modulation of learning, memory, neural plasticity and neurogenesis. *Brain Behav. Immun.* 25, 181–213. doi: 10.1016/j.bbi.2010.10.015

Zhu, M., Wang, X., Hjorth, E., Colas, R. A., Schroeder, L., Granholm, A. C., et al. (2016). Pro-resolving lipid mediators improve neuronal survival and increase abeta42 phagocytosis. *Mol. Neurobiol.* 53, 2733–2749. doi: 10.1007/s12035-015-9544-0

Zimmer, L., Delpal, S., Guilloteau, D., Aioun, J., Durand, G., and Chalon, S. (2000). Chronic n-3 polyunsaturated fatty acid deficiency alters dopamine vesicle density in the rat frontal cortex. *Neurosci. Lett.* 284, 25–28. doi: 10.1016/S0304-3940(00)00950-2

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BRIEF RESEARCH REPORT
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NVP-BEZ235 (Dactolisib) Has Protective Effects in a Transgenic Mouse Model of Alzheimer's Disease

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Alzheimer's disease (AD) is a neurodegenerative disease and the main cause of dementia. Its major symptom is memory loss, which is a result of neuronal cell death, which is accompanied by neuroinflammation. Some studies indicate the overactivation of the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt)/mechanistic target of rapamycin (mTOR) pathway in this disease, being, thus, a potential target for pharmacological treatment. Here, we used a transgenic mouse model of AD that expresses a mutant amyloid-β precursor protein (T41 mice) to investigate the effects of dactolisib (alternative name: NVP-BEZ235, abbreviation BEZ), a dual PI3K/mTOR inhibitor. Ten-months-old T41 animals were treated for 14 days with BEZ or vehicle *via* oral gavage and then submitted to social memory, open field and contextual conditioned fear tests. Hippocampal slices were prepared and $A\beta_{1-42}$ content, NeuN, Iba-1, CD68 and GFAP were evaluated. Tissues were further processed to evaluate cytokines levels through cytometric bead array. The treatment with BEZ (5 mg/kg) reduced social memory impairment in T41 mice. However, BEZ did not have any effect on altered Aβ levels, NeuN, or GFAP staining. The drug reduced the CD68/Iba-1 ratio in CA3 region of hippocampus. Finally, BEZ diminished IL-10 levels in T41 mice. Thus, although its mechanisms are not clear, BEZ protects against memory impairment, reduces microglial activation and reestablishes IL-10 levels, revealing beneficial effects, which should be further investigated for the treatment of AD.

Keywords: Alzheimer's disease, dactolisib, NVP-BEZ235, neurodegeneration, neuroinflammation, PI3K, mechanistic target of rapamycin

INTRODUCTION

Alzheimer's disease (AD) is the leading cause of dementia and is characterized as a progressive neurodegenerative disease (Romberg et al., 2012), whose main risk factor is aging (O'neill, 2013). The main sign of AD is memory loss (El Haj and Kessels, 2013), which is directly related to hippocampal and cortical dysfunctions (Wirth et al., 2013).

Abbreviations: AD, Alzheimer's disease; Aβ, amyloid-β; APP, amyloid precursor protein; BEZ, NVP-BEZ235; BSA, bovine serum albumin; CBA, cytometric bead array; CA, cornus ammonis; DG, dentate gyrus; i.p., intraperitoneal; PBS, phosphate buffered saline; SEM, standard error of the mean; TBS, tris buffered saline; TBST, TBS-Tween-20; T41, Tg(Thy1-APPSweLon)41Ema.

Neuronal toxicity, which occurs as a consequence of amyloid-β (Aβ) accumulation and tau hyperphosphorylation, results in synaptic loss, neuronal death, and brain atrophy (Lazzari et al., 2015; Kocahan and Dogan, 2017), especially in the entorhinal cortex and hippocampus (Hampel et al., 2002; Holtzman et al., 2011). Synaptic density decrease observed in mesial temporal regions in the early stages of AD correlates with cognitive deficits, since these regions are responsible for the formation and storage of new information (Brouillette, 2014).

Another important phenomenon that occurs in AD is neuroinflammation, which can be mediated by glial cells, especially microglia (Hurley and Tizabi, 2013). Despite evidences that microglia initially removes and degrades Aβ (Krabbe et al., 2013), they lose this ability with the progression of the disease, but they are still capable of producing proinflammatory cytokines (Hickman et al., 2008). Hypertrophic reactive astrocytes are also a hallmark of AD (Frost and Li, 2017), and they are found associated with Aβ in the brain (Chun and Lee, 2018). Alongside with microglia, astrocytes can mediate the clearance of Aβ (Cai et al., 2017; Garwood et al., 2017). The increase of several inflammatory cytokines induce activation of these glial cells, which can contribute to Aβ overgeneration, metabolic misbalance, and problems associated with glutamatergic dysfunction and excitotoxicity (Ourdev et al., 2015; Chen et al., 2016; Cai et al., 2017; Gonzalez-Reyes et al., 2017).

Different pathways contribute to the maintenance and progression of AD. The phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt)/mechanistic target of rapamycin (mTOR) signaling pathway regulates cell metabolism, growth, and survival (Kitagishi et al., 2012), being also fundamental for healthy aging. Several studies have shown that in AD early stages, abnormal and continuous activation of PI3K/Akt/mTOR signaling occurs, with increased phosphorylation of mTOR, contributing to disease progression and cognitive decline (Bhaskar et al., 2009; O'neill, 2013). PI3Kγ inhibition and the double inhibition of PI3K and mTOR after Aβ intracerebral injection reduced pathological changes associated with AD (Passos et al., 2010; Bellozi et al., 2016). Importantly, inhibitors of PI3K and mTOR enzymes have been developed in order to elucidate their participation in several diseases and as a possible treatment strategy (Mukherjee et al., 2012). Both enzymes can be inhibited by dactolisib (alternative name: NVP-BEZ235, abbreviation BEZ), a drug that has undergone several clinical trials for the treatment of different tumor types (Maira et al., 2008; Clinicaltrials.Gov, 2015).

Considering that the PI3K/Akt/mTOR pathway is involved in neuroinflammation and neurodegeneration, it is important to establish its roles in AD. Although we have recently demonstrated that the dual inhibition of PI3K and mTOR by BEZ reverses neuropathological changes induced by Aβ (Bellozi et al., 2016), it is important to investigate the effects of the drug in other models, such as transgenic animals, which better resemble the pathological condition (Bilkei-Gorzo, 2014). Therefore, we studied the potential neuroprotective effects of BEZ in a transgenic mouse model that overexpresses APP.

MATERIAL AND METHODS

Drugs

The following substances were used in this study: NVP-BEZ235 (BEZ; LC Laboratories, Woburn, EUA), 10% ketamine hydrochloride (Syntec, Brazil), and 2% xylazine hydrochloride (Syntec, Brazil).

Animals

All procedures were approved by the institutional Ethic Committee on Animal Use (protocol 159/2012) and followed the NIH guide for the care and use of laboratory animals. Experiments were conducted using 10-months-old male transgenic Tg (Thy1-APPSweLon) 41Ema (T41) mice and their wild-type (WT) littermates (Faizi et al., 2012). Male T41 mice were kindly donated by Prof. Tony Wyss-Coray (Glenn Center for aging animal facility, Stanford, USA), and mated with C57Bl/6 female, from Animal Care Facilities of Federal University of Minas Gerais. Animals were kept under controlled room temperature (24°C), under 12 h: 12 h light-dark cycle, with free access to food and water.

Animals Genotyping

We performed DNA extraction from the tail of the animals followed by polymerase chain reaction (PCR) in the presence of primers specific for the mutant APP and electrophoresis in agarose gel to identify the mutant sequences in the region of 364 base pairs.

Experimental Protocol

Animals were treated by oral gavage with 5 or 25 mg/kg of BEZ, diluted in 1-metyl 2-pirrolidone 10% in PEG 300, or vehicle, once a day for 14 days. According to the genotype (WT or T41) and the treatment (BEZ or vehicle), the animals were divided into five groups: WT + Vehicle, WT + BEZ 25 mg/kg (WT + BEZ 25), T41 + Vehicle, T41 + BEZ 5 mg/kg (T41 + BEZ 5), and T41 + BEZ 25 mg/kg (T41 + BEZ 25). The doses of BEZ were chosen based on previous published data (Bellozi et al., 2016). Animals were weighed every day before drug administration and the volume of vehicle and drug solution administered was 4 ml/kg of animal weight. Behavioral tests started 30 min after the administration of the drug or vehicle.

Social Memory Test

Social memory test was performed on the 11th day of treatment, in an acrylic box measuring 60 cm × 40 cm × 23 cm, subdivided in three chambers of equal size, with communication to each other through passages. For habituation, WT + Vehicle (n = 11), WT + BEZ 25 (n = 10), T41 + Vehicle (n = 6), T41 + BEZ 5 (n = 6), and T41 + BEZ 25 (n = 6) animals were introduced for 5 min into the central chamber, with both passages closed. To perform the test, two juveniles C57Bl/6 animals, obtained from a different source of the test animals, were used. One min after the habituation phase, the first juvenile was introduced into one of the lateral chambers, inside a compartment. The passages were opened for 10 min. Then, the test animal was driven to the central compartment, the passages were closed, and a new C57Bl/6 juvenile animal was introduced

into the other side chamber, inside another compartment. One min after the second phase, the passages were opened and the interaction of the test animal with both juveniles was evaluated for further 10 min. Each trial (the three phases of the test) was performed for each animal before starting the next. The percentage of time spent in the compartment with the new juvenile animals was calculated using the formula: 100*[time exploring compartment with new juvenile animal/(time exploring compartment with old animal + time exploring compartment with new juvenile animal)] (Nadler et al., 2004; Kobayashi and Chen, 2005). Tests were recorded and videos were analyzed using ANY-maze software version 4.99.

Open Field Test

Open field test was performed on the 12th day of treatment. Animals were introduced in the center of a 30cm diameter open field during 10 min, and the total traveled distance was assessed. Experimental groups and sample sizes were the same used for social memory test.

Contextual Conditioned Fear Test

On the 13th day of treatment, animals were habituated for 3 min in a conditioning fear chamber containing a grid bottom, dimensions 23 cm × 20 cm × 21 cm. After that, a cycle of 3 shocks of 600 mA for 2 s was started, with intervals of 30, 60, and 40 s, respectively. After the last shock, animals were left into the chamber for 1 min more. On the following day, they were reintroduced into the chamber, and the freezing time was measured during 5 min (Roy et al., 2016). Experimental groups and sample sizes were the same used for social memory test.

Intracardiac Perfusion, Brain Slice Preparation, and Tissue Dissection

On the last day of treatment, 1 hour after drug administration, a subgroup of animals [WT + Vehicle (n = 7), T41 + Vehicle (n = 6) and T41+BEZ 5 (n = 6)] was intraperitoneally anesthetized with ketamine (80 mg/kg) and xylazine (8 mg/kg) and perfused with PBS. Then, animals were decapitated, brains were removed, stored in PFA 4% overnight, and subsequently moved to a 30% sucrose solution until complete saturation. Brains were frozen, stored at −80°C (Gage et al., 2012), and posteriorly sliced into 30-µm-thick sections at −20°C with the aid of a cryostat.

Another subgroup of animals [WT + Vehicle (n = 11), T41 + Vehicle (n = 6) and T41 + BEZ 5 (n = 5)] had their hippocampus carefully dissected 1 h after drug administration, and stored at −80°C, until the day of analysis.

Histological Analysis

Free-floating slices from perfused animals were incubated with citrate buffer at 70°C for 1 h for antigen retrieval, followed by blocking solution [BSA (4%), Triton X (0.5%) in TBS] for 1 h. Then, the primary antibodies rabbit anti-Fox3/NeuN (1:800; EnCor, USA); rabbit anti-Aβ $_{1-42}$ (1:1,600; 1-42 specific; D9A3A, Cell Signaling, USA), mouse anti-GFAP (1:800; Cell Signaling, USA), rabbit anti-Iba-1 (1:500; Wako, Japan), or rat anti-CD68 (1:500; Bio-Rad, USA) were added and incubated overnight. On the next day, the secondary antibodies donkey antirabbit (1:1,000; Alexa Fluor 594, Invitrogen, USA), goat antimouse (1:1,000, Alexa Fluor 488, Life Technologies, USA)or goat antirat (1:1,000, Alexa Fluor 488, Life Technologies, USA) were added for 1 h. Slices were mounted in gelatinized slides and coverslipped with Fluoromount media (Sigma-Aldrich, USA).

Immunostaining was analyzed under a Zeiss fluorescence microscope in 20X/0.4NA magnification lens. In order to obtain a broad and representative perspective of the whole region that was being evaluated, separated slices ranging from −2.06 to −2.54 mm relative to bregma were used. In order to make a proper comparison, equivalent regions containing similar portions were chosen for all the groups. Three slices per animal were used. One picture per region (CA1, CA3, or DG) of each of the three slices were taken. Each picture contained the oriens, pyramidal, and lacunous-molecular layers in the case of CA1 and CA3 regions. In the case of DG, the molecular, granular and polymorphic layers were included in the images and all of them were analyzed together. Each picture had dimension of 710 µm × 530 µm (1388 × 1040 pixels) and resolution of 1.96 pixels/µm. The boundaries of the CA1, CA3, and DG layers of hippocampus were determined by anatomical delimitation as previously established (George Paxinos, 2001). Photomicrographs of stained fluorescence were quantified with the aid of ImageJ software (NIH), and the whole picture was used for quantification. To perform immunostaining analysis, images were converted to 8-bits type, then the threshold method with default algorithm was applied, followed by the percentage of area occupied, mean intensity or integrated density quantification by the analyze particle method, with size (pixels^2) of 0-infinity range and circularity of 0.0–1.0 range. For GFAP, NeuN, Iba-1, and CD68 staining, threshold level was selected as automatically provided by the software. For $A\beta_{1-42}$ staining, it was established a minimum size of 20 pixels (9 µm) with the analyze particle tool in order to avoid the detection of unspecific objects. The integrated density (IntDen) reflects the product between the area and the mean gray value and it was used in addition to mean intensity. In order to evaluate the percentage of Iba-1-stained area counterstained by CD68, CD68/Iba-1 ratio was obtained by the ratio between CD68 and Iba-1 stained areas. Finally, the number of Iba-1^+ cell clusters was counted using 10X (0.25 of numerical aperture) magnification photomicrographs.

Cytokine Analysis

Hippocampi from dissected animals were homogenized in 200 µl of a buffer containing protease inhibitors (0.4 M NaCl; 0.05% Tween 20; 0.5% BSA; 0.1 mM phenylmethylsulfonyl fluoride; 0.1 mM benzethonium chloride; 10 mM EDTA; 20 IU aprotinin in PBS). Total proteins were measured by Bradford method (Bradford, 1976) and analyzed by cytometric bead array (CBA) with the Th1/Th2 kit (BD, USA) to detect IL-2, IL-4, IL-5 IL-6, IFN-γ, TNF-α, IL-10, and IL-17A. All the procedures followed manufacturer's instructions.

Statistical Analysis

Statistical analysis was performed using the statistical software graphpad prism 6.0 and statistica 7.0. Quartile extreme test for identification of outlier values was applied on the results, and extreme values were removed before analysis with the aid of the

interquartile range method. body weight data were analyzed by two-way Analysis of Variance (ANOVA), followed by Newman-Keuls Posttest. Behavioral, biochemical, and histological data were analyzed by one-way ANOVA followed by Newman-Keuls post-test. the data were presented as mean ± standard error of the mean (SEM). The level of significance was set At $P < 0.05$.

RESULTS

BEZ Does Not Alter Body Weight

We first tested whether BEZ would alter body weight, in the same groups of animals submitted to behavioral tests. We did not observe differences between experimental groups ($F(4,12) = 0.804$, $p = 0.545$) and there was no interaction between variables ($F(44,132) = 1.290$, $p = 0.137$). However, there was effect of the variable time, with reduction of the mean body weight in all experimental groups ($F(11,132) = 2.123$; $p = 0.022$; **Figure 1A**).

BEZ Rescues Social Memory in T41 Mice

Memory loss is the main sign of AD. Therefore, we aimed to evaluate T41 animal's cognition in the social memory test. T41 mice explored for less time the new juveniles compared to WT + Vehicle and WT + BEZ 25 groups. Importantly, BEZ 5 mg/kg significantly reversed this memory impairment in T41 mice [$F(4,33) = 3.596$; $p = 0.014$; **Figure 1B**]. We did not observe any difference in total distance traveled in the apparatus between

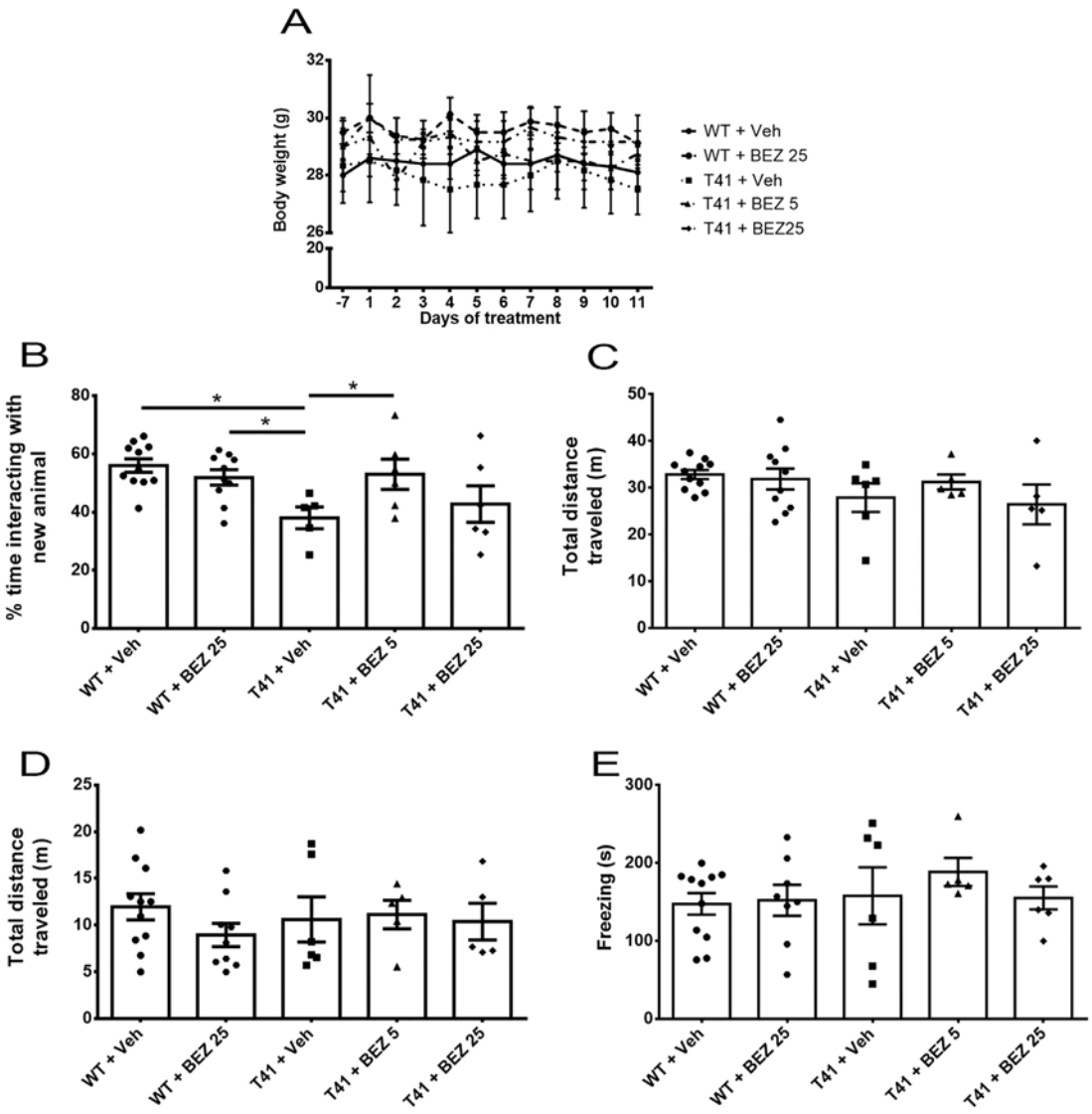

FIGURE 1 | Effects of BEZ treatment on mice weight, memory, and locomotor activity. **(A)** Lines represent the quantification of BEZ treatment effect on animal's weight; **(B)** Bar graphs represent the quantification of BEZ treatment effect on animal's social memory; **(C)** Total traveled distance during the social memory test; **(D)** Total traveled distance in open field test; and **(E)** freezing time in the contextual conditioned fear test. WT + Vehicle (n = 11), WT + BEZ 25 (n = 10), T41 + vehicle (n = 6), T41 + BEZ 5 (n = 6) and T41 + BEZ 25 (n = 6). *$p < 0.05$ (ANOVA followed by Newman-Keuls test).

groups [F(4,32) = 1.308; ns; **Figure 1C**]. Mean ± SEM of the total time of exploration of each compartment in 1st and 2nd sessions and the total distance traveled in the 1st session can be found in **Supplementary Figure 1**.

BEZ Does Not Change Locomotor Activity in T41 Mice

Memory loss may be accompanied by locomotor changes. Thus, we also evaluated animal's locomotor activity in an open field. Treated and nontreated T41 mice did not exhibited any locomotor changes when compared with WT treated or nontreated groups [F(4,31) = 0.577; ns; **Figure 1D**].

T41 Mice Do Not Reveal Altered Behavior in Contextual Conditioned Fear Test

Contextual conditioned fear test was used to assess memory through another behavioral paradigm. However, T41 mice did not present memory impairment in this task and both BEZ 5 and 25 mg/kg did not change the behavior [F(4,31) = 0.494; ns; **Figure 1E**].

BEZ Does Not Change the Increase in $A\beta_{1-42}$ Plaque Load in the Hippocampus of T41 Mice

Considering that the higher dose of BEZ did not alter the behavior tasks, we further evaluated the effect of the lower dose in different parameters associated with AD. Since we observed that BEZ rescued social memory in T41 mice, we investigated whether this effect was related to changes in Aβ plaque load. $A\beta_{1-42}$ was increased in CA1 (F(2,15) = 5.496; p = 0.0162), in CA3 (F(2,15) = 16.47; p = 0.0002) and in the DG (F(2,15) = 5.606; p = 0.0152) of the hippocampus of T41 mice treated with vehicle. BEZ treatment did not reverse the increased $A\beta_{1-42}$ load observed in T41 mice (**Figures 2A, B**).

BEZ Does Not Alter the Immunostaining of NeuN in the Hippocampus of T41 Mice

In order to further investigate the possible neuroprotective effect of BEZ, we evaluated its effect in NeuN immunostaining. There was no alteration in NeuN stained area in CA1 (F(2,16) = 1.798; ns), CA3 (F(2,16) = 2.252; ns), and DG (F(2,16) = 0.2116; ns) regions of the hippocampus, when comparing WT and T41 nontreated or treated mice (**Figures 2C, D**).

T41 Mice Present Astrocytosis in the Hippocampus, Which Is Not Altered by BEZ

Astrocytosis is a common event observed in AD that may also contribute to the progression of the disease. Therefore, we evaluated whether T41 mice presented changes in GFAP intensity and whether BEZ would alter this scenario. There was an increase in the GFAP mean intensity (F(2,14) = 5.132; p = 0.021) and GFAP IntDen (F(2,14) = 4.077; p = 0.040) in CA1 in nontreated T41 mice, as compared with their WT littermates. In CA3 region, we observed only a trend towards an increase in GFAP mean intensity (F(2,15) = 2.757; ns) but an increase in GFAP IntDen (F(2,14) = 5.468; p = 0.017) in nontreated T41 group, as compared to nontreated WT group. In the DG, both GFAP mean intensity (F(2,15) = 4.044; p = 0.039) and IntDen (F(2,14) = 4.762; p = 0.026) were significantly increased in nontreated T41 mice, in comparison with WT. We also noticed that the treatment did not reverse the increase in neither GFAP mean intensity nor GFAP IntDen found in nontreated T41 mice. In addition, we observed an increase in GFAP IntDen in CA3 of treated T41 mice, as compared with nontreated WT mice (**Figures 2E–G**).

BEZ Partially Reduces the Increased Microglial Activation in the Hippocampus of T41 Mice

Microglia activation may be a result of neurodegeneration, as well as it could also contribute to the progression of the disease. Since CD68 has been suggested as a lysosomal marker highly expressed in activated macrophages/microglia, and Iba-1 is a marker of microglia, we evaluated whether BEZ would also reduce the ratio between CD68 and Iba-1 immunostaining. Importantly, we did not notice CD68 staining out of Iba-1^+ cells. There was an increase in CD68/Iba-1 ratio in CA1 (F(2,12) = 7.796; p = 0.0068), CA3 (F(2,10) = 55.14; p < 0.0001), and DG (F(2,12) = 4.775; p = 0.0298) layers in both T41 nontreated and treated mice. Importantly, BEZ reduced the increase in CD68/Iba-1 ratio in CA3 layer of T41 mice. In addition to the CD68/Iba-1 ratio, we also counted the number of Iba-1^+ cell clusters in each layer. We considered as a cluster every Iba-1^+ cell agglomerate, which probably occurs around the plaques. The number of cell clusters was increased in CA1 (F(2,12) = 7.267; p = 0.0086) and in DG (F(2,12) = 6.222; p = 0.014) layers in both T41 nontreated and T41 treated mice in comparison with the WT littermates. In CA3 layer, there was an increase in the number of cell clusters in T41 nontreated mice, as compared to WT nontreated mice (F(2,12) = 8.00; p = 0.0062; p < 0.01). However, there was no post-test difference between T41 treated mice and WT nontreated mice or between T41 treated and T41 nontreated mice (**Figure 3**).

BEZ Reduces Hippocampal IL-10 in T41 Mice, But No Other Cytokines Are Changed

Finally, we evaluated the effects of BEZ on cytokines levels. Among the cytokines evaluated (**Supplementary Table 1**), only IL-10 was found to be increased in T41 mice, which was reversed by treatment with BEZ [F(2,13) = 4.079; p < 0.05; **Figure 4**].

DISCUSSION

In the present study, we demonstrated that BEZ reversed memory impairment and levels of hippocampal IL-10 in 10-months-old T41 mice. Besides, it reduced CD68/Iba-1 immunostaining ratio in CA3 region of these transgenic animals.

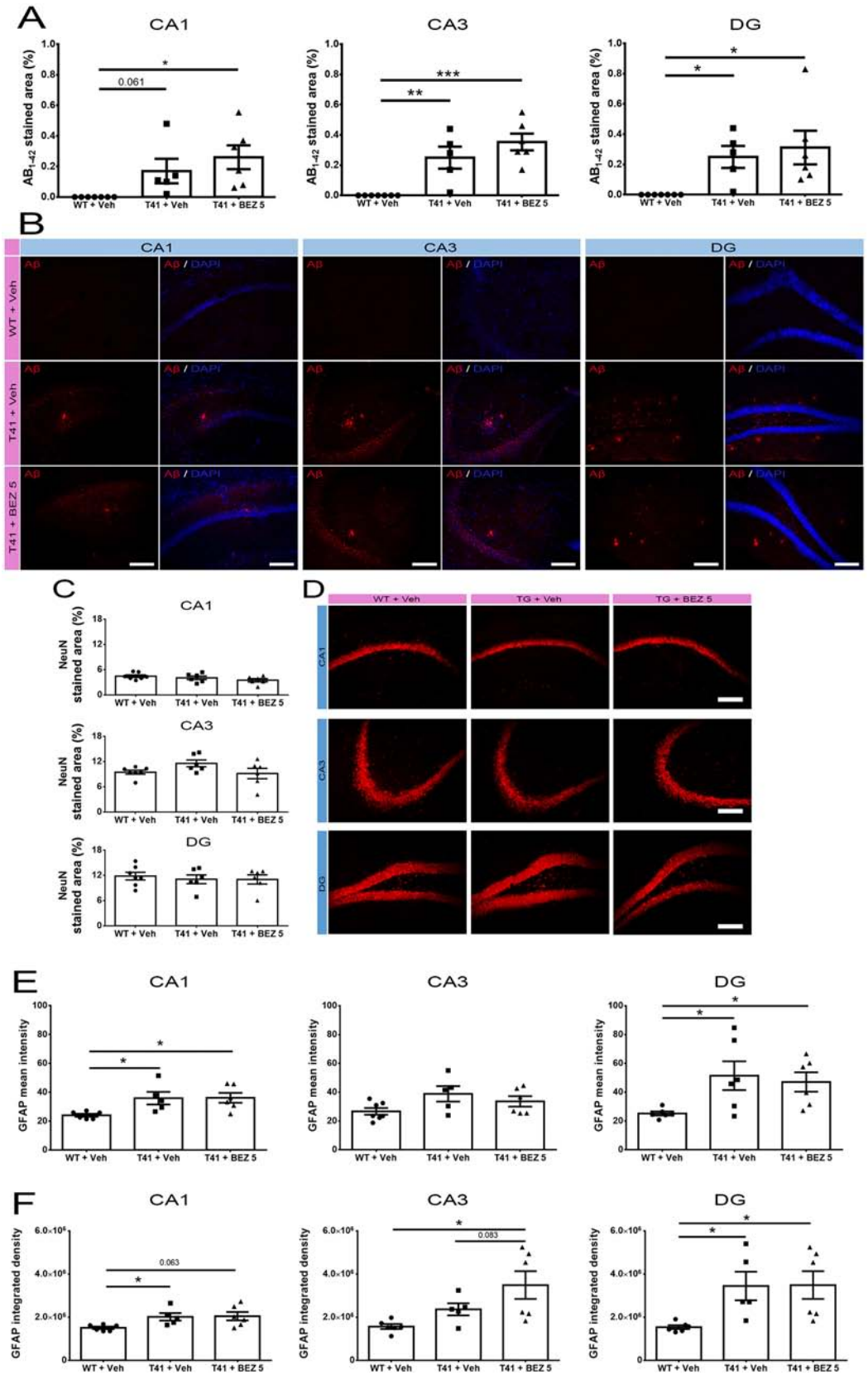

FIGURE 2 | Continued

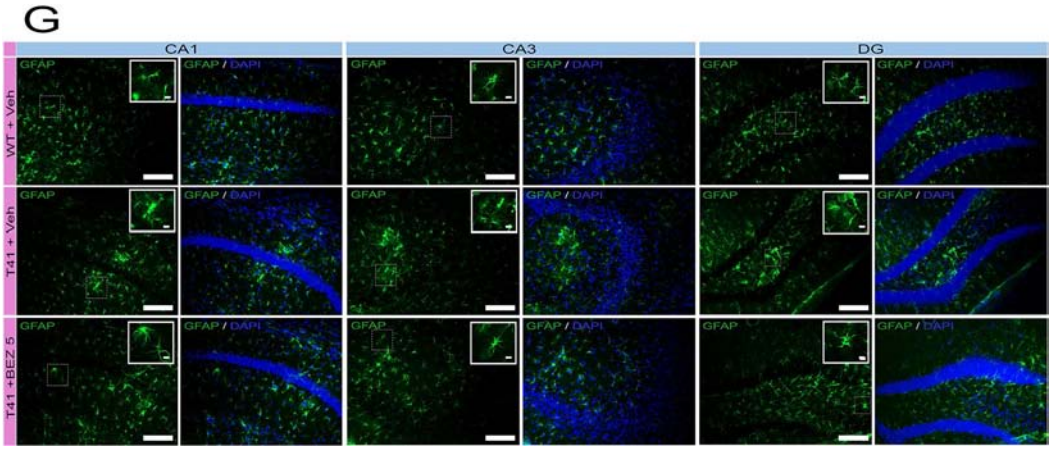

FIGURE 2 | Effects of BEZ treatment on Aβ, NeuN, and GFAP immunostaining. **(A**, **B)** Bar graphs represent the quantification of BEZ treatment effect on Aβ; **(C**, **D)** NeuN; **(E**–**G)** GFAP immunostaining. WT + Vehicle (n = 7), T41 + vehicle (n = 6), and T41 + BEZ 5 (n = 6). Representative images of Aβ **(B)**, NeuN **(D)**, and GFAP **(G)** immunostaining in CA1, CA3, and DG of animals. Inset images are a magnified portion to show detail (40× objective). *p < 0.05, **p < 0.01, and ***p < 0.001 (ANOVA followed by Newman-Keuls test). Scale bar for 20× objective = 125 μm. Scale bar for 40× objective = 10 μm.

Increased Akt activation and mTOR phosphorylation have been reported in brains of AD patients, which is associated with a disrupted clearance of Aβ and tau, synaptic loss, and cognitive decline (Heras-Sandoval et al., 2014). Thus, the adequate control of PI3K/Akt/mTOR pathway activation might have a potential to ameliorate AD associated features. The dual inhibition of PI3K and mTOR with BEZ 5mg/kg reduced memory loss in T41 mice, without, however, changing the locomotor parameter. The higher BEZ dose did not induce significant memory improvement in this mice strain aged 10 months old. The ineffectiveness of this dose should be evaluated in future studies. It was previously demonstrated that the inhibition of PI3K with LX2343 ameliorates memory loss in APP/PS1 transgenic mouse (Guo et al., 2016). Moreover, the inhibition of mTOR with rapamycin rescues memory impairment (Spilman et al., 2010), since it regulates autophagy leading to a reduction of APP levels, processing and metabolites production, and oxidative stress (Tramutola et al., 2018). Although further studies are necessary, these mechanisms may be related to the BEZ effects in the present study.

PI3K and mTOR inhibition have neuroprotective effects in different mice models of neurodegeneration (Malagelada et al., 2010; Bellozi et al., 2016; Saliba et al., 2017). In animal models of AD, administration of rapamycin reduces the accumulation of Aβ, leading to the reduction of synaptic neurotransmission dysfunction (Spilman et al., 2010; Singh et al., 2017). Furthermore, dual inhibition of PI3K and mTOR with BEZ reduces memory impairment induced by intrahippocampal injection of Aβ, which is associated with reduced neurodegeneration and reduced microglial activation (Bellozi et al., 2016). In the present investigation, 14 days of treatment with BEZ did not evidence changes in $A\beta_{1\text{-}42}$ immunostaining, when compared with T41 animals, suggesting that the cognitive improvement mediated by BEZ is related to another mechanism, instead of the modulation of Aβ processing. Since it was previously demonstrated that BEZ reduces Aβ-induced neurodegeneration (Bellozi et al., 2016), we evaluated NeuN immunostaining in T41 mice to assess its potential protective effect. We evaluated CA1, CA3, and DG, since these regions are involved in memory and the neuronal circuits involving them may be affected in AD (Brouillette, 2014; Moorthi et al., 2015). Nevertheless, we did not observe changes in the density of neurons, as indicated by the percentage of stained area with anti-NeuN antibody, nor did BEZ alter this parameter. However, further estimation of the total $NeuN^+$ cells could contribute to the understanding of the possible neuroprotective effect of the tested drug.

Aβ accumulation also leads to neuroinflammation in AD, which is primarily mediated by microglia and astrocytes (Giovannini et al., 2002; Hurley and Tizabi, 2013; Janota et al., 2015). Herein, we observed astrocytosis in hippocampi of T41 animals. It is important to note that GFAP is also expressed by radial glia-like stem cells in the DG. However, BEZ did not induce significant effects in GFAP expressing cells in T41 mice. In addition, we also evaluated the microglia profile. It is known that microglia response can significantly contribute to the chronic neuroinflammation, which has strong relationship with the progression of AD (Li et al., 2018; Nordengen et al., 2019). BEZ reduced the increased CD68/Iba-1 ratio in CA3 region of transgenic animals, suggesting an interference in microglial activation, since CD68 has been suggested as a lysosomal marker highly expressed in activated macrophages/microglia (Graeber and Streit, 2010; Lehmann et al., 2016; Navarro et al., 2018). Moreover, despite treatment with BEZ did not significantly reduce the number of Iba-1 cell clusters in CA3 of T41 mice, there were no differences between the treated T41 group and WT animals. Despite the decrease in the CD68/Iba-1 ratio induced by BEZ was restrained to CA3 region of T41 mice, it is worth highlighting that the treatment was performed for only 14 days. Thus, although it is possible to speculate that the drug may interfere with microglia activity

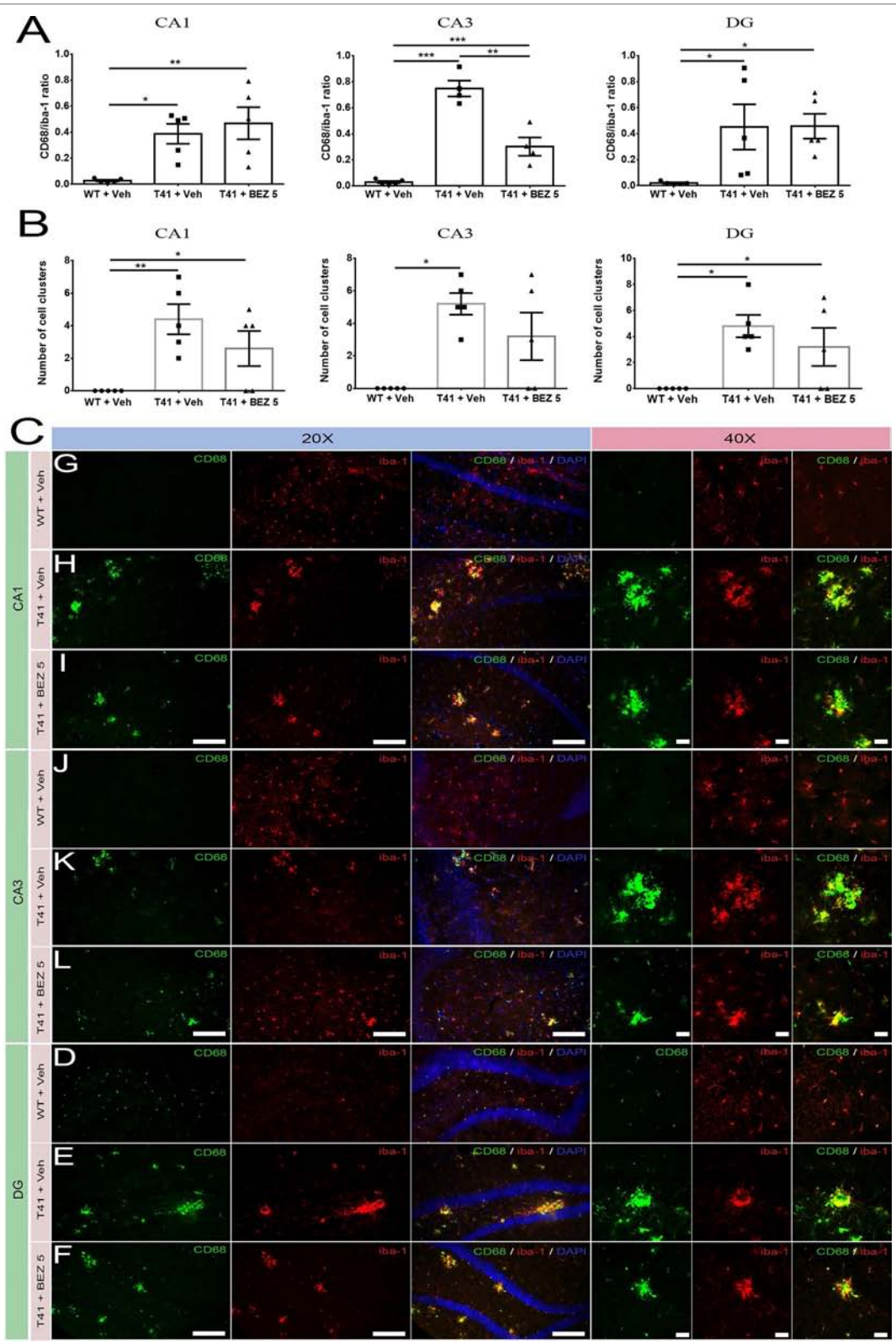

FIGURE 3 | Effects of BEZ treatment on CD68 and Iba-1 immunostaining ratio. Bar graphs represent the quantification of BEZ treatment effect on ratio between CD68 and Iba-1 immunostaining **(A)** or the number of Iba-1$^+$ cell clusters **(B)** in CA1, CA3, and DG. Representative images are shown in 20× and 40× magnitude **(C)**. For CD68 and Iba-1 immunofluorescence, it was used WT + Vehicle (n = 5), T41 + vehicle (n = 5), and T41 + BEZ 5 (n = 5). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ (ANOVA followed by Newman-Keuls test). Scale bar for 20× objective = 125 μm. Scale bar for 40× objective = 10 μm.

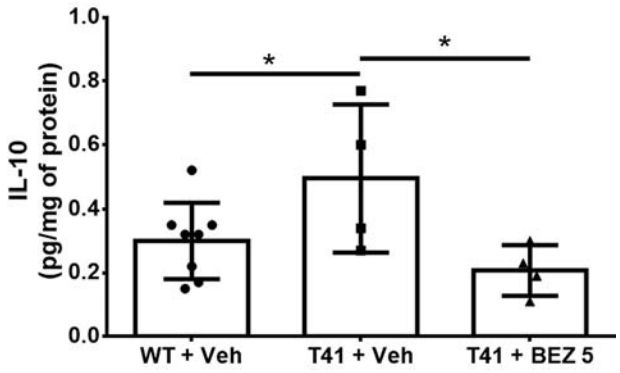

FIGURE 4 | Effects of BEZ treatment on mice hippocampal IL-10 levels. Bar graph represent the quantification of the effect of BEZ treatment on IL-10 levels in the hippocampus. WT + Vehicle (n = 11), T41 + vehicle (n = 6), and T41 + BEZ 5 (n = 5). *p < 0.05 (ANOVA followed by Newman-Keuls test).

and neuroinflammatory process, this pharmacological effect should be further investigated. It is equally relevant to point out the limitations of bidimensional (2D) evaluations, such as the lack of volume data and the reduction of information regarding objects in the three-dimensional (3D) structure (Boyce et al., 2010; Fujisawa et al., 2015), which could be further used to corroborate the present histological findings.

Altered cytokine gene expression and protein levels are also associated with the neuroinflammatory process of AD (Strauss et al., 1992; Janelsins et al., 2008; Morimoto et al., 2011), having protective or nonprotective roles (Su et al., 2016; Zheng et al., 2016). Interestingly, we observed elevated levels of IL-10 in the hippocampus of T41 mice, which was reversed by BEZ. Our finding corroborates previous reports of elevated IL-10 levels in patients (Asselineau et al., 2015) and AD mouse models (Jin et al., 2008). However, in our previous study BEZ increased IL-10 hippocampal levels (Bellozi et al., 2016), a difference that may be related to the age of the animal and stimuli used. Although it is assumed that IL-10 has an antiinflammatory role, its function in AD is still controversial, since it could be either protective or deleterious (Bellozi et al., 2016; Lobo-Silva et al., 2016). We did not find alterations in the other evaluated cytokines in the hippocampus of neither nontreated nor treated T41 animals, as compared to WT mice, which might probably be due to the age.

The effects observed in the present study were not due to body weight change, since the alteration of this parameter along the time was less than 5% and there was no difference between the groups of animals. When comparing the present study results with the model using a hippocampal injection of Aβ, we must consider that the transgenic model better mimics AD, leading to Aβ accumulation in the whole brain (Rockenstein et al., 2001). Herein, a 14-day treatment in a transgenic mouse model expressing a mutant APP was already enough to detect an important cognitive improvement and some benefits related to neuroinflammation, albeit it was not able to change other pathological features. Thus, other mouse models, longer treatment duration or an earlier intervention should be considered to fully understand the potential of BEZ in the treatment of AD.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

All procedures were approved by the institutional Ethic Committee on Animal Experimentation from Federal University of Minas Gerais (CEUA/UFMG) under the protocol number 159/2012. Procedures are in agreement with the Ethical Principles in Animal Experimentation, adopted by CEUA/UFMG, and followed the National Institutes of Health guide for the care and use of Laboratory animals.

AUTHOR CONTRIBUTIONS

PB, BF, and AO designed the study. IO did the animal genotyping. PB was responsible for animal breeding, performing the treatments, behavioral tasks, removal of tissues, intracardiac perfusion, and slices and tissues preparation. LO helped in behavioral tasks and intracardiac perfusion. GG did histological staining. Cytokines dosages were done by ÉV and PB. Results were analyzed by PB and GG. Article was written by PB, GG, BF, and AO. Authors PB, GG, FR, BF, and AO revised the data and discussed and corrected the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2019.01345/full#supplementary-material

REFERENCES

Asselineau, D., Benlhassan, K., Arosio, B., Mari, D., Ferri, E., Casati, M., et al. (2015). Interleukin-10 production in response to amyloid-beta differs between slow and fast decliners in patients with alzheimer's disease. *J. Alzheimers Dis.* 46, 837–842. doi: 10.3233/JAD-142832

Bellozi, P. M., Lima, I. V., Doria, J. G., Vieira, E. L., Campos, A. C., Candelario-Jalil, E., et al. (2016). Neuroprotective effects of the anticancer drug NVP-BEZ235 (dactolisib) on amyloid-beta 1-42 induced neurotoxicity and memory impairment. *Sci. Rep.* 6, 25226. doi: 10.1038/srep25226

Bhaskar, K., Miller, M., Chludzinski, A., Herrup, K., Zagorski, M., and Lamb, B. T. (2009). The PI3K-Akt-mTOR pathway regulates Abeta oligomer induced neuronal cell cycle events. *Mol. Neurodegener.* 4, 14. doi: 10.1186/1750-1326-4-14

Bilkei-Gorzo, A. (2014). Genetic mouse models of brain ageing and Alzheimer's disease. *Pharmacol. Ther.* 142, 244–257. doi: 10.1016/j.pharmthera.2013.12.009

Boyce, R. W., Dorph-Petersen, K. A., Lyck, L., and Gundersen, H. J. (2010). Design-based stereology: introduction to basic concepts and practical approaches for estimation of cell number. *Toxicol. Pathol.* 38, 1011–1025. doi: 10.1177/0192623310385140

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248–254. doi: 10.1016/0003-2697(76)90527-3

Brouillette, J. (2014). The effects of soluble Abeta oligomers on neurodegeneration in Alzheimer's disease. *Curr. Pharm. Des.* 20, 2506–2519. doi: 10.2174/13816128113199990498

Cai, Z., Wan, C. Q., and Liu, Z. (2017). Astrocyte and Alzheimer's disease. *J. Neurol.* 264, 2068–2074. doi: 10.1007/s00415-017-8593-x

Chen, Y. L., Wang, L. M., Chen, Y., Gao, J. Y., Marshall, C., Cai, Z. Y., et al. (2016). Changes in astrocyte functional markers and beta-amyloid metabolism-related proteins in the early stages of hypercholesterolemia. *Neuroscience* 316, 178–191. doi: 10.1016/j.neuroscience.2015.12.039

Chun, H., and Lee, C. J. (2018). Reactive astrocytes in Alzheimer's disease: A double-edged sword. *Neurosci. Res.* 126, 44–52. doi: 10.1016/j.neures.2017.11.012

Clinicaltrials.Gov (2015). *ClinicalTrials.gov* [Online]. Available: https://clinicaltrials.gov [Accessed 2014].

El Haj, M., and Kessels, R. P. (2013). Context memory in Alzheimer's disease. *Dement. Geriatr. Cognit. Dis. Extra.* 3, 342–350. doi: 10.1159/000354187

Faizi, M., Bader, P. L., Saw, N., Nguyen, T. V., Beraki, S., Wyss-Coray, T., et al. (2012). Thy1-hAPP(Lond/Swe+) mouse model of Alzheimer's disease displays broad behavioral deficits in sensorimotor, cognitive and social function. *Brain Behav.* 2, 142–154. doi: 10.1002/brb3.41

Frost, G. R., and Li, Y. M. (2017). The role of astrocytes in amyloid production and Alzheimer's disease. *Open Biol.* 7, 1–14. doi: 10.1098/rsob.170228

Fujisawa, S., Yarilin, D., Fan, N., Turkekul, M., Xu, K., Barlas, A., et al. (2015). Understanding the three-dimensional world from two-dimensional immunofluorescent adjacent sections. *J. Pathol. Inform.* 6, 27. doi: 10.4103/2153-3539.158052

Gage, G. J., Kipke, D. R., and Shain, W. (2012). Whole animal perfusion fixation for rodents. *J. Vis. Exp.* 65, 3564. doi: 10.3791/3564

Garwood, C. J., Ratcliffe, L. E., Simpson, J. E., Heath, P. R., Ince, P. G., and Wharton, S. B. (2017). Review: Astrocytes in Alzheimer's disease and other age-associated dementias: a supporting player with a central role. *Neuropathol. Appl. Neurobiol.* 43, 281–298. doi: 10.1111/nan.12338

George Paxinos, K. B. J. F. (2001). *The Mouse Brain in Stereotaxic Coordinates*. San Diego: ACADEMIC PRESS.

Giovannini, M. G., Scali, C., Prosperi, C., Bellucci, A., Vannucchi, M. G., Rosi, S., et al. (2002). Beta-amyloid-induced inflammation and cholinergic hypofunction in the rat brain in vivo: involvement of the p38MAPK pathway. *Neurobiol. Dis.* 11, 257–274. doi: 10.1006/nbdi.2002.0538

Gonzalez-Reyes, R. E., Nava-Mesa, M. O., Vargas-Sanchez, K., Ariza-Salamanca, D., and Mora-Munoz, L. (2017). Involvement of Astrocytes in Alzheimer's Disease from a Neuroinflammatory and Oxidative Stress Perspective. *Front. Mol. Neurosci.* 10, 427. doi: 10.3389/fnmol.2017.00427

Graeber, M. B., and Streit, W. J. (2010). Microglia: biology and pathology. *Acta Neuropathol.* 119, 89–105. doi: 10.1007/s00401-009-0622-0

Guo, X. D., Sun, G. L., Zhou, T. T., Xu, X., Zhu, Z. Y., Rukachaisirikul, V., et al. (2016). Small molecule LX2343 ameliorates cognitive deficits in AD model mice by targeting both amyloid beta production and clearance. *Acta Pharmacol. Sin.* 37, 1281–1297. doi: 10.1038/aps.2016.80

Hampel, H., Teipel, S. J., Alexander, G. E., Pogarell, O., Rapoport, S. I., and Moller, H. J. (2002). In vivo imaging of region and cell type specific neocortical neurodegeneration in Alzheimer's disease. Perspectives of MRI derived corpus callosum measurement for mapping disease progression and effects of therapy. Evidence from studies with MRI, EEG and PET. *J. Neural Transm. (Vienna)* 109, 837–855. doi: 10.1007/s007020200069

Heras-Sandoval, D., Perez-Rojas, J. M., Hernandez-Damian, J., and Pedraza-Chaverri, J. (2014). The role of PI3K/AKT/mTOR pathway in the modulation of autophagy and the clearance of protein aggregates in neurodegeneration. *Cell Signal* 26, 2694–2701. doi: 10.1016/j.cellsig.2014.08.019

Hickman, S. E., Allison, E. K., and El Khoury, J. (2008). Microglial dysfunction and defective beta-amyloid clearance pathways in aging Alzheimer's disease mice. *J. Neurosci.* 28, 8354–8360. doi: 10.1523/JNEUROSCI.0616-08.2008

Holtzman, D. M., Morris, J. C., and Goate, A. M. (2011). Alzheimer's disease: the challenge of the second century. *Sci. Transl. Med.* 3, 77sr71. doi: 10.1126/scitranslmed.3002369

Hurley, L. L., and Tizabi, Y. (2013). Neuroinflammation, neurodegeneration, and depression. *Neurotox. Res.* 23, 131–144. doi: 10.1007/s12640-012-9348-1

Janelsins, M. C., Mastrangelo, M. A., Park, K. M., Sudol, K. L., Narrow, W. C., Oddo, S., et al. (2008). Chronic neuron-specific tumor necrosis factor-alpha expression enhances the local inflammatory environment ultimately leading to neuronal death in 3xTg-AD mice. *Am. J. Pathol.* 173, 1768–1782. doi: 10.2353/ajpath.2008.080528

Janota, C. S., Brites, D., Lemere, C. A., and Brito, M. A. (2015). Glio-vascular changes during ageing in wild-type and Alzheimer's disease-like APP/PS1 mice. *Brain Res.* 1620, 153–168. doi: 10.1016/j.brainres.2015.04.056

Jin, J. J., Kim, H. D., Maxwell, J. A., Li, L., and Fukuchi, K. (2008). Toll-like receptor 4-dependent upregulation of cytokines in a transgenic mouse model of Alzheimer's disease. *J. Neuroinflammation* 5, 23. doi: 10.1186/1742-2094-5-23

Kitagishi, Y., Kobayashi, M., Kikuta, K., and Matsuda, S. (2012). Roles of PI3K/AKT/GSK3/mTOR Pathway in Cell Signaling of Mental Illnesses. *Depress. Res. Treat* 2012, 752563. doi: 10.1155/2012/752563

Kobayashi, D. T., and Chen, K. S. (2005). Behavioral phenotypes of amyloid-based genetically modified mouse models of Alzheimer's disease. *Genes Brain Behav.* 4, 173–196. doi: 10.1111/j.1601-183X.2005.00124.x

Kocahan, S., and Dogan, Z. (2017). Mechanisms of alzheimer's disease pathogenesis and prevention: the brain, neural pathology, N-methyl-D-aspartate receptors, tau protein and other risk factors. *Clin. Psychopharmacol. Neurosci.* 15, 1–8. doi: 10.9758/cpn.2017.15.1.1

Krabbe, G., Halle, A., Matyash, V., Rinnenthal, J. L., Eom, G. D., Bernhardt, U., et al. (2013). Functional impairment of microglia coincides with Beta-amyloid deposition in mice with Alzheimer-like pathology. *PloS One* 8, e60921. doi: 10.1371/journal.pone.0060921

Lazzari, C., Kipanyula, M. J., Agostini, M., Pozzan, T., and Fasolato, C. (2015). Abeta42 oligomers selectively disrupt neuronal calcium release. *Neurobiol. Aging* 36, 877–885. doi: 10.1016/j.neurobiolaging.2014.10.020

Lehmann, M. L., Cooper, H. A., Maric, D., and Herkenham, M. (2016). Social defeat induces depressive-like states and microglial activation without involvement of peripheral macrophages. *J. Neuroinflammation* 13, 224. doi: 10.1186/s12974-016-0672-x

Li, J. W., Zong, Y., Cao, X. P., Tan, L., and Tan, L. (2018). Microglial priming in Alzheimer's disease. *Ann. Transl. Med.* 6, 176. doi: 10.21037/atm.2018.04.22

Lobo-Silva, D., Carriche, G. M., Castro, A. G., Roque, S., and Saraiva, M. (2016). Balancing the immune response in the brain: IL-10 and its regulation. *J. Neuroinflammation* 13, 297. doi: 10.1186/s12974-016-0763-8

Maira, S. M., Stauffer, F., Brueggen, J., Furet, P., Schnell, C., Fritsch, C., et al. (2008). Identification and characterization of NVP-BEZ235, a new orally available dual phosphatidylinositol 3-kinase/mammalian target of rapamycin inhibitor with potent in vivo antitumor activity. *Mol. Cancer Ther.* 7, 1851–1863. doi: 10.1158/1535-7163.MCT-08-0017

Malagelada, C., Jin, Z. H., Jackson-Lewis, V., Przedborski, S., and Greene, L. A. (2010). Rapamycin protects against neuron death in in vitro and in vivo models of Parkinson's disease. *J. Neurosci.* 30, 1166–1175. doi: 10.1523/JNEUROSCI.3944-09.2010

Moorthi, P., Premkumar, P., Priyanka, R., Jayachandran, K. S., and Anusuyadevi, M. (2015). Pathological changes in hippocampal neuronal circuits underlie age-associated neurodegeneration and memory loss: positive clue toward SAD. *Neuroscience* 301, 90–105. doi: 10.1016/j.neuroscience.2015.05.062

Morimoto, K., Horio, J., Satoh, H., Sue, L., Beach, T., Arita, S., et al. (2011). Expression profiles of cytokines in the brains of Alzheimer's disease (AD) patients compared to the brains of non-demented patients with and without increasing AD pathology. *J. Alzheimers Dis.* 25, 59–76. doi: 10.3233/JAD-2011-101815

Mukherjee, B., Tomimatsu, N., Amancherla, K., Camacho, C. V., Pichamoorthy, N., and Burma, S. (2012). The dual PI3K/mTOR inhibitor NVP-BEZ235 is a potent inhibitor of ATM- and DNA-PKCs-mediated DNA damage responses. *Neoplasia* 14, 34–43. doi: 10.1593/neo.111512

Nadler, J. J., Moy, S. S., Dold, G., Trang, D., Simmons, N., Perez, A., et al. (2004). Automated apparatus for quantitation of social approach behaviors in mice. *Genes Brain Behav.* 3, 303–314. doi: 10.1111/j.1601-183X.2004.00071.x

Navarro, V., Sanchez-Mejias, E., Jimenez, S., Munoz-Castro, C., Sanchez-Varo, R., Davila, J. C., et al. (2018). Microglia in Alzheimer's disease: activated, dysfunctional or degenerative. *Front. Aging Neurosci.* 10, 140. doi: 10.3389/fnagi.2018.00140

Nordengen, K., Kirsebom, B. E., Henjum, K., Selnes, P., Gisladottir, B., Wettergreen, M., et al. (2019). Glial activation and inflammation along the Alzheimer's disease continuum. *J. Neuroinflammation* 16, 46. doi: 10.1186/s12974-019-1399-2

O'neill, C. (2013). PI3-kinase/Akt/mTOR signaling: impaired on/off switches in aging, cognitive decline and Alzheimer's disease. *Exp. Gerontol.* 48, 647–653. doi: 10.1016/j.exger.2013.02.025

Ourdev, D., Foroutanpay, B. V., Wang, Y., and Kar, S. (2015). The Effect of Abeta(1)(-)(4)(2) Oligomers on APP Processing and Abeta(1)(-)(4)(0) Generation in Cultured U-373 Astrocytes. *Neurodegener. Dis.* 15, 361–368. doi: 10.1159/000438923

Passos, G. F., Figueiredo, C. P., Prediger, R. D., Silva, K. A., Siqueira, J. M., Duarte, F. S., et al. (2010). Involvement of phosphoinositide 3-kinase gamma in the neuroinflammatory response and cognitive impairments induced by beta-amyloid 1-40 peptide in mice. *Brain Behav. Immun.* 24, 493–501. doi: 10.1016/j.bbi.2009.12.003

Rockenstein, E., Mallory, M., Mante, M., Sisk, A., and Masliaha, E. (2001). Early formation of mature amyloid-beta protein deposits in a mutant APP transgenic model depends on levels of Abeta(1-42). *J. Neurosci. Res.* 66, 573–582. doi: 10.1002/jnr.1247

Romberg, C., Mctighe, S. M., Heath, C. J., Whitcomb, D. J., Cho, K., Bussey, T. J., et al. (2012). False recognition in a mouse model of Alzheimer's disease: rescue with sensory restriction and memantine. *Brain* 135, 2103–2114. doi: 10.1093/brain/aws074

Roy, D. S., Arons, A., Mitchell, T. I., Pignatelli, M., Ryan, T. J., and Tonegawa, S. (2016). Memory retrieval by activating engram cells in mouse models of early Alzheimer's disease. *Nature* 531, 508–512. doi: 10.1038/nature17172

Saliba, S. W., Vieira, E. L., Santos, R. P., Candelario-Jalil, E., Fiebich, B. L., Vieira, L. B., et al. (2017). Neuroprotective effects of intrastriatal injection of rapamycin in a mouse model of excitotoxicity induced by quinolinic acid. *J. Neuroinflammation* 14, 25. doi: 10.1186/s12974-017-0793-x

Singh, A. K., Kashyap, M. P., Tripathi, V. K., Singh, S., Garg, G., and Rizvi, S. I. (2017). Neuroprotection through rapamycin-induced activation of autophagy and PI3K/Akt1/mTOR/CREB Signaling against amyloid-beta-induced oxidative stress, synaptic/neurotransmission dysfunction, and neurodegeneration in adult rats. *Mol. Neurobiol.* 54, 5815–5828. doi: 10.1007/s12035-016-0129-3

Spilman, P., Podlutskaya, N., Hart, M. J., Debnath, J., Gorostiza, O., Bredesen, D., et al. (2010). Inhibition of mTOR by rapamycin abolishes cognitive deficits and reduces amyloid-beta levels in a mouse model of Alzheimer's disease. *PloS One* 5, e9979. doi: 10.1371/journal.pone.0009979

Strauss, S., Bauer, J., Ganter, U., Jonas, U., Berger, M., and Volk, B. (1992). Detection of interleukin-6 and alpha 2-macroglobulin immunoreactivity in cortex and hippocampus of Alzheimer's disease patients. *Lab. Invest.* 66, 223–230.

Su, F., Bai, F., and Zhang, Z. (2016). Inflammatory Cytokines and Alzheimer's Disease: a review from the perspective of genetic polymorphisms. *Neurosci. Bull.* 32, 469–480. doi: 10.1007/s12264-016-0055-4

Tramutola, A., Lanzillotta, C., Barone, E., Arena, A., Zuliani, I., Mosca, L., et al. (2018). Intranasal rapamycin ameliorates Alzheimer-like cognitive decline in a mouse model of Down syndrome. *Transl. Neurodegener.* 7, 28. doi: 10.1186/s40035-018-0133-9

Wirth, M., Madison, C. M., Rabinovici, G. D., Oh, H., Landau, S. M., and Jagust, W. J. (2013). Alzheimer's disease neurodegenerative biomarkers are associated with decreased cognitive function but not beta-amyloid in cognitively normal older individuals. *J. Neurosci.* 33, 5553–5563. doi: 10.1523/JNEUROSCI.4409-12.2013

Zheng, C., Zhou, X. W., and Wang, J. Z. (2016). The dual roles of cytokines in Alzheimer's disease: update on interleukins, TNF-alpha, TGF-beta and IFN-gamma. *Transl. Neurodegener.* 5, 7. doi: 10.1186/s40035-016-0054-4

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REVIEW
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The Role of Intercellular Adhesion Molecule-1 in the Pathogenesis of Psychiatric Disorders

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Intercellular adhesion molecule-1 (ICAM-1) is a transmembrane glycoprotein that is overexpressed in many pathological states. Although, like many other immune molecules, ICAM-1 plays only a limited role in the abundant concert of the immune response, it may be more important than we realize. In the central nervous system (CNS), ICAM-1 is expressed in microglial cells and astrocytes and in endothelial cells in the white and gray matter of the human forebrain. It is of particular interest in psychiatric disorders for two reasons: It has a key function for the blood–brain barrier, which plays an important role in the biology of psychiatric disorders, and it is a marker for inflammation. Although the blood level of soluble ICAM-1 (sICAM-1) might be lower in acute unmedicated schizophrenia, it has been reported to be increased in many other psychiatric conditions, such as major depression, bipolar disorder, and dementia. In bipolar disorder, high sICAM levels were found during both the depressed and the manic states and also during the euthymic phase (the free interval), possibly indicating that sICAM is a trait marker. High sICAM-1 blood levels have also been found in depression comorbid to a somatic disease state. Interestingly, sICAM-1 levels also increase during aging. Some studies investigated sICAM-1 levels in the cerebrospinal fluid of psychiatric disorders and ICAM-1 expression in postmortem CNS tissue of psychiatric patients and found that the overall duration and duration of the chronic phase of the psychiatric disorder seem to play a role in both. Moreover, confounders, such as antipsychotic and antidepressive medication, have to be considered. sICAM-1 levels seem to be associated with hypopermeability or hyperpermeability of the blood-brain barrier and thus to influence the communication between the CNS immune system, represented by glia cells, and the peripheral immune system. The balance between the influx and efflux of immune molecules into and out of the CNS may be one of the pinpoints in psychiatric disorders, in particular in the chronic phase, e.g., in schizophrenia. This aspect, however, needs further intense research, in particular to enable researchers to develop therapeutic principles based on an immune/inflammatory approach.

Keywords: intercellular adhesion molecule-1, adhesion molecule, schizophrenia, depression, bipolar disorder, immunity, psychoimmunology

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INTRODUCTION

Psychotropic drugs such as antidepressants and antipsychotics act on serotonergic, noradrenergic, dopaminergic, and glutamatergic neurotransmission and are effective and well tolerated in the majority of patients. However, around one third of patients with depression are resistant to treatment and even fewer recover. In patients with schizophrenia, the effects of antipsychotics are often unsatisfactory because these drugs cannot prevent the chronic phase. These examples indicate that pathological mechanisms beyond those involving neurotransmitters may play an important role in psychiatric disorders and that research focused on neurotransmitters has reached its limits.

One important argument for the necessity of widening the view on the role of inflammation and the immune system in psychiatric disorders is that no pathogenetic cause has been found for the known dysfunction of monoaminergic neurotransmission, despite intense genetic and biochemical research over the last 40 years. Inflammation and immune dysfunction have direct and indirect influences on neurotransmission and thus may at least partly explain the biological dysfunctions in psychiatric disorders. For example, an activation of the inflammatory response system is well documented in schizophrenia and major depressive disorder (MDD) (Maes et al., 1992; Müller et al., 1993; Maes, 1994; Rothermundt et al., 2001; Myint et al., 2005; Müller and Bechter, 2013). Two meta-analyses on the role of cytokines clearly revealed similar changes in pro- and anti-inflammatory cytokines in various psychiatric disorders, such as MDD, schizophrenia, and bipolar disorder, in both the blood and the cerebrospinal fluid (CSF) (Goldsmith et al., 2016; Wang and Miller, 2018), although they also showed differences between disorders in the levels of the inflammatory markers C-reactive protein, interleukin (IL)-1, and tumor necrosis factor-α (Goldsmith et al., 2016; Wang and Miller, 2018). Other examples are that levels of the pro-inflammatory cytokine IL-6 during childhood predict the risk for later psychosis or depression (Khandaker et al., 2014) and that inflammatory or autoimmune diseases predict the risk for later schizophrenia or mood disorders (Benros et al., 2011; Benros et al., 2013).

In general, the inflammatory response system appears to be activated in psychiatric disorders, but the levels of the different markers vary across studies. The immune system is a highly divergent and differentiated system composed of numerous interconnected molecules. Therefore, at first it may seem unwarranted to discuss the role of just one molecule in psychiatric disorders. Nevertheless, this review will take a closer look at intercellular adhesion molecule-1 (ICAM-1), one of five intercellular adhesion molecules, because it is a well-characterized molecule that has important functions in the process of inflammation and has been studied in various psychiatric disorders as a representative of a pro-inflammatory immune response. It is of special interest because studies indicate that it is differentially expressed in different psychiatric disorders. This review will focus on the role of ICAM-1 in schizophrenia, depression, bipolar disorder, and dementia. It will not discuss ICAM-1 in anxiety disorders because research on immune parameters in this disorder is rare and no studies have been published on ICAM-1 in anxiety disorders.

FUNCTION OF ICAM-1 AND SOLUBLE ICAM-1

ICAM-1 is an immunoglobulin (Ig)-like transmembrane glycoprotein that is overexpressed on the endothelial lumen in many pathological states (Springer, 1990; Muro, 2007; Lawson and Wolf, 2009). It is ~100 kDa in size and belongs to the immunoglobulin supergene family. The membrane-bound form of ICAM-1 serves as a counter receptor for the β_2-integrins, CD11a/CD18 (LFA-1) and CD11b/CD18 (Mac-1), found on leukocytes. Interactions with membrane-bound ICAM-1 facilitate leukocyte transmigration across the endothelium of many cell types. A soluble form of the molecule, soluble intercellular adhesion molecule-1 (sICAM-1), is found in serum and other body fluids, including CSF (Schwarz et al., 1998). sICAM-1 may be generated by proteolytic cleavage and/or alternative splicing of mICAM-1 messenger RNA (Ramos et al., 2014). Like membrane-bound ICAM-1, sICAM-1 interacts with LFA-1/Mac-1 to compete with leukocyte binding to membrane-bound ICAM-1 (Tsakadze et al., 2006) and to stimulate leukocytes (Schmal et al., 1998). Besides endothelial cells, membrane-bound ICAM-1 is primarily expressed not only in microglia cells but also in astrocytes of the central nervous system (CNS), e.g., in the white and gray matter of the human forebrain (Lee and Benveniste, 1999). ICAM-1 expression in the CNS is often associated with glial fibrillary acidic protein (GFAP)-immunoreactive astrocytes (Miguel-Hidalgo et al., 2007); i.e., there is a co-localization of ICAM-1 and GFAP. ICAM-1 is of interest in psychiatric disorders for two reasons: 1) It has a key function in the blood–brain barrier (BBB), which plays an important role in the biology of psychiatric disorders by regulating the movement of molecules from the peripheral body, in particular components of the immune system, into and out of the CNS (see 2.1), and 2) it is a marker for inflammation.

Soluble ICAM-1, a circulating form of ICAM-1, arises from alternative splicing and proteolytic cleavage of membrane-bound ICAM-1 (Ramos et al., 2014). An interesting biological process associated with ICAM-1 overexpression during inflammation and its engagement by leukocytes is that of endothelial release of the ectodomain of ICAM-1, which can then circulate as sICAM-1 (Witkowska and Borawska, 2004; Lawson and Wolf, 2009). The literature describes sICAM-1 as an inflammatory marker and regulator that can promote the inflammatory response (Witkowska and Borawska, 2004; Lawson and Wolf, 2009). Serum levels of sICAM-1 appear to be low in healthy individuals, but they increase in many pathologies and are associated with disease progression and severity, for example, in cancer, cardiovascular disease, immune syndromes, and maladies involving chronic inflammation (Witkowska and Borawska, 2004; Lawson and Wolf, 2009). This association is not merely circumstantial, and sICAM-1 appears to be functionally involved in these diseases (Witkowska and Borawska, 2004). *In vitro* studies have shown a direct correlation between levels of shedded sICAM-1 and cell-surface ICAM-1, suggesting that increased levels of sICAM-1 in the CSF—as in the blood—are indicative of upregulation of surface-bound ICAM-1 in the brain (Leeuwenberg et al., 1992).

When discussing the role of ICAM-1 in psychiatric disorders, it must be noted that increased expression and shedding of ICAM-1 is observed in a wide range of diseases, only a few of which are neuropsychiatric. Inflammation, however, is a general condition of the human body, and the mechanisms of inflammation in psychiatric conditions parallel those of inflammatory disorders in non-psychiatric ones. A specific, important aspect of ICAM-1 in psychiatric disorders might be its role at the BBB.

ICAM-1 and the BBB

In healthy states, there is an ongoing but limited communication between the CNS and peripheral immune system. In some CNS diseases, however, a disturbance or breakdown of the BBB leads to an uncontrolled invasion of components of the peripheral immune system into the CNS, where they are primed, e.g., by microglial cells; after re-entering the periphery, they act to activate or inhibit the peripheral immune system. Pro-inflammatory molecules lead to an activation of brain-derived cells, including microglia and astrocytes; these two cell types almost completely surround the capillary endothelial cells that make up the BBB and modulate the barrier *via* effects on these endothelial cells (Benveniste, 1992). During acute inflammation, immune cells invade the CNS parenchyma through the disturbed BBB, i.e., through the endothelium of the small vessels and the tight junctions of astrocytes around the vessels. Activation of endothelial cells and increased expression of ICAM-1 result in breakdown of the BBB and increase leukocyte recruitment, adhesion, and infiltration. This immune cell invasion is mediated by cytokines, chemokines, adhesion molecules, and other mediators of inflammation, and ICAM-1 is a key molecule in this process. The physiological purpose of this process is to identify and eliminate antigens in the CNS. For example, infections with a virus that does not activate glial cells have a considerably more unfavorable course than do infections with a virus that does stimulate an immune response that involves astrocytes (Lewandowski et al., 1994).

Abnormalities in the CSF that indicate a disturbance of the BBB are described in at least 20% to 30% of psychiatric patients. One study found that the BBB was disturbed in 27% of patients with schizophrenia, for example, and IgG was produced intrathecally in 15% of these patients (IgG was measured by the relatively "rough" method of analyzing albumin and IgG in the CSF) (Müller and Ackenheil, 1995). Interestingly, the amount of IgG in the CSF correlated significantly with the psychopathology of schizophrenia, in particular with negative symptoms. In MDD, pathological changes in the CSF were observed in about 25% to 30% of patients (Hampel et al., 1995). Much more severe disturbances of the BBB are seen in acute, fulminant inflammatory processes, e.g., bacterial or viral meningitis or encephalitis. The signs of a mild inflammatory process in a range of mental disorders prompted Müller and Bechter to formulate the "mild encephalitis hypothesis" (Müller and Bechter, 2013). The significant correlation between psychopathology and IgG levels indicates close links between immune and disease processes, at least in schizophrenia.

ICAM-1 IN PSYCHIATRIC DISORDERS

As described above, sICAM-1 can be determined in body fluids, whereas ICAM-1 expression is analyzed in tissue, including CNS tissue. The results of sICAM-1 and ICAM-1 evaluations in different psychiatric disorders are described below.

Schizophrenia

A study of the *peripheral blood* in schizophrenia showed lower sICAM-1 levels in the blood of unmedicated patients than in healthy controls (Schwarz et al., 2000). During antipsychotic treatment lasting about 12 weeks, the study found a trend toward an increase in sICAM-1 and detected a relationship between sICAM-1 blood levels and schizophrenic psychopathology: Negative symptoms correlated positively with sICAM-1 levels, indicating that higher levels of sICAM-1 are associated with a more severe schizophrenic negative syndrome (Schwarz et al., 2000). The view that sICAM-1 levels are influenced by antipsychotic medication is supported by a study on the ICAM-1 ligand leucocyte function antigen-1 (LFA-1), which found that LFA-1 expression on leucocytes increased significantly during antipsychotic therapy (Müller et al., 1999). A recent study assessed sICAM-1 (and additional inflammatory adhesion molecules) in the plasma of 78 patients with schizophrenia or schizoaffective disorder and 73 healthy controls (Cai et al., 2018); interestingly, the authors found that sICAM-1 was significantly elevated in schizophrenia. However, the paper gives no information on antipsychotic treatment, so that a role of medication in the increase in sICAM-1 levels cannot be excluded. Another study described an association of sICAM-1 plasma levels with the stage of schizophrenia: In the early stage of schizophrenia, levels of sICAM-1 did not differ from those in healthy controls, but in the late stage of the disease, levels were higher than those in controls (Stefanovic et al., 2016). Of interest is that there was a significant relationship between sICAM-1 levels (but not levels of vascular cell adhesion molecule, VCAM-1, a cell adhesion molecule expressed on blood vessels) with clinical features; i.e., in the early stage of schizophrenia, sICAM-1 was related to the severity and type of current psychopathology, whereas in the late stage, it was associated with the progression of the disorder (Stefanovic et al., 2016). In the early stage of schizophrenia, patients with higher acute sICAM-1 levels had a less favorable treatment response. Similarly, in the late stage of schizophrenia, patients with a longer duration of untreated psychosis and shorter lifetime exposure to treatment had higher sICAM-1 levels compared with the early episode. In addition, higher daily chlorpromazine-equivalent doses applied at the initiation of the psychotic episode and maintained to the remission phase resulted in significantly lower sICAM-1 levels in patients in the late stage of the disease (Stefanovic et al., 2016). These findings are only partly in line with those of Schwarz and colleagues reported above (Schwarz et al., 2000). Many studies have described an increase of pro-inflammatory markers in patients with a longer duration of disease and longer treatment period. A possible explanation why Stefanovic and colleagues did not find decreased sICAM-1 in unmedicated patients with schizophrenia

may be that the patients in their study were in different clinical and psychopathological states.

Levels of **sICAM-1 in the CSF** better reflect the environmental condition of the CNS than do blood levels. However, valid CSF studies are lacking. A small study of sICAM-1 levels in the CSF of schizophrenia patients found significantly lower levels than those in healthy controls and in patients with a non-inflammatory neurological disorder (Schwarz MJ, unpublished results). Another study showed that sICAM-1 levels in the CSF are related to a disturbance of the BBB (Schwarz et al., 1998), a finding that supports the role of ICAM-1 in the BBB.

A recent study in schizophrenia patients estimated **ICAM-1 expression in the brain**. ICAM-1 mRNA expression in the prefrontal cortex was compared in a "high inflammation" schizophrenia subgroup—defined *a priori* acording to a cluster of cortical pro-inflammatory cytokine mRNAs in the patients—a low inflammation subgroup, and healthy controls. The study found that mRNA expression was higher in the "high inflammation" subgroup than in the "low inflammation" subgroup and controls (Cai et al., 2018). The role of antipsychotic medication and other confounding factors, however, needs to be evaluated in further studies.

An immunogenetic influence of the ICAM **G241A** or **A469G polymorphisms** in schizophrenia could not be detected, and no difference in sICAM-1 levels was found (Riedel et al., 2003). In healthy control persons carrying the polymorphic A allele (G241A), however, markedly lower sICAM-1 serum levels were found than in carriers of the homozygous GG wild type (G241G) ($p < 0.004$) (Kronig et al., 2005).

An overview of studies on ICAM-1 in schizophrenia is provided in **Table 1**.

MDD

In the past decade, a huge number of studies have been published on pro- and anti-inflammatory markers, and a role for inflammation in at least a subgroup of depressed patients is well established. Interestingly, the role of ICAM-1 has been poorly evaluated, in particular in MDD patients without co-morbidities. Because accompanying depressive states often occur in somatic disorders and especially in inflammatory disorders—one of the starting points for research on the role of inflammation in depression—the function of ICAM-1 has often been studied in depressed states comorbid to somatic disorders. Moreover, because aging is associated with an increase in the pro-inflammatory immune state in healthy people (immunosenescence) (Fulop et al., 2017), research has focused more on the role of ICAM-1 in elderly people.

Various research groups have studied *blood levels of sICAM-1*, as well as of other adhesion molecules, in late-life depression. A recently published meta-analysis of data from 43,600 participants aged >40 years, which included 9,203 people with depression, found an association between higher sICAM-1 levels and depression (van Agtmaal et al., 2017). Another study examined the relationship between sICAM-1 and depression and found significantly higher sICAM-1 levels in MDD patients after a 3-day wash-out of antidepressants than in age- and sex-matched healthy controls (Baghai et al., 2018). The authors took into account a medication and treatment effect because they described a trend toward an increase in sICAM-1 levels during treatment before discharge. Nevertheless, a 3-day wash-out may be too short to exclude an effect of medication on sICAM-1. So far, six different meta-analyses have presented pooled odd ratios. These analyses showed a significant association between high

TABLE 1 | ICAM-1 in schizophrenia: overview of findings.

Author	Source	n	Method	Result
Cai et al., 2018	Prefrontal cortex tissue	37 schizophrenia/schizoaffective psychosis no information on medication vs 37 healthy controls	PCR	Higher ICAM-1 mRNA expression ($p < 0.05$)
	Plasma	78 schizophrenia/schizoaffective (medicated) vs 73 healthy controls	Luminex	Significantly higher sICAM-1 levels ($p < 0.01$)
Müller et al., 1999	Serum	45 schizophrenia patients (unmedicated) 22 schizophrenia patients (medicated)	FACS	ICAM-1 ligand leucocyte function antigen-1 expression on leucocytes increased significantly during antipsychotic therapy
	CSF	32 schizophrenia patients (CSF)		
Schwarz et al., 1998	CSF	40 schizophrenia patients (medicated)	ELISA	Significant relationship of SICAM-1 to blood–CSF barrier
Schwarz et al., 2000	Serum	36 unmedicated schizophrenia 36 medicated schizophrenia 38 healthy controls	ELISA	Trend toward significantly lower sICAM-1 levels in unmedicated and medicated schizophrenia patients; increase of sICAM-1 during treatment
	CSF	18 schizophrenia (CSF) ICAM-1 in schizophrenia: overview on findings		Significant positive correlation of sICAM-1 with negative symptoms and duration of disease
Stefanovic et al., 2016	Serum	80 schizophrenia (medicated) (40 early stage, 40 late stage) 80 matched healthy controls	ELISA	sICAM-1 levels normal in early-stage schizophrenia, higher in late-stage schizophrenia. Related to disease severity and cognitive and excitement symptoms in early stage. Related to disease duration in late stage

ICAM-1, intercellular adhesion molecule-1; CSF, cerebrospinal fluid; sICAM-1, soluble ICAM-1; FACS, fluorescence-activated cell sorting.

levels of sICAM-1 (and some other adhesion molecules) and symptoms of depression in different samples of elderly people (Lesperance et al., 2004; Dimopoulos et al., 2006; Thomas et al., 2007; van Sloten et al., 2014; Tchalla et al., 2015; van Dooren et al., 2016). Most of the participants were not diagnosed with depression, but depression scores were higher in people with higher sICAM-1 levels (odds ratio 1.58, 95% confidence interval 1.28–1.96). Functionally, the authors interpreted the results as showing that generalized microvascular dysfunction is associated with depression. Multiple markers of microvascular dysfunction, including endothelial plasma markers and markers of cerebral small vessel disease, were cross-sectionally associated with a higher level of depressive symptoms and depressive disorder. These meta-analyses support the view that higher sICAM levels are associated with more severe psychopathology, here with symptoms of depression.

sICAM-1 blood levels in depressive disorder comorbid with a somatic disease, e.g., diabetes, have been studied in different conditions. An often-studied psychoneuroimmunological model is treatment with interferon-alpha. In interferon-alpha–treated patients, an increase in sICAM-1 levels was observed in parallel to an increase in depression scores, and significantly higher sICAM levels were found in more severely depressed patients (Schaefer et al., 2004). sICAM-1 blood levels were also examined in a study that aimed to investigate whether biomarkers of inflammation were associated with symptoms of depression in individuals with recently diagnosed diabetes (Herder et al., 2017). The authors were also interested to investigate whether such associations may differ by diabetes type. In contrast to the findings in people with type 2 diabetes, in people with type 1 diabetes, serum levels of sICAM-1 were positively associated with the score on the German version of the Center for Epidemiological Studies Depression Scale (Allgemeine Depressionsskala, Langversion).

CSF levels of sICAM-1 (and other pro-inflammatory markers) were assessed in patients after CNS trauma and found to predict the risk for developing a posttraumatic depressive syndrome (Juengst et al., 2015), providing further evidence of the close relationship between sICAM-1 levels and the psychopathology of depression.

The *CNS expression of sICAM-1* may best reflect the relationship between the psychopathological state of depression and the role of ICAM-1. One interesting study compared the expression of ICAM-1 in CNS tissue in patients with unipolar and bipolar depression, patients with schizophrenia, and healthy controls (Thomas et al., 2004). The study found significant increases in the expression of ICAM-1 (and additional CAMs) in the gray matter of the dorsolateral prefrontal cortex (DLPFC) in the unipolar depression group but no comparable differences between groups in the anterior cingulate cortex (ACC) or occipital cortex (Thomas et al., 2004). It also found a non-significant increase in ICAM-1 in the white matter of the DLPFC in the unipolar depression group (but no increase in the other areas of the brain or of VCAM-1 in any area). Paired tests showed the specificity for the DLPFC in the unipolar depression group only (Thomas et al., 2004). Another group of researchers estimated the expression of ICAM-1 in the choroid plexus (ChP), a highly vascularized tissue in the CNS that produces CSF, does not have a BBB and is an interface between the peripheral and central immune responses (Devorak et al., 2015). The authors found lower ICAM-1 expression in suicide victims with depression than in controls. Because ICAM-1 is expressed by choroid epithelial cells and is thought to support immune cell trafficking in the ChP, this finding might be an indication of decreased immune cell trafficking through the BBB in depression and suicide. ICAM-1 in the choroidal epithelium is also known to be upregulated in response to exposure to acute pro-inflammatory molecules *in vitro*. The observed downregulation of ICAM-1 expression in the ChP may represent a compensatory mechanism that functions to counteract (chronically) increased activation of pro-inflammatory signaling pathways elicited by chronically elevated cytokine levels, which are frequently observed in depressed patients. A decrease in immune cell trafficking through the BBB from the periphery into the CNS or vice versa could consequently limit central and/or peripheral pro-inflammatory signaling (Devorak et al., 2015). A converse interpretation of the finding might be that under certain psychopathological conditions the BBB shows hypopermeability and thus does not allow adequate molecular interchange between the CNS and peripheral immune system, so that immune activation in the CNS cannot be cleared.

Bipolar Disorder

As mentioned in the above discussion of the role of ICAM-1 in depression, the majority of studies to date did not examine individuals with a diagnosis of depression, but with a depressed state of varying severity. Accordingly, they did not differentiate between different types of depression, e.g., recurrent depression or bipolar depression. However, some studies have evaluated individuals with bipolar disorder, during either a manic or a euthymic state.

One study found a weak positive correlation between sICAM-1 levels and the scores on the Yale Mania Rating Scale in patients in a manic state (Turan et al., 2014). In their study in patients with bipolar disorder (n = 83), another group of researchers described higher blood levels of sICAM-1 (and higher IL-6, but lower tumor necrosis factor-α and lower sVCAM) in both acute and remission phases of the disorder than in age-, sex-, and body-mass–matched healthy controls (n = 73) (Pantovic-Stefanovic et al., 2016). Similarly, another study found significantly higher blood levels of sICAM-1 in euthymic bipolar patients than in healthy controls (Reininghaus et al., 2016). This study did not find an association with smoking, sex, or the duration of disease, but patients in a progressive stage of the disease had higher levels of sICAM-1 than did those in an earlier stage. Higher sICAM-1 levels were also found in patients on atypical antipsychotics, whereas prophylactic treatment with lithium or antiepileptics showed no association with sICAM-1 levels (Reininghaus et al., 2016). This finding is in line with earlier results showing signs of an immune activation during the euthymic phase (or free interval) of bipolar patients (Müller et al., 1993). It also corresponds with an interesting finding in a sample of healthy people (without a psychiatric diagnosis) who were grouped according to their affective temperament: In a multiple linear regression model, sICAM-1 blood levels were not related to affective states per se but to the state severity of manic symptoms measured with the Yale Mania Rating Scale (Ivkovic et al., 2017).

In CNS tissue, increased expression of ICAM-1 was described in both the gray and white matter of the ACC in bipolar patients compared with controls (gray: $p = .001$; white: $p < .001$) and schizophrenia patients (gray: $p = .016$; white: $p = .025$), and modestly increased expression was described in the white matter of the ACC in bipolar patients compared with patients with unipolar depression ($p = .049$). No differences were found in the DLPFC (Thomas et al., 2004).

Dementia

Aging is the main risk factor for Alzheimer's disease (AD), and a dramatic increase in extravascular ICAM-1 (associated with GFAP-immunoreactive astrocytes) is seen in the orbitofrontal cortex in normal aging. This increase may contribute to an enhanced risk for CNS inflammatory processes during aging (Miguel-Hidalgo et al., 2007). Decades ago, ICAM-1 was found to be increased in age-related neurodegenerative diseases (Akiyama et al., 1993). Accordingly, high ICAM-1 levels can be expected in AD. Besides localizing to the brain vasculature, in AD ICAM-1 also localizes to amyloid plaques, the biological hallmark of the disease, forming extravascular aggregates (Verbeek et al., 1994). It has been shown that these ICAM-1 immunoreactive aggregates are mostly absent in non-demented control individuals, even in the presence of normal vascular ICAM-1 immunoreactivity (Lee and Benveniste, 1999). However, it is still unclear whether age-related changes in extravascular ICAM-1 immunoreactivity occur in the CNS of normal, non-demented individuals, even though inflammatory processes in the CNS are well known to increase with aging (Bodles and Barger, 2004) and increases observed in neurodegenerative disorders could be age related. In animal models of brain injury, levels of mRNA for ICAM-1 and inflammatory cytokines increase more in old than in young rats (Kyrkanides et al., 2001), in parallel with increased immunostaining of GFAP in astrocytes. Furthermore, in humans, there seems to be an age-related increase in astrocytic GFAP immunoreactivity in the cerebral cortex (Prolla and Mattson, 2001) that might be paralleled by changes in key inflammatory molecules, particularly ICAM-1. In fact, astrocytes express ICAM-1 immunoreactivity *in vitro* and *in vivo* after brain damage (Lee and Benveniste, 1999).

The direct association between ICAM-1 and neurodegeneration has not yet been intensely studied. An *analysis of plasma sICAM-1* in patients with AD or Lewy body dementia (LBD) revealed significantly higher sICAM-1 levels in both patient groups than in healthy controls ($p < 0.001$). *In the CSF*, however, only LBD patients showed higher sICAM-1 levels (and significantly lower sVCAM-1 levels) than AD patients and healthy controls ($p < 0.001$) (Nielsen et al., 2007). As expected, the study found a strong correlation between sICAM-1 levels and BBB permeability in the AD patients and controls, but not in the LBD patients. A further evaluation of the data revealed a significant correlation between CSF sICAM-1 and kynurenic acid, a metabolite of tryptophan/kynurenine metabolism that has N-methyl-d-aspartate antagonistic effects and is driven by pro-inflammatory cytokines. Interestingly, the levels of kynurenic acid significantly correlated with the AD biomarker phosphorylated tau, supporting the association between sICAM-1 CSF levels and neurodegeneration markers in AD (Wennstrom et al., 2014).

A large study in nearly 800 individuals showed that CSF levels of sICAM-1 (and other markers of neuroinflammation) were increased not only during the dementia stages of AD, but already during the preclinical and prodromal stages of the disease. High levels of sICAM-1 were associated with increased CSF levels of total tau and phosphorylated tau. These associations were found in both Aß-positive and Aß-negative individuals, although the association was stronger in the former. The results were similar for associations between phosphorylated tau and ICAM-1. High levels of sICAM-1 were also associated with cortical thinning (primarily in the precuneus and superior parietal regions) and with subsequent cognitive deterioration in patients without dementia, as measured with the Clinical Dementia Rating Scale Sum of Boxes. Finally, higher levels of CSF ICAM-1 increased the risk of developing AD dementia in patients without dementia (Janelidze et al., 2018). The authors concluded that neuroinflammation and cerebrovascular dysfunction are early events that occur at presymptomatic stages of AD and contribute to disease progression.

In postmortem tissue of demented patients, in AD an increased expression of ICAM-1 was detected in amyloid plaques and astrocytes around plaques (Akiyama et al., 1993). A recent study of 143 cases of LBD determined levels of ICAM-1 (and the anti-inflammatory molecule CD200) in postmortem CNS tissue, in particular in the temporal and cingulate cortex, and found that high levels of ICAM-1 expression correlated with the density of amyloid plaques and neurofibrillary tangles; moreover, there was a strong positive correlation between phosphorylated tau levels and ICAM-1 (Walker et al., 2017). In this study, ICAM-1 expression correlated more with the typical AD pathology (and phosphorylated tau as a general marker of neurodestruction) than with the typical LBD hallmarks.

DISCUSSION/CONCLUSION

In recent decades, psychoneuroimmunological research has come into the focus of biological psychiatry, and it is now widely accepted that mild inflammation plays a role in many different psychiatric disorders. The data indicate that ICAM-1 may be a key molecule in various psychiatric disorders. This view is supported by findings that ICAM-1 is related to clinical features of schizophrenia, depression, and bipolar disorder and to specific neurodegeneration markers in dementia. The results of studies on sICAM-1 are more consistent than those of studies on several other adhesion molecules, such as VCAM-1, ICAM-3, platelet endothelial cell adhesion molecule-1, and others, possibly because sICAM-1 is biologically more stable and easier to measure. ICAM-1 has more diverse functions than sICAM-1. On the one hand, it is a marker for inflammation in the periphery and CNS, and on the other, it reflects the permeability of the BBB and the communication between the CNS and peripheral immune system.

Decreased levels of sICAM-1, as partly described in schizophrenia, may result from dysfunctional neuroendocrine-immune communication, wherein an adequate immune response is not mounted or, alternatively, neuroinflammation

is prolonged. A decrease in immune cell trafficking through the BBB from the periphery into the CNS or vice versa could consequently limit central or peripheral pro-inflammatory signaling or both (Devorak et al., 2015). Another interpretation of this finding might be that under certain psychopathological conditions the BBB is hypopermeable, which prevents adequate molecular interchanges between the CNS and peripheral immune systems and does not allow immune activation in the CNS to be cleared. Increased levels of sICAM-1 reflect an at least partial breakdown of the BBB, with the invasion of pro-inflammatory molecules into the CNS. This invasion is necessary to clear an infectious or inflammatory process in the CNS; however, once the process is complete, the inflammatory process must be downregulated; i.e., the number of anti-inflammatory molecules in the CNS and at the BBB must be increased. A disturbance of this regulatory process might be the key factor in diverse psychopathological states.

As a final point, one has to ask whether the increase in sICAM-1 levels and expression of ICAM on tissue reflect an unspecific process and why this increase in sICAM-1 is specifically associated with different disorders, including depression, schizophrenia, dementia, or bipolar disorder. The answer is provided by postmortem studies of CNS tissue, which reveal that different CNS regions are involved in the neuroinflammatory process in different psychopathological states, e.g., the DLPFC in depression and the ACC in bipolar disorder. *Ex vivo* neuroimaging, e.g., positron emission tomography, may be able to provide additional information in answer to these questions, but to date, no ligand for ICAM has been developed. The differences in the neuroinflammatory process between disorders may also explain why anti-inflammatory treatment has beneficial therapeutic effects in some psychiatric disorders, such as MDD and schizophrenia (Müller, 2015), and prophylactic effects in dementia (Müller et al., 2015).

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NM, the sole author, was responsible for all aspects of the manuscript.

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REFERENCES

Akiyama, H., Kawamata, T., Yamada, T., Tooyama, I., Ishii, T., and McGeer, P. L. (1993). Expression of intercellular adhesion molecule (ICAM)-1 by a subset of astrocytes in Alzheimer disease and some other degenerative neurological disorders. *Acta Neuropathol.* 85 (6), 628–634. doi: 10.1007/BF00334673

Baghai, T. C., Varallo-Bedarida, G., Born, C., Hafner, S., Schule, C., Eser, D., et al. (2018). Classical risk factors and inflammatory biomarkers: one of the missing biological links between cardiovascular disease and major depressive disorder. *Int. J. Mol. Sci.* 19 (6), 1740–1752. doi: 10.3390/ijms19061740

Benros, M. E., Nielsen, P. R., Nordentoft, M., Eaton, W. W., Dalton, S. O., and Mortensen, P. B. (2011). Autoimmune diseases and severe infections as risk factors for schizophrenia: a 30-year population-based register study. *Am. J. Psychiatry* 168 (12), 1303–1310. doi: 10.1176/appi.ajp.2011.11030516

Benros, M. E., Waltoft, B. L., Nordentoft, M., Ostergaard, S. D., Eaton, W. W., Krogh, J., et al. (2013). Autoimmune diseases and severe infections as risk factors for mood disorders: a nationwide study. *JAMA Psychiatry* 70 (8), 812–820. doi: 10.1001/jamapsychiatry.2013.1111

Benveniste, E. N. (1992). Inflammatory cytokines within the central nervous system: sources, function, and mechanism of action. *Am. J. Physiol.* 263 (1 Pt 1), C1–16. doi: 10.1152/ajpcell.1992.263.1.C1

Bodles, A. M., and Barger, S. W. (2004). Cytokines and the aging brain - what we don't know might help us. *Trends Neurosci.* 27 (10), 621–626. doi: 10.1016/j.tins.2004.07.011

Cai, H. Q., Catts, V. S., Webster, M. J., Galletly, C., Liu, D., O'Donnell, M., et al. (2018). Increased macrophages and changed brain endothelial cell gene expression in the frontal cortex of people with schizophrenia displaying inflammation. *Mol. Psychiatry* 44 (1), 75–83. doi: 10.1038/s41380-018-0235-x

Devorak, J., Torres-Platas, S. G., Davoli, M. A., Prud'homme, J., Turecki, G., and Mechawar, N. (2015). Cellular and molecular inflammatory profile of the choroid plexus in depression and suicide. *Front. Psychiatry* 6, 138. doi: 10.3389/fpsyt.2015.00138

Dimopoulos, N., Piperi, C., Salonicioti, A., Mitsonis, C., Liappas, I., Lea, R. W., et al. (2006). Elevation of plasma concentration of adhesion molecules in late-life depression. *Int. J. Geriatr. Psychiatry* 21 (10), 965–971. doi: 10.1002/gps.1592

Fulop, T., Larbi, A., Dupuis, G., Le Page, A., Frost, E. H., Cohen, A. A., et al. (2017). Immunosenescence and inflamm-aging as two sides of the same coin: friends or foes? *Front. Immunol.* 8, 1960. doi: 10.3389/fimmu.2017.01960

Goldsmith, D. R., Rapaport, M. H., and Miller, B. J. (2016). A meta-analysis of blood cytokine network alterations in psychiatric patients: comparisons between schizophrenia, bipolar disorder and depression. *Mol. Psychiatry* 21 (12), 1696–1709. doi: 10.1038/mp.2016.3

Hampel, H., Müller-Spahn, F., Berger, C., Haberl, A., Ackenheil, M., and Hock, C. (1995). Evidence of blood-cerebrospinal fluid-barrier impairment in a subgroup of patients with dementia of the Alzheimer type and major depression: a possible indicator for immunoactivation. *Dementia* 6 (6), 348–354. doi: 10.1159/000106969

Herder, C., Furstos, J. F., Nowotny, B., Begun, A., Strassburger, K., Mussig, K., et al. (2017). Associations between inflammation-related biomarkers and depressive symptoms in individuals with recently diagnosed type 1 and type 2 diabetes. *Brain Behav. Immun.* 61, 137–145. doi: 10.1016/j.bbi.2016.12.025

Ivkovic, M., Pantovic-Stefanovic, M., Petronijevic, N., Dunjic-Kostic, B., Velimirovic, M., Nikolic, T., et al. (2017). Predictive value of sICAM-1 and sVCAM-1 as biomarkers of affective temperaments in healthy young adults. *J. Affect Disord.* 207, 47–52. doi: 10.1016/j.jad.2016.09.017

Janelidze, S., Mattsson, N., Stomrud, E., Lindberg, O., Palmqvist, S., Zetterberg, H., et al. (2018). CSF biomarkers of neuroinflammation and cerebrovascular dysfunction in early Alzheimer disease. *Neurology* 91 (9), e867–ee77. doi: 10.1212/WNL.0000000000006082

Juengst, S. B., Kumar, R. G., Failla, M. D., Goyal, A., and Wagner, A. K. (2015). Acute inflammatory biomarker profiles predict depression risk following moderate to severe traumatic brain injury. *J. Head Trauma Rehabil.* 30 (3), 207–218. doi: 10.1097/HTR.0000000000000031

Khandaker, G. M., Pearson, R. M., Zammit, S., Lewis, G., and Jones, P. B. (2014). Association of serum interleukin 6 and C-reactive protein in childhood with depression and psychosis in young adult life: a population-based longitudinal study. *JAMA Psychiatry* 71 (10), 1121–1128. doi: 10.1001/jamapsychiatry.2014.1332

Kronig, H., Riedel, M., Schwarz, M. J., Strassnig, M., Moller, H. J., Ackenheil, M., et al. (2005). ICAM G241A polymorphism and soluble ICAM-1 serum levels: evidence

for an active immune process in schizophrenia. *Neuroimmunomodulation* 12 (1), 54–59. doi: 10.1159/000082364

Kyrkanides, S., O'Banion, M. K., Whiteley, P. E., Daeschner, J. C., and Olschowka, J. A. (2001). Enhanced glial activation and expression of specific CNS inflammation-related molecules in aged versus young rats following cortical stab injury. *J. Neuroimmunol.* 119 (2), 269–277. doi: 10.1016/S0165-5728(01)00404-0

Lawson, C., and Wolf, S. (2009). ICAM-1 signaling in endothelial cells. *Pharmacol. Rep.* 61 (1), 22–32. doi: 10.1016/S1734-1140(09)70004-0

Lee, S. J., and Benveniste, E. N. (1999). Adhesion molecule expression and regulation on cells of the central nervous system. *J. Neuroimmunol.* 98 (2), 77–88. doi: 10.1016/S0165-5728(99)00084-3

Leeuwenberg, J. F., Smeets, E. F., Neefjes, J. J., Shaffer, M. A., Cinek, T., Jeunhomme, T. M., et al. (1992). E-selectin and intercellular adhesion molecule-1 are released by activated human endothelial cells in vitro. *Immunology* 77 (4), 543–549.

Lesperance, F., Frasure-Smith, N., Theroux, P., and Irwin, M. (2004). The association between major depression and levels of soluble intercellular adhesion molecule 1, interleukin-6, and C-reactive protein in patients with recent acute coronary syndromes. *Am. J. Psychiatry* 161 (2), 271–277. doi: 10.1176/appi.ajp.161.2.271

Lewandowski, G., Hobbs, M. V., and Bloom, F. E. (1994). Alteration of intracerebral cytokine production in mice infected with herpes simplex virus types 1 and 2. *J. Neuroimmunol.* 55 (1), 23–34. doi: 10.1016/0165-5728(94)90143-0

Maes, M. (1994). Cytokines in major depression. *Biol. Psychiatry* 36 (7), 498–499. doi: 10.1016/0006-3223(94)90652-1

Maes, M., Stevens, W., DeClerck, L., Bridts, C., Peeters, D., Schotte, C., et al. (1992). Immune disorders in depression: higher T helper/T suppressor-cytotoxic cell ratio. *Acta Psychiatr. Scand.* 86 (6), 423–431. doi: 10.1111/j.1600-0447.1992.tb03292.x

Miguel-Hidalgo, J. J., Nithuairisg, S., Stockmeier, C., and Rajkowska, G. (2007). Distribution of ICAM-1 immunoreactivity during aging in the human orbitofrontal cortex. *Brain Behav. Immun.* 21 (1), 100–111. doi: 10.1016/j.bbi.2006.05.001

Müller, N. (2015). "Inflammation and Immunomodulation as Therapeutic Approaches in Schizophrenia and Depression – State of the Art," in *Immunology and Psychiatry: From basic research to therapeutic applications*. Eds. N. Müller, A.-M. Myint, and M. J. Schwarz (New York: Springer), 351–369. doi: 10.1007/978-3-319-13602-8_17

Müller, N., and Ackenheil, M. (1995). Immunoglobulin and albumin content of cerebrospinal fluid in schizophrenic patients: relationship to negative symptomatology. *Schizophr. Res.* 14 (3), 223–228. doi: 10.1016/0920-9964(94)00045-A

Müller, N., and Bechter, K. (2013). The mild encephalitis concept for psychiatric disorders revisited in the light of current psychoneuroimmunological findings. *Neurol. Psychiatry Brain Res.* 19 (3), 87–101. doi: 10.1016/j.npbr.2013.04.004

Müller, N., Hofschuster, E., Ackenheil, M., Mempel, W., and Eckstein, R. (1993). Investigations of the cellular immunity during depression and the free interval: evidence for an immune activation in affective psychosis. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 17 (5), 713–730. doi: 10.1016/0278-5846(93)90055-W

Müller, N., Riedel, M., Hadjamu, M., Schwarz, M. J., Ackenheil, M., and Gruber, R. (1999). Increase in expression of adhesion molecule receptors on T helper cells during antipsychotic treatment and relationship to blood–brain barrier permeability in schizophrenia. *Am. J. Psychiatry* 156 (4), 634–636.

Müller, N., Krause, D. L., Schwarz, M. J., Weidinger, E., and Reinisch, V. M. (2015). "The role of inflammation in Alzheimer's disease," in *Immunology and Psychiatry: From Basic Research to Therapeutic Applications*. Eds. N. Müller, A.-M. Myint, and M. J. Schwarz (Cham Heidelberg New York: Springer), 313–336. doi: 10.1007/978-3-319-13602-8_15

Muro, S. (2007). "Intercellular adhesion molecule-1 and vascular adhesion molecule-1," in *Endothelial Biomedicine*. Ed. W. C. Aird (New York: Cambridge University Press), 1058–1070. doi: 10.1017/CBO9780511546198.118

Myint, A. M., Leonard, B. E., Steinbusch, H. W., and Kim, Y. K. (2005). Th1, Th2, and Th3 cytokine alterations in major depression. *J. Affect Disord.* 88 (2), 167–173. doi: 10.1016/j.jad.2005.07.008

Nielsen, H. M., Londos, E., Minthon, L., and Janciauskiene, S. M. (2007). Soluble adhesion molecules and angiotensin-converting enzyme in dementia. *Neurobiol. Dis.* 26 (1), 27–35. doi: 10.1016/j.nbd.2006.11.011

Pantovic-Stefanovic, M., Petronijevic, N., Dunjic-Kostic, B., Velimirovic, M., Nikolic, T., Jurisic, V., et al. (2016). sVCAM-1, sICAM-1, TNF-alpha and IL-6 levels in bipolar disorder type I: acute, longitudinal and therapeutic implications. *World J. Biol. Psychiatry* 19 (sup2), S41–S51. doi: 10.1080/15622975.2016.1259498

Prolla, T. A., and Mattson, M. P. (2001). Molecular mechanisms of brain aging and neurodegenerative disorders: lessons from dietary restriction. *Trends Neurosci.* 24 (11 Suppl), S21–S31. doi: 10.1016/S0166-2236(01)00005-4

Ramos, T. N., Bullard, D. C., and Barnum, S. R. (2014). ICAM-1: isoforms and phenotypes. *J. Immunol.* 192 (10), 4469–4474. doi: 10.4049/jimmunol.1400135

Reininghaus, E. Z., Lackner, N., Birner, A., Bengesser, S., Fellendorf, F. T., Platzer, M., et al. (2016). Extracellular matrix proteins matrix metallopeptidase 9 (MMP9) and soluble intercellular adhesion molecule 1 (sICAM-1) and correlations with clinical staging in euthymic bipolar disorder. *Bipolar Disord.* 18 (2), 155–163. doi: 10.1111/bdi.12380

Riedel, M., Kronig, H., Schwarz, M. J., Engel, R. R., Sikorski, C., Kuhn, K. U., et al. (2003). Investigation of the ICAM-1 G241A and A469G gene polymorphisms in schizophrenia. *Mol. Psychiatry* 8 (3), 257–258. doi: 10.1038/sj.mp.4001320

Rothermundt, M., Arolt, V., Peters, M., Gutbrodt, H., Fenker, J., Kersting, A., et al. (2001). Inflammatory markers in major depression and melancholia. *J. Affect Disord.* 63 (1-3), 93–102. doi: 10.1016/S0165-0327(00)00157-9

Schaefer, M., Horn, M., Schmidt, F., Schmid-Wendtner, M. H., Volkenandt, M., Ackenheil, M., et al. (2004). Correlation between sICAM-1 and depressive symptoms during adjuvant treatment of melanoma with interferon-alpha. *Brain Behav. Immun.* 18 (6), 555–562. doi: 10.1016/j.bbi.2004.02.002

Schmal, H., Czermak, B. J., Lentsch, A. B., Bless, N. M., Beck-Schimmer, B., Friedl, H. P., et al. (1998). Soluble ICAM-1 activates lung macrophages and enhances lung injury. *J. Immunol.* 161 (7), 3685–3693.

Schwarz, M. J., Ackenheil, M., Riedel, M., and Müller, N. (1998). Blood-cerebrospinal fluid barrier impairment as indicator for an immune process in schizophrenia. *Neurosci. Lett.* 253 (3), 201–203. doi: 10.1016/S0304-3940(98)00655-7

Schwarz, M. J., Riedel, M., Ackenheil, M., and Müller, N. (2000). Decreased levels of soluble intercellular adhesion molecule-1 (sICAM-1) in unmedicated and medicated schizophrenic patients. *Biol. Psychiatry* 47 (1), 29–33. doi: 10.1016/S0006-3223(99)00206-1

Springer, T. A. (1990). Adhesion receptors of the immune system. *Nature* 346 (6283), 425–434. doi: 10.1038/346425a0

Stefanovic, M. P., Petronijevic, N., Dunjic-Kostic, B., Velimirovic, M., Nikolic, T., Jurisic, V., et al. (2016). Role of sICAM-1 and sVCAM-1 as biomarkers in early and late stages of schizophrenia. *J. Psychiatr. Res.* 73, 45–52. doi: 10.1016/j.jpsychires.2015.11.002

Tchalla, A. E., Wellenius, G. A., Sorond, F. A., Travison, T. G., Dantoine, T., and Lipsitz, L. A. (2015). Elevated circulating vascular cell Adhesion Molecule-1 (sVCAM-1) is associated with concurrent depressive symptoms and cerebral white matter Hyperintensities in older adults. *BMC Geriatr.* 15, 62. doi: 10.1186/s12877-015-0063-7

Thomas, A. J., Davis, S., Ferrier, I. N., Kalaria, R. N., and O'Brien, J. T. (2004). Elevation of cell adhesion molecule immunoreactivity in the anterior cingulate cortex in bipolar disorder. *Biol. Psychiatry* 55 (6), 652–655. doi: 10.1016/j.biopsych.2003.10.015

Thomas, A. J., Morris, C., Davis, S., Jackson, E., Harrison, R., and O'Brien, J. T. (2007). Soluble cell adhesion molecules in late-life depression. *Int. Psychogeriatr.* 19 (5), 914–920. doi: 10.1017/S1041610206004728

Tsakadze, N. L., Sithu, S. D., Sen, U., English, W. R., Murphy, G., and D'Souza, S. E. (2006). Tumor necrosis factor-alpha-converting enzyme (TACE/ADAM-17) mediates the ectodomain cleavage of intercellular adhesion molecule-1 (ICAM-1). *J. Biol. Chem.* 281 (6), 3157–3164. doi: 10.1074/jbc.M510797200

Turan, C., Kesebir, S., Suner, O., and Are, I. C. A. M. (2014). VCAM and E-selectin levels different in first manic episode and subsequent remission? *J. Affect Disord.* 163, 76–80. doi: 10.1016/j.jad.2014.03.052

van Agtmaal, M. J. M., Houben, A., Pouwer, F., Stehouwer, C. D. A., and Schram, M. T. (2017). Association of microvascular dysfunction with late-life depression: a systematic review and meta-analysis. *JAMA Psychiatry* 74 (7), 729–739. doi: 10.1001/jamapsychiatry.2017.0984

van Dooren, F. E., Schram, M. T., Schalkwijk, C. G., Stehouwer, C. D., Henry, R. M., Dagnelie, P. C., et al. (2016). Associations of low grade inflammation and endothelial dysfunction with depression - The Maastricht Study. *Brain Behav. Immun.* 56, 390–396. doi: 10.1016/j.bbi.2016.03.004

van Sloten, T. T., Schram, M. T., Adriaanse, M. C., Dekker, J. M., Nijpels, G., Teerlink, T., et al. (2014). Endothelial dysfunction is associated with a greater depressive symptom score in a general elderly population: the Hoorn Study. *Psychol. Med.* 44 (7), 1403–1416. doi: 10.1017/S0033291713002043

Verbeek, M. M., Otte-Holler, I., Westphal, J. R., Wesseling, P., Ruiter, D. J., and de Waal, R. M. (1994). Accumulation of intercellular adhesion molecule-1 in senile plaques in brain tissue of patients with Alzheimer's disease. *Am. J. Pathol.* 144 (1), 104–116.

Walker, D. G., Lue, L. F., Tang, T. M., Adler, C. H., Caviness, J. N., Sabbagh, M. N., et al. (2017). Changes in CD200 and intercellular adhesion molecule-1 (ICAM-1) levels in brains of Lewy body disorder cases are associated with amounts of Alzheimer's pathology not alpha-synuclein pathology. *Neurobiol. Aging* 54, 175–186. doi: 10.1016/j.neurobiolaging.2017.03.007

Wang, A. K., and Miller, B. J. (2018). Meta-analysis of cerebrospinal fluid cytokine and tryptophan catabolite alterations in psychiatric patients: comparisons between schizophrenia, bipolar disorder, and depression. *Schizophr. Bull.* 44 (1), 75–83. doi: 10.1093/schbul/sbx035

Wennstrom, M., Nielsen, H. M., Orhan, F., Londos, E., Minthon, L., and Erhardt, S. (2014). Kynurenic Acid levels in cerebrospinal fluid from patients with Alzheimer's disease or dementia with lewy bodies. *Int. J. Tryptophan. Res.* 7, 1–7. doi: 10.4137/IJTR.S13958

Witkowska, A. M., and Borawska, M. H. (2004). Soluble intercellular adhesion molecule-1 (sICAM-1): an overview. *Eur. Cytokine Netw.* 15 (2), 91–98.

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Quercetin Alleviates LPS-Induced Depression-Like Behavior in Rats *via* Regulating BDNF-Related Imbalance of Copine 6 and TREM1/2 in the Hippocampus and PFC

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Quercetin is a polyphenol with multiple biological activities, and results of our preliminary study showed that it could shorten the immobility time of mice in the forced swimming test and tail suspending test. The aim of this study was to investigate its effects on the behavioral performance of lipopolysaccharide (LPS)-challenged rats and explore the potential mechanism. The results showed that intragastrical administration of quercetin (40 mg/kg) could improve the bodyweight gain of LPS-challenged rats, increase the saccharin preference index in the saccharin preference test and the novel arm preference index in the Y-maze, and decrease the immobility time in the FST. However, it showed no significant effect on the performance of LPS-challenged rats in the Morris water maze and the plasma concentrations of nesfatin-1, C-reactive protein (CRP), and IL-6. Results of western blot showed that the expression levels of BDNF, Copine 6, p-TrkB, and the triggering receptors expressed on myeloid cells (TREM) 1 were decreased in both the hippocampus and the prefrontal cortex (PFC) of LPS-challenged rats, while the expression of TREM2 was increased. The protein expression of synapsin-1 was decreased in the hippocampus without significant changes in the PFC. These imbalance protein expressions could be balanced by treatment with quercetin. The results suggested that quercetin could alleviate LPS-induced depression-like behaviors and impairment of learning and memory in rats, the mechanism of which might be involved with regulating the BDNF-related imbalance expression of Copine 6 and TREM1/2 in the hippocampus and the PFC.

Keywords: quercetin, nesfatin-1, brain derived neurotrophic factor (BDNF), Copine 6, the triggering receptors expressed on myeloid cells (TREMs), synapsin-1

INTRODUCTION

Depression is a prevalent and recurrent mental abnormality that affects human health and economical development worldwide. It has been reported that during 2013–2016, 8.1% of the American adults aging 20 and above suffer from depression in a given 2-week period (Brody et al., 2018). Since first discovered in 1950s, most of the antidepressants are designed to correct the imbalances of neurotransmitters, which are taken as the key factor of regulating mind and emotion. Unfortunately, they are not effective for all the depression patients, and almost all the classical antidepressants share the same defect of long latency. Although the finding of the robust and rapid-onset antidepressant effects of ketamine has shifted the direction of antidepressants research and development (Dhir, 2017), and been taken as the single most important advance in the treatment of depression in over 50 years (Singh et al., 2017), it should be taken into account of the adverse effects including not only the cardiovascular symptoms, but also hallucinations, confusion, and irrational behaviors. Thus, it is urgent to further investigate the pathogenesis of depression and explore new effective antidepressants with less side effects and shorter acting period.

Inflammation has been long taken as one of the cornerstones in the development of depression (Jeon and Kim, 2017). Higher incidence of depression has been found in patients with infection, and depression patients present increased levels of pro-inflammatory cytokines and decreased level of brain-derived neurotrophic factor (BDNF) (Felger and Lotrich, 2013). C-reactive protein (CRP) and Interleukin 6 (IL-6) are common inflammatory markers, and elevated levels of CRP and IL-6 are associated with increased risk for psychological distress and depression (Wium-Andersen et al., 2013; Khandaker et al., 2014). Consistently, results of the animal studies reported that administration of lipopolysaccharide (LPS) could significantly elevate the IL-6 mRNA expression in both the brain and the spleen (Szot et al., 2017), resulting in depressive-like behavior and hippocampal microglial activation (Yamawaki et al., 2018). Moreover, the elevated plasma concentrations of CRP and IL-6 in patients with major depressive disorder are reported to be positively related to the increased plasma nesfatin-1 level (Xia et al., 2018), which is an anorexigenic molecule localized widely in the brain and peripheral tissues with multiple biological activities including regulation of feeding and mood (Weibert et al., 2019). Consistently, in our previous study (Ge et al., 2015a), administration with nesfatin-1 could induce depression-like behaviors, accompanied with the increased plasma concentrations of IL-6 and CRP. Moreover, the antidepressant-like effect of ibuprofen, which is one of the common used nonsteroidal anti-inflammatory drugs (NSAIDs), has demonstrated in animal studies (Mesripour et al., 2019; Seo et al., 2019). These findings support an overlapping pathobiology between inflammation and depression (Merali et al., 2003).

It is important to notice that the disruption of BDNF/TrkB signaling pathway and impairment of neuronal plasticity have been implicated in the pathogenesis of depressive disorders (Erickson et al., 2012). Hippocampus and prefrontal cortex (PFC) are key brain regions involved in the pathogenesis of depression and the antidepressant effects. Reduced serum BDNF levels, together with altered expression of BDNF and its high-affinity receptor tropomyosin receptor kinase B (TrkB) in the hippocampus and PFC, have been found in depression state and could be reversed by effective treatment with antidepressants (Gawali et al., 2016; Song et al., 2019; Seo et al., 2019). Results of our previous studies have suggested a positive relationship between the hippocampal expression of BDNF and Copine 6, synaptotagmin I, and synapsin I in stressed rats (Han et al., 2018). Copine 6 is another important molecular that plays a vital role in regulation of synaptic plasticity (Reinhard et al., 2016), *via* alternating the neurotransmission process (Liu et al., 2018) or morphology (Burk et al., 2018). Knockout of Copine 6 could induce a deficiency of hippocampal long time potentiation (LTP) and learning and memory in mice (Xu et al., 2015). However, little is known about their alternations in LPS-challenged rats.

The triggering receptors expressed on myeloid cells (TREMs) are a family of activating receptors, potentially may be manipulated to alter the inflammatory response (Bouchon et al., 2001). Inflammatory conditions that alter the balance in TREM expression could exert an important influence on homeostatic activity (Owens et al., 2017), resulting in different outcomes in different diseases (Konishi and Kiyama, 2018). Recent years, much attention has been paid to the role of TREM1/2 in the neuropsychiatric diseases including Alzheimer's disease (AD), although the results are not always consistent (Jiang et al., 2016; Suarez-Calvet et al., 2019; Jiang et al., 2017; Sayed et al., 2018). In our previous research, the protein expressions of TREM2 in the hippocampus are different between rats aging 2 months and 6 months, accompanied with a different performance in behavior tasks. These results suggest that the function of TREMs could be affected by a variety of factors, such as disease duration and stage (Jay et al., 2017).

Querctin is a polyphenol derived from many kinds of plants with multiple activities including antiinflammation and neuroprotective effects. Our previous results demonstrated that it could bind to beta amyloid (Aβ)1-40 and Aβ1-42 in monomer or fibril state. Recently, it has been reported that quercetin could protect against stress-induced anxiety- and depression-like behavior and improve memory in male mice (Samad et al., 2018). Consistently, results of our preliminary study showed that it could shorten the immobility time of mice in the forced swimming test (FST) and tail suspending test, though the mechanism remains unclear.

The aim of the present study is to investigate the effects of quercetin on LPS-induced depression-like behavior and explore its potential mechanism, especially its effect on the BDNF-related protein expression of the key molecules in the hippocampus and PFC.

MATERIALS AND METHODS

Drugs

Quercetin was provided by Haoyang Biotechnology Co, LTD. (Xi'an, Shanxi, China) with the purity 95%, and the structure of

quercetin is shown in **Figure 1**. Fluoxetine Hydrochloride (Prozac) was purchased from Eli Lilly Pharmaceuticals. Ibuprofen was the production of Renfu pharmaceutical co., LTD (Yichang, Hubei, China).

Animals

Fourty-five male Sprague-Dawley rats, aged 2 months, were purchased from Anhui Experimental Anhui Center of China. The rats were assigned randomly to five groups including an unhandled control group (Control), a LPS-injection group (Model), a ibuprofen treatment group (240 mg/kg), a fluoxetine treatment group (5 mg/kg), and a quercetin treatment group (40 mg/kg), with nine rats in each group. After a 7 days' adaptive breeding, rats were administered intragastrically the drugs daily with the dose mentioned above, while equal volume of 0.5% carboxymethyl cellulose (CMC) were given to rats in control group and model groups. During the 3th–5th day, 0.5 mg/Kg LPS (from Escherichia coli, serotype 055:B5, Sigma-Aldrich) was administered through intraperitoneal injection to rats in model and drug-treated groups, and equivalent sterile normal saline solution was administered to rats in control group. The behavioral experiments were put into operation from the 5th day. The schedule of the experimental design is shown in **Figure 2**.

Rats were raised and maintained under a 12-h light-dark cycle and housed 4–5 per cage with free access to food and water. The ambient temperature was maintained at 21°C–22°C with 50%–60% relative humidity. The experimental procedures were approved by the animal Care and Use committee at Anhui Medical University and complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. (NIH publication No.85-23, revised 1985)

Behavioral Tests

Behavioral tests were performed in a soundproof room with a neutral environment. All the rats in this study including the control ones received all the behavior tasks one by one. As the following description order, the first one was the saccharin preference test (SPT), followed by the open field test (OFT), then Y-maze and Morris water maze (MWM), and the last one

FIGURE 1 | The structure of quercetin.

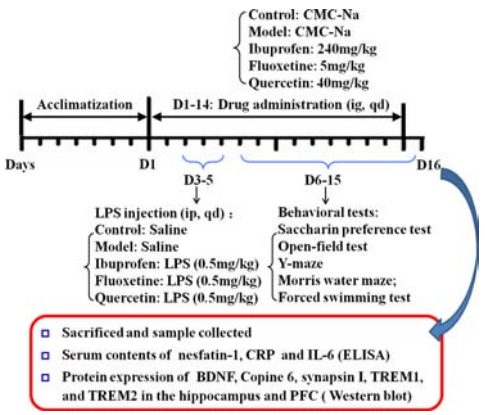

FIGURE 2 | Schedule of the experimental design.

was the FST. All the tests were carried out between 08:30 and 12:30, one hour after intragastric administration of solvent or drug, with matching between the groups. The observers were blind to the treatment. The behavioral tests were monitored and recorded by a digital camera interfaced to a computer running the ANY-maze video imaging software (Stoleting Co.,Wood Dale, IL, USA).

Saccharin Preference Test

The SPT is one of the routine methods to evaluate the anhedonic behavior in rodents. After a 12-h period of food and water deprivation, all rats were housed solely and had free access to two bottles containing either plain water or 2% saccharin solution. After 6 h, the volumes of water and saccharin consumed were measured. The percentage of saccharin solution ingested, which is also named as saccharin preference index (SPI), is a measure to assess the sensitivity of rats to hedonia.

Open Field Test

The OFT provides synchronous measures of locomotion, exploration, and anxiety. The apparatus of the test consisted of a black square arena with a length of 100 cm and a height of 30 cm. The floor was marked with white grids dividing it into 16 equally sized squares. During a 5-min observation period, rats were placed at one corner of the apparatus facing the wall. The total distance, duration in the center, and the frequencies of rearing and grooming were recorded. Rats were returned to their housed cages after test, and the open field was cleaned with 75% alcohol and permitted to dry between tests.

Y-Maze

Y-maze consists of three identical arms with an angle of 120° between each other. Each arm was 10 cm × 48 cm × 20 cm (length× width× height). They were randomly designated as the start arm, the novel arm, and the familiar arm. The novel arm was blocked during the 1st trail but open during the 2nd trail, while the other two arms were always open. The maze was cleaned with 75% alcohol and permitted to dry between tests. The Y-maze test consisted of two trials separated by an intertrail

interval to assess spatial recognition memory. The first trail allowed the rats 10 min to explore the start arm and the other arm. Twenty-four hours later, the second trail was conducted. The rat was placed back in the same starting arm, with free access to all the three arms for 5 min, and the percentage of the duration in the novel arm to that in both the novel and familiar arms was taken as the novel arm preference index.

Morris Water Maze

MWM was used to test the spatial learning and memory. The maze was consisted of black circular pool (diameter 160 cm, height 50 cm, filled with water to 30 cm high containing black edible pigment at 21°C–22°C). A black circular platform (9 cm in diameter) was 2.0 cm below the water line in the center of one quadrant, and remained in the same position. Several constant and large visual cues surrounded the tank at a height of 60 cm to facilitate orientation.

The task consisted of a 3-day acquisition phase with four trails administered each day and a 1-day memory retention test phase. During the acquisition phase, the rat was placed in the water facing the wall at one random start location of four (north, south, east, and west, locating at equal distances from each other on the pool rim). Each rat was allowed to find the submerged platform within 60 s, and rest on it for 30s. If the rat failed to find the hidden platform within 60 s, it was guided to the platform and allowed to remain there for 30 s. The procedure was repeated for all the four start locations.

On the 4th day (the test phase), memory retention was determined in a single 60 s probe trial. The underwater platform was removed. The rats were placed into water from the opposite quadrant of the platform, facing the wall, and were permitted to explore the environment for 60 s *ad libitum*. Performance parameters of each rat including total swim distance, mean swim velocity, and the duration in each quadrant were monitored and recorded.

Forced Swimming Test

The behavioral cylinder was 60 cm high with the diameter 25cm, filled with 30 cm of water and maintained at 24°C–25°C. The immobility time was recorded and rats were considered immobile when they did not make any active movements.

Measurement of the Plasma Concentration of Nesfatin-1, IL-6, and CRP

Twenty-four hours after the last behavioral test, the rats were deeply anesthetized with chloral hydrate, and the blood was drawn from abdominal aorta. The plasma concentrations of nesfatin-1, IL-6, and CRP were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Nesfatin-1: Huamei Biotech. Co., LTD., Wuhan, China; IL-6 and CRP: Yuanye Biotech. Co., LTD., Shanghai, China) based on the manufactures' instructions.

Western Blot Assays

The hippocampus and the PFC of three rats from each group were promptly dissected, frozen in liquid nitrogen, and stored at −80°C. The tissue was homogenized in radio immuniprecipitation assay (RIPA) buffer (50mM Tris-HCl, pH 7.4, 0.1% SDS, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, and 1 mM Na3Vo4). A protease inhibitor cocktail (Roche, IN, USA) and the phosphatase inhibitor PhosSTOP (Roche, IN, USA) were added before homogenization. The protein content was measured using a Lowry Protein Assay Kit (Meiji Biotech Co., Ltd., Shanghai, China). The same quantity (50 μg) of protein from each sample was loaded and separated by 15% SDS-PAGE and then transferred onto a polyvinylidene difluoride membrane (Amersham Biosciences, UK). The membrane was blocked with 5% skim milk for 1 h; incubated with antibodies targeting BDNF (1:1000; Abcam, Cambridge, UK), TrkB (1:10000; Abcam, Cambridge, UK), phosphorylated TrkB (p-TrkB) (1:10000; Abcam, Cambridge, UK), Copine 6 (1:1,000; Santa Cruz Biotechnology, Inc., Delaware, USA), synapsin-1 (1:1000; ImmunoWay, Newark, Delaware, USA), TREM-1 (1:1000; ImmunoWay, Newark, Delaware, USA), TREM-2 (1:1000; ImmunoWay, Newark, Delaware, USA), or β-actin (1:1000; Zhongshan Biotechnology, INC, Beijing, China) at 4°C overnight, and then incubated with a horseradish peroxidase-conjugated secondary antibody (1:2000) at 37°C for 2 h. The blots were developed with the Easy Enhanced Chemiluminescence Western Blot Kit (Pierce Biotechnology, Rockford, IL, USA). The protein bands were scanned and analyzed using Image J software (NIH). The TrkB/TrkB ratio was observed, and the expression of other proteins was normalized to β-actin and analyzed.

Statistical Analysis

All statistical analyses were performed using SPSS (version 12.0.1, SPSS Inc., Chicago, IL, United States). Data are expressed as the means ± SEM and $P < 0.05$ was considered statistically significant. Between-group effects on the escaping latency in the MWM task were analyzed by repeated measures ANOVA with group and time as the factors. Statistical analyses of the effect of quercetin on other parameters were carried out using ANOVA followed by LSD posthoc tests. The correlation analysis was performed by Pearson's correlation test.

RESULTS

Quercetin Administration Improved the Decreased Bodyweight Gain in LPS-Challenged Rats

Figure 3 shows the bodyweight of the rats in different group before and after the test. As shown in **Figure 3A**, the bodyweight increased in all the groups, indicating that all the rats were growing. However, the bodyweight gain of the model ones were less than that of the control rats ($F[4, 40] = 4.795$, $P = 0.031$), and this change could be reversed by treatment with quercetin ($F[4, 40] = 4.795$, $P = 0.005$) or fluoxtine ($F[4, 40] = 4.795$, $P = 0.001$) (**Figure 3B**).

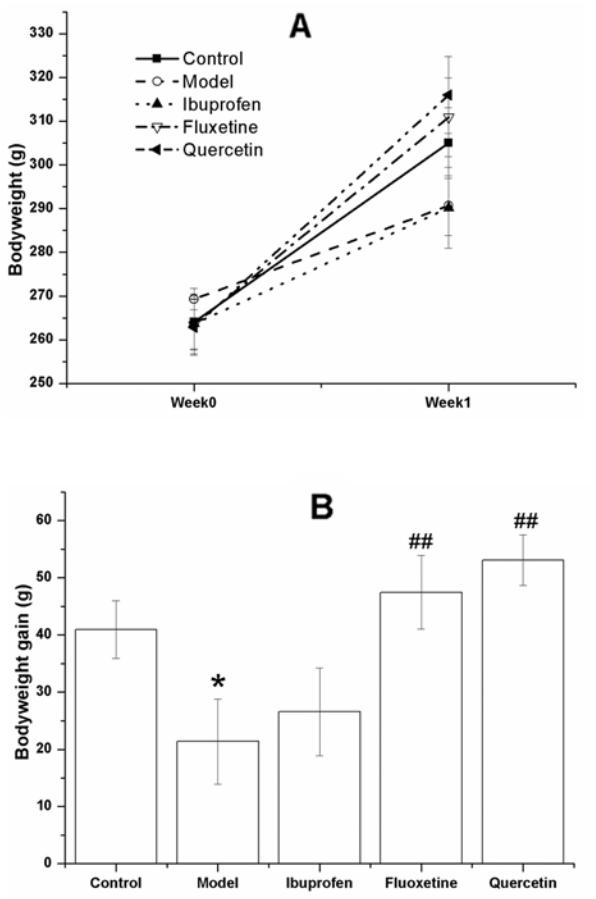

FIGURE 3 | Effect of quercetin on the bodyweight and bodyweight gain in lipopolysaccharide (LPS)-challenged rats.The data are presented as the mean ± SEM, with nine rats in each group.There was no significant difference among groups with regard to the bodyweight **(A)**, but the bodyweight gain of the model rats in LPS-challenged group were less than that of the control ones, and this change could be reversed in the quercetin- and fluoxetine-treated group **(B)**. $^{*}P < 0.05$, compared with the control group; $^{\#\#}P < 0.01$, compared with the model group.

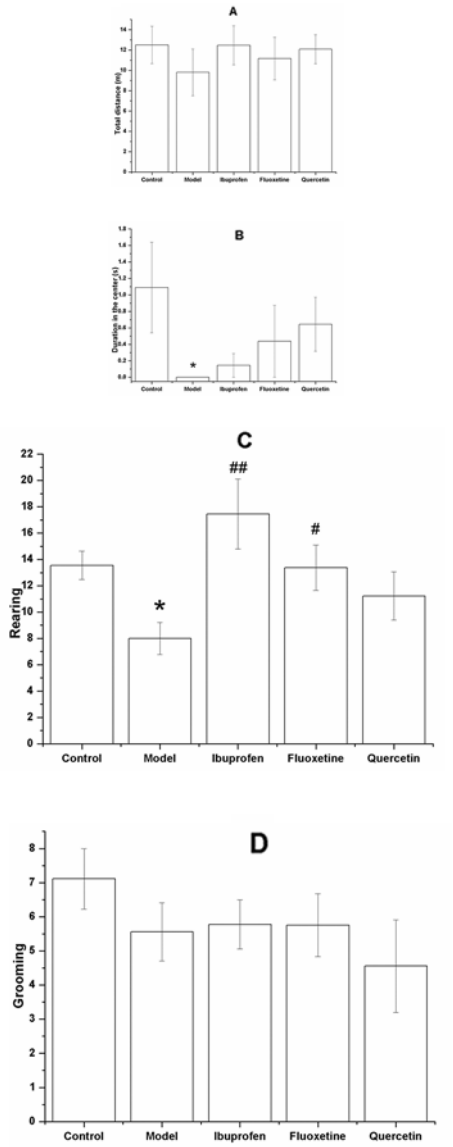

FIGURE 4 | Effect of quercetin on the performance of lipopolysaccharide (LPS)-challenged rats in the open field test (OFT).The data are presented as the mean ± SEM, with nine rats in each group.In the OFT, there was no significant difference among groups with respect to the total moving distance **(A)** and grooming frequency **(D)**, but the duration in the center **(B)** and the rearing frequency **(C)** were significant decreased in the LPS-challenged group. Treatment with quercetin could increase the rearing frequency **(C)**.$^{*}P < 0.05$, compared with the control group; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$, compared with the model group.

Quercetin Administration Alleviated the Depression-Like Behavior in LPS-Challenged Rats

Figure 4 shows the performance of rats in the OFT. Although there was no significant difference among groups with regards to the total distance ($F[4, 40] = 0.377$, $P = 0.317$; **Figure 4A**) and the grooming frequency ($F[4, 40] = 0.918$, $P = 0.264$; **Figure 4D**), the LPS-challenged rats spent less time in the center ($F[4, 40] = 1.601$, $P = 0.030$; **Figure 4B**) and showed less rearing frequency ($F[4, 40] = 3.869$, $P = 0.031$; **Figure 4C**), and treatment with ibuprofen ($F[4, 40] = 3.869$, $P < 0.001$; **Figure 4C**) or fluoxetine ($F[4, 40] = 3.869$, $P = 0.042$; **Figure 4C**) could significantly increase the rearing frequency. Compared with that in the control group, the model rats showed less SPI in the SPT ($F[4, 40] = 4.809$, $P = 0.041$; **Figure 5A**) and longer immobility time in the FST ($F[4, 40] = 4.829$, $P = 0.039$; **Figure 5B**). However, treatment with quercetin, fluoxetine, and ibuprofen could reverse the changes of SPI and immobility time ($P < 0.05$ or $P < 0.01$).

Results of the Pearson's correlation test showed that the immobility time in the FST was negatively correlated to the sucrose preference index ($r = -0.375$, $P = 0.012$; **Figure 5C**) and the bodyweight gain ($r = -0.322$, $P = 0.031$; **Figure 5D**).

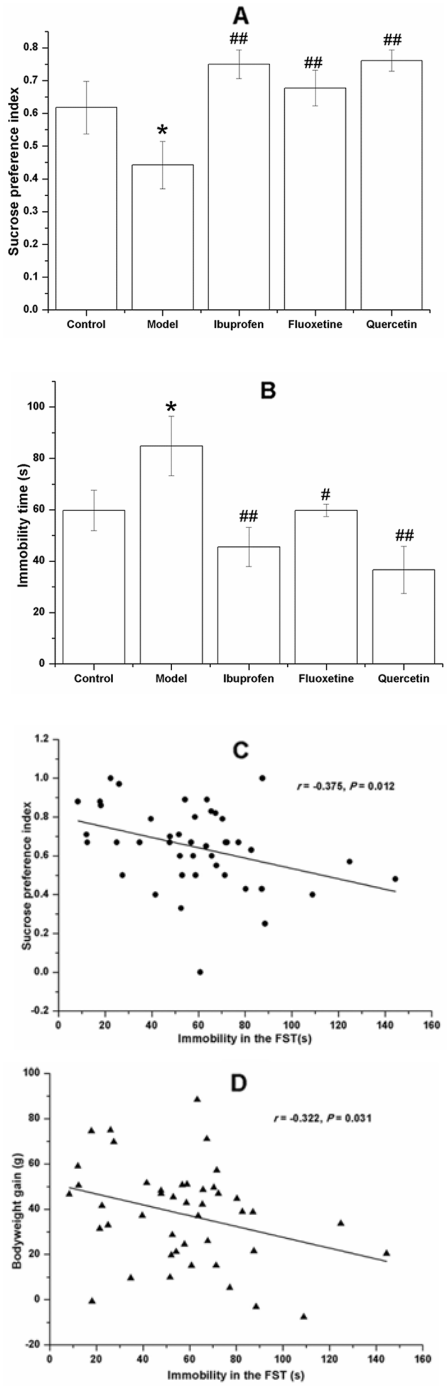

FIGURE 5 | Effect of quercetin on the performance of lipopolysaccharide (LPS)-challenged rats in the saccharin preference test (SPT) and forced swimming test (FST). The data are presented as the mean ± SEM, with nine rats in each group. Compared with that in the control group, the sucrose preference index of rats in LPS-challenged group was lower **(A)** and the immobility time in the FST **(B)**. These changes could be reversed by treatment with quercetin, fluoxetine, and ibuprofen. The immobility time in the FST was negatively correlated to the sucrose preference index **(C)** and the bodyweight gain **(D)**. $^{*}P < 0.05$, compared with the control group; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$, compared with the model group.

Quercetin Administration Improved the Performance of LPS-Challenged Rats in Y-Maze Without Significant Influence in the MWM

For all the rats studied in the experiment, the escape latency declined with each day during the acquisition phase, as is shown in **Figure 6A**. Analyzing the latency using repeated measures ANOVA with experimental treatment as the between-subject factor and training days as the within-subject factor, the results showed that only the training days ($F[2, 39] = 65.426$, $P < 0.01$), but not the experimental treatment ($F[4, 40] = 2.077$, $P = 0.102$) had a significant effect on the escape latency, without a significant interaction between each other ($F[8, 80] = 1.888$, $P = 0.073$). In the probe trail, there was no significant difference among groups with regard to the duration in the target quadrant ($F[4, 40] = 0.136$, $P = 0.968$; **Figure 6B**).

In the Y-maze task, the LPS-challenged rats spent less time in the novel arm than the controls ($F[4, 40] = 2.099$, $P = 0.011$; **Figure 6C**), together with a decreased novel arm preference ($F[4, 40] = 2.669$, $P = 0.008$; **Figure 6D**). These changes could be significantly reversed by treatment of fluoxetine or ibuprofen ($P < 0.05$). Treated with quercetin could increase the decreased novel arm preference index induced by LPS-challenge ($F[4, 40] = 2.669$, $P = 0.028$; **Figure 6D**).

Quercetin Administration Did Not Change the Plasma Concentration of Nesfatin-1, CRP, and IL-6 in LPS-Challenged Rats

As shown in **Figure 7**, the plasma concentrations of nesfatin-1 ($F[4, 40] = 3.170$, $P = 0.003$; **Figure 7A**), CRP ($F[4, 40] = 2.610$, $P = 0.039$; **Figure 7B**), and IL-6 ($F[4, 40] = 2.442$, $P = 0.005$; **Figure 7B**) were all remarkably increased in the model group, and there was no significant change after treatment with ibuprofen, fluxetine, or quercetin.

Results of the Pearson's correlation test showed that the plasma CRP concentration was negatively correlated to the novel arm preference index ($r = -0.315$, $P = 0.042$; **Figure 7C**), and the plasma IL-6 concentration was positively correlated to the immobility time in the FST ($r = 0.365$, $P = 0.016$; **Figure 7D**).

Quercetin Administration Balanced the Protein Expression of BDNF, P-TrkB/TrkB, Copine 6, Synapsin-1, TREM1, and TREM2 in the Hippocampus and PFC of LPS-Challenged Rats

Figure 8 shows the key protein expression in the hippocampus and the PFC of rats in all the groups. As compared with that in the control group, the protein expression of BDNF, p-TrkB/TrkB, Copine 6, and TREM1 were decreased in both the hippocampus and the PFC of LPS-challenged rats, while the expression of TREM2 were increased, and the protein expression of synapsin-1 were decreased in the hippocampus without significant changes in the PFC. These changes could be balanced by treatment of quercetin.

Apart from the negative correlation between the protein expression of TREM1 and TREM2 (Hippocampus: $r = -0.635$,

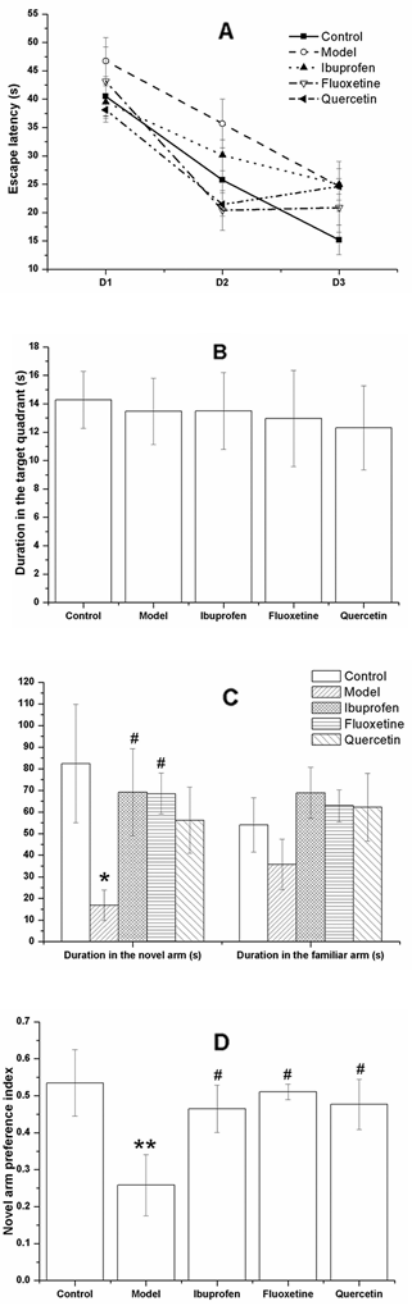

FIGURE 6 | Effect of quercetin on the performance of lipopolysaccharide (LPS)-challenged rats in the Morris water maze (MWM) and Y-maze. The data are presented as the mean ± SEM, with nine rats in each group. The escape latency of all the rats was declined with each day during the acquisition phase **(A)**. Results of repeated measures showed that the training days, but not the experimental treatment had a significant effect on the escape latency. In the probe trail, there was no significant difference among groups of the duration in the target quadrant **(B)**. In the Y-maze, the duration or rats in the novel arm was decreased in the model group but significantly increase in the ibuprofen- and fluoxetine- treated group **(C)**. Treatment with quercetin could reverse the decreased novel arm preference index of LPS-challenged rats **(D)**. $^{*}P < 0.05$ and $^{**}P < 0.001$, compared with the control group; $^{\#}P < 0.05$, compared with the model group.

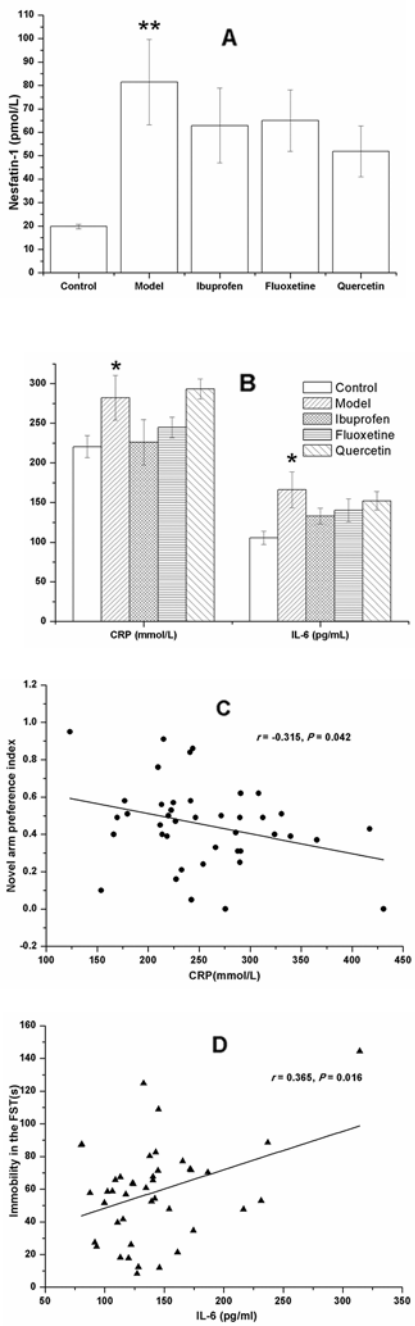

FIGURE 7 | Effect of quercetin on the plasma concentrations of nesfatin-1, C-reactive protein (CRP), and IL-6 in lipopolysaccharide (LPS)-challenged rats. The data are presented as the mean ± SEM, with nine rats in each group. Compared with that of the control rats, the plasma concentrations of nesfatin-1, CRP, and IL-6 were all remarkably increased in the model group. There was no significant difference between the model group and the three groups with treatment of ibuprofen, fluoxetine, or quercetin **(A**, **B)**. Results of the Pearson's correlation test showed that the plasma CRP concentration was negatively correlated to the novel arm preference index **(C)**, and the plasma IL-6 concentration was positively correlated to the immobility time in the forced swimming test (FST) **(D)**. $^{*}P < 0.05$ and $^{**}P < 0.001$ compared with the control group.

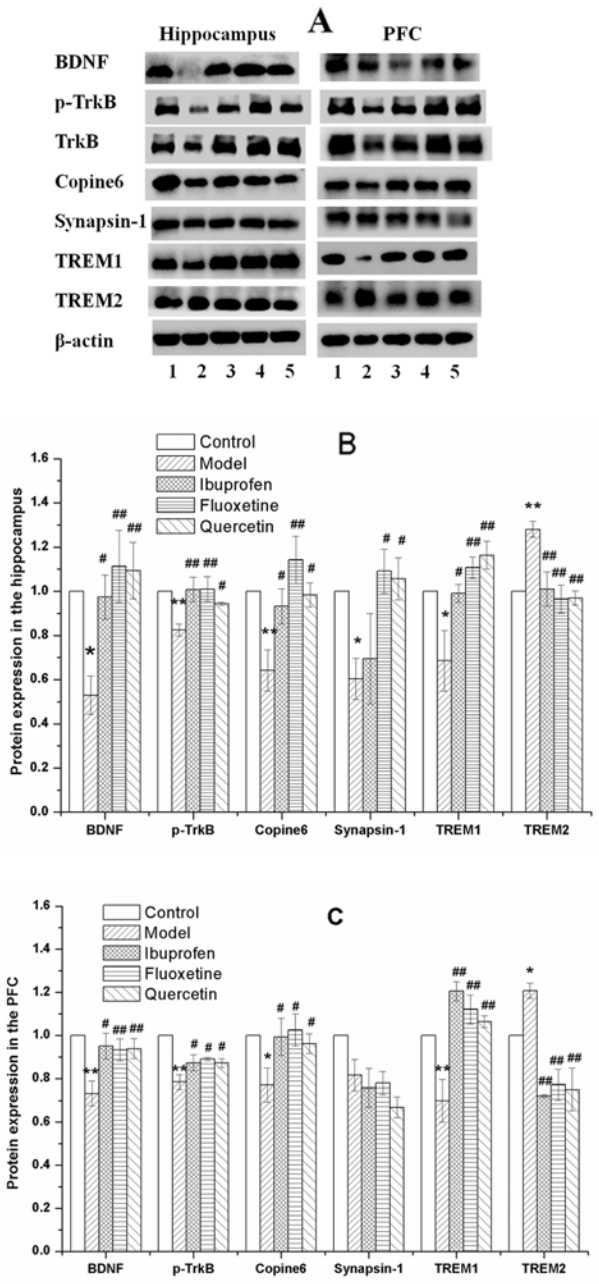

FIGURE 8 | Effect of quercetin on the protein expression of brain derived neurotrophic factor (BDNF), Copine 6, TREM1, TREM2, and Synapsin-1 in the hippocampus and prefrontal cortex (PFC) of lipopolysaccharide (LPS)-challenged rats **(A)** shows a typical graph, and **(B)** and **(C)** show the statistical analysis of western blotting results. The data in **(B)** and **(C)** are presented as the mean ± SEM, with n = 3 for each group. The protein expression of BDNF, Copine 6, and TREM1 were decreased in both the hippocampus and the PFC of LPS-challenged rats, while the expression of TREM2 were increased, and the protein expression of synapsin-1 were decreased in the hippocampus without significant changes in the PFC. These changes could be balanced by treatment of ibuprofen, fluoxetine, and quercetin. $^{*}P < 0.05$ and $^{**}P < 0.001$, compared with the control group; $^{\#}P < 0.05$ and $^{\#\#}P < 0.001$, compared with the NAFLD model group. 1 control; 2 Model; 3 Ibuprofen; 4 Fluoxetine; 5 Quercetin.

$P = 0.011$, **Figure 9A**; PFC: $r = -0.758$, $P = 0.001$, **Figure 9B**), results of the Pearson's correlation test showed that the protein expression of BDNF was positively correlated to that of Copine 6 (Hippocampus: $r = 0.923$, $P < 0.001$, **Figure 9C**; PFC: $r = 0.603$, $P = 0.017$, **Figure 9D**) and TREM1 (Hippocampus: $r = 0.592$, $P = 0.020$, **Figure 9E**; PFC: $r = 0.547$, $P = 0.035$, **Figure 9F**), while negatively correlated to that of TREM2 (Hippocampus: $r = -0.516$, $P = 0.049$, **Figure 9G**; PFC: $r = -0.547$, $P = 0.035$, **Figure 9H**). A positive correlation was found between the expression of BDNF to that of synapsin-1 in the hippocampus ($r = 0.613$, $P = 0.015$), but not in the PFC ($r = -0.060$, $P = 0.832$).

DISCUSSION

In the present study, our results showed that quercetin could alleviate the depression-like behaviors induced by intraperitoneal injection of LPS in rats, as indicated by the increased SPI in the SPT and decreased immobility time in the FST. Moreover, the mechanism of its antidepressant-like effects might be associated with its ability in regulating the BDNF-related imbalance of Copine 6 and other synaptic plasticity-related protein expression in the hippocampus and PFC. Additionally, although administration of quercetin could not improve the performance of LPS-challenged rats in the MWM task, it increased the novel arm preference index in the Y-maze.

Inflammation is an important pathogenic factor involved with the development of depression. LPS is a common agent used as inflammatory stimuli. In spite of the different experimental protocol with different dose, frequency or administrating route, it has been reported that LPS-challenged rodents present depression-like behavior (Szot et al., 2017) (Wickens et al., 2017; Yamawaki et al., 2018) with a sex-specific Effect (Millett et al., 2019). In line with these findings, in our present study, LPS-challenged rats showed less bodyweight gain, less exploratory behavior, anhedonia, and despair behaviors. Using directional heading error as the evaluation index, Kupferschmid and his colleagues reported a selective, age-dependent spatial learning impairment in LPS-administered rats (Kupferschmid and Therrien, 2017). In the present study, although there was no significant difference among groups in the MWM tasks, the LPS-challenged rats showed a decline of novel arm preference index in the Y-maze. Additionally, results of the Pearson's correlation test showed a close relationship among the different behavioral indicators. These results indicated again that LPS could be widely used to investigate the relationship between inflammation challenge and the impairment of neuropsychiatric behaviors including anxiety, depression, and memory deficit. As a common used NASID, the antidepressant effect of ibuprofen has been demonstrated in several animal models and human studies (Saleh et al., 2014; Kohler et al., 2015; Mesripour et al., 2019; Seo et al., 2019). Consistently, our results showed that treatment with ibuprofen could increase the rearing frequency and SPI of LPS-challenged rats, together with the decrease of immobility time in the FST. The results suggested again the close relationship between inflammation and depression.

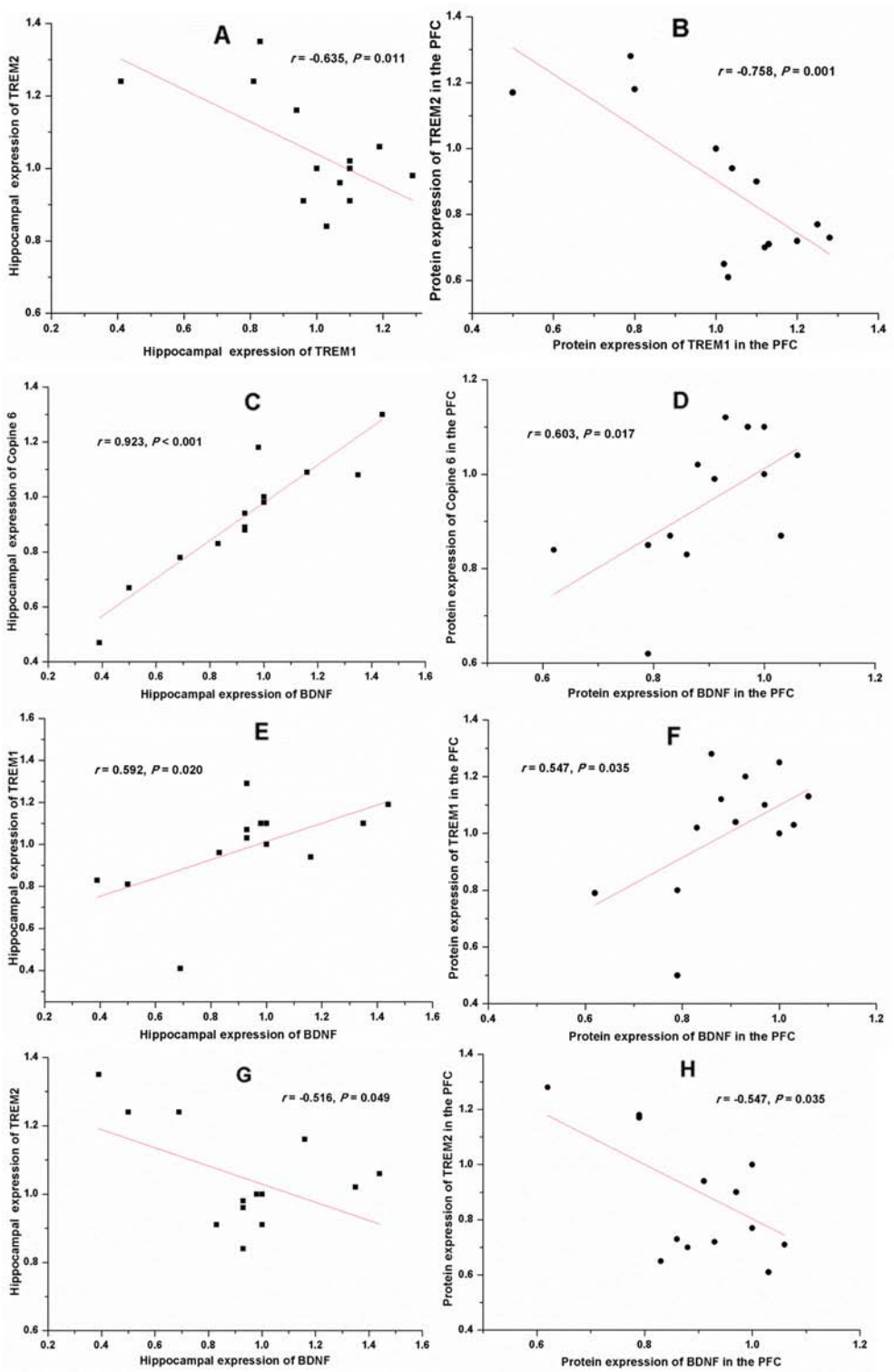

FIGURE 9 | Correlation analysis of the protein expression of brain derived neurotrophic factor (BDNF), Copine 6, TREM1, and TREM2 in the hippocampus and the prefrontal cortex (PFC). A negative correlation was found between the protein expression of TREM1 and TREM2 in both the hippocampus **(A)** and the PFC **(B)**. The protein expression of BDNF was positively correlated to that of Copine 6 **(C**, **D)** and TREM1 **(E**, **F),** while negatively correlated to that of TREM2 **(G**, **H).**

As an established antidepressant, fluoxetine was selected as a positive control in the present study. Consistent with its clinical application, fluoxetine has also been demonstrated to prevent LPS-induced increase in the immobility time in the FST and tail suspension tests (Taniguti et al., 2019). Unsurprisingly, in the present study, rats in fluoxetine-administered group showed improvement in both bodyweight gain and depression-like behaviors. Similar with the results of fluoxetine, administration of quercetin could also improve the bodyweight gain, increase the SPI in the SPT, and decrease the immobility time in the FST, although it had no effect on the performance in the OFT. Together with the findings that quercetin could reduce the immobility time of mice in the FST and tail suspend test in our preliminary study (attached in the **Supplementary Materials**) and relieve the depression-like behavior of stressed mice (Samad et al., 2018), our results suggested again the antidepressant effect of quercetin. Moreover, our results showed a task-related improvement effect of quercetin on the learning and memory.

Raised inflammatory markers are reported in depressed patients and taken as an important mediating factor for behavior, neural plasticity and brain structure (Valkanova et al., 2013; Lotrich, 2015). There is increasing evidence that inflammatory cytokines can induce or worsen depressive symptoms (Horowitz and Zunszain, 2015). Consistently, it has been reported in animal studies that LPS challenge could induce an increase of pro-inflammatory cytokine response (Ge et al., 2015b; Szot et al., 2017). In line with these findings, our results proved that plasma concentrations of CRP and IL-6 were remarkably increased in LPS-challenged rats. Moreover, our results showed a close relationship between the plasma inflammatory indicators and the behavioral performance. Furthermore, in line with the report that LPS could induce an elevation of plasma nesfatin-1 concentration in rats (Stengel et al., 2011), the plasma concentration of nesfatin-1 of LPS-challenged rats was also increased. These results suggested again the relationship between inflammation and depression. However, administration of quercetin could not reverse the increased plasma concentrations of nesfatin-1, CRP, and IL-6. Although we did not detect their alternation in the brain, our results indicated the hypothesis that there would be other mechanisms underlying the antidepressant-like effect of quercetin.

BDNF is well recognized for its neuroprotective functions, *via* binding and activating its high affinity receptor TrkB. The alternation of BDNF/TrkB signaling pathway has been demonstrated to play a key role in the pathophysiology of depression and in the therapeutic mechanisms of antidepressant (Zhang et al., 2016). In the present study, the protein expressions of BDNF, p-TrkB/TrkB, Copine 6, and synapsin-1were all reduced in the hippocampus and PFC of LPS-challenged rats, and a positive relationship was found between the protein expression of BDNF and Copine 6 or synapsin-1. Taking together with their important role in linking neuropsychiatric behaviors to synaptic plasticity (Jangra et al., 2014; Reinhard et al., 2016), these results indicated that BDNF-related imbalance expression of Copine-6 and synapse plasticity-associated proteins play a vital role in the depression-like behavior induced by LPS challenge. However, treatment with fluoxetine, ibuprofen, or quercetin could reverse these alternations. Therefore, it is plausible to infer that, apart from the reported mechanisms including modulating cytokines production and inhibiting oxidative stress (Sah et al., 2011), the antidepressant-like effect of quercetin might be associated with its ability in regulating the BDNF-related imbalance of key proteins expression involved in neuroinflammation and neuroplasticity.

The TREM family proteins are cell surface receptors with important roles in regulation of myeloid cell inflammatory activity, and different TREM receptors appear to have contrasting roles in controlling myeloid immune activity (Owens et al., 2017). Therefore the relative and coordinated regulation of their expression is important to understand, though the inconsistent reports made it more complicated. Although TREM-1 are demonstrated to amplify inflammation in sepsis (Bouchon et al., 2001), soluble TREM-1 has been taken as an antiinflammatory mediator in sepsis (Giamarellos-Bourboulis et al., 2006; Gibot and Massin, 2006). It has been reported that TREM2 can promote microglial cell survival by activating the Wnt/β-catenin signaling pathway (Zheng et al., 2017), and accelerate the removal of Aβ to reduce oxidative stress damage in hippocampal neurons (Fan et al., 2019). Upregulating the expression of TREM-2 could inhibit the apoptosis of hippocampal neurons (Liu et al., 2019), ameliorate neuropathology, and rescue spatial cognitive impairment (Jiang et al., 2014). Loss of TREM2 function increases amyloid seeding but reduces plaque-associated ApoE (Parhizkar et al., 2019), and aggravates spatial learning impairment in P301S transgenic mice (Jiang et al., 2015). However, it has also been reported that the ameliorative effect of TREM2 overexpression in microglia on the behaviors and neuropathological injuries including Aβ aggregation, neuroinflammation, and loss of neurons and synapses (Jiang et al., 2014). Similarly, Inconsistent or contrary effects of TREM2 on the microglial injury response and tau pathology were also reported (Bemiller et al., 2017) (Leyns et al., 2017), and even the effect would be varied by partial or complete loss of TREM2 function (Sayed et al., 2018). Additionally, different expression of TREM2 has also been found in the hippocampus between rats aging 2 months and 6 months in our previous study. In the present study, the LPS-challenged rats presented not only depression-like behaviors and impairment of learning and memory, but also the decreased expression of TREM1 and increased expression of TREM2 in the hippocampus and PFC. Despite the small sample size, our results suggested a negative relationship between the protein expression of TREM1 and that of TREM2. Moreover, these alternated expressions of TREM1 and TREM2 could be reversed by treatment of quercetin. These results suggested again the important role of TREMs in regulating the neurological behaviors and the potential effect of quercetin, although more details should be investigated thoroughly in the future.

There are several limitations in our study. Firstly, the multiple behavioral tasks were carried out in the same cohort of rats. Although the confounding factors including time, place, order

have been taken into account and controlled, we cannot exclude absolutely the interactions among the behavioral tasks. Secondly, due to the limited data of continuous variables, the Pearson correlation test were carried out based on all the data together. Although it could increase the sample size and enlarge the variation of the observation indicators, making it more convenient and reliable to observe the relationship between the changes of the indicators. However, we cannot exclude the limitation by the influence of different treatments. More detailed investigation should be taken in our further study.

Taken together, our study revealed that quercetin could alleviate LPS-induced depression like behaviors and impairment of learning and memory in rats, the mechanism of which might be partly associated with its ability in regulating the BDNF-related imbalance expression of Copine 6 and TREM1/2 in the hippocampus and PFC.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The experimental procedures were approved by the animal Care and Use committee at Anhui Medical University and complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publication No. 85-23, revised 1985).

AUTHOR CONTRIBUTIONS

J-FG designed the study and wrote the protocol. H-RL wrote the first draft of the manuscript. KF and X-XC managed the literature searches and the statistical analyses. KF, L-LH, CJ, A-QD, and HL performed animal model experiments. X-XC and X-RG performed the gene expression experiments and wrote parts of the manuscript. All authors contributed to and have approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2019.01544/full#supplementary-material

REFERENCES

Bemiller, S. M., McCray, T. J., Allan, K., Formica, S. V., Xu, G., Wilson, G., et al. (2017). TREM2 deficiency exacerbates tau pathology through dysregulated kinase signaling in a mouse model of tauopathy. *Mol. Neurodegener.* 12, 74. doi: 10.1186/s13024-017-0216-6

Bouchon, A., Facchetti, F., Weigand, M. A., and Colonna, M. (2001). TREM-1 amplifies inflammation and is a crucial mediator of septic shock. *Nature* 410, 1103–1107. doi: 10.1038/35074114

Brody, D. J., Pratt, L. A., and Hughes, J. P. (2018). *Prevalence of Depression Among Adults Aged 20 and Over: United States, 2013-2016* (1-8: NCHS data brief).

Burk, K., Ramachandran, B., Ahmed, S., Hurtado-Zavala, J. I., Awasthi, A., Benito, E., et al. (2018). Regulation of Dendritic Spine Morphology in Hippocampal Neurons by Copine-6. *Cereb. Cortex* 28, 1087–1104. doi: 10.1093/cercor/bhx009

Dhir, A. (2017). Investigational drugs for treating major depressive disorder. *Expert Opin. Invest. Drugs* 26, 9–24 doi: 10.1080/13543784.2017.1267727

Erickson, K. I., Miller, D. L., and Roecklein, K. A. (2012). The aging hippocampus: interactions between exercise, depression, and BDNF. *Neuroscientist* 18, 82–97. doi: 10.1177/1073858410397054

Fan, Y., Ma, Y., Huang, W., Cheng, X., Gao, N., Li, G., et al. (2019). Up-regulation of TREM2 accelerates the reduction of amyloid deposits and promotes neuronal regeneration in the hippocampus of amyloid beta1-42 injected mice. *J. Chem. Neuroanat.* 97, 71–79. doi: 10.1016/j.jchemneu.2019.02.002

Felger, J. C., and Lotrich, F. E. (2013). Inflammatory cytokines in depression: neurobiological mechanisms and therapeutic implications. *Neuroscience* 246, 199–229. doi: 10.1016/j.neuroscience.2013.04.060

Gawali, N. B., Bulani, V. D., Chowdhury, A. A., Deshpande, P. S., Nagmoti, D. M., and Juvekar, A. R. (2016). Agmatine ameliorates lipopolysaccharide induced depressive-like behaviour in mice by targeting the underlying inflammatory and oxido-nitrosative mediators. *Pharmacol. Biochem. Behav.* 149, 1–8. doi: 10.1016/j.pbb.2016.07.004

Ge, J. F., Xu, Y. Y., Qin, G., Peng, Y. N., Zhang, C. F., Liu, X. R., et al. (2015a). Depression-like behavior induced by Nesfatin-1 in rats: involvement of increased immune activation and imbalance of synaptic vesicle proteins. *Front. Neurosci.* 9, 429. doi: 10.3389/fnins.2015.00429

Ge, L., Liu, L., Liu, H., Liu, S., Xue, H., Wang, X., et al. (2015b). Resveratrol abrogates lipopolysaccharide-induced depressive-like behavior, neuroinflammatory response, and CREB/BDNF signaling in mice. *Eur. J. Pharmacol.* 768, 49–57. doi: 10.1016/j.ejphar.2015.10.026

Giamarellos-Bourboulis, E. J., Zakynthinos, S., Baziaka, F., Papadomichelakis, E., Virtzili, S., Koutoukas, P., et al. (2006). Soluble triggering receptor expressed on myeloid cells 1 as an antiinflammatory mediator in sepsis. *Intensive Care Med.* 32, 237–243. doi: 10.1007/s00134-005-0017-1

Gibot, S., and Massin, F. (2006). Soluble form of the triggering receptor expressed on myeloid cells 1: an antiinflammatory mediator? *Intensive Care Med.* 32, 185–187. doi: 10.1007/s00134-005-0018-0

Han, Y. X., Tao, C., Gao, X. R., Wang, L. L., Jiang, F. H., Wang, C., et al. (2018). BDNF-related imbalance of copine 6 and synaptic plasticity markers couples with depression-like behavior and immune activation in CUMS rats. *Front. Neurosci.* 12, 731. doi: 10.3389/fnins.2018.00731

Horowitz, M. A., and Zunszain, P. A. (2015). Neuroimmune and neuroendocrine abnormalities in depression: two sides of the same coin. *Ann. N.Y.Acad. Sci.* 1351, 68–79. doi: 10.1111/nyas.12781

Jangra, A., Lukhi, M. M., Sulakhiya, K., Baruah, C. C., and Lahkar, M. (2014). Protective effect of mangiferin against lipopolysaccharide-induced depressive and anxiety-like behaviour in mice. *Eur. J. Pharmacol.* 740, 337–345. doi: 10.1016/j.ejphar.2014.07.031

Jay, T. R., Hirsch, A. M., Broihier, M. L., Miller, C. M., Neilson, L. E., Ransohoff, R. M., et al. (2017). Disease progression-dependent effects of TREM2 deficiency

in a mouse model of Alzheimer's disease. *J. Neurosci. Off. J. Soc Neurosci.* 37, 637–647. doi: 10.1523/JNEUROSCI.2110-16.2016

Jeon, S. W., and Kim, Y. K. (2017). Inflammation-induced depression: its pathophysiology and therapeutic implications. *J. Neuroimmunol.* 313, 92–98. doi: 10.1016/j.jneuroim.2017.10.016

Jiang, T., Tan, L., Zhu, X. C., Zhang, Q. Q., Cao, L., Tan, M. S., et al. (2014). Upregulation of TREM2 ameliorates neuropathology and rescues spatial cognitive impairment in a transgenic mouse model of Alzheimer's disease. *Neuropsychopharmacol. Off. Publ. Am. Coll. Neuropsychopharmacol.* 39, 2949–2962. doi: 10.1038/npp.2014.164

Jiang, T., Tan, L., Zhu, X. C., Zhou, J. S., Cao, L., Tan, M. S., et al. (2015). Silencing of TREM2 exacerbates tau pathology, neurodegenerative changes, and spatial learning deficits in P301S tau transgenic mice. *Neurobiol. Aging* 36, 3176–3186. doi: 10.1016/j.neurobiolaging.2015.08.019

Jiang, T., Zhang, Y. D., Gao, Q., Zhou, J. S., Zhu, X. C., Lu, H., et al. (2016). TREM1 facilitates microglial phagocytosis of amyloid beta. *Acta Neuropathol.* 132, 667–683. doi: 10.1007/s00401-016-1622-5

Jiang, T., Wan, Y., Zhang, Y. D., Zhou, J. S., Gao, Q., Zhu, X. C., et al. (2017). TREM2 overexpression has no improvement on neuropathology and cognitive impairment in aging APPswe/PS1dE9 mice. *Mol. Neurobiol.* 54, 855–865. doi: 10.1007/s12035-016-9704-x

Khandaker, G. M., Pearson, R. M., Zammit, S., Lewis, G., and Jones, P. B. (2014). Association of serum interleukin 6 and C-reactive protein in childhood with depression and psychosis in young adult life: a population-based longitudinal study. *JAMA Psychiatry* 71, 1121–1128. doi: 10.1001/jamapsychiatry.2014.1332

Kohler, O., Petersen, L., Mors, O., and Gasse, C. (2015). Inflammation and depression: combined use of selective serotonin reuptake inhibitors and NSAIDs or paracetamol and psychiatric outcomes. *Brain Behav.* 5, e00338. doi: 10.1002/brb3.338

Konishi, H., and Kiyama, H. (2018). Microglial TREM2/DAP12 Signaling: A Double-Edged Sword in Neural Diseases. *Front. Cell. Neurosci.* 12, 206. doi: 10.3389/fncel.2018.00206

Kupferschmid, B. J., and Therrien, B. A. (2017). Spatial learning responses to Lipopolysaccharide in adult and aged rats. *Biol. Res. Nurs.* 20, 32–39. doi: 10.1177/1099800417726875

Leyns, C. E. G., Ulrich, J. D., Finn, M. B., Stewart, F. R., Koscal, L. J., Remolina Serrano, J., et al. (2017). TREM2 deficiency attenuates neuroinflammation and protects against neurodegeneration in a mouse model of tauopathy. *Proc. Natl. Acad. Sci. U. S. A.* 114, 11524–11529. doi: 10.1073/pnas.1710311114

Liu, P., Khvotchev, M., Li, Y. C., Chanaday, N. L., and Kavalali, E. T. (2018). Copine-6 binds to SNAREs and selectively suppresses spontaneous neurotransmission. *J. Neurosci. Off. J. Soc Neurosci.* 38, 5888–5899. doi: 10.1523/JNEUROSCI.0461-18.2018

Liu, A. H., Chu, M., and Wang, Y. P. (2019). Up-regulation of Trem2 inhibits hippocampal neuronal apoptosis and alleviates oxidative stress in epilepsy *via* the PI3K/Akt pathway in mice. *Neurosci. Bull.* 35, 471–485. doi: 10.1007/s12264-018-0324-5

Lotrich, F. E. (2015). Inflammatory cytokine-associated depression. *Brain Res.* 1617, 113–125. doi: 10.1016/j.brainres.2014.06.032

Merali, Z., Brennan, K., Brau, P., and Anisman, H. (2003). Dissociating anorexia and anhedonia elicited by interleukin-1beta: antidepressant and gender effects on responding for "free chow" and "earned" sucrose intake. *Psychopharmacology* 165, 413–418. doi: 10.1007/s00213-002-1273-1

Mesripour, A., Shahnooshi, S., and Hajhashemi, V. (2019). Celecoxib, ibuprofen, and indomethacin alleviate depression-like behavior induced by interferon-alfa in mice. *J. Complement. Integr. Med.* doi: 10.1515/jcim-2019-0016

Millett, C. E., Phillips, B. E., and Saunders, E. F. H. (2019). The Sex-specific Effects of LPS on Depressive-like Behavior and Oxidative Stress in the Hippocampus of the Mouse. *Neuroscience* 399, 77–88. doi: 10.1016/j.neuroscience.2018.12.008

Owens, R., Grabert, K., Davies, C. L., Alfieri, A., Antel, J. P., Healy, L. M., et al. (2017). Divergent Neuroinflammatory Regulation of Microglial TREM Expression and Involvement of NF-kappaB. *Front. Cell. Neurosci.* 11, 56. doi: 10.3389/fncel.2017.00056

Parhizkar, S., Arzberger, T., Brendel, M., Kleinberger, G., Deussing, M., Focke, C., et al. (2019). Loss of TREM2 function increases amyloid seeding but reduces plaque-associated ApoE. *Nat. Neurosci.* 22, 191–204. doi: 10.1038/s41593-018-0296-9

Reinhard, J. R., Kriz, A., Galic, M., Angliker, N., Rajalu, M., Vogt, K. E., et al. (2016). The calcium sensor Copine-6 regulates spine structural plasticity and learning and memory. *Nat. Commun.* 7, 11613. doi: 10.1038/ncomms11613

Sah, S. P., Tirkey, N., Kuhad, A., and Chopra, K. (2011). Effect of quercetin on lipopolysaccharide induced-sickness behavior and oxidative stress in rats. *Indian J. Pharmacol.* 43, 192–196. doi: 10.4103/0253-7613.77365

Saleh, L. A., Hamza, M., El Gayar, N. H., Abd El-Samad, A. A., Nasr, E. A., and Masoud, S. I. (2014). Ibuprofen suppresses depressive like behavior induced by BCG inoculation in mice: role of nitric oxide and prostaglandin. *Pharmacol. Biochem. Be.* 125, 29–39. doi: 10.1016/j.pbb.2014.07.013

Samad, N., Saleem, A., Yasmin, F., and Shehzad, M. A. (2018). Quercetin protects against stress-induced anxiety- and depression-like behavior and improves memory in male mice. *Physiol. Res.* 67, 795–808. doi: 10.33549/physiolres.933776

Sayed, F. A., Telpoukhovskaia, M., Kodama, L., Li, Y., Zhou, Y., Le, D., et al. (2018). Differential effects of partial and complete loss of TREM2 on microglial injury response and tauopathy. *Proc. Nat. Acad. Sci. U.S. A.* 115, 10172–10177. doi: 10.1073/pnas.1811411115

Seo, M. K., Lee, J. G., and Park, S. W. (2019). Effects of escitalopram and ibuprofen on a depression-like phenotype induced by chronic stress in rats. *Neurosci. Lett.* 696, 168–173. doi: 10.1016/j.neulet.2018.12.033

Singh, I., Morgan, C., Curran, V., Nutt, D., Schlag, A., and McShane, R. (2017). Ketamine treatment for depression: opportunities for clinical innovation and ethical foresight. *Lancet Psychiatry* 4, 419–426. doi: 10.1016/S2215-0366(17)30102-5

Song, T., Wu, H., Li, R., Xu, H., Rao, X., Gao, L., et al. (2019). Repeated fluoxetine treatment induces long-lasting neurotrophic changes in the medial prefrontal cortex of adult rats. *Behav. Brain Res.* 365, 114–124. doi: 10.1016/j.bbr.2019.03.009

Stengel, A., Goebel-Stengel, M., Jawien, J., Kobelt, P., Tache, Y., and Lambrecht, N. W. (2011). Lipopolysaccharide increases gastric and circulating NUCB2/nesfatin-1 concentrations in rats. *Peptides* 32, 1942–1947. doi: 10.1016/j.peptides.2011.07.006

Suarez-Calvet, M., Morenas-Rodriguez, E., Kleinberger, G., Schlepckow, K., Araque Caballero, M. A., Franzmeier, N., et al. (2019). Early increase of CSF sTREM2 in Alzheimer's disease is associated with tau related-neurodegeneration but not with amyloid-beta pathology. *Mol. Neurodegener.* 14, 1. doi: 10.1186/s13024-018-0301-5

Szot, P., Franklin, A., Figlewicz, D. P., Beuca, T. P., Bullock, K., Hansen, K., et al. (2017). Multiple lipopolysaccharide (LPS) injections alter interleukin 6 (IL-6), IL-7, IL-10 and IL-6 and IL-7 receptor mRNA in CNS and spleen. *Neuroscience* 355, 9–21. doi: 10.1016/j.neuroscience.2017.04.028

Taniguti, E. H., Ferreira, Y. S., Stupp, I. J. V., Fraga-Junior, E. B., Doneda, D. L., Lopes, L., et al. (2019). Atorvastatin prevents lipopolysaccharide-induced depressive-like behaviour in mice. *Brain Res. Bull.* 146, 279–286. doi: 10.1016/j.brainresbull.2019.01.018

Valkanova, V., Ebmeier, K. P., and Allan, C. L. (2013). CRP, IL-6 and depression: a systematic review and meta-analysis of longitudinal studies. *J. Affect. Disord.* 150, 736–744. doi: 10.1016/j.jad.2013.06.004

Weibert, E., Hofmann, T., and Stengel, A. (2019). Role of nesfatin-1 in anxiety, depression and the response to stress. *Psychoneuroendocrinology* 100, 58–66. doi: 10.1016/j.psyneuen.2018.09.037

Wickens, R. A., Ver Donck, L., MacKenzie, A. B., and Bailey, S. J. (2017). Repeated daily administration of increasing doses of lipopolysaccharide provides a model of sustained inflammation-induced depressive-like behaviour in mice that is independent of the NLRP3 inflammasome. *Behav. Brain Res.* 352, 99–108. doi: 10.1016/j.bbr.2017.07.041

Wium-Andersen, M. K., Orsted, D. D., Nielsen, S. F., and Nordestgaard, B. G. (2013). Elevated C-reactive protein levels, psychological distress, and depression in 73, 131 individuals. *JAMA Psychiatry* 70, 176–184. doi: 10.1001/2013.jamapsychiatry.102

Xia, Q. R., Liang, J., Cao, Y., Shan, F., Liu, Y., and Xu, Y. Y. (2018). Increased plasma nesfatin-1 levels may be associated with corticosterone, IL-6, and CRP levels in patients with major depressive disorder. *Clin. Chimica Acta Int. J. Clin. Chem.* 480, 107–111. doi: 10.1016/j.cca.2018.02.004

Xu, Y. Y., Ge, J. F., Qin, G., Peng, Y. N., Zhang, C. F., Liu, X. R., et al. (2015). Acute, but not chronic, stress increased the plasma concentration and hypothalamic mRNA expression of NUCB2/nesfatin-1 in rats. *Neuropeptides* 54, 47–53. doi: 10.1016/j.npep.2015.08.003

Yamawaki, Y., Yoshioka, N., Nozaki, K., Ito, H., Oda, K., Harada, K., et al. (2018). Sodium butyrate abolishes lipopolysaccharide-induced depression-like behaviors and hippocampal microglial activation in mice. *Brain Res.* 1680, 13–38. doi: 10.1016/j.brainres.2017.12.004

Zhang, J. C., Yao, W., and Hashimoto, K. (2016). Brain-derived Neurotrophic Factor (BDNF)-TrkB Signaling in Inflammation-related Depression and Potential Therapeutic Targets. *Curr. Neuropharmacol.* 14, 721–731. doi: 10.2174/1570159x14666160119094646

Zheng, H., Jia, L., Liu, C. C., Rong, Z., Zhong, L., Yang, L., et al. (2017). TREM2 Promotes Microglial Survival by Activating Wnt/beta-Catenin Pathway. *J. Neurosci. Off. J. Soc Neurosci.* 37, 1772–1784. doi: 10.1523/JNEUROSCI.2459-16.2017

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Corrigendum: Quercetin Alleviates LPS-Induced Depression-Like Behavior in Rats *via* Regulating BDNF-Related Imbalance of Copine 6 and TREM1/2 in the Hippocampus and PFC

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An author name was incorrectly spelled as "Fang Ke." The correct spelling is "Ke Fang".

The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

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