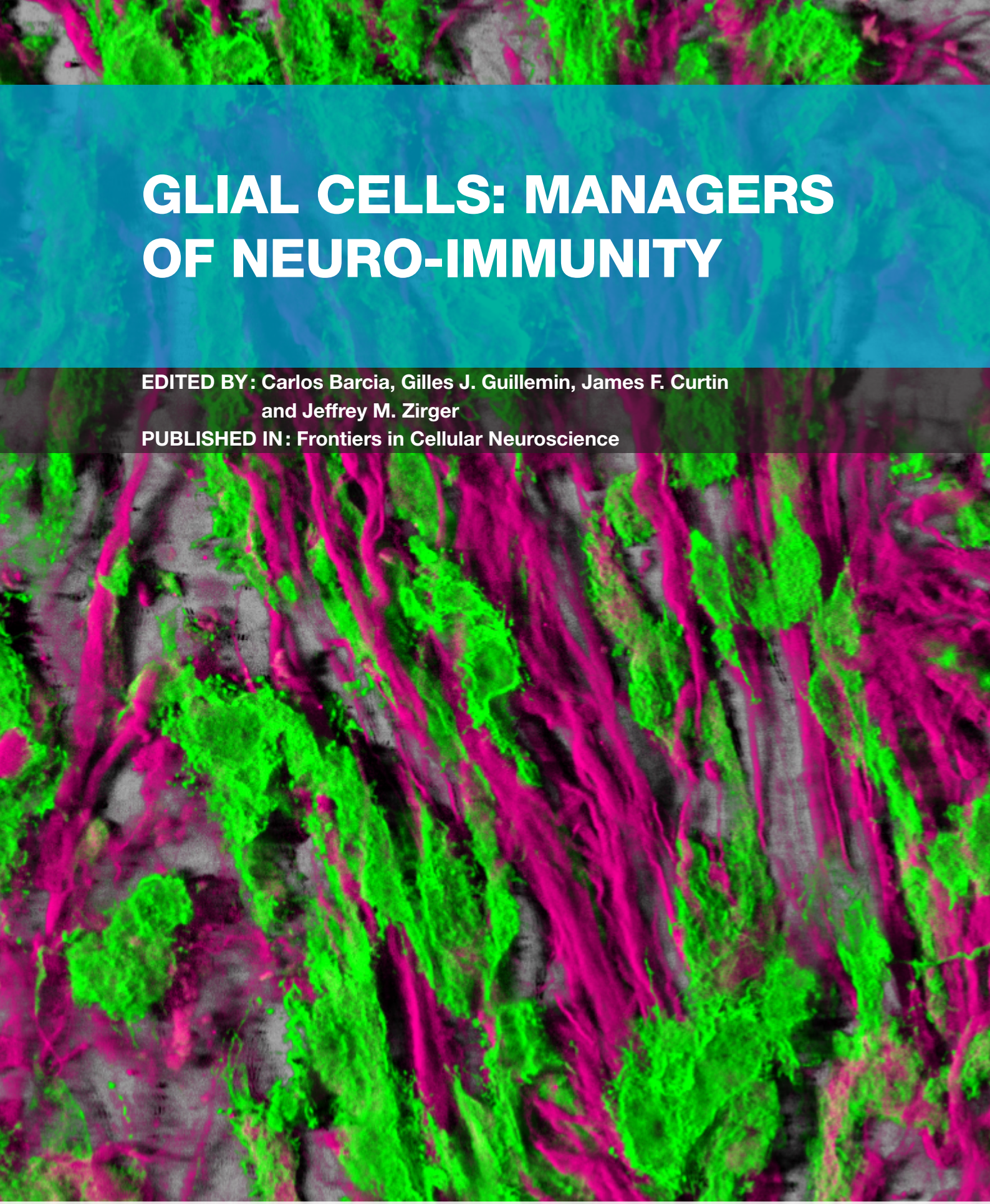

GLIAL CELLS: MANAGERS OF NEURO-IMMUNITY

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GLIAL CELLS: MANAGERS OF NEURO-IMMUNITY

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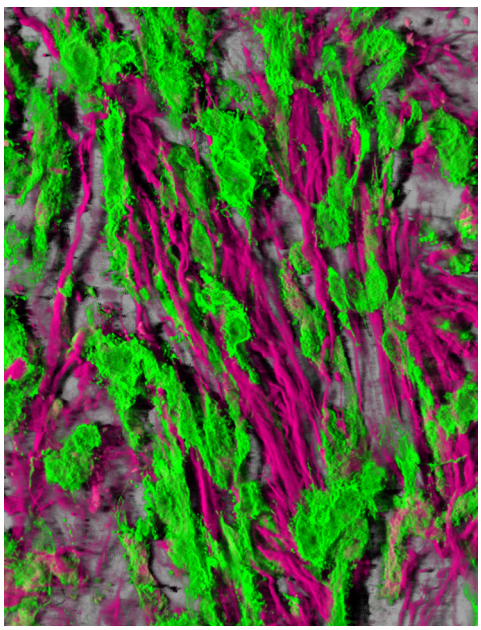

Cover Caption: Three-dimensional rendering of a confocal imaging performed in a biopsy of human glioblastoma. Microglia/macrophages, marked with Iba-1 (green) are populating the tumor tissue, evidenced by elongated Vimentin positive glioma cells (pink). Confocal image scanned with confocal microscope (ZEISS LSM 700) and 3D reconstruction performed with specialized software (Imaris, Bitplane).
Image by: Núria Barba and Carlos Barcia, Institut de Neurociències, Universitat Autònoma de Barcelona, Barcelona, Spain

Immune responses within the brain are still scarcely explored. Nerve tissue damage is accompanied by the activation of glial cells, primarily microglia and astroglia, and such activation is responsible for the release of cytokines and chemokines that maintain the local inflammatory response and actively recruit lymphocytes and monocytes to the damaged areas. Theoretically, these responses are designed to repair the brain damage. However, alterations, or a chronic perpetuation of these responses may underlie a number of neuro-pathologies. It is thought that each inflammatory scenario within the brain have a specific biochemical footprint characterized by the release of

determined cytokines, chemokines and growing factors able to define particular immunological responses. Alongside, glial cells transform their cell body, become larger and develop higher number of branches adopting an active morphological phenotype.

These changes are related with the search of interactions with other cells, such as bystander resident cells of the brain parenchyma, but also cells homing from the blood stream. In this process, microglia and astrocytes communicates with other cells by the formation of specific intercellular connections that are still poorly understood. These interactions are complex and entail the arrangement of cytoskeletal compounds, secretory and phagocytic domains. In this particular crosstalk there is a two-way communication in which glial cells and target cells come together establishing interfaces with specific information exchange. This way, glial cells orchestrate the particular response recruiting cellular subsets within the central nervous system and organizing the resolution of the brain damage.

In this Frontiers Research Topic, we compile a selection of articles unfolding diverse aspects of glial-derived inflammation, focused on neurodegenerative diseases and other nervous system disorders, with special emphasis on microglia/macrophages as leading actors managing neuro-immunity.

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EDITORIAL
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Editorial: Glial Cells: Managers of Neuro-Immunity

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Keywords: glia, neuroinflammation, neuroimmunology, microglia, astroglia, T cells

The Editorial on the Research Topic

Glial Cells: Managers of Neuro-Immunity

After many decades of study in the field of Neuroscience that were mostly centered on the neuron there is a mounting interest in the study of the function of the glial cells in many aspects and functions of the central nervous system. The involvement of glial cells in neuroimmunity is one of the critical pieces within this puzzle, and one that entails great complexity. An increasing number of publications shows that resident astroglia and microglia are the real managers of immune responses, orchestrating chemokine and cytokine release, blood cell infiltration, and promotion of angiogenesis, etc. Moreover, each disease and neuroinflammatory scenario seems to have its own distinct biochemical characteristics and glial phenotype. Classical definitions of resting and activated microglial cells or pro-inflammatory and anti-inflammatory phenotypes are recognized today as oversimplified models of glial cell functions and have since been surpassed by more defined and precise characterizations. The present Frontiers Research Topic (FRT) is a great example of this, since the study of different scenarios reflects diverse modes of glial activation and distinct complexities.

We present here a selection of articles, both original research and reviews, solving clinical, and basic aspects of the biology of glial cells in neuro-inflammatory and neuro-immune scenarios.

A good number of manuscripts in this FRT shows the importance of glial cell-derived inflammation on neurodegenerative diseases. Particularly, Ben Haim and colleagues, from Escartin's lab, show a compelling review on the peculiar, and still poorly understood, roles of astrocytes in neurodegenerative diseases, unfolding the signaling pathways toward reactivity (Ben Haim et al.). Von Bernhardi et al., review the roles of glial cells in neurodegeneration, but focused on Alzheimer's disease and particularly discussing the effects of the cytokine TGFβ (von Bernhardi et al.). Herrera et al. center their attention on another major neurodegenerative disorder, Parkinson's disease, and how stress and glucocorticoids may interact and play important roles in modulating microglial activation (Herrera et al.). Yuste and colleagues, give us an interesting view of the role of nitric oxide in neurodegenerative diseases from a glial-derived inflammatory perspective (Yuste et al.). Vieira et al., review the glial reaction triggered in multiple system atrophy (MSA) focused on the α-synuclein-mediated activation (Vieira et al.).

Other papers are centered on Multiple Sclerosis. Crowley et al., for instance, present an original paper characterizing the roles of Baclofen, a well-known GABA B receptor agonist used clinically, for regulating TLR3 and TLR4 signaling in murine glial cells and peripheral monocytes obtained from Multiple Sclerosis patients (Crowley et al.). Huseby and colleagues review another aspect, focusing their manuscript on the amplification of the neuroinflammatory response due to glial cells-T cell interactions (Huseby et al.). Almolda et al. from González and Castellano's lab,

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also review the topic of glia-lymphocyte crosstalk but compellingly covering other pathological scenarios, suggesting that microglial cells are able to acquire a phenotype of dendritic cells (Almolda et al.).

We also include articles reflecting that inflammatory glial response is involved in mental and psychiatric alterations, which include frontotemporal dementia associated with amyotrophic lateral sclerosis (ALS), reviewed by Radford et al. and the pathogenesis of delirium, reviewed by Sfera et al.

Due to its patent roles in neuro-immunity, microglial cells and brain macrophages are the main protagonists of many of the papers included in this FRT. We would like to highlight the appealing work on the *in vivo* characterization of microglial engulfment of dying neurons presented by Morsch et al., which represents a fine piece of basic science (Morsch et al.). From a clinical point of view, Spanos et al., review the roles of microglia in brain infection, particularly in CNS tuberculosis and how this may affect future therapeutic strategies (Spanos et al.). Following the focus on microglia, Perrotta et al., present an interesting perspective article on the importance of the microglia-glioma cells crosstalk in hormone and immune-derived response in glioma (Perrotta et al.). Particularly important for glioma is the modification of the phenotype, which varies from the classically activated to pro-tumoral phenotype. In this context of phenotype modulation, Kopitar-Jerala reviews the novel role of the cystatin, statin B, in modulating microglial cells toward a pro and anti-inflammatory response (Kopitar-Jerala). In a different scenario, López-Pedrajas et al. report here that cocaine-treated rats show microglial activation in the cerebellum, suggesting that glial reaction may have important implications in motor control during drug addiction (Lopez-Pedrajas et al.).

Importantly, for the field of the long-term maintenance of the neuro-immune response, an interesting original article, Cao et al., reports that prior activation of microglia during embryo development may have consequences in the susceptibility to inflammation in the life of the newborns (Cao et al.). This advocates for the importance of glial cells driving lasting immune responses. Finally, Bas et al., report that infiltrated macrophages participate in the repair of spiral ganglion cells and neurons forming the cochlear nerve, having important implications for successful cochlear implant surgery (Bas et al.).

We believe that this comprehensive collection of articles contains valuable information that will contribute to the knowledge on how glial cells drive the management of neuro-immunity. As the editorial team, we hope you enjoy reading, *Carlos Barcia, Gilles J Guillemin, James F. Curtin, Jeffrey M. Zirger.*

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Elusive roles for reactive astrocytes in neurodegenerative diseases

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Astrocytes play crucial roles in the brain and are involved in the neuroinflammatory response. They become reactive in response to virtually all pathological situations in the brain such as axotomy, ischemia, infection, and neurodegenerative diseases (ND). Astrocyte reactivity was originally characterized by morphological changes (hypertrophy, remodeling of processes) and the overexpression of the intermediate filament glial fibrillary acidic protein (GFAP). However, it is unclear how the normal supportive functions of astrocytes are altered by their reactive state. In ND, in which neuronal dysfunction and astrocyte reactivity take place over several years or decades, the issue is even more complex and highly debated, with several conflicting reports published recently. In this review, we discuss studies addressing the contribution of reactive astrocytes to ND. We describe the molecular triggers leading to astrocyte reactivity during ND, examine how some key astrocyte functions may be enhanced or altered during the disease process, and discuss how astrocyte reactivity may globally affect ND progression. Finally we will consider the anticipated developments in this important field. With this review, we aim to show that the detailed study of reactive astrocytes may open new perspectives for ND.

Keywords: astrocyte reactivity, neuron-astrocyte interactions, neuroinflammation, Alzheimer's disease, Huntington's disease, amyotrophic lateral sclerosis, Parkinson's disease

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Introduction

Astrocytes become reactive in response to virtually all pathological conditions in the central nervous system (CNS), both following acute injuries (stroke, trauma) and during progressive diseases (tumors, epilepsy and ND see **Box 1** and **Table 1**). Astrocyte reactivity is observed in many mammalian and bird species. In non-mammalian species, which have low number of or no parenchymal astrocytes, it is unclear whether *bona fide* astrocyte reactivity exists (Appel, 2013). Yet, in lampreys, newts and frogs, astrocyte-like cells react to injury and form a glial bridge promoting axonal regeneration (Bloom, 2014). In Drosophila, glial cells with some typical astrocyte functions display strong phagocytic activity and morphological changes following neuronal degeneration (Freeman, 2015).

Astrocyte reactivity involves morphological, transcriptional and functional changes that we will try to cover in this review. For the sake of clarity, we will focus primarily on Alzheimer's (AD) and Huntington's diseases (HD), as well as amyotrophic lateral sclerosis (ALS) and Parkinson's disease (PD). In particular, we aim to illustrate that astrocyte reactivity is a shared and central feature in ND that requires further characterization.

BOX 1 | Terminology and definitions

"Neuroinflammation" defines the state of reactivity of astrocytes and microglia induced by pathological conditions. It may be associated with the recruitment of peripheral macrophages and lymphocytes. Reactive astrocytes and microglia mediate the innate immune responses in the brain (Heneka et al., 2014).

In this review, the term "***astrogliosis"*** will not be used because it implies the notion of astrocyte proliferation. In fact, in most injury or disease models, astrocytes do not proliferate (see Section Do Reactive Astrocytes Proliferate in ND?) and thus, reactive astrogliosis is a confounding term.

"***Astrocyte reactivity***" or "***reactive astrocytes"*** refer to astrocytes that respond to any pathological condition in the CNS. Astrocytes are considered reactive when they become hypertrophic and overexpress the intermediate filament GFAP. This "minimal definition" of reactive astrocytes is thus based on the two most universal hallmarks of reactivity, but this does not exclude that additional transcriptional, morphological and functional changes occur in a disease-specific manner, as discussed later in this review. Astrocyte reactivity involves the activation of a transcriptional program triggered by specific signaling cascades (see Section How Do Astrocytes Become Reactive?) that results in long-lasting changes in morphology and function, persisting over several hours, days or even decades. This should be distinguished from **"activated astrocytes,"** which are stimulated by exposure to neurotransmitters, for example. This transient response involves intracellular Ca^{2+} on the millisecond to second time scale, and is sometimes accompanied by subtle morphological changes (Bernardinelli et al., 2014), but not long term increases in GFAP gene expression or morphological hypertrophy.

"***Glial scar***" is a specific form of astrocyte reactivity, which is irreversible and involves major morphological remodeling of reactive astrocytes along the disrupted parenchyma.

"Resting astrocytes" will be used to denote astrocytes that are not reactive. However, it does not mean that astrocytes are inactive; instead, it refers to a non-disease state, or a "homeostically active" state.

TABLE 1 | Reactive astrocytes are found in vulnerable brain regions in animal models and patients with ND.

		When	Where	References	Comments
AD	Patients	Before clinical symptoms. GFAP levels increase with Braak stage	Entorhinal cortex and hippocampus. Gradual progression to temporal, frontal and parietal lobes	Simpson et al., 2010; Carter et al., 2012	
	Murine models	May start before amyloid deposition. Prominent when plaques are formed	Primarily around amyloid plaques. (Brain region depends on the model)	Heneka et al., 2005; Duyckaerts et al., 2008; Olabarria et al., 2010	Astrocytes located far from plaques are atrophied in 3xTg-AD mice
HD	Patients	Already visible at grade 0 in putamen.	Primarily in caudate and putamen. Later in motor cortex, globus pallidus, thalamus, hippocampus	Vonsattel et al., 1985; Faideau et al., 2010	
	Murine models	Late or no reactivity	Striatum	Tong et al., 2014; Ben Haim et al., 2015	Strong reactivity in models with neuronal death (Lenti-Htt82Q or toxins)
ALS	Patients	Before motor symptoms	Ventral and dorsal horns in the spinal cord. Lateral descending corticospinal tracts, subcortical white matter, cortical gray matter in the brain	Maragakis and Rothstein, 2006; Philips and Robberecht, 2011	
	Murine models	Before motor symptoms	Pattern similar to that in patients	Hall et al., 1998; Barbeito et al., 2004; Maragakis and Rothstein, 2006	
PD	Patients	Follows dopaminergic cell death	*Substantia nigra*, correlates with the severity of neuronal loss	Forno et al., 1992; Damier et al., 1993	
	MPTP- monkeys	Follows dopaminergic cell death	*Substantia nigra*	Barcia et al., 2004	
	MPTP-mice 6-OHDA rats	Follows microglial activation, peaks at 4–5 days after intoxication	*Substantia nigra* and striatum	Sheng et al., 1993; Kohutnicka et al., 1998; Hirsch and Hunot, 2009	

It has been very difficult to distinguish the contribution of astrocytes from that of microglia because they usually become reactive in concert and both are involved in neuroinflammation (see definitions in **Box 1**). However, they have quite different functions in the brain in normal conditions; therefore, they may also play different roles during ND. Cell-type specific approaches based on viral vectors or transgenesis offer a unique opportunity to understand the roles of reactive astrocytes (Davila et al., 2013). In this review, we will focus on reactive astrocytes in ND. Excellent reviews recently published on microglia in ND can be found elsewhere (Hanisch and Kettenmann, 2007; Heneka et al., 2014).

Reactive Astrocytes in ND: Definitions and General Considerations

A Brief History

In 1856, Rudolf Virchow first described "neuroglia" as a connective tissue with embedded nerve cells (Virchow, 1856). The development of microscopic and histological techniques by Camilo Golgi, Santiago Ramón y Cajal and Pio del Rio Hortega later revealed the morphology of astrocytes and their extraordinary diversity (Somjen, 1988; Kettenmann and Ransom, 2004). The first description of astrocyte reactivity also dates from the nineteenth century, when Virchow reported that the spinal cord tissue was more fibrillar in neurosyphilis patients than in healthy individuals (Weigert, 1895; Oberheim et al., 2008). The concept of astrocyte reactivity truly emerged with the discovery of the intermediate filament (IF) protein GFAP (Eng et al., 1971) and the development of immunohistological staining for this protein (Eng et al., 2000). Strong GFAP expression in astrocytes became the hallmark of reactivity (Bignami and Dahl, 1976), even though other IF such as vimentin and nestin are also upregulated by reactive astrocytes.

Morphological Changes

Another cardinal feature of astrocyte reactivity is hypertrophy, which was reported by early neuropathologists. Reactive astrocytes display an enlarged cell body and processes (Wilhelmsson et al., 2006). In addition, astrocyte arborization is reorganized with reactivity: the number of primary processes changes (Wilhelmsson et al., 2006) or they polarize toward the site of injury (Bardehle et al., 2013) or toward amyloid plaques in AD (see below).

Less is known about the thin distal processes in astrocytes called perisynaptic processes (PAP), which contact synapses. PAP are dynamic and they influence synaptic transmission in physiological conditions (Oliet et al., 2001; Genoud et al., 2006; Bernardinelli et al., 2014). It is experimentally challenging to monitor morphological changes in PAP that are smaller than the diffraction limit. New microscopy techniques will allow the study of PAP with higher resolution in both resting and reactive astrocytes (Panatier et al., 2014).

Astrocytes occupy separate and non-overlapping spatial domains (Bushong et al., 2002). This organization seems fairly insensitive to reactivity during ND because an increase in domain overlap occurs only after severe insults such as recurring epilepsy, but not in AD models (Oberheim et al., 2008).

Reactive Astrocytes in Commonly Studied ND

AD is the most common form of dementia, characterized by cognitive deficits including learning impairment and memory loss (Querfurth and Laferla, 2010). The brains of AD patients display extracellular amyloid depositions composed of amyloid β (Aβ) peptides and intracellular neurofibrillary tangles formed by hyperphosphorylated Tau protein. AD is characterized by severe neuronal loss; primarily located in the hippocampus and the entorhinal cortex (Querfurth and Laferla, 2010). More than 100 transgenic mouse models of AD are now available (Duyckaerts et al., 2008, see also www.alzforum.org). Most involve the expression of mutated amyloid precursor protein (APP), presenilin 1 (PS1), PS2 and/or Tau, and they replicate some neuropathological features and functional alterations of AD as well as memory deficits (Gotz and Ittner, 2008). Astrocyte reactivity can be detected in the brain of AD patients with imaging and proteomic techniques before the onset of symptoms (Owen et al., 2009; Carter et al., 2012). Similarly, foci of reactive astrocytes are detected at early stages in some mouse models, even before amyloid deposition (Heneka et al., 2005). Reactive astrocytes are usually found around amyloid plaques (Nagele et al., 2003; Wyss-Coray et al., 2003, **Table 1**). However, plaques can also be devoid of reactive astrocytes and patches of reactive astrocytes may be found in the absence of plaques in patients (Simpson et al., 2010). In addition, atrophied astrocytes may be located at a distance from plaques in some mouse models (Olabarria et al., 2010, see **Tables 1**, **2**).

HD is a fatal genetic ND caused by an autosomal dominant mutation, involving the expansion of glutamine (Q) repeats in the protein huntingtin (Htt). HD patients present psychiatric, cognitive and motor symptoms, the most characteristic being progressive chorea (Vonsattel et al., 1985). HD is characterized by the extensive loss of GABAergic neurons in the caudate and putamen (striatum) and by mutant Htt (mHtt) aggregates. Many HD transgenic mouse models exist, which were generated by expressing mHtt under the endogenous Htt promoter (knock-in) or using various transgenic constructs (see **Table 2**). Astrocyte reactivity is an early feature of HD because GFAP immunoreactivity is detected in the striatum of presymptomatic carriers and it increases with disease progression (Faideau et al., 2010). Strikingly, no clear evidence of astrocyte reactivity exists in most HD models (Tong et al., 2014; Ben Haim et al., 2015, **Table 1**). Instead, HD astrocytes show functional alterations (see Section What Do Reactive Astrocytes Do or Fail to Do During ND?) in the absence of the main features of reactivity (hypertrophy and high GFAP expression).

ALS is characterized by the progressive loss of upper motor neurons in the motor cortex and lower motor neurons in the spinal cord and brainstem, resulting in progressive muscle atrophy, weakness and spasticity (Rothstein et al., 1992). Murine models overexpressing mutated forms of superoxide dismutase 1 (mSOD1), identified in familial forms of ALS, develop progressive motor neurodegeneration that mimics the pathogenic features of ALS (Turner and Talbot, 2008, **Table 2**). More recently, new genetic loci associated with familial ALS have been identified, like the *43 kDa transactivation-response DNA-binding protein (TDP-43)*, and new mouse models are being developed (Robberecht and Philips, 2013). Reactive astrocytes are observed in both ALS patients and models (**Table 1**). They appear in vulnerable regions and the degree of reactivity correlates with the level of neurodegeneration (Barbeito et al., 2004).

PD is characterized by the loss of dopaminergic neurons in the substantia nigra (SN), resulting in dopamine deficiency in the striatum and alteration of the basal ganglia circuitry. This causes major motor symptoms, such as akinesia, bradykinesia, tremor, rigidity and postural instability (Agid, 1991), as well as non-motor alterations such as cognitive fluctuations (Witjas

TABLE 2 | Main behavioral, cellular, and molecular features of several mouse models of ND.

Disease modeled	Model	Species	Genetic construct	Promoter	Lifespan	Major symptoms	Histopathological features	Vulnerable brain regions	Original references
AD	APP/PS1dE9	Mouse	Mo/hu APP_{Swe}, Hu PSEN1 deltaE9	MoPrP	Normal	Spatial memory impairment Reduced LTP	Extracellular Aβ depositions No extensive neuronal death	Hippocampus Cortex	Jankowsky et al., 2004
	3xTg-AD	Mouse	$HuAPP_{Swe}$, Mo $PSEN1^{M146V}$, Hu $MAPT^{P301L}$,	MoThy1.2 (for APP and MAPT); endogenous moPSEN1 promoter (for PSEN1)	Normal	Spatial memory impairment Reduced basal synaptic transmission and LTP	Extracellular Aβ depositions Tau pathology in CA1 neurons No extensive neuronal death	Subiculum Hippocampus Entorhinal cortex	Oddo et al., 2003
	Tg2576	Mouse	$HuAPP_{Swe}$	Hamster PrP	Normal	Spatial memory impairment Reduced LTP	Extracellular Aβ depositions No extensive neuronal death	Hippocampus Frontal, entorhinal and occipital cortex	Hsiao et al., 1996
	APPPS1	Mouse	Mo/hu APP_{Swe}; Hu $PSEN1^{L166P}$	Mo Thy1	Normal	Spatial memory impairment Reduced LTP	Extracellular Aβ depositions Local neuronal death in the dentate gyrus Altered dendritic spine morphology	Hippocampus Cortex	Radde et al., 2006
HD	R6/2	Mouse	Exon 1 of huHtt with 150 CAG	1 kb huHtt	10–13 weeks	Altered motor coordination and gait Involuntary movements Seizures Cognitive deficits (selective discrimination learning, contextual fear conditioning) Alteration of MSN electrophysiology	NII Striatal atrophy No extensive neuronal death Alteration of MSN morphology	Striatum Motor cortex	Mangiarini et al., 1997
	Hdh140	Mouse	Knock-in of Mo/hu exon 1 Htt with 140 CAG	Endogenous moHtt promoter	Normal	Altered motor coordination Alteration of MSN electrophysiology	NII Striatal atrophy Low expression of neuronal transcripts	Striatum Motor cortex	Menalled et al., 2003
	zQ175	Mouse	Knock-in of Mo/hu exon 1 Htt with 175 CAG (derived from Hdh140)	Endogenous moHtt promoter	Normal	Altered motor coordination Cognitive deficits (selective discrimination learning)	NII Striatal atrophy Low expression of neuronal transcripts	Striatum Motor cortex	Menalled et al., 2012
	BACHD	Mouse	Full length huHtt with 97 CAG/CAA	HuHtt	Normal	Altered motor coordination Alteration of MSN electrophysiology	Extranuclear aggregates Striatal and cortical atrophy No extensive neuronal death	Striatum Motor cortex	Gray et al., 2008
	Lenti-Htt82Q (lentiviral-based)	Mouse	Lentiviral vector encoding 171 first amino acids of huHtt with 82 Q	PGK	Normal	Behavioral alterations not tested in mice	Cytoplasmic and nuclear inclusions MSN loss	Striatum	De Almeida et al., 2002

(Continued)

TABLE 2 | Continued

Disease modeled	Model	Species	Genetic construct	Promoter	Lifespan	Major symptoms	Histopathological features	Vulnerable brain regions	Original references
ALS	SOD1G93A	Mouse	HuSOD1^{G93A}	HuSOD1	5 months	Weight loss Hindlimb weakness Progressive paralysis	Loss of ventral SC motor neurons Degeneration of ventral root axons SOD1 inclusions in the SC and brain	Spinal cord Motor cortex	Gurney et al., 1994
	SOD1G93A	Rat	HuSOD1^{G93A}	HuSOD1	3–4 months	Abnormal gait Quick hindlimb paralysis Weight loss Muscle atrophy and denervation	Loss of ventral SC motor neurons Degeneration of ventral root axons Vacuolar degeneration SOD1 inclusions in the SC and brain	Spinal cord Brain	Howland et al., 2002
PD	MPTP	Mouse	N/A	N/A	N/A	Altered rearing and coordination	Loss of DA neurons Reduced dopamine levels in striatum	SNpc Striatum	Heikkila et al., 1984
	MPTP	Non-human primate	N/A	N/A	N/A	Bradykinesia, rigidity, tremor	Loss of DA neurons Reduced dopamine levels in caudate-putamen	SNpc Caudate putamen	Burns et al., 1983

Common mouse models of ND were included in the table if they are mentioned at least twice in the text. APPSwe, KM670/671NL (Swedish mutation); DA, dopaminergic. Hu, human; Mo, mouse; MSN, medium-sized spiny neurons; NII, neuronal intranuclear inclusions; PGK, phosphoglycerate kinase; PrP, Prion Protein; Q, glutamine, SNpc, Substantia nigra pars reticulata.

et al., 2002). The first animal models of PD were based on toxins that specifically induce the degeneration of dopaminergic neurons in the SN pars compacta (SNpc) in rodents or primates (e.g., 6-hydroxydopamine [6-OHDA], 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine [MPTP] or rotenone). Transgenic mice harboring genes mutated in familial PD (α-synuclein, leucine-rich repeat kinase 2…) were subsequently developed (Beal, 2010, see **Table 2**). The involvement of microglial cells in PD has been more extensively studied than that of astrocytes. Yet, astrocyte reactivity is detected in the SNpc of patients with PD, individuals intoxicated with MPTP and in animal models (Forno et al., 1992; Hirsch and Hunot, 2009, **Table 1**).

Do Reactive Astrocytes Proliferate in ND?

The original definition of astrocyte reactivity included the notion of proliferation. The idea that reactive astrocytes proliferate is based on the misleading observation that the number of GFAP$^+$ cells increases after injury (Dimou and Gotz, 2014). Most astrocytes in the adult mouse CNS express GFAP at undetectable levels under physiological conditions. Upon injury or disease, reactive astrocytes upregulate GFAP, leading to an increased number of GFAP$^+$ cells. Recent evidence based on BrdU incorporation or Ki67 labeling reveals that astrocyte proliferation is very limited, especially in ND. The exact value depends on the model, age and detection method. For example, reactive astrocytes do not proliferate in the APP/PS1dE9 mouse model of AD (Kamphuis et al., 2012), and represent less than 3% of total proliferating cells in the APPPS1 mouse model of AD (Sirko et al., 2013) and less than 7% in a model of ALS (Lepore et al., 2008a). Proliferating astrocytes account for only 1% of total gray matter astrocytes in APPPS1 mice (Sirko et al., 2013). In the temporal cortex of AD patients, GFAP$^+$ cells were carefully quantified by co-labeling with ubiquitous astrocyte markers (glutamine synthase [GS] or aldehyde dehydrogenase 1 family, member L1). This analysis confirms that the high density of GFAP$^+$ cells is explained by enhanced GFAP expression and cortical atrophy (Serrano-Pozo et al., 2013).

Can Astrocyte Reactivity be Reproduced *In vitro*?

With the development of *in vitro* systems to study astrocytes (McCarthy and De Vellis, 1980), it became possible to study reactive astrocytes in a dish. Human astrocytes can also be grown *in vitro*, either from fetuses or biopsies (Sharif and Prevot, 2012) or generated from induced pluripotent stem cells, including from patients (Krencik and Ullian, 2013). Generally, primary astrocytes are exposed to cytokines such as interleukins (IL), tumor necrosis factor alpha (TNFα) and interferon gamma (IFNγ), which induce many transcriptional and functional changes (Sofroniew, 2009; Sofroniew and Vinters, 2010). Describing them all is beyond the scope of this review. The main limitation to *in vitro* studies is that astrocytes in a dish show signs of reactivity, even in the absence of stimulus. They express high levels of GFAP and usually have a flat, polygonal morphology, very different from the bushy morphology observed *in situ*. This precludes the identification of the hallmarks of astrocyte reactivity. Co-culture with neurons triggers a stellate morphology and low GFAP expression, suggesting that neurons

release factors that maintain astrocytes in a resting state (see Section The Molecular Triggers of Reactivity). More recently, new methods have been developed to reduce astrocyte reactivity *in vitro*, such as exposure to heparin-binding EGF-like growth factor (Foo et al., 2011) or 3D polymer matrix (Puschmann et al., 2013). Given the above-mentioned limitations, we will focus on results obtained in animal models, or even more relevant, in patient brains.

How Do Astrocytes Become Reactive?

The Molecular Triggers of Reactivity

Astrocyte reactivity is triggered by any alteration in brain homeostasis. Astrocytes are equipped with many receptors and intracellular signaling cascades to respond quickly to changes in their environment (Buffo et al., 2010; Burda and Sofroniew, 2014). They express many receptors, including pattern recognition receptors (PRR), that detect abnormal signals in the extracellular space (viral or bacterial molecules, serum proteins, aggregated proteins such as Aβ…), increased concentrations of some molecules (cytokines, chemokines, purines) and even the absence of "normal" signals from neighboring cells (growth factors, neurotransmitters…) (Buffo et al., 2010; Burda and Sofroniew, 2014; Kigerl et al., 2014). Indeed, astrocytes, like microglia, seem to be actively maintained in a resting state. For instance, knocking out fibroblast growth factor (FGF) receptors or β1 integrin (a subunit of the integrin receptor family that binds extracellular matrix molecules) in astrocytes, results in astrocyte reactivity in absence of pathological stimuli (Robel et al., 2009; Kang et al., 2014).

Extracellular molecules inducing astrocyte reactivity have primarily been studied in acute injury models involving scar formation (Burda and Sofroniew, 2014). Such acute lesions involve the breach of the blood-brain-barrier (BBB) and infiltration of immune cells. By contrast, these events occur very progressively in ND, if ever (Zlokovic, 2008). Therefore, although some molecular triggers are shared between acute injuries and ND, molecules such as endothelins or serum proteins are probably not involved in the initiation of astrocyte reactivity in ND.

The exact molecular triggers that occur during the initial stages of ND, before significant neurodegeneration takes place, are unknown. It is probable that glial cells (both astrocytes and microglial cells) can detect even mild neuronal dysfunction (altered neurotransmission, release of stress signals, and abnormally folded proteins). Indeed, astrocytes are very well positioned at the tripartite synapse to detect abnormal synaptic activity and microglial cells permanently monitor the brain parenchyma. Once activated by such signals, glial cells further release active molecules to set up a reactive state. For example, purines, pro-inflammatory cytokines and growth factors may be released by reactive astrocytes and, in even larger amounts by activated microglia (Buffo et al., 2010, see Section Release of Active Molecules).

Importantly, in ND, mutant proteins (e.g., mHtt, mSOD1) may be directly expressed by astrocytes or toxic proteins (Aβ, hyperphosphorylated tau, α-synuclein) can be taken up by astrocytes and activate them (see **Figure 1**). Lentiviral-mediated expression of mHtt specifically in striatal astrocytes increases GFAP expression and induces cellular hypertrophy (Faideau et al., 2010). Similarly, the expression of $SOD1^{G86R}$, α-$synuclein^{A53T}$, or tau (either WT or P301L mutant) in astrocytes, induces their reactivity (Gong et al., 2000; Dabir et al., 2006; Gu et al., 2010). The precise molecular mechanisms linking the accumulation of intracellular toxic proteins in astrocytes to reactivity remain to be characterized (see **Figure 1**). Cytosolic PRR that can detect intracellular "danger associated molecular patterns" have been described in microglial cells, but much less is known about their role in astrocytes (Heneka et al., 2014).

Other molecular triggers of reactivity (e.g., cytokines, growth factors, purines), bind to their cognate receptors at the astrocyte membrane and activate various intracellular signaling cascades. These include the Janus Kinase/Signal Transducer and Activator of Transcription (JAK/STAT) pathway, the Nuclear Factor of Kappa light polypeptide gene enhancer in B-cells (NF-κB) pathway, the calcineurin (CN) pathways and the Mitogen-Activated Protein Kinase (MAPK) pathway (**Figure 1**).

The JAK/STAT3 Pathway

The JAK/STAT3 pathway is a ubiquitous cascade that predominantly mediates cytokine signaling in cells. It regulates the expression of genes involved in many functions including cell growth, proliferation, differentiation and inflammation. Cytokines of the interleukin family (e.g., IL-6, ciliary neurotrophic factor [CNTF] and leukemia inhibitory factor) signal through specific cell-surface receptors possessing the glycoprotein 130 receptor (gp130) subunit. Upon binding, they trigger the assembly of multimeric receptors, leading to the phosphorylation and nuclear translocation of STAT3, and the transcription of its target genes (Levy and Darnell, 2002 and see **Figure 1** for a detailed description of the pathway). Interestingly, the JAK/STAT3 pathway also controls the onset of astrogliogenesis during brain development (He et al., 2005), by promoting the expression of mature astrocyte genes such as *gfap* and *S100*β (Kanski et al., 2014).

It is well established that the JAK/STAT3 pathway mediates astrocyte reactivity and scar formation in models of acute injuries (Okada et al., 2006; Herrmann et al., 2008). Fewer studies have been performed in ND models. Phospho-STAT3 (pSTAT3) is detected in the nucleus of reactive astrocytes (as well as microglia and motor neurons) in the spinal cord of mouse models and patients with ALS (Shibata et al., 2009, 2010). STAT3 accumulates in the nucleus of reactive astrocytes in the hippocampus of transgenic mouse models of AD and in the striatum of murine and primate models of HD (Ben Haim et al., 2015). Activation of the STAT3 pathway seems to be a universal feature of astrocyte reactivity in ND models, shared between disease models, brain regions and animal species (**Figure 2**). Pharmacological inhibition of JAK2 in the MPTP mouse model of PD significantly decreases pSTAT3 and GFAP levels, suggesting that the JAK/STAT3 pathway is required to induce astrocyte reactivity (Sriram et al., 2004). However, this pathway is active in all brain cells; therefore,

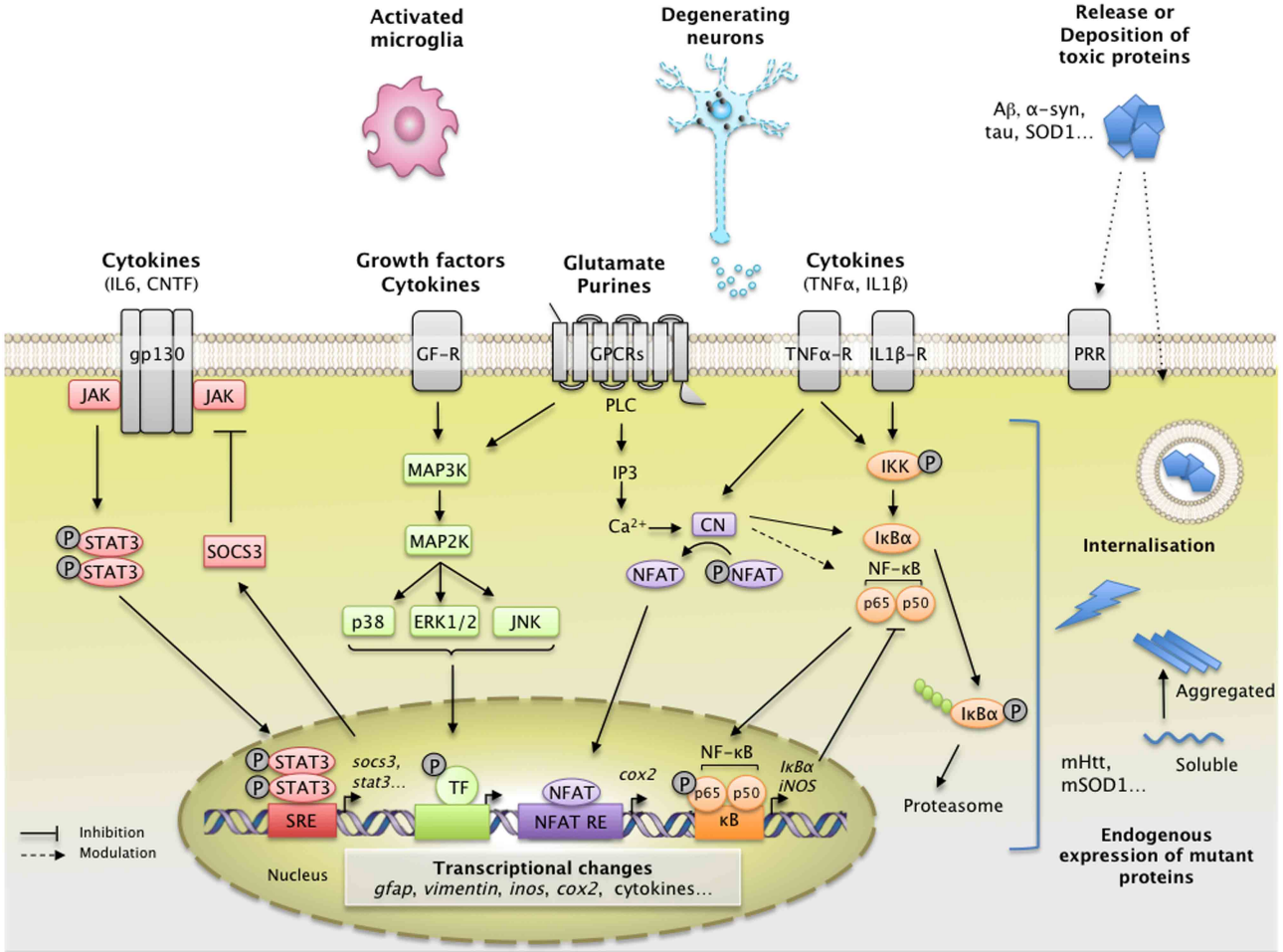

FIGURE 1 | Main extracellular stimuli and intracellular signaling pathways leading to astrocyte reactivity in ND. Dysfunctional neurons, activated microglia, and astrocytes themselves release a wide range of molecules, which bind specific receptors at the astrocyte plasma membrane. These signals activate intracellular pathways such as the JAK/STAT3 pathway (in red), the NF-κB pathway (in orange), the CN/NFAT pathway (in purple), or the MAPK pathway (in green). **The JAK/STAT3 pathway** is activated by interleukins such as IL-6 or CNTF. Upon cytokine binding, the kinase JAK is activated and STAT3 is recruited to the gp-130 receptor. JAK phosphorylates STAT3, which dimerizes and translocates to the nucleus, where it binds consensus sequences (STAT responsive element, SRE) in the promoter region of its target genes. In astrocytes, the JAK/STAT3 pathway regulates the transcription of *gfap, vimentin,* and *stat3* itself. STAT3 also induces the expression of SOCS3, the endogenous inhibitor of the JAK/STAT3 pathway, which mediates an inhibitory feedback loop. **The NF-κB pathway** is activated by pro-inflammatory cytokines such as TNFα and IL-1β. The canonical NF-κB pathway involves the activation of the IKK complex by receptor-bound protein kinases, leading to the phosphorylation of IκBα, the master inhibitor of NF-κB. Upon phosphorylation, IκBα is polyubiquinated and targeted to the proteasome for degradation. The NF-κB subunits p50 and p65 then translocate to the nucleus, where they activate the transcription of various target genes such as inducible nitric oxide synthase and *cox2*. Like the JAK/STAT3 pathway, NF-κB induces the transcription of its own inhibitor, IκBα. **The CN/NFAT pathway** is activated by cytokines such as TNFα or by glutamate. CN is a Ca^{2+}-dependent phosphatase with many regulatory effects on the NF-κB pathway depending on the initial trigger and cellular context. CN also activates NFAT by dephosphorylation. NFAT binds specific promoter sequences and activates the expression of target genes (*cox2*). **The MAPK pathway** is activated by growth factors and cytokines which initiate a phosphorylation cascade. Upon activation, ERK1/2, p38 and c-jun also regulate gene transcription through the activation of a specific set of transcription factors (TF). ND are characterized by intracellular and/or extracellular depositions of pathologic proteins (such as Aβ, Tau, and mHtt, which are shown in blue). In ND, pathological proteins can either be endogenously expressed or internalized by astrocytes. They represent "danger associated molecular patterns" that bind specific pattern recognition receptors (PRR) at the membrane or within astrocytes. These abnormal proteins can interfere with intracellular signaling pathways, activating or inhibiting various signaling proteins (represented as lightning, see Section Additional levels of Complexity and **Figure 3**). The precise molecular mechanisms involved in astrocytes are mostly unknown. These complex signaling cascades strongly affect the astrocyte transcriptome and lead to astrocyte reactivity. Abbreviations: NFAT RE, NFAT responsive element; GF-R, Growth factor receptor; GPCR, G-protein coupled receptor.

non-specific effects of JAK2 inhibitors on other cell types cannot be ruled out. To overcome this limitation, we used lentiviral vectors to overexpress suppressor of cytokine signaling 3 (SOCS3), the endogenous inhibitor of the JAK/STAT3 pathway, selectively in astrocytes of the adult mouse brain. SOCS3 overexpression prevented the nuclear accumulation of STAT3

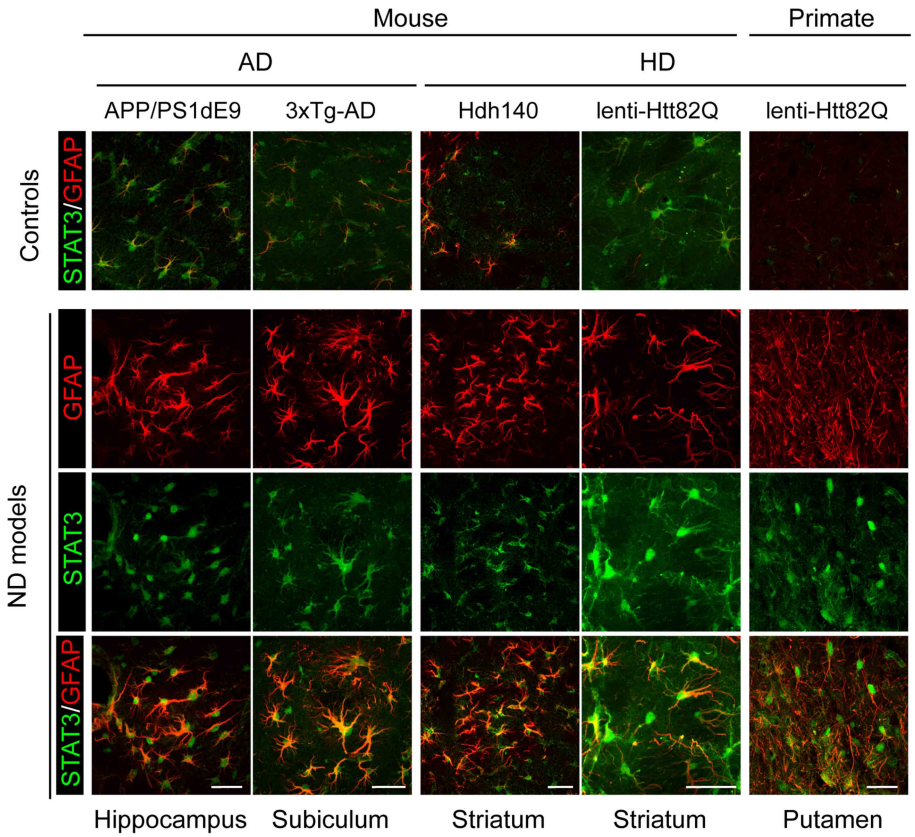

FIGURE 2 | Activation of the JAK/STAT3 pathway is a common feature of astrocyte reactivity in various ND models. Images of brain sections from several models of AD and HD (APP/PS1dE9 mice, 3xTg-AD mice, Hdh140 mice, and the murine and primate lenti-Htt82Q-based models of HD). For all ND models, STAT3 (green) accumulates in the nucleus of reactive astrocytes labeled with GFAP (red) in specific vulnerable regions (as indicated at the bottom). The first line shows the merged STAT3 (green)/GFAP (red) staining in age-matched control animals for each model. Scale bars: 20 μm (mouse) and 40 μm (primate). Adapted from Ben Haim et al. (2015).

and GFAP upregulation in mouse models of ND. Furthermore, SOCS3-expressing astrocytes displayed a resting morphology, showing that the JAK/STAT3 pathway is responsible for astrocyte reactivity in these models (Ben Haim et al., 2015). Interestingly, a recent paper showed that the *Drosophila* ortholog of STAT3 also modulates the reactivity of glial cells following injury (Doherty et al., 2014). Therefore, the JAK/STAT3 pathway is a conserved and central pathway for astrocyte reactivity.

The NF-κB Pathway

The NF-κB pathway is another pathway associated with neuroinflammation. It is involved in many cellular processes including immune responses, inflammation, cell division and apoptosis (Mattson and Meffert, 2006 see **Figure 1** for a detailed description of the pathway). This pathway is activated by several known pro-inflammatory agents (e.g., lipopolysaccharide [LPS], IL-1β, TNFα) (Kaltschmidt et al., 2005). The NF-κB pathway is found activated during ND. Following microinjection of Aβ1-42 oligomers into the rat cortex, NF-κB activation is detected in some $GFAP^+$ astrocytes, along with cyclooxygenase 2 (COX2) and IL-1β, two NF-κB target genes (Carrero et al., 2012). NF-κB accumulates in astrocyte nuclei in the R6/2 model of HD (Hsiao et al., 2013) and in the spinal cord of ALS patients (Migheli et al., 1997). However, experiments involving an NF-κB-GFP reporter construct in ALS mice demonstrate that this pathway is predominantly active in microglial cells in the spinal cord (Frakes et al., 2014). In fact, the NF-κB pathway seems to be active in many cell types other than reactive astrocytes during ND. NF-κB is activated in dopaminergic neurons in the SNpc of PD patients (Hunot et al., 1997), in peripheral immune cells in patients with HD (Trager et al., 2014) and, in hippocampal and entorhinal cortex neurons of AD patients, but not in glial cells (Terai et al., 1996; Kaltschmidt et al., 1997; Ferrer et al., 1998). Overall, this ubiquitous cascade is found activated in various cell types including astrocytes but these observations do not prove that NF-κB is required for astrocyte reactivity. In a mouse model of ALS, Crosio et al. found that inhibiting NF-κB signaling selectively in astrocytes only transiently attenuated their reactivity at the onset of disease (Crosio et al., 2011).

Overall, the NF-κB pathway is activated in ND and plays a key role in microglial activation, but this cascade does not seem essential to initiate astrocyte reactivity. Further studies in other models are needed to understand the role of NF-κB pathway in astrocyte reactivity during ND.

The Phosphatase Calcineurin

The Ca^{2+}/calmodulin-dependent serine/threonine phosphatase CN regulates gene expression by modulating transcription factors such as nuclear factor of activated T-cells (NFATs) and NF-κB (Furman and Norris, 2014). CN is a ubiquitous protein, although it is expressed at high levels in the brain. It regulates growth, differentiation and various cellular processes in T-cells, osteoclasts and myocytes (Hogan et al., 2003 and see **Figure 1** for a detailed description of the pathway).

CN is activated in inflammatory conditions. Several studies have linked the CN/NFAT pathway to astrocyte reactivity, in particular in AD. Indeed, CN immunoreactivity is high in reactive astrocytes in aged mice and around amyloid plaques both in AD patients and mouse models (Furman and Norris, 2014). Activated NFAT1 and 3, two downstream targets of CN, are found in both neurons and astrocytes in AD brains (Abdul et al., 2009).

The effects of CN on astrocyte reactivity are extremely complex and context-dependent because CN can both trigger and prevent reactivity (Furman and Norris, 2014). On the one hand, overexpression of constitutively active CN (caCN) in primary rat hippocampal astrocytes induces morphological and transcriptional changes reminiscent of astrocyte reactivity *in vivo* (Norris et al., 2005). Viral-mediated overexpression of VIVIT, a blocking peptide that inhibits NFAT, attenuates astrocyte reactivity around amyloid depositions in APP/PS1dE9 mice (Furman et al., 2012). But on the other hand, caCN expression in astrocytes reduces GFAP induction following brain injury or LPS injection (Fernandez et al., 2007), and in APP/PS1 mice (Fernandez et al., 2012). This discrepancy between the pro- and anti-reactivity action of CN may be controlled by its signaling partners (Fernandez et al., 2012; Furman and Norris, 2014): its downstream targets (NF-κB vs. NFAT for example) as well as its activators. Indeed, Aβ and IGF-1 both activate CN in cultured astrocytes, but they have opposite effects on the NF-κB pathway (Pons and Torres-Aleman, 2000; Lim et al., 2013).

Overall, CN appears to modulate rather than induce astrocyte reactivity. Whether the effects of CN are conserved in other models and ND remains to be assessed.

The MAPK Pathway

The binding of growth factors (such as FGF, EGF, and TGFα), cytokines or extracellular matrix proteins to their specific cell-surface receptors activates the MAPK pathway (Jeffrey et al., 2007). This is mediated by the activation of small GTP-ase proteins (RAS) and the successive phosphorylation of MAP3K, MAP2K, and MAPK. There are three main phosphorylation cascades, with p38, ERK1/2 or JNK as downstream effectors (see **Figure 1** for a detailed description of the pathway). All result in the activation of different transcription factors by phosphorylation. Cellular stress and extracellular matrix proteins such as integrins activate the c-jun N-terminal kinase (JNK) cascade, whereas cytokines such as IL-1β activate p38 (Jeffrey et al., 2007).

The MAPK pathway is activated in many cell types in ND patients and models. Reactive astrocytes contain active forms of p38, JNK and ERK in mouse models and/or in patients with ALS (Migheli et al., 1997; Tortarolo et al., 2003; Bendotti et al., 2004; Chung et al., 2005). However, in ALS mice, p38 is also activated in motor neurons and microglial cells (Tortarolo et al., 2003). Similarly, p38 and JNK are activated both in neurons and reactive astrocytes in the brain of patients with various tauopathies (Ferrer et al., 2001). In AD patients, phosphorylated forms of p38 are observed in neurons and glial cells around plaques (Hensley et al., 1999) but only in microglial cells in a mouse model (Koistinaho et al., 2002). Several MAPK inhibitors have been tested in an attempt to reduce neuroinflammation in pathological conditions; however, they are thought to act on reactive microglia (Kaminska et al., 2009).

Overall, although the MAPK pathway is activated in many cell types in ND patients and mouse models, to the best of our knowledge, there is no evidence showing that it is directly involved in the initiation of astrocyte reactivity in ND.

Additional Levels of Complexity

Interactions between Pathways

There are many levels of crosstalk between these intracellular signaling pathways (**Figures 1**, **3**). For instance, depending on the specific cellular environment, STAT3 and NF-κB pathways may interfere with each other through direct physical interaction, perform reciprocal inhibition through their respective inhibitors, or cooperate in the regulation of transcription of target genes (Grivennikov and Karin, 2010; Oeckinghaus et al., 2011). Similar interactions between STAT3 and ERK have been reported *in vitro* (Jain et al., 1998). In astrocyte cultures, the stimulation of purinergic receptors by specific agonists results in STAT3 phosphorylation, suggesting crosstalk between STAT3 and purinergic signaling cascades (Washburn and Neary, 2006). Similarly, several members of the MAPK family may interact with the NF-κB pathway. For example, p38 is a co-factor for NF-κB activation (Hoesel and Schmid, 2013). However, most of these mechanisms were described in cell lines, using cytokine stimulation or expression of constitutively active mutant proteins. Whether these interactions occur in reactive astrocytes *in vivo* remains to be demonstrated, especially in ND.

In addition, transcription factors such as STAT3 can also bind non-consensus sequences and interact with co-factors or epigenetic regulators, which represent an additional level of transcriptional regulation (Hutchins et al., 2013). Zamanian et al. recently showed that astrocyte reactivity induced by LPS injection or ischemia in the mouse brain induces the expression of hundreds of genes (Zamanian et al., 2012). Only a subset of these genes was common between the two models, illustrating the diversity of the transcriptional changes that may occur in reactive astrocytes, depending on the trigger and cellular environment (see **Figure 3**).

MicroRNA

MicroRNAs (miRNA) are non-coding RNA involved in the post-transcriptional regulation of gene expression. Several studies have linked changes in miRNA expression to astrocyte reactivity

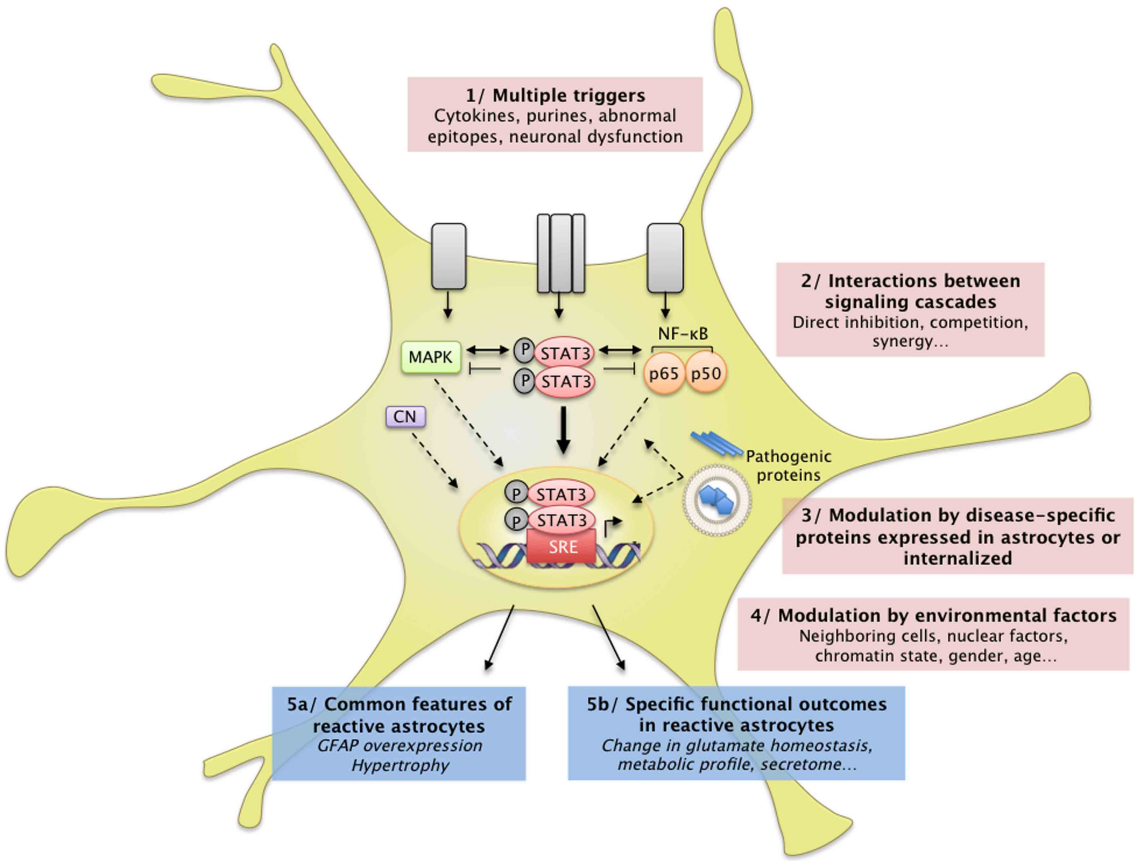

FIGURE 3 | Complex interactions between intracellular cascades result in a unique pattern of astrocyte reactivity in ND. (1) Many signals can trigger astrocyte reactivity (see Section How do Astrocytes become Reactive? and **Figure 1**) such as cytokines, purines or abnormal epitopes like aggregated proteins. Astrocytes may also react to the absence of "resting signals" from neighboring cells, which occurs with neuronal death in ND. (2) These molecular signals are detected by specific receptor complexes at the astrocyte membrane, which activate several signaling cascades such as the JAK/STAT3 pathway, the NF-κB pathway, and the MAP kinase pathway (see also **Figure 1**). These pathways and their effectors interact either directly or indirectly, in the cytoplasm, in the nucleus, or on DNA promoter regions (see Section Additional Levels of Complexity). Recent studies support the idea that these cascades eventually converge on the JAK/STAT3 pathway, triggering a transcriptional program of reactivity in astrocytes. This program is modulated at several levels. (3) Disease-specific proteins (mHtt, mSOD1) endogenously expressed in astrocytes as well as internalized aggregated proteins (Aβ) can directly interfere with these signaling cascades or with transcriptional activity. (4) Several environmental factors may also affect the transcriptional program in reactive astrocytes, such as nuclear factors (other transcription factors, chromatin state) and environmental factors (age, sex). In the brain, dialog with other cell types or the specific brain regions involved is another potential level of modulation. These complex signaling cascades result in (5a) common features of astrocyte reactivity such as the upregulation of GFAP and cellular hypertrophy and (5b) disease-specific outcomes. This scheme illustrates how several signals can converge on a central signaling cascade that, in turn, is modulated in a disease- and environment-specific manner to produce a particular functional outcome.

(Bhalala et al., 2013). For example, the expression of particular miRNA increases in parallel with markers of reactivity, in the brains of patients and models of AD (Li et al., 2011) and in mouse astrocytes after spinal cord injury (Bhalala et al., 2012). Furthermore, miR145 reduces GFAP expression (Wang et al., 2015), whereas miR181 controls the expression of several cytokines in astrocytes (Hutchison et al., 2013). These miRNAs can also modulate signaling cascades including the NF-κB (Cui et al., 2010) and JAK/STAT pathways (Witte and Muljo, 2014). Overall, miRNAs, by regulating gene networks, add another level of control to astrocyte reactivity, which requires further investigation in ND.

The Effects of Mutant Proteins

Interestingly, mutant proteins involved in familial forms of ND may also interfere directly with intracellular cascades and thus affect signaling that reaches the nucleus (**Figure 3**). For example, wild-type Htt plays a role in NF-κB nuclear transport (Marcora and Kennedy, 2010) and mHtt impairs NF-κB signaling in astrocytes (Chou et al., 2008). In addition, mHtt interacts with the inhibitor of κB kinase (IKK) and inhibits IKK activity and NF-κB signaling (Khoshnan et al., 2004). Finally, ND are associated with dysfunction of the ubiquitin proteasome system (UPS, see Section Processing of Mutant Proteins), which is responsible for the degradation of IκB, the master inhibitor of

NF-κB (Oeckinghaus et al., 2011, **Figure 1**). JAK proteins can also be targeted to the UPS by SOCS3 (Kershaw et al., 2014). Alterations in the UPS may thus indirectly influence the activity of several signaling cascades within astrocytes.

In conclusion, astrocyte reactivity can be triggered by many extracellular or intracellular signals. Although several signaling pathways are activated in reactive astrocytes, they seem to converge on the JAK/STAT3 pathway (**Figure 3**). Other cascades such as the NF-kB pathway or CN may regulate, rather than induce, astrocyte reactivity in ND. These signaling pathways result in massive transcriptional changes that may affect many astrocyte functions.

What Do Reactive Astrocytes Do or Fail to Do during ND?

Insights from Cytokine-induced Astrocyte Reactivity

As a first attempt to elucidate the functional changes occurring in reactive astrocytes, cytokines and pro-inflammatory agents were overexpressed directly in the brain to induce reactivity. This was achieved by the injection of recombinant proteins, viral vector-mediated gene transfer or through transgenic mice overexpressing the protein of interest. Clearly, many functional changes are triggered by exposure to these molecules (Sofroniew, 2014). In addition to astrocytes, microglial cells also become activated and peripheral immune cells can be recruited within the brain parenchyma. Interestingly, reactivity can be selectively induced in astrocytes, leaving microglial cells virtually unaffected, by overexpressing the cytokine CNTF (Lavisse et al., 2012), or through the genetic ablation of β1-integrin in astrocytes (Robel et al., 2009). Such approaches have contributed to identify functional changes occurring in reactive astrocytes, including changes in glutamate homeostasis (Escartin et al., 2006; Beurrier et al., 2010), energy metabolism (Escartin et al., 2007; Carrillo-De Sauvage et al., 2015) and K^+ homeostasis (Seidel et al., 2014; Robel et al., 2015); see Liberto et al. (2004) for a general review.

More relevant to ND, experiments have also been performed with disease-causing agents like Aβ or mHtt to decipher the functional changes occurring in reactive astrocytes during ND.

Insights from Disease Models

In the following paragraphs, we will present several astrocyte functions that are known to be modified by reactivity, instead of describing each ND separately, to illustrate the existence of shared mechanisms between several ND.

Glutamate Homeostasis

Alteration of glutamate uptake is probably one of the best documented and earliest described dysfunction of astrocytes in ND (Maragakis and Rothstein, 2004; Soni et al., 2014). Indeed, astrocytes are responsible for most glutamate uptake at synapses, through transporters encoded by *excitatory amino acid transporter gene 1 and 2* (EAAT1 and 2, also called GLAST and GLT1 in rodents) (Danbolt, 2001). Inefficient glutamate uptake leads to over-stimulation of glutamate receptors, which causes excitotoxic cell death in neurons. Excitotoxicity is a well-described pathological mechanism in ND (Maragakis and Rothstein, 2004).

Pioneering work from the Rothstein laboratory showed that glutamate transport is impaired in synaptosomes from patients with ALS (Rothstein et al., 1992). EAAT2 protein levels are low in the spinal cord and motor cortex of patients with familial or sporadic ALS (Rothstein et al., 1995) and animal models expressing mSOD1 (Bendotti et al., 2001; Howland et al., 2002), even before neuronal loss (Howland et al., 2002).

EAAT2 mRNA (Arzberger et al., 1997) and protein (Faideau et al., 2010) levels are also decreased in the caudate and putamen of patients with HD, depending on the disease stage (Faideau et al., 2010). In the prefrontal cortex of patients with HD, the uptake of glutamate is significantly impaired (Hassel et al., 2008). Such alterations are reproduced in mouse and fly models of HD (Lievens et al., 2001, 2005). Importantly, selective expression of mHtt in striatal astrocytes is sufficient to reduce GLT-1 expression, alters glutamate uptake and is associated with the dysfunction of striatal neurons (Faideau et al., 2010) and motor abnormalities (Bradford et al., 2009). Thus, alteration of glutamate uptake in astrocytes contributes to the neuronal toxicity observed in HD.

Alteration of glutamate homeostasis is also thought to contribute to the pathogenesis of AD. Binding of [^{3}H] aspartate (a transportable analog of glutamate that does not bind to glutamate receptors) is reduced in the midfrontal cortex of patients with AD (Masliah et al., 1996). In a transgenic mouse model of AD, aspartate binding and glutamate transporter levels are lower than in WT littermates (Masliah et al., 2000). However, mRNA levels of glutamate transporters were unaffected in this model, suggesting that post-transcriptional modifications are involved. Indeed, in protein lysates from AD brains and Aβ-treated synaptosomes, EAAT2 is oxidized, which may impair its function (Lauderback et al., 2001). In addition, alternative EAAT2 splice variants with reduced glutamate transport capability (Scott et al., 2011) and abnormal detergent-insoluble EAAT2 (Woltjer et al., 2010) are found in vulnerable brain regions of AD patients. Finally, in brain slices, $A\beta_{1-42}$ treatment results in the internalization of GLT-1, which reduces glutamate clearance by astrocytes (Scimemi et al., 2013). Therefore, transcriptional, post-transcriptional and post-translational mechanisms account for the dysregulation of EAATs in AD.

The low expression and poor functionality of EAATs appears to be a truly universal feature of ND. Expression of the human tau protein under the GFAP promoter decreases EAAT expression in the brainstem and the spinal cord and impairs glutamate transport in synaptosomal preparations from the spinal cord (Dabir et al., 2006). The selective expression of α-synuclein^{A53T} in astrocytes also reduces GLT-1 levels in both pre-symptomatic and symptomatic mice and triggers the death of dopaminergic neurons in the SNpc (Gu et al., 2010).

Once taken up from the synaptic cleft by astrocytes, glutamate is metabolized into glutamine by GS. Glutamine is then transported back to neurons and used for the production of glutamate and GABA (Danbolt, 2001). GS expression is low in the temporal cortex of patients with AD (Le Prince et al., 1995) and

in the hippocampus of 3xTg-AD mice (Olabarria et al., 2011). GS mRNA levels are also lower in R6/2 mice (Lievens et al., 2001) and in BACHD mice (Boussicault et al., 2014) than in WT littermates. In the mouse hippocampus, the reduction in GS expression in reactive astrocytes triggers GABA depletion and neuronal hyper-excitability (Ortinski et al., 2010). Therefore, alterations of the glutamate-glutamine cycle may directly contribute to neuronal dysfunction in ND.

Altogether, it is well established that glutamate homeostasis is altered in ND, both in patients and animal models. In addition to its action on synaptic receptors, glutamate also serves as a metabolic signal to promote glucose uptake (Pellerin and Magistretti, 1994). Therefore, any alteration of glutamate homeostasis is likely to affect brain energy metabolism.

Energy Metabolism

Astrocytes are involved in complex metabolic interactions with neurons (Allaman et al., 2011). Their strategic location at the interface between intracerebral blood vessels and synapses make them ideally positioned to deliver neurons with blood-borne metabolic substrates, according to their energy needs (see Belanger et al., 2011, for review). It is still unclear how the morphological changes associated with reactivity affect blood vessels coverage and metabolite uptake by astrocytes. Several metabolic pathways such as the glutamate-glutamine cycle, cholesterol metabolism and glutathione production, are compartmentalized between neurons and astrocytes. This confers astrocytes with a pivotal regulatory role. Energy deficits are a common hallmark of various ND (Lin and Beal, 2006), suggesting that some metabolic interactions are altered when astrocytes become reactive in ND.

Glucose metabolism

Glucose is by far the preferred energy substrate for the brain. According to the astrocyte-to-neuron lactate shuttle hypothesis, in conditions of increased neuronal activity, more glucose is taken up by astrocytes and oxidized through glycolysis and redistributed to neurons in the form of lactate (Pellerin and Magistretti, 1994). Positron emission tomography (PET) imaging shows that cerebral glucose metabolism is impaired in HD and AD patients (Grafton et al., 1992; Fukuyama et al., 1994). However, it is not known whether such metabolic deficits originate from reactive astrocytes.

A comprehensive autoradiography analysis suggests that regional glucose metabolism is reorganized in aged BACHD mice (decreased glucose uptake in the striatum, increased in the hypothalamus). Neuron-astrocyte insert co-cultures were performed to identify the cellular origin of such deficits. They showed that expression of mHtt in astrocytes does not affect their own rate of glucose uptake. However, it impairs glucose uptake in neurons, regardless of their genotype, suggesting that HD astrocytes indirectly regulate neuronal metabolism by diffusible factors (Boussicault et al., 2014). In ALS, lactate was identified as one of the molecules that are released differently when astrocytes are reactive. $SOD1^{G93A}$ mice express lower levels of the astrocytic lactate transporter Slc16a4 than WT mice, which reduces lactate release in the spinal cord. Decreased lactate production is also observed in spinal astrocytes from familial ALS patients (Ferraiuolo et al., 2011), and may be deleterious to neurons relying on this metabolic supply.

Astrocyte metabolism can be studied by NMR on brain extracts after i.p. injection of ^{13}C-labeled acetate in animal models because this compound is metabolized preferentially by astrocytes (see Section Reactive Astrocytes as Biomarkers). Astrocytic "hyper-metabolism," characterized by high incorporation of ^{13}C into metabolic intermediates is observed in the brain of 7-month old 3xTg-AD mice (Sancheti et al., 2014) and in the cortex of a mouse model of a tauopathy (Nilsen et al., 2013). But opposite changes are observed in the frontal cortex in a rat model of AD (McGill-R-thy1-APP) (Nilsen et al., 2014). Also in favor of a decreased metabolic activity in reactive astrocytes, the transfer of glutamine to glutamate is reduced in 3xTg-AD mice (Sancheti et al., 2014). Similar conflicting effects of Aβ on astrocyte oxidative metabolism were reported in culture (see Allaman et al., 2010, and references therein). Overall, the metabolic changes occurring in reactive astrocytes during ND are quite contrasted, depending on the ND, the animal model and the stage of the disease considered.

Besides glucose uptake, many studies report mitochondrial dysfunction in ND (Lin and Beal, 2006). Some studies suggest that not only neurons display mitochondrial failure in ND, but reactive astrocytes too. Motori et al. performed an elegant imaging study of reactive astrocytes following stab wound injury. They reported that mitochondria undergo more fission events in reactive than in resting astrocytes (Motori et al., 2013). The exposure of pro-inflammatory cytokines had the same effect on mitochondria *in vitro* and resulted in impaired respiratory activity and reactive oxygen species (ROS) production (Motori et al., 2013). Transcriptomic analysis of astrocytes from AD patients indicates that mitochondrial genes, such as those involved in tricarboxylic acid cycle, are expressed at lower levels than in astrocytes of age-matched control individuals (Sekar et al., 2015). The exposure of astrocytes to Aβ reduces their mitochondrial membrane potential, which activates toxic enzymes such as poly (ADP-ribose) polymerase 1 and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, a potent pro-oxidant enzyme (Abeti et al., 2011). Similarly, mitochondrial respiration is altered in astrocytes isolated from the spinal cord of ALS rats, probably because of increased oxidative stress (Cassina et al., 2008). Therefore, although transient beneficial changes occur in reactive astrocytes during ND, overall, they display altered metabolism that may result in ROS production (see Section Antioxidants and ROS).

Cholesterol metabolism

Cholesterol, the most common steroid in humans, is a structural component of cell membranes and a precursor of steroid hormones. It also contributes to synapse formation and neuronal activity; therefore, defects in cholesterol homeostasis may have severe consequences on brain function (Pfrieger and Ungerer, 2011). Cholesterol synthesis and degradation are highly compartmentalized in astrocytes and neurons, respectively (Pfrieger and Ungerer, 2011). In particular, astrocytes express

high levels of apolipoprotein E (ApoE) that carries cholesterol to neurons (Bu, 2009).

The ε4 allele of the *ApoE* gene is the major risk factor for sporadic AD. It increases the probability of developing AD by a factor of 3–4 (Corder et al., 1993). ApoE influences the pathogenesis of AD at multiple levels, by regulating cholesterol metabolism, APP processing and Aβ clearance (Bu, 2009). ApoE is a chaperone for the binding of Aβ to the low density lipoprotein receptor or low density lipoprotein receptor-related protein 1 on astrocytes (Koistinaho et al., 2004). This is an important route for Aβ clearance (see Section Processing of Mutant Proteins). In astrocytes from aged APP/PS1dE9 mice, there is a widespread reduction in the expression of enzymes and transporters linked to cholesterol metabolism including ApoE, suggesting a decrease capacity to clear Aβ in these mice (Orre et al., 2014).

Cholesterol biosynthesis is low in the brain of several mouse models of HD (Valenza et al., 2010). In primary astrocytes from HD mice, mRNA levels of genes for cholesterol biogenesis and efflux are substantially lower than in control astrocytes. In addition, lower amounts of ApoE are secreted by HD *in vitro* and it forms smaller lipoprotein particles in the cerebrospinal fluid of HD mice (Valenza et al., 2010). The impairment in cholesterol biosynthesis correlates with the number of CAG repeats, the amount of mHtt (Leoni and Caccia, 2014) and is eventually toxic to HD neurons (Valenza et al., 2015).

Connexin-based networks of astrocytes

Astrocytes form multicellular networks connected by their gap junctions composed of connexins. These networks are involved in K^+ buffering but also deliver metabolic substrates to active synapses (Rouach et al., 2008).

The expression of astrocyte connexin (Cx) and astroglial coupling through gap junction channels is changed in reactive astrocytes in ND (Giaume et al., 2010). Cx43 expression is high in the caudate of HD patients (Vis et al., 1998) and around amyloid plaques in the cortex of AD patients (Nagy et al., 1996). Similarly, Cx43 expression is higher in the spinal cord of $SOD1^{G93A}$ mice (Cui et al., 2014), in two mouse models of AD (Mei et al., 2010) and in the MPTP model of PD (Rufer et al., 1996), than in their respective controls. Cx30 expression is also altered in ND models and patients, although the direction of the change is context dependent. Cx30 is expressed at low levels in the striatum of a rat and primate pharmacological model of PD (Charron et al., 2014) but is highly expressed in a mouse model of AD (Mei et al., 2010) and in AD patients (Nagy et al., 1996). However, in most cases, the functional effects on the astrocyte network and especially on metabolite trafficking was not assessed (Escartin and Rouach, 2013). Increased coupling may be beneficial for the delivery of metabolites. However, Cx also form hemichannels, through which several active molecules or gliotransmitters are released (Giaume et al., 2010; Bosch and Kielian, 2014). High Cx expression in reactive astrocytes in ND may thus lead to the excessive release of ATP or glutamate (Bosch and Kielian, 2014). This would maintain microglial cells in an active state and cause excitotoxicity in nearby neurons (see Section Release of Active Molecules and **Figure 4**).

Ion Homeostasis

Buffering of K^+

Astrocytes buffer K^+ by specific channels and transporters, which are enriched in PAP and vascular endfeet. The maintenance of K^+ homeostasis by astrocytes is essential for synaptic transmission and appears to be altered in ND. The mRNA expression of several K^+ channels is lower in astrocytes isolated from APP/PS1dE9 than from WT mice (Orre et al., 2014). Protein levels of Kir4.1, an inward rectifier K^+ channel, are decreased in the spinal cord of ALS mice (Kaiser et al., 2006) and in the striatum of R6/2 and zQ175 mice, two models of HD (Tong et al., 2014). Restoration of Kir4.1 levels through viral gene transfer in striatal astrocytes improves some of the neurological features in R6/2 mice (Tong et al., 2014). Importantly, astrocytes do not display the hallmarks of reactivity in these HD mice (see **Table 1**), suggesting that astrocytes can be dysfunctional before being "fully" reactive.

Ca^{2+} homeostasis

Several studies have reported alterations of Ca^{2+} homeostasis in reactive astrocytes in ND models, especially in AD (Vincent et al., 2010). Spontaneous Ca^{2+} transients are more frequent in slices from Tg2576 mice overexpressing APP than in controls (Pirttimaki et al., 2013). Hyperactive Ca^{2+} transients and waves can also be observed by two-photon live imaging with Ca^{2+} dyes in several mouse models of AD (Takano et al., 2007; Kuchibhotla et al., 2009; Delekate et al., 2014). Astrocytes from $SOD1^{G93A}$ ALS mice also display enhanced Ca^{2+} transients following stimulation of mGluR5 receptors (Martorana et al., 2012) or exposure to ATP (Kawamata et al., 2014). Store-operated accumulation of Ca^{2+} in the endoplasmic reticulum may be responsible for these altered Ca^{2+} responses (Kawamata et al., 2014).

Deregulation of Ca^{2+} in reactive astrocytes may elicit profound changes in various Ca^{2+}-dependent processes such as intracellular signaling cascades, proteolysis and gliotransmitter release.

Release of Active Molecules

Astrocytes interact with neighboring cells by releasing many molecules involved in cell-to-cell signaling, trophic support or antioxidant defense. The overall "secretome" of astrocytes is strongly altered by their reactivity (**Figure 4**). Some of these neuroactive molecules like glutamate, purines or GABA are neurotransmitters, and are thus called gliotransmitters when released by astrocytes (Araque et al., 2014).

Gliotransmitters

The release mode of gliotransmitters and their physiological relevance is a matter of intense debate (Araque et al., 2014; Sloan and Barres, 2014). The study of gliotransmission *in situ* is particularly difficult because most of these molecules are also released by neurons.

Glutamate is one of the best studied gliotransmitters. FRET-based imaging shows that cultured astrocytes release glutamate in response to recombinant Aβ1–42 or Aβ isolated from the brain of AD patients. This phenomenon is Ca^{2+}-dependent and is deleterious to neighboring neurons (Talantova et al., 2013). Glutamate release elicited by mechanical stimulation is more

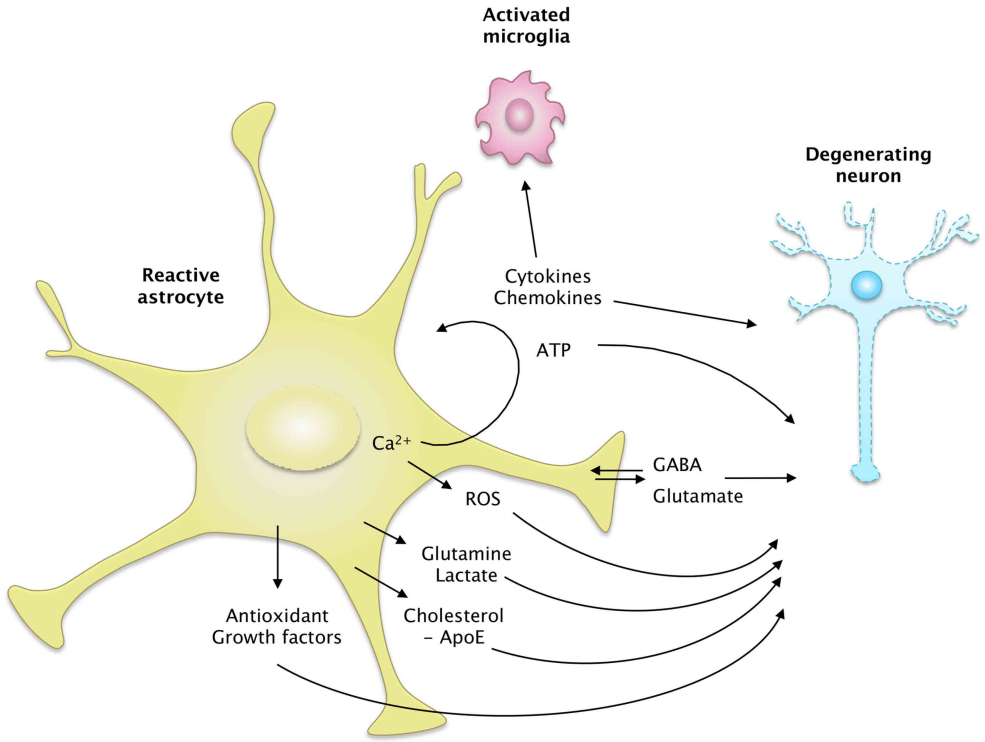

FIGURE 4 | The secretome of reactive astrocytes. Astrocytes secrete many active molecules that influence neuronal survival and synaptic activity. Reactivity affects the pattern of secreted molecules, and thus alters neuron-astrocyte communications. In ND, reactive astrocytes may secrete higher levels of antioxidants, such as glutathione and its precursors or metabolic substrates. These changes would promote neuron survival. However, reactive astrocytes may also release fewer trophic molecules such as cholesterol, growth factors or glutamine and produce more ROS than resting astrocytes. The regulation of glutamate and GABA homeostasis may also be altered by reactivity, due to a change in their release but also their uptake. Intracellular Ca^{2+} levels are deregulated in ND, which may stimulate the release of gliotransmitters such as glutamate and ATP. Reactive astrocytes also produce more cytokines, which activate microglial cells or act as paracrine factors, maintaining glial cells in a chronically reactive state.

important in cortical astrocytes isolated from BACHD mice than in their WT counterparts (Lee et al., 2013). Interestingly, the cytokine TNFα plays many regulatory roles at the excitatory synapse; it directly scales synaptic transmission and potentiates glutamate release by astrocytes (see Santello and Volterra, 2012, for review). TNFα levels are elevated in patients and animal models of ND (see Section Cytokines and Inflammatory Molecules), which may stimulate the non-physiological release of glutamate by reactive astrocytes. Unexpectedly, glutamate release in response to TNFα is impaired in hippocampal slices from AD mice harboring numerous Aβ plaques and reactive astrocytes (Rossi et al., 2005). The authors hypothesized that the intracellular cascades downstream from the TNFα receptor were altered in reactive astrocytes in this model (Rossi et al., 2005). Overall, additional studies are still needed, especially *in vivo*, to determine precisely how the release of glutamate by reactive astrocytes is changed in ND, and how it modulates synaptic transmission (Agulhon et al., 2012).

GABA is yet another gliotransmitter that was recently implicated in ND. Reactive astrocytes release more GABA than resting astrocytes, which contributes to cognitive impairment in two mouse models of AD (Jo et al., 2014; Wu et al., 2014). Excessive GABA released by reactive astrocytes results in the tonic inhibition of dentate gyrus granule cells in the hippocampus of AD mice. Inhibition of GABA synthesis or pharmacological blockade of GABA transporters restores synaptic plasticity and memory deficits in these mice (Jo et al., 2014; Wu et al., 2014). By contrast, GABA release by astrocytes appears to be defective in HD. Electrophysiological recordings on slices show that both $GABA_A$ (postsynaptic) and $GABA_B$ (presynaptic) currents are lower in R6/2 and zQ175 mice than in control mice. This results in a lower GABA-mediated tonic inhibition of striatal neurons. Pharmacological manipulation of the GABA transporter-3 (GAT-3), which is preferentially expressed by astrocytes, suggests that HD astrocytes have an impaired capacity to release GABA through GAT-3 (Wojtowicz et al., 2013).

Purines are another class of gliotransmitters, comprising ATP and its metabolite adenosine, which is generated extracellularly by ectonucleotidases. Stimulation of primary astrocyte cultures with Aβ induces the release of ATP (Jung et al., 2012) via Cx43 hemichannels (Orellana et al., 2011). Recently, it was shown that reactive astrocytes around amyloid plaques in the cortex of APPPS1 mice release more ATP via hemichannels than their WT counterparts. ATP, degraded into adenosine, acts as an autocrine signal on astrocyte P2Y1 receptors and elicits Ca^{2+} hyperactivity (Delekate et al., 2014). Cultured astrocytes from SOD^{G93A} mice

also release more ATP than those from WT mice, which is toxic to co-cultured motor neurons (Kawamata et al., 2014).

Cytokines and inflammatory molecules

The levels of pro-inflammatory cytokines are higher in vulnerable brain regions and in the cerebrospinal fluid in ND patients than in healthy individuals (Lucin and Wyss-Coray, 2009; Heneka et al., 2014). However, many cell-types such as activated microglia or peripheral immune cells may produce these molecules. Transcriptional analysis performed on laser-captured $GFAP^+$ reactive astrocytes from APP/PS1dE9 mice reveal that these cells express high levels of several cytokines (Orre et al., 2014). The number of genes induced and the fold-increase in expression are higher in astrocytes than in microglial cells, showing that reactive astrocytes may contribute significantly to the production of cytokines during AD. However, the absolute expression level of these cytokines remains lower in reactive astrocytes than in microglia. Some of these cytokines act as recruiting signals for peripheral immune cells or promote BBB permeability (Farina et al., 2007; Sofroniew, 2015). In ALS, a major increase in the transcription of inflammatory molecules is well established, including in astrocytes derived from both familial and sporadic forms of the disease (Haidet-Phillips et al., 2011). These astrocytes are toxic to motor neurons in co-culture systems.

In microglia, the maturation of some cytokines like IL-1β is operated in the cytosol by the inflammasome. Aβ phagocytosis activates the NOD-like receptor protein (NLRP) 3 inflammasome in these cells (Halle et al., 2008), thereby linking the internalization of pathologic proteins with the release of pro-inflammatory cytokines in ND. Only two recent studies suggest that in astrocytes, stimulation of the inflammasome also triggers IL-1β production (Minkiewicz et al., 2013; Zeis et al., 2015).

Finally, reactive astrocytes overexpress molecules of the complement system in AD mice (Orre et al., 2014), which can alter dendrite morphology, Ca^{2+} homeostasis and excitatory synaptic responses in neurons, at least *in vitro* (Lian et al., 2015). In AD and HD patients, components of the complement system are overexpressed (Singhrao et al., 1999; Lian et al., 2015); however they are not necessarily produced by astrocytes only.

Trophic factors

Astrocytes secrete various factors exhibiting trophic effects on neurons, such as growth factors (e.g., CNTF, brain-derived neurotrophic factor [BDNF], nerve growth factor [NGF], FGF), neurosteroids, and adhesion molecules involved in neurite outgrowth (Muller et al., 1995; Sofroniew and Vinters, 2010). Inadequate synthesis and release of such factors may contribute to neuronal toxicity observed in HD. The expression of mHtt in primary cultures of cortical astrocytes impairs BDNF production in astrocytes. Levels of mature BDNF in the medium are thus low under these conditions, which limits neurite development of primary cortical neurons (Wang et al., 2012). Similarly, transcription and release of the chemokine (C-C motif) ligand 5 (CCL5/RANTES), which promotes neurite outgrowth and neuronal survival, is also impaired by the expression of mHtt in cultured astrocytes (Chou et al., 2008). CCL5/RANTES accumulates in the cytosol of astrocytes in HD patients and in two mouse models of HD (Chou et al., 2008). Although reactive astrocytes secrete more trophic factors such as NGF in ALS rodent models and patients (Pehar et al., 2004; Ferraiuolo et al., 2011), it may nonetheless have unexpected detrimental consequences on nearby neurons. Indeed, vulnerable motor neurons in ALS express the specific p75 neurotrophin receptor isoform, and its stimulation by NGF triggers apoptosis instead of trophic actions (Pehar et al., 2004).

Antioxidants and ROS

Astrocytes are important for defense against ROS because they express many detoxifying enzymes and transporters (Vargas and Johnson, 2009; Allaman et al., 2011). They produce high levels of antioxidants for neurons, including ascorbic acid (AA, also known as vitamin C), glutathione and its precursors. The antioxidant action of astrocytes is crucial for neurons, because oxidative respiration produces high levels of ROS. Indeed, oxidative stress contributes to neuronal dysfunction in several ND (Belanger et al., 2011). The expression of many detoxifying enzymes and transporters are controlled by the master regulator NF-E2 related factor-2 (Nrf2), a transcription factor which translocates to the nucleus and binds specific promoter sequences in response to oxidative stress (Vargas and Johnson, 2009). Reactive astrocytes found in the spinal cord of early symptomatic ALS rats show high levels of Nrf2 expression and nuclear translocation (Vargas et al., 2005). Although high Nrf2 activity may be beneficial for neurons exposed to oxidative stress, this endogenous antioxidant response does not offer sufficient protection. Indeed, Nrf2 activity can be further enhanced in astrocytes by genetic manipulation, which improves disease outcome in animal models of ALS (Vargas et al., 2008), PD (Chen et al., 2009; Gan et al., 2012), and HD (Calkins et al., 2010).

The release of AA by astrocytes is altered in HD (Rebec, 2013). In the R6/2 mouse model of HD, extracellular AA levels are lower than in age-matched WT mice but only during behavioral activity (Rebec et al., 2002). Accordingly, mHtt expression in astrocytes (and not in neurons) is sufficient to trigger oxidative stress in neurons by diffusible factors (Boussicault et al., 2014). In fact, reactive astrocytes may not only produce fewer antioxidant molecules during ND, they may also release more pro-oxidant factors. Exposure to Aβ stimulates the pentose phosphate pathway in astrocytes *in vitro*; yet they release more ROS than in control conditions and are toxic to co-cultured neurons even without physical contact (Allaman et al., 2010). Furthermore, reactive astrocytes overexpress inducible NO synthase (NOS), including in the brain of AD patients (Heneka et al., 2014). Aβ peptides also cause a loss of mitochondrial membrane potential in astrocytes, which is associated with the activation of NADPH oxidase and excessive ROS production (Abramov et al., 2004). Accordingly, mitochondria from mSOD1 astrocytes produce large amounts of superoxide radicals, causing motor neuron death in co-culture, which is prevented by pre-incubation with antioxidants and NOS inhibitors (Cassina et al., 2008). Similarly, rodent astrocytes expressing a mutant form of TDP43 induce nitrosative stress in motoneurons and kill them (Rojas et al., 2014). Overall, ROS production by reactive astrocytes exposed

to toxic or mutant disease-specific proteins seems to be another deleterious mechanism common to several ND.

Processing of Mutant Proteins

Aggregation of intra- or extra-cellular misfolded proteins is a central feature of ND. However, the exact role of aggregate formation is still debated. Soluble forms of mutant proteins are now considered to be the most toxic forms, and their aggregation may be instead a protective mechanism that prevents them from interfering with important intracellular partners (Ross and Poirier, 2004).

Misfolded proteins are degraded by two major intracellular pathways: autophagy and the UPS. Autophagy involves the formation of intra-cytoplasmic vesicles that may also envelop organelles. Engulfed elements are completely degraded by proteases such as cathepsins after fusion with a lysosome. Alternatively, the UPS forms a protease complex to which proteins are addressed by specific ubiquitin tags. Both pathways are altered in ND (Dantuma and Bott, 2014; Ghavami et al., 2014).

The UPS has been extensively studied in neurons in models of ND and is even a target of neuroprotection (Margulis and Finkbeiner, 2014; Popovic et al., 2014). Much less is known about the UPS in astrocytes (Jansen et al., 2014). Protein aggregates are mainly found in neurons, suggesting that astrocytes are more efficient than neurons at handling toxic proteins (Jansen et al., 2014). Indeed, a study based on a reporter system showed that the UPS is more active in glial cells than in neurons *in vitro* and *in vivo* (Tydlacka et al., 2008). During ND, the UPS in astrocytes may become less efficient than in healthy conditions because UPS subunits are down-regulated in astrocytes from AD patients (Simpson et al., 2011). In addition, during ND, reactive astrocytes may express a specific form of the proteasome, called the immunoproteasome, which is formed by the cytokine-inducible subunits β1i, β2i, and β5i. The immunoproteasome is detected in reactive astrocytes around amyloid plaques in AD patients and APP/PS1dE9 mice (Orre et al., 2013) and in the spinal cord of SOD^{G93A} mice (Puttaparthi and Elliott, 2005). The immunoproteasome is involved in antigen presentation (Jansen et al., 2014), but its functional role in reactive astrocytes during ND is not yet known. Invalidation of the B1i subunit of the immunoproteaseome does not influence disease outcome in SOD^{G93A} mice (Puttaparthi et al., 2007).

In AD, reactive astrocytes play yet another role in the clearance of extracellular Aβ. More than a decade ago, it was shown that astrocytes are able to internalize amyloid plaques and Aβ peptides (Funato et al., 1998; Nagele et al., 2003; Wyss-Coray et al., 2003). They do so by phagocytosis or by internalizing Aβ bound to membrane receptors, including ApoE receptors (Koistinaho et al., 2004; Thal, 2012, see Section Cholesterol Metabolism). The astrocytic protein ApoE also promotes Aβ extrusion through the BBB or along the perivascular space (Bu, 2009, and see Section Cholesterol Metabolism). Intracellular vesicles containing Aβ are addressed to lysosomes for degradation and the enhancement of lysosomal biogenesis selectively in astrocytes attenuates amyloid-related disease in a mouse model of AD (Xiao et al., 2014).

Aβ may also be degraded extracellularly, and astrocytes produce some Aβ-degrading enzymes such as insulin-degrading enzyme (IDE), neprilysin or matrix metalloproteinase 2 and 9 (MMP9). Neprilysin and IDE are overexpressed in reactive astrocytes in contact with plaques in AD brains (Apelt et al., 2003; Dorfman et al., 2010) and MMP9 is overexpressed in mouse models of AD (Yan et al., 2006). However, reactive astrocytes may eventually become overwhelmed as the disease progresses because they undergo cell lysis and form extracellular deposits containing neuronal-derived Aβ peptides (Nagele et al., 2003).

Alternatively, it was suggested that reactive astrocytes may contribute to Aβ production by overexpressing β-site APP cleaving enzyme 1 (BACE1), the rate limiting enzyme for Aβ production. Strong BACE1 expression is observed in reactive astrocytes in patients and several mouse models of AD, and following exposure to pro-inflammatory cytokines, which can directly activate the BACE1 promoter (Cole and Vassar, 2007). However, it is unknown how the amount of Aβ produced by astrocytes compares with the large pool of Aβ generated by neurons.

Regarding HD, we found that blocking astrocyte reactivity by overexpressing SOCS3 significantly promoted the formation of mHtt aggregates in the mouse striatum (Ben Haim et al., 2015). A recent study performed in *Drosophila* also reported that reactive glia are able to phagocyte mHtt expressed in neurons (Pearce et al., 2015). These results suggest that reactive astrocytes may participate in the processing of mHtt and its aggregation in neurons, but the exact molecular mechanisms need to be established.

How Do Reactive Astrocytes Globally Contribute to ND?

We have seen that many changes occur in astrocytes during ND, which makes it extremely difficult to get a clear view of their impact on the disease. In addition, the reactive status of astrocytes is not always directly reported in studies. Therefore, to evaluate the overall contribution of reactive astrocytes to ND, experimental designs that interfere with astrocyte reactivity provide a valuable insight into this difficult question (**Table 3**).

Intermediate Filament KO

Given that the upregulation of IF is a hallmark of astrocyte reactivity, transgenic mice knockout (KO) for GFAP or Vim or double KO were initially generated and tested in acute injuries. Disruption of reactive astrocyte cytoskeleton was also studied in ND models and was found to shorten the lifespan of the $SOD1^{H46R}$ mouse model of ALS (Yoshii et al., 2011). Studies involving the genetic ablation of GFAP and vimentin in APP/PS1dE9 mice gave conflicting results, with one reporting increased amyloid load and dystrophic neurites (Kraft et al., 2013), and another showing no effect on amyloid load in the cortex (Kamphuis et al., 2015). However, knocking out IF affects several basal functions in astrocytes (Shibuki et al., 1996) and results in many transcriptional changes (Kamphuis et al., 2015).

TABLE 3 | Main genetic approaches to block reactive astrocytes in mouse models of ND.

Approach	Construct	ND	ND model	Effect on astrocyte reactivity	Effects on disease outcomes	References
Disruption of cytoskeleton in reactive astrocytes	gfap$^{-/-}$	ALS	SOD1^{H46R} mice	No change in vimentin protein levels No data on astrocyte morphology	Shorter lifespan No effect on motor symptoms	Yoshii et al., 2011
	gfap$^{-/-}$ vimentin$^{-/-}$	AD	APP/PS1dE9 mice	Lower astrocyte hypertrophy	Higher amyloid load More dystrophic neurites	Kraft et al., 2013
					No effect on amyloid load	Kamphuis et al., 2015
Ablation of proliferating astrocytes	gfap-tk	ALS	SOD1^{G93A}	No change in the number of GFAP^{+} cells in the ventral SC No data on astrocyte morphology	No effect on survival, disease onset, duration No effect on motor function No effect on neuronal loss	Lepore et al., 2008a
Inhibition of the JAK/STAT3 pathway	lenti-socs3	HD	Lenti-Htt82Q	Lower GFAP and vimentin expression (mRNA and protein) Resting-like morphology	No effect on neuronal loss More mHtt aggregates	Ben Haim et al., 2015
Inhibition of the NF-kB pathway	hGFAP-Cre x IKKβ $^{fl/fl}$	ALS	SOD1^{G93A} mice	No data on astrocyte phenotype	No effect on survival No effect on motor performances	Frakes et al., 2014
	AAV-IκBα-SR	ALS	SOD1^{G93A} mice	No data on astrocyte phenotype	No effect on survival No effect on motor performances No effect on neuron survival *in vitro*	Frakes et al., 2014
	hGFAP-IκBα-DR	ALS	SOD1^{G93A} mice	Temporary lower number of GFAP^{+} cells (at disease onset)	No effect on survival No effect on motor performances	Crosio et al., 2011
	lenti-DN-IKKγ	HD	R6/2 mice	No data on astrocyte phenotype	Improved motor and cognitive deficits, Less severe MSN atrophy	Hsiao et al., 2013
Inhibition of CN/NFAT signaling	AAV-VIVIT	AD	APP/PS1dE9	Trend of lower GFAP levels (protein) Reduced astrocyte hypertrophy	Improved cognitive deficits Improved synaptic transmission Lower amyloid load	Furman et al., 2012
Constitutive activation of CN	mGFAP-caCN	AD	APP/PS1	Fewer GFAP^{+} cells Lower GFAP levels (protein and mRNA) Reduced astrocyte hypertrophy around plaques	Reduced cognitive deficits Lower amyloid load	Fernandez et al., 2012

Abbreviations: AAV, adeno-associated viral vector; caCN, constitutively active form of calcineurin; DN, dominant negative; IκBα, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha; IKKβ, IκBα kinase alpha; MSN, medium-sized spiny neurons; SC, spinal cord; SR, super repressor; tk, thymidine kinase; DR, degradation resistant.

Upregulation of IF is only a hallmark of reactivity; therefore, their removal from astrocytes will not necessarily block other molecular cascades associated with reactivity.

Ablation of Proliferative Astrocytes

Another strategy developed to evaluate the contribution of reactive astrocytes to CNS injury is the ablation of proliferating astrocytes. This approach involves the expression of the viral enzyme thymidine kinase (TK) under the GFAP promoter, in presence of the drug ganciclovir (Bush et al., 1999). Ganciclovir is metabolized by TK-expressing cells into a base analog that blocks DNA replication, thus inducing the death of proliferating cells. This system has been extensively used to evaluate the effects of glial scar formation in acute injury models (Sofroniew, 2009). These studies demonstrate that glial scar-forming astrocytes act as a barrier to limit immune cell extravasation in the CNS parenchyma. However, in the progressive SOD1^{G93A} mouse model of ALS, ablation of proliferating astrocytes has no effect on disease outcomes (Lepore et al., 2008a), probably because of the small number of proliferating astrocytes in this model (see Section Do Reactive Astrocytes Proliferate in ND?).

Manipulation of Intracellular Signaling Pathways

A third strategy to study reactive astrocytes is to block intracellular signaling pathways controlling reactivity.

The JAK/STAT3 Pathway

Several transgenic mice have been generated to block the JAK/STAT3 pathway in reactive astrocytes. They are based on the conditional KO of STAT3 following the expression of the Cre recombinase under the GFAP or nestin promoter (Okada et al., 2006; Herrmann et al., 2008). Most of these studies have focused on acute injuries with glial scar formation and found that reactive astrocytes mainly exert beneficial functions. By contrast, few studies have investigated the contribution of the JAK/STAT3 pathway in reactive astrocytes in ND, using pharmacological or viral-based approaches. In the MPTP mouse model of PD, pharmacological inhibition of JAK2 reduces astrocyte reactivity. However, this inhibitor does not influence tyrosine hydroxylase levels in the striatum, suggesting that reactive astrocytes do not contribute to dopaminergic loss in this model (Sriram et al., 2004). In a mouse model of HD, viral-mediated overexpression of SOCS3 in reactive astrocytes did not influence neuronal death but promoted the formation of mHtt aggregates (Ben Haim et al., 2015). This intriguing result suggests that reactive astrocytes affect the processing and aggregation of mHtt, which is key pathological mechanism in HD (see Section Processing of Mutant Proteins).

The NF-κB Pathway

In ALS, two independent studies reported that inhibition of the NF-κB pathway in reactive astrocytes does not influence disease phenotype in $SOD1^{G93A}$ mice (Crosio et al., 2011; Frakes et al., 2014). To block this pathway in astrocytes, Frakes et al. crossed mice KO for IKKβ in astrocytes ($GFAP\text{-}Ikkb^{fl/fl}$) with $SOD1^{G93A}$ mice or they overexpressed a dominant negative form of IκBα (AAV-IκB-SR) in astrocytes by viral gene transfer. The inhibition of the NF-κB pathway in reactive astrocytes did not influence motor neuron survival in culture or in the spinal cord of $SOD1^{G93A}$ mice (Frakes et al., 2014), probably because this pathway is mainly active in microglia.

The role of the NF-κB pathway in reactive astrocytes has also been studied in HD. A dominant negative form of IKKγ (DN-IKKγ) was overexpressed by lentiviral gene transfer in the striatum of R6/2 mice to block NF-κB signaling. DN-IKKγ overexpression improved motor performance and prevented shrinkage of striatal neurons in HD mice (Hsiao et al., 2013). However, DN-IKKγ expression was not restricted to astrocytes and may thus have acted in other cell types such as microglia.

The CN/NFAT Pathway

CN is activated upon inflammatory stimulation and regulates gene expression through the transcription factors NFATs and NF-κB (Furman and Norris, 2014). Expression of caCN in astrocytes of APP/PS1 mice reduces astrocyte reactivity, Aβ levels and the number of amyloid plaques. These effects are associated with improved cognitive functions (Fernandez et al., 2012). The beneficial effects of CN are mediated by the inhibition of the NF-κB pathway and subsequent production of pro-inflammatory cytokines (Fernandez et al., 2012). Viral-mediated gene transfer of the blocking peptide VIVIT was used to inhibit NFAT signaling in hippocampal astrocytes in APP/PS1dE9 mice (Furman et al., 2012). VIVIT limited astrocyte hypertrophy, prevented the accumulation of Aβ and improved synaptic plasticity and cognitive functions in AD mice (Furman et al., 2012). These results suggest that reactive astrocytes play detrimental roles in AD. However, VIVIT may be secreted by infected astrocytes; therefore, it is not possible to exclude the involvement of other cell types, especially because CN is permanently activated in neurons in the Tg2576 mouse model of AD (D'Amelio et al., 2011).

In conclusion, several approaches have been used to determine the contribution of reactive astrocytes to ND progression. The overall picture is still unclear because reactive astrocytes have been shown to be beneficial, detrimental or to have no effect, depending on the experimental approach chosen, the molecular target (e.g., IF, signaling cascades) and the disease model (**Table 3**). Some of these approaches rely on pharmacological inhibitors or transgenic mice lacking cell-type specificity or that might involve developmental effects (with non-inducible Cre expression for example). To better delineate the roles of reactive astrocytes in ND, it will be interesting to target pivotal signaling cascades and to use cell-type specific and versatile tools like viral vectors to interfere with astrocyte reactivity in different ND models.

Ongoing Questions, Future Directions

Heterogeneity of Reactive Astrocytes

One of the next challenges in the field is to deal with the functional heterogeneity of astrocytes. Indeed, like neurons, astrocytes display remarkable heterogeneity regarding their density, morphology (Emsley and Macklis, 2006), transcriptional profile (Bachoo et al., 2004), and expression of transporters, channels, receptors and transcription factors (Matyash and Kettenmann, 2010). Astrocyte reactivity is also quite heterogeneous both between and within brain regions (Anderson et al., 2014). Such heterogeneity is best explored in acute injury models, because injury can be inflicted in different brain regions. In the spinal cord for example, astrocytes from the ventral horn do not migrate into a dorsal stab wound, even for very close lesions (Tsai et al., 2012). Even within the same sub-region of the mouse cerebral cortex, astrocytes respond heterogeneously to stab wound injury. A very elegant study based on live two-photon microscopy demonstrated that almost all astrocytes become hypertrophic and overexpress GFAP following injury; however, some had their processes polarized toward the lesion, others proliferated (less than 15%), and some remain static (Bardehle et al., 2013). In ND, reactive astrocytes in contact with plaques have a more pronounced reactive morphology than those at a distance, which correlates with larger transcriptional changes (Orre et al., 2014). In the 3xTg-AD model of AD, astrocytes at distance from plaques may even be atrophic (Olabarria et al., 2010). Overall, the heterogeneity of reactive astrocytes at the regional, sub-regional and cellular level needs to be thoroughly investigated in animal models and patients taking advantage

of modern techniques such as two-photon microscopy and cell-specific transcriptomic analysis. Indeed, it remains unclear how such heterogeneity is established during development or disease and how it contributes to the local vulnerability of neighboring neurons in ND (Molofsky et al., 2012).

Reactive Astrocytes in the Clinics

Reactive Astrocytes as Biomarkers

Given that astrocytes are able to sense even mild neuronal dysfunction and to become reactive, they represent attractive biomarkers for the diagnosis and monitoring of ND. Reactive astrocytes can be imaged in brain slices or in living mice by the expression of a reporter gene (GFP, luciferase) under the control of the GFAP promoter (see O'Brien et al., 2013, for a complete review). For clinical applications, non-invasive image techniques to monitor reactive astrocytes are still under development.

PET provides a way to quantify neuroinflammation through radiolabeled tracers that bind to glial cells. The most common target for neuroinflammation is the peripheral benzodiazepine receptor or translocator protein 18 kDa (TSPO) (see Chauveau et al., 2008). Activated microglia express high levels of TSPO but, as recently demonstrated, reactive astrocytes also overexpress this protein (Lavisse et al., 2012). Therefore, TSPO radioligands do not discriminate between reactive microglia and reactive astrocytes, but they are nonetheless a valuable imaging approach to identify early neuroinflammation in ND patients (Venneti et al., 2006). Radiotracers that target astrocyte metabolism, such as [1-^{11}C]-octanoate (Kuge et al., 2000) and [2-^{18}F]-fluoroacetate (Marik et al., 2009) have also been evaluated in models of glioblastoma and ischemia. It remains to be established whether they can detect progressive astrocyte reactivity in ND patients. Another molecular target is monoamine oxidase B (MAO-B), which is highly expressed in reactive astrocytes. The binding of ^{11}C-DED, a MAO-B radioligand is high in patients with ALS (Johansson et al., 2007), and patients with mild cognitive impairment or AD (Carter et al., 2012). However, this enzyme is also found in serotonergic neurons, which could contribute to this signal. Overall, new PET radiotracers with higher specificity for reactive astrocytes are needed and recent transcriptomic studies on reactive astrocytes may help to identify new targets.

Nuclear magnetic resonance (NMR) techniques are an attractive alternative to monitor astrocyte reactivity *in situ*. Increased T1 relaxation time is observed by magnetic resonance imaging (MRI) in acute models of ischemia and excitotoxicity. Arundic acid, an inhibitor of astrocyte reactivity, normalizes it, but the molecular basis for such changes in NMR signals is unclear (Sibson et al., 2008). NMR-spectroscopy (MRS) allows the quantification of abundant brain metabolites, including myo-inositol, glutamine and choline which are enriched in glial cells. In a model of selective astrocyte reactivity in the rat brain, myo-inositol and choline levels are higher whereas glutamine levels are lower than in controls, suggesting that reactivity leads to the complex re-structuring of metabolic pathways (Carrillo-De Sauvage et al., 2015). High concentrations of myo-inositol are also commonly observed in ND models and patients, which correlates with neuroinflammation (Choi et al., 2007). However, the exact contribution of reactive astrocytes to these NMR signals is unknown because of the concomitant activation of microglial cells or other pathological events in ND.

More cellular selectivity may be achieved by MRS techniques after the infusion of ^{13}C-labeled metabolic substrates such as glucose or acetate. Indeed, acetate is preferentially oxidized by astrocytes and its metabolic fate can be monitored by MRS (De Graaf et al., 2011). Furthermore, the rate of astrocytic tricarboxylic acid cycle and of the glutamate/glutamine cycle can be estimated by modeling (Lebon et al., 2002). ^{13}C-acetate injection coupled with *ex vivo* MRS analysis was performed recently in several rodent models of AD (see Section Cholesterol Metabolism). ^{13}C-MRS may be translated to the clinics although it remains quite an expensive and sophisticated approach (Ross et al., 2003).

Reactive Astrocytes as Therapeutic Targets

The above-mentioned changes in reactive astrocytes make these cells alternative or complementary therapeutic targets to neurons for ND (Escartin and Bonvento, 2008). For example, strategies enhancing glutamate uptake in astrocytes may prevent excitotoxicity, which is common to all ND (Soni et al., 2014). High-throughput screening identified β-lactam antibiotics as potent inducers of glutamate uptake by astrocytes (Rothstein et al., 2005). The β-lactam antibiotic ceftriaxone is neuroprotective *in vitro* and *in vivo* in models of ALS (Rothstein et al., 2005) and HD (Miller et al., 2008). A phase I clinical trial with ceftriaxone in ALS patients gave promising results, but they were not confirmed in the phase II-III stage (Cudkowicz et al., 2014). Another astrocyte-based therapeutic strategy involves grafting astrocyte progenitors close to vulnerable neurons to provide them with global support (Lepore et al., 2008b). Interestingly, some pharmacological agents tested or used in clinics to target neurons may also affect astrocyte functions. Indeed, neurons and astrocytes share many membrane receptors, transporters and signaling pathways. For example, activators of the Nrf2 pathway like curcumin may enhance antioxidant defense in the brain by acting within astrocytes (Vargas and Johnson, 2009).

Therapeutic strategies tested so far for ND have largely focused on neurons and have been mostly unsuccessful to date (Huang and Mucke, 2012; Wild and Tabrizi, 2014). Only symptomatic treatments are offered to patients and their efficacy of some decreases with disease progression (e.g., acetylcholine esterase inhibitors for AD, L-DOPA supplementation for PD). No treatment truly prevents neurons from degenerating. In light of their many actions on neurons, strategies targeting reactive astrocytes may effectively sustain neuronal function and hence survival during ND. However, given the complex changes that occur in reactive astrocytes during ND, complete ablation of astrocyte reactivity may be counterproductive because these cells also display beneficial adaptative changes during disease. Identifying the complex interplay between shared intracellular pathways mediating reactivity and disease specific signals may enable the design of selective therapeutic cocktails to engage reactive astrocytes in protective actions (**Figure 3**).

Conclusions

Overall, this review illustrates the multifaceted and complex roles of reactive astrocytes during ND. Astrocyte reactivity appears as a conserved response that is initially beneficial but is later corrupted by disease-specific alterations. Huge progress has been made recently as a result of the heightened interest in glial cells, and the development of innovative and cell type-specific approaches. However, these cells remain enigmatic, and many aspects of their physiology need to be clarified. Although the molecular pathways leading to astrocyte reactivity during ND have been described, it is crucial to elucidate what disease-, region- and environmental-specific mechanisms control the functional outcomes associated with astrocyte reactivity (**Figure 3**). In any case, considering reactive astrocytes as key partners in neuronal dialog during ND opens new avenues for neuroscience and biomedical research.

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HYPOTHESIS AND THEORY
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Role of TGFβ signaling in the pathogenesis of Alzheimer's disease

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Aging is the main risk factor for Alzheimer's disease (AD); being associated with conspicuous changes on microglia activation. Aged microglia exhibit an increased expression of cytokines, exacerbated reactivity to various stimuli, oxidative stress, and reduced phagocytosis of β-amyloid (Aβ). Whereas normal inflammation is protective, it becomes dysregulated in the presence of a persistent stimulus, or in the context of an inflammatory environment, as observed in aging. Thus, neuroinflammation can be a self-perpetuating deleterious response, becoming a source of additional injury to host cells in neurodegenerative diseases. In aged individuals, although transforming growth factor β (TGFβ) is upregulated, its canonical Smad3 signaling is greatly reduced and neuroinflammation persists. This age-related Smad3 impairment reduces protective activation while facilitating cytotoxic activation of microglia through several cellular mechanisms, potentiating microglia-mediated neurodegeneration. Here, we critically discuss the role of TGFβ-Smad signaling on the cytotoxic activation of microglia and its relevance in the pathogenesis of AD. Other protective functions, such as phagocytosis, although observed in aged animals, are not further induced by inflammatory stimuli and TGFβ1. Analysis *in silico* revealed that increased expression of receptor scavenger receptor (SR)-A, involved in Aβ uptake and cell activation, by microglia exposed to TGFβ, through a Smad3-dependent mechanism could be mediated by transcriptional co-factors Smad2/3 over the MSR1 gene. We discuss that changes of TGFβ-mediated regulation could at least partially mediate age-associated microglia changes, and, together with other changes on inflammatory response, could result in the reduction of protective activation and the potentiation of cytotoxicity of microglia, resulting in the promotion of neurodegenerative diseases.

Keywords: aging, cytokines, Glia, MAPK, NFκB, neurodegenerative diseases, neuroinflammation, transforming growth factor-β

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OVERVIEW: GLIAL CELLS AND NEUROINFLAMMATION

Homeostasis of the nervous system is maintained by the finely tuned interaction of glial cells and neurons, involving a complex network of signaling pathways. Inflammation, a primarily beneficial process mediated by the activation of glia in response to injury, illness or infection, allows for the elimination of harmful stimuli and the repair of damaged tissue. However, this process can become dysregulated, when the activating stimulus cannot be removed, or in the context of a maintained inflammatory environment, as observed in aging (von Bernhardi et al., 2010).

Thus, neuroinflammation can also be a self-perpetuating deleterious response, with persistent activation of glia, sustained release of inflammatory mediators, and increased oxidative and nitrosative stress; becoming a source of additional injury to host cells. Chronic neuroinflammation plays a role in a number of neurodegenerative diseases (Block and Hong, 2005; von Bernhardi et al., 2007; Gao and Hong, 2008), inducing neuronal injury.

Microglia are the brain resident innate immune system (Hemmer et al., 2002; Ransohoff and Perry, 2009; Rivest, 2009). When stimulated, microglia activate and change their functional properties (Liu et al., 2001; von Bernhardi and Eugenin, 2004; Lue et al., 2010). They can sense and respond to a wide range of stimuli, including central nervous system (CNS) trauma, ischemia, infection, toxic, and autoimmune insults (Kreutzberg, 1996; Streit, 2002; Kim and de Vellis, 2005; Schwab and McGeer, 2008; Lue et al., 2010; von Bernhardi et al., 2010). In fact, microglia are activated in virtually all CNS diseases (Kreutzberg, 1996; Hanisch and Kettenmann, 2007; Neumann et al., 2009). They are the main producers of a broad spectrum of inflammatory mediators, such as eicosanoids, cytokines (Nakamura, 2002; Kim and de Vellis, 2005; Tichauer et al., 2007), chemokines, reactive oxygen species (ROS), nitric oxide (NO)·, small metabolite mediators, and proteases (α-antichymotrypsin and α-antitrypsin) (Benveniste et al., 2001; Nakamura, 2002; Streit, 2002; Li et al., 2007; Tichauer et al., 2007; Neumann et al., 2009; Lue et al., 2010).

Other glial cell type, astrocytes, which will not be discussed in this work, share several functions with microglia. They are important for neurotrophic support and metabolism, synaptic regulation and several other functions, in addition to their participation in β-amyloid (Aβ) *clearance* (Rossner et al., 2005; Murgas et al., 2012). Inflammatory mediators regulate the innate immune defense, induce bystander damage, and modify synaptic function (Aldskogius et al., 1999; Selkoe, 2002; Di Filippo et al., 2008) according to environmental conditions (Li et al., 2007; von Bernhardi, 2007). Depending on the activation context, microglia secrete inflammatory cytokines such as interleukin 1β (IL1β), tumor necrosis factor α (TNFα) and interferon gamma (IFNγ), and reactive species (Kettenmann et al., 2011), as well as regulatory cytokines like interleukin 10 (IL10) and transforming growth factor β (TGFβ1; Nakajima et al., 2007; Sierra et al., 2007; Welser-Alves and Milner, 2013). Inflammatory cytokines trigger the production of several inflammatory factors that could affect neuronal function. In response to IFNγ, for example, glia produce NO· by up-regulation of inducible nitric oxide synthase (iNOS) and release superoxide radicals ($O_2 \cdot^-$) by a nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase mediated mechanism (Hu et al., 1995; Calabrese et al., 2007). Neuroinflammation affects neuron-glia crosstalk and establishes interactions with oxidizing agents through redox sensors in enzymes, receptors, and transcription factors, all of which can affect neuronal function (Liu et al., 2012), inducting neurodegeneration (Raj et al., 2014). Oxidative stress, in turn, further increases inflammatory cytokines, creating a vicious cycle (Rosales-Corral et al., 2010), with profound impact in cell homeostasis and survival (Satoh and Lipton, 2007).

Astrocyte and microglia activation occur through the phosphorylation of MAPKs and the activation of nuclear factor *kappa* B (NFκB) pathway, inducing the expression of inflammatory mediators (Van Eldik et al., 2007; Glass et al., 2010; Heneka et al., 2010). MAPKs include extracellular signal-regulated protein kinases (ERKs) and stress activated protein kinases c-Jun NH2-terminal kinase (JNK) and P38. Activated MAPKs exert their actions both in the cytoplasm and translocating into the nucleus, phosphorylating transcription factors. Noteworthy, ERK and P38 appear to be key actors in the production of free radicals (Bhat et al., 1998; Marcus et al., 2003; Qian et al., 2008). The ERK pathway is regulated by pro- and anti-inflammatory cytokines, determining the timing of microglia activation (Saud et al., 2005; Glass et al., 2010). In addition, P38 is involved in the production of NO· by up-regulating iNOS (Saha et al., 2007; Munoz and Ammit, 2010), and enhances the expression of inflammatory cytokines, such as TNFα, through both transcriptional and post-transcriptional mechanisms. P38 can induce transcription of the TNFα gene by increasing activator protein-1 (AP-1) activity (Spriggs et al., 1992) and enhances its production by increasing the stability and translation of TNFα mRNA (Dean et al., 2004).

Activation of microglia shows a broad functional spectrum associated with specific expression patterns of cytokines and their receptors (Town et al., 2005). Depending on the stimuli they receive, they show different activation profiles (Gordon, 2003; Martinez et al., 2008; Mosser and Edwards, 2008), including: (i) classical activation (M1 activation), which under certain conditions will be cytotoxic; (ii) alternative phagocytic/neuroprotective (M2 activation; Gordon, 2003; Martinez et al., 2008); or (iii) regulatory activation (Mosser and Edwards, 2008). Activation of interferon-regulatory factor 5 (IRF5), defines commitment to the M1 macrophage lineage (Satoh et al., 2010), while IRF4 controls M2 polarization (Satoh et al., 2010; Krausgruber et al., 2011). In M2 macrophages, activation of NFκB-p50 appears to be associated with the inhibition of M1-polarizing genes (Porta et al., 2009). M2-type induction, through secretion of IL4, IL10 and TGFβ, promotes humoral immune responses and down-regulates M1 responses, inhibiting many macrophage inflammatory functions (Town et al., 2005). A third group, regulatory macrophages, arises at later stages and have a primary role limiting inflammation (Mosser, 2003; Lucas et al., 2005; Mosser and Edwards, 2008).

AGE-RELATED CHANGES OF MICROGLIAL CELLS

The term "inflamm-aging" was coined in reference to the state of mild chronic inflammation (Franceschi et al., 2000) observed in aged individuals, functionally characterized by a reduced capability to deal with stressing stimuli. The age-related immune changes, known as immune-senescence (Larbi et al., 2008), would be also induced by cumulative low-level inflammation, which induces changes in gene expression related to inflammation and immune response (Lee et al., 2000; de Magalhães et al., 2009), increases plasmatic levels of inflammatory cytokines

(Singh and Newman, 2011), and activates inflammatory intracellular pathways (Helenius et al., 1996).

In aged animals, protein homeostasis is impaired at multiple levels, including chaperone-mediated protein folding and stability, protein trafficking, protein degradation and autophagy. A major consequence of these impairments is the aggregation of abnormal proteins, which are related to neurodegenerative diseases, such as Parkinson's disease (PD) and Alzheimer's disease (AD; Taylor and Dillin, 2011). Aged microglia undergo multiple functional changes (reviewed in von Bernhardi et al., 2015), impacting the neuronal environment and promoting development of cognitive impairments (**Figure 1**). Among these changes, microglial cell production of ROS and inflammatory cytokines could contribute to the onset of chronic neurodegenerative diseases (von Bernhardi, 2010). Decline of lysosomal and mitochondrial functions results in an exacerbated generation of ROS and inflammatory mediators by microglia. Moreover, aged microglia show a decreased ability to phagocytose Aβ in comparison with young microglia (Floden and Combs, 2011).

In aging, activated microglia remain as the principal source of inflammatory molecules and oxidative products of the CNS (Pawate et al., 2004; Qin et al., 2005; Hayashi et al., 2008). Both basal production of IL-6 and lipopolysaccharide (LPS)-induced secretion of IL-6 and IL1β are higher in aged microglia than in younger cells (Ye and Johnson, 1999; Sierra et al., 2007). In fact, mild stimulatory events or minor injuries, otherwise innocuous, could induce a robust and potentially damaging response. Thus, a stimulus that normally would trigger a protective response, in presence of age-related impairment of regulation could determine a persistent activation, associated, for example, to oxidative stress (von Bernhardi, 2007; Herrup, 2010). Similarly, aged microglia become more inflammatory than their younger counterparts upon systemic inflammatory stimulation, exacerbating damage (Combrinck et al., 2002; Cunningham et al., 2005; Godbout et al., 2005; Sierra et al., 2007). Accordingly, when exposed to endotoxin, microglia derived from adult mice secrete high amounts of ROS, whereas those from young animals mostly produce NO (Tichauer et al., 2014). Those effects depend, at least partly, on the upregulation of Toll-like receptors (TLRs), increased expression of the TLR4 co-receptor CD14 (Letiembre et al., 2007), changes in TLR4 signaling, and changes on the expression profile of scavenger receptors (SRs; Yamamoto et al., 2002; Hickman et al., 2008).

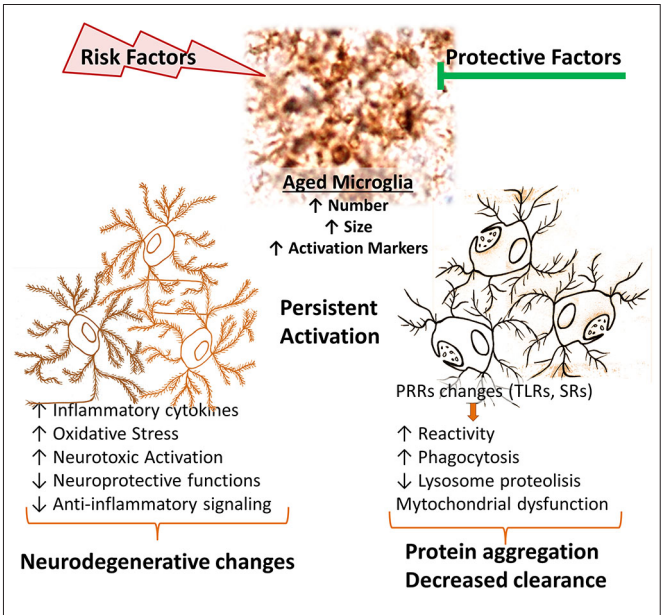

FIGURE 1 | Age-related changes of microglial cell function. In aged brains, there is an increased number, size and activation of microglia. Age-related microglia changes depend both on gained and lost functions. Diverse stimuli or injury processes can further promote an inflammatory environment, promoting cytotoxic microglial cell activation. Aged microglia show increased basal phagocytic activity, although a reduced capacity to induce phagocytosis when stimulated, together with reduced lysosomal activity, resulting in a decreased clearance activity. They also have increased production of inflammatory cytokines and reactive species. Those changes result in a shift of balance towards decreased protective functions and an increased neurotoxicity. PRRs, pattern recognition receptors; SRs, scavenger receptors; TLRs, Toll-like receptors.

The activation of TLRs, CD14, and SRs by specific ligands is associated with microglial cell activation (Godoy et al., 2012; Murgas et al., 2012; Nakajima et al., 2007), production of inflammatory mediators, and uptake of macromolecules, including Aβ (Alarcón et al., 2005). There is conflicting evidence regarding the effect of age on phagocytosis. In contrast with reports indicating that microglia from aged mice have a decreased ability to phagocytose Aβ compared with young mice (Floden and Combs, 2011), we observed that basal phagocytosis of aged microglia is slightly increased compared with that from young mice, but phagocytosis fails to be induced by TGFβ (Tichauer et al., 2014) or LPS (Cornejo et al., 2014). Class A SR (SR-A) appears to play a key role for Aβ internalization by microglia (Chung et al., 2001) and degradation by cathepsin B (Yang et al., 2011), and for activation of microglia (Cornejo and Von Bernhardi, 2013). SR-A participates in the phagocytosis of Aβ and other anionic molecules, leading to the production of ROS (El Khoury et al., 1996). In AD, microglia expressing SR-A have been observed in close association with senile plaques (Honda et al., 1998; Bornemann et al., 2001). SR-A inhibition appears to increase Aβ burden in the brain of AD patients, potentially promoting neurotoxic effects and disease progression (Frenkel et al., 2013). The expression of these receptors decreases in the brain of aging animal models of AD (Hickman et al., 2008). Age-related changes in the expression of receptors involved in inflammatory activation could account for part of the function impairment of microglia, and provide insight regarding cell phenotypes that could play a role in the pathophysiological changes leading to neurodegenerative diseases. Given the various protective functions served by microglia, rather than seeking the inhibition of microglia, the regulation of those receptors involved in microglia activation could reduce some of the deleterious effects secondary to age-related microglial cell dysfunction.

MICROGLIA AND ALZHEIMER'S DISEASE

Neuropathology in AD is characterized by the deposition of Aβ plaques and neurofibrillary tangles, constituted

by hyper-phosphorylated tau, in the brain parenchyma (Hardy and Selkoe, 2002), intimately associated with activated microglia and astrocytes (Kim and de Vellis, 2005; Jellinger, 2006; Heneka and O'banion, 2007; von Bernhardi, 2007; von Bernhardi et al., 2010), and loss of synapses and neurons (Uylings and de Brabander, 2002). Worth mentioning, Aloise Alzheimer already stated in the early 1900's that plaques and tangles probably were markers of an upstream process rather than the disease cause (Davis and Chisholm, 1999).

Microglial cell reactivity to Aβ and phagocytic activity are modulated by astrocytes, attenuating the cytotoxic response of microglia (DeWitt et al., 1998; von Bernhardi and Ramírez, 2001). However, modulation is abolished when microglia exposed to Aβ was previously primed (von Bernhardi and Eugenin, 2004), condition in which microglia show increased cytotoxicity, Aβ precursor protein (APP) synthesis, Aβ aggregation, and impairment of the uptake and degradation of Aβ compared with non-activated microglia (Rogers et al., 2002; von Bernhardi et al., 2007; Ramírez et al., 2008).

P38 appears to be involved in several pathological processes of AD. P38 becomes activated at early stages of the disease (Pei et al., 2001; Sun et al., 2003), being one of the kinases that phosphorylates specific sites in tau (Feijoo et al., 2005; Churcher, 2006). Inhibition of P38 abolishes Aβ-induced neuronal death *in vitro* (Zhu et al., 2005). P38 and NFκB appear to have a critical role for glial cell activation. Activation of those pathways are involved in Aβ-mediated induction of NO and TNFα production by glia (O'Neill and Kaltschmidt, 1997; Akama et al., 1998; Saha et al., 2007; Munoz and Ammit, 2010), which correlates with Aβ-induced cognitive impairment (Tran et al., 2001; Wang et al., 2005; He et al., 2007; Medeiros et al., 2007). Stimulation by Aβ induces a transient phosphorylation of P38, and a slower activation of NFκB (Flores and von Bernhardi, 2012) depending on the up-regulation of the transcriptional activity of NFκB by P38 (Saha et al., 2007), which contributes to neuroinflammation by activating AP-1 and by stabilizing mRNA and enhancing activity of NFκB. Production of TNFα and NO have different temporal profiles, in agreement with the early induction of cytokines by Aβ that appears to be needed for the subsequent induction of iNOS expression (Akama and Van Eldik, 2000).

The "Glial Dysfunction Hypothesis"

The consideration that brain innate immune response can be involved in the genesis of neurodegenerative diseases (Nguyen et al., 2002; Bjorkqvist et al., 2009; von Bernhardi et al., 2010), lead to re-consider the role of Aβ and propose glia as a leading factor in AD pathology (**Figure 2**; von Bernhardi, 2007). However, for most scientists who adhere to the "amyloid cascade hypothesis", Aβ is viewed as the cause of AD and neuroinflammation is considered just a consequence of glia activation (Akiyama et al., 2000; Heneka and O'banion, 2007; Hirsch and Hunot, 2009).

Astrocytes modulate microglia cytotoxicity and phagocytosis of Aβ (von Bernhardi and Ramírez, 2001). TGFβ1, secreted by astrocytes and neurons among other cells, regulates microglia activation, reducing release of inflammatory cytokines and reactive species (Chen et al., 2002; Mittaud et al., 2002; Herrera-Molina and Von Bernhardi, 2005; Herrera-Molina et al., 2012), protecting neuronal cells *in vitro* (Hu et al., 1995; Lieb et al., 2003; Herrera-Molina and Von Bernhardi, 2005) and promoting phagocytosis (Wyss-Coray et al., 2001). However, chronically activated microglia show a reduced response to such a modulation (von Bernhardi and Eugenin, 2004), showing instead an increased cytotoxicity and impaired uptake of Aβ (von Bernhardi et al., 2007; Ramírez et al., 2008). Regulation by TGFβ1 depends on a Smad3-mediated mechanism (Flores and von Bernhardi, 2012; Tichauer and von Bernhardi, 2012). Age-related inhibition on the activation of Smad has a profound effect on the regulation of microglia by TGFβ (Tichauer et al., 2014).

In the context of the "glial cell dysregulation hypothesis" neurotoxicity is not viewed as a consequence of hyperactive but rather of "mis-active", dysfunctional microglia (von Bernhardi, 2007). Solid evidence show that adequately activated microglia are needed as scavenger cells in the CNS, participating for example in Aβ clearance (Paresce et al., 1996;

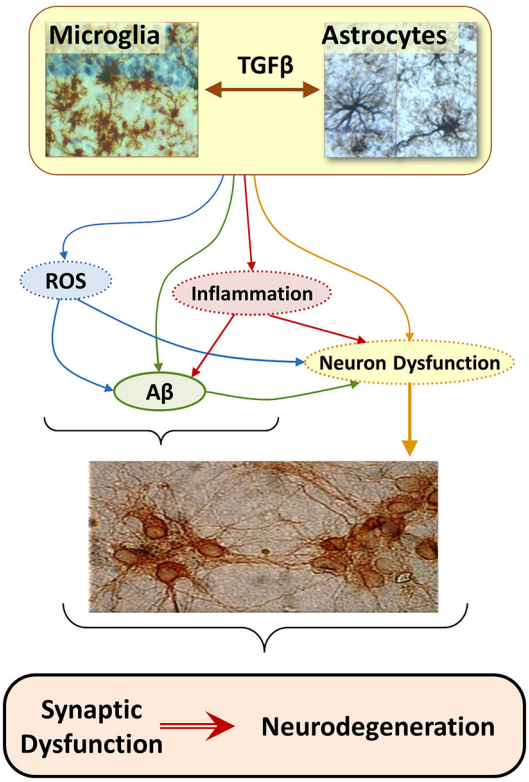

FIGURE 2 | The "Glial Cell Dysregulation Hypothesis" for Alzheimer's disease (AD). The glial cell dysregulation hypothesis proposes that AD has its cause on changes on the activation and impaired regulation of microglia, which become increasingly cytotoxic decreasing their protective functions. Microglia is under the regulation of astrocytes which, among other factors, secrete TGFβ. Inflammatory activation, secondary to aging and to certain forms of pathological stimuli, can result in glial cell dysregulation. Dysregulated glia, though the abnormal release of cytokines, reactive species, and other mediators, contributes to the increased expression of Aβ precursor protein (APP) and aggregation of Aβ, as well to functional and degenerative changes of neurons, perpetuating abnormal activation of glia, synaptic dysfunction and cell damage.

Alarcón et al., 2005). However, lost response to normal regulatory feedback and/or an impaired ability to clear Aβ (Paresce et al., 1997; von Bernhardi, 2007), could lead microglia to develop predominantly cytotoxic features, establishing an inflammatory environment with increased oxidative stress, conditions that are amyloidogenic (Gabuzda et al., 1994; Wang et al., 2004), and promote neuron dysfunction (**Figure 2**). Thus, microglia, initially protective, would become chronically activated and show an exacerbated reactivity, contributing to brain cytotoxicity and neurodegeneration (Nguyen et al., 2002; Wyss-Coray and Mucke, 2002; Saud et al., 2005).

Regulation of glial cell activation appears to be impaired under sustained inflammatory stimulation (Ramírez et al., 2008), as those observed in the aged brain (Tichauer et al., 2014). Whereas inflammatory activation of glia by Aβ is relatively mild in culture, it is markedly potentiated in primed glia (von Bernhardi et al., 2007). Likewise, attenuation of microglia reactivity by astrocytes is greatly reduced when glia are exposed to inflammatory conditions (von Bernhardi and Eugenin, 2004). The priming of glia, rather than Aβ, could be the main trigger for abnormal glia activation in response to a stimulus that normally would not produce a sustained robust activation, a condition we named "dysregulated glia" (von Bernhardi, 2007). In that sense, in contrast to microglia normally reacting mildly when exposed to Aβ, microglia have an enhanced activation under chronic inflammatory conditions. Enhanced activation in turn could result in an increased cytotoxicity (von Bernhardi et al., 2015).

ROLE OF TGFβ IN THE "GLIAL CELL DYSREGULATION HYPOTHESIS"

TGFβ is present in three isoforms, TGFβ1, TGFβ2 and TGFβ3. Astrocytes secrete preferentially TGFβ1. Increased production of TGFβ1 in response to inflammatory conditions is one of the regulatory mechanisms secondary to cell activation (Herrera-Molina and Von Bernhardi, 2005) that limits the temporal and spatial extent of neuroinflammation (Ramírez et al., 2005; Saud et al., 2005), and neurotoxicity (Eyupoglu et al., 2003). The modulation exerted by TGFβ1 is mediated by the activation of Smad3, which is down regulated in AD patients (Colangelo et al., 2002) and aged mice (Tichauer et al., 2014), and the activation of ERK (Saud et al., 2005), which also appears to be neuroprotective under certain conditions (Zhu et al., 2002, 2004). In addition to Smad, dynamic regulation of PI3K and MAPK, which are activated as part of the TGFβ signaling pathway (**Figure 3**) as well as with other inflammatory cytokines, can be key factors for cell viability and the regulation of inflammation. In the following sections, we will discuss how inhibition of Smad3 pathway and increased levels of TGFβ, as observed in aging,

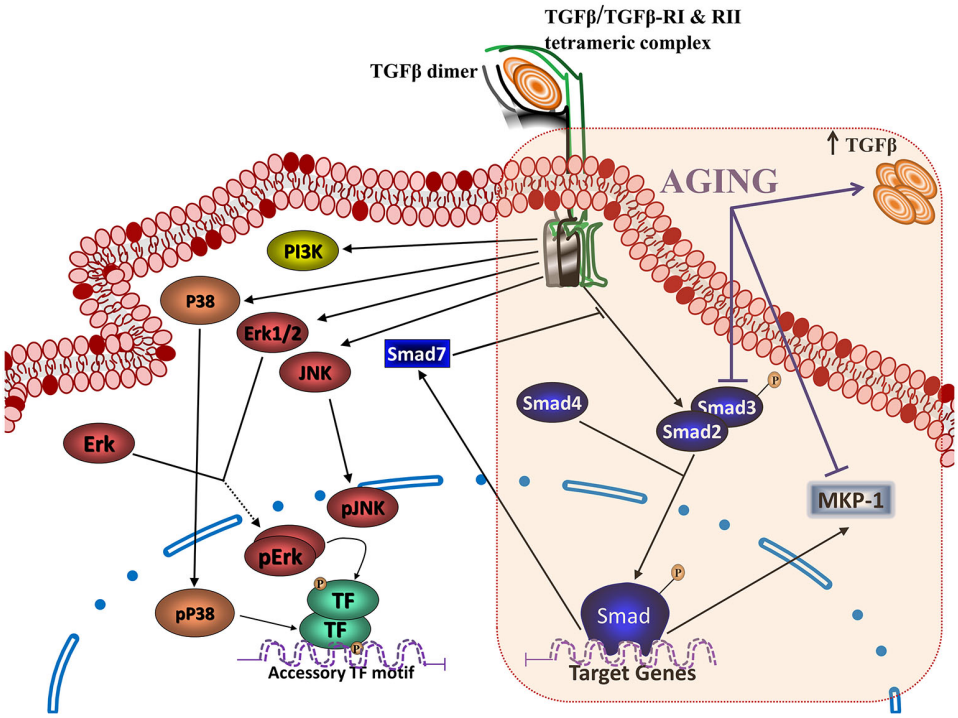

FIGURE 3 | Transforming growth factor β (TGFβ) signaling pathways and aging. Binding of TGFβ to the type II TGFβ receptor dimer (TGFβRII) triggers recruitment of type I receptor dimer (TGFβRI), generating the heterotetrameric TGFβ receptor capable of activating the intracellular signaling pathways for TGFβ action. The activation of this complex activates (a) the canonical TGFβ signaling, with the phosphorylation of Smads 2 and/or 3 dimers, which bind to Smad 4 and translocate into the nucleus to regulate gene transcription, together with the activation of (b) non-canonical TGFβ signaling, which includes activation of MAPKs (ERK, extracellular signal-regulated protein kinase; JNK, c-Jun NH2-terminal kinase; and P38) and PI3K. Aging results in several changes on TGFβ signaling, including an increased production of TGFβ, as well as inhibition of the Smad pathway and activation of the phosphatase MAPK phosphatase (MKP-1).

could modify regulatory signals, leading to glia dysregulation. The lack of inhibition of microglia inflammatory activation by TGFβ could result in cytotoxicity and neurodegenerative changes as those observed in AD. Impairment of TGFβ-Smad3 signaling could reduce the capability of microglia to deal with injury, inhibiting beneficial responses while inducing progression towards a more inflammatory state in the aging brain (Franceschi et al., 2000). This neuroinflammatory state could favor the development of age-related neurodegenerative diseases (Larbi et al., 2008), affecting the regulation of several inflammatory signaling pathways as we will discuss on the following sections.

TGFβ SIGNALING PATHWAYS AND THEIR REGULATION

TGFβ1 is a pleiotropic cytokine and a potent regulator of neuroinflammation and cytotoxicity. Many beneficial effects depend on the regulation of microglial cell activity by TGFβ1 (Hu et al., 1995; Lieb et al., 2003; Herrera-Molina and Von Bernhardi, 2005). In the brain, TGFβ1 is associated with neuroprotection in excitotoxicity, hypoxia, and ischemia, as well as with interfering with cell death cascades induced by compounds such as Aβ (Caraci et al., 2011). TGFβ1 secreted at the injury site promotes microglia recruitment, allowing for an efficient removal of the noxious stimulus. Stimulation of hippocampal cultures with LPS and IFNγ increases the secretion and activation of TGFβ1 (Uribe-San Martén et al., 2009). TGFβ1 secreted by hippocampal neurons and astrocytes *in vitro* (Ramírez et al., 2005; Tichauer et al., 2007) and microglia (Welser-Alves and Milner, 2013) decreases release of inflammatory mediators, O_2^- and NO by microglia (Chen et al., 2002; Mittaud et al., 2002; Herrera-Molina and Von Bernhardi, 2005; Saud et al., 2005; Herrera-Molina et al., 2012), and increases viability of neurons (Hu et al., 1995; Lieb et al., 2003; Herrera-Molina and Von Bernhardi, 2005). Inhibition of LPS-induced macrophage and microglia activation by TGFβ1 is regulated in a Smad3-dependent manner (Werner et al., 2000; Le et al., 2004). The same mechanism is also involved in astrocyte-mediated neuroprotection against N-methyl-D-aspartate (NMDA)-induced neuronal injury (Docagne et al., 2002; Katayama et al., 2010) that results from the sustained activation of the ERK pathway, playing also a pivotal role in astrogliosis (Chu et al., 2004).

TGFβ signaling pathway (**Figure 3**) is activated when TGFβ interacts and induces assembly of an heterotetrameric receptor, containing two serine/threonine kinase receptors, type II and type I (Rahimi and Leof, 2007). In mammals, there are five type II receptors, TβRII, ActR-II, ActR-IIB, BMPR-II, AMHR-II, and seven type I receptors, the *activin receptor-like kinases 1-7 (ALK1-7*; Rahimi and Leof, 2007). In canonical TGFβ signaling pathway, ligand binding induces type II receptor to phosphorylate and activate type I receptor, which then phosphorylates receptor activated members of the Smad family (*R-Smad*). TGFβ activates the phosphorylation of Smad2 and Smad3, their assembly with a Smad common-partner, Smad4, and the nuclear translocation of the heterotrimeric complex (Smad2/Smad2/Smad4, Smad3/Smad3/Smad4 or Smad2/Smad3/Smad4). In the nucleus, the complex interacts with AGAC enriched Smad binding elements (SBE) on the DNA, acting as a transcriptional co-activator (Wrighton et al., 2009). They bind to specific sequences where they can activate or inhibit transcription, regulating gene expression of target genes associated with inflammatory activation (Schmierer and Hill, 2007; Heldin and Moustakas, 2012), including that of Smad 7 (Ross and Hill, 2008) that belongs to a third type, the inhibitory Smads (*I-Smads*: Smad6/7), which are an endogenous inhibitory system.

Smad pathways act as co-factors coupled to master transcriptional factors to direct gene expression in a cell-specific manner (Mullen et al., 2011). The main regulation of TGFβ signaling occurs on Smad. For example, Smad2/3 can be acetylated by transcriptional co-activators such as p300 and CREB-binding protein (CBP) in a TGFβ-dependent way (Simonsson et al., 2006; Tu and Luo, 2007). This post-transduction modification favors binding of the Smad complex to DNA, and the transcription of its target genes (Simonsson et al., 2006). Also, phosphorylation of Smad3 mediated by CDK2 and CDK4 inhibits its transcriptional activity (Liu, 2006; Buxton and Duan, 2008), and Smad3-PIAS (*protein inhibitor of activated STAT*) interaction suppresses Smad3 activation by TGFβ and favors its SUMOilation (Imoto et al., 2003). These and others post-transduction modifications, such as MAPKs-mediated phosphorylation (see below), can also regulate Smad activity and therefore TGFβ-mediated transcription (Ross and Hill, 2008).

In addition to TGFβ-mediated Smad activation, TGFβ activates a complex Smad-independent (TGFβ-non canonical pathways) signaling pathway (Weiss and Attisano, 2013; **Figure 3**). Non canonical signaling includes MAPK pathways ERK, P38 and JNK, and PI3K/Akt (Derynck and Zhang, 2003; Weiss and Attisano, 2013), and participate in many biological processes such as cell cycle inhibition, immunosuppression and neuroprotection, among others (Bosco et al., 2013).

REGULATION OF INFLAMMATION-RELATED GENES BY TGFβ

TGFβ orchestrates the expression of numerous genes associated with inflammation and the immune response. In the nervous system, the TGFβ pathway is involved in the regulation of genes associated with cell cycle, cell proliferation, preservation of neural progenitor cells, oligodendroglia and neuronal differentiation, neuron survival and function, and the several neurotransmission-related genes (Kandasamy et al., 2014). TGFβ has a role in adult neurogenesis (He et al., 2007), and in the differentiation of adult neural progenitor cells, inducing the expression of several voltage-dependent channel subunits (Kcnd3, Scn1b, Cacng4, and Accn1) and other neuronal proteins, such as Cadps, Snap25, Grik4, Gria3, Syngr3, Gria4, doublecortin (DCX), Nrxn1, Sept8, and Als2cr3, suggesting that TGFβ participates, at least in part, in the induction of a functional

neuronal phenotype, (Kraus et al., 2013). In addition, TGFβ participates in cell migration by modulating the expression of cell adhesion proteins such as nCAM, integrin α3, αV and β1 (Siegenthaler and Miller, 2004; Milner, 2009).

TGFβ regulates MHC class II expression in astrocytes, (Johns et al., 1992). It also regulates the expression of some constituents of its own pathway, including receptors type I TGFβ receptor (TGFβRI) and type II TGFβ receptor (TGFβRII), Smad7 and Smad3 (Ma et al., 2007; Qin et al., 2009). Microglia treated with TGFβ show a reduced expression of the immune mediators CCL3, CCL2, IL1a, IL1rl2, CCR5 and CD11c (Abutbul et al., 2012; Butovsky et al., 2014), and upregulation of CX3CR1, CSF3 and TLR3 expression (Chen et al., 2002; Butovsky et al., 2014), as well as SRs, which will be discussed in a later section.

AGE-RELATED CHANGES ON THE TGFβ PATHWAY

In the brain, TGFβ favors cell survival, modulating the expression of Bad, Blc-2 and Bcl-x1 as a mechanism of neuroprotection against apoptosis (Dhandapani and Brann, 2003). TGFβ also participates in the regulation of temporal transition between early and late phases of neurogenesis and the regulation of the stem cell potency (Dias et al., 2014). Increased levels of TGFβ have been reported in several brain areas, including the hippocampus and hypothalamus, during aging (Bye et al., 2001; Werry et al., 2010). Cells showing increased production of TGFβ apparently do not include neurons, since TGFβ transcripts level are severely reduced in aged neurons (de Sampaio e Spohr et al., 2002).

Aging also affects the circadian variation of TGFβ expression. It has been reported a loss of the diurnal pattern of TGFβ expression, as well as a loss of the day/night expression of activated Smad3 compared with the pattern observed in young animals (Beynon et al., 2009) that have profound functional effects. In addition, the pedunculopontine (PPT) nucleus of aged rats, a structure related to sleep and cognitive functions, shows an over activation of the TGFβ-Smad signaling pathway that appears to be involved in sleep-related memory impairment in aging (George et al., 2006).

There are age-related changes on TGFβ signaling at several levels. Some depend on changes on the level or the pattern of secretion of TGFβ, or in its canonical signaling pathway, Smad (**Figure 3**). Others depend on changes on the interaction of TGFβ with other inflammatory mediators or their transcription factors, such as IFNγ and NFκB, or on regulatory components, such as MAPK phosphatases (MKP-1). Finally, there are age-related changes on its regulation on cellular processes, as observed with stem cells. The various changes will be discussed on the following sections.

Aging-Related Inhibition of the TGFβ-Smad Pathway

TGFβ1, produced by astrocytes *in vitro*, decreases microglial NO and ROS production induced by LPS and IFNγ (Herrera-Molina and Von Bernhardi, 2005). Induction of both NO and ROS is prevented by TGFβ1 in neonatal, but not in adult animals. Therefore, response to inflammatory stimulation appears to become more oxidative, and for that reason, potentially more cytotoxic in aged animals (Tichauer et al., 2014). Moreover, modulation by TGFβ1 also is abolished in microglia obtained from animals previously exposed to inflammatory conditions (Tichauer et al., 2014).

TGFβ-Smad pathway is very important on the regulatory and neuroprotective effect of TGFβ1 (Derynck and Zhang, 2003), being involved in the induction of the quiescent phenotype of microglia (Abutbul et al., 2012). Activation of the TGFβ1-Smad3 pathway induces glial cells to produce MKP-1, a phosphatase exerting negative regulation on inflammatory activation that inhibits Aβ-induced MAPK and NFκB signaling (**Figure 4**), and decreases production of TNFα and NO (Flores and von Bernhardi, 2012). MKP-1 appears to preferentially dephosphorylate P38 and JNK, but it also dephosphorylates ERK in some cell types (Liu et al., 2007; Boutros et al., 2008).

TGFβ1-dependent regulatory mechanisms are impaired in aging. Aged microglia show a basal activated status, which has been linked to neuronal damage, cognitive impairment, and an increased susceptibility to neurodegenerative diseases, such as AD (Block et al., 2007). Age-related alteration of TGFβ pathway includes changes in TGFβ release, Smad3 activation, and on microglial response induced by inflammatory stimuli in the hippocampus of aged mice, as well as abolition of TGFβ-induced phagocytosis (Tichauer and von Bernhardi, 2012) by aged microglia (Tichauer et al., 2014). In addition, Smad2/3 expression pattern is altered, showing increased expression of Smad3 in aging. In contrast, Smad2 (Deltaexon3), a splice form of Smad2 that directly binds to the DNA, is highly expressed prenatally and in early postnatal life, but it diminishes with aging (Ueberham et al., 2009).

Both age and inflammatory status affect the amount and phosphorylation of Smad3 protein in mice hippocampus (Tichauer et al., 2014). Whereas 2-month old mice show a robust increase of Smad3 in the hippocampus after a systemic inflammatory stimulus, 12-month old animals maintain Smad3 at its increased basal level (**Figure 3**). Similarly, phosphorylation (activation) of Smad is not induced by inflammation in old animals (Tichauer et al., 2014). The activation of the Smad pathway in young animals could depend on the effective elevation of TGFβ1 levels induced by inflammatory stimulation (Wynne et al., 2010). The induction of Smad3 expression could depend on the activation of MAPK1 (Ross et al., 2007). In contrast, in adult mice, increased basal levels of TGFβ1 (Colangelo et al., 2002; Lukiw, 2004) maintains elevated Smad3, becoming unresponsive to new inflammatory stimulation. Increased levels of TGFβ1 with a reduced activation of Smad signaling can result in an unbalance between the various TGFβ1 activated pathways (Schmierer and Hill, 2007). Furthermore, considering that the non-Smad TGFβ1 pathways MAPKs and PI3K, also participate in inflammatory activation signal transduction, and their activation is not abolished in aged mice, inhibition of Smad could abolish the regulatory effect of TGFβ1 on inflammation, facilitating the cytotoxic activation of glia. The partial inhibition of TGFβ1-Smad3 signaling in aging could explain the persistent activation of microglia and mild

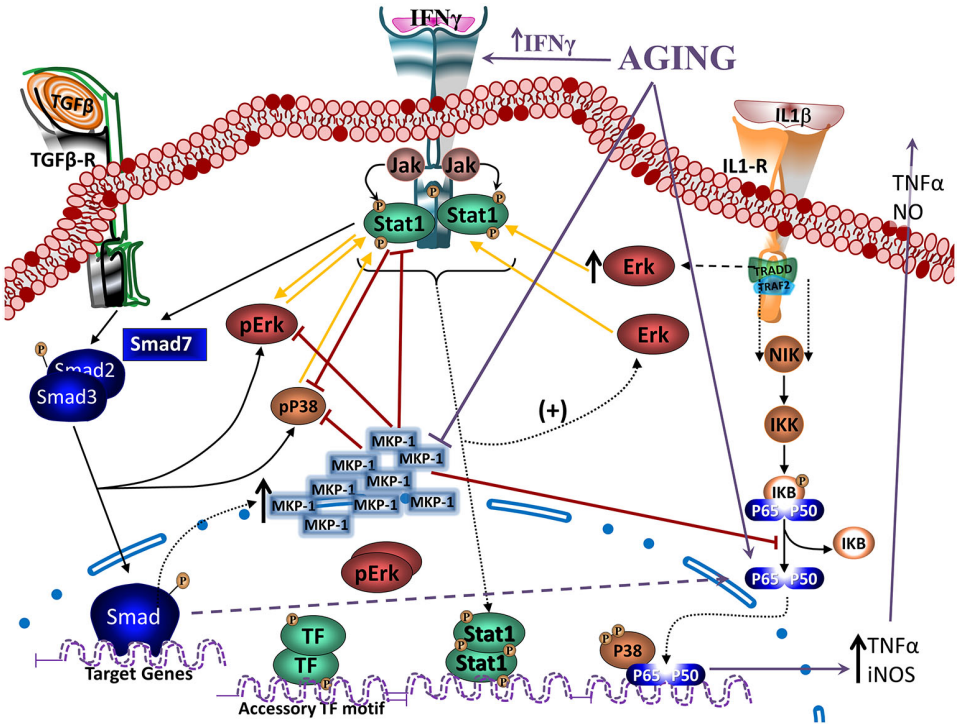

FIGURE 4 | Regulation of Janus activated kinase (JAK)-Stat and nuclear factor kappa B (NFκB) signaling by TGFβ through MKP-1. Modulation of Interferon gamma (IFNγ)- and Interleukin 1β (IL1β)- induced signaling pathways (as models of inflammatory activation) by TGFβ1 reveals, at least partially, the anti-inflammatory effects of TGFβ1. IFNγ activates JAK- signal transducer and activator of transcription-type-1 (STAT1) pathway, increasing pSTAT1tyr, pERKs and to a lesser extent pP38. ERK and P38 MAPKs potentiate STAT1 activation by phosphorylation of a serine residue. IFNγ-induced signaling pathway inhibits Smad signaling by increasing synthesis of its endogenous inhibitor Smad7. TGFβ1 is also able to activate MAPKs. Particularly, TGFβ1 can activate ERK and P38 and also, through the activation of the Smad3 pathway, induces an increase in MKP-1 expression. Particularly in microglia, TGFβ1 inhibits IFNγ-induced STAT1 activation via a MKP-1-mediated inhibition of ERK1/2. Cytokines, such as IL1β induces activation of glial cells and the production of TNFα and nitric oxide (NO) through the activation of P38 and NFκB pathways. TGFβ1, by inducing an increase in MKP-1 levels, inhibits P38 and NFκB pathways, reducing production of TNFα and NO. In aging, there are increased levels of IFNγ, inhibiting activation of the TGFβ1-Smad signaling pathway, as well as reducing induction of MKP-1, which will result in a decreased regulation of MAPKs and NFκB pathways. An alternative pathway observed in aging leads to atypical TGFβ1 signaling that through inducing sequestering of IκB, induces activation of NFκB. Furthermore, age-related inflammation and increased production of reactive oxygen species (ROS) are strong activators of NFκB.

neuroinflammation, regardless the elevated levels of TGFβ1 in aged mice. The lost ability to modulate microglia activation, together with the increased ROS production by aged animals, could result in a predominantly cytotoxic activation.

Age-Related Changes on TGFβ and NFκB

The transcription factor NFκB is a robust candidate for showing age-dependent changes due to its role in the regulation of immunity, inflammation, and cell death (Adler et al., 2007). Blockade of NFκB in aged mice has been reported to reverse the gene expression program and cell morphology, "rejuvenating" old mice (Adler et al., 2008). Robust evidence in a variety of cell and animal based experimental systems show that oxidative stress and inflammation are strong inducers of NFκB activation (Muriach et al., 2014). They are frequently associated with aging, and are involved in the pathophysiology of several chronic diseases observed in aged individuals, like diabetes and AD. In fact, Aβ can be a strong inducer of NFκB in neuron cell death via the induction of intracellular ROS (Lee et al., 2005b; Valerio et al., 2006), and through tumor necrosis factor receptor 1 (TNFR1) signaling, which result in neuronal apoptosis (Li et al., 2004; Valerio et al., 2006). Inhibition of these pathways could be beneficial in the treatment of neurodegenerative diseases, including AD (Lee et al., 2005a,b; Munoz et al., 2007; Paris et al., 2007; Wang et al., 2008a). The effect appears to involve attenuation of Aβ-induced activation of ERK1 and P38 MAPKs, which are upstream NFκB signaling pathway (Pannaccione et al., 2005; Valerio et al., 2006).

TGFβ1 can also promote inflammatory activity under certain conditions. Yan et al. showed that TGFβ1 injection to the hypothalamus resulted in inflammatory NFκB signaling. Activation is via the TGFβ-R2 receptors expressed on neurons of the medial basal hypothalamus. It induces formation of RNA stress granules that accelerate the decay of IκBα, resulting in activation of NFκB (Yan et al., 2014). Although the work was oriented to understanding mechanisms of diabetes, similar conditions are also observed in aging.

In addition to this novel mechanism of TGFβ-dependent activation of NFκB, aging also shows reduction of MKP-1 (see subsection "Age-Related Changes on TGFβ and MKP-1") that impairs inhibitory regulation over NFκB and MPAKs, potentiating cell reactivity, inflammatory activation, and

potentially oxidative stress and cytotoxicity. In addition, increased production of ROS and neuroinflammation will result in an independent activation of NFκB.

Age-Related Changes on TGFβ and IFNγ

IFNγ is a potent activator of microglia (Ng et al., 1999; Klegeris et al., 2005). IFNγ increases in the aged brain although its endogenous cell source remains unidentified (Lyons et al., 2011). The main signaling pathways induced by IFNγ are signal transducer and activator of transcription-type-1 (STAT1) and MAPKs (**Figure 5**; Blanchette et al., 2003; Platanias, 2005; Gough et al., 2008). STAT1 is activated by a Janus activated kinase (JAK)-dependent phosphorylation on tyrosine Y701 ($pSTAT1^{tyr}$) and then translocates into the nucleus to induce the expression of target genes. Full transcriptional activity requires a second phosphorylation on serine S727 ($pSTAT1^{ser}$; Wen et al., 1995).

STAT1 is a key signaling pathway involved in the up-regulation of iNOS and NO· production (Dell'Albani et al., 2001; Gough et al., 2008). Inhibition of ERK1/2 and P38 decrease IFNγ-induced $pSTAT1^{ser}$, which correlates with a reduction in NO· production. Decrease of $pSTAT1^{ser}$ and NO· production is additive when both MAPK are inhibited, indicating that ERK1/2 and P38 are needed for full activation of the STAT1 pathway in glia (**Figure 4**), and other cell types (Blanchette et al., 2003; Platanias, 2005; Gough et al., 2008). In contrast, $O_2\cdot^-$ production induced by IFNγ depends on increased levels of pERK1/2, but not pP38 (Bhat et al., 1998; Dang et al., 2003).

IFNγ suppresses TGFβ signaling through up-regulation of the inhibitory Smad7 (Ulloa et al., 1999), and there is a reciprocal regulatory interaction between TGFβ1 and IFNγ activated pathways (**Figure 4**). TGFβ1 released by hippocampal cells induce a transient increase of pERKs and a persistent increase of pP38, decreasing IFNγ-induced $O_2\cdot^-$ and NO· production by glia (Herrera-Molina and Von Bernhardi, 2005), decreasing activation of STAT1 and ERK by IFNγ (**Figure 5**). Also, after persistent stimulation, IFNγ decreases TGFβ1 induced P38 signal transduction (Herrera-Molina et al., 2012). IFNγ-TGFβ1 crosstalk regulates the production of radical species through the modulation of STAT1, ERK1/2 and P38 activation. Co-treatment with TGFβ1 and IFNγ results in decreased IFNγ-induced pERK1/2, $pSTAT1^{ser}$, $pSTAT1^{tyr}$, total STAT1 and also reduces induction of P38 activation by TGFβ1. Suppression of $pSTAT1^{ser}$ appears to be mediated by a TGFβ1-induced decrease of pERKs.

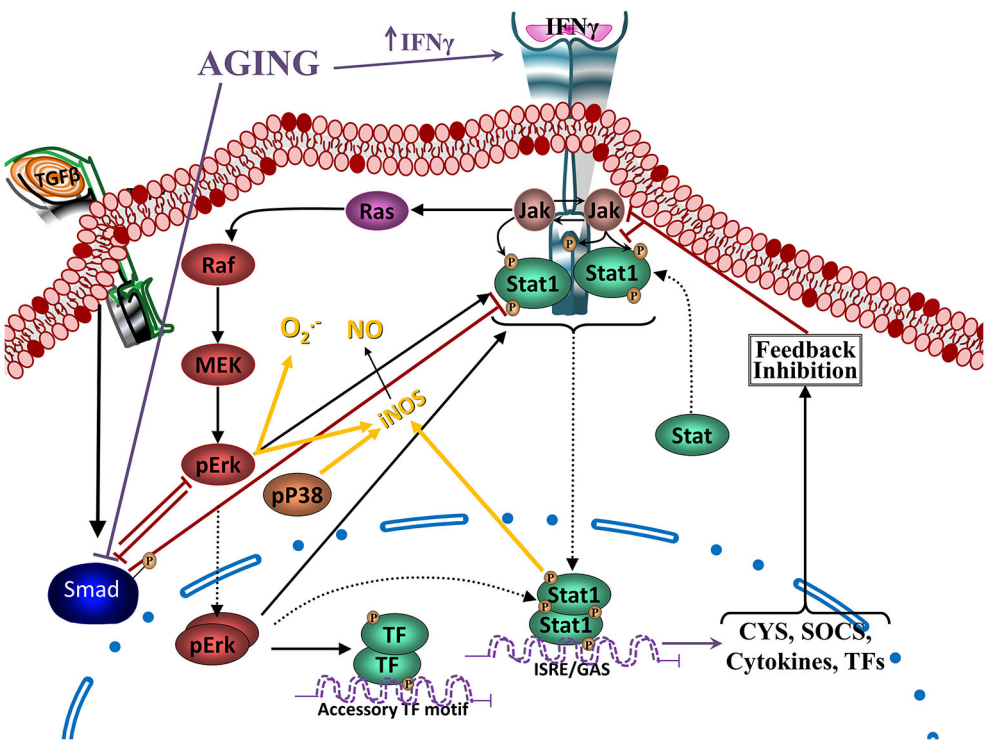

FIGURE 5 | Reciprocal regulation of TGFβ and Jak-Stat signaling. Crosstalk between IFNγ- and TGFβ1- induced signaling pathways. IFNγ activates JAK-STAT1 pathway, increasing pSTAT1tyr, which will translocate to the nucleus and activate transcription of several cytokines and other mediators and receptors involved in inflammatory activation. In addition, it will also activate pERKs and to a lesser extent pP38. ERK and P38 MAPKs potentiate STAT1 activation and induce inducible nitric oxide synthase (iNOS), increasing production of NO, as well as production of O_2^-. IFNγ-induced signaling pathway inhibits TGFβ1-Smad signaling by inducing its endogenous inhibitor Smad7 (see **Figure 4**) and through inhibition by ERK. On the other hand, TGFβ1, through the activation of the Smad3 pathway, inhibits IFNγ-induced STAT1 activation via a MKP-1-mediated inhibition of ERK1/2 and iNOS expression, reducing production of NO and O_2^-. In aging, there are increased levels of IFNγ, inhibiting activation of the TGFβ1-Smad signaling pathway, as well as a reduced activation of TGFβ1-Smad, which will result in a reduced regulation of MAPKs (and NF-κB pathways as shown in the previous figure).

In contrast, inhibition of pSTAT1^{tyr} depends on the decrease of total STAT1 mediated by TGFβ1 (**Figure 5**). Increased MKP-1 activity appears to be responsible for the reduction of IFNγ-induced activation of glia induced by TGFβ1 co-treatment. In fact, transfection with MKP-1 siRNA significantly reduces modulation of IFNγ-induced NO· production by TGFβ1. Thus, MKP-1 induction appears to be responsible of the effects on MAPK pathways and link them with those observed on the STAT1 pathway (**Figure 4**; Flores and von Bernhardi, 2012; Herrera-Molina et al., 2012). The regulatory interaction between TGFβ1 and IFNγ has been well described in tissue repair *in vivo*. IFNγ null mice show increased amounts of TGFβ1 and activation of TGFβ1 signaling, indicating that IFNγ exerts negative regulation of TGFβ1 activity (Ishida et al., 2004). On the other hand, TGFβ1 null mice has elevated levels of IFNγ and STAT1 activation, and iNOS and NO· production, indicating that absence of TGFβ1 results in the deregulation of IFNγ pathway and its target genes (McCartney-Francis and Wahl, 2002).

Age-related increase of IFNγ and changes on MPK-1 regulation could be key elements for the increased activation of microglia in aged animals. Aged animals show increased levels of IFNγ, directly potentiating inflammatory signaling and further inhibiting the Smad pathway through the induction of Smad7. On the other hand, decreased activation of TGFβ-Smad3 by both age-related changes and increased IFNγ, further reduce MKP-1 induction, suppressing the regulatory effect of TGFβ on inflammatory activation.

Age-Related Changes on TGFβ and MKP-1

Among the molecular mechanism underlying the anti-inflammatory and neuroprotective effects of TGFβ1, negative regulation of MAPK signaling, key inducer of glial cell activation, is exerted by a group of MKP. In the brain, induction of MKP-1 expression in response to anti-inflammatory molecules has been demonstrated for both astrocytes and microglia (Eljaschewitsch et al., 2006; Lee et al., 2008). TGFβ1 increases MKP-1 in glia, and other cells (Jono et al., 2003; Tong and Hamel, 2007), induction that is not affected by the presence of inflammatory conditions. Increased MKP-1 reduces the activation of P38 and NFκB pathways and decreases the NO and TNFα production induced by Aβ (Flores and von Bernhardi, 2012). siRNA transfection targeting MKP-1 attenuates the effects of TGFβ1, causing a significant amelioration of the modulation of Aβ-induced TNFα and NO production by TGFβ1 (Flores and von Bernhardi, 2012). Furthermore, MKP-1 null mice show increased P38 and JNK activity and cytokine and NO production, suggesting that this phosphatase serves as an immune regulator (Liu et al., 2007; Boutros et al., 2008).

Induction of MKP-1 is mediated by the Smad3 pathway (**Figure 4**). Smad3 inhibition greatly reduces TGFβ1-mediated MKP-1 induction, suggesting that it is a transcriptional target for Smad3, and results in a significant amelioration of the inhibition of TNFα and NO production. MKP-1 stability and enzymatic activity can be regulated through phosphorylation and acetylation, respectively (Liu et al., 2007; Boutros et al., 2008). Although other mechanisms are involved in the regulation of the production of inflammatory mediators by TGFβ1, Smad3-mediated MKP-1 induction is a novel manner of TGFβ1 action on glia that supports its anti-inflammatory role. Increased MKP-1 levels appears also to be the mechanism of action for other anti-inflammatory molecules, such as glucocorticoids (Kassel et al., 2001; Jang et al., 2007; King et al., 2009), and it has been demonstrated that this phosphatase participates in STAT1 dephosphorylation (Venema et al., 1998).

Age-related decrease on MKP-1 secondary to the inhibition of the Smad pathway results in the impairment of the inhibitory regulation on NFκB and MAPK pathways, potentiating inflammatory activation and cytotoxicity.

Effect of TGFβ on Stem Cells and Aging

As the brain ages, TGFβ has important roles both in neuronal survival and in the promotion of stem cell quiescence (Kandasamy et al., 2014). In the hippocampus, TGFβ appears to potentiate the survival and proliferation of intermediate progenitor cells in the dentate gyrus of aged mice, by a Smad3-dependent mechanism (Tapia-González et al., 2013). Regarding regulation of neural stem cells in the aged brain (Dias et al., 2014), TGFβ lengthens G1 phase of the cell cycle in activated stem cells, impairing cell cycle progression of neural progenitors and neurogenesis (Daynac et al., 2014). Because of those effects, blockade of TGFβ signaling could improve neurogenesis in the aged brain (Pineda et al., 2013).

Although TGFβ serves key role in neuronal surveillance and stem cell proliferation, most of the cellular changes induced by aging have been described in glial cells. Expression of TGFβ by oligodendrocytes is reduced in aging, condition that interferes with oligodendrocytes recruitment and reduces remyelination (Hinks and Franklin, 2000). In aged microglia and astrocytes, TGFβ expression shows a regional specificity. TGFβ signaling increases after brain infarct in aged individuals (Doyle et al., 2010). In contrast, microglia and astrocytes located close to the leptomeninges show reduced TGFβ expression with age, reduction that could have a role on the increased permeability of leptomeninges during systemic inflammation (Wu et al., 2008).

TGFβ SIGNALING AND ITS ROLE IN ALZHEIMER DISEASE

There is evidence that impaired TGFβ signaling could be involved in the pathogenesis of AD. AD patients show decreased plasmatic levels of TGFβ1 (Mocali et al., 2004; Juraskova et al., 2010), but increased levels in cerebrospinal fluid (Blobe et al., 2000; Tarkowski et al., 2002), and within Aβ plaques (Burton et al., 2002). Brains of AD patients have reduced levels of TGFβRII (Tesseur et al., 2006). Reduced levels of Smad3 and impairment of Smad3 signaling have been observed in the AD brain, associated with increased Aβ accumulation, Aβ-induced neurodegeneration and neurofibrillary tangle formation (Luterman et al., 2000; Colangelo et al., 2002; Katsel et al., 2005; Tesseur et al., 2006). In addition to decreased expression of Smad3 in hippocampi of AD patients, hippocampal neurons show increased levels of

activated Smad2 (Lee et al., 2006), along with alterations in the subcellular localization of phosphorylated Smad2/3 (Colangelo et al., 2002; Lee et al., 2006), which remains in the cytoplasm of neurons, instead of translocating into the nucleus (Lee et al., 2006; Ueberham et al., 2006). The ectopic localization of activated Smads in AD could be attributed to another pathological feature observed in AD, the hyperphosphorylation of tau. Hyperphosphorylated tau is associated with the sequestration of activated Smad2/3, and the disruption of TGFβ signaling (Baig et al., 2009). Both reduced presence of TGFβRII and defects on Smad are associated with inhibition of TGFβ signaling.

The TGFβ pathway exerts direct regulation over some of the pathological features of AD, since it upregulates the expression of the APP in normal human astrocytes by a Smad4-dependent mechanism (Burton et al., 2002). TGFβ also favors stabilization of the APP mRNA by binding it to a RNA-protein complex that reduces the rate of APP mRNA decay (Amara et al., 1999). In addition, TGFβ1 has been implicated on the promotion of amyloid angiopathy in frontal cortex and meninges (Wyss-Coray et al., 1997, 2000; Mazur-Kolecka et al., 2003), and on the increased production of Aβ by astrocytes in APP/TGFβ1 transgenic mice (Lesne et al., 2003). Notoriously, this amyloid angiopathy appears at a younger age when the overexpression of TGFβ1 in astrocytes occurs in the absence of SR-A (TGFβ1/SR-A$^{-/-}$ mice) (Lifshitz et al., 2013), which lead us to further inquire on the expression of that receptor, as discussed on the next two sections. Furthermore, those effects appear to depend on the activation of astrocytes that stimulate the production of APP due to the presence of a TGFβ1 response element in the 5'UTR of APP. Moreover, Tg2576 mice with a dominant negative TGFβ1-receptor II that blocks Smad2/3 signaling show a conspicuous reduction of amyloid deposits in the brain (Town et al., 2008).

In contrast with those potentially deleterious effects of TGFβ1, increased TGFβ1 has been also associated with a lower burden of Aβ in the parenchyma, which correlates with an increased microglia activation. Several reports show that TGFβ1 has anti-amyloidogenic roles, reducing the Aβ burden and inhibiting the formation of neuritic plaques, effects that appear to be mediated by the promotion of microglia-mediated Aβ degradation (Wyss-Coray et al., 2001). The neuroprotective role of TGFβ1 against Aβ toxicity has been studied *in vitro* and *in vivo* models of AD (Prehn et al., 1996; Caraci et al., 2008). Furthermore, TGFβ1 Smad3 also inhibits the production of radical species induced by inflammatory stimuli, and induces phagocytosis of Aβ *in vitro* (Tichauer and von Bernhardi, 2012).

However, induction of phagocytosis is lost as animals age (von Bernhardi et al., 2011). Smad3 pathway is altered in microglia from adult mice, affecting the induction of Aβ phagocytosis and the modulation of radical species production by TGFβ1.

The uncoupling of TGFβ1 signal transduction pathway could result in an altered pattern of microglial activation and reduced clearance of amyloid; effects that in fact are observed in aging and in AD. Impairment of TGFβ signaling can potentiate neuroinflammation, favoring neuronal dysfunction and neurodegenerative changes (Tesseur and Wyss-Coray, 2006). Reduced TGFβ-Smad3 signaling results in age-related neuroinflammation and neurodegeneration and in increased accumulation of Aβ (Tesseur et al., 2006). The accumulation of Aβ could depend on a reduction of its clearance, and be mediated by the reduced expression of SR-A by glia (Tichauer and von Bernhardi, 2012). These changes could facilitate cytotoxic inflammation and neurodegenerative diseases in aging (von Bernhardi et al., 2011). If accumulation of Aβ depends indeed on impaired clearance, it could situate Aβ as the result of disease progression instead of being its primary cause, as we propose in the "microglial cell dysregulation" hypothesis for AD.

TGFβ REGULATES SR-A

The effect of TGFβ is cell and tissue specific. Whereas TGFβ reduces expression of some SRs in circulating macrophages, TGFβ increases expression of SR-A, while decreases expression of SR-BI by microglia (Tichauer and von Bernhardi, 2012). Given the relevance of SRs as well as other pattern recognition receptors (PRRs) on the inflammatory activation and the scavenger function of microglia, changes on the expression of these receptors could have a profound effect on microglial cell activation (von Bernhardi, 2007).

In addition to phagocytosis, SR-A is also involved in the regulation of glia activation (Murgas et al., 2014). Accordingly, the use of SR-A antagonists appears to improve the phenotypic features of AD (Handattu et al., 2009) by reducing microglial activation (Handattu et al., 2013). These results support the idea that SR-A activity could be part of the molecular mechanism involved in glial cell activation. In contrast to most SRs, SR-A expression is not necessarily downregulated, but can be increased by its ligands (Nikolic et al., 2011). In addition, binding of ligands to SRA recruits SRs to the membrane surface by a PI3K-activated mechanism (Cholewa et al., 2010). In macrophage cell lines, like THP-1 and J774A.1 (Bottalico et al., 1991; Nishimura et al., 1998; Draude and Lorenz, 2000; Argmann et al., 2001; Michael et al., 2012) and human monocytes (Draude and Lorenz, 2000), TGFβ1 reduces SR-A expression, through a mechanism that depends on Smad-2 (Michael et al., 2012). In addition, it has been also reported that induction of TGFβ1 by statin treatment, abolish induction of SR-A by inflammatory stimuli, in a mechanism mediated by ERK activation (Baccante et al., 2004).

In addition of increasing their expression of SR-A, TGFβ1 also increases Aβ uptake by microglia, an effect that is prevented by the Smad3 inhibitor SIS3 (Tichauer and von Bernhardi, 2012). Studies by our laboratory have also shown that induction of Aβ phagocytosis by TGFβ1 is decreased in aged mice (Tichauer et al., 2014). Reduction of induction of Aβ phagocytosis, together with decreased expression of SR-A in the brain of aged APP/PS1 mice (Hickman et al., 2008), suggest the existence of an altered regulation of SR-A expression in aging. Given its participation in Aβ uptake, SR-A impairment could be involved in the accumulation of Aβ during aging, with a mechanism associated with TGFβ1-Smad3 signaling (Tichauer and von Bernhardi, 2012), and could be related with AD pathogenesis.

GENE TRANSCRIPTION REGULATION BY TGFβ SR-A AS A MODEL

Given our observation of increased protein expression of SR-A in microglia exposed to TGFβ through a Smad3-dependent mechanism (Tichauer et al., 2014), we performed *in silico* analyses of human genomic data to determine if the effect could be mediated by the transcriptional co-factors Smad2/3 over the MSR1 gene coding SR-A. As shown in **Figure 6A**, MSR1 gene is located at chromosome 8 and its transcription commonly generates three main splicing variants (**Figure 6B**). Analysis of ChIP-Seq data generated by Kim et al. revealed a significant peak of binding of Smad2/3 and Smad4 at the intron 7 of the MSR1 gene when human embryonic stem cells (hESC) were treated with activin (**Figure 6C**), a TGFβ receptor agonist (Kim et al., 2011). In addition, the MSR1 gene is ubiquitously bound by CTCF (**Figure 6D**), a protein factor having a key role in chromatin topological regulation (Ong and Corces, 2014) and previously described as a robust transcriptional repressor (Holwerda and De Laat, 2013) in various cell lines. The chromatin state segmentation data shows that the CTCF binding site is located in a genomic insulator region that is surrounded by highly repressed chromatin (**Figure 6E**), and is correlated with the presence of a topological domain predicted from Hi-C experiments (**Figure 6F**; Dixon et al., 2012). The presence of a CTCF peak in the MSR1 gene and the fact this gene is in a topologic domain suggest that MSR1 is highly repressed in most cell types (**Figures 6D–F**). This could explain the tissue specificity of this gene, which is almost exclusively present on the monocyte-macrophage lineage (Christie et al., 1996; Godoy et al., 2012), and astrocytes (Alarcón et al., 2005).

We also analyzed data of DNase sensitivity and ChIP-Seq available at WashU Epigenome Browser (Zhou et al., 2013), finding that H1-hESC cells differentiated into mesenchymatic

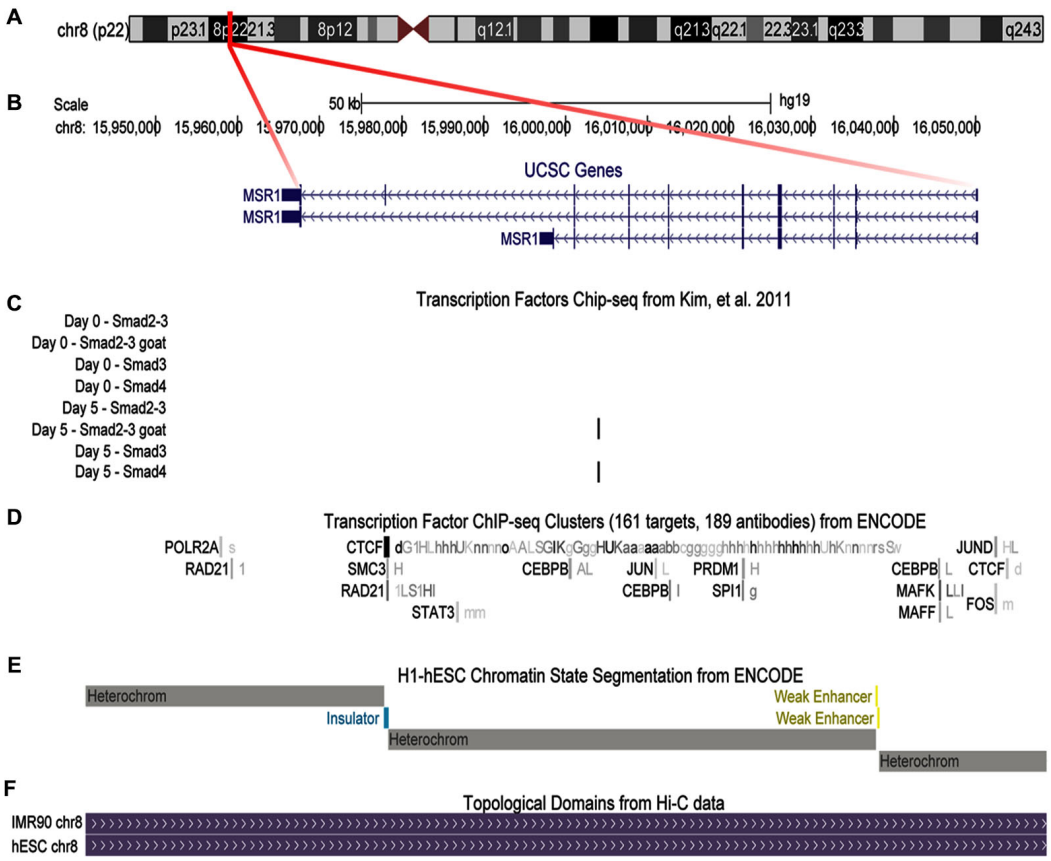

FIGURE 6 | Binding of transcriptional co-factors Smad2/3 and 4 to the MSR1 gene. SR-A gene structure is shown using UCSC gene annotation, ChIP-Seq available data, and chromatin structure associated to this gene. **(A)** Ideogram of chromosome 8. The region labeled in red shows the MSR1 gene location. **(B)** MSR1 gene structure, indicating the three most common isoforms. Arrows indicate the direction of transcription and boxes symbolize exons. **(C)** ChIP-Seq data for Smads using four different antibodies before activin treatment (day 0) and 5 days after treatment of human embryonic stem cells (hESC; Kim et al., 2011); black bars show significant signals of ChIP-Seq indicating Smad2/3 and Smad 4 binding to MSR1 gene after activin treatment (day 5). **(D)** ChIP-Seq results provided by ENCODE project for 161 transcription factors from various cell lines. Boxes represent ChIP-Seq significant signal for a specific transcription factor. The darkness of the box is proportional to the maximal signal intensity of ChIP-Seq observed in a cell line, shown next to the box, in a lowercase letter, cells where this signal was found significant. **(E)** Chromatin segmentation state data generated by ENCODE project for H1-hESC (Aad et al., 2011); gray boxes represent heterochromatin sites, yellow is used for enhancer sites, and blue are for insulators. **(F)** Genomic topological domains detected by using Hi-C data in hESC and IMR90 cell lines (Dixon et al., 2012).

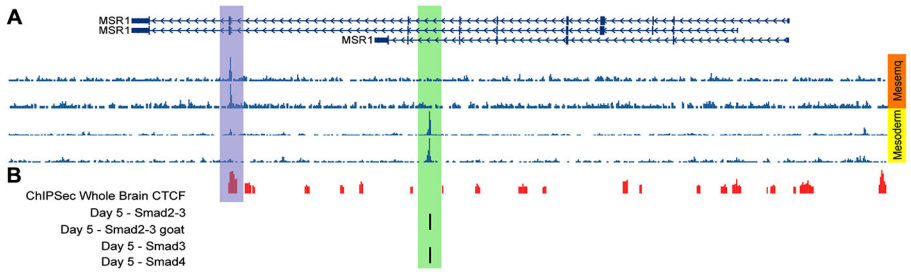

FIGURE 7 | DNase sensitive sites matching with CTCF binding sites after activin treatment. (A) DNase hypersensitivity data in H1-hESC cells differentiated into mesenchymal cells (orange block) and treated with activin to differentiate H1-hESC cells into mesoderm (yellow block); different rows correspond to different experiments. **(B)** ChIP-Seq data for CTCF in the whole brain (red bars) and for Smad 2/3 and 4 (black bars; Kim et al., 2011).

cells present a hypersensitivity peak to DNase coincidental to the CTCF binding site (**Figure 7A**). In contrast, after treatment of H1-hESC cells with activin to differentiate them into mesoderm, the DNase hyper-sensitivity peak related to CTCF disappears, but a new DNase hypersensitivity peak is observed, which match with the Smad2/3 and Smad 4 binding site detected by the previous ChIP-Seq data analysis (**Figures 7A,B**; Kim et al., 2011). This shift between those two DNase hypersensitivity peaks after activin treatment leads us to infer that TGFβ pathway activation interferes with the binding of CTCF and might promote a topological rearrangement that induces MSR1 transcription by a mechanism mediated by Smad2/3/4. This hypothesis is currently been experimentally tested by our group.

The brain of AD patients reveal changes on the methylation pattern of some genes described as susceptible to be involved in AD, such as PSEN1 y APOE, and in genes related to homeostasis of gene methylation, like MTHRF and DNMT1

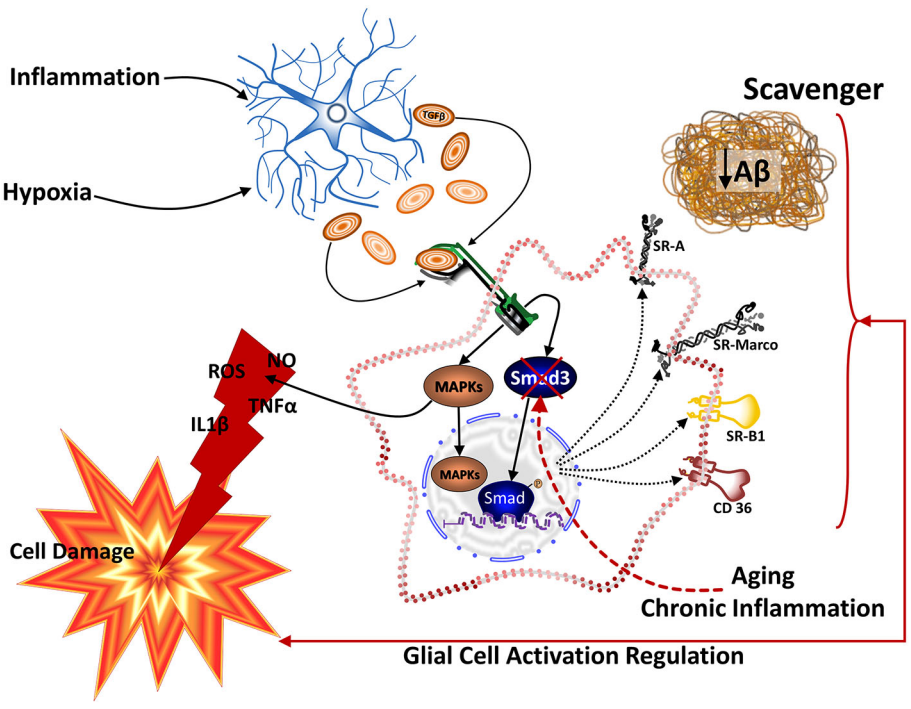

FIGURE 8 | Model for TGFβ1-Smad3 pathway regulation in aged microglia. Diverse stimuli, including inflammatory stimulation and hypoxia, induce astrocytes to secrete TGFβ1. Binding of TGFβ1 to its receptor results in the activation of the Smad3 pathway, as well as MAPKs and PI3K signaling in microglia. Thus, TGFβ1 regulates the inflammatory activation of microglia in addition to modifying the SRs profile expressed by the cell. SRs appear to be involved both in the uptake of Aβ and in the activation of glial cells. Several of the effects of TGFβ1 on cell viability, reduction of inflammatory cytokines and reactive species, and expression of SRs depend on the activation of Smad3. In aging or after exposure to chronic inflammatory conditions, canonical activation of Smad3 is greatly reduced, whereas MAPKs remains activatible. As result of this change on TGFβ1 signaling, microglia show increased cytotoxicity, undergo changes on their expression of SRs and decrease their Aβ clearance. Thus, reduced TGFβ1-Smad3 activity on aged microglia appears to impair the beneficial effect of TGFβ1.

(Wang et al., 2008b). This study also identified age-specific epigenetic changes, suggesting that epigenetics could have a role in the development of AD (Wang et al., 2008b). Given that possibility, a future challenge is to assess the existence of epigenetic changes on the MSR1 gene or on genes related with the TGFβ pathway during aging and in AD. Changes potentially affecting SR-A expression or function as the brain ages would affect inflammatory activation and Aβ uptake by glia.

CONCLUDING REMARKS

Age dependent changes such as microglia mis-activation, production of ROS, and decreased proteasome activity could establish the grounds for microglial cell dysfunction, leading to cytotoxicity and accumulation of Aβ or other protein aggregates. The combined effect of various age-associated changes, in addition to the individual endophenotypic condition and diverse environmental stimuli can initiate the vicious circle of cytotoxic activation of microglia.

Innate immune response, with microglia as the pivotal player, is recognized to have a profound immune-modulatory and reparative potential. However, chronic activation and dysregulation of microglia can lead to deleterious effects, inducing malfunction and damage of the CNS. Microglia activation appears to undergo different phases depending on their environmental and functional context. Whereas inhibition of microglia can be beneficial at a certain phase of disease progression it can become detrimental at another. A critical area of research would be to understand their activation process, developing pharmacologic tools directed towards selected properties of microglia. That would be a major improvement respect the present approach of turning off microglial cell activation as a whole, which likely has a major bearing in the limitations of past thinking about immunoinhibitory drugs for neurodegenerative diseases.

Our interest in identifying protective and regulatory pathways, to potentiate them while inhibiting cytotoxic activation of microglia, lead us to study the effect of TGFβ on microglia function in aging and various inflammatory conditions. TGFβ-Smad3 is involved in many protective functions of microglia, and shows major changes with aging. Our working model (**Figure 8**) shows that upon activation by various stimuli, TGFβ binds to its receptor activating a complex signaling pathways that includes activation of both the canonical Smad pathway as well as the non-canonical MAPKs and PI3K. As result of the activation of those pathways, among many changes, there will be changes on the expression pattern of SRs, the phagocytic activity and the production of inflammatory cytokines and other inflammatory and oxidative stress mediators. In aged microglia, increased amounts of TGFβ will act upon its receptor. However, secondary to age-related changes or chronic inflammation, the activation of Smad3 pathway is inhibited. Inhibition of Smad3 activation in the context of increased TGFβ levels shifts the regulatory signaling towards a dysregulated inflammatory activation, potentially leading to the impairment of protective response, development of an increased cytotoxicity and to neurodegenerative changes. Thus, increased neuroinflammation, decreased Aβ clearance and impaired cell viability could be consequence of the impaired TGFβ signaling.

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frontiers in Cellular Neuroscience

REVIEW
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Relevance of chronic stress and the two faces of microglia in Parkinson's disease

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This review is aimed to highlight the importance of stress and glucocorticoids (GCs) in modulating the inflammatory response of brain microglia and hence its potential involvement in Parkinson's disease (PD). The role of inflammation in PD has been reviewed extensively in the literature and it is supposed to play a key role in the course of the disease. Historically, GCs have been strongly associated as anti-inflammatory hormones. However, accumulating evidence from the peripheral and central nervous system have clearly revealed that, under specific conditions, GCs may promote brain inflammation including pro-inflammatory activation of microglia. We have summarized relevant data linking PD, neuroinflamamation and chronic stress. The timing and duration of stress response may be critical for delineating an immune response in the brain thus probably explain the dual role of GCs and/or chronic stress in different animal models of PD.

Keywords: corticosterone, glucocorticoids, microglia, neuroinflammation, neurodegeneration, stress, Parkinson's disease

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Role of Inflammation in Parkinson's Disease

Parkinson's disease (PD) is a neurodegenerative disorder second only to Alzheimer's disease (AD) in prevalence. It is characterized by the loss of the dopaminergic neurons in the substantia nigra (SN) and the accumulation of α-synuclein (α-syn) and other proteins in intracellular proteinaceous aggregates called Lewy bodies (LB). Familiar forms of PD, usually appearing under the age of 40 and accounting for no more than 5% of cases, are due to mutations in a reduced number of genes including *SNCA, PINK1, PARKIN* and *LRRK2* among few others. Idiopathic forms, usually affecting people from 65 years old, have an obscure etiology; mitochondrial dysfunction, toxins, oxidative stress, infections, decrease of trophic factors, impairment of the ubiquitine-proteosome system, metabolic alterations, inflammation and the accumulative effect of a number of susceptibility genes have been proposed to explain the initiation and development of this form, which accounts for 95% of cases.

Neuroinflammation

Neuroinflammation seems to be an underlying process in many cases of PD. In McGeer et al. (1988) reported the presence of reactive microglia and inflammatory macrophages as well as proinflammatory cytokines in SN postmorten samples from PD patients. Considering the

brain was believed to have immune privilege,these inflammatory signs were thought to be a response from the microglial system to neuronal death. The brain is no longer considered to be immunoprivileged; in fact, infiltration of lymphocytes into the brain parenchyma of PD patients has been demonstrated (Brochard et al., 2009); the role of T lymphocytes in PD will be reviewed in "Chronic Stress and Parkinson's Disease in Humans" Section).

It is now thought that neuroinflammation could be a triggering mechanism of neuronal death. Inflammatory animal models based on the injection of proinflammatory compounds as LPS, thrombin or tissue plasminogen activator within the SN have shown that the induction of an inflammatory process can induce the death of dopaminergic neurons (Castaño et al., 1998, 2002; Herrera et al., 2000; Kim et al., 2000; Carreño-Müller et al., 2003; de Pablos et al., 2005, 2006; Tomás-Camardiel et al., 2005; Hernández-Romero et al., 2008; Villarán et al., 2009; Argüelles et al., 2010). Evidence supporting the inflammatory hypothesis of neurodegeneration also comes from studies showing the expression of a bunch of inflammatory markers within the brain including specific proteins, pro-inflammatory cytokines and markers of active glial cells (for a schematic review of the effects of LPS on neurons and glial cells found by our group, see **Figure 1**). An altered expression of immune signaling-related transcripts have been described in early stages of PD in a study of microarray analysis of nucleated blood cells (Soreq et al., 2008). Epidemiological studies evidence the protective effect of several nonsteroidal anti-inflammatory drugs, whereas genetic studies show that polymorphisms in some pro-inflammatory cytokines may influence the risk of developing PD (Klegeris et al., 2007). Some studies have shown that classical steroid anti-inflammatory drugs, such as dexamethasone (Castaño et al., 2002), as well as drugs used for quite different goals, such as minocycline (Tomás-Camardiel et al., 2004) and simvastatin (Hernández-Romero et al., 2008), are able to reduce the inflammatory process and neuronal death induced by LPS. Thus, it seems that the pro-inflammatory hypothesis is not merely possible but likely. The question here is how such an inflammatory process is initiated within the brain and endlessly self-sustained.

Not all brain structures exhibit a similar sensitivity to pro-inflammatory compounds; whereas the SN seems to be very sensitive, the hippocampus appears to be resistant to LPS (Espinosa-Oliva et al., 2011). This could be in part related to differences in the number of microglial cells between brain structures (Lawson et al., 1990). However, an inflammatory process induced for LPS can be developed in the hippocampus of stressed animals (Espinosa-Oliva et al., 2011), suggesting that the inflammatory responses can be modulated. Unfortunately, the factors triggering this brain inflammatory response are yet not known.

Dopaminergic neurons seem to be especially sensitive to a number of factors that can induce cell damage and eventually cell death. Dying neurons release substances that are recognized by glial cells, activating them. Amongst the substances released by damaged/dying dopaminergic neurons, this review will focus on three of them: dopamine (DA), neuromelanin (NM) and α-syn.

Dopamine

DA is the neurotransmitter that defines the dopaminergic phenotype. It is synthesized from tyrosine in a two-reaction process catalyzed by the enzymes tyrosine hydroxylase (TH, the key enzyme in the synthetic pathway of DA) and aromatic amino acid decarboxylase (AADC), which transform tyrosine in L-3,4-dihydroxyphenylalanine (L-DOPA) and then L-DOPA into DA, respectively. The loss of DA is the hallmark of PD and explains most of the motor alterations of this disease. Thus, replenishing DA levels is a major therapeutic target in PD. This is achieved by the combined administration of L-DOPA, the precursor of DA, and an inhibitor of peripheral AADC; unfortunately, L-DOPA cannot be freely administered because many patients develop L-DOPA-induced dyskinesias. After its release in synapses and signaling on its receptors, DA can be taken up by glial cells and also by the presynaptic neurons through the specific DA transporter. Uptaken DA can be revesiculated in neurons by the vesicular monoamine transporter 2, but part of it can remain free in the cytoplasm, where it is metabolized by the monoamine oxidase enzyme to yield 3,4-dihydroxyphenylacetic acid. Free cytoplasmic DA can also autooxidize, forming DA quinones and semiquinones able to react with cysteines residues to form adducts that impair the function of proteins; for example, DJ-1, a redox sensor protein involved in recessive forms of familial PD, is covalently modified by DA and highly reactive DA quinones thus impairing DJ-1 function (Girotto et al., 2012). High cytosolic concentrations of free DA can lead to oxidative stress as well as to its interaction with α-syn, both of them initiating the neurodegenerative process (Mosharov et al., 2006). Strikingly, DA seems to play a role in the inflammatory response induced by the intranigral injection of LPS, since experimental depletion of DA caused by the inhibition of TH by α-methyl-p-tyrosine (α-MPT) prevents glial activation and loss of dopaminergic neurons (de Pablos et al., 2005). Bypassing TH inhibition (using the combination of L-DOPA plus an inhibitor of the peripheral AADC enzyme, just the composition of several anti-parkinsonian therapies as SINEMED® or MADOPAR®, for example) reverses the protective effect induced by α-MPT. Furthermore, depletion of DA content by α-MPT is able to reduce the infiltration of peripheral macrophages as well as the increase of microglial cells induced by the injection of 6-hydroxydopamine (6-OHDA) within the SN (de Pablos et al., 2014). Thus, DA could have a relevant role in sustaining inflammation and lymphocyte recruitment within the brain; in fact, DA could be involved in the degeneration of dopaminergic neurons induced by inflammatory processes.

DA, however, may play a beneficial role controlling systemic inflammation. In a recent article, Yan et al. (2015) show that DA inhibits the activation of the NLRP3 inflammasome through D1 receptor (DRD1) signaling. DRD1 activation stimulates adenylate cyclase activity; the resulting cAMP binds to NLRP3, promoting its ubiquitination by the E3 ubiquitin ligase MARCH7. Degradation of the ubiquitinated NLRP3 prevents the

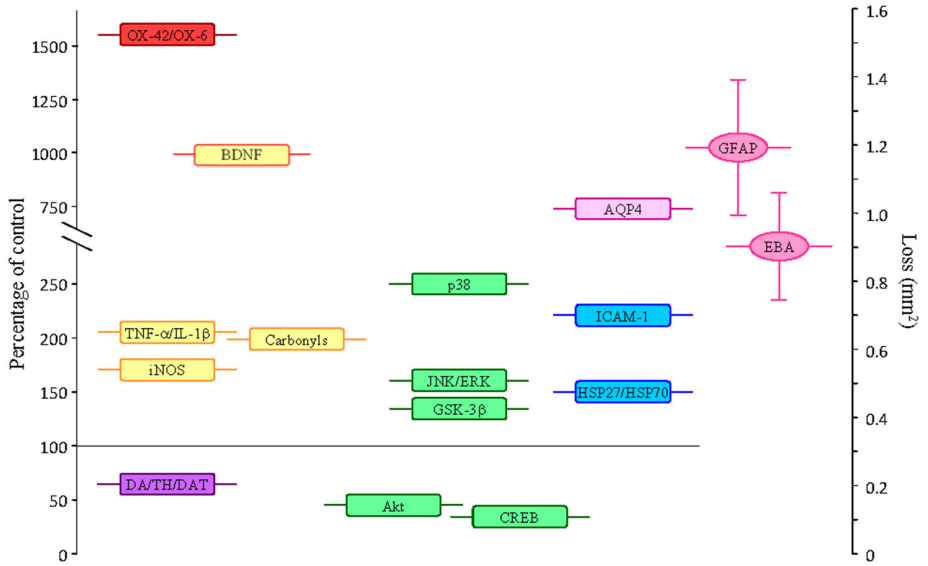

FIGURE 1 | Average values of some parameters measured in the SN (as percentage of controls) after the single intranigral injection of 2 μg of LPS. Parameters that increase: OX-42/OX-6, density of activated microglial cells; amounts of the proinflammatory cytokines TNF-α and IL-1β; the inducible nitric oxide synthase (iNOS) enzyme; the amount of carbonyl groups (oxidized proteins); the expression of BDNF (this neurotrophin is associated to cell survival, but it can have a damaging role under the oxidative conditions induced by LPS); the phosphorylated (active) forms of the MAP kinases p38, JNK, ERK and GSK-3β (associated with promotion of apoptosis); the expression of AQP4; the adhesion molecule ICAM-1; the heat shock proteins (HSP)-27 and 70. Parameters that decrease: DA/TH/DAT, dopamine content, neurons expressing tyrosine hydroxylase and dopamine transporter; the phosphorylated forms of the MAP kinase Akt and the transcription factor CREB (cell surviving signals). Alterations on the expression of GFAP and the endothelial barrier antigen (EBA), as area lacking expression (in mm^2), are also shown. Loss of expression of GFAP and EBA is associated to BBB damage.

inflammasome-dependent inflammation. Acting on DRD1, DA is even able to prevent systemic inflammation induced by LPS, proving that DA has an important role as a messenger in the periphery. Consequently, these authors point out to DRD1 as a potential target for the treatment of inflammatory processes driven by NLRP3.

Neuromelanin

NM is a dark, complex endogenous polymer derived from DA that stains dopaminergic neurons and give the name to the SN; loss of pigmentation is evident in the SN of PD patients due to the loss of dopaminergic neurons. Free melanin was already observed in the SN of postmorten samples from PD patients studied for McGeer et al. (1988). Several studies have shown that NM is able to induce microglial activation, initiating neuroinflammation and neurodegeneration of dopaminergic neurons (Wilms et al., 2003; Zecca et al., 2008; Karlsson and Lindquist, 2013; Nie et al., 2013). Viceconte et al. (2015) has shown that synthetic NM is able to activate microglial cells, both *in vitro* and *in vivo*, through a mechanism mediated by caspase-8 and caspase-3/7. They found *in vivo* upregulation of the M1 marker CD16/32 induced by NM, as well as chemotactic response to NM for the microglial-derived cell line BV2, accompanied by features of microglial activation as morphological changes, increase of oxidative stress and induction of pattern recognition receptors as TLR2, NOD2 and CD14. Thus, release of NM from damaged/dying dopaminergic neurons can trigger a self-induced cycle of neuron damage, release of NM and neuroinflammation with dire consequences for these neurons.

Alpha-Synuclein

As it was mentioned above, some cases of familiar PD are due to mutations in a number of genes related with mitochondrial function, so as neurons lose their capacity to produce the required amount of energy and thus die, activating and attracting glial cells. In other cases, polymorphisms in inflammatory genes seems to be associated with the disease, including pro-inflammatory cytokines such as TNF-α and IL-1β and their receptors (Krüger et al., 2000; McGeer et al., 2002; Schulte et al., 2002; Wahner et al., 2007; Wu et al., 2007; Nie et al., 2013), as well as TREM2 (Rayaprolu et al., 2013). An increasing number of GWAS studies have provided possible genetic associations with the disease, as that found for HLA for example (Hamza et al., 2010), supporting the role of the immune system in the development of PD.

A great proportion of these hereditary forms of PD are related to α-syn, a presynaptic protein involved in vesicle transport among other functions not fully known (Maroteaux et al., 1988). α-syn aggregation seems to be an important event in the development of PD (Marques and Outeiro, 2012; Kalia et al., 2013). Several point mutations in the *SNCA* gene are directly related to familial forms of PD (Polymeropoulos et al., 1997; Krüger et al., 1998; Zarranz et al., 2004; Lesage et al., 2013; Proukakis et al., 2013). Over-expression of wild type α-syn due to duplication or even

triplication of the gene is also related with PD, suggesting that an excessive amount of the normal protein is also involved in damaging processes (Singleton et al., 2003; Ross et al., 2008).

As mentioned above, interactions between α-syn and DA might play an important role in the death of dopaminergic neurons: oligomeric forms of α-syn can produce leakage of DA from presynaptic vesicles into the cytosol (Volles et al., 2001), and high cytosolic concentrations of DA may in turn promote the accumulation of α-syn protofibrils (Conway et al., 2001). Wild-type α-syn can be modified by DA, resulting in the inhibition of chaperone-mediated autophagy (CMA), which impairs degradation of α-syn and other substrates of CMA (Martinez-Vicente et al., 2008). Furthermore, neuron-released α-syn fibrils that have been phagocytized by microglia can induce the release of IL-1β through the activation of the NLRP3 inflammasome (Codolo et al., 2013). It is well known that IL-1β is one of the main and most abundant pro-inflammatory cytokines affecting inflammatory processes (Dinarello, 2010), having harmful effects on dopaminergic neurons (Ferrari et al., 2006; Koprich et al., 2008). The role of IL-1β on inflammation and dopaminergic neurodegeneration will be discussed later. All these evidence suggest that interactions between α-syn and DA might trigger a self-sustained loop leading to neuronal death.

Braak et al. (2003) postulated the hypothesis that pathology of PD in the brain could start in structures such as the olfactory nucleus and the dorsal motor nuclei, progressing from them to the midbrain and cortex by the spreading of α-syn seeds. The possible mechanism for cell-to-cell transmission of pathogenic proteins in neurodegenerative diseases has been beautifully revised by Guo and Lee (2014). In brief, naked-protein oligomers seeds can be released from neurons, reaching other neurons directly through the plasma membrane or entering by endocytosis. Seeds can also be released within exosomes that fuse with the receiving cell membrane. Intracellular transfer by nanotubes connecting cells is also hypothesized. It is now clear that α-syn can be found outside cells (for a review, see Guo and Lee, 2014), and several studies have shown that α-syn activates microglia into a pro-inflammatory state (for a very interesting and deep review on microglial activation by α-syn, see Sanchez-Guajardo et al., 2015). Thus, α-syn might be an answer to the question stated above: an inflammatory process can be initiated within the brain and endlessly self-sustained upon activation of microglia by extracellular α-syn. However, some questions regarding neuroinflammation and α-syn are still under debate, as whether neuroinflammation is causing α-syn misfolding and aggregation, or the role of neuroinflammation favoring or delaying the cell-to-cell transfer of α-syn; namely, if α-syn aggregation is a cause of neuroinflammation or just a consequence of it (Lema Tomé et al., 2013). In any case, neuroinflammation and α-syn aggregation form a positive feedback loop that increases protein aggregation and neuronal damage in PD. Strikingly, the release of α-syn from neurons, with its consequent arrival to other neurons, can be initiated outside the brain.

Given the significant role of inflammation in PD pathogenesis, and considering the influence of stress/glucocorticoids (GCs) in microglia polarization, we will next discuss most relevant aspects of stress and microglia activation as well as their likely influence on PD.

Stress

Organisms, both simple and complex, have developed mechanisms to deal with the different challenges that occur during their lifetime. Stress is a condition of human experience and also an important factor in the onset of several diseases, including cardiovascular, metabolic and neuropsychiatric diseases (Renard et al., 2005; Musazzi et al., 2010). Stress is a term commonly used to describe internal states or feelings and, as a consequence, the interpretation of its meaning is often subjective, making it difficult to assess how it impacts upon our body (Miller and O'callaghan, 2005).

The term stress would be difficult to understand without the concepts developed by the physiologists Claude Bernard and Walter Cannon. Whilst the former described the concept of internal environment (Millieu Interieur), the latter introduced the term homeostasis to define the combination of physiological mechanisms that together maintain the stability of the internal environment. Homeostasis is achieved via different systems which cooperatively counteract internal or external factors that tend to modify it. Based on these concepts, Selye (1946) coined the term *stress* as a "general adaptation syndrome". Of note, the cortico-adrenal activation is the most important physiological aspect of stress. Selye would futher introduce the term *stressor* as the stimulus that causes the syndrome, narrowing the definition of stress as the body's response to such stimulus. However, although numerous scientific articles on stress have been published in recent decades, the definition of stress remains complex, controversial and somehow ambiguous.

Under stressful situations, humans mobilize physiological resources to respond to these situations in the so-called stress response (Van de Kar and Blair, 1999). Different variables influence the individual response to these threats. Among these stand stressors (depending on the type, severity or repetition of events), individual factors (such as vulnerability, emotional stability and coping styles) and environmental variables. The interaction of these variables can lead to various stress responses from the physiological point of view, in which brain (information processing), autonomic (innate system) and neuroendocrine (adaptative system) activation are involved. Although they are individual systems, in practice their functions overlap at some point. All these responses constantly feedback the body to increase, maintain or decrease the stress response.

Systems Activated by Stress

When an organism is stressed, two different systems are activated: the Sympathetic-adrenal-medullary system (SMA) and the Hypothalamic-pituitary-adrenal system (HPA). Initially, a threatening stimulus excites a receptor leading this information to the brain in the form of nerve impulses. The sensory

information is then projected on the associative thalamic nuclei from where it continues its journey to the cerebral cortex (Van de Kar and Blair, 1999), which is responsible for modulating the sensory processing and identify if there is a threatening stimulus or not. The activation is promoted by the action of the reticular formation that starts the general excitation. Amongst other things, the anterior cingulate cortex changes the priorities of attention and concentration (Botvinick et al., 2001), whilst the frontal cortex generates the plan of priorities for attention skills and working memory (Rushworth et al., 2004).

If not valued as stressful, the body's response would involve the implementation of normal, specific homeostatic mechanisms that are appropriate for the particular situation. If it is considered stressful, the emergency mechanisms that constitute the stress response (activation of SMA and HPA axes, autonomic arousal and neuroendocrine activation) would become activated (Finsterwald and Alberini, 2014). This may occur because the stimulus is qualitatively stressful for all individuals of a particular species or because it exceeds a certain threshold of intensity.

When the nerve-center activation appears to have peaked, autonomic activation occurs, activating the SMA axis. This, in turn, prepares the body to cope with the external threat and to facilitate state of fight-or-flight response, so that the internal environment is maintained uniform (homeostasis; Finsterwald and Alberini, 2014).

Once the reticular formation has begun the process of general activation, the hypothalamus is excited. The hypothalamus is responsible for controlling the functions of the autonomic nervous system and the endocrine system, organizing survival behaviors such as fighting, feeding, fleeing and reproduction (Maggi et al., 2015). After the arrival of the information to the hypothalamus, the primary and quick response will cause the release of catecholamines (noradrenaline and adrenaline) from the hypothalamus and the sympathetic pathway. These catecholamines are responsible for putting the body on alert in preparation for "fight–or-flight". Some of the observable physiological effects include tachycardia, increased blood pressure, sweating and dilated pupils (Ursin and Olff, 1993). Finally, the neuroendocrine response is activated, initiated by the HPA axis when neurons in the paraventricular nucleus (PVN) of the hypothalamus secrete a peptide called corticotropin releasing factor (CRH). This and other related hormones enter the private circulatory system connecting the hypothalamus with the anterior pituitary. Within seconds, CRH activates the pituitary by inducing the release of adrenocorticotropic hormone (ACTH; Leng and Russell, 1998). Once released, ACTH enters into the blood flow and stimulates the adrenal cortex to release GCs: cortisol, hydrocortisone and corticosterone. Mineralocorticoids, such as deoxycorticosterone and aldosterone, alongside sex steroids, such as progesterone, are also released. The release of GCs in stressful situations aims to raise the level of glucose in the blood, helps to convert fats into energy, increases blood flow, and stimulates behavioral responses; at the same time it inhibits unnecessary vegetative activities such as digestion, growth, reproduction and immune system (Stratakis and Chrousos, 1995), to prepare the body to deal with the emergency.

Corticoids

Glucocorticoid receptors (GRs) are ligand dependent transcription factors that regulate the expression of several genes. The appropriate modulation of its expression is critical for maintenance of cellular homeostasis. Genomic actions of steroids on CNS are mediated by two types of receptors (de Kloet et al., 1987, 1993): the glucocorticoid receptor type II (GR) and the mineralocorticoid receptor type I (MR). Both differ from each other in their specificity and affinity for ligands, their location and their function. Inactivated GRs have a predominantly cytoplasmic localization. Free receptor forms a complex with a system of chaperone proteins (heat shock protein or hsp90, hsp70 and hsp40) or with FKBP52 (immunophilins; Rajapandi et al., 2000; Galigniana et al., 2001; Pratt et al., 2001). These proteins confer to the receptor high affinity for the hormone and dissociate from the receptor upon binding the hormone. However, these proteins also seem to be involved in the movement of GRs from the cytoplasm to the nucleus along microtubules (Galigniana et al., 2001; Pratt et al., 2001). When the hormone binds to the receptor, the complex undergoes a conformational change that mediates its activation and facilitates the binding of specific DNA sequences to regulatory or promoter regions of genes. GCs therefore control gene expression by activating or inhibiting specific genes.

MR primarily binds to GCs such as corticosterone and mineralocorticoids such as aldosterone, presenting high affinity for both, whereas GRs have relatively low affinity for corticosterone and higher affinity for cortisol and various synthetic GCs such as dexamethasone. MRs are present in target organs for mineralocorticoids such as kidney, intestine and salivary glands, where they regulate the osmotic balance and salt intake. At the central level, MRs are particularly expressed in the hippocampus, septum, amygdala, olfactory bulb and in regions of the cerebral cortex (de Kloet et al., 1993). Within the hippocampal formation, they abound in the CA1 and CA3 areas as well as in the dentate gyrus, and they have also been found in lesser amounts in the nucleus of the solitary tract. On the other hand, GRs present a more widespread distribution and are therefore present in all tissue types. However, within the CNS, they are more abundant in the limbic system (septum and hippocampus), in PVN and in brainstem monoaminergic areas.

The distinct affinity of both receptors for corticosterone determines a different degree of occupation depending on circulating levels of GCs. Thus, when the concentration of GCs is high, as occurs in rats during the night or after stressful situations, GRs are the receptors involved. In contrast, MRs are involved during the light phase, when they play a modulatory role in the basal levels of the HPA axis (de Kloet et al., 1987). At the peripheral level, almost all tissues possess GRs and, in a lesser extent, MRs (Munck et al., 1984). The varying distribution of these receptors in tissues allows GCs to act in different metabolic processes that are essential for body homeostasis. Finally, this general vegetative excitation returns to the brain, initiating a feedback on internal receptors in the body. This feedback may result in a further increase in the overall excitation (sympathetic stimulation) and, conversely,

a relaxation (parasympathetic stimulation) by decreasing the excitation.

The effects of GCs on the stress response are important and necessary. Physiological changes associated with stress include mobilization of energy to maintain brain and muscle function, (which sharpens and focuses the attention of the perceived threat), increased cerebral perfusion rates and local cerebral glucose utilization, enhanced cardiovascular output and respiration, redistribution of the blood flow, and modulation of immune system (Carrasco and Van de Kar, 2003; Filipović and Pajović, 2009). However, its long-term activation can have harmful effects on health, such as increased blood pressure, muscle tissue damage, atherosclerosis, diabetes, inhibition of immunological responses (Sorrells and Sapolsky, 2007; Tank and Lee Wong, 2015), and even damage in brain structures such as the hippocampus (Sapolsky, 1986; McEwen, 1999).

Models of Stress

Depending on the time of exposure, different stress models have been developed (Jaggi et al., 2011). Exposure to a stressor can last from seconds to days or weeks, and the effects of stress on the endocrine system depend on the duration of the exposure and its impact. If the stressful stimulation is repeated and prolonged in time, it is considered chronic stress and has been associated with important psychological and physiological conditions in humans.

Two types of chronic stress can be distinguished: continuous chronic stress and intermittent chronic stress. In chronic continuous stress, the animal may be successively subjected to stress for days or weeks, whereas in intermittent chronic stress animals are exposed for a period of weeks to a stressful situation, with daily exposure time ranging from minutes to hours. As an example of a model of continuous stress, we could mention social stress in rodents. This is particularly noticeable when the aggressive behavior of the male is enhanced by the presence of females (Blanchard et al., 1993).

The stress model most used experimentally is the intermittent chronic stress model, in which animals are exposed to a stressful situation on daily basis. In the literature, the most commonly used models are exposure to cold, restriction of movement (in a tube), forced swimming and electric foot shock among others (Katz et al., 1981; Armario et al., 1988; Riccio et al., 1991). The chronic variable stress is another model of chronic stress that shares characteristics with the two types of chronic models listed above; the animals are daily exposed to different random stressors, making it difficult to predict the arrival of a particular stimulus and avoiding the consequent adaptation (Katz et al., 1981; Armario et al., 1985, 1988).

Dual Roles of Glucocorticoids in Microglia Activation and Polarization

From a historical perspective, GCs are strongly associated with anti-inflammatory hormones, as exemplified by the 1950 Nobel Prize in Physiology or Medicine, awarded jointly to Edward Calvin Kendall, Tadeus Reichstein and Philip Showalter Hench *"for their discoveries relating to the hormones of the adrenal cortex, their structure and biological effects"*. In keeping with this view, the action of GCs in the brain could be solely seen as inhibitors of pro-inflammatory microglia under conditions of brain inflammation. However, accumulating evidence from the peripheral and the central nervous system have revealed that, under specific conditions, GCs may promote brain inflammation including the pro-inflammatory activation of microglia. It is, however, important to highlight that GC actions do not necessarily have to fully mimic stress actions. From studies in the peripheral system, it has been established that the timing and duration of the stress response may be critical for delineating an immune response (Sorrells et al., 2009). If stress takes place before immune activation, it may sensitize (prime) immune cells for a greater response. In this section, we will briefly summarize literature demonstrating anti- and pro-inflammatory actions of GCs on brain. Special attention will be paid to the pro-inflammatory actions of either GCs or stress in the CNS, considering their relevance in the course of different neurodegenerative diseases.

Pro- and Anti-Inflammatory Actions of GCs

As already mentioned, the timing of GC administration may be critical in finally determining a pro- or an anti-inflammatory action. Supporting this idea, it has been shown that *in vitro* treatment of microglia with GCs decreases the ability of these cells to produce interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) in response to stimulation with lipopolysaccharide (LPS; Tanaka et al., 1997). In similar conditions, dexamethasone suppresses LPS-induced nuclear factor kappa B (NF-κB) activation in the brain (Glezer et al., 2003). Interestingly, NF-κB has been involved in the resolution of inflammation (Lawrence and Fong, 2010; Taoufik et al., 2011). Similarly, GCs suppress microglial toxic radical production (Colton and Chernyshev, 1996; Drew and Chavis, 2000) and cell proliferation (Woods et al., 1999). Exogenous corticosterone abolishes cytokine gene expression in microglia (Blais et al., 2002; Nadeau and Rivest, 2003). Together, these studies point out that GCs are able to directly inhibit microglia function and suggest a possible role for GCs in the regulation of microglia function *in vitro*. Stress-related GCs, when applied after an inflammatory challenge, are also anti-inflammatory. Thus, post-LPS acute stress decreases the production of pro-inflammatory cytokines (Goujon et al., 1995). Systemic LPS has been associated to GCs increase. Interestingly, adrenalectomy potentiates the increase of brain cytokines produced by systemic LPS. Consequently, GCs released upon LPS treatment inhibit the inflammatory response triggered by LPS.

Even though GCs may exert immunosuppressive actions on microglia, it is becoming evident that they may also play opposite roles, this effect being particularly evident if GCs or stress are applied before a pro-inflammatory challenge. The administration of GCs prior to a systemic LPS challenge exacerbates brain inflammation in terms of up-regulation of mRNA expression of interleukin-1β (IL-1β) and TNF-α in the hippocampus (Frank et al., 2010). However, under the same experimental conditions, GCs diminishes the expression of pro-inflammatory cytokines when given 1 h after LPS

challenge (Frank et al., 2010). In this study, GCs act as pro-inflammatory agents at 2 and 24 h before LPS challenge, thus suggesting that the previous exposure to GCs could prime microglia (Frank et al., 2010). To demonstrate that microglia is the target of GC action, the authors isolated microglia from GC-pretreated rats and further challenged it *ex vivo* with LPS. Under these conditions, GCs treatment further upregulated MHCII and toll-like receptor-4 (TLR-4), and produced more IL-1β and TNF-α (Frank et al., 2010), supporting the hypothesis that GCs sensitizes brain microglia to subsequent pro-inflammatory stimuli. The pro-inflammatory actions of GCs may be neurotoxic under conditions associated to neural damage and inflammation. The effect of GCs under conditions of excitotoxicity induced by intrahippocampal kainic acid injections are associated to pyramidal CA3 degeneration and glial response (Dinkel et al., 2003). In this study, GCs increased the number of hippocampal immune cells including microglia, up-regulated mRNA and protein expression of IL-1β and TNF-α, and increased the kainate-induced lesions in the CA3 region (Frank et al., 2010). Further evidence that GCs exacerbate the microglial pro-inflammatory response under conditions of excitotoxicity derived from hippocampal cultures, where GCs exacerbates KA-induced expression of pro-inflammatory cytokines (Macpherson et al., 2005). Different stress paradigms, when applied before an inflammatory challenge, have also been shown to sensitize pro-inflammatory microglia. Thus, stress exposure to inescapable tail shock (Johnson et al., 2002, 2003, 2004) potentiated the expression of pro-inflammatory mediators (i.e., IL-1β, TNF-α and inducible nitric oxide synthase, iNOS) in hippocampus, hypothalamus and frontal cortex, as well as the sickness response (i.e., fever) produced by a peripheral injection of LPS given 24 h after the stressor regimen. Similar results were found after 14 daily sessions of unpredictable chronic stress (Munhoz et al., 2006) in terms of expression of pro-inflammatory mediators (TNF-α, IL-1β, iNOS). Subacute stress also induces GR-dependent microglia proliferation (Nair and Bonneau, 2006). In this study, the authors used a murine restraint stress model known to elevate GC levels. Stress maintained for 6 days increased microglia proliferation *in vivo*. This stress-induced proliferation of microglia is mediated by GCs since RU486 (or mifepristone), a potent inhibitor of GR activation, prevented this effect (Nair and Bonneau, 2006). Besides, N-methyl-d-aspartate (NMDA) receptor blockage prevented the proliferating stimulus induced by stress. Further work is needed to shed light into the role of NMDA receptors in microglia proliferation since NMDA receptor expression is mostly located on neurons. A paradigm of inescapable shock 24 h before microglia isolation from hippocampal tissue, and further plating and treatment with LPS, was used to test if microglia was the neuroimmune substrate responsible for the stress-induced pro-inflammatory response (Frank et al., 2007). This experimental design showed that stress primes microglia to a subsequent inflammatory challenge. Besides, it was found that stress downregulated CD200, a neuronal glycoprotein that maintains microglia in a quiescent state of activation (Hoek et al., 2000).

We and others have demonstrated that chronic stress potentiate the microglial pro-inflammatory response (de Pablos et al., 2006, 2014; Tynan et al., 2010; Espinosa-Oliva et al., 2011; Wohleb et al., 2011; Hinwood et al., 2012). Psychological chronic stress-induced inflammatory changes in microglia/macrophages were blocked by propranolol, an indication of an active role of β-adrenergic receptors in stress-induced microglial pro-inflammatory response (Wohleb et al., 2011). GRs but not MRs seem to have a central role in stress-induced sensitization of microglia. Blockage of GRs prevents stressor-induced enhancement of cytokine release (Munhoz et al., 2006). We and others have demonstrated that blockage of GRs prevents stress-induced microglial activation (Nair and Bonneau, 2006; de Pablos et al., 2006; Espinosa-Oliva et al., 2011). Similar results have been found using inhibitors of GCs synthesis (Nair and Bonneau, 2006). These studies suggest that GRs are key mediators in modulating the activation state of microglia.

The role of IL-1β in the stress-induced sensitization of proinflammatory immune response and GC responses to a subsequent immune challenge has been analyzed (Johnson et al., 2004). Intracisternal administration of IL-1 receptor antagonist 1 h before tail shock completely prevented the stress-induced enhancement in central and pituitary IL-1β and plasma IL-6 release following LPS challenge (Johnson et al., 2004). Central human recombinant IL-1β administration significantly enhanced elevated central IL-1β levels and plasma corticosterone following LPS challenge. This study demonstrates a central role of IL-1β in mediating microglia brain priming to subsequent immune challenge. More efforts have been made to uncover how GCs sensitizes brain microglia to further pro-inflammatory stimuli, thus becoming neurotoxic. Within this context, and considering the central role of IL-1β in modulating GC-derived immune responses, the NLRP3 inflammasome (nucleotide-binding domain, leucine-rich repeat, pyrin domain containing proteins) appears as an attractive target where GCs may exert its pro-inflammatory action (**Figure 2**). NLRP3 senses various stimuli ranging from ATP, nigericin, uric acid crystals, *Escherichia coli* and *Citrobacter rodentium* (Gurung and Kanneganti, 2015). Activation of the NLRP3 inflammasome is tightly regulated and requires two independent signals: a priming step is first needed to drive transcription of inflammasome machinery. This first signal is usually facilitated through activation of TLRs or nucleotide-binding oligomerization-domain protein 2 (NOD2) to initiate the NF-κB signaling pathway. It should be first highlighted that GCs increase the activity of the transcription factor NF-κB (Hermoso et al., 2004; Smyth et al., 2004; Munhoz et al., 2006). Blockage of TLR2 and TLR4 prevents stress-induced priming of the microglial pro-inflammatory response, thus supporting the notion that pro-inflammatory actions of GCs relies, at least in part, on TLRs signaling (Weber et al., 2013). Once the machinery of NLRP3 inflammasome is synthesized due to the priming response, subsequent activation, referred to as "signal 2", results in its oligomerization and inflammasome assembly, eventually leading to caspase-1-dependent cleavage and secretion of pro-IL-1β and pro-IL-18 (**Figure 2**). GC-induction of NLRP3 could serve as a mechanism for

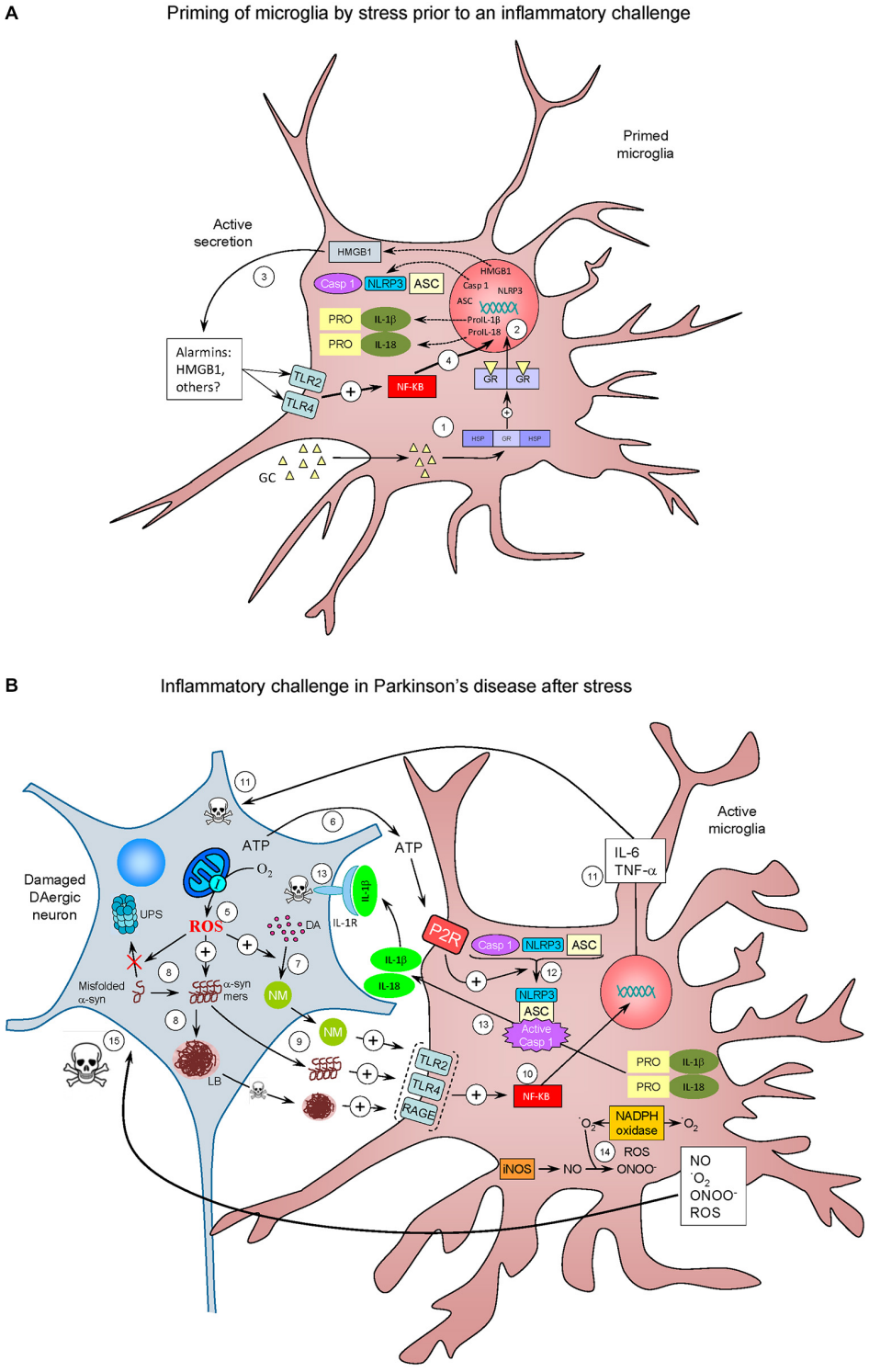

FIGURE 2 | The realm of vicious cycles. (A) Microglia priming by stress prior to an inflammatory challenge. (1) Stress increases glucocorticoids (GCs) levels, which enter the microglial cell. Once inside, GCs can bind mineralocorticoid and Glucocorticoid receptors (GRs) or both (here referred to as GR). GR are normally bound to HSPs. Upon binding, HSPs are released from GR to further translocate to the nucleus and induce transcriptional machinery (2) that lead to active secretion of HMGB1 (3), an alarmin well-known for being an endogenous true-ligand of Toll-like receptors (TLR), including TLR2 TLR4. TLR activation is known to activate NF-κB (4), thus inducing transcription of the NLRP3 inflammasome machinery (NLRP3, ASC and caspase 1) along with expression of pro Il-1α and pro IL-18 proteins (caspase-1 substrates). Under these conditions, microglial cells become primed for a subsequent inflammatory challenge. **(B)** Inflammatory challenge in Parkinson's disease (PD) after stress.

(*Continued*)

FIGURE 2 | Continued
Several factors (genetic and/or non genetic) may drive nigral DAergic neurons to an altered state in which ROS production (for example) are increased (5). Degenerating cells release different factors, including ATP (6), a well known inducer of NLRP3 assembly. ROS may induce an excessive production of the dark polymer neuromelanin (NM) (7), as well as impairs degradation of misfolded α-synuclein (α-syn) by the ubiquitin proteasome system (UPS) and potentiate the formation of α-syn oligomers (α-syn-mers) and Lewy bodies (LB), the most distinctive histopathological feature of PD (8). NM, α-syn oligomers and LB (this after neuronal death) are released from the dying DAergic neurons and recognized for different PRRs including TLR2, TLR4 and RAGE (9). Again, this leads to NF-κB activation and translocation to the nucleus (10), with the consequent transcription of pro-inflammatory genes (11); pro-inflammatory cytokines as TNF-α and IL-6 may induce neuronal death (11). ATP released from the damaged DAergic neurons activate the purinergic receptor (P2R) on microglial cells, leading to the assembly of the NLRP3 inflammasome and caspase-1 activation (12). Activated caspase 1 cleaves pro-Il-1α and pro-IL18 to IL-1β and IL-18, which are then released (13). IL-1β is known to bind IL-1R on DAergic neurons, which may contribute to cell death (13). Activation of microglia is accompanied by increased activity of different ROS- and RNS-producing enzymes such as iNOS, NADPH oxidase and myeloperoxidase (not shown) (14). NADPH oxidase catalyzes the production of superoxide anion ($\bullet O_2$) from oxygen in response to different pro-inflammatory stimuli. $\bullet O_2$ reacts with NO (mainly derived from upregulation of iNOS) to produce peroxynitrite (ONOO-), the most reactive free radical, thus inducing nitrosative stress. Peroxynitrite can both initiate and sustain a toxic loop eventually leading to neuronal damage (15), establishing a self-perpetuating process of neuroinflammation and neurodegeneration.

stress- and GC-induced priming of neuroinflammatory processes (**Figure 2**). GCs induce NLRP3 mRNAs and proteins in primary macrophages (Busillo et al., 2011). More interesting, these authors found that dexamethasone enhanced the ATP-induced release of IL-1β, an effect that was prevented by RU486, thus suggesting that GCs may sensitize macrophages-derived proinflammatory response by regulating NLRP3 expression and function (Busillo et al., 2011). This was confirmed by using a caspase-1 inhibitor, which completely inhibited the ATP-dependent and GC-enhanced release of IL-1β (Busillo et al., 2011). Subsequent studies suggested a similar effect of GCs on microglial cells (Frank et al., 2014). In this study, microglia was isolated from adrenalectomized rats and treated with different doses of GCs to be subsequently challenged with systemic LPS. A dose-response effect was found in terms of Iba-1, MHCII, NF-κBIα and NLRP3, in a concentration dependent manner. Activity of the NLRP3 inflammasome was not measured in this study. It would be interesting to test the effect of GC treatment on ATP or nigericin-induced NLRP3 assembly, caspase-1 activation and IL-1β release. Considering that ATP is released by injured or dying cells, these findings may explain why GCs enhances the immune response. Injured and dying cells release danger-associated molecular patterns (DAMPs). DAMPs and pathogen-associated molecular patterns (PAMPs) are detected by TLRs, thus activating the immune system (**Figure 2**).

Stress-induced release of DAMPs represents an attractive molecular link between stress and priming of brain microglia. Supporting this view, it has been demonstrated that inescapable tail shock induced high mobility group box-1 (HMGB-1), which is considered an archetypical alarmin (Frank et al., 2015) in the hippocampus, along with NLRP3 up-regulation (Weber et al., 2015). Intracisternal administration of BoxA, an HMGB-1 antagonist prior to stress, prevented the LPS-induced up-regulation of NLRP3 and proinflammatory cytokines in isolated cultured microglia (Weber et al., 2015). Interestingly, microglia isolated from animals subjected to inescapable tail stress was found to actively secrete HMGB-1, thus bringing a rationale of how acute/chronic stress or GCs may lead to release of one or different DAMPs, thus increasing the inflammatory response to a subsequent pro-inflammatory stimulus. **Figure 2** highlights more potential mechanistic events linking chronic stress and PD.

Effect of Chronic Stress in Animal Models of Parkinson's Disease

Animal models of PD based on neurotoxins and the manipulation of genes implicated in this disease have been important in elucidating aspects as the molecular basis of dopaminergic neurons death or the importance of misfolded proteins in neurodegeneration, respectively (Dauer and Przedborski, 2003). Although research has deepened into the pathology and symptoms of PD, the factors involved in the onset and course of this disease remain to be characterized. Taken into account the pro-inflammatory effects of GCs on the CNS and the important role that inflammation plays in the development of PD, stress have been considered to influence not only the diversity in symptoms and course of PD within different patients, but also their individual responses to medication after the onset of the disease (Foley et al., 2004).

With regards to the effects of stress in different animal models of PD, recent studies have demonstrated that stress and high corticosterone levels can exacerbate nigral neuronal loss and motor symptoms in these animals (Smith et al., 2008; Kelly et al., 2012; de Pablos et al., 2014). This suggests that the impediment of functional and structural compensation generated by stress may be enhancing neurodegenerative processes and thus may represent a key factor in the pathogenesis of PD.

One of the first studies about the relationship between PD and stress showed that chronic stress and elevated corticosterone levels increased neurodegenerative events and motor deficits in a rat model of PD (Smith et al., 2008). It is generally acknowledged that intracranial injection of 4 mg/mL of 6-OHDA induces DA depletion (Kostrzewa and Jacobowitz, 1974; de Pablos et al., 2014). They evaluated the effects of both chronic restraint stress and oral treatment with corticosterone, a hormone released under stress conditions, on motor impairments and neurodegenerative processes in the unilateral rat model of PD. The authors showed that chronic psychological stress, such as that produced by daily restraint, and elevated corticosterone levels can separately lead to impaired skilled limb use in both naïve and 6-OHDA DA-depleted rats. Furthermore, in the 6-OHDA injected group, stress and corticosterone treatments also disturbed limb coordination and altered exploratory behavior, which was accompanied by an acceleration of the

neurodegenerative processes and neuronal loss in the SN. All these findings point out that stress can increase functional deficits and thus accelerate the loss of DA-producing neurons (Smith et al., 2008).

Methamphetamine (METH) is known to cause damage to dopaminergic nerve terminals in the striatum, producing loss of TH and DA, and an activation of microglia and astroglia (Seiden et al., 1976; Hotchkiss et al., 1979; Bowyer et al., 1994; Miller and O'callaghan, 1994; O'callaghan and Miller, 1994; Ladenheim et al., 2000; Thomas et al., 2004; Fleckenstein et al., 2007; Krasnova and Cadet, 2009). To suppress this METH-related neuroinflammation, mice were pre-treated with corticosterone, both acutely (30 min before METH) and chronically (1 week before METH; Kelly et al., 2012). Not only both treatments failed to prevent neuroinflammation responses to METH, but the striatal neuroinflammatory response to METH was enhanced when mice were chronically pre-treated. This effect was further accompanied by enhanced astrogliosis and dopaminergic neurotoxicity. Furthermore, chronic pre-treatment also sensitized frontal cortex and hippocampus to induce a neuroinflammatory response to METH. These levels of chronic corticosterone are associated with high physiological stress, suggesting that chronic corticosterone therapy or sustained physiological stress may sensitize neuroinflammation and neurotoxicity responses produced by pro-inflammatory drugs as seen on METH (Kelly et al., 2012).

In another study showing the negative effects of stress/GCs (de Pablos et al., 2014), the authors observed that chronic stress increased both microglial activation and the expression of pro-inflammatory markers in an animal model of PD based on the intranigral injection of LPS. Importantly, they reported a higher inflammatory response in stressed animals which was associated with an increased death rate in dopaminergic neurons of the SN. To test whether this effect was related to GCs, they pretreated the animals with the GR inhibitor RU486. When the animals received this treatment, both microglial overactivation and the subsequent neuronal death in response to LPS were prevented, once again suggesting a potential negative role of stress/GCs in PD (de Pablos et al., 2014).

Anti-Inflammatory Effects of GCs

However, besides their negative effects, GCs are the most powerful endogenous immunosuppressors via inhibiting the transcription of genes involved in the innate immune response, such as the pro-inflammatory transcription factor NF-κB (McKay and Cidlowski, 1999). Within this context, animals treated with RU486 showed a rapid and severe neurodegeneration after the intracerebral infusion of LPS (Nadeau and Rivest, 2003). They attributed this effect to impaired levels of cytokines in the cerebral environment, in particular TNF-α, as the inhibition of its biological activity was able to abolish the neurotoxic effect of LPS injection. According to these data, GCs could play a role as modulators of the innate immune system in the CNS; therefore, an imbalance on their immunosuppressive activities may lead to cerebral damage (Nadeau and Rivest, 2003).

As mentioned before, GCs exert their actions mostly through ubiquitously expressed type II GRs. Since several reports have hypothesized a link between GC-GR responses and PD pathogenesis, several studies have attempted to elucidate the role of these receptors in regulating dopaminergic neurodegeneration (Morale et al., 2004; Ros-Bernal et al., 2011). Ros-Bernal et al. (2011) performed 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) injections in mice in which GRs had been selectively knocked-out in either macrophages/microglia or dopaminergic neurons. MPTP treatment unleashed an increase in CNS GRs that was followed by a rapid increment in the number of microglia colocalizing these receptors. Mice lacking GRs in microglia but not in dopaminergic neurons experienced a higher loss of dopaminergic neurons after MPTP intoxication that could not be prevented by corticosterone. The increased death of dopaminergic neurons observed in the former was also correlated with reductions in DA uptake and DA levels in the striatum. Moreover, the absence of microglial GRs increased microglial reactivity and continuous activation. The regulatory role of GRs was confirmed by a higher expression of pro-inflammatory genes, such as TNF-α, alongside a decrease in anti-inflammatory genes, as IL1-R2. Together, these findings point out the potential role for the GC-GR system in the development of PD, and how GR dysfunction in PD may result in a subsequent chronic inflammatory reaction generating a positive feedback (Ros-Bernal et al., 2011).

Morale et al. (2004) injected MPTP in transgenic mice that constitutively expressed GR antisense RNA from early embryonic life. This produced a deficiency in GRs. They found this deficiency exacerbated the MPTP-induced toxicity to dopaminergic neurons, so that an increased loss of tyrosine hydroxylase positive nigral neurons and a decrease of DA levels in the striatum were observed. In these mice, microglia produced higher nitrite levels than in wild-type mice; these increases occurred before the loss of dopaminergic function. This shows the importance of GRs in the anti-inflammatory and immunosuppressive effects of GCs, and establishes a relation between impairment function of GRs and vulnerability to MPTP (Morale et al., 2004).

A question arising from these studies is how acute and chronic stress can trigger opposite effects in terms of brain inflammation and associated neurodegeneration. The paradoxical actions of GCs on microglial activation may be associated with differences in pharmacological approaches, experimental models or steroid hormone concentrations (Glezer et al., 2003; Macpherson et al., 2005). However, as mentioned earlier in this review, the timing and duration of stress response may be critical for modulating the immune response (Sorrells et al., 2009). Although evidence for a causal relationship between stress and PD has not been defined, stress could be playing an important role in its pathogenesis. GRs density differs throughout distinct brain structures and, interestingly, it is increased in regions involved in motor control, such as the motor cortex, basal ganglia and cerebellum (Ahima and Harlan, 1990; Ahima et al., 1992). This might in turn enhance their susceptibility to the effects of stress in both

humans (Maki and Mcilroy, 1996) and rats (Metz et al., 2001, 2005).

Therefore, stress might represent a critical variable to be consider in the progression of neurodegenerative events underlying PD, where it can contribute to early onset of the motor symptoms in the presymptomatic stage of the disease as well as worsen the symptoms of PD once the disease has been diagnosed (Smith et al., 2008).

Chronic Stress and Parkinson's Disease in Humans

Within the medical community, it is generally accepted that chronic stress can be a contributing factor in the development of mental illness, metabolic diseases and immune system suppression, amongst many other physiological dysfunctions. For instance, it has been recently discovered a genetic relationship between anxiety and hypertension through microRNA regulation of the cholinergic pathway (Hanin et al., 2014). Higher risk of developing a neurological disease after prolonged emotional stress has already been established in AD (Rothman and Mattson, 2010). Although this relationship has not been established yet in PD, some studies prove that there is a higher prevalence of PD in people suffering stress. For instance, depression, as well as other chronic stress situations, increase the prevalence of PD (Schuurman et al., 2002) and seems to be related to severe symptoms like dyskinesias, disturbed sleep, and bradyphrenia (Pålhagen et al., 2008). The relationship between these two pathologies is not well-defined yet. Some studies estimate a 50% prevalence of depression in PD patients compared to 11% in non-PD patients (Hemmerle et al., 2012). Depression has also been proposed as a symptom for some subtypes of PD (Brown et al., 2011). However, this poorly understood relationship is due to an overlap of symptoms between both diseases. Defining depression as a previous stage, a concomitant disease, a non-motor symptom or an emotional stress factor is complicated because depression does not follow the same rhythm as other symptoms during PD progression. A possible explanation may be a deficit in neurotransmitters such as norepinephrine (Espay et al., 2014) or serotonin (Fox et al., 2009), suggesting a complementary field of action of chronic stress in PD through these neurotransmitters (Fitzgerald, 2014).

The idea of emotional stress being implicated in PD onset has already been supported in some PD patients who previously suffered the Holocaust (Salganik and Korczyn, 1990). Former American prisoners of war have roughly twice the rate of death due to PD (Page and Tanner, 2000). During World War I the term "shell shock" (currently known as post-traumatic stress disorder) was coined to describe those soldiers affected by a diversity of mental disorders resulting from combat stress (Crocq and Crocq, 2000). In most severe cases, many of the symptoms observed often remarkably resembled those of PD (Linden et al., 2013; Djamshidian and Lees, 2014). Among the motor symptoms recorded, they found mask-like expressions, resting tremors, postural instability, bradykinesia (slow movements), rigidity and freezing. Since intense traumatizing_events can trigger immediate "shell shock" symptoms resembling PD, it is reasonable to think that mild, but chronic stress may also elicit "shell shock"-like symptoms such as PD in the long run.

Chronic stress can induce pro-inflammatory cytokine and chemokine networks. These networks in turn cause a prolonged activation of the HPA axis (Haddad et al., 2002; McEwen, 2003) creating a cycle that exacerbates inflammation. TNF levels are increased during long-lasting stress periods in healthy volunteers (Visnovcova et al., 2015). Increased concentration of TNF has been found also in the SN of PD patients (Hirsch and Hunot, 2009). Further, it has been found a significant correlation between TNF levels and non-motor symptoms such as cognition, depression and disability in PD patients (Menza et al., 2010). On the other hand, regulatory T cells ($CD4^+$ $CD25^+$ $FoxP3^+$) are responsible of maintaining self-tolerance and controlling immune responses. In this sense, it was found a 48% reduction in the proportion of regulatory T cells in post-traumatic stress disorder patients vs. controls (Sommershof et al., 2009). Similarly, a study reported lower $CD4^+/CD8^+$ ratios and a 24% reduction of $CD4^+$ $CD25^+$ cells (reaching up to a 64% reduction for $CD4^+$ $CD25^{high}$ cells) in PD patients (Baba et al., 2005). DA by itself is able to suppress regulatory T cells and activate resting effector T cells ($CD4^+$ $<<<$ $CD8^+$), suggesting an important link between stress, lymphocytes and PD (Levite, 2015).

A review on the literature about the causes of PD concluded that emotional trauma could be a causative agent (Schwab and Zieper, 1965). In that article, the authors cited two specific cases. In one, an individual learned via telegram that his son had died in a plane that was shot down; in another, a woman saw her husband being killed in a car accident. In both cases, symptoms of PD emerged within hours, although such signs had never been previously detected. There is also a reported case of a woman who experienced sudden onset PD 1 week after discovering that her husband was involved in an affair (Zou et al., 2013). However, another study with more than 13,500 patients showed that the risk of PD was up to 42% lower among men that had experienced several major life events than those without any. No other correlation was found for women (Rod et al., 2010). The authors explained these results in part due to the fact that PD patients tend to be more cautious and have premorbid personalities, minimizing risk exposure, which could reflect an inherited tendency. In this sense, frontotemporal dementia and parkinsonism has been linked to chromosome 17 (Hong et al., 1998) and some forms of PD are inherited. They also argue that not all individuals respond equally to major life events.

Therefore, major life events do not necessarily reflect perceived stress by the individual and its allostatic load, meaning the wear and tear effect on the body (McEwen, 2007). Chronic stress develops from accumulation of little everyday life events and should, therefore, be approached differently in studies involving stress and PD. Some authors have established that stressors, such as job-related events, economic hardship, disappointment in love, loss of relatives and friends and social

isolation, may be risk factors for developing PD due to the important role they play in the mental state of the individuals affected (Smith et al., 2002; Salmon, 2006; Hemmerle et al., 2012). The risk of parkinsonism in different occupations has been studied and authors found that those with daily exposure to dangerous and/or stressful situations are common occupations of people who suffer from PD (Goldman et al., 2005; Tanner et al., 2009). In keeping with this view, Indiana became in 2009 the first state in the U.S.A. to recognize PD as a line of duty disability among firemen, policemen and EMS responders. Moreover, according to another study with almost ten thousand participants, vital exhaustion (a psychological response when a person is unable to solve or adapt to the source of stress) may be a pre-motor marker of neurodegeneration leading to PD (Clark et al., 2013). A symptom of vital exhaustion is unusual fatigue. Interestingly, mental fatigue, a typical feature of patients with chronic fatigue syndrome, can be found in up to 70% of PD patients at some stage of the illness (Friedman et al., 2011). Chronic fatigue syndrome can increase some classical PD symptoms in depressed patients including slow thinking (bradyphrenia), psychomotor retardation or psychomotor agitation (Lane et al., 1991; Djamshidian and Lees, 2014). In fact, abnormalities in walk movement were detected in patients with chronic fatigue syndrome (Boda et al., 1995), suggesting a possible motor dysfunction similar to that seen in PD patients. Stress can increase the symptoms of PD, such as tremor after episodes of anxiety or anger (Djamshidian and Lees, 2014).

Although typical clinical features of PD include motor symptoms, the disease also include some non-motor disturbances, such as autonomic dysfunction as well as neuropsychiatric problems such as depression, mood changes and pain. It is known that stress can transiently increase motor symptoms of PD, and a positive association between cortisol and gait deficits has been shown (Charlett et al., 1998). Several studies also discuss how stress may deteriorate the symptoms of PD patients, such as bradykinesia and akinesia, the difficulty to initiate movements, sudden motor blocks (freezing), and tremor (Schwab and Zieper, 1965). Moreover, stress can also affect some nonmotor signs of PD. Individuals with PD have reduced hedonic responses after exposure to emotional stress (Macht et al., 2007). These authors demonstrated that hedonic responses of PD patients were reduced by stress, being this effect independent from depressive mood. The authors suggested that the ability to respond in an emotionally positive manner to external stimuli may be reduced in PD.

Stress has also been considered to influence not only the diversity in symptoms and course of PD within different patients, but also their individual responses to medication after the onset of the disease (Foley et al., 2004). This might depend on the extension of dopaminergic damage, so that stress could make abruptly appear an altered behavior that was hidden in a preclinical phase. Supporting this, experiments on rats showed that drugs enhancing dopaminergic function reversed neurological deficits induced by stress, while harmful effects of stress were increased by dopaminergic antagonists (Snyder et al., 1985).

However, the greatest risk factor for PD appears to be age, since the symptoms of PD emerge preferentially after the age of 65. In this regard, it is important to note that dysfunctions in the stress response develop during the aging process. Hence, as an organism ages, the response of the HPA axis to stress becomes hyperactive and less efficient to return to former homeostatic conditions, thus exposing brain cells to higher levels of GCs for longer periods of time (Stein-Behrens et al., 1992). Taking into account that another factor in the late onset of PD is the increased vulnerability of DA neurons to insults, the deregulation of the HPA axis might render cells in the aged brain more susceptible to degeneration in the face of subsequent stress.

High levels of cortisol have been found in PD patients (Hartmann et al., 1997; Charlett et al., 1998; Djamshidian and Lees, 2014). High levels of cortisol provoked by a dysfunctional HPA axis have been associated to dopaminergic cell loss and motor disability (Müller and Muhlack, 2007, 2008). However, a relative diurnal decrease of 22% in cortisol secretion in PD patients vs. controls has been reported (Hartmann et al., 1997). They postulated that a decreased expression of hippocampal MRs in PD patients may explain such reduction (Hartmann et al., 1997). All these data point out that stress could be an important factor in the development of PD. Measurement of stress hormones such as salivary cortisol levels (Djamshidian and Lees, 2014), inflammatory markers such as cytokines (Hirsch and Hunot, 2009), and the roles of physical exercise and cognitive behavioral therapy are potential lines of future research that may further clarify the role of stress in PD.

Concluding Remarks

Accumulating evidence demonstrates how stress-related GCs may sensitize microglia to subsequent pro-inflammatory challenges, thus enhancing the brain inflammatory response (**Figure 2**). Excessive pro-inflammatory microglia activation may be neurotoxic, thus bringing a rationale between chronic stress and the progression of different neurodegenerative diseases, particularly PD. The potential involvement of brain inflammation in the etiology of PD is well established. Consequently, stress-related GCs may be an important contributing factor to modulate the long-term brain inflammatory response, including the appearance of neurotoxic microglia. Since midbrain dopaminergic neurons are especially sensitive to pro-inflammatory microglia, the impact of chronic stress in the etiology and course of PD deserves a special attention.

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frontiers in Cellular Neuroscience

REVIEW
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Implications of glial nitric oxide in neurodegenerative diseases

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Nitric oxide (NO) is a pleiotropic janus-faced molecule synthesized by nitric oxide synthases (NOS) which plays a critical role in a number of physiological and pathological processes in humans. The physiological roles of NO depend on its local concentrations, as well as its availability and the nature of downstream target molecules. Its double-edged sword action has been linked to neurodegenerative disorders. Excessive NO production, as the evoked by inflammatory signals, has been identified as one of the major causative reasons for the pathogenesis of several neurodegenerative diseases. Moreover, excessive NO synthesis under neuroinflammation leads to the formation of reactive nitrogen species and neuronal cell death. There is an intimate relation between microglial activation, NO and neuroinflammation in the human brain. The role of NO in neuroinflammation has been defined in animal models where this neurotransmitter can modulate the inflammatory process acting on key regulatory pathways, such as those associated with excitotoxicity processes induced by glutamate accumulation and microglial activation. Activated glia express inducible NOS and produce NO that triggers calcium mobilization from the endoplasmic reticulum, activating the release of vesicular glutamate from astroglial cells resulting in neuronal death. This change in microglia potentially contributes to the increased age-associated susceptibility and neurodegeneration. In the current review, information is provided about the role of NO, glial activation and age-related processes in the central nervous system (CNS) that may be helpful in the isolation of new therapeutic targets for aging and neurodegenerative diseases.

Keywords: nitric oxide, neuroinflammation, neurodegenerative disorders, neuronal nitric oxide, neuronal death

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Abbreviations: AD, Alzheimer's disease; BBB, blood-brain barrier; cGMP, guanosine 3′,5′-cyclic monophosphate; CNS, central nervous system; DA, dopamine; eNOS, endothelial nitric oxide synthase; GSH, glutathione; GSNO, S-nitrosoglutathione; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; LTP, long term potentiation; MS, multiple sclerosis; NMDA, N-Methyl-D-aspartate; NO, nitric oxide; NOS, nitric oxide synthase; nNOS, neuronal nitric oxide synthase; NSAIDs, Non-steroidal anti-inflammatory drugs; PD, Parkinson's disease; RONS, reactive oxygen and nitrogen species; sGC, soluble guanylyl cyclase.

Introduction

Nitric oxide (NO) was discovered as an endothelium-derived relaxing factor more than two decades ago, and since then, its participation in a widening number of pathways has been continuously reported. There is increasing evidence showing that alterations in the NO signaling may be related with different diseases as it plays a key role in diverse neurodegenerative-associated processes such as neuronal death, necrosis, apoptosis and autophagy (Calabrese et al., 2007). In particular, it has been suggested that S-nitrosylation is involved in the pathogenesis of various neurodegenerative disorders including Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), multiple sclerosis (MS) and Alzheimer's disease (AD). The neuroinflammation that characterize these pathologies is largely associated with the production of NO; it is the aim of this review to describe how these NO-induced outcomes are produced, as well as trying to explain why they are important in the context of neurodegeneration. Further understanding of how imbalanced NO metabolism can contribute to neuronal cell death is determinant to formulate achievable strategies for the prevention and treatment of neurodegenerative disorders. Moreover, as NO acts as a double-edged sword contributing both positively and negatively, or even simultaneously, to these diseases it is important to disentangle the effects of this molecule in order to attempt rational interventions towards them.

NO Signaling Pathways

In mammals, NO is mainly synthesized by nitric oxide synthases (NOS) through the conversion of L-arginine to NO and L-citrulline (Knowles and Moncada, 1994). Traditionally, three isoforms of NOS have been identified in central nervous system (CNS): NOS1 or neuronal NOS (nNOS), NOS2 or inducible NOS (iNOS) and NOS3 or endothelial NOS (eNOS; Alderton et al., 2001). These three isoforms differs in their activity patterns: (i) nNOS localizes to synaptic spines, astrocytes and the loose connective tissue surrounding blood vessels in the brain; (ii) iNOS is a calcium (Ca^{2+})-independent isoform not constitutively expressed by astrocytes and microglia but these glial cells often expressed this isoform in pathological conditions such in response to inflammatory stimuli (Saha and Pahan, 2006); and (iii) eNOS is present in both cerebral vascular endothelial cells and in motor neurons (Estévez et al., 1998). The activity of iNOS is tightly associated with its expression levels and is induced during cell inflammatory response while nNOS and eNOS activities depend on intracellular Ca^{2+} levels and their CNS expression.

To date, soluble guanylyl cyclase (sGC) is the most accepted physiological receptor described for NO. This receptor is formed by α and β subunits together with a prosthetic heme group with a ferrous iron. The binding of NO to this receptor activates the C-terminal catalytic domain, which produces guanosine 3′,5′-cyclic monophosphate (cGMP; Stamler et al., 1997). This enzyme activity is critically affected by redox status as the oxidation of the heme moiety on the β-subunit turns the enzyme sensitive to NO. There are other mechanisms by which oxidative stress may compromise this cGMP synthetic pathway (**Figure 1**). For instance, reactive oxidant peroxynitrite ($ONOO^-$) induced by NO in the presence of superoxide ($O2^-$) results in a dysfunctional uncouple variety of NOS that produces $O2^-$ rather than NO under oxidative stress (Xia et al., 1998; Sasaki et al., 2008).

In response to NO, sGC activity increases more than 200 fold. The increased level of cGMP activates selected pathways to induce cellular responses (Stamler et al., 2001). Briefly, the cGMP produced by NO-activated sGC directly interacts with its downstream effectors, such as cGMP dependent kinase (cGKI or PKG), cyclic nucleotide gated (CNG) channels and cGMP dependent phosphodiesterase (PDEs). There is great scientific agreement about the nNOS and eNOS implication in NO production in skeletal and cardiac muscle (Kobzik et al., 1994; Sartoretto et al., 2011). This endogenously produced NO can promote two physiological functions differentiated by its cGMP-dependence: (i) to induce relaxation through the cGMP signaling pathway (Balligand et al., 1993; Mohan et al., 1996); and (ii) to modulate increases in contraction independent of cGMP concentration (Kobzik et al., 1994). The activation of such elements is the preferred mechanism by which NO mediates most of its physiological effects including vascular smooth muscle tone and motility, phototransduction and maintaining fluid and electrolyte homeostasis (Palmer et al., 1987; Bredt et al., 1990; Knowles and Moncada, 1994).

However, emerging evidence suggests the participation of NO in another signaling mechanism: "S-nitrosylation" of target proteins. S-nitrosylation is a non-enzymatic post-translational modification consisting in a covalent addition of a NO group to a cysteine thiol/sulfhydryl (RSH). This S-nitrosylation participates in a huge number of physiological events including those implicated in muscular contraction (Xu et al., 1998), cellular trafficking (Ozawa et al., 2008), circulation (Singel and Stamler, 2005) and apoptotic pathways (Benhar et al., 2008; Cho et al., 2009). Coherent with this, a main implication of ryanodine receptor 1 (RyR1) has been found in the activation and S-nitrosylation of Ca^{2+} release channel in sarcoplasmic reticulum of skeletal muscle by low concentration of NO (Eu et al., 2000).

Based on these studies, a possible involvement of S-nitrosylation in neuronal function has been suggested in the brain, particularly in the cerebellar Purkinje cell layer and dentate gyrus where RyR1 messenger RNA (mRNA) is mostly prominent (Mori et al., 2000). However, more studies have to be performed to corroborate a preponderant S-nitrosylation pathway in CNS and its implication in neuroinflammatory processes and neurological disorders.

Neuroinflammation and Nitric Oxide

Neuroinflammation represents the coordinated cellular response to tissue damage and is characterized by the microglial release of pro-inflammatory factors such as cytokines, proteases and toxic free radicals. The progress associated with neuroinflammation can be acute or chronic, while the appropriate regulation of this general process facilitates recovery, uncontrolled

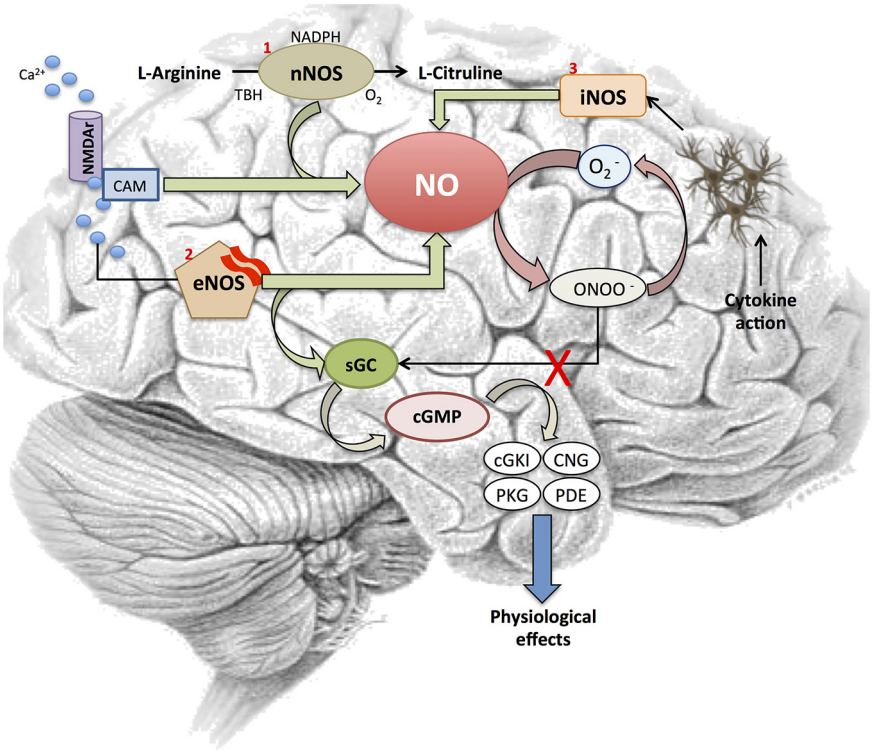

FIGURE 1 | Nitric oxide signaling pathway. Figure shows different steps in the NO signaling cascade and its interaction with several elements involved in the signaling pathway. NO is synthetized by two Ca^{2+}-dependent or one independent Ca^{2+}-mediated processes. First, (1) NOS1 or neuronal NOS (nNOS)-catalyzed reaction converts L-arginine into L-citrulline in the presence of O_2, nicotinamide adenine dinucleotide phosphate (NADPH) and tertiary-butyl hydroperoxide (TBH) after the activation of the NMDA receptor by Ca^{2+}. Also, (2) intracellular Ca^{2+} activates eNOS to release NO from brain microvessels. This NO binds to soluble guanylyl cylclase (sGC) receptors, which trigger a cGMP-dependent pathway and interacts with its downstream effectors (cGKI, CNG, PKG, PDE), the ultimate mediators of the NO's physiological response. In addition, sGC is also critically affected by redox status. NO initiates the synthesis of $ONOO^-$ when O_2^- is present, which results in a dysfunctional uncouple variety of nitric oxide synthases (NOS) that produces O_2^- rather than NO. Finally, (3) NO is synthesized following the transcriptional expression of a Ca^{2+}-independent iNOS isoform in glial cells, astrocytes and microglia after cytokine exposure.

neuroinflammation can induce a secondary injury. The main purpose of acute neuroinflammation is to remove the source of harm in order to restore the brain to a healthy condition. However, a maintained response is known to induce neuronal dysfunction and death (McGeer et al., 2003).

Neuroinflammation has been demonstrated to be closely associated with the pathogenesis of several psychiatric illnesses and neurodegenerative diseases like AD, PD and Huntington's disease (Bales et al., 2000; Hunot and Hirsch, 2003; Doorduin et al., 2009; Silvestroni et al., 2009; Dobos et al., 2010; Rao et al., 2010). Moreover, there is evidence showing that this condition is detectable years before significant loss of neurons occurs (Frank-Cannon et al., 2009; Fuhrmann et al., 2010; Ratai et al., 2011), hence its relevance in the context of neurodegenerative disorders. This paradigm is supported by several studies showing that a long-term treatment with Non-steroidal anti-inflammatory drugs (NSAIDs) may have a preventative effect in neurodegenerative diseases as the above mentioned (McGeer and McGeer, 2007; Wahner et al., 2007).

Neuroinflammation-induced cell death is often derived from the long-term impact caused by the increase of reactive oxygen and nitrogen species (RONS), which play a major role in eliciting apoptotic cell death through irreversible oxidative or nitrosative injury to neuronal elements (Nakagawa and Yokozawa, 2002). The brain is highly susceptible to oxidative stress due to its imbalance between an efficient antioxidant defense system and its capacity to generate oxidative species. As a matter of fact, the brain presents low levels of glutathione (GSH) and moderate activity of the antioxidant enzymes catalase, superoxide dismutases (SODs) and GSH peroxidase. On the contrary, the elevated levels of ascorbic acid, the high concentration of transition metals such as copper and iron, and the huge aerobic metabolism contribute to the generation of oxidative (ROS/RNS) species eliciting necrotic neuronal death (**Figure 2**).

There is an intimate relation between glial activation and neuroinflammation in the human brain. The presence of activated microglia was initially considered as a sensitive marker to identify potential sites of tissue destruction (Mosley et al., 2006; Galea et al., 2007). Even more, the engagement of astrocytes and endothelial cells, the observed activation of microglia and its implication in neuroinflammatory processes, have been widely

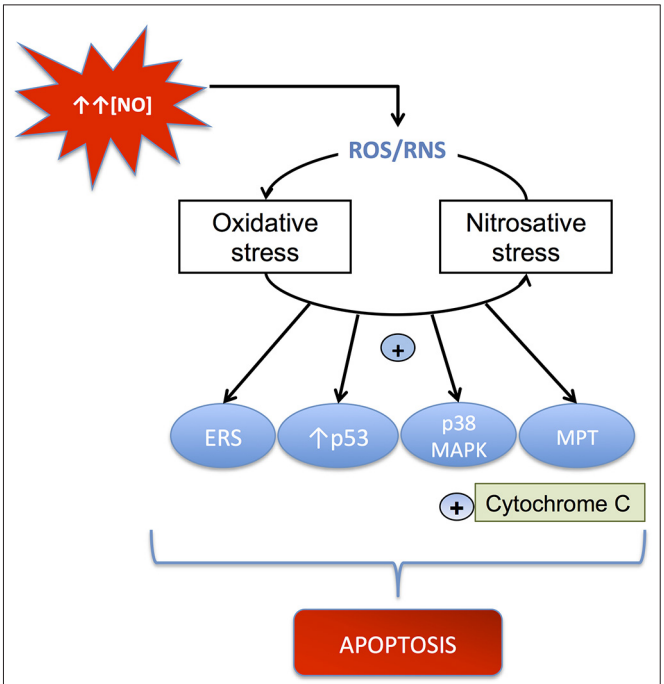

FIGURE 2 | Mechanisms through which ROS/RNS cause apoptosis. NO can induce oxidative and nitrosative stress, which activates mitochondrial apoptosis by several pathways, including: (i) stimulation of mitochondrial permeability transition (MPT); (ii) up-regulation of p53; (iii) activation of the p38 MAP kinase pathway; and (iv) induction of endoplasmic reticulum stress (ERS). All these processes produce cytochrome c release and apoptosis.

demonstrated to derive from the affection of neuronal viability through a persistent RONS generation.

The role of NO in neuroinflammation has been determined in animal models associated with excitotoxicity processes induced by glutamate accumulation and microglial activation. Released NO from activated microglia acts at the presynaptic site blocking the reuptake of glutamate, thus inducing the activation of N-Methyl-D-aspartate (NMDA) receptors and facilitating neuronal death (Rao et al., 2007, 2012; Kim et al., 2009). The regulation of iNOS isoform, highly implicated in neuroinflammatory processes associated with glial cells, takes place at the transcriptional level. Several transcription factors are implicated in trans-activation of iNOS gene, among them the nuclear factor *k*-light-chain-enhancer of activated B cells (NF-κB) is the most important one. Without the inhibition from the NF-κB p50:p65 arresting protein (IκB) NF-κB translocates to the nucleus and binds κB elements in iNOS promoter (Davis et al., 2005; Kanarek et al., 2010). In glial cells, this NF-κB-mediated iNOS expression triggers several pathways related with RONS formation, caspase and nNOS signaling activity and mevalonate production. It has been demonstrated that chronic NMDA administration up-regulates the levels of proinflammatory IL-1β, TNF-α, glial fibrillary acidic protein (GFAP) and iNOS in rat brains (Chang et al., 2008). Altogether these findings suggest that there is cross-talk between neuroinflammation and excitotoxicity that involves NO release and iNOS up-regulation in the brain.

However, it seems clear that NO modulation of inflammatory processes requires other mechanisms besides microglial cytokine release. The interaction with key regulatory pathways might be one of these mechanisms. Coherently, it is known that NO inhibits NF-κB activation thus controlling inflammation both in muscular cell lines (Hattori et al., 2004) and through release by nitrooxyphenyl acetylsalicylate (NO-ASA), a non-steroidal anti-inflammatory drug (NO-NSAID) in cancer cell lines (Kashfi and Rigas, 2005). Moreover, S-nitrosylation of NF-κB protein was the mechanistic role for NO-action resulting in diminished binding of this protein to DNA for transcriptional activation (Chattopadhyay et al., 2010).

Historically, astrocytes function in CNS injuries was reduced to maintain ionic homeostasis and participate in glial scar formation and tissue repair, which limits inflammation and promotes tissue repair, secreting nerve growth factors (Simard and Nedergaard, 2004). However, since NO and IL-1β are also produced by activated astrocytes, recent studies support an important active role of these cells in neuroinflammation and the neurodegeneration associated with dysregulations in NF-κB pathway and in NO production. Both dysregulations associates with a crosstalk between lipid mediators, such as sphingosin, and signaling inflammatory cytokines (Spiegel and Milstien, 2011).

Nitric Oxide and Neurodegenerative Diseases

Selective neuronal death is typical of most neurodegenerative diseases including PD, AD, ALS and MS (Guix et al., 2005). The participation of oxidative stress in the development of several neurodegenerative disorders has been largely documented (Calabrese et al., 2007), with NO suggested as a starring character (Chabrier et al., 1999). This relationship was evidenced by the fact that increased nitration of protein aggregates was prominent in different synucleinopathies and tauopathies (Duda et al., 2000; Horiguchi et al., 2003).

The augmented nitration of proteins can be initiated by an increase in the production of NO during neuroinflammation and the generation of free radicals by dysfunctional mitochondria, which are commonly observed in various neurodegenerative disorders (Guix et al., 2005; Pacher et al., 2007). Moreover, it has been demonstrated that NO is able to activate molecular elements, such as cyclooxygenase (COX; Mollace et al., 2005), which is typically up-regulated in brain cells under inflammatory conditions (Mancuso et al., 2007). In addition, the combination of NO and free radicals like the superoxide anion will result in the formation of highly reactive peroxynitrite. Peroxynitrite can then nitrate tyrosine residues on proteins to 3-nitrotyrosine, induce lipid peroxidation, and cause DNA damage (Ischiropoulos and Beckman, 2003).

NO is especially harmful under pathological conditions involving the production of RONS (Wahner et al., 2007) and $ONOO^-$ formation. Nitrotyrosination inhibits tyrosine phosphorylation and hence affects the signal transduction pathways of growth factor (Jonnala and Buccafusco, 2001). Moreover, the presence of nitrotyrosination has been described in several neurodegenerative diseases linked to oxidative stress,

such as AD (Guix et al., 2012), PD (Good et al., 1998) and ALS (Cookson and Shaw, 1999; Smith and Lassmann, 2002).

In sum, diverse stimuli ranging from neuronal impaired pathway-associated products to environmental toxins can trigger glial dysregulations. In neurodegenerative diseases, alterations derived from overactivated glia, microglia and astroglia are particularly present.

Alzheimer's Disease

The accumulation of β amyloid (Aβ) plaques and neurofibrillary tangles are the histopathological gold-standard hallmark for AD diagnosis. Together with these, the contribution of neuroinflammatory processes to the aging brain and the development of Alzheimer's and other neurodegenerative diseases is also well documented (Zhang et al., 2013; Mosher and Wyss-Coray, 2014). However, the exact mechanism by which microglial activation is disturbed in AD is still not completely understood.

Accordingly, a critical role of NO in the development of AD has been suggested, as neuronal cell loss, neuronal injury and protein misfolding are reported to occur as a consequence of NO overproduction (Nunomura et al., 2001; Nakamura and Lipton, 2011; Swerdlow, 2011). Significantly, nitrated form of protein tau has been reported in NFTs and neuritic plaques in brains of AD patients as well (Reynolds et al., 2006).

There is evidence that link NO production with mitochondrial dysfunction and neuroinflammation, especially as regards glial response (Jekabsone et al., 2007). Moreover the pro-inflammatory and toxic effects of amyloid in neurons co-cultured with glia are hampered by iNOS inhibitors (Brown, 2007).

Furthermore, it has been suggested that microglial nicotinamide adenine dinucleotide phosphate (NAPDH) oxidase complex is the major source of ROS in the brain (Wilkinson and Landreth, 2006).

The imbalance produced by the detoxification of ROS prompts an increase in oxidative stress that has proved to be involved in several excitotoxicity processes (Ferrer et al., 2010). S-nitrosylation has also been implicated in AD (Lipton et al., 1993), exhibiting a modulatory effect on glutamatergic NMDA receptors (Lipton, 2007b). Over-stimulation of NMDA receptors may produce an excessive Ca^{2+} influx that can generate ROS and activate excitotoxicity processes that lead to cell death. Moreover, this excitotoxicity that has been suggested as a mediator of neurotoxicity in this neurodegenerative disorder (Lipton, 2007a), and specifically in neurons, may also activate nNOS and induce NO overproduction (Gu et al., 2010; **Figure 3**).

Studies in cell cultures suggest that glutamate-induced cell death after NO release and ROS results from the inhibition of mitochondrial respiration caused by glial activation (Beckman et al., 1994; Loihl and Murphy, 1998; Bal-Price and Brown, 2001). Interestingly, Aβ stimulates the production of NO, which turns to be also a proinflammatory marker of microglial activation. There is evidence of an interaction between Aβ aggregates and microglia, as it was demonstrated that the later binds to the former through membrane receptors such as TLR2, 4, 6, and 9 (Bamberger et al., 2003), and this has been suggested as

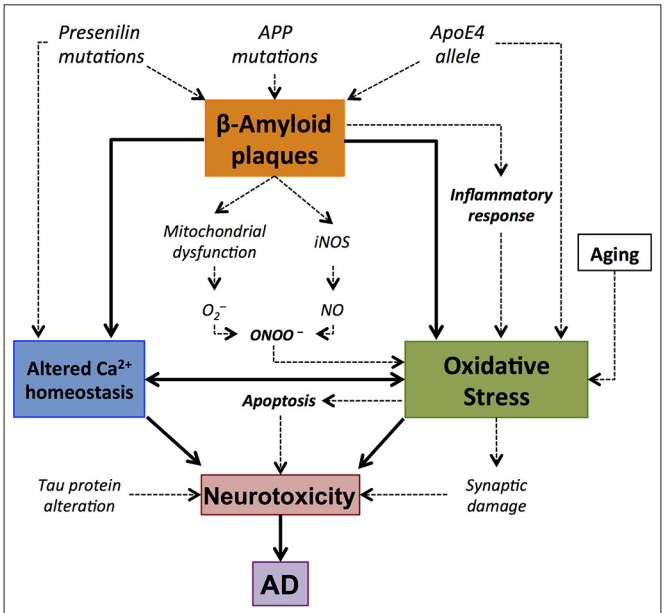

FIGURE 3 | Central role of amyloid β-peptide (Aβ) in the oxidative stress elements involved in Alzheimer's disease (AD). Accumulation of Aβ plaques results in oxidative stress. This oxidative stress might result from the implication of different pathways, such as mithocondrial dysfunction or inflammatory response, and it is manifested by synaptic damage and alterations in Ca^{2+} homeostasis. This may lead to apoptotic processes that result in the death of the cell and neurotoxicity. This is consistent with the concept of Aβ-associated oxidative stress and neurodegeneration in AD brain.

a constituent of the inflammatory process in this disease. In addition, there is evidence of Nod-like receptor (NLR) family, pyrin domain containing 3 (NLRP3), activation in a mouse model of AD (Lambert et al., 2009). Interestingly, NLRP3 is a modulator of IL-1β, a proinflammatory cytokine which production is also stimulated by the presence of Aβ plaques, together with other inflammatory markers, including IL-6 and tumor necrosis factor alpha (TNF-α; Jekabsone et al., 2006; Jimenez et al., 2008).

The description of the mechanism by which Aβ increases the production of NO is still incomplete. One explanation is that progressive Aβ accumulation disrupts the Ca^{2+} homeostasis causing the before mentioned rises in NO (Cetin et al., 2013). Also, an increase of lipid peroxidation in the cellular membrane has been observed in the presence of Aβ accumulation (Xie et al., 2010). Another source of Aβ-induced increase in oxidative stress is the known interaction between both APP and amyloid plaques with mitochondrial proteins, which leads to alterations in normal function (Spuch et al., 2012).

Nitrosative stress has also been associated with pathological alterations (Nakamura and Lipton, 2011) since this process increases the aggregation of Aβ in early stages of AD, impairing the formation of hippocampal long-term potentiation (LTP; Kummer et al., 2011; Thiabaud et al., 2013). In addition, increasing oxidative stress in cultured hippocampal neurons led to nitrotyrosination of presenilin protein (PSEN1), which finally induced an enhancement of total Aβ (Guix et al., 2012). Interestingly, AD brains show similar increased levels

of nitrotyrosinated PSEN1 in comparison with age-matched controls (Guix et al., 2012), which remarks the relevance of oxidative stress to the neuroinflammatory process and the progress of pathophysiological hallmarks in this disease.

Parkinson's Disease

PD is an age-related neurodegenerative disease characterized by a prominent loss of dopaminergic neurons in the substantia nigra (SN; Dauer and Przedborski, 2003; Danielson and Andersen, 2008). The loss of DA modulation triggers a complex series of neurochemical, anatomical, and electrophysiological alterations that lead to persistent changes in striatal neurons and their signaling pathways (Wang and Pickel, 2002; Bamford et al., 2004; Picconi et al., 2004). Significant evidence indicates that chronic inflammatory response, mainly triggered by activated microglia and astroglia has a crucial role among the pathogenic mechanisms contributive to degeneration of dopaminergic neurons (McGeer et al., 1988, 2003; Barcia et al., 2004; Benner et al., 2008).

As in AD, the NO-induced glial activation has also a detrimental effect related to PD. The pathophysiology of microglial activation due to increases in oxidative stress causes an increased uptake of manganese inside the cell, which has been linked to the density of microglial cells especially in the basal ganglia (Gonzalez-Cuyar et al., 2014).

Substantial evidence demonstrates the involvement of NO in the degeneration of dopaminergic neurons in the SNpc (Jenner, 2003) and along the nigrostriatal pathway (Duncan and Heales, 2005; Zhang et al., 2006). It has also been demonstrated that nitrotyrosination can inhibit tyrosine hydroxylase (Kuhn and Geddes, 2002), and it is known that monoamine oxidase B (MAO-B) generates H_2O_2 during the catecholamine metabolism (Tipton, 1967). This is interesting because the activity of MAO-B, which is located in the mitochondrial membrane, is increased in aged population (Bhaskaran and Radha, 1983). This source of increased oxidative stress has been suggested as a risk factor for the development of PD (Jenner, 2003).

Neuroinflammation can be induced by several factors, such as exposure to either infectious agents or toxicants. Compounds with such proinflammatory characteristics have been recognized from some time now as significant contributors to the pathogenesis of PD. This is coherent with studies showing that the inhibition of complex I of the mitochondrial electron transport by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) can cause human parkinsonism, and increased nitrotyrosine in Lewy bodies and oxidative damage (Beal, 1998, 2002). Moreover, rotenone, a widely used pesticide, has proved to cause a syndrome in rats that mimics typical pathology displayed by PD patients (He et al., 2003), including microglial activation and the presence of proinflammatory markers in the brain (Li et al., 2012).

A potential role of NO and NOS isoforms in the pathophysiology of PD has been emphasized. Increases in iNOS expression and NO-mediated modulation of the mitochondrial apoptotic pathway have also been observed after injection of lipopolysaccharide (LPS) or 6-OHDA in the SN and striatum in different experimental models of PD (Singh et al., 2005). It is worth mentioning that nNOS overexpression and the formation of peroxynitrite in polymorphonuclear leukocytes have been reported in PD patients (Gatto et al., 2000; Gilgun-Sherki et al., 2001). Interestingly, this peroxynitrite exposure has been also linked to the formation of α-synuclein aggregates (Souza et al., 2000). This is important, given that nitrated α-synuclein seems to contribute to the increased ROS production, decreased adenosine triphosphate (ATP) production, and degeneration of dopaminergic neurons, as well as to microglial activation, a reduction in the number of T-cells and increased cell death (Murray et al., 2003; Guix et al., 2005; **Figure 4**).

However, regardless all these data, the exact contribution of NO-dependent mechanisms to neurodegeneration and neuroinflammation in PD is still not completely understood. One of the proposed ideas is the production of ROS during the normal metabolism of dopamine (DA). In the human SNpc, the oxidation product of DA may polymerize to form neuromelanin, which has proved effective in aggravating the degenerative process through neuroinflammation (Zecca et al., 2008). On the other hand, although data from preclinical and clinical studies suggest that neuroinflammation could be a hallmark of the progression of the disease from an early asymptomatic stage (Lee et al., 2009). An elevated level of inflammatory cytokines, such as TNF-α and IL-6 NO-associated (Wilms et al., 2007) was found in the *post mortem* brain of PD patients. Importantly, an up-regulation of the genes encoding for these inflammatory cytokines, COX-2 and iNOS was observed in microglial cells from PD patients (Knott et al., 2000; Saha and Pahan, 2006). In sum, although later studies have shed light on the etiopathology and neuroinflammatory processes associated with PD, more studies are have to be developed since the exact mechanism through which neuroinflammation and NO-associated pathways contribute to the development or progress of PD remains elusive.

Interestingly, the S-nitrosylation of parkin, another element significantly implicated in familial form of PD, has shown to interfere with the protective properties of this protein. Briefly, parkin acts as a transcriptional repressor of p53 (da Costa et al., 2009). What various studies demonstrate is that the presence of oxidative stress in the form of S-nitrosylation diminishes parkin's protective features (Chung et al., 2004; Sunico et al., 2013). Concretely, the addition of ubiquitin on specific substrates that parkin provides is impaired in the presence of NO. This evidence suggests that S-nitrosylation is directly implicated in the pathophysiology of PD by impairing the protective role of parkin.

Multiple Sclerosis

MS is an inflammatory disease in which the insulating myelin of SNC is damaged (Duncan and Heales, 2005). Of unknown etiology, this disease is characterized by an infiltration of inflammatory mononuclear cells into the CNS through a damaged blood-brain barrier (BBB), which causes the release of inflammatory and cytotoxic mediators, including NO (Smith and Lassmann, 2002). This neuroinflammation elicits the infiltration of T lymphocytes, the recruitment of macrophages, astrocytic damage and the local activation of microglia (Gay et al., 1997; Nikić et al., 2011). Although there is a strong

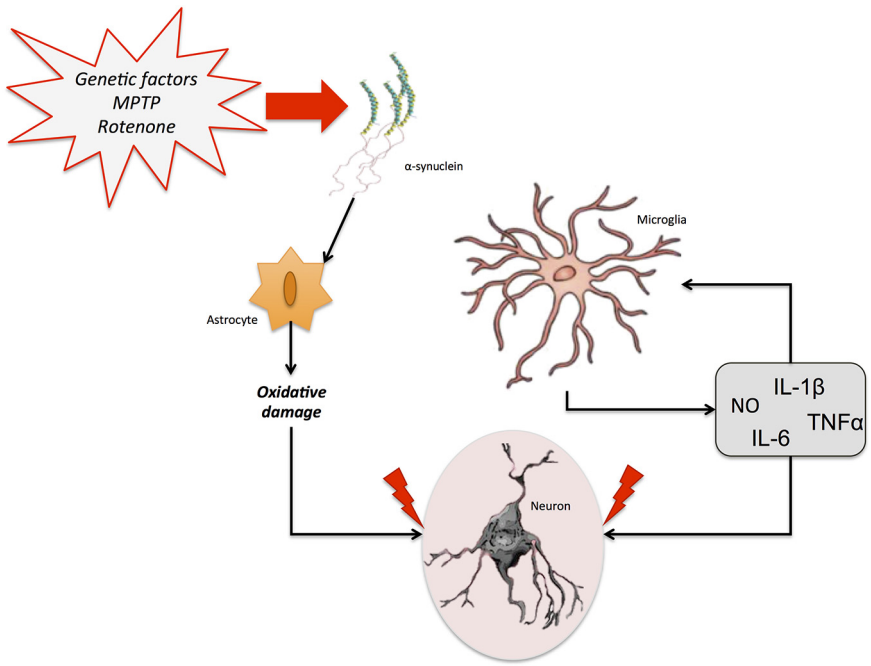

FIGURE 4 | Neuroinflammation in Parkinson's disease (PD). Different genetic and/or environmental factors such as parkin mutations or MTPT exposure lead to the accumulation of α-synuclein aggregates in the brain. This accumulation triggers the activation of glial cells. The proinflammatory cytokines freed by astrocytes and microglia stimulate the release of several neuroinflammatory markers, including NO, IL-6, IL-1β, and TNF-α, which can promote neuronal death and aggravate the neurodegenerative process.

correlation between neuroinflammation and axonal damage, the exact mechanism of this damage has to be elucidated (**Figure 5**).

Importantly, since NO expression participates in the homeostatic maintenance of the BBB permeability, this molecule may have an essential role in MS. Additionally in this BBB breakdown different pathways might be involved inducing oligodendrocyte injury and loss of neuronal function (Smith and Lassmann, 2002). Although there is considerable evidence showing that the three NOS isoforms are involved in the pathophysiology of MS (Wu and Tsirka, 2009; AlFadhli et al., 2013), iNOS would play, together with cyclic guanosine monophosphate (Janigro et al., 1994; Hurst and Fritz, 1996; Mayhan, 1999) and the overproduction of RNS (Mayhan, 1999; Kean et al., 2000; Spitsin et al., 2000; Winkler et al., 2001), a particularly crucial role specially at the beginning of this disease (Duncan and Heales, 2005).

The first direct evidence of glial implication in this disease was the demonstration of an altered mitochondrial function, following the inhibition of microglial respiratory chain in an animal model of demyelination (Zielasek et al., 1995; Lu et al., 2000). In these studies, a tendency for impaired Nicotinamide adenine dinucleotide (NADH) dehydrogenase activity and a compensatory increase in cytochrome oxidase in chronic active MS plaques was also demonstrated. More recently, another role of microglia in MS has been clarified since the phagocytosis of neuronal debris, the result of neuronal and axonal damage, would contribute to ongoing neurodegeneration in this disease (Huizinga et al., 2012).

Initially, astrocytes have been assigned a secondary role in the lesion formation and repair in MS. However, recent literature has implicated astrocytes in both lesion development and repair depending on the lesion stage and topography (Brosnan et al., 1994). Additionally, increased iNOS and mRNA has been identified in astrocytes in *post mortem* studies (Bagasra et al., 1995), and the MS-associated characteristic of reversible axonal conduction blockade has also been demonstrated in *in vitro* conditions (Redford et al., 1997). However, iNOS reactivity in hypertrophic astrocytes has only been described in acute but not chronic MS lesions (Brosnan et al., 1994; Liu et al., 2001) associated with peroxynitrite overproduction. More recently, different studies have shown the contribution of brain-derived neurotrophic factor (BNDF)-dependent NO release (Colombo et al., 2012) and the NF-κB pathway associated with NO production and astrocytes activation to the regulation of cytokine and chemokine expression. Interestingly, both markers have been related to the regulation of the severity and progression of the disease (Brambilla et al., 2009).

Another crucial step for the recruitment of leukocytes to the CNS and evolution of MS pathology is the chemokine expression by microglia and macrophages. Associated with this expression and in response to interleukin release astrocytes are able to regulate the production of different chemokines such as C-X-C motif chemokine 12 (CXCL12; Calderon et al., 2006). Recently it was demonstrated that the excessive expression of iNOS is

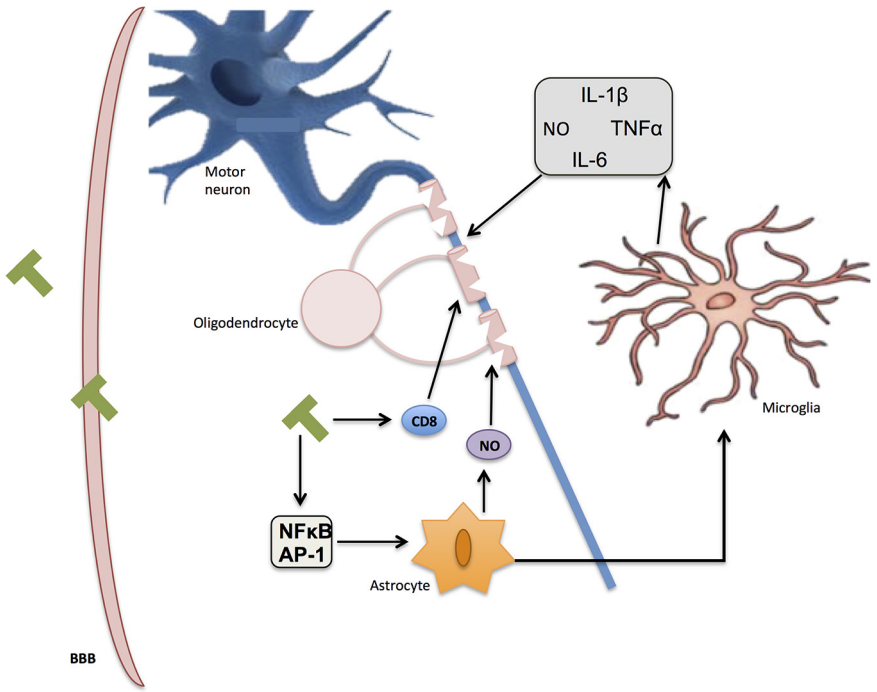

FIGURE 5 | Inflammation in multiple sclerosis (MS). Immune T-cells bypassing the blood-brain barrier (BBB) affect oligodendrocyte structure and activate glial response through NF-κB and AP-1. Reactive oxygen species (ROS) and nitric oxide (NO) secretion by activated microglia and astrocytes further contribute to myelin damage, axon degradation, and ultimate neuronal death.

able to decrease the expression level of CXCL12 gene, which has been implicated in the restriction of immune cell invasion to the CNS and the neuroinflammation limit in animal model of MS (Petković et al., 2013). This suggests that down-regulating NO release and maintaining CXCL12 expression within the CNS could be a potential therapeutic approach to MS.

Amyotrophic Lateral Sclerosis

ALS is mainly characterized by a progressive degeneration of motor neurons in the CNS that results in weakness, paralysis, and death (Long and Nguyen, 2013). The exact mechanism triggering this disorder is not totally understood, but within the primary hypotheses put forth to explain motor neuron degeneration, oxidative stress counts among the preferred theories (Rothstein, 2009).

There is evidence in mouse models of ALS that the administration of non-selective NOS inhibitors reduces motor neuron degeneration (Hyun et al., 2003). Accordingly, *post mortem* examinations of brains from patients with ALS show high levels of NO metabolites (Boll et al., 2003), together with clear protein and DNA damage caused by oxidation (Agar and Durham, 2003; Kato et al., 2005).

Another line of evidence proposes astroglial cells, specifically astrocytes, as the primary generator of NO-derived molecules contributing to both ALS initiation and development (Pehar et al., 2006; D'Amico et al., 2013). In this sense, some evidence demonstrates that astrocytes exposed to NO promote apoptosis of embryonic motor neurons (Cassina et al., 2002). Moreover, peroxynitrite has proved to also affect protein activity by oxidizing amino acid residues, as seen in nitrotyrosine, which is found in the CNS of both ALS patients (Abe et al., 1997; Bruijn et al., 1997) and ALS-mice models (Casoni et al., 2005; Yoshino and Kimura, 2006). Hence, peroxynitrite-mediated tyrosine nitration has been suggested as key for triggering neuronal degeneration in ALS (Beckman and Crow, 1993; Peluffo et al., 2004).

In addition, one of the genetic features found in more than 20% of ALS patients is an alteration in the gene encoding for the enzyme SOD-1, an intrinsic antioxidant (Reaume et al., 1996). Studies *in vitro* show that, differently from the augmentation in NO release found in normal SOD-1, mutant cells for this gene express fewer levels of NO (Cookson et al., 2002). This is coherent with the evidence indicating an association of this gene with the familial form of ALS (Conwit, 2006). Interestingly, the reaction of $ONOO^-$ with the mutant form of SOD-1 has shown to have an effect on protein nitrotyrosination (Beckman and Crow, 1993), which is coherent with data obtained from clinical studies (Abe et al., 1995).

Together, all these data support the idea of a prominent role of oxidative harm as one of the principal cellular mechanisms of motor neuron degeneration (Beckman and Esteves, 2006; **Figure 6**).

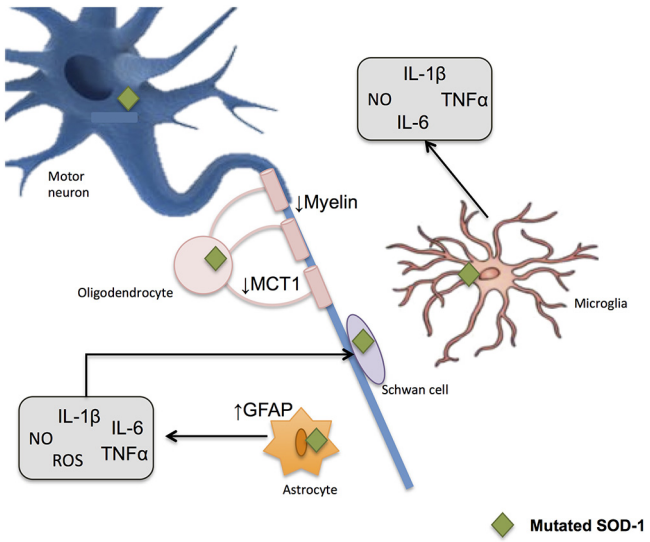

FIGURE 6 | Glial-induced neuroinflammation and neurotoxicity in amyotrophic lateral sclerosis (ALS). Reactive astrocytes contribute to the degenerative process by influencing the activity of microglial and immune cells. An up-regulation of filament glial fibrillary acidic protein (GFAP) takes place and astrocytes increase the release of proinflammatory markers including NO and ROS. When mutated SOD1 accumulates within microglia, the later generates substances potentially harmful to other cells, thus potentiating neurotoxicity. Demyelinization and progressive loss of cholesterol is also observed after oligodendrocyte damage. These glial cells show a reduction in the monocarboxylate transporter 1 (MCT1), which in turn difficult the energy supplies to the neuron.

Conclusions and Perspectives

NO plays multiple roles in the nervous system and glial regulated pathways associated with neuroinflammation and neurodegenerative diseases. Under physiological conditions, it contributes to regulating proliferation, survival, and differentiation of neurons. It is involved in synaptic activity, neural plasticity, and cognitive function (i.e., memory); it also exerts long-lasting effects through regulation of transcription factors and modulation of gene expression. However, RNS generation also brings modifications to critical cysteine residues in proteins, including Snitrosylation or nitrotyrosination.

If the physiological control of this signaling pathway fails, the pathological effects of NO and other RNS lead to (or are involved in) neuroinflammation and neurodegeneration processes. The NO-associated products resulted from the activation of glial response (either astrocyte or microglia) appear to especially contribute to the excitotoxic process that leads to neuronal death in several pathologies. More concretely, neurons appear particularly vulnerable to the effects of nitrosative stress. Susceptibility to NO and peroxynitrite exposure may depend on factors such as the intracellular antioxidants and stress resistance signaling pathways. Thus, NO redox signaling and modulation of the adaptive cellular stress responses, being released by glial cells or activating them, require further research to develop predictive means to deal with the increasing number of age-related neuropathological conditions.

Collectively, among NOS isoforms implicated in glial response related with neurodegenerative diseases, nNOS is the most implicated in a wide range of functions and pathologies with pleiotropic effects. In view of its ubiquitous expression in the CNS, there are extensive and unique chances for nNOS to interact with other neuronal elements, such as microglial and astroglial cells, thus exerting appropriate functional properties. Given increased nNOS activity and expression in many diseases, inhibiting nNOS might have putative therapeutic effects, among which anti-inflammatory properties can be hypothesized. Unfortunately, it will be a preferable means to interfere with specific pathway, for example, uncoupling nNOS–PSD95 interactions (Cao et al., 2005) since to date is impossible to inhibit nNOS directly without disturbing vital physiological functions and produce side effects.

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MINI REVIEW
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Neuroinflammation in Multiple System Atrophy: Response to and Cause of α-Synuclein Aggregation

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Multiple system atrophy (MSA) is a progressive neurodegenerative disease presenting with combinations of autonomic dysfunction, parkinsonism, cerebellar ataxia and/or pyramidal signs. Oligodendroglial cytoplasmic inclusions (GCIs) rich in α-synuclein (α-syn) constitute the disease hallmark, accompanied by neuronal loss and activation of glial cells which indicate neuroinflammation. Recent studies demonstrate that α-syn may be released from degenerating neurons to mediate formation of abnormal inclusion bodies and to induce neuroinflammation which, interestingly, might also favor the formation of intracellular α-syn aggregates as a consequence of cytokine release and the shift to a pro-inflammatory environment. Here, we critically review the relationships between α-syn and astrocytic and microglial activation in MSA to explore the potential of therapeutics which target neuroinflammation.

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INTRODUCTION: MULTIPLE SYSTEM ATROPHY AND α-SYNUCLEIN

Multiple System Atrophy (MSA) is a complex progressive neurodegenerative disease which affects 3.4–4.9 cases/100,000 with 0.6–0.7/100,000 new cases each year. It has no significant gender bias with disease onset typically over the age of 60 and a mean survival of ∼7.9 years following diagnosis (Watanabe et al., 2002; Wenning et al., 2013; Longo et al., 2015). Although there are no strong genetic determinants, studies have associated MSA cases with the H1 haplotype of *MAPT*, also linked to tauopathies such as Progressive Supranuclear Palsy (PSP; Vilariño-Güell et al., 2011), *COQ2* loss of function linked mutations (Multiple-System Atrophy Research Collaboration, 2013) and hexanucleotide repeat expansions in *C9orf72* (also associated with Amyotrophic Lateral Sclerosis and Frontotemporal Dementia; Goldman et al., 2014).

Abbreviations: α-syn, α-synuclein; CMA, Chaperone-mediated autophagy; CNP, Cyclic nucleotide phosphodiesterase; CNS, Central nervous system; CSFs, Colony-stimulating factors; DLB, Dementia with Lewy bodies; ER, Endoplasmic reticulum; GCI, Glial cytoplasmic inclusion; GFAP, Glial fibrillary acidic protein; HSP, Heat shock protein; IDO, Indoleamine 2,3-dioxygenase; IL, Interleukin; iNOS, Inducible nitric oxide synthase; LPS, Lipopolysaccharide; MBP, Myelin basic protein; MSA, Multiple system atrophy; MSC, Mesenchymal stem-cells; NADPH, Nicotinamide adenine dinucleotide phosphate hydroxylase; NF-κB, Nuclear factor kappa B; NO, Nitric oxide; PD, Parkinson's disease; PET, Positron Emission Tomography; PGE2, Prostaglandin E2; PLP, Proteolipid protein; PSP, Progressive Supranuclear Palsy; ROS, Reactive oxygen species; SNARE, Soluble NSF Attachment Protein receptor; SNpc, Substantia nigra pars compacta; TGF, Transforming growth factor; TH, Tyrosine hydroxylase; TLR, Toll-like receptors; TNF, Tumour necrosis factor; YFP, Yellow fluorescent protein.

Clinically, a predominance of Parkinsonism (MSA-P) or Cerebellar Ataxia (MSA-C) plus a heterogenous combination of pyramidal signs, autonomic and urogenital dysfunctions may be detected (Longo et al., 2015). Due to this complex phenotype, definite MSA diagnosis requires autopsy to detect glial cytoplasmic inclusions (GCIs) immunopositive for α-synuclein (α-syn) and neurodegeneration in striatonigral or olivopontocerebellar structures (Lantos, 1998; Trojanowski and Revesz, 2007; Gilman et al., 2008).

Alpha-synuclein is a 14.4 kDa protein of predominant neuronal pre-synaptic location where it is believed to chaperone the assembly of synaptic vesicles for exocytosis via interaction with synaptotagmin (SNARE complex component) and has characteristic conformational plasticity. It normally exists as a soluble monomer/tetramer in equilibrium with a membrane-bound α-helical multimer (Narayanan and Scarlata, 2001; Tong et al., 2009; Burré et al., 2014). However, for reasons not yet fully elucidated, α-syn may misfold into abnormal dimers, oligomers, or fibrils/protofibrils that aggregate and constitute the pathological hallmark of several neurodegenerative conditions, including Parkinson's disease (PD) and Dementia with Lewy Bodies (DLB), where they primarily occur in neurons (McKeith et al., 2005; Shulman et al., 2011). As mature human oligodendrocytes do not express α-syn normally (Miller et al., 2005), the origin of α-syn glial aggregates in MSA is unclear, whether as a consequence of primary oligodendrogliopathy followed by neuronal degeneration or a neuronal α-synucleinopathy leading to glial inclusions (Nishie et al., 2004; Wenning et al., 2008). Thus, it has been hypothesized that intercellular transmission of α-syn might be occurring via mechanisms such as endocytosis, direct penetration, micropinocytosis, pore formation, nanotube tunneling or diffusion (Ubhi et al., 2011; Konno et al., 2012). Nevertheless, the proposed mechanisms of release and subsequent cellular uptake suggest that at α-syn pathology transmission may occur in a putative prion-like manner (Prusiner et al., 2015).

In addition to α-syn rich GCIs as the central pathological feature, MSA also exhibits neuronal loss and strong neuroinflammation which both correlate with the density of inclusions and disease duration (Gai et al., 2003; Ozawa et al., 2004; Ahmed et al., 2012) as well as expression of inflammatory markers (Chen et al., 2015). Neuroinflammation is a dynamic response that involves changes in glial cell morphology, number, function and concomitant production of signaling molecules (O'Callaghan et al., 2008; Shastri et al., 2013). In the context of neurodegenerative diseases, persistent intra- and extracellular imbalances (such as those caused by misfolded proteins, oxidative stress, and neuronal death) are known to trigger and chronically perpetuate this response, which is dominated by microglia and astrocytes (Takeuchi, 2013). Gliosis is the term that indicates the phenotypic changes of glia and is exemplified in **Figures 1A–C**, where activated astrocytes and microglia co-localize with GCI pathology. This manuscript explores the role of α-syn and its relationship to neuroinflammation mediated by astrocytes and microglia in MSA.

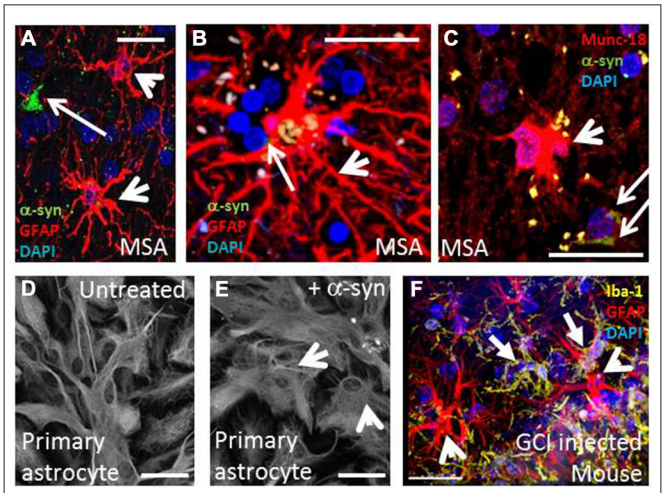

FIGURE 1 | Multiple system atrophy is characterized by widespread oligodendroglial α-syn inclusion bodies, astrogliosis and microgliosis. **(A,B)** MSA putamen **(A)** and visual **(B)** showing activated astrocytes (arrowheads, GFAP, red) in close proximity to GCIs (arrows, α-syn, green). **(C)** A subset of activated astrocytes are also intensely immunopositive for the exocytic vesicle marker, munc18 (Radford et al., 2015). **(D,E)** Rat primary astrocyte cells adopt activated morphology when treated with α-syn. **(E)** compared to control cells **(D)** Scale bars, 20 μm. **(F)** Frequent activated microglia (solid arrow, yellow, Iba-1) and activated astrocytes (red, GFAP) occur near to the site of GCI injection in unilateral-lesioned mice (Radford et al., 2015). Scale bar, 30 μm.

α-SYNUCLEIN TOXICITY AND SPREADING

That α-syn is the pathogenic root of neurodegeneration in MSA has been given further credence by the discovery of *SNCA* mutations in α-synucleinopathies (mostly PD), but where G51D and A53E α-syn mutations have been described in patients with atypical parkinsonism and may overlap with MSA (Polymeropoulos et al., 1997; Krüger et al., 1998; Zarranz et al., 2004; Lesage et al., 2013; Proukakis et al., 2013; Pasanen et al., 2014). In MSA, it remains unclear which α-syn form/s is the principal mediator of toxicity but their compact and insoluble structure allows them to resist intracellular cleavage, accumulate and disrupt otherwise normal downstream processes (e.g., the ubiquitin-proteasome system, synaptic exocytosis, mitochondrial metabolism and ER-Golgi transport; Burré et al., 2015). Multiple modes of α-syn toxicity are reviewed elsewhere, including membrane permeabilization by annular oligomers and disrupting protein degradation pathways by inhibition of the proteasome and autophagy (Cuervo et al., 2004; Winner et al., 2011; Daturpalli et al., 2013; Roberts and Brown, 2015), but it is accepted that this α-syn-induced dysfunction can lead to the death of central nervous system (CNS) cells that defines neurodegeneration (Radford et al., 2014).

In addition, the exchange of amorphous α-syn between neurons and glial cells via exo- and endocytosis characterizes the cell-to-cell transmission and uptake that may ultimately lead to pathology spread (Reyes et al., 2014), with evidence for unconventional exocytosis (independent from ER-Golgi) and exosomes as mechanisms of release (Emmanouilidou et al., 2010;

Jang et al., 2010). Key studies have recently provided evidence for α-syn transmission to occur in a prion-like manner. Thus, α-syn isolated post-mortem from MSA cases was transferred to HEK cells (modified to express the A53T mutation and tagged with yellow fluorescent protein, namely α-syn 140*A53T-YFP), that were then found to increase aggregate formation and increase YFP aggregate expression from the infected single-cells group, when compared to untransfected group (indicating a *de novo* prion formation in the transfected group; Woerman et al., 2015). In line with these findings, when brain extracts from MSA cases were injected intracerebrally in transgenic mice (M83 carrying the A53T mutation, namely tgM83), phenotypic changes (e.g., dysmetria and circling behavior) manifested 100–150 days post-inoculation in the homozygous group and pathological aggregates of phosphorylated α-syn and astrogliosis were detected in regions including brainstem. Interestingly, tgM83 mice inoculated with PD homogenates did not exhibit specific α-syn deposition or manifest clinical alterations significantly different from those of controls (Prusiner et al., 2015). These findings provide experimental evidence for MSA as a possible prion disease, with different α-syn strains being able to spread and promote tissue pathology in contrast with those of PD.

NEUROINFLAMMATION: ASTROCYTES, MICROGLIA AND α-SYNUCLEIN IN MSA

Astrocytes are key players in CNS homeostasis and pathology and are involved in a wide array of functions including modulating CNS immunity and inflammation, synaptic pruning and degradation of neuronal organelles (De Keyser et al., 2008; Sofroniew and Vinters, 2010; Chung et al., 2013; Hostenbach et al., 2013; Davis et al., 2014; Ben Haim et al., 2015). In MSA, the influence of α-syn on astrogliosis has been investigated by several studies. Treatment of primary astrocytes with α-syn promoted astrogliotic changes, as shown in **Figures 1D,E** (Radford et al., 2015). In astrocytes transfected with an inhibitor of endocytic vesicle formation (dominant negative dynamin-1 K44A mutant) and co-cultured with neuron-derived cell lines expressing α-syn, endocytosis was shown as a mechanism for direct uptake, which strongly correlates with the production of cytokines (such as IL-1α, -1β, -6, -18), colony-stimulating factors (CSF-1, -2, -3), and chemokines [(CCL-3, -4, -5, -12, -20), (CXCL-1, -2, -5, -10, -11, -12, -16)] (Lee et al., 2010). Moreover, in transgenic mice overexpressing oligodendroglial α-syn, exposure to oxidative stress (using the mitochondrial inhibitor 3-nitropropionic acid) led to astrogliosis and degeneration in close proximity to GCIs (Stefanova et al., 2005). Accordingly, the morphometric analysis of human cases and mouse models of MSA reveals that the degree of astrogliosis increased with proximity to α-syn deposits, as seen in **Figures 1A,B** (Song et al., 2009; Radford et al., 2015).

Microglia account for approximately 10% of all brain cells and derive from a primitive myeloid lineage of macrophages that migrate to cerebral regions during intrauterine life, after which they are distributed unevenly throughout the brain hemispheres, mostly concentrating in the hippocampus, basal ganglia and substantia nigra (Prinz et al., 2011). Normally, in the healthy brain, resident microglia adopt a resting (surveillant) phenotype, which is maintained by feedback of signaling molecules such as neuronal fractalkine and astrocytic glial-derived neurotropic factor, with perturbations of homeostasis triggering microglial activation into effector phenotypes, namely M1 and M2 (Tang and Le, 2015). This requires interaction of the noxious stimulus with immune response receptors such as complement factors, pattern recognition receptors and scavenger receptors (Husemann et al., 2002; Scheffel et al., 2012). Once activated, the M1 phenotype produces pro-inflammatory and cytotoxic molecules, such as TNF-α, IL-6, IL-1β, superoxide, NO, reactive oxygen species (ROS) and excitatory amino acids, which can induce more neuronal damage and progression of cellular dysfunction (Kettenmann et al., 2011). The other activated state, M2, also manifests phagocytic activity, but performs anti-inflammatory responses through release of IL-10 and transforming growth factor beta (TGF-β). It may also be induced by anti-inflammatory cytokines (e.g., IL-13 and IL-14) and acts in tissue repair via release of growth factors such as major histocompatibility complex 5, monocyte chemoattractant protein 1 and insulin-like growth factor 1 (Colton and Wilcock, 2010; Welser-Alves and Milner, 2013).

Morphologically, cellular hypertrophy and branching are the most commonly described changes in microgliosis which, depending on nature and intensity of the damage, may be detected as early as minutes to hours after acute injuries, with rapid process extension occurring in an ATP-dependent manner through P2Y12 receptors (Jensen et al., 1999; Davalos et al., 2005; Nimmerjahn et al., 2005; Parkhurst and Gan, 2010). In primary mesencephalic neuron-glia culture systems, extracellular α-syn was shown to be directly phagocytosed by microglia producing microgliosis, upregulation of NADPH oxidase and secretion of ROS, enhancing neurodegeneration (Zhang et al., 2005). Microgliosis can be identified clustering around α-syn years to decades after α-syn accumulation (Ishizawa et al., 2004; Graeber and Streit, 2010), or colocalizing with α-syn-rich neurons after direct stereotactic injection of α-syn ribbons or fibrils (Peelaerts et al., 2015).

In the extracellular environment, the abnormal presence of α-syn can be sensed and internalized by glial cells, leading to a cascade of reactive gliosis, secretion of pro-inflammatory cytokines and subsequent cell recruitment; characterizing the amplification of a localized deposit of protein. Localized micro- and astrogliosis resulted from injection of purified GCI material into mouse medial forebrain bundle after 23 days, as shown in **Figure 1F** (Radford et al., 2015). In microglia, pathogen pattern recognition receptors in the membrane surface enable the initial identification of foreign structural motifs on multiple arrays of pathogens (in the case of infectious disease), but they are also capable of recognizing changes in homeostatic cellular conditions and endogenous molecules, such as misfolded proteins in neurodegenerative diseases (Stefanova et al., 2005; Block et al., 2007). In particular, the Toll-like receptors (TLRs) 2 and 4 are known to interact with α-syn. In a cellular model, purified microglial cultures from brains of wild type ($TLR4^{+/+}$) and deficient ($TLR4^{-/-}$) postnatal mice were treated with wild-type and abnormal α-syn forms (fibrillar, truncated). The

results revealed prolific microgliosis in the TLR4$^{+/+}$ groups, increased phagocytic activity, upregulation of nuclear factor-kappa B (NF-κB), and increased production of CXCL1, IL-6, and TNF-α. Furthermore, TLR4-deficient microglia showed a reduced production of ROS upon α-syn treatments. In line with these findings, both human cases and transgenic mouse models of MSA also exhibit upregulation of TLRs (Béraud et al., 2011; Brudek et al., 2013; Fellner et al., 2013).

The temporal relationship between astro- and microgliosis in MSA is still poorly understood, as preclinical studies cannot reproduce the long timescale of MSA pathogenesis. Clinical radiology could overcome this limitation but, to date, tagging neuroinflammation with specific markers (Gerhard et al., 2003) has not been performed (Schrag et al., 1998; Schocke et al., 2002; Ozawa et al., 2004; Watanabe et al., 2004; Brooks et al., 2009; Chandran and Stoessl, 2014). Despite limitations related to preclinical and clinical assessment of neuroinflammation, it may be that, because glial cells express no or very little α-syn, glial uptake of α-syn occurs and α-syn triggers the neuroinflammatory process, which may then operate in waves of incremental feed-forward damage. This process, combined with the prion-like behavior of α-syn, implicates the role of neuroinflammation in worsening/perpetuating MSA. **Figure 2** represents schematically the interplay between α-syn aggregation and release, astrogliosis and microgliosis and the feedback of pro-inflammatory factors that may in turn result in additional neuronal stress.

It is known that extracellular α-syn may directly act upon astrocytes, microglia and oligodendrocytes. However, to date, there is a lack of studies specifically addressing gliosis as a trigger for α-syn misfolding and/or release (Croisier et al., 2005). Because of their role in surveillance and in reaction to pathogens, microglia may exert an indirect effect on α-syn by secreting a variety of toxic factors, which disrupt basic intracellular protein degradation systems and ultimately affect α-syn dynamics (Fellner and Stefanova, 2013). For example, it is known that α-syn oxidation and nitration inhibits chaperone-mediated autophagy (CMA; Kiffin et al., 2004; Martinez-Vicente et al., 2008; Xilouri et al., 2009, 2013) and that α-syn phosphorylation alters macroautophagy (Tenreiro et al., 2014) which, unlike CMA, can eliminate larger protein species, such as oligomers and aggregates (Engelender, 2012; Tanik et al., 2013). Moreover, neuron-glia cultures treated with lipopolysaccharide (LPS), a potent stimulator of microgliosis, have increased H_2O_2-mediated chemoattraction towards α-syn aggregates (Ejlerskov et al., 2015), which can be enhanced by pre-injection of LPS in an oxidative-stress (rotenone) mouse model (Tien et al., 2013). Although a unified body of data is required to define a single model of MSA, the overproduction of cytotoxic by-products by microgliosis may contribute to α-syn misfolding/aggregation.

MSA may also share common pathological features with the tauopathy PSP, although astrocytes display a degenerative phenotype in PSP tissue rather than a reactive one (Togo and Dickson, 2002; Radford et al., 2015). Studies with microglial fractalkine receptor deficient hTau*Cx3cr1*$^{-/-}$ mice have shown that enhanced microglial activation led to accelerated tau pathology and could be transferred to non-transgenic recipient mice by adoptive microglia; which was blocked by the interleukin1 receptor antagonist, Kineret (Bhaskar et al., 2010; Maphis et al., 2015). Recently, microglia have been shown to be directly linked to hTau propagation between non-synaptically connected neuronal populations *in vivo* via exosome release following phagocytosis of hTau (Asai et al., 2015). As most experiments to date have used models of PD to address α-syn diseases, future experiments will need to focus on specific MSA animal and cell culture models, such as by direct injection of purified GCI material (Radford et al., 2015), that may better mimic the disease's specific cellular features (Halliday and Stevens, 2011), especially to further elucidate the role of both astrocytes and microglia in α-syn misfolding/aggregation and spreading.

NEUROINFLAMMATION AS A THERAPEUTIC TARGET

Due to the possible cyclic nature of α-syn aggregation/release and gliosis in MSA, interventions that target neuroinflammation have the potential to slow the progression of disease and increase quality of life. Recent studies have approached α-synucleinopathies, including MSA, by use of immunotherapy (Valera and Masliah, 2013). For example, short immunogenic peptides mimicking the C-terminus of α-syn were administered to MBP-α-syn trangenic mice, followed by measurements of inducible anti-α-syn antibodies, cellular and tissue outcomes over time. Interestingly, as the peptides used did not carry the native epitope but instead a variation of it, this approach did not produce autoimmunity reactions via T-cells, leading to a beneficial prolonged response. Moreover, the induced antibodies were able to cross the blood-brain barrier where they could detect intracellular α-syn (monomer, oligomers and aggregates) after being internalized by microglia, oligodendrocytes and astrocytes. This reduced α-syn colocalization in oligodendrocytes and astrocytes but not in microglia, suggesting increased microglial uptake whilst preserving the oligodendrocyte population and decreasing demyelination, neuronal death and motor deficits (Mandler et al., 2015). Indeed, as in animal models of α-synucleinopathy the microglial response occurs prior to neuronal loss (Sanchez-Guajardo et al., 2015), strategies such as pre-immunization with α-syn peptides or approaches aimed at priming the CNS against α-syn immune insult may provide a glial memory and lessen subsequent neuroinflammatory responses to therapeutic benefit. Moreover, as activated microglia could participate actively in the spread of α-syn pathology, therapies that promote microgliosis to facilitate the clearance of extracellular pathological protein aggregates may have unwanted side effects. Furthermore, a recent study has shown that peripheral vector administration of the protease neurosin could degrade extracellular α-syn and thereby may reduce microglial and astrocyte activation (Spencer et al., 2015).

Some studies also aimed at microgliosis as a therapeutic target. For example, the inhibition of pro-inflammatory (iNOS or NAPH oxidase) enzymes from activated microglia is followed by decreased degeneration of neurons upon treatments with 7-nitroindazole and apocynin (Gao et al., 2003). Also, the treatment of the PLP-α-syn mouse model with minocycline

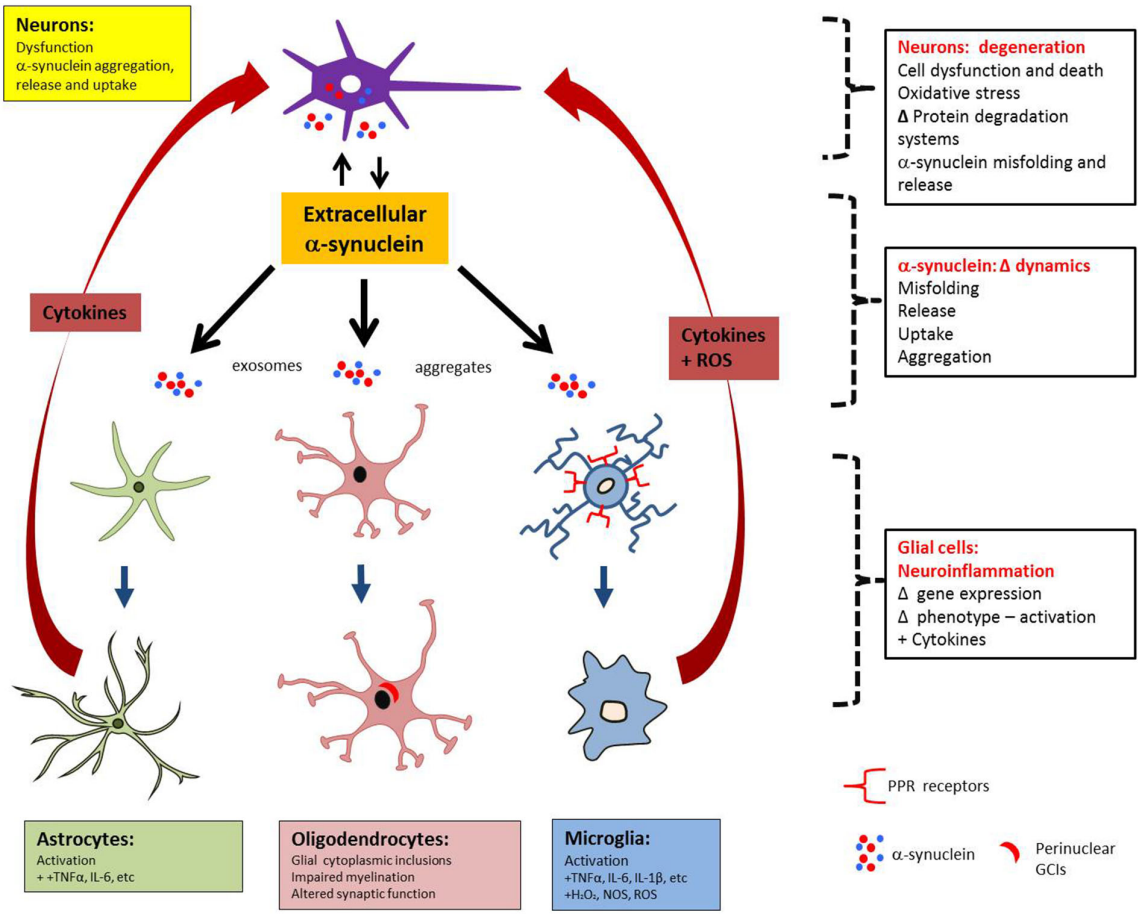

FIGURE 2 | MSA pathology may spread between anatomically connected regions as a result of reciprocal rounds of α-syn release and neuroinflammation. Neuronal dysfunction can lead to α-syn aggregation and release of α-syn aggregates, which can then interact directly with astrocytes and microglia to mediate activation. In turn, the release of pro-inflammatory factors by activated glia can act back on neurons to cause stress, thereby stimulating the formation and release of additional α-syn aggregates.

decreased the density of activated microglia in the SNpc, reduced iNOS and TLR4-immunoreactivity and reduced dopaminergic degeneration (Stefanova et al., 2007).

As their differentiation capacity and immunomodulatory properties can address both neurodegeneration and neuroinflammation, mesenchymal stem-cells (MSCs) have been investigated as therapeutic options. In a study using the transgenic PLP mouse model of MSA (Stemberger et al., 2011), intravenous transplantation of MSCs promoted neuroprotection in the SNpc (as determined by TH+ neurons in the treated group) and down regulation of cytokines (IL-1α, IL-2, Il-10, TGF-β1 and TNF-α) in midbrain-brainstem lysates 4 weeks post-injection. Similarly, in the double-toxin-induced MSA-P mouse model, treatment with human MSC (hMSC) improved motor behavior (pole-descending test), increased neuronal survival (TH- and NeuN-positive markers), and decreased astro- and microgliosis (anti-Iba1 and anti-GFAP immunostaining, respectively) in the SNpc and striatum (Park et al., 2011). Lastly, a clinical trial using autologous MSC transplantation in patients with MSA-C improved symptoms severity [as observed on the baseline unified MSA rating scale (UMSARS)], and attenuated the declines of cerebral glucose metabolism and gray matter density (assessed by neuroimaging) along a 360-day follow-up (Lee et al., 2012). The immunosuppressive actions of MSC are believed to operate in a non-MHC-restricted manner, for example via secretion of soluble factors such as TGF-β1, PGE2 and indoleamine 2,3-dioxygenase (IDO; Krampera et al., 2006b). As glial cells are known to use IDO in the conversion of tryptophan to kynurenine, the finding that interferon-γ can upregulate the enzyme points to the kynurenine pathway as another therapeutic target in neuroinflammation related and non-related to MSCs (Krampera et al., 2006a). Recently, mice lacking anti-inflammatory interferon-β were shown to develop motor and cognitive deficits and α-syn pathology similar to a DLB/PD phenotype (Ejlerskov et al., 2015). This strengthens the link between dysfunctional inflammation and α-synucleinopathies and indicates dysfunction in inflammation can induce α-syn toxicity and *vice versa*.

Therapies targeting neuroinflammation in other α-syn disease models may warrant investigation in MSA. One

example is the induction of heat shock proteins (HSPs) which are known to act via multiple pathways such as protein misfolding, neuroinflammation and mitochondrial oxidative phosphorylation. Recently, a HSP inducer, carbenoxolone, was shown to decrease astrogliosis, pro-inflammatory cytokines and oxidative stress in a rotenone model of PD (Thakur and Nehru, 2015). Additionally, HSPs are intimately involved in the degradation of α-syn by CMA (Wong et al., 2013; Vijayakumaran et al., 2015) and their induction may provide dual benefits by reducing neuroinflammation and toxic α-syn aggregates. The renin-angiotensin system may also be targeted to reduce the microglial inflammatory response via angiotensin II antagonists (Labandeira-Garcia et al., 2011). Microglial β-nicotinamide adenine dinucleotide phosphate oxidase 2 inhibitors may be useful by decreasing microglial O_2- generation (Zhang et al., 2014). Other alternatives may be to combat oxidative stress, such as by the naturally occurring Acetyl-L-Carnitine (Singh et al., 2015). Counterbalancing neuroinflammation in glia and immune cells with naturally occurring lipids or hormonal modulation (Herrera et al., 2015; Skaper et al., 2015) may also be promising therapeutic strategies for MSA.

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frontiers in Cellular Neuroscience

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Modulation of TLR3/TLR4 inflammatory signaling by the $GABA_B$ receptor agonist baclofen in glia and immune cells: relevance to therapeutic effects in multiple sclerosis

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The $GABA_B$ receptor agonist, baclofen, is used to treat muscle tightness and cramping caused by spasticity in a number of disorders including multiple sclerosis (MS), but its precise mechanism of action is unknown. Neuroinflammation drives the central pathology in MS and is mediated by both immunoreactive glial cells and invading lymphocytes. Furthermore, a body of data indicates that the Toll-like receptor (TLR) family of innate immune receptors is implicated in MS progression. In the present study we investigated whether modulation of $GABA_B$ receptors using baclofen can exert anti-inflammatory effects by targeting TLR3 and(or) TLR4-induced inflammatory signaling in murine glial cells and human peripheral blood mononuclear cells (PBMCs) isolated from healthy control individuals and patients with the relapse-remitting (RR) form of MS. TLR3 and TLR4 stimulation promoted the nuclear sequestration of NF-κB and pro-inflammatory cytokine expression in murine glia, while TLR4, but not TLR3, promoted pro-inflammatory cytokine expression in PBMCs isolated from both healthy donors and RR-MS patients. Importantly, this effect was exacerbated in RR-MS patient immune cells. We present further evidence that baclofen dose-dependently attenuated TLR3- and TLR4-induced inflammatory signaling in primary glial cells. Pre-exposure of PBMCs isolated from healthy donors to baclofen attenuated TLR4-induced TNF-α expression, but did not affect TLR4-induced TNF-α expression in RR-MS patient PBMCs. Interestingly, mRNA expression of the $GABA_B$ receptor was reduced in PBMCs from RR-MS donors when compared to healthy controls, an effect that might contribute to the differential sensitivity to baclofen seen in healthy and RR-MS patient cells. Overall these findings indicate that baclofen differentially regulates TLR3 and TLR4 signaling in glia and immune cells, and offers insight on the role of baclofen in the treatment of neuroinflammatory disease states including MS.

Keywords: glia, TLR, multiple sclerosis, GABA, baclofen, inflammation, innate immunity

Introduction

Autoimmunity drives the development of multiple sclerosis (MS), involving central nervous system (CNS) infiltration of immune cells, myelin degradation, reactive changes in glia and axonal loss (Compston and Coles, 2002). The innate immune system has received much interest as having a defined role in the progression and/or etiology of MS (O'Brien et al., 2008; Downer, 2011). Innate immunity is regulated by complex mechanisms involving pattern-recognition receptors (PRRs) that recognize molecular signatures of microbes. Intracellular signaling triggered by PRRs leads to transcriptional expression of inflammatory mediators that coordinate the elimination of pathogens (Akira et al., 2006). If left unchecked, or not tightly regulated, dysregulation of this system can lead to conditions such as sepsis and autoimmunity (Lehnardt, 2010).

Toll-like receptors (TLRs) belong to the family of signaling PRRs (Jeannin et al., 2008) that initiate innate immune reactions by activating transcription factors such as nuclear factor (NF)-κB, in addition to inducing the expression of interferons (IFNs) and cytokines. Thus far, 12 functional TLRs have been identified in mice, and 10 in humans (O'Neill, 2004; Kawai and Akira, 2006). TLRs are localized in endosomal compartments (including TLR3, TLR7, TLR8, TLR9), or are cell membrane-bound (as with all other TLRs; O'Neill, 2004), and couple to specific signaling cascades, inducing gene transcription and controlling immune processes, with this specificity reliant on the TLR adaptor proteins recruited (O'Neill, 2004). All TLRs (with the exception of TLR3), recruit the adaptor myeloid differentiation factor 88 (MyD88; Medzhitov et al., 1998). TLR3 (and TLR4) induces MyD88-independent signaling to couple to NF-κB via Toll-Interleukin-1 Receptor (TIR)-domain-containing adaptor-inducing IFN-β (TRIF) protein. TLRs are key players in CNS diseases, and with respect to MS, key roles of TLRs have been shown in murine models of MS (Touil et al., 2006), while the expression of TLRs characterized on immune cells and CNS glia and neurons (Nishimura and Naito, 2005).

Gamma amino butyric acid (GABA) is the major inhibitory amino acid neurotransmitter in the brain (Cobb et al., 1995). GABA exerts its effects through the ionotropic receptors, $GABA_A$/$GABA_{A-\rho}$ and the metabotropic receptor $GABA_B$ (Bormann, 2000; Campagna-Slater and Weaver, 2007; Pinard et al., 2010), and GABA receptor transcripts are present in neurons, glia (Lee et al., 2011; Vélez-Fort et al., 2012), and immune cells (Bhat et al., 2010). There is a growing body of evidence pointing to the key role of GABA receptors in neuroinflammation (Ziegler et al., 1980; Tyagi et al., 2015), and recent reports has identified that modulation of the GABAergic system occurs in MS (Han et al., 2008; Carmans et al., 2013). Indeed, GABA insufficiency has been identified in MS patients (Demakova et al., 2003), while intrathecal administration of the $GABA_B$ receptor agonist, baclofen, is used as a treatment strategy to control spasticity in patients with MS (Gunnarsson and Samuelsson, 2015). Furthermore, enhancing endogenous GABA via administration of the GABA-degrading enzyme GABA-transaminase (GABA-T), has been shown to be protective in an experimental autoimmune encephalomyelitis (EAE) mouse model (Carmans et al., 2013), indicating that increasing endogenous GABA also has therapeutic potential in the murine model of MS. In support of these *in vivo* findings, *in vitro* studies have shown that baclofen reduces TLR4-induced release of pro-inflammatory cytokines from primary murine microglia (Kuhn et al., 2004), indicating that cross talk may exist between the GABAergic and TLR systems, with relevance to inflammatory signaling events.

$GABA_B$ receptors are metabotropic G_i/G_o-coupled receptors (Padgett and Slesinger, 2010) which are distributed throughout the CNS and periphery (Ong and Kerr, 1990; Hyland and Cryan, 2010). $GABA_B$ receptors can function to regulate ion channels (activate K^+ and inhibit Ca^{2+} channels) and cellular signaling (adenylate cyclase, MAPK; Kornau, 2006; Jiang et al., 2012), limit the release of neurotransmitters (GABA, glutamate; Pinard et al., 2010; Gassmann and Bettler, 2012), and dampen depolarisation induced by excitatory neurotransmitters. $GABA_B$ receptors are implicated in a variety of neurodegenerative, neuroinflammatory, and pathophysiological disorders including depression, spasticity, pain, and schizophrenia (Reyes-García et al., 2007; Bjurstöm et al., 2008; Garcia-Oscos et al., 2012). Given previous reports linking TLR's and the $GABA_B$ receptor subtype with neuroinflammation, particularly with relevance to MS pathogenesis, we sought to explore the impact of baclofen (the $GABA_B$ receptor agonist) on TLR3 and TLR4-induced inflammatory signaling both centrally and in the periphery, using murine glial cells and human peripheral blood mononuclear cells (PBMCs) isolated from healthy individuals and newly-diagnosed patients with the relapsing-remitting (RR) form of MS patients. This study identifies baclofen as a differential regulator of TLR3 and TLR4 signaling in glia and immune cells, and offers insight on the role of baclofen in regulating the innate immune response in cellular pathology associated with MS.

Materials and Methods

Preparation of Primary Mixed Glial Cultures and Treatments

Primary mixed glia were prepared from the whole brain of 1-day-old C57/BL6 mice and plated (at 5×10^5 cells/ml) as previously described (Downer et al., 2010). All experiments were performed under a license issued by the Health Products Regulatory Authority (Ireland) and in accordance with the European Directive 86/609/EEC and the guidelines laid down by the Animal Experimentation Ethics Committee of University College Cork. After 14 days in culture, mixed glia were treated with the TLR4 ligand LPS (100 ng/ml; Sigma–Aldrich, UK), the TLR3 ligand Poly(I:C) (10 μg/ml; InvivoGen, France) or vehicle (sterile H_2O) for timepoints ranging from 10 min–24 h. In a second series of experiments, mixed glia were exposed to the $GABA_B$ agonist baclofen (10, 30, and 100 μM; Sigma–Aldrich, Germany) for 30 min prior to LPS (100 ng/ml;

30 min and 24 h) or Poly(I:C) (10 μg/ml; 6 h and 24 h) exposure.

Patients and Blood Samples

Healthy donors and RR-MS patients attending outpatient clinics at the Mercy University Hospital, Cork, were recruited for this study. Written informed consent was obtained from each participant and the study received ethical approval from the Clinical Research Ethics committee of the Cork Teaching Hospitals (CREC). Patient recruitment into the study was via a Senior Consultant Neurologist and patients had to meet the revised MacDonald diagnostic criteria for clinically defined MS (Polman et al., 2011) including patient history, clinical signs and symptoms, physical examination, and adjunctive diagnostic tools including MRI. The Disability Status Scale scores were taken using the Expanded Disability Status Scale (EDSS) by a Senior Consultant Neurologist in an outpatient clinic. All confirmed MS patients had a RR form of MS as defined by the revised McDonald criteria. Disease severity was scored at time of collection using the Kurtzke's (1983) EDSS. Patients with RR-MS were clinically stable and naïve to disease modifying therapies including IFN-β, glatiramer acetate, fingolimod, teriflunomide, dimethyl fumarate, natalizumab, and alemtuzumab. PBMCs were collected from venous blood of healthy controls participants (mean age 30.3 ± 1.4 years; $n = 16$) and RR-MS patients (mean age 39.3 ± 3.2 years; $n = 8$) by way of venipuncture as previously described (Downer et al., 2013b). Details of the patient demographics are presented in **Figure 3A**. Human PBMCs were prepared from heparinized venous whole blood samples (50 ml per donor) from individuals by density separation over Lymphoprep™ (Axis-Shield, Norway). PBMCs were plated (1×10^6 cells/ml) on 24-well plates prior to treatment. Plasma samples were separated following centrifugation, aliquoted, and stored at −80°C for subsequent analysis via ELISA.

Cytokine Analysis in Culture Supernatants

Mixed glia (5×10^5 cells/ml) and PBMCs (1×10^6 cells/ml) were seeded overnight in 24-well plates. Cells were incubated with LPS (100 ng/ml) or Poly(I:C) (10 μg/ml) for timepoints ranging from 10 min–24 h. Mixed glia/PBMCs were also pre-exposed (30 min) to baclofen (10, 30, and 100 μM) prior to LPS (100 ng/ml; 24 h) or Poly(I:C) (10 μg/ml; 24 h) exposure. Supernatants were assayed for tumor necrosis factor-α (TNF-α) and IL-8 concentration by ELISA according to manufacturer's instructions (Duoset, R&D Systems, Abingdon, UK).

Quantitative Real-Time PCR

RNA was extracted from PBMCs (untreated) using a NucleoSpin® RNAII isolation kit (Macherey-Nagel Inc., Geschäftsführer, Germany). The concentration of RNA was determined using a UV/V is spectrophotometer. cDNA synthesis was performed on 1 μg RNA using a High Capacity cDNA RT Kit (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's instructions. Equal amounts of cDNA were used for RT-PCR amplification. Real-time PCR primers were delivered as "Taqman® Gene Expression Assays" containing forward and reverse primers, and a FAM-labeled MGB Taqman probe for the target gene (Applied Biosystems). $GABA_{B1}$ primers were used to assess the expression of the target gene (Taqman Gene Expression Assay no. Hs00181306_m1). cDNA (1:4 dilution) was prepared and real-time PCR performed using Applied Biosystems 7300 Real-time PCR System. cDNA was mixed with qPCR™ Mastermix Plus (Applied Biosystems) and the respective gene assay in a 25 μl volume (10 μl of diluted cDNA, 12.5 μl Taqman® Universal PCR Mastermix, 1.25 μl target primer and 1.25 μl 18S). Eukaryotic 18S rRNA was used as an endogenous control and expression was conducted using a gene expression assay containing forward and reverse primers and a VIC-labeled MGB Taqman probe (#4319413E; Applied Biosystems, USA). Samples were run in duplicate and 40 cycles were run as follows: 10 min at 95°C and for each cycle, 15 s at 95°C and 1 min at 60°C. Gene expression was calculated relative to the endogenous control and analysis was performed using the $2^{-\Delta\Delta CT}$ method. In all experiments no change in relative 18S rRNA expression between treatment groups was observed.

TNF-α and IL-8 Measurement in Plasma

Plasma samples from healthy donors and RR-MS patients were analyzed for concentrations of TNF-α and IL-8 by ELISA (Duoset, R&D Systems, UK) according to manufacturer's instructions.

Immunocytochemistry

Mixed glia (5×10^5 cells/ml) and PBMCs (1×10^6 cells/ml) were seeded overnight on 13-mm diameter coverslips coated with poly-l-lysine (Sigma–Aldrich) and grown for 24 h. Cells were incubated with LPS (100 ng/ml) or Poly(I:C) (10 μg/ml) for timepoints ranging from 10 min–24 h. Mixed glia/PBMCs were also pre-exposed (30 min) to baclofen (10, 30 and 100 μM) prior to LPS (100 ng/ml; 30 min) or Poly(I:C) (10 μg/ml; 6 h) exposure. Cells were then fixed in ice-cold methanol for 10 min, permeabilized with 0.2% Triton X-100 (Thermo Fisher Scientific, Waltham, IL, USA) in PBS for 10 min at room temperature and blocked with 10% goat serum (Vector Laboratories, Peterborough, UK) for 2 h. Cells were treated overnight at 4°C with rabbit polyclonal NF-κB p65 antibody (1:200 in 5% goat serum; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Cells were then washed and incubated with goat anti-rabbit Alexa488 secondary antibody (1:1000 in 5% goat serum; Invitrogen, Dublin, Ireland) and 4′,6-diamidino-2-phenylindole (DAPI; 1.5 μg/ml) in PBS, washed, and mounted (Vectashield; Vector Laboratories). Cells were imaged using an Olympus IX70 inverted microscope. The fluorescence intensity in the nucleus of individual cells stained for NF-κB p65 was measured using the Image J analysis software (Rasband,WJ, http://rsb.info.nih.gov/ij/). The relative fluorescence intensity was calculated as the intensity after subtraction of the background noise. For each treatment, six coverslips were stained and five fields of view were captured per coverslip. Negative control experiments were performed by replacing the primary antibody with PBS and using equal gain settings during acquisition and analysis.

Statistical Analysis

Data were analyzed using one-way or two-way analysis of variance (ANOVA) as appropriate. When analysis by ANOVA indicated significance ($P < 0.05$), the *post hoc* Student Newman–Keuls test was used. Correlation tests were performed using two-tailed Spearman (non-parametric) correlation coefficient. Data are expressed as means ± SEs of the mean (SEM).

Results

TLR4 Activation Time-Dependently Increases Nuclear NF-κB p65 Expression and TNF-α Release in Mixed Glia

TLR4 and TLR3 were initially targeted given their involvement in EAE progression (Touil et al., 2006) and evidence that their expression is dysregulated in MS lesions (Bsibsi et al., 2002). To initially characterize the impact of TLR4 stimulation on pro-inflammatory signaling in primary murine mixed glial cells, we temporally assessed the impact of LPS on the distribution of the NF-κB p65 subunit in response to LPS treatment. Firstly, mixed glia were stimulated with LPS for various timepoints ranging from 10 min–24 h, and the localization of endogenous p65 was assessed by immunofluorescence (**Figures 1A,B**). In vehicle-treated glial cells, p65 is predominantly cytoplasmic, as evidenced by the detection of 488-conjugated immunocomplexes outside of the DAPI-stained regions (**Figure 1B**). Stimulation of glia with LPS time-dependently promoted the accumulation of p65 in the nucleus, peaking at 30 min and 1 h post-LPS exposure (**Figures 1A,B**). We next characterized the impact of TLR4 activation on TNF-α protein production in primary mixed glia. LPS time-dependently enhanced TNF-α expression in primary murine mixed glial cells, with mean maximal stimulatory effects on protein expression observed at 6–24 h (**Figure 1C**), indicating that TLR4 stimulation promotes inflammatory signaling in primary mouse glial cells in our culture system.

TLR3 Activation Enhances Nuclear NF-κB p65 Expression and TNF-α Release in Mixed Glia

To assess the impact of TLR3 stimulation on inflammatory signaling events in mixed glial cells, we next assessed the effect of poly(I:C) exposure on the distribution of the NF-κB p65 subunit. Primary murine glial cells were incubated with poly(I:C) for timepoints ranging from 10 min–24 h, and the localization of the p65 subunit assessed by immunofluorescence (**Figures 1D,E**). TLR3 stimulation time-dependently promoted the accumulation of NF-κB in the nucleus, peaking at 6 and 8 h post-poly(I:C) exposure (**Figures 1D,E**). This temporal profile differed in response to TLR4 stimulation, where maximal sequestration of NF-κB p65 was observed at 30 min and 1 h post-LPS treatment (**Figure 1A**). Poly(I:C) significantly enhanced TNF-α expression in mixed glial cells, with mean maximal stimulatory effects on protein expression observed at 24 h (**Figure 1F**). This indicates that TLR3 and TLR4 activation in primary glia robustly enhances pro-inflammatory signaling in primary murine glial cells.

Effect of the $GABA_B$ Receptor Agonist Baclofen on TLR4-Induced NF-κB-p65 Nuclear Expression and Pro-Inflammatory Cytokine Release in Mixed Glia

We next examined the impact of the $GABA_B$ agonist baclofen on TLR4-induced inflammatory signaling events in primary mixed glia. Glial cultures were pre-treated (30 min) with baclofen (10, 30, and 100 μM) prior to LPS exposure (30 min) and nuclear expression of NF-κB-p65 measured by fluorescence microscopy (**Figures 2A,B**). Stimulation of glia with LPS promoted the accumulation of p65 in the nucleus (**Figure 2A**). Pre-exposure to baclofen (at 30 μM) significantly attenuated the LPS-induced accumulation of p65 in the nucleus, while pre-incubation of glia with the $GABA_B$ agonist at concentrations of 10 μM ($p = 0.09$) and 100 μM ($p = 0.85$), failed to significantly impact LPS-induced nuclear p65 sequestration (**Figures 2A,B**). Similarly, **Figure 2C** demonstrates that baclofen attenuated LPS-induced TNF-α production in glia at concentrations of 10 and 30 μM, although this did not reach statistical significance. These findings suggest that the proclivity of baclofen to impact TLR4 signaling in primary mixed glia is concentration dependent.

$GABA_B$ Receptor Activation Modulates TLR3-Induced NF-κB-p65 Nuclear Expression and TNF-α Expression in Glia

Given that $GABA_B$ activation modulates TLR4-induced signaling in glia, we next assessed the impact of baclofen on TLR3-induced inflammatory signaling in glial cells. Pre-incubation of glia with baclofen dose-dependently attenuated poly(I:C)-induced nuclear sequestration (at 6 h) of NF-κB, reaching significance at 100 μM (**Figures 2D,E**). Baclofen reduced poly(I:C)-induced TNF-α production in glia at concentrations of 100 μM, although this did not reach statistical significance (**Figure 2F**). These findings suggest that $GABA_B$ activation regulates TLR3 signaling to NF-κB in glia in a concentration-dependent manner.

Demographic Data of Human Participants

A total of 24 subjects were assessed consisting of healthy control participants ($n = 16$) and newly diagnosed RR-MS patients ($n = 8$). All RR-MS patients were drug naïve at the time of inclusion. Mean disease duration in the RR-MS cohort was 1.2 ± 0.3 years and subjects had mild to moderate disability as reflected by EDSS scores of 2.6 ± 0.4 on average. The mean age of the 24 participants who enrolled in the investigation was 33.1 ± 7.8 years (range 23–40 years in control cohort; range 31–54 years in RR-MS cohort) and 46% of the sample was female. The overall demographics of the participants are reported in **Figure 3A**.

Cytokine and Chemokine Analysis in Human Plasma

To determine if RR-MS is associated with alterations in peripheral inflammatory signature, we initially assessed plasma levels of the inflammatory chemokine IL-8 and cytokine TNF-α in plasma from control and RR-MS groups. No significant difference in plasma IL-8 ($p = 0.94$) and TNF-α ($p = 0.28$) concentration

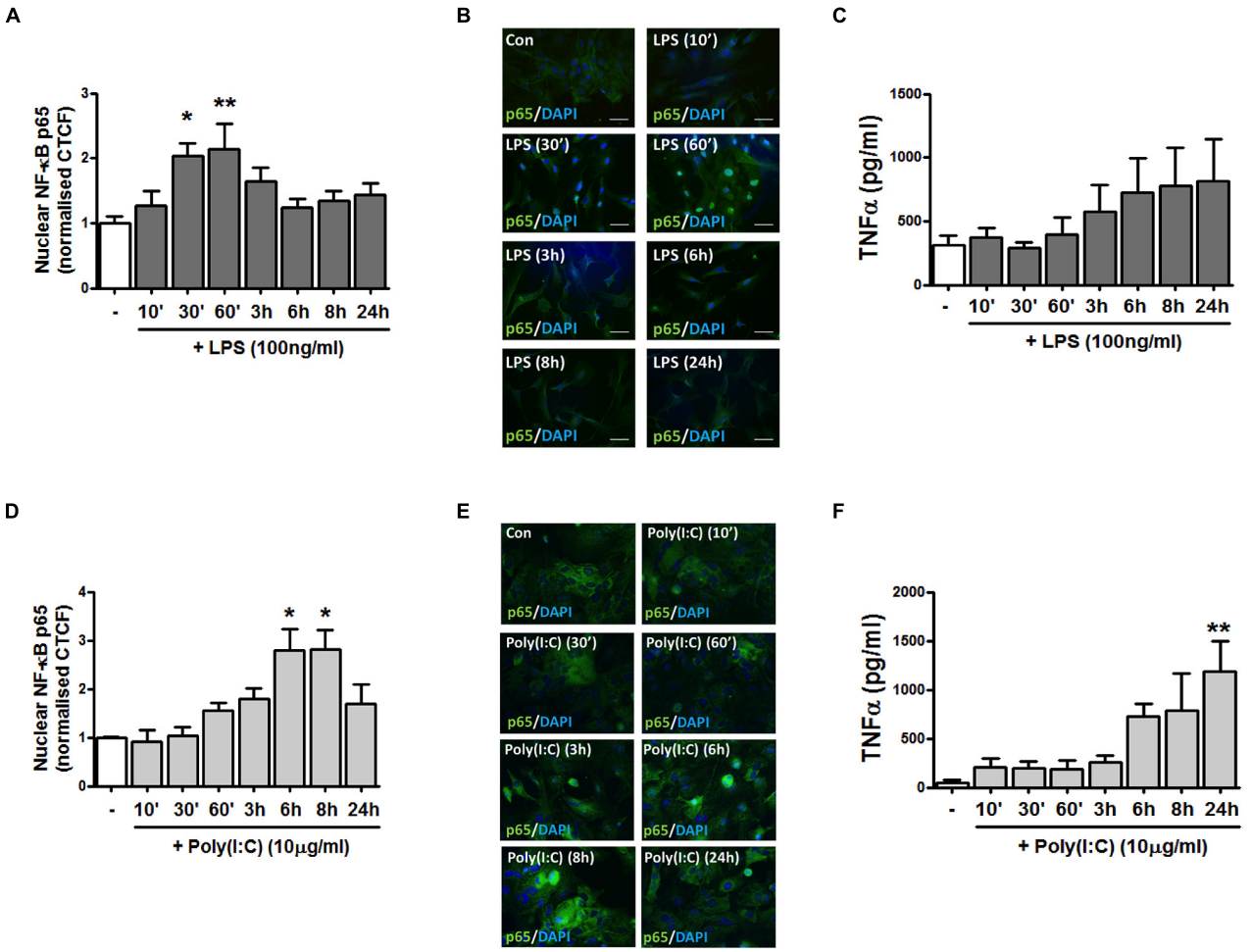

FIGURE 1 | TLR4 and TLR3 stimulation promotes NF-κB p65 distribution to the nucleus and pro-inflammatory TNF-α expression in primary mixed glial cell cultures. (A,D) The relative fluorescence intensity (corrected total cell fluorescence; CTCF) of nuclear NF-κB p65 subunit was determined using Image J to assess the subcellular localization of p65 in mixed glial cultures following exposure to **(A)** LPS and **(D)** poly(I:C) for the indicated time periods. **(B,E)** Immunofluorescent images assessing the subcellular localization of p65 in mixed glial cultures following exposure to **(B)** LPS and **(E)** poly(I:C) for the indicated time periods. Nuclei were stained with DAPI, and images obtained using an Olympus IX70 inverted microscope equipped with the appropriate filter sets. Data are presented as the mean ± S.E.M and are representative of six animals. Scale bar = 20μm. Mixed glial cells were incubated with **(C)** LPS and **(F)** poly(I:C) (timepoints from 10 min–24 h) and supernatants were analyzed for TNF-α production using ELISA. Data are presented as the mean ± SEM of triplicate determinations and are representative of six animals. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with vehicle-treated cells.

was found between groups (**Figure 3B**). Detection rates and mean values of plasma TNF-α and IL-8 are summarized in **Figure 3B**. In addition, no clear correlation between the levels of plasma cytokines and participant age was determined in either RR-MS patient or control groups (two-tailed Spearman non-parametric correlation coefficient; **Figures 3C,D**), indicating that participant age had no impact on peripheral cytokine/chemokine expression. We next assessed the impact of gender on plasma cytokine and chemokine levels. Interestingly, compared to male control subjects, the expression levels of TNF-α and IL-8 were significantly reduced in female control subjects (**Figure 3E**). No significant difference was determined between males vs. females in the RR-MS cohort (**Figure 3F**). This data indicates that the level of cytokine/chemokine expression in healthy individuals is higher in males than females, but that this gender difference is not apparent in the RR-MS cohort.

$GABA_B$ Expression in Human PBMCs

To determine the expression profile of the metabotropic receptor $GABA_B$ in human PBMCs, and whether this profile was altered in disease, we assessed the expression of $GABA_{B1}$ subunit in PBMCs isolated from healthy volunteers and RR-MS patients. Detectable levels of the $GABA_{B1}$ subunit was determined in human PBMCs, with RR-MS associated with a reduction (twofold) in endogenous $GABA_{B1}$ expression when compared to PBMCs isolated from healthy subjects (**Figure 3G**). This indicates that downregulation of $GABA_B$ receptors occurs in PBMCs isolated from newly diagnosed RR-MS patients, when compared to immune cells from healthy subjects.

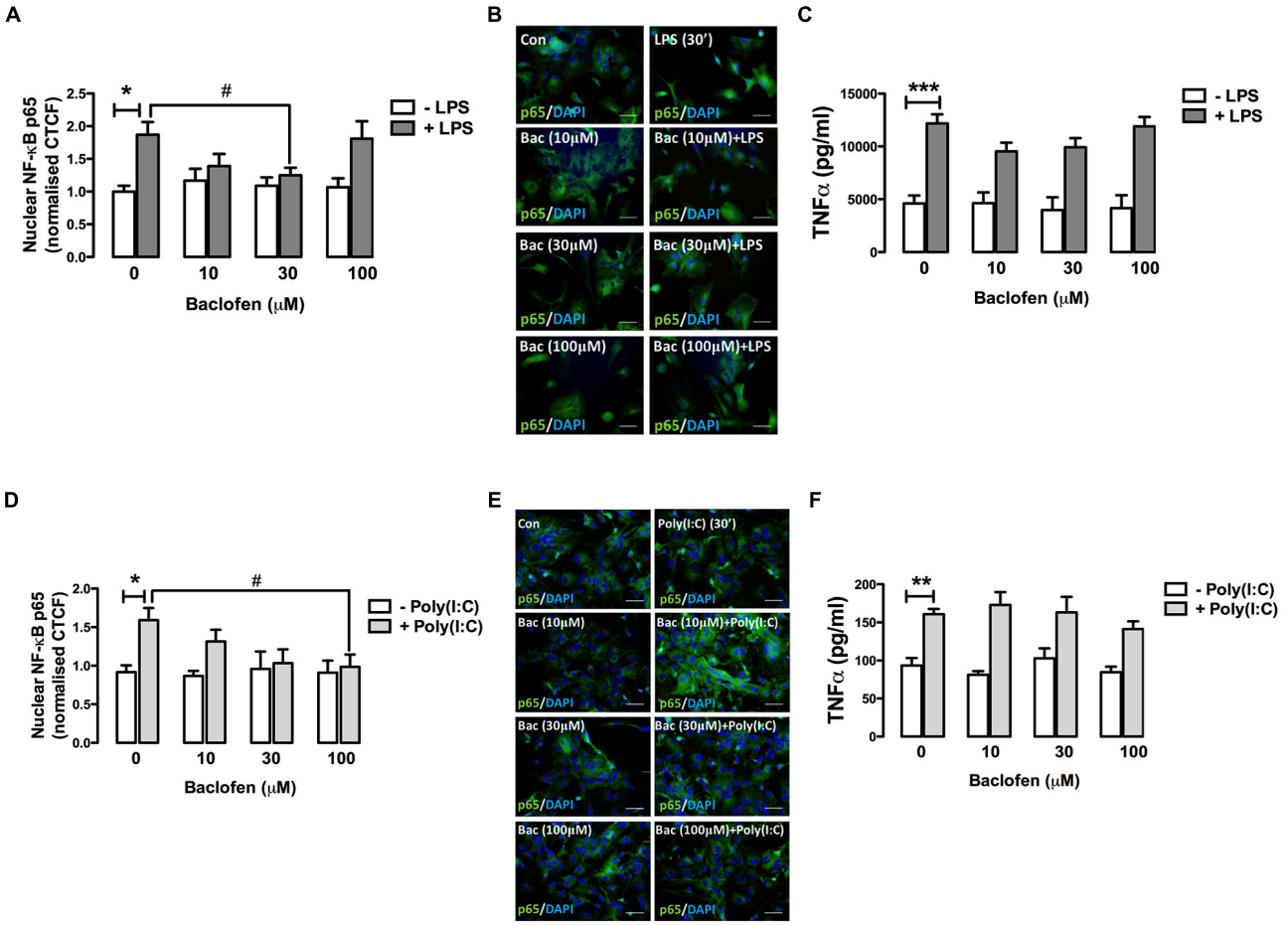

FIGURE 2 | $GABA_B$ receptor activation modulates TLR4- and TLR3-induced NF-κB-p65 nuclear expression and TNF-α expression release in glia. (A,D) CTCF of nuclear NF-κB p65 subunit in mixed glial cultures following exposure to **(A)** LPS (30 min) and **(D)** poly(I:C) (6 h) in the absence and presence of baclofen (10, 30, and 100 μM). **(B,E)** Immunofluorescent images demonstrating the subcellular localization of p65 in glia following exposure to **(B)** LPS ± baclofen and **(E)** poly(I:C) ± baclofen. Nuclei were stained with DAPI, and images obtained using an Olympus IX70 inverted microscope equipped with the appropriate filter sets. Data are presented as the mean ± SEM and are representative of six animals. Scale bar = 20 μm. Mixed glial cells were pre-incubated with baclofen (10, 30, and 100 μM) prior to **(C)** LPS and **(F)** poly(I:C) (both at 24 h) and supernatants analyzed for TNF-α expression by ELISA. Data are presented as the mean ± SEM of triplicate determinations from six animals. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with vehicle-treated cells. $\#p < 0.05$ compared to cells treated with LPS or Poly(I:C) in the absence of baclofen.

Effect of TLR3 and TLR4 Stimulation on PBMCs Isolated from Control and RR-MS Cohorts

Given the defined role of TLR3 and TLR4 in EAE progression (Touil et al., 2006), in addition to evidence that their expression is up-regulated in MS lesions (Bsibsi et al., 2002), we next set out to assess the impact of TLR3 and TLR4 stimulation on inflammatory responses of immune cells isolated from control and RR-MS patients. Firstly, supernatants from unstimulated PBMCs isolated from RR-MS patients displayed enhanced TNF-α (**Figures 4A,C**) and IL-8 (**Figures 4B,D**) expression, when compared to PBMCs from healthy subjects, indicating the RR-MS patient PBMCs display an enhanced endogenous inflammatory signature. LPS enhanced TNF-α production in PBMCs from both groups (**Figure 4A**), and importantly the production of TNF-α was exacerbated in PBMCs prepared from the RR-MS group, compared with the control cohort (**Figure 4A**). In terms of IL-8 expression, two-way ANOVA revealed a significant influence of disease status on IL-8 levels [$F_{(1,30)} = 4.773$, $p = 0.0369$], but no significant influence of TLR4 stimulation on overall variation [$F_{(2,30)} = 0.7174$, $p = 0.4962$] (**Figure 4B**). However, *post hoc* analysis revealed a trend toward a statistically significant increase in IL-8 release following LPS treatment in PBMCs isolated from healthy controls ($p = 0.0612$), but not in RR-MS patients. Interestingly, PBMCs isolated from healthy subjects and RR-MS patients were unresponsive to poly(I·C) stimulation, both in terms of TNF-α and IL-8 production (**Figures 4C,D**), indicating that PBMCs from control individuals and RR-MS patients are refractory to TLR3 stimulation, in terms of TNF-α and IL-8 signaling.

A

Clinical characteristics of MS patients and healthy controls

Clinical category	HC	MS
n	16	8
Sex (Female/Male)	6/10	5/3
Age (years)	30.3 (±1.4)	39.3 (±3.2)
EDSS	n/a	2.6 (±0.4)
Disease duration (years)	n/a	1.2 (±0.3)

Data are expressed as mean (standard error of mean); HC: Healthy controls; MS: Multiple Sclerosis; EDSS: Expanded Disability Status Scale

B

Detection rates and values (pg/ml) of plasma cytokines

Clinical category	HC		MS	
	Detection rate	Mean (SEM)	Detection rate	Mean (SEM)
IL-8	16/16	22.50 (±1.26)	8/8	22.64 (±1.71)
TNF-α	10/16	325.70 (±95.07)	6/8	176.40 (±78.93)

The table includes mean values of cytokines that could be detected. HC: Healthy controls; MS: Multiple Sclerosis;

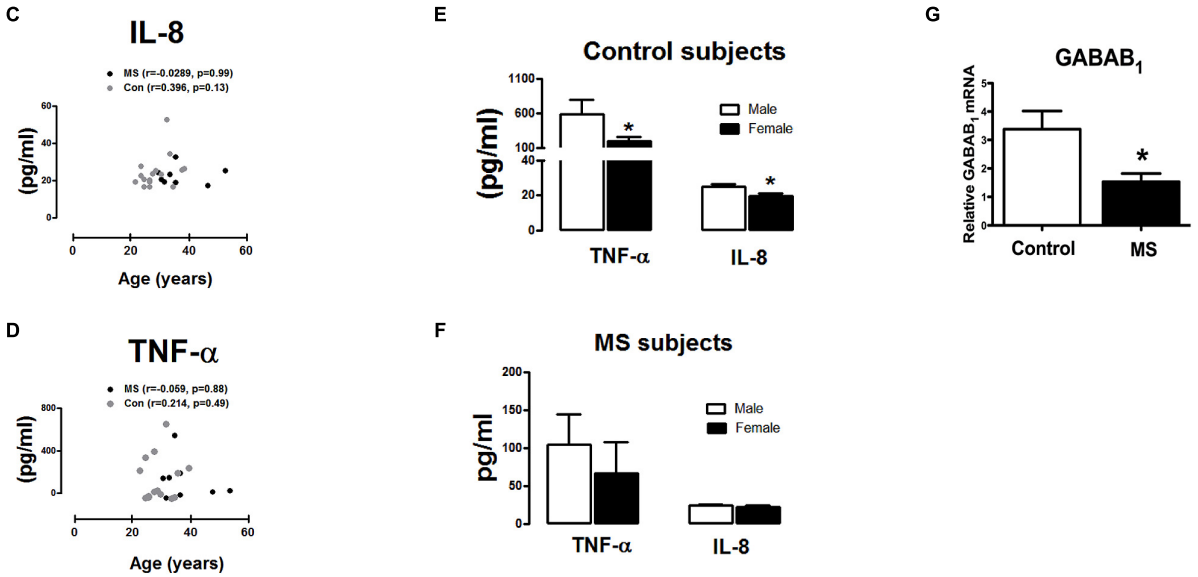

FIGURE 3 | Demographic and clinical characteristics of MS patients and control subjects included in the study. (A) Demographics of healthy control participants and RR-MS patients. Expanded Disability Status Scale (EDSS). **(B)** Plasma levels of IL-8 and TNF-α in control (n = 16) and MS (n = 8) cohort. Detection rates and mean plasma expression values of IL-8 and TNF-α are summarized. **(C,D)** Correlation between IL-8/TNF-α plasma levels and age of study participants. Correlation plots demonstrating **(C)** IL-8 and **(D)** TNF-α plotted against subject age. No correlation was observed between age in control and MS groups analyzed (two-tailed Spearman non-parametric correlation coefficient, correlation r-values, and significance p-values, are indicated in the graphs). Levels of TNF-α and IL-8 in **(E)** healthy control participants and **(F)** MS patients in plasma obtained from male (n = 13) and female (n = 11) participants. **(G)** Peripheral blood mononuclear cells (PBMCs) isolated from healthy subjects and newly diagnosed RR-MS patients were assessed for $GABA_B$ mRNA expression, cDNA generated and assayed by quantitative real-time PCR for levels of $GABAB_1$ mRNA. Data are presented as the mean ± SEM of triplicate determinations. *$p < 0.05$ compared with male control subjects **(E)**. *$p < 0.05$ compared with all control subjects **(G)**.

Baclofen Attenuates TLR4-Induced TNF-α Cytokine Release in Human PBMCs Isolated from Healthy, but not RR-MS, Subjects

Given that $GABA_B$ activation modulates TLR4-induced signaling in primary mixed glia (**Figure 2**), and that TLR4 stimulation enhances TNF-α signaling in PBMCs from control and RR-MS patients (**Figure 4**), we next assessed the proclivity of baclofen to modulate TLR4-induced TNF-α release in PBMCs isolated from healthy subjects and RR-MS patients. PBMCs from healthy subjects (**Figure 5A**) and RR-MS patients (**Figure 5B**) were pre-treated (30 min) with baclofen (10, 30, and 100 μM) prior to LPS exposure (24 h) and TNF-α expression assessed. PBMCs isolated from healthy subjects were responsive to LPS with an increase in TNF-α release observed, whereas baclofen modestly attenuated this (**Figure 5A**). In contrast, pre-exposure (30 min) to baclofen prior to LPS exposure (24 h) failed to significantly impact TLR4-induced TNF-α expression in PBMCs from RR-MS patients at each dose tested (**Figure 5B**). Importantly, this indicates a differential sensitivity of cells from healthy and RR-MS subjects in terms of the ability of baclofen to modulate TLR-induced inflammatory signaling, which may have implications in terms of the design of $GABA_B$ receptor therapeutics to treat neuroinflammatory disorders including MS.

Discussion

Understanding the mechanisms underlying neuroinflammatory processes in MS may pave the way to novel therapeutic strategies. Data herein demonstrate that the $GABA_B$ receptor agonist baclofen has the proclivity to differentially target

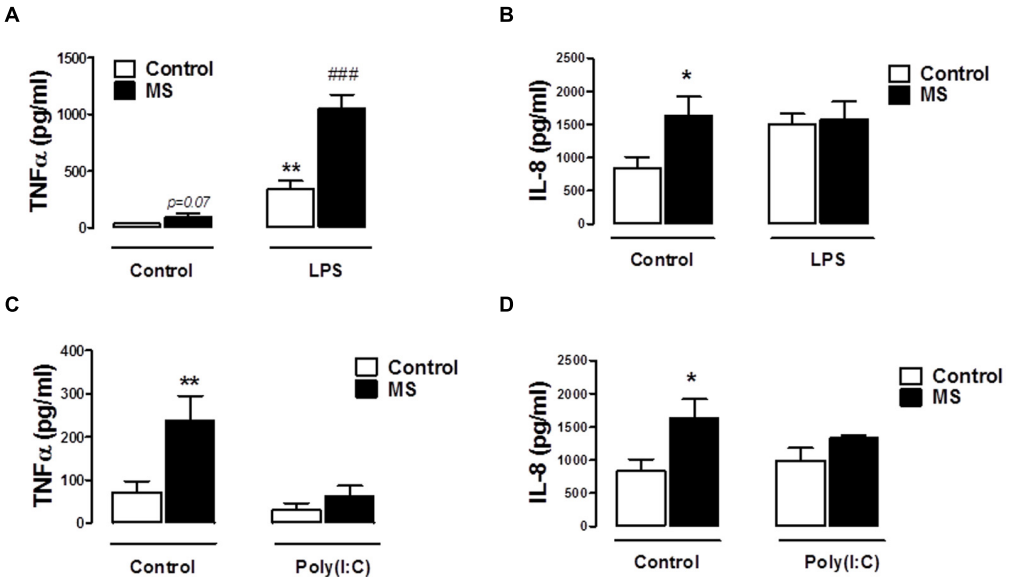

FIGURE 4 | Peripheral blood mononuclear cells from RR-MS patients display enhanced inflammatory signaling. PBMCs prepared from healthy subjects and MS patients were seeded into 24-well plates and treated with vehicle, LPS (100 ng/ml) or poly(I·C) (10 μg/ml) for 24 h. Supernatants were analyzed for TNF-α **(A,C)** and IL-8 **(B,D)** production using ELISA. Data are presented as the mean ± SEM of triplicate determinations. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with vehicle-treated PBMCs from healthy subjects. $^{###}p < 0.001$ compared with vehicle-treated cells from MS patients.

TLR4 and TLR3 inflammatory signaling events both in CNS glial cells, and in peripheral immune cells. Baclofen reduced TLR3/4-induced nuclear NF-κB and TNF-α (albeit modestly) expression in glial cells, with distinct dose-dependency identified for these effects. $GABA_B$ receptor activation attenuated TLR4-induced TNF-α expression in PBMCs isolated from healthy individuals, while baclofen had no impact on TLR4 signaling in RR-MS patient PBMCs. Furthermore, PBMCs from RR-MS patients expressed lower levels of the $GABA_B$ receptor mRNA. Significantly, we also determined that immune cells from the RR-MS patient group displayed hypersensitivity to LPS exposure in terms of TNF-α expression.

TLR family members are expressed in cells of the CNS (Downer et al., 2013a; Nakano et al., 2015) and TLR ligands regulate CNS inflammation (Lehnardt, 2010). We initially targeted TLR3 and TLR4 signaling given the role of these receptors in neuroinflammatory events linked with MS (Bsibsi et al., 2002; Touil et al., 2006). Both NF-κB activation and pro-inflammatory cytokine expression are known downstream signaling events following ligation of TLR3 and TLR4 (Moynagh, 2005), and signaling via both cascades is perturbed following TLR3 (Cavassani et al., 2008) and TLR4 (Devaraj et al., 2011) knockout. We examined the ability of LPS and poly(I:C) to couple to TLR4 and TLR3 inflammatory signaling in primary glia, respectively. Data presented herein indicate that LPS and poly(I:C) enhanced NF-κB activation and TNF-α cytokine expression in glia, with the kinetics of this response mirroring data described elsewhere (Park et al., 2006; Horvath et al., 2008).

This study highlights the anti-inflammatory potential of baclofen in glial cells, by virtue of its inhibitory effects on the NF-κB-TNF-α pathway induced by TLR3/4 activation. This is consistent with data elsewhere indicating that baclofen reduces LPS-induced production of IL-6/IL-12 in mouse microglia (Kuhn et al., 2004), and reduces phospho-p65-NF-κB and IL-6/TNF-α expression in human astrocytes and microglial cells stimulated with IFN-γ/LPS (Lee et al., 2011). $GABA_B$ receptors are expressed on astrocytes and microglia (Charles et al., 2003), and evidence indicates that glial $GABA_B$ receptors are functional (Kuhn et al., 2004; Oka et al., 2006). Given the expression and functional activity of $GABA_B$ receptors on glia, alongside evidence that baclofen negatively regulates TLR3/4-induced inflammatory signaling, it is likely that $GABA_B$ receptors play an integral role in the course of neuroinflammation by targeting glia. The ability of baclofen to target TLR3/4-induced inflammatory signaling in glia *in vitro* may contribute to an anti-inflammatory efficacy *in vivo*, and this will be the focus of further studies.

Interestingly, different effects of $GABA_B$ receptor modulation on the immune system have been indicated, suggesting that GABA is a regulator of immune cell activity and inflammation. Indeed, GABA receptors have been identified on neutrophils, macrophages, dendritic cells, and T-cells (Rane et al., 2005; Jin et al., 2013), and cells of the immune system possess the metabolic machinery for the synthesis of GABA (Auteri et al., 2015). Given the evidence that GABA is a neuroimmune modulator, alongside our findings that baclofen modulates TLR-induced inflammatory signaling in central glial cells, we next assessed the impact of baclofen on TLR signaling in peripheral immune cells. Firstly, our findings indicate that TLR4 activation promotes TNF-α and IL-8 cytokine expression in human PBMCs, and these findings are consistent with data elsewhere (Kaplin et al., 2009; Downer et al., 2013b). Importantly, data presented herein indicate an

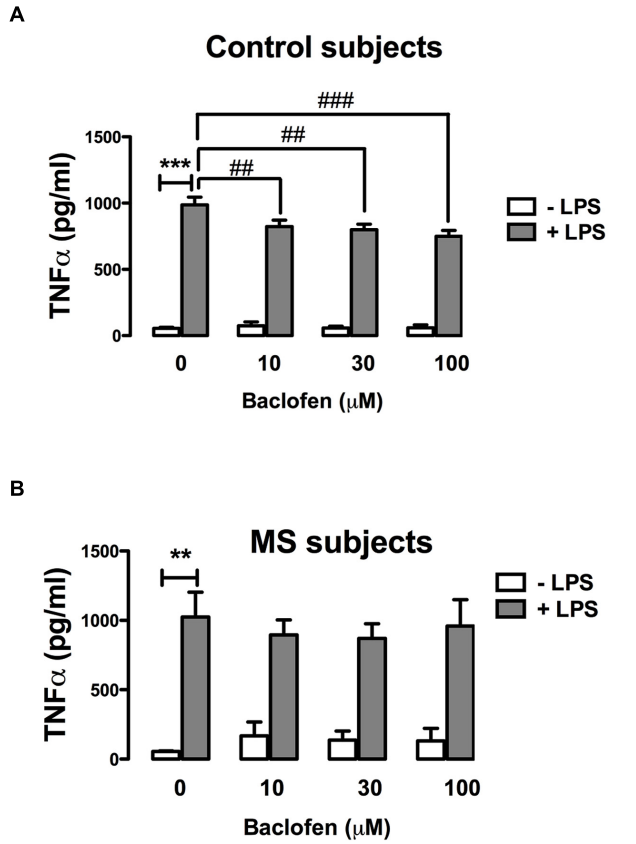

FIGURE 5 | $GABA_B$ activation attenuates TLR4-induced TNF-α production in PBMCs from healthy, but not MS, subjects. PBMCs isolated from **(A)** healthy subjects and **(B)** MS patients were seeded in 24-well plates and pre-treated with baclofen (10, 30, and 100 μM) in the absence and presence of LPS (100 ng/ml) for 24 h. Supernatants were analyzed for TNF-α production using ELISA. Data are presented as the mean ± SEM of triplicate determinations. $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with vehicle-treated cells. $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ compared to cells treated with LPS in the absence of baclofen.

anti-inflammatory propensity of baclofen in human immune cells, demonstrating that baclofen blunts LPS-induced TNF-α expression in peripheral immune cells from healthy subjects. This finding is in line with data demonstrating that exposure of human PBMCs to baclofen ameliorates phytohemagglutinin-induced TNF-α release (Duthey et al., 2010). A significant finding here is that this is the first direct evidence that baclofen can target TLR signaling in human PBMCs, and hence has relevance across a range of neuroimmune disorders.

Our results identify that PBMCs isolated from healthy subjects were responsive to baclofen, with a significant, albeit moderate, decrease in LPS-induced TNF-α expression observed following baclofen treatment. In contrast, baclofen failed to regulate TLR4-induced TNF-α expression in PBMCs from RR-MS subjects. This is significant as intrathecal administration of baclofen can be used to treat spasticity in MS, in addition to spinal cord injury, cerebral palsy, and acquired brain injury (Gunnarsson and Samuelsson, 2015). Furthermore, there is evidence of a loss of GABAergic neurons at lesion sites in post-mortem MS brain (Redondo et al., 2014). The differential sensitivity of cells from healthy and RR-MS subjects to baclofen indicates that MS drives a desensitizing signal in terms of $GABA_B$ receptor function. Indeed, our findings indicate that downregulation of $GABA_B$ receptors occurs in PBMCs in newly diagnosed RR-MS patients, which may govern the differential sensitivity to baclofen exposure seen in healthy and MS patient cells. In support of this, the level of GABA is reduced in the blood serum of patients with MS as compared to the controls (Demakova et al., 2003). In addition, baclofen treatment has been shown to reduce $GABA_B$ receptor density in rat spinal cord (Kroin et al., 1993), which may be postulated as a molecular mechanism for development of tolerance. It is especially interesting to note that treatment failure has been shown with baclofen in MS patients (Stroet et al., 2013), and the inability of baclofen to target TLR4-induced pro-inflammatory cytokine production might contribute to these effects.

Whether the effects of baclofen identified in the present and other studies are a direct result of its action at the $GABA_B$ receptor is not yet entirely clear, particularly given the recent discovery that baclofen, and several $GABA_B$ receptor antagonists, are allosteric modulators of CXCR4, a chemokine receptor involved in neuroimmune crosstalk (Guyon et al., 2013). Indeed, determining whether the effects of baclofen that were observed in this study are specifically due to its action at the $GABA_B$ receptor rather than at CXCR4 will be the focus of future studies. Such studies will be challenging because several $GABA_B$ receptor antagonists also act as allosteric modulators of CXCR4 (Guyon et al., 2013). Future studies aimed at identifying $GABA_B$ receptor antagonists that do not affect CXCR4 activity will be required in order to address this question directly. Nevertheless, since baclofen and some $GABA_B$ receptor antagonists exert the same effects on CXCR4 activity (Guyon et al., 2013), then it would be expected that prevention of the effects of baclofen by a $GABA_B$ receptor antagonist would be independent of CXCR4 activity (since both baclofen and the $GABA_B$ receptor antagonists behave as CXCR4 antagonists). Alternatively, experiments could examine the effects of baclofen under conditions whereby CXCR4 expression or activity is altered, however, such experiments might be complicated by the modulation of inflammatory mediators by CXCR4 itself. Despite these limitations, there is accumulating evidence that many components of the GABA neurotransmitter system, including not only the $GABA_B$ receptor but also the $GABA_A$ receptor, can have anti-inflammatory effects (Song et al., 1998; Bhat et al., 2010; Lee et al., 2011).

The rationale behind the present study was to determine if baclofen could target TLR-induced signaling in RR-MS, particularly given the growing body of data indicating that the TLR system is a key player in MS pathogenesis (O'Brien et al., 2008; Downer, 2011). However, intrathecal administration of baclofen is used to manage spasticity in patients with a range of conditions including spinal cord injury, cerebral palsy and acquired brain injury in addition to MS, and hence in future studies it would be interesting to compare the effects of baclofen on the PBMCs of patients with other neurological conditions.

The concentrations of cytokines/chemokines are altered in MS (Ubogu et al., 2006), suggesting that cytokine/chemokine signatures may indicate disease progression in patient groups (O'Connell et al., 2014). Given that evidence indicates that TNF-α (Sharief and Hentges, 1991) and IL-8 (Lund et al., 2004) are higher in serum of patients with MS, we examined the relative expression of TNF-α and IL-8 in plasma isolated from newly diagnosed RR-MS patients. Our analysis revealed no difference in plasma IL-8/TNF-α between control and RR-MS groups. Systemic inflammation in MS is strongly associated with periods of intense relapse (Confavreux et al., 2000), hence it is plausible that alterations in peripheral cytokines in MS may be phase dependent. In support of this, Mellergard et al. (2010) indicate that MS patients with EDSS measurements in the range comparable to the RR-MS cohort assessed in present study demonstrate no alteration in plasma TNF-α when compared to healthy subjects (Mellergard et al., 2010).

Neuroinflammatory changes develop with age, with defects in the immune system identified in elderly individuals (Allman and Miller, 2005). In addition, an increased plasma level of pro-inflammatory cytokines has been demonstrated in elderly, compared to young, individuals (Forsey et al., 2003). Our findings indicate no clear correlation between the levels of plasma cytokine/chemokine and participant age in either RR-MS or control groups, indicating that the age of subjects did not impact cytokine/chemokine profiles. Interestingly, compared to male control subjects, the expression levels of TNF-α and IL-8 were significantly reduced in female control subjects. This indicates that particular expression profiles of cytokines/chemokines may be gender specific, which is confirmed elsewhere (Asai et al., 2001).

Marked alterations in the expression profile of TLRs has been determined in MS lesions in human brain samples, in the brain samples from mice that have undergone EAE, and in CSF cells isolated from MS patients (Marta, 2009). With this in mind we investigated the effect of TLR4 and TLR3 stimulation on the pro-inflammatory cytokine and chemokine, TNF-α and IL-8. PBMCs without stimulation, as well as after stimulation with LPS, displayed increased production of TNF-α and IL-8 in the RR-MS sample cohort, when compared to the control group. These results suggest that immune cells from the RR-MS patient group have an enhanced endogenous inflammatory signature. In addition, RR-MS patient PBMCs displayed hypersensitivity to LPS exposure in terms of TNF-α expression. TLR4 expression is upregulated in CNS lesions in mice following EAE and in PBMCs from RR-MS and secondary progressive (SP) MS patients (Andersson et al., 2008), which in support of our study, indicates that TLR4 signaling participates in an innate immune response that may shape the inflammatory response in both forms of MS. It is intriguing that the hypersensitivity of RR-MS patient cells to LPS is only relevant in the context of TNF-α induction, since LPS shows comparable efficacy in inducing IL-8 in cells from healthy and RR-MS patients. Hence, any form of TLR4 hypersensitivity that may exist in MS patient cells appears to be restricted to the pathway leading to TNF-α expression, and further studies will probe the intracellular signaling events in patient cells.

Our studies also probed the effects of TLR3 stimulation on TNF-α/IL-8 in PBMCs. Surprisingly, PBMCs isolated from healthy donors and RR-MS patients did not respond to TLR3 stimulation by enhancing TNF-α or IL-8 expression, suggesting that the TLR3 pathway leading to cytokine and chemokine expression may be desensitized in our participant cohorts. Indeed, we have previously demonstrated that the TLR3-IFN-β pathway is desensitized in MS patient PBMCs, suggesting that MS patients may be pre-sensitized to viral infection showing some form of TLR3 tolerance. However, the non-responsiveness of healthy donor PBMCs to poly(I·C) is in line (Wesch et al., 2006) and in contrast (Myles et al., 2012) to findings elsewhere in studies which investigated freshly isolated PBMCs; differences in dose and timecourse for treatment regimen used with poly(I:C) in the present study may underlie the differences observed.

Although baclofen demonstrates therapeutic effects in MS, the mechanism(s) of action are poorly understood. We present evidence that the innate arm of the immune system is a target for baclofen anti-inflammatory action, and demonstrate that baclofen differentially targets TLR4 and TLR3 inflammatory signaling events both in primary murine glial cells and in PBMCs isolated from human blood samples. Baclofen can exert anti-inflammatory properties at specific doses, by down-regulating TLR-induced activation of NF-κB and induction of pro-inflammatory cytokines. Significantly, our findings also indicate that inflammatory signaling, and sensitivity to TLR4 stimulation, was enhanced in PBMCs of patients with RR-MS, highlighting that TLR4 may play a role at least in the RR form of MS pathogenesis. We targeted this particular patient cohort because they were newly-diagnosed and naïve to disease modifying therapies. However, investigating TLR functioning and its modulation by baclofen in patients with secondary chronic progressive MS would be an important future study. Overall, the present study provides novel insight into the cellular effects of targeting central and peripheral $GABA_B$ receptors in the modulation of TLR functioning.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fncel.2015.00284

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frontiers in Cellular Neuroscience

MINI REVIEW
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Role of T cell—glial cell interactions in creating and amplifying central nervous system inflammation and multiple sclerosis disease symptoms

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Multiple Sclerosis (MS) is an inflammatory disease of the Central Nervous System (CNS) that causes the demyelination of nerve cells and destroys oligodendrocytes, neurons and axons. Historically, MS has been thought of as a T cell-mediated autoimmune disease of CNS white matter. However, recent studies have identified gray matter lesions in MS patients, suggesting that CNS antigens other than myelin proteins may be involved during the MS disease process. We have recently found that T cells targeting astrocyte-specific antigens can drive unique aspects of inflammatory CNS autoimmunity, including the targeting of gray matter and white matter of the brain and inducing heterogeneous clinical disease courses. In addition to being a target of T cells, astrocytes play a critical role in propagating the inflammatory response within the CNS induced NF-κB signaling. Here, we will discuss the pathophysiology of CNS inflammation mediated by T cell—glial cell interactions and its contributions to CNS autoimmunity.

Keywords: T cell, autoimmunity, glial fibrillary acidic protein, multiple sclerosis, astrocytes, experimental autoimmune encephalomyelitis, cerebellum

Myelin-Specific T Cell Responses in MS and EAE

Multiple Sclerosis (MS), an inflammatory T cell-mediated autoimmune disease, is the most common neurological disease of young adults. MS causes the demyelination of nerve cells and destroys oligodendrocytes, neurons and axons (Frohman et al., 2006; Lassmann et al., 2007), with highly variable clinical manifestations. Such clinical manifestations of MS often include hyperreflexia, ataxia, spasticity and visual defects (Noseworthy et al., 2000; Keegan and Noseworthy, 2002; Hafler et al., 2005; Frohman et al., 2006; McFarland and Martin, 2007), and in some cases there are sensory defects and partial or complete paralysis. In the majority of patients, disease manifests as relapsing-remitting cycles of impairment, usually converting over time to a chronic progressive stage; 10–15% of patients present with disease that is progressive from onset (Sospedra and Martin, 2005; Frohman et al., 2006; McFarland and Martin, 2007; Steinman, 2009).

MS is thought to be primarily a CD4 T cell-mediated disease. Susceptibility to MS is genetically linked to major histocompatibility complex (MHC) genes and genes associated with T cell activation and homeostasis; however, the strongest genetic linkage occurs with certain alleles of MHC class II, which suggests a direct relationship between autoreactive

$CD4^+$ T cells and MS disease development in humans (Hillert and Olerup, 1993; Fogdell-Hahn et al., 2000; Sospedra and Martin, 2005). $CD4^+$ T cells, in particular those that secrete IL-17, are considered to play an important role in the induction of central nervous system (CNS) autoimmunity (Korn et al., 2009). The identification of genes involved in CD4 T-cell differentiation and activation through genome wide association studies (GWAS) have further supported a role for CD4 T cells in the pathogenesis of MS (Patsopoulos et al., 2011).

The ability of myelin-reactive CD4 T cells to cause experimental autoimmune encephalomyelitis (EAE) further supports the hypothesis that myelin-reactive CD4 T cells have a central role in MS disease pathogenesis (Kuchroo et al., 2002; Sospedra and Martin, 2005; Ercolini and Miller, 2006; Hafler et al., 2007; Goverman, 2009; Steinman, 2009). MS-like clinical symptoms can be induced in animals by immunization with CNS proteins, as well as peptides derived from these CNS proteins, including myelin basic protein (MBP), proteolipid protein (PLP) and myelin oligodendrocyte glycoprotein (MOG; Ben-Nun et al., 2014). In addition, the adoptive transfer of activated CNS protein-specific CD4 T cells into naïve mice can induce paralytic diseases, allowing for *in vivo* study of the migratory behavior of pathogenic T cells (Jäger et al., 2009; Arima et al., 2012; Odoardi et al., 2012). However, it is unlikely that CD4 T cells are the sole mediators of disease pathogenicity, as treatments specifically targeting these cells limit neither the rate of disease relapses nor the formation of new lesions. In contrast, therapies that deplete or inhibit CNS infiltration of all lymphocyte subsets have been more successful (Lindsey et al., 1994; van Oosten et al., 1996; Rice et al., 2005).

Accumulating evidence strongly suggests that CD8 T cells also contribute to MS disease. Studies have shown that CD8 T cells are found in MS plaques—these cells are often oligoclonal, accumulate over time and can outnumber CD4 T cells regardless of the stage of activity or disease (Booss et al., 1983; Traugott et al., 1983; Hauser et al., 1986; Babbe et al., 2000; Lucchinetti et al., 2000; Frohman et al., 2006; Lassmann et al., 2007; Huseby et al., 2012). Though the antigen specificity of CNS infiltrating CD8 T cells remains unclear, a role for CD8 T cells in MS is further supported by the finding that particular MHC class I alleles can contribute to disease susceptibility (Cree et al., 2010; Healy et al., 2010).

Both a pathogenic or protective role for CNS-infiltrating CD8 T cells has been proposed. Myelin-specific CD8 T cells that are capable of killing neuronal cells *in vitro* have been isolated from MS patients (Tsuchida et al., 1994; Dressel et al., 1997; Medana et al., 2001; Crawford et al., 2004; Zang et al., 2004), which supports the hypothesis that CD8 T cells play a pathogenic role in the MS disease process. Further in support of this hypothesis, CD8 T cells specific for myelin proteins, including MBP, MOG, and PLP, have been shown to be pathogenic in several animal models of CNS disease (Huseby et al., 2001a; Sun et al., 2001; Ford and Evavold, 2005; Friese et al., 2008; Anderson et al., 2012). The clinical symptoms induced by such CNS-reactive CD8 T cells can be diverse. For example, mice carrying activated MBP-specific CD8 T cells succumb to a non-paralytic, acute demyelinating CNS autoimmunity that is clinically and histologically different than those of classic CD4-EAE. These atypical-EAE disease pathologies have similarities to MS patients with upper motor neuron disease (Huseby et al., 2001a). In contrast, experiments with MOG- and PLP-specific CD8 T cells resulted in CNS disease symptoms similar to classical EAE (Sun et al., 2001; Ford and Evavold, 2005; Friese et al., 2008; Anderson et al., 2012). These data suggest that myelin-specific CD8 T cells may contribute to some of the disease heterogeneity observed in MS patients.

Conversely, other studies have suggested that CD8 T cells may be suppressive during the MS disease process. CD8 T cell clones that can lyse myelin-specific CD4 T cells have been detected in MS patients (Chou et al., 1992; Zhang et al., 1993; Correale et al., 2000), and longitudinal magnetic resonance imaging (MRI) analysis has shown a negative correlation between the percentage of Tc2 cytokine-producing CD8 T cells in the periphery of MS patients and the development of lesions (Killestein et al., 2003). Moreover, protective MHC class I alleles have been identified through GWA studies, suggesting a relationship between autoreactive regulatory $CD8^+$ T cells and MS disease development (International Multiple Sclerosis Genetics Consortium et al., 2011). In animal models, early studies found that polyclonal CD8 T cells can limit disease severity and relapses of CD4 T cell-mediated EAE (Jiang et al., 1992; Koh et al., 1992). The ability of CD8 T cells to regulate CNS autoimmune disease may occur by CD8 T cells targeting activated CD4 T cells through the recognition of peptide displayed on MHC class I and Ib molecules, as well as by secreting IL-10 and other anti-inflammatory soluble mediators (Jiang and Chess, 2006; Goverman, 2009; Kim and Cantor, 2011; Ortega et al., 2013). Thus, different subsets of CD8 T cells, like their CD4 counterparts, likely play pathogenic and immuno-regulatory roles in MS (Huseby et al., 2012).

Gray Matter Lesions in MS and EAE

MS has traditionally been thought of as a disease that targets myelin proteins within the white matter of the CNS. Recent findings indicate, however, that this may not always be the case. Using advanced MRI techniques, multiple investigators have identified gray matter lesions in MS patients that appear at the earliest stages of disease and accumulate over time (Lucchinetti et al., 2000, 2011; Peterson et al., 2001; Bo et al., 2003; Frohman et al., 2006; Calabrese et al., 2007; Lassmann et al., 2007; Fisher et al., 2008; Ontaneda et al., 2012). The presence of T cells within gray matter lesions of MS patients suggests that T cells reactive to antigens other than myelin proteins may contribute to MS disease progression. One potential cellular target of gray matter disease is astrocytes, which reside within the white and gray matter of the CNS. Astrocytes normally express low levels of MHC, however levels increase during inflammation (Wong et al., 1984; Ransohoff and Estes, 1991; De Keyser et al., 2010).

Autoreactive T cells must avoid negative selection within the thymus and be exported to the peripheral T cell repertoire in order to contribute to the CNS autoimmune disease

process. Though myelin proteins, the prototypical targets of encephalogenic CD4 T cells, are primarily expressed behind the blood-brain barrier, some myelin peptide epitopes are expressed and presented in the thymus. Developing T cells that are reactive to these ligands can be subject to thymic deletion or be skewed towards low avidity or suppressive responses. These findings have lead to a differential avidity model for the development of encephalogenic T cells: strong avidity T cells targeting myelin epitopes that are presented in the thymus undergo negative selection whereas weak avidity T cells that target these same epitopes or strong avidity T cells that target myelin epitopes that are only expressed within the CNS are exported into the mature T cell repertoire and can induce autoimmunity (Liu et al., 1995; Harrington et al., 1998; Targoni and Lehmann, 1998; Huseby et al., 1999, 2001b; Klein et al., 2000; Kuchroo et al., 2002). The expectation is that T cells which target astrocytes or other CNS cell types will follow similar rules for development as those identified for T cells that target myelin.

Two proteins predominately expressed in astrocytes, Glial fibrillary acidic protein (GFAP) and S100β, have been studied as targets for autoreactive T cells. GFAP, an intermediate filament protein, is an archetypal astrocyte-specific antigen that is expressed throughout the gray matter and white matter of the brain and spinal cord (Middeldorp and Hol, 2011). GFAP is also expressed in some peripheral tissues including the thymus, intestine and pancreas, though expression levels are lower in these tissue types (Zelenika et al., 1995). In MS lesions, the expression level of GFAP increases and peptides derived from GFAP are presented by MHC class I and class II molecules (Nait-Oumesmar et al., 2007; Fissolo et al., 2009; Linker et al., 2009). S100β, a calcium binding protein, is also expressed within astroglia present within the gray and white matter of the CNS (Zimmer et al., 1995). Although both proteins are also expressed outside of the CNS, including at a low level within the thymus, T cell responses to these proteins indicate that immune tolerance towards these antigens is incomplete.

The adoptive transfer of $CD4^+$ T cells reactive to GFAP or to S100β into rodents induces a strong inflammatory response within the spinal cord and throughout the entire CNS, including the cerebral cortex and the retina of the eye, with particularly severe inflammation observed in the gray matter (Kojima et al., 1994, 1997). These experiments demonstrate that T cell responses to non-myelin antigens are capable of being pathogenic in models of CNS autoimmunity. Compellingly, $CD4^+$ S100β-specific T cells have been isolated from MS patients, as well as from healthy controls, indicating astrocyte-specific T cells are present in the mature T cell repertoire and may contribute to the disease process (Schmidt et al., 1997).

GFAP-Specific CD8 T Cells can Induce Relapsing/Remitting CNS Autoimmunity

The observation that CD8 T cells are present within gray matter lesions of MS patients (Peterson et al., 2001; Bo et al., 2003; Calabrese et al., 2007; Lassmann et al., 2007; Fisher et al., 2008; Lucchinetti et al., 2011; Ontaneda et al., 2012) inspired us to study astrocyte-specific CD8 T cells. We chose the astrocyte protein GFAP as the target antigen because GFAP expression and GFAP-peptide presentation by MHC class I and II molecules are increased within MS lesions (Nait-Oumesmar et al., 2007; Fissolo et al., 2009; Linker et al., 2009). Furthermore, although GFAP-specific T cells isolated from MS patients have not been studied, GFAP-specific CD8 T cells have been isolated from patients with type 1 diabetes, indicating that human T cells with this reactivity pattern populate the peripheral T cell repertoire (Standifer et al., 2006). CD8 T cells that target astrocytes and neurons have also been suggested in Rasmussen encephalitis (Schwab et al., 2009).

We have recently found that C57BL/6 mice carry CD8 T cells reactive to $GFAP_{264–272}$ presented by H2-D^b. We constructed TCR Tg mice expressing the GFAP-specific CD8 T cell clone, BG1 (BG1 mice), to follow the fate of naïve GFAP-specific T cells. To determine if BG1 mice maintain quiescence to GFAP over their lifetime, a cohort of WT, $Rag1^{-/-}$ and $Gfap^{-/-}$ BG1 mice were analyzed for clinical signs of CNS disease as they aged. We observed that BG1 mice do not maintain ignorance of GFAP: ∼50% of WT BG1 mice and 100% of $Rag^{-/-}$ BG1 mice succumb to spontaneous clinical signs of CNS autoimmunity by 6 months of age. The majority of diseased BG1 mice develop balancing defects, lethargy, uneven gait and ataxia—such symptoms are referred to as atypical disease (Sasaki et al., 2014)—whereas some diseased mice also succumb to mild ascending flaccid paralysis—such symptoms are referred to as classical EAE (Stromnes and Goverman, 2006). The atypical disease symptoms that develop in BG1 mice reflect the locations within the CNS that is targeted; BG1 mice develop lesions showing prominent glial responses within the cerebellum, mid-brain and spinal cord early in a spontaneous disease course that includes both white matter and gray matter (**Figure 1**).

The BG1 CD8 effector T cell populations that target the CNS during spontaneous CNS disease phenotypically resemble anti-viral tissue-resident memory (T_{RM}) cells that populate peripheral tissues following viral challenges (Schenkel and Masopust, 2014). Functionally, only low frequencies of CD8 T cells within the CNS are capable of producing IFNγ, IL-17 or granzyme B (GZB), indicating that many of the BG1 CD8 T cells present within the brain are not classic effector CD8 T cells. These data suggest that BG1 CD8 T cells that spontaneously enter into the brain interact with astrocytes to induce their differentiation into auto-reactive T_{RM}, without gaining inflammatory cytokine expression or cytotoxic effector functions. Nevertheless, these auto-reactive T_{RM} CD8 T cells can induce severe inflammation, glial responses and clinical disease symptoms. In contrast to CNS disease induced by auto-reactive T_{RM} CD8 T cells, disease induced by classic IFNγ-producing pro-inflammatory CD8 T cells demonstrates severe ataxia and lethargy within 7 days, a disease pattern highly similar to those induced by *in vitro* or Vac-activated MBP-specific CD8 T cells (Huseby et al., 2001a; Sasaki et al., 2014). These differences in CNS disease pathologies suggest that different auto-reactive CD8 T cell lineages induce distinct CNS disease phenotypes, thereby contributing to MS disease heterogeneity. This hypothesis is consistent with studies of encephalogenic CD4 T cells. Through the observation of

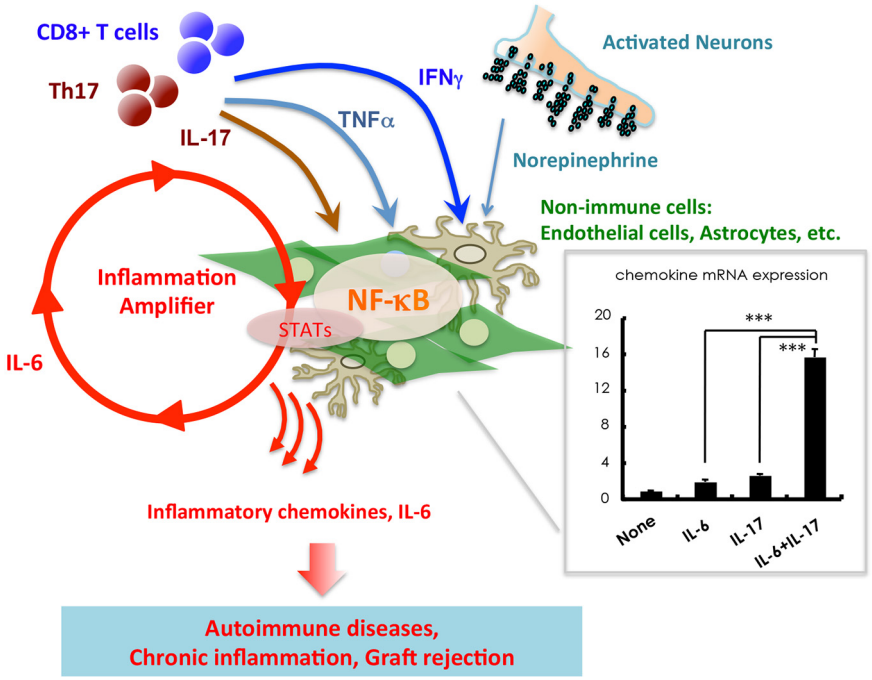

FIGURE 1 | Stimulation of non-immune cells including endothelial cells and astrocytes with IL-17, TNFα, IFNγ, and IL-6 from T cells induces a synergistic effect on the production of inflammatory chemokines such as CCL20 and IL-6. An imaginary figure is shown. The synergistic effect requires the simultaneous activation of two transcription factors, NF-κB and STATs, in non-immune cells. Various soluble factors, including neurotransmitters from activated neurons, augment the inflammation amplifier by activating or sustaining the activation of NF-κB and STATs. Mean ± SD are shown. ***$p < 0.001$.

CD4 T cells responding to different neuroantigens and different priming protocols, it has been demonstrated that the effector lineage and activation status of CD4 T cells within the CNS influence the location of lesions within the CNS, the severity of the acute disease as well as the overall clinical outcome (Kawakami et al., 2004; Jäger et al., 2009; Pierson et al., 2012).

In both WT and Rag1$^{-/-}$ BG1 mice, spontaneous clinical symptoms begin as episodic bouts of functional impairment, with many mice displaying severe CNS dysfunction and then remitting to unobservable clinical symptoms. Rag1$^{-/-}$ BG1 mice, however, develop more severe bouts of disease, and have more relapses than WT BG1 mice, with the majority progressing to a chronic disease stage. The observed differences in the frequency and severity of spontaneous disease between WT BG1 and Rag1$^{-/-}$ BG1 mice suggests that GFAP-specific CD8 T cells are subject to extrinsic sources of immune regulation. To genetically map the lymphocytes that regulate GFAP-specific CD8 T cells, IA$^{b}\beta^{-/-}$ (MHC II-deficient) and μMT$^{-/-}$ (B cell-deficient) BG1 mice were generated. Spontaneous CNS disease in IA$^{b}\beta^{-/-}$ BG1 mice was similar in frequency and severity to WT BG1 mice. In contrast, μMT$^{-/-}$ BG1 mice were found to be highly susceptible to spontaneous CNS disease, with ∼80% of μMT$^{-/-}$ BG1 mice developing chronic clinical disease, a fundamentally distinct disease course as compared to the relapsing-remitting disease most often observed in WT BG1 and Rag$^{-/-}$ BG1 mice (Sasaki et al., 2014). Thus, GFAP-specific CD8 T cell-mediated spontaneous relapsing-remitting and chronic disease is associated with the infiltration of tissue resident memory-like CD8 T cells into the CNS parenchyma and is regulated by polyclonal B cells. How B cells regulate CD8 T cell CNS autoimmunity, inflammation and disease remission is currently unknown.

Does the Inflammation Amplifier Regulate Relapsing/Remitting CNS Disease?

In addition to immune cells, we have demonstrated that non-immune cells, including vascular endothelial cells and glial cells, play critical roles in the induction of chronic inflammatory diseases such as EAE. Glial cells of the CNS can secrete large quantities of chemokines, growth factors and IL-6 in response to inflammatory stimuli, all of which can activate the NF-κB and STAT signaling pathways (Ogura et al., 2008; Atsumi et al., 2014). This induction of inflammation, mediated by IL-17, TNFα, IFNγ, IL-6 or various neurotransmitters, is synergistically enhanced when both the NF-κB and STAT signaling pathways are induced in glial cells. We termed this synergistic effect the inflammation amplifier (Atsumi et al., 2014). Importantly, clinical symptoms of EAE, and several additional chronic inflammatory diseases, are significantly improved in mice unable to activate the inflammation amplifier (Ogura et al., 2008; Arima et al., 2012; Lee et al., 2012; Murakami et al., 2013; Harada et al., 2015). These findings indicate that the inflammation amplifier has a central role in chronic inflammatory diseases.

The inflammation amplifier is regulated by the production of several neurotransmitters, including norepinephrine and ATP. These findings led us to hypothesize that the inflammation amplifier may link the onset and severity of CNS diseases to mental and physical stress. Indeed, regional neural activity created by gravity of the Earth on the soleus muscles enhances chemokine expressions within the CNS, resulting in inflammation occurring around the dorsal vessels of the fifth lumbar cord, during early stages of EAE. CNS inflammation and the upregulation of chemokine expression can similarly be induced artificially using electric stimulation of peripheral muscles, formally demonstrating that neuronal activity can regulate the inflammation amplifier *in vivo* (Arima et al., 2012). These phenomena have been termed the "gateway reflex" as these neural stimulations can create "gateways" for immune cells to enter into the CNS (Kamimura et al., 2013; Sabharwal et al., 2014).

Future Studies

The inflammation amplifier can be turned on or off in response to acute inflammation, as well as to mental and physical stress. Thus, the temporal regulation of NF-κB and STAT signaling pathways in glial cells may regulate episodic cycles of relapsing/remitting clinical disease in MS patients. Mechanistically, one way this may occur is by recruiting or limiting immune cell migration through the "gateway" present within the spinal cord and potentially through other sites within the brain. Clarifying these mechanisms, and identifying how different immune cell lineages and subsets respond to and regulate the inflammation amplifier, will provide insights into the pathogenesis of relapsing/remitting CNS diseases, and identify drug-targetable molecular pathways that can be exploited to minimize MS disease relapses.

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REVIEW
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Are Microglial Cells the Regulators of Lymphocyte Responses in the CNS?

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The infiltration of immune cells in the central nervous system is a common hallmark in different neuroinflammatory conditions. Accumulating evidence indicates that resident glial cells can establish a cross-talk with infiltrated immune cells, including T-cells, regulating their recruitment, activation and function within the CNS. Although the healthy CNS has been thought to be devoid of professional dendritic cells (DCs), numerous studies have reported the presence of a population of DCs in specific locations such as the meninges, choroid plexuses and the perivascular space. Moreover, the infiltration of DC precursors during neuroinflammatory situations has been proposed, suggesting a putative role of these cells in the regulation of lymphocyte activity within the CNS. On the other hand, under specific circumstances, microglial cells are able to acquire a phenotype of DC expressing a wide range of molecules that equip these cells with all the necessary machinery for communication with T-cells. In this review, we summarize the current knowledge on the expression of molecules involved in the cross-talk with T-cells in both microglial cells and DCs and discuss the potential contribution of each of these cell populations on the control of lymphocyte function within the CNS.

Keywords: antigen presentation, lymphocyte, dendritic cells, co-stimulatory signals, MHCs, B7, purine nucleotides, CD39

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INTRODUCTION

The central nervous system (CNS) has been considered for many years as an organ immunologically isolated from the peripheral immune system, on one hand due to the presence of the blood brain barrier (BBB) and the absence of lymphatic vessels (Perry, 1998) and, on the other hand, by the fact that skin grafts and the direct inoculation of viruses, bacteria or antigens in the nervous parenchyma did not induce an immune response (Medawar, 1948; Barker and Billingham, 1977; Stevenson et al., 1997; Matyszak and Perry, 1998). Nevertheless, in the last decade, an increasing number of studies has demonstrated that the CNS is not only immune-competent, but it also actively interacts with cells of the peripheral immune system (Aloisi et al., 2000; Becher et al., 2000; Steinman, 2004; Almolda et al., 2011b; Gonzalez et al., 2014), which can be recruited to the nervous parenchyma under specific circumstances (Ransohoff et al., 2003; Engelhardt and Ransohoff, 2005; Becher et al., 2006; Engelhardt, 2006, 2008).

With all of these studies in mind, it is easy to think that the isolated view of the CNS has drastically changed toward a more active scenario, in which a situation of active immune tolerance is continuously maintained within the CNS. Different mechanisms have been reported to contribute to this active tolerance, including the constitutive expression of FasL, a receptor involved in the death of infiltrated immune cells (Bechmann et al., 1999; Flugel et al., 2000) and the local production of anti-inflammatory mediators such as indolamine 2,3-dioxygenase,

in response to the interaction with pro-inflammatory lymphocytes (Kwidzinski et al., 2005). The presence of some populations of cells, such as macrophages and dendritic cells (DCs), located in strategic areas of the CNS such as the meninges and the choroid plexus, may play a key function in the initiation and regulation of immune responses. Nowadays, then, the CNS is considered as an immune-privileged site, rather than immune-isolated (Ousman and Kubes, 2012; Ransohoff and Engelhardt, 2012).

INFILTRATION OF LYMPHOCYTES IN THE CNS UNDER PATHOLOGICAL SITUATIONS

The infiltration of lymphocytes within the CNS parenchyma is a common hallmark in many pathological conditions (Rezai-Zadeh et al., 2009; Anderson et al., 2014) such as VIH (Petito et al., 2003) and West Nile virus infection (Glass et al., 2005); neurodegenerative diseases such as Parkinson's disease (Brochard et al., 2009) and amyotrophic lateral sclerosis (Holmoy, 2008); acute lesions like facial nerve axotomy (Raivich et al., 1998), entorhinal cortex lesion (Babcock et al., 2008), stroke (Schroeter et al., 1994; Gelderblom et al., 2009) and ischemia (Gelderblom et al., 2009) or autoimmune processes such as experimental autoimmune encephalomyelitis (Dittel, 2008; Almolda et al., 2011a). While in some circumstances lymphocyte infiltration has been related to protective functions, as occurs in the facial nerve axotomy paradigm (Serpe et al., 1999), the West Nile virus infection (Glass et al., 2005) and amyotrophic lateral sclerosis (Beers et al., 2008; Chiu et al., 2008), in other circumstances lymphocyte infiltration has been shown to contribute to the exacerbation of the pathology. This is the case of Parkinson's disease (Brochard et al., 2009), VIH virus infection (Petito et al., 2003), stroke (Yilmaz et al., 2006) and some autoimmune diseases (Dittel, 2008).

Due to the fact that T-cells are not able to recognize soluble antigens, they need the help of specialized cells, the so-called antigen presenting cells (APCs), which through antigen presentation mechanisms can capture, process and present pathogen and viral antigens and other strange structures for recognition by T-cells. Depending on the pattern of cytokine secretion, the functions and the molecules that drive their differentiation, different subtypes of T-helper lymphocytes are identified (Reinhardt et al., 2006; Takatori et al., 2008; Sun and Zhang, 2014). Classical classification considers two different subtypes: T-helper 1 (Th1) lymphocytes, which secrete pro-inflammatory cytokines such as interferon-γ (IFN-γ) or tumoral necrosis factor-α (TNF-α) and Th2 lymphocytes, which produce anti-inflammatory cytokines such as interleukin-4 (IL-4) and interleukin-10 (IL-10). Therefore, Th1 accumulation has been usually considered as an inflammatory event, whereas presence of Th2 has been related to the down-regulation of the inflammatory response. However, a growing accumulation of evidence has changed this simple paradigm based on the presence/absence of Th1/Th2, as other subpopulations of Th cells have been discovered, among them, effector T-cells including Th17, Th22, Th9, T-follicular helper (Tfh) cells with the capacity to secrete different cytokines (Cosmi et al., 2014), but also regulatory T-cells such as T-regulatory (Treg) and Tr1, whose principal function is to maintain the immune system homeostasis and the tolerance to self-antigens (Bluestone and Tang, 2005; Eltzschig et al., 2012; Piccioni et al., 2014). Two different subtypes of Treg are currently identified: the natural Treg (nTreg) and the induced Treg (iTreg) (Horwitz et al., 2008; Curotto de Lafaille and Lafaille, 2009; Piccioni et al., 2014). The nTregs, defined as CD4+CD25+Foxp3+ cells, are generated in the thymus during the maturation of T-cells by recognition of self-peptides with intermediate affinity, whereas the iTregs are produced in secondary lymphoid organs (spleen and lymph nodes) from naïve CD4+Foxp3- T-cells under both homeostatic conditions and in the presence of inflammation, infection or allergy after stimulation with TGF-β (Piccioni et al., 2014). Due to their capacity to suppress immune responses, the participation of Tregs in the evolution of acquired immune responses in the CNS, especially those related to autoimmunity, has generated much attention in the last several years. In this sense a remarkable accumulation of Tregs in cerebral gliomas (Grauer et al., 2007), ischemic stroke (Stubbe et al., 2012) and in some experimental models of encephalomyelitis such as EAE (McGeachy et al., 2005; Kohm et al., 2006; Korn et al., 2007) has been reported.

The discovery of all of these subtypes of lymphocytes with putative new functions in the promotion and modulation of the acquired immune response and their still-unknown interactions with resident CNS cells, specially microglia, has contributed to becoming aware that the scenario of the neuroimmune response could be even more complicated than previously thought.

ACTIVATED MICROGLIA ARE CONSIDERED THE MAIN APC IN THE CNS

Microglial cells are considered the sole representative of the immune system within the CNS parenchyma. The precise origin of microglia during development still remains under debate, although emerging evidence reported that yolk-salk primitive precursors are the principal source (Ginhoux et al., 2010, 2013; Schulz et al., 2012). Studies in bone-marrow chimera and parabiotic mice indicated that these yolk-salk precursors invade the CNS parenchyma through the blood vessels around embryonic Day 9 in mice, corresponding to the vascularization process, and contribute substantially to the maintenance of microglial cells in the adult (Ginhoux et al., 2010). However, alternative routes of entry for microglial precursors, including the ventricles and meninges, have been identified (Cuadros and Navascues, 1998; Dalmau et al., 1998, 2003; Navascues et al., 2000). Whether these different routes of entry are linked to different populations of microglial precursors with different functions is an interesting field that is still unsolved.

Microglial cells are equipped with a broad range of receptors in their plasma membrane that allows them to sense subtle changes in the micro-environment (Kettenmann et al., 2011;

Hanisch, 2013; Kierdorf and Prinz, 2013). Microglial cells play very important roles in healthy, normal CNS, not only during the post-natal period, where they contribute to the elimination of synaptic structures (Pont-Lezica et al., 2011; Tremblay et al., 2011; Harry and Kraft, 2012), but also in the adult, where they are continuously scanning their local micro-environment (Davalos et al., 2005; Nimmerjahn et al., 2005; Kierdorf and Prinz, 2013; Castellano et al., 2015). When the homeostasis of the CNS is perturbed as a result of injury or disease, microglial cells become rapidly activated, acquiring a specific phenotype totally dependent on the environment in which they are activated and the specific stimulus that drives their activation (Kettenmann et al., 2011; Gonzalez et al., 2014; Chen and Trapp, 2015). Activated microglia can rapidly proliferate and increase the expression or *de novo* express a multitude of different molecules and secrete a plethora of substances such as cytokines, chemokines and trophic factors, all of which make them able to modulate both the innate and the acquired immune responses within the CNS (Ransohoff and Cardona, 2010; Kettenmann et al., 2011; Eggen et al., 2013; Goldmann and Prinz, 2013; Casano and Peri, 2015).

Recognition of the T-cell receptor (TCR) on the surface of T-lymphocytes by the major histocompatibility complexes (MHCs) located on the surface of the APCs, MHC-I in the case of CD8+T-cytotoxic lymphocytes and MHC-II for CD4+T-helper cells, constitutes the first signal of the antigen-presenting mechanism related to the activation of T-cells (Lanzavecchia, 1997; Abbas et al., 2010). Co-stimulation, the second signal involved in this mechanism, is based on the binding of diverse receptors and counter-receptors expressed on the surface of both APC and T-cells (Nurieva et al., 2009) and is essential for a complete antigen presentation, as expression of MHCs in the absence of co-stimulation leads to the apoptosis or anergy of T-cells (Kishimoto and Sprent, 1999). A multitude of co-stimulatory pairs of molecules, which can be classified into two main families (the B7/CD28 and the TNFR families), have been reported in the immune system, exerting different effects on the activation/deactivation of T-cells (Sharpe, 2009) and driving the final outcome and function of T-cells.

Expression of MHCs in Microglia

Resident glial cells, principally microglia, can establish a cross-talk with infiltrated T-cells regulating their recruitment, activation and function within the CNS (Gonzalez et al., 2014). Although in healthy CNS microglial cells do not express MHCs (Kreutzberg, 1996; Perry, 1998), it is well known that, when activated in pathological conditions, they showed a wide number of phenotypic changes (Ransohoff and Cardona, 2010; Kettenmann et al., 2011; Prinz et al., 2014), including *de novo* expression of these molecules (Kreutzberg, 1996; Perry, 1998). Therefore, many authors consider microglial cells as the principal APC within the CNS parenchyma (Aloisi, 2001; Carson, 2002; Raivich and Banati, 2004; Graeber and Streit, 2010). Expression of MHC-II in activated microglia *in vivo* has been reported after a wide variety of CNS injuries including LPS injection (Xu and Ling, 1995; Ng and Ling, 1997), ischemia and kainic acid injection (Finsen et al., 1993), graft *vs.* host disease (Sedgwick et al., 1998), facial nerve axotomy (Streit et al., 1989; Villacampa et al., 2015), entorhinal cortex lesion (Bechmann et al., 2001; Kwidzinski et al., 2003a) and different models of EAE (Almolda et al., 2010).

Expression of Co-stimulatory Molecules in Microglia

While the expression of MHCs has been extensively reported in activated microglia, only a limited number of studies have addressed the question of whether activated MHC-II+ microglia simultaneously express co-stimulatory molecules (Summarized in **Table 1**).

The B7/CD28 Family

The pair of co-stimulatory molecules with the major relevance in the activation of T-cells, and therefore the most extensively studied in the organism, is that formed by receptors B7.1/B7.2 (CD80/CD86) on the surface of APCs and their counter-receptors CD28 and CTLA-4 on the surface of T-cells. The binding of B7.1 or B7.2 to CD28 induces T-cell proliferation and cytokine secretion, whereas binding of these same receptors to CTLA-4 induces the inhibition of T-cell activity, promoting the down-regulation of the immune response (Sansom, 2000; Sharpe and Freeman, 2002). Specifically in the CNS, *de novo* expression of B7.1 and/or B7.2 has been reported in microglial cells after entorhinal cortex lesion (Bechmann et al., 2001; Kwidzinski et al., 2003b), peripheral nerve injury (Rutkowski et al., 2004), facial nerve axotomy (Bohatschek et al., 2004), cuprizone-induced demyelination (Remington et al., 2007) and models of autoimmunity such as EAE and Theiler's virus encephalomyelitis (Issazadeh et al., 1998; Juedes and Ruddle, 2001; Mack et al., 2003; Raivich and Banati, 2004; Almolda et al., 2010, 2011b).

Recently, other members of the B7 co-stimulatory molecules family have been described in the immune system, including B7-H2 (ICOS-L), B7-H1 (PD-L1), B7-DC (PD-L2), B7H3 (CD276), B7H4, B7S3 and BTNL (Sharpe, 2009; Chen and Flies, 2013). The ICOS-ICOSL pathway has important roles in the fine-tuning of effector T-cell functions and the control of T-cell tolerance (Nurieva et al., 2009). Although the presence of ICOS+ T-cells has been reported in the CNS of EAE-induced mice (Rottman et al., 2001), to-date, no studies on the expression of its ICOSL ligand on microglia or any other CNS resident cells are available. PD-1 is another receptor gaining attention, due to its crucial role in maintaining peripheral immune tolerance (Nurieva et al., 2009). PD-1 has been shown to be a negative regulator of T-cell responses, expressed at low levels on the surface of T, B and natural killer T-cells, and further induced upon activation. PD-1 has two counter-receptors that are expressed on the surface of APCs, PD-L1 and PD-L2 also called B7H1 and B7DC, respectively (Nurieva et al., 2009). The few reports addressing the expression of this molecule in the CNS demonstrated PD-L1 expression in both activated microglia after middle-cerebral artery occlusion (Ren et al., 2011; Bodhankar et al., 2013), coronavirus infection (Phares et al., 2009), Theiler's murine encephalomyelitis (Duncan and Miller, 2011; Jin et al., 2013) and EAE (Schreiner et al., 2008),

TABLE 1 | Principal co-stimulatory molecules from the B7/CD28 and TNFR family.

	Effect on T-cell	T-cell	APC	Determined in microglia	Experimental model	Reference
B7/CD28 family	Stimulation	CD28	B7.1/B7.2	+	PPT	Bechmann et al., 2001 Kwidzinski et al., 2003b
					Peripheral nerve injury	Rutkowski et al., 2004
					FNA	Bohatschek et al., 2004
					Cuprizone	Remington et al., 2007
	Inhibition	CTLA-4			EAE models	Issazadeh et al., 1998 Juedes and Ruddle, 2001 Mack et al., 2003 Raivich and Banati, 2004 Almolda et al., 2010 Almolda et al., 2011b
	Stimulation	ICOS	B7H2 (ICOS-L)	n.d.	–	–
	Inhibition	PD-1	B7H1 (PD-L1)	+	MCAO	Ren et al., 2011 Bodhankar et al., 2013
					Coronavirus infection	Phares et al., 2009
					TMEV	Duncan and Miller, 2011 Jin et al., 2013
					EAE models	Schreiner et al., 2008
					PPT	Lipp et al., 2007
			B7DC (PD-L2)	n.d.	–	–
	Inhibition	TLT-2	B7H3	n.d.	–	–
	Inhibition	Unknown	B7H4	n.d.	–	–
	Inhibition	Unknown	B7S3	n.d.	–	–
	Inhibition	Unknown	BTNL	n.d.	–	–
TNFR family	Stimulation	CD40-L	CD40	+	Microglial cultures	Tan et al., 1999 Qin et al., 2005 Lin et al., 2009 Lin and Levison, 2009 Vidyadaran et al., 2009
					Ageing	Griffin et al., 2006 Simpson et al., 2007
					Epilepsy	Sun et al., 2008
					Alzheimer's disease	Togo et al., 2000 Town et al., 2001 Tan et al., 2002a
					ALS	Okuno et al., 2004
					Neurodegeneration	Ke et al., 2005
					HIV infection	D'Aversa et al., 2002, 2005
					EAE models	Becher et al., 2001; Ponomarev et al., 2006
					TMEV	Olson et al., 2001
					MS	Vogel et al., 2013
	Stimulation	OX40	OX40-L	n.d.	–	–
	Stimulation	CD27	CD70	n.d.	–	–

The table summarized the different molecules studied in the context of microglial cells (+). n.d indicates that the expression has not been determined specifically in microglia. PPT, perforant pathway transection; FNA, facial nerve axotomy; EAE, experimental autoimmune encephalomyelitis; MCAO, middle cerebral artery occlusion; TMEV, Theiler's induced encephalitis; ALS, amyotrophic lateral sclerosis; MS, multiple sclerosis.

and in astrocytes after entorhinal cortex lesion (Lipp et al., 2007). Moreover, the blockade of PD-1 signaling enhances EAE severity (Salama et al., 2003) suggesting an outstanding role in the control of CNS pathologies.

To our knowledge, no studies regarding the expression of B7H3, B7H4, B7S3 or BTNL specifically in microglia are, until present, available in the literature.

The TNFR Family

Additionally, a second family of co-stimulatory receptors, the TNFR family, has been reported in the immune system. Various members, including pairs CD40/CD40L, OX40L/OX40, and CD70/CD27, expressed on APCs and T-cells, respectively, form this family (Watts, 2005; Sharpe, 2009). Among them, CD40 is the only molecule studied in the context of microglial activation (Chen et al., 2006). CD40 expression in activated microglia has been described not only *in vitro* in many cell-lines activated with IFN-γ, LPS or β-amyloid protein (Tan et al., 1999; Qin et al., 2005; Lin and Levison, 2009; Lin et al., 2009; Vidyadaran et al., 2009) but also *in vivo* during physiological aging (Griffin et al., 2006; Simpson et al., 2007) and under pathological situations such as epilepsy (Sun et al., 2008), Alzheimer's disease (Togo et al., 2000; Town et al., 2001; Tan et al., 2002b), amyotrophic lateral sclerosis (Okuno et al., 2004), neurodegeneration induced by thiamine deficiency (Ke et al., 2005), human HIV (D'Aversa et al., 2002, 2005), different animal models of autoimmunity such as EAE (Becher et al., 2001; Ponomarev et al., 2006) and Theiler's murine encephalomyelitis (Olson et al., 2001) and MS (Vogel et al., 2013). Moreover, inhibition of CD40 in microglia results in the attenuation of β-amyloid pathology (Tan et al., 2002a) and the reduction of EAE severity (Becher et al., 2001; Ponomarev et al., 2006), pointing toward this molecule as a good candidate for therapeutic interventions in these specific CNS pathologies.

Altogether, these studies indicate that, although so far it seems that microglial cells may be the principal APC within the CNS, in the coming years it will be necessary to inquire about the expression of some other markers related to the antigen-presenting mechanism described in professional DCs and, until now, not explored in the context of microglial activation.

OTHER MOLECULES EXPRESSED BY MICROGLIA THAT CAN BE INVOLVED IN THE COMMUNICATION WITH T-CELLS

Recent studies indicate that CD39 and CD73, some of the molecules that mediate the immunosuppressive activity of Treg lymphocytes (Deaglio et al., 2007), are also expressed in specific subtypes of APCs and may be involved in the suppressive activity of these cells. Specifically, a subtype of DCs induced by IL27 has been shown to increase expression of CD39 and exert protective functions in EAE (Mascanfroni et al., 2013). CD39 and CD73 (also known as NDPase and 5′ nucleotidase, respectively) are enzymes involved in the hydrolysis of extracellular ATP to ADP/AMP and to adenosine. CD39-deficiency in DCs has been shown to ameliorate the course of EAE by reducing the number of Th1 and Th17 effector cells (Mascanfroni et al., 2013). The precise mechanism by which CD39 regulates T-cell responses is not clear, although it is proposed to be mediated by a reduction in the ATP levels producing a down-regulation of the inflammasome activity (Eltzschig et al., 2012), a multiprotein-assembled complex involved in the initiation of the immune innate responses (Vanaja et al., 2015).

Expression of both CD39 and CD73 in the membrane of microglial cells has been extensively reported to regulate ATP levels within the CNS, in both healthy situations and after damage (Castellano et al., 2015). Therefore, it is easy to suggest that regulation of the expression of those enzymes in activated microglia take part in modulating the final outcome of infiltrated T-cells.

PRESENCE OF DENDRITIC CELLS IN THE CNS

Dendritic cells are considered to be the professional APCs in the immune system (Guermonprez et al., 2002). They are derived from hematopoietic stem cells in the bone marrow that gives rise to early precursors called the Common Myeloid Precursor (CMP). CMPs, in turn, originate the formation of two different precursors, the Granulocyte/Monocyte precursors (G/Ms) and the Macrophage/DC precursors (M/DPs). From M/DPs, the common DC progenitors, the pre-DC precursors and the plasmacytoid DCs are sequentially formed. Pre-DC precursors egress into the blood circulation and populate different organs, including the skin, heart, lung and spleen, becoming conventional DCs (Liu and Nussenzweig, 2010). As both DCs and macrophages derived from the same precursors most of the markers and functions of these two populations are similar.

Although the parenchyma of the normal CNS are devoid of the so-called professional DCs, these cells are abundant in the meninges, the choroid plexus (McMenamin, 1999; McMenamin et al., 2003), the perivascular space and the juxtavascular parenchyma (i.e., the neuropil just beyond the glia limitants) (Prodinger et al., 2010). These locations are considered strategically well-positioned for the communication with blood-circulating pathogens or T-cells, supporting a role of DCs in the control of the entry gates to the brain and thus in the regulation of immune surveillance in the CNS during homeostasis. With aging, the number of DCs increases markedly in the perivascular space, meninges and choroid plexuses, and has even been found into the brain parenchyma (Stichel and Luebbert, 2007; Kaunzner et al., 2010). The presence of CNS parenchymal DCs has also been reported in different neuroinflammatory situations (McMahon et al., 2006; Colton, 2012; D'Agostino et al., 2012), including infections (Fischer and Reichmann, 2001), traumatic brain injury (Israelsson et al., 2010), ischemia (Kostulas et al., 2002; Reichmann et al., 2002; Felger et al., 2009; Gelderblom et al., 2009), excitotoxicity (Newman et al., 2005) and some diseases such as amyotrophic lateral sclerosis (Henkel et al., 2004), multiple sclerosis (Plumb et al., 2003; Serafini et al., 2006)

and EAE (Matyszak and Perry, 1996; Serafini et al., 2000; Fischer and Reichmann, 2001; Santambrogio et al., 2001; Santambrogio and Strominger, 2006; Almolda et al., 2010, 2011b).

FUNCTION OF DCS IN THE CNS

Numerous works (McMahon et al., 2006; Colton, 2012; D'Agostino et al., 2012) emphasize the possible relevance of DCs in the CNS immunosurveillance as well as the function they can play in neuroinflammatory situations. However, the specific contribution of those cells is still not well-understood.

The actual knowledge regarding the function of DCs in the brain come from studies using the inoculation of different types of DCs into the CNS under different circumstances. Thus, it has been shown that subcutaneous administration of bone marrow DCs before EAE-induction prevents EAE development in rats (Huang et al., 2000). Other studies reported that intraparenchymal inoculation of tolerogenic DCs, induced by TNF-α treatment, prevents or delays EAE onset, whereas immunogenic DCs administration increases the severity of this disease (Zozulya et al., 2009).

All together, these studies have demonstrated the potential of DCs to serve as potent vehicles to induce tolerance and open a door to new therapeutic strategies to modulate CNS disease. A question not yet addressed in this kind of studies is how these DCs interact with both glial cells and blood–borne infiltrated cells. Research in this field in the coming years is vital to understand the molecular and cellular mechanisms involved in the regulation of immune responses in the CNS.

ARE CNS PARENCHYMAL DCS AUTHENTIC DCS OR ARE THEY A SUBTYPE OF ACTIVATED MICROGLIA?

In addition to the poor knowledge on the role of DCs in the immune responses within the CNS, one of the issues that generate more controversy is the origin of parenchymal DCs observed in a wide range of neuroinflammatory situations

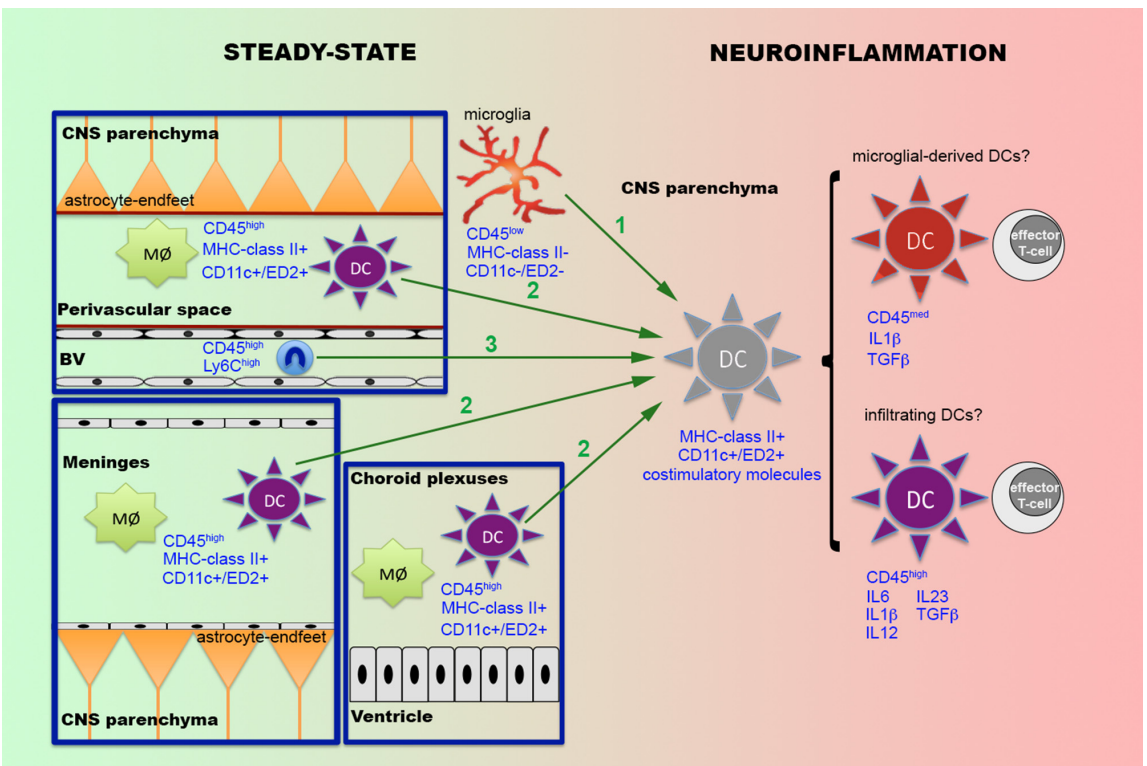

FIGURE 1 | Putative origins of parenchymal dendritic cells during neuroinflammatory conditions. In the CNS, under steady-state conditions a population of professional DCs expressing MHC-class II and CD11c or ED2 are found in the meninges, the choroid plexuses and the perivascular space, where they coexist with specific subpopulations of resident macrophages (Mø). Under specific neuroinflammatory conditions, such as infections, traumatic brain injury or EAE, DCs have been also reported within the CNS parenchyma. Different possibilities are suggested to explain the origin of these parenchymal DCs (green arrows). The first possibility (1) is that parenchymal DCs are derived from activated microglia. The second possibility (2) is that parenchymal DCs come from the recruitment of either perivascular or meningeal or both DC populations. The third possibility (3) is that DCs come from infiltrated monocytes (Ly6C^{high}). Recent research indicates that parenchymal DCs are constituted by two different populations of cells, one becoming from microglia and the other infiltrated from the periphery. Although both populations of parenchymal DCs present the ability to activate T-cells, the fact that they display a distinct phenotype, characterized principally by changes in the levels of CD45 and the pattern of cytokine secretion, suggest that they may play different roles in the regulation of the immune response.

(**Figure 1**). One possibility suggested by some authors is that the perivascular or meningeal DCs observed in the healthy brain are recruited to inflammatory sites within the CNS parenchyma (McMahon et al., 2006). Alternatively, other authors supported the idea that parenchymal DCs observed during neuroinflammatory conditions come from infiltrated monocytes (Ifergan et al., 2008), which under the influence of specific molecules such as GM-CSF, differentiate to DCs (Ashhurst et al., 2014). Supporting this idea, an alternative developmental circuit occurring after the MDP precursors involves monocytes as precursors of inflammatory DCs in peripheral organs (Dominguez and Ardavin, 2010; Liu and Nussenzweig, 2010). Infiltration of monocytes is a common event in many of the above-mentioned neuroinflammatory situations in which DCs have been described in the CNS parenchyma (Zhu et al., 2007; Serbina et al., 2008; Mildner et al., 2009). Furthermore, systemic administration of GM-CSF in EAE-induced mice mobilizes Ly6C^{high}-circulating monocytes that migrate to the CNS parenchyma and are converted into DCs (King et al., 2009). Nevertheless, later studies have demonstrated that intraparenchymal infusion of GM-CSF not only promotes the apparition of DC precursors recruited from the periphery but also induces the emergence of a second population of DCs derived from the CNS with an inhibitory phenotype (Hesske et al., 2010), supporting the idea that DCs not only immigrate from the periphery but may also be derived from local CNS cells.

In this regard, several lines of evidence, including *in vitro* studies (Fischer and Reichmann, 2001; Butovsky et al., 2007) and neuroinflammatory situations such as ischemia (Kostulas et al., 2002) and EAE (Fischer and Reichmann, 2001; Almolda et al., 2011b; Wlodarczyk et al., 2014), support the hypothesis that parenchymal DCs are derived from the differentiation of local cells, probably microglia, on the basis that the expression of some of the surface antigens commonly used for the identification of DCs, such as CD11c, MHCII and CD86, are found in activated microglial cells. In addition, a study using the CD11c-GFP mouse, which expresses the GFP protein under the control of the CD11c promoter, the pan-marker of DCs, has reported the presence of CD11c+ cells not only in the choroid plexuses and perivascular space but also in the juxtavascular parenchyma of non-lesioned CNS (Prodinger et al., 2010). Interestingly, these authors found that almost all CD11c+ cells in the juxtavascular parenchyma presented markers of microglial cells such as Iba1 and CD11b, indicating that, presumably, a subpopulation of microglial cells is able to express DC markers in steady-state conditions. Even more, an interesting study (Anandasabapathy et al., 2011) using the Flt3-treatment, a transcription factor involved in the generation of DCs (Waskow et al., 2008; Kingston et al., 2009), to induce the expansion of DCs in transgenic mice carrying the EYFP fluorescent protein under the control of the CD11c promoter, demonstrated the presence of two different populations of CD11c+ cells within healthy CNS. These two populations corresponded to a population of EYFP+ cells located in the choroid plexuses and meninges whose number increased after Flt3 treatment and another discrete population of EYFP+ cells located in CNS parenchyma with ramified morphology whose number remains stable after the treatment. Flow cytometry studies of these two populations demonstrated that the EYFP+ cells in the choroid plexuses and meninges presented a profile of CD45^{high}/MHCII+ DCs, whereas those EYFP+ cells in the parenchyma corresponds to CD45^{int}/MHCII- microglial cells (Anandasabapathy et al., 2011). Furthermore, other works (Wlodarczyk et al., 2014) using flow cytometry for different DCs markers have reported the existence of two populations of DCs in EAE-induced animals *in vivo*: CD11c+ DCs and CD11c+ microglia. Interestingly, both populations showed a similar ability to induce T-cell proliferation *in vitro* but, once activated, those T-cells showed a different cytokine profile, suggesting that both populations can play different functions in T-cell activation (Wlodarczyk et al., 2014).

Altogether, these studies indicate, as previously suggested by other authors (Ghosh, 2010), that in addition to professional DCs located in meninges, choroid plexuses and the perivascular space, there is a population of microglial cells that, according to environmental cues, can acquire the phenotype of DCs and consequently may act as professional APCs. One issue to be resolved is if these parenchymal DCs that come from microglia develop the same functions as other DCs or, conversely, if both populations in the CNS have different roles regulating the immune response.

CONCLUDING REMARKS

Current research suggests that the net effect of the acquired immune response within the CNS must depend not only on the number of lymphocytes and APCs, but must also be directly related to the specific subtype of infiltrated lymphocytes, the particular phenotype of the APC in each situation and the specific micro-environment in which the communication between these two cells takes place. Whether the principal intercomunicators in the cross talk with T-cells are microglial cells, professional DCs or both is an intriguing question, still under discussion, and should be subject to thorough investigation. Research to help clarify the question of the origin and a more complete characterization of the phenotype and function of parenchymal DCs in CNS will offer a more comprehensive understanding of the role played by these cells during the evolution of neuroinflammatory processes.

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MINI REVIEW
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The established and emerging roles of astrocytes and microglia in amyotrophic lateral sclerosis and frontotemporal dementia

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Amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) are two progressive, fatal neurodegenerative syndromes with considerable clinical, genetic and pathological overlap. Clinical symptoms of FTD can be seen in ALS patients and *vice versa*. Recent genetic discoveries conclusively link the two diseases, and several common molecular players have been identified (*TDP-43, FUS, C9ORF72*). The definitive etiologies of ALS and FTD are currently unknown and both disorders lack a cure. Glia, specifically astrocytes and microglia are heavily implicated in the onset and progression of neurodegeneration witnessed in ALS and FTD. In this review, we summarize the current understanding of the role of microglia and astrocytes involved in ALS and FTD, highlighting their recent implications in neuroinflammation, alterations in waste clearance involving phagocytosis and the newly described glymphatic system, and vascular abnormalities. Elucidating the precise mechanisms of how astrocytes and microglia are involved in ALS and FTD will be crucial in characterizing these two disorders and may represent more effective interventions for disease progression and treatment options in the future.

Keywords: amyotrophic lateral sclerosis, frontotemporal dementia, astrocyte, microglia, neuroinflammation, phagocytosis, glymphatic, vasculature

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INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a fatal and rapidly progressing multisystem neurodegenerative syndrome, characterized by the degeneration of the motor neurons (MNs) in the motor cortex, brainstem and spinal cord (Hardiman et al., 2011). Symptoms present first as focal upper and/or lower MN dysfunction of a skeletal muscle group which progressively deteriorates, ultimately spreading to other muscle groups (Ravits, 2014). Disease progression is rapid,

Abbreviations: ALS, Amyotrophic lateral sclerosis; Aβ, Amyloid beta; AQP4, Aquaporin 4; CNS, Central Nervous System; CSF, Cerebrospinal Fluid; FTD, Frontotemporal dementia; FUS, Fused in Sarcoma; GRN, Progranulin; iPSC, Inducible Pluripotent Stem Cell; MRI, Magnetic Resonance Imaging; MN, Motor Neuron; NF, κB, Nuclear Factor-kappa B; OPTN, Optineurin; PET, Positron Emission Tomography; PFN1, Profilin 1; RNAi, RNA interference; SOD1, Superoxide Dismutase 1; SPECT, Single Positron Emission Computed Tomography; SQTSM1, Sequestosome 1; Tau, Microtubule Associated Protein Tau; TBK1, Tank Binding Kinase 1; TREM2, Triggering Receptor Expressed on Myeloid cells 2; TDP, 43-TAR DNA-binding 43; VCP, Valosin-Containing Protein.

with 50% of patients dying due to respiratory complications within 3 years of symptom onset (Kiernan et al., 2011). Over the last 25 years, it has become increasingly apparent that ALS shares significant overlap with another progressive and fatal neurodegenerative syndrome Frontotemporal dementia (FTD). Up to 50% of patients with ALS develop FTD symptoms and approximately 15% of FTD patients display MN dysfunction typical of ALS (Ng et al., 2015). Besides this clinical connection, ALS and FTD also share significant genetic and pathological overlap (Bennion Callister and Pickering-Brown, 2014), represented in **Figure 1**. However, the causal mechanism/s of both syndromes are currently unknown and treatment is largely symptomatic (Hardiman et al., 2011; Piguet et al., 2011).

Five years ago, large hexanucleotide repeat expansions (∼100–1600 G_4C_2 repeats) of intronic regions of the *C9ORF72* gene were discovered in sporadic and familial forms of ALS and FTD (Renton et al., 2014). These studies provided seminal evidence for a direct molecular link between these two conditions. The repeat expansions are now recognized as the most common known mutation in both familial and sporadic ALS and FTD. Expansions have been identified in up to 40% and 25% of familial cases and ∼6% and 7% of sporadic or seemingly non-inherited forms of ALS and FTD respectively (Robberecht and Philips, 2013; Renton et al., 2014). Multiple other genes have also been linked to ALS and FTD. Mutations in the genes *TARDBP* and fused in sarcoma (*FUS*), which respectively encode for the proteins TDP-43 and FUS, are associated with ∼9% of familial, 2% of sporadic cases of ALS and rarely in FTD (Renton et al., 2014). The *MAPT* gene encodes microtubule associated protein tau (tau) and mutations have been identified in ∼2–11% of familial FTD cases (Sieben et al., 2012). *SOD1* is another gene that is strongly associated with ALS with mutations found in 12–20% of familial and 1–2% of sporadic cases (Al-Chalabi et al., 2012). With the exception of *MAPT* these genes are not segregated to neurons and are expressed by glia and various other cell types, which suggests a multicellular pathogenesis.

The presence of ubiquitinated, cytoplasmic inclusions in neurons and some glia is a pathological hallmark shared by the two disorders (Ng et al., 2015). **Figure 1B** shows the distribution of inclusion pathology seen in both ALS and FTD. In ∼95% of ALS and 50% of FTD cases, these inclusions are predominately comprised of TDP-43. FUS protein inclusions are found in ∼1% and 10% of ALS and FTD cases respectively (Mackenzie et al., 2010). Tau inclusion pathology is more characteristic of FTD (∼40% of cases) and is only found rarely in cases of ALS (Dickson et al., 2011; Ng et al., 2015). At the other end of the spectrum, SOD-1 inclusion pathology is seen in ∼2% of ALS cases and is incredibly rare in FTD with only one case reported (Bennion Callister and Pickering-Brown, 2014). Taken together, the pathogenic and genetic features represent a clear commonality between ALS and FTD, which are now believed to exist on a phenotypic continuum (Ling et al., 2013).

In addition to protein inclusions, another feature of ALS and FTD neuropathology is reactive gliosis, which is characterized by astrocytic hypertrophy and microglial proliferation (Al-Chalabi et al., 2012; Ng et al., 2015). Reactive gliosis is an indicator of neuroinflammation (Streit et al., 2004) and occurs in areas of neuronal loss and inclusion pathology in ALS and FTD (Brettschneider et al., 2012). Studies over the past 15 years have strongly indicated that ALS and FTD propagate via multiple cell types, with reactive gliosis being heavily implicated (Ilieva et al., 2009). Astrocytes and microglia in particular have been shown to be associated with disease progression and spreading (Philips and Robberecht, 2011).

Various pathways have been implicated to contribute to ALS and FTD neurodegeneration, including inflammation, RNA toxicity and altered splicing/expression (DNA/RNA homeostasis), and cytoskeletal, vascular and protein dysfunction (Lagier-Tourenne and Cleveland, 2009; Garbuzova-Davis et al., 2012; Ravits et al., 2013). Both microglia and astrocytes can be compromised through a variety of these signaling pathways that result in deregulated glia–motor neuron communication. However, the precise contribution of glial cells and their exact involvement in ALS and FTD pathology is currently under intense investigation. Here we aim to summarize the established and novel implications of astrocyte and microglia in ALS and FTD, identifying key aspects of the neuroinflammatory involvement, microglia phagocytosis, defective waste clearance and circulatory dysfunction.

THE PATHOGENIC ROLE OF NEUROINFLAMMATORY GLIA IN ALS

Inflammatory glia have been repeatedly reported in animal models of FTD (see Roberson, 2012). Yet, current models of tau pathology describe clinically a very heterogeneous group, including FTD and Parkinsonism, and our focus in this review will therefore be on the neuropathological characterization of inflammatory glia in ALS. Significant insight into the pathogenic role of glia in disease progression has been revealed through allografted chimeric and conditional knock out studies using ALS mutant SOD-1 rodent models (see Robberecht and Philips, 2013). These studies have convincingly shown that both astrocytes and microglia/myeloid progenitors significantly influence the progression of neurodegeneration in these models. More recent studies have added weight to the existing evidence that astrocytes contribute to ALS progression by utilizing mice xenografted with human glial progenitors generated from induced pluripotent stem cells (iPSCs). Grafted glial progenitors from patient iPSCs with familial *SOD1* mutations differentiated into astrocytes and induced MN degeneration and motor deficits in WT mice. Progenitors from healthy individuals without ALS linked mutations did not contribute to an ALS phenotype (Chen et al., 2015). In another study, iPSC derived glial progenitors from healthy individuals (lacking ALS mutations) formed astrocytes that increased the survival of MNs when transplanted at disease onset in a mutant SOD-1 mouse model (Kondo et al., 2014). Such *in vivo* studies provide important insight into the pathogenic role of ALS patient glia and demonstrate a

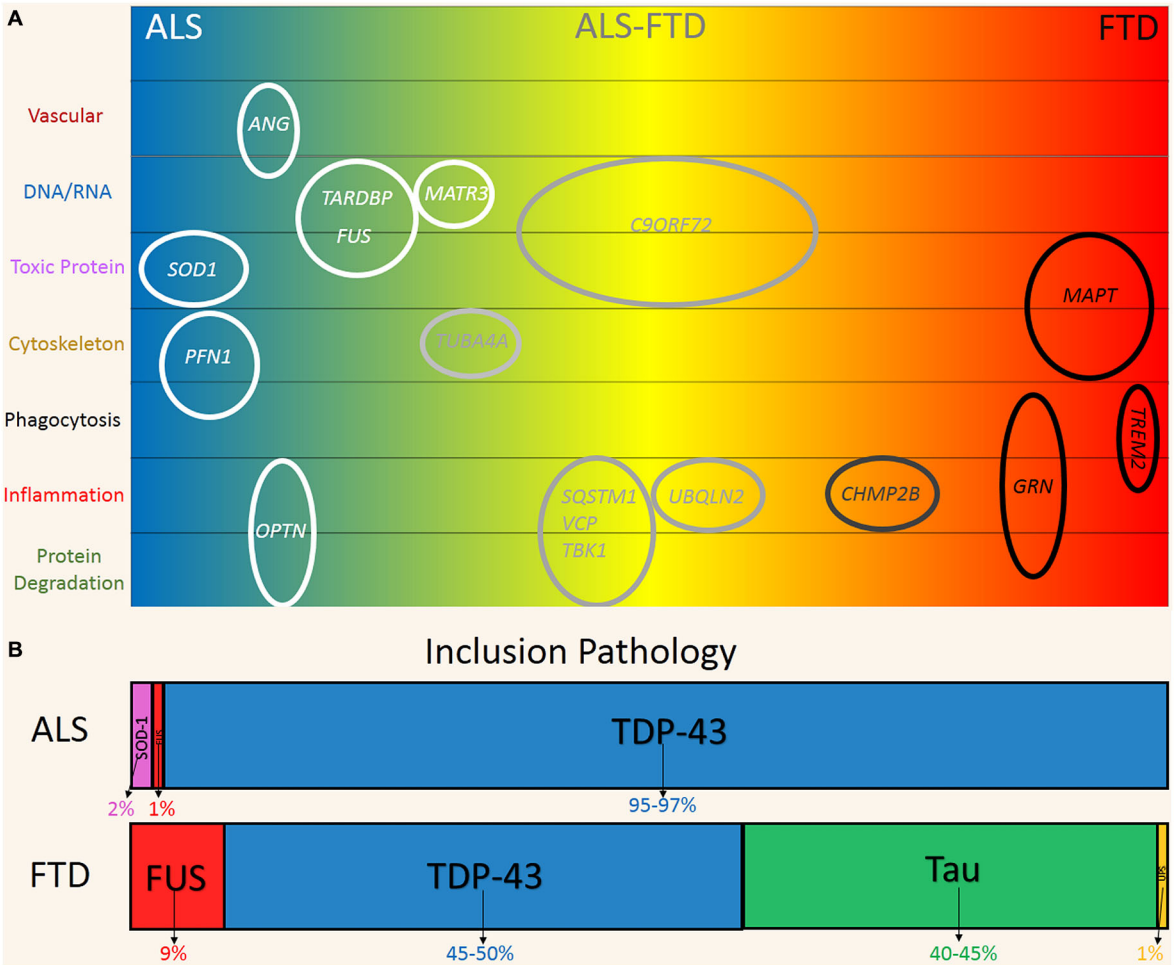

FIGURE 1 | Genetic and pathological overlap between amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD). (A) Familial and sporadic genetic mutations were linked to the clinical phenotypes on the ALS (blue) and FTD (red) spectrum. Genes are plotted according to their hypothesized mechanism in relation to disease (top to bottom). **(B)** Pathological protein inclusions are a hallmark of ALS and FTD, reflecting the significant overlap on the disease spectrum. FUS (Red) and TDP-43 (Blue) inclusions are found in both ALS and FTD. Predominate SOD1 (Pink) and Tau (Green) is more indicative of ALS and FTD respectively. FTD-UPS (Yellow) is found in ~1% of cases and represent cases of familial *CHMP2B* mutations.

potential mechanism of how glia can influence the progression of neurodegeneration (i.e., modifying the molecular phenotype and function).

Notably, astrocytes retrieved from post-mortem central nervous system (CNS) of familial (*SOD1* and unidentified) and sporadic cases were also neurotoxic to co-cultured MNs (Haidet-Phillips et al., 2011; Re et al., 2014). While the necrotic environment of post-mortem tissue has to be considered, another study reported that astrocytes generated from sporadic and familial (*C9ORF72* and *SOD1*) ALS iPSCs were also toxic to co-cultured MNs (Meyer et al., 2014). These findings correlate with studies that report the neurotoxicity of glia derived from SOD-1 transgenic mice (Di Giorgio et al., 2007; Nagai et al., 2007). Collectively, this glia-induced neurotoxicity suggests a common mechanism in both sporadic and familial ALS.

Different experimental approaches further suggest that astrocytes are neurotoxic to MNs in the context of ALS. A recently reported ubiquitous RNAi knockdown of TDP-43 in mice led to severe neurodegeneration and an ALS phenotype. The study revealed a greater knockdown of TDP-43 in astrocytes compared to MNs, significant astrogliosis and marked upregulation of lipocalin-2 expression in reactive astrocytes (Yang et al., 2014). Lipocalin-2 is a feature of inflammatory astrocytes (Zamanian et al., 2012) and can enhance reactive astrogliosis via autocrine signaling (Lee et al., 2009). Specific knockout of TDP-43 in cortical and MNs in mice produced a less severe phenotype, which further highlights the contribution of multiple cell types in ALS and FTD (Wu et al., 2012b; Iguchi et al., 2013).

Interestingly, overexpression of ALS associated mutant TDP-43 driven by an astrocytic promoter was sufficient to cause MN degeneration in rats and was also associated with marked up-regulation of lipocalin-2 in reactive astrocytes (Tong et al., 2013). Overexpression of mutant TDP-43 in rat

neurons also induced gliosis and lipocalin-2 upregulation in surrounding reactive astrocytes. The analysis of post-mortem frontal cortex of FTD patients likewise revealed an increase in lipocalin-2, and that recombinant lipocalin-2 was exclusively toxic to cultured neurons (Bi et al., 2013). Huang et al. (2014) reported that inducible overexpression of mutant TDP-43 can increase lipocalin-2 expression in cultured astrocytes and while pathological mutations can lead to alterations in RNA homeostasis similar to those seen in knockdown studies. Further studies are clearly needed to understand the mechanism of pathological TDP-43 and the ambiguous role of lipocalin-2 in ALS and FTD pathogenesis and its potential as a therapeutic target or biomarker for assessing neuroinflammation.

Recent advances in patient neuroimaging have allowed direct visualization of neuroinflammation such as gliosis. Positron Emission Tomography (PET) and Single Positron Emission Computed Tomography (SPECT) or Magnetic Resonance Imaging (MRI) in patients targeting activated microglial receptors or astrocytic metabolites have shown gliosis throughout various symptomatic stages of ALS and FTD while absent in non-disease controls (Cagnin et al., 2004; Chiò et al., 2014). This, along with data from animal models, strongly indicates that gliosis is unlikely to represent a specific event only seen in post-mortem tissue at the end stage of ALS and FTD. More recently, modalities that specifically evaluate astrocyte metabolism (e.g., radiopharmaceutical acetate derivatives; Marik et al., 2009; Ouyang et al., 2014) could be used to monitor astrogliosis in patients more accurately. New imaging ligands such as the modern translocator protein ligands allow for tracking microglia activation with higher specificity and reduced radiation dosage (Corcia et al., 2012). Collectively, these rapidly improving technologies are revealing important information regarding the involvement of astrocytes and microglia in various stages of degeneration in ALS and FTD patients.

THE ROLE OF GLIAL PHAGOCYTOSIS IN ALS AND FTD

Astrocytes have been found to highly express an array of phagocytic receptors and actively contribute to this process by phagocytosing synapses and axonal mitochondria in the developing and adult CNS (Chung et al., 2013; Davis et al., 2014). Nonetheless, microglial cells have been shown to be the main culprit for phagocytosis and synaptic pruning that is crucial to CNS function by removing potentially toxic debris and the reorganization of the CNS connectome (Neumann et al., 2009; Xavier et al., 2014). The altered phagocytic activity of microglia has been implicated in multiple neurodegenerative disorders. This link has been highlighted through three discoveries of genetic mutations in phagocytosis-related genes in ALS and FTD patients (**Figure 2A**). *TREM2* is exclusively expressed by microglia in the CNS (Colonna, 2003; Thrash et al., 2009) and missense variants have been recognized as a risk factor for ALS, FTD, Alzheimer's and Parkinson's disease (Rayaprolu et al., 2013; Cady et al., 2014; Harms et al., 2014). Furthermore, recessive mutations in *TREM2* are also associated to an orphan neurodegenerative condition known as Nasu-Hakola Syndrome with patients presenting with lytic bone cysts, atypical FTD and psychiatric dysfunction (Kaneko et al., 2010). Interestingly homozygous and compound heterozygous mutations are linked to a familial FTD-like disorder without bone involvement (Kaneko et al., 2010; Guerreiro et al., 2013; Borroni et al., 2014). These mutations in *TREM2* are proposed to confer loss of TREM2 protein function, which causes decreased microglial phagocytosis and altered inflammatory responses (Kleinberger et al., 2014; Wang et al., 2015). Additionally, dysfunctional microglial phagocytosis is directly linked to FTD via mutations in *GRN* (progranulin) and may confer elevated risk of developing Alzheimer's disease and ALS (Petkau and Leavitt, 2014). Progranulin is expressed by neurons and microglia and following secretion can act as a neuroinflammatory modulator (Petkau et al., 2010), and facilitate microglial recognition of apoptotic cells and potentially toxic elements such as amyloid beta (Aβ; Pickford et al., 2011; Minami et al., 2014). Also, loss of function mutations in *PFN1* (profilin 1) have been identified in familial ALS (Wu et al., 2012a) and profilin has been shown to be essential in regulating actin dynamics necessary for phagocytosis, phagosome formation and is upregulated in microglia following insult (Pearson et al., 2003; Dong et al., 2004; Kim et al., 2012). Research into how these *PFN1* mutations influence microglia function will be crucial to understanding the pathogenicity of those mutations. While progranulin and profilin 1 mutations are likely to impact multiple cell types (especially neurons), cell specific transcriptome analysis of the mouse cortex indicates that all three genes are highly transcribed in microglia (Zhang et al., 2014). Taken together, these studies highlight a potential link between reduced microglial phagocytic capacity and the development of neurodegeneration, ALS and particularly FTD and is represented in **Figure 2A**.

Phagocytosis is incomplete without the intracellular breakdown of engulfed material. Autophagy is an essential component of this internal degradation inside phagocytes as autophagosome-lysosome fusion is crucial to break down this debris. Alterations to this pathway are directly implicated in ALS, ALS-FTD, FTD and/or Multisystem Proteinopathy pathogenesis through mutations to *OPTN*, *SQST1*, *VCP* and the recently discovered *TBK1* (Renton et al., 2014; Freischmidt et al., 2015). These genes are vital to autophagosome formation, maturation and therefore crucial in LC3-assisted phagocytosis and intracellular waste clearance (Tresse et al., 2010; Seto et al., 2013). Interestingly these genes are also involved in inflammation as they can regulate Nuclear Factor-kappaB (NF-κB) signaling (Pomerantz and Baltimore, 1999; Asai et al., 2002; Zhu et al., 2007; Duran et al., 2008; Tresse et al., 2010; Seto et al., 2013). NF-κB is one of the major regulators of neuroinflammatory activation of glia (Zamanian et al., 2012) and its induction is seen in post-mortem ALS tissue, mutant SOD-1 and TDP-43 models (Migheli et al., 1997; Swarup et al., 2011a,b; Frakes et al., 2014). Any defects to these genes would potentially impact upon the function

A

Gene	*TREM2*	*GRN*	*PFN1*
CNS Expression	Microglia	Neurons & Microglia	Ubiquitous, Enriched in Microglia
Homozygous Mutations	Nasu-Hakola Syndrome, Atypical FTD-Like Syndrome	Neuronal Ceroid Lipofuscinosis	-
Compound Heterozygous Mutations	Atypical FTD-like Syndrome	-	-
Heterozygous Mutations	Risk Factor for ALS, AD, FTD & PD	FTD Risk Factor/Disease Modifier ALS, AD & MS	ALS

Normal

	Phagocytosis	Inflammation	Cell Motility
TREM2	↓	↑	–
GRN	↓	↑	–
PFN1	↓	–	↓

B

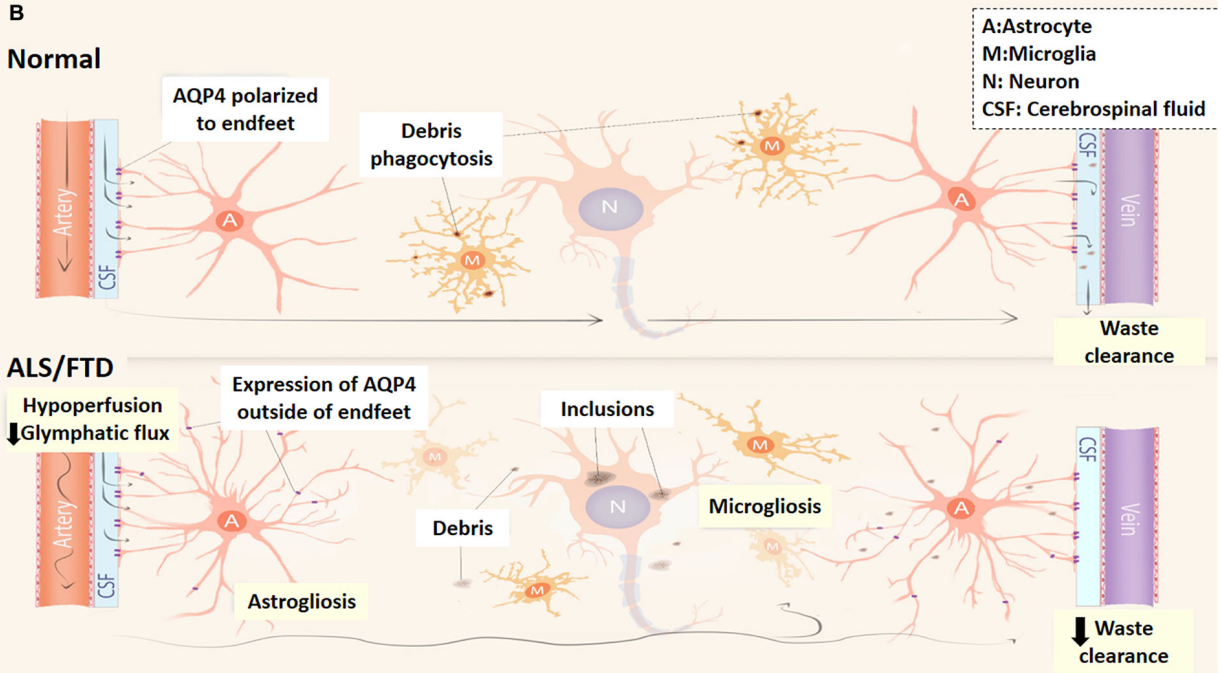

FIGURE 2 | Phagocytic dysfunction and the glymphatic pathway and its (potential) involvement in ALS and FTD. **(A)** Three genes (*TREM2, GRN* and PFN1) which link microglial phagocytic dysfunction to ALS and FTD and their effect on microglial phenotype. All three are predicted loss of function mutations which decrease the phagocytic capacity of microglia. Depending on the type of mutation/s to these genes different neurodegenerative conditions can arise, while variants cause increase risk of developing neurodegenerative conditions or worsen prognosis. Neuronal Ceroid Lipofuscinosis is a type of neurodegenerative lysosomal disorder which has been reported in patients *PRGN* null patients (Petkau and Leavitt, 2014). AD, Alzheimer's disease; PD, Parkinson's disease; MS, multiple sclerosis. **(B)** In the normal CNS, CSF circulates in a perivascular compartment driven by arterial pulse pressure. Astrocytic endfeet cover the perivascular space and facilitate movement of CSF into the parenchyma largely via AQP4. This fluid flow through the interstitial space allows the removal of debris from the extracellular space before draining into venous perivascular compartments. Microglia also remove potentially toxic waste via phagocytosis and dysfunctional microglial phagocytosis is linked to ALS and FTD pathogenesis (see **A**). In the CNS of ALS and FTD patients, glymphatic function and microglial phagocytosis may be compromised and contribute to neurodegeneration. Reactive astrocytes conceivably lose AQP4 polarization and express it elsewhere. This may lead to turbulent flow through the interstitium. Cerebral vascular function is reduced in patients which could potentially lead to decreased pressure for glymphatic function. A, astrocytes; M, microglia; N, neuron; CSF, cerebrospinal fluid.

of innate immune cells (Deretic et al., 2013), particularly those segregated in the CNS like microglia and astrocytes. Determining how these genes are expressed, regulated and function in astrocytes and microglia will provide important insights into the neurodegenerative mechanisms underlying ALS and FTD.

THE EMERGING ROLE OF THE GLYMPHATIC SYSTEM AND VASCULAR FUNCTION IN ALS AND FTD

While phagocytosis of apoptotic neurons and cellular debris is a major pathway for removal of toxic substances within the CNS, the glymphatic system has recently emerged as a different clearance pathway with important immune functions (Iliff et al., 2012). The glymphatic system mediates circulation of cerebrospinal fluid (CSF) and exchange of interstitial fluid to remove extracellular waste (such as Aβ and tau proteins) and distribute compounds such as glucose, lipids, and neuromodulators to the CNS (Thrane et al., 2013; Xie et al., 2013; Iliff et al., 2014). The glymphatics run parallel to the CNS vasculature in a paravascular space enclosed by astrocytic endfeet (**Figure 2B**). Accordingly, the glymphatic system (reviewed in Jessen et al., 2015) relies heavily upon the vasculature in order to function as pressure differentials between arteries and veins propel the CSF through the CNS parenchyma (Iliff et al., 2013). The bulk of glymphatic flow through the CNS is facilitated by aquaporin-4 (AQP4), a water transporter specific to astrocytes in the CNS and polarized to their endfeet (Iliff et al., 2012; Papadopoulos and Verkman, 2013). Interestingly, elevated AQP4 with loss of astrocytic endfeet depolarization has been reported in transgenic mutant SOD-1 rat models and reactive astrocytes have been shown to up-regulate AQP4 elsewhere in the astrocytic arbor apart from the endfeet (Bataveljić et al., 2012; Papadopoulos and Verkman, 2013). Glymphatic flow significantly increased during non-rapid eye movement sleep and was largely controlled by norepinephrine (which modulates arousal) acting upon astrocytic α-adrenoceptors (Xie et al., 2013; Paukert et al., 2014). Increased levels of norepinephrine have been observed in the CSF, plasma and spinal cord tissue of ALS patients (Brooks et al., 1980; Bertel et al., 1991) and norepinephrine CSF levels were positively correlated with the severity of dementia in FTD (Engelborghs et al., 2008). It has been hypothesized that increased CSF levels of norepinephrine could lead to decreased glymphatic function, while any aberrant expression of AQP4 could potentially create turbulent convective flux through the CNS interstitium, ultimately leading to decreased removal of neurotoxic metabolites (Kress et al., 2014; Jessen et al., 2015). MRI imaging techniques allow live-imaging of the glymphatic system (Iliff et al., 2013; Yang et al., 2013) and are a novel approach to detect flow abnormalities in the glymphatic system in ALS and FTD patients. Recent studies have identified lymphatic vessels present in the dura mater, which drains CNS interstitial fluid via the glymphatic system and CSF from the subarachnoid space (Aspelund et al., 2015; Louveau et al., 2015). As T cells are implicated in the progression of ALS patients and animal models (Philips and Robberecht, 2011) this would reflect a novel way for lymphocytes to monitor and interact with CNS tissue via the glymphatic system and potentially influence neuroinflammatory events in ALS and FTD.

Glymphatic function is intimately linked to vascular flow via the parallel anatomy and requirement of pressure differentials created by blood flow. During development and in the mature CNS, astrocytes and microglia are crucial to complex signaling cascades and angiogenesis necessary for cerebrovascular function (see Abbott et al., 2006). Interestingly, two genes involved in vascular function have been linked to ALS. Mutations in *ANG* (angiogenin) have been found to segregate with both familial and sporadic forms of ALS and Parkinson's disease (Greenway et al., 2006; van Es et al., 2011). Angiogenin was enriched and secreted by MNs with paracrine effects exclusively on astrocytes *in vitro* (Skorupa et al., 2012, 2013). *VEGFa* promoter haplotypes causing decreased expression also infer a greater risk of ALS. VEGFa is predominately expressed by astrocytes in the CNS (Zhang et al., 2014) and decreased VEGFa levels significantly reduce survival in mutant SOD-1 mice (Lambrechts et al., 2003). While decreased expression is a greater risk for ALS and can cause MN degeneration due to reduced ischemic tolerance (Oosthuyse et al., 2001), increased VEGFa expression by reactive astrocytes due to NF-κB-dependent pathways leads to greater infiltration of peripheral immune cells and blood brain barrier (BBB) breakdown in multiple sclerosis (MS) mouse models (Argaw et al., 2012; Chapouly et al., 2015). This highlights a potential dual role for VEGFa in neurodegeneration and how inappropriate control of VEGFa expression is associated with various forms of neurodegeneration. Further implicating the involvement of vascular defects, compromised blood brain and spinal cord barriers have been observed in post-mortem ALS and FTD tissue (De Reuck et al., 2012; Garbuzova-Davis et al., 2012). Early dysfunction of the blood spinal cord barrier has been shown to contribute to early MN damage in transgenic ALS-mutant SOD-1 mice (Zhong et al., 2008; Winkler et al., 2014). Further, patient cerebral perfusion neuroimaging studies have noticed hypoperfusion abnormalities in areas that correlate with neurodegeneration in ALS and FTD (Martin et al., 2001; Du et al., 2006; Zhong et al., 2008; Chiò et al., 2014; Winkler et al., 2014). Altogether these studies implicate vascular dysfunction in the pathogenesis of ALS and FTD.

CONCLUSION

Research over the last decades has established that astrocytes and microglia play crucial roles in the development and/or progression of ALS and FTD through their complex interactions. Recent advances in iPSC technology have highlighted that glia secrete toxic factors that can trigger neurodegeneration. New gene discoveries have implicated that defects in glial phagocytic and neuroinflammatory activity are associated with neurodegeneration. There is now emerging evidence suggesting that non-inflammatory glial properties associated with vascular fluid flow and waste clearance have important roles in disease pathogenesis. Collectively, an emerging body of recent literature highlights the critical role of microglia and astrocytes in the etiology of ALS and FTD.

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Delirium from the gliocentric perspective

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Delirium is an acute state marked by disturbances in cognition, attention, memory, perception, and sleep-wake cycle which is common in elderly. Others have shown an association between delirium and increased mortality, length of hospitalization, cost, and discharge to extended stay facilities. Until recently it was not known that after an episode of delirium in elderly, there is a 63% probability of developing dementia at 48 months compared to 8% in patients without delirium. Currently there are no preventive therapies for delirium, thus elucidation of cellular and molecular underpinnings of this condition may lead to the development of early interventions and thus prevent permanent cognitive damage. In this article we make the case for the role of glia in the pathophysiology of delirium and describe an astrocyte-dependent central and peripheral cholinergic anti-inflammatory shield which may be disabled by astrocytic pathology, leading to neuroinflammation and delirium. We also touch on the role of glia in information processing and neuroimaging.

Keywords: immunity, acethylcholine, volume transmission, AQP-4, extracellular space

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Is Delirium an Astropathy?

The search for cellular and molecular underpinnings of delirium became important as it was demonstrated that after an episode of delirium in elderly, there is a 63% probability of developing dementia at 48 months compared to 8% in patients without delirium[1] (Pandharipande et al., 2013). Furthermore, an association was shown between delirium and increased mortality, length of hospitalization, cost, and discharge to extended stay facilities (Witlox et al., 2010).

Examined exclusively from the neuronal standpoint, delirium is often described as a neurobehavioral syndrome. In this article we take a gliocentric approach and look at the role of astrocytes in the pathophysiology of delirium. Observed from this angle, delirium is as much a neuro as a gliobehavioral disorder marked by a global cholinergic deficit and a malignant inflammation. We believe that both hypo-cholinergia and inflammation are symptoms of astrocytic failure. Furthermore, aside from reconciling neuroinflammation and cholinergic deficit in the pathogenesis of delirium, adopting a glial perspective explains the astrocytic origin of most delirium markers.

Acethylcholine (ACh) signaling was suggested to engender a potent cholinergic anti-inflammatory shield (CAIS), protecting against peripheral and central inflammation (Tracey, 2009).

[1] American Delirium Society. https://www.americandeliriumsociety.org/about-delirium/healthcare-professionals, available on line.

CAIS operates in the extracellular space (ECS) both peripherally and centrally by blocking the release of pro-inflammatory mediators (Shaked et al., 2009; Han et al., 2014). CAIS consists of extracellular ACh biosynthesis, its diffusion through the interstitium and action on alpha-7 nicotinic ACH receptors (nAChR) expressed on the peripheral and central immune cells (macrophages, natural killer cells, lymphocytes, astrocytes and microglia), as well as on neuronal and endothelial cells in the CNS (Perry and Teeling, 2013; **Figure 1**).

The brain is not protected from peripheral pro-inflammatory mediators, as it was believed in the past. In fact, recent studies demonstrate that peripheral cytokines cross routinely the intact blood brain barrier and interact with brain cells. Despite their entry into the CNS, the peripheral pro-inflammatory cytokines are inactivated by CAIS, thus preventing the seeding of inflammation into the brain.

We propose that CAIS is engendered by the astrocytes by secretion of choline acetyltransferase (ChAT), the ACh synthesizing enzyme, into the ECS and by regulation of the interstitial fluid (ISF) volume.

Astrocytes maintain brain water homeostasis by regulating the ISF volume. They accomplish this as their membranes are more permeable to water than those of other brain cells (8). This unique property of astrocytes is based on aquaporine-4 (AQP-4) water channels expressed in large numbers on their end feet processes. In fact, astrocytes express the largest number of AQP-4 receptors in the entire CNS (Nagelhus and Ottersen, 2013). This unique property of their membranes confers astrocytes a sponge-like ability to absorb and release water as needed, thus regulating ISF volume (Gundersen, 2013). With the same token, this property renders astrocytes vulnerable to cytotoxic edema with resultant

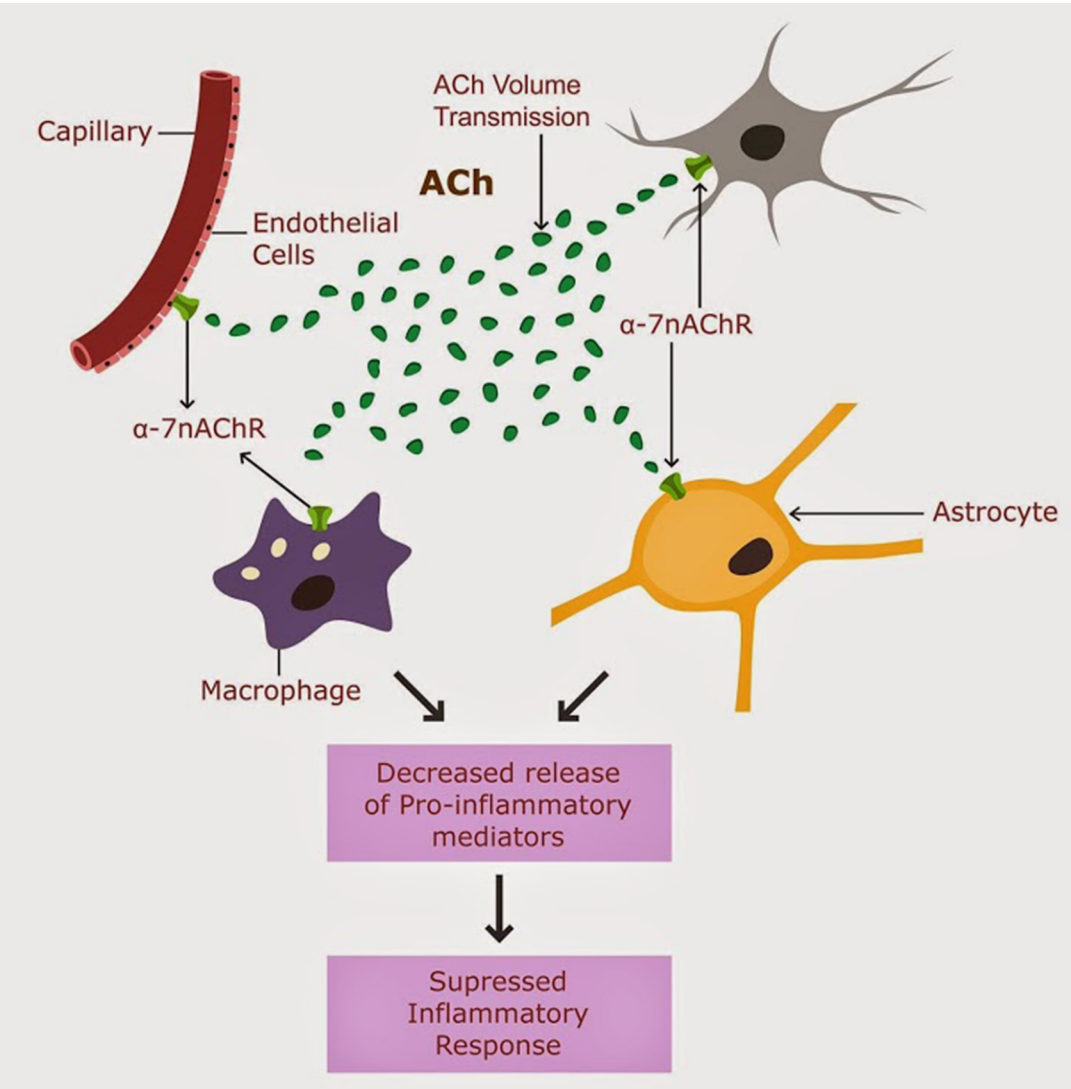

FIGURE 1 | CAIS is operational in the CNS, and involves ACh action of alpha 7 nAChRs expressed on astrocytes, neurons, microglia and brain macrophages (Tyagi et al., 2010).

ISF hypovolemia. As a result of swelling, astrocytes become insufficient, thus unable to secrete ChAT into the ECS which disables CAIS, unleashing neuroinflammation. Moreover, ISF hypovolemia disables beta-amyloid clearance, cluttering the ECS, and disabling CAIS further. Indeed, astrocyte swelling and accumulation of molecular waste in the interstitium was described in several neuro-psychiatric conditions including: sepsis-induced delirium (SID), stroke, traumatic brain injury, brain tumors and metastases, meningitis, brain abscesses, water intoxication, altitude sickness, malignant hypertension, hypoglycemia, and metabolic encephalopathies (Thrane et al., 2014).

We hypothesize that delirium is the clinical manifestation of astrocytic failure caused by astrocytic edema. Edema is the result of AQP-4 water channels' overexpression on astrocytic membranes. We hypothesize further that failed astrocytes are unable to secrete ChAT into the ECS, disabling CAIS, while the resultant ISF hypovolemia disables beta-amyloid clearance, further impairing CAIS. In the absence CAIS protection, uncontrolled neuroinflammation flares up possibly resulting in delirium. To illustrate this hypothesis, we envision astrocytes as the firefighters of the brain. They keep the fire (neuroinflammation) under control, by maintaining enough water reserves into the interstitial space. In the absence of this water, fire flares up, resulting in uncontrolled neuroinflammation and delirium.

The Brain-Immune Interface

The fact that the brain is an immunologically privileged organ is a myth. Recent studies demonstrate a continuous two-way communication between the CNS and the peripheral immune system (Perry and Teeling, 2013). For example, it was demonstrated that a cardiac arrest can induce alterations in the central cholinergic signaling, including reduction of ChAT and vesicular ACh transporter mRNA (Norman et al., 2011; Zhao et al., 2013). Conversely, global cerebral ischemia often induces peripheral inflammation (Zhan et al., 2010). This inflammatory response can be pharmacologically reversed by the administration of selective alpha 7 nAChR agonists such as GTS-21, suggesting a role for selective nicotinic agonists in delirium (de Jonge and Ulloa, 2007; Kox et al., 2011).

Alpha 7 nAChRs agonistic ligands are currently being investigated in the treatment of sepsis, inflammation, dementia and schizophrenia (Han et al., 2014). In addition, CAIS was shown to prevent obesity-induced inflammation and insulin-resistance (Lakhan and Kirchgessne, 2011). It is well known that muscarinic M3 receptors in the pancreas are involved in the metabolic syndrome (Duttaroy et al., 2004). This beneficial effect of ACh can be reversed by the administration of alpha 7 nAChR antagonists (Kox et al., 2011). Furthermore, the action of ACh on selective muscarinic receptors was demonstrated to modulate cellular proliferation and is therefore of interest in various cancers (Spindel, 2012; Ferretti et al., 2013). Interestingly, an aberrant neuronal cell cycle entry was suggested in the pathogenesis of Alzheimer's disease (AD; Stieler et al., 2001).

Biological Markers: Not too Specific for Delirium, but Specific for Astrocytes

Most biological markers of interest in delirium can be traced to astrocytes. It is well established that Matrix Metaloprotease-9 (MMP-9; Yin et al., 2006), S 100B protein (Tateishi et al., 2006) and glial fibrillary acidic protein (GFAP; Brownell et al., 1991) are secreted by astrocytes. Procalcitonin (McGrane et al., 2011), a newly identified marker of delirium caused by sepsis is expressed on neurons, astrocytes, microglia and oligodendrocytes (Ojeda et al., 2006).

A recent CSF proteomic analysis identified four categories of markers in delirium. Interestingly, they can be traced to the astrocytes as well (Poljak et al., 2014). They include: acute phase proteins, granins, serine protease inhibitors, and apolipoproteins (references in parenthesis demonstrate glial origin).

Acute phase proteins: lipocalins (Jang et al., 2013) (includes alpha-1-acidic glycoprotein), alpha-2 microglobulin (Bauer et al., 1988), fibrinogen (Pichler et al., 1999; Hsiao et al., 2013), alpha-1 antitrypsin (Gollin et al., 1992), alpha-1 antichymotrypsin (Gollin et al., 1992), transferrin (Qian et al., 1999), complement component 3 (Maranto et al., 2008), and haptoglobin (Lee et al., 2002).

Interestingly, alpha 1-acidic glycoprotein was shown to decrease brain cellular edema after experimental stroke, possibly by down-regulating AQP-4 receptors (Pichler et al., 1999).

Granins: secretogranin 3 (Paco et al., 2010).

Serine protease inhibitors: occur both in neurons and glial cells (Buisson et al., 1998).

Apolipoproteins: Apo A1 (Ito et al., 2002), ApoJ (DeMattos et al., 2001), ApoE (DeMattos et al., 2001).

These markers point to glial pathology in delirium and are in line with our hypothesis on astrocytic failure.

Astrocytes and ChAT Secretion

At the cellular level, ACh biosynthesis occurs in two distinct brain compartments: the neuronal bodies of brain cholinergic tracts and the ECS of CNS (Vijayaraghavan et al., 2013). Regardless of their origin, ACh manufacture depends on the availability of its synthesizing enzyme ChAT. Secreted by astrocytes into the ECS, this enzyme ensures a constant presence of ACh in the interstitium (Vijayaraghavan et al., 2013). **Figure 1**. This ambient ACh is crucial for proper CAIS functioning. Previously it was assumed that ACh could not participate in long distance intercellular signaling because of the presence of its hydrolyzing enzymes, acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) in the ECS. Recent studies reveal that ACh can "survive" in the ECS and participate in long distance signaling despite physiological levels of AChE and BuChE because it can be synthesized *ad hoc* with astrocyte-secreted ChAT (Vijayaraghavan et al., 2013).

Diminished ACh signaling in the ECS disables CAIS and this event is marked by an immediate compensatory up-regulation of alpha 7 nAChRs, the first sign of inflammation. We believe that alpha 7 nAChRs up-regulation is the first measurable marker

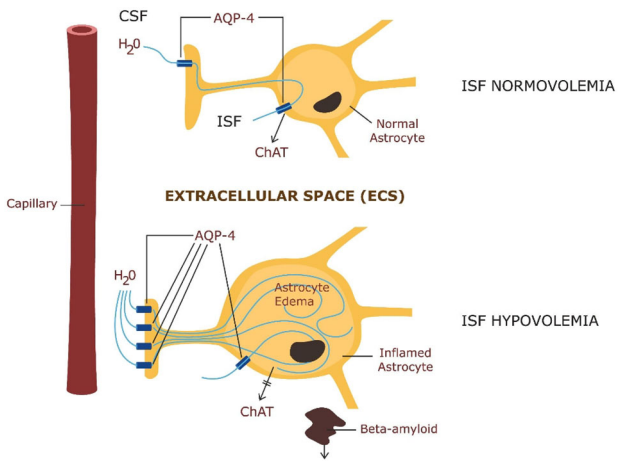

FIGURE 2 | ABOVE: Astrocytic physiology—intra-parenchymal CSF exchange with the interstitial fluid (ISF) via AQP-4 channels and ChAT secretion. BELOW: Pathology—astrocytic edema: up-regulation of AQP-4 in astrocytic end-feet with impairment of ChAT secretion and beta amyloid clearance.

of astrocytic failure. Indeed, a recent study demonstrates that increased astrocytic expression of alpha 7 nAChRs is positively correlated with the extent of neuropathological alterations in AD (Yu et al., 2012). Measuring ECS level of ChAT may provide a biological marker of pending inflammation as it expresses alpha 7 nAChRs up-regulation.

Astrocytes contribute to beta-amyloid clearance either directly, by phagocytosis, or indirectly by the glymphatic system (Nagele et al., 2003; Lasagna-Reeves and Kayed, 2011; Sokolowski and Mandell, 2011; Iliff et al., 2012). We believe that swollen, failed astrocytes may be inefficient in disposing of beta amyloid and other molecular waste, leading to their accumulation and neuroinflammation. Indeed, studies in AD reveal that excess beta-amyloid in the ECS alters cholinergic signaling by activating BuChE, decreasing secretion of astrocytic ChAT and up-regulating alpha 7 nACh receptors on astrocytic membranes (Vijayaraghavan et al., 2013; Malmsten et al., 2014).

Beta amyloid clearance via the glymphatic system is highly dependent on the intra-parenchymal water exchange between the cerebrospinal fluid (CSF) and the ISF, occurring via AQP-4 channels in astrocytic end-feet (Yang et al., 2013) **Figure 2**.

Accumulation of beta-amyloid and low ACh were documented in delirium (Hshieh et al., 2008) and led to the emission of the cholinergic hypothesis in the pathogenesis of this condition. However, since cholinesterase inhibitors showed only mild or no efficacy in delirium, this hypothesis seemed to have been invalidated (van Eijk et al., 2010; Brinker et al., 2014).

Astrocytic failure hypothesis offers an alternative explanation that does not necessarily invalidate the cholinergic hypothesis. On the contrary, since they do not restore astrocytic secretion of ChAT, cholinesterase inhibitors are not expected to beneficial in delirium. In the absence of ACh biosynthesis in the ECS, inhibiting its hydrolyzing enzymes would not resurrect CAIS function. On the other hand, co-administration of cholinesterase inhibitors with drugs capable of restoring the extracellular water volume (such as agonists of alpha 7 nAChRs, direct AQP-4 blockers or indirect ones, such as erytropoietin) may be salutary.

Is there a Water Connectome?

Being more permeable to water, astrocytes serve as brain fluid reservoirs where water can be stored and also retrieved as needed (Gundersen, 2013). The high AQP-4 expression on their membranes enables astrocytes to move water in and out of the neuropile. Studies done three decades ago show that water homeostasis is dependent on the neuropile activity (Dudek and Rogawski, 2005). For example, during the day (when information processing is usually more intense), water is shifted into the astrocytic compartment, while during the night (when there is less information processing), water is shifted back into the interstitium (Xie et al., 2013). This water movement was hypothesized to help beta amyloid clearance during sleep (Brinker et al., 2014).

Water can cross cellular membranes slowly by diffusion or co-transport with other substances, but the quickest modality of water transport occurs via AQP-4 receptors which abound on astrocytic membranes (Gundersen, 2013). The high permeability of astrocytic membranes and the sponge-like properties of these cells render neuroimaging possible. Diffusion tensor imaging (DTI) visualizes water diffusion across membranes which can be either unrestricted (isotropy) or restricted by the myelin sheath (anisotropy). Since water does not diffuse through the myelin, but follows it, this technique is used for tracing white matter tracts (Lazar et al., 2003; Lazar, 2010).

We suggest that DTI could be utilized for assessing glial integrity by adapting its algorithm to the areas of highest isotropy. Since astrocytes are the most water permeable cells in the brain, the highest CNS isotropy must coincide anatomically with astrocytes.

Recent data point to the fact that DTI signal changes, attributed earlier to white matter tracts and axonal myelination, may in fact mirror astrocytic expression of AQP-4 receptors (Meng et al., 2004; Tourdias et al., 2009), astrocytic edema (Harsan et al., 2007; Fukuda and Badaut, 2012), ISF volume (Fukuda and Badaut, 2012), or glial scars (Budde et al., 2011).

In its journey through the brain fluid compartments, water carries along signaling molecules that comprise an extra-cellular communication platform described as volume transmission (VT; Agnati et al., 1995). Does this signaling platform fathom a liquid connectome? Furthermore, since it mirrors the water flow along white matter tracts, is the connectome in general a liquid connectome? It was proposed that point-to-point synaptic transmission engenders a quick and precise mode of information processing, such as the one necessary for playing tennis or piano, while the diffuse and widespread communication platform by water signaling may engender more pervasive functions such as awareness, attention, mood, circadian rhythm or cognition (Gualtieri, 2002; Taber and Hurley, 2014; Vizi et al., 2010). As

these functions are known to "wax and wane" in delirium, can they be part of an astrocyte-centered information processing? Recent studies imply that astrocytic networks are crucial for information integration and cholinergic plasticity (Pereira and Furlan, 2010; Takata et al., 2011). A recent study demonstrated that ACh signaling is highly correlated with gamma oscillations on the EEG, which are believed to facilitate the somatosensory cortex plasticity (Rodriguez et al., 2004). This may suggest an astrocytic-based cognition, possibly complementary to the rapid, point-to-point information processing established by neurons.

Conclusions

Memory characterizes both, cellular immunity and human cognition. Both systems are adapted to interact with the surrounding world and to protect from it. Both are capable of distinguishing "self" from the environment ("non-self; Lampron et al., 2013). Since nature rarely wastes ideas, immunologic memory may be a distant phylogenetic ancestor of human cognition. Controlled inflammation and cellular proliferation may benefit hippocampal neuroplasticity and long term potentiation. Out of control, inflammation, on the other hand, may lead to sepsis, while aberrant cellular proliferation to cancer. With the same token, activation of cellular proliferation in non-dividing cells, like neurons, may lead to aberrant cell cycle entry, resulting in apoptosis. This may be the case in AD.

Both astrocytes and microglia are part of the innate immune system which responds to the early stages of infection by triggering non-specific, high collateral damage, inflammatory responses, which are later expected to die down, as the high precision, specific, adaptive immune system is activated. Failure of the innate system to shut down may result in "cytokine storms" encountered in sepsis and sepsis-induced delirium (SID). On the other hand, intact astrocytes with proper CAIS calm down the innate immune system, containing inflammatory responses. Failed astrocytes unleash exaggerated inflammation which may lead to delirium. Engel and Romano (1959) described delirium as "brain failure"; we think of it as a primary astrocytic failure with secondary neuronal involvement. Since astrocytic suffering occurs prior to neuronal damage, identifying it early may offer a window of opportunity for preventive interventions that could salvage the cognitive domain before the occurrence of neuronal apoptosis.

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frontiers in Cellular Neuroscience

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In vivo characterization of microglial engulfment of dying neurons in the zebrafish spinal cord

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Microglia are specialized phagocytes in the vertebrate central nervous system (CNS). As the resident immune cells of the CNS they play an important role in the removal of dying neurons during both development and in several neuronal pathologies. Microglia have been shown to prevent the diffusion of damaging degradation products of dying neurons by engulfment and ingestion. Here we describe a live imaging approach that uses UV laser ablation to selectively stress and kill spinal neurons and visualize the clearance of neuronal remnants by microglia in the zebrafish spinal cord. *In vivo* imaging confirmed the motile nature of microglia within the uninjured spinal cord. However, selective neuronal ablation triggered rapid activation of microglia, leading to phagocytic uptake of neuronal debris by microglia within 20–30 min. This process of microglial engulfment is highly dynamic, involving the extension of processes toward the lesion site and consequently the ingestion of the dying neuron. 3D rendering analysis of time-lapse recordings revealed the formation of phagosome-like structures in the activated microglia located at the site of neuronal ablation. This real-time representation of microglial phagocytosis in the living zebrafish spinal cord provides novel opportunities to study the mechanisms of microglia-mediated neuronal clearance.

Keywords: zebrafish, apoptosis, necrosis, imaging, M1, M2, glia, neuron

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Introduction

Microglia are the resident macrophages of the CNS and play crucial roles in mediating immune-related functions (Barron, 1995; Hanisch and Kettenmann, 2007; Graeber and Streit, 2010; Ransohoff and Cardona, 2010). Microglia patrol the entire vertebrate nervous system, where they can detect the presence of apoptotic and damaged neurons, and consequently engulf these cells to minimize the spread of neuronal debris. This microglial activity requires fast-acting communication between the two cell types, such that microglia are primed for rapid response to a variety of stimuli (such as dying neurons). However, many of the fundamental mechanisms that regulate the detection of injured neurons and subsequent microglial activation during phagocytosis still remain elusive. Short-term microglial activity is generally accepted to serve a neuroprotective role, while chronic activation has been implicated as a potential pathogenic mechanism in neurodegenerative disorders (Block et al., 2007). *In vitro* studies over the last decade have established the morphological transformations that microglia undergo during injury and disease,

characterized by the transformation from a ramified morphology to an ameboid appearance, in a process termed "microglial activation" (Hanisch and Kettenmann, 2007; Kettenmann et al., 2011; Michaelis et al., 2015). Accordingly, microglia are now established as key players in the CNS, with their activation and inflammatory profile a major hallmark of CNS injury and neurodegenerative diseases (Block et al., 2007).

Importance of Clearance of Dying Cells by Microglia

With a turnover of billions of cells as part of the normal homeostasis each day, prompt and efficient clearance is necessary to prevent secondary necrosis of dying cells and immune responses to autoantigens (Henson, 2005; Nagata et al., 2010). Irrespective of whether these cells are generated as part of normal development, tissue maintenance, or as part of adult neurogenesis, a striking feature of this phagocytic activation of microglia is that it occurs in an immunologically silent manner. This is in contradiction to the classical paradigm of microglial activation in disease states, where microglial activation is associated with inflammatory behavior such as cytokine expression (Smith et al., 2012). Microglial clearance of dying neurons can be distinguished into four steps: (1) "Find me" signals that attract microglia toward dying cells (Peter et al., 2010); (2) the dying cells then promote their fate through "eat me" signals that can be recognized by specific receptors on the microglia (Gardai et al., 2006); (3) physical interaction through engulfment of the dying cell by microglia (Ravichandran and Lorenz, 2007); and (4) the phago-lysosomal digestion of the engulfed cellular debris (Kinchen et al., 2008; Kinchen and Ravichandran, 2008).

Advantages of Zebrafish for Real-time Imaging of Microglia Function

In vitro studies have contributed extensively to improving our understanding of microglial function. However, certain questions can only be addressed using an *in vivo* system in which normal cell composition, morphology, and dynamics are preserved. This is particularly important when working with microglia, as these cells respond to even very small changes in the CNS, with their morphological properties often being used as readout of pathological conditions. Live-imaging in mice, with surgically thinned skulls, has opened up a new era of glial research as it has revealed for the first time that "resting" microglia are actually highly dynamic and use their long cellular extensions to scan the surrounding environment (Davalos et al., 2005; Nimmerjahn et al., 2005). More recently, the use of zebrafish for live-imaging studies of the nervous system has advanced the study of microglial activation toward elucidating specific molecular mechanisms involved in the engulfment of neurons (Peri and Nüsslein-Volhard, 2008; Sieger et al., 2012; Sieger and Peri, 2013; Mazaheri et al., 2014). Zebrafish offer unique advantages, including their small size and transparency (facilitating high resolution live-imaging microscopy), relatively easy and inexpensive maintenance, and ex-utero development (Westerfield, 2000). In accordance with their mammalian counterparts, zebrafish microglia are dynamic cells that form a non-overlapping network (with discrete domains) within the zebrafish spinal cord (Eyo and Dailey, 2013). Moreover, microglia, neurons and organelles of the microglial phagocytic pathway can be simultaneously labeled with spectrally distinct fluorophores, allowing live imaging of the entire microglial population in order to study the interaction between neurons and microglia (Li et al., 2012). Importantly, the molecular and cellular machineries of microglia to recognize and engulf dying neurons are conserved across vertebrates (Peri and Nüsslein-Volhard, 2008).

In this study, we have used time-lapse confocal microscopy to reveal that "resting" microglia in zebrafish are highly dynamic in the spinal cord in their non-activated form. In response to selective ablation of an individual spinal motor neuron, a single microglia migrates toward the site of injury within several minutes and subsequently ingests neuronal debris from the dying cell. Collectively, these studies provide new insight into the essential clearance of dying neurons in the spinal cord by microglia.

Materials and Methods

Fish Maintenance and Transgenic Lines

Zebrafish (*Danio rerio*) were maintained at 28°C in a 13 h light and 11 h dark cycle. Embryos were collected by natural spawning and raised at 28.5°C in E3 solution according to standard protocols (Westerfield, 2000). Experimental protocols were approved by Macquarie University Animal Ethics Committee (Zebrafish models of neural disorders; protocol no. 2012/050).

The behavior of "activated" microglia was studied between 48 hours post fertilization (hpf) and 5 days post fertilization (dpf). In order to allow high-resolution confocal live-imaging of individual neuron-microglia interactions, we utilized the following previously characterized zebrafish lines: *Tg(mpeg1:GAL4,UAS:mCherry) (gl22Tg)*, referred to as *mpeg1:mCherry* in the text (Ellett et al., 2011); *rwTg(isl1:GFP)*, referred to as *islet1:GFP* in the text (Higashijima et al., 2000); *Tg(met:GAL4,UAS:EGFP) (ed6)*, referred to as *cmet:GFP* in the text (Hall et al., 2007); s1020t*Et(-0.6hsp70l:GAL4-VP16) (s1020t) and Tg(UAS:Kaede)*, referred to as *s1020t:Kaede* in the text (Scott et al., 2007). Expression constructs and novel transgenic lines were generated using the Tol2 kit (Kwan et al., 2007). *Tg(mnx1:mKOFP2-CAAX) (mq7Tg)* (Flanagan-Steet et al., 2005; Arkhipova et al., 2012; Acosta et al., 2014), referred to as *mnx1:mKO2* in the text was generated using recombined p5E-mnx1 (-6 to -2869bp mnx1, Arkhipova et al., 2012), pME-mKO2caax (synthesised by GeneArt), p3E-pA (Kwan et al., 2007) and pDest-Tol2-pA2-acrys-EGFP (Berger and Currie, 2013). *Tg(-3.5ubb:secAnnexinV-mVenus) (mq8Tg)*, referred to as *ubiq:secAnnexinV-mVenus* in the text was generated using recombined p5E-ubb (Mosimann et al., 2011), pMEsecAnnexinA5-NS (Addgene ID 67718), p3E-mVenus (ID 67719) and pDEST-Tol2-pA2 (Kwan et al., 2007). pME-secAnnexinA5-NS was based on the initial design of Van Ham et al. (2010, 2012). It incorporates the human

ANXA5 fused to a mammalian codon optimized consensus secretion signal. The *ubiq:secAnnexinV-mVenus* fish line expresses fluorescent AnnexinV ubiquitously throughout the zebrafish and allows the detection of any cell that expresses phosphatidylserine (PS) on the outer leaflet of the plasma membrane. PS is normally constrained to the inner leaflet of the plasma membrane and gets exposed to the outer leaflet in various conditions, including oxidative stress and apoptosis (Kuan et al., 2000; Valencia and Morán, 2001).

UV Ablation

Targeted ablation of individual neurons was achieved using the 405 nm UV laser of the Leica SP5 confocal microscope. Through an initial z-stack the middle plane of the soma of the neuron of interest was determined and set for UV ablation. The internal Leica FRAP software was used to manually outline the ablation region of interest (ROI; generally a circle or ellipse covering 30–50% of the cell body). For ablation the laser power levels were set between 50–80% of the maximal 405 nm laser power and the "zoom-in" function of the FRAP software was applied to maximize dwell time and laser ablate precisely the outlined area. Dwell time of the laser for neuronal ablation was generally set in a range of ∼60s to assure immediate ablation of the neuron (irreversible loss of fluorescence) and to trigger microglial response. For photoconversion and dose-dependency measurements laser power levels were set between 15 and 90% and dwell time between 10 and 60s.

Microscopy and Imaging

For live-imaging, fish 2–5 dpf were anesthetized in 0.01% tricaine (w/v) and embedded in 1.5% (w/v) low-melting point agarose in glass-bottomed dishes (Westerfield, 2000). When the agarose was cooled down to room temperature the embedded fish were covered with E3 plus 0.01% (w/v) tricaine. The experiments were terminated by adding tricaine solution (4g/L) to the dish. Time-lapse imaging was started approximately 45 min after embedding and was carried out on an upright Leica SP5 confocal microscope at room temperature (22–24°C) with 40 × (NA 0.8) and 63 × (NA 0.9) water objectives and the argon laser lines (458, 476, 488, 496, and 514 nm) or tunable white-light laser (470–670 nm) respectively. A sub-set of preparations was imaged to count and measure the movement of the "surveying" microglia using a 10 × (NA 0.3) water objective. Usually, z-stacks spanning 10–15 planes (approximately 10–30 μm) were imaged (2–5 min; depending on size) every 3–8 min and collapsed to maximum intensity projections using ImageJ or Fiji software (http://imagej.nih.gov/; http://fiji.sc/Fiji). Images were brightness and contrast adjusted for visualization and illustration. Videos were created using the ImageJ export function or the Imaris software (Bitplane Imaris, Switzerland) for rendered visualizations.

Analysis

ImageJ or Fiji software was used to analyze microglia size, movement, and time-course. Maximum intensity projections (MIP) of the time-lapse recordings were used to measure the area and movement of microglia staining as described previously (Tse et al., 2014). Briefly, MIP were thresholded and the area determined using the "Analyse–Measure" function in ImageJ at relevant time points. To measure the movement of the microglia, a straight line was drawn between the center of the cell at the different time points and the distance measured and added. Average speed of microglia was calculated by dividing the maximum distance by the time taken to capture the relevant z-stacks. Statistical analysis was performed with GraphPad Prism software (GraphPad Software, CA, USA). Microglial sizes before and after ablation were taken from the same cells and differences between the means were evaluated using a paired *t*-test. Microglia speed was averaged from microglia surveying the spinal cord and activated after ablation. Differences in speed, volume, and surface area were evaluated using an unpaired *t*-test. For all statistical tests significance was taken as $P < 0.05$. Unless otherwise indicated the data were symmetrically distributed with equivalent variances and values are presented as the mean ± standard error of the mean (SEM). Numbers of fish used for analysis were designated as N and number of cells as n.

Imaris Rendering

3-dimensional visualization and quantification of macrophages was performed using Imaris v7.7.2 (Bitplane, Switzerland). Morphometrics of *mpeg1:mCherry* microglia were rendered and tracked over time using the automated surfaces function. Images were smoothed at a detail level of 0.4 μm and a threshold established by background subtraction (local contrast) of 1.5 μm. Surfaces below 10 voxels were filtered from the algorithm. For 4D surface tracking the autoregressive motion algorithm was employed (gap closed and a maximum distance of 50 μm). The same parameters were used for each quantified image set. Only cells fully within the Z volume were included for analysis.

Results

In vivo Live-imaging of Spinal Motor Neurons and Microglia in Zebrafish

We firstly characterized the behavior of microglia in the uninjured spinal cord in *mpeg1:mCherry* zebrafish. This line was chosen as the Gal4 regulatory element causes mosaic expression of mCherry in microglia, allowing us to confidently visualize individual microglia. Accordingly, we observed a non-overlapping distribution of fluorescent microglia throughout the spinal cord (on average 12 microglia ± 1.4; $N = 7$). The mpeg1-fluorescent cells were on average 240 μm^2 (±15.4 μm^2; $n = 27$; $N = 21$) in size and displayed several features that are characteristic of microglia in the zebrafish and mouse brain (Nimmerjahn et al., 2005; Ellett et al., 2011; Svahn et al., 2013). Hence, spinal microglia exhibited a branched morphology, with filopodia-like processes and bulbous-tipped processes that extended and retracted over minutes (**Figure 1**). A subset of microglial cells (∼30%) showed a highly dynamic behavior, patrolling up and down the spinal cord. This behavior was obvious even in the absence of an "activating" trigger and without characteristic phagocytic activities, such as engulfment of neuronal structures or rapid extension/retraction of their

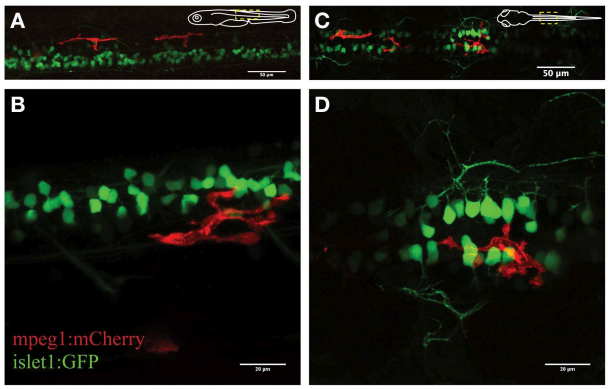

FIGURE 1 | Visualization of microglial activity in the spinal cord of 3 day old transgenic zebrafish expressing GFP-positive neurons (*islet1:GFP*) and mCherry-positive microglia (*mpeg1:mCherry*). (A) Overview (lateral) of the spinal cord and **(B)** enlarged lateral view of spinal neurons and a single microglia. **(C)** Dorsal view of the spinal cord microglia and **(D)** enlarged dorsal view. Schematic inserts in **(A,C)** depict orientation of the fish and outline the presented area.

processes. These motile microglia traveled at an average speed of 1.5 µm/min, averaging distances of 98 µm per hour (±7.7 µm; $n = 18$; $N = 7$) and a maximum of 241 µm within less than 2 h.

Selective Ablation of Individual Motor Neurons within the Spinal Cord

Microglia constantly and efficiently scan for any alterations in their microenvironment, ranging from changes in neuronal activity to signals of damage-associated processes (Nimmerjahn et al., 2005; Ransohoff and Perry, 2009). We applied UV laser ablation (405 nm) to the soma of spinal motor neurons to selectively induce a localized microglial response in the living zebrafish spinal cord. UV ablation of the fluorescent-labeled neurons (*cmet:GFP*) reproducibly led to selective death of the targeted neuron within minutes to hours (**Figure 2**). The time-course of neuronal death was dependent upon parameters such as laser-power, ablation size and dwell time. Ablated and dying neurons showed characteristic morphological changes, including the shrinkage of the cell soma with intact membrane structures (**Figures 2A,B**), and progressive anterograde degeneration (blebbing) commencing at the targeted soma and continuing along the axon over time (**Figures 2C–D**; **Supplementary Video 1**).

To validate this process of neuronal ablation and the specificity of this UV laser ablation method, we used transgenic zebrafish expressing the Kaede fluorophore in spinal neurons (*s1020t:Kaede*; Scott et al., 2007). Kaede is a photo convertible fluorophore that changes fluorescence from green to red after exposure to UV light (Ando et al., 2002). UV laser targeting the soma of an individual neuron with different laser power levels revealed the tuneable nature of this approach for selectively inducing immediate or delayed death of individual neurons. Accordingly, ablation with a laser power of 30% for 10 s led to the efficient photo-conversion of only a single green Kaede neuron amongst a dense cluster of other Kaede-labeled motor neurons in the spinal cord (**Figures 3A,B**). Importantly, this laser power resulted in no cell death of the targeted or surrounding neurons within the next 2 h. On the other hand, using high laser power at a near maximum of 95%, resulted in immediate ablation of the targeted neuron (**Figures 3C,D** white circle) and collateral photo conversion of surrounding neurons within a radius of approximately 20 µm (**Figure 3D**). Even though these surrounding neurons were exposed to some UV irradiation, we never observed any unspecific death within the direct proximity of the laser ablation site. Increasing the duration (longer dwell time) of the UV laser ablation with different laser power did not result in further spread of the photoconversion and was maintained in a radial diameter of approximately 25 µm (**Supplementary Figure S1**).

In summary, our high resolution time-lapse imaging laser ablation demonstrated neuronal blebbing, observed as the development of bead-like formations along the axon and granular disintegration distal to the site of UV-induced injury, and ultimately death of the neuron (**Figure 2**). Reducing the laser power further provided an opportunity to stress the neuron without inducing death (**Figures 3A,B**), whereas raising the laser power to near maximal levels resulted in the immediate death of the targeted neuron with minimal effect on the surrounding cells (**Figures 3C,D**). Consequently, this approach of targeting neurons with varying intensities of UV light represents a novel and reliable method to selectively induce stress or death to individual spinal (motor) neurons in the living zebrafish.

Dying Motor Neurons Express Apoptotic Markers Associated with Phagocytic Signaling

When cells undergo stress or death, they release "eat-me" signals that mediate the rapid recognition and engulfment of these dying neurons and neuronal debris to avoid a spread of inflammation. Eat-me signals, such as the phospholipid phosphatidylserine (PS) act as crucial detection signals for the recognition and ultimately the efficient digestion of these cells (Davalos et al., 2005; Takahashi et al., 2005; Ravichandran, 2011; Brown and Neher, 2014). We utilized AnnexinV, a protein that binds to PS lipids exposed on apoptotic cells (Vermes et al., 1995), to visualize this apoptotic/phagocytic signaling process *in vivo*. We generated a stable transgenic line (*ubiq:secAnnexinV-mVenus*; see Materials and Methods) and crossed these fish with the *mnx1:mKO2* motor neuron line to selectively use this reporter to detect dying neurons in the spinal cord. UV ablation of motor neurons in these double-labeled fish showed the same consistent morphological changes of somal degeneration and axonal blebbing as described earlier (compare **Figure 4** and **Figure 2**; **Supplementary Video 2**). Moreover, within several minutes after ablation we detected the activation and accumulation of AnnexinV at the neuron (**Figure 4B**, yellow channel), firstly at the targeted soma site and then progressively along the axon toward the more distal parts of the neuron. As the neuron degenerated, AnnexinV-labeled fragments of neuronal debris were observed around the ablation site (**Figures 4D–F**).

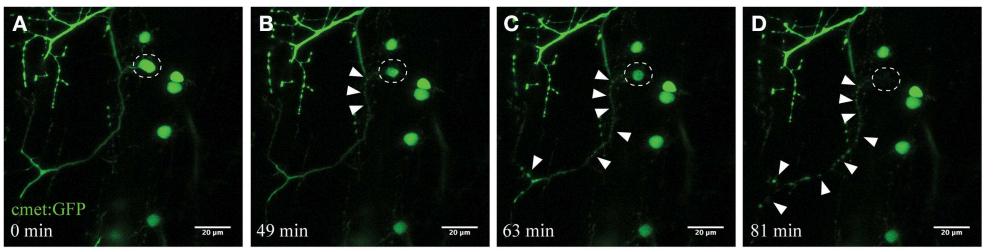

FIGURE 2 | Time-course imaging of the neurodegeneration of UV-ablated spinal neurons. (A–D) UV-irradiation of a single spinal neuron (*cmet:GFP*; **A**; circle) resulted in the soma of the neuron shrinking over time, followed by axonal fragmentation (**B**; arrowheads). This axonal degeneration radiated anterogradely toward the distal end of the axon **(C)**, until finally the fluorescence in the soma disappears and the entire axon shows "blebbing" **(D)**. Scale bars = 20 μm. **Supplementary Video 1** shows the time-lapse video of this process.

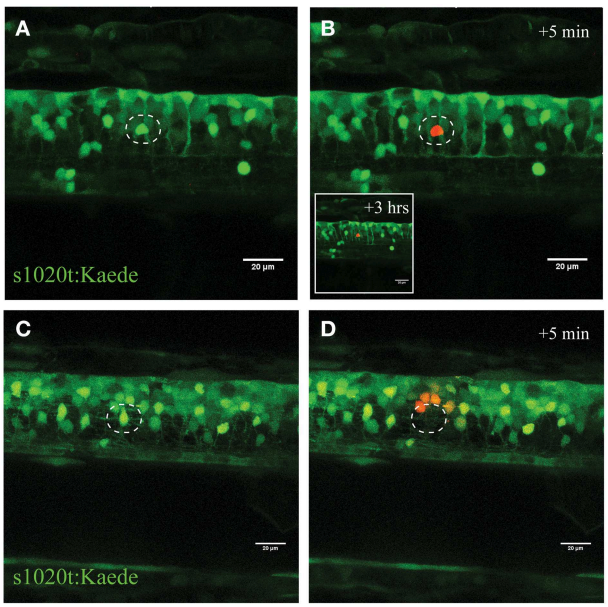

FIGURE 3 | Confirmation of single-cell UV irradiation through the activation of photoconvertable Kaede inside a motor neuron. (A–D) UV irradiation of neurons labeled with the photoconvertable fluorophore Kaede. **(A)** Photoconversion of a single neuron (circle) with a laser power of 30% for 10 s led to photoconversion of Kaede (from green to red) in only the targeted individual neuron (**B**; insert illustrates the intact neuron 3 h post conversion). Ablation of a single neuron (**C**; circle) with a higher laser power (95% for 10 s) resulted in immediate disappearance of that neuron **(D)**, and subsequent photoconversion of Kaede in a small number of surrounding neurons in a radius of approximately 20 μm. Scale bars = 20 μm.

Activated Microglia Engulf and Accumulate Neuronal Debris

To specifically visualize the engulfment of neuronal remnants from dying neurons, we used a triple labeled fish (*islet1:GFP; mpeg1:mCherry; ubiq:secAnnexinV-mVenus*) to visualize this process following our UV ablation approach. AnnexinV-mVenus accumulated in the phagocytic vesicles of the activated microglia after neuronal ablation (**Figure 5**). Over a time frame of approximately 2 hours, AnnexinV accumulated within the cytoplasm of the microglial cell and subsequently was transported away from the ablation site.

Microglia Rapidly Respond to, and Phagocytose Dying Spinal Motor Neurons

In order to characterize the phagocytosis of dying neurons via microglial engulfment in the spinal cord, we generated transgenic lines in which zebrafish expressed together green fluorescent neurons (*islet1:GFP*) and a subset of red microglia (*mpeg1:mCherry*; **Figures 1**, **5**, **6**). The microglial population in these fish showed a ramified morphology that was reminiscent of reports from the (fish) brain (Peri and Nüsslein-Volhard, 2008; Svahn et al., 2013). Consistent with our *mpeg1* fish line that expressed fluorescence in a subset of microglia (Gal4:UAS), UV laser ablation of some neurons did not lead to a response of the fluorescent microglia. In approximately 60% of our ablations we did not observe subsequent microglia engulfment, conceivably because non-fluorescent microglia would have responded to ensure the immediate uptake of the neuronal debris. In successful experiments, time-lapse imaging revealed that soon after UV laser ablation of a single motor neuron, individual microglia underwent dramatic changes in morphology by extending and retracting processes, moving toward the site of the ablated neuron and changing to a spherical shape within minutes (**Figure 6**). The typical first response was for microglia to extend phagocytic protrusions toward the ablated neuron body, therefore shifting the whole microglia body toward the lesion site. This process took on average 27 min until complete engulfment of the ablated soma was achieved (**Table 1**). Notably, this process was characterized by a remarkable increase in the dynamic behavior of the microglia as it moved toward the dying neuron (**Supplementary Video 4**). Within minutes, activated microglia doubled their speed (2.7 μm/min vs. 1.5 μm/min in the "surveying" state; **Table 1**; **Figures 6H–K**), covered substantial distances of 33–94 μm toward the lesion site (72.8 μm on average), and decreased in size significantly to form a round amoeboid body as it engulfed the neuron remnants (128.9 μm^2 vs. 229.4 μm^2 before activation; **Table 1**; **Figures 6H–K**).

Interestingly, occasionally multiple microglia showed clear signs of activation by rushing toward the lesion site. However, in all cases only a single fluorescent microglia (the first) remained

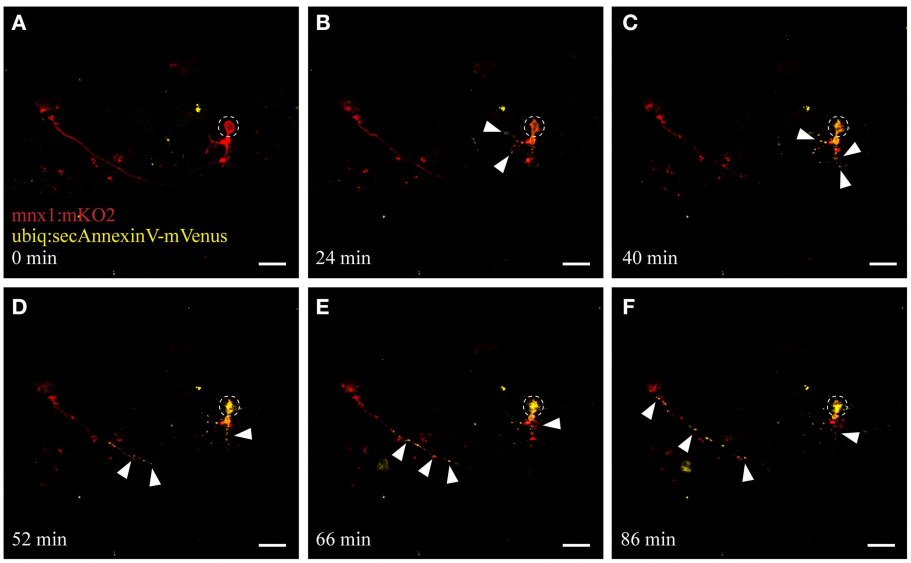

FIGURE 4 | UV ablation activates annexinV-mediated neuronal apoptosis. UV ablation of a single neuron in a transgenic fish expressing the neuronal marker *mnx1:mKO2* as well as the apoptotic marker AnnexinV (*ubiq:secAnnexinV-mVenus*). Shortly, after UV ablation of the soma of the neuron (**A**; circle), phosphatidylserine (PS) gets switched to the outer leaflet of the plasma membrane and trapped by the AnnexinV marker indicated by the yellow fluorescence (**B,C**, arrowheads). Throughout the time-course of neuronal degeneration, AnnexinV puncta (yellow fluorescence) were present within the degenerating cell body **(C,D)** as well as along the axon, progressing anterogradely from the site of ablation **(D–F)**. Scale bars = 20 μm. **Supplementary Video 2** shows the time-lapse video of this process.

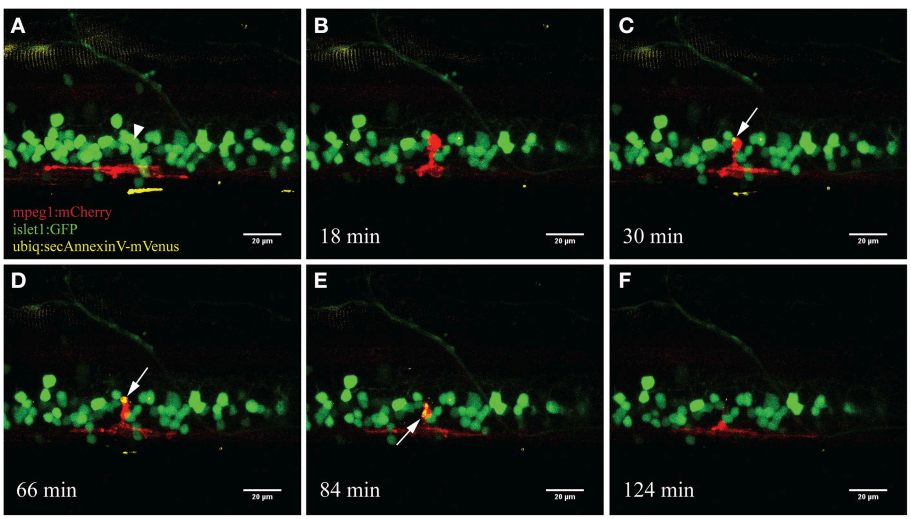

FIGURE 5 | Accumulation of the apoptotic marker AnnexinV within microglia. Upon ablation of a neuron in the spinal cord (**A**; arrowhead) the surrounding microglia moved toward the ablation site **(B)**. After several minutes the fluorescently labeled marker AnnexinV lights up within the microglia (**C**; arrow). AnnexinV accumulation within the microglia increases over time **(D)**, till the phagocytic debris gets transported away from the ablation site **(E)** and conceivably degraded resulting in disappearance of the fluorescence (**F**). Scale bars = 20 μm. **Supplementary Video 3** shows the time-lapse video of this process.

at the site of injury to clear the neuronal debris. Over the period of several hours during which microglial clearance took place (on average 251 min until the microglia moved away again; ±34.6 min; $n = 4$; $N = 4$), other microglia would inspect the site in close proximity without interfering with the ongoing clearance, seemingly as if they were aware of the ongoing phagocytosis by another microglia (**Supplementary Video 4**). The mechanism(s) governing this selective uptake of a dying neuron by a single macrophage in the presence of activation signals remain unknown. Microglia that were clearing a lesion site remained in their activated spherical shape for up to 2 h (average of 86 min; ±22.6 min; $n = 4$; $N = 4$). Upon

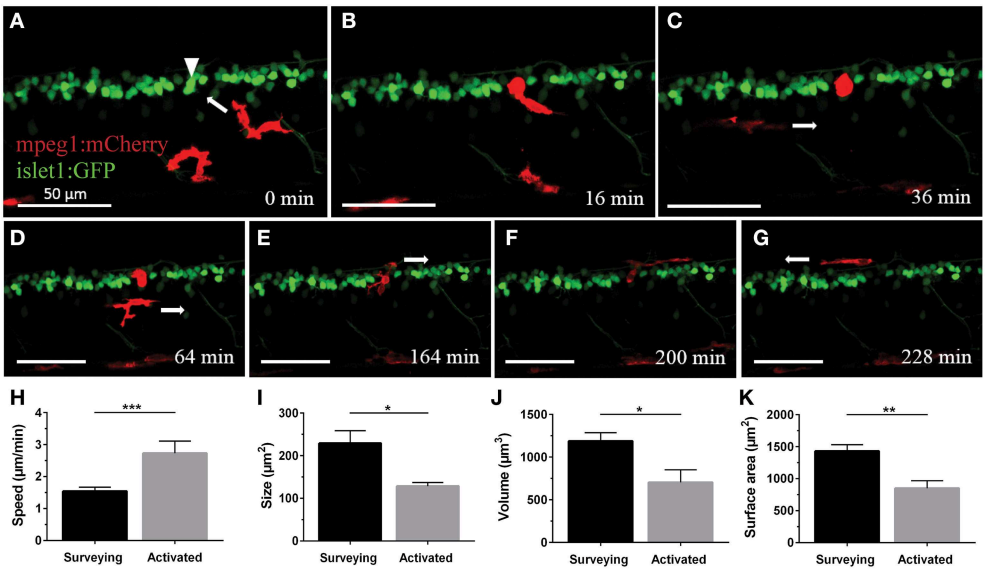

FIGURE 6 | Microglia rapidly respond and are recruited to the site of neuronal ablation. UV ablation of a GFP-expressing spinal neuron (**A**; arrowhead) resulted in only one of two surrounding microglia (red) relocating and phagocytosing the dying neuron **(B,C)**. Over a period of several hours, other microglia pass by the phagocytosing microglia **(D)**, and after approximately 2.5 h the phagocytosing glial cell changes morphology back to a stellate morphology **(E)**, indicating termination of the phagocytosis process **(F,G)**. **Supplementary Video 4** shows the time-lapse video of this process. Scale bars = 50 µm. **(H–K)** Quantitative analysis of morphometric changes in microglial response to dying spinal neurons. Microglia undergo significant changes in morphology **(I–K)** and speed **(H)** upon activation. $*p < 0.05$ paired Student's *t*-test; $n = 6–7$; $N = 6–7$; 2D ImageJ analysis **(H)**; $***p < 0.001$ unpaired Student's *t*-test; $n = 7–22$; $N = 6–9$; 2D ImageJ analysis **(I)**; $*p < 0.05$; $**p < 0.01$ unpaired Student's *t*-test; $n = 4–13$; $N = 4–10$; 3D Imaris analysis **(J,K)**.

completion of clearance the microglial cell transitioned back to a branched morphology by extending processes forth and back again, and migrating away from the ablation site (**Figures 6E–G**; **Supplementary Video 4**).

3D Modeling of Phagocytosing Microglia

Confocal live-imaging alone provides only limited morphometric information upon phagocytosing microglia. Using three-dimensional rendering of time-lapse responses (Imaris), we found repeatedly that phagocytosing microglial cells extend bulbous-like protrusions tipped with phagocytic cups (**Figure 7**). For example, we observed a single microglia engulfing the neuronal remnants of an UV-ablated spinal neuron through formation of a phagosome-like structure (10 µm in size; **Figure 7B**, **Supplementary Video 6**) that cannot readily be resolved through standard confocal microscopy (**Figure 7A**; **Supplementary Video 5**). Importantly, this live-imaging observation is in line with scanning electron microscopy data showing that indeed phagosomes form a tight fitting around apoptotic particles (Krysko et al., 2003, 2006). Collectively, our data strongly demonstrates that microglia (in the zebrafish spinal cord) form bulbous-like phagocytic cups to engulf neuronal remnants, equivalent to the phagocytic behavior of mammalian microglia in the brain. Morphometric rendering analysis of a subset of these microglial cells with Imaris confirmed the speed and distances reported above (**Table 1**). It furthermore revealed that these microglial cells condensed in size during activation, demonstrated by a ~40% reduction in their average volume and surface area (1189.5 μm^3 vs. 703.8 μm^3; 1433.3 μm^2 vs. 852.6 μm^2; **Table 1**; **Figures 6H–K**).

Discussion

We have studied the nature of microglial behavior through live-imaging approaches in the spinal cord of zebrafish. We found that microglia in the spinal cord are dynamic, constantly migrating through the spinal cord, seemingly surveying the environment for signs of disturbance. Accordingly, we show that microglia rapidly respond to the ablation of a single spinal neuron. We establish here that microglia immediately recognize dying neurons (most likely through "eat-me" signals like Phosphatidylserine and AnnexinV recognition) and effectively clear the neuronal debris by engulfing the lesion site. This single-cell resolution analysis combined with 3D rendering methods has uncovered that microglia efficiently ingest apoptotic material via the formation of cup-shaped phagosome-like structures.

Previous work has shown that Mpeg1 is expressed by the early myeloid originating macrophages that colonize the CNS throughout development (Herbomel et al., 2001; Ellett et al., 2011; Svahn et al., 2013). Svahn and colleagues have shown that the Mpeg1-labeled cells are the same macrophages that are under control of the *apoE* promoter and that have been observed in previous zebrafish live-imaging studies (Herbomel et al., 2001; Peri and Nüsslein-Volhard, 2008; Svahn et al., 2013). In our analysis, we observed that a subset of microglia from day 2 onwards actively survey the spinal cord by covering substantial

TABLE 1 | Morphometric characteristics of microglia before and after activation in response to UV-ablated spinal neurons.

	"Surveying state"				"Activated state"				
		SEM	n=	N=		SEM	n=	N=	P
ImageJ ANALYSIS									
Average size microglia in spinal cord (μm^2)	244.4	±15.4	27	21					
Average number of microglia (n)	12	±1.4		7					
Average distance moved per hour (μm)	97.8	±7.7	18	7					
Average size (μm^2)	229.4	±29.3	7	7	128.9	±8.2	6	6	*a
Average speed (μm/min)	1.5	±0.1	22	7	2.7	±0.3	7	7	***b
Average time to engulf (min)					27.3	±1.9	7	7	
Average distance moved toward ablation site (μm)					72.8	±8.7	7	7	
IMARIS ANALYSIS									
Average volume microglia (μm^3)	1189.5	±97.9	13	10	703.8	±148.0	4	4	*b
Surface area (μm^2)	1433.3	±98.0	13	10	852.6	±115.9	4	4	**b
Average speed (μm/min)	1.4	±0.27	4	4	2.5	±0.2	4	4	*b
Distance moved (from start to area ablated, μm)					72.3	±7.5	4	4	

*Numbers of fish were designated as N and number of cells as n. P-values indicate statistical significance ([a]paired t-test; [b]unpaired t-test). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.*

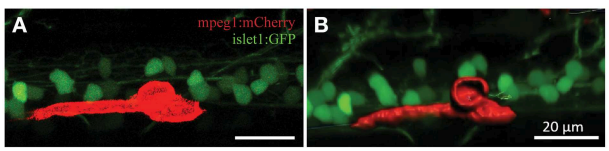

FIGURE 7 | 3D rendering demonstrates dynamic morphological features of activated microglia. Confocal live-imaging alone provides basic morphometric information of microglia phagocytosing an UV-ablated spinal neuron **(A)**. Imaris 3D rendering of this same single microglia revealed the formation of a phagosome-like structure **(B)**, which presumably facilitates engulfment of the neuronal remnants. **Supplementary Videos 5**, **6** show the time-lapse videos of this process for comparison. Scale bars = 20 μm.

distances (98 μm on average per hour). While we cannot exclude that temperature and/or anesthetics had an influence on microglial motility and function, other *in vivo* studies in mice and zebrafish have also reported a highly dynamic nature of microglia branching with speeds similar to what we have shown here (1.47 μm/min, Nimmerjahn et al., 2005; 2.5 μm/min, Peri and Nüsslein-Volhard, 2008; 0.3 μm/min, Svahn et al., 2013).

To study microglial responses to dying neurons in real-time, we used a UV laser ablation technique to induce targeted neuronal injury. Photoconversion experiments of the Kaede fluorophore revealed that this method induces dose-dependent cell death with little scattering to the surrounding tissue (**Figures 2**, **3**; **Supplementary Figure S1**). Different types of laser have been used in previous zebrafish studies to induce injury to the kidney, heart, and brain as well as prompting thrombosis (Jagadeeswaran et al., 2006; Johnson et al., 2011; Sieger et al., 2012; Matrone et al., 2013). In Drosophila and epithelial cells, Soustelle and colleagues applied a very similar UV ablation approach to the one described here (Soustelle et al., 2008). However, we are not aware of any studies that have selectively demonstrated single-cell stress/death through UV-irradiation in the zebrafish spinal cord. While most studies have not characterized the type of (programmed) cell death they elicited through laser ablation, Matrone and colleagues reported histological changes suggestive of necrosis and apoptosis following localized laser injury of the heart (Matrone et al., 2013). Other studies demonstrated increased caspase activity and TUNEL-staining in the developing zebrafish embryo after severe stress conditions such as UV irradiation (Yabu et al., 2001). Accordingly, our studies using single-cell resolution imaging of spinal motor neurons confirmed the anterograde degeneration of the UV targeted neurons, and revealed rapid recruitment of AnnexinV, a marker of phosphatidylserine-positive apoptotic cells within minutes after ablation (**Figure 4**; **Supplementary Video 2**). Throughout the next hours the formation of PS-positive fragments became obvious along the axonal projections. Equally, cellular blebbing (fragmentation of cellular bodies) at the soma and the axons occurred consistently in our experiments when we stressed the neuron with intermediate laser power. Based upon these parameters, we demonstrate that the programmed cell death that we have triggered with our UV ablation approach has characteristic features of apoptotic cell death.

Microglia undergo complex interactions with other glial cells and neurons upon stress or injury. Most studies have focused upon microglial behavior in the brain during development or after injury. In our work, we have focused on the spinal cord of the zebrafish and have developed a robust method to study the behavior of microglia under normal physiological conditions. We show that after UV ablation the apoptotic marker AnnexinV accumulates in the phagocytic vesicles of the activated microglia (**Figure 5**). Importantly, when we selectively trigger microglial activation to visualize the immediate response to neuronal death, we show that microglia increase their speed significantly upon detection of neuronal injury, doubling their speed for a short period of time to reach the site of injury (**Figure 6**; **Table 1**).

Since our approach allows the observation of microglial behavior before and after manipulation in the same experiment, we propose that this acceleration in movement is a clear example of specific activation of this microglia. It is important to note that our studies utilized an approach incorporating mosaic fluorophore labeling of microglia. Accordingly, in about 60% of imaging studies we did not observe a microglia phagocytose a neuron, presumably because the microglia was non-labeled. This indicates that the imaging itself did not activate the labeled microglia. While noting that there might be differences between microglial behavior in the brain vs. the spinal cord, our data are in accordance with a previous study in zebrafish brain which reported microglial migration rates of 2.5–3.5 μm/min toward apoptotic cells (Mazaheri et al., 2014).

An important advancement of our study is the ability to observe the response of individual microglia to the degeneration of a single spinal motor neuron. Our experiments consistently reveal that it is always only one microglia that ultimately phagocytoses the neuronal debris of the dying neuron. Indeed, following ablation of a single spinal neuron we have observed rapid recruitment of a single microglia, yet other microglia rush toward the lesion site, stop or slow down in close proximity but then pass by, seemingly as if they know that the degenerating neuron is already engulfed by another glial cell (**Figure 6**; **Supplementary Video 4**). This behavior is a level of complexity above the chemotactic diffusion model of attraction that has been reported previously, where injections of a bolus of ATP into the brain attract multiple microglia to the site of injection (Davalos et al., 2005; Sieger et al., 2012). Our studies suggest a higher order of regulation, such that microglia can sense when they are required to respond to a dying neuron or when no response is required. It will be interesting to differentiate the different molecular profiles of "resting" and "activated" glial cells in more detail in future studies.

The morphometric characteristics of microglial phagocytosis have primarily been identified through histological approaches. Although other studies have previously reported the (two-dimensional) formation of phagosome-like structures during microglial ingestion (Peri and Nüsslein-Volhard, 2008), the three-dimensional visualization of these phagosomes has, to the best of our knowledge, not been demonstrated in live-imaging studies. Precise visualization of the process of internalization of apoptotic bodies by macrophages was limited to scanning electron microscopy studies (Krysko et al., 2006). By applying 3D rendering techniques we consistently observed the formation of tight-fitting phagosome-like structure around the soma of dying neurons (**Figure 7**; **Supplementary Video 6**). Thus, this method provides a significant advancement in the visualization of live-imaging microglial phagocytosis to unambiguously demonstrate the formation of a phagosomal cup-shaped structure to capture neuronal fragments.

Combination of the visualization and ablation techniques with markers of inflammation in future studies will further advance our knowledge of microglia activation and homeostasis. Understanding these processes is critical as microglial clearance is symptomatic for many neurodegenerative diseases including motor neuron disease, where glial activation has been shown to contribute to the death of motor neurons (Philips and Robberecht, 2011).

Author Contributions

Conceived and designed the experiments: MM and RC. Performed the experiments: MM and RR. Analyzed the data: MM and RR. Contributed reagents/materials/analysis tools: MM, RR, RC, AL, ED, AB, TH, and NC. Provided technical assistance: MM, RR, RC, AL, ED, AB, TH, and NC. Wrote the paper: MM and RC. All authors contributed to revision of the article, and approved the final version of the manuscript.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fncel.2015.00321

Supplementary Video 1 | A GFP-expressing spinal motor neuron is UV ablated, resulting in the rapid loss of GFP from the soma and blebbing of the axonal remnants (video time course is 90 min, 5 min per frame).

Supplementary Video 2 | A single spinal motor neuron (red) was targeted by UV laser ablation. The ubiquitously expressed apoptotic marker AnnexinV (yellow) appears at the ablation site within ∼12 min, indicating induction of apoptotic-like cell death in this spinal neuron (4 min/frame, overall duration ∼2 h).

Supplementary Video 3 | After UV ablation of a spinal neuron (green) a fluorescently labeled microglia (red) gets activated to take up the neuronal debris via phagocytosis. Our apoptotic reporter fish *ubiq:secAnnexinV-mVenus* shows consequently the accumulation and transport of the fluorescently labeled apoptotic debris (yellow) within the microglia.

Supplementary Video 4 | UV ablation of a GFP-expressing spinal cord motor neuron (green) results in immediate microglia (red) response. One of the two surrounding microglia quickly relocates toward the ablated neuron and starts to phagocytose the remnants of the dying neuron. This activated microglia displays a profound change in morphology (from a ramified appearance to a spherical shape). After approximately 2.5 h the phagocytosing glial cell reverts back to a ramified morphology, indicating termination of the phagocytosis process. Interestingly only one microglia actively phagocytoses the dying neuron while other microglia pass by and ignore the site of neuronal ablation, even after the phagocytosing microglia has departed (6 min/frame, overall duration ∼10 h).

Supplementary Video 5 | UV ablation of a single spinal cord motor neuron (green) triggers an immediate microglia (red) response. A single microglia migrates towards the ablation site and phagocytoses the neural remnants (5 min/frame, overall duration ∼3.5 h). The 3D rendered version can be found at **Supplementary Video 6**.

Supplementary Video 6 | Imaris 3D-rendering of the same microglia response shown at Supplementary Video 5. This method clearly reveals the formation of a phagosome-like structure (5 min/frame).

Supplementary Figure S1 | UV laser irradiation induces neuronal damage in a dose-dependent manner. UV laser ablation with different laser power (**A**: 15%; **B**: 30%; **C**: 60%, and **D**: 90%) of the s1020t:Kaede expressing neurons (green) in the spinal cord shows the focal spread of the UV laser, indicated by the photoconversion (red). Using a low laser power **(A)** results in focused Kaede photoconversion (from green to red fluorescence) limited to a single targeted neuron (upper row: lateral view; lower row: dorsal view). Higher laser power **(B–D)** results in radial diffusion of the UV laser of approximately 40–50 μm. All laser ablation sites were conducted using a circular region of interest of ∼1.5 μm in diameter, focused upon an individual soma of a neuron, with a laser dwell time of ∼60 seconds in each experiment. Scale bar = 20μm.

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frontiers in Cellular Neuroscience

REVIEW
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Microglia are crucial regulators of neuro-immunity during central nervous system tuberculosis

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Mycobacterium tuberculosis (*M. tuberculosis*) infection of the central nervous system (CNS) is the most devastating manifestation of tuberculosis (TB), with both high mortality and morbidity. Although research has been fueled by the potential therapeutic target microglia offer against neurodegenerative inflammation, their part in TB infection of the CNS has not been fully evaluated nor elucidated. Yet, as both the preferential targets of *M. tuberculosis* and the immune-effector cells of the CNS, microglia are likely to be key determinants of disease severity and clinical outcomes. Following pathogen recognition, bacilli are internalized and capable of replicating within microglia. Cellular activation ensues, utilizing signaling molecules that may be neurotoxic. Central to initiating, orchestrating and modulating the tuberculous immune response is microglial secretion of cytokines and chemokines. However, the neurological environment is unique in that inflammatory signals, which appear to be damaging in the periphery, could be beneficial by governing neuronal survival, regeneration and differentiation. Furthermore, microglia are important in the recruitment of peripheral immune cells and central to defining the pro-inflammatory milieu of which neurotoxicity may result from many of the participating local or recruited cell types. Microglia are capable of both presenting antigen to infiltrating $CD4^+$ T-lymphocytes and inducing their differentiation—a possible correlate of protection against *M. tuberculosis* infection. Clarifying the nature of the immune effector molecules secreted by microglia, and the means by which other CNS-specific cell types govern microglial activation or modulate their responses is critical if improved diagnostic and therapeutic strategies are to be attained. Therefore, this review evaluates the diverse roles microglia play in the neuro-immunity to *M. tuberculosis* infection of the CNS.

Keywords: microglia, *Mycobacterium tuberculosis*, central nervous system, cytokines, chemokines, neurotoxicity, neuroprotection, tuberculosis meningitis

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Abbreviations: BCG, Bacillus Calmette-Guérin; CCL, chemokine C-C motif ligand; CD, cluster of differentiation; CNS, central nervous system; CNS-TB, tuberculosis of the central nervous system; CSF, cerebrospinal fluid; CXCL, C-X-C motif chemokine; DC-SIGN, dendritic cell-specific intercellular adhesion molecule grabbing nonintegrin; ERK, extracellular-signal-regulated kinases; G-CSF, granulocyte colony stimulating factor; GM-CSF, granulocyte macrophage stimulating factor; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; IL, interleukin; MCP, monocyte chemotactic protein; MHC, major histocompatibility complex; MMP, matrix metallometalprotease; *M. tuberculosis*, *Mycobacterium tuberculosis*; NADPH, nicotinamide adenine dinucleotide phosphate; NOS, Nitric Oxide Synthase; PRR, pattern recognition receptor; ROS, reactive oxygen species; $sPLA_2$, secretory phospholipase A_2; TB, Tuberculosis; TLR, toll like receptor; TNF, Tumor necrosis factor; ICAM, intracellular adhesion molecule.

Introduction

Microglia, from the literal Greek "*small glue*", are one of three glial cell types found within the brain and spinal cord. As arguably the most prominent immune effector cells of the central nervous system (CNS), microglia simultaneously exhibit a potential for neurotoxicity. Found in numbers comparable to neurons, microglia comprise 0.5–16.6% of the total human CNS cell population, varying by anatomical site and with higher densities in white matter (Mittelbronn et al., 2001). Microglia are isolated within the cerebral parenchyma by the blood-brain barrier (BBB), thereby serving as the first line of defense against intra-cerebral infections such as *Mycobacterium tuberculosis* (*M. tuberculosis*), causative pathogen of tuberculosis (TB).

It is known that TB in the CNS (CNS-TB) generates host-orchestrated tissue destruction by infiltrating monocytes (Price et al., 2001; Lee et al., 2004). Although defined as the facultative phagocytic myeloid cells of the CNS, microglia are distinct from the macrophages located within the subarachnoid space, choroid plexus, meninges and perivascular spaces (Mittelbronn et al., 2001; Guillemin and Brew, 2004). Differential molecular expression, a unique "spiny" morphology, an experimentally-useful resilience to ionizing radiation and a unique blend of the phagocytic behavior of innate immune cells with the trophic nature of glia, define microglia as being a highly unique cell-type (Flaris et al., 1993; Ulvested et al., 1994; Giulian et al., 1995; Aarum et al., 2003; Guillemin and Brew, 2004; Balentova et al., 2015). This creates difficulty in relating the well-studied interactions between macrophage and *M. tuberculosis* of the periphery to the role microglia might play in CNS infections—an interaction which has, unfortunately, formed the basis of our understanding of microglia in CNS-TB infection to date.

Polarized views of beneficial and harmful results of active microglia have not clarified the perception of the diverse roles of microglia during CNS pathology (Glezer et al., 2007; Hanisch and Kettenmann, 2007; Sierra et al., 2013). On one hand, microglia are viewed positively as the initiators and sustainers of acute neuroinflammation where they are responsible for pathogen identification, the subsequent clearance of infection, insult repair and the restoration of cerebral homeostasis. Such trophic roles stand in stark contrast to their capacity for robust immune activation and their accountability for the consequent neuropathology of chronic inflammation. Therefore, this review aims to describe and critically evaluate the potential roles of microglia in the pathogenesis of *M. tuberculosis* infection of the CNS, a better understanding of which is critical for improved diagnostic and therapeutic technologies.

Tuberculosis of the Central Nervous System

In 2013, an estimated 1.5 million people succumbed to TB, making it second only to HIV as the largest cause of infectious mortality. Global incidence remains high, estimated at 9 million cases (World Health Organisation (WHO), 2014). One in three people are thought to be latently infected, carrying a lifetime risk of developing active, transmissible disease. Extra-pulmonary TB accounts for 15–20% of all cases prior to the HIV pandemic (Mehta et al., 1991; Kulchavenya, 2014). Approximately 3–10% of all extra-pulmonary TB cases in developed countries exhibit CNS involvement (Rieder et al., 1990; Houston and Macallan, 2014), with far higher prevalence likely in developing countries bearing the brunt of the HIV pandemic (Berenguer et al., 1992; Leeds et al., 2012). Although CNS-TB represents just 1% of the global TB burden (Cherian and Thomas, 2011), it is the severest form of TB owing largely to its difficulty in diagnosis (Karstaedt et al., 1998; Marais et al., 2010), and high mortality and morbidity even after appropriate management (Afghani and Lieberman, 1994; Cheng et al., 2002)—with children and the immunosupressed being vulnerable, yet not presenting atypically (Dubé et al., 1992; Farinha et al., 2000; Nelson and Zunt, 2011). In African adults, approximately one in three cases of bacterial meningitis is attributable to *M. tuberculosis* infection, with fatality in almost two out of every three patients (Woldeamanuel and Girma, 2013).

The propensity for disseminated disease depends upon both bacterial and host-specific factors. Two retrospective studies found significant associations between strain-patterning and CNS infection (Arvanitakis et al., 1998; Click et al., 2012), whilst a Brazilian study using Restriction Fragment Length Polymorphism analysis concluded that risk factors for dissemination are more host-dependent (Gomes et al., 2013). A meta-analysis concluded that age, sex, and lifestyle habits, in addition to immunological factors (but not, interestingly HIV status), contributed towards the probability of extra-pulmonary TB (Webster and Shandera, 2014). This indicates that host factors are also critical to the pathogenesis of extra-pulmonary TB, as disease often results from either exaggerated or inefficient host-responses. Therefore, studying the role of host cells, such as microglia, is just as important as studying the pathobiology of the infectious agent.

Clinically, CNS-TB has been studied through the use of various imaging techniques, resulting in a hierarchical classification system. Primary classification is on the basis of the infection affecting the spinal cord or the cerebrum, then further sub-classified by the diffuse or localized nature of the infection, and finally, anatomically by the precise nidus of infection (Jinkins et al., 1995; Bernaerts et al., 2003). TB meningitis (TBM) is a diffuse infection of the leptomeninges, characteristically affecting the brain in a basal fashion (Thwaites and Hien, 2005). However, infection of the pachymeninges has also been described (Bernaerts et al., 2003). Direct infection of the brain parenchyma does occur, during which the *M. tuberculosis* bacilli breech the BBB. Localized infections of the parenchyma may result in a tuberculoma, an abscess, or focal cerebritis, whilst more diffuse parenchymal infections are, by definition, encephalitic. Such a diverse spectrum of cerebral infections has been explained through a unifying pathogenic theory, built largely upon the seminal studies by Rich et al. who posited that, following hematogenous deposition of bacilli into the parenchyma, the subsequent tuberculoma ruptures into the cerebral spinal fluid and adjacent brain structures

become infected (Rich and Mccordock, 1933; Donald et al., 2005). However, many questions are left unanswered. For instance, what are the differential host mechanisms regulating BBB penetration by *M. tuberculosis*, and, if the parenchyma is required to be infected prior to cerebral dissemination, what immunological factors could potentially be mediating the disease. These questions lead one to consider the most renowned immunological effector cells of the CNS, the microglia, as the missing link in this paradigm.

Microglia

Origin and Maintenance

Historically, microglia were considered derivatives of invading pia, or malleable neuroectodermal elements (Rezaie and Male, 2002). Subsequent studies recognized their origin from mesoderm; borne from bone-marrow progenitors that seed the brain parenchyma (Hess et al., 2004). The resemblance of microglia to macrophages in surface antigen expression, as well as both phagocytic and cytotoxic effector functions hinted, particularly, at a myeloid origin. Although many experiments failed to provide definitive proof of such a myeloid heritage, mice lacking the myeloid-specific transcription factor *PU1.1* also lacked microglia (Beers et al., 2006). Later, primitive microglia were identified as erythromyeloid precursors arising from the yolk sack very early in embryogenesis (Alliot et al., 1999; Ginhoux et al., 2010; Schulz et al., 2012; Kierdorf et al., 2013). It was originally hypothesized that continual replenishment of the microglia population occurred into adulthood via peripheral recruitment of circulating monocytes, followed by subsequent differentiation steps. However, although murine monocytes have been shown to invade the CNS amidst insult (Andersson et al., 1992) and microglia demonstrate the potential to differentiate into either CNS-macrophage or dendritic cell profiles *in vitro* (Santambrogio et al., 2001), evidence for monocyte to microglial differentiation in the developed CNS is lacking (Ajami et al., 2007; Ginhoux et al., 2010). Thus, these studies suggest that microglia, upon successful CNS-seeding of their progenitors in early development, act as an independent, self-renewing population into adulthood (Ginhoux et al., 2010).

Populations and Phenotypes

Microglial cells may be classified by location or functional morphology. Juxtavascular microglia, which contribute to the glia limitans by incorporating processes between those of astrocytes, are found adjacent to and migrate along penetrating cerebral arteries (Lassmann et al., 1991; Grossmann et al., 2002; Mathiisen et al., 2010). Microglia not in contact with the CNS microvasculature contribute to the parenchymal population. Perivascular antigen-presenting macrophages, ensheathed within the basal lamina and replenished by bone marrow progenitors (Hickey and Kimura, 1988; Hickey et al., 1992), are sometimes referred to as perivascular "microglia." The opinion that true microglia are "macrophages of the CNS" is perhaps simplistic; advances in monocyte-macrophage immunology, such as the introduction of the M1-M2 paradigm or classical vs. alternative activation have been extrapolated from peripheral to central (i.e., microglial) immunological processes (Mittelbronn, 2014).

Nevertheless, microglial morphological plasticity reflects a specific yet stereotypical, graded spectrum of functional states. Ramified or "branched" microglia are a resting but highly active, baseline phenotype continually palpating the local microenvironment with cytoplasmic processes; searching for pathogens, signs of injury or homeostatic disturbances. Ramified microglia are well known for their potential to up-regulate the constitutive expression of both major histocompatibility complex (MHC) classes, amongst many other immune molecules (Leong and Ling, 1992; Ford et al., 1995; Olah et al., 2011). Activated microglia (upon encounter of injury or pathogen) typically display an amoeboid phenotype through cytoplasmic contraction. Such microglia are defined functionally by migration to the site of interest (Carbonell et al., 2005), proliferation (Giordana et al., 1994), discretional phagocytosis of self or non-self constituents (Magnus et al., 2001; Rogers and Lue, 2001; Shams et al., 2003), cytokine and chemokine expression (Hanisch, 2002), and induction of reactive oxygen species (ROS; Colton et al., 1996; Wang et al., 2004; Long et al., 2006). Such morphological diversity is further amplified by regional variations in molecular expression (de Haas et al., 2008), and evidence suggesting that microglial activation may simultaneously generate an immune-regulatory phenotype (Liao et al., 2012; Selenica et al., 2013).

Microglia-*Mycobacterium tuberculosis* Interactions

Microglia in Context: the Cellular and Biochemical Milieu

Microglia rely heavily on a complex system of *in vivo* signals from the surrounding cellular and biochemical milieu for both activation and modulation. Although microglia are the principal CNS cells infected by *M. tuberculosis* (Peterson et al., 1995a; Rock et al., 2005; Yang et al., 2007), other CNS-specific cells that display potential for *M. tuberculosis* infection include astrocytes and neurons (Rock et al., 2005; Randall et al., 2014). When human astrocytes and microglia were challenged with *M. tuberculosis in vitro*, Rock et al. observed a 15% and 76% bacilli uptake respectively (Rock et al., 2005). Teasing apart these complex cellular interactions, both amongst the different CNS cell-types and with the immune system, remains an outstanding step towards fully understanding the molecular pathogenesis of CNS-TB.

The most striking feature of microglial activation in general is the rapidity at which it occurs, suggesting a potential role of diminishing neuronal inhibitory signals in producing swift immune responses. Randall et al. (2014) were the first to observe that neurons can be infected by *M. tuberculosis* and, in response, contribute immunologically to the inflammatory state by secreting IL-1β, IL-6, and IL-10 (Randall et al., 2014). Although neither the extent to which this occurs *in vivo*

during human CNS-TB pathogenesis nor the consequences of such infections on neuronal electrical or immunological signaling has been fully investigated, this introduces the possibility that altered neuron-microglia interactions (either in diminishing inhibitory signals, or increasing activation signals) as well as the direct participation of other CNS cell-types promote the pro-inflammatory milieu that drives chronic inflammation and culminates in subsequent host-pathology.

Mycobacterium tuberculosis Recognition, Internalization and Microglial Activation

Microglia possess a unique repertoire of innate-immune and neuro-specific receptors, including pattern-recognition receptors (PRRs); the broad class of molecules used to identify pathogen-associated molecular patterns for self vs. non-self distinction. Although some of these receptors are of importance in macrophage recognition of *M. tuberculosis*, further experimental evidence is required to confirm their role in the microglial response to *M. tuberculosis* (**Table 1**).

Internalization of *M. tuberculosis* bacilli by human microglia is dependent on CD14 – a monocyte differentiation antigen which binds to lipopolysaccharide (LPS) with Toll-like receptor 4 (TLR4; Wright et al., 1990). Peterson et al. (1995a) observed a 64% and 62% reduction in non-opsonized tubercle bacilli uptake in the presence of anti-CD14 monoclonal antibodies and soluble CD14 ligand, respectively (Peterson et al., 1995a). On the other hand, Shams et al. (2003) found that CD14 does not mediate entry of *M. tuberculosis* into human peripheral blood mononuclear cells (Shams et al., 2003), while others observed a CD14-dependant and regulated internalization of *M. bovis* (Khanna et al., 1996; Sendide et al., 2005). Dectin-1 and TLR2 have been recognized as key mediators of macrophage activation by *M. tuberculosis* (Yadav and Schorey, 2006). Yang et al. used combinations of well-characterized TLR2 antigen, dectin-1 antagonists and TLR2-deficient mice to show that *M. tuberculosis* bacilli recognition by microglia occurs via an as yet unidentified pathogen recognition mechanism involving identification of a heat-stable *M. tuberculosis* bacilli antigen (Yang et al., 2007). Such recognition could possibly be orchestrated by other PRR's or perhaps activate through alteration of microglial-specific ion channels (Kettenmann et al., 1990; Prinz et al., 1999), which have been shown to be modulated by both cytokine signals and pathogen associated molecular patterns such as LPS (Nörenberg et al., 1994). Interestingly, Lambert et al. has shown the induction of dendritic cell-specific intercellular adhesion molecule grabbing nonintegrin (DC-SIGN) in human microglia when treated with GM-CSF, IL-4, and LPS (Lambert et al., 2008). DC-SIGN is a known PRR expressed by DC as part of the innate immunity for the recognition of *M. tuberculosis* (Tailleux et al., 2003), therefore one cannot exclude the potential recognition of *M. tuberculosis* by the induced microglia using DC-SIGN.

Whilst most pathogens attempt to avoid host immunity, it is generally accepted that tubercle bacilli actively seek internalization by host macrophages in which they have developed strategies to survive. Of all the parenchymal CNS cell types, microglia could, theoretically, demonstrate preferential infection by *M. tuberculosis* due to their similarity with monocytes, as evidenced by their tendency to associate with more bacilli per cell than astrocytes (Rock et al., 2005). Microglia internalize virulent *M. tuberculosis* more rapidly and efficiently than less virulent strains. Upon internalization, tubercle bacilli are found in sparse, but densely packed "vacuoles" (Curto et al., 2004). A number of studies report *M. tuberculosis* bacilli retaining reproductive potential within infected microglia; providing a cerebral niche for persistence and a possible mechanism for subsequent reactivation should a state of immune-suppression be acquired (Peterson et al., 1995b; Curto et al., 2004; Cannas et al., 2011).

To illustrate the complexity of immune-modulatory signals, microglia have diminished phagocytic capacity when treated with anti-CD14 antibodies, opiate antagonists and pertussis toxin; indicating a G-protein dependent mechanism (Peterson et al., 1995b). Although opiate abuse has been associated with CNS-TB development, Peterson et al. (1995b) observed an enhanced phagocytic capacity of primary fetal microglia when pre-exposed to a morphine concentration of 10^{-}M—reporting a higher proportion of phagocytically active microglia and greater *M. tuberculosis* burdens (Peterson et al., 1995b). Mu receptors have also been implicated in the control of microglial chemotaxis, suggesting that morphine's anti-inflammatory action is due to a reduction in microgliosis rather than diminished microglial activity (Chao et al., 1997).

Microglial activation by *M. tuberculosis* has been largely studied through cytokine and chemokine as opposed to morphological or transcriptional responses. Messenger RNA and protein expression studies by Qin et al. (2015) suggest that a classically activated, M1 phenotype is induced and an M2-like phenotype reduced in microglia following exposure to *Mycobacterium*-challenged macrophage culture medium (Qin et al., 2015). Although such studies need to be verified in a human model, several important observations can be made. Firstly, this illustrates that microglia, even when not directly infected, can be activated and respond immunologically to infections by tubercle bacilli. Secondly, macrophage infection by *M. tuberculosis* may not only incite a pro-inflammatory microglial phenotype, but their persistent infection may prevent either the conversion or reversion of microglia to a more M2-like phenotype. This has further potential applications to the underlying pathophysiology of CNS-TB as macrophage infection could: precede CNS-parenchymal infection; contribute towards a breakdown of the BBB for peripheral immune recruitment or enhance bacilli breech; contribute towards either the activation or persistence of a pro-inflammatory milieu through direct communication with microglia.

Furthermore, patterns of microglial activation differ not only between organisms, but also between virulent and avirulent strains of mycobacteria—revealing the high degree of specificity with which microglia respond to pathogens (Curto et al., 2004; Cannas et al., 2011). Curto et al. challenged the "all-or-nothing" dogma of microglial activation by observing a stronger inhibition of both IL-1 and IL-10 in *M. tuberculosis*-microglial

TABLE 1 | Correlation between innate receptors on macrophages and microglia that have demonstrated importance in *M. tuberculosis* infections.

Microglial PRR	Macrophage recognition of *M. tuberculosis*	Microglial recognition of *M. tuberculosis*	References
TLR2	+	Unknown	Drennan et al. (2004) and Kielian et al. (2005)
TLR4	+	Unknown	Abel et al. (2002)
TLR9	+	Unknown	Bafica et al. (2005)
CD14	–	+	Peterson et al. (1995a), Means et al. (1999), and Shams et al. (2003)
CR3 (CD11b/CD18)	+	Unknown	Melo et al. (2000)

Comparing the experimentally determined importance of innate immune receptors in M. tuberculosis infection of macrophages and microglia. "+" indicates confirmed importance, "–" indicates no importance determined whilst "unknown" indicates a lack of experimental evidence altogether

infections with more virulent strains (Curto et al., 2004). This finding suggests that *M. tuberculosis* infection initiates a rigorous transcription profile that enhances the expression of certain molecules whilst simultaneously suppressing the expression of others. Therefore, microglial effector mechanisms are tightly regulated, pathogen-specific responses that appear to also be virulence-specific, and such effector profiles, or a dysregulation in certain profiles, may yet be correlated with either a propensity to neuropathology, or a heightened resistance to it.

Cytokine Effector Responses Orchestrated by Microglia

Microglia are known to be capable of secreting a wide range of cytokines and chemokines (**Figure 1**). The cytokine levels in TBM are distinct from meningitis caused by other microbes (Mastroianni et al., 1998). Studies in cerebrospinal fluid of patients with CNS-TB indicate significantly elevated concentrations of sTNFR-75, sTNFR-55, IFN-γ, and IL-10, and persistently elevated levels of TNF-α—even following therapeutic interventions (Mastroianni et al., 1997). Other immunological molecules, confirmed experimentally, to be secreted by microglia following *M. tuberculosis* stimulation include: IL-1α, IL-1β, IL-6; IL-10, IL-12p40, TNF-α, G-CSF, GM-CSF, CCL2, CCL5, and CXCL10 (Curto et al., 2004; Rock et al., 2005; Yang et al., 2007; Cannas et al., 2011; **Table 2**). In comparison, Rock et al. found *M. tuberculosis*-challenged astrocytes to have a much narrower cytokine-chemokine response—detecting modest levels of CXCL10 only (Rock et al., 2005).

TNF, a pro-inflammatory cytokine, demonstrates paradoxically destructive and protective roles in CNS and peripheral *M. tuberculosis* infection, owing to transmembrane or soluble forms binding to either of two receptors. Microglia encountering *M. tuberculosis*, generate an initial burst of TNF-α, followed by a sustained decline thereafter (Curto et al., 2004). TNF-α acts on other microglia, possibly in an autocrine fashion, to induce NADPH production of H_2O_2, and thus drive microglial proliferation—an attempt either to promote neuroinflammation or limit its sequelae (Mander et al., 2006). TNF-α has been shown to have both protective (Nawashiro et al., 1997) and harmful (Barone et al., 1997) effects in cerebral ischemia, and alone induces necrotic changes in cerebral endothelial cells. After which, microglia could contribute towards forming a secondary BBB (Claudio et al., 1994). Multi-nucleated giant cell formation following *M. bovis* is initiated by TNF-α in swine microglia (Peterson et al., 1996), making TNF-α a critical contributor to the formation of tuberculomas and the subsequent seclusion of mycobacteria. TNF-α induces expression of chemokines within the CNS, including intercellular adhesion molecule (ICAM-1), important in leukocyte recruitment to the brain during bacterial infection (Engelhardt et al., 1994). Although TNF-α appears to be the most potent ICAM-1 inducer within the CNS, it acts in concert with other molecules in the recruitment of leukocytes into the nervous system (Claudio et al., 1994; Shrikant et al., 1994; Glabinski et al., 2003). Interestingly, neurotoxicity has been attributed to the synergism of TNF-α with IL-1β on NO generation by astrocytes (Chao et al., 1995). Moreover, the importance of TNF has been further supported by the neutralization study, leading to *M. tuberculosis* dissemination causing severe CNS-TB (Seong et al., 2007; Lynch and Farrell, 2010).

IL-1α and IL-1β, members of the pyrogenic IL-1 family of cytokines, are both believed to act through IL-1RI with an accessory protein, and compete for binding with IL-1Rα. Although secreted, a strong inhibition of IL-1α expression was observed when microglia were infected with more virulent mycobacteria (Curto et al., 2004; Cannas et al., 2011). Given the importance of IL-1α in a pulmonary granulomatous response (Kasahara et al., 1988), both the initial parenchymal cell type and the virulence of the mycobacteria infection may dictate the IL-1α levels within the CNS, and thus define both the form and course of the infection. IL-1β has also been shown to be secreted by both neurons and microglia upon encountering *M. tuberculosis* (Cannas et al., 2011; Randall et al., 2014), and is known to induce microglial proliferation via the same mechanism as TNF-α (Mander et al., 2006). Recombinant IL-1β injected into rat brain induces both astrocyte proliferation and stimulates blood vessel growth (Giulian et al., 1988), and has been shown to initiate transcription of type II NOS (Liu et al., 1996).

Astrocytes play an important role in complementing and counteracting the adverse effects of IL-1α and IL-1β by secreting G-CSF and GM-CSF in response (Tweardy et al., 1990). Murine

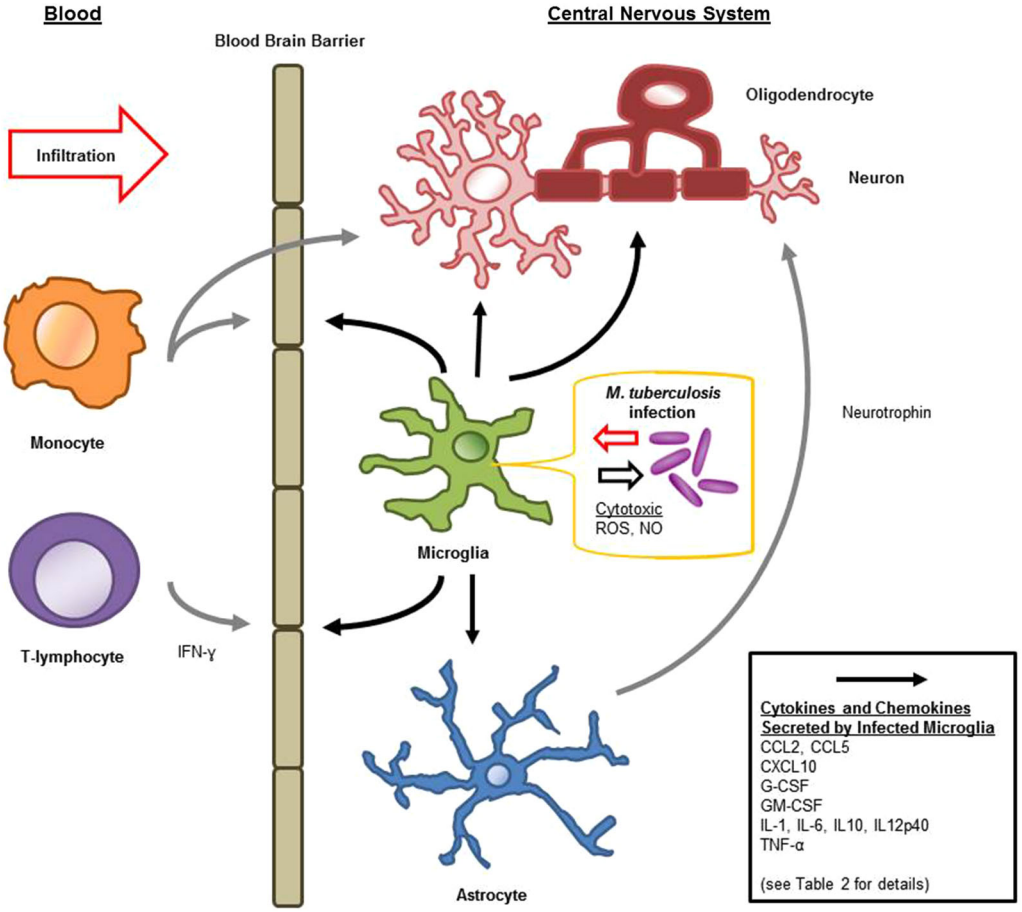

FIGURE 1 | Schematic diagram of microglial immune responses and interactions during *M. tuberculosis* infection in the central nervous system (CNS).

TABLE 2 | Experimentally confirmed cytokine and chemokine expression by microglia during *M. tuberculosis* infection summarizing the experimental evidence for microglial cytokine secretion in response to *M. tuberculosis* stimulation.

Molecule	Microglia line	*M. tuberculosis* strains	Experimental model	Reference
CCL2/ MCP1	Human Fetal	H37Rv	Human	Rock et al. (2005)
CCL5/RANTES	Human Fetal	H37Rv	Human	Rock et al. (2005)
CXCl10	Human Fetal	H37Rv	Human	Rock et al. (2005)
G-CSF	Murine BV-2	H37Rv	Murine	Cannas et al. (2011)
GM-CSF	Murine BV-2	H37Rv	Murine	Cannas et al. (2011)
IL-1	Human Fetal	N.C 0741708	Human	Curto et al. (2004)
IL-1α	BV-2	H37Rv	Murine	Cannas et al. (2011)
IL-1β	BV-2; Human Fetal	H37Rv	Murine, Human	Rock et al. (2005) and Cannas et al. (2011)
IL-10	Human Fetal	N.C 0741708	Human	Curto et al. (2004)
IL-12p40	BV-2	H37Rv	Murine	Yang et al. (2007)
IL-6	BV-2; Human Fetal	H37Rv	Murine, Human	Rock et al. (2005) and Yang et al. (2007)
TNF-α	BV-2; Human Fetal	H37Rv; N.C 0741708;H37Rv	Murine, Human	Curto et al. (2004), Yang et al. (2007) and Cannas et al. (2011)

microglia have also been shown to secrete G-CSF and GM-CSF when infected with *M. tuberculosis*, which, unlike IL-1 or IL-2, drives microglial-specific proliferation via JAK/STAT pathways (Lee et al., 1994; Liva et al., 1999), amoeboid differentiation, and stimulates debris clearance (Giulian and Ingeman, 1988; Cannas et al., 2011). GM-CSF may facilitate bacilli containment by augmenting the neutrophilic phagocytosis of bacilli (Fleischmann et al., 1986) and enhancing the

bactericidal mechanisms of macrophages (Blanchard et al., 1991)—but whether G-CSF contributes to collateral neurotoxic damage through infiltrating leukocyte activation remains to be investigated. Some systemic symptoms of CNS-TB may be due to GM-CSF negatively affecting food intake and positively affecting energy expenditure (Reed et al., 2005). G-CSF, like GM-CSF, is mainly known for its hematopoietic effects. Recently, however, it has been recognized that G-CSF plays important roles in the induction of immune tolerance, including redirection of T-lymphocytes to a Th2 phenotype (Pan et al., 1995; Sloand et al., 2000), and of specific importance in *M. tuberculosis* immunity, a decline in IFN-γ secretion (Sloand et al., 2000). G-CSF and GM-CSF not only augment phagocyte cell survival, but also highly neurotrophic factors: decreasing cortical ischemic damage, improving neuronal survivability and contributing towards neuronal regeneration (Kim et al., 2004; Schneider et al., 2005). Thus, microglia do not only orchestrate and lead the host immune response (and through which, may participate in neurotoxicity), but may have additional roles in neuronal protection and recovery.

Microglia secrete IL-6 in direct response to *M. tuberculosis* (Yang et al., 2007) and, through TNF-α, promote its additional expression by astrocytes (Sawada et al., 1992), and serves to dampen the TNF-inducible expression of VCAM-1 within the CNS (Oh et al., 1998). IL-6, well-known for its primary importance in B-lymphocyte differentiation (Burdin et al., 1995), has secondary, neuron-specific effects. IL-6 induces neurotrophin secretion from astrocytes in a region-specific pattern: (März et al., 1999) this not only disrupts the IFN-γ-induced expression of MHC class II by microglia (Neumann et al., 1998), but has been shown to increase the survival of dissociated neurons (Thier et al., 1999). Monocytes cultured from Chinese individuals with rs1800796GG polymorphisms produced less IL-6, which also granted these individuals a reduced risk of pulmonary TB (Zhang et al., 2012). This correlates with observations that *M. tuberculosis* bacilli maximize IL-6 production from macrophages to antagonize IFN-γ-induced autophagy, increasing the longevity of their macrophage host and thereby extending intracellular persistence (Dutta et al., 2012). Although not yet investigated, IL-6 may thus be used by the tubercle bacilli to extend the lifespan of their CNS-specific host-cells, including (but not limited to) microglia and neurons (Randall et al., 2014).

Neurons infected with *M. tuberculosis* act as a source of IL-10 which is generally accepted as an anti-inflammatory cytokine. In TB specifically, its principal role is considered to be the regulation of Th1 responses and thus, opposing IFN-γ production (Jamil et al., 2007). In the CNS, IL-10 diminishes MHC class II receptor expression on microglia but not astrocytes, and reduces the proliferative response induced by glial interactions with effector T-lymphocytes (Frei et al., 1994). IL-10 may also silence cytokine production by infiltrating monocytes (de Waal Malefyt et al., 1991). IL-10 represents an important mechanism by which the body protects itself from CNS-autoimmunity through Th1 attenuation (Bettelli et al., 1998; Fillatreau et al., 2002). Furthermore, Curto et al. observed inhibition of IL-10 expression by microglia in more virulent *M. tuberculosis* infection, uncovering one possible mechanism driving a pro-inflammatory response, and potentially the basis of pathogenicity between mycobacteria within the CNS (Curto et al., 2004). Considering IL-10 alone suggests possible contributory mechanisms in TB neuropathology: loss of neuron-to-effector inhibition, which results in spurious immune activation; a relative resistance of microglia in some individuals, be the cause acquired or inherited, resulting in autonomous immune activation; or a relatively pro-inflammatory milieu (to which multiple cells contributed) as ultimately generating host-mediated tissue damage.

Potential Neurotoxicity

The inflammatory damage found in the CNS amidst infections, ranging from HIV (Garden, 2002) to bacterial meningitis (Gerber and Nau, 2010) has, of all the CNS-specific cells-types, been attributed largely to microglia. However, microglia are not the only cell type to produce increased MMPs (matrix metalloproteinases) in response to TB, and neither do microglia act in isolation. It is, in fact, the contribution of other cell types towards a pro-inflammatory milieu that appears to drive the secretion of destructive compounds. For example, *M. tuberculosis*-activated monocytes release factors into the local microenvironment that rapidly stimulate microglia to produce MMP-1 and MMP-3, well known to induce tissue damage through degradation of various matrix-associated proteins (Green et al., 2013). The dependency on monocyte-priming of microglia was reproduced in other studies, where it was reported that significantly greater MMP-1, MMP-3, and MMP-9 synthesis is observed in microglia co-cultured in *M. tuberculosis*-infected monocyte culture medium as opposed to those microglia exposed to *M. tuberculosis* bacilli alone (Green et al., 2010). Astrocytes are an additional source of MMPs within the CNS, and have been shown to secrete significantly more MMP-9 in a monocyte-dependent fashion as do microglia (Harris et al., 2007). Such data suggests that it is miscommunication or dysregulation between many cell types, performing exaggerated but physiological activities, that result in CNS pathology, rather than individual cellular populations (like microglia) inducing pathology autonomously.

Although it has not been studied directly in the context of *M. tuberculosis*, microglia are known to demonstrate cytotoxic behavior towards oligodendrocytes—a possible component of demyelinating tuberculous diseases. One such rarer form of CNS-TB, Tuberculous (allergic) encephalopathy, usually occurs in vulnerable populations with a preceding or concurrent tuberculous infection (Bernaerts et al., 2003). The broad pathognomonic features of this complication are diffuse white matter destruction, occurring with or without clinical meningism in an individual exposed to TB, and has been classically attributed to a delayed hypersensitivity reaction towards tuberculoprotein (Udani and Dastur, 1970; Dastur, 1986). Activated microglia are capable of lysing oligodendrocytes via a NO-dependent mechanism requiring membrane-bound TNF-α (Merrill

et al., 1993). Alternatively, a more novel mechanism whereby microglia destroy oligodendrocytes involves a local spike in extracellular glutamate and excitotoxic cellular death (Domercq et al., 2007). Regardless of the precise mechanisms, such microglial-oligodendrocyte interactions are well worth further investigation and may very well broaden the understanding of CNS-TB.

Also, many of the signaling molecules used by microglia are also potential sources of collateral neurotoxicity. Central to microglial pro-inflammatory activation pathways is secretory phospholipase A_2 ($sPLA_2$; Yang et al., 2009), a compound shown to be a culprit of toxicity in neurons (Kolko et al., 1996, 1999) and potential contributor to neural damage amidst CNS-TB. Studies in lungs have demonstrated the regulatory role of $sPLA_2$ in inflammation through the induction of cytokine production and cellular recruitment (Granata et al., 2005, 2006). Yang et al. has shown in the CNS that $sPLA_2$ is essential for *M. tuberculosis*-dependent ROS (in particular, H_2O_2 and superoxide) generation in microglia via increased NADPH oxidase activity, which further initiates MAPK signaling of the pro-inflammatory response (Yang et al., 2007). Tumor necrosis factor alpha (TNF-α) and IL-6 secretion are positively regulated by ERK1/2 and p38, but p38 alone negatively regulates IL-12p40 generation (Yang et al., 2007). IL-12 is critically important for the protective granulomatous, antigen-specific Th1 and $CD8^+$ T-lymphocyte responses to *M. tuberculosis* infection (Hölscher et al., 2001). Hence, many of the pro-inflammatory programs initiated by microglia require potentially cytotoxic compounds.

Another potential source of cytotoxicity in CNS-TB is associated with the treatment of adjunctive corticosteroids. Its success is often attributed solely to the modulation of microglial pro-inflammatory cytokine activity, and thus used as evidence for the destructive nature of microglia. However, a double-blinded randomized control trial found that adjunctive corticosteroid use in patients with TB meningitis had improved mortality, but not morbidity (Thwaites et al., 2004). Furthermore, there was no difference in cytokine levels within the CNS of CNS-TB patients treated with corticosteroids (Claudio et al., 1994) and those who remained untreated (Mastroianni et al., 1997).

IL-1β and TNF-α are factors secreted from microglia that drive the production of MMP-2 and MMP-9 from astrocytes; their production reduced through glucocorticoids (Gottschall and Deb, 1996). Experiments involving dexamethasone demonstrate two main mechanisms by which the effects of corticosteroids in CNS-TB may be explained. Firstly, production of IL-1β, IL-6, and TNF-α by *M. tuberculosis*-stimulated microglia is significantly reduced (Rock et al., 2005). Secondly, dexamethasone reduces MMP-1 and MMP-3 production within the CNS (which could alternatively be explained by the reduction of its TNF-α and IL-1β, as these promote MMP secretion) (Green et al., 2010). However, microglia are not the only sources of these cytokines in the CNS, and thus to either fully achieve anti-inflammatory effects *in vivo* or to prove the beneficial effects of adjunct steroid use relating only to microglia attenuation, it is necessary to look at these cells in a much broader context. In other words, microglia could still respond to cytokines from additional, upstream sources, even if their own autocrine or paracrine responses have been suppressed.

Blood-Brain Barrier Permeability and Immune Recruitment

Cytokines are generally considered inducers of BBB permeability for the influx of peripheral immune constituents (**Figure 1**). IL-6 and TNF-α increase cerebral endothelial cell permeability both *in vitro* and *in vivo* (Bamforth et al., 1996; Duchini et al., 1996). IL-1β interference of the BBB is associated with inter-endothelial pores, as well as leukocyte recruitment and hemorrhage (Claudio et al., 1994). Although these secreted products of microglia compromise the integrity of the BBB, this may facilitate additional activation signals: such as ATP by means of purinergic receptors, or complement through complement receptors (Lynch et al., 2004; Davalos et al., 2005). Microglia have been shown to internalize many extravasated proteins during BBB compromise (Claudio et al., 1994). Furthermore, as a component of the glia limitans capable of migration and immune activation, microglia not immediately responding to the intra-cerebral threats, may play a role in protecting the CNS when it is at its most vulnerable; compensating for the altered BBB permeability (Claudio et al., 1994).

Chemokines produced by *M. tuberculosis*-challenged microglia include CCL2, CCL5, and CXCL10 (Rock et al., 2005). CCL2 (also known as MCP-1) is essential for the cellular response in *M. tuberculosis* infection, recruiting leukocytes (in particular, monocytes, and T-lymphocytes) to the sites of infection or injury (Babcock et al., 2003; Hasan et al., 2005). Similarly, CCL5 has shown particular importance in recruiting T-lymphocytes in pulmonary granulomas (Berenguer et al., 1992; Vesosky et al., 2010), and CXCL10 (from microglia and *M. tuberculosis*-challenged astrocytes) is likely important in helper T-lymphocyte trafficking (Fife et al., 2001; Rock et al., 2005). Along with this importance in cellular recruitment into the CNS, CCL2 has shown additional inflammation-modulating and protective activities. CCL2 deficient mice have more pronounced pro-inflammatory responses from astrocytes (Semple et al., 2010). Both CCL2 and CCL5 increase neuronal resilience to neurotoxicity in various experimental settings (Bruno et al., 2000; Madrigal et al., 2009). Given that microglia express PRR's, are preferentially infected by *M. tuberculosis* and secrete an array of immunologically-relevant molecules, it is likely that they are critical regulators of chemokine receptors within the CNS, hence regulating both the trafficking and the state of activation of peripheral immune components.

Throughout the course of mycobacterial infections of the CNS, infiltrating cell populations change, with significant recruitment of innate $CD11b^+$ cells, $CD11c^+$ cells, and $CD4^+$ T-lymphocytes (Lee et al., 2009). Of these cell types recruited to the infected CNS, T-lymphocytes and monocytes have the best-characterized roles in TB. Within macrophages, *M. tuberculosis* evade intracellular killing by means of multiple mechanisms,

including phago-lysosome exploitation and disruption of CD4+ T-lymphocyte interactions by a reduction of MHC Class II expression (Noss et al., 2001; Vergne et al., 2005). Although this may hold true for infiltrating monocytes, who could be the actual agents of neurotoxicity, the uniqueness of microglia makes it difficult to assume similar escape mechanisms within or MHC class II evasion in glia, and highlights the importance of gaining an experimental, rather than a purely hypothetical understanding, of such processes.

Microglia form an important mediator between innate and adaptive immune responses within the CNS. Microglial stimulation by *M. tuberculosis* induces the rapid expression of the co-stimulatory molecule CD137 for the activation of infiltrating T-lymphocytes (Curto et al., 2004). Numerous studies have highlighted the importance of a T-cell response against *M. tuberculosis*, in particular the importance of a robust Th1 response (Salgame, 2005). For instance, Lienhardt et al. found that African TB patients had not only reduced proxies of a Th1 response, but exhibited an inferior capacity to suppress a Th2 response (Lienhardt et al., 2002). Taking this into account, it is of utmost importance to appreciate microglia as a source of IL-12 amidst *M. tuberculosis* infection, which may play a role in polarizing local Th1 responses (Yang et al., 2007). Furthermore, IFN-γ treated microglia rapidly express MHC class II, through which they present antigen to helper T-lymphocytes (Frei et al., 1987; Steiniger and Van Der Meide, 1988) (as previously discussed, microglia may play roles in the regression thereof, too). IFN-γ, interestingly, also provides a mechanism to keep microglia in check by inducing apoptotic pathways (Spanaus et al., 1998; Badie et al., 2000). Although microglia are weaker antigen presenting cells compared to macrophages, they are perfectly adapted to the delicate CNS: stimulating the Th1 differentiation of T-lymphocytes without inducing their proliferation (Carson et al., 1999).

A number of meta-analyses have confirmed the efficacy of BCG against CNS-TB (Rodrigues et al., 1993; Colditz et al., 1994; Trunz et al., 2006). Whether this protective effect is due to preventing the bacilli from reaching the CNS, or through CNS-specific immune augmentation, remains to be uncovered. In a murine study of intracerebral BCG infection, Lee et al. report that microglia are the eminent TNF-α producers, with additional sources including infiltrating $CD4^+$ T-lymphocytes (Lee et al., 2009). Given that microglia have the capacity to differentiate $Foxp3^+CD4^+$ T-regulatory lymphocytes (Ebner et al., 2013), and that unique IFN-γ^+IL-17^+ T-lymphocytes have been identified with protective immunity characterized by the $Foxp3^+CD4^+$ T-regulatory phenotype (Colditz et al., 1994), it is not unreasonable to assume that microglia may be involved in mediating the CNS efficacy of BCG against *M. tuberculosis*.

Conclusion

To improve patient outcomes following CNS infections by *M. tuberculosis*, more research needs to be conducted on the mechanism of *M. tuberculosis* identification and internalization within the CNS, mechanisms of persistence within microglia, the nature of each cytokine or chemokine secreted by microglia, and the means by which other CNS specific cells responding to or infected by *M. tuberculosis* govern microglial activation and modulate their responses. The neurological environment is unique in that inflammatory signals, which may appear to be damaging in the periphery, may in fact be beneficial in the CNS by governing neuronal survival, regeneration and differentiation.

In conclusion, microglia are the understudied arbiters of initiating, maintaining within acceptable limits, and attenuating the immune responses to CNS-TB, and may even be critical in mediating the protection or recovery from such responses. During *M. tuberculosis* infection, microglia are essentially the conductors of a tightly regulated immune symphony, and may well be a missing link towards fully understanding the molecular pathogenesis of CNS-TB.

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Hormones and immunity in cancer: are thyroid hormones endocrine players in the microglia/glioma cross-talk?

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Accumulating evidence indicates that the endocrine and immune systems engage in complex cross-talks in which a prominent role is played by thyroid hormones (THs). The increase of resident vs. monocyte recruited macrophages was shown to be an important effector of the TH 3,3′,5′-Triiodo-L-thyronine (T3)-induced protection against inflammation and a key role of T3 in inhibiting the differentiation of peripheral monocytes into macrophages was observed. Herein, we report on the role of T3 as a modulator of microglia, the specialized macrophages of the central nervous system (CNS). Mounting evidence supports a role of microglia and macrophages in the growth and invasion of malignant glioma. In this respect, we unveil the putative involvement of T3 in the microglia/glioma cell communication. Since THs are known to cross the blood-brain barrier, we suggest that T3 not only exerts a direct modulation of brain cancer cell itself but also indirectly promotes glioma growth through a modulation of microglia. Our observations expand available information on the role of TH system in glioma and its microenvironment and highlight the endocrine modulation of microglia as an important target for future therapeutic development of glioma treatments.

Keywords: thyroid hormones, microglia, macrophages, glioma, tumor microenvironment, hypothyroidism, tumor growth

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Introduction

The specialized macrophages of the central nervous system (CNS), namely microglia, constitute 5–20% of total glial cells (Ransohoff and Perry, 2009; Kettenmann et al., 2011; Saijo and Glass, 2011). The lineage relationship between microglia and peripheral macrophages is well established (Yang et al., 2010; Saijo and Glass, 2011); it has been recently suggested that microglia originate from macrophages migrating into the CNS during early embriogenesis and that microglial cell population can locally expand in CNS (Ginhoux et al., 2010; Saijo and Glass, 2011). Our understanding of the key factors and molecular mechanisms responsible for microglia development and function is however still incomplete. In a healthy environment, resting microglia displays low expression levels of inflammatory molecules, but when activated, microglial cells abandon their ramified surveiling morphology, become ameboid, acquire phagocytic functions and migrate to the injured site to release inflammatory molecules (Polazzi and Monti, 2010; Saijo and Glass, 2011). Generally, microglial cells act as the primary

responding cells for infectious and traumatic stimuli although their activation may also result in pathological forms of inflammation that contribute to the progression of neurodegenerative diseases (Glass et al., 2010; Perry et al., 2010; Saijo and Glass, 2011; Assi et al., 2013).

Studies of peripheral macrophages have led to the development of the concept of two different macrophage activation states, i.e., the "classically activated" (M1) and "alternatively activated" (M2) ones (Murray and Wynn, 2011; Sica and Mantovani, 2012). The "classically activated" macrophages express pro-inflammatory cytokines, mediate defense of the host from a variety of bacteria, protozoa and viruses, and have roles in anti-tumor immunity. The "alternatively activated" macrophages have anti-inflammatory, pro-tumoral function and regulate wound healing. It is generally assumed that macrophages activation *in vivo* represents extreme of a continuum in a universe of activation states and mixed phenotypes and coexistence of cells in different activation states have been observed in preclinical/clinical conditions (Sica and Mantovani, 2012). These concepts might also be applicable in the case of microglia which has activation states similar to that of macrophages and exhibits functional plasticity during activation states (Saijo and Glass, 2011). However, the associations between distinct activation states and pathology are less well defined and may differ from those of macrophages in peripheral tissues (Ghosh and Chaudhuri, 2010; Yang et al., 2010; Saijo and Glass, 2011; Wei et al., 2013).

Similarly to other tissues, brain cancers are complex ecosystems composed of many interacting elements. The communication between the tumor cells and the surrounding cells helps to drive the process of tumor progression and the shaping of its complexity. Increasing evidence indicates that what is happening inside the tumor cell occurs also under exogenous stimuli arising around tumor cells (Albini and Sporn, 2007; Joyce and Pollard, 2009; Charles et al., 2012; Goubran et al., 2014; Klemm and Joyce, 2015). Beyond cancer cells, microglia, astrocytes, the extracellular matrix and soluble factors influence the tumor invasion, angiogenesis, cell proliferation/apoptosis also having profound effects on the efficacy of cancer therapies (Albini and Sporn, 2007; Joyce and Pollard, 2009; Charles et al., 2012; Goubran et al., 2014; Klemm and Joyce, 2015; Gutmann, 2015). In the case of malignant gliomas, a primary CNS cancers arising from glial cells, our understanding of the role of microenvironmental cells has lagged behind the discovery that monocytes are the most likely source of all brain macrophages and that microglia and macrophages may account for a large amount of total cell populations in brain tumors (Watters et al., 2005; Saijo and Glass, 2011; Gutmann, 2015). In this regard, glioma tissue shows high levels of infiltrating microglia, localized diffusely throughout the tumor, rather than to the areas of necrosis (Yang et al., 2010; Charles et al., 2012). Although once previously thought to play an anti-tumorigenic role, microglia has recently emerged as important element in the progression and growth of glioma through diverse mechanisms (Ghosh and Chaudhuri, 2010; Yang et al., 2010; Saijo and Glass, 2011; Zhai et al., 2011; Charles et al., 2012; Jacobs et al., 2012; Wei et al., 2013; da Fonseca and Badie, 2013; Gutmann, 2015). Glioma-associated microglia produce plenty of cytokines, chemokines, interleukins, and growth factors, which can either shape a more permissive tumor microenvironment or directly trigger glioma cell growth and invasion. In particular, by inducing new blood vessel formation and/or changes in the extracellular matrix microglia may create indirectly a supportive soil that further enhances glioma growth or invasion. Alongside microglia-released soluble factors may increase directly glioma stem cell or astrocytoma cell proliferation, survival, and/or invasion. In addition, glioma-infiltrating microglial cells appear incapable of inducing an effective anti-tumor T cell response, strongly supporting the fact that microglias promote tumor growth by facilitating immunosuppression of the tumor microenvironment. Of notice, glioma cells may over-rule the normal defensive role of microglial cells and confine them into an immune-depressive boundary. In this context, the elucidation of the microglia-glioma ecosystem can provide useful information for manipulation of the glioma microenvironment in a therapeutic perspective, i.e., to generate a specific and durable anti-glioma immune response.

Thyroid Hormones and Macrophages/Microglia

The endocrine and immune systems engage in complex cross-talks. Hormones and endocrine transmitters bind to immune system cells, thus modifying immune cell functions and tuning immune responses (Dorshkind and Horseman, 2000; Kelley et al., 2007; Barnard et al., 2008; Butts and Sternberg, 2008; Rivest, 2010; Carlton et al., 2012). In this respect, growing evidence indicates that the thyroid hormones (THs) 3,3′,5′-Triiodo-L-thyronine (T3) and L-thyroxine (T4) are important modulator factors of immune cells, including peripheral macrophages (Khansari et al., 1990; Rosa et al., 1995; Forner et al., 1996; Rittenhouse and Redei, 1997; Ortega et al., 1999; Dorshkind and Horseman, 2000; El-Shaikh et al., 2006; Klecha et al., 2006; Mascanfroni et al., 2008; Mazzoccoli et al., 2010; De Vito et al., 2011; Chen et al., 2012). Recently we identified a homeostatic link between T3 and the pathophysiological role of macrophages (Perrotta et al., 2014). In particular, our *in vitro* results indicate a negative role of T3 in triggering the differentiation of mouse circulating monocytes into macrophages. T3 was also shown to induce macrophages to display a "classically activated" signature, as revealed by the expression analysis of surface proteins and cytokine release, as well as the experiments on cell migratory ability (chemotaxis) and phagocytosis. Interestingly, the analysis of gene markers in macrophages treated with T3 revealed a somehow "classically activated"/"alternatively activated" mixed phenotype thus suggesting that the switching induced by T3 is very complex. *In vivo* results demonstrated that circulating T3 increased the content of the resident macrophages in the mouse peritoneal cavity while reducing the content of the recruited monocyte-derived cells. Additionally, T3 significantly protected mice against endotoxemia: decreased T3 levels increased the recruited (potentially damaging) cells while the restoring of T3 levels decreases recruited and increases resident (potentially beneficial) cells (Perrotta et al., 2014). Although macrophages

were historically considered to be derived from the blood monocyte reservoir, numerous studies have since demonstrated that, under steady-state conditions, resident tissue macrophage populations are largely maintained through local proliferation (Yona and Jung, 2010). Inflammatory insults, however, result in the rapid recruitment of blood-borne precursors to the respective tissue macrophage compartment (Yona and Jung, 2010). In this line, our data suggest that T3 contributes to limit inflammation by promoting the proliferation of peritoneal macrophages *in situ*, while inhibiting the potentially damaging cell recruitment from monocyte cell pools, in a context not fully explained by the "classically activated"/"alternatively activated" framework (Perrotta et al., 2014).

The influence of thyroid imbalance on microglial development was firstly identified in 2001 when hypothyroidism was found to slow markedly the progressive elaboration of microglial processes in the developing rat forebrain and increases in T3 levels accelerate them (Lima et al., 2001). In addition, *in vitro* and *in vivo* analyses revealed that T3 increases the number of microglia cell bodies, promotes microglia survival (but not the proliferation) and enhances growth of their processes (Lima et al., 2001). These results indicate that THs promote the growth and morphological differentiation of cortical microglia during development. Accordingly, it has been recently shown that hypothyroidism prominently reduces the processes of microglia in the hippocampus of diabetic rats (Nam et al., 2013).

Thyroid Hormones at the Interplay Between Microglia and Glioma Cells

An aspect that is worth pursuing to understand better the interplay between the immune system and glioma is the role of the endocrine system since both contribute with an integrated action in the maintenance of the body defense against tumors. For instance, hormone dysregulations may determine the efficacy of chemo- or immuno-modulatory therapies likely affecting the tumor microenvironment (Mazzoccoli et al., 2010; ThyagaRajan and Priyanka, 2012; Armaiz-Pena et al., 2013; Goubran et al., 2014). In order to get new insight on the possible role of T3 in the regulation of microglia/glioma cross-talk we used here a retroviral-immortalized cell line, the N9 microglia line, and the GL261 murine model of malignant glioma as previously reported (Davis et al., 2006; Zhang et al., 2009, 2011; Liu et al., 2011; Zhai et al., 2011). The N9 microglia is derived from mouse brain and shares many phenotypical characteristics with primary mouse microglia, also maintaining the crucial properties of *in vivo* microglia (Stansley et al., 2012). N9 and GL261 cell lines were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% heat inactivated fetal bovine serum, 2 mM glutamine, 100 UI/ml penicillin and 100 μg/ml streptomycin (Euroclone, Milano, Italy) at 37°C, 5% CO_2 in an humidified atmosphere. During treatments, cells were exposed to THs-depleted medium (Perrotta et al., 2014). T3 (Sigma-Aldrich, Saint Louis, MO, USA) was added to the cell medium for 24 h at the concentration of 1 μM, giving maximal receptor occupancy in macrophages (Perrotta et al., 2014). Parallel cultures were maintained with T3 vehicle and used as a control. As shown in the western blot experiment of **Figure 1A**, the levels of proliferating cell nuclear antigen (PCNA) in N9 microglia did not change in the presence of T3, further confirming that T3 was not coupled to microglia proliferation (Lima et al., 2001). We then set-up an indirect co-culture experimental procedure in which GL261 cells were plated in the bottom wells with or without N9 cells and T3 in the top wells. Using this system we observed a significant increase in GL261 cell proliferation in the presence of T3 and N9 cells when compared to GL261 with N9 only (about 49%), while T3 had no effect on GL261 cell number in the absence of N9 (**Figure 1B**). These results were confirmed by the analysis of PCNA protein expression (**Figure 1C**). Although THs (especially T4) were suggested to be a growth factor for different glioma cells *in vitro* (Davis et al., 2006; Lin et al., 2009), in our experimental settings T3 itself did not affect GL261 proliferation. Accordingly, similar concentrations of T3 did not modify PCNA levels in GL261 cells (Davis et al., 2006). In this respect, T3 effect on cell growth appears to be dependent on the type of glioma tumor cell line (Liappas et al., 2011).

The activation of signal transducers and activators of transcription 3 (STAT3) has been proposed to play an anti-tumor immunity role (Yu et al., 2014), and indeed activation of STAT3 in N9 cells increased GL261 growth (Zhang et al., 2009). Interestingly, it is becoming apparent that STAT3 is an important molecular player that allows glioma cells to promote the activity of microglia; reciprocally microglia facilitate tumor survival, growth and the spread of glioma cells (Zhang et al., 2009; Wu et al., 2010; Wei et al., 2013; da Fonseca and Badie, 2013). The inhibition of STAT3 function in tumor microglia may thus potentially be used as an immunotherapy approach for gliomas. We reported here an activatory role of T3 on STAT3 of microglia since N9 treatment with T3 resulted in elevated levels of STAT3 phosphorylation when compared to control (**Figure 1D**). In addition, as shown in real-time quantitative PCR experiments of **Figure 1E**, treatment of N9 microglia with T3 increased the mRNA expression of chemokine (C-X-C motif) ligand (CXCL) 9 and CXCL10 by 15.9 and 3.4 fold, respectively, vs. untreated control. Similar results were obtained in mouse peripheral macrophages (Perrotta et al., 2014). Chemokines constitute a significant portion of the modulatory messengers that can be released by activated microglia and interact with specific transmembrane G protein-coupled receptors (Hanisch, 2002). Of interest, both *in vitro* and *in vivo* experiments using different glioma tumors, including GL261 cells, indicated CXCL9 and CXCL10 (which bind to their endogenous receptor CXCR3) as key ligands promoting the growth of glioma (Liu et al., 2011). In this respect, different evidence indicates CXCR3 as an independent prognostic factor for glioblastoma patients and promotes an invasive phenotype (Pu et al., 2015).

Taken together our results indicate that T3 promotes GL261 glioma growth through a modulation of N9 microglia and that T3 effects involve the modulation of soluble factors released by microglia. From a mechanistic point of view, we suggest that STAT3 activation and the release of CXCL9/10 are suitable candidates to answer the question of how microglia

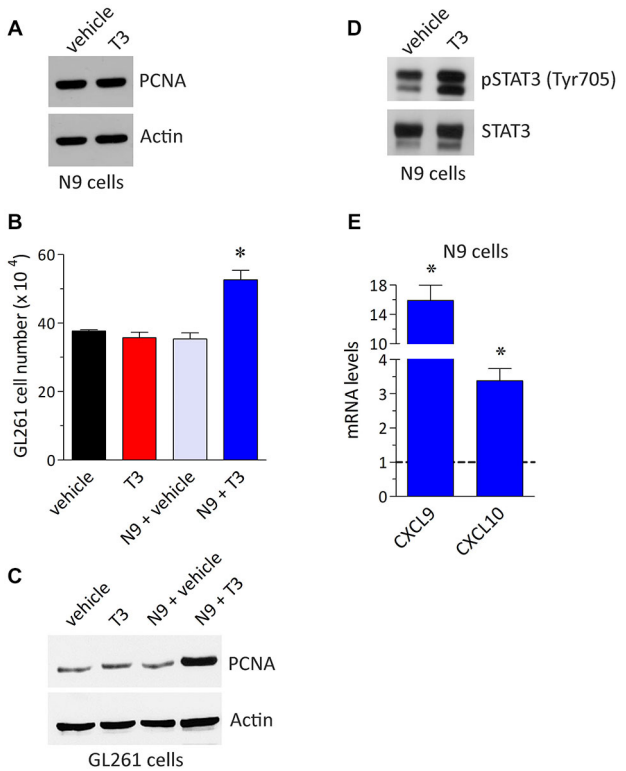

FIGURE 1 | T3 induces glioma cell growth by a direct action on microglia. (A) Expression of the proliferation marker proliferating cell nuclear antigen (PCNA) in N9 cells plated in the absence and in the presence of T3 (1 μM, 24 h). The Western blot analysis was performed as described previously (Armani et al., 2007; Cervia et al., 2007, 2013; Bizzozero et al., 2014; Cazzato et al., 2014; De Palma et al., 2014; Perrotta et al., 2014) using the mouse monoclonal anti-PCNA (PC-10) and the goat polyclonal anti-actin (I-19) (internal standard) primary antibodies (Santa Cruz Biotechnology, Dallas, TX, USA). The image is representative of results obtained from three different experiments ($n = 3$). **(B)** GL261 cell number in co-culture experiments. The experimental setting was in agreement with a previous report (Zhai et al., 2011), with minor corrections. Briefly, GL261 cells were seeded in the bottom wells of Costar transwell plates (24-mm diameter insert, 0.4 μM pore size, polycarbonate membrane; Corning Life Sciences, Corning, NY, USA) with or without N9 cells in the top wells (1:1 N9:GL261), both in the absence or in the presence of T3 (1 μM). Cell concentration after 24 h cultures was measured by counting trypan blue-excluding cells with TC20 Automated Cell Counter (Bio-Rad, Hercules, CA, USA), as described previously (Cervia et al., 2013; Perrotta et al., 2014). Each histogram represents the data obtained from 3–6 different experiments ($n = 3$–6). The results were expressed as means ± SEM. *$P < 0.001$ vs. the other values, using one-way ANOVA followed by the Tukey's multiple comparison post-test (GraphPad Prism; GraphPad Software, La Jolla, CA, USA). **(C)** Western blot analysis of PCNA in GL261 cells co-cultured as described above. The image is representative of results obtained from three different experiments ($n = 3$). **(D)** STAT3 phosphorylation in N9 cells plated in the absence and in the presence of T3 (1 μM, 24 h). The Western blot analysis was performed using the rabbit polyclonal anti-phospho STAT3 (Tyr705) and the anti-STAT3 primary antibodies (Cell Signaling Technology, Danvers, MA, USA). The image is representative of results obtained from three different experiments ($n = 3$). **(E)** Real-time quantitative PCR experiments of mRNA levels for CXCL9 and CXCL10 in N9 cells in the presence of T3 (1 μM, 24 h). Experiments were performed as previously detailed (Cervia et al., 2008, 2012, 2013; Charles et al., 2012; Bizzozero et al., 2014; Cazzato et al., 2014; De Palma et al., 2014; Perrotta et al., 2014).

(*Continued*)

FIGURE 1 | Continued

Primer pairs: CXCL9, 5′-TCCTTTTGGGCATCATCTTCC-3′ (forward) and 5′-TTTGTAGTGGATCGTGCCTCG-3′ (reverse); CXCL10 5′-TCCTTGTCCTCCCTAGCTCA-3′ (forward) and 5′-ATAACCCCTTGGGAAGATGG-3′ (reverse) (Primmbiotech, Milano, Italy). Values are expressed as the fold change over control (untreated N9 cells). Each histogram represents the data obtained from three different experiments ($n = 3$) run in triplicate. The results were expressed as means ± SEM. $P < 0.05$ vs. respective control (one-way ANOVA followed by the Tukey's multiple comparison post-test).

supports glioma growth. This hypothesis, however, needs to be verified by different experimental approaches using, for example, pharmacological and/or genetic manipulations. This may also help to fully understand the signaling pathway mediating T3 actions. Indeed, STAT3 and its downstream effectors may act in parallel with different transduction mechanisms. Also, the possibility that soluble factors other than chemokines may be involved in the modulation of glioma growth cannot be excluded. At present, the pathological significance of T3-microglia-glioma axis *in vivo* remains to be established. The study of this complex issue and its molecular players appears of great interest and might highlight targets for future therapeutic development of glioma treatments based on endocrine modulation of microglia.

Relevance of Thyroid Hormones in Glioma Therapy

There is increasing evidence that alterations in TH system are common events in cancer (Aranda et al., 2009; Moeller and Führer, 2013). However, our current understanding of the effects of THs on cancer cells reflects a rather complex picture and conflicting results mainly obtained in *in vitro* and *in vivo* animal models have also been reported. Indeed, in addition to the studies describing that THs can function as tumor suppressors, other reports support the concept that THs can enhance carcinogenesis, thus suggesting a dual role of THs (Aranda et al., 2009; Moeller and Führer, 2013).

Although no unequivocal association between thyroidal status and human cancer has been demonstrated, epidemiology and clinical studies strongly support a generalized tumor-promoting effects of THs and suggest the possibility that thyroid function/dysregulations influence the outcome of tumor therapy (Hercbergs et al., 2010; Ashur-Fabian et al., 2013; Moeller and Führer, 2013). In this respect, hypothyroidism is associated with a favorable outcome in several cancer types (Hercbergs et al., 2010; Moeller and Führer, 2013). In brain tumors, the concentration and metabolism of THs found in human tissues are altered thus suggesting that changes in circulating levels of THs may be related to malignant progression of gliomas (Nauman et al., 2004). In addition, treatment-induced hypothyroidism in glioma patients significantly improves survival and response to tamoxifen (Hercbergs et al., 2003, 2010; Moeller and Führer, 2013). Also, the successful long-term tumor response to medically induced chemical hypothyroidism in conjunction with carboplatinum chemotherapy of an adult patient with glioma

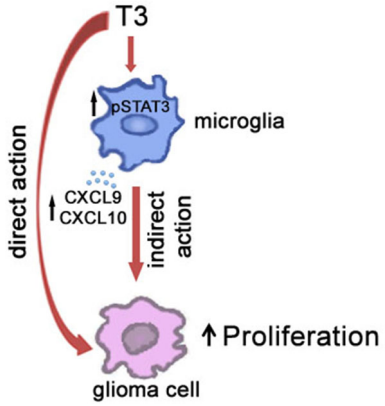

FIGURE 2 | Schematic illustration of the role of T3 in the cross-talk between microglia and glioma cells in the tumor microenvironment.

was recently reported (Ashur-Fabian et al., 2013). Yet, it is still possible that hypothyroidism is only a surrogate marker for treatment efficacy and does not positively influence treatment outcome by itself (Moeller and Führer, 2013).

Conclusion and Outlook

Malignant gliomas are aggressive, highly invasive, and neurologically destructive tumors considered to be among the deadliest of human cancers. Three decades of intensive research and a variety of chemotherapy regimes, radiotherapy and surgical approaches have been trialed and investigated, however the prognosis for patients with malignant glioma has not changed significantly (Desjardins et al., 2005; Taylor, 2010; Talibi et al., 2014). This has stimulated active research in multiples areas and the advent of new treatment strategies.

The emerging recognition of the roles of microglia in health and disease has stimulated substantial efforts to define more clearly the regulatory mechanisms that control their functions. With respect to CNS pathological remodeling induced by dysregulation of plasmatic levels of THs, the characterization of the physiologic factors that regulate the establishment of the microglial/glioma network is challenging. It has been previously hypothesized that changes in the host stroma associated with hypothyroidism rather than a direct receptor-mediated action on the tumor cells may be responsible for THs-induced modulation of tumor growth (Martínez-Iglesias et al., 2009a,b). In this context, since THs are known to cross the blood-brain barrier and microglial cells are CNS targets of THs, it is reasonable to assume that T3, beside a direct modulation of brain cancer cell itself, influences the relationship of tumor cells with stroma cells (**Figure 2**). In particular, our suggestion that T3 indirectly promotes glioma growth through a modulation of microglia, deserves further consideration and may help to understand better the role of T3 dysregulations in brain tumorigenesis. Given the important clinical impact of glioma tumors, clariyfying T3-induced microenvironment regulations may open the field to significant advances in the identification of possible new strategies to cancer therapy thus translating the role of thyroid gland status into clinical cancer cell biology.

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REVIEW
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The Role of Stefin B in Neuro-inflammation

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Stefin B (cystatin B) is an endogenous cysteine cathepsin inhibitor localized in the cytosol, mitochondria and nucleus. Its expression is upregulated upon macrophage activation and cellular stress. Mutations in the gene of stefin B are associated with the neurodegenerative disease known as Unverricht-Lundborg disease (EPM1). It was reported that early microglial activation precedes neuronal loss in the brain of the stefin B-deficient mice, implying a role of the inhibitor at the cross-talk between microglia and cerebellar cells. Detailed analysis of microglial activation in stefin B-deficient microglia showed a significantly higher proportion of both pro-inflammatory M1 and anti-inflammatory M2 microglia in stefin B-deficient mouse brain compared with control mice. In our recent work, we demonstrated that stefin B-deficient mice were significantly more sensitive to the lethal lipopolysaccharide (LPS)-induced sepsis, due to increased caspase-11 expression and secreted higher amounts of pro-inflammatory cytokines IL-1β and IL-18. Upon LPS stimulation, stefin B was targeted into the mitochondria, and the lack of stefin B resulted in the increased destabilization of the mitochondrial membrane potential and mitochondrial superoxide generation. The increased caspase-11 gene expression and better pro- inflammatory caspase-1 and -11 activation determined in stefin B deficient bone marrow-derived macrophages resulted in enhanced non-canonical inflammasome activation. Since signaling pathways in macrophages could be compared to the ones in microglia we propose that inflammasome activation could play an important role in the pathogenesis of EPM1.

Keywords: cystatins, EPM1, inflammation, microglia, TLR, NLRP3 inflammasome, ROS

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INTRODUCTION

Inflammation is a protective and tightly regulated immune response to tissue damage or pathogen invasion (Chovatiya and Medzhitov, 2014). In the central nervous system (CNS), this process is known as neuroinflammation and is characterized by the activation of the microglia and astrocytes population (Aguzzi et al., 2013). The innate immune response is triggered upon the recognition of pathogen-associated molecular patterns (PAMPs), derived from invading pathogens, and danger-associated molecular patterns (DAMPs), induced as a result of endogenous stress, by pattern-recognition receptors (PRRs; Akira et al., 2006). Activation of PRRs by PAMPs or DAMPs triggers signaling cascades that promote gene transcription by nuclear factor-κB (NF-κB), activator protein 1 (AP1), and interferon regulatory factors (IRFs) and results in the production of pro-inflammatory cytokines, interferons, and other pro-inflammatory proteins (Akira et al., 2006; Kawai and Akira, 2009). DAMPs correspond to endogenous ligands that are released by dying or damaged cells after cellular stress and can be recognized by PRRs such as membrane-bound toll-like receptors (TLRs)

or cytosolic nucleotide-binding domain and leucine-rich repeat-containing (NLR), the RIG-I-like receptor (RLR), the AIM2-like receptor (ALR; Medzhitov, 2007; Moresco et al., 2011; Franchi et al., 2012).

In the CNS, PRRs are primarily expressed by microglia, macrophages and astrocytes. These receptors are either membrane-bound and sense extracellular or endosomally located signals (TLRs) or are located within the cytoplasm and sense intracellular signals (NLRs). Recently, it was proposed that TLRs have an important role in the crosstalk between neurons and glial cells in the CNS. TLR signaling was linked to neurogenesis, it was also found to be involved in the pathogenesis of neurodegenerative diseases (Heneka et al., 2014). Only cytosolic receptors are involved in the formation of inflammasomes. The inflammasome is an intracellular multimolecular complex for the activation of inflammatory caspases-1 and -11 which leads into the cleavage and secretion of IL-1β and IL-18 and cell death called – pyroptosis (Martinon and Tschopp, 2007; Lamkanfi and Dixit, 2014). Caspases-1 and -11 both induce pyroptosis, but only caspase-1 processes IL-1β and IL-18 (Kayagaki et al., 2011). The nucleotide binding and oligomerization domain-like receptor family pyrin domain containing 3 (NLRP3) inflammasome, which is composed of NLRP3, the adaptor molecule apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) and the cysteine protease caspase-1, is one of the most studied inflammasomes with responses to various endogenous and exogenous danger signals (Latz et al., 2013). The priming step, that up-regulates NLPR3 pro-IL-1β gene expression, provides TLR signaling (Bauernfeind, 2009). Once primed, NLRP3 can respond to its stimuli and assemble the NLRP3 inflammasome. Stimuli that induce NLRP3 inflammasome assembly include ATP, pore-forming toxins, crystalline substances, nucleic acids, hyaluronan, and fungal, bacterial, or viral pathogens (Latz et al., 2013; Lamkanfi and Dixit, 2014). It has been proposed that phagocytosis of crystalline or particulate structures triggers lysosomal destabilization and subsequent release of the lysosomal cathepsins into the cytosol, and subsequently activates NLRP3 inflammasome (Halle et al., 2008). However, it is not known yet if the cathepsins interact directly with the inflammasome or the process involves molecules activated by the cathepsins. Recent studies have revealed a role for reactive oxygen species (ROS) of mitochondrial origin in the promotion of NLRP3 inflammasome activation (Nakahira et al., 2011; Zhou et al., 2011). Several reports showed that caspase-8 localizes and binds to ASC specks, indicating that caspase-8 is an important component of the inflammasome complex (Man, 2013; Sagulenko, 2013). In addition to the canonical [lipopolysaccharide (LPS) and ATP] NLRP3 inflammasome activation, a non-canonical inflammasome activation was described (Kayagaki et al., 2011; Rathinam et al., 2012; Broz and Monack, 2013). Canonical inflammasomes convert procaspase-1 into the catalytically active enzyme, whereas an undefined non-canonical inflammasome promotes activation of procaspase-11 (Lamkanfi and Dixit, 2014). The mouse caspase-11 (gene name *Casp4)* has 46% similarities to caspase-1 and is orthologous to human caspases-4 and -5 (Wang et al., 1996; Kajiwara, 2014). Non canonical inflammasomes could be activated by Gram-negative, but not by Gram-positive, bacteria, indicating that a specific factor from Gram-negative bacteria – LPS is required (Broz et al., 2012; Rathinam et al., 2012). In addition, caspase-11 detected intracellular LPS and some intracellular bacteria, directly mediate cell death and IL-1α secretion by a TLR4-independent mechanism (Hagar et al., 2013; Lamkanfi and Dixit, 2014). The non-canonical inflammasome pathway caspase-11 can interact with caspase-1 and forms a heterodimeric complex. It could induce a lytic cell death similarly to caspase-1; however, it cannot by itself trigger IL-1β/-18 processing (Wang et al., 1998; Kayagaki et al., 2011). Only caspase-11-deficient mice, but not caspase-1-deficient mice were protected from endotoxic shock (Wang et al., 1998; Kayagaki et al., 2011). The CNS is particularly sensitive to IL-1β and IL-18 signaling because multiple neural cell types in the CNS express receptors for these cytokines (Allan et al., 2005; Alboni et al., 2010).

The goal of the present review is to describe recent advances in neuroinflammation and the role of stefin B in the process.

CELLS OF THE IMMUNE SYSTEM IN CNS

Microglia are CNS resident myeloid cells of embryonic hematopoietic origin and comprise approximately 12% of cells in the brain (Aguzzi et al., 2013). Other CNS resident cells descend from neuroepithelial stem cells and are categorized as neurons and macroglia, with glia further subdivided into astrocytes and oligodendrocytes.

Astrocytes maintain CNS homeostasis and provide neuronal support in healthy conditions; moreover, astrocytes can undergo diverse phenotypic changes that may be protective or causative with regard to pathology (Sofroniew and Vinters, 2010). Astrocytes can produce numerous inflammatory molecules like cytokines, chemokines, growth factors, and nitric oxide (NO). Analysis of astrocyte transcriptome profiles indicates that astrocyte exposure either *in vivo* or *in vitro* to PAMPs such as LPS turns astrocyte transcriptome changes toward pro-inflammatory and potentially cytotoxic profiles (Hamby, 2012; Zamanian, 2012). Although astrocytes may undergo diverse phenotypic changes and secrete pro-inflammatory molecules, a recent study reported that NLRP3 inflammasome was expressed and functional only in mouse brain microglia, but not in astrocytes (Gustin et al., 2015). However, microglial–astrocyte interactions are important in the CNS innate immunity.

Microglia is a unique myeloid cell population, derived from primitive myeloid progenitors that arise before embryonic day 8, before vascularization or definitive hematopoiesis in the embryo (Ginhoux et al., 2010). Its density varies by brain region, they are localized mostly in the grey matter, with the highest concentrations being found in the hippocampus, olfactory telencephalon, basal ganglia, and substantia nigra (Lawson et al., 1990). Upon localization, microglia acquires a compact or ramified phenotype (Lawson et al., 1990; Block et al., 2007). In their resting state microglia have ramified morphology, and monitor the brain environment. In response

to immunological stimuli or brain injury the cells are activated (Saijo and Glass, 2011). Activated microglia acquire a compact phenotype and up-regulate several surface molecules like major histocompatibility complex (MHC) molecules, chemokine receptors and several other markers (Rock, 2004). Under other circumstances, however, microglia become over-activated and can induce significant and highly detrimental neurotoxic effects by the excess production of a large array of cytotoxic factors such as superoxide (Colton and Gilbert, 1987), NO (Moss and Bates, 2001), and tumor necrosis factor-α (TNFα; Lee et al., 1993). In some cases, microglial responses could also be protective to the CNS (Lalancette-Hebert et al., 2007; Vinet et al., 2012). Gene expression and morphological changes associated with microglial activation have been extensively studied (Prinz and Priller, 2014). Several TLRs are expressed on the microglial membrane and signaling induced by TLR activation results in production of neurotoxicity and could contribute to the microglial response to neuronal damage. Activation of TLR2, TLR4, and TLR9 induces microglial production of NO through multiple ligands (Ebert, 2005). TLR9 recognizes single-stranded unmethylated CpG-DNA (bacterial DNA), which stimulates an increase in the production of microglial NO and TNFα (Olson and Miller, 2004; Ebert, 2005). TLR4 together with CD14 is implicated in brain inflammation and microglial activation in response to endotoxemia (Chakravarty and Herkenham, 2005). Monocyte-derived macrophages are classified as M1, M2a, M2b, and M2c subsets (Gordon and Taylor, 2005; Geissmann et al., 2010). It is possible that microglia also transcribe activation-dependent genes, like macrophages. Both microglia and macrophages share several similarities, they are both myeloid-derived cells; however, there are also some differences between the two cell types. Some common markers used for microglial identification such as CD11b, CD11c, and CX3CR1, could be found in microglial cells as well as in monocytes, macrophages, and dendritic cells. The difference in the expression level of cell membrane tyrosine phosphatase CD45 may be used to discriminate $CD45^{low}$ microglia from $CD45^{high}$ blood-derived cells by flow cytometry (Sedgwick et al., 1991; Ford et al., 1995). However, the signaling pathways in NLRP3 inflammasome activation are comparable between macrophages and microglia (Halle et al., 2008). Microglia clear apoptotic cells and are involved in both elimination and maintenance of synapses, they use their fine processes to monitor for dysfunctional synapses, which they are able to eliminate by phagocytosis (Wake et al., 2009). They also promote synaptic activity by secretion of brain-derived neurotrophic factor (BDNF), a molecule that is essential for learning-dependent synapse formation (Parkhurst, 2013). Moreover, microglia could modulate adult neurogenesis in the brain (Vukovic et al., 2012). Some studies suggested that microglial cells not only have a scavenger role during development but can also promote the death of some neuronal populations (Marin-Teva, 2004). Several studies have reported NLRP3 activation in microglia or CNS macrophages, although NLRP3 has also been proposed to function in neurons (Compan, 2012; Ramos, 2012). The activation mechanisms reported for NLRP3 activation in macrophages, such as ROS production, K^+ efflux and endosomal rupture, also apply to NLRP3 activation within microglia (Halle et al., 2008; Hoegen, 2011; Heneka, 2013). Not only caspase-1, but also caspase-11 is expressed in microglial cells and could contribute to inflammasome activation (Lee et al., 2001; Kim et al., 2003).

CYSTATINS IN INFLAMMATION

Cystatins were initially characterized as inhibitors of lysosomal cysteine cathepsins, however, in recent years some alternative functions for cystatins have been proposed. Cystatins possessing inhibitory function are members of three families, family I (stefins), family II (cystatins), and family III (kininogens; Kopitar-Jerala, 2006; Turk et al., 2008).

The cystatins (cysteine proteinase inhibitor) are reversible and tight-binding inhibitors of the papain (C1) and legumain (C13) families of cysteine proteases and have significant similarities in amino acid sequence (Barrett, 1981; Barrett et al., 1986). The inhibitory profile of a particular cystatin is rather specific, despite significant sequence homologies (Turk et al., 2008). Type 1 cystatins – stefins are mostly intracellular cystatins present in the cytosol and the nuclei (Abrahamson et al., 1986). They are single-chain polypeptides ∼100 amino acid residues long, are synthesized without signal peptides and do not possess any disulfide bonds or carbohydrate side-chains. Recently, we reported the mitochondrial localization of stefin B (Maher et al., 2014a). Type 2 cystatins are mainly extracellular, secreted proteins. They are synthesized with 20–26 residue long signal peptides and most of them are found in physiologically relevant concentrations in body fluids (Abrahamson et al., 1986; Kopitar-Jerala, 2006; Turk et al., 2008). They contain disulphide bridges and may be phosphorylated (Laber et al., 1989). Type II cystatins also possess a second reactive site for inhibition of the C13 family of cysteine proteases (legumain; Alvarez-Fernandez et al., 1999). Cystatin C was found upregulated in the serum of patients with autoimmune diseases like systemic lupus erythematosus (Lertnawapan et al., 2012). Moreover, cystatin F was found abundant in the cells of the immune system: macrophages and dendritic cells and the cells involved in target cell killing (NK cells and cytotoxic T cells (CTLs; Halfon, 1998; Ni et al., 1998; Obata-Onai et al., 2002). It was also found in the microglial cells and monocyte/macrophages in the CNS. Cystatin F is expressed as a di-sulfide-linked dimer (Cappello et al., 2004) and translocated to endolysosomes where it regulates cathepsin activity. Cystatin F transport to endolysosomes depends on its *N*-linked glycosylation and it was reported that the secreted dimeric cystatin F could be internalized and activated by the mannose-6-phosphate receptor system (Colbert et al., 2009). After proteolytic removal of its N-terminal part, cystatin F becomes a potent inhibitor of cathepsin C with the potential to regulate pro-granzyme processing and cell cytotoxicity (Hamilton et al., 2008). Recently, we demonstrated that cathepsin V in IL-2 stimulated NK cells could process cystatin F (Maher et al., 2014b). In cytotoxic cells, cystatin F, therefore, appears as a key regulator of granzyme processing and consequently cell cytotoxicity.

Type 3 cystatins are high molecular weight (60–120 kDa) multidomain proteins and have three tandemly repeated type 2-like cystatin domains (Salvesen et al., 1986). The mammalian cystatins belonging to this type are the kininogens (Ohkubo et al., 1984). Cystatins in immune cells have been reported to participate in the release of nitric oxide, phagocytosis, and expression of cytokines (Kopitar-Jerala, 2006; Magister and Kos, 2013; Maher et al., 2014a).

STEFIN B AND EPMI 1

Stefin B belongs to the type one cystatins and is located in the cytosol, mitochondria, and nucleus where it protects cells from the detrimental release of the lysosomal cysteine cathepsins. In the nucleus, stefin B interacts with nucleosomes, specifically with histones H2A.Z, H2B, and H3 and cathepsin L (Ceru et al., 2010). Goulet et al. (2004) has shown that only shorter procathepsin L isoforms translocate to the nucleus and stimulate processing of the CUX1 transcription factor at the G_1/S transition of the cell cycle. Stefin B-deficient mouse embryonic fibroblasts entered S phase earlier than wild type mouse embryonic fibroblasts. In contrast, increased expression of stefin B in the nucleus delayed cell cycle progression in T98G cells. The delay in cell cycle progression was associated with the inhibition of cathepsin L in the nucleus, as judged from the decreased cleavage of the CUX1 transcription factor (Ceru et al., 2010). Moreover, we have shown that stefin B overexpression in the nucleus delayed not only cell cycle progression, but also caspase activation (Sun et al., 2012). Mutations in the gene encoding stefin B (either through a multiplied repeat unit in the promoter or through point mutations in the structural gene) are present in both alleles of the gene in patients with a form of progressive myoclonus epilepsy of Unverricht-Lundborg type (EPM1; Pennacchio et al., 1996; Lalioti et al., 1997; Pennacchio et al., 1998). EPM1 is an autosomal recessively inherited neurodegenerative disease, characterized by the cerebellar granule neurons apoptosis, progressive ataxia and myoclonic epilepsy (Joensuu et al., 2008). In lymphoid cells of EPM1 patients, increased cathepsin activity, due to reduced expression of stefin B was reported (Rinne et al., 2002), we determined increased overall cathepsin activity in untreated, as well as in classically activated stefin B-deficient bone marrow-derived macrophages (BMDMs) compared to WT cells (Maher et al., 2014c).

MOUSE MODEL OF EPM1

Stefin B-deficient mice develop myoclonic seizures by one month of age and progressive ataxia by six months of age (Pennacchio et al., 1998). Houseweart et al. (2003) reported that the removal of cathepsin B from stefin B-deficient mice greatly reduced the neuronal apoptosis, but did not rescue the ataxia and seizure phenotype. Moreover, stefin B deficiency was implicated in the impaired redox homeostasis, resulting in a pronounced oxidative stress-induced cell death and neurodegeneration (Lehtinen et al., 2009). Thymocytes from stefin B deficient mice were significantly more sensitive to apoptosis induced with the inhibitor of protein kinase C, staurosporin (Kopitar-Jerala et al., 2005). Manninen et al. examined in detail the spatiotemporal dynamics of the brain atrophy in stefin B-deficient mice (Manninen et al., 2014). They showed progressive but non-uniform volume loss of the stefin B-deficient mouse brains, indicating that different neuronal populations possess distinct sensitivity to the damage, as a consequence of stefin B deficiency. The authors suggested that the white matter damage in the brain of stefin B-deficient mice was secondary to glial activation and neurodegeneration (Manninen et al., 2014). Another report showed that the early microglial activation precedes neuronal loss in the brain of the stefin B deficient mice, implying a role of the inhibitor at the cross-talk between microglia and cerebellar cells (Tegelberg et al., 2012).

Joensuu et al. (2014) analyzed the gene expression changes in the cerebellum of pre-symptomatic and symptomatic stefin B -deficient mice and in cultured stefin B-deficient cerebellar granule cells. Already in the cerebellum of pre-symptomatic stefin B-deficient mice (7 days after the birth), multiple changes in gene expression related to synapse maturation, development, and function during postnatal maturation were observed. More prominent changes were reported in the GABAergic signaling pathway (Joensuu et al., 2014). GABA plays a central role in controlling neuronal development and connectivity and defective GABAergic signaling in the cerebellum of stefin B deficient mice underlines a mechanism for ataxia in these mice (Grusser-Cornehls and Baurle, 2001). At a later stage (30 days after the birth), in symptomatic stefin B-deficient mice, the authors reported the upregulation of immune response genes, in line with the results showing early glial activation that preceded neuronal degeneration (Tegelberg et al., 2012). Moreover, Joensuu et al. (2014), reported the upregulation of the genes involved in cell cycle progression, in stefin B-deficient granule neurons. We have shown that the interactions of stefin B with cathepsin L in the nucleus influence cell cycle progression into the S phase (Ceru et al., 2010). We cannot exclude the possibility that the impaired cathepsin regulation in the synapses could lead to morphological and functional changes observed in stefin B-deficient mice (Joensuu et al., 2014). For example, cathepsin B-like immunoreactivity was observed at synaptic sites and myristoylated-alanine-rich C-kinase substrate (MARCKS), a known substrate of cathepsin B, was specifically degraded in response to intense NMDA receptor stimulation (Graber et al., 2004). Previously, we reported the increased cleavage of MARCKS in the brains and macrophages of stefin B-deficient mice, when compared to cells and tissue from control wild-type animals (Kopitar-Jerala and Turk, 2007).

STEFIN B, EPM1, AND INNATE IMMUNE RESPONSE

The increased expression of inflammatory genes indicates that neuro-inflammation, together with neuronal dysfunction, plays a crucial role in pathology of EPM1. Pro-inflammatory chemokines and cytokines, highly expressed in symptomatic stefin B-deficient mice were reported to lower the seizure threshold and may thus

contribute to recurrent excitation in epilepsy (Devinsky et al., 2013). Okuneva et al. (2015) reported significantly higher stefin B mRNA expression in microglia than in neurons or astrocytes, which is in line with our observation that stefin B is highly upregulated in activated macrophages (Maher et al., 2014c). In pre-symptomatic stefin B-deficient mice compared to control animals the ratio of M1/M2 microglia is skewed towards M2 type, but towards M1 type in symptomatic mice. In addition, a heightened expression of both pro-inflammatory inducible nitric oxide synthase (iNOS), anti-inflammatory arginase 1 (ARG1) and chemokine release was detected (Okuneva et al., 2015). Interestingly, MHCII surface expression was suppressed. We have reported that IFN-γ and LPS-activated stefin B-deficient BMDMs produced higher amounts of NO, and expressed more iNOS than WT BMDMs. IL-10 is a potent anti-inflammatory cytokine that is crucial for dampening the inflammatory response after pathogen invasion and acts to protect the host from excessive inflammation (Fiorentino et al., 1991). We showed decreased expression of IL-10 in BMDMs of stefin B deficient mice, due to impaired STAT3 signaling (Maher et al., 2014c). IL-10 plays an essential role in mediating inflammatory processes not only in the cells of immune system, but also in the brain (Zocchia et al., 1997). It has been demonstrated that it increases the survival of cerebellar granule cells by blocking caspase-3-like activity (Bachis et al., 2001). It is tempting to speculate that the decreased IL-10 expression in stefin B-deficient mice could contribute to the increased apoptosis in the cerebellum in EPM1.

In the developing mouse cerebellum, Purkinje cells die and a majority of these neurons are engulfed by microglial cells. Interestingly, apoptosis of Purkinje cells in the cerebellum was strongly reduced by selective elimination of microglia and superoxide produced by microglia cells (Marin-Teva, 2004). In our recent work we showed that stefin B-deficient mice were significantly more sensitive to the lethal LPS-induced endotoxemia due to increased caspase-11 expression. The

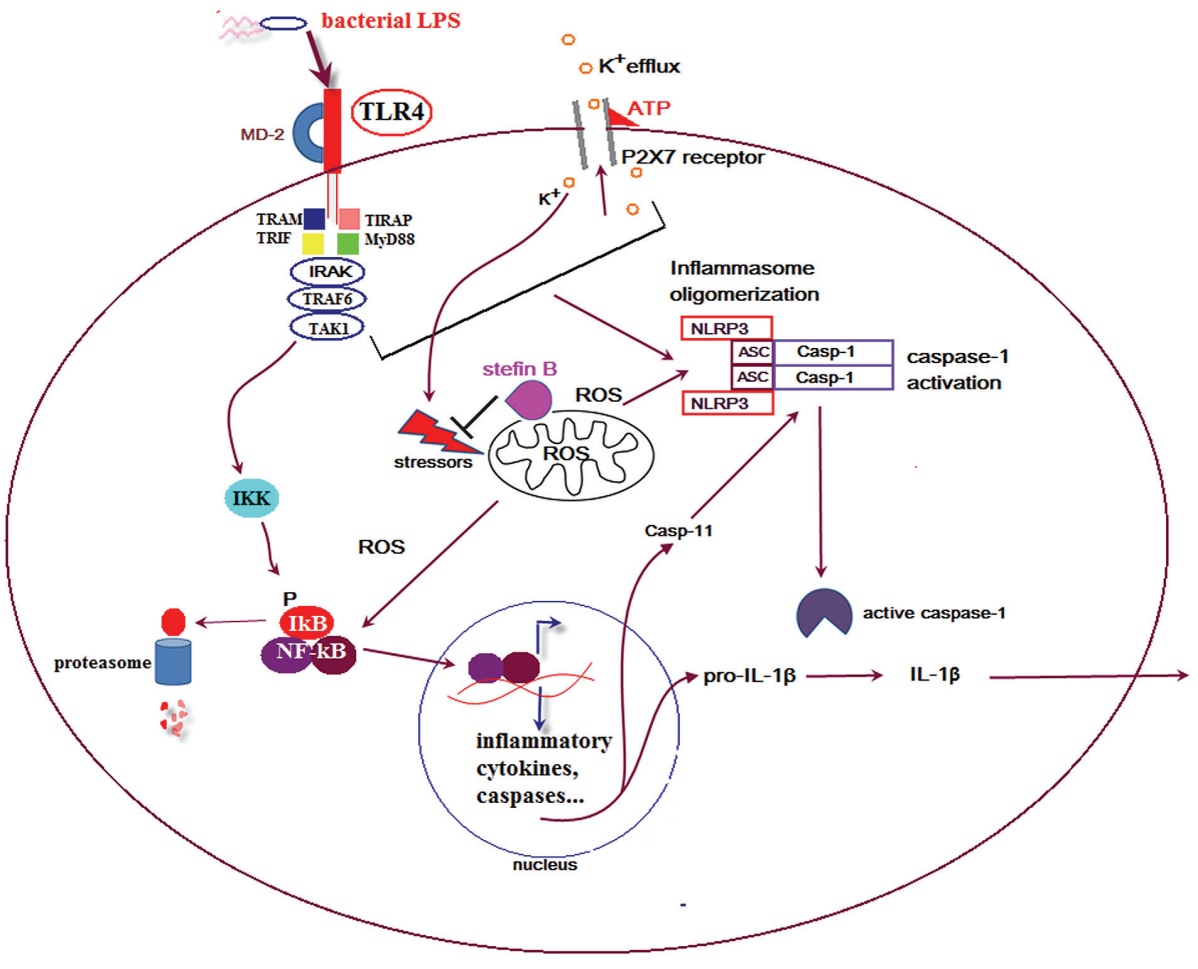

FIGURE 1 | Proposed model for the role of stefin B in non canonical inflammasome activation. Upon lipopolysaccharide (LPS) stimulation stefin B is translocated from cytosol into mitochondria and protects mitochondrial membrane integrity. Stefin B deficiency resulted in the breakdown of mitochondria membrane potential and increased mtROS generation. The consequence of the increased mtROS detected in stefin B-deficient bone marrow-derived macrophages (BMDMs) upon LPS stimulation, was the increased nuclear factor-κB (NF-κB) activation and caspase-11 expression. Increased caspase-11 expression resulted in increased inflammasome activation and pro-inflammatory IL-1β and IL-18 secretion.

increased caspase-11 gene expression and better caspase-1 and -11 processing determined in stefin B-deficient BMDMs resulted in enhanced IL-1β and IL-18 processing and secretion (**Figure 1**). The increased cathepsin activity determined in stefin B deficient BMDMs was not essential for inflammasome activation, since treatment of BMDMs with the cathepsin inhibitor E-64d did not influence caspase-1 activation and IL-1β secretion. Upon LPS stimulation, stefin B was targeted to mitochondria, and the lack of stefin B resulted in the increased destabilization of mitochondrial membrane potential and mitochondrial ROS generation (Maher et al., 2014a). The induction of ROS in microglia may therefore play an important role in non-canonical inflammasome activation and cell death in the cerebellum in disease.

CONCLUSION

This review summarizes recent discoveries that may contribute to the understanding of the role of stefin B in neuro-inflammation. Several studies, each from a different angle, have contributed a piece of the puzzle, a process we are trying to understand. Stefin B-deficient mice have proven to be a valuable tool to explore the function of the protein in the pathology of disease. During the past couple of years, several new data from microarray experiments, histology, as well as magnetic resonance imaging have revealed that neuro-inflammation is an essential process in the pathology of EPM1. Biochemical experiments in macrophages have contributed some hints regarding the signaling pathways in inflammasome activation. Signaling pathways in macrophages were compared to the ones in microglia and the expression of caspase-11 was strongly induced by activation of rat glial cells, as well as in astrocytes, with interferon-γ and LPS (Hur et al., 2001). The expression of caspase-11 in microglia may play an important role in non-canonical inflammasome activation and cell death in the cerebellum in disease. However, some questions still remain and some more pieces need to be added to complete the whole picture. Additional experiments will reveal if the inflammasome activation and caspase-11 expression are part of the pathology of EPM1.

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frontiers in Cellular Neuroscience

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Cocaine promotes oxidative stress and microglial-macrophage activation in rat cerebellum

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Different mechanisms have been suggested for cocaine neurotoxicity, including oxidative stress alterations. Nuclear factor kappa B (NF-κB), considered a sensor of oxidative stress and inflammation, is involved in drug toxicity and addiction. NF-κB is a key mediator for immune responses that induces microglial/macrophage activation under inflammatory processes and neuronal injury/degeneration. Although cerebellum is commonly associated to motor control, muscular tone, and balance. Its relation with addiction is getting relevance, being associated to compulsive and perseverative behaviors. Some reports indicate that cerebellar microglial activation induced by cannabis or ethanol, promote cerebellar alterations and these alterations could be associated to addictive-related behaviors. After considering the effects of some drugs on cerebellum, the aim of the present work analyzes pro-inflammatory changes after cocaine exposure. Rats received daily 15 mg/kg cocaine i.p., for 18 days. Reduced and oxidized forms of glutathione (GSH) and oxidized glutathione (GSSG), glutathione peroxidase (GPx) activity and glutamate were determined in cerebellar homogenates. NF-κB activity, CD68, and GFAP expression were determined. Cerebellar GPx activity and GSH/GSSG ratio are significantly decreased after cocaine exposure. A significant increase of glutamate concentration is also observed. Interestingly, increased NF-κB activity is also accompanied by an increased expression of the lysosomal mononuclear phagocytic marker ED1 without GFAP alterations. Current trends in addiction biology are focusing on the role of cerebellum on addictive behaviors. Cocaine-induced cerebellar changes described herein fit with previosus data showing cerebellar alterations on addict subjects and support the proposed role of cerebellum in addiction.

Keywords: cerebellum, cocaine, nuclear factor kappa B, oxidative stress, mononuclear phagocyte

Abbreviations: GFAP, glial fibrillary acidic protein; GSH, glutathione; GSSG, oxidized glutathione; IκB, inhibitory kappa B; MP, mononuclear phagocyte; NAc, nucleus accumbens; NF-κB, nuclear factor kappa B; ROS, reactive oxygen species; VTA, ventral tegmental area.

Introduction

Cocaine effects have been widely studied in neural areas traditionally related with drug addiction e.g., frontal cortex, NAc, and VTA among others. Cerebellum has been typically involved in functions related to motor control, from balance to fine motor tuning. However, some studies indicate that cerebellum could be involved in higher cognitive processes (Ramnani, 2006; Izawa et al., 2012). Even more, there are evidences supporting the idea that cerebellum is also involved with cocaine addiction (Anderson et al., 2006; Sim et al., 2007; Vazquez-Sanroman et al., 2015).

Much of the interest in the role of the cerebellum in cognition is based on the presence of anatomical pathways connecting the cerebellum and dorsolateral prefrontal cortex (Middleton and Strick, 1994). In addition, cerebellar lesions lead to behavioral changes characterized by executive function impairments such as motor planning, verbal fluency, and changes in personality among others. Moreover, it has been suggested that some of these changes implicate altered functional connections between cerebellum and prefrontal, posterior parietal, temporal, and limbic cortices (Jiménez-Rivera et al., 2000).

Recent studies indicate that cocaine exposure promotes molecular and structural cerebellar alterations (Palomino et al., 2014; Moreno-López et al., 2015) and fitting with this, some neuroimaging studies show how cocaine alters cerebellar functions (Anderson et al., 2006; Sim et al., 2007).

There is a growing body of evidence implicating oxidative stress in the pathogenesis and development of addiction to cocaine and other substances (Uys et al., 2011; Sordi et al., 2014). Despite oxygen is essential for aerobic life, and particularly central nervous system exhibits the highest oxygen consumption rate (20% from total O_2), excessive amounts of ROS derived from cell activity can result deleterious for cells. As other addictive substances, cocaine promotes oxidative stress in specific areas closely implicated in the circuitry of addiction such as NAc, frontal cortex, and hippocampus (Dietrich et al., 2005; Muriach et al., 2010; Jang et al., 2014). Surprisingly there is no literature about how cocaine modulates oxidative stress in cerebellum.

As occurs during inflammatory processes, ROS recruit inflammatory responses and microglia/macrophage, referred as MP, represents one of the most relevant actors during this process (Aguzzi et al., 2013). During pathological processes, MP releases cytokines, trophic factors, and even ROS (Krasnova and Cadet, 2009), and as reviewed by McNally et al. (2008) these pro-inflammatory factors, such as ROS and cytokines, impair astrocytic glutamate reuptake, resulting neurotoxic for cells. Stressing this hypothesis, cocaine exposure leads to up-regulation of pro-inflammatory mediators such as cytokines and chemokines, or astroglia/microglia activation (Renthal et al., 2009; Piechota et al., 2010; Blanco-Calvo et al., 2014).

In addition, one of the most relevant roles of MP is that related to phagocytosis of pathogens, degenerating cells and debris (Schafer et al., 2013). Apart from this defense rol, resident microglia is also involved in other physiological processes such as neuronal activity modulation, synaptic regulation, learning, and memory (Wake et al., 2009; Tremblay et al., 2011; Pascual et al., 2012; Blank and Prinz, 2013). Microglia has been also implicated in the development of cocaine addiction (Del Olmo et al., 2007; Porter et al., 2011). Despite of there are controversial results about MP activation during cocaine exposure (Little et al., 2009; Narendran et al., 2014), psychostimulant drugs activate specific components of the response, following both acute and chronic psychostimulant exposure (Yamamoto et al., 2010). Several researchers state that methamphetamine induces microglial activation in the brain (Thomas et al., 2004). Moreover, reactive microgliosis (estimated as microglia/macrophage activation) has been detected in several brain regions of methamphetamine addicts even after years of abstinence (Sekine et al., 2008). On this line, some reports indicate that cerebellar MP activation induced by cannabis or ethanol could be associated to cerebellar impairments (Cutando et al., 2013; Drew et al., 2015). These alterations fit with the proposed implication of cerebellum in the development of addictive-related behaviors.

Nuclear factor kappa B is a central mediator of human immune response (Pahl, 1999), and acts as sensor of oxidative stress situations (Schreck et al., 1992). In addition, NF-κB-DNA binding activity and transcription are regulated by various forms of synaptic activity (Albensi and Mattson, 2000; Freudenthal et al., 2004). In fact, O'Riordan et al. (2006) provide evidence suggesting that hippocampal NF-κB is activated by metabotropic glutamate receptors and Willard and Koochekpour (2013) proposed that NF-κB-mediated glutamate signaling plays a role in many neuronal processes where synaptic remodeling and plasticity are critical, e.g., learning and memory. Moreover, activation of group II mGluRs by glutamate promotes TNF release and neurotoxicity by activating NF-κB signaling (Lee, 2013).

Nuclear factor kappa B has been proposed as target of drugs such as ethanol or cocaine. Thus, increased NF-κB activity in the NAc after cocaine exposure has been demonstrated (Ang et al., 2001; Russo et al., 2009). Furthermore, ethanol exposure induces NF-κB activity in the mouse brain, which in turn, induces the transcription of pro-inflammatory immune genes, increasing expression of cytokines, proteases, and oxidases (Qin et al., 2008; Mayfield et al., 2013).

So, after considering that cocaine increases oxidative stress in several brain areas and the relevant role of NF-κB in oxidative stress, inflammation, and addiction. This report is addressed to investigate the oxidative cerebellar-related misbalance induced by cocaine giving more support to the emerging hypothesis that implicates cerebellum in addiction.

Materials and Methods

Experimental Design

Male Wistar rats weighing 300 g (Charles River Laboratories SA, Barcelona, Spain) were used for the experiment ($n = 20$). Rats were individually caged and maintained in a 12 h light/dark cycle with controlled temperature (20–25°C) and relative humidity (60%). Animals had access to food and water *ad libitum*. All the experimental procedures were carried out according to the

European Union regulation (order 86/608/CEE) and approved by the committee on animal care of the UCH-CEU with reference number 11/022.

The experiment lasted 18 days. In this period, the animals were separated into two groups, control and cocaine. Cocaine was daily administered by intraperitoneal injection at a dose of 15 mg/Kg in saline, and control animals received same saline volume (0.9%) in the intraperitoneal cavity.

Rats were sacrificed on the last day by cervical dislocation (previously anesthetized with pentobarbital). Brains were removed and cerebellum was dissected and divided into two parts. One part was used to measure oxidative stress markers and glutamate concentration. Samples used to analyze the oxidative stress were homogenized in 0.1 M PB (HK_2O_4P 0.1 M; H_2KO_4P 0.1 M) pH 7.0 at 4°C.

Homogenates were centrifuged at 6,000 rpm 2 min and the supernatant was stored at −20°C until used for protein determination and glutathione peroxidase (GPx) enzyme activity. In the case of GSH, GSSG, and glutamate, immediately after centrifugation, the supernatant was acidified with 20% of perchloric acid (Panreac, Spain) and stored at –20°C until the determination of these parameters.

Protein content was measured by means of the Lowry method (Lowry et al., 1951) to allow expression of the biochemical results taken into account the protein content of each sample.

Samples for western blot analysis and NF-κB activity. Nuclear fraction was separated from cytoplasmic fraction using the following protocol. Cerebellar tissue was homogenized on working solution A (Hepes 10 mM, KCl 5 mM, EDTA 0.1 mM, EGTA 0.1 mM, Ditiotreitol 100 mM, IGEPAL 0.05%, Complete 1x, NaF 10 mM, Na_3V0_4 200 mM). The samples were centrifuged at 850*g* for 10 min at 4°C. The supernatant was separated from the pellet and stored. 400 μl of working solution A were mixed, incubated 15 min at 4°C and centrifuged at 10,000*g* for 30 s at 4°C. The supernatant was collected and stored (cytoplasmic fraction). Hundred and fifty microliter of the working solution C were added to the remaining pellet (Hepes 20 mM, NaCl 0.4 M, EDTA 1 mM, EGTA 1 mM, Ditiotreitol 100 mM, Complete 1x, NaF 10 mM, Na_3V0_4 200 mM) and incubated again 15 min at 4°C. The sample was centrifuged at 10,000*g* for 5 min at 4°C. The collected supernatant was the nuclear fraction. Both cytoplasmic (used for western blot analysis) and nuclear fractions (used to measure NF-κB activity) were stored at −20 for later use.

Animals assigned for immunohistochemical procedures were perfused with saline followed by 4% paraformaldehyde solution in 0.1 M phosphate buffer (PB), pH 7.4. Brains were post-fixed in paraformaldehyde solution for 24 h and then placed in a 30% sucrose solution for 24 h. Thirty micrometer thick sections were obtained using cryostat.

Oxidative Stress

Reduced GSH, GSSG, and glutamate concentrations were quantified following the method of Reed et al. (1980), based in the reaction of iodoacetic acid with the thiol groups followed by a chromophore derivatization of the amino groups with Sanger reactant (1-fluoro-2,4-dinitrobencene), giving rise to derivates which are quickly separated by means of high-performance liquid chromatography (HPLC).

Glutathione peroxidase activity, which catalyzes the oxidation by H_2O_2 of GSH to its disulfide (GSSG), was assayed spectrophotometrically as reported by Lawrence et al. (1978) toward hydrogen peroxide, by monitoring the oxidation of NADPH at 340 nm. The reaction mixture consisted of 240 mU/mL of GSH disulfide reductase, 1 mM GSH, 0.15 mM NADPH in 0.1 M potassium phosphate buffer, pH 7.0, containing 1 mM EDTA and 1 mM sodium azide; a 50 μL sample was added to this mixture and allowed to equilibrate at 37°C for 3 min. Reaction was started by the addition of hydrogen peroxide to adjust the final volume of the assay mixture to 1 mL.

Western Blot Analysis

Samples were resolved on 10% SDS polyacrylamide gels and transferred to nitrocellulose membrane. Membranes were blocked in 5% skim milk in T-TBS buffer and 0.1% Tween 20, for 1 h and were incubated thereafter with the primary antibody overnight at 4°C. Primary antibodies used were ED1 (a lysosomal protein which is overexpressed during inflammatory challenge, and is used as a marker to confirm microglial activation), peroxidase β-Actin (Sigma–Aldrich, Alcobendas, Spain) caspase 3 (a pro-apoptotic protein; Cell Signaling, Barcelona, Spain) and GFAP one of the major intermediate filament proteins of mature astrocytes (Dako, Denmark). Peroxidase-coupled secondary antibodies were used for primary antibody detection by incubating membranes 1 h at room temperature. (anti-mouse Thermo Fisher Scientific, Madrid, Spain; anti-rabbit, Santa Cruz, California, EEUU) Finally, the signal was detected with ECL developing kit (Amersham Biosciences, UK). Blots were quantified by densitometry using Quantity One software and the results were represented in density units.

Ba/F3 cells (murine interleukin-3 dependent pro-B cell line) were used as positive control for caspase-3 activation.

CD68 (ED1) Immunohistochemistry

Cerebellar CD68 expression was examined by immunohistochemistry. Sections were rinsed with 0.01M PBS, pH 7.0 and blocked with 30% H_2O_2 for 20 min followed by incubation overnight with a primary rabbit anti-CD68 (ED1) (Abcam, Cambridge, UK; dilution 1:500 in PBS with 0.3% Triton X-100 and 5% normal goat serum). Sections were rinsed in PBS and incubated at room temperature shaking for 1 h in 0.4% biotinylated anti-rabbit IgG. Finally, sections were rinsed and re-incubated with avidin-biotin complex and reaction was developed with DAB.

Images were captured with a CCD camera (Coolsnap FX Color; Roper Scientific).

Nuclear Factor Kappa B Activity

To determine NF-κB activity in the nuclear fraction, an ELISA-based kit to detect and quantify transcription factor activation was used, TransAM NF-κB (Active Motif, Rixensart, Belgium). Results were represented as arbitrary units.

Terminal Deoxynucleotidyl Transferase Biotin-dUTP Nick end Labeling

Transferase biotin-dUTP nick end labelling (TUNEL) assay was performed by an *in situ* cell death detection kit (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's instructions. DNAase reaction was used for positive control labeling.

Statistical Analysis

Results are presented as mean values ± SE. Statistical significance was assessed by Students *t*-test. The level of significance was set at $p < 0.05$.

Results

Antioxidant Defenses are Decreased in the Cerebellum After 18 days of Cocaine Administration in Rats

Cerebellar homogenates were processed for HPLC determination. Cerebellar GSH levels remained unaltered (data not shown) whereas GSSG levels presented a statistically significant increase compare to control GSSG levels (**Figure 1A**). Interestingly, the GSH/GSSG ratio was statistically significant lower in cerebella from cocaine treated animals compared to control animals (**Figure 1B**). GPx is the enzyme that converts GSH to GSSG by reducing hydrogen peroxide (H_2O_2) to water. Cocaine treated group presented statistically significant lower cerebellar levels of GPx than control groups (**Figure 1C**).

Astrocytic and Microglial/Macrophage Response

ED1 antigen or CD68 is a lysosomal protein expressed during inflammatory processes by both microglia and macrophages. Cerebellar CD68 expression was determined by western blot. As shown in **Figure 2A**, ED1 expression was statistically significant increased in cocaine treated rats when compared to control group.

Fitting with this result, ED1-IHC technique shows almost unappreciable rounded ED1 positive cells sparse and randomly located in the cerebellar cortex of control animals. However, cerebella from cocaine treated animals present more and rounded ED1 positive cells with evident perivascular location (**Figure 2B**).

In order to investigate the possibility of a pro-inflammatory response, glial fibrilary acidic protein (GFAP) was analyzed by western blot. This astrocytic protein is over expressed under inflammatory-related responses. Surprisingly no GFAP differences could be set between groups (**Figure 2C**).

Glutamate Concentration

Total glutamate (extra-cellular + intra-cellular) was measured by HPLC from cerebellar homogenates. As shown in **Figure 3**, 18 days of cocaine administration induced a statistically significant increase on cerebellar glutamate concentration compared to control rats.

Nuclear Factor Kappa B Activity

Cytoplasmic IκB factor inhibits NF-κB. NF-κB inducing stimuli activate the IκB kinase complex that phosphorylates IκB. IκB degradation exposes the NF-κB DNA-binding domain allowing its nuclear translocation regulating NF-κB target genes.

NF-κB activity from the nuclear fraction was statistically significant increased in cerebella of cocaine treated rats when compared to control rats (**Figure 4**).

Apoptotic Cell Death: Caspase 3 Expression and TUNEL

No signal of pro-apoptotic protein caspase-3 was observed in cerebellum after 18 days of cocaine exposure (**Figure 5A**).

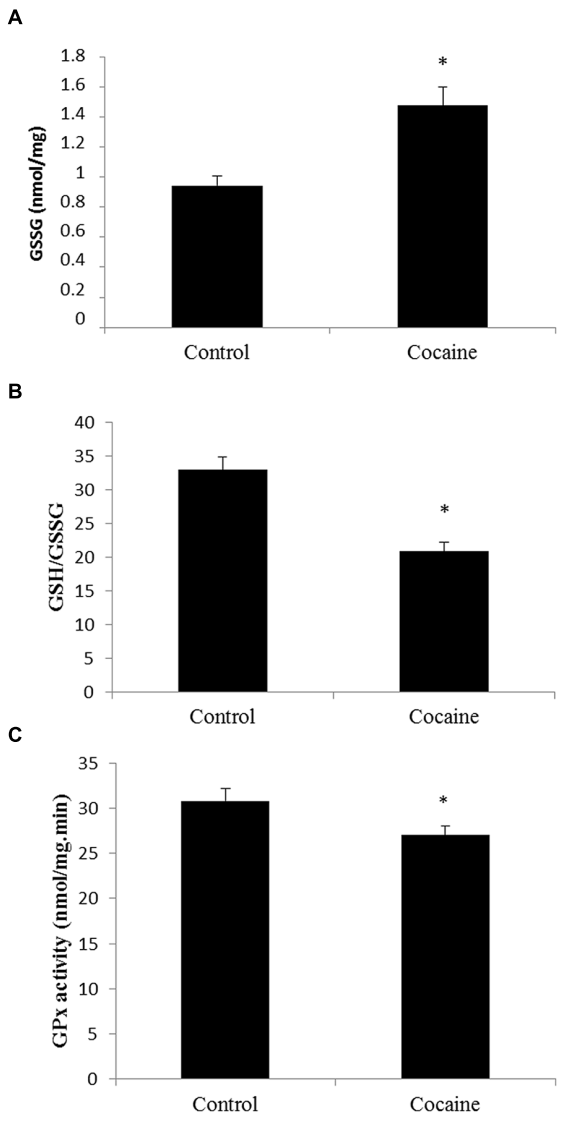

FIGURE 1 | Effect of cocaine administration on cerebellar antioxidant defenses. (A) GSSG concentration (nmol/mg prot) *$p < 0.05$ vs. control group. **(B)** GSH/GSSG ratio *$p < 0.05$ vs. control group. **(C)** GPx activity (nmol/mg.min) *$p < 0.05$ vs. control group.

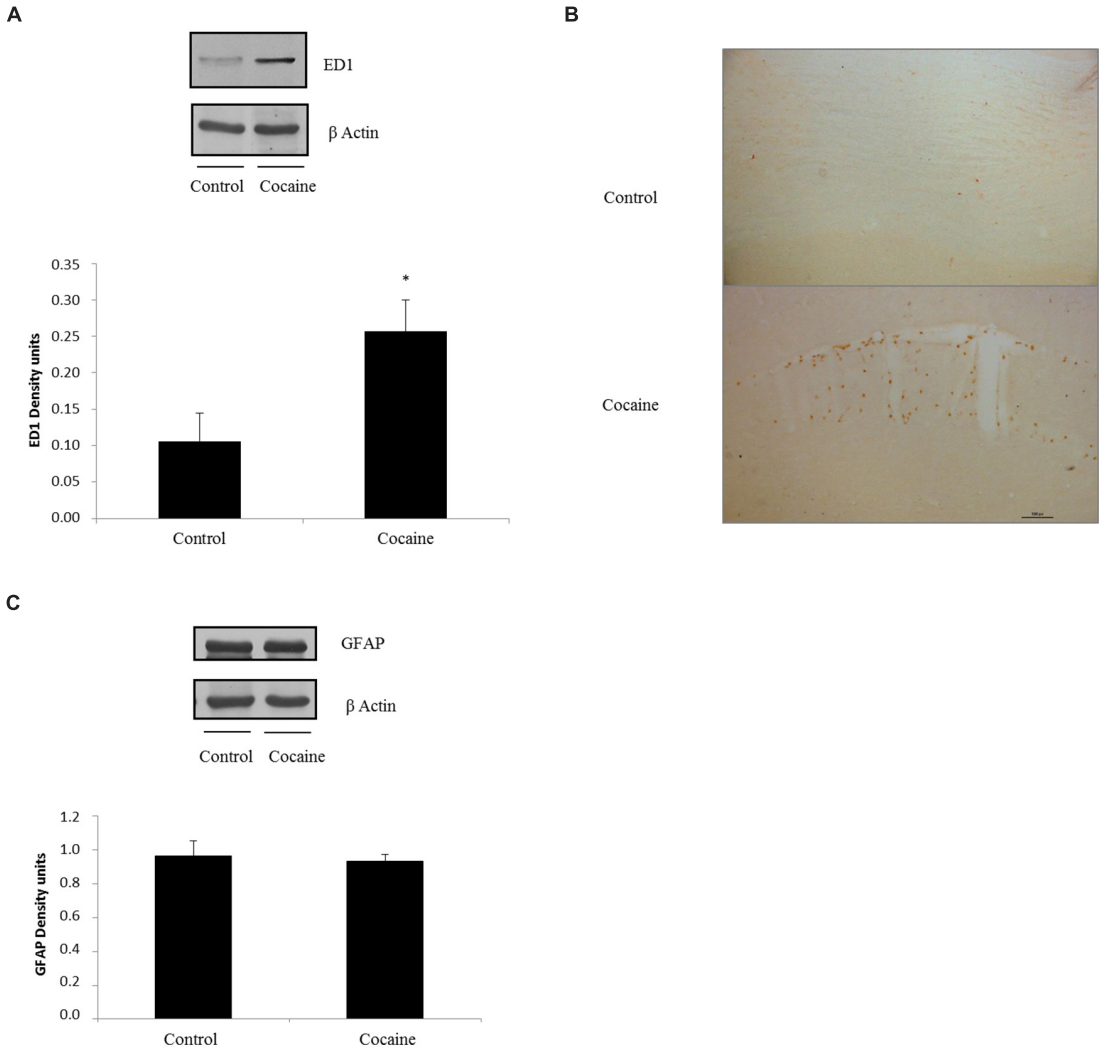

FIGURE 2 | ED1 (68 kDa) and β-Actin (42 kDa) western blot dots in rat cerebellum. Representation of ED1 (density units) in cerebellum showing a significant ED1 increase in cerebellar samples of cocaine-treated rats (*$p < 0.05$ vs. control group; **A**). ED1 immunohistochemistry from control and cocaine-treated cerebellar samples. Little or null ED1 positive cells can be observed in control samples, whereas noticeable ED1 positive cells can be found around cerebellar vessels **(B)**. GFAP (50 kDa) and β-Actin (42 kDa) western blot dots in control and cocaine-treated cerebellar samples, with GFAP representation (as density units). No differences can be set between control and cocaine-treated rats suggesting a lack of astrocytic response after cocaine exposure **(C)**.

However, because caspase-3 is activated after its cleavage, TUNEL determination was conducted in cerebellar samples in order to find apoptotic cell death. As observed in TUNEL positive control sample (DNAase reaction) profuse TUNEL positive labeled cells can be found. Little or null TNEL positive labeling could be demonstrated in control or cocaine treated groups (**Figure 5B**).

Discussion

Mononuclear Phagocyte Activation and Glutamate Concentration

Mononuclear phagocyte activation has been related with psychiatric diseases (Holstege et al., 2008; Chen et al., 2010). Addiction is included as a mental disorder and therefore several studies are currently focusing on the structural and molecular CNS alterations during drug consumption and addiction. In this sense, large evidence indicates that different drugs from ethanol to psychostimulants (e.g., cocaine) promote microglial activation (Thomas et al., 2004; Little et al., 2009; Yamamoto et al., 2010; Raineri et al., 2012; Cutando et al., 2013; Drew et al., 2015). It is well documented that cocaine exposure affects microglia (Hayashi and Su, 2003), up-regulating pro-inflammatory mediators such as cytokines with astroglia/microglia activation (Renthal et al., 2009; Piechota et al., 2010; Blanco-Calvo et al., 2014).

Cocaine challenge resulted in a marked increase of cerebellar ED1. Fitting with this, ED1 is overexpressed during inflammatory

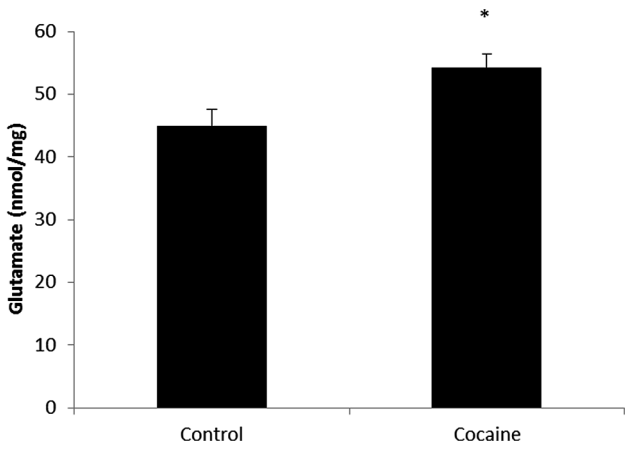

FIGURE 3 | Microglial-macrophage activation is closely related to extracellular glutamate over-drive. HPLC cerebellar glutamate levels (nmol/mg of total protein) in control and cocaine-treated rats. Total cerebellar glutamate levels are increased after cocaine administration. (*$p < 0.05$ vs. control group).

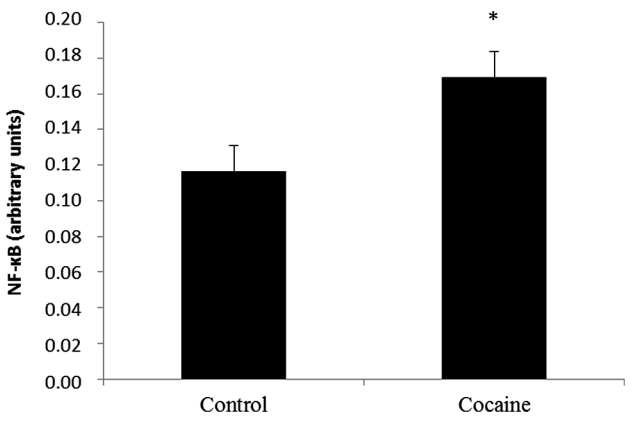

FIGURE 4 | Nuclear factor kappa B is a redox-sensitive nuclear factor involved in the control of immune-inflammatory responses. Cerebellar NF-κB activity assay (arbitrary units) from control and cocaine- treated animals (18 days) showing a significant increase of NF-κB activity in cocaine-treated rats (*$p < 0.05$ vs. control group).

challenge, and it is used as marker to confirm MP activation (Graeber et al., 1990). Furthermore, ED1 seems to correlate with the capacity of postnatal microglia to engulf synapses (Schafer et al., 2013). After consider the typical phagocytic role of microglia during inflammation-related processes and the role of microglia in remodeling neural contacts during learning and memory processes (Felger and Miller, 2012; Blank and Prinz, 2013), it seems plausible that cocaine-induced cerebellar MP activation might be related to these aforementioned cerebellar changes after cocaine exposure.

Fitting with the increased cerebellar glutamate levels found, cerebellar glutamatergic activation has been also described after cocaine exposure (McFarland et al., 2003; Palomino et al., 2014). Although synaptic glutamate results of high interest for addiction and learning-memory processes, the present report focus attention on extra-synaptic glutamate, in view of its relevance on inflammation and its implication with mental disorders (Müller and Schwarz, 2007). In this sense, MP activation is closely related to extracellular glutamate over-drive leading to neurotoxicity and neural remodeling. So, the finding of increased cerebellar glutamate levels fits with CD68 over expression and lends support to previous reports on cocaine-related cerebellar alterations in overt behavior and cognition (Jiménez-Rivera et al., 2000). One relevant observation is that related to cerebellar function after cocaine exposure. As previously reported, no differences could be found on targeting-directionality in the Morris water maze test after the same cocaine challenge (Muriach et al., 2010). So there are not evidences of cocaine-related cerebellar alterations after this experimental paradigm in terms of motor-related functions.

Antioxidant Defenses and Apoptotic Markers

Some reports indicate redox status misbalance after cocaine treatment in several brain areas (Dietrich et al., 2005; Muriach et al., 2010; Uys et al., 2011). Interestingly and as novelty, this is the first report showing cerebellar oxidative alterations after cocaine challenge. It is well known that oxidative stress causes cellular damage and eventually cell death (Calabrese et al., 2007). Because caspase-3 levels were undetectable after cocaine challenge and caspase-3 is active after its cleavage, TUNEL assay was developed. The lack of TUNEL labeling indicates a lack of apoptotic cell death, suggesting that apoptosis is not promoted after cocaine exposure in cerebellum. Fitting with this, other reports show the absence of apoptosis after cocaine exposure in brain (Dominguez-Escriba et al., 2006; Muriach et al., 2010). Controversially, some reports indicate that cocaine exposure induces apoptosis in different tissues (Cerretani et al., 2012; Blanco-Calvo et al., 2014). The discussion about what dose or duration can promote cell death (apoptotic or not) is so far from the goal of this work and probably the aforementioned differences could be explained depending on the tissue, cocaine concentration/duration, animal model, etc. In fact, diverse published data are conducted with different cocaine doses and duration. For this work, cocaine dose and duration was chosen from previous published works (Ishikawa et al., 2009; Schroeder et al., 2009; Muriach et al., 2010).

Dopamine auto-oxidation (Numa et al., 2008) has been typically accepted as cocaine-induced ROS source. However, dopamine is not the main neurotransmitter in cerebellum, particularly present in vermis (Melchitzky and Lewis, 2000). Therefore, the observed antioxidant defense decrease could be due to other origins. Cocaine-induced vasoconstriction may decreases cerebellar blood flow, leading to hypoxia increasing ROS (Kaufman et al., 1998; Gottschalk and Kosten, 2002; Pae et al., 2005). Additionally, it has been described that cocaine increases brain temperature, which is a reliable indicator of metabolic neural activation (Kiyatkin and Brown, 2004) and thus, cocaine-enhanced metabolism can increase ROS (Brookes et al., 2004). Finally and fitting with the results shown herein, the decrease of antioxidant defenses could be due to MP activation, since activated microglia and macrophages can

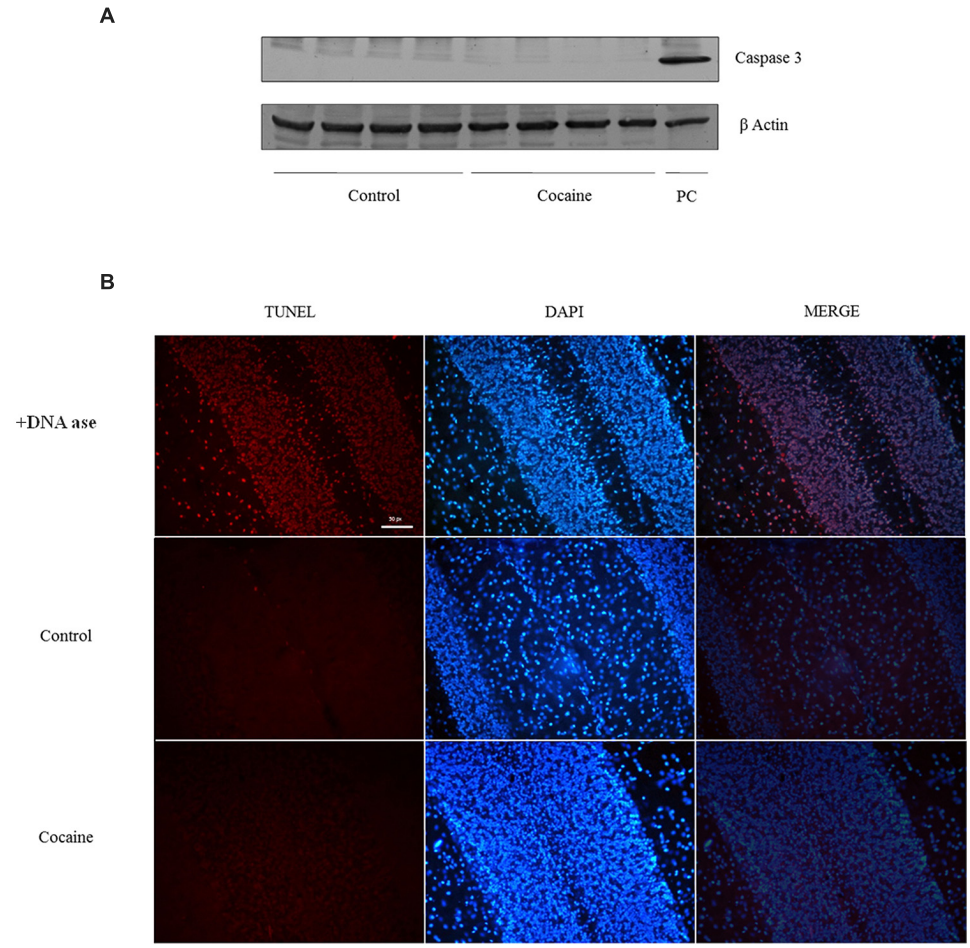

FIGURE 5 | Caspase-3 (17/19 kDa) and β-Actin (42 kDa) western blot analysis in cerebellum (A; Ba/F3 cells were used as positive control of caspase 3 activation). TUNEL assay on cerebellum: +DNA ase reaction (used as positive control of TUNEL) exhibits profuse TUNEL-positive cell labeling (red dye). Minimal or null TUNEL-positive labeled cells could be demonstrated in control or cocaine-treated cerebellar samples **(B)**.

produce ROS after LPS stimulus (Marín-Teva et al., 2004; Di Penta et al., 2013). In conclusion, despite there are multiple ways by which cocaine potentially promotes oxidative stress. The findings shown herein indicate that cocaine-induced cerebellar MP activation is accompanied by antioxidant defense decay, suggesting an unusual pro-inflammatory response since GFAP is unaltered. Future studies must be addressed to resolve this issue.

NF-κB is Activated After Cocaine Exposure

Nuclear factor kappa B is a redox-sensitive nuclear factor involved in the control of immune-inflammatory responses, developmental processes, and apoptosis. NF-κB is a key regulator of inflammation and secondary injury processes. In fact, several members of the NF-κB family are considered essential regulators of cellular activities associated with inflammation/chemokine production (Ghosh and Hayden, 2008). Stimuli such as cytokines, free radicals, ultraviolet irradiation, bacterial or viral antigens and glutamate increase NF-κB-DNA binding promoting chemokine-cytokine gene transcription (Schreck et al., 1992; Meffert and Baltimore, 2005). Although NF-κB is expressed in many cells, NF-κB is transcriptionally active primarily in glia (Mao et al., 2009). Astrocytes, monocytes, and microglia express high levels of NF-κB under pathological situations, this transcription factor is the key one involved in induction of innate immune genes in microglia and other monocyte-like cells (Mattson and Camandola, 2001; Crews et al., 2011). The lack of GFAP over expression may suggest that astrocytes are not directly involved in this NF-κB activation.

Nuclear factor kappa B activation seems to mediate some processes in cocaine addiction, particularly in the NAc, hippocampus, or frontal cortex (Ang et al., 2001; Russo et al., 2009), but nothing is known regarding other brain areas, such as the cerebellum. Cerebellar cocaine-induced NF-κB activation is accompanied by a significant decrease of the antioxidant defense and by increased microglial/macrophage ED1 expression. In line with this, increased p65 NF-κB activity accompanied by ROS production and cytokine expression has been also demonstrated in cocaine-treated microglia (Yao et al., 2010). Moreover, ROS promotes microglial NF-κB activation (Block et al., 2007).

Relationship between NF-κB, Glutamate Concentration and Antioxidant Defenses

It is well known that NMDA receptors activate NF-κB (Lipsky et al., 2001; Munhoz et al., 2008). Therefore, the increase observed in NF-κB activity, could be due to the enhanced glutamate concentration observed after cocaine administration. Moreover, Barger et al. (2007) reported that the glutamate release from activated microglia is an indirect consequence of GSH depletion. Thus, it seems that if cocaine increases cerebellar glutamate concentration, it could be associated to GSH depletion. On the other hand, the activation of NMDA and AMPA receptor subtypes causes the mobilization of free cytosolic calcium, and the excess of intracellular calcium results in ROS generation (Carriedo et al., 1998).

Cocaine promotes oxidative cerebellar misbalance with increased ED1 expression, as estimation of mononuclear phagocytic activity. In addition, NF-κB activation and increased glutamate levels strongly suggest a pro-inflammatory process underlying mechanism after cocaine exposure. Future studies would be addressed to investigate the role and meaning of this cocaine-induced cerebellar ED1 over expression and whether these molecular and cellular modifications may lead to perpetuate neural circuitries involved in addiction.

Author Contributions

MM, JB, and FR conceived and designed the study. RL-P, DT, and MS-V performed the experiments. IA, MS-V, and DR-L were responsible for the biochemical analysis. BM, RL-P, and DR-L performed the western blot analysis. JB, IA, and BM were responsible for the statistical analyses. LV-G and RL-P performed IHC procedures, RL-P and DR-L designed the figures. RL-P, JB, MM, and FR wrote the manuscript.

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Fetal microglial phenotype *in vitro* carries memory of prior *in vivo* exposure to inflammation

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Objective: Neuroinflammation *in utero* may result in life-long neurological disabilities. The molecular mechanisms whereby microglia contribute to this response remain incompletely understood.

Methods: Lipopolysaccharide (LPS) or saline were administered intravenously to non-anesthetized chronically instrumented near-term fetal sheep to model fetal inflammation *in vivo*. Microglia were then isolated from *in vivo* LPS and saline (naïve) exposed animals. To mimic the second hit of neuroinflammation, these microglia were then re-exposed to LPS *in vitro*. Cytokine responses were measured *in vivo* and subsequently *in vitro* in the primary microglia cultures derived from these animals. We sequenced the whole transcriptome of naïve and second hit microglia and profiled their genetic expression to define molecular pathways disrupted during neuroinflammation.

Results: *In vivo* LPS exposure resulted in IL-6 increase in fetal plasma 3 h post LPS exposure. Even though not histologically apparent, microglia acquired a pro-inflammatory phenotype *in vivo* that was sustained and amplified *in vitro* upon second hit LPS exposure as measured by IL-1β response *in vitro* and RNAseq analyses. While NFKB and Jak-Stat inflammatory pathways were up regulated in naïve microglia, heme oxygenase 1 (*HMOX1*) and Fructose-1,6-bisphosphatase (*FBP*) genes were uniquely differentially expressed in the second hit microglia. Compared to the microglia exposed to LPS *in vitro* only, the transcriptome of the *in vivo* LPS pre-exposed microglia showed a diminished differential gene expression in inflammatory and metabolic pathways prior and upon re-exposure to LPS *in vitro*. Notably, this desensitization response was also observed in histone deacetylases (*HDAC*) *1, 2, 4*, and *6.* Microglial calreticulin/LRP genes implicated in microglia-neuronal communication relevant for the neuronal development were up regulated in second hit microglia.

Discussion: We identified a unique $HMOX1_{down}$ and FBP^{up} phenotype of microglia exposed to the double-hit suggesting interplay of inflammatory and metabolic pathways.

Our findings suggest that epigenetic mechanisms mediate this immunological and metabolic memory of the prior inflammatory insult relevant to neuronal development and provide new therapeutic targets for early postnatal intervention to prevent brain injury.

Keywords: brain, neuroinflammation, bioinformatics, RNAseq, sheep, metabolism, cytokines, epigenetics

Introduction

Brain injury acquired antenatally remains a major cause of long-term neurodevelopmental sequelae (Saigal and Doyle, 2008). There is growing clinical and experimental evidence for maternal and fetal infection acting via systemic and neuroinflammation to cause fetal brain injury or contributing to *in utero* asphyxial brain injury with consequences for postnatal health (Hagberg et al., 2002; Rees and Inder, 2005; Wang et al., 2006; Gotsch et al., 2007; Murthy and Kennea, 2007; Fahey, 2008).

In humans, the main cause of fetal inflammation is chorioamnionitis, a frequent condition affecting 10% of all pregnancies and up to 40% of preterm births. Chorioamnionitis is associated with ∼nine-fold increased risk for cerebral palsy spectrum disorders with life lasting neurological deficits and an increased risk for acute or life-long morbidity and mortality (Fahey, 2008; Agrawal and Hirsch, 2012; Fishman and Gelber, 2012).

In addition to short-term brain damage, neuroimmune responses to *in utero* infection may also have long-term health consequences, the "second hit" hypothesis: In adults, exposure to inflammatory stimuli can activate microglia (glial priming, reviewed in Billiards et al., 2006; Karrow, 2006; Bilbo and Schwarz, 2009; Bilbo and Tsang, 2010; Ajmone-Cat et al., 2013; Bolton et al., 2014).

We hypothesized that an inflammatory response induced by lipopolysaccharide (LPS) will result in microglial activation reflecting neuroinflammation. To test the "second hit" hypothesis, we developed a protocol to culture fetal sheep microglia and re-expose them to LPS under *in vitro* conditions allowing a more mechanistic study of their phenotype.

Materials and Methods

Ethics Statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The respective *in vivo* and *in vitro* protocols were approved by the Committee on the Ethics of Animal Experiments of the Université de Montréal (Permit Number: 10-Rech-1560).

Anesthesia and Surgical Procedure

We instrumented pregnant time-dated ewes at 126 days of gestation (dGA, ∼0.86 gestation) with arterial, venous and amniotic catheters and ECG electrodes (Frasch et al., 2007). Ovine singleton fetuses of mixed breed were surgically instrumented with sterile technique under general anesthesia (both ewe and fetus). In case of twin pregnancy the larger fetus was chosen based on palpating and estimating the intertemporal diameter. The total duration of the procedure was approximately 2 h. Antibiotics were administered to the mother intravenously (trimethoprim sulfadoxine 5 mg/kg body weight) as well as to the fetus intravenously and into the amniotic cavity (ampicillin 250 mg). Amniotic fluid lost during surgery was replaced with warm saline. The catheters exteriorized through the maternal flank were secured to the back of the ewe in a plastic pouch. For the duration of the experiment the ewe was returned to a metabolic cage, where she could stand, lie and eat *ad libitum* while we monitored the non-anesthetized fetus without sedating the mother. During postoperative recovery antibiotic administration was continued for 3 days. Arterial blood was sampled for evaluation of maternal and fetal condition and catheters were flushed with heparinized saline to maintain patency.

In vivo Experimental Protocol

Postoperatively, all animals were allowed 3 days to recover before starting the experiments. On these 3 days, at 9.00 am 3 mL arterial plasma sample were taken for blood gasses and cytokine analysis. Each experiment commenced at 9.00 am with a 1 h baseline measurement followed by the respective intervention as outlined below. FHR and arterial blood pressure was monitored continuously (CED, Cambridge, UK, and NeuroLog, Digitimer, Hertfordshire, UK). Blood samples (3 mL) were taken for arterial blood gasess, lactate, glucose, and base excess (ABL800Flex, Radiometer) and cytokines at the time points 0 (baseline), +1 (i.e., after LPS administration), +3, +6, +24, +48, and +54 h (i.e., before sacrifice at day 3). For the cytokine analysis, plasma was spun at 4°C (4 min, 4000 g, Eppendorf 5804R, Mississauga, ON), decanted and stored at −80°C for subsequent ELISAs. After the +54 h (Day 3) sampling, the animals were sacrificed with an overdose of barbiturate (30 mg pentobarbital sodium, Fatal-Plus; Vortech Pharmaceuticals, Dearborn, MI) and a post mortem was carried out during which fetal gender and weight were determined. The fetal brain was then perfusion-fixed with 250 mL of cold saline followed by 250 mL of 4% paraformaldehyde and processed for histochemical analysis or dissected for cell culture (details see *in vitro* microglia culture paragraph). Fetal growth was assessed by body, brain, liver, and maternal weights.

Nine fetuses were used as controls receiving NaCl 0.9%. Twelve fetuses received LPS (400 ng/fetus/day) derived from *E. coli* (Sigma L5293, from *E. coli* O111:B4, ready-made solution containing 1 mg/ml of LPS) were administered intravenously to fetuses on days 1 and 2 at 10.00 am to mimic high levels of endotoxin in fetal circulation over several days as it may occur in chorioamnionitis.

In vitro Microglia Culture Protocol

Fetal sheep brain tissues were obtained during sheep autopsy after completion of the experiment for *in vitro* study (**Figure 1A**). The non-instrumented, untreated twins were designated "naïve" (N_C, no LPS exposure *in vivo*) and N_L when exposed to LPS *in vitro* for the first time. Instrumented animals that received LPS *in vivo* (SH_C) were used for 2nd hit LPS exposure *in vitro* (SH_L). Fetal sheep microglia culture protocol was adapted from an established human adult and fetal microglia culture protocol that was modified to include a myelin removal step following high-speed centrifugation (Durafourt et al., 2013). Briefly, fetal sheep cells were plated on poly-L-lysine (PLL)-coated tissue culture flasks at a concentration of 2×10^6 cells /ml in DMEM with 5% heat-inactivated fetal bovine serum (Gibco, Canada Origin), 1% penicillin/ streptomycin, and 1% glutamine (5% DMEM), in which microglia are preferable to grow (Durafourt et al., 2013). Cells were allowed to incubate for seven days at 37°C, 5% CO_2, followed by media change by centrifugation and addition of re-suspended cells back to the culture flask. Cells were continued to incubate for seven more days with 5% DMEM at 37°C, 5% CO_2, before floating cells were collected. Carefully collecting the floating microglia to avoid contamination with astrocytes and oligodendrocytes, the cells were incubated in 24-well plates at 1×10^5 cells/1.82 cm^2 surface area with 1 mL of 5% DMEM for another 4–5 days, and then treated with or w/o LPS (100 ng/ml, Sigma L5024, from *E. coli* O127, B8) for 6 h. Cell conditioned media were collected for cytokine analysis, 0.5 ml TriZol were added per well for RNA extraction.

To verify microglia purity, a portion of floating cells were cultured in 24-well plates at above conditions for flow cytometry analysis, cell morphology was documented with light microscopy (see Supplementary Material). Another portion of floating cells were plated into Lab-Tek eight well chamber glass slide (Thermo Scientific) and treated with or w/o LPS for immunocytochemistry

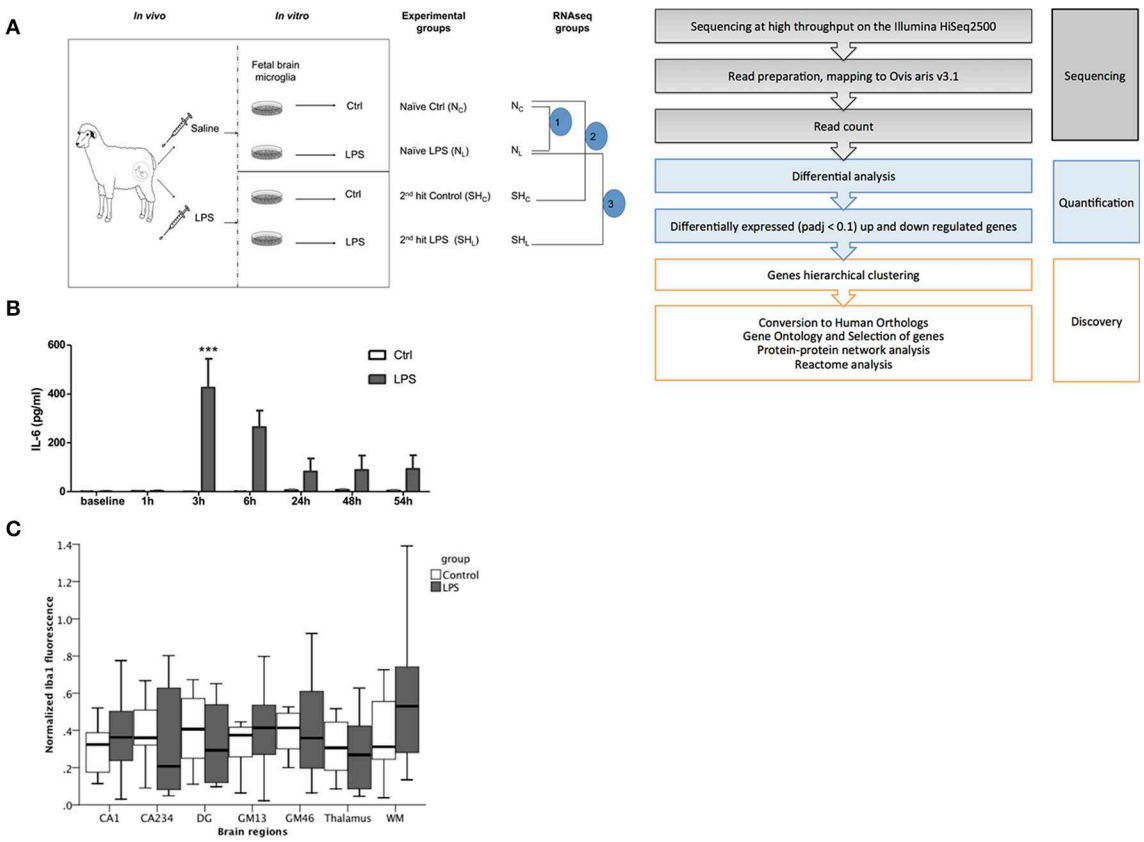

FIGURE 1 | Fetal sheep *in vivo* LPS exposure causes a systemic inflammation. (A) Experimental design. *In vivo, in vitro* and RNAseq experiments are illustrated. *In vivo* study, Control (saline) and LPS; *in vitro* study, cultured cells derived from *in vivo* Control animal, named as Naïve, whereas cells derived from LPS-exposed animal named as 2nd hit (second hit, SH), there are four experimental groups: naïve Control (N_C), naïve LPS (N_L), 2nd hit control (SH_C), and 2nd hit LPS (SH_L), respectively. For RNAseq data comparisons, we first compared pair 1 ($n = 6$) Control (N_C) vs. LPS-exposed naïve microglia (N_L); then pair 2 ($n = 4$) naïve control (N_C) vs. 2nd hit Control (SH_C); and finally pair 3 ($n = 4$) naïve LPS-exposed microglia (N_L) vs. 2nd hit LPS-exposed microglia (SH_L). **(B)** IL-6 levels peaked at 3 h in fetal sheep plasma following LPS administration *in vivo* (***$P < 0.001$). *In vivo* Control group, $n = 9$, *in vivo* LPS group, $n = 12$. Blood samples were collected in heparinized syringe from fetal arterial catheter, plasma was obtained by centrifugation. A sheep specific IL-6 ELISA was performed to measure the cytokine levels. **(C)** No evidence of Iba1+ fetal *brain* microglia inflammatory response to LPS exposure *in vivo*. Normalized Iba1 + signal (microglia) fluorescence in six randomly chosen high power fields per brain region is shown in hippocampus [CA1, CA234, and dentate gyrus (DG) subregions], cortical gray matter (GM) layers GM13, GM46, white matter (WM), and thalamus. GEE model for prediction of Iba1+ normalized signal intensity: group main effect $p = 0.62$; brain region main effect $p < 0.001$; brain region*group interaction $p = 0.13$.

analysis, in this experiment, some wells of astrocytes cultured at DMEM with 10% FCS were included for comparison.

Measurements of Inflammatory Responses

Measurement of Cytokines in Plasma and Cell Culture Media

Cytokine concentrations in plasma (IL-6) and cell culture media (IL-1β) were determined by using an ovine-specific sandwich ELISA. Briefly, 96-well plates (Nunc Maxisorp, high capacity microtitre wells) were pre-coated with the capture antibody, the mouse anti sheep monoclonal antibodies (IL-6, MCA1659; IL-1β, MCA1658, Bio Rad AbD Serotec) at a concentration 4 μg/ml on ELISA plates at 4°C for overnight, after 3 times wash with washing buffer (0.05% Tween 20 in PBS, PBST), plates were then blocked for 1 h with 1% BSA in PBST for plasma samples or 10% FBS for cell culture media. Recombinant sheep proteins (IL-6, Protein Express Cat. no 968-305; IL-1 β, Cat. no 968-405) were used as ELISA standard. All standards and samples (50 μl per well) were run in duplicate. Rabbit anti sheep polyclonal antibodies (detection antibody IL-6, AHP424; IL-1β, AHP423, Bio Rad AbD Serotec) at a concentration of 4 μg/ml were applied in wells and incubated for 30 min at room temperature. Plates were washed with washing buffer for 5–7 times between each step. Detection was accomplished by assessing the conjugated enzyme activity (goat anti-rabbit IgG-HRP, dilution 1:5000, Jackson ImmunoResearch, Cat. No 111-035-144) via incubation with TMB substrate solution (BD OptEIA TMB substrate Reagent Set, BD Biosciences Cat. No 555214), color development reaction was stopped with 2 N sulphuric acid. Plates were read on ELISA plate reader at 450 nm, with 570 nm wavelength correction (EnVision 2104 Multilabel Reader, Perkin Elmer). The sensitivity of IL-6 ELISA for plasma was 16 pg/ml, the sensitivity of IL-1b ELISA for media was 41.3 pg/ml, respectively. For all assays, the intra-assay and inter-assay coefficients of variance was <5 and <10%, respectively.

Immunofluorescence Imaging Analysis

Complete brain was taken from the fetus during necropsy after perfusion and immediately immersed in 4% PFA for 48–72 h. The tissue sample was then washed and stored in 1× PBS buffer changed daily for 3 days. Finally, the brain was stored in 70% ethanol until further processing. All the brain tissue samples were kept at 4°C when they were in liquid. The fetal brains were cut into two equal halves of left and right hemispheres, and then sliced coronally and placed into cassettes to be processed with Leica TP 1020 Automatic Tissue Processor (Leica Instruments, Mussloch, Germany). The tissues were embedded in paraffin with Leica EG 1160 Paraffin Embedding Center (Leica Instruments, Mussloch, Germany). Five-micrometer slices were obtained from slicing the embedded tissue samples with the Leica RM2145 Rotary Microtome (Leica Instruments, Mussloch, Germany), and mounted on the Fisherbrand Colorfrost Plus microscope slides (Fischer Scientific). The sectioned brain tissue samples went through de-paraffinization with CitroSolv (Fischer Scientific), 100, 95, 70, and 50% ethanol at room temperature, and antigen retrieval with 10 mM citrate buffer at pH 6 before being washed with water and 1× PBS, and blocked by Background Sniper Blocking Reagent (Biocare Medical, Cat. No BS966JJ). Then the sections were incubated with the primary antibody (Iba1, rabbit polyclonal antibody 4, 1:250 dilution, Wako, Cat No. 019-19741) for 1 h, followed by washing with 1× PBS and incubation with secondary antibody (Alexa Fluor 568 goat anti-rabbit IgG, 1:400 dilution, Life Technologies, Cat no A-11011) for 30 min in the dark. After that, the sections were washed again with 1× PBS, and the nuclei were counterstained with DAPI (1:4000 dilution, Sigma D-9564). Finally, the sections were cover-slipped with Fisherfinest Premium Cover glass (22 × 50-1, Fisher Scientific) and Fluoromount-G (SouthernBiotech, Cat no 0100-01) mounting medium, and viewed after 24 h of drying. Widefield fluorescence microscopy was performed on the stained brain tissue samples with a Zeiss Axiovert 200 M inverted microscope (Jena, Germany), at the magnification of 40× using a HBO100 mercury-arc lamp as a light source. The images were captured using a Zeiss Axiocam HRm (high-resolution monochrome) CCD (charged-coupled device) camera. Six high power field (HPF) images at 40× magnification were obtained for each animal. Multichannel imaging was used with the Iba1 channel and the DAPI channel for obtaining the pictures used for macrophage quantification. Appropriate ranges of color were selected showing positive contiguous cytoplasmic staining as a criterion for microglia cell count scoring which were then applied uniformly to calibrated images for all brain regions (**Figure 2**). Scoring was performed in a blinded fashion to experimental groups. To normalize for cell density Iba1+ signal over the whole area measured (100 sq micron) was divided by the respective optical intensity values for each HPF according to Lin et al. (2000).

RNAseq Approach

To extract and quantify RNA, total RNA was extracted from cultured microglia using TRIzol Reagent (Life Technologies). To obtain enough RNA, same treatment cells were pooled in one replicate. RNA quantity and quality (RNA integrity number, RIN) was determined by using a RNA Nano Chips (Agilent RNA 6000 Nano Chips) with Agilent 2100 BioAnalyzer. All samples had a RIN-value ranging from 6 to 8.5, except for one sample having RIN = 5.5 but an acceptable 84% of transcripts mapped, which did not affect the read count for this sample.

A total of eight samples from four set of replicates were selected for RNA sequencing at high throughput, of which three replicates were derived from *in vivo* control fetal sheep and one replicate was from *in vivo* LPS-exposed (second hit) fetal sheep. RNAseq libraries were prepared by using Illumina TruSeq RNA Sample Preparation v2 kit (Illumina) and quality control was performed on the BioAnalyzer. Single-end 50-bp sequencing was performed at high throughput on an Illumina HiSeq2500 at the CHU Ste-Justine Core Facility Sequencing Platform. Raw data and RNAseq data discussed in this publication were deposited on NCBI and are accessible online with the GEO accession number GSE71037.

RNAseq Data Analysis

Reads alignment to the reference genome

To maximize the amount of genes covered, raw data were mapped to the reference genome of the sheep *Ovis aris* v3.1

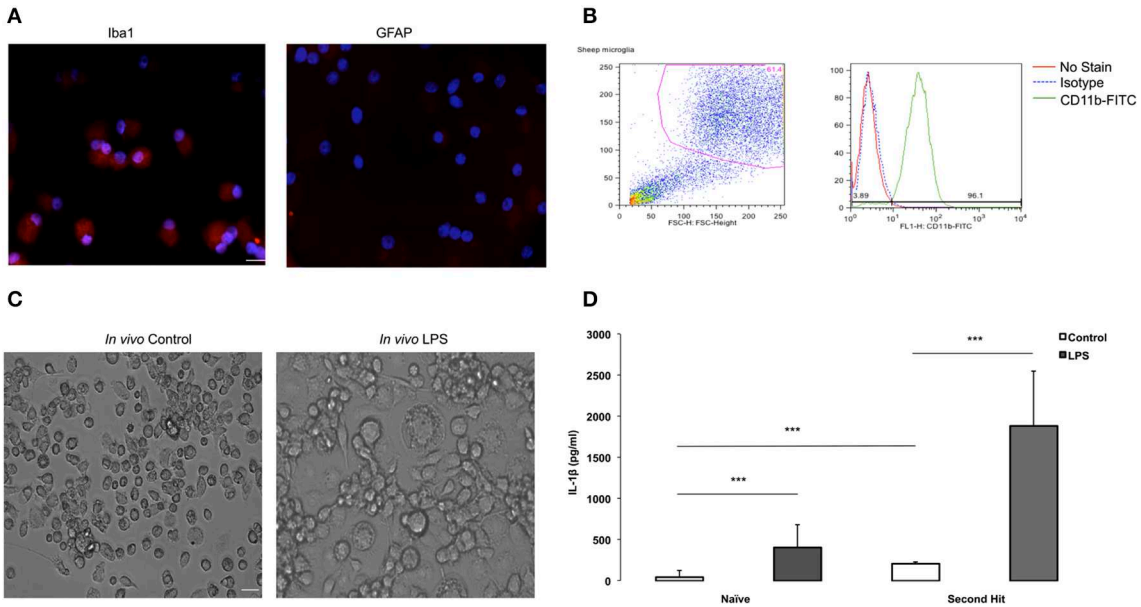

FIGURE 2 | Purity validation of fetal sheep brain primary microglia in cultures and LPS second hit. (A) Photomicrographs (ICC) confirming cell purity. Iba1+ staining in microglia vs. undetectable GFAP signal in microglia indicating no contamination with astrocytes in the culture. Microglia were cultured in eight-well chamber slide with DMEM +5% FCS for 4–7 days. Microglia were collected from the floating fraction and stained for Iba1 and GFAP. Scale bar = 50 μm. Magnification 40× for both images. **(B)** Purity of fetal sheep brain primary microglia cultures was verified by flowcytometry. After several days in culture, fetal sheep microglia were scraped from the wells using a cell scraper and blocked for 30 min using normal mouse IgG and 10% human serum. Cells were then stained using a FITC-conjugated monoclonal bovine anti-CD11b (1:40, Bio-Rad) on ice for 20 min. Cells were washed in FACS buffer and analyzed using a BD FACSCalibur and FlowJo software. **(C)** Microglia from *in vivo* LPS exposed brain appear more aggregated *in vitro* than microglia derived from *in vivo* controls. Microglia were cultured in 24-well plates with DMEM +5% FCS for 4–7 days, when images were taken. Cells were extracted from a twin control fetal brain and an *in vivo* LPS exposed fetal brain (Magnification 20× for both images). **(D)** Effect of "second-hit" *in vitro* LPS treatment on microglial phenotype. BOTTOM: IL-1β concentration in conditioned media of microglia derived from fetal sheep brain that were exposed to LPS vs. saline *in vivo* (***$P < 0.0001$). Cultures from *in vivo* LPS-exposed (SH), $n = 4$, cultures from *in vivo* Control (Naïve), $n = 10$. Cell culture media supernatant was obtained by centrifugation upon cell culture termination. A sheep specific IL-1β ELISA was performed to measure cytokine levels in cell culture media. *In vitro*, at baseline, microglia secreted more IL-1β in the *in vivo* LPS group (SH_C) than in naïve Control (N_C). LPS re-exposure (SH_L) further increased IL-1β vs. naïve LPS (N_L) by ∼4.6-fold.

from NCBI and Ensembl (GCA_000298735.1) as transcriptome reference. Index of the reference fasta file were built with Bowtie2 (Langmead and Salzberg, 2012), we then trimmed the adaptor of the fastQ files with TrimGalore, and mapped reads to the reference with Tophat2 (Kim et al., 2013). From the aligned reads from Tophat2, the number of reads per gene were counted with HTseq and assembled into a matrix containing the read count of each gene per sample (Anders et al., 2015).

Normalization and transcriptome analysis

Among packages available to test for differential expression, DESeq2 provides methods suited for the use of replicates; it uses negative binomial generalized linear models and estimates dispersion and logarithmic fold changes. We used DESeq2 to normalize the dataset, generated $\log_2$-fold changes and adjacent *P*-values (p_{adj}) and therefore, to find differentially expressed (DE) genes in microglia (Love et al., 2014). We first compared N_C to N_L to understand gene expression in naïve microglial cells after *in vitro* exposure to LPS. Then, due to the lack of replicates, we were not able to compare second hit microglial cells to their respective second hit control. Instead, we compared the genetic expression difference in response to a pre-exposure *in vitro* to LPS in N_C vs. SH_C. Finally, we assessed genetic expression in SH_L compared to N_L. A gene was considered differentially expressed (DE) if its adjacent *p*-value was strictly lower than 0.1. Pools of DE up and down regulated genes were clustered and visualized in heat maps, generated in *R* using the $\log_2$ normalized counts and the heatmap 0.2 method of the gplots library (Warnes et al., 2009).

Gene selection and Gene Ontology (GO)

The sheep genome is not yet supported by most gene ontology platforms, therefore, downstream analyses were performed with orthologs in the human genome Homo sapiens. ToppGenes and ToppCluster (Chen et al., 2009; Kaimal et al., 2010) were used to test for functional annotation enrichment analyses of biologic process and pathway with a false discovery rate correction of 0.05 (Franceschini et al., 2013). Gene Ontology was then performed with Gorilla and significant networks ($P < 0.03$) were selected for further discussion (Bauer et al., 2008; Eden et al., 2009).

Quantitative Real-Time PCR Analysis

The expression profiles of candidate genes were validated by real-time qRT-PCR. Total RNAs were subjected to cDNA synthesis

using a QuantiTech Rev. Transcription Kit (Qiagen). *HMOX1* and *FBP* mRNA were quantified by qRT-PCR using a QuantiFast SYBR Green PCR Kit (Qiagen) with STRATAGENE 3000 P, mRNA relative expression was calculated by the $2^{-\Delta\Delta Ct}$ method over housekeeping gene *GAPDH* compared to baseline (Livak and Schmittgen, 2001). Sheep specific *HMOX1* primers were designed with primer3 (Untergasser et al., 2012) and *FBP* primers were designed using Integrated DNA Technologies online tool and listed in **Table 1**.

Statistical Analyses

Generalized estimating equations (GEE) modeling was used to assess the effects of LPS while accounting for repeated measurements on fetal blood gasses and acid-base status, plasma and *in vitro* cytokines, cardiovascular responses [AR(1) correlation matrix to account for temporal structure] and *in vivo* Iba1+ fluorescence (independent correlation matrix to deal with the spatial distribution of Iba1+ fluorescence across the brain regions). We used a linear scale response model with LPS and time or brain regions as predicting factors to assess their interactions using maximum likelihood estimate and Type III analysis with Wald Chi-square statistics. Correlation analysis was performed using Spearman correlation coefficient. SPSS Version 21 was used for these analyses (IBM SPSS Statistics, IBM Corporation, Armonk, NY). Significance was assumed for $p < 0.05$. Results are provided as means ± SEM. Not all measurements were obtained for each animal studied.

Results

In vivo Studies

Cohorts' Characteristics

Maternal venous blood gasses, pH, and lactate did not significantly change during the experiments and were within physiological range throughout the experiment for both groups. Maternal and fetal cohort's characteristics are summarized in **Table 2**. Gestational age at time of the experimental day 1 averaged 130 days ± 1.3 dGA (term 145 dGA). Overall, mother and fetus were considered healthy based upon a physical examination and laboratory data collected.

Clinical-chemical Data

Clinical-chemical data, reported elsewhere, (Durosier et al., 2015) are summarized in **Table 2** and were within physiological range for both groups. We found significant time*LPS interactions for pH ($P = 0.03$), pO_2, pCO_2, lactate, and BE (all $P < 0.001$).

TABLE 1 | Primers of quantitative real time PCR analysis of HMOX1 and FBP.

Gene name	Forward	Reverse
HMOX1	CACCAAGTTCAAGCAGCTGT	CAACCCTGCGAGAAATGTCC
FBP	CGAATGTGACGGGAGATCAA	GGCATGTTTGTCTTCTTCTGAC
GADPH	TGAGATCAAGAAGGTGGTGAAG	GCATCGAAGGTAGAAGAGTGAG

Cardiovascular Analysis

As reported (Durosier et al., 2015), we found time-LPS interactions for mean arterial blood pressure and fetal heart rate responses ($P = 0.015$ and $P < 0.001$, respectively).

Plasma Cytokines Response to LPS

In vivo LPS exposure resulted in a peak of IL-6 at 3 h compared to baseline. We detected time-LPS interaction for fetal plasma IL-6 ($P < 0.001$, **Figure 1B**).

In vivo LPS Effect on Neuroinflammation

To assess the effect of *in vivo* LPS exposure on neuroinflammation *in situ* we measured microglial activation as Iba1+ immunofluorescence signal. We found a significant brain region main effect ($p < 0.001$), but no group main effect ($p = 0.62$) and no significant brain region∗group interaction ($p = 0.13$) (**Figure 1C**). Thus, *in vivo* LPS exposure did not cause any measurable neuroinflammation as can be seen with higher doses of LPS using the same microglia marker (Keogh et al., 2012; Kuypers et al., 2013).

Overall, fetuses responded to the *in vivo* LPS exposure with signs of moderate sepsis as evident by the changes observed with arterial blood gas, pH, lactate, but with no signs of cardiovascular decompensation. Despite the systemic response to the LPS challenge, we observed no signs of neuroinflammation *in vivo*.

In vitro Studies

Having established a moderate LPS-induced *in vivo* fetal systemic inflammation paradigm without overt neuroinflammation *in situ*, we next aimed to test the functional properties of microglia exposed to LPS *in vivo* in an *in vitro* setting allowing characterization of microglial cytokine secretion and transcriptome profiles in response to LPS.

Primary Fetal Sheep Microglia Culture

In vitro studies were conducted in primary cultures derived from six controls (naïve) and from two *in vivo* LPS-exposed animals (SH). We were able to perform 1–2 *in vitro* replicates per each animal depending on cell numbers obtained.

TABLE 2 | Maternal and fetal *in vivo* clinical characteristics.

Characteristics	Maternal*	Fetal**
Averaged body weight (kg)	76 ± 11	3.8 ± 0.9
Gender: control group, male		5/9
Gender: LPS group, male		4/12
Parity: control group		7/9
Parity: LPS group		3/12
pO_2 (mmHg)	54 ± 6	20 ± 1
pCO_2 (mmHg)	41 ± 2	52 ± 2
pH	7.44 ± 0.01	7.37 ± 0.04
Lactate (mmol/L)	0.7 ± 0.2	1.5 ± 0.9
BE (mmol/L)	1.1 ± 0.2	3.3 ± 2.3

*Values averaged over the course of the experiment, mean ± SEM.
**Baseline characteristics averaged for control and LPS groups.

We identified oligodendrocytes and neurons in the initial cell isolation in addition to microglia and astrocytes (data not shown). To enrich for microglia we subjected the cells to a second step as detailed in Methods. To verify cell culture purity, we performed immunofluorescence staining with a microglia marker confirming that the isolated primary microglia was very high (**Figure 2A**), whereas an astrocyte marker, GFAP, was absent from the cell population. To further verify cell purity, flow cytometry CD11b-FITC antibody was performed resulting in 96% of cultured cells are CD11b+ (**Figure 2B**), further indicating that a highly pure microglia population was obtained.

We used the purified highly enriched microglia cultures to pursue the second hit paradigm, i.e., how these cells behave *in vitro* in dependence on previous *in vivo* LPS exposure (**Figure 1A**). We found that microglia from *in vivo* LPS exposed fetal brain differ in morphology, showing more aggregation or clumping compared to naïve microglia (**Figure 2C**). This finding indicates that these microglia might have already been activated by LPS exposure *in vivo*.

Next, we investigated cytokine secretion properties of these cells in the absence or presence of LPS. For IL-1β, we found that *in vitro* LPS administration resulted in increased IL-1β in microglia compared to control cell cultures; this IL-1β response was potentiated by 4.6-fold in cells derived from animals with *in vivo* LPS exposure: 1884 ± 481 pg/ml vs. 406.14 ± 193 pg/ml (all $p < 0.001$, **Figure 2D**). Moreover, even in the absence of LPS *in vitro*, at baseline, microglia from the *in vivo* LPS group secreted more IL-1β (208.1 ± 16.63 pg/ml vs. 44.97 ± 59.21 pg/ml) with the fold increase being concordant with the level of gene expression increase (all $P < 0.001$, **Table 3**). Other pro-inflammatory cytokines of interest such as IL-6 and TNF-α were undetectable in cell-conditioned media (ELISA data not shown). Our findings suggest that a pro-inflammatory microglial phenotype acquired during *in vivo* exposure to LPS is sustained *in vitro* (second hit paradigm).

RNAseq Approach

General overview of the whole transcriptome sequencing

To explore the genomic landscape of fetal sheep microglia, we sequenced the transcriptome of naïve and "second hit" microglia. As a quality control, we tested the expression levels of GFAP and TNFα across our three comparisons, and confirmed that all cells in our platform shared the same gene expression characteristics of microglia (**Table 3**). As a control measure for cell purity, our data confirmed the presence of TGF-β1 in each sample, as previously reported (Butovsky et al., 2014). To further confirm cell purity and the findings on protein level (ELISA), our transcriptome analysis showed a 1.654-fold increase of IL-1β ($\log_2 = 0.726$) between naïve and second hit LPS-exposed microglia (**Table 3**, respectively N_L and SH_L).

Firstly, we compared gene expression between the naïve controls and naïve LPS-exposed microglial cells. We found 258 differentially expressed genes ($p_{adj} < 0.1$), among which, 205 genes were up regulated and 53 were down regulated. We selected relevant differentially expressed genes with ToppCluster ($\log P > 4.00$) based on their role in the immune response (**Figures 3A,B**).

Then, to better understand the effect of an *in vivo* pre-exposure to LPS on biological processes, we compared gene expression between the naïve and second hit controls, i.e., N_C and SH_C, respectively. We found 6642 differentially expressed genes, among which, we identified 4112 up regulated and 2530 down regulated genes. Selection of relevant genes with ToppCluster ($\log P > 4.00$) showed that up regulated genes in SH_C are mainly composed of *GABA* genes and genes responsible for calcium, potassium, and second messengers transport. Differentially expressed down regulated genes comprised the genes of the NF-κB signaling pathway and the *HMOX1* gene, responsible for iron metabolism ($\log_2 = -4.462$ and $p_{adj} = 4.22 \times 10^{-19}$).

Finally, in an effort to discover the differences in response between N_L and SH_L, we compared gene expression of the N_L set of three replicates and SH_L ($n = 1$). We identified a total of six differentially expressed genes: five were up regulated and one gene, *HMOX1*, was strongly down regulated ($HMOX1_{down}$, $\log_2 = -4.303$ and $p_{adj} = 8.13 \times 10^{-2}$). Among the five differentially up regulated genes identified, Fructose-1,6-bisphosphatase (*FBP*) was uniquely differentially expressed in second hit LPS-exposed microglia (FBP^{up}, $\log_2 = 4.057$ and $p_{adj} = 9.40 \times 10^{-2}$). The expression profile of *HMOX1* and *FBP* were assessed by quantitative real-time PCR (qRT-PCR). The results showed that the expressions of *HMOX1* and *FBP* were consistent with the expressions from the transcriptome analyses (**Figures 4A,B**). The roles of these genes are discussed below.

Discussion

We established for the first time an *in vivo–in vitro* endotoxin double-hit mammalian microglia experimental model to mimic multiple perinatal neuroinflammation episodes. The isolation of viable and highly purified microglia populations from *in vivo* LPS-exposed brain allowed an *in vitro* characterization of this cell type. Our most striking discovery was that the fetal inflammatory microglial phenotype acquired during *in vivo* exposure to LPS, even if not histologically apparent, is sustained and potentiated *in vitro* upon re-exposure to LPS. The subsequent RNA sequencing of the microglial genome revealed a unique $HMOX1_{down}$ and FBP^{up} phenotype of microglia exposed to the double-hit, suggesting interplay of inflammatory and metabolic pathways.

In vivo–In vitro Model of Perinatal Inflammation Double-hit

Intrauterine exposure to inflammatory stimuli may switch innate immunity cells such as macrophages and microglia to a reactive phenotype ("priming"). Confronted with renewed inflammatory stimuli during labor or postnatally (especially in preterm neonates in the intensive care unit), such sensitized cells can sustain a chronic or exaggerated production of proinflammatory cytokines associated with neurodevelopmental deficits persisting into adulthood (double-hit hypothesis) (Larouche et al., 2005; Spencer et al., 2006; Wang et al., 2006).

TABLE 3 | Gene expression summary in naïve (one time exposure to LPS *in vitro*) and second hit (exposed once *in vivo* and second time *in vitro*) microglia.

Relevance	Gene name (Common name)	Naïve microglia	Second hit control	Second hit
Activated mitochondrial biogenesis	SLC2A4	6.386	**8.911**	0.821
	PPARGC1A	4.842	**5.835**	1.466
Adipocytokine signaling pathway	CAMKK1	−1.110	0.511	−0.205
	CAMKK2	0.660	1.206	0.453
Adiponectin	ADIPOQ	2.422	**4.588**	1.137
Adrenoceptor alpha 1A	ADRA1A	2.572	**4.687**	−0.471
AMPK signaling pathway	PRKAA1	0.169	0.218	−0.643
	PRKAA2	2.776	**4.784**	1.774
	PRKAB1	−0.189	−1.319	−2.603
	PRKAB2	0.861	0.179	0.066
	PRKAG1	−0.379	**−3.249**	−2.168
	PRKAG2	−0.405	−1.194	−0.513
	PRKAG3	−1.329	0.099	−0.050
B-cell development and survival	TNFSF13B (BAFF)	2.562	−0.955	−1.823
Calcium binding protein 39	CAB39	0.279	**−1.520**	−1.583
	CAB39L	−0.076	−1.092	−1.106
Esterase enzyme	ACHE	−0.036	−0.978	−0.698
	BCHE	−0.475	**−3.197**	−1.782
Fractalkine/CX3CR1 axis and biological signature of microglial cells	CX3CR1	2.017	3.079	0.880
	CX3CL1	0.618	−0.443	0.076
	ITGAM (CD11b)	0.130	−0.113	−1.083
	IL1B	7.578	**1.766**	0.726
Fructose-1,6-Biphosphate	FBP	−0.792	1.465	**4.057**
Gluconeogenesis and glucolysis	ALDOA	−0.106	−0.485	0.355
	ALDOB	−1.659	−1.842	−2.033
	ALDOC	−0.266	−0.717	0.328
	PFKP	−0.461	−0.228	0.686
	GPI	−0.940	**−2.001**	−0.363
Growth, proliferation, fate determination, development, immunity	ELAVL1	−0.252	−0.986	−0.895
	CCNA1	2.574	**6.259**	2.854
	CCNA2	0.143	0.098	0.686
	IRF9 (p48)	0.407	**−1.286**	−0.641
	PIM1	3.108	−0.815	−2.762
	EP300 (CBP)	0.784	0.638	−1.094
	CREBBP (CBP)	0.881	0.752	−0.835
	CISH (CIS)	7.170	2.217	−2.097
HDAC genes: potential epigenetic regulators	HDAC1	**2.271**	0.145	0.676
	HDAC10	−0.242	−0.840	0.116
	HDAC11	−0.214	0.867	0.812
	HDAC2	−0.299	**−2.746**	−2.423
	HDAC3	0.045	−0.692	0.321
	HDAC4	1.292	1.502	−0.691

(Continued)

TABLE 3 | Continued

Relevance	Gene name (Common name)	Naïve microglia	Second hit control	Second hit
	HDAC5	−0.869	0.333	0.501
	HDAC6	**−0.688**	−0.126	−0.430
	HDAC7	−0.109	0.643	−0.486
	HDAC8	0.336	−0.889	−2.510
	HDAC9	0.732	1.556	−1.816
Increased FFA oxidation	CPT1A	−0.260	0.631	0.100
	CPT1B	0.496	1.140	0.304
	CPT1C	0.608	0.315	0.503
Inhibit cell growth and protein synthesis	RPS6KB1	−0.078	−1.659	−1.444
	RPS6KB2	−0.569	**−1.900**	−0.634
	EIF4EBP1	−0.822	**−2.609**	−1.533
	PPARG2	**−2.093**	**−2.107**	−0.656
Inhibit protein synthesis	EEF2	−0.005	0.210	0.421
	EEF2K	−0.394	0.208	−0.321
Initiators of the JAK-Stat pathway	JAK1	0.213	−1.404	−2.112
	JAK2	2.289	0.000	−1.430
	JAK3	**2.965**	**2.121**	−0.920
	TYK2	0.948	−0.296	−0.929
	STAT1	−0.136	−0.825	−0.563
	STAT2	1.276	−1.321	−2.852
	STAT3	0.660	**−2.050**	−0.889
	STAT5A	3.365	−0.188	−2.798
	STAT5B	1.554	−0.436	−1.093
Insulin signaling pathway	IGF1	1.601	2.264	1.177
	IGF1R	−0.125	−0.489	−0.838
	IRS1	1.241	**2.665**	0.344
	IRS4	4.420	**7.086**	1.953
Iron metabolism and/or anti-inflammatory	HMOX1	**−2.686**	**−4.462**	**−4.303**
	NRF-2	0.855	−1.235	−1.225
JNK/P38 MAPK	MAPK8 (JNK)	0.544	−2.918	−1.173
	MAPK9 (JNK)	−0.266	**−2.257**	−2.853
	MAPK10 (JNK)	1.492	3.024	−0.262
	MAPK12 (P38)	−0.905	−0.708	0.458
	MAPK13 (P38)	3.294	3.173	0.171
	MAPK14 (P38)	−0.848	−0.612	0.113
Leptin	LEP	5.033	**7.429**	−0.143
LRP phagocytosis signaling	LRP1B	4.522	**6.380**	0.336
	LRP2	4.860	**6.571**	1.410
	LRP6	**1.052**	**1.850**	0.157
Lymphocyte adhesion, T-cell costimulation	ICAM-1	4.055	−0.181	−2.801
Lymphoid-tissue homing	CCL21	18.917	20.916	0.368
	CCL19	5.439	5.328	−0.321
mTOR signaling pathway	RHEB	−0.257	**−3.516**	−2.292

(Continued)

TABLE 3 | Continued

Relevance	Gene name (Common name)	Naïve microglia	Second hit control	Second hit
	AKT1S1	0.908	−0.657	−1.496
	MTOR	−0.107	−0.234	−1.066
	RPTOR	0.298	0.361	−0.625
Myeloiesis and B-cell lymphopoiesis	CXCL12 (SDF-1alpha)	−0.545	1.552	0.268
NF-kB signaling and inflammation	RELB	1.503	−1.389	−1.227
	NFKB	**2.676**	−0.323	−0.200
	NFKBIA	**2.578**	**−1.934**	−1.546
	NFKB1 (p50)	2.569	−0.801	−2.673
	RELA (p65)	−0.031	**−2.098**	−2.268
	PTGS2	5.166	−0.609	−2.168
	TNF	4.990	0.028	−2.743
	PTGS2	5.166	−0.609	−2.168
	IL8	4.779	**−2.847**	−3.988
	IL1B	7.578	**1.766**	0.726
	TNFAIP3	2.628	−0.542	−3.162
Nitric oxide (NO) and NO production	NOS1	6.201	**8.416**	1.233
	NOS1AP	0.386	−0.599	−1.393
	NOS2	5.951	**3.039**	−1.958
	NOS3	**5.266**	**7.637**	1.112
P53	TP53	−0.554	−0.379	−1.539
	MDM4	3.489	−13.081	−1.120
PIK3-Akt signaling pathway	PIK3CA	0.018	**−1.584**	−1.561
	PIK3CB	1.793	−0.972	−1.750
	PIK3CG	1.950	1.106	−0.094
	PIK3R1	1.330	**−1.533**	−1.884
	PIK3R3	1.083	2.066	−1.507
	PIK3R5	4.343	**2.695**	−0.635
	PDPK1	0.915	0.946	−0.606
	AKT2	−0.335	−0.857	−1.328
	AKT3	0.350	0.104	−1.292
	TSC1	1.099	0.940	−0.386
	TSC2	0.219	0.587	1.182
Quality control	TGFBR1	0.327	0.158	−0.943
	TGFβ	−0.419	**−1.798**	−0.690
	GFAP	−1.044	−5.512	−3.709
	ITGAM (CD11b)	0.130	−0.113	−1.083
	CD40	4.656	**2.831**	1.352
	IBA1 (AIF1)	0.060	0.208	0.956
Serine/threonine kinase 11	STK11	**−0.178**	**−1.553**	−1.491
STE20-related kinase adaptor alpha	STRADA	**−0.536**	**−1.252**	−1.293
	STRADB	**−0.506**	**−2.816**	−3.582
Tak1 protein	MAP3K7	−0.057	−0.809	−2.136
Toll-like receptor 4	TLR4	0.807	**−2.451**	−3.749
	LY96 (MD-2)	1.213	0.263	0.340
	LBP	4.472	**5.990**	0.172

(Continued)

TABLE 3 | Continued

Relevance	Gene name (Common name)	Naïve microglia	Second hit control	Second hit
Transcription factors	C-JUN	0.795	**−1.539**	−2.385
	C-FOS (FOS)	**−3.072**	−1.635	−0.317
	NFKB	**2.676**	−0.323	−0.200
	CREB1	0.782	−0.228	0.190
	ATF4 (creb TF)	0.968	−0.319	0.163
	CEBPB (CEBP)	0.586	−0.993	−1.451

Bold values correspond to a significant log_2 fold change ($p_{adj} < 0.1$).
Differential analysis of the count data was done with the DESeq2 package; up regulated genes are highlighted in red and down regulated genes are highlighted in blue. Values in the column "naïve cells" correspond to fold change from the naïve controls (N_C) to LPS-exposed microglia (N_L). In the same way, we compared second hit control (SH_C) to the N_C. Fold changes are summarized in the column "second hit control." Values in the "second hit" column represent the fold change from N_L to second-hit, i.e., LPS re-exposed, (SH_L) microglia.

Experimentally induced inflammation in chronically instrumented non-anesthetized fetal sheep is a well-established *in vivo* model of fetal physiology (Prout et al., 2010, 2012). Primary microglia cultures in different species have been reported for decades (Stansley et al., 2012). We integrated both *in vivo* and *in vitro* models into a new, hybrid system adding the layer of the whole transcriptome analysis using RNAseq analyses. The chief advantage of the new *in vivo–in vitro* model presented here is that it allows us to examine microglia responses to LPS-induced double-hit inflammation *in situ* and *in vitro* on integrative physiological, protein and genomic levels, and in a physiologically and clinically meaningful context. This approach has the potential to uncover hitherto unseen relationships between brain and immune system on different scales of organization in the perinatal stage of development, which might accelerate discovery of new treatment strategies.

In vivo, our experimental cohort's morphometric, arterial blood gasses, acid-base status and cardiovascular characteristics were within physiological range and representative for late-gestation fetal sheep as a model of human fetal development near term (Frasch et al., 2007; Rurak and Bessette, 2013). As reported elsewhere (Durosier et al., 2015), the effect of the low LPS dose we administered on the arterial blood gasses, acid-base status and cardiovascular responses is compatible with a mild septicemia (mild compensated metabolic acidemia and hypoxia) evidenced by a transient rise of IL-6 at 3 h without overt shock, i.e., without cardiovascular decompensation. Similar levels of systemic IL-6 have been reported (Prout et al., 2010).

In vitro, we developed a new microglia isolation protocol that combines the human adult and fetal brain microglia isolation protocols (Durafourt et al., 2013) and successfully collected a highly enriched microglia population. The use of a modified cell isolation approach from fetal brain tissue is mainly due to the higher degree of myelination in the adult human brain compared to that of a near-term fetus (Durafourt et al., 2013). We were able to attain high purity of microglia, which we validated by flow cytometry and immunocytochemistry. Moreover, RNAseq showed a consistent and constant low level of expression of the astrocyte marker GFAP further confirming cell purity.

In this study, the morphology of microglia from *in vivo* LPS exposed fetal brain was distinguished by more aggregation or clumping and less ramification compared to naïve microglia (Suzuki et al., 2006; Henkel et al., 2009). This suggests that microglia exposed to LPS *in vivo* may have been already activated before plating in cultures.

Single-hit LPS Exposure *In vitro* Results in Up Regulation of Inflammatory Pathways JAK-STAT and NFκB and Down Regulation of Metabolic Pathways

Gene ontology analyses of DE genes in N_L microglia revealed an up regulation of inflammatory pathways NFκB, PIK3-Akt, and Jak-STAT. Interestingly, this was accompanied by a down regulation of metabolic pathways in LPS-induced inflammatory response (**Figures 3A,B** and **Table 3**). These findings may be explained, at least in part, by the emerging role of energy-sensing AMPK signaling in microglia, which links the inflammatory and metabolic regulatory networks (Frasch, 2014), We will return to this observation in Section Double-hit LPS Exposure of Microglia *In vivo* and *In vitro* Is Uniquely Characterized By a $HMOX1_{down}/FBP^{up}$ Phenotype.

Among differentially expressed genes selected, NFKB ($log_2 = 2.676$ and $p_{adj} = 4.58 \times 10^{-2}$) and JAK3 ($log_2 = 2.965$ $p_{adj} = 2.49 \times 10^{-3}$) were up regulated in N_L microglia. We then investigated the expression of genes involved in the NFκB and JAK-Stat pathways; our data showed that IL1B ($log_2 = 7.578$), TNF ($log_2 = 4.990$), NFKBIA ($log_2 = 2.578$ and $p_{adj} = 9.09 \times 10^{-2}$), and RELB ($log_2 = 1.503$) were up regulated in N_L microglia. Gene ontology analysis revealed down regulation of the energy consuming processes and up regulation of energy conserving processes, as evidenced by the down regulation of genes related to glycolysis (*GPI*) and up regulation of gluconeogenesis (*FBP*) and the insulin signaling pathway. Furthermore, Gene Ontology of up regulated genes revealed that the GO term "immune system process" clustered key genes of inflammatory pathways, such as, JAK3, NFKBIA, and NFKBIB (GO:0002376 and $P = 9.56 \times 10^{-8}$). Differentially expressed down regulated genes also affected "the immune system process" (GO:0002376 and $P = 3.24 \times 10^{-4}$) and cellular response to metal ion (GO: 0071248 and $P = 6.05 \times 10^{-6}$). *HMOX1* and *FOS* clustered in both GO terms underlying the potential role of *HMOX1* in the immune system in relation with *FOS*. Analysis of all down regulated genes showed that the "metabolic process"

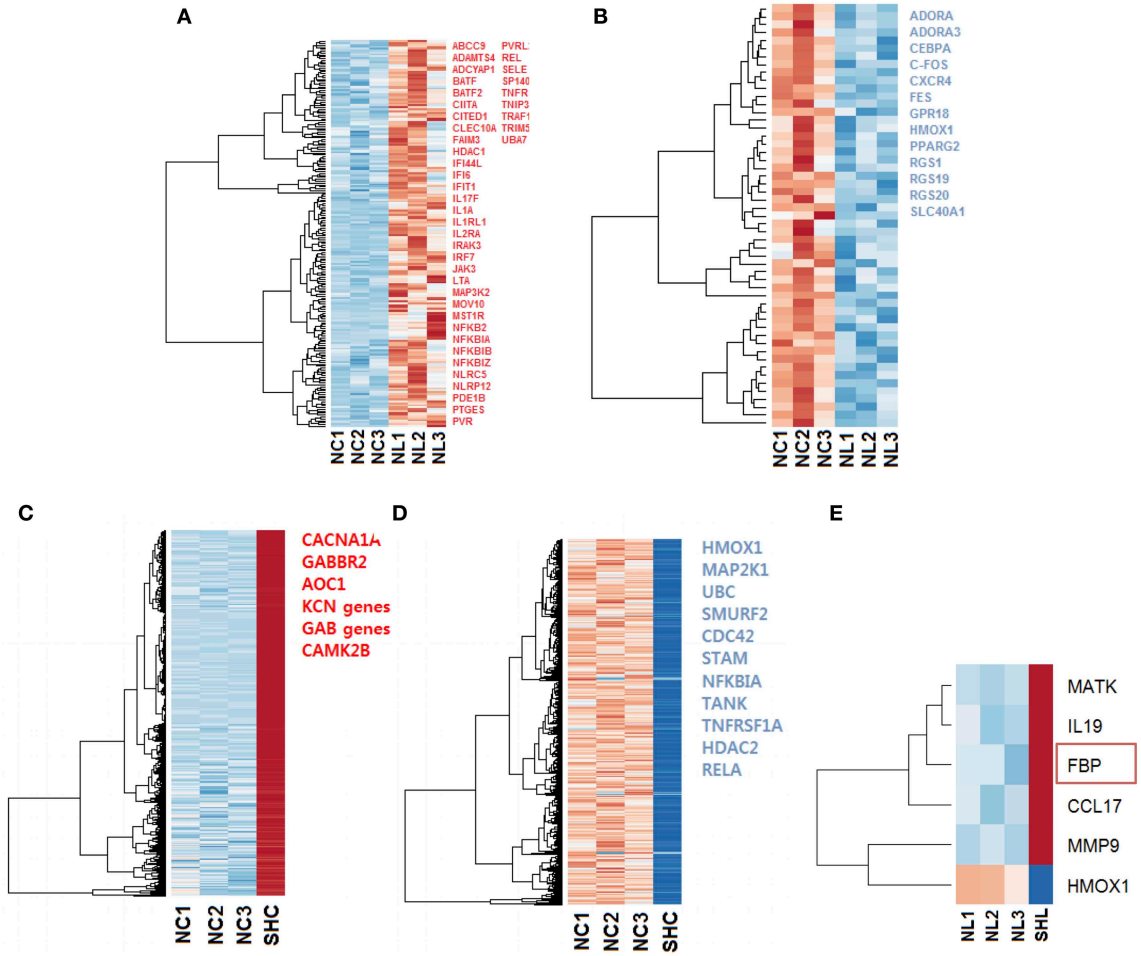

FIGURE 3 | Heat maps of the gene expression in microglia cells exposed to LPS. Selected up regulated (red) and down regulated (blue) genes are listed; genes were selected with ToppCluster based on their significance in the immune response (log$P$ > 4.00) **(A)** Heat map of 205 differentially expressed (p_{adj} < 0.1) up regulated genes (red) in N_L microglia. **(B)** Heat map of 53 differentially expressed down regulated genes in N_L microglia, among selected genes indicated in blue, *HMOX1* was strongly differentially expressed ($\log_2 = -2.686$ and $p_{adj} = 3.09 \times 10^{-8}$). In both up and down regulated genes, we observed a different behavior for N_{L3} that did not affect our differential analysis. **(C)** Heat map of the 4112 most differentially expressed and up regulated genes in SH_C compared to N_C cells. Selected genes with ToppCluster (log$P$ > 4.00) include GABA receptor genes and genes related to the transport of ion, Calcium, Sodium, and Potassium. IL1B was up regulated with $\log_2 = 1.766$ ($p_{adj} = 1.53 \times 10^{-3}$) corresponding to a 3.40-fold increase **(D)** Among the 2530 most differentially expressed and down regulated genes identified, *HMOX1* was significantly down regulated ($\log_2 = -4.462$ and $p_{adj} = 4.22 \times 10^{-19}$). The reported genes were selected with ToppCluster (log$P$ > 4.00) for their implication in the inflammatory response. **(E)** Differentially expressed up and down regulated genes in SH_L compared to N_L microglia. *HMOX1* was the only down regulated gene ($\log_2 = -4.303$, $p_{adj} = 8.13 \times 10^{-2}$). When comparing common genes in SH_C and SH_L cells, the gene *FBP* was unique to SH_L (red rectangle). NC, Naïve control microglia; NL, Naïve LPS-exposed microglia; SHC, Second hit control microglia; SHL, Second hit LPS-exposed microglia.

(GO:0008152 and $P = 2.38 \times 10^{-8}$) was also globally affected (data not shown).

Double-hit LPS Exposure of Microglia *In vivo* and *In vitro* is Uniquely Characterized By a $HMOX1_{down}/FBP^{up}$ Phenotype

Interestingly, *HMOX1* gene expression showed a strong down regulation in SH_L and SH_C by four-fold (**Table 3**, **Figures 3D,E**). The level of expression of *HMOX1* was higher in SH_C than in SH_L ($\log_2 = -4.462$ and $p_{adj} = 4.22 \times 10^{-19}$; $\log_2 = -4.303$ and $p_{adj} = 8.13 \times 10^{-2}$, respectively). Such differences in response patterns were observed in other genes as well suggesting a memory of inflammation induced by pre-exposure to LPS *in vivo*.

HMOX1 role in microglia is yet to be fully understood. Across the three group comparisons (**Figure 1A**), *HMOX1* was significantly down regulated and *in vivo* pre-exposure to LPS seemed to further enhance the down regulation of *HMOX1* in response to second-hit *in vitro* LPS stimulation. We confirmed by RT-PCR that transcript amounts of *HMOX1* are low in N_L and SH_L microglia (**Figures 4A,B**). *HMOX1* was suggested to play an anti-inflammatory role in LPS-induced murine adult cell line macrophages via the activation of the Nrf2/ARE pathway (Ye et al., 2014). Pre-treatment with Oroxylin A, an inhibitor of

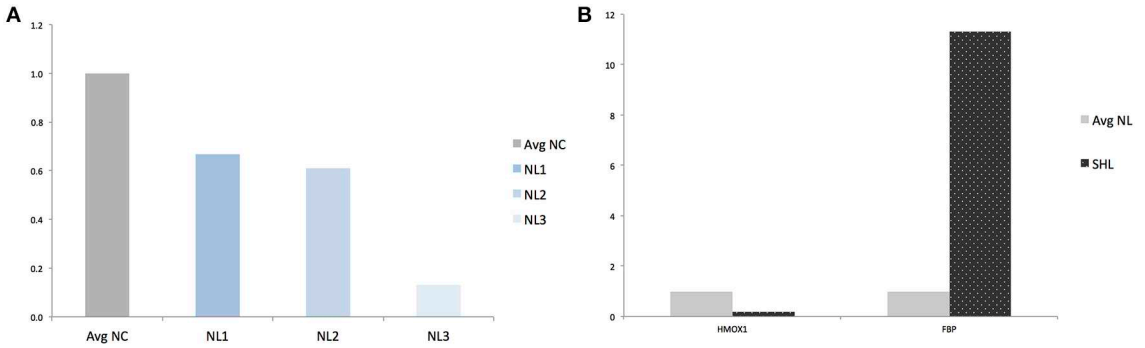

FIGURE 4 | Validation of RNAseq findings by qRT-PCR analysis of *HMOX1* and *FBP*. (A) Quantification of HMOX1 in each N_L microglia compared to averaged-N_C. **(B)** Quantification of HMOX1 and FBP in SH_L compared to averaged-N_L microglia. Avg, Averaged; NC, Naïve control microglia; NL, Naïve LPS-exposed microglia; SHL, Second hit LPS-exposed microglia.

LPS-induced mRNA, substantially increased the levels of *NRF-2* and heme oxygenase 1. The response of SH_C and SH_L compared to their single-hit microglia counterpart N_L showed that *HMOX1* and *NRF-2* had a greater down regulation after pre-exposure to LPS *in vivo* (**Table 3**), supporting the potential role of *HMOX1* in the inflammatory response and as a determinant of microglial phenotype.

While the role of *FBP* in inflammation is unclear, its neuroprotective effect in brain injury models was suggested through various mechanisms. During hypoxia, *FBP* supports ATP production via stimulation of glycolysis which results in maintenance of normal intracellular calcium levels via the phospholipase-C dependent MAP kinase signaling pathway (Bickler and Kelleher, 1992; Fahlman et al., 2002). When comparing gene expression in SH_C (**Figures 3C,D**), we observed that genes responsible for the transport of calcium, potassium and second messengers were also differentially expressed and up regulated.

It was previously reported that FBP dose-dependently suppressed LPS-induced nitric oxide (NO) production, and higher FBP doses were also associated with decreased levels of the transcription factor activator protein AP-1 in primary neonatal murine microglia cultures (Kim et al., 2012). We confirmed this observation in our comparison of SH_L to SH_C, wherein we observed that up regulation of *FBP* was concordant with lower expression of *NOS1AP* ($\log_2 = -1.393$) suggesting lower production of NO. We also observed that lower FBP transcripts amount in N_L was accompanied by higher expression level of NOS production related genes (**Table 3**). The authors observed that FBP had an effect on the binding of transcription factors to DNA: FBP diminished the binding of AP-1 to DNA, but NFKB and CREB did not seem affected. We found down regulation of *AP-1* ($\log_2 = -2.385$) and a slight down regulation of *NFKB* and *CEBP*, though *CREB1* remained unaffected. In the SH_L, our results confirmed that DNA binding nuclear factors were not strongly down regulated upon higher transcript level of *FBP*. The authors suggested that FBP inhibits iNOS expression by blocking the JNK/p38 MAPK pathway. We confirmed that *JNK* related genes may have lower expression level, however we did not observe any marked difference for *P38*.

A common theme within the newly found $HMOX1_{down}/FBP^{up}$ phenotype appears to be its memory of the "energy restoring direction" following *in vivo* exposure to LPS. This metabolic effect is evidenced for example by up regulation of AMPK, insulin, growth arrest processes, mitochondrial biogenesis signaling pathways and down regulation of mTOR signaling pathway and such energy consuming processes as cell growth and protein synthesis (cf. **Table 3**).

Does *In vivo* Endotoxin Exposure Induce Transcriptome Memory of Inflammation in Fetal Microglia Mediated By Epigenetic Mechanisms?

Pre-exposure to LPS *in vivo* affected globally the transcriptome of microglia (**Table 3**). We observed that SH_C microglia had a diminished response in gene expression of inflammatory pathways NF-κB, JAK-Stat, and PIK3-Akt compared to the behavior of the N_L microglia; this phenomenon was sustained in SH_L microglia. As mentioned in Section Double-hit LPS Exposure of Microglia *in vivo* and *In vitro* Is Uniquely Characterized By a $HMOX1_{down}/FBP^{up}$ Phenotype, this was also true for the metabolic pathways. This desensitization was also observed in histone deacetylase 1 (*HDAC1 and 6*), which was DE up regulated by two-fold in N_L ($\log_2 = 2.271$ and $p_{adj} = 8.58 \times 10^{-2}$), and up regulated by less than one-fold in SH_C and SH_L ($\log_2 = 0.676$ and $\log_2 = 0.145$, respectively, **Table 3**). This *HDAC1* profile was accompanied by a less than one-fold down regulation of *HDAC6* ($\log_2 = -0.688$ and $p_{adj} = 8.75 \times 10^{-2}$) followed again by desensitization in microglia exposed to LPS *in vivo*. *HDAC4* was 2.5-fold up regulated ($\log_2 = 1.292$ and $p_{adj} = 0.133$) in N_L vs. N_C microglia followed also by desensitization in the comparison to the *in vivo* pre-exposed microglia; meanwhile, *HDAC2* showed a less than one-fold down regulation ($\log_2 = -2.746$ and $p_{adj} = 2.30 \times 10^{-4}$) in N_C vs. SH_C microglia, with no detectable change in microglia exposed to LPS *in vitro* only or upon double-hit exposure. In parallel, *HMOX1* was down regulated by four-fold in SH_L and SH_C, and by two-fold in N_L microglia, and did not seem to have a diminished response in SH_L. These findings underscore the potential role of *HDAC1, 2, 4*, and *6* in the memory of the

in vivo exposure to inflammation in line with the histone code hypothesis (Jenuwein and Allis, 2001).

In light of the putative epigenetic mechanisms underlying our findings of single- and double-hit LPS signatures in microglial transcriptomes, it remains to be tested whether these signatures are indeed unique to LPS or apply more widely for perinatal exposures to other stressors, such as the psychosocial stress, e.g., caused by fear (Shapiro et al., 2013; Monteleone et al., 2014; Metz et al., 2015). Forced-swim stress applied over 4 days in adult male mice induced changes lasting at least two following weeks in neuronal acetylcholine esterase (AChE) expression via an epigenetic mechanism of hypoacetylation, with near-exclusive enrichment of HDAC4, and hypermethylation of histone H3K9 at a specific promoter of AChE (mP1c) with resulting suppression of the mE1c exon expression levels (Meshorer et al., 2002; Sailaja et al., 2012). Interestingly, a non-exclusive increase of HDAC-1, 2, and 7 was also detected. Animals showed anxiety-like behavior and this behavior as well as the AChE chromatine structure and the entire HDAC enrichment profile were reversed by NaBu, an HDAC inhibitor; the restoration of mE1c expression level was however due to HDAC4 inhibition entirely. AChE-R is the alternative splicing soluble variant of AChE-S in neurons; AChE-R production increases under various stress influences (Soreq and Seidman, 2001). This splicing switch can be induced by short-lasting (minutes) stress exposures, but can then last for weeks as shown in adult neuronal and hippocampal slice cultures (Meshorer et al., 2002; Sailaja et al., 2012). NaBu restored this splicing switch with regard to reduction in AChE-R, although the renewed increase of AChE-S variant was incomplete compared to the non-stressed animals. This finding is particularly interesting, as it sheds a new light on how stress may modulate inflammation via epigenetic mechanisms impacting the pro-inflammatory AChE. AChE inhibition restricts inflammation not only in the peripheral organs, but also in the brain (Pollak et al., 2005). The incomplete restoration of AChE-S suggests a complex regulatory network controlling AChE-S/AChE-R ratio in response to stress. Ultimately, such shifts in AChE presence in intercellular space may have long-lasting effects on cholinergic transmission with regard to cognition (cf. Section Microglial LRP-mediated Neuronal Phagocytosis May Be Enhanced By *In utero* Exposure to Inflammation) and neuroinflammation. Adding to the complexity of epigenetic regulation of cholinergic signaling and neuroinflammation, microRNA (miRNA)-132 has been shown in adult murine model and cell lines to potentiate cholinergic anti-inflammatory signaling in the periphery, myeloid cells in particular, and in the brain by inhibiting AChE expression (Shaked et al., 2009). The role of miRNA-132 in microglia is not yet known, but the evidence is growing for the overall importance of miRNA signaling in determining the polarization and phenotype of microglia and myeloid cells in general (Ponomarev et al., 2013).

Fear represents a model system to study chronic impact of stress on epigenome and cardiovascular system (Shenhar-Tsarfaty et al., 2015). As noted above, this approach may relate conceptually to our current findings bringing together the effects of *in vivo* endotoxin exposure as a stressor on the brain's microglial transcriptome and the cardiovascular system. Interestingly, changes in miRNA-608 activity on AChE binding sites in the brain (e.g., due to single nucleotide polymorphisms, SNPs) concomittantly raise levels of anxiety and blood pressure in adult mice and humans by decreasing the inhibition of AChE expression, while reducing CDC42 and IL-6 levels, important pro-inflammatory mediators (Hanin et al., 2014). This link between epigenetic signaling mechanisms, stress, and cardiovascular system is further strengthened by the recent study, in adult humans showing synergistic effects of fear as a stressor on heart rate and inflammation with cholinergic signaling playing a central role in modulating both systems (Shenhar-Tsarfaty et al., 2014, 2015). We found an increase of heart rate and a slight drop of blood pressure within the time frame of the IL-6 peak following LPS injection to the ovine fetus (Durosier et al., 2015). However, this effect appeared to dissipate at 54 h following the initial LPS exposure. Still, our experimental design does not allow drawing conclusions whether such intrauterine exposure to low-dose endotoxin concentrations may induce lasting cardiovascular changes along with alterations in innate immune responses upon repeated exposure to inflammatory stimuli. This remains subject of future studies. Interestingly, *BCHE*, but not *ACHE*, showed DE and less than one-fold down regulation ($\log_2 = -3.197$ and $p_{adj} = 6.86 \times 10^{-3}$) in N_C vs. SH_C microglia, with no detectable change in microglia exposed to LPS *in vitro* only or upon double-hit exposure; both *BCHE* and *ACHE* were also less than one-fold down regulated, but not differentially expressed in all other comparisons. In this regard, the potential role of serum cholinesterases as easily accessible biomarkers of neuroimmune function, along with heart rate variability monitoring, present an attractive opportunity to translate these insights into bedside applications to improve perinatal health outcomes (Durosier et al., 2015; Lake et al., 2014; Shenhar-Tsarfaty et al., 2014).

In summary, microglia pre-exposed to inflammation *in vivo* seem to acquire a memory of inflammation that reflects on the transcriptome by an overall decreased response in inflammatory pathways while the production of the pro-inflammatory cytokine IL-1β is up regulated. In light of the above discussion, our findings lend support to the notion of an inflammation memory in SH_C sustained in SH_L microglia that may be mediated by epigenetic regulatory processes involving histone acetylation and miRNA signaling. The intriguing link to the metabolic processes and cardiovascular system also deserves attention in future studies. Additional mechanistic studies (knockout, knockdown, or overexpression) are needed to validate these observations.

Microglial LRP-Mediated Neuronal Phagocytosis May be Enhanced By In utero Exposure to Inflammation

Calreticulin (CRT) exposure on the surface of viable or apoptotic neurons is required for their phagocytosis via low-density lipoprotein receptor-related protein (LRP) receptors on LPS-stimulated primary culture rat microglia (Fricker et al., 2012). We found that the gene *LRP6* is significantly up regulated after LPS exposure *in vitro* in N_L microglia ($\log_2 = 1.052$ and $p_{adj} = 2.76 \times 10^{-2}$) and the activation of *LRP6* is sustained *in vitro* in SH_C microglia ($\log_2 = 1.850$ and $p_{adj} = 5.58 \times 10^{-3}$), i.e., after the LPS exposure *in vivo*. *LRP1B* ($\log_2 = 6.380$ and $p_{adj} =$

5.66×10^{-5}) and *LRP2* ($\log_2 = 6.571$ and $p_{adj} = 4.24 \times 10^{-11}$) were also strongly up regulated in SH_C microglia. *LRP1B* and *LRP2* showed a four-fold up regulation in N_L microglia, however, adjacent *p*-values were not consistent to support this observation.

We show that a single LPS exposure *in vivo* or *in vitro* suffices to up regulate *LRP* genes suggesting that *in utero* exposure to inflammation may alter microglial—neuronal communication making CRT expressing neurons vulnerable to LRP-mediated phagocytosis. Our data does not allow validating the idea that double hit exposure to an inflammatory stimulus enhances up regulation of microglial *LRP*, because we could not test directly SH_L vs. SH_C (cf. Section Methodological Considerations, Discussion on limitations of RNAseq approach). Future studies, should estimate genetic expression profile of SH_L compared to SH_C.

Methodological Considerations

We could not detect any *in situ* neuroinflammation using Iba1, a well-established myeloid cell marker in sheep and other species. Despite the lack of overt neuroinflammation seen *in situ* we demonstrated a pattern of LPS-induced systemic IL-6 cytokine production *in vivo* and microglial IL-1β cytokine secretion *in vitro*. This further supports the notion that even subtle LPS exposures *in utero in vivo* may polarize microglia toward a neuroinflammatory phenotype without or with secondary re-exposure to an inflammatory stimulus. The LPS-triggered rise of IL-6 in plasma is in line with animal and human studies at this developmental stage (Duncombe et al., 2010; Chan et al., 2013). Microglia *in vitro* have been shown to secrete IL-1β preferentially when challenged with LPS, while IL-6 secretion is a hallmark of cultured astrocytes in rat (Gottschall et al., 1994). Our findings are consistent with literature and further support the cell culture purity.

In parallel to our team, the feasibility of creating a mixed primary fetal ovine brain culture has been recently, demonstrated (Weaver-Mikaere et al., 2012). We have advanced this work by focusing on late rather than mid-gestation fetuses and creating primary pure microglial culture rather than mixed culture. This allowed us to then study the microglia-specific effects of the double hit *in vivo/in vitro* LPS exposure on the secretion profile of the inflammatory cytokine IL-1β and the high-throughput transcriptome.

In this study, we did not discriminate between the various phenotypes of the endogenous microglia as well as the microglia recruited to the brain during the inflammatory process via the blood brain barrier, whose permeability increases under conditions of hypoxia/ischemia and fetal inflammatory response (Hutton et al., 2007, 2008; Butovsky et al., 2014; Yamasaki et al., 2014; Greter et al., 2015; Sadowska et al., 2015). Considering the mild, low-dose LPS exposure, we speculate that no recruitment of peripheral monocytes was triggered. However, we cannot state with certainty whether the microglial memory of inflammation was entirely newly established upon *in vivo* LPS exposure, or certain pre-existing sub-populations of microglia responded differentially to the endotoxin; another possibility needing validation remains that progenitor cells from the periphery differentiated accordingly. Hence, future studies, perhaps using single cell RNAseq, will elucidate whether the "memory" is entirely newly established, carried by a subpopulation of endogenous or periphery-recruited microglia. Isolating single cells and expanding them in culture may be another approach to test these hypotheses.

In our approach, we used DESeq2 to normalize read counts and identify differentially expressed genes. DESeq2 was specifically designed to estimate differential expression in a dataset containing replicates for both control and treatment samples. Our method used a large number of animals allowing us to have replicates for naïve control and LPS-exposed microglia. However, a limitation of our RNAseq analysis is the lack of replicates for SH_C and SH_L preventing us from comparing SH_C to SH_L directly. Other platforms meant to analyze samples without replicate could have been used here. However, we chose not to disrupt the analytical pipeline and keep the statistical analysis consistent throughout the analysis. Despite quality control measures prior to sequencing, the sample N_{L3} had a different expression pattern than the two other N_L samples. We believe it is not related to RNA quality, and may have been due to environmental or other physiological conditions of the animal that we were not aware of at the time of the experiment. In interrogating the differential gene expression, we have ensured that the partially deviating pattern observed in sample N_{L3} did not confound our findings (**Figures 3A,B**).

Conclusions

Inflammatory microglial phenotype acquired during *in vivo* exposure to LPS is sustained and potentiated *in vitro* upon re-exposure to LPS. We identified a unique $HMOX1_{down}$ and FBP^{up} phenotype of microglia exposed to the double-hit. Our results also suggest that microglia may have acquired *in vivo* a memory of inflammation regulated by an epigenetic process that should be confirmed by further epigenomic studies. This model allows studying mechanisms of fetal neuroinflammation *in utero in vivo* and *in vitro* to identify potential therapeutic targets for early postnatal intervention to prevent brain injury.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fncel.2015.00294

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frontiers in Cellular Neuroscience

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Spiral ganglion cells and macrophages initiate neuro-inflammation and scarring following cochlear implantation

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Conservation of a patient's residual hearing and prevention of fibrous tissue/new bone formation around an electrode array are some of the major challenges in cochlear implant (CI) surgery. Although it is well-known that fibrotic tissue formation around the electrode array can interfere with hearing performance in implanted patients, and that associated intracochlear inflammation can initiate loss of residual hearing, little is known about the molecular and cellular mechanisms that promote this response in the cochlea. *In vitro* studies in neonatal rats and *in vivo* studies in adult mice were performed to gain insight into the pro-inflammatory, proliferative, and remodeling phases of pathological wound healing that occur in the cochlea following an electrode analog insertion. Resident Schwann cells (SC), macrophages, and fibroblasts had a prominent role in the inflammatory process in the cochlea. Leukocytes were recruited to the cochlea following insertion of a nylon filament in adult mice, where contributed to the inflammatory response. The reparative stages in wound healing are characterized by persistent neuro-inflammation of spiral ganglion neurons (SGN) and expression of regenerative monocytes/macrophages in the cochlea. Accordingly, genes involved in extracellular matrix (ECM) deposition and remodeling were up-regulated in implanted cochleae. Maturation of scar tissue occurs in the remodeling phase of wound healing in the cochlea. Similar to other damaged peripheral nerves, M2 macrophages and de-differentiated SC were observed in damaged cochleae and may play a role in cell survival and axonal regeneration. In conclusion, the insertion of an electrode analog into the cochlea is associated with robust early and chronic inflammatory responses characterized by recruitment of leukocytes and expression of pro-inflammatory cytokines that promote intracochlear fibrosis and loss of the auditory hair cells (HC) and SGN important for hearing after CI surgery.

Keywords: neuro-inflammation, fibrosis, pathology, cochlea, cochlear implant, Schwann cells, macrophages, spiral ganglion neurons

Introduction

Cell regeneration (Löwenheim et al., 1999; Stone and Rubel, 2000; Kawamoto et al., 2003; Levic et al., 2007; Chen et al., 2013; Mizutari et al., 2013; Shi et al., 2013) and stem cells (Ito et al., 2001; Li et al., 2003; Martinez-Monedero and Edge, 2007; Koehler et al., 2013; Bas et al., 2014) are emerging therapies that aim to restore hearing in patients with deafness. Despite promising results, these novel therapies will take a long time to reach clinical application due to concerns regarding both safety and efficacy. Cochlear implantation is still one of the best options for patients with unserviceable hearing.

Cochlear implantation can restore hearing perceptions in patients with significant sensorineural hearing loss (SNHL) by bypassing the auditory hair cells (HC) and directly stimulating the spiral ganglion neurons (SGN). Non-traumatic cochlear implantation can be associated with preservation of auditory HCs of the apical and middle turns of the cochlea, which are important for low and even mid-frequency residual hearing, respectively. Significant gains in hearing in quiet and in noise as well as improvements in music perception are associated with residual "acoustic" hearing preservation following cochlear implantation (Mowry et al., 2012). Another important factor associated with better hearing outcomes following CI surgery is preventing excessive cochlear fibrosis, because fibrosis in the cochlea can negatively impact electrode impedance and "electrical" hearing perception with CIs (Hughes et al., 2001; Choi and Oghalai, 2005; Jia et al., 2011; Wolfe et al., 2013; Mosca et al., 2014). Although there have been many triumphs in CI research, long-term residual hearing preservation and prevention of fibrous tissue formation around an electrode array are still major challenges in cochlear implantation (Santa Maria et al., 2013).

Hearing outcomes after CI surgery depend on the health of residual auditory HCs, SGNs, and the factors that support their survival. Auditory HCs and their supporting cells within the organ of Corti (OC) secrete neurotrophic factors that support and help maintain SGN viability (Santa Maria et al., 2014). In addition, the health of Schwann cells (SC, glial cells of the peripheral nervous system) that reside within the SGN fibers is also an important component of hearing after implantation. SCs are responsible for myelination of type 1 SGNs, assuring an insulating sheath around spiral ganglia axons for rapid propagation of action potentials from the cochlea to the cochlear nucleus within the central nervous system (Romand and Romand, 1990). Similar to auditory HCs and supporting cells of the OC, SCs also cross-communicate with SGNs through the expression of neurotrophins and their receptors to promote SGN homeostasis (Hansen et al., 2001). A loss of sensory auditory HCs and SCs can reduce the number and activity of afferent SGNs, which are crucial for hearing in cochlear implantation (Roehm and Hansen, 2005). Therefore, traumatic cochlear implantations can magnify post-operative electrical-acoustic hearing impairments through synergistic losses of auditory HCs and SGNs. The application of "soft" atraumatic surgical techniques in CI surgery and the development of electrode arrays designed to reduce friction and trauma in the cochlea can reduce auditory HC and SGN losses, thereby improving post-operative "electrical-acoustic" hearing outcomes (Coco et al., 2007; Bas et al., 2012a; Mowry et al., 2012).

Electrode array insertions into the cochlea can initiate loss of auditory HCs and SGN fibers through direct mechanical injury and expression of intracochlear inflammatory cascades that are detrimental to their survival. Through these mechanisms, an aberrant wound healing response is activated in the cochlea that leads to fibrosis. By understanding the linkages between electrode insertion trauma (EIT), inflammation, and fibrosis formation, therapeutic strategies can be developed against these signaling pathways to prevent auditory HC death, loss of SGNs, and fibrotic deposition to improve post-implantation hearing outcomes.

Using adult mouse *in vivo* and neonatal rat *in vitro* models of electrode analog insertion trauma (EIT) (Bas et al., 2012b), the molecular and cellular mechanisms involved in the inflammatory, proliferative and remodeling phases of wound healing within the cochlea and their role in fibrosis were investigated. The early inflammatory response characterized by inflammatory cell infiltration was studied using the *in vitro* model. An *in vivo* model was used to investigate both early and late phases of the inflammatory response as well as contributions of the proliferative and remodeling phases of pathological wound healing to fibrosis and scar formation after EIT. In summary, a robust neuro-inflammatory response occurs after EIT, which leads to impressive amounts of cell proliferation, tissue remodeling, and fibrosis in the cochlea *in vitro* and *in vivo*.

Materials and Methods

Animals

For the *in vitro* section, 3 or 4 day old (P3–P4) Sprague Dawley rat pups were used (Charles River Laboratories, Wilmington, MA, USA). For the *in vivo* studies 1.5–2 month old Balb/c mice of either sex were used (The Jackson Laboratory, Bar Harbor, ME). The mice were housed in sterile cages in a Virus Antigen Free facility from the Division of Veterinary Resources of University of Miami and were fed sterilized standard diet and water *ad libitum*.

In Vitro

P3–P4 rat pups are anesthetized with ice for 15 min and then decapitated. The otic capsules were dissected using a surgical microscope and placed in cold and sterile phosphate saline buffer (PBS). Cochleae were randomly assigned to each experimental group: control (no EIT and no treatment), EIT, and EIT + dexamethasone (DXM) treatment. To simulate EIT in experimental cochleae, a 0.2 mm diameter monofilament fishing line (Cajun Line; W.C. Bradley Co., OK, USA) was introduced three to four times through a small (∼0.3 mm diameter) cochleostomy that was created with a sharpened #5 Dumont forcep next to the round window membrane. With this technique, a high angle (110–150°) and depth of insertion (2 mm) into the *scala tympani* was achieved (Bas et al., 2012b). All cochleae are then incubated for 10 min in PBS. Subsequently, whole OC with lateral wall tissues were harvested and cultured in serum-free culture media consisting of Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA, USA) supplemented with glucose (final concentration at 6 g/L), 1% of

N-1 supplement (Sigma Aldrich, St. Louis, MO, USA), penicillin G (30 U/mL), and either saline or DXM (20 μg/ml, D1756, Sigma Aldrich). The spleens of the pups were also harvested and kept in DMEM at 4°C. Leukocytes were isolated from spleen at 24 h and incubated in a culture dish for 2 h at 37°C. Supernatant was discarded and the adhered cells were collected. An aliquot of the cells was analyzed by Flow Cytometry (LSR-II, BD Biosciences, San Jose, CA) to confirm 90–95% enrichment in the monocytes population. To assess leukocyte recruitment and invasion into injured cochlear tissues, the monocytes were labeled with QTracker 655 (Life Technologies, Carlsbad, CA), re-suspended in PBS containing 2% FBS, incubated for 1 h at 37°C, and exposed to cochlear tissues (whose media was replaced for PBS + 2% FBS) prior to acquisition of images. For gene expression studies, the monocytes were re-suspended in serum free culture media and placed in inserts for indirect co-culture with the cochlear tissues. Both monocytes and cochlear tissues were collected at 72 h after co-culture, washed with cold PBS and stored in Trizol (Life Technologies, arlsbad, CA) at −80°C until further processing.

Imaging and Analysis of Leukocyte Behavior in the Damaged Cochlea Microenvironment

Four cochlear tissue explants (i.e., OC with lateral wall tissues) were used for each condition with a total of 3 independent replicates. Conditions were control, EIT, and EIT + DXM. Sequential images of each group were taken every 15 s for 20 min with a 20× lens in a Zeiss LSM 700 confocal upright microscope. ImageJ was used to analyze the images. Manual tracking (Manual Track plugin) was performed for 25–30 random cells in each sample. The trajectory and distance that the leukocytes traveled were measured. The numbers of leukocyte-leukocyte and leukocyte-tissue interactions were counted during this time period and the durations of these interactions were also documented in a double-blinded manner. The distance and trajectory of each cell was calculated as follow. $Distance = \sqrt{(X_n - X_0)^2 + (Y_n - Y_0)^2}$, where (x_n, y_n) are the coordinates for the last time point (n) and (x_0, y_0) are the origin coordinates. $Trajectory = \sum_0^n \sqrt{\left(X_i - X_{(i-1)}\right)^2 + \left(Y_i - Y_{(i-1)}\right)^2}$, where $X_i = X - X_0$ and $Y_i = Y - Y_0$ for each coordinate and $\left(X_{(i-1)}, Y_{(i-1)}\right)$ are the coordinates for the previous time point. For display purposes, the TrackMate plugin was used. For display purposes, the TrackMate plugin was used. For leukocyte-leukocyte and leukocyte-cochlear tissue interaction counts and duration of the interactions, each sequence of images was divided in 4 quadrants (or regions of interest, 320 × 320 μm) and 1–80 cells were analyzed in each region.

Gene Expression Analysis of the Indirect Co-cultured Leukocytes and Injured Cochlear Tissues

Six cochlear tissue explants (i.e., OC with lateral wall tissues) were used in each group and 4 independent replicates were done. Groups were control, EIT, and EIT + DXM. Leukocytes were removed from leukocyte-and-cochlear tissue co-cultures and analyzed separately. RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. RNA purity and concentration were determined by the absorbance at 260 and 280 nm using a Nano Drop ND-1000 (Thermo Fisher Scientific, Waltham, MA). iScript kit (Bio-Rad, Hercules, CA, USA) was used to synthesize the cDNA. Quantitative real-time PCR was performed in duplicate by using iQ SYBR Green Supermix (Bio-Rad) on a iCycler Real-Time CFX96 Detection System (Bio-Rad). The mRNA levels were normalized against β-actin (a housekeeping gene). The primers were designed based on the cDNA sequences obtained from Ensembl Genome Browser (http://www.ensembl.org).

The primers used were: Chemokine *Ccl2* (ENSRNOT00000009448, NM031530) forward 5′-TAATGCCCCACTCACCTGCT-3′, reverse 5′-AGGTGCTGAAGTCCTTAGG-3′; *Sele*, (ENSRNOT00000076757, NM138879) forward 5′-GAGATCTACATCCAAAGACC-3′, reverse 5′-CTTTACATTCAACCACATGGC-3′; *Sell* (ENSRNOT00000003733, NM019177) forward 5′- CAGTGTCAGTATGTGATCC-3′, and reverse 5′-GACATATTGGACTAGGAC-3′; *Icam1*, (ENSRNOT00000028066, NM012967) forward 5′-CTGTGTATTCGTTCCCAGAGC-3′ and reverse 5′-GATCGAAAGTCCGGAGCT-3′; *Vcam1* (ENSRNOT00000019377, NM012889) forward 5′-GACATCTACTCATTCCCTAAGG-3′ and reverse 5′-GGAGGTGTAGACTTGTAGT-3′; *Il10* (ENSRNOT00000006246, NM012854) forward 5′-TAAGGGTTACTTGGGTTGC-3′ and reverse 5′-CACCTTTGTCTTGGAGCTT-3′; *Tgfb1* (ENSRNOT00000028051, NM_021578) forward 5′-CGGACTACTACGCCAAAGAA-3′ and reverse 5′-TCAAAAGACAGCCACTCAGG-3′; *Actb* (ENSRNOT00000042459, NM031144) forward 5′-CGTTGACATCCGTAAAGACC-3′, and reverse 5′-AGCCACCAATCCACACAGAG-3′ (Sigma Aldrich). Real-time PCR was performed using the following parameters: 3 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Melting curves were also performed to ensure primer specificity and evaluate for any contamination. Relative changes in mRNA levels of genes were assessed using the ΔΔCt method and normalized to the house-keeping gene β-actin (*Actb*) and then to the expression levels of the control group.

In Vivo

Twenty adult mice were randomly assigned to each selected time point and group. The animals were anesthetized with a Ketamine (40 mg/kg)-Xylazine (5 mg/kg) cocktail. Buprinorphine (0.05 mg/kg) was administrated at this time and 2 times a day for 2 more days. The eye blink response to a light touching of the cornea and a toe pinch withdrawal response was used to determine the depth of the anesthesia, which was kept a surgical plane. We also looked at any changes in both rate and depth of inspirations in the breathing pattern of the anesthetized mice.

The post-auricular hair was shaved and the area cleaned with iodine. Artificial tears were added to each eye to prevent corneal dryness. Five microliter of 1% lidocaine was used for local anesthesia. A post auricular incision was performed behind the experimental ear and extended ventrally to the rostral neck skin. The subcutaneous connective tissues were separated to expose the deep structures. The ear canal, the sternocleidomastoid muscle and the facial nerve were identified. The bulla that

encloses the middle and inner ears was visualized below the facial nerve. A self-retaining retractor was used to maintain exposure. The soft tissue structures overlying the temporal bone were removed to expose the bulla. A small hole was opened in the bulla and ∼2 mm of sterile nylon monofilament (∼0.2 mm diameter) was introduced through a puncture in the round window membrane and left in place. Tissue glue was used to plug the hole made into the bulla and the skin was sutured closed with nylon sutures and cleaned once more with topical iodine. The contralateral ear was used as an internal control. The animals were returned to clean cages fitted with a water circulating warming pad and checked periodically during the post-anesthesia recovery period. The animals were euthanized with inhaled CO_2 at 1, 3, 7, 14, or 30 days after the implantation. The cochleae were kept either in Trizol at −80°C for mRNA processing or in freshly prepared 4% paraformaldehyde at 4°C for immunostaining processing.

Genes Involved in Fibrosis

Cochleae from adult mice exposed to unilateral cochlear implantation for 7 days were utilized. Pooled samples from five adult mice non-implanted cochleae were compared to five implanted cochleae; experiments were replicated three times. The cochleae that were preserved at −80°C in Trizol (Life Sciences, Carlsbad, CA) were thawed in a bath of ice and homogenized. Total RNA was extracted following the manufacturer's protocol. The quantity and quality of RNA was measured. RT^2 First Strand Kit for cDNA synthesis and RT^2 SYBR Green qPCR Mastermix with a RT^2 Profiler PCR Array for Mouse Fibrosis genes (all from Qiagen, Valencia, CA, USA) were used in the gene expression studies. Data analysis of the PCR Array was performed with Qiagen's web based software using the ΔΔCt method. The raw data was normalized to housekeeping genes (*Actb* and *Gusb*). Paired experiments were run and the average of the fold change between implanted cochleae and contralateral unoperated control was calculated.

Immunohistology

Cochleae from mice exposed to unilateral cochlear implantation for 1, 3, 7, 14 and 30 days were utilized ($N = 4$ or 5 per condition). The cochleae kept in 4% in paraformaldehyde were transferred to 10% EDTA buffered in PBS at pH 6 and kept in gentle rotation for 7 days. The cochleae were washed in PBS and passed through a sucrose gradient from 5 to 30%. Cochleae were then frozen in O.C.T. compound media (Tissue-Tek, Sakura Finetek USA, Inc, Torrance, CA, USA) and cryosections were performed parallel to the central plane of the modiolus of the cochleae. Slides were washed in PBS and kept in a blocking-permeabilizating media (normal serum, 1% Triton x-100 in PBS) for 1 h. After this time, samples were incubated at 4°C overnight with either of the following primary antibodies: rabbit anti-Arginase I (sc-20150, Santa Cruz Biotech, Dallas, TX, USA), rabbit anti-Interleukin 1β pAb (sc-7884, Santa Cruz Biotech, Dallas, TX, USA) or rabbit anti-ITGA4 (A0696, Neobiolab, Woburn, MA, USA). The slides were washed 3 times with PBS and incubated for 90 min at room temperature with anti-F4/80-FITC (ab60343, Abcam, Cambridge, MA, USA) for Arg1 and IL-1β or Phalloidin-FITC (Sigma-Aldrich, St Louis, MO, USA), and the secondary antibody anti-rabbit IgG Alexa 633. After 3 more washes the specimens were stained with DAPI, washed and cover-slipped with anti-fade mounting media. The sections were observed under a Zeiss LSM 700/confocal upright microscope. Images of different areas of the cochlea were acquired and later constructed. ImageJ was used to analyze the images, histograms of red and green channels for each region of interest (i.e., lateral wall, organ of Corti, spiral ganglion, cochlear nerve, and wound site) were recorded.

Histological Distribution of the Fibrotic Tissue

Selected slides from cochleae cryosections were stained using a Masson's Trichrome Stain Kit (Polysciences, Inc, Warrington, PA, USA) following the manufacturer's instructions. Slides were then dehydrated in alcohol gradient, cleared with Histo-Clear (National Diagnostics, Charlotte, NC) and mounted with Cytoseal XYL (Richard-Allan Scientific, Campus Dr, Kalamazoo, MI). The specimens were observed under a Zeiss Axiovert 200 microscope with a ×10 lens and the mosaic images were stitched together afterwards from individual images.

Statistics

One-Way ANOVA and Tukey's Multiple Comparison test were utilized for *in vitro* studies: leukocyte distance covered, trajectory of leukocytes, counts of leukocyte-leukocyte and leukocyte-tissue interactions, and duration of interactions. The same test was applied for the *in vitro* gene expression study. Two-Way ANOVA followed by Bonferroni *post-hoc* tests were used to analyze the relative and absolute fluorescence intensities from the adult cochleae immunolabeling studies (*in vivo* studies). In all graphs the results are expressed as mean values ± S.D. In the study of genes involved in fibrosis in adult mice cochleae, the results are expressed as mean values ± S.D. Genes from implanted cochleae that demonstrated differences of ≥2 mean fold changes over control values were considered up regulated. Mean fold changes from control values ≤0.5 were considered down-regulated.

Results

Increased Leukocyte Recruitment and Cell-Cell Interaction in Damaged Cochlear Tissues

Cell movement analyses: **Figure 1** shows the analysis of tracked leukocytes exposed to EIT cochlear tissue explants. The trajectory (a, 94.7 ± 2.5, $N = 250$ cells, $p < 0.001$) and distance (b, 14.48 ± 1.8, $N = 249$ cells, $p < 0.001$) covered by these tracked leukocytes were significantly reduced compared with leukocytes co-cultured with undamaged control cochlear tissue explants (a, 191.8 ± 8.7, $N = 239$ cells; distance, b, 96.9 ± 5.9, $N = 249$ cells). The behavior of leukocytes was assessed in EIT cochlear tissue explants following DXM treatment (20 μg/ml), a synthetic steroid known to inhibit expression of pro-inflammatory cytokines, chemokines, and cell adhesion molecules in other tissue types. Leukocyte response in DXM treated EIT cochlear tissue explants was similar

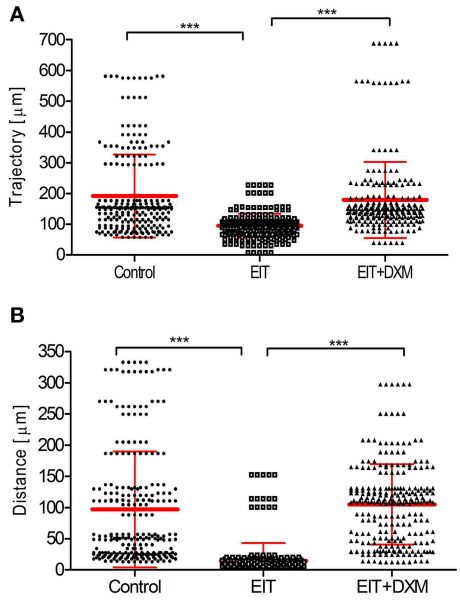

FIGURE 1 | Analysis of tracked cells for direct leukocyte and cochlear tissue co-cultures. (A) Leukocyte trajectory and **(B)** leukocyte distance covered. Comparison are between EIT-cochlear tissues and undamaged controls and with EIT pre-treated with Dexamethasone (DXM, 20 μg/ml). $N = 239 - 250$ cells/group, One-Way ANOVA and Tukey's Multiple Comparison Test was used for statistical analysis $^{***}p < 0.001$.

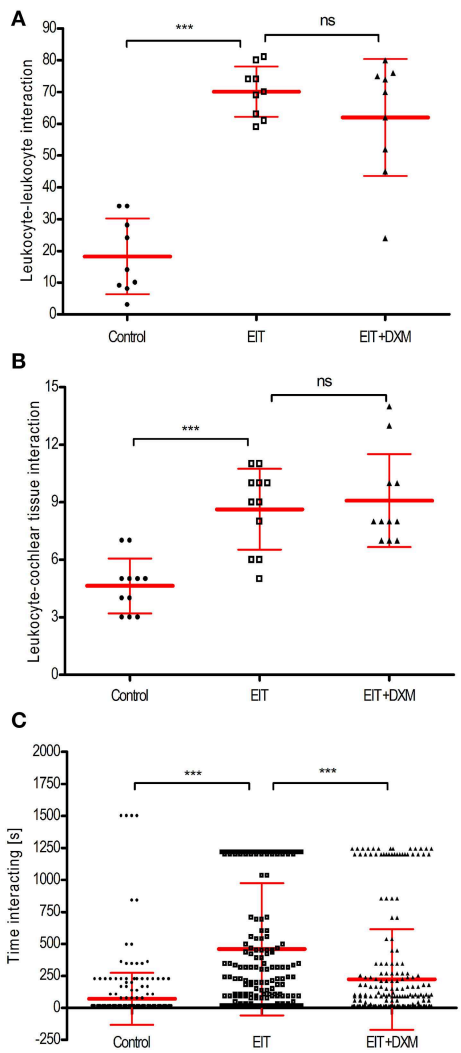

FIGURE 2 | Cell-cell interactions analysis for direct leukocyte and cochlear tissue co-cultures. (A) Number of leukocyte-leukocyte interactions in leukocytes and cochlear tissues co-cultures; **(B)** number of leukocyte-cochlear cells interactions in co-cultures and **(C)** time average that leukocytes interact. Comparisons are between EIT-cochlear tissues and undamaged controls and with EIT pre-treated with dexamethasone (DXM, 20 μg/ml). In upper and middle graphs, the number of cells interacting was averaged from 9 to 11 quadrants or regions of interest. For the bottom panel, $N = 278$ cells/group. One-Way ANOVA and Tukey's Multiple Comparison Test was used for statistical analysis $^{***}p < 0.001$, ns non-significant p-value.

to responses observed in undamaged control explants and significantly different from EIT cochlear tissues (DXM treated: trajectory, a: 178.6 ± 8.0, $N = 239$ cells, $p < 0.001$ compared to EIT; distance, b: 105.2 ± 4.1, $N = 249$ cells, $p < 0.001$ compared to EIT). Videos of leukocyte recruitment and interaction in the three different groups of cochlear tissue explants can be viewed in the supplemental data section (Supplemental Movies 1–6).

Each sequence of images was divided in quadrants or regions of interest (320×320 μm). We found a significant increase in the number of leukocyte-leukocyte (a, 70 ± 3, $N = 9$ regions of interest, $p < 0.001$) and leukocyte- cochlear tissue (b, 9 ± 1, $N = 11$ regions of interest, $p < 0.001$) interactions between the leukocytes co-cultured with the EIT explant group compared to the undamaged cochlear tissue explants (leukocyte-leukocyte, a: 18 ± 4, $N = 9$ regions of interest, and leukocyte-cochlear tissue, b: 5 ± 1, $N = 11$ regions of interest) (**Figures 2A,B**). DXM treatment did not affect the number of interactions between leukocytes and leukocyte-cochlear tissue explants, when compared to the EIT group of explants (DXM treated: leukocyte-leukocyte, a: 62 ± 6, $N = 9$ regions of interest, $p > 0.05$; leukocyte-cochlear tissue, b: 9 ± 1, $N = 11$ regions of interest, $p > 0.05$ The duration of interactions between leukocytes in the EIT group of explants was significantly increased compared to values obtained in the undamaged control group of explants (458.20 ± 30.95 EIT vs. 71.76 ± 12.15 control explants, $N = 278$, $p < 0.001$) (**Figure 2C**). Interestingly, even though DXM did not alter the number of cell-cell interactions between leukocytes and leukocyte-and-cochlear tissue, the time that the leukocytes remained in contact was significantly shorter than durations observed in the EIT group of explants (c, DXM treated: 222.70 ± 23.53, $N = 278$).

Cochlear Tissues and Leukocytes Overexpress Chemokines and Cell Adhesion Molecules in Response to an Electrode Analog Insertion-induced Trauma *in Vitro*

EIT injury in cochlear tissues is associated with increased mRNA levels for the potent chemokine *Ccl2* in both cochlear tissue (4.76 ± 0.21, $N = 4$, $p < 0.001$) and leukocytes that were co-cultured with EIT cochlear tissues (2.27 ± 0.12, $N = 4$, $p <$

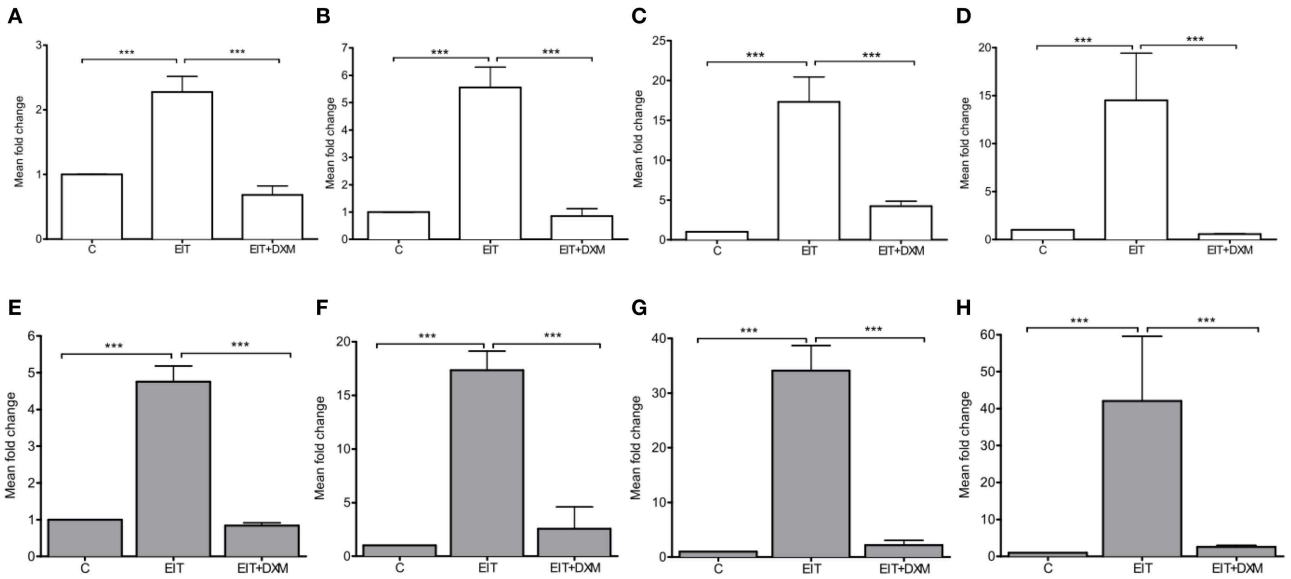

FIGURE 3 | Gene expression studies *in vitro*. The experiments were performed in indirect leukocyte and cochlear tissue co-cultures. The top panel shows the gene expression levels for leukocytes (white), where **(A)** *Ccl2*, **(B)** *Sell*, **(C)** *Icam,* and **(D)** *Tgfb1*. The bottom (gray) panel shows the gene expression levels in cochlear tissues, i.e., organ of Corti and lateral wall tissues, where **(E)** *Ccl2*, **(F)** *Sele*, **(G)** *Vcam,* and **(H)** *Tgfb1*. Comparisons are between EIT-cochlear tissues and undamaged controls and with EIT pre-treated with Dexamethasone (DXM, 20 μg/ml). Six animals per group were included in each set of experiments and a total of 4 independent experiments were carried out. $N = 4$, One-Way ANOVA and Tukey's Multiple Comparison Test was used for statistical analysis $^{***}p < 0.001$.

0.001), when compared to mRNA levels for this chemokine in the undamaged control group (**Figures 3A,B**). A rise in the transcript levels that encode for the cell adhesion molecules *Vcam1* (34.11 ± 2.29, $N = 4$, $p < 0.001$) and *Sele* (17.34 ± 0.89, $N = 4$, $p < 0.001$) was observed in EIT cochlear tissues when compared to the undamaged control explants. Similarly, an increase in *Icam1* (17.31 ± 1.58, $N = 4$, $p < 0.001$) and *Sell* (5.55 ± 0.37, $N = 4$, $p < 0.001$) mRNA levels was observed in the co-cultured leukocytes from the EIT group of explants, when compared to uninjured explants. In addition, *Tgfb1*, a growth factor released by SCs and macrophages upon nerve injury, was overexpressed in both cochlear tissues (42.02 ± 8.77, $N = 4$, $p < 0.001$) and leukocytes (14.50 ± 2.46, $N = 4$, $p < 0.001$) in the EIT explant group, when compared to the uninjured control group. DXM treatment (20 μg/ml) of EIT cochlear tissues and leukocytes demonstrated significant reductions in *Ccl2, Vcam1, Sele, Icam1, Sell*, and *Tgfb1* gene expression levels when compared to EIT injured cochlear tissue and leukocytes ($p < 0.05$; **Figure 3**).

Fibrosis-related Gene Expression Levels *in Vivo*

Real-time PCR: Gene expression profiling for the proliferative/fibrogenic process in cochlear tissue harvested from adult mice exposed to unilateral cochlear implantation for 7 days was performed (**Table 1**). In order to maintain consistency with the literature, genes with fold change = 2 in EIT cochlear tissue when compared to expression levels of contralateral un-operated cochleae were considered up-regulated. Gene expression levels ≤0.5 when compared to control cochleae were considered down-regulated. Consistent with previous publications of peripheral nerve injury and our *in vitro* gene expression data, there were increased expression levels of of Th1 (i.e., *Il1a* and *Il1b*) and Th-2 (i.e., *Il4* and *Il13*) types of cytokines in EIT cochlear tissues. The chemokines *Ccl3* and *Ccl12* responsible for the recruitment of leukocytes to the wound site were also up-regulated in implanted cochlear tissues. Higher levels of mRNA for platelet-derived growth factor beta polypeptide (*Pdgfb*), a protein that promotes proliferation, differentiation, and migration were observed in cochlear tissues traumatized by the monofilament insertion. Vascular endothelial growth factor A (*Vegfa*), which is a member of the PDGF family and promotes angiogenesis and vascular permeability, was also up-regulated in implanted tissues. mRNA levels for the integrin subunits α1 (*Itga1*), α2 (*Itga2*), α3 (*Itga3*), αv (*Itgav*), and β6 (*Itgb6*) were higher in EIT cochlear tissues when compared to contralateral ears, while *Itgb3* levels were lower in implanted cochleae.

Similar to the *in vitro* study results, *Tgfb1* gene expression levels were also higher in the adult implanted cochleae compared to the contralateral control cochleae at 7 days. Interestingly, *Tgfb3* and its receptor *Tgfbr2*, which are associated with scarless wound healing were down-regulated in EIT cochlear tissues. mRNA expression levels for enzymes involved in extracellular matrix (ECM) remodeling such as *Mmp2, Mmp3, Mmp13, Mmp14*, Urokinase-type plasminogen activator (*Plau*), *Serpine1, Serpinh1, Timp1, and Timp2* were all increased following EIT, relative to un-implanted cochlear tissues. High gene expression levels for hepatocyte growth factor (*Hgf*, a protein involved in angiogenesis and tissue regeneration) and ECM components smooth muscle α-2 actin (*Acta2*), collagen type 1 (*Col1a2*) and 3 (*Col3a1*) were observed in traumatized cochleae when

TABLE 1 | Gene expression profile of the proliferative/fibrogenic process at 7 days post-cochlear implantation in adult mice.

	Mean fold	S.D.		Mean fold	S.D.
Mmp3	35.80	10.38	*Snai1*	1.79	0.27
Ccl12	31.52	7.97	*Ltbp1*	1.75	0.35
Timp1	17.25	5.25	*Il5*	1.62	0.17
Col3a1	15.97	8.82	*Dcn*	1.61	0.31
Ccl3	12.74	4.71	*Tnf*	1.59	0.19
Col1a2	8.50	2.70	*Itgb8*	1.56	0.27
Il13	8.32	3.00	*Ccr2*	1.51	0.14
Il13ra2	6.04	1.41	*Edn1*	1.42	0.47
Serpine1	5.50	3.19	*Stat6*	1.42	0.43
Mmp14	5.21	2.93	*Stat1*	1.38	0.18
Il1a	5.12	0.83	*Smad2*	1.37	0.13
Fasl	4.95	1.32	*Timp3*	1.33	0.41
Itgb6	4.93	3.33	*Nfkb1*	1.31	0.20
Il1b	4.05	0.65	*Cebpb*	1.30	0.63
Il4	4.05	1.40	*Agt*	1.28	0.66
Timp2	3.89	0.78	*Eng*	1.25	0.46
Thbs2	3.75	1.35	*Smad6*	1.25	0.63
Jun	3.67	0.45	*Ccl11*	1.20	0.23
Lox	3.40	1.33	*Tgif1*	1.19	0.13
Mmp13	3.19	1.30	*Serpina1a*	1.19	0.21
Serpinh1	2.92	1.75	*Tgfbr1*	1.18	0.21
Hgf	2.79	1.11	*Smad4*	1.16	0.14
Itga2	2.73	0.55	*Plat*	1.15	0.18
Plau	2.58	1.39	*Cxcr4*	1.09	0.25
Ctgf	2.45	0.75	*Smad3*	1.08	0.28
Itgav	2.38	0.88	*Myc*	1.02	0.20
Mmp2	2.33	1.13	*Akt1*	1.00	0.32
Itga3	2.29	1.16	*Timp4*	0.99	0.21
Vegfa	2.22	0.69	*Thbs1*	0.97	0.19
Itga1	2.14	0.99	*Tgfb2*	0.95	0.17
Acta2	2.12	0.81	*Egf*	0.93	0.27
Grem1	2.11	1.41	*Itgb5*	0.93	0.35
Tgfb1	2.03	0.28	*Pdgfa*	0.81	0.11
Pdgfb	2.01	0.95	*Sp1*	0.81	0.08
Itgb1	1.95	0.25	*Smad7*	0.79	0.30
Bmp7	1.93	0.54	*Ilk*	0.71	0.07
Mmp9	1.81	0.51	*Mmp8*	0.64	0.09
Cav1	1.80	0.32	*Mmp1a*	0.63	0.18
Ifng	1.79	0.27	*Itgb3*	0.62	0.06
Il10	1.79	0.27	*Bcl2*	0.50	0.02
Inhbe	1.79	0.27	*Tgfb3*	0.50	0.06
Plg	1.79	0.27	*Tgfbr2*	0.47	0.10

normalized to control cochleae. Lysyl oxidase (*Lox*, a protein coding gene that is known to mediate the cross-linking of the ECM proteins collagen and elastin), thrombospondin-2 (*Thbs2*; mediator of cell-cell and cell-matrix interactions), Gremlin-1 [*Grem1*, an antagonist of the bone morphogenetic proteins (BMP)] and *Jun* (proto-oncogene) were all over-expressed in cochlear tissues traumatized by EIT, when compared to the unoperated control group. In injured cochleae, Fas ligand (*Fasl*; initiator of the extrinsic pathway of cell death) was upregulated and *Bcl2* (anti-apoptotic protein involved in mitochondrial cell death) levels were significantly reduced relative to control cochleae.

Early Expression of Integrin 4α Following an Electrode Analog Insertion Trauma

Integrin 4α (ITGA4) is a receptor that is expressed after peripheral nerve injury, binds to fibronectin, and is important for neuron regeneration and cell-matrix interactions during leukocyte recruitment. Fluorescence intensities were measured in cross sections of cochleae at 1, 3, 7, 14, and 30 days after EIT after immunofluorescence labeling for ITGA4. Relative fluorescence intensity values were obtained by normalizing data to contralateral un-operated cochleae of these same areas (**Figures 4–6**). There was increased ITGA4 expression at 1 and 3 days post-implantation, which rapidly dropped at 7 days in lateral wall tissues, OC, spiral ganglion, and site of monofilament insertion. ITGA4 expression either returned to baseline (lateral wall and OC), decreased (site of monofilament insertion), or increased (spiral ganglia) 14 days after implantation (**Figures 4**, **5**).

Cell-cell junctions are comprised of cadherin, catenins, and filamentous actin (f-actin) cytoskeleton. Together, f-actin and myosin II form stress fibers, which are contractile bundles important for cell adhesion; they are abundant in endothelial cells, epithelial cells, and myofibroblasts. As a result, f-actin remodeling can affect the integrity of cell-cell junctions and destabilize the epithelial barrier, rendering leukocytes access to surrounding tissues. Reorganization of the actin cytoskeleton and loss of cell-cell adhesions are also seen in epithelial-mesenchymal transition, a phenomenon that occurs in fibrosis and wound healing (Haynes et al., 2011). A significant reduction in f-actin levels in lateral wall, spiral ganglion and cochlear nerve at 3–7 days *in vivo* contrasts with an increase on this stress fibers component in the area where the electrode-analog was placed (**Figures 4**, **6**).

Macrophages and Schwann Cells Involvement in Early and Chronic Inflammatory Responses

Glial cells are non-neuronal cells that are important for myelin formation. They also surround neurons, ground them to the ECM, supply nutrients to neurons, and can destroy and remove pathogens in their environment. Macroglia are large glial cells of the peripheral nervous system that specialize in phagocytosis; SCs are a type of macroglial cell that provides myelination to axons in the peripheral nervous system remove cellular debris through phagocytosis to promote regeneration of nerves (Haack and Hynes, 2001; Gardiner, 2011). Although they can be supportive for neuron regeneration, activated SCs assume many cellular responsibilities, some that are detrimental to nerves. Following nerve injury, SCs release a number of pro-inflammatory cytokines (such as IL-1β) that contribute to the neuro-inflammatory response (Shamash et al., 2002; Tofaris et al., 2002). Similar to SCs, monocytes also demonstrate cytotoxic and cell protective properties. Monocytes can also transform into

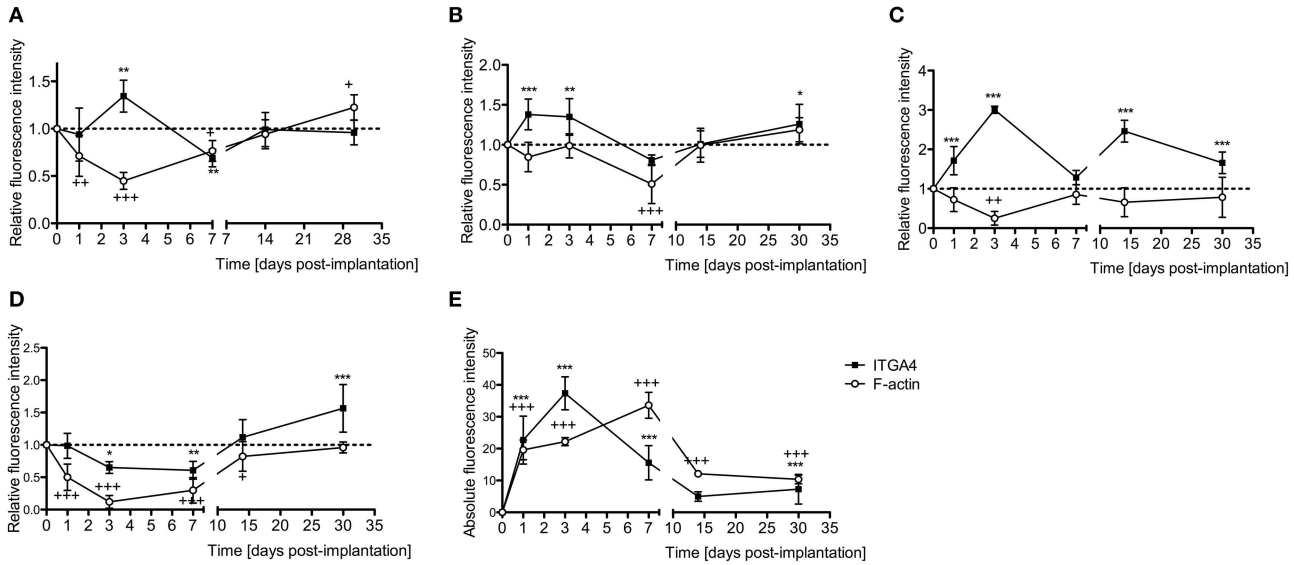

FIGURE 4 | Time-course immunofluorescence analysis for integrin 4α and f-actin levels in different areas of adult mice implanted cochleae. **(A)** Lateral wall tissues (see **Figure 6**, spiral ligament, SL), **(B)** organ of Corti, **(C)** spiral ganglion, **(D)** cochlear nerve, and **(E)** wound site. Three to four specimens for each time point and were analyzed. ImageJ was used to analyze the images, histograms of red and green channels for each region of interest i.e., lateral wall, organ of Corti, spiral ganglion, cochlear nerve, and wound site images were recorded. $N = 8$ each time point, Two-Way ANOVA followed by Bonferroni posttests was used to analyze the relative and absolute fluorescence intensities. $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$ and $+++p < 0.001$, $++p < 0.01$, $+p < 0.05$ represent the p-values for ITGA4 and F-actin, respectively.

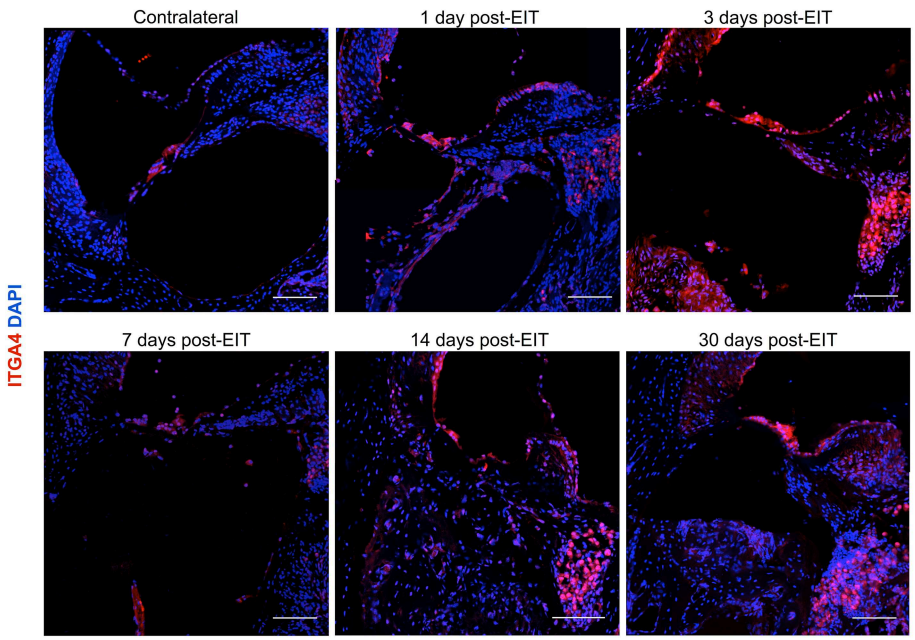

FIGURE 5 | Immunofluorescence images for integrin α4 (ITGA4) in red and cell nuclei in blue. Cross sections corresponding to a contralateral cochlea (bottom), and cochleae at 1, 3, 7, 14, and 30 days post-implantation. The sections were observed under a confocal microscope. Mosaic images of different areas of the cochlea were acquired and later constructed. Three to four specimens were analyzed for each time point. Scale bars = 100 μm.

M1 and M2 tissue macrophages. While M1 macrophages of the classical pathway secrete pro-inflammatory cytokines (such as IL-1β), monocytes that are primed by the alternative pathway (Th-2) or M2 macrophages release anti-inflammatory cytokines that promote cell survival and regeneration and express Arginase 1 (Arg1) (Martinez et al., 2008; Stout, 2010; Ydens et al., 2012).

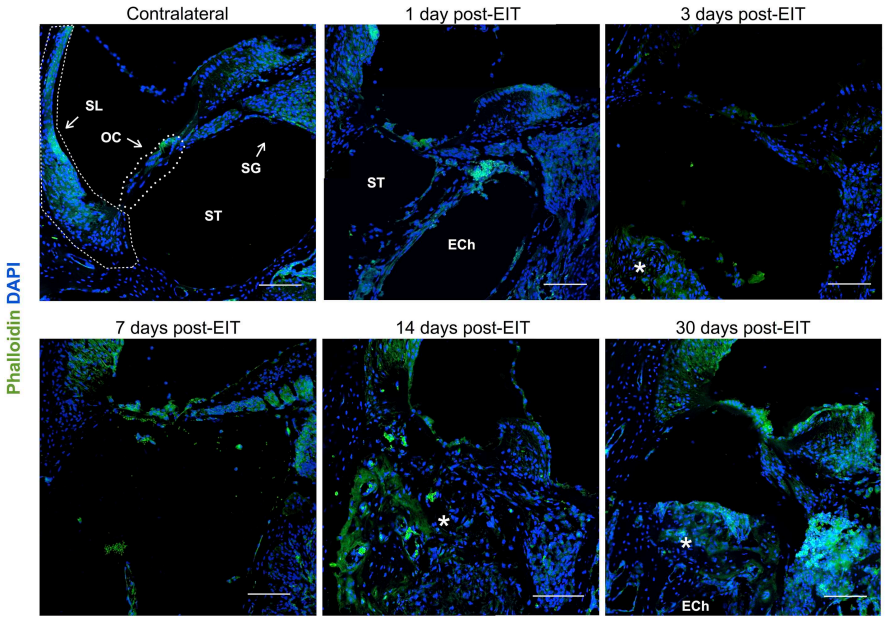

FIGURE 6 | Immunofluorescence images for f-actin in green and cell nuclei in blue. Cross sections corresponding to a contralateral cochlea (bottom), and cochleae at 1, 3, 7, 14, and 30 days post-implantation. The sections were observed under a confocal microscope. Mosaic images of different areas of the cochlea were acquired and later constructed. Annotations for the different structures of the cochlea are in the contralateral photograph. Spiral ligament, SL; organ of Corti, OC; Scala tympanica, ST; Spiral ganglion, SG. In the implanted cochleae: electrode analog channel, ECh; an asterisk marks hyperproliferative tissue in the Scala tympanica. Three to four specimens were analyzed for each time point. Scale bars = 100 μm.

Therefore, macrophage and SC activity can be indirectly studied using markers for IL-1β and Arg1.

Relative fluorescence intensities (**Figure 7**) for IL-1β (a pro-inflammatory cytokine, indirect marker used for the classical activation pathway of M1 macrophages and SCs), Arg1 (indirect marker for the alternative pathway activated M2 macrophages), and F4/80 (marker for monocytes, macrophages and microglia) were calculated and normalized as described in the previous section. Immunofluorescence images at days 1, 3, 7, 14, and 30 post-implantation are shown for F4/80 (**Figure 8**), IL-1β (**Figure 9**), and Arg1 (**Figure 10**). Progressive increases in monocyte/macrophage infiltration (F4/80) and IL-1β production were seen in the lateral wall tissues over time, reaching maximum levels at 14 and 30 days post-implantation. In the OC, the IL-1β levels increased on day 14 and then remained stable until day 30. Interestingly, monocyte/macrophage infiltration and the levels of Arg1 showed a biphasic pattern of expression with one peak at 3 days and the second at 14 days. After 1 month post-implantation, the levels of Arg1 remained higher than IL-1β and the intensity of F4/80 staining for monocytes/macrophages/microglia was reduced. Results from the spiral ganglion area and wound site (area of monofilament insertion) were similar, i.e., Arg1 levels and monocyte/macrophage /microglia invasion rose rapidly at 1 day post-implantation with maximum levels detected at 7 days post-implantation. In the spiral ganglion, the levels of Arg1 predominated over IL-1β levels, peaking at day 7, indicating involvement of M2 macrophages. Expression of IL-1β and Arg1 in the wound site (area of electrode insertion) overlapped at all times, suggesting that both M1 and M2 macrophages are present. In the cochlear nerve, there is a progressive increase in monocytes/macrophages infiltration and IL-1β expression, beginning at post-implantation day 3.

Excessive Deposition of Fibrotic Tissue after an Electrode Analog Insertion Trauma

Cochleae implanted with a monofilament were processed with antibodies for α-smooth muscle actin (myofibroblast marker) and Collagen type 1A 30 days after implantation and compared to contralateral control cochleae. The fibrous tissue that formed around the electrode array (O-shaped ring) demonstrated presence of myofibroblasts and expression of Collagen type 1A, while unoperated cochleae did not demonstrate any staining for these markers (**Figure 11**).

Cochleae from adult mice that received unilateral cochlear implantation *in vivo* were harvested 30 days after implantation, stained with Masson's trichrome and examined for the presence of scar tissue (**Figure 12**). Implanted cochleae demonstrated blue staining for collagen fibers in the scala tympani (the area of the monofilament insertion). Sections obtained from contralateral unoperated cochleae did not demonstrate blue staining for collagen. The magnitude of the scar thickness can be appreciated in **Figure 12**.

Discussion

Cochlear implantation can restore hearing in patients with significant HL by electrically stimulating the neurons of SG. Preservation of residual auditory HCs and hearing and

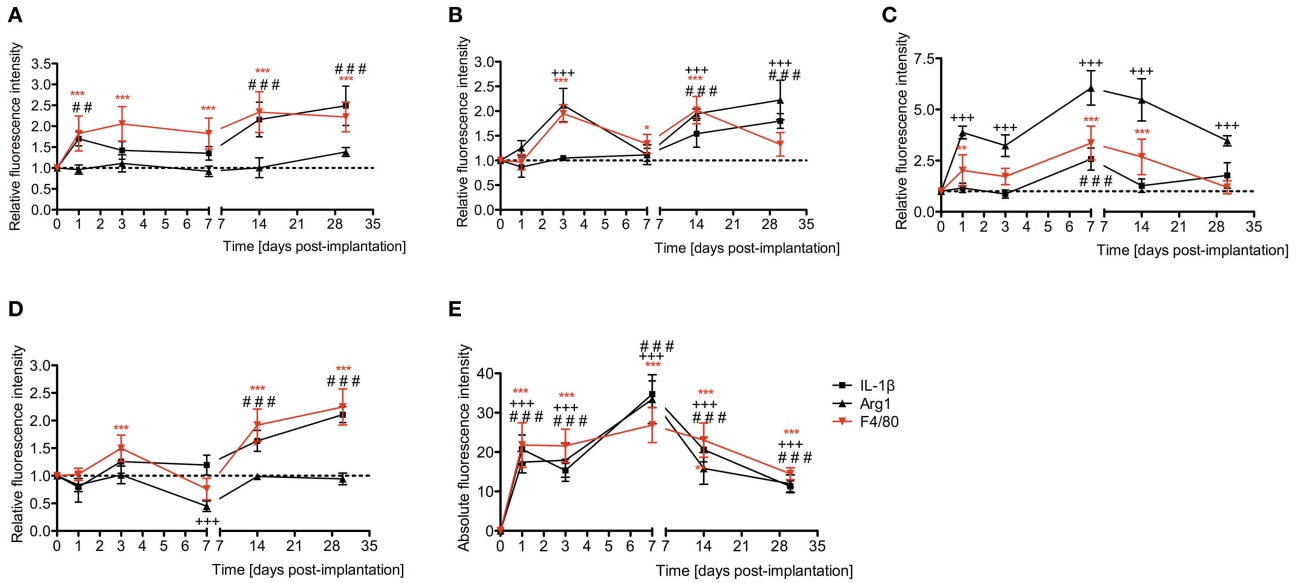

FIGURE 7 | Time-course immunofluorescence analysis for IL-1β, Arg1, and presence of macrophages/microglia (F4/80 positive cells) levels in different areas of the implanted cochleae. (A) Lateral wall tissues (marked as SL, spiral ligament in the immunofluorescence images), **(B)** organ of Corti (OC, see **Figure 8**), **(C)** spiral ganglion (SG), **(D)** cochlear nerve, and **(E)** wound site (asterisk in **Figure 8**). ImageJ was used to analyze the images, histograms of red and green channels for each region of interest i.e., lateral wall, organ of Corti, spiral ganglion, cochlear nerve, and wound site images were recorded. $N = 8$ each time point, Two-Way ANOVA followed by Bonferroni posttests was used to analyze the relative and absolute fluorescence intensities. $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$, $+++p < 0.001$, and $###p < 0.001$, $##p < 0.01$ represent the p values for F4/80, Arg1 and IL-1β, respectively.

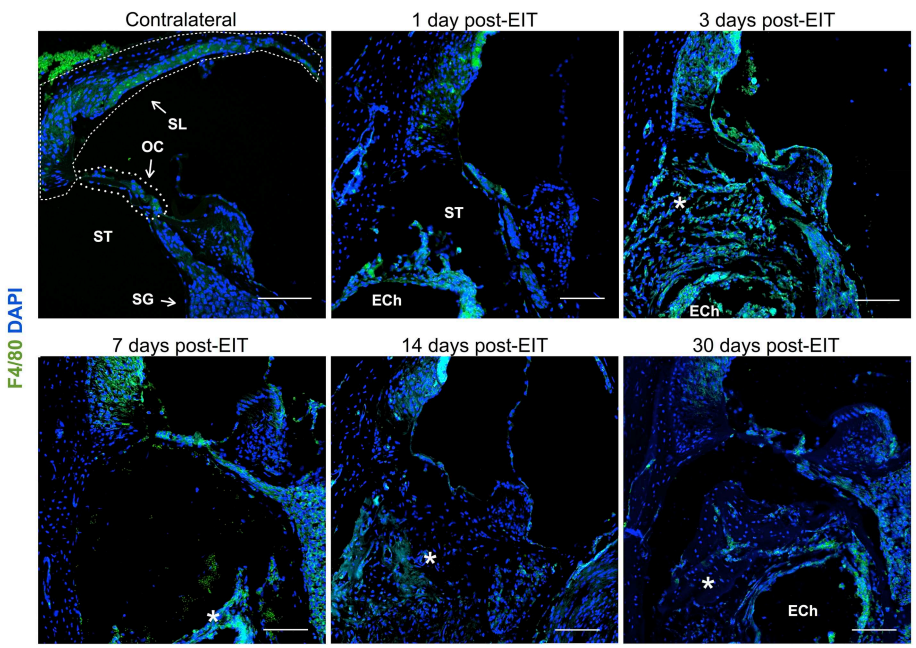

FIGURE 8 | Immunofluorescence images for F4/80 (monocytes/macrophages/microglia, green) and nuclei (blue). Representative micrographs of cross sections correspond to a contralateral cochlea (bottom), and cochleae at 1, 3, 7, 14, and 30 days post-implantation. A heavy influx of monocytes/macrophages/microglia can be observed at 3 days in the newly formed tissue en sheathing electrode analog as well as in the lateral wall, organ of Corti, and to a lessen extend to the spiral ganglion area. The sections were observed under a confocal microscope. Images of different areas were acquired and stitched together afterwards. Annotations for the different structures of the cochlea are in the contralateral photograph. Spiral ligament, SL; organ of Corti, OC; Scala tympanica, ST; Spiral ganglion, SG. In the implanted cochleae: electrode analog channel, ECh; an asterisk marks hyperproliferative tissue in the Scala tympanica. Four to five specimens were analyzed for each time point. Scale bars = 100 μm.

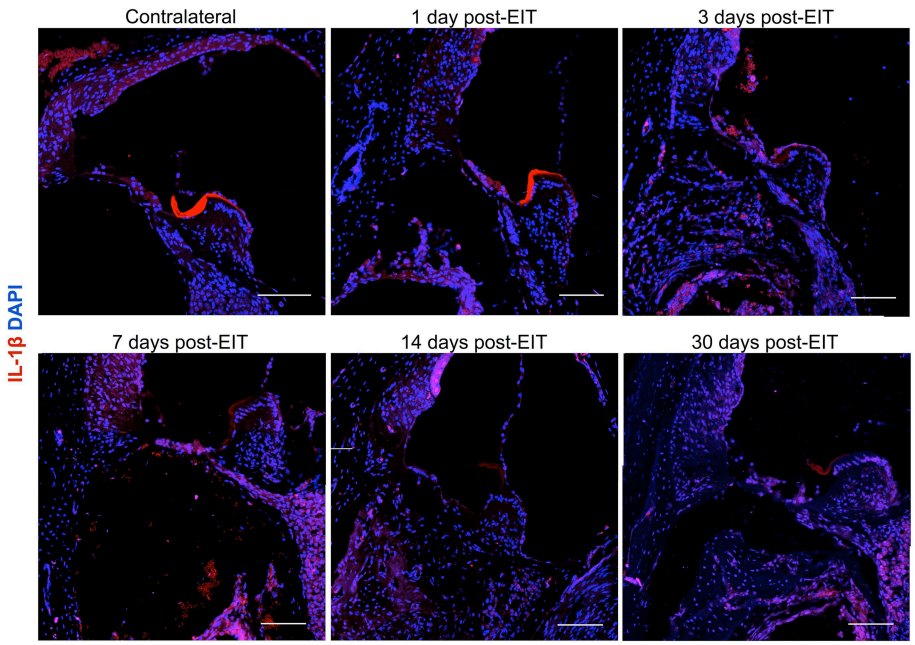

FIGURE 9 | Immunofluorescence images for interleukin-1β (IL-1β, red), and nuclei (blue). Representative micrographs of cross sections correspond to a contralateral cochlea (bottom), and cochleae at 1, 3, 7, 14, and 30 days post-implantation. A strong red signal at 7 days post-implantation, especially in the spiral ganglion area and wound site, reveals a severe neuro-inflammatory response. The sections were observed under a confocal microscope. Images of different areas were acquired and stitched together afterwards. Four to five specimens were analyzed for each time point. Scale bars = 100 μm.

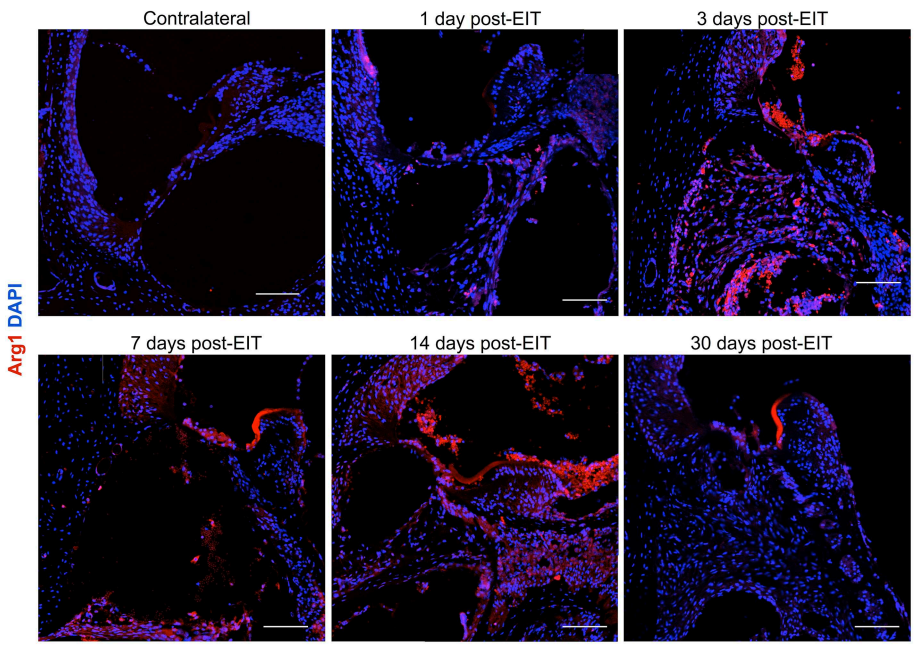

FIGURE 10 | Immunofluorescence images for Arginase I (Arg1, red) and nuclei (blue). Representative micrographs of cross sections are from a contralateral cochlea (bottom), and cochleae at 1, 3, 7, 14, and 30 days post-implantation. Note that red blood cells are accumulated over the organ of Corti area, these cells are auto-fluorescent due to hemoglobin fluorescence. An increase in Arg1 levels at 7 days post- implantation, especially in the spiral ganglion area and wound site, indicates healing process. The sections were observed under a confocal microscope. Images of different areas were acquired and stitched together afterwards. Four to five specimens were analyzed for each time point. Scale bars = 100 μm.

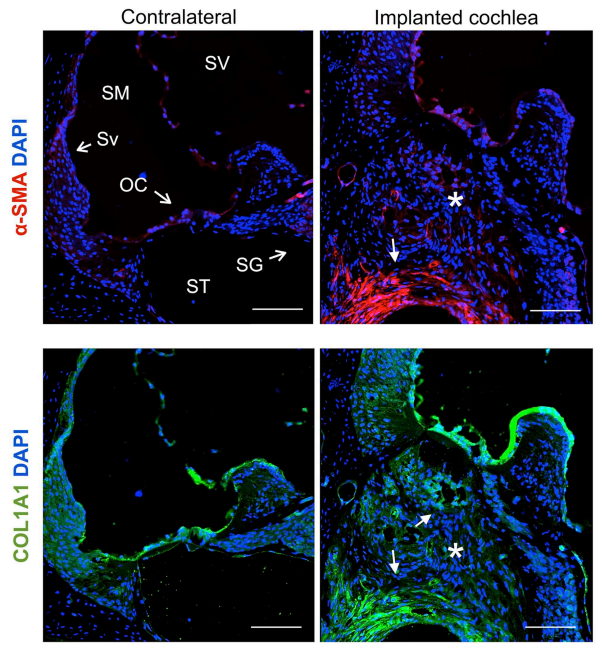

FIGURE 11 | Immunofluorescence images for the myofibroblast marker α-smooth muscle actin (α-SMA, red), Collagen type 1A (COL1A1, green) and nuclei (blue). Representative micrographs of cross sections correspond to a contralateral cochlea and a cochlea implanted for 1 month. Cells from the fibrotic tissue enclosing the electrode analog stain positive for α-SMA and COL1A1, asterisks indicate the presence of fibrotic tissue. The sections were observed under a confocal microscope. Images of different areas were acquired and stitched together afterwards. Scale bars = 100 μm.

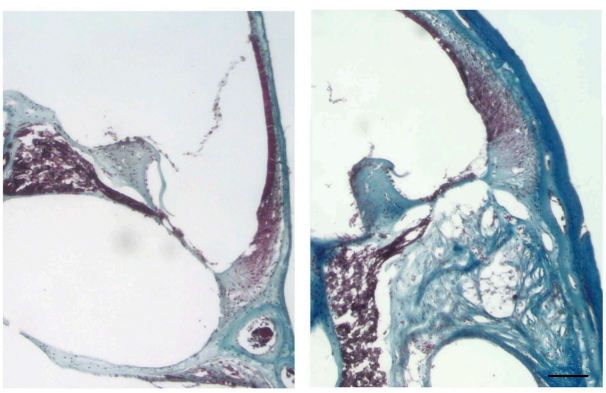

FIGURE 12 | Masson's trichrome staining for the presence of scar tissue. Representative micrographs of section of a contralateral non-implanted cochleae on the left, section of implanted cochlea on the right. Collagen fibers stained blue, nuclei stained black and cytoplasm and erythrocytes stained red. Images of different areas were acquired and stitched together afterwards. Four contralateral and four implanted cochleae were used. Scale bar = 100 μm.

reducing injury to SGNs during and after implantation can improve hearing outcomes with electrical-acoustic stimulation (Mowry et al., 2012). However, CI surgery initiates a strong inflammatory response in the cochlea that can promote loss of SGNs and remaining auditory HCs that are crucial for hearing perception. In addition, the inflammatory response that is produced following cochlear implantation promotes fibrotic tissue deposition around the electrode array, which can also impair electrode impedance and post-implantation hearing outcomes (Hughes et al., 2001; Choi and Oghalai, 2005; Jia et al., 2011; Wolfe et al., 2013; Mosca et al., 2014). Removal of cochlear fibrosis is nearly impossible without causing significant trauma in the inner ear that can promote more inflammation and fibrotic deposition. In cases of CI device failure and need for explantation and re-implantation, intracochlear osteoneogenesis and excessive fibrosis make CI electrode insertion extremely challenging and sometimes impossible (Côté et al., 2007). Descriptions of the trauma caused by electrode insertion and associated fibrosis and new bone formation in human temporal bones can be found in the literature (Eshraghi et al., 2003; Fayad et al., 2009). The basal portion of the cochlea is most affected by EIT induced fibrosis and osteoneogenesis. The wound healing response that leads to fibrosis and new bone formation has not been well characterized following cochlear implantation. By understanding the pathophysiologic mechanisms of EIT and wound healing in the cochlea, different therapeutic strategies can be investigated to modulate fibrosis in the inner ear and prevent loss of SGNs and residual auditory HCs following cochlear implantation.

Wound healing in peripheral nerve demonstrates similarities to cutaneous tissues (Mutsaers et al., 1997; Velnar et al., 2009; Stout, 2010; Ydens et al., 2012). In injured peripheral nerve, a strong inflammatory and proliferative response occurs at the site of injury and is followed by a late remodeling phase. SCs of injured nerve rapidly release a number of pro-inflammatory cytokines, chemokines, and cell adhesion molecules that promote recruitment of inflammatory cells to the wounded area (Shamash et al., 2002; Tofaris et al., 2002). Monocytes are recruited to the injured nerve, where they transform into M1 and M2 macrophages. M1 macrophages have debride bacteria, damaged tissues, and cellular debris and release reactive radicals and pro-inflammatory cytokines (such as TNF-α and IL-1β) that are toxic to surrounding tissue during the acute and chronic inflammation. M2 macrophages secrete anti-inflammatory cytokines and factors important for nerve regeneration and tissue remodeling (Martinez et al., 2008; Stout, 2010). Both resident activated macrophages and SCs contribute to myelin down-regulation and phagocytosis (Romand and Romand, 1990). Phagocytosis, however, can be a double-edged sword in peripheral nerve injury. Although removal of apoptotic cells and debris is thought to be part of a homeostatic mechanism to prevent or delay cell death signals from spreading, phagocytosis of myelin and damaged SGNs can adversely affect the propagation of action potentials critical to auditory processing (Hurley et al., 2007).

Peripheral nerve injury can also lead to inflammation and injury more proximally. Similar to macrophages (Stout, 2010) and microglia from the central nervous system, astrocytes (the most abundant macroglial cell of the central nervous system) can exhibit polarized phenotypes induced by either classical or alternative activation in response to peripheral nerve injury (Reichert et al., 1994; de Waele et al., 1996). Reactive astrocytes have been found within the cochlear nucleus following a labyrinthectomy (Jang et al., 2013).

These classical activated macro-glia cells show neurotoxic effects and express *Il1b, Inos, Tnfa,* and *Cxcl10* genes. M1 macrophages and SCs of the classical pathway express pro-inflammatory cytokines such as IL-1β and M2 macrophages and astrocytes (activated with IL-4 and IL-13) of the alternative pathway express other regenerative and anti-apoptotic factors such as *Il10, Arg1, Mrc1, Il1ra, Fizz1*, and *Ym1*. (Perry and Gordon, 1988; Haack and Hynes, 2001; Vogelezang et al., 2001; Martinez et al., 2008; Stout, 2010; Ydens et al., 2012).

Subsequently, a proliferative phase predominates after the initial inflammatory phase of wound healing in nerve injury. Fibroblasts and activated macrophages produce large amounts of matrix proteins (fibrins and collagens) creating an extracellular matrix (ECM) that acts as a network where cell-cell (through cadherins and different cell adhesion molecules) and cell-matrix (through integrins) interactions promote migration, growth, and cellular differentiation (e.g., myofibroblast formation) (de Waele et al., 1996; Stout, 2010; Ydens et al., 2012). Fibronectin (i.e., a component of the ECM) is rapidly upregulated upon injury to peripheral nerves either by fibroblasts or endothelial cells. De-differentiated SCs in injured nerve express integrin α4β1, a receptor protein that binds fibronectin and promotes axonal regeneration and SC proliferation (Jessen and Mirsky, 1999, 2002; Haack and Hynes, 2001; Vogelezang et al., 2001; Gardiner, 2011). Although integrin α4β plays a role in nerve regeneration, the expression of α4 integrin can also mediate influx of immune cells to the injured nerve through VCAM1 binding, propagating the inflammatory response (Rose et al., 2002). After proliferation, remodeling occurs at the injured nerve, inflammation and angiogenesis declines, and scarring matures.

The wound healing process that occurs in the cochlea following EIT parallels descriptions in peripheral nerve injury. A robust inflammatory response occurs following EIT. Injured cochlear tissues express a number of pro-inflammatory factors and chemoattractants such as chemokine *Ccl2*, cell adhesion molecules *Sele* and *Vcam1*, and growth factor *Tgfb1* 3 days after EIT *in vitro* (**Figure 3**). Cultured monocytes migrate to the site of cochlear injury and express other pro-inflammatory cytokines, chemokines, and factors at 3 days *in vitro* (*Ccl2, Sell, Icam1,* and *Tgfb1*; **Figures 1–3**). In our hands, DXM did not affect the number of interactions between leukocytes and leukocyte-tissue cells, however a decrease in the duration of these interactions was observed. Similarly, other authors (Mancuso et al., 1995; Tailor et al., 1997) have already reported that DXM affects selectively the leucocyte emigration process, but not the rolling or adhesion processes in response to chemoattractants onto the microvascular tissues of the rat mesentery. Therefore, a reduction in the time of interaction may affect the leukocytes trans-endothelial emigration. Particularly in the OC, spiral ganglia, and site of EIT *in vivo*, monocytes transform into M1 macrophages that secrete other inflammatory factors (such as *IL-1ß)* and M2 macrophages that express regenerative and anti-apoptotic factors (such as *Arg1*; **Figures 6–8**). Increased *Arg1* expression in the spiral ganglia may in part be due to infiltrating M2 or SCs activated by the alternative pathway, promoting nerve regeneration (Reichert et al., 1994; Martinez et al., 2008; Stout, 2010; Ydens et al., 2012; Jang et al., 2013). The lateral wall, OC, spiral ganglia, and site of EIT all express integrin 4α (receptors for fibronectin important nerve regeneration and cell-matrix interactions) during the early wound healing process (**Figures 4**, **5**) that persists in the OC, spiral ganglia, and wound bed 30 days after implantation *in vivo*. Fibroblasts and differentiated myofibroblasts proliferate in the cochlea after EIT injury and new collagen is deposited in the wound bed around the electrode analog *in vivo* (**Figure 9**) (Van De Water et al., 2013). F-actin was up regulated in the area of a monofilament insertion in mouse cochleae *in vitro*, suggesting there is reorganization of the actin skeleton and remodeling of the ECM (**Figures 4**, **6**). Scar forms and matures around the electrode array in the cochlea during the remodeling phase of wound healing (**Figure 12**). Macrophage activity still persists at high levels in the wound bed (site of EIT) at 30 days *in vivo*, suggesting an atypical chronic inflammatory response and a possible immune response against a foreign body (the electrode analog).

Increased gene expression for leukocyte chemoattractants (*Ccl3* and *Ccl12*), pro-angiogenesis factors (*Vegfa, Hgf*), integrin subunits important for collagen deposition (*Itga1, Itga2, Itga3),* enzymes responsible for ECM remodeling (*Mmp2, Mmp3, Mmp13, Mmp14, Plau, Serpine1, Serpinh1, Timp1, Timp2*) (La Fleur et al., 1996), components of the ECM (*Acta2, Col1a2, Col3a1)*, and mediators of ECM cross-linking and interactions (*Lox, Thbs2*) were also demonstrated in cochlear tissue following EIT *in vivo*, supporting histologic findings of wound healing in the cochlea (**Table 1**). *Tgfb3* and its receptor *Tgfbr2*, which are associated with scar-less wound healing, were down-regulated in EIT cochlear tissues, representing a possible therapeutic target for prevention of fibrotic scar in future studies.

In summary, cochlear implantation promotes a wound healing response in the cochlea characterized by an inflammatory phase (i.e., expression of pro-inflammatory cytokines, chemokines, and chemoattractants, leukocyte infiltration, and macrophage activation), a proliferative phase (i.e., angiogenesis, fibroblast and differentiated myofibroblast proliferation, collagen deposition, synthesis of ECM, and scar formation), and a remodeling phase (i.e., turnover of the ECM and maturation of the scar). How trauma and inflammation from EIT can initiate loss of auditory HCs and SGNs is well-described in the literature (Roehm and Hansen, 2005; Bas et al., 2012b). However, the molecular and cellular mechanisms involved in the proliferative and remodeling phases following EIT are not entirely known. The results of this study confirm key events in the inflammatory process following EIT and offer a detailed description of the proliferative and remodeling cascades of wound healing in the cochlea. A chronic inflammatory response characterized by macrophage activity is evidenced long after initial cochlear trauma and likely represents a foreign body reaction to the electrode analog (nylon filament) and a source of pro-inflammatory factors that can be detrimental to remaining HCs and viable SGNs long-term. Furthermore, fibrotic deposition in the cochlea following EIT was also confirmed and associated signaling cascades were depicted in this study. By understanding these pro-inflammatory, proliferative, and remodeling phases of wound healing in the cochlea following EIT, therapeutic strategies can be developed and tested to mitigate losses of auditory HCs and SGNs from a robust inflammatory response, reduce fibrosis that can interfere

with electrode impedance, and alter chronic inflammatory changes in the inner ear from a foreign body reaction in efforts to improve residual hearing preservation, electrical-acoustic hearing outcomes, and quality of life after cochlear implantation.

Compliance with Ethical Standards

Ethical approval: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the US National Institutes of Health guidelines and with the approval of the University of Miami Institutional Animal Care and Use Committee.

Author Contributions

SG participated in the analysis of the data from the *in vitro* and *in vivo* section, where she was blinded to the different groups. MA participated in the processing of the adult mice cochleae for histology. CD participated in the molecular genetic studies and helped to draft the manuscript. JB helped with the design and management of the project and the draft of the manuscript. TV and AE helped to draft the manuscript and provided important inputs during the data analysis. EB conceived of the study, and participated in its design and coordination and helped to draft the manuscript. 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: http://journal.frontiersin.org/article/10.3389/fncel.2015.00303

Supplemental Movie 1 | Cell tracking of leukocytes co-cultured with undamaged control group of organ of Corti and lateral wall tissues. Four cochlear tissue explants (i.e., organ of Corti and lateral wall tissues) were used, with a total of 3 independent replicates. Sequential images of each group were taken every 15 s for 20 min with a 20× lens in a Zeiss LSM 700/ confocal upright microscope. ImageJ with Trackmate plugin was used to make the movies. The detected cells are included in a magenta circle and the line color codes are in function of the velocity for that particular cell. A rainbow color code displays dark blue lines for slow moving cells and red for fast moving cells (3.2 μm/s). Scale bar = 100 μm.

Supplemental Movie 2 | Raw sequential images for Supplemental Movie 1.

Supplemental Movie 3 | Cell tracking of leukocytes co-cultured with EIT group of organ of Corti and lateral wall tissues. Four cochlear tissue explants were used, with a total of 3 independent replicates. Sequential images of each group were taken every 15 s for 20 min with a 20× lens in a Zeiss LSM 700/ confocal upright microscope. ImageJ with Trackmate plugin was used to make the movies. The detected cells are included in a magenta circle and the line color codes are in function of the velocity for that particular cell. A rainbow color code displays dark blue lines for slow moving cells and red for fast moving cells (3.2 μm/s). Scale bar = 100 μm.

Supplemental Movie 4 | Raw sequential images for Supplemental Movie 3.

Supplemental Movie 5 | Cell tracking of leukocytes co-cultured with EIT pre-treated with dexamethasone (20 μg/ml) group of organ of Corti and lateral wall tissues. Four cochlear tissue explants were used, with a total of 3 independent replicates. Sequential images of each group were taken every 15 s for 20 min with a 20× lens in a Zeiss LSM 700/ confocal upright microscope. ImageJ with Trackmate plugin was used to make the movies. The detected cells are included in a magenta circle and the line color codes are in function of the velocity for that particular cell. A rainbow color code displays dark blue lines for slow moving cells and red for fast moving cells (3.2 μm/s). Scale bar = 100 μm.

Supplemental Movie 6 | Raw sequential images for Supplemental Movie 5.

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