IDENTIFICATION OF MULTIPLE TARGETS IN THE FIGHT AGAINST ALZHEIMER'S DISEASE

EDITED BY: Patrizia Giannoni, Silvia Fossati, Sylvie Claeysen and Elena Marcello

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IDENTIFICATION OF MULTIPLE TARGETS IN THE FIGHT AGAINST ALZHEIMER'S DISEASE

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EDITORIAL
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Editorial: Identification of Multiple Targets in the Fight Against Alzheimer's Disease

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Editorial on the Research Topic

Identification of Multiple Targets in the Fight Against Alzheimer's Disease

INTRODUCTION

This Research Topic is a collection of 20 articles that depict a broad representation of the most impactful advances in Alzheimer's disease (AD) comprehension and therapeutic openings. As it clearly emerges from recent literature, AD is a complex pathology with many different phenotypes and heterogeneous clinical settings. Although in a minority of cases genetic mutations have been linked to its development, for the vast majority of AD patients the triggering event remains to be elucidated. Main hallmarks such as amyloid beta (Aβ) plaques and neurofibrillary tangles have been identified, but understanding their exact role on cognitive consequences, timing of appearance, mechanisms of toxicity, and interplay required years of studies, with many questions still remaining unanswered. To note, the events possibly driving the development of such pathological signs are diverse and much more numerous than expected. For this reason, scientists have been directing their efforts to improving the understanding of the pathways involved in the toxicity mechanisms observed. Indeed, only a profound knowledge of the full process bringing to the different pathological phenotypes will help in figuring out effective treatments and/or preventive actions. With this concept in mind we have developed the present topic, which aims at giving a far-reaching picture of our actual knowledge, from the etiology of AD to mechanistic insights and possible new targets of intervention. Authors present their latest discoveries on a variety of breakthrough AD-related subjects such as inflammation, microbiome, hormones, Aβ production and catabolism, and neurovascular unit (NVU) alterations. Notably, the importance of reliable biomarkers as well as the paramount role of global approaches for the treatment/prevention of AD (see multi-interventions), are granted and finely discussed. We present here a summary of the main fields covered by this collection that we believe will constitute critical hints for future research development.

APP METABOLISM

According to the amyloid hypothesis, the increase in brain Aβ levels is a central event in AD pathogenesis. The soluble Aβ oligomers trigger synaptic dysfunction and, thereby, early cognitive deficits. Synaptic failure can occur also in the olfactory bulb leading to olfactory deficits that can be detected in many AD patients. Li et al. revealed a critical mechanism underlying olfactory dysfunction in AD showing that Aβ deposition induces morphological and functional changes in

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the synapses of the olfactory processing network in APP/PS1 mice during aging.

Aβ results from the proteolysis of the Amyloid Precursor Protein (APP). β-secretase BACE1 and γ-secretase concerted action on APP releases Aβ peptide, while the α-secretase ADAM10 (A Disintegrin and metalloproteinase domain-containing protein 10) cleaves APP within Aβ sequence, thereby preventing its generation. Promoting the α-cleavage of APP not only precludes the formation of Aβ but also increases the release of the neuroprotective sAPPα fragment. This can be achieved by acitretin, an activator of the α-secretase ADAM10, as assessed by dos Santos Guilherme et al. in the 5XFAD mouse model and in humans.

Even though APP metabolism has been studied in detail, it is still a central question in AD. Haytural et al. highlighted the technical concerns related to a potentially non-specific western blotting band at the expected molecular weight of APP-derived fragments, which calls for precaution when analyzing proteins of this size in human brain tissue. García-González et al. emphasized the importance of the comprehension of APP processing since APP can be a common substrate for several proteases. Among them, MT-MMPs (Membrane-type Metalloproteinases) are at the crossroads of pathological events involving not only amyloidogenesis, but also neuroinflammation and synaptic failure, thus opening up new research perspectives in AD.

NEUROINFLAMMATION

Neuroinflammation is definitely a hot-topic and one of the most studied fields in AD and related dementias in recent years. As summarized in the review by Hemonnot et al., many aspects of inflammation are still not completely understood. Microglial cells, main players in inflammatory reactions, are complex and heterogeneous cells. Several activation states exist and a fine-tuned characterization of AD stages is needed in order to design effective preventive and/or curative microglial-targeting interventions. One main aspect that slows down our full understanding of neuroinflammation is represented by the numerous mechanisms that might contribute to its development and progression. Hemonnot et al. presented an analysis of recent GWAS studies and described major genes implicated in microglial cells regulation, such as Apolipoprotein E (APOE) and TREM2 (triggering receptor expressed on myeloid cells 2). Finally, the authors proposed the purinergic signaling as one possible target of intervention for microglial state modulation. Another potential pharmacological target was evidenced by Adorni et al., that highlighted the role of proprotein convertase subtilisin/kexin type 9 (PCSK9) not only in cholesterol homeostasis, but also in neuroinflammation. Finally, Cerovic et al. evidenced in their review the strong link between gut microbiota (GM) and neuroinflammation development and exacerbation. The limitations of experimental protocols constitute a significant obstacle to which researchers are trying to find innovative solutions. Indeed, laboratory models only partially reproduce the complex reality of human AD inflammation. This is true for the microbiome status of animals living in animal facilities, which is far from the "real life" status, transgenic models (Hemonnot et al.) that focus in most cases on specific and limited AD features, and many other constraints, such as the fact that marked immunological differences exist between animals and humans as well as between genders. All these aspects need to be addressed and investigated to globally understand the role of neuroinflammation in AD and develop selective strategies.

HORMONES

Sex hormonal variations are a strongly debated risk factor for AD development and, as pointed out recently, they might interplay with other hormones in a refined modulation of neurocognitive functions. Different models have been proposed to study the peri-menopause influence on AD development. Marongiu suggested the accelerated ovarian failure as an optimal model to mimic the human process. This model could indeed be used to understand the molecular pathways implicated in sex hormone-related changes increasing AD risk. Rahman et al. underlined in their review the remarkable variety of pathophysiological conditions that are directly regulated by estrogen or that could be related to an alteration of its levels, like cardiovascular diseases (CVD). In this case, the increased risk of CVD is linked to an altered level of cholesterol, which is frequently observed in association with menopause. Indeed, estrogen seems to play a role in a variety of pathologies going from diabetes to depression, including infections and chronic inflammation. An aspect that should be particularly well-evaluated is the timing of a possible intervention in order to re-establish a hormonal balance. Evidences coming from recent studies are discussed and conclusions are pointing to the need of clinical trials and research investigations considering gender differences and leading to precision medicine approaches capable of analyzing the complexity of a patient clinical history. One of the gender differences that has been investigated concerns the consequences of chronic stress, which could be impacted by the activity of gonadal hormones (Rahman et al.). Indeed, AD has been proposed as a stress-related disorder by Canet et al.. In their up-to-date review, the authors evidenced how a dysregulation of the hypothalamic-pituitary-adrenal axis (HPA axis) has been observed in AD patients and could therefore represent a target for intervention. Molecules acting on the HPA axis hormones and/or receptors are already in clinical trials for other pathologies and the research for AD treatments could take advantage of such information.

NEUROVASCULAR UNIT

The neurovascular unit (NVU) is a functional brain unit composed my multiple cell types, including endothelial cells, smooth muscle cells, pericytes, and glial cells. The NVU is

responsible for maintaining brain homeostasis by regulating permeability of the blood brain barrier (BBB), as well as clearance of unwanted products (such as Aβ) from the brain. Giannoni et al. emphasized in their review how the regulation of BBB permeability is of outmost importance for brain health, and its deregulation is associated with AD and related vascular dementias through multiple mechanisms, including auto-antibody responses and altered gut microbiome. The manuscript by Nizari et al. evaluated the effect of the loss of cholinergic innervation of components of the NVU in hippocampus and cortex, suggesting that cortical arteries are more affected by cholinergic denervation than hippocampal arteries, and pointing to a differential regulation of neurovascular responses in different brain areas. In regard to clearance mechanisms, Aldea et al. proposed through sophisticated mathematical modeling that localized contraction of smooth muscle cells generates the force that drives intramural periarterial drainage of Aβ and other soluble metabolites in the brain.

Circulating blood cells and vesicles may also contribute to BBB function and neurovascular regulation. Interestingly, Espinosa-Parrilla et al. reviewed the link between platelet aging and multiple neurodegenerative diseases including AD, discussing the role of platelets as drivers of protein dysfunctions, and the potential clinical significance of platelets and related miRNAs as peripheral biomarkers of neurodegenerative diseases.

MULTI-INTERVENTIONS

Norwitz et al. highlight how AD might be the result of different dysregulations that interplay with each other in a complex and heterogenous matrix. In particular, they focus on the Wnt-signaling, α-synuclein and diabetes hypothesis, that might all contribute at different points and in different ways to the toxicity of the major AD hallmarks, Aβ and tau. In this context, AD therapies engaging simultaneously several molecular actors of the disease might achieve better efficacy in clinical trials. This multiplicity of action can be obtained by multi-target-directed ligands (MTDLs). In the present topic, Cuadrado-Tejedor et al. presented the compound CM-695, a dual inhibitor of histone deacetylase 6 (HDAC6) and phosphodiesterase 9 (PDE9). This MTDL demonstrated a therapeutic effect upon chronic administration to Tg2576 mice. In the same line, Hatat et al. developed an innovative molecule with triple activity of potential therapeutic interest against AD: –activation of serotonin type 4 (5-HT_4) receptors, –inhibition of 5-HT_6 receptors and –inhibition of acetylcholinesterase (AChE). Acute administration of this compound to mice prevented the memory deficits induced by scopolamine. Pleiotropic synergistic effects can also be demonstrated with molecules targeting α-secretase activity, such as acitretin. Indeed, dos Santos Guilherme et al. demonstrated that this molecule increased cerebrospinal fluid levels of interleukin 6 (IL6). Whether this effect is a direct effect of the molecule on an unknown target or is a consequence of retinoid control on inflammation has to be further investigated.

BIOMARKERS

Facing the complexity and the heterogeneity of AD pathology, early, reliable, and easy to access biomarkers will be fundamental for effective therapies. Combining the current knowledge and routinely used biomarkers, such as Aβ and tau quantification and imaging, Younes et al. identified changepoints of these biomarkers far before the clinical symptoms. The future goal would be being able to point out individuals likely to progress to AD as they age. New biomarkers could help to develop an accurate prediction. One strategy could be to implement biomarkers tracking cellular metabolism such as nicotinamide adenine dinucleotide (NAD+). In this Research Topic, Grant et al. presented the fate of NAD+ and its metabolites following an intravenous infusion of NAD+ in a human cohort. As gut microbiota composition seems to be altered in AD patients and to have an impact on amyloid pathology (Cerovic et al.), another strategy to identify innovative biomarkers could be to follow GM composition. Would the huge amount of data collected by metagenomic analysis of bacterial taxa abundancy be informative to point out predictive, stratifying or prognostic biomarkers? This remains to be demonstrated. The success might come from the quantification of bacteria-derived metabolites such as bile acids or short chain fatty acids (SCFA).

CONCLUDING REMARKS

This series of articles is representative of the complexity of AD pathology. It clearly underlines the importance of transdisciplinary studies that are linking multiple aspects of the pathology or, as pointed out in many reviews, of different subtypes of AD etiology and development. The sharing of pathological features with other diseases, although complex to analyze, may also open numerous treatment possibilities. For exemple, Deering Brose et al. suggest the use of hydroxyurea, an FDA-approved ribonucleotide reductase inhibitor currently prescribed to treat cancer, to ameliorate the cognitive deficits in AD. The use of repurposed drugs can notably accelerate clinical trials bringing to novel therapeutics through a relatively rapid protocol. The example of hydroxyurea, which is tested in a down syndrome mouse model but suggested for a broad range of neurodegenerative diseases (Deering Brose et al.), highlights how future drugs may be able to target pathways shared by multiple pathologies.

AUTHOR CONTRIBUTIONS

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

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ORIGINAL RESEARCH
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Cerebrovascular Smooth Muscle Cells as the Drivers of Intramural Periarterial Drainage of the Brain

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The human brain is the organ with the highest metabolic activity but it lacks a traditional lymphatic system responsible for clearing waste products. We have demonstrated that the basement membranes of cerebral capillaries and arteries represent the lymphatic pathways of the brain along which intramural periarterial drainage (IPAD) of soluble metabolites occurs. Failure of IPAD could explain the vascular deposition of the amyloid-beta protein as cerebral amyloid angiopathy (CAA), which is a key pathological feature of Alzheimer's disease. The underlying mechanisms of IPAD, including its motive force, have not been clarified, delaying successful therapies for CAA. Although arterial pulsations from the heart were initially considered to be the motive force for IPAD, they are not strong enough for efficient IPAD. This study aims to unravel the driving force for IPAD, by shifting the perspective of a heart-driven clearance of soluble metabolites from the brain to an intrinsic mechanism of cerebral arteries (e.g., vasomotion-driven IPAD). We test the hypothesis that the cerebrovascular smooth muscle cells, whose cycles of contraction and relaxation generate vasomotion, are the drivers of IPAD. A novel multiscale model of arteries, in which we treat the basement membrane as a fluid-filled poroelastic medium deformed by the contractile cerebrovascular smooth muscle cells, is used to test the hypothesis. The vasomotion-induced intramural flow rates suggest that vasomotion-driven IPAD is the only mechanism postulated to date capable of explaining the available experimental observations. The cerebrovascular smooth muscle cells could represent valuable drug targets for prevention and early interventions in CAA.

Keywords: lymphatic, brain, vasomotion, multi-scale model, poroelastic, Alzheimer's disease, cerebral amyloid angiopathy, perivascular drainage

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1. INTRODUCTION

The brain lacks a conventional lymphatic system, which in the rest of the body is responsible for removing waste products and excess fluid (Aspelund et al., 2015; Louveau et al., 2015; Bakker et al., 2016). Of high interest is the clearance of soluble amyloid-beta (Aβ) proteins that are released by neurons into the surrounding extracellular spaces (i.e., interstitium) following normal synaptic activity (Tarasoff-Conway et al., 2015). Inefficient removal of Aβ from the brain leads to parenchymal amyloid plaques and cerebral amyloid angiopathy (CAA), commonly seen in the

commonest form of dementia, Alzheimer's disease (AD) (Selkoe, 2001; Ross and Poirier, 2005; Cupino and Zabel, 2014). CAA describes the accumulation of Aβ initially within the basement membranes (BMs) of vascular smooth muscle cells (VSMCs) of cortical and leptomeningeal arteries, but also within the BM of cortical capillaries (with or without recruitment of other vessels). Although CAA has been almost invariably reported in AD, the exact causes of the onset of CAA remain unclear (Charidimou et al., 2012). The hemorrhages and ischemic lesions associated with CAA result in cognitive impairment and dementia (Attems et al., 2011; Reijmer et al., 2015). Currently, 47 million people worldwide suffer from dementia and there is no effective curative or preventive intervention. Aging highly increases the risk for CAA and dementia and, considering increased longevity, the occurrence of dementia by 2050 is evaluated to 131 million people (Prince et al., 2016). This increases the urgency to explore potential unconventional clearance mechanisms for Aβ that may contribute toward maintaining the homeostasis of the brain (Tarasoff-Conway et al., 2015; Bakker et al., 2016).

Aβ produced by neurons is degraded by enzymes (Farris et al., 2003; Marr et al., 2003), transported into the blood via lipoprotein receptor related protein (LRP)-1 (Deane et al., 2008) or cleared along the walls of capillaries and arteries (Hawkes et al., 2014). The Aβ transport along the wall of arteries, generally termed perivascular drainage, has been a subject of considerable controversy over the last few years. The pathways for the elimination of interstitial fluid (ISF) and solutes (including Aβ) from the brain parenchyma have recently been reviewed by Abbott et al. (2018) and Hladky and Barrand (2018). Early experiments by Szentistvanyi et al. (1984) showed that the major pathway by which radioactive solutes drain from the brain to cervical lymph nodes is along the walls of cerebral arteries in the direction counter to the blood flow. This pathway was later located in the BMs interposed between the VSMCs from the tunica media of cerebral arteries (Carare et al., 2008), i.e., the Intramural Peri-Arterial Drainage (IPAD) pathways, effectively the lymphatic drainage routes of the brain (Carare et al., 2014; Morris et al., 2014). An alternative viewpoint has also been postulated which states that, following influx of cerebrospinal fluid (CSF) and solutes from the subarachnoid space into the brain parenchyma, the CSF-ISF mixture is cleared along the walls of intracortical veins, back into CSF (Iliff et al., 2012). However, apart from the original paper, there is little support for this para-venous clearance mechanism. Other studies that injected soluble tracers intracerebrally (Carare et al., 2008; Arbel-Ornath et al., 2013) or into the CSF (Albargothy et al., 2018) have found no evidence of tracer presence along the intracerebral para-venous pathways, but rather along the walls of cerebral arteries, specifically within the IPAD pathways.

Here, we are concerned with shedding light on the mechanisms responsible for transport of ISF and Aβ along the IPAD pathways. The IPAD pathways were mapped by Carare et al. (2008) based on the following observations: soluble tracers injected into the brain parenchyma (i) rapidly enter the BM of capillaries and (ii) are progressively observed in BM in the tunica media of intracerebral arterioles and arteries, and later on, in the walls of leptomeningeal arteries (see **Figure 1**). Extensive work has been done for assessing the transport of solutes out of the brain along the IPAD pathways during aging (Hawkes et al., 2011) and under various physiological and pathological conditions, such as the presence of CAA (Arbel-Ornath et al., 2013; Hawkes et al., 2014), possession of APOE4 (Zekonyte et al., 2016), consumption of a high-fat diet (Hawkes et al., 2015) and after ischemic stroke (Arbel-Ornath et al., 2013). All those experiments showed that soluble tracers injected into the brain interstitium reach the BM of intracerebral and leptomeningeal arteries and their distribution within the arterial wall resembles the pathological deposition of Aβ in CAA (Weller et al., 1998; Preston et al., 2003). These findings indicate that soluble Aβ can be removed from the brain tissue along the IPAD pathways, in the opposite direction to arterial pulsations, and that failure of this clearance mechanism results in the vascular deposition of Aβ as CAA.

Improvement of IPAD holds great promise for treatment of CAA. However, significant advancement is unlikely until the underlying mechanisms of IPAD, including the motive force, are elucidated. The observation that no periarterial drainage occurs following cardiac arrest suggested that arterial pulsations, derived from the heartbeat, might drive IPAD in the brain (Carare et al., 2008). Driving net intramural periarterial flow in the opposite direction to the arterial pulse, as shown in **Figure 1**, appears to be physically difficult. Several mechanisms that could allow periarterial clearance to occur in the reverse direction to arterial pulsations have been mathematically modeled, all of them assuming the arterial pulse to be the motive force for IPAD (Schley et al., 2006; Coloma et al., 2016; Sharp et al., 2016; Diem et al., 2017). Previous suggestions included some degree of attachment between solutes and BM (Schley et al., 2006), different flexible structures within the BM (Sharp et al., 2016) and a valve mechanism (Diem et al., 2017), that would be needed to generate greater resistance to forward periarterial flow than to retrograde periarterial flow (against the direction of arterial pulse). The presence of flexible and valve-like structures within the wall of cerebral arteries still awaits experimental confirmation. More importantly, Diem et al. (2017) have shown that arterial pulsations are incapable of driving intramural fluid flow rates out of the brain of physiological significance, even with valve-like structures within the BM. The very long wavelength of the arterial pulse is inadequate to induce pressure gradients large enough to generate periarterial flow rates comparable with experimental observations (Carare et al., 2008; Arbel-Ornath et al., 2013). Forces other than cardiac pulsations must therefore drive IPAD in the brain and the quest for other candidates is still open.

With this in mind, we propose that the forces generated by cerebral VSMCs can drive IPAD in the brain by acting upon the deformable BM. The VSMCs are contractile cells embedded within the arterial wall and, under physiological conditions, generate a basal vascular tone that is maintained by a combination of various stimuli (e.g., arterial pressure, shear stress, neuronal metabolic activity and several types of innervation) (Cipolla, 2009). Deviations from the basal vascular tone result in significant variations in the diameter of arteries. The evoked vasomotor response is able to spread along the

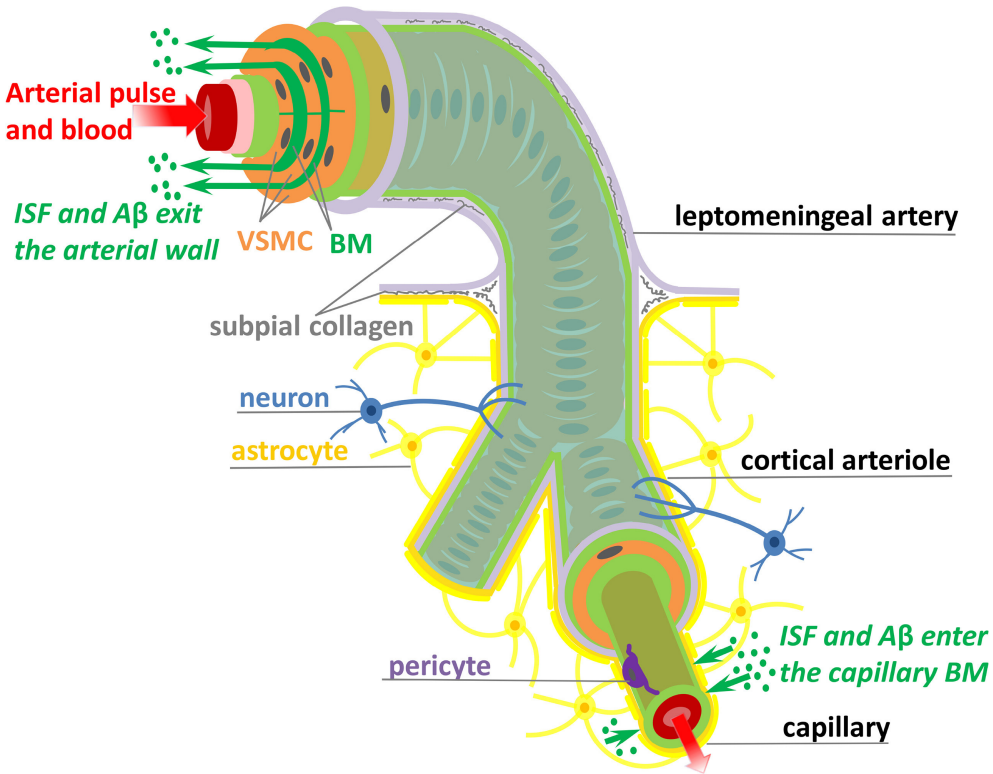

FIGURE 1 | Schematic representation (not to scale) of the IPAD pathways. Soluble Aβ (green dots) and ISF from the brain interstitium enter the BM of capillaries and flow upstream toward large arteries through the BM (dark green) positioned between VSMCs (orange). Transport along the IPAD pathways is shown by the green arrows which are against the direction of arterial pulse and blood flow (red arrow). Three concentric layers of VSMCs are pictured at the larger extreme of the artery, while, for the sake of simplicity, only the outer most layer of VSMCs is shown along the length of the artery. The artery is wrapped in a pial sheath (light purple) and innervated by neurons (blue). At the capillary level, the endothelial BM (light green) is fused with the BM of glia limitans (dark yellow) secreted by astrocytes (yellow); they also interact with brain interstitium (due to gap junctions between the end-feet of astrocytes), allowing entrance of ISF and Aβ in the vascular wall (Morris et al., 2016; Weller et al., 2018). VSMC, vascular smooth muscle cell; BM, basement membrane; endothelium (red), internal elastic lamina (pink), inner and outer BM (light green), pericyte (purple), and subpial collagen (gray).

artery length, as well as across branch points (Welsh et al., 2018). The conducted vasomotor response may be induced by vasoactive drugs or it may occur spontaneously. The spontaneous rhythmic oscillations of vascular tone are known as vasomotion (Nilsson and Aalkjær, 2003; Aalkjær et al., 2011). The initial, local contractions of the VSMCs are propagated over macroscopic distances as a contraction wave that is linked to the calcium waves mediated via intercellular gap-junctions (Seppey et al., 2009; Pradhan and Chakravarthy, 2011). Vasomotion is independent of pulse rate and respiration and has been observed in the vascular beds of numerous tissues, including the cerebral tissue (Fujii et al., 1990, 1991; Gokina et al., 1996; Mayhew et al., 1996; Obrig et al., 2000; Filosa et al., 2004; Vetri et al., 2007). Vasomotion has been previously modeled as a mechanism aiding the transport of blood (Carew and Pedley, 1997; Ursino et al., 1998) and oxygen (Goldman and Popel, 2001; Hapuarachchi et al., 2010) from blood vessels to various tissues in the body. Di Marco et al. (2015) reviewed the ways in which cerebral vasomotion may be hindered in AD and mentioned that vasomotion could act as a secondary input to the motive force of arterial pulse-driven IPAD. A systematic contribution of the cerebral VSMCs to the periarterial drainage of fluid out of the brain has not been previously inspected, neither experimentally nor by any modeling technique.

Proposed mechanism: vasomotion-driven IPAD. We propose the hypothesis of vasomotion-driven IPAD (denoted V-IPAD) and explain below the underlying mechanisms for this potential clearance process. The position of the contractile VSMCs within the artery wall appears to be ideal for allowing an immediate effect of their contractions on the conformation of the adjoining BM, which is a specialized sheet of extracellular matrix filled with interstitial fluid (see **Figure 2**). In other words, the BM is a soft fluid-filled poroelastic medium whose pores could be closed and reopened during VSMC contraction and relaxation, respectively. In this way, the contractions of two layers of VSMCs will squeeze the interposed BM, pushing fluid out of its pores in the direction of the contraction wave. In order to clear any soluble material from the brain interstitium via the IPAD pathways, the vasomotion wave must propagate from the intracerebral arterioles toward the large arteries on the surface of the brain. The V-IPAD mechanism bears some analogy to an old-fashioned mangle or a wringer which squeezes water

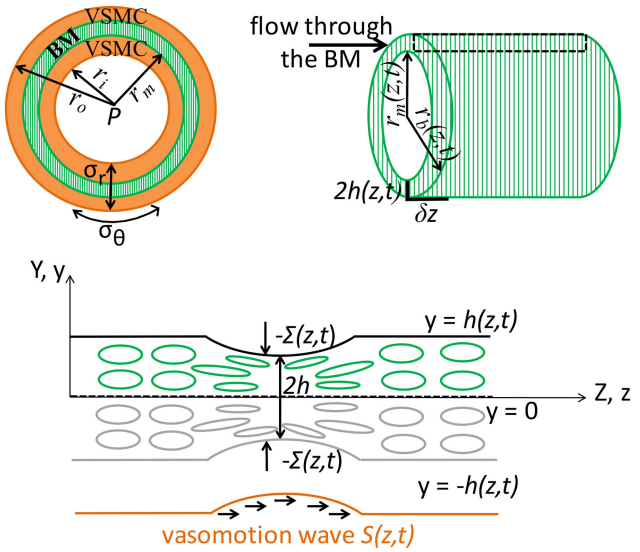

FIGURE 2 | Schematic representation (not to scale) of the V-IPAD model. A leptomeningeal artery is modeled as a long thick-walled cylinder with uniform material properties along its length, maintained at a constant intraluminal pressure P and constant longitudinal stretch and exposed to active contractions of VSMCs. The top layer shows the arterial cross section with a layer of BM (green compartment) embedded in the wall **(Left)** and the longitudinal section of the BM **(Right)**. For simplicity reasons, only one layer of BM is considered at the middle of the wall and the two layers of the VSMCs surrounding the BM are assumed to behave identically. The remaining wall components are not shown, but their effect on the wall elasticity is captured by the radial (σ_r) and circumferential stress (σ_θ). The deformed inner radius, middle radius and outer radius of the arterial wall are denoted by r_i, r_m, and r_o, respectively. The position of the inner layer bounding the BM is assumed at r_m and, owing to stress continuity across the wall, the radial stress σ_r at that point represents the external compressive stress Σ which acts on the BM, i.e., $\Sigma = \sigma_r(r = r_m)$; thereby, the arterial wall model is coupled with the BM model. Both r_m and Σ depend on the prescribed vasomotion wave $S(z, t)$ generated by the contractile VSMCs and are determined from the arterial wall model. The position of the outer layer bounding the BM is assumed at r_b with $r_b = r_m + 2h$, where $2h$ denotes the whole BM thickness. Since the BM thickness is significantly smaller than the arterial radius, its upper half is assumed to behave identically to its lower half. The deformed thickness $h = h(z, t)$ of the upper-half BM is determined by solving the BM model in the Cartesian system (y, z) and this is justified by the relationship $r_b = r_m(z, t) + 2y$ where $2y \ll r_m$ and $2h \ll r_m$. The area delimited by the rectangular is illustrated in the bottom schematic: a sheet of BM modeled as a poroelastic compartment whose fluid-filled pores are squeezed during the VSMC contractions, under the direct actions of $-\Sigma(S(z, t))$, driving the fluid out of the BM pores in the direction of the vasomotion wave.

out of soft materials such as towels. Other parts of the human body, such as the digestive system, use wave-like muscular contractions to propel the content of a tube (e.g., food along the gastro-intestinal tract); this process is also known as peristaltic pumping. We note that the nomenclature of "peristalsis" has also been used in modeling studies of perivascular drainage driven by the heart-derived arterial pulse (Bilston et al., 2003; Wang and Olbricht, 2011; Sharp et al., 2016), rather than by the VSMCs. However, the vasomotion wave has significantly different properties than the arterial pulse. For example, vasomotion has a wavelength of several millimeters, which is at least two orders of magnitude lower than that of arterial pulsations. In addition, the arterial pulse has an approximate frequency of 1 Hz, while the vasomotion frequency, although varies with tissue type and species, is commonly taken to be 0.1 Hz (Nilsson and Aalkjær, 2003; Aalkjær et al., 2011). Hence, comparison between the current work and previous studies should be made with caution because both the arterial pulse and the vasomotion wave are commonly modeled as sinusoidal waves.

Here, we test the role of cerebral vasomotion in the clearance of fluid from the brain by developing a novel physiologically-based multiscale model of a middle cerebral artery (MCA); this model is denoted the V-IPAD model and presented in section 2.1 (see details in Aldea, 2017). The V-IPAD model couples two models: (i) the arterial wall model which captures the biomechanics of an active elastic cerebral artery and (ii) the BM model which yields the fluid flow rates, along the IPAD pathways, generated by the activation of the VSMCs. We emphasize that this is the first model treating the BM as a fluid-filled poroelastic medium, rather than just a fluid-filled channel (Schley et al., 2006; Coloma et al., 2016) or a fluid-filled porous medium (Wang and Olbricht, 2011; Diem et al., 2017).

The poroelastic BM is a biphasic material composed of: (i) a porous and elastic solid matrix (e.g., the extracellular matrix of BM proteins) and (ii) a fluid component (e.g., the ISF) that occupies the connected pores, an example being a water filled bath sponge. Where the solid matrix deforms in response to an external load it transmits force (in the form of a pressure) to the fluid filling the pores; this subsequently leads to changes in the permeability of the solid matrix to fluid flow as, for example, the pores close up. As it will be shown, the elastic deformations of the poroelastic BM, induced by the contractile VSMCs, are critical in assuring high net fluid flow rates along the IPAD pathways, without the need of any intramural valves; such a behavior could not be obtained in a purely porous, underformable material. Models of poroelasticity have been widely applied to biological materials in applications ranging from fluid movement in bone (Cowin, 1999), to tumor growth (Roose et al., 2007) and biomechanics of brain tissue (Goriely et al., 2015).

The mathematical details of the V-IPAD model are given in section 2. The simulation results are presented in section 3, followed by their discussion in section 4. We have written the paper such that the sections 3 and 4 can be read prior to section 2. In section 4, we also discuss suitable animal models (e.g., hyperhomocystaeinaemia (HHcy) mouse models) for testing experimentally the V-IPAD hypothesis. In **Supplementary Material 1**, we present in detail the arterial wall model which follows popular hyperelastic arterial models from the literature (Rachev and Hayashi, 1999; Kalita and Schaefer, 2008), while in **Supplementary Material 2**, we explain the numerical implementation of the BM-model. Finally, our pilot experimental data from the HHcy animal models are shown in **Supplementary Material 3**.

2. MATERIALS AND METHODS

In this section, we first describe the governing equations of the V-IPAD model and then explain their physiological significance. The V-IPAD model consists of two coupled models: (i) the BM model for the vasomotion-induced intramural periarterial flows through the deformable poroelastic BM, coupled to (ii) the arterial wall model for the elastic response of a rat MCA. We recall that the latter model is presented in detail in **Supplementary Material 1**, so below we focus on the novel BM model. We also present a concise formulation of the BM model that is solved numerically in **Supplementary Material 2**. The full derivation of the BM model can be found in Aldea (2017). The parameters used in the V-IPAD model are given in **Table 1**.

2.1. Lubrication Model of the Poroelastic BM

The aim of this model is to quantify the amount of fluid eliminated from the brain tissue along the intramural vascular BM as a consequence of muscular contractions of cerebral arteries. The intramural vascular BM is modeled as a slowly varying sheet of width $2h$, running through the wall of a cylindrical vessel which is itself centered along the z-axis, undergoing axisymmetric deformation (i.e., no θ-dependence), as illustrated in **Figure 2**. On its top and bottom boundaries (both assumed impermeable), the BM is exposed to compressive stresses dependent on the contractile activity of the VSMCs. Given its anatomical properties (e.g., a thin, extracellular matrix of fibrous proteins), the BM is treated as deformable spongy material filled with interstitial fluid. More specifically, the BM is modeled as a fluid-filled poroelastic medium comprised of a porous solid phase (the matrix of proteins) denoted by the superscript "s" and a fluid phase (interstitial fluid) denoted by the superscript "f." The pores in the solid matrix provide a path for the movement of fluid. Since the BM thickness ($\approx 0.4\ \mu$m) is significantly smaller than the arterial radius ($\approx 100\ \mu$m), we assume that its upper half behaves identically to its lower half. For visual purposes, the following notation is adopted: $2H$ is the undeformed thickness of the BM and $2h$ is the deformed thickness of the BM.

The poroelastic BM is a compressible elastic medium subjected to deformations in response to an external compressive stress and to changes in fluid pressure in the pores of the matrix. Specifically, the external compressive stress, denoted Σ, is a known input function of time and position, i.e., $\Sigma = \Sigma(z,t)$; it depends on the contractile oscillations of the VSMCs and its value is previously determined from the elastic analysis of an active cerebral artery, rather than just being a prescribed function from the literature (see **Supplementary Material 1** and Aldea, 2017). $\Sigma(z,t)$ affects the fluid flow through the BM by inducing deformations of its boundaries. Thus, the system depends on time only through the boundary conditions. Accounting for the symmetry of the system, as illustrated in **Figure 2**, it suffices to solve the BM model in the upper half plane in order to determine the deformed BM thickness (Aldea, 2017).

2.1.1. Governing Equations

In general, solving a three-dimensional poroelastic model results in a highly complex system of equations. However, by exploiting the disparity between the length scales of the contracting arterial wall [BM thickness ($0.4\ \mu$m) $\ll$ arterial radius ($100\ \mu$m) $\ll$ wavelength of muscular contractions ($2000\ \mu$m)], we derive a simplified version of the BM model by using a lubrication approximation (see details in Aldea, 2017). Thereby, it is conceivable to assume that the variations in the radial direction of the BM are weak and the variables of the system, at leading order, have only one spatial dependence, e.g., the axial z-dependence. This also makes it sensible to assess the BM model in a two-dimensional Cartesian system (y,z), where the Cartesian y-coordinate is related to the cylindrical r-coordinated by the

TABLE 1 | Dimensional parameters.

Parameter	Value	Unit	Description	References
H	0.20	μm	Thickness of upper-half BM	
L_a	2,000	μm	Artery length	Bell et al., 2013
L_S	10	mm	Computational system length	
ϕ^s_*	0.25	1	Solid volume fraction	Candiello et al., 2010
ϕ^f_*	0.75	1	Fluid volume fraction	Candiello et al., 2010
μ_S	3,700	Pa	Lamé parameter "lymphatic"	Heppell et al., 2013
λ_S	8,600	Pa	Lamé parameter "lymphatic"	Heppell et al., 2013
k_*	10^{-2}	μm^2	BM permeability	Heppell et al., 2013
κ	4/3	1	Parameter	Wirth et al., 2010
η	10^{-3}	$Pa \cdot s$	ISF viscosity	Syková and Nicholson, 2008
S_m	10^5	Pa	Maximum vascular tone	Rachev and Hayashi, 1999
A	1	1	Amplitude activation wave	
λ_w	2,000	μm	Vasomotion wavelength	$c_w \cdot T$
T	10	s	Vasomotion period	Aalkjær et al., 2011
c_w	200	μm $\cdot$ s^{-1}	Average wave speed	Duling and Berne, 1970
				Dietrich et al., 1996; Seppey et al., 2009

relationship $r_b = r_m(z,t) + 2y$, where $2y \ll r_m$ and $2h \ll r_m$; here, the expressions $r = r_b(z,t)$ and $r = r_m(z,t)$ describe the radial position of the outer and inner layer bounding the BM, respectively, as illustrated in **Figure 2**.

In order to quantitatively determine the fluid flow rate $Q_{BM}(z,t)$ through the BM, as an effect of VSMC activity, we first need to solve the system described below in Equations (1–5) for the following five variables: the deformed thickness of the BM $h(z,t)$, the volume fraction of fluid $\phi^f(z,t)$, the fluid velocity in the z-direction $v_z^f(z,t)$, the pore pressure in the basement membrane $p(z,t)$ and the principal effective Cauchy stress $\sigma_y^e(z,t)$ (i.e., a measure of the force per unit area acting on a surface element in the deformed BM); t is time and z is the position along the z-axis. The full derivation of this lubrication model of the poroelastic BM is given in Aldea (2017). However, in section 2.1.2, we provide a more intuitive derivation of this model based on the physiology of the BM system. The governing equations are:

$$\frac{\partial(\phi^f h)}{\partial t} + \frac{\partial}{\partial z}(\phi^f v_z^f h) = 0, \tag{1}$$

$$-\frac{\partial \phi^f}{\partial t} + (1-\phi^f)\frac{1}{h}\frac{\partial h}{\partial t} = 0, \tag{2}$$

$$\phi^f v_z^f = -\frac{k(h)}{\eta}\frac{\partial p}{\partial z}, \tag{3}$$

$$\sigma_y^e - p = \Sigma(z,t), \tag{4}$$

$$\sigma_y^e = f\left(\frac{h}{H}\right), \tag{5}$$

where η is the fluid viscosity and k is the deformation dependent permeability of the porous medium (details in Equation 6). H denotes the undeformed thickness of the upper half BM and Σ denotes the external constrictive stress; these two terms are the input of the BM model. The function $f\left(\frac{h}{H}\right)$ relates the stress in the BM to its deformation and is derived from a given strain energy function. The reader is referred forward to Equations (8–9) for the particular forms of the stress-strain relationship and the strain energy function used in this work.

2.1.2. Physiological Interpretation of the System (1–5)

The physiological significance of the BM model is outlined below (Aldea, 2017).

Equations (1, 2) represent conservation of fluid and solid mass, respectively, in the deformed configuration of the system. Assuming that the BM is comprised only of fluid and solid phases, the volume fractions ϕ^s (solid) and ϕ^f (fluid) satisfy $\phi^s + \phi^f = 1$.

Equation (3) is the lubrication approximation of Darcy's law which relates the interstitial fluid velocity to the pore pressure gradient, the fluid viscosity and the deformation-dependent permeability. Various functions for deformation-dependent permeability have been discussed in the literature and, here, we choose the model described in Markert (2005) which stands for a large range of material compression, as well as distension,

$$k(h) = k_*\left(\frac{\frac{h}{H} - \phi_*^s}{1-\phi_*^s}\right)^\kappa, \tag{6}$$

where k_* is the permeability of the undeformed BM, κ is a positive parameter and ϕ_*^s is the volume fraction of the solid in the fluid-filled reference configuration.

Applying an asymptotic analysis (details in Aldea, 2017) shows that, at leading order, the only dimension of the BM that changes significantly is its thickness, meaning that the term $\frac{h}{H}$ represents the Jacobian of the system which gives the change in the volume of the BM. Equations (3, 6) account for the fact that any change in the volume of the BM will affect its porosity and, subsequently, its permeability to fluid flow. The state of zero porosity is reached when $\frac{h}{H} = \phi_*^s$, meaning that the BM pores are fully closed.

Equation (4) represents the force-balance equation derived from the conservation of momentum. A close look at the elasticity of an arterial segment shows that Σ is the radial stress at the radial position of the BM (see **Figure 2**, **Supplementary Material 1**, and Aldea, 2017), so Equation (4) follows from the continuity of radial stress across the BM. In **Supplementary Figures 1**, **2**, we plot the dependence of the radial stress at the middle of the wall on the magnitude of VSMC activation, i.e., $\Sigma(S)$, where S acts as a parameter describing the local level of contractile activity of VSMCs. Here, we model the propagation of the muscular contractions of the VSMCs with the following wave form of the vascular tone

$$S(z,t) = A \cdot \sin^2\left(\frac{\pi}{\lambda_w}(z - c_w t)\right) \cdot S_m, \tag{7}$$

which was previously used for the muscular activation wave of the ureter wall by Carew and Pedley (1997). For the purposes of this study, the vascular tone wave $S(z,t)$ represents the vasomotion wave from the V-IPAD model, having the same pattern as the arterial vasomotion wave observed experimentally (Bouskela and Grampp, 1992; Mayhew et al., 1996; Haddock and Hill, 2005; Vetri et al., 2007; Rayshubskiy et al., 2014). S_m reflects the physically observed maximal contraction of the VSMCs (Rachev and Hayashi, 1999) and A is the amplitude of change in the maximal muscular contraction. The magnitude of S_m has not been experimentally confirmed in the cerebral muscular arteries. Hence, S_m is taken in accordance with the value reported in carotid arteries (Rachev and Hayashi, 1999). λ_w and c_w denote the wavelength and the speed of the vasomotion wave, respectively; t is time and z is the spatial coordinate. The wavelength λ_w of cerebral vasomotion has not been experimentally measured, but can be calculated by dividing the wave velocity c_w by the wave frequency (or equally by multiplying the wave velocity with the time period of the vasomotion wave). The propagation velocities of the vasomotor response available in the literature describe the induced vasomotor response of arteries, rather than the (spontaneous) vasomotion wave (Duling and Berne, 1970; Dietrich et al., 1996; Seppey et al., 2009).

Finally, Equation (5) represents the constitutive equation, i.e., the stress-strain relationship for the hyperelastic BM. This

means that the principal effective Cauchy stress σ_y^e is derived from a strain energy function (here denoted W_{BM}) that describes the elastic behavior of the material under particular deforming conditions. Given the geometrical setup of the BM model, in which compressive forces are only significant in the y-direction, the relationship between the principal stress in the y-direction and the corresponding deformation is given (in terms of a strain energy function) by

$$\sigma_y^e(h) = f\left(\frac{h}{H}\right) = \frac{\partial W_{BM}(\frac{h}{H})}{\partial \lambda_y(\frac{h}{H})}. \tag{8}$$

Here, λ_y is the principal stretch ratio in the y-direction and is defined as $\lambda_y(\frac{h}{H}) = \frac{h}{H}$, considering that the only dimension of the BM that changes significantly is its thickness. The choice of W_{BM} requires careful attention. Limited by the lack of experimental data on the elasticity of the cerebrovascular BM, a minimum number of material parameters able to describe the physiological response is desired. With this in mind, we choose the neo-Hookean model with only two material parameters from the study of Wirth et al. (2010), in which various permeability functions (e.g., Equation 6) and hyperelasticity constitutive laws were combined for modeling choked (or limited) flows through biological poroelastic materials. Hence, within this lubrication model, the elastic response of the poroelastic BM to the external constrictive stress is captured by the strain energy function

$$W_{BM}\left(\frac{h}{H}\right) = \frac{\mu_s}{2}\left(\left(\frac{h}{H}\right)^2 - 1 - 2(1-J_*)\log\frac{\frac{h}{H}-J_*}{1-J_*}\right) + \frac{1}{2}\left(\lambda_s - \mu_s\frac{J_*}{1-J_*}\right)\left(\frac{h}{H}-1\right)^2, \tag{9}$$

which is plotted in **Figure 3**. The material parameters μ_s and λ_s denote the first and second Lamé parameters, respectively. $H \cdot J_*$ represents the minimum possible deformed thickness h of the membrane. For example, if the solid matrix of proteins is also incompressible, J_* equals ϕ_*^s when all the pore spaces are closed. In other words, J_* represents the lower limit of physical validity of the employed elastic model, meaning that the BM cannot be compressed to zero volume with finite energy (W_{BM} diverges as $\frac{h}{H} \to J_*$). For the Lamé parameters given in **Table 1**, the BM behavior is denoted by the word "lymphatic" and the corresponding W_{BM} from Equation (9) is plotted in **Figure 3**, showing that it takes a fast increasing energy to deform the BM once its pores are nearly shut (as $\frac{h}{H}$ approaches J_*). The Cauchy stress σ_y^e is obtained according to Equation (8) and shown in **Figure 3**. If the BM were a more deformable material (denoted "spongy"), then its pores could be nearly shut during the maximum activation of the VSMCs, e.g., by a compressive stress of 6 kPa (**Figure 3** and details in section 4.2).

2.1.3. Concise Formulation of the BM Model

The system of Equations (1–5) is simplified by replacing Equations (2, 3) in Equation (1), which yields the non-linear equation for the deformed upper-half BM

$$\frac{\partial h}{\partial t} = \frac{\partial}{\partial z}\left(h\frac{k(h)}{\eta}\frac{\partial p(h, \Sigma(z,t))}{\partial z}\right), \tag{10}$$

recalling that $k(h)$ is given by Equation (6) and $p = \sigma_y^e(h(z,t)) - \Sigma(S(z,t))$. Equation (10) requires one initial condition and two boundary conditions.

The BM is considered initially undeformed and uniform along the vessel, i.e.,

$$h|_{t=0} = H, \quad 0 \le z \le L_s, \tag{11}$$

where $z = 0$ represents the proximal end of the BM, while $z = L_s$ represents the distal end of the BM. Subsequently, both the deformation of the BM induced by the external compressive stress and the resulting flow through the BM, are investigated.

The ends of the BM are assumed to be at the same pressure (i.e., the pressure drop along the BM between the two ends of the vessel does not drive a significant flow)

$$p|_{z=0} = p|_{z=L_s} = 0, \quad t > 0. \tag{12}$$

Equation (10) plays a paramount role in the dynamics of the system and is solved for $h(z,t)$ for a given $\Sigma(S(z,t))$ and W_{BM}. Once h is determined, all the other variables of the system can be calculated given their dependency on h. Finally, the fluid flow rate through the poroelastic BM is calculated as shown below.

2.1.4. Volumetric Flow Rate Through the Poroelastic BM

An infinitesimal element of the cerebral artery is considered, as illustrated in **Figure 2**. The annular region represents the poroelastic BM. Radial symmetric deformation of the artery is assumed and the deformation of BM depends solely on the external compressive stress induced by the vasomotion wave, i.e., on $\Sigma(S(z,t))$. The total flow rate of fluid through the infinitesimal volume of BM, generated by the vasomotion-induced deformation of the BM boundaries, is calculated by using the differential form of the Darcy's law and recalling that the cross sectional area of the BM is $2\pi r_m(2h)[\mu m^2]$; hence, it follows that

$$Q_{BM}(z,t) = -2\frac{2\pi r_m h k(h)}{\eta}\frac{\partial p}{\partial z}, \tag{13}$$

where $Q_{BM}(z,t)[\mu m^3 \cdot s^{-1}]$ is the volumetric flow rate through the entire thickness of the BM and $r_m[\mu m]$ denotes the radial position of the BM. Here, the extra factor of 2 appears because we have only been considering the upper half of the BM in our model from sections 2.1.1–2.1.3. It is emphasized that r_m is a function of the wave of vascular tone, i.e., $r_m = r_m(S(z,t))$, and its form is not prescribed, but is rather determined from the arterial wall model in **Supplementary Material 1**.

The net flow rate over one cycle of vasomotion is

$$\bar{Q}_{BM} = \frac{1}{T}\int_0^T Q_{BM} dt, \tag{14}$$

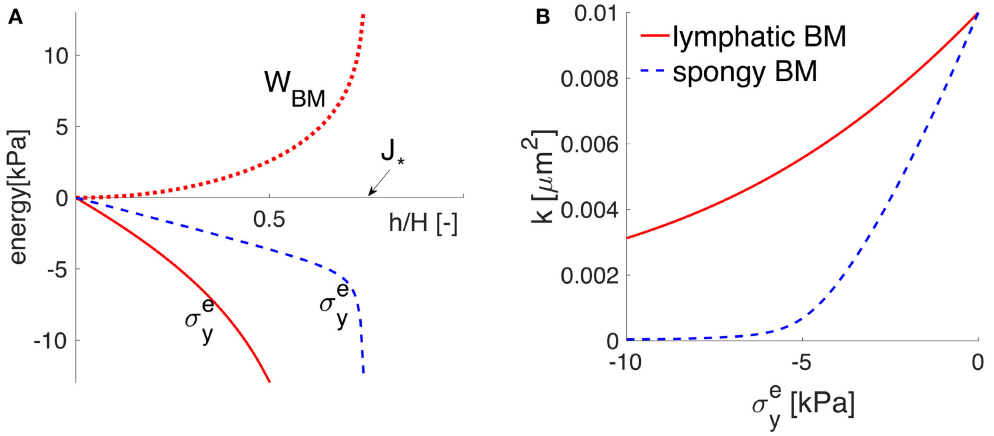

FIGURE 3 | The elastic response of the poroelastic BM. The potential behavior of an idealized "spongy" material is sketched in dotted blue line, while that of the "lymphatic" BM considered throughout this study is plotted in solid red line. **(A)** The strain energy function $W_{BM} = W_{BM}(h/H)$ in the upper (positive) part and the principal Cauchy stress $\sigma_e^y = \sigma_e^y(h/H)$ in the lower (negative) part, as functions of the stretch h/H. The values of h/H (smaller than one) show that the BM thickness is compressed by the normal elastic stress σ_e^y until reaching the physically-valid lower limit J_*. **(B)** the stress-permeability relationship, according to which the BM permeability k decreases non-linearly with an increases in the compressive normal stress σ_e^y.

where T represents the time period of the vasomotion wave. The parabolic Equation (10) is solved with the method of lines as described in **Supplementary Material 2**. All the common diagnostic checks were performed to ensure that the numerical method conserves the mass of the system and the errors are acceptably small. The convergence of the employed numerical method is demonstrated in **Supplementary Figure 3**.

3. RESULTS

We have considered the cerebral artery to be an idealized vessel (e.g., thick-walled cylinder) with uniform material properties determined experimentally by Bell et al. (2013). For reasons of simplicity, we have accounted for a single layer of BM positioned at the middle of the arterial wall and modeled it as a fluid-filled poroelastic material with a slow-varying thickness, as illustrated in **Figure 2**. We have assumed the BM boundaries to be impermeable, such that the fluid cannot escape from this compartment, guaranteeing in this way conservation of fluid. The artery deforms due to the non-zero intraluminal pressure and longitudinal stretching, as well as due to VSMC contractions (i.e., active mechanical response). The biomechanical behavior of three MCAs was investigated in detail in **Supplementary Material 1** and the results corresponding to an artery exposed to a physiological intraluminal pressure of 13.3 kPa and an axial stretch of 1.07 are presented below. The deformation of the artery wall triggers the flow in the BM, but the BM is small enough such that it does not alter the elastic stresses in the wall. Hence, there is only one-way coupling, allowing for the elastic analysis of the artery to be handled independently of the BM.

Rather than prescribing the deformation of the arterial wall, we have first solved the arterial wall model from **Supplementary Material 1** in order to assess the variations in arterial diameter as a consequence of the local VSMC contractions, by maintaining both the intraluminal pressure and the axial stretch constant. Once the deformed inner radius of the artery is determined, the deformation and the corresponding stresses at any spatial point within the wall can be calculated. Secondly, by assuming continuity of stress across the wall, we can take the radial stress (denoted σ_r) at the middle of the arterial wall as the external constrictive stress (denoted Σ) acting on the BM, i.e., $\sigma_r(r = r_m) = \Sigma$, where r_m is the deformed radius r at the middle of the arterial wall (see **Figure 2** and **Supplementary Figure 1**).

Furthermore, the spatiotemporal contractile oscillations of the cerebral VSMCs, i.e., the vasomotion wave, are prescribed by the wave form of the vascular tone $S(z, t)$ with units of stress, as shown in **Figure 4** and defined in Equation (7). The values of S were determined elsewhere (Rachev and Hayashi, 1999) based on experimentally recorded pressure-diameter curves of arteries, under VSMC contraction and control conditions. Thereby, $S = 0$ kPa reflects the purely passive mechanical response of the arterial wall when the VSMCs are fully relaxed, while $S = 100$ kPa is taken for maximal muscular contraction. In terms of spatiotemporal properties of the system, we consider the frequency of vascular oscillations to be around 0.1 Hz (i.e., they repeat every 10 s) and investigate the particular case in which the wavelength of the vasomotion wave is comparable with the characteristic length of a MCA. Moreover, for reasons of simplicity, we assume a uniform system and one that is sufficiently long in order to encompass several vasomotions waves simultaneously (see **Table 1**). This latter consideration allows for evaluation of the solution to our poroelastic problem far from the ends of the computational spatial grid where rapid variations in the solution may occur.

Both the deformation of the middle wall of the artery, $r_m = r_m(S(z, t))$, and the corresponding radial stress, $\Sigma = \Sigma(S(z, t))$,

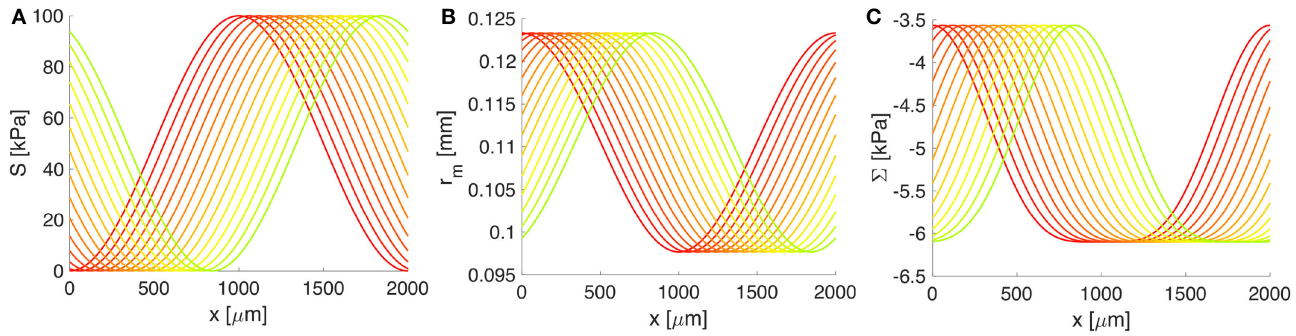

FIGURE 4 | The response of the artery wall to muscular oscillations over one wavelength. **(A)** The vasomotion wave, **(B)** the radial position of the BM, and **(C)** the constrictive stress acting on the BM. Both r_m and Σ depend on $S(z, t)$ and serve as input in the BM model. Progressive change from the red to the green curve shows increase in time; only 4 s from one vasomotion cycle of 10 s are illustrated.

depend on the vascular tone $S(z, t)$ through the arterial wall model (see details in **Supplementary Material 1**). In turn, these quantities serve as input in the BM model (see **Figure 4**). A whole cycle of vasomotion includes both the contraction and the relaxation of VSMCs and the corresponding oscillation between the maximal and minimal radius of the artery has an amplitude of approximately 20%, as pictured in **Figure 4**. In addition, from **Figure 4**, it is obvious that the highest negative value of Σ (i.e., the strongest constrictive stress on the BM) is generated during maximal muscular contraction. On the other hand, the lowest value of Σ corresponds to the case of fully relaxed VSMCs and is non-zero due to stresses generated by the passive load bearing components of the wall of the artery (e.g., collagen and elastin fibers) during inflation and axial stretching of the artery.

3.1. The VSMCs as the Pump for IPAD in the Brain

The spatiotemporal variations in cerebral vascular tone influence the fluid flow through the BM by inducing deformations of its top and bottom boundaries via the radial constrictive stress $\Sigma(S(z, t))$. The fluid movement through the BM is governed by Darcy's law. The change in the BM volume affects its porosity and, subsequently, its permeability to fluid flow, according to Equations (3, 6). In this particular scenario, it has been allowed for the fluid volume fraction to drop to zero due to finite compressive forces that reduce the BM pores and, as a consequence, obstruct the path for fluid drainage; this behavior is similar to squeezing shut a fluid-filled sponge.

The response of the poroelastic BM to the external constrictive stress is described by the stress-stretch relationship from Equation (8), which is derived using the strain energy function for neo-Hookean poroelastic materials from Wirth et al. (2010). The ability of the chosen strain energy function from Equation (9) to describe a non-linear elastic behavior for a general large range of deformations is shown in **Figure 3** and further discussed in section 4.2. The cerebrovascular BM considered here has similar properties to the interstitium of the systemic lymphatic system (Smillie et al., 2005) and to the interstitium of the brain (Heppell et al., 2013), therefore it appears as a physiologically reasonable assumption. From **Figures 3**, **5**, it is obvious that the compressive stress generated during maximum VSMC activation (e.g., 6 kPa) decreases the permeability of the BM by 50% compared to its undeformed state. As the BM pores are squeezed by the contractile VSMCs, the reduced BM permeability prevents high reverse flows (in the direction opposing the vasomotion wave), making the VSMCs an efficient pump for driving fluid along the IPAD pathways.

3.2. Non-zero Net Intramural Periarterial Flow Rates During One Cycle of Vasomotion

Owing to the assumed symmetry of the system pictured in **Figure 2**, the vasomotion-induced BM deformation has been determined by solving Equation (10) for the upper-half part of the BM. The BM deformations propagate longitudinally and vary in time, with characteristics specific to the vasomotion wave (e.g., a time period of 10 s or, equally, a frequency of 0.1 Hz). The evolution of the BM thickness $h(z, t)$ and permeability $k(z, t)$, as well as the resulting fluid flow rate $Q_{BM}(z, t)$ through the BM are shown in **Figure 5**. The vasomotion-induced fluid flow rate through the entire BM compartment, calculated with Equation (13), depends on the radial deformation of the artery wall, the fluid viscosity, the deformation-dependent permeability of the poroelastic BM and the pore pressure gradient. Given that one vasomotion cycle incorporates both the contraction and the relaxation of the VSMCs, positive (i.e., in the direction of the vasomotion wave) as well as negative flows (i.e., in the reverse direction of the vasomotion wave) occur at different times at a given spatial point of the BM. Although high intramural flow rates of nearly 4000 $\mu m^3 \cdot s^{-1}$ are obtained, the net flow rate during one cycle of vasomotion is only 360 $\mu m^3 \cdot s^{-1}$. The positive value indicates that the net intramural fluid flow rate during one cycle of vasomotion is always in the direction of the vasomotion wave (a consequence of the much reduced BM permeability as it is squeezed; Aldea, 2017).

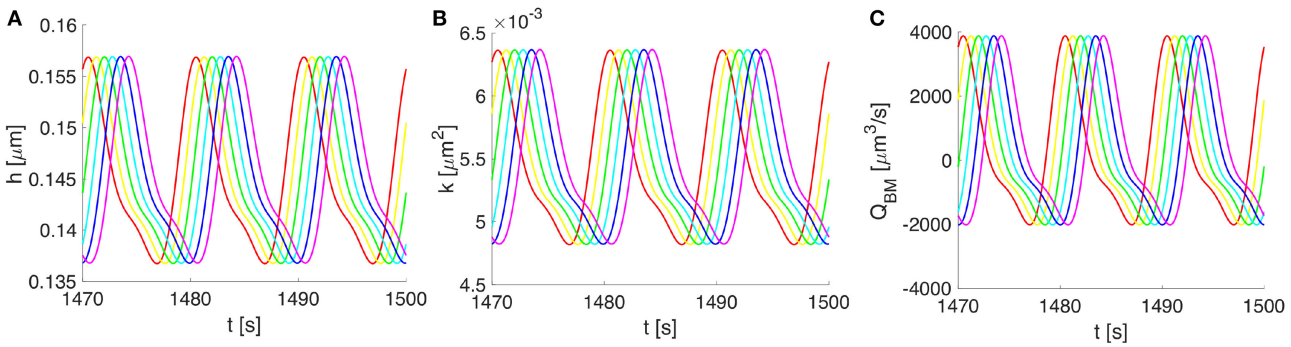

FIGURE 5 | Vasomotion-induced deformation and flows along the BM for three cycles of vasomotion. **(A)** Oscillations in time of the upper-half thickness of the BM, **(B)** deformation dependent permeability of the BM, and **(C)** the corresponding volumetric flow rate along the whole compartment of BM. Progressive change in color from red to magenta shows six distinct spatial points over 750 μm (out of a 2,000 μm wavelength).

4. DISCUSSION AND CONCLUSION

The small dimension of the clearance pathways of the brain (e.g., 10–400 nm) and their anatomical position within the deep cortical layers make the investigation of brain clearance challenging to conduct in living humans and even in animal models. Physiologically-based mathematical and computational models represent an alternative tool for analysing potential clearance mechanisms of the brain, likely generating new mechanistic insights (Goriely et al., 2015; Holter et al., 2017). Since the motive force for IPAD has remained unresolved (Diem et al., 2017), we have proposed and tested with *in silico* modeling the idea of vasomotion-driven IPAD (denoted V-IPAD). According to the V-IPAD hypothesis, the contractile VSMCs of cerebral arteries act as the drivers of IPAD in the brain by inducing BM deformations and, subsequently, net intramural fluid flows in the direction of the vasomotion wave. To our knowledge, this is the first quantitative study that explores the contribution of the cerebral VSMCs in the drainage of fluid from the brain. Although the exact physiological roles of vasomotion remain elusive, the most common view is that vasomotion serves as an auxiliary mechanism in tissue perfusion, especially under hypoperfusion conditions (Nilsson and Aalkjær, 2003). The theoretical results from this study indicate another potential role for vasomotion, namely the clearance of fluid and soluble metabolites from the brain along the IPAD pathways.

4.1. Experimental Evidence Supporting the V-IPAD Hypothesis

The most solid evidence for the V-IPAD hypothesis probably comes from the experiments of Beach et al. (2000) who found significant accumulation of Aβ within the abluminal BM of VSMCs as a result of cortical cholinergic deafferentation (i.e., interruption of acetylcholine neurotransmitter to the cerebral blood vessels and other cells of the brain). The authors found it difficult to explain why the Aβ deposition was predominantly in the wall of arterioles and only sparsely in the brain tissue as amyloid plaques. Here, we propose that the cerebrovascular accumulation of Aβ , observed in Beach et al. (2000), resulted from impaired activity of the VSCMs. As the vascular tone of cerebral arteries is modulated by a rich cholinergic innervation (Hamel, 2006), the cholinergic deafferentation induced in the study of Beach et al. (2000) may have hindered the VSMC activity and, subsequently, their contribution to the clearance of Aβ along the BMs that act as the IPAD pathways.

Inefficient periarterial drainage was observed during ischemic stroke (i.e., focally disrupted blood perfusion to the brain) in Arbel-Ornath et al. (2013). Since the contractile filaments of the arterial VSMCs are significantly damaged after 15–45 min of ischemia (Kwon et al., 2002), altered contractile activity of the VSMCs of the vessels deprived of blood flow seems likely and this could explain the impaired periarterial drainage along occluded vessels observed by Arbel-Ornath et al. (2013). On a similar note, Carare et al. (2008) observed no perivascular drainage following cardiac arrest. Further evidence that indicates a link between the cerebral vascular tone and IPAD comes from Maki et al. (2014) where the vasoactive drug, Cilostazol, was administrated into the mouse brain, leading to enhanced periarterial drainage.

4.2. The Most Likely Motive Force for IPAD in the Brain

The simulated amplitude of oscillations in arterial diameter (e.g., 20%), induced by the contracting and relaxing VSMCs, is in line with the experimental observations of vasomotion in large cerebral arteries (Fujii et al., 1990, 1991; Rayshubskiy et al., 2014) and is considerably larger than the amplitude of oscillations induced by systolic pulsations (e.g., on average 3%, Bedussi et al., 2018). Comparison of the resulting intramural fluid flow rates with previous studies is given in **Table 2**. The net fluid flow rate of 360 $\mu m^3 \cdot s^{-1}$ generated by the V-IPAD mechanism along only one BM layer is five orders of magnitude higher than the net periarterial flow rate induced by arterial pulsations (Diem et al., 2017). The studies of Asgari et al. (2015, 2016) also yielded perivascular flow rates driven by arterial pulsations, but in a distinct perivascular space positioned

TABLE 2 | Comparison of results between the V-IPAD model and previous models.

Study	Space	Thickness [μm]	Net flow rate [$\mu m^3 \cdot s^{-1}$]
V-IPAD model	Poroelastic BM	0.4	360
Diem et al., 2017	Porous BM	0.2	$1.12 \cdot 10^{-3}$
Asgari et al., 2015	PVS	1	$2.94 \cdot 10^{-2}$
Asgari et al., 2016	PVS	10	$3.72 \cdot 10^{-1}$

The driving force in the V-IPAD model is the vasomotion wave, while in the models of Diem et al. (2017), Asgari et al. (2015), and Asgari et al. (2016) is the arterial pulse. The net flow rates represent the absolute values. BM is the compartment within the arterial wall, while PVS denotes the perivascular space between the arterial wall and the glial layer of the brain.

between the arterial wall and glial layer of the brain. Nonetheless, their findings offer additional proof that arterial pulsations are incapable of generating significant perivascular flow rates out of the brain. The values reported by Asgari et al. (2015, 2016) differ from those reported by Diem et al. (2017) due to the fact that the former authors assumed the thickness of the perivascular space up to two orders of magnitude higher than in the model of Diem et al. (2017). Here, the BM thickness is taken to be 0.4 μm in the undeformed, hydrated state and decreases to 0.27 μm during its compression by the maximally activated VSMCs. It is worth remarking that when comparison with other studies is made, it should be kept in mind that others may have defined the net flows out of the brain as negative flows relative to the direction of arterial pulsations.

Compared with the arterial pulse, the cerebral vasomotion is a more reasonable driving force of IPAD because its wavelength is orders of magnitude smaller than that of the arterial pulse wave. The wavelength of vasomotion is calculated based on its frequency and velocity. While the frequency of vasomotion is commonly reported with values centred at 0.1 Hz, its velocity is less well known. However, existing data allow a reasonable estimate. For example: (i) rat arterial strips elicited vasomotion with a velocity of 100 μm/s (Seppey et al., 2009), while (ii) hemodynamic oscillations propagating on the surface of the human brain (probably related to vasomotion) had an average velocity of 800 μm/s (Noordmans et al., 2018). Even when the higher end of velocities is considered, the resulting wavelength of cerebral vasomotion (e.g., 8 mm) is still two orders of magnitude smaller than that of the human arterial pulse wave (e.g., 1 m, calculated based on a velocity of 1 m/s and a frequency of 1 Hz, Stefanovska, 2007). In particular, vasomotion wavelength appears comparable with the length of the cerebral arteries; this feature favors development of pressure gradients that are able to drive significant net ISF flows along the IPAD pathways.

Despite the fact that the V-IPAD mechanism promotes significantly higher net intramural periarterial flow rates than the previously suggested mechanisms (see **Table 2**), it is essential to examine the relative contribution of V-IPAD in the global picture of brain clearance. The model we have designed yields the fluid flow rate along only one BM compartment surrounding the arterial VSMCs. The total number of BMs contributing to IPAD in the whole brain is currently unknown, so instead we consider a smaller block of gray matter and its vascular supply which can be relatively easier to assess. Based on the *in vivo* imaging of the surface arterial vascular network in the rodent brain (Schaffer et al., 2006), minimum 8 surface arteries branching from the MCA can be detected in an area measuring 4 × 3 mm. It is reasonable to assume that the penetrating arterioles arising from the surface arterial network extend through the entire depth of the cortex (1 mm in the rat brain Vetreno et al., 2017). In this way, a brain volume measuring 4 × 3 × 1 mm appears to be supplied (blood through the lumen) and cleared (ISF and soluble metabolites through the artery wall) by at least 8 surface arteries branching from the MCA. Assuming that each of these surface arteries has on average 3 BM compartments (Lee, 1995), a total of 24 BMs clears a block of gray matter of 12 mm^3, leading to a net ISF flow rate of 720 $\mu m^3 \cdot s^{-1} \cdot mm^{-3}$ (calculated as 24 · 360 $\mu m^3 \cdot s^{-1}$/12 mm^3) or, equally, 0.04 $\mu l \cdot min^{-1} \cdot g_{brain}^{-1}$. Owing to the lack of consistent experimental data on the passive and active mechanical response of small cerebral arteries, we have considered the vascular response of the branches of MCA to be identical to that of the MCA itself when estimating the total net ISF flow rate drained from a block of gray matter along the IPAD pathways. We note that investigation of IPAD in the MCA is itself an important goal, because the MCAs, together with the Circle of Willis, may be seen as a bottleneck in the outflow of ISF and solutes toward the cervical lymph nodes along the IPAD pathways. Therefore, it is of high significance to evaluate the likely strength of IPAD drainage along the MCAs. The proposed mechanism can also explain vasomotion-driven IPAD in smaller cerebral arteries with at least two layers of VSMCs. Adaptation of the V-IPAD model to intracerebral arterioles with only one layer of VSMCs will be addressed in a future study.

How physiologically-significant is the estimated net ISF flow rate along the IPAD pathways? The exact amount of ISF that requires removing from the brain interstitium along the IPAD pathways vs. other routes (e.g., convection through the cerebral extracellular spaces toward the CSF Abbott, 2004 or the recently proposed glymphatic system; Iliff et al., 2012) has been widely debated (Hladky and Barrand, 2014). By comparison with the average value for ISF flow rate from the brain (e.g., 0.2 $\mu l \cdot min^{-1} \cdot g_{brain}^{-1}$), which falls within the range of lymph flow rates and was estimated by Szentistvanyi et al. (1984), our estimated fluid flow rate induced by the V-IPAD mechanism (e.g., 0.04 $\mu l \cdot min^{-1} \cdot g_{brain}^{-1}$) has a 20% contribution to the clearance of cerebral ISF secreted at the blood-brain barrier. The amount of ISF transported by the V-IPAD mechanism may prove sufficient to maintain a physiological Aβ concentration within the artery wall by diluting the soluble Aβ that is not cleared at the blood-brain barrier via LRP1 transporters (escaping instead along the BM of cerebral arteries).

The elastic properties of the BM and the pattern of the vasomotion wave prove crucial in inducing net intramural fluid flow rates of physiological importance. If the cerebrovascular

BM has the purpose of removing most of the cerebral soluble metabolites, its properties will likely have evolved to improve clearance of the brain and, hence, the BM may be a more deformable material than the one simulated here. The behavior of the BM may resemble the elastic response of the "spongy" material illustrated in **Figure 3**, which could undergo more significant compression of its pores, thereby hindering almost completely the reverse intramural flow. Such a behavior should make the pumping action of cerebral VSMCs even more effective, leading to higher net intramural fluid flow rates than the ones simulated here. This encourages future experimental assessment of the biomechanics of the contractile cerebral VSMCs and their BMs in order to explore the full potential of the V-IPAD mechanism. In the light of the values given in **Table 2**, the vasomotion wave appears as the best candidate proposed to date for the motive force for IPAD in the brain because it has a more appropriate amplitude and wavelength to drive fluid out of the brain at physiologically more significant flow rates than the arterial pulse. This conclusion holds for all the arterial segments described in **Supplementary Material 1** (with material parameters in **Supplementary Table 2**), considering that the resulting net intramural flow rates are 380 $\mu m^3 \cdot s^{-1}$ and 444 $\mu m^3 \cdot s^{-1}$ when the artery experiences an axial stretch of 1.09 and 1.13, respectively.

4.3. The Direction of the V-IPAD Mechanism

We have shown that the oscillations in the contractile state of the cerebral VSMCs are sufficiently powerful to promote net intramural flow rates of notable magnitude for efficient drainage of fluid along the IPAD routes, but the predominant direction of vasomotion remains elusive. Within the context of IPAD of the brain, the vasomotion wave must propagate from penetrating arterioles toward leptomeningeal arteries on the surface of the cortex. Direct investigations of the cerebral cortex in humans when awake showed distinct regions of pial arterioles exhibiting slow sinusoidal vascular oscillations, with a frequency of $\approx$ 0.1 Hz, which propagated as spatial waves across the cortex (Rayshubskiy et al., 2014). Spontaneous, low-frequency oscillations of cerebral arteries within the range 0.1–1 Hz, presumably resulting from vasomotion, were also reported in mice when awake, propagating over several hundred micrometers across the cortex (Drew et al., 2011). Remote communication of local vascular responses (e.g., vasodilation) of arterioles to their parent arteries is common during cerebral functional hyperemia, in order to assure an optimal blood supply to the highly active brain region (Iadecola et al., 1997). Considering the complexity of communication between the active neurons and their supplying blood vessels during cerebral functional hyperemia, the cerebral arterial network seems to be empowered with properties that allow vasomotion to propagate in the optimal direction for IPAD (Secomb and Pries, 2002; Girouard and Iadecola, 2006; Sweeney et al., 2018). Additional experimental recordings of the spatial pattern of vasomotion is required and *in vivo* optical imaging (e.g., two-photon microscopy, optical intrinsic signal imaging) of contractile cerebral vascular networks (Nishimura et al., 2007; Chen et al., 2011; Rayshubskiy et al., 2014) will prove useful in such an endeavor.

It has recently been shown that the permeability of the brain interstitium is too low to allow development of any substantial bulk flow at physiological intracerebral pressure differences (Jin et al., 2016; Holter et al., 2017). Soluble metabolites can thus only leave the brain tissue by either diffusion toward the CSF compartments or by bulk flow along less resistant pathways such as the perivascular pathways of blood vessels (Abbott, 2004; Bakker et al., 2016). However, diffusion is only able to operate effectively over very short distances (Syková and Nicholson, 2008). It is not immediately obvious how the vasomotion wave will improve the flow of fluid and soluble metabolites along the other proposed para-vascular clearance pathways, such as the pial-glial basement membranes (Albargothy et al., 2018) or the para-venous pathways (Iliff et al., 2012). According to the recent review of Welsh et al. (2018), there is no evidence of conducted vasomotor responses along venules and veins, or from veins to the arterial system through the capillary network. This indicates that vasomotion can only act as a driving force for periarterial drainage. Furthermore, if the artery wall acts as a two-way traffic system for (i) the efflux of ISF and soluble parenchymal metabolites along the IPAD pathways and (ii) the convective influx of CSF into the brain parenchyma along the pial-glial basement membranes, then two distinct motive forces are required. With respect to the latter, it has been demonstrated by Asgari et al. (2016) that the arterial pulsations propagating into the brain are not sufficient to drive significant net para-vascular flows along arteries, but they may enhance local mixing and diffusion of solutes in the CSF along para-vascular pathways. It remains an open question which exact physical forces are responsible for this influx of CSF into the brain parenchyma. In order for separate longitudinal flows (in opposite directions) to occur along both the IPAD and para-vascular pathways, the vascular BM, adjacent to the VSMCs, needs to be separated from the CSF by an impermeable (or highly resistant) membrane. The pial layer could serve this purpose, but its exact permeability needs further experimental characterization, as it may not only vary between species, but also between intracerebral and extracerebral regions (Alcolado et al., 1988; Abbott et al., 2018). Here, we have shown that vasomotion can drive net ISF flow along the vascular BM positioned between two impermeable layers of VSMCs.

4.4. Contributors to the Pathogenesis of CAA

While direct evidence is still missing, the aforementioned experiments (Beach et al., 2000; Arbel-Ornath et al., 2013; Maki et al., 2014) strongly support the proposed hypothesis that the activity of the cerebral VSMCs may be a key element for IPAD in the brain and, subsequently, in preventing the onset of CAA. The contractile and relaxing abilities of the VSMCs are weakened in old arteries (Tümer et al., 2014). The cholinergic innervation

of cerebral arteries also decreases with age and in the presence of AD, thus impairing vessel dilatation (Tong and Hamel, 1999; Van Beek and Claassen, 2011). Moreover, the transport of Aβ out of the brain interstitium across the blood-brain barrier also becomes inefficient with age (Shibata et al., 2000; Deane et al., 2009), leading to an elevated burden of intracerebral Aβ that requires removal along alternative routes, presumably along the IPAD pathways. The cumulative effect of all these age-driven changes may translate into an altered response of the VSMCs and, consequently, into a diminished motive force for IPAD, increasing in this way the likelihood of CAA.

Various hypotheses have been advanced to explain CAA. It has been proposed that the Aβ deposited in the walls of arteries in CAA was derived from VSMCs (Vinters, 1987). However, production of Aβ by VSMCs does not explain the deposition of Aβ in the walls of capillaries that lack VSMCs. Furthermore, transgenic mice in which neurons overproduce Aβ develop CAA, both in cortical and leptomeningeal arteries; this suggests that parenchymal Aβ is transported from the brain tissue along the walls of arteries where it accumulates due to amyloid overload of the drainage pathways (Calhoun et al., 1999). It has also been proposed that the origin of Aβ in the walls of arteries in CAA is from the CSF due to convective influx along para-arterial pathways (Iliff et al., 2012). However, subsequent studies have shown that tracers from the CSF enter the brain along pial-glial basement membranes (Morris et al., 2016) and drain from the brain along the IPAD pathways (Albargothy et al., 2018). The most feasible mechanism for the pathogenesis of CAA, therefore, appears to be protein elimination failure angiopathy due to age-related failure of IPAD (Carare et al., 2013).

4.5. Potential Therapeutic Implications

The impact of this study opens avenues for targeting vasomotion toward the facilitation of IPAD and prevention of CAA. This is key for the successful outcome of current immunization trials against Aβ in which plaques are solubilized, but Aβ becomes entrapped in the IPAD pathways, increasing the severity of CAA and most likely resulting in amyloid-related imaging abnormalities (Holmes et al., 2008; Sperling et al., 2012; Weller et al., 2015). Moreover, as neurovascular therapeutic approaches in patients with mild cognitive impairment and AD are developed, e.g., the clinical trial with Cilostazol (Saito et al., 2016), the potential role of cerebral VSMCs in the periarterial clearance of Aβ from the brain may be worth considering in greater detail. The role of the VSMCs in the efficiency of IPAD in the brain could be tested experimentally in animal models with endogenous dysfunction of the VSCMs, which could be induced by dietary interventions (Gooch and Wilcock, 2016) or, in a more specific manner, by genetic mutations (Lee et al., 2012).

Using transgenic models with overproduction of mutant amyloid-beta peptides has many advantages in basic understanding of mechanism of disease (Klohs et al., 2014) but has also led to problems regarding translation into therapies (Jäkel et al., 2017). One model that may be more suitable, as there are no transgenic manipulations, is that of HHcy (Gooch and Wilcock, 2016). The HHcy mouse model develops physiologically relevant cerebrovascular pathology including microhemorrhages, vascular fibrosis, cerebral hypoperfusion and, most importantly, cognitive impairment (Hainsworth et al., 2016; Sudduth et al., 2017). The HHcy model is a dietary intervention that drives the folate cycle and methionine metabolic pathways to accumulate homocysteine through the elimination of vitamins B6, 9, and 12, and enrichment with methionine. The levels of homocysteine achieved in this model equate to moderate HHcy for a mouse. Our pilot data from HHcy models presented in **Supplementary Material 3** show a strong trend of reduced expression of α-smooth muscle actin in the HHcy mouse model compared to the control group (see **Supplementary Figure 4**). This suggests that the normal contractile function of the VSMCs may be impaired in the HHcy cases. In the future stages of our experiments, we will investigate the efficiency of IPAD in the HHcy mouse model.

Drugs such as cholinesterase inhibitors, commonly prescribed in the treatment of Alzheimer's disease, have enhanced high cognitive functions in demented patients who responded to treatment (Birks, 2006). Under the cholinergic-vasculature hypothesis, Claassen and Jansen (2006) have proposed that the observed benefits may be an outcome of the direct action of cholinesterase inhibitors on blood vessels. It therefore remains an interesting question how various vasoactive agents acting on the cerebral VSMCs affect not only the cerebral blood flow, but also the perivascular drainage of Aβ out of the brain.

In conclusion, this study has theoretically proven that the vasomotion wave initiated by the contractile VSMCs of cerebral arteries can represent the motive force for IPAD in the brain, shifting the view from a heart-driven clearance of fluid and solutes from the brain to an intrinsic mechanism of cerebral arteries. The V-IPAD model is the first one that quantitatively examines the contribution of the contractile cerebral VSMCs in the clearance of fluid from the brain, proposing a physically plausible driving force for IPAD. The model offers mechanistic insights into the behavior of the cerebrovascular BM and its interaction with the adjacent VSMCs. Once additional experimental data about the active response of cerebral arteries are provided, the V-IPAD model could be extended in order to improve its accuracy and simulate the effect of cerebral vascular aging on the IPAD of the brain. The hypothesis proposed and tested here stimulates further experimental investigation of the contribution of cerebral VSMCs in the clearance of fluid and solutes from the brain, particularly as these cells represent an accessible therapeutic target for CAA.

ETHICS STATEMENT

The study was approved by the University of Kentucky Institutional Animal Care and Use Committee and conformed to the National Institutes of Health's Guide for the Care and Use of Laboratory Animals.

AUTHOR CONTRIBUTIONS

RA, GR, and RC formulated the hypothesis. RA and GR formulated and solved the model. RC and DW designed

the experimental pilot study, obtained, and analyzed the experimental data. All authors contributed to the draft of the manuscript, read, and approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnagi.2019.00001/full#supplementary-material

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Impairment of Dendrodendritic Inhibition in the Olfactory Bulb of APP/PS1 Mice

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Olfactory dysfunction is an early event in Alzheimer's disease (AD). However, the mechanism underlying the AD-related changes in the olfactory bulb (OB) remains unknown. Granule cells (GCs) in the OB regulate the activity of mitral cells (MCs) through reciprocal dendrodendritic synapses, which is crucial for olfactory signal processing and odor discrimination. Nevertheless, the relationships between the morphological and functional changes of dendrodendritic synapses, particularly the local field potentials (LFPs) as a consequence of olfactory disorders in patients with AD have not been investigated. Here, we studied the morphological and functional changes induced by dendrodendritic inhibition in GCs onto MCs in the OB of amyloid precursor protein (APP)/PS1 mice and age-matched control mice during aging, particular, we focused on the effects of olfactory disorder in the dendrodendritic synaptic structures and the LFPs. We found that olfactory disorder was associated with increased amyloid-β (Aβ) deposits in the OB of APP/PS1 mice, and those mice also exhibited abnormal changes in the morphology of GCs and MCs, a decreased density of GC dendritic spines and impairments in the synaptic interface of dendrodendritic synapses between GCs and MCs. In addition, the aberrant enhancements in the γ oscillations and firing rates of MCs in the OB of APP/PS1 mice were recorded by multi-electrode arrays (MEAs). The local application of a $GABA_AR$ agonist nearly abolished the aberrant increase in γ oscillations in the external plexiform layer (EPL) at advanced stages of AD, whereas a $GABA_AR$ antagonist aggravated the γ oscillations. Based on our findings, we concluded that the altered morphologies of the synaptic structures of GCs, the dysfunction of reciprocal dendrodendritic synapses between MCs and GCs, and the abnormal γ oscillations in the EPL might contribute to olfactory dysfunction in AD.

Keywords: Alzheimer's disease, olfactory dysfunction, olfactory bulb, dendrodendritic inhibition, γ oscillations

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INTRODUCTION

Alzheimer's disease (AD), the most common neurodegenerative disorder, results in severe memory and learning impairments (Wei et al., 2010). Olfactory deficits have been proposed as a marker of AD because reductions in odor detection thresholds, recognition, and identification occur earlier than dementia in many patients (Wesson et al., 2010; Wu et al., 2013). The driving force of AD pathology is hypothesized to be the formation of toxic amyloid-β (Aβ) peptides that are

cleaved from amyloid precursor protein (APP), the subsequent development of neurofibrillary tangles (NFTs), and the resulting, cascade of secondary pathologies (Lachén-Montes et al., 2016). Therefore, studies aiming to reveal the relationship between neuropathological Aβ deposition and olfactory dysfunction have the potential to provide information about early AD pathology, and, ultimately, early diagnosis.

Olfaction involves various processes including sensory neuron inputs to the olfactory bulb (OB), decoding in the primary olfactory cortex, and ultimately transmission to downstream neurons in the amygdala, hippocampus, hypothalamus and nucleus accumbens (Wachowiak and Shipley, 2006; Wesson et al., 2010). The OB constitutes the first relay of the olfactory system (Lepousez and Lledo, 2013). In the OB, the excitatory sensory inputs from excitatory sensory neurons to mitral cells (MCs) trigger the release of glutamate from their lateral dendrites onto the dendrites of granule cells (GCs), and this section mediates the transmission of the GABAergic inhibition back to MCs (Isaacson and Strowbridge, 1998; Lepousez and Lledo, 2013). The recurrent and lateral inhibition supported by dendrodendritic reciprocal synapses between the dendrites of MCs' and GCs' mediates key roles in sensory processing, such as the gain control and odor selectivity of MC responses, which are crucial for proper odor discrimination (Abraham et al., 2010; Tan et al., 2010). As the largest population of interneurons in the OB, GCs are involved in the synchronization and establishment of the slow temporal firing patterns of MC activity (Schild, 1988; Friedrich and Laurent, 2001; Nusser et al., 2001; Lledo and Lagier, 2006). The function of GCs in inhibiting MCs has been studied using various approaches. However, researchers have not yet determined whether the morphology and function of dendrodendritic reciprocal synapses between GCs and MCs in the OB are impaired in patients with AD.

MCs extend lateral dendrites in the external plexiform layer (EPL) that are contacted by pedunculated, headed spines arising from the distal apical dendrites of GCs, which results in the establishment of the reciprocal dendrodendritic synapses between GCs and MCs (Phillips et al., 1963; Rall et al., 1966; Price and Powell, 1970). Reciprocal dendrodendritic synapses between GCs and MCs are involved in the generation of γ oscillations (40–100 Hz) in the OB (Lagier et al., 2007). As one band of the local field potential (LFP) oscillations in the OB, γ oscillations are induced by odorants and reflect the synchronized spike discharges from principal neurons (Beshel et al., 2007; Lagier et al., 2007; Lepousez and Lledo, 2013; Osinski and Kay, 2016). Notably, γ oscillations are observed in awake and anesthetized animals, as well as in brain slices *in vitro*. However, the role of γ oscillations in sensory coding, particularly in patients with AD, remains unclear.

In the present study, we identified the relationship between Aβ deposits within the olfactory processing network and odor perception in APP/PS1 mice during aging. In addition, we investigated the morphological and functional changes in the reciprocal dendrodendritic synapses by employing a multi-electrode array (MEA) to illustrate the network-level dynamics in response to pharmacological stimulation under excessive Aβ depositions in APP/PS1 mice at sequential age stages. The results showed that Aβ deposition induced morphological and functional changes in the dendrodendritic synapses in the EPL, and thus, contribute to a more in-depth illustration of the mechanism of olfactory disorders in AD.

MATERIALS AND METHODS

Animals

B6/JNju-Tg (APPswe, PSEN1dE9)/Nju (APP/PS1) mice, which coexpress human PS1 encoding the E9 deletion and mouse APP containing humanized APP and Swedish mutations (K594N, M595L), were employed in this study. The mice were bred and maintained within the animal facility at the Laboratory Animal Center of Zhejiang University, Chinese Academy of Sciences. Age-matched nontransgenic littermate, C57BL/6Nju (C57) mice, were used as controls. The animals were housed with three-five same-sex littermates per cage under standard conditions (20–22°C; 40%–60% humidity; 12-h light/dark cycle; and *ad libitum* access to water and food). All animal experiments were carried out in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals (NIH Publication No. 85-23, revised 1996), and the protocols were approved by the Institutional Animal Care and Use Committee of Zhejiang University. We studied 3–4-month-old (mo), 6–7-mo and 9–10-mo APP/PS1 mice and C57 mice to examine the possible contributions of accumulating Aβ deposits on olfaction over time. Both female and male mice were used in all the experiments. The ratio of female and male mice was approximately 1:1. No differences were observed between female and male mice.

Buried Food Test

A buried food test, which measures how rapidly an overnight-fasted animal locates a small piece of familiar palatable food, was performed as previously published described with minor modifications (Hu et al., 2016). Briefly, at approximately 24 h prior to testing, the 3–4-mo, 6–7-mo and 9–10-mo APP/PS1 and age-matched C57 mice were weighed and subjected to a food-restricted diet. On the testing day, all the mice were habituated to the testing room for 1 h prior to testing, and the mice were then allowed to acclimate to the cage for 5 min before being transferred to an empty clean cage. A small piece (10 mm cube) of the same food that the mouse was fed daily was then randomly placed in a random corner of a clean mice cage with ∼3 cm of woodchip bedding. Before the mouse was transferred, a small piece (10-mm cube) of the same food that the mouse was fed daily was placed ∼1 cm beneath the bedding in the clean mice cage. The experimental mouse was then placed in the testing cage at a constant distance from the hidden food. The time it takes the mice to find the food was recorded, and whether the food was consumed was also noted. If the mouse failed to find the buried food within 5 min, the test was stopped, and the latency score was recorded as 300 s. Twelve mice from each group were used in the buried food test.

Fine Olfactory Discrimination Test

The fine olfactory discrimination test was used to measure the olfactory discrimination ability of the mice by associating olfaction with taste aversion. The test was conducted using previously published protocols (Enwere et al., 2004; Zhu et al., 2014). After the buried food test, the same mice were separated into individual cages and deprived of water for 24 h. Each individual mouse was subjected to two stages of testing, a training stage and a testing stage, to obtain each data point. The training experiment was designed to encourage the mice to associate mango smells with palatable drinks and almond smells with bitterness. For the first training stage, a mixture of 10 μl of double-distilled water and 1 μl of mango extract (Mgo) was placed in a sterile 35 × 10-mm dish to allow the mice to habituate to the Mgo smell. The combination of distilled water and Mgo, which served as a reward for response, was designated [+]. The mice were allowed 2 min to find [+]. Thirty seconds after the mouse finished drinking the solution, a fresh [+] solution was provided. In the trials, the amount of Mgo was sequentially increased to 2.5, 4, 5.5, 7 and 8.5 μl. We repeated the last trial five times, and for the sixth trial, we presented the mice with 8.5 μl of almond extract (ALM) with 10 μl of a 1% denatonium benzoate (DB) solution (Sigma-Aldrich, St. Louis, MO, USA). The combination of ALM and DB was designated [−]. The experimental mice found the [−] solution extremely aversive, learned to associate the bitter taste with the smell of ALM because DB is extremely bitter, and subsequently avoided drinking the [−] solutions. An additional four trials were conducted with the [−] solution to ensure that the mice had learned the association between bitter taste and the smell of ALM. In the testing stage, the mouse was presented with two dishes, one of the dishes mainly contained [+], and the other contained [−]. For example, a 60:40 ratio of the odor components in task indicates that the composition ratio of Mgo in distilled water to almond in 1% DB in one dish was 60:40, and that in the other dish was 40:60. Successful discrimination occurred if the mouse drank [+] and did not perform any the following behaviors: (a) chose [−] rather than [+] or (b) chose both [+] and [−] within 30 s. A task was designated a total failure if the mouse tasted the [−] first. The mice that did not choose within 2 min were excluded from the analysis. Ten trials were conducted in each testing stage. In the additional testing sessions, which incorporated decreased contents of Mgo and ALM in [+] and [−], respectively, an appropriate training stage using the new [+] and [−] solutions was included prior to the testing stage.

Morris Water Maze Test

The Morris water maze (MWM) test, which was conducted according to previously described protocols (Guo et al., 2017), was performed to test the spatial learning and memory of the mice. After experiencing the buried food and fine olfactory discrimination tests, the same mice were subjected to the MWM test. To test their spatial acquisition abilities, all the mice were trained to find the platform and underwent four trials per day for five consecutive days. During the acquisition phase trials, if the mouse was able to escape onto the hidden platform within 90 s it was allowed to stay on the platform for 5 s. If the mouse failed to find the hidden platform within 90 s, it was guided to the platform by the experimenter and allowed to remain on the platform for 15 s to help it remember the platform's location. The probe trial was performed 24 h after the last acquisition trial to access the retention of the spatial memory. In this phase, the hidden platform was removed, and the mice were allowed to swim for 90 s in the pool. The numbers of platform crossings were recorded using a computerized tracking system.

Tissue Preparation

After the MWM tests, six randomly selected mice from each group were sacrificed under deep anesthesia with sodium pentobarbital (50 mg/kg) and perfused with 25 ml of 0.01 M phosphate-buffered saline (PBS, pH 7.4) and then with 100 ml of 4% paraformaldehyde in 0.1 M PB solution (pH 7.4). The OBs were removed, postfixed in the same fixative for 2 h and then cryoprotected for 36 h at 4°C in 0.1 M PB containing 30% sucrose. Olfactory sections (25 μm) were cut coronally using a freezing microtome (Leica CM2100, Germany) at −20°C and collected in a cryoprotectant fluid containing 30% ethanediol and 30% glycerinum in 0.01 M PBS. The olfactory sections were then stored at −20°C for immunofluorescence staining and Nissl staining. Six mice from each group that underwent the behavioral tests were anesthetized, perfused transcardially with ice-cold PBS (pH 7.4), and fixed with 0.5% paraformaldehyde. The brain tissues were then submerged in a Golgi-Cox solution containing 5% potassium dichromate, 5% mercuric chloride and 5% potassium chromate in distilled water. A new set of mice from each group were used for electron microscopy, Western blotting and MEA assays.

Immunofluorescence Staining

The olfactory sections of each group stored at −20°C were washed three times with PBS, blocked with 10% normal goat serum in PBS, and incubated with primary antibodies against Aβ (6E10, Invitrogen, Carlsbad, CA, USA, 1:500) overnight at 4°C. The sections were then rinsed with PBS and incubated with secondary antibodies conjugated to Alexa Fluor 549 (Invitrogen Technologies, 1:500) for 2 h at room temperature. The sections were subsequently rinsed twice with PBS, mounted on gelatin-coated glass slides with fluorescent sealant and cover-slipped. The sections were subsequently observed and imaged with a laser-scanning confocal microscope (Olympus FV1000, Japan). The photomicrographs were saved as TIFs and quantitatively analyzed using ImageJ software.

Nissl Staining

For Nissl staining, the olfactory sections from each group were rinsed three times with 0.01 M PBS, mounted on gelatin-coated glass slides and naturally dried at room temperature overnight. The sections were defatted by incubation with 75% ethanol at 37°C for 2 h, stained within 0.1% cresyl violet solution for 10 min at room temperature, and rinsed with water. The sections were then sequentially incubated with 70% ethanol (3 s), 80% ethanol (3 s), 90% ethanol (3 s), 95% ethanol (3 s), absolute ethanol I (3 s), absolute ethanol II (5 min), xylene I (10 min) and xylene

II (30 min). Permount was added to the slides, and the slides were then covered with coverslips. Images were captured using an Olympus microscope.

Golgi-Cox Staining

The brain tissues of each group were submerged in Golgi-Cox solution in the dark for 8 days, and the solution was replaced every 2–3 days. The brains were then dehydrated with a 30% sucrose solution, and the OBs were mounted on the vibratome platform and sectioned at 150 μm. The sections were rinsed twice (5 min each) with distilled water to remove any traces of the impregnating solution. The sections were dehydrated by incubation in 50% alcohol for 5 min, incubated with an ammonia solution (3:1, ammonia: distilled water) for 10 min, rinsed twice with distilled water, and incubated with 5% sodium thiosulfate solution for 10 min in the dark. The sections were then rinsed twice with distilled water, sequentially dehydrated in 50% ethanol I (5 min), 50% ethanol II (5 min), 70% ethanol (5 min), 80% ethanol (5 min), 95% ethanol (5 min), 100% ethanol I (5 min), add 100% ethanol II (10 min), cleared with xylene I (10 min) and xylene II (30 min) and mounted on gelatinized slides using Permount. A confocal microscope was then used to obtain the spine density in apical dendrites of GCs. A Z-stack of the optical section was captured using an Olympus FV1000 instrument with an 60× oil-immersion objective lens and a 10× optical zoom. When capturing the fluorescent signals, an excitation wavelength of 559 nm was used, and the light path was adjusted to mirror and receive the reflected light. For the analyses of the dendritic spine density and the neuron maturity, we adopted the criteria detailed in the published literature (Matsuda and Hisatsune, 2017). The dendritic spines of GCs were divided into mature spines (mushroom and thin spines) and immature spines (filopodia and stubby spines) according to their morphology. In this study, all the dendritic spines were categorized and quantified manually as immature or mature.

Western Blotting

Western blotting was conducted as described previously (Yang et al., 2016). Six OBs were dissected from APP/PS1 mice and age-matched C57 mice at different ages and then lysed using RIPA buffer supplemented with an EDTA-free protease inhibitor cocktail and phosphatase inhibitors. Twenty micrograms of total protein from each group were separated on 10% SDS-PAGE gels and transferred to PVDF membranes. Protein-bound PVDF membranes were incubated with rabbit anti-postsynaptic density 95 (anti-PSD95), β-actin (1:1,000, Cell Signaling Technology, Danvers, MA, USA) synaptophysin and synapsin I antibodies (1:1,000, Sigma, USA) overnight at 4°C. the membranes were then washed with TBST for 15 min, incubated with a horseradish peroxidase-conjugated goat anti-rabbit antibody (1:2,000; Cell Signaling Technology, Danvers, MA, USA) for 2 h, and then washed with TBST. The membranes were subsequently processed for detection using the ECL system. The protein levels were quantified by assessing their optical densities using Quantity One software and are expressed as ratios relative to β-actin.

Electron Microscopy

Six mice in each group were anesthetized, and their brains were removed. The OB was sliced coronally into 0.2-mm slices using a vibrating slicer and then postfixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 12 h. After three washes with 0.1 M PBS (10 min each), the OB slices were exposed to 1% osmium tetroxide for 2 h, washed several times with water, and dehydrated with a gradient series of alcohol solutions (2 × 10 min with 50%, 2 × 10 min with 70%, 2 × 10 min with 90%, and 2 × 10 min with 100%). The sections were subsequently embedded in epon resin, and randomly selected ultrathin sections were stained with uranyl acetate and lead citrate. Three slides per animal and three fields within each granule layer and EPL per slide were randomly selected to quantify the number of synapses and measure the thickness of the PSD. Each field was imaged at 26,500× magnification using a transmission electron microscope (Tecnai G2 F20 S-TWIN, FEI). The number of synapses and the thickness of the PSD in the granule layer and EPL were analyzed in 15 images from each mouse by an experimenter who was blinded to the treatment and genotype using Image Pro Plus 6.0 software.

MEA Assay

Six mice from each group were deeply anesthetized, and their brains were rapidly excised from their skulls and submerged in ice-cold cutting solution containing 2.34 mM sucrose, 5 mM KCl, 1.25 mM NaH_2PO_4, 5 mM $MgSO_4$, 26 mM $NaHCO_3$, 25 mM glucose, and 1 mM $CaCl_2$ for 5 min. Slices were cut from the brain samples using a vibratome (Leica Microsystems, Germany) while the samples were submerged in a chamber filled with ice cold cutting solution. The OB was cut into 250-μm coronal sections, and the tissue slices were allowed to recover in an oxygenated artificial cerebrospinal fluid (ACSF) solution (119 mM NaCl, 2.5 mM KCl, 1.0 mM NaH_2PO_4, 26.2 mM $NaHCO_3$, 11 mM glucose, 1.3 mM $MgSO_4$, and 2.5 mM $CaCl_2$) at 34°C for 0.5 h and then incubated at 28°C for at least for 1 h. OB slices were transferred to an MEA chip (60 Square MEA200/50iR-Ti-gr), continuously perfused with oxygenated ACSF, and saturated with 95% O_2/5% CO_2 (pH 7.4 and 325 mOsm/kg) at 28°C at a rate of 1 ml/min. All the experiments were conducted through a 6-min recording in ACSF. In addition, control slices that were continuously treated with ACSF after the initial 5 min showed no change in frequencies, sites of initiation, and level of propagation. We treated the OB slices with a $GABA_A$ receptor antagonist (bicuculline, 10 μM) and an agonist (muscimol, 100 μM) to assess the synaptic events between GCs and MCs in response to pharmacological stimulation. The drugs were perfused over the slices through gravity at a rate of 3 ml/min. The chemicals were acquired from Tocris Bioscience (Ellisville, MO, USA) and Sigma-Aldrich Canada (Oakville, ON, Canada). The neuronal network activity was recorded using an MEA (MEA2100-System, Reutlingen, Germany) with 60 platinum electrodes, a 50-μm electrode diameter and a 200-μm interelectrode spacing. An OB slice was positioned over the array such that the slice was in close contact with the microelectrode array electrodes throughout the recording. The electrical activity

recorded from each channel was digitized at a sampling rate of 25 kHz and acquired using a MEA2100 amplifier (Multi Channel Systems, Reutlingen, Germany). The temperature in the recording chamber was constantly monitored and maintained at 28°C by a heated perfusion cannula (TC02, Reutlingen, Germany).

Experimental Design and Statistical Analysis

The data were analyzed and performed using GraphPad Prism version 6.0 (GraphPad software). The escape latency data obtained from the MWM test were analyzed by two-way repeated-measures analysis of variance (ANOVA) with Bonferroni post-tests. For the rest of the data, the statistical significance of the differences in the means between two groups was assessed by Mann-Whitney or two-tailed unpaired *t*-tests, as appropriate. A value of $p < 0.05$ was considered to indicate statistical significance. All the data are expressed as the means ± standard errors of the means (SEMs). In all the figures, $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

RESULTS

Olfactory Dysfunction Occurs Earlier Than Cognitive Impairment in APP/PS1 Mice

We performed two behavioral tests to assess whether the olfaction function of APP/PS1 mice was altered: the buried food test and the olfactory discrimination test. In the buried food test, the time each individual mouse required to find the hidden food was recorded (**Figure 1A**). As shown in

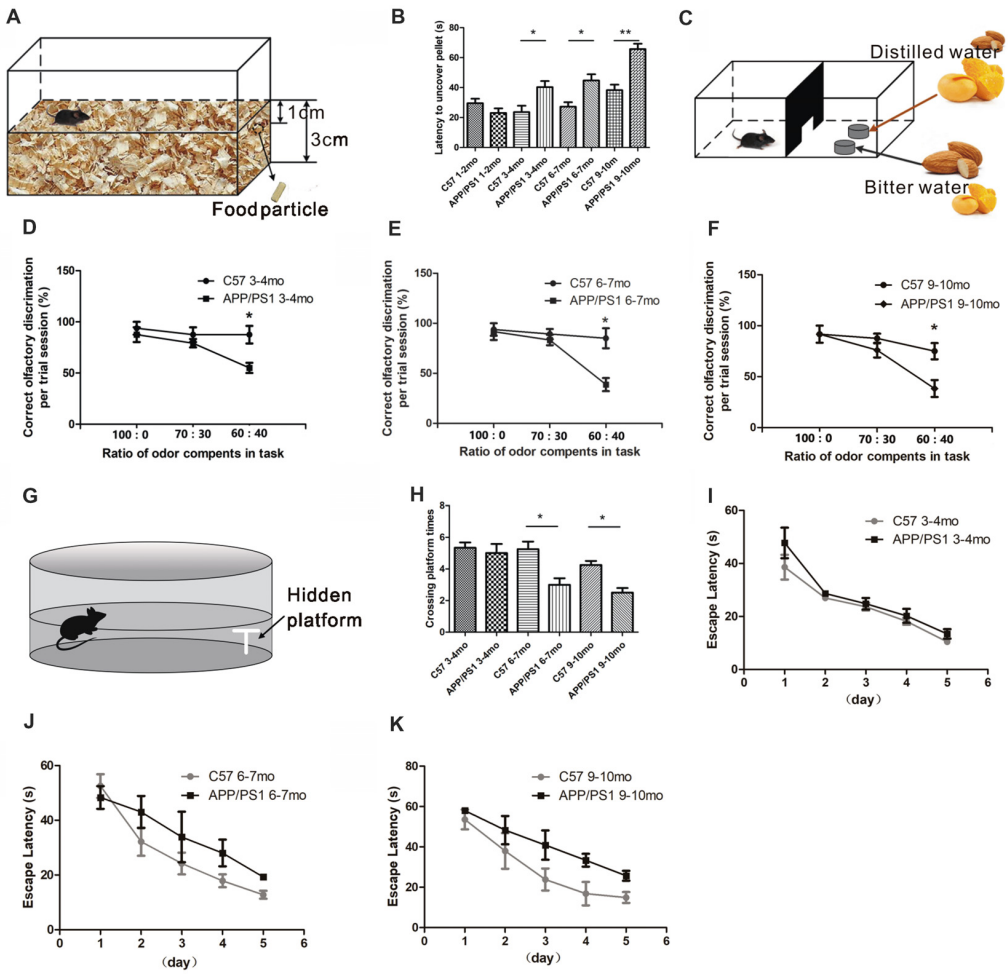

FIGURE 1 | Atypical olfactory and cognitive behaviors of amyloid precursor protein (APP)/PS1 mice. **(A)** Design of the buried food test. **(B)** Quantitative statistical analysis of latency of 3–4-, 6–7 and 9–10-month-old (mo) APP/PS1 and C57 mice to find the hidden food particle. **(C)** Design of the fine odor discrimination test. Mice were deprived of water for 24 h and trained to identify distilled water. In one dish, the distilled water was coupled with an odor mixture of mango and almond, in which mango was the main component. The other dish contained bitter water with an odor mixture in which almond was the main component. Different odor proportions of mango and almond were used in the performance test. **(D–F)** Performance of APP/PS1 and C57 mice at 3–4 **(D)**, 6–7 **(E)**, and 9–10 months of age **(F)**. **(G)** Design of the Morris water maze (MWM) test. **(H)** Quantitative statistics of the crossing platform times of APP/PS1 and C57 mice at 3–4, 6–7 and 9–10 months of age. **(I–K)** Escape latencies of APP/PS1 and C57 mice at 3–4 **(I)**, 6–7 **(J)** and 9–10 months of age **(K)**. The data are presented as the means ± standard errors of the means (SEMs). $^{*}p < 0.05$; $^{**}p < 0.01$.

Figure 1B, the 3–4-mo ($p < 0.05$), 6–7-mo ($p < 0.05$) and 9–10-mo ($p < 0.01$) APP/PS1 mice needed more time to find the hidden food than the age-matched C57 mice, indicating an impairment in their olfactory performance. The same groups of mice that were subjected to the hidden food test were subsequently subjected to the olfactory discrimination test. In the olfactory discrimination test, the mice were acquainted with mango flavor mixed with distilled water and with an almond flavor mixed with bitter water. The olfactory capabilities of the mice were determined based on their ability to distinguish between mixtures containing different fractions of mango and almond smells, which have distinct aromas (**Figure 1C**). The accuracy of the discrimination between mixtures containing more than 60% of either mango flavor or almond odor based on their smells was comparable. The variable trends in the results from the olfaction discrimination test were consistent with those from the buried food test. Compared with the C57 mice, the 3–4-mo ($p < 0.05$, **Figure 1D**), 6–7-mo ($p < 0.05$, **Figure 1E**) and 9–10-mo ($p < 0.05$, **Figure 1F**) APP/PS1 mice showed decreases in the numbers of correct olfactory discrimination responses per trial, indicating that the level of olfactory discrimination became increasingly impaired as the animals aged. Thus, APP/PS1 mice exhibited olfactory dysfunction at 3–4 months of age.

We then evaluated the hippocampal-dependent spatial learning and memory abilities of APP/PS1 and age-matched C57 mice at sequential age stages using the MWM test (**Figure 1G**). In this test, the escape latencies of the mice were measured during the 5-day acquisition phase, and the time of platform site crossings was recorded in the probe trial. There were no significant differences in the crossing plate times between the APP/PS1 and age-matched C57 mice at 3–4 months of age ($p < 0.05$, **Figure 1H**). In addition, no significant differences in escape latency were found in the acquisition phase trials between the APP/PS1 and C57 mice at 3–4 months of age ($F = 3.883$; $p = 0.0628 > 0.05$, **Figure 1I**), indicating that the 3–4-mo APP/PS1 and control mice have equal abilities to encode and remember the spatial coordinates of the platform. At 6–7 months of age, significantly decreased crossing plate times in probe trial ($p < 0.05$, **Figure 1H**) and a tendency to require a longer time to find the platform ($F = 0.2689$; $p = 0.1049 > 0.05$, **Figure 1J**) were observed in the APP/PS1 mice compared with the control mice, indicating that the spatial memory of APP/PS1 mice was impaired. The 9–10-mo APP/PS1 mice needed a longer time to find the platform ($F = 9.12$; $p = 0.0056 < 0.01$, **Figure 1K**) and exhibited fewer crossings over the former platform site than the control mice ($p < 0.05$, **Figure 1H**). The above-mentioned results showed that the spatial discrimination learning and memory impairments in APP/PS1 mice started to occur at 6–7 months of age. Based on the results from the behavioral tests, the 3–4-mo APP/PS1 mice suffered olfactory deficits but not cognitive impairments. We thus hypothesized that the 3–4-mo APP/PS1 mice can be

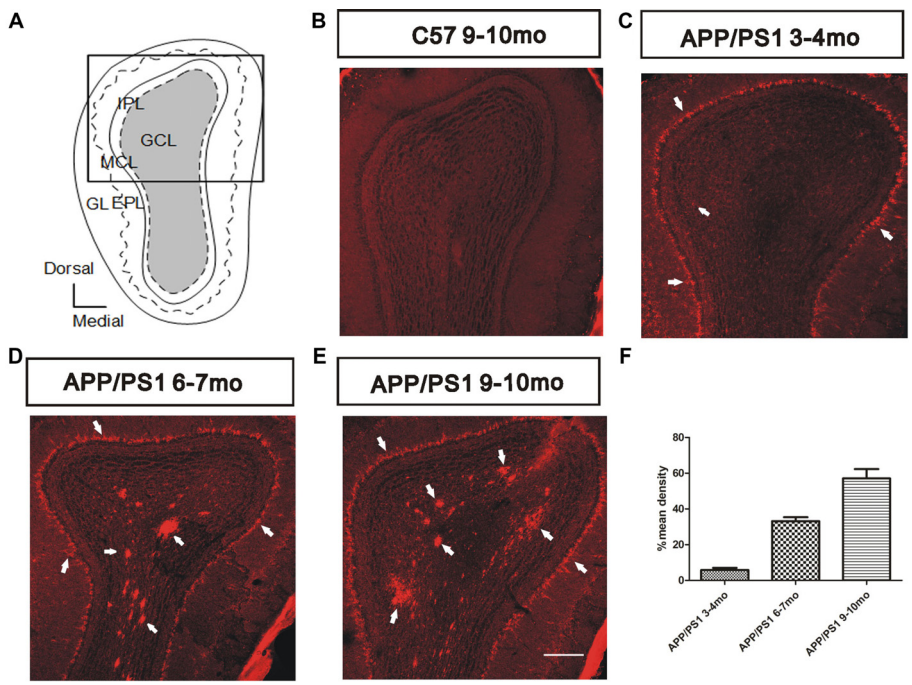

FIGURE 2 | Spatiotemporal pattern of amyloid-β (Aβ) depositions in the olfactory bulb (OB) of APP/PS1 mice. **(A)** Image of the OB coronal plate indicating the bulbar cell layers [glomerular layer (GL), external plexiform layer (EPL), MCL, inner plexiform layer (IPL) and granule cell layer (GCL)]. **(B)** No Aβ was observed in the OB of C57 mice at 9–10 months of age. **(C–E)** Representative images of Aβ staining (white arrows) in the OB of APP/PS1 mice at 3–4, 6–7 and 9–10 months of age. Scale bars = 200 μm in (**E**; applies to **B–E**). **(F)** The mean percentage density of deposited Aβ was elevated in the GCL within the OB cell layers of APP/PS1 mice. The error bars indicate the SEMs.

considered to be at a relative early stage of AD. In addition, the 9–10-mo APP/PS1 mice suffered olfactory deficits and cognitive impairments and were considered representative of late-stage AD.

Deposition of Soluble Aβ Aggregates in the Olfactory Bulb

According to recent evidence, soluble Aβ is strongly correlated with olfactory dysfunction in patients with AD (Wu et al., 2013; Wang et al., 2016). We characterized Aβ deposition in APP/PS1 mice through immunostaining for Aβ using the anti-Aβ antibody 6E10 (1:2,000 diluted) to investigate the spatiotemporal pattern of Aβ aggregates in the OB (**Figure 2A**). The results showed gradual increases in the amount, degree of aggregation, and spatial distribution of Aβ deposits from the outer to the inner layers in APP/PS1 mice with increasing age and negative staining for Aβ in 9–10-mo C57 mice (**Figure 2B**). At 3–4 months of age, the MCL developed obvious Aβ deposits (**Figure 2C**). In addition, Aβ began to accumulate in the GC layer (GCL) and was secreted into the extracellular space (**Figure 2C**). At 6–7 months of age, the Aβ burden spread throughout the GCL (**Figure 2D**), and an increased Aβ burden was observed in all layers in 9–10-mo APP/PS1 mice (**Figure 2E**). In both 6–7-mo and 9–10-mo APP/PS1 mice, Aβ was almost exclusively located within the GCL and MCL in APP/PS1 mice (**Figure 2F**), and only rare Aβ deposits were observed in the glomerular layer (GL), EPL, or inner plexiform layer (IPL).

Effect of Aβ Exposure on the Laminar Organization of the OB

Nissl staining for Nissl bodies was performed to visualize the structure of the OB and thus explore the effects of Aβ depositions on the overall morphology of the OB. A distinct laminar organization, with intact glomeruli and clear layers, was observed in the OB. During aging, the morphology of the OB remained unchanged in APP/PS1 mice compared with the age-matched C57 mice (**Figures 3A–F,A1–F1**).

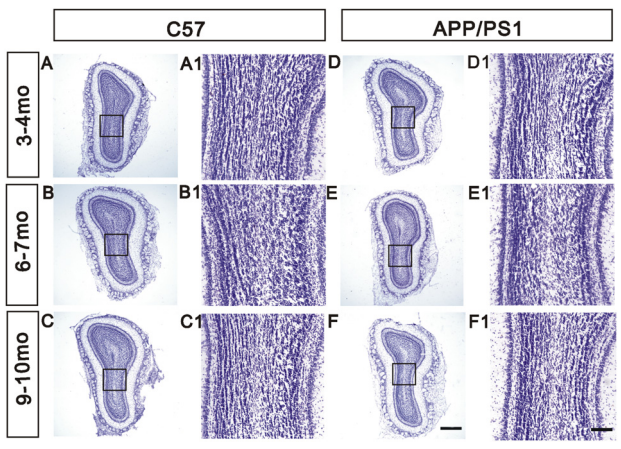

FIGURE 3 | Unchanged laminar organization of the OB in APP/PS1 mice. **(A–F)** No significant change in the laminar organization of the OB was observed between APP/PS1 and age-matched C57 mice at different ages. **(A1–F1)** High-magnification images showing the structure of the GCL. Scale bars = 500 μm in (**F**; applies to **A–F**) and 100 μm in (**F1**; applies to **A1–F1**).

The Dendritic Spine Density of GCs Is Reduced in the OB of APP/PS1 Mice

We analyzed the dendritic spine density of GCs in the OB through Golgi staining to investigate the mechanisms through which Aβ mediates olfactory deficits in APP/PS1 mice. The morphology of the dendritic spines in GCs in the adult OB is heterogeneous based on the dendrite distributions and dendro-dendritic synaptic partners (Nagayama et al., 2014; McDole et al., 2015). An examination of the dendritic spine density of GCs indicated a qualitatively significant decrease in the APP/PS1 mice compared with the age-matched C57 mice (**Figure 4A**). The dendritic spine density of GCs in APP/PS1 mice tended to decrease at 3–4 months of age ($p > 0.05$, **Figure 4B**), but significant decreases were observed in both 6–7-mo ($p < 0.05$, **Figure 4B**) and 9–10-mo ($p < 0.001$, **Figure 4B**) APP/PS1 mice compared with their age-matched control mice. The spines of GCs were classified as mature and immature, and confocal observations revealed that the mature spine density was approximately the same in 3–4-mo APP/PS1 and C57 mice ($p > 0.05$, **Figure 4C**), but significantly impaired in 6–7- and 9–10-mo APP/PS1 mice compared with their age-matched C57 mice ($p < 0.05$, **Figure 4C**). Compared with that in the age-matched control mice, the immature spine density of GCs in APP/PS1 mice tended to decline at 3–4 and 6–7 months of age ($p > 0.05$, **Figure 4D**) but was significantly reduced at 9–10 months of age ($p < 0.01$, **Figure 4D**). We further explored the molecular mechanisms underlying Aβ overexpression-induced olfactory deficits. Specifically, a Western blotting analysis was performed to examine the levels of several synaptic proteins, including a postsynaptic protein (PSD95) and two presynaptic proteins (synaptophysin and synapsin I), and the results revealed that their expression was gradually decreased in APP/PS1 mice compared with C57 mice (**Figures 4E–H**).

Amyloid-Beta Overexpression Impairs Synaptic Ultrastructure Parameters

An electron microscopy analysis was performed to investigate possible changes in the morphology of the reciprocal dendrodendritic synapses in the EPL. Specifically, we examined various synaptic components, including the minor axis diameter of the synaptic vesicle, the synaptic cleft size, and the thickness of PSD, to demonstrate the distinctive features of reciprocal dendrodendritic synapses. At 3–4 months of age, the synaptic vesicles surrounding the synaptic membrane were transparent and clear in both APP/PS1 and C57 mice ($p > 0.05$, **Figure 5A**). However, the presynaptic and postsynaptic membrane structures of asymmetric synapses, which are considered excitatory synapses, were damaged. At 6–7 and 9–10 months of age, an unclear and broken membrane structure and blurred boundaries for the synaptic vesicles were observed in APP/PS1 mice. Significant differences in the synaptic cleft ($p > 0.05$, **Figure 5B**) and the diameters of synaptic vesicles ($p > 0.05$, **Figure 5C**)

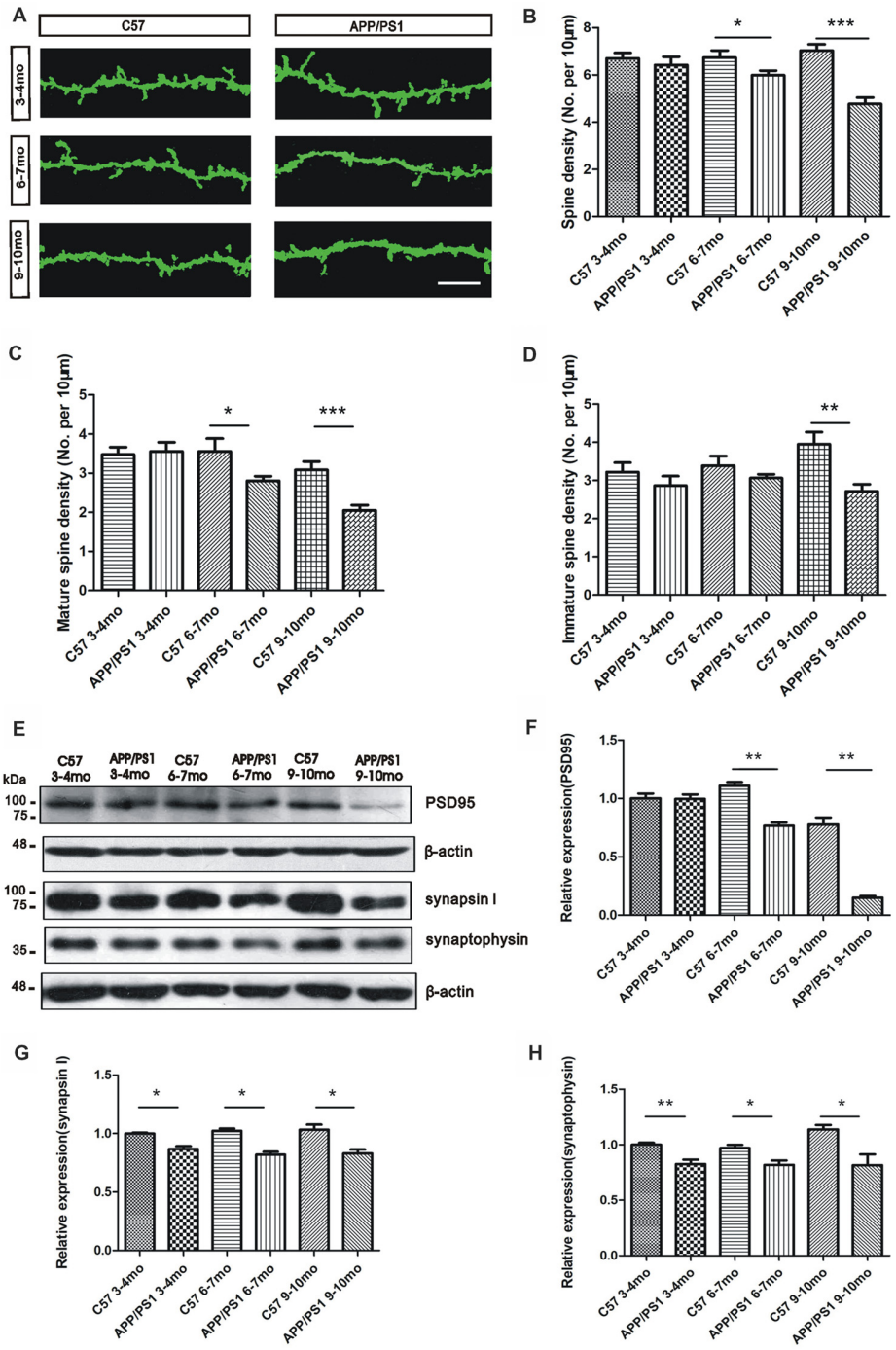

FIGURE 4 | Reduced dendritic spine density of GCs in APP/PS1 mice. **(A)** Representative images of Golgi-stained apical dendritic spines of GCs in APP/PS1 and C57 mice at 3–4, 6–7 and 9–10 months of age. Scale bar = 10 μm. **(B–D)** Quantitative analysis of the dendritic spine density **(B)**, mature spine density **(C)** and immature spine density **(D)** from randomly selected dendritic segments of GCs in APP/PS1 and age-matched C57 mice. **(E–H)** Western blotting assays of postsynaptic density95 (PSD95; **F**), synapsin I **(G)** and synaptophysin **(H)** in the OB; the levels of these proteins were reduced in APP/PS1 mice compared with age-matched C57 mice. The data are expressed as the means ± SEMs. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

were not observed between APP/PS1 and C57 mice. However, compared with the C57 mice, the thickness of the PSD ($p < 0.05$, **Figure 5D**) in asymmetric synapses was thinner in 3–4-, 6–7-, and 9–10-mo APP/PS1 mice. We subsequently examined the structures of the symmetric synapses in the EPL (**Figure 5E**), which are also known as inhibitory synapses, and the thickness of the presynaptic membrane was similar to that of the postsynaptic membrane. The integrated structures of synaptic membranes

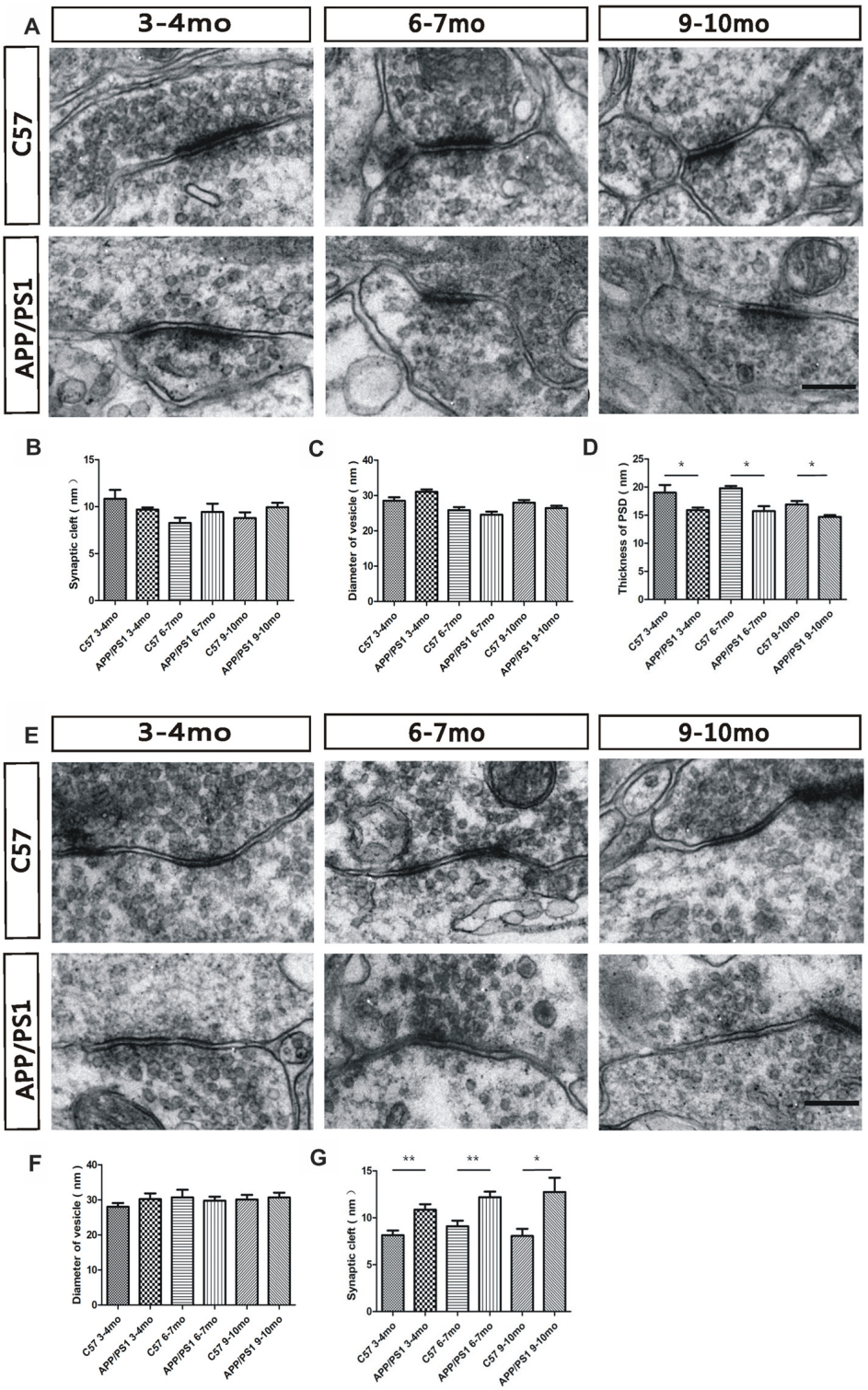

FIGURE 5 | Ultrastructural characterization of dendrodendritic synapses in the EPL. **(A)** The morphology of asymmetric synapses between GC dendrites and mitral cell (MC) dendrites in the EPL of APP/PS1 and C57 mice. **(B,C)** The synaptic clefts and diameter of the vesicles in asymmetric synapses were not significantly different between APP/PS1 and age-matched C57 mice. **(D)** The APP/PS1 mice showed a reduced thickness of the PSD in asymmetric synapses compared with the age-matched C57 mice. **(E)** Morphological structures of symmetric synapses between GC dendrites and MC dendrites in the EPL of APP/PS1 and C57 mice. **(F)** The diameters of the vesicles in symmetric synapses were not significantly different between APP/PS1 and age-matched C57 mice. **(G)** The synaptic clefts were wider in APP/PS1 mice than in age-matched C57 mice. The data are presented the means ± SEMs. The scale bar represents 200 nm. $^{*}p < 0.05$; $^{**}p < 0.01$.

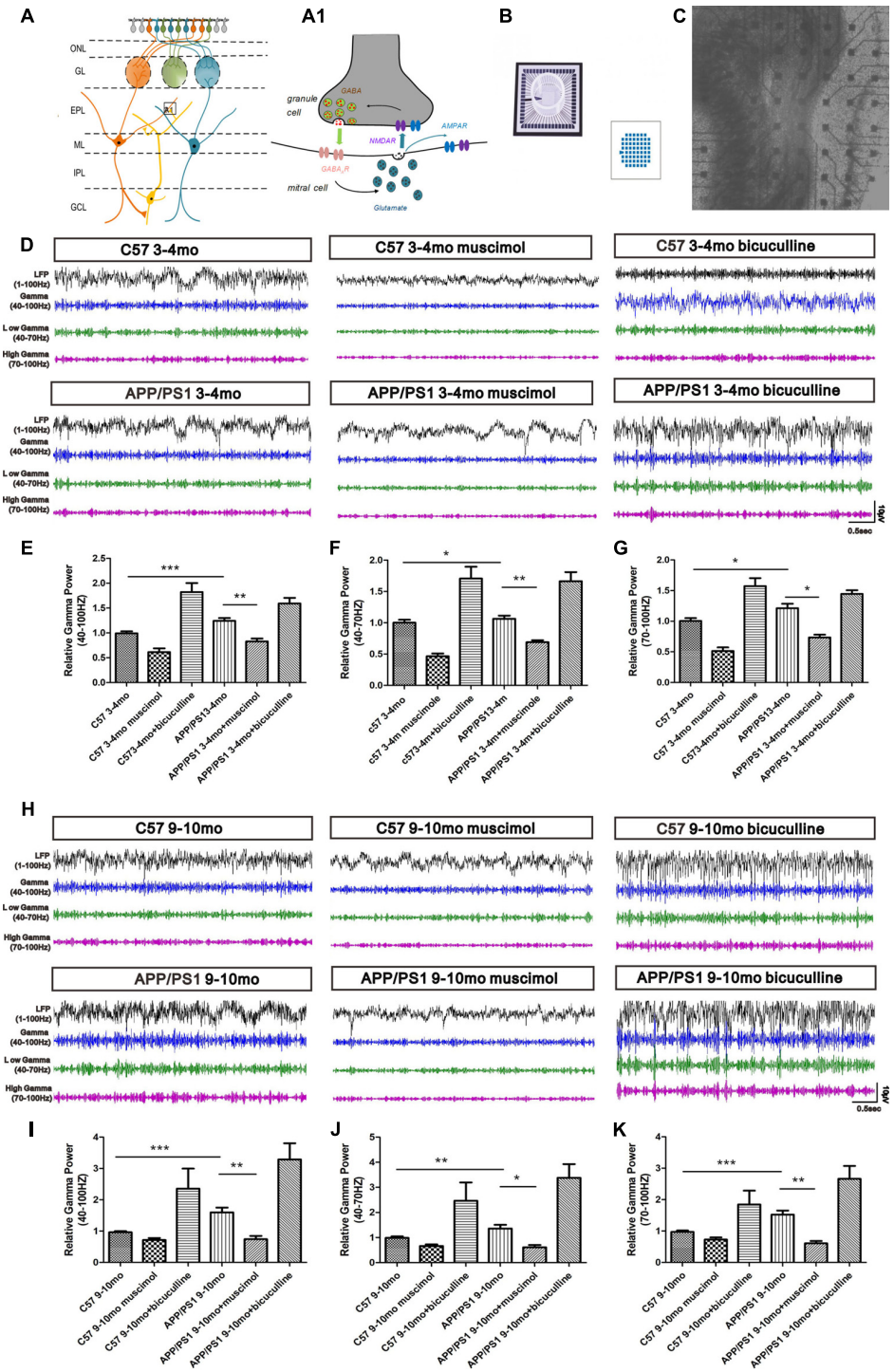

FIGURE 6 | Spontaneous γ oscillations in the EPL of OB slices. **(A)** Schematic showing the locations of MCs and GCs and the dendrodendritic synapses between MC and GC dendrites. Dendrites of MCs receive signals from olfactory sensory neuron termini in the GCL and form synaptic connections with GCs in the EPL. **(A1)** Action potential (AP) propagation in the MC lateral dendrites release glutamate (black vesicles) to activate postsynaptic AMPA and NMDA receptors in the GC dendrites. In turn, NMDA receptors trigger GABA release (red vesicles) and postsynaptic activation of $GABA_AR$ in MCs. In addition, glutamate released from MCs also activates extrasynaptic glutamate autoreceptors on MC lateral dendrites, resulting in spontaneous excitatory transmission. **(B)** Example of a 60-channel multi-electrode array (MEA). **(C)** Photograph of an OB slice placed on an MEA chip; the subregions of the OB are indicated. **(D)** Representative traces of local field potential (LFP) signals (1–100-Hz bandwidth) and filter traces (γ, low-γ and high-γ) of the EPL in the C57 group, APP/PS1 group, APP/PS1 + muscimol group and

(*Continued*)

FIGURE 6 | Continued
APP/PS1 + bicuculline group at 3–4 months of age. **(E–G)** Muscimol weakened the aberrantly increased γ oscillations in 3–4-mo APP/PS1 and C57 mice. Bicuculline enhanced the aberrantly elevated γ oscillations in 3–4-mo APP/PS1 and C57 mice. **(H)** Representative traces of LFP signals (1–100-Hz bandwidth) and filter traces of the EPL in 9–10-mo C57 and APP/PS1 mice. **(I–K)** Muscimol weakened the aberrantly increased γ oscillations in 9–10-mo APP/PS1 and C57 mice, and bicuculline enhanced the aberrantly elevated γ oscillations in 9–10-mo APP/PS1 and C57 mice. The data are presented as the means ± SEMs. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

and the approximate diameters of the synaptic vesicles in APP/PS1 mice were similar to those of the C57 mice ($p > 0.05$, **Figure 5F**). However, the APP/PS1 mice displayed a wider synaptic cleft than the age-matched C57 mice (**Figure 5G**). These results demonstrate morphological alterations in the reciprocal dendrodendritic synapses in the EPL of APP/PS1 mice, which potentially indicates changes in the function of synapses between GCs and MCs in the presence of excess Aβ depositions.

Pharmacological Characterization of γ Oscillations in APP/PS1 Mice

In the OB slices, the LFPs in the EPL are induced by dendrodendritic excitation/inhibition between GCs and MCs (**Figures 6A,A1**). The LFP signals in the OB are composed of bursts of γ oscillations (40–100 Hz), which are split into two subbands, the low-γ (40–70 Hz) band and the high-γ (70–100 Hz) band. We investigated the LFPs in the OB through a spontaneous exploration of 3–4-mo and 9–10-mo APP/PS1 and C57 mice using a MEA to assess the effect of Aβ on γ oscillations (**Figures 6B,C**). The 3–4-mo APP/PS1 mice were in the early phase of olfaction disorder, and the 9–10-mo APP/PS1 mice suffered serious olfaction disorder. The trend in the variations in γ oscillations was similar in the 3–4-mo and 9–10-mo experimental mice and reflected the aberrant synaptic transmission in both the early and late stages of AD.

However, the difference between APP/PS1 and control mice at 9–10 months of age was greater than that at 3–4 months of age. At 3–4 months of age, increased γ, low-γ and high-γ oscillations were observed in the EPL of APP/PS1 mice compared with their age-matched C57 mice (**Figures 6D–G**). Pharmacological treatments with a $GABA_AR$ agonist (muscimol) significantly reduced the γ oscillations, and treatment with an antagonist (bicuculline) notably increased the γ oscillations (**Figures 6D–G**). The γ, low-γ and high-γ oscillations in the EPL of 9–10-mo APP/PS1 mice were substantially enhanced compared with those in their age-matched C57 mice (**Figures 6H–K**). Treatment with $GABA_AR$ agonist (muscimol) reduced the γ, low-γ and high-γ oscillations in APP/PS1 mice, whereas the $GABA_AR$ antagonist (bicuculline) boosted the γ, low-γ and high-γ oscillations (**Figures 6H–K**).

Amyloid-Beta Overexpression Induces the Overexcitability of MCs

To explore the effect of Aβ deposition on the spontaneous activity of MCs, a MEA was used to record the MC spontaneous firing rate of spontaneous action potentials (sAPs). The results showed that MCs in APP/PS1 mice at 3–4 ($p < 0.01$, **Figures 7A,B**) and 9–10 months of age ($p < 0.001$, **Figures 7C,D**) displayed a relatively higher spontaneous firing rate of sAP than their age-matched control mice (**Figures 7A–D**), indicating the hyperactivation of MCs in both the early and late stage of AD.

DISCUSSION

In this study, we investigated the structural and functional changes in the reciprocal dendrodendritic synapses between GCs and MCs in the OB of APP/PS1 mice. During aging, increases in the Aβ deposition in the GCL induced a gradual intensification of olfactory deficits. A reduced dendritic spine density in GCs, decreased protein levels of synapse markers in the OB, and damaged reciprocal dendrodendritic synaptic structures were observed in the APP/PS1 mice. Functionally, disrupted γ oscillations and increased firing rates of MCs were observed in the OB of APP/PS1 mice. The morphological and functional changes in the reciprocal dendrodendritic synapses suggested that the impaired inhibition of MCs from GCs might be a critical mechanism underlying olfactory dysfunction in patients with AD.

As the most common neurodegenerative disorder in the elderly, AD is clinically characterized by progressive declines in memory and learning symptoms, which occur later than olfactory deficit (Djordjevic et al., 2008; Wu et al., 2013; Hu et al., 2017). Olfaction, including the detection threshold, odor identification and odor discrimination, is one of the most important sensations in mammals (Lepousez and Lledo, 2013). AD is a progressive neurodegenerative disease induced by multiple factors, such as apolipoprotein E genetic risk variants, excessive levels of phosphorylated tau protein, immunoinflammatory responses and Aβ deposition, all of which contribute to AD pathogenesis through the Aβ-dependent pathway (Chen et al., 1996; Yu et al., 2014; Moosavi et al., 2015). Most scholars believe that Aβ is the common pathway to AD and is caused by diverse stimuli that play critical roles in the pathogenesis of AD. In addition, it has been hypothesized that Aβ deposits in the olfactory system promote AD pathology in rodents (Wesson et al., 2010, 2011). *In vivo*, excessive Aβ depositions within the hippocampus lead to learning and memory deficits (Kim et al., 2013; Birnbaum et al., 2015; Lei et al., 2016; Wei et al., 2018). Similarly, Aβ depositions in the OB affected olfactory-related behavioral performances in APP/PS1 mice. As the main etiology of AD, the expression and location of Aβ deposits play critical roles in the pathological progression of AD. The degree of Aβ aggregates that accumulate in cognitive cortices through the olfactory pathway during aging is associated with the degree of olfactory dysfunction, ranging from the odor detection threshold to spatial memory (Wu et al., 2013). Based on the results from the olfactory behavioral tests, the APP/PS1 mice displayed progressively aggravated olfactory deficits at 3–4 months of age, when nonfibrillar Aβ depositions were mainly detected within MCs but slightly observed within the GCL. The Aβ depositions in the

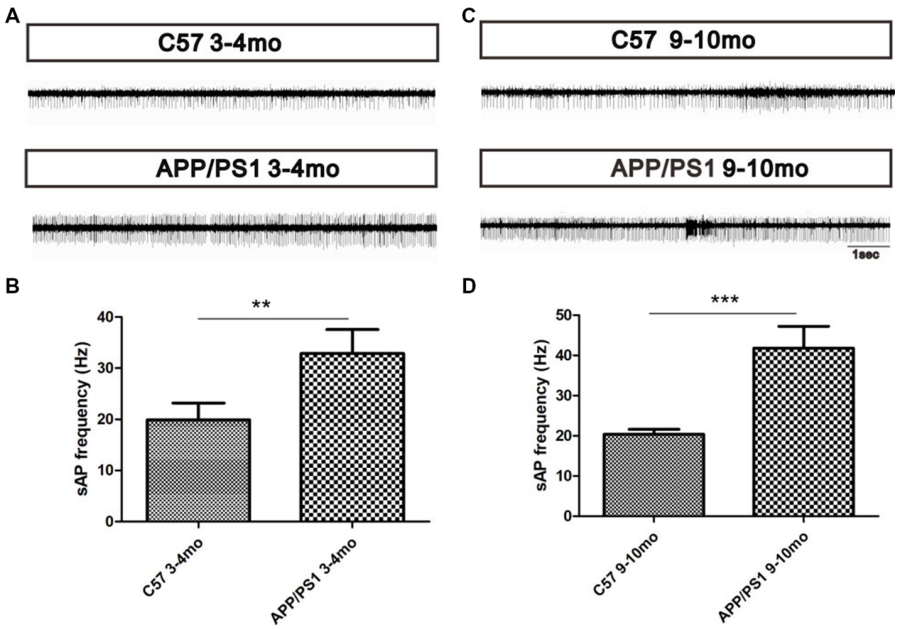

FIGURE 7 | Increased MC spontaneous firings in APP/PS1 mice. **(A)** Spontaneous firing rates of MCs in APP/PS1 and C57 mice at 3–4 months of age. **(B)** Quantitative analysis of spontaneous firing rates of MCs in APP/PS1 and C57 mice at 3–4 months of age. **(C)** Spontaneous firing rates of MCs in APP/PS1 and C57 mice at 9–10 months of age. **(D)** Quantitative analysis of spontaneous firing rates of MCs in APP/PS1 and C57 mice at 9–10 months of age. The data are presented as the means ± SEMs. $^{**}p < 0.01$; $^{***}p < 0.001$.

OB of APP/PS1 mice at 3–4 months of age precedes previous reports of Aβ in the entorhinal cortex and hippocampus, which are involved in learning and memory (Wu et al., 2013; Vasavada et al., 2015; Misiak et al., 2017), and this finding might explain why the 3–4-mo mice suffered olfactory deficits but did not experience memory impairments. The APP/PS1 mice suffered serious olfactory deficits at 6–7 and 9–10 months of age, when the Aβ depositions were mainly accumulated in the GC and MC layers. The GC-mediated regulation of MCs in the OB constitutes the basis for olfactory information processing and transmission processes. Whether the dendritic morphology of GCs and the GC-mediated regulation of MCs in the presence of excessive Aβ deposition were the focus of our research.

In the OB, the functional regulation of GCs is essential for the normal processing of odor information (McDole et al., 2015). In addition, the dendritic morphology of GCs endows the spines with various functional properties (Jiang et al., 2015). Dendritic spines are highly plastic structures that are capable of undergoing adaptive morphological and physiological changes, both during development and in adulthood (Engert and Bonhoeffer, 1999; Tada and Sheng, 2006; Yoshihara et al., 2009; Bosch and Hayashi, 2012; McDole et al., 2015). The dendrite morphology is regulated by many factors, including, PSD95 which can regulate the structure and function of dendritic spines in the brain (Engert and Bonhoeffer, 1999). The neurotransmitter release of synaptic vesicles at nerve terminals involves synaptic vesicle-associated proteins, including synaptophysin and synapsin I, which are related to synaptic transmission and synaptic plasticity in neural networks (Pieribone et al., 1995; Zhang et al., 2016; Chai et al., 2017). Our results showed nonsignificant changes in the dendritic spine density and in the numbers of mature and immature dendritic spines of GCs and decreased protein levels of synaptophysin and synapsin I in 3–4-mo APP/PS1 mice. The results implied that decreased synaptic vesicle-associated protein levels preceded the impairments in the synaptic structure and density. However, the APP/PS1 mice started to show olfactory deficits at 3–4 months of age. We hypothesized that the dendritic spines that form synapses could survive but might have been damaged for a long time. This finding indicated that the impaired synaptic transmission caused by decreased levels of synaptic vesicle-associated proteins, which could be verified by aberrant increases in γ oscillations, might play a key role in abnormal olfactory information integration in the OB and olfactory deficits at the early stage of AD. The 6–7-mo and 9–10-mo APP/PS1 mice showed a gradual accumulation of Aβ depositions within the OB and an aggravated AD pathology, and these effects were accompanied by gradual decrease in the synaptic protein levels and dendritic spine density of GCs, which suggested a decreased number of inhibitory synapses and a weaker inhibitory effect of GCs on MCs in the presence of excessive Aβ depositions. The decreased expression of synaptic-associated proteins and the altered morphology of dendrites of GCs in APP/PS1 mice indicated GC dysfunction in the presence of excess deposited Aβ.

In the OB, GCs establish most of their connections with MCs through ubiquitous dendrodendritic synapses, known as "reciprocal synapses" (Schoppa and Urban, 2003; Shepherd et al., 2007; Bardy et al., 2010), and these reciprocal synapses consist of a presynaptic site of an asymmetrical synapse from

MCs to interneurons and a postsynaptic site of a symmetrical synapse that reciprocally connects interneurons to mitral/tufted cells (Isaacson and Strowbridge, 1998). At the early stage of olfactory deficit, pathological changes in the reciprocal synaptic structures in the EPL of APP/PS1 mice were observed, and these changes were followed by more severe lesions with increasing age. At the late stage of AD, the asymmetrical synapses from MCs to interneurons suffered serious damage, such as blurred membrane boundaries and a decreased thickness of the PSD, suggesting aberrant excitatory transmission from MCs to GCs. The integrated structures of synaptic membranes, the approximate diameters of synaptic vesicles, a wider synaptic cleft in the symmetrical synapses, and the decreased dendritic spine density of GCs observed at the late stage of AD suggest a decrease in the efficiency of the recurrent GC-mediated inhibition of MCs. The interaction between MCs and GCs has been postulated to produce network oscillations in the OB that are associated with synchronous MC firing (Ravel et al., 2003; Beshel et al., 2007; Lagier et al., 2007). Impaired ultrastructural parameters of dendrodendritic synapses were always accompanied by abnormal synaptic transmission in APP/PS1 mice.

Synaptic transmission between dendrites is a major contributor to olfactory processing. In the OB, the glutamate released from MC dendrites excites the dendrites of GCs, and the excited dendrites mediates the GABAergic inhibition of MCs by releasing GABA (Lagier et al., 2007). $GABA_A$Rs are ligand-gated Cl^- channels that mediate most of the fast inhibitory action of GABA in the central nervous system (CNS; Pallotto and Deprez, 2014). The affinity of the binding of GABA to $GABA_A$Rs influence GABAergic synaptic transmission during olfaction processing. LFP oscillations in the mammalian OB represent coordinated neural activity that is dynamically regulated during olfactory processing. The γ rhythms strongly depend on the behavioral context and odor quality (Kay et al., 2009), and the γ oscillations in the OB are functionally related to the discrimination of overlapping odor input patterns (Beshel et al., 2007; Lepousez and Lledo, 2013). The γ oscillations reflect the synchronized spike discharges from MCs and local network activity (Schoppa, 2006; Sohal et al., 2009; Zhang et al., 2018). The γ oscillations rely on the dendrodendritic microcircuit between MCs and GCs and not on other synaptic interactions, such as gap-junction coupling or interneuron-interneuron connections (Lepousez and Lledo, 2013). The long-range synchronization between remote MCs operates selectively in the low-γ rhythms, whereas high-γ rhythms are spatially more restricted and represent the local dendrodendritic interactions (Lepousez and Lledo, 2013). At the early stage of AD, the γ oscillations in the EPL of APP/PS1 mice were significantly increased. Moreover, the older mice suffered serious olfactory dysfunction accompanied by an aberrant augmentation of γ oscillations in the EPL. The aberrantly increased γ oscillations in APP/PS1 mice indicated disrupted synchronized spike discharges from MCs, impaired synaptic transmission between GCs and MCs and abnormalities in olfactory processing in the OB. The application of a $GABA_A$R agonist (muscimol) resulted in reduced γ oscillations, and a $GABA_A$R antagonist (bicuculline) induced enhanced γ oscillations. The pharmacological treatments indicated that the regulation of the GC-mediated inhibition of MCs through modulation of the activities of $GABA_A$Rs plays a key role in synaptic transmission between MCs and GCs and affects the γ oscillations. Moreover, a pharmacological decrease in the GC-mediated inhibition of MCs and an increase in the excitatory/inhibitory balance in MCs through the application of a $GABA_A$R antagonist enhance the activity of MCs and long-range γ synchronization in a timely manner. Manipulations that aberrantly increase excitation/inhibition can reportedly impair odor mixture discrimination and slow the time required to discriminate between related odors (Lepousez and Lledo, 2013). Therefore, the enhanced γ oscillations in APP/PS1 mice might be a consequence of an increased excitatory/inhibitory balance in MCs due to a decreased GC-mediated inhibition of MCs. An increase in the GC-mediated inhibition of MCs and a decrease in the excitation/inhibition of MCs by $GABA_A$R agonist (muscimol) could reduce the enhanced γ oscillations in APP/PS1 mice. The findings that pharmacologically treatment with a $GABA_A$R agonist (muscimol) resulted in reduced γ oscillations support the hypothesis that altered GABAergic inhibition might underlie the aberrant γ oscillations in APP/PS1 mice.

MCs are necessary for generating spontaneous γ oscillations and for mediating the increased γ oscillations (Lepousez and Lledo, 2013). As the primary output neurons in the OB, MCs delivery the processed olfactory information in the OB to an advanced olfactory CNS. In the present study, the increased firing activity of MCs in APP/PS1 mice indicated abnormalities in olfactory information integration and transmission, which was consistent with olfactory disorder. The overexcitability of MCs was consistent with the neuronal hyperexcitability in higher-order cortical regions of AD patients and transgenic AD model mice and has been reported to be caused by Aβ (Wesson et al., 2011; Xu et al., 2015; Hu et al., 2017). At the early stage of AD, Aβ deposits mainly accumulated in MCs, although a small mount was detected within GCs. The decreased levels of synaptic vesicle-associated protein and the increased synaptic cleft of symmetrical synapses between GCs and MCs indicated aberrant synaptic transmission, which was consistent with the aberrant γ oscillations. In addition, the increased firing rates of MCs indicated the hyperexcitability of MCs and a failure of GC-mediated regulation. We hypothesized that the hyperexcitability of MCs and the observed aberrant synaptic transmission might contribute to the olfactory disorder observed early in AD. At the late stage of AD, excessive Aβ deposits accumulated in the OB, particularly within the GCs and MCs. In the OB, the dendrites of GCs, whose soma are located in the GCL, can regulate the activities of MCs through interaction with the dendrites of GCs. In addition, normal GC structure is essential for maintaining the normal activities of MCs. The decreased dendritic spine density of GCs in the presence of excessive Aβ deposits provides morphological evidence for a weakened inhibitory control of MCs by GCs. In addition, impaired structures of dendrodendritic synapses between GCs

and MCs indicate damaged excitatory interactions between MCs and GCs and a reduced efficiency of inhibitory synapses to MCs. Aberrantly increased γ oscillations, which rely on the GC-mediated inhibition of MCs, verify the weakened GC-mediated inhibition of MCs (Lepousez and Lledo, 2013). Based on the morphological changes of GCs and dendrodendritic synapses, the MC hyperexcitability might be the result of Aβ toxicity-induced neurotoxicity within MCs and weakened inhibition of MCs by GCs. Based on the above-described results, we speculate that aberrant excitatory synaptic transmission and preserved inhibitory synaptic transmission might decrease the excitatory/inhibitory balance of MCs and enhance γ oscillations in APP/PS1 mice. In addition, the Aβ toxicity-induced MCs hyperexcitability signify aberrant olfactory information processing and transmission, which might contribute to olfactory dysfunction.

Taken together, our findings illustrated abnormal morphological changes in dendrodendritic synapses and disturbed local dendrodendritic neuronal circuits between GCs and MCs in the presence of excessive Aβ deposits, and these effects affected odorant processing and resulted in abnormal output signals from MCs. In addition, the impairments in the local inhibitory circuits and the excitatory/inhibitory balance lead to aberrantly enhanced γ oscillations, which might be responsible for the altered responses manifested as olfactory disorders in the AD mice. Our findings of local microcircuit impairments between GCs and MCs in the OB will help researchers obtain a better understanding of the synaptic mechanisms underlying early olfactory dysfunction in patients with AD. Additionally, the pharmacological and physiological manipulation of MC inhibition in patients with AD might provide a potential therapeutic strategy for this disease.

STUDY LIMITATIONS AND FUTURE DIRECTIONS

We verified the impaired structure and function of dendrodendritic synapses in the presence of excessive Aβ deposits and demonstrated that abnormal dendrodendritic inhibition in MCs might contribute to olfactory dysfunction. However, whether and how Aβ deposits induce impaired dendrodendritic inhibition of MCs from GCs and thereby lead to olfactory deficits in APP/PS1 mice require further investigation. In our preliminary study, pharmacological treatments with $GABA_AR$ agonist weakened the aberrantly increased γ oscillations. Therefore, we will utilize other methods in the future to strengthen the transmission of dendrodendritic inhibition from GCs onto MCs *in vivo* and the effects on olfactory behaviors in APP/PS1 mice.

CONCLUSION

In summary, this study showed that APP/PS1 mice at sequential age stages exhibited gradual reductions in the dendritic spine density of GCs and continuous impairments in the synaptic interface parameters of the dendrodendritic synapses between GCs and MCs in the presence of excessive Aβ deposits. In addition, we observed aberrantly enhanced γ oscillations in the OB of APP/PS1 mice. Taken together, the data illustrate the structural and functional changes in the GC-mediated dendrodendritic inhibition of MCs in APP/PS1 mice and might help elucidate the mechanism of olfactory dysfunction in AD.

AUTHOR CONTRIBUTIONS

XY and SLin conceived and designed the study. LS, JW, XW, JL and CT acquired the data and prepared the figures. WL and SLi analyzed the data and wrote the manuscript.

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Identifying Changepoints in Biomarkers During the Preclinical Phase of Alzheimer's Disease

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Objective: Several models have been proposed for the evolution of Alzheimer's disease (AD) biomarkers. The aim of this study was to identify changepoints in a range of biomarkers during the preclinical phase of AD.

Methods: We examined nine measures based on cerebrospinal fluid (CSF), magnetic resonance imaging (MRI) and cognitive testing, obtained from 306 cognitively normal individuals, a subset of whom subsequently progressed to the symptomatic phase of AD. A changepoint model was used to determine which of the measures had a significant change in slope in relation to clinical symptom onset.

Results: All nine measures had significant changepoints, all of which preceded symptom onset, however, the timing of these changepoints varied considerably. A single measure, CSF t-tau, had an early changepoint (34 years prior to symptom onset). A group of measures, including the remaining CSF measures (CSF Abeta and phosphorylated tau) and all cognitive tests had changepoints 10–15 years prior to symptom onset. A second group is formed by medial temporal lobe shape composite measures, with a 6-year time difference between the right and left side (respectively nine and 3 years prior to symptom onset).

Conclusion: These findings highlight the long period of time prior to symptom onset during which AD pathology is accumulating in the brain. There are several significant findings, including the early changes in cognition and the laterality of the MRI findings. Additional work is needed to clarify their significance.

Keywords: preclinical Alzheimer's disease, biomarkers, changepoints, shape analysis, cognitive assessment, CSF assessment

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INTRODUCTION

Accumulating evidence indicates that the underlying neuropathological mechanisms associated with Alzheimer's disease (AD) begin a decade or more before the emergence of mild cognitive impairment (MCI) (Sperling et al., 2011). This has led to an increasing interest in understanding the order and magnitude of biomarker changes during this 'preclinical' phase of AD.

A hypothetical model has been proposed describing the order in which biomarkers change across the spectrum of AD (Jack et al., 2013). It has, however, been challenging to effectively test this model since most longitudinal studies that have enrolled cognitively normal individuals and collected relevant measures have limited follow-up. Additionally, studies with limited follow-up tend to lack a sufficient number of clinical outcomes (i.e., number of cases who progress to MCI) and therefore have limited power for statistical analyses designed to determine the timing of biomarker changes during preclinical AD.

Such analyses are feasible using data from the BIOCARD study, in which participants were cognitively normal when first enrolled, a wide range of informative measures were collected at baseline, and some participants have now been followed for over 20 years. The availability of these measures when the subjects were cognitively normal, and the unusually long duration of follow-up, allows the examination of the timing of biomarker changes during preclinical AD.

The primary goal of the analyses described here was to identify changepoints in measures based on cerebrospinal fluid (CSF), magnetic resonance imaging (MRI), and cognitive testing, obtained from a cohort of cognitively normal individuals, a subset of whom subsequently progressed to the symptomatic phase of AD. This study was approved by the Johns Hopkins Medicine Institutional Review Board.

MATERIALS AND METHODS

Study Design

The BIOCARD study, the study from which these data were drawn, was initiated at the National Institutes of Health (NIH) in 1995. While at the NIH, subjects were administered a neuropsychological battery and clinical assessments annually. MRI scans, CSF, and blood specimens were obtained approximately every 2 years. The study was stopped in 2005 for administrative reasons and re-established at Johns Hopkins University (JHU) in 2009, at which point the annual clinical and neuropsychological assessments were reinitiated. Bi-annual collection of CSF and MRI scans was re-established in 2015, and the acquisition of positron emission tomography (PET) scans using Pittsburgh Compound B (PiB) was begun. Tau PET imaging was initiated in 2017 (see **Figure 1** for a schematic representation of the study design). This paper is based on CSF and MRI data collected during the 1995–2005 period and neuropsychological tests during the 1995–2013 period.

Qualified researchers may obtain access to all de-identified clinical and imaging data used for this study.

Selection of Participants

Recruitment was conducted by the staff of the Geriatric Psychiatry branch of the intramural program of the National Institute of Mental Health. At baseline, all participants completed a comprehensive evaluation at the NIH, consisting of a physical, neurological and psychiatric examination, an electrocardiogram, standard laboratory studies, and neuropsychological testing. Individuals were excluded from participation if they were cognitively impaired or had significant medical problems such as severe cerebrovascular disease, epilepsy or alcohol or drug abuse.

A total of 349 individuals were initially enrolled in the study, after providing written informed consent. By design, approximately 75% of the participants had a first degree relative with dementia of the Alzheimer type. The analyses presented here are based on data from 290 subjects who were cognitively normal at baseline and had complete observations on the baseline variables of interest. Subjects were excluded from analyses for the following reasons: (1) subjects had not yet re-enrolled in the study or had withdrawn ($n = 29$); (2) Subjects were below 40 years old at the beginning of study ($n = 20$). Not all biomarkers were available for every subject and the actual number of subjects actually used for each run of the model was smaller: 256 for CSF, 270 for MRI and 281 for cognitive tests, for which we also excluded subjects who only had one battery of tests, to allow for a more reliable practice effect correction (see Statistical Analysis).

Of the 290 subjects included in these analyses, 209 subjects remained cognitively normal at their last visit and 81 subjects were diagnosed with MCI or dementia due to AD by the time of their last visit. The demographic characteristics of the subjects in the analysis are shown in **Table 1**, which are similar to the characteristics of the cohort as a whole. Most of the subjects who became symptomatic over time still meet criteria for MCI and all but a very small number ($n = 3$) have a clinical diagnosis consistent with AD. Follow-up of the cohort is continuing and the goal is to get autopsies on as many participants as possible. The accuracy of the clinical-pathological diagnoses has been 92% to date.

Consensus Diagnostic Procedures

Clinical and cognitive assessments were completed annually at the NIH initially and subsequently at JHU, as noted above. A consensus diagnosis for each study visit was established by the staff of the BIOCARD Clinical Core at JHU (prospectively for subjects evaluated starting in 2009 and retrospectively for subjects evaluated at the NIH). This research team included: neurologists, neuropsychologists, research nurses and research assistants. During each study visit, each subject had received a comprehensive cognitive assessment and a Clinical Dementia Rating (CDR), as well as a comprehensive medical evaluation (including a medical, neurologic and psychiatric assessment). For the cases with evidence of clinical or cognitive dysfunction, a clinical summary was prepared that included information about demographics, family history of dementia, work history, past history of medical, psychiatric and neurologic disease, current medication use and results from the neurologic and psychiatric evaluation at the visit. The reports of clinical symptoms from the CDR interview with the subject and collateral source (e.g., spouse, child, friend) were summarized, and the results of the neuropsychological testing were reviewed.

The diagnostic process for each case was handled in a similar manner. Two sources of information were used to determine if the subject met clinical criteria for the syndromes of MCI or dementia: (1) the CDR interview conducted with the subject and the collateral source was used to determine if there was evidence that the subject was demonstrating changes in cognition

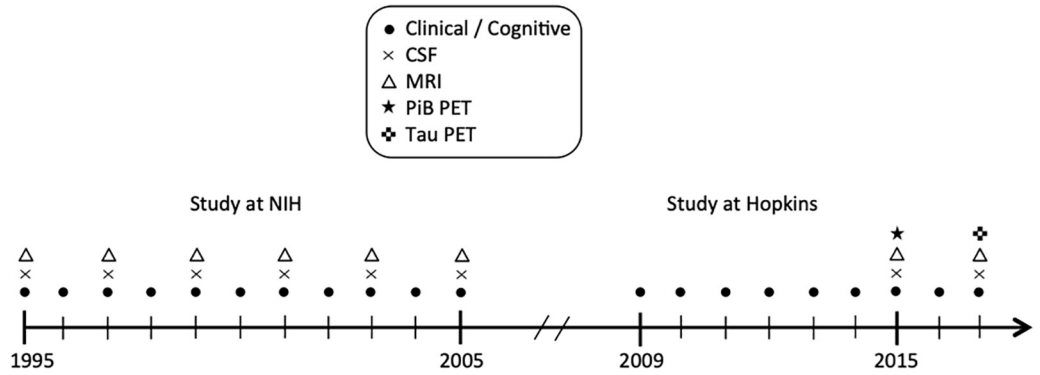

FIGURE 1 | Schematic representation of the study design.

TABLE 1 | Baseline characteristics of the participants included in the analyses in comparison to the cohort as a whole.

Variable	Cohort as a whole	Subjects in Analysis
Subjects in Analysis	349	290
Age, mean years (*SD*)	57.3 (10.4)	58.8 (8.5)
Gender, % females	57.6%	58.3%
Education, mean years (*SD*)	17.0 (2.4)	17.1 (2.3)
Ethnicity, % Caucasians	97.1% (0.9%)	89.3%
% ApoE-4 carriers	33.6%	35.4%
MMSE, mean score (*SD*)	29.5 (0.9)	29.5 (0.8)

ApoE-4, apolipoprotein E-4; MMSE, Mini-Mental State Exam.

in daily life, (2) cognitive tests scores (and their comparison to established norms) were used to determine if there was evidence of significant decline in cognitive performance over time. If a subject was deemed to be impaired, the decision about the likely etiology of the syndrome was based on the medical, neurologic, and psychiatric information collected at each visit, as well as medical records obtained from the subject, where necessary. More than one etiology could be endorsed for each subject (e.g., AD and vascular disease). One of four possible diagnostic categories was selected at each visit for each subject: (1) Normal, (2) Mild Cognitive Impairment, (3) Impaired Not MCI or (4) Dementia. The decision about the estimated age of onset of clinical symptoms was determined separately, and was based on responses from the subject and collateral source during the CDR interview regarding approximately when the relevant clinical symptoms began to develop. These diagnostic procedures are comparable to those implemented by the Alzheimer's Disease Centers program supported by the National Institute on Aging.

The estimated age of onset of clinical symptoms was based primarily on a semi-structured interview with the subject and the collateral source. The staff conducting the consensus diagnoses were blinded to the CSF and imaging measures.

Within the context of this study, the diagnosis of Impaired Not MCI typically reflected contrasting information from the CDR interview and the cognitive test scores (i.e., the subject or collateral source expressed concerns about cognitive changes in daily life but the cognitive testing did not show changes, or vice versa, the test scores provided evidence for declines in cognition but neither the subject nor the collateral source reported changes in daily life).

Selection Criteria for Variables Included in the Analyses

The changepoint analyses presented here include variables from the three primary domains evaluated in the BIOCARD study, obtained when subjects were first enrolled. These domains include: (1) cognitive test scores, (2) CSF values, and (3) MRI measures. In order to be as parsimonious as possible, we based the selection of which specific variables should be included in the analyses on findings from prior publications (Albert et al., 2014) that examined each of these measures in relation to time to onset of clinical symptoms. A total of 9 measures were included, as described below.

Cognitive Assessments

The annual, comprehensive neuropsychological battery covered all major cognitive domains, including memory, executive function, language, visuospatial ability, attention, speed of processing and psychomotor speed (see Albert et al., 2014 for the complete battery). We selected four cognitive measures to include in the changepoint analyses, as these four measures were significant in the multivariate Cox models examining the association between baseline performance and time to onset of clinical symptoms: (1) Digit Symbol Substitution Test from the Wechsler Adult Intelligence Scale – Revised; (2) Logical Memory – delayed recall from the Wechsler Memory Scale – Revised; (3) Verbal Paired Associates – Immediate recall from the Wechsler Memory Scale - Revised; and (4) Boston Naming Test.

CSF Assessments

Cerebrospinal fluid specimens were collected over time at the NIH (1995–2005) but were later analyzed at a single point in time by investigators at JHU. The CSF specimens collected from the participants were analyzed using the xMAP-based AlzBio3 kit [Innogenetics] run on the Bioplex 200 system. CSF specimens were analyzed in triplicate on the same plate. The AlzBio3 kit

contains monoclonal antibodies specific for Aβ1-42 (4D7A3), t-tau (AT120), and p-tau181p (AT270), each chemically bonded to unique sets of color-coded beads, and analyte-specific detector antibodies (HT7, 3D6). Calibration curves were produced for each biomarker using aqueous buffered solutions that contained the combination of the three biomarkers at concentrations ranging from 54 to 1,799 pg/ml for synthetic Aβ1-42 peptide, 25–1,555 pg/ml for recombinant tau, and 15–258 pg/ml for a tau synthetic peptide phosphorylated at the threonine 181 position (i.e., the p-tau181p standard). Each subject had all samples (run in triplicate) analyzed on the same plate. The intra-assay coefficients of variation (CV) for plates used in this study were: 7.7% ± 5.3 ($A\beta_{1-42}$); 7.1% ± 4.9 (t-tau); 6.3% ± 4.8 ($p\text{-}tau_{181}$). Interassay (plate-to-plate) CVs for a single CSF standard run on all plates used in this study were: 8.9% ± 6.5 ($A\beta_{1-42}$); 4.7% ± 3.3 (t-tau), and 4.3% ± 3.18 ($p\text{-}tau_{181}$). Compared with studies using the same kits and platforms, our absolute results are at the median levels for $A\beta_{1-42}$, t-tau, and $p\text{-}tau_{181}$. The CVs, plate-to-plate variability, and the dynamic range of our assays are well within published norms (Mattsson et al., 2009; Shaw et al., 2009).

Three CSF variables were generated from these analyses: (1) Abeta 42, (2) total tau (t-tau), and (3) phosphorylated tau (p-tau) (Moghekar et al., 2013).

MRI Assessments

The MRI scans acquired from the participants were obtained using a standard multi-modal protocol with a GE 1.5T scanner. The coronal scans employed an SPGR (Spoiled Gradient Echo) sequence (TR = 24, TE = 2, FOV = 256 × 256, thickness/ gap = 2.0/0.0 mm, flip angle = 20, 124 slices). The scans were processed with a semi-automated method, using region-of-interest large deformation diffeomorphic metric mapping (ROI-LDDMM) techniques (Miller et al., 2013). More precisely, the MRI volumetric regions of interest (ROI) included the entorhinal cortex, hippocampus, and amygdala. For each of the three ROI, landmarks were placed manually in each MRI scan to mark the boundaries of the ROI, following previously published protocols [see Csernansky et al. (1998) and Miller et al. (2013) for the hippocampus, Munn et al. (2007) for the amygdala, and Miller et al. (2013) for the entorhinal cortex]. Next, a group template for the entorhinal cortex, hippocampus, and amygdala was created, based on the set of baseline MRI scans. The same set of landmarks was placed into this group template as in the individual subject scans. ROI-LDDMM procedures were then used to map the group template to the individual subject scans, using both landmark matching (Csernansky et al., 2000) and volume matching (Beg et al., 2005). The resulting segmented binary images for the entorhinal cortex, hippocampus and amygdala were used to calculate the volume of each structure, by hemisphere, by summing the number of voxels within the volume.

A medial temporal lobe composite was used in the present analyses, based on an average of the entorhinal cortex, hippocampus and amygdala. [Prior analyses showed that this composite is more strongly associated with CSF alterations that are an early marker of AD than the individual MRI measures taken separately (Gross et al., 2017).] The measurements from the right and left hemisphere were examined separately.

The volumetric measurements of the entorhinal cortex, hippocampus and amygdala were normalized for head size by including total intracranial volume (ICV) as a covariate (Sanfilipo et al., 2004). ICV was calculated using coronal SPGR scans in Freesurfer 5.1.0 (Segonne et al., 2004).

Statistical Analysis

Overview

The overall goal of the changepoint analyses was to determine if each of the measures selected for analysis had a significant changepoint in relation to time to onset of clinical symptoms and, if so, the timing of these changepoints with respect to one another. The model used in these analyses has previously been applied to MRI data in this cohort in order to establish the order in which changes occur in the volume, thickness and shape of medial temporal lobe regions during preclinical AD (Younes et al., 2014). A more advanced version of the model (Tang et al., 2017) is applied here to the full range of biomarkers available in the study.

The changepoint is represented in the model as a significant change in slope (see **Figure 2**). The model uses all of the available data (both from subjects who remained normal as well for those who progressed to MCI) in order to estimate the changepoint. The main features of the model are as follows:

1. Time is measured relative to the clinical onset time of the disease (even though age is included as a covariate). This means that if a subject has been diagnosed with MCI 10 years after another, the time scale for the latter is shifted 10 years to the right compared with the former.
2. Clinical onset times for normal subjects, which are not observed, are treated as missing data, therefore assuming "right censoring." The model therefore assumes that every subject will ultimately get the disease if they were to live indefinitely. A prior model of disease onset is used in conjunction.

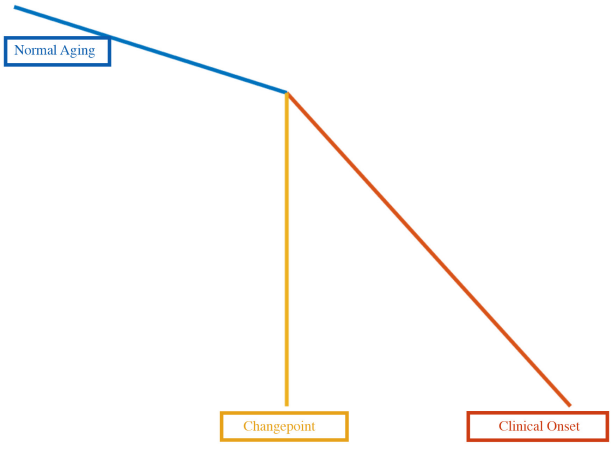

FIGURE 2 | Schematic representation of the changepoint model.

3. The model assumes linearity in the measures as a function of age, with the change of slope at the changepoint. It is slightly different from the sigmoidal model of Jack et al. (2013), in which biomarkers smoothly transition from a low-abnormality plateau to a high-abnormality plateau in an S-shaped curve. Since the data in the study pertain to individuals who were cognitively normal at baseline and remained normal or who progressed from normal to MCI, the model assumes that the subjects are either in the low-abnormality range or in a transition phase, thus not requiring adaptation to an S-shaped function which might allow for two changepoints.
4. All models included age and gender as covariates and a constant random effect. Education was included as an additional covariate for the cognitive measures; intracranial volume and left-handedness was included as an additional covariate for the MRI measures.
5. We also corrected for the impact of a practice effect on cognitive tests (Rabbitt et al., 2004; Zehnder et al., 2007; Vivot et al., 2016) by introducing a covariate that depends on the number of tests taken in the past, defined by $z_{practice} = 1 - 2^{-k}$, when a test is taken for the kth time.
6. For cognitive tests, we also limited our analyses to subjects that had at least two measurements over the course of the study. (This restriction was not applied to other biomarkers.)
7. CSF t-tau and p-tau were transformed to logarithmic scale in the analyses. For robustness, a constraint ensuring non-negative slopes in the regression model was applied.
8. The model can project the changepoint forward in time as well as backward, sometimes allowing for a changepoint that precedes the initiation of data collection. Although the goal is to identify a changepoint preceding the onset of symptoms, this two-phase model allows for the changepoint and clinical onset of symptoms to coincide.

As shown in **Figure 3**, the model organizes the estimated time of symptom onset for the biomarker values along a broken line. The model fits the data so that the subjects with less abnormal values (e.g., higher test scores) tend to be on the left side of the curve and therefore to have a longer estimated time to clinical symptom onset.

Mathematical Description

Let n denote the number of subjects in the study. For subject k, we assume p_k observations of a scalar biomarker, denoted $y_{k,1}, \ldots, y_{k,p_k}$, at ages $t_{k,1}, \ldots, t_{k,p_k}$. Let $T_1,\ldots,T_n$ denote the subjects' ages at the end of the study. Typically: $T_k > t_{k,p_k}$ (age at last biomarker measurement). Let U_k denote the age at MCI onset, which is observed only if $U_k \leq T_k$.

Finally, let $z_{k,1}, \ldots, z_{k,p_k}$ denote additional covariates, such as gender, education level, intracranial volume, etc. (Each z_k may be a vector.) Let η_k denote a constant random effect associated with each subject and $\varepsilon_{k,1}, \ldots \varepsilon_{k,p_k}$ a random noise associated with each observation. They are modeled as Gaussian variables with respective variances τ^2 and σ^2. A prior distribution is used for U_k, and modeled as a Gaussian with mean m_1 = 93 years and standard deviation σ_1 = 14.5 years. (This distribution was learned from an independent dataset.) Details on the estimation procedure leading to these values can be found in Tang et al. (2017). Importantly, this distribution represents a clinical onset time applicable to the whole population (including people who will not get AD during their lifetime). Onset times for the diseased population (i.e., conditional to onset prior to death) would be significantly smaller.

The changepoint model is

$$y_{k,j} = a + b_1 t_{k,j} + b_2 U_k + c \max(t_{k,j} - S_k, 0) + \beta^T z_{k,j} + \eta_k + \varepsilon_{k,j}$$

where $S_k = \max(U_k - \Delta, 20)$ (in years) is the changepoint, the largest of Δ years before onset or 20 years.

This is a two-phase regression model. The biomarker first follows a linear trajectory (phase I)

$$y_{k,j} = a + b_1 t_{k,j} + b_2 U_k + \beta^T z_{k,j} + \eta_k + \varepsilon_{k,j}$$

for $t_{k,j} < S_k$ and then switches (with a continuous transition) to the model (phase II)

$$y_{k,j} = a + (b_1 + c) t_{k,j} + b_2 U_k - c S_k + \beta^T z_{k,j} + \eta_k + \varepsilon_{k,j}$$

which is still linear, now with slope $b_1 + c$.

The null hypothesis model assumes the phase I model over all times, or equivalently that $c = 0$.

The changepoint parameter is estimated using posterior means defined as follows. For each fixed Δ, the model parameters are estimated by maximum likelihood, and the value of the log-likelihood $\ell(\Delta)$ is computed. The estimator for Δ is then defined by

$$\hat{\Delta} = \frac{\sum_\Delta \Delta (e^{\ell(\Delta)} - e^{\ell_0})}{\sum_\Delta (e^{\ell(\Delta)} - e^{\ell_0})}$$

where the sum is over a finite number of Δ between 0 and 100, and ℓ_0 is the log-likelihood for the null hypothesis of no changepoint.

Validation

P-values and confidence intervals are estimated using bootstrap techniques. The bootstrap method estimates standard errors based on random resampling of the data with replacement; it can be a more reliable method of calculating standard errors and statistical significance than parametric methods (Efron, 1979). For *p*-values, a general model is fitted, residuals are estimated, then resampled to reconstruct a model satisfying the null hypothesis (hence with $c = 0$). For confidence intervals, the approach is similar, but the full estimated model is used for reconstruction. We used 1,000 bootstrap samples for each estimation. A median absolute deviation was calculated for each changepoint in order to provide a robust estimate of the standard deviation, estimated as the median of the absolute value of the difference from the median of the sample as a whole.

Additionally, a "precedence graph" was developed using the variables for which significant changepoints were calculated

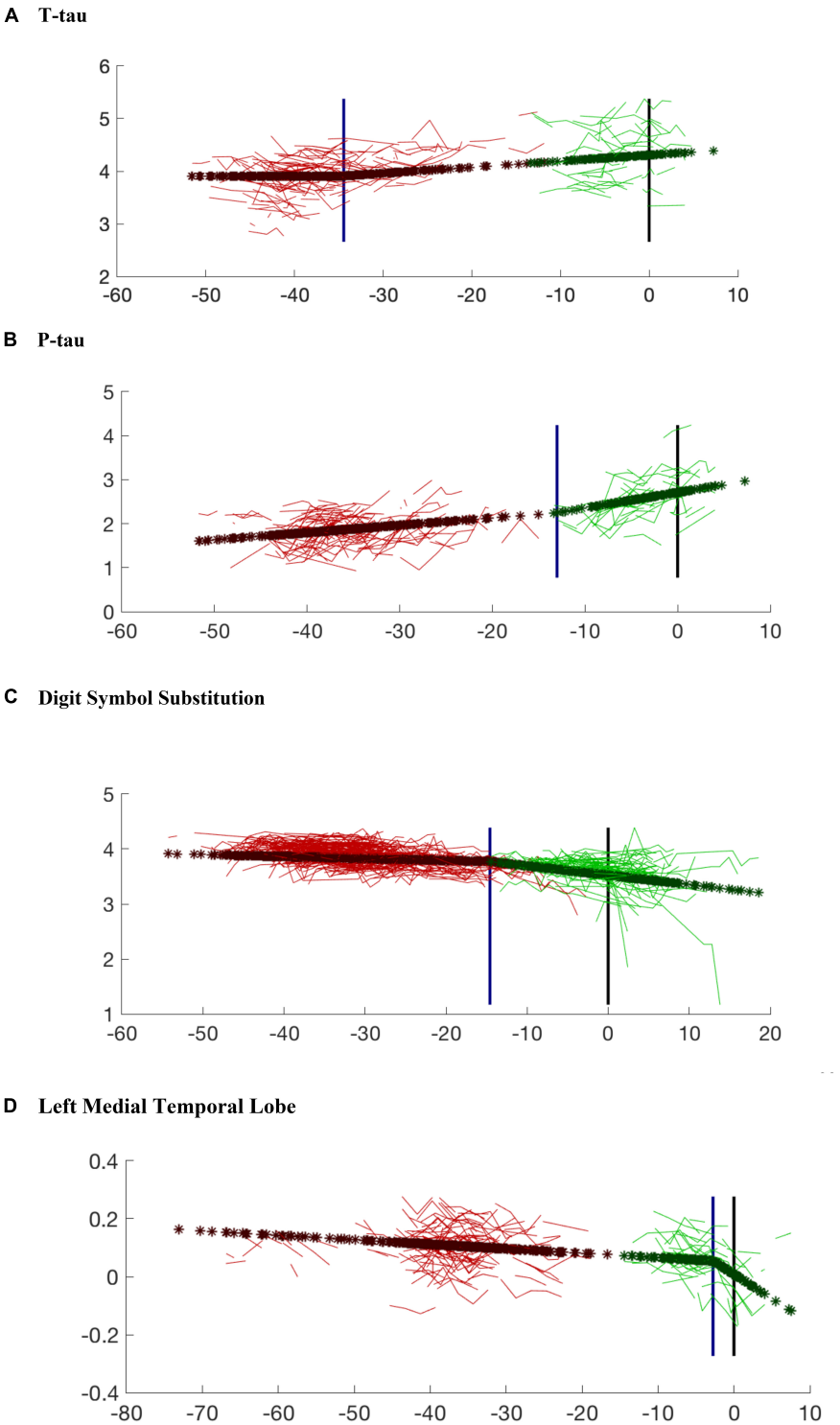

FIGURE 3 | Model prediction for each variable compared with the observed data. The variables shown in the figures include: **(A)** CSF t-tau; **(B)** CSF p-tau; **(C)** Digit Symbol Substitution Test; **(D)** Left Medial Temporal Lobe Volume. The red lines are the observed data for the subjects who remained cognitively normal. The green lines represent individuals who progressed to cognitive impairment. Dark red stars (and dark green stars, respectively) are the model predictions for the same subjects for whom observed data are presented. The blue vertical line marks the estimated changepoint. The black vertical line marks the estimated onset of clinical symptoms. The age of onset for the subjects who remained cognitively normal was imputed via Bayesian prediction. Note that the *x*-axis values for cognitively normal subjects are based on an estimated clinical onset time (since the "true one" is right-censored), using the posterior mean of its distribution given the observed data. This explains the gap that can be observed in some graphs between actual and censored observations, since the latter lacks the statistical variability around the estimated posterior mean.

(n = 8). Each of the measures were compared with one another using a bootstrap technique to determine the fraction of bootstrap samples for which the changepoint estimates for one measure were found to be earlier than the other. More precisely, precedence between two modalities A and B, with estimated changepoints $\widehat{\Delta}_A$ and $\widehat{\Delta}_B$, is assessed by computing the probability

$$P(\widehat{\Delta}_A \leq \widehat{\Delta}_B),$$

this probability being itself estimated using bootstrap resampling (i.e., for the sampling distribution). Because this probability requires to sample from the joint distribution of $\widehat{\Delta}_A$ and $\widehat{\Delta}_B$, bootstrap samples are generated consistently across modalities, and the corresponding normalized frequency are computed over 1,000 replicas. This means that if, in order to reconstitute a bootstrap sample for visit time t' in modality A, one has used the original residual computed at visit time t, then the corresponding original residual from the same visit time t in modality B will be used, whenever possible, to reconstitute a bootstrap sample at time t' for B. (When these two modalities have not been measured together at the considered visit times, the bootstrap samples are created independently.)

Groups of variables were computed using hierarchical clustering, based on precedence probability vectors, in order to provide a more concise representation of the changepoints with respect to one another. Arrows were drawn between measures when the confidence for one changepoint was earlier than the other at least 75% of the time.

Null Hypothesis Model

The model with $c = 0$, which corresponds to our null hypothesis of no changepoint related to disease onset takes the form

$$y_{k,j} = a + b_1 t_{k,j} + b_2 U_k + \beta^T z_{k,j} + \eta_k + \varepsilon_{k,j}.$$

Importantly, it includes a disease effect, through the use of the onset time as a partially observed covariate. While our primary focus here is on changepoint, there is certainly an interest in testing the significance of the hypothesis$b_2 \neq 0$, with respect to the "double-null" model

$$y_{k,j} = a + b_1 t_{k,j} + \beta^T z_{k,j} + \eta_k + \varepsilon_{k,j},$$

which, this time, includes no disease related effect. A significant value of b_2, say, with $b_2 > 0$, implies a lower value of the biomarker for earlier cognitive onsets.

Accounting for a Normal Changepoint

The changepoint in the proposed model is specified in terms of "time before disease onset" and does not include the possibility of such a change being due to normal aging. One of the difficulties in trying to account for both effects (let us call them disease vs. normal changepoint) is that if one of them is strong enough and not corrected for, it may induce significance when testing for the other effect even if that one is not present. On the other hand, correcting for an effect that is not present may reduce the power for detecting the other effect, even if the latter is present. For clarity and to simplify the exposition, we have focused our model and results on a single changepoint measured against disease onset. To be complete, however, we also explored a model in which a correction for a normal changepoint is included (which will therefore be more conservative for the detection of a change associated with disease). This model includes one additional covariate taking the form $\max(t_{kj}-\delta, 0)$ where δ is a subject-independent age measuring the normal changepoint. To simplify the estimation process, this time δ is computed first (using maximum likelihood for a model without disease changepoint, which in this case only includes random effects as hidden variables), and then plugged into the general model.

RESULTS

Like most statistical results, significant tests reflect a possible association between two factors and any further interpretation (including, in particular, conclusions about cause and effect) can only be expressed as plausible hypotheses, consistent with the results, with other evidence and maybe prior beliefs. For our model, significant results provide a credible indication that a change of regime in the biomarker occurs some number of years before clinical onset. One of the possible interpretations is indeed that the changepoint marks an effect of the disease, which happens before its onset can be detected. Another, however, is that the change is non-pathological, but that its timing is correlated with the disease onset. Statistics alone cannot determine which one is more likely to reflect reality.

The results of the changepoint analyses for each of the nine variables examined are summarized in **Table 2**. As can be seen, all of the variables had a significant changepoint. The changepoints varied widely across the years preceding symptom onset. **Figures 3**, **4** provide graphical representations of the model predictions.

The earliest changepoint is for CSF t-tau, that is estimated at approximately 34 years. Significantly later in time, we estimate changepoints for two cognitive markers: The Logical Memory Delayed Recall (15.4 years prior to symptom onset), and the Digit Symbol Substitution Test (14.6 years prior to symptom onset). They are followed a couple of years later by the other two cognitive measurements: Boston Naming Test (13.2 years prior to clinical symptom onset) and Paired Associates Immediate Recall (11.3 years prior to symptom onset), with CSF p-tau in between (13.0 years prior to clinical symptom onset) and CSF abeta a little later (9.6 years prior to symptom onset). Imaging markers come next, with a 6-year difference between the changepoints estimated on the right (8.8 years prior to clinical symptom onset) and on the left (2.8 years prior to clinical symptom onset) medial temporal lobe volumes. This arrangement is summarized in **Figure 5** showing the precedence graph between these variables, in which arrows are placed only when the changepoint order could be estimated with enough reliability, as measured via bootstrap resampling.

All markers except CSF t-tau were significant for rejecting the double-null hypothesis of no effect of the cognitive onset time on the marker, with p-values given by 0.047 (left MTL), 0.004 (right MTL), 0.016 (CSF abeta), 0.007 (CSF p-tau) and

TABLE 2 | Results of changepoint analysis for CSF, MRI and cognitive variables.

Variable Category/Name	Significance of normal changepoint *p*-value	Estimated number of years of changepoint prior to symptom onset [75% CI]	Median absolute deviation of changepoint
Cerebrospinal fluid			
Abeta	<0.001	9.6 [6.4–12.8]	1.6
p-tau	0.024	13.0 [6.0–20.1]	3.1
t-tau	0.027	34.4 [24.9–44.0]	3.8
Magnetic Resonance Imaging			
Medial Temporal Lobe (L)	<0.001	2.8 [1.9–3.6]	0.2
Medial Temporal Lobe (R)	0.002	8.8 [6.0–11.6]	1.5
Cognitive Test Scores			
Digit Symbol Substitution	<0.001	14.6 [11.5–17.7]	1.2
Logical Memory Delayed	<0.001	15.4 [12.6–18.1]	1.6
Paired Associates Delayed	<0.001	11.3 [8.4–14.2]	1.2
Boston Naming Test	0.001	13.2 [10.3–16.2]	1.7

less than 0.001 for all cognitive markers. We found $b_2 > 0$ for all markers (indicating a smaller value of the marker for earlier onsets), except for CSF p-tau, for which this value was negative.

Variables that were significant for normal changepoints (*p*-value less than 0.05) at fixed age in the considered biomarkers for CSF p-tau (age: 51.3), CSF t-tau (age: 64.2 years), Digit Symbol Substitution (age: 74.8), Logical Memory Delayed (age: 67) and right medial temporal lobe (age: 48.6 years). Introducing this normal changepoint in the model as an additional covariate had limited impact on the significance and value of the disease changepoint times.

DISCUSSION

The changepoint analyses presented here lead to several conclusions. First, the changepoint for CSF t-tau occurs several decades prior to the onset of clinical symptoms. Second, the changepoints seen in the rest of the variables appear to reflect a cascade of events in which multiple measures are changing a decade prior to the onset of clinical symptoms. Third, there is a significant difference in the vulnerability of the right vs. the left medial temporal lobe. Several of these findings diverge from the hypothesized ordering of biomarkers in the model proposed by Jack et al. (2013), as well as the hypothetical stages proposed in the NIA/AA Working Group Report, both of which propose that cognitive change follows significant accumulation of amyloid and tau (Sperling et al., 2011). Our results describe a more complex ordering, in which some cognitive effects were found to predate changepoints in CSF abeta. It is of course possible that changes in amyloid occur at a time too early to be detectable in our model, or with a different slope associated with the disease, which is not addressed here.

To gain further insights into these findings, we looked in greater detail at the two cognitive tests with very early timepoints. **Figure 3A** (which shows the model regression on t-tau after removal of covariate and random effects) indicates that, after a first phase during which t-tau is flat, the protein appears to accumulate starting about 34 years before onset. This is a large gap, but, as already remarked, these results do not inform us on the pathological nature of this increase, but rather on the fact that an event/changepoint seems to happen for CSF t-tau accumulation with a timing that can be associated with clinical impairment several decades later. In other terms, while this changepoint appears to be associated with the onset of disease, it does not necessarily correspond to an early effect. These findings also highlight the differential relationship between CSF t-tau and p-tau during the evolution of AD, although p-tau and t-tau tend to be highly correlated. This difference is emphasized in the recent AD biomarker "framework" (Jack et al., 2018), which argued that p-tau is more closely related to the pathophysiology of AD, with CSF p-tau levels correlating with neurofibrillary tangle pathology in AD patients. By comparison, elevations in t-tau are also seen in other diseases and are reflective of more general levels of neurodegeneration.

The difference in the changepoint for the right and left medial temporal has been presaged by prior reports that have examined the individual regions within the MTL separately. For example, we previously reported that both the right entorhinal cortex and amygdala, when measured at baseline, were significantly related to time to onset of symptoms, whereas measures on the left were not (Soldan et al., 2015). Further studies are needed to determine why this differential vulnerability may occur.

It is important to acknowledge the limitations of these analyses. First, the wide confidence intervals for the CSF assays, particularly for CSF t-tau and p-tau, limit the ability to narrow down the changepoint for this important biomarker. The variability of CSF assays has been an acknowledged challenge in the field for some time, reflected by international efforts to develop improved methods (Mattsson et al., 2011). Newer assays are currently under development (Chang et al., 2017) raising the possibility that measures with less variability will be soon available that will permit more accurate changepoint estimates, with narrower confidence intervals. Second, while the sample size used here is sufficient to generate findings

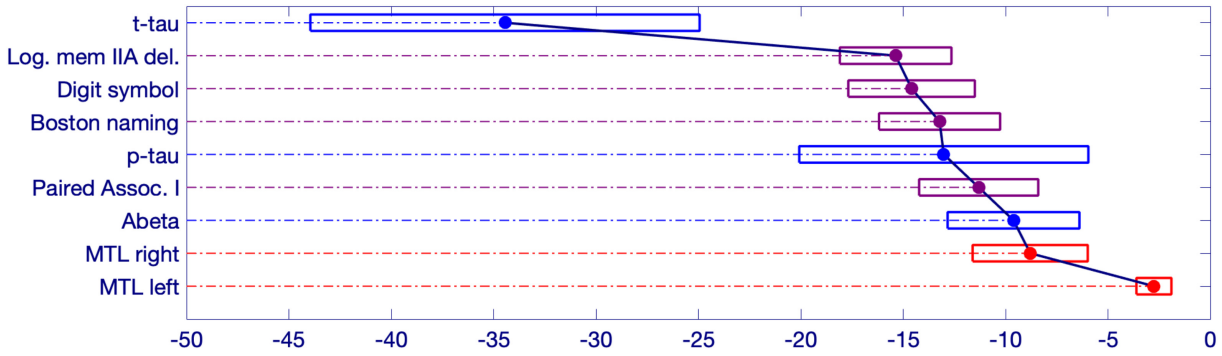

FIGURE 4 | Schematic representation of significant changepoint results in relation to symptom onset. The estimated onset of clinical symptoms is represented by the value of 0 at the bottom right side of the figure. The numbers to the left of the 0 represent the estimated number of years prior to symptom onset for the changepoint of each variable. The width of each box represents a bias-corrected 75% confidence interval for the estimated value of each variable.

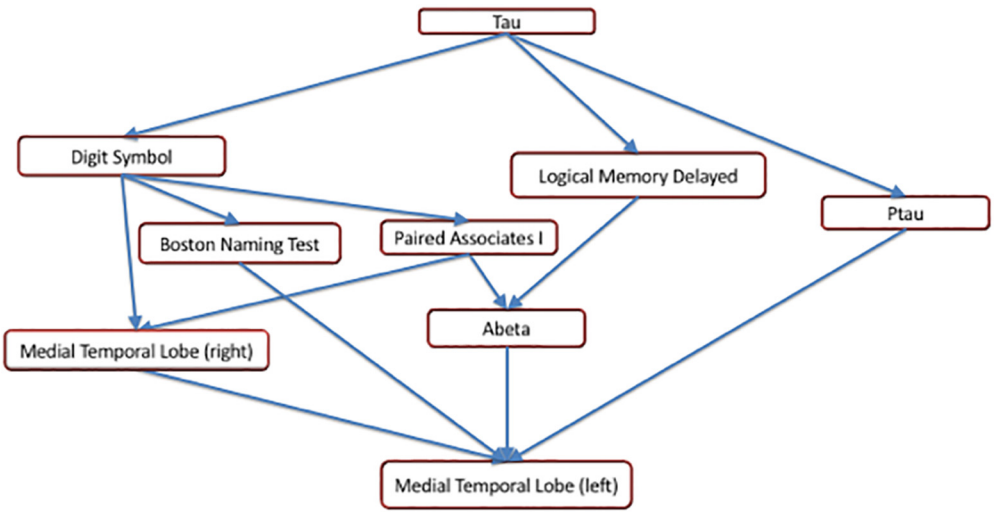

FIGURE 5 | A precedence graph representing the order of the changepoints among the variables with significant changepoints. An arrow between groups of variables indicates that, more than 75% of the time, the changepoint for the variable represented as the 'source' was found to be earlier than the changepoint for the variable represented as the 'target,' using bootstrap samples. The groupings of the variables were computed using hierarchical clustering within each modality, based on the precedence probability vectors. Arrows that can be inferred by transitivity are not shown for clarity.

with substantial statistical significance, the width of most of the 75% confidence intervals is between 5 and 10 years, some of them being even greater. Under the assumption that an increase in sample size may reduce the confidence intervals, we have established a consortium of five sites around the world that are collecting comparable data (Gross et al., 2017). We plan to apply this changepoint model to data gathered from across the sites, which will greatly increase the sample size. Third, the model itself incorporates assumptions that may limit its applicability. For example, a two-phase linear model assumes some continuity of the biomarkers before and after changepoint, since it only accounts for a change of slope. A very abrupt change, for example, would be imperfectly approximated by the model and may result in a loss of power in the likelihood ratio test. Sublinear or hyperlinear evolutions before or after changepoints may have a similar effect. There could also be more than one changepoint, which is not handled by the analysis thus far. Additionally, the estimates of the changepoint are generally more stable when the likelihood ratio test p-value is small, and for small changepoints. Lastly, the changepoint analysis presented here is for univariate biomarkers, and therefore it has been applied separately to each of the variables. While this approach can, in theory, be extended to the multivariate case, such an extension presents statistical challenges, which are currently under investigation.

As these findings emphasize, identifying biomarker changepoints during the preclinical phase of AD remains challenging. Extrapolating the implications of changepoints to predictive models that might identify individuals likely to progress to AD in later life is yet another step beyond the estimation of changepoints. The efforts underway to develop

improved treatments for AD offer the hope that when accurate prediction on an individual basis is possible, effective therapeutic interventions will be available.

DATA AVAILABILITY

Publicly available datasets were analyzed in this study. This data can be found here: http://www.biocard-se.org/.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of Johns Hopkins Medicine Institutional Review Boards with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Johns Hopkins Medicine Institutional Review Boards.

AUTHOR CONTRIBUTIONS

LY conducted the analyses and drafted the manuscript. AM played a major role in acquiring the data. MA interpreted the data and revised the manuscript. AS revised the manuscript for intellectual content. CP revised the manuscript for intellectual content. MM interpreted the data and revised the manuscript.

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Hydroxyurea Improves Spatial Memory and Cognitive Plasticity in Mice and Has a Mild Effect on These Parameters in a Down Syndrome Mouse Model

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Down syndrome (DS), a genetic disorder caused by partial or complete triplication of chromosome 21, is the most common genetic cause of intellectual disability. DS mouse models and cell lines display defects in cellular adaptive stress responses including autophagy, unfolded protein response, and mitochondrial bioenergetics. We tested the ability of hydroxyurea (HU), an FDA-approved pharmacological agent that activates adaptive cellular stress response pathways, to improve the cognitive function of Ts65Dn mice. The chronic HU treatment started at a stage when early mild cognitive deficits are present in this model (~3 months of age) and continued until a stage of advanced cognitive deficits in untreated mice (~5–6 months of age). The HU effects on cognitive performance were analyzed using a battery of water maze tasks designed to detect changes in different types of memory with sensitivity wide enough to detect deficits as well as improvements in spatial memory. The most common characteristic of cognitive deficits observed in trisomic mice at 5–6 months of age was their inability to rapidly acquire new information for long-term storage, a feature akin to episodic-like memory. On the background of severe cognitive impairments in untreated trisomic mice, HU-treatment produced mild but significant benefits in Ts65Dn by improving memory acquisition and short-term retention of spatial information. In control mice, HU treatment facilitated memory retention in constant (reference memory) as well as time-variant conditions (episodic-like memory) implicating a robust nootropic effect. This was the first proof-of-concept study of HU treatment in a DS model, and indicates that further studies are warranted to assess a window to optimize timing and dosage of the treatment in this pre-clinical phase. Findings of this study indicate that HU has potential for improving memory retention and cognitive flexibility that can be harnessed for the amelioration of cognitive deficits in normal aging and in cognitive decline (dementia) related to DS and other neurodegenerative diseases.

Keywords: Down syndrome, trisomy, hydroxyurea, adaptive stress response, neurodegeneration, nootropic effect, reference memory, episodic-like memory

INTRODUCTION

Individuals with Down syndrome (DS) have a partial or complete extra copy of human chromosome 21 (trisomy 21; HSA21). DS is the most common aneuploidy compatible with survival, occurring in 1 out of 691 live births (Centers for Disease Control and Prevention [CDC], 2017). People with DS commonly display intellectual disability, hypotonia, and delayed language and speech development (Mundy et al., 1995; Pavarino Bertelli et al., 2009). The DS brain is characterized by a small cerebellum, reduced neurogenesis, dendritic hypotrophy, and altered neurotransmitter and receptor systems (Das et al., 2013; Stagni et al., 2015). Degeneration of basal forebrain cholinergic neurons, increased microglial activation, cognitive decline, and Alzheimer's disease neuropathology and dementia develop with age (Bimonte-Nelson et al., 2003).

One of a multitude of DS-associated deficiencies that recently attracted more attention is the dysregulation of the adaptive cellular stress response. It involves several interconnected signaling pathways, including mitochondrial bioenergetics, autophagy, the antioxidant response, and the unfolded protein response. Trisomic cells from DS or DS mouse models exhibit mitochondrial dysfunction, increased oxidative stress and damage, and mTOR pathway hyperactivation leading to reduced autophagy (Busciglio et al., 2002; Helguera et al., 2013; Cenini et al., 2014; Perluigi et al., 2014; Tramutola et al., 2015, 2016; Liu et al., 2017). Many disomic genes are dysregulated as a result of trisomy, and these are enriched for genes related to oxidative stress and mitochondrial function, including *NFE2L2*-associated genes (Helguera et al., 2013). Further, brain tissue from individuals with DS exhibits reduced autophagosome formation, reducing the ability of the brain to clear damaged proteins and organelles (Di Domenico et al., 2013). Importantly, these effects on the proteostasis network are found in individuals with DS years before age-related cognitive decline and Alzheimer's-like dementia are detected. This represents a large pharmaceutical treatment window to delay and/or improve the long-term cognitive function of individuals with DS. There is no available treatment to improve intellectual disabilities or age-related dementia in individuals with DS. Improvement in the pathways of adaptive stress response may present a novel therapeutic opportunity and is particularly attractive as treatments targeting these pathways have already been approved for non-DS related clinical use.

Hydroxyurea (HU), an FDA-approved ribonucleotide reductase inhibitor, is known to improve cellular homeostasis through stimulation of mitochondrial bioenergetics, autophagy, and the antioxidant response (Brose et al., 2012). In our previous work we showed that *in vitro*, HU protects primary rat hippocampal neurons against increased oxidative stress, mitochondrial stress, and excitotoxicity (Brose et al., 2018). *In vivo*, HU treatment of *APPswe/PS1dE9* mice, an Alzheimer's disease model, ameliorated deficits in spatial memory tested in a hippocampus-dependent Morris water maze (MWM) task (Brose et al., 2018). We hypothesized that HU treatment would improve cognitive deficits in a mouse model of DS, as well. To test our hypothesis, we chose the Ts65Dn mouse model which is trisomic for the distal portion of mouse chromosome 16 (MMU16) containing approximately 94 genes orthologous to HSA21. Ts65Dn mice have brain dysmorphology, transcriptional and biochemical changes as well as cognitive deficits that mirror several anomalies observed in individuals with DS (Davisson et al., 1993; Reeves et al., 1995; Kahlem et al., 2004). This is the most widely used DS mouse model to date for the preclinical study of therapeutic treatments for DS.

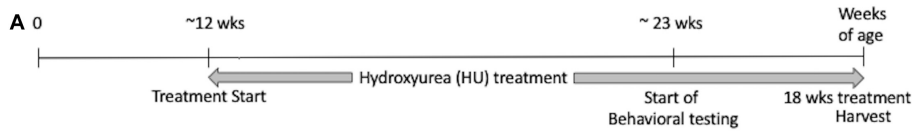
The Ts65Dn model has been used extensively in different behavioral tests, including the Y-maze, novel object recognition test, MWM, and fear conditioning (Das et al., 2013; Dutka et al., 2015; Olmos-Serrano et al., 2016). Consistent among different research groups, the Ts65Dn mice have severely impaired spatial learning and memory, measurable by their inability to learn and remember the location of the hidden platform in the MWM. This deficit is correlated with significant impairment of long term potentiation in the dentate gyrus of the hippocampus (Siarey et al., 1997; Belichenko et al., 2004; Ruparelia et al., 2012). Behavioral tests with consistently reproducible DS-related phenotypes, such as the MWM, have been used to evaluate the ability of pharmacological interventions to improve cognitive measures in DS mouse models (Reeves et al., 1995; Moran et al., 2002; Stasko and Costa, 2004; Das and Reeves, 2011; Velazquez et al., 2013). The usual caveats of using an animal model in the preclinical stage of testing must, of course, be applied to the Ts65Dn model. From a genetic perspective, it has been known since the model was created that it is trisomic for only about 60% of mouse orthologs of Hsa21 genes. More recently, whole genome sequencing has documented the presence of a number of trisomic genes whose orthologs are on human chromosomes other than 21 (Duchon et al., 2011; Reinholdt et al., 2011).

To test our hypothesis, we treated Ts65Dn mice with HU and monitored cognitive performance using a battery of water maze tasks originally designed to detect changes in different types of memory in normal aging and in aging aggravated by Aβ amyloidosis in AD mouse models (Jankowsky et al., 2005; Savonenko et al., 2005). These tasks have been used successfully to test several different experimental treatments (Laird et al., 2005; Savonenko et al., 2009; Chow et al., 2010; Tabatadze et al., 2010) with sensitivity wide enough to detect deficits as well as improvements in spatial memory. Our experiments demonstrated that chronic HU treatment resulted in mild but significant improvements of cognitive deficits in the Ts65Dn mice, while in wild type control mice the treatment had clear nootropic effects, significantly facilitating learning and memory.

MATERIALS AND METHODS

Study Design

This study was carried out in accordance with the recommendations of the NIH Guide for the Care and Use of Laboratory Animals and the Johns Hopkins University Institute of Animal Care and Use Committee. The protocol was approved by the Johns Hopkins University Institute of Animal Care and Use Committee. Ts65Dn mice were maintained by our laboratory as an advanced intercross on a C57BL/6J x C3H/HeJ F_n background.



B

Genotype +/- Treatment	Females	Males	Total
Euploid	7	5	12
Euploid + HU	7	5	12
Ts65Dn	10	5	15
Ts65Dn + HU	11	4	15
Totals	35	19	54

FIGURE 1 | Study design. **(A)** A proof-of-concept study to monitor the preventative effects of hydroxyurea (HU) on cognitive function in Ts65Dn mice. HU (30 mg/kg/day dissolved in water) treatment started at ∼12 weeks of age and continued for at least 10 weeks before behavioral testing began. Mice were harvested after 18 weeks of HU treatment. **(B)** Number of cases per genotype per treatment. During the study two untreated male euploid mice and one untreated Ts65Dn male died.

No mice homozygous for the *Pde6b(rd)1* retinal degeneration mutant allele were used in this study. Investigators performing the testing were blind to genotype. The study design is shown in **Figure 1**. This study began with 54 mice: 15 Ts65Dn mice (10 females, 5 males), 15 Ts65Dn mice treated with HU (11 females, 4 males), 12 euploid littermates (7 females, 5 males), 12 euploid littermates treated with HU (7 females, 5 males). Three untreated mice died during this study, two male euploid mice and one male Ts65Dn mouse.

Drug Treatment and Dose Selection

The dosage of HU was based on previous studies focused on the effects of hydroxyurea in different mouse models. Sickle cell disease mouse models were given 50 mg/kg/day of HU to induce fetal hemoglobin and reduce leukocyte–endothelial interactions (Lebensburger et al., 2012). *APPswe/PS1dE9* mice were treated with 45 mg/kg/day to improve spatial memory in the MWM (Brose et al., 2018). Hydroxyurea is an FDA-approved drug for several diseases. Humans with sickle cell disease, HIV, or psoriasis are treated with 15 – 35 mg/kg/day (Medscape, 2018). Based on this information, we used a conservative dose of 30 mg/kg/day of HU in their drinking water for 18 weeks. The HU was refreshed weekly. Control groups received water alone (untreated) for 18 weeks. Water intake per cage was monitored weekly. HU crosses the blood-brain barrier; its brain uptake clearance is 0.10 microl/g/s in mice (Dogruel et al., 2003; Bihorel et al., 2006; Syvanen et al., 2007).

This is the first proof-of-concept study of hydroxyurea treatment in the Ts65Dn model. The treatment started at a stage when only early mild cognitive deficits are present (Olmos-Serrano et al., 2016). In this study, the mice were approximately 3 months of age (mean ± SEM = 11.8 ± 0.3 weeks, range 7–14 weeks) when HU treatment began. Mouse handling and behavioral testing began after at least 10 weeks of HU treatment (range 10–13 weeks). Treatment was continued throughout behavioral testing. After 18 weeks of HU treatment, the mice were euthanized and their organs harvested.

Behavioral Testing

Behavioral testing started when mice reached approximately 6 months of age (23.3 ± 1.8 weeks; see **Supplementary Figure S1**). Mice were handled for several minutes a day for four consecutive days at least 1 week prior to the start of behavioral testing to familiarize them with handling. Two HU-treated Ts65Dn mice were excluded from behavioral testing due to cataracts (one male, one female) and one female HU-treated Ts65Dn mouse was excluded from the RRWM and RAWM because of low body weight (less than 19 g). Tests were performed in the order described below. ANY-maze 5.1 behavioral tracking software (Stoelting Co.; Wood Dale, IL, United States) was used for video-recording and executing each test.

Water Maze Tasks

Water maze tasks were performed as described before (Jankowsky et al., 2005; Savonenko et al., 2005) with small modifications. For all water maze tasks, the water was 20–22°C and made opaque by adding non-toxic white tempera paint to hide a rectangular platform (10 cm × 10 cm) 2 cm below the water surface. The mice were tested in groups of 8–10 mice with an inter-trial interval of 20 min. Each trial was performed for all mice in a group before starting the next trial. If the platform was not found, the mouse was guided visually by placing a finger on top of the platform or by manually guiding the mouse to the platform. Mice were dried between trials and returned to a dry waiting cage.

Platform Pretraining

Mice were first taught to find a platform as an escape from a small pool of opaque water (55 cm diameter) over five trials. For the first trial, each mouse was placed directly next to the platform. For trial 2, each mouse was placed one to two inches away from the platform. For trials 3–5, each mouse was placed halfway between the platform and the wall of the pool.

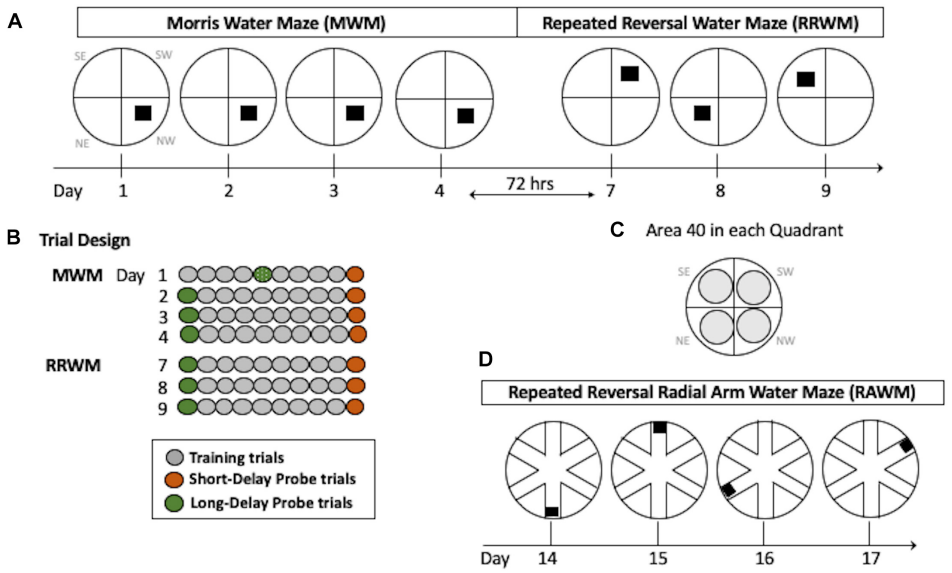


FIGURE 2 | Water maze paradigms. **(A)** The MWM task was performed for four consecutive days with the hidden platform in the same location. Seventy-two hours after Day 4, the RRWM task was started in which the location of the hidden platform was changed each day for three days as depicted. **(B)** Daily trial design. Each day, mice had 8 training trials. In addition, two daily probe trials (at the beginning and the end of the day) were introduced in which the platform was lowered for a variable interval (30–45 s). Since mice were naive, on day 1 the first probe trial of the day was performed after four training trials. **(C)** Areas 40 cm in diameter (gray circles) are shown for each quadrant of the water maze. These areas were used to calculate spatial preferences in the MWM and the RRWM probe trials (see Stat Analyses section for more details). Day designations 1–17 correlate to chronological experimental days. Quadrant designations in **(A,C)** panels: NE, northeast; NW, northwest; SW, southwest; and SE, southeast. **(D)** The RAWM task was performed for four consecutive days consisting of 6 trials. The location of the hidden platform was changed daily.

Straight Swim Pretraining

A platform hidden 2 cm below opaque water was placed at the end of a straight alley (15 × 110 cm). For five trials, each mouse was placed in the end of the alley opposite the platform and given 60 s to find the platform. If the platform was not found, the mouse was guided visually as above. The purpose of this and the previous task was to check for possible deficits in swimming abilities as well as to train the mice to find and stay on a hidden platform as preparation for the subsequent tasks.

Classic Morris Water Maze (MWM)

This task requires incremental learning of a constant platform location over multiple days of training using the same set of spatial cues. Learning in this task results in formation of long-lasting reference memory (Morris, 2001; Nonaka et al., 2017). The duration of the task was 4 days with the order of the trials as shown in **Figure 2**. For each trial, the mouse was placed in a 110 cm pool facing the wall in a randomly predetermined quadrant other than the quadrant containing the platform. Inter-trial intervals were approximately 20 min. Training trials were 60 s long with the hidden platform in its upright position two centimeters below the water surface and available for a mouse to climb on. If a mouse did not find the platform during a training trial it was either visually or manually guided to the platform. Probe trials were conducted with the platform in its lowered position and unavailable for climbing for a variable interval (30–45 s). At the end of this interval, the collapsed platform was returned to its raised position to maintain the same response-reinforcement contingency as in the platform trials, allowing the use of probe trials repeatedly without the effect of extinction (Markowska et al., 1993; Andreasson et al., 2001; Micheau et al., 2004). The probe trials were conducted at the beginning and the end of a daily session, and therefore they assessed memory following short (30 min) and long delays (24 h, the first trial of each daily session except Day 1; **Figure 2**). The last probe trial for this task was run after a 72-h delay and this trial initiated the testing in the RRWM task (see below and **Figure 2**). This design of the probe trials increases sensitivity of the task by detecting deficits or improvements in short- vs. long-term spatial memory as well as accessing memory acquisition at different stages of spatial learning (Andreasson et al., 2001).

Repeated Reversal Water Maze (RRWM)

In contrast to the reference memory task (MWM), where mice are required to remember the same platform location over several days of training, the RRWM task challenged episodic-like memory as mice need to keep changing their memory representation for the environment by discriminating the information by "what," "where" and "when" categories (Morris, 2001; Nonaka et al., 2017). This task was performed similarly to the MWM except the platform location was changed daily for three testing days (**Figure 2**). Each day consisted of a probe trial, followed by eight training trials, and a final probe trial. The start location for each trial was randomly predetermined and the probe trials were always started in a location that the platform had not been in the previous testing day.

Radial Arm Water Maze (RAWM)

After completion of the RRWM task, a radial maze enclosure was placed into the same pool and the mice were required to find the hidden platform in one of the six arms of the radial water maze (**Figure 2D**). Similar to the repeated reversals, the position of the platform was changed daily. Originally sought as only a working memory task, RAWM also includes episodic-like features and, similar to the repeated reversals task, requires use of "where" and "when" categories in an integrated and flexible manner (Savonenko et al., 2005). Procedural aspects of the RAWM task were similar to the previous ones since mice used the same set of spatial cues as in the previous water maze tasks and learned a new platform location every day as in the repeated reversals task (**Figure 2**). For a total of 4 days, each mouse performed six 120 s trials; each trial was started in a randomly predetermined arm. In addition to recording by the AnyMaze software, the tester documented the arms the mouse entered before finding the platform during each trial. Errors were counted as entries into the arms that do not contain the platform or as entries to a correct arm (containing the platform) with a failure to find the platform.

Statistical Analyses

The data were analyzed using the statistical package STATISTICA 13 (TIBCO Software Inc, Palo Alto, CA, United States) and a minimal level of significance $p < 0.05$. Based on our previous studies using the three-tier battery of water maze tasks, the primary outcomes were chosen to ensure the sensitivity of the tests without inflating the number of comparisons. The outcome measures were latency and distance to find the platform, swim speed (all tasks), percent of time spent in Areas 40 (MWM and RRWM), and number of errors (RAWM). The Areas 40 were the areas with a 40 cm diameter centered on the platform locations used in the MWM and RRWM tasks (**Figure 2C**). The sum of all Areas 40 represented only 67% of the pool area that allowed statistical analyses of all four areas without violating degrees of freedom. Therefore, the chance level of swimming in any one of the four Areas 40 was 16%. The data were initially analyzed using three-way repeated measures analyses of variance (RM-ANOVA) with Sex and Group (EU, EU+HU, TS, TS+HU) as independent factors and a repeated measure, RM (trials, blocks, arms, etc.). Sex × Group, Group × RM, and Sex × RM interactions were set as orthogonal.

Only one outcome measure, the latency to find the platform, was significantly modified by Sex because male mice swam with shorter latencies/higher swimming speed. To avoid this interference, we report distance traveled to the platform instead of latency. Importantly, no other than latency outcome measures yielded significant effects of Sex or interactions of Sex with Group or RM. Based on these findings, the data were reported based on results of two-way RM-ANOVAs with Group as a single main factor. The LSD *post hoc* test was applied to significant Group or Group × RM interactions to evaluate differences between four sets of means: EU vs. EU+HU; EU vs. TS; EU vs. TS+HU; TS vs. TS+HU. Two-tailed *t*-test was used to analyze differences from chance levels. The summary of statistical analyses is presented in **Table 1**. The details of statistical results including *F*, df, and *post hoc* tests are presented in **Supplementary Tables S1–S3**. Data in figures represent means ± standard error of means (SEM) unless otherwise noted.

RESULTS

To determine the cognitive differences between Ts65Dn mice and their euploid littermates and to more sensitively detect possible effects of pharmacological treatment with HU on cognitive function, untreated and HU-treated trisomic Ts65Dn mice and euploid littermates were tested in the three-tier battery of water maze tasks. The serial water maze design measures differences in spatial learning and allows for a differential assessment of reference memory, episodic-like memory, and working memory (Jankowsky et al., 2005; Savonenko et al., 2005). In addition, the design of the probe trials with a collapsible platform increased the sensitivity of the tasks by detecting deficits or improvements in short- vs. long-term spatial memory as well as accessing memory acquisition at different stages of spatial learning (Andreasson et al., 2001). Each mouse performed a 4-day classic MWM task followed by 3 days of repeated reversals (RRWM) and a 4-day repeated reversal in a radial water maze (RAWM) (**Figure 2**). During daily probe trials, the hidden platform was lowered for a variable interval (30–45 s) and then raised to maintain the same response-reinforcement contingency as in the platform trials (Markowska et al., 1993; Micheau et al., 2004). Probe trials performed shortly after the training trials monitored short-term memory. Probe trials performed after an overnight delay and before the training trials monitored long-term memory. The mice were 11.8 ± 0.3 weeks old at the start of HU treatment and 23.3 ± 1.8 weeks old at the time of behavioral testing. **Figures 1**, **2** depict our study design and schedule of behavioral testing. **Table 1** and **Supplementary Tables S1–S3** summarize the statistical results for the water maze tasks.

Classic Morris Water Maze (MWM)

The MWM was used to test spatial reference memory acquisition and retention (Morris, 1984; Vorhees and Williams, 2006, 2014). During the first day of MWM training, all four groups of mice improved their ability to locate the platform as indicated by a decrease in the distance swum to find the platform across training trials (**Figure 3A** and see **Table 1** for statistical analysis). As expected, however, Ts65Dn mice traveled significantly greater distances to find the hidden platform as compared to euploid littermates indicating a spatial learning deficit. HU treatment did not significantly affect the performance of either genotype (**Figure 3A**). These differences between Ts65Dn and control mice observed during Day 1 of the MWM persisted through all 4 days of training in this reference memory task (**Figure 3B**). HU-treatment did not improve the Ts65Dn-related deficit (**Figure 3B**).

To test for spatial memory retention, short-delay (30 min) and long-delay (24 h) probe trials were introduced on each training day (**Figure 2**). In the short-delay probe trials, both control euploid groups (EU and EU+HU) increased their preference to the NW Area 40 platform area as training progressed

TABLE 1 | Summary of statistical analyses for the Morris Water Maze (MWM), repeated reversal water maze (RRWM), and the repeated reversal radial arm water maze (RR-RAWM).

Figure	Task	Measurement	RM-ANOVA	Fisher's LSD *post hoc*
3A	MWM	Distance to platform by trial Day 1	$p_{trial} < 1 \times e^{-6}$	EU or EU + HU vs. TS or TS + HU $p < 0.004$
3B	MWM	Distance to platform across days	$p < 1 \times e^{-6}$	EU or EU + HU vs. TS or TS + HU $p < 1 \times e^{-6}$
3C	MWM	Percent time in NW Area 40 during 30-min short-delay probe trial	$p < 1 \times e^{-6}$	EU or EU + HU vs. TS or TS + HU $p \leq 0.022$
3C	MWM	Percent time in NW Area 40 during 30-min short-delay probe trial day 1 vs. day 4	$p = 0.006$	$p_{EU,EU + HU,TS + HU} \leq 0.016$ $p_{TS} = 0.238$
3D	MWM	Percent time in NW Area 40 during 30-min short-delay probe trial, day 4	$p = 6 \times e^{-6}$	EU or EU + HU vs. TS or TS + HU $p \leq 2 \times e^{-5}$
3D	MWM	Percent time in NW Area 40 during 30-min short-delay probe trial on day 4 compared to chance level	$p = 6 \times e^{-6}$	NW_{EU}, $NW_{EU + HU}$, or $NW_{TS + HU}$ $p \leq 0.022$ and $NW_{TS + HU}$ $p = 0.180$
3E	MWM	Percent time in NW Area 40 during 24-h long-delay probe trial	$p = 6 \times e^{-6}$	EU vs. TS or TS + HU $p \leq 2 \times e^{-4}$
3F	MWM	Percent time in NW Area 40 after 72-h delay	$p = 0.010$	EU + HU vs. EU, TS, or TS + HU $p \leq 3.9 \times e^{-4}$
3F	MWM	Percent time in NW Area 40 after 72-h delay compared to other quadrant areas	$p = 1.5 \times e^{-3}$	$NW_{EU + HU}$ vs. NE, NW, or SW $p \leq 1.2 \times e^{-5}$
4A	RRWM	Distance to platform per trial	$p < 1 \times e^{-6}$	EU or EU + HU vs. TS or TS + HU $p \leq 0.004$; TS + HU vs. TS $p = 0.044$
4B	RRWM	Average distance trials 4–8	$p < 1 \times e^{-6}$	TS + HU vs. TS $p = 0.108$TS or TS + HU $p \leq 0.108$
4C	RRWM	Percent time in Area 40 platform areas during 30-min short-delay probe trials	$p < 1 \times e^{-6}$	EU or EU + HU vs. TS or TS + HU $p \leq 5.6 \times e^{-4}$
4D	RRWM	Percent time in the previous day's Area 40 platform area, 24-h long-delay probe trials	$p < 1 \times e^{-6}$	EU or EU + HU vs. TS or TS + HU $p \leq 0.011$; EU + HU vs. EU $p \leq 4.2 \times e^{-4}$
5A	RR-RAWM	Distance to platform per trial	$p_{group} = 0.388$ $p_{trial} < 1 \times e^{-6}$	
5B	RR-RAWM	Average distance trials 4–6	$p = 0.016$	EU vs. TS $p = 0.018$; TS + HU vs. TS $p = 0.115$
5C	RR-RAWM	Average arm entry errors per trial	$p_{trial*group} < 1 \times e^{-6}$	
5D	RR-RAWM	Average arm entry errors trials 4–6	$p < 1.3 \times e^{-3}$	EU, EU + HU, or TS + HU vs. TS $p \leq 0.03$
5E	RR-RAWM	Errors due to swimming in previous day's platform location, trial 1	$p = 3.1 \times e^{-3}$	EU or EU + HU vs. TS or TS + HU $p \leq 0.040$
5F	RR-RAWM	Average previous platform errors trial 1	$p = 3.2 \times e^{-3}$	EU or EU + HU vs. TS or TS + HU $p \leq 0.040$

EU, euploid; EU + HU, HU-treated euploid; TS, Ts65Dn; TS + HU, HU-treated Ts65Dn; NW, northwest. See detailed statistical results in ***Supplementary Tables S1–S3****.*

(**Figure 3C**, **Table 1**, and **Supplementary Table S1**). In contrast, performance of Ts65Dn mice remained close to the chance level (16%) throughout the entire period of testing in the MWM task (**Figures 3C,D**). This was consistent with the deficit in acquisition of spatial memory observed in the TS group during the training trials (**Figures 3A,B**). The performance of HU-treated Ts65Dn mice (TS+HU) was also significantly worse compared to euploid littermates (**Figure 3C**). However, in contrast to the TS group, TS+HU mice significantly increased the time spent in the NW Area 40 platform area between Day 1 and Day 4 (**Figure 3C**). An assessment of the final accuracy of spatial reference memory (Day 4, **Figure 3D**) showed that the time spent by HU-treated Ts65Dn mice in the NW Area 40 platform area was significantly higher than the chance level (**Supplementary Table S1**). These data indicate that despite the dramatic deficits observed in the acquisition of spatial reference memory in Ts65Dn mice, HU-treatment significantly improved the accuracy of their memory when tested during the short-term delay.

In the probe trials with longer delays (24 or 72 h), none of the Ts65Dn groups showed performance better than the chance level (**Figures 3E,F**). Notably, the data from the 24-h delay probe trials indicated that the euploid mice successfully retained the location of the hidden platform (**Figure 3E**). However, only the HU-treated euploid mice showed significant recall of the platform location after a 72-h delay (**Figure 3F**). This finding implicates a nootropic effect of HU in control mice. Overall, HU-treatment most significantly improved the short-term memory of the HU-treated Ts65Dn mice (Day 4) and the long-term reference memory of the euploid mice.

Repeated Reversal Water Maze (RRWM)

During the RRWM the hidden platform was moved to a different pool quadrant each day for 3 days to assess the cognitive ability to learn a new platform location daily, cognitive plasticity (**Figure 2A**). The results of the 72-h MWM probe trial, the first probe trial of the RRWM task, demonstrated that the HU-treated euploid mice were the only group of mice to remember the location of the platform from the previous week's testing. Thus, in contrast to other groups, HU-treated euploid mice had not only to learn the new platform location, but also inhibit visiting the previous platform. Despite this additional complexity, the performance of HU-treated euploid mice in the training trials was indistinguishable from the untreated euploid mice (**Figures 4A,B**). Both groups of Ts65Dn mice swam a

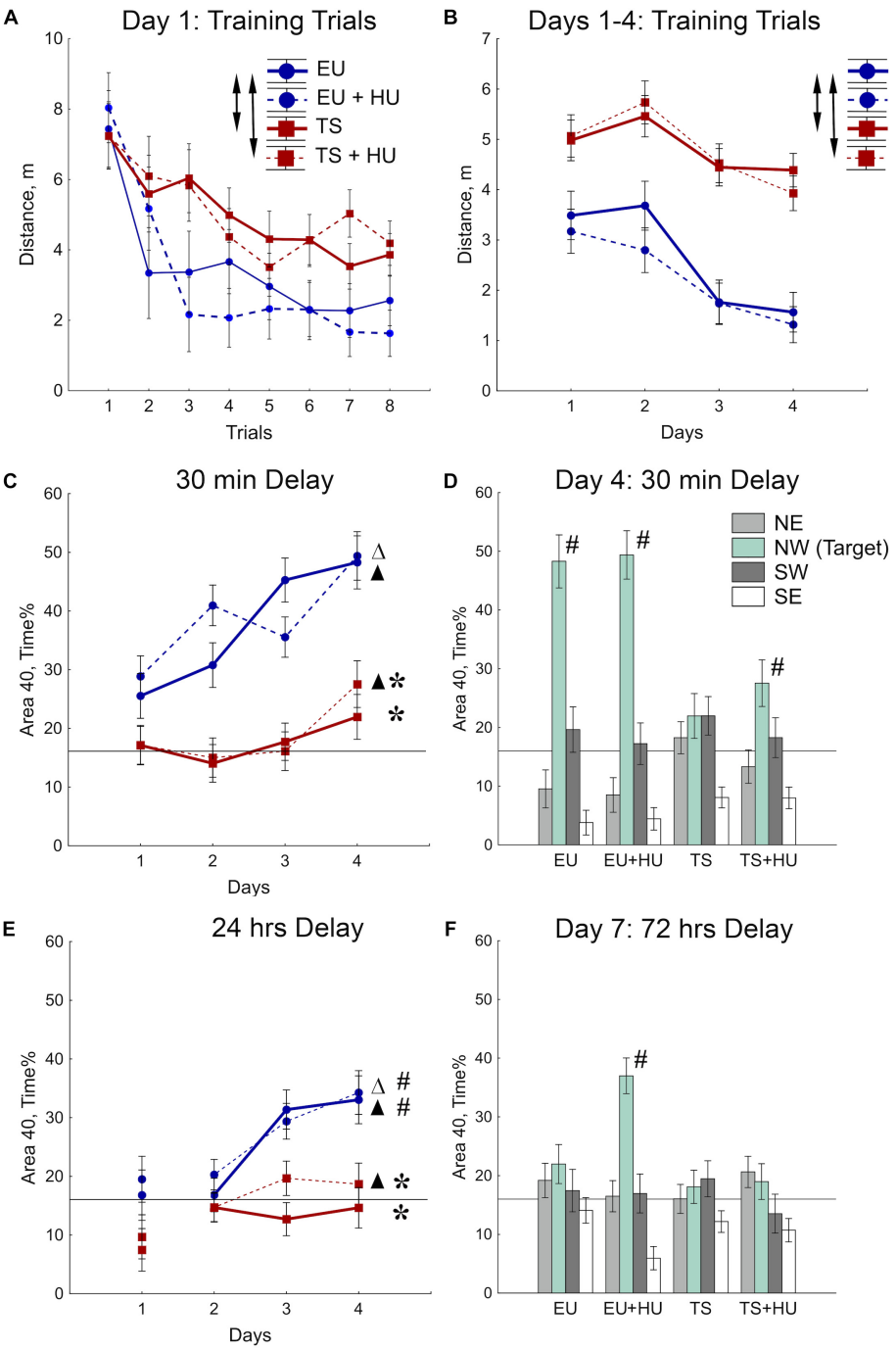


FIGURE 3 | Trisomic Ts65Dn mice display deficits in spatial learning and memory in the MWM task. HU treatment marginally improved deficits in Ts65Dn mice and facilitated memory retention in control mice. **(A)** Day 1 learning dynamics. Group averages for distance traveled to the hidden platform during eight training trials on day 1. **(B)** Average distance traveled to the hidden platform across days. **(C)** Short-term probe trials conducted after a 30-min delay. Percent of time spent in the Area 40 platform area during the last probe trial of days 1–4. **(D)** Spatial preferences for different Areas 40 in the water maze. Percent of time spent in each Area 40 is shown for probe 2 (30-min delay) on Day 4 of the MWM task. **(E)** Long-term probe trials conducted after a 24-h delay. Percent of time spent in the NW Area 40 platform area during the first probe trial of days 1–4. **(F)** Spatial preferences for different Areas 40 after a 72-h delay. Arrows in **(A,B)** indicate significant differences from EU group ($p < 0.005$, LSD *post-hoc* test applied to significant main effect of Group in RM-ANOVA, see statistical results in **Table 1**). Asterisks in **(C,E)** indicate significant differences from EU group ($p < 0.005$, LSD *post-hoc* tests applied to a set of means at particular levels of Group × RM interaction in RM-ANOVA, **Table 1**). Triangles in **(C,E)** indicate significant differences between Day 1 and Day 4 ($p < 0.05$, LSD *post-hoc* test, **Table 1**). Pound signs in **(D–F)** indicate significant differences from the chance level for the NW Area 40 ($p < 0.025$, two-tailed *t*-test). Solid lines in **(C–F)** represent the chance level of performance during probe trials (16%). EU, euploid, $n = 10$. EU + HU, HU-treated euploid, $n = 12$. TS, Ts65Dn, $n = 14$. TS + HU, HU-treated Ts65Dn, $n = 13$. NE, northeast; NW, northwest; SE, southeast; SW, southwest.

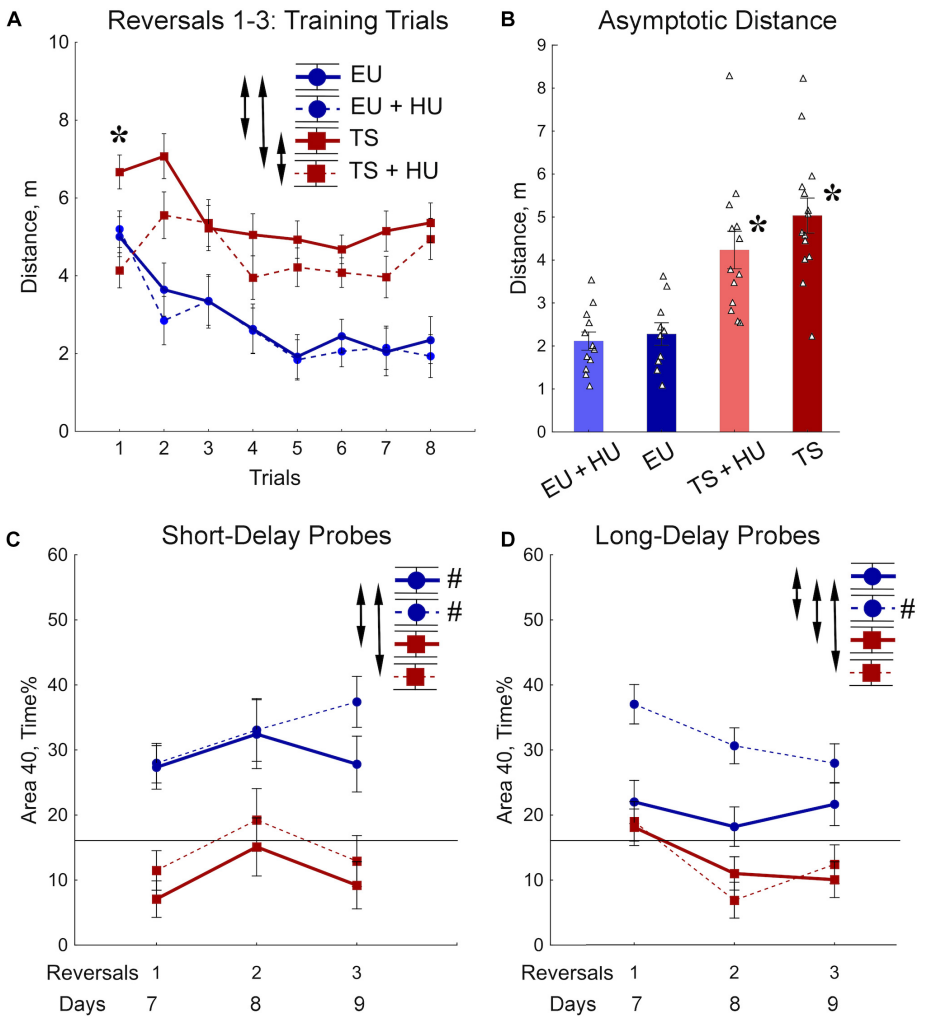


FIGURE 4 | Trisomic Ts65Dn mice display deficits in acquisition of memory for new platform locations in the RRWM task. HU-treatment improved retention of new memories in euploid mice. **(A)** The group means of the distance traveled to the new platform locations averaged per trial across days. **(B)** Asymptotic level of performance shown as the distance averaged across trials 4–8. No effect of trial was observed during this period of training (**Table 1**). **(C)** Percent of time spent in the area 40 cm in diameter around a new platform location during the short-term probe trials with a 30-min delay. **(D)** Percent of time spent in the Area 40 surrounding the previous day's platform as assessed in the long-term probe trials with a 72-h delay for reversal 1 and a 24-h delay for reversals 2 and 3. Arrows in panels **(A,C,D)** indicate significant differences from EU group ($p < 0.05$, LSD *post-hoc* test applied to significant main effect of Group in RM-ANOVA, see statistical results in **Table 1**). Asterisks in **(A,B)** – significant differences from EU group ($p < 0.005$, LSD *post hoc* test applied to main Group effect in one-way ANOVA, **Table 1**). Pound signs in **(C,D)** indicate significant differences between an average preference to new Area 40 platform locations and a chance level ($p < 0.025$, two-tailed *t*-test). Solid lines in **(C,D)** represent the chance level of performance during probe trials (16%). EU, euploid, $n = 10$. EU + HU, HU-treated euploid, $n = 12$. TS, Ts65Dn, $n = 14$. TS + HU, HU-treated Ts65Dn, $n = 12$.

significantly greater distance than control euploid mice to reach the new platform location (**Figure 4A**). Notably, HU-treatment resulted in mild but significant amelioration of the learning deficits in Ts65Dn mice (**Figure 4A**, **Table 1**, and **Supplementary Table S2**). These between-group differences were modulated across the training trials. In particular, the ameliorating effect of HU-treatment in Ts65Dn mice was the most pronounced in the first trial after the new platform location was introduced (**Supplementary Table S2**). Later in the training when the asymptotic level of performance was reached (Trials 4–8), the advantage of HU-treatment in Ts65Dn mice became marginal (Trials 4–8, **Figure 4B**).

Short- (30 min) and long-delay (24 h) probe trials revealed dramatic deficits in memory for the new platform location in both the untreated and HU-treated Ts65Dn mice (**Figures 4C,D**). These deficits were characterized by significantly poorer performance as compared to the euploid mice as well as by an inability to retain any spatial preference for a platform location higher than the chance level (16%; **Figures 4C,D**). These findings were consistent with the impairment in memory acquisition observed in trisomic mice during training trials (**Figure 4B**). In both groups of euploid mice, the retention of memory for the new platform location was above the chance level when tested shortly after the training trials (30 min short-delay; **Figure 4C**).

However, after the 24 h long-delay, untreated euploid mice failed to show spatial preference. Notably, HU-treated euploid mice spent significantly more time in the Area 40 platform areas than untreated controls, and their performance was considerably above the chance level even after the 24 h long-delay (**Figure 4D**). Thus, the data from the RRWM task indicated that trisomic mice were unable to learn this complex episodic-like memory task. HU-treatment resulted only in a mild improvement of learning deficits in trisomic mice that did not result in better short- or long-term memory. In the euploid mice, HU-treatment significantly improved long-term memory of the platform locations without interfering with behavioral flexibility in acquisition of new spatial memories.

Repeated Reversal Radial Arm Water Maze (RAWM)

All four groups of mice were tested in a repeated reversal RAWM. This task requires cognitive flexibility to learn a new platform location daily, similar to the RRWM. However, the RAWM protocol does not require procedural learning to stay around the platform location when the platform is absent as do the probe trials of the MWM and RRWM. In the RAWM task, the mice climb onto the platform as soon as they find it. Analyses of distance and number of errors before finding the platform revealed similar patterns of results for both variables (**Figures 5A–D**). The between-group differences were modulated across the training trials (**Figures 5A,C**, **Table 1**, and **Supplementary Table S3**). During the orientation trial, when a new location of the platform was first presented, the euploid mice showed longer distances and a higher number of errors than trisomic mice. During the asymptotic phase of training (trials 4–6), the between-group differences were characterized by significant deficits observed in untreated Ts65Dn mice as compared to untreated euploid controls. Notably, for both distance and the number of errors measured (**Figures 5B,D**), HU-treated Ts65Dn mice were not statistically distinguishable from euploid controls, indicating beneficial effects of the HU-treatment. Furthermore, HU-treated Ts65Dn mice made significantly fewer errors than did their untreated trisomic littermates (**Figure 5D**).

To determine if the longer distances and larger number of errors made by euploid mice during the first trials were due to their memory of the previous day's platform location, the percentage of errors due to entry into the arm that contained the platform on the previous training day was analyzed (**Figures 5E,F**). Indeed, the number of visits to the previous day's platform location was significantly greater for untreated and HU-treated euploid mice than Ts65Dn or HU-treated Ts65Dn mice. Moreover, in contrast to trisomic mice, the number of visits to the previous day's platform was significantly higher than the chance level (16.6%; **Figure 5F**). None of the genotypes were significantly affected by HU-treatment in this measurement of performance (**Figures 5E,F**). Overall, the RAWM results suggest that HU-treatment in trisomic Ts65Dn mice improved their acquisition of spatial memory for a new platform location but did not improve the deficits in long-term memory retention.

DISCUSSION

Here we demonstrate that hydroxyurea (HU), an FDA-approved pharmacological agent known to induce the adaptive cellular stress response, can improve acquisition and retention of spatial memory in mice. One of the main novel findings is that HU treatment significantly improves long-term retention of reference and episodic-like memory in control mice, which is consistent with a robust nootropic effect. The beneficial effects of HU treatment in Ts65Dn mice are significant but limited. HU treatment only partially ameliorates some of the deficits exhibited by 6 month-old Ts65Dn mice in reference and episodic-like memories. These results also show the value of using the three-tier water maze design to more sensitively assess the cognitive deficits present in Ts65Dn mice and to better evaluate the therapeutic potential of pharmacological agents such as HU on learning and memory in both wildtype and genetically modified mice.

Since individuals with DS have more difficulty with working memory and episodic long-term memory than with implicit reference memory (Liogier d'Ardhuy et al., 2015), testing DS models and potential treatments using experimental designs that assess different memory systems has translational benefits. The three-tier water maze testing (Savonenko et al., 2005; Borchelt and Savonenko, 2008) begins with the classic MWM, a task that tests spatial reference memory and is the most widely used task to document spatial learning and memory deficits in Ts65Dn (Reeves et al., 1995; Moran et al., 2002; Seo and Isacson, 2005; Das et al., 2013; Dutka et al., 2015). Here we incorporated daily memory probe trials at the start and end of each MWM training session to monitor memory acquisition and retention after short and long delays. This schedule of probe trials revealed that despite significant improvements in the distance to reach the platform observed in Ts65Dn mice within a daily training session, there was no significant acquisition of spatial memory when tested at the end of daily training. These data indicate that the improvement observed in trisomic mice during training trials cannot solely be attributed to spatial learning but rather to engagement of non-spatial adaptive strategies. The robust deficit of Ts65Dn mice in the acquisition of spatial memory prevented incremental daily increases in reference memory as measured by the 4-day long MWM. However, HU-treatment produced mild but significant benefits in Ts65Dn by improving the short-term retention of spatial information as measured by a significant increase in time spent in the Area 40 platform area at the end of the MWM training compared to the chance level.

Less documented in Ts65Dn mice are deficits in spatial episodic-like and working memories. The repeated reversals task (RRWM) introduces an additional challenge of cognitive flexibility for learning a new platform location every day. A recent study, using a similar behavioral paradigm of reversal learning, indicated that diminished cognitive flexibility is the most robust cognitive impairment in Ts65Dn mice (Olmos-Serrano et al., 2016). Here, we show that at the stage of severe deficits in the acquisition of reference memory (discussed above) trisomic mice are unable to demonstrate behavior consistent with episodic-like memory. The consequences of this deficit were further

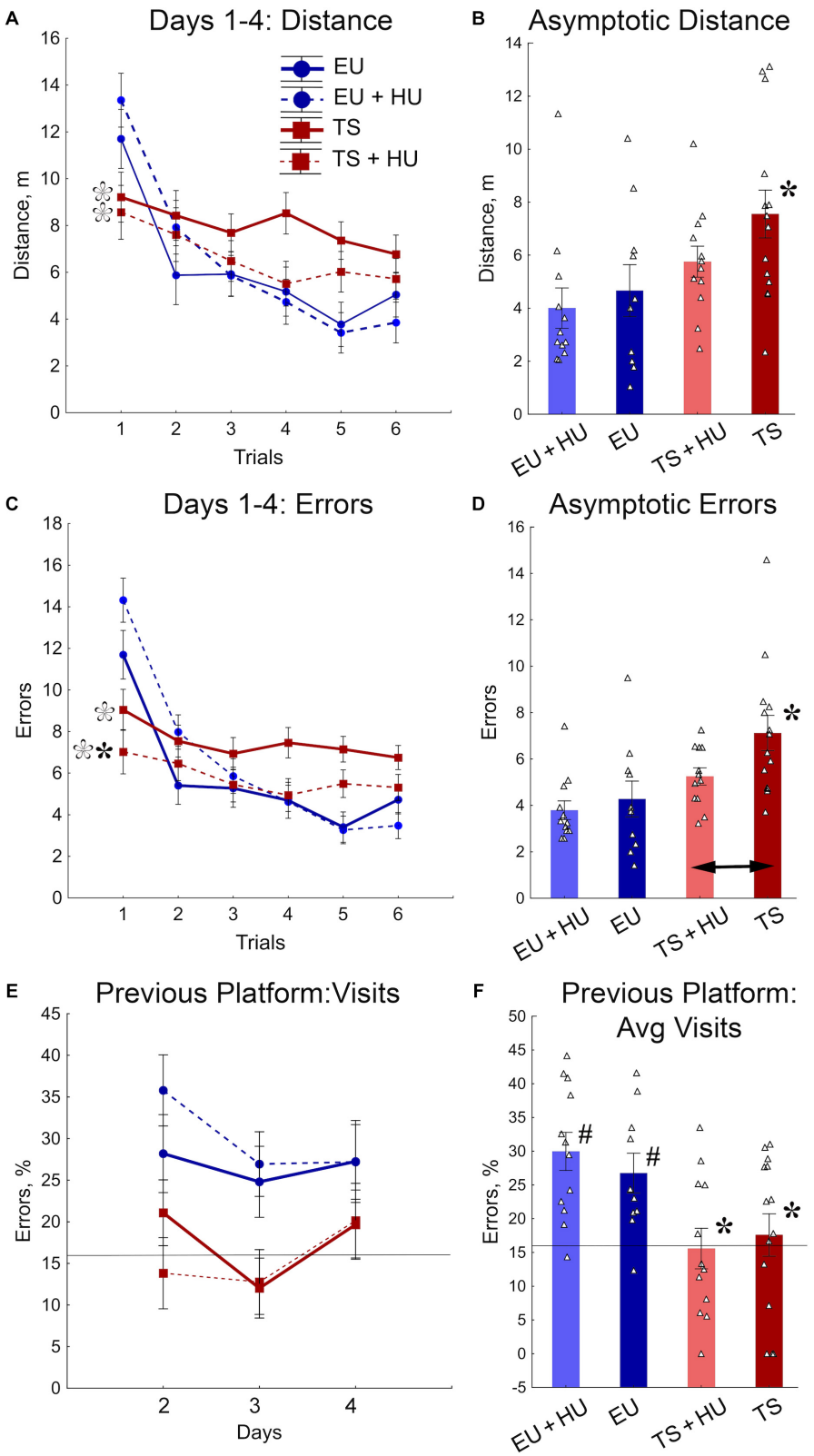


FIGURE 5 | HU-treatment ameliorated deficits of trisomic Ts65Dn mice in acquisition of memory for new platform locations in the RAWM task. **(A)** Average distance to the platform per trial averaged across days. **(B)** Asymptotic performance assessed as average distance for trials 4–6. **(C)** Average number of errors per trial

(Continued)

FIGURE 5 | Continued
averaged across days. **(D)** Asymptotic performance assessed as average number of errors for trials 4–6. **(E)** Percent of errors due to visits to the previous day's platform location during trial 1 (total number of entries to previous day's platform arm/total number of arm entries × 100%). **(F)** Group means for percent of errors due to visits to the previous day's platform location (as shown in **E**) averaged across days 1–3. Filled asterisks in **(B–D)** indicate significant differences from EU group ($p < 0.02$, LSD *post hoc* test applied to main Group effect in one-way ANOVA, **Table 1**). Empty asterisks indicate significant differences from EU + HU group ($p < 0.01$) and were added to panels **(A,C)** to explain significant effects of Group in trial 1 (one-way ANOVA, **Table 1**). Arrow in **(D)** indicates significant differences between TS and TS + HU groups ($p < 0.05$, LSD *post hoc* test applied to significant main effect of Group in ANOVA, **Table 1**). Pound signs in **(F)** indicate significant differences between levels of errors and a chance level (16.6%; $p < 0.010$, two-tailed *t*-test). Solid lines in **(E,F)** represent the chance level of performance (16.6%). $n = 10$. EU + HU, HU-treated euploid, $n = 12$. TS, Ts65Dn, $n = 14$. TS + HU, HU-treated Ts65Dn, $n = 12$.

detected in the radial water maze (RAWM) as an increase in the number of errors and distance swum to find the new locations of the hidden platform. Considering the severity of the deficits observed in Ts65Dn mice in both episodic-like memory tasks, the finding that HU treatment partially counteracted such severe cognitive impairment is remarkable. The beneficial effects of HU-treatment in the RRWM were limited to training trials, in particular the 1st trial after the mice were introduced to a new platform location (immediately after the 1st probe trial). In the RAWM, the beneficial effects of HU-treatment extended to more training trials which resulted in significant improvements of asymptotic performance levels. Some procedural differences between RRWM and RAWM tasks might support more sensitive detection of behavioral responses to HU-treatment in the latter task. The RAWM helps to circumvent thigmotaxic behavior that the Ts65Dn mice tend to show in a circular pool (Vorhees and Williams, 2006; Altafaj et al., 2013). One of the other main differences with RRWM is the lack of probe trials in the RAWM which bypasses the requirement of instrumental learning to stay around the platform when it is not readily available (Savonenko et al., 2005; Borchelt and Savonenko, 2008). Summarizing the observations from all three water maze tasks, the most common characteristic of cognitive deficits observed in trisomic mice was their inability to rapidly acquire new information for long-term storage, a feature akin to episodic-like memory. Despite the severity of cognitive impairments, HU-treatment mildly but significantly ameliorated these deficits in trisomic mice.

Multiple compounds have been tested for the ability to improve DS cognitive deficits in mouse models and human clinical trials (reviewed in Hart et al., 2017). Tested treatments have targeted neurogenesis (fluoxetine and lithium), *N*-methyl-D-aspartic acid (NMDA) receptor functioning (memantine), neurotrophin production, oxidative stress (vitamin E), and Alzheimer's neuropathology (Lockrow et al., 2009; Bianchi et al., 2010; Netzer et al., 2010; Rueda et al., 2010, 2012; Begenisic et al., 2014). Additionally, a single postnatal dose of a sonic hedgehog pathway agonist, SAG, has been shown to normalize the morphology of the cerebellum as well as improving spatial learning and memory performance of Ts65Dn mice; a single injection of SAG on the day of birth normalizes learning in the MWM and its physiological correlate, long term potentiation, in adult mice (Das et al., 2013).

There are several possible reasons why the HU treatment in our study yielded only small improvements. Since this was the first study of this compound in a DS model, parameters for HU treatment have not been optimized. Changing the dosage and/or starting HU treatment at an earlier age may improve the efficacy of HU. Also, some brain abnormalities occur prenatally in DS, and therefore, neurodevelopmental defects are present at birth (Stagni et al., 2015). Most of neurogenesis occurs during the prenatal period. However, neurogenesis in the cerebellum does not stop until approximately 2.5 weeks after birth and is slowly ongoing in the dentate gyrus of the hippocampus. The effects of HU at the doses used here have not been studied *in utero*. Assuming prenatal HU treatment is safe, HU treatment may have the largest effect if started *in utero* or may have a larger effect on cerebellar and hippocampal development if administered neonatally (Stagni et al., 2015).

The pathways altered by HU may not have a significant effect on the structural and neurodevelopmental defects that are already present when the treatment started (∼3 month-old Ts65Dn mice). Some of the cognitive deficits experienced by individuals with DS are due to structural and developmental abnormalities. However, additional neuronal changes and decreases in cognition also occur during the lifetime of an individual with DS. In fact, approximately half of all individuals with DS will exhibit the neuropathology of Alzheimer's-associated dementia by 60 years old (Head et al., 2012). This highlights the potential for therapeutic improvement of age-related cognitive decline in DS. In a mouse model, basal forebrain cholinergic (BFC) degeneration begins to occur at 6 months of age and continues throughout adulthood (Hunter et al., 2004; Kelley et al., 2014). Since BFC neurons are important for a variety of processes including learning/memory and attention (Hangya et al., 2015; Harrison et al., 2016), beginning HU treatment earlier and assessing the effects of HU treatment on a wider range of age-related cognitive deficits (1–12 months) may be informative for understanding a window of opportunity for HU treatment to be effective.

In our previous study, HU-treatment ameliorated the deficits in spatial reference memory in a model of Alzheimer's disease, *APPswe/PS1dE9* mice (Brose et al., 2018). In contrast to that model, the efficacy of HU treatment in Ts65Dn mice was much more limited. The *APPswe/PS1dE9* mice, when tested in the MWM task with similar protocols (Savonenko et al., 2005; Borchelt and Savonenko, 2008; Liu et al., 2008), have less severe deficits in reference memory than that observed in trisomic mice. Although the absolute levels of memory measures in the *APPswe/PS1dE9* mice can be lower than in control mice starting from 6 to 8 months of age, their spatial preferences remain higher than chance level up to 18 months of age indicating robust reference memory. In contrast, the Ts65Dn mice tested at approximately 5–6 months of age in this study are not able to acquire any spatial preferences under similar protocols.

The differences in the level of cognitive disability between the two disease models can be one of the reasons for diminished efficacy of HU treatment in the 5–6 month old Ts65Dn mice.

One of the unexpected findings of this study is the powerful nootropic effect of HU treatment observed in control mice. The HU-treated euploid mice were the only group of mice to accurately remember the platform location 72 h after the last day of the MWM training. The intact memory for the previous platform location increased the complexity of reversal learning for this group as they needed to re-write their memories to learn the new platform location. Nevertheless, the acquisition of memory for the new platform location in HU-treated euploid mice was as efficient as in their untreated littermates. Furthermore, HU-treated euploid mice successfully remembered the new reversed locations of the platform when tested 24 h later, a feature unreachable by untreated control mice. These nootropic effects of HU observed in the control mice were consistent with facilitation of memory retention in constant (reference memory) as well as time-variant conditions (episodic-like memory). Considering that episodic memory is particularly sensitive to aging, the data on HU nootropic effects suggest that this treatment may be beneficial to prevent such aging-related cognitive declines. Therefore, HU may be therapeutic for age-related dementia in DS and neurodegenerative diseases such as Alzheimer's and Parkinson's disease. Although we did not examine the molecular effects of HU in this study, we showed previously that HU treatment of cultured rat hippocampal neurons attenuated the loss of cell viability of neurotoxins that increase oxidative, metabolic, and excitotoxic stress, characteristics of neurodegenerative diseases. The neurotoxins tested included hydrogen peroxide, glutamate, rotenone, and amyloid beta peptide 1–42 (Brose et al., 2018). Furthermore, HU treatment of rat hippocampal neurons attenuated reductions of mitochondrial function induced by hydrogen peroxide treatment. Neurodegenerative disorders also exhibit defects in components of the adaptive cellular stress response pathways, pathways known to be upregulated by HU in human fibroblasts (Brose et al., 2012). Since neurodegenerative disorders tend to occur later in life, this leaves a large treatment window. The ability to diagnose these diseases at an early stage could be critical to treatment outcome.

CONCLUSION

For future studies of therapeutic agents in Ts65Dn mice, we suggest the use of the three-tier water maze design because of its sensitivity to detect the effects of pharmacological agents on spatial learning and different types of memory including reference, episodic-like and working memories. Further studies in trisomic animal models should be completed to determine if a different dosage, different schedule or an earlier start of HU treatment may result in better efficacy. Additionally, the nootropic effects of HU on reference and episodic-like memory suggest the expansion of the therapeutic studies of HU to other neurological disorders with learning and memory deficits. The average lifespan of individuals with DS continues to increase. Identifying therapeutic treatments that improve the cognitive abilities of individuals with DS or delay cognitive decline offers a significant opportunity to positively affect the lives of DS individuals.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the NIH Guide for the Care and Use of Laboratory Animals and the Johns Hopkins University Institute of Animal Care and Use Committee. The protocol was approved by the Johns Hopkins University Institute of Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

RB, AS, KS, and RR contributed to conception and design of the study protocol. RB and BD performed the experimental protocols and collected the data. RB and AS analyzed and interpreted the data. RB and AS wrote the manuscript. All authors contributed significantly to preparation of the manuscript and have read and approved of the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnagi.2019.00096/full#supplementary-material

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Proprotein Convertase Subtilisin/Kexin Type 9, Brain Cholesterol Homeostasis and Potential Implication for Alzheimer's Disease

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Alzheimer's disease (AD) has been associated with dysregulation of brain cholesterol homeostasis. Proprotein convertase subtilisin/kexin type 9 (PCSK9), beyond the known role in the regulation of plasma low-density lipoprotein cholesterol, was first identified in the brain with a potential involvement in brain development and apoptosis. However, its role in the central nervous system (CNS) and in AD pathogenesis is still far from being understood. While *in vitro* and *in vivo* evidence led to controversial results, genetic studies apparently did not find an association between PCSK9 loss of function mutations and AD risk or prevalence. In addition, a potential impairment of cognitive performances by the treatment with the PCSK9 inhibitors, alirocumab and evolocumab, have been excluded, although ongoing studies with longer follow-up will provide further insights. PCSK9 is able to affect the expression of neuronal receptors involved in cholesterol homeostasis and neuroinflammation, and higher PCSK9 concentrations have been found in the cerebrospinal fluid (CSF) of AD patients. In this review article, we critically examined the science of PCSK9 with respect to its modulatory role of the mechanisms underlying the pathogenesis of AD. In addition, based on literature data, we made the hypothesis to consider brain PCSK9 as a negative modulator of brain cholesterol homeostasis and neuroinflammation and a potential pharmacological target for treatment.

Keywords: PCSK9 (proprotein convertase subtilisin/kexin type 9), Alzheimer, cholesterol, apolipoprotein E, neuron, brain, cognitive, apoE receptors

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INTRODUCTION

The Proprotein convertase subtilisin/kexin type 9 (PCSK9), acts as one of the major regulators of cholesterol homeostasis, by mediating the degradation of hepatic low density lipoprotein receptors (LDLr) (Macchi et al., 2019). Interestingly, PCSK9 was firstly identified in the brain where its expression in primary embryonic telencephalon cells was maximal between embryonic days 13–15, a gestational period characterized by intense neurogenesis (Seidah et al., 2003). In zebrafish, but not in mice, specific knockdown of PCSK9 mRNA led to a general disorganization of cerebellar

neurons and loss of hindbrain-midbrain boundaries, with an end result of embryonic death (Poirier et al., 2006).

In this review article, we will focus on the role of PCSK9 at the cerebral level with particular attention on its potential involvement in neuronal functions and Alzheimer's disease (AD) pathogenesis. We also critically examined the possibility to consider PCSK9 as a modulator of brain cholesterol homeostasis and inflammation and a potential pharmacological target for neurodegenerative disorders.

BRAIN CHOLESTEROL HOMEOSTASIS

Cholesterol is one of the most important molecules in brain physiology (Chang et al., 2017): it is an important component of myelin, it is involved in neuronal development, synaptogenesis, outgrowth of neuritis, maintenance and repair of damaged membranes (Dietschy, 2009). Due to the presence of the blood-brain barrier (BBB), the brain relies on *in situ* local cholesterol synthesis (Björkhem and Meaney, 2004). In fact, cholesterol cannot cross the BBB, unlike its side-chain oxidized metabolites, 24S-hydroxycholesterol and 27-hydroxycholesterol (Björkhem et al., 2019). Central nervous system (CNS) cells are able to synthesize cholesterol but adult neurons progressively loose this capacity and become dependent on cholesterol provided from astrocytes (Dietschy and Turley, 2004; Saito et al., 2009). Depletion of neuronal cholesterol leads to excess tau phosphorylation, changes in β-amyloid (Aβ) peptides metabolism, neural oxidative stress reactions, ultimately resulting in neurodegeneration, as demonstrated in *ex vivo* rat hippocampus slices (Koudinov and Koudinova, 2005). The transport of cholesterol from astrocytes to neurons is warranted by peculiar molecules and receptors that cooperate in a coordinated manner. Cholesterol produced from astrocytes undergoes cholesterol efflux to Apolipoprotein E (ApoE)-containing particles through the activity of transporters, such as the ATP binding cassette transporters A1 (ABCA1), G1 (ABCG1) and G4 (ABCG4) (Chen et al., 2013). Subsequently, cholesterol transported by such particles, that resemble plasma HDL in composition and size, is finally incorporated into neurons by the particles binding to specific receptors, such as the LDL receptor (LDLr), the LDL receptor-related protein 1 (LRP1), the VLDL receptor (VLDLr) and the ApoE receptor 2 (ApoEr2) (Bu, 2009). Concerning the latter two, PCSK9 increases their degradation (Poirier et al., 2008; Canuel et al., 2013), implying PCSK9 in cerebral cholesterol homeostasis. This hypothesis is strengthened by *in vivo* findings showing that LDLr expression is reduced by PCSK9 during brain development and after transient ischemic stroke (Rousselet et al., 2011). It is therefore conceivable that the degrading activity of PCSK9 on lipoprotein receptors listed above may translate in a reduced cholesterol uptake by neurons, with potential deleterious consequences (Koudinov and Koudinova, 2005). However, not all data are consistent with this hypothesis. Liu et al. (2010) found that PCSK9 did not affect the expression of LDLR, VLDLR and apoEr2 in the mouse brain (Liu et al., 2010). These discrepancies highlight the need for further studies to dissect out the involvement of PCSK9 on brain cholesterol homeostasis.

PCSK9 AND ALZHEIMER'S DISEASE PATHOGENESIS

Alterations of CNS cholesterol homeostasis are associated with various neurodegenerative disorders, including AD (Sato and Morishita, 2015; Arenas et al., 2017). Genomic-wide association (GWAS) studies have identified several loci involved in lipid metabolism among AD susceptible genes (Lambert et al., 2013; Dong et al., 2017). A striking example of this association is the ε4 allele of the APOE gene encoding ApoE, the main apolipoprotein mediating the transport of cholesterol in the CNS (Mahoney-Sanchez et al., 2016). The E4 isoform is undoubtedly one of the most predictive factors for AD onset (Liu et al., 2013). However, recent studies have identified other genes involved in lipid metabolism, such as BIN1, CLU, PICALM, ABCA7, ABCA1, ABCG1 and SORL1 (Dong et al., 2017; Picard et al., 2018). From a molecular point of view, the cerebral cholesterol accumulates in lipid rafts, membrane microdomains where the processing of the amyloid precursor protein (APP; Picard et al., 2018) occurs, leading to deposition of insoluble fragments of Aβ in brain parenchyma. At this regards, it has been found that cholesterol promotes amyloidogenesis by providing structural stability to membrane-adjacent lipid rafts (Vetrivel and Thinakaran, 2010). Consequently, modulation of cholesterol content in lipid rafts is able to affect deposition of Aβ.

The few and controversial data on PCSK9 and AD are summarized in **Table 1**. Concerning neuronal apoptosis, a pro-apoptotic activity of PCSK9 may occur through the upregulation of caspases or the reduction of the ApoEr2 levels (Wu et al., 2014). In $APOE^{(-/-)}$ mice fed with a high-fat diet, the hippocampal neuronal apoptosis was associated with an increase of PCSK9 expression (Zhao et al., 2017). Consistently, silencing of PCSK9 attenuates the neuronal apoptosis induced by cerebral ischemia reducing brain damage in mice (Wang et al., 2018). Conversely, a preventive action of PCSK9 on neuronal apoptosis may occur through the decrease in Aβ generation (Wu et al., 2014). In addition, the direct effect of PCSK9 on Aβ processing is still unresolved. In its absence, mice show increased expression of the β-site amyloid precursor protein-cleaving enzyme 1 (BACE1), the protease producing toxic Aβ that accumulates in neuritic plaques of AD brains. This effect translates in an increased total Aβ brain deposition: PCSK9 overexpression in mice reduced BACE1 levels (Jonas et al., 2008). On the other hand, in brain-damaged rats, the administration of a small molecule inhibiting PCSK9 prevented dendritic spine loss by attenuating the aggregation of Aβ and neuroinflammation (Apaijai et al., 2019). No evidence that PCSK9 regulates BACE1 levels or APP processing in the brain of mice has been reported by other authors (Liu et al., 2010; Fu et al., 2017). To the best of our knowledge, no data are available on the potential influence of PCSK9 on tau phosphorylation, another peculiar hallmark of AD pathogenesis.

The impact of PCSK9 on neurocognitive performances in pre-clinical models has been indirectly suggested by the observation that deletion of the LRP1, which is sensitive to the degrading action of PCSK9 (Canuel et al., 2013), leads to a reduced Aβ clearance and to cognitive deficits in mice

TABLE 1 | Summary of studies investigating the involvement of PCSK9 in AD pathogenesis.

Study	Experimental model	Involvement of PCSK9 in AD	Effect on cholesterol metabolism and inflammation
Wu et al. (2014)	Mice model	PCSK9 promotes neuronal apoptosis	Reduced apoEr2
Wu et al. (2014)	Mice model	PCSK9 prevents neuronal apoptosis and decreases Aβ generation	
Zhao et al. (2017)	Mice model	Hyperlipidaemia induces neuronal apoptosis by increasing PCSK9 and BACE1 expression	Increased lipid accumulation in the hippocampus
Wang et al. (2018)	Mice model	Inhibition of PCSK9 attenuates the neuronal apoptosis	Reduced ApoEr2 in hippocampus and cortex
Jonas et al. (2008)	Cellular models	Absence of PCSK9 induces Aβ production while its overexpression reduces BACE1 levels	Reduced LDLr
Apaijai et al. (2019)	Rats	PCSK9 inhibitor administration attenuates Aβ aggregation	Reduced number of $CD11b^{+}/CD45^{high}$ microglia
Liu et al. (2010)	Mice model	PCSK9 does not regulate BACE1 levels or APP processing in the brain	No effect on LDLr, VLDLr and apoEr2
Fu et al. (2017)	Cellular and mice models	PCSK9 does not regulates APP processing in brain	No effect on LDLr
Reynolds et al. (2010) Shibata et al. (2005) Benn et al. (2017) Mefford et al. (2018) Paquette et al. (2018)	Human (genetic studies)	PCSK9 gene variants are not associated to the risk ratio for AD	
Zimetti et al. (2017)	Human	PCSK9 levels are increased in CSF of AD patients	Correlation between PCSK9 and apoE4. PCSK9 levels higher in APOE ε4 carriers
Courtemanche et al. (2018)	Human	PCSK9 levels are increased in CSF of AD and non-AD neurodegenerative patients	CSF PCSK9 is positively correlated with AD biomarkers

Abbreviations: AD, Alzheimer's Disease; BACE1, β-site amyloid precursor protein-cleaving enzyme 1; Aβ, Amyloidβ; CSF, cerebrospinal fluid; apoE, apolipoprotein E; LDLr, low density lipoprotein receptor; VLDLr, very low density lipoprotein receptor; apoEr2; CHO, Chinese hamster ovary; APP, amyloid precursor protein.

(Storck et al., 2016). Consistently, mice lacking the LDLr show signs of impaired learning and memory (Mulder et al., 2004), reduced hippocampal cell proliferation and synapses formation (Mulder et al., 2007).

Lipoprotein Receptors Target of PCSK9 and Their Role in Alzheimer's Disease

Besides APOE, several cholesterol homeostasis related genes have been investigated for their association with AD (Wollmer, 2010) and some of them are targeted by PCSK9 (**Figure 1**). LRP1 is an ApoE receptor, and both positive and negative results on its genetic association with AD have been reported (Kang et al., 1997). LRP1 expression is reduced in total brain and brain capillaries with increasing age and even more reduced in AD (Kang et al., 2000; Shibata et al., 2000; Silverberg et al., 2010). Highly validated genetic risk factors for AD, like the APOE allele, may be linked to a reduced clearance of Aβ *via* LRP1 (Bell et al., 2007; Deane et al., 2008). LRP1 mediates Aβ clearance from the brain into the circulation at the BBB (Deane et al., 2009) and brain endothelial-specific LRP1 deletion elevates soluble brain Aβ, leading to aggravated spatial learning and memory deficits (Storck et al., 2016). Thus, LRP1 plays a pivotal role in the metabolism of the ApoE-Aβ complex and ApoE may compete with Aβ for the interaction with LRP1, resulting in impaired Aβ clearance (Verghese et al., 2013). LRP1 is also involved in the hepatic clearance of Aβ (Sagare et al., 2007). Because LRP1 is expressed in different cell types, including neurons, astrocytes and vascular cells in the brain, its levels may be altered differently in AD (Donahue et al., 2006; Ruzali et al., 2012). Indeed, LRP1 levels are decreased in neurons but increased in vascular cells or astrocytes that are proximate to amyloid plaques in AD brains (Arélin et al., 2002; Donahue et al., 2006; Ruzali et al., 2012). PCSK9 may induce the degradation of LRP1 in different cell types, including hepatocytes (Canuel et al., 2013) and vascular cells (Ferri et al., 2012, 2016b). Since PCSK9 is expressed in neurons and in vascular cells, it may influence the LRP1 levels in these cell types (Poirier et al., 2009). Thus, the observed increased PCSK9 cerebrospinal fluid (CSF) concentrations in AD (Zimetti et al., 2017) may determine a higher turnover of LRP1 on different cell types, thus affecting the Aβ elimination *via* the BBB.

The LDLr is a major ApoE receptor in the brain and genetic studies aiming at identifying its link to AD are controversial, although one large study found an association in men but not in women (Lendon et al., 1997; Zou et al., 2008). In a mouse model of AD, LDLr demonstrated a beneficial effect *via* enhancement of Aβ clearance (Kim et al., 2009), suggesting its promising association with the risk for AD and consequently the involvement of PCSK9.

VLDLr can be involved in the clearance of ApoE-Aβ complexes (Helbecque and Amouyel, 2000). However, a meta-analysis of genetic studies conducted on the VLDLr gene polymorphic triplet (CGG) repeat in the 5-UTR showed contradictory results, being a protective factor for AD in Caucasians and as a risk factor in Asian people (Llorca et al., 2008). In addition, genetic association with AD was described in one study but not confirmed in two replication studies (Taguchi et al., 2005). Thus, the direct association of VLDLr and AD still needs to be proven.

The scavenger receptor CD36 is involved in fibrillar Aβ-mediated microglial activation and consecutive activation of an innate immune response (Coraci et al., 2002; Moore et al., 2002; Bamberger et al., 2003). PCSK9 leads to increased CD36 expression in macrophages and microglial-like cells (Ding et al., 2018), suggesting that PCSK9 may regulate both the CD36-mediated clearance of Aβ and the innate host response to Aβ and oxidized-LDL (oxLDL) in brain cells. Indeed, CD36 acts as a co-receptor for the toll-like receptors (TLRs) heterodimerization, an essential step for the initiation of the inflammatory signals and microglia-dependent neurodegeneration (Stewart et al., 2010). Consistently, PCSK9 elicits a proinflammatory effect on macrophages (Ricci et al., 2018) and the administration of a PCSK9 inhibitor leads to a neuroinflammation attenuation in mice models (Apaijai et al., 2019).

Finally, our group reported that human recombinant PCSK9 inhibits the ABCA1-mediated cholesterol efflux in macrophages (Adorni et al., 2017). In this regard, data on the influence of ABCA1 in AD are conflicting as well. For example, carriers of the R219K SNP in the ABCA1 gene shown 33% lower total cholesterol in CSF compared to non-carriers. This allele is also associated with a delay of disease onset by 1.7 years on average (Wollmer et al., 2003). This suggests that a genetic variability of ABCA1 influences the development of AD, possibly by interfering with CNS cholesterol homeostasis. Nevertheless, an association of the same ABCA1 variant R219K with increased AD risk has been described (Rodríguez-Rodríguez et al., 2007). Similarly, additional evidence reported the association between genetic variants of ABCA1 gene with either reduced or increased AD risk (Katzov et al., 2004; Nordestgaard et al., 2015; Beecham et al., 2018). Specifically, a genetic study involving more than 90,000 subjects evidenced a significant association between ABCA1 loss-of-function mutation and 41% increased risk of AD (Nordestgaard et al., 2015). Nevertheless, also for ABCA1, several negative studies have been published, such as a meta-analysis where no association has been found between R219K, I883M and R1587K polymorphisms and risk of AD (Jiang et al., 2012). The involvement of ABCA1 in AD has been investigated in ABCA1 knock-out mice cross-bred with an amyloid pathology of AD. The absence of ABCA1 determined a higher amyloid load in the brains (Koldamova et al., 2005; Wahrle et al., 2005, 2008), although other similar experiments failed to show an effect of ABCA1 on amyloid pathology (Hirsch-Reinshagen et al., 2005, 2007). Since ABCA1 regulates ApoE levels and the transfer of cholesterol from the glial to the neuronal compartment (Wahrle et al., 2004), its role on AD may involve PCSK9, which affects ABCA1 expression (Adorni et al., 2017). Consistent with this hypothesis, a strong decrease in ApoE levels in both the cortex and CSF, together with an impairment of its lipidation, has been described in ABCA1 knock-out mice (Wahrle et al., 2004). Interestingly, CSF samples extracted from AD patients have lower *ex-vivo* capacity to promote ABCA1-

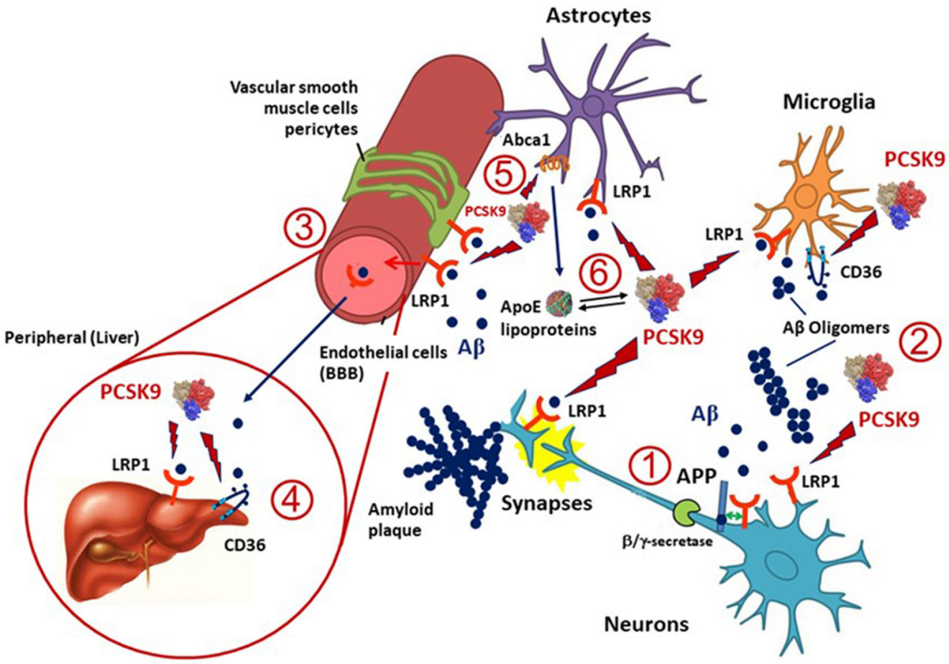

FIGURE 1 | Potential implication of proprotein convertase subtilisin/kexin type 9 (PCSK9) in amyloid β (Aβ) clearance in Alzheimer's disease (AD). LDL receptor-related protein 1 (LRP1), expressed in microglia, neurons, astrocytes and pericytes, and CD36, mainly present in microglia, are the two main lipoprotein receptors involved in Aβ clearance and are potentially targeted by PCSK9. (1) LRP1 may also influence the production of Aβ from amyloid precursor protein (APP) in neurons through a direct protein-protein interaction or competition with the α/β-secretase cleavage of APP. (2) Once Aβ is released into the extracellular space in the brain can form amyloid plaques or oligomers and LRP1 or CD36 can mediate its cellular uptake by neurons, microglia, astrocytes, vascular smooth muscle cells, pericytes and endothelial cells. (3) A portion of Aβ may be transported through LRP1 at the blood-brain barrier (BBB) and reversed into the blood, thus PCSK9 may also interfere with this process. (4) Both LRP1 and CD36 expressed in the liver might also help the clearance of Aβ from the blood, and PCSK9 may affect this pathway by reducing their expression levels in hepatocytes. (5) Apolipoprotein E (ApoE), which is mainly produced and secreted from astrocytes in the brain, is lipidated by ATP binding cassette transporters A1 (ABCA1) to supply cholesterol/lipids to neurons and other cells through LRP1 and CD36. PCSK9 has been shown to downregulate the expression of ABCA1, thus opening to a possible modulation of the release of ApoE containing lipoproteins and thus LRP1- or CD36-mediated Aβ metabolism. Indeed, ApoE isoforms may affect LRP1-mediated Aβ metabolism by directly interacting with Aβ or competing with Aβ for receptor binding. (6) ApoE lipoprotein may also interact with PCSK9 hence influencing its action on LRP1 and CD36.

mediated cholesterol efflux compared to controls (Yassine et al., 2016). Finally, a further level of complexity could be the possible binding between PCSK9 and apoE-containing lipoproteins as previously described for LDL (Tavori et al., 2013), Lipoprotein (a) (Tavori et al., 2016) and HDL (Ferri et al., 2016a; Ruscica et al., 2018).

PCSK9 and Alzheimer's Disease in Humans

The genetic studies conducted so far in humans (**Table 1**) are not conclusive on the impact of PCSK9 mutations on AD. Although Wollmer (2010) first identified PCSK9 among the cholesterol-related genes that have been matched with AD genes listed in the AlzGene database, no association was found between PCSK9 polymorphism and the risk of AD onset, neither in a Japanese nor in a Swedish cohort study (Shibata et al., 2005; Reynolds et al., 2010). Consistently, in a recent Mendelian randomization analysis, PCSK9 loss-of-function mutations were not associated to a rise in the risk of AD [Hazard Ratio (HR) = 0.50; $p = 0.37$; Benn et al., 2017].

To a negative conclusion came also the results of genetic studies among African American REGARDS (Reasons for Geographic and Racial Differences in Stroke) participants with and without the PCSK9 loss-of-function variants C697X or Y142X. The presence of these variants did not affect the primary endpoint of the study, i.e., the neurocognitive performance (Mefford et al., 2018). In another study conducted in French Canadian subjects, carriers of the PCSK9 loss of function mutations, R46L and InsLEU, did not differ from non-carriers as either AD prevalence or age of disease onset (Paquette et al., 2018).

In humans, PCSK9 has been detected in CSF even though at a much lower concentration compared to plasma (Chen et al., 2014). CSF PCSK9 concentrations appeared to be constant throughout the day, thus not undergoing the typical diurnal pattern of plasma PCSK9 (Persson et al., 2010) and suggesting a differential mechanism of PCSK9 regulation in the peripheral and central body compartments (Chen et al., 2014).

In a previous work, we demonstrated increased levels of PCSK9 in the CSF of AD patients with the highest levels in APOE ε4 carriers (Zimetti et al., 2017). These data suggest an involvement of PCSK9 in the disease and a pathophysiological link with APOE4 that deserves further investigations. Our

observation has been confirmed by Courtemanche et al. (2018), however, they show a trend for increased CSF PCSK9 levels also in non-AD neurodegenerative disease, confirming a link to the neurodegenerative process but not specifically to AD.

PCSK9 Inhibitors and Neurocognitive Disorders

Plasma cholesterol under the PCSK9 inhibitor (alirocumab, evolocumab or bococizumab) treatment reached very low levels (mean level 30 mg/dL in the FOURIER trial and 25 mg/dL in ODYSSEY LONG-TERM and SPIRE trials) (Robinson et al., 2015; Ridker et al., 2017; Sabatine et al., 2017). These observations raised some concerns about the potential side effects related to the clinical use of PCSK9 inhibitors. In particular, some clinical trials highlighted a potential association between treatment with PCSK9 monoclonal antibodies and cognitive adverse events (Robinson et al., 2015; Sabatine et al., 2015). However, such disorders were often self-reported and occurred in very few patients with pre-existing medical conditions or other confounders, as emerged from the results of an analysis made by the FDA (Food and Drug Administration Briefing Document, 2015a,b). In order to better clarify this issue, a recent study prospectively and objectively evaluated the effect of the PCSK9 inhibitor evolocumab on cognitive functions (Giugliano et al., 2017a). In this study, a total of 1,204 subjects with mean age of 65 years, that did not present neurological disorders on treatment with evolocumab or placebo, were followed for 1.6 years without evidencing any association with adverse cognitive effects (Giugliano et al., 2017b). This result was further confirmed by a recent meta-analysis (Bajaj et al., 2018; Harvey et al., 2018). However, considering the short follow-up period of the EBBINGHAUS, a 5-year extension of the FOURIER trial will provide further findings on neurocognitive functions[1].

The lack of an evident effect by PCSK9 inhibitors on cognitive functions is very likely explained by the BBB presence, with the consequence that high or low levels of cholesterol in the circulation are not likely to have direct effects on lipid level in the brain (Olsson et al., 2017). With this respect, in the genetic study with carriers of PCSK9 loss-of-function variants, lifelong exposure to low levels of LDL-cholesterol (LDL-C) was not indeed associated with neurocognitive effects (Mefford et al., 2018; Paquette et al., 2018).

Moreover, BBB limits the access of both PCSK9 (Rousselet et al., 2011) and more so of monoclonal antibodies, such as alirocumab or evolocumab to the CNS. Under the conditions where the integrity of BBB is intact, the presence of tight junctions prevents the transcellular route for diffusion of antibodies across the capillary. Therefore, in general, the antibodies penetration into the brain has been estimated to be about 0.1%, both in humans and animals (Tabrizi et al., 2010). In some pathological conditions, such as diabetes, the BBB might be compromised (Rom et al., 2019). However, some indications ruling out the possibility that the antibodies cross the BBB in such conditions, come from the EBBINGHAUS study, which also involves diabetic subjects (37.2%), and in which no variation of cognitive functions was observed (Giugliano et al., 2017b).

Thus, it would be of great interest to evaluate the effect of small molecules PCSK9 inhibitors capable of crossing the BBB.

CONCLUSION

Although several extrahepatic effects of PCSK9 beyond LDL-C (Stoekenbroek et al., 2018) have been identified and well characterized, its role in the brain and the potential involvement in CNS diseases is still under investigation. In this regard, pathophysiological studies on pre-clinical models of AD led to controversial results, leaving open the question of the potential implication of PCSK9 in the disease pathogenesis. Furthermore, the few genetic studies available focused only on PCSK9 genetic variants leading to loss-of-function mutations and are not supportive of an association between PCSK9 and AD risk (Mefford et al., 2018; Paquette et al., 2018). Notably, in all of the studies, only plasma PCSK9 concentrations have been evaluated, while PCSK9 cannot cross the BBB and its regulation may be different in the central and periphery body compartments. Based on the observation made by ourselves and others regarding increased PCSK9 levels in the CSF of AD patients and considering that PCSK9 may interfere with CNS cholesterol transport by degrading the neuronal ApoE-receptors responsible for astrocyte-derived cholesterol uptake, it is conceivable to hypothesize a PCSK9-induced impairment of cholesterol supply to neurons occurring in AD. The consequences of this cholesterol-depletion would include a loss of neuronal physiological functions and ultimately neurodegeneration. In addition, PCSK9 may contribute to exacerbate neuroinflammation, possibly acting on the receptors CD36 and TLR4 (Stewart et al., 2010). These hypotheses, that are being tested by our research group, may set the basis for testing new pharmacological approaches, including existing small molecules potentially able to cross the BBB by either diffusion or transporter-mediated processes, differing from the anti- PCSK9 antibodies. These molecules, by restoring the physiological brain cholesterol transport from astrocytes to neurons in CNS and by attenuating the neuroinflammation through CD36-TRL4 pathway inhibition, might clear the path for a potential future innovative AD therapy.

AUTHOR CONTRIBUTIONS

FZ and MA wrote the first draft of the review and prepared the table. NF, MR and FB wrote sections of the review article. NF made the figure. All authors critically revised the text and all approved the submitted version.

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frontiers in Aging Neuroscience

ORIGINAL RESEARCH
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A Novel *in vivo* Anti-amnesic Agent, Specially Designed to Express Both Acetylcholinesterase (AChE) Inhibitory, Serotonergic Subtype 4 Receptor (5-HT_4R) Agonist and Serotonergic Subtype 6 Receptor (5-HT_6R) Inverse Agonist Activities, With a Potential Interest Against Alzheimer's Disease

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This work describes the conception, synthesis, *in vitro* and *in vivo* biological evaluation of novel Multi-Target Directed Ligands (MTDL) able to both activate 5-HT_4 receptors, block 5-HT_6 receptors and inhibit acetylcholinesterase activity (AChE), in order to exert a synergistic anti-amnesic effect, potentially useful in the treatment of Alzheimer's disease (AD). Indeed, both activation of 5-HT_4 and blockage of 5-HT_6 receptors led to an enhanced acetylcholine release, suggesting it could lead to efficiently restoring the cholinergic neurotransmission deficit observed in AD. Furthermore, 5-HT_4 receptor agonists are able to promote the non-amyloidogenic cleavage of the amyloid precursor protein (APP) and to favor the production of the neurotrophic protein sAPPα. Finally, we identified a pleiotropic compound, [1-(4-amino-5-chloro-2-methoxyphenyl)-3-(1-(3-methylbenzyl)piperidin-4-yl)propan-1-one fumaric acid salt (**10**)], which displayed *in vivo* an anti-amnesic effect in a model of scopolamine-induced deficit of working memory at a dose of 0.3 mg/kg.

Keywords: Alzheimer's disease, acetylcholinesterase, 5-HT_4 receptors, 5-HT_6 receptors, MTDL

Abbreviations: AD, Alzheimer's disease; ACh, acetylcholine; AChE, acetylcholinesterase; AChEI, acetylcholinesterase inhibitor; DPZ, donepezil; MTDL, multi-target directed ligands.

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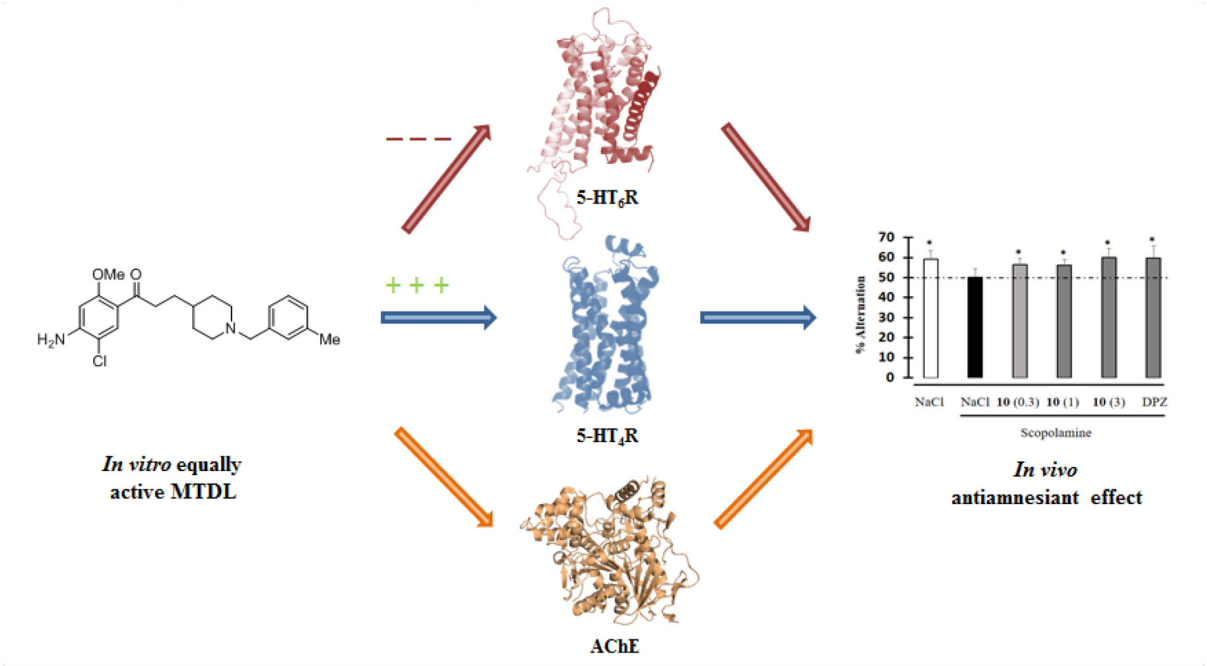


GRAPHICAL ABSTRACT | Design of a MTDL able *in vitro* to both inactivate 5-HT6R and AChE and to activate 5-HT4R and finally displaying *in vivo* antiamnesiant effect.

INTRODUCTION

The pathogenesis of Alzheimer's Disease (AD) is complex and related to the abnormality and dysfunction of multi-systems. Thus, to be potentially more effective, a treatment might consider more than a single target such as acetylcholinesterase (AChE) the main focus of marketed AD drugs.

Within this framework, the design of some pleiotropic ligands known as Multi-Target Directed Ligands (MTDL; Cavalli et al., 2008) appears as a promising approach to tackle the complex origin of the disease as demonstrated recently for several G Protein-Coupled Receptors (GPCRs) and enzymes (Dolles and Decker, 2017; Dolles et al., 2018). We recently described compound **1** (donecopride), which is currently a novel preclinical drug candidate exhibiting both an *in vitro* dual-binding site AChE inhibitory activity and a serotonergic subtype 4 receptor (5-HT_4R) agonist effect leading to *in vivo* procognitive and anti-amnesic effects in mice (**Figure 1**; Lecoutey et al., 2014; Rochais et al., 2015). Indeed, 5-HT_4R agonists are able to promote the "non-amyloidogenic" cleavage of the amyloid precursor protein (APP) by α-secretase, inducing the decrease in amyloid-β peptide (Aβ) production in primary neurons (Lezoualc'h, 2007; Russo et al., 2009), the release of soluble and neuroprotective sAPPα protein (Cho and Hu, 2007), and the *in vivo* improvement of memory in rodents (Lelong et al., 2003; Nirogi et al., 2018).

5-HT_4R is a GPCR. Interestingly, another serotonin GPCR, the serotonergic subtype 6 receptor (5-HT_6R) appears as a valuable target to treat cognitive impairments in the field of neurodegenerative disorders, notably AD (Karila et al., 2015). In fact, its blockade confers to 5-HT_6R antagonists procognitive effects (Benhamú et al., 2014). Among these 5-HT_6R antagonists, **2** (idalopirdine) was studied in phase 3 of clinical trials (Wilkinson et al., 2014), and **3** (landipirdine), a dual antagonist of the 5-HT_6 and 5-HT_{2A} receptors is currently under investigation in the field of Parkinson Disease (**Figure 1**; Ellis and Fell, 2017).

The procognitive activity of 5-HT_6R antagonists is probably mediated by modulation of neurotransmitters' release. Indeed, 5-HT_4R activation enhances the liberation of acetylcholine (Kilbinger and Wolf, 1992; Consolo et al., 1994), dopamine (Steward et al., 1996; Lucas et al., 2001) and serotonin (Ge and Barnes, 1996), while blockade of 5-HT_6R enhances the liberation of acetylcholine (Shirazi-Southall et al., 2002; Riemer et al., 2003; Hirst et al., 2006; Marcos et al., 2006; Zhang et al., 2007) and glutamate (Dawson et al., 2000, 2001). Consequently, 5-HT_6R antagonists can improve cognition since they limit the activation of the mTOR pathway (de Bruin and Kruse, 2015). The acute administration of 5-HT_4R agonists or 5-HT_6R antagonists leads to procognitive effects. However, recently we demonstrated that chronic 5-HT_4R activation or chronic 5-HT_6R blockade, also improved memory performances in object recognition test in mice (Quiedeville et al., 2015). These ligands are active at lower doses than those needed for an acute effect and furthermore, seem to be devoid of side effects in these conditions. We thus considered that co-modulation of these two receptors could represent a valuable strategy against memory deficits in AD (Claeysen et al., 2015; Lalut et al., 2017).

Therefore, we recently reported the synthesis and the *in vivo* procognitive effect displayed by the dual compound **4** with *in vitro* 5-HT_4R agonist and 5-HT_6R antagonist effects (**Figure 2**; Yahiaoui et al., 2016). Based on the evidence that 5-HT_4R, 5-HT_6R and AChE are all valuable therapeutic targets in AD treatment, the present work aimed at designing, starting from our dual compound **4** and the benzyl analog of donecopride **5a** (Rochais et al., 2015), novel MTDLs associating 5-HT_4R agonist, 5-HT_6R antagonist and AChE inhibitory balanced activities (**Figure 2**).

EXPERIMENTAL SECTION

Chemistry

General Materials and Methods

All chemical reagents and solvents were purchased from commercial sources and used without further purification. Melting points were determined on a STUART SMP50 melting point apparatus. ^{1}H, ^{13}C and ^{19}F NMR spectra were recorded on a BRUKER AVANCE III 400MHz with chemical shifts expressed in parts per million (in chloroform-*d*, methanol-d_4 or DMSO-d_6) downfield from TMS as an internal standard and coupling in Hertz. IR spectra were recorded on a Perkin-Elmer BX FT-IR apparatus using KBr pellets. High resolution mass spectra (HRMS) were obtained by electrospray on a BrukermaXis. The purities of all tested compounds were analyzed by LC−MS, with the purity all being higher than 95%. Analyses were performed using a Waters Alliance 2695 as separating module (column XBridge C18 2.5 μM/4.6 × 50 mM) using the following gradients: A (95%)/B (5%) to A (5%)/B (95%) in 4.00 min. This ratio was held during 1.50 min before return to initial conditions in 0.50 min. Initial conditions were then maintained for 2.00 min (A = H_2O, B = CH_3CN; each containing HCOOH: 0.1%). MS were obtained on a SQ detector by positive ESI.

Procedures for the Deprotection and *N*-alkylation of *tert*-butyl 4-[3-(4-amino-5-chloro-2-methoxyphenyl)-3-oxo-propyl]piperidine-1-carboxylate (8)

Procedure A: to a solution of compound **8** in DCM (5 mL) was added TFA (1 mL) dropwise. The reaction mixture was stirred at room temperature for 1 h and then concentrated under reduced pressure. Evaporation of the solvent provided a light-yellow oil, which was thereafter dissolved in 1,4-dioxane (30 mL). To the resulting solution, 20 equivalent of K_2CO_3

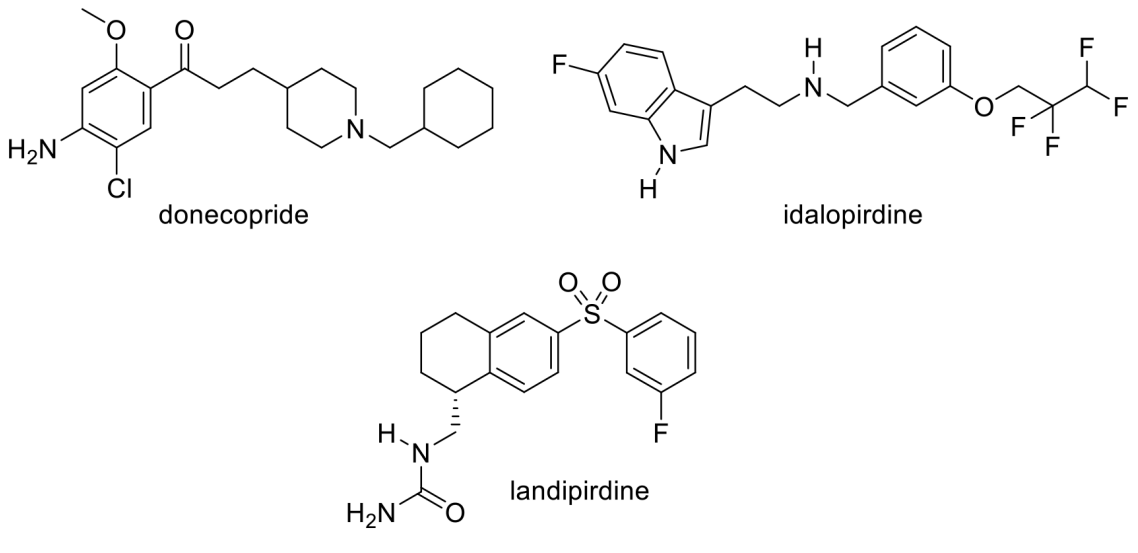


FIGURE 1 | Structure of donecopride, idalopirdine and landipirdine.

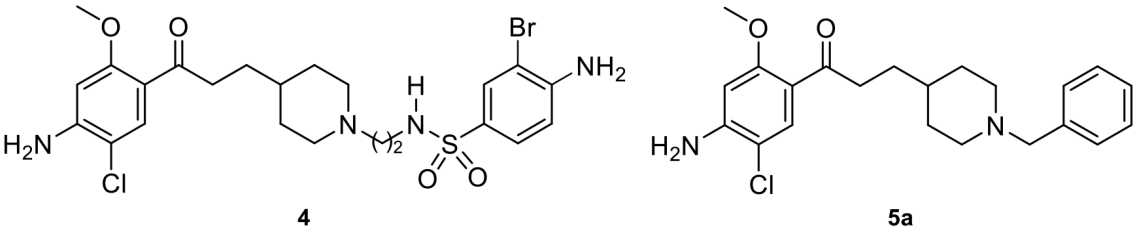


FIGURE 2 | Structure of compounds **4** and **5a**.

were added and thereafter 1.2 equivalent of commercially available benzylbromide derivative was added. The reaction mixture was then stirred at reflux until the full consumption of the starting material. The mixture was then concentrated *in vacuo*, diluted with water and extracted twice with EtOAc. The combined organic phases were washed with brine, dried over $MgSO_4$, filtrated and concentrated under pressure. The crude product was thereafter purified by silica column flash chromatography using the appropriate eluting system.

Procedure B: to a solution of compound **8** in DCM (5 mL/mmol) was added TFA (1 mL/mmol) dropwise. The reaction mixture was stirred at room temperature for 15 min and then concentrated under reduced pressure. Evaporation of the solvent provided a light-yellow oil, which was thereafter dissolved in DMF (10 mL/mmol). To the resulting solution, 10 equivalent of K_2CO_3 were added and thereafter 1.2 equivalent of commercially available benzylbromide derivative was added. The reaction mixture was then stirred at 110°C until the full consumption of the starting material. The mixture was then concentrated *in vacuo*, diluted with EtOAc and washed twice with brine. The organic layer was dried over $MgSO_4$, filtrated and concentrated under pressure. The crude product was thereafter purified by silica column flash chromatography using the appropriate eluting system.

1-(4-Amino-5-chloro-2-methoxyphenyl)-3-(1-benzyl)piperidin-4-yl)propan-1-one (5a; Rochais et al., 2015)

Following procedure A. Pale yellow oil (23%); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.71 (s, 1H), 7.19 (m, 5H), 6.18 (s, 1H), 4.39 (s, 2H), 3.76 (s, 3H), 3.45 (s, 2H), 2.81 (m, 4H), 1.90 (*br* t, *J* = 10.2 Hz, 2H), 1.61 (*br* d, *J* = 9.2 Hz, 2H), 1.52 (q, *J* = 6.9 Hz, 2H), 1.22 (m, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 199.2, 159.5, 147.7, 137.7, 132.3, 129.5 (2C), 128.2 (2C), 127.1, 118.9, 111.3, 97.5, 63.3, 55.6, 53.7 (2C), 40.8, 35.4, 31.9 (2C), 31.4; HRMS (m/z) calcd for $C_{22}H_{28}ClN_2O_2$ $[M + H]^+$ 387.183382, found 387.183120.

1-(4-Amino-5-chloro-2-methoxyphenyl)-3-(1-(2-chlorobenzyl)piperidin-4-yl)propan-1-one (5b)

Following procedure B. Pale yellow solid (33%); mp 125.4°C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.78 (s, 1H), 7.48 (dd, *J* = 7.7, 1.6 Hz, 1H), 7.32 (dd, *J* = 7.8, 1.4 Hz, 1H), 7.22 (dt, *J* = 7.5, 1.4 Hz, 1H), 7.16 (dt, *J* = 7.7, 1.8 Hz, 1H), 6.25 (s, 1H), 4.47 (s, 2H), 3.83 (s, 3H), 3.59 (s, 2H), 2.92–2.88 (m, 4H), 2.08–2.03 (m, 2H), 1.69–1.67 (m, 2H), 1.62–1.57 (m, 2H), 1.30–1.23 (m, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 199.3, 159.6, 147.8, 136.5, 134.3, 132.3, 130.8, 129.4, 128.0, 126.7, 119.1, 111.4, 97.7, 59.7, 55.7, 54.1 (2C), 41.1, 35.7, 32.5 (2C), 31.5; MS (m/z) $[M + H]^+$ 421.61–423.63; HRMS (m/z) calcd for $C_{22}H_{27}Cl_2N_2O_2$ $[M + H]^+$ 421.1450, found 421.1462.

1-(4-Amino-5-chloro-2-methoxyphenyl)-3-(1-(2-bromobenzyl)piperidin-4-yl)propan-1-one (5c)

Following procedure B. Pale brown solid (43%); mp 118.1°C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.78 (s, 1H), 7.51 (dd, *J* = 7.9, 1.1 Hz, 1H), 7.48 (dd, *J* = 7.3, 1.5 Hz, 1H), 7.26 (dt, *J* = 7.5, 1.1 Hz, 1H), 7.08 (dt, *J* = 7.8, 1.7 Hz, 1H), 6.25 (s, 1H), 4.49 (s, 2H), 3.82 (s, 3H), 3.56 (s, 2H), 2.92–2.88 (m, 4H), 2.09–2.04 (m, 2H), 1.69–1.67 (m, 2H), 1.62–1.57 (m, 2H), 1.28–1.25 (m, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 199.3, 159.6, 147.8, 138.2, 132.7, 132.3, 130.8, 128.3, 127.3, 124.7, 119.0, 111.3, 97.6, 62.2, 55.7, 54.1 (2C), 41.1, 35.7, 32.5 (2C), 31.4; MS (m/z) $[M + H]^+$ 465.59–467.59–469.59; HRMS (m/z) calcd for $C_{22}H_{27}BrClN_2O_2$ $[M + H]^+$ 465.0944, found 465.0945.

1-(4-Amino-5-chloro-2-methoxyphenyl)-3-(1-(2-fluorobenzyl)piperidin-4-yl)propan-1-one (5d)

Following procedure B. Pale yellow solid (56%); mp 115°C; ^{1}H NMR (399.75 MHz, $CDCl_3$) δ 7.77 (s, 1H), 7.36 (td, *J* = 7.6, 1.8 Hz, 1H), 7.22 (m, 1H), 7.10 (td, *J* = 7.6, 1.2 Hz, 1H), 7.01 (m, 1H), 6.24 (s, 1H), 4.47 (s, 2H), 3.82 (s, 3H), 3.56 (s, 2H), 2.88 (m, 4H), 2.00 (*br* t, *J* = 10.7 Hz, 2H), 1.67 (*br* d, *J* = 9.3 Hz, 2H), 1.58 (m, 2H), 1.27 (m, 3H); ^{13}C NMR (100.53 MHz, $CDCl_3$) δ 199.1, 161.2 (d, *J* = 244.5 Hz), 159.5, 147.7, 132.2, 131.7 (d, *J* = 4.6 Hz), 128.6 (d, *J* = 8.0 Hz), 125.0 (d, *J* = 14.7 Hz), 123.8 (d, *J* = 3.4 Hz), 118.9, 115.2 (d, *J* = 22.5 Hz), 111.2, 97.5, 63.3, 55.6 (2C), 53.6 (2C), 40.9, 35.5, 32.3, 31.3, 29.7; ^{19}F NMR (376.10 MHz, $CDCl_3$) δ −117.75 (s); MS (m/z) $[M + H]^+$ 405.56–407.55; HRMS (m/z) calcd for $C_{22}H_{27}ClFN_2O_2$ $[M + H]^+$ 405.1745, found 405.1747.

1-(4-Amino-5-chloro-2-methoxyphenyl)-3-(1-(2-iodobenzyl)piperidin-4-yl)propan-1-one (5e)

Following procedure B. Pale yellow solid (44%); mp 106°C;^{1}H NMR (400 MHz, $CDCl_3$) δ 7.81 (dd, *J* = 7.8, 1.1 Hz, 1H), 7.78 (s, 1H), 7.44 (d, *J* = 7.4 Hz, 1H), 7.30 (dt, *J* = 7.4, 1.1 Hz, 1H), 6.92 (dt, *J* = 7.6, 1.7 Hz, 1H), 6.25 (s, 1H), 4.48 (s, 2H), 3.83 (s, 3H), 3.50 (s, 2H), 2.92–2.88 (m, 4H), 2.11–2.06 (m, 2H), 1.69–1.67 (m, 2H), 1.62–1.57 (m, 2H), 1.31–1.25 (m, 3H) ^{13}C NMR (100 MHz, $CDCl_3$) δ 199.2, 159.6, 147.8, 141.0, 139.4, 132.3, 130.4, 128.7, 128.1, 119.0, 111.3, 100.7, 97.7, 67.0, 55.8, 54.0 (2C), 41.1, 35.7, 32.4 (2C), 31.4; MS (m/z) $[M + H]^+$ 513.59–515.61; HRMS (m/z) calcd for $C_{22}H_{27}ClIN_2O_2$ $[M + H]^+$ 513.0806, found 513.0824.

1-(4-Amino-5-chloro-2-methoxyphenyl)-3-(1-(2-methylbenzyl)piperidin-4-yl)propan-1-one (5f)

Following procedure B. Yellow-pale solid (45%); mp 119°C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.78 (s, 1H), 7.37 (m, 1H), 7.13 (m, 1H), 6.25 (s, 1H), 4.44 (*br* s, 2H), 3.84 (s, 3H), 3.41 (s, 2H), 2.91–2.83 (m, 4H), 2.35 (s, 3H), 1.95 (*br* t, *J* = 11.6 Hz, 2H), 1.67–1.53 (m, 4H), 1.28–1.18 (m, 3H);^{13}C NMR (100 MHz, $CDCl_3$) δ 199.3, 159.5, 137.4, 137.1, 132.2, 130.1, 129.7, 126.8, 125.4, 119.0, 111.2, 97.6, 61.1, 55.6, 54.1, 41.0, 35.8, 32.5, 31.4, 19.3; MS (m/z) $[M + H]^+$ 401.65–403.64; HRMS (m/z) calcd for $C_{23}H_{30}ClN_2O_2$ $[M + H]^+$ 401.1996, found 401.1994.

1-(4-Amino-5-chloro-2-methoxyphenyl)-3-(1-(2-benzyloxybenzyl)piperidin-4-yl)propan-1-one (5g)

Following procedure B. Yellow oil (16%); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.78 (s, 1H), 7.46–7.44 (m, 2H), 7.40–7.37 (m, 3H),

7.32 (m, 1H), 7.21 (dt, *J* = 7.9, 1.6 Hz, 1H), 6.96–6.91 (m, 2H), 6.24 (s, 1H), 5.08 (s, 2H), 4.47 (s, 2H), 3.82 (s, 3H), 3.62 (s, 2H), 2.95–2.87 (m, 4H), 2.05–2.00 (m, 2H), 1.68–1.66 (m, 2H), 1.61–1.56 (m, 2H), 1.31–1.26 (m, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 199.3, 159.6, 157.1, 147.8, 137.5, 132.3, 130.9, 128.6 (2C), 128.0, 127.8, 127.3 (3C), 120.7, 119.1, 112.0, 111.3, 97.7, 70.2, 56.7, 55.7, 54.0 (2C), 41.1, 35.7, 32.5 (2C), 31.5; MS (m/z) $[M + H]^+$ 493.65–495.66; HRMS (m/z) calcd for $C_{29}H_{34}ClN_2O_3$ $[M + H]^+$ 493.2258, found 493.2254.

1-(4-Amino-5-chloro-2-methoxyphenyl)-3-(1-(2-hydroxybenzyl)piperidin-4-yl)propan-1-one (5h)

Following procedure B. Yellow-brown oil (12%); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.78 (s, 1H), 7.15 (dt, *J* = 8.0, 1.3 Hz, 1H), 6.95 (d, *J* = 7.3 Hz, 1H), 6.80 (d, *J* = 8.1 Hz, 1H), 6.76 (dt, *J* = 7.4, 1.1 Hz, 1H), 6.25 (s, 1H), 4.45 (s, 2H), 3.84 (s, 3H), 3.68 (s, 2H), 3.00–2.97 (m, 2H), 2.90 (t, *J* = 7.8 Hz, 2H), 2.10–2.07 (m, 2H), 1.76–1.73 (m, 2H), 1.63–1.58 (m, 2H), 1.33–1.25 (m, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 198.9, 159.6, 158.3, 147.8, 132.4, 128.7, 128.6, 122.0, 119.1, 119.0, 116.2, 109.9, 97.7, 61.8, 55.8, 53.4 (2C), 40.8, 35.3, 32.2 (2C), 31.1; MS (m/z) $[M + H]^+$ 403.60–405.58; HRMS (m/z) calcd for $C_{22}H_{28}ClN_2O_3$ $[M + H]^+$ 403.1788, found 403.1788.

1-(4-Amino-5-chloro-2-methoxyphenyl)-3-(1-(3-bromobenzyl)piperidin-4-yl)propan-1-one (5i)

Following procedure A. Yellow-pale oil (34%); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.78 (s, 1H), 7.47 (*br* t, *J* = 1.6 Hz, 1H), 7.36 (*br* dt, *J* = 1.6, 7.6 Hz, 1H), 7.23 (*br* dt, *J* = 1.6, 7.6 Hz, 1H), 7.16 (t, *J* = 7.6 Hz, 1H), 6.25 (s, 1H), 4.44 (s, 2H), 3.84 (s, 3H), 3.44 (s, 2H), 2.89 (*br* t, *J* = 7.8 Hz, 2H), 2.84 (*br* d, *J* = 10.8 Hz, 2H), 1.93 (*br* t, *J* = 11.0 Hz, 2H), 1.74–1.64 (m, 2H), 1.59 (*br* q, *J* = 6.9 Hz, 2H), 1.34–1.19 (m, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 199.2, 159.6, 147.8, 141.3, 132.4, 132.1, 130.1, 129.8, 127.9, 122.5, 119.2, 111.4, 97.7, 63.0, 55.8, 54.0, 41.0, 35.7, 32.4, 31.4; HRMS (*m*/*z*) calcd for $C_{22}H_{27}BrClN_2O_2$ $[M + H]^+$ 465.093894, found 465.093548.

1-(4-Amino-5-chloro-2-methoxyphenyl)-3-(1-(3-fluorobenzyl)piperidin-4-yl)propan-1-one (5j)

Following procedure B. White solid (48%); mp 95°C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.78 (s, 1H), 7.25 (m, 1H), 7.06 (m, 2H), 6.92 (m, 1H), 6.25 (s, 1H), 4.44 (s, 2H), 3.84 (s, 3H), 3.46 (s, 2H), 2.87 (m, 4H), 1.94 (*br* t, *J* = 11.1 Hz, 2H), 1.66 (m, 2H), 1.58 (m, 2H), 1.27 (m, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 199.1, 162.9 (d, *J* = 243.7 Hz), 147.7, 141.5 (d, *J* = 6.7 Hz), 132.2, 129.5 (d, *J* = 8.1 Hz), 124.6 (d, *J* = 2.9 Hz), 118.9, 115.8 (d, *J* = 21.1 Hz), 113.7 (d, *J* = 20.8 Hz), 111.3, 97.6, 62.9, 55.6, 53.9, 40.9, 35.5, 32.3, 31.3; ^{19}F NMR (376.10 MHz, $CDCl_3$) δ −113.97 (s); MS (m/z) $[M + H]^+$ 405.58–407.57; HRMS (m/z) calcd for $C_{22}H_{27}ClFN_2O_2$ $[M + H]^+$ 405.1745, found 405.1745.

1-(4-Amino-5-chloro-2-methoxyphenyl)-3-(1-(3-methylbenzyl)piperidin-4-yl)propan-1-one (5k)

Following procedure A. Yellow-pale oil (41%); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.78 (s, 1H), 7.19 (t, *J* = 7.4 Hz, 1H), 7.13 (*br* s, 1H), 7.09 (*br* d, *J* = 7.6 Hz, 1H), 7.06 (*br* d, *J* = 7.2 Hz, 1H), 6.25 (s, 1H), 4.44 (s, 2H), 3.83 (s, 3H), 3.45 (s, 2H), 2.96–2.80 (m, 4H), 2.34 (s, 3H), 1.93 (*br* t, *J* = 10.4 Hz, 2H), 1.73–1.52 (m, 4H), 1.36–1.17 (m, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 199.3, 159.6, 147.8, 138.3, 137.9, 132.4, 130.2, 128.1, 127.8, 126.6, 119.2, 111.4, 97.7, 63.7, 55.8, 54.0, 41.0, 35.7, 32.3, 31.5, 21.5; HRMS (*m*/*z*) calcd for $C_{23}H_{30}ClN_2O_2$ $[M + H]^+$ 401.1994, found 401.1992.

1-(4-Amino-5-chloro-2-methoxyphenyl)-3-(1-(3-methoxybenzyl)piperidin-4-yl)propan-1-one (5l)

Following procedure A. Colorless oil (27%); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.78 (s, 1H), 7.22 (t, *J* = 8.0 Hz, 1H), 6.93–6.85 (m, 2H), 6.79 (ddd, *J* = 0.8, 2.4, 8.0 Hz, 1H), 6.25 (s, 1H), 4.44 (s, 2H), 3.83 (s, 3H), 3.81 (s, 3H), 3.47 (s, 2H), 2.95–2.81 (m, 4H), 1.94 (*br* t, *J* = 10.2 Hz, 2H), 1.75–1.52 (m, 4H), 1.36–1.17 (m, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 199.3, 159.7, 159.6, 147.7, 140.2, 132.4, 129.2, 121.8, 119.2, 114.9, 112.5, 111.4, 97.7, 63.5, 55.8, 55.4, 54.0, 41.0, 35.7, 32.4, 31.5; HRMS (*m*/*z*) calcd for $C_{23}H_{30}ClN_2O_3$ $[M + H]^+$ 417.193947, found 417.193907.

1-(4-Amino-5-chloro-2-methoxyphenyl)-3-(1-(3-aminobenzyl)piperidin-4-yl)propan-1-one (5m)

Following procedure A. Hygroscopic solid (36%); ^{1}H NMR (400 MHz, CD_3OD) δ 7.66 (s, 1H), 7.17 (t, *J* = 7.8 Hz, 1H), 6.87–6.70 (m, 3H), 6.69 (s, 2H), 6.44 (s, 1H), 4.11 (s, 2H), 3.85 (s, 3H), 3.43 (*br* d, *J* = 12.4 Hz, 2H), 3.05–2.76 (m, 4H), 1.95 (*br* d, *J* = 14.0 Hz, 2H), 1.73–1.28 (m, 5H).

1-(4-Amino-5-chloro-2-methoxyphenyl)-3-(1-(3-nitrobenzyl)piperidin-4-yl)propan-1-one (5n)

Following procedure A. Yellow oil (80%); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.18 (*br* t, *J* = 1.6 Hz, 1H), 8.09 (*br* d, *J* = 8.0 Hz, 1H), 7.78 (s, 1H), 7.66 (*br* d, *J* = 7.6 Hz, 1H), 7.47 (t, *J* = 8.0 Hz, 1H), 6.26 (s, 1H), 4.45 (s, 2H), 3.84 (s, 3H), 3.55 (s, 2H), 2.90 (*br* t, *J* = 7.8 Hz, 2H), 2.83 (*br* d, *J* = 11.6 Hz, 2H), 1.99 (*br* t, *J* = 10.2 Hz, 2H), 1.78–1.64 (m, 2H), 1.60 (*br* q, *J* = 6.8 Hz, 2H), 1.35–1.19 (m, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 199.2, 159.6, 148.4, 147.8, 141.4, 135.2, 132.4, 129.2, 123.9, 122.2, 119.1, 114.4, 97.7, 62.6, 55.8, 54.1, 41.0, 35.6, 32.4, 31.4; HRMS (*m*/*z*) calcd for $C_{22}H_{27}ClN_3O_4$ $[M + H]^+$ 432.168460, found 432.168262.

1-(4-Amino-5-chloro-2-methoxyphenyl)-3-(1-(4-fluorobenzyl)piperidin-4-yl)propan-1-one (5o)

Following procedure B. Yellow pale solid (32%); mp 128°C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.78 (s, 1H), 7.26 (m, 2H), 7.06 (m, 2H), 6.98 (m, 2H), 6.25 (s, 1H), 4.48 (s, 2H), 3.83 (s, 3H), 3.43 (s, 2H), 2.86 (m, 4H), 1.91 (*br* t, *J* = 10.6 Hz, 2H), 1.66 (*br* d, *J* = 8.8 Hz, 2H), 1.58 (m, 2H), 1.25 (m, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 199.1, 161.9 (d, *J* = 243.0 Hz), 159.5, 147.7, 134.3 (d, *J* = 3.0 Hz), 132.2, 130.7 (d, *J* = 7.9 Hz, 2C), 118.9, 114.9 (d, *J* = 21.1 Hz, 2C), 111.2, 97.5, 62.7, 55.6, 53.8, 40.9, 35.6, 32.3, 31.3 ; ^{19}F NMR (376.1 MHz, $CDCl_3$) δ −113.97 (s); MS (m/z) $[M + H]^+$ 405.62–407.62; HRMS (m/z) calcd for $C_{22}H_{27}ClFN_2O_2$ $[M + H]^+$ 405.1745, found 405.1740.

1-(4-Amino-5-chloro-2-methoxyphenyl)-3-(1-(4-hydroxybenzyl)piperidin-4-yl)propan-1-one (5p)

Following procedure B. Yellow oil (13%); ^{1}H NMR (400 MHz, CD_3OD) δ 7.64 (s, 1H), 7.14–7.12 (m, 2H), 6.74 (m, 2H), 6.43

(s, 1H), 3.84 (s, 3H), 3.44 (s, 2H), 2.93–2.87 (m, 4H), 2.04–1.99 (m, 2H), 1.72–1.70 (m, 2H), 1.57–1.51 (m, 2H), 1.32–1.22 (m, 4H); ^{13}C NMR (100 MHz, CD_3OD) δ 201.0, 161.6, 158.1, 151.7, 132.9, 132.4 (2C), 128.2, 117.9, 116.0 (2C), 111.6, 98.1, 63.7, 56.1, 54.4 (2C), 41.7, 36.6, 32.6 (3C); MS (m/z) $[M + H]^+$ 403.65–405.66; HRMS (m/z) calcd for $C_{22}H_{28}ClN_2O_3$ $[M + H]^+$ 403.1788, found 403.1786.

1-(4-Amino-5-chloro-2-methoxyphenyl)-3-(1-(4-methylbenzyl)piperidin-4-yl)propan-1-one (5q)

Following procedure B. White solid (20%); mp 97.0°C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.77 (s, 1H), 7.20 (d, *J* = 7.9 Hz, 2H), 7.11 (d, *J* = 7.9 Hz, 2H), 6.24 (s, 1H), 4.49 (*br* s, 2H), 3.81 (s, 3H), 3.47 (s, 2H), 2.90–2.86 (m, 4H), 2.33 (s, 3H), 1.96–1.91 (m, 2H), 1.68–1.65 (m, 2H), 1.60–1.55 (m, 2H), 1.33–1.23 (m, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 199.2, 159.6, 147.8, 136.7, 135.0, 132.3, 129.5 (2C), 128.9 (2C), 118.9, 111.3, 97.6, 63.2, 55.7, 53.8, 53.5, 41.0, 35.6, 32.2 (2C), 31.4, 21.2; MS (m/z) $[M + H]^+$ 401.66–403.65; HRMS (m/z) calcd for $C_{23}H_{30}ClN_2O_2$ $[M + H]^+$ 401.1996, found 401.1996.

N-(3-((4-(3-(4-Amino-5-chloro-2-methoxyphenyl)-3-oxopropyl)piperidin-1-yl)methyl)-4-bromophenyl) acetamide (5r)

Following procedure A. Yellow-pale oil (70%); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.77 (s, 1H), 7.96 (*br* s, 1H), 7.52 (dd, *J* = 2.4, 8.8 Hz, 1H), 7.47–7.39 (m, 2H), 6.25 (s, 1H), 4.50 (s, 2H), 3.82 (s, 3H), 3.50 (s, 2H), 3.01–2.77 (m, 4H), 2.16 (s, 3H), 2.05 (*br* t, *J* = 10.8 Hz, 2H), 1.72–1.61 (m, 2H), 1.57 (*br* q, *J* = 6.8 Hz, 2H), 1.35–1.18 (m, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 199.4, 168.7, 159.7, 147.9, 138.8, 137.5, 133.0, 132.3, 121.6, 120.0, 118.9, 118.8, 111.3, 97.7, 62.0, 55.8, 54.1, 41.1, 35.7, 32.5, 31.4, 24.7; HRMS (*m/z*) calcd for $C_{24}H_{30}BrClN_3O_3$ $[M + H]^+$ 522.115358, found 522.114977.

1-(4-Amino-5-chloro-2-methoxyphenyl)-3-(1-(2-fluoro-5-nitrobenzyl)piperidin-4-yl)propan-1-one (5s)

Following procedure B. Yellow solid (35%); mp 133.4°C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.36 (dd, *J* = 6.1 Hz, *J* = 2.8 Hz, 1H), 8.13 (ddd, *J* = 8.9, 4.3 Hz, *J* = 3.0 Hz, 1H), 7.78 (s, 1H), 7.15 (t_{app}, *J* = 8.8 Hz, 1H), 6.25 (s, 1H), 4.45 (s, 2H), 3.84 (s, 3H), 3.59 (s, 2H), 2.91–2.84 (m, 4H), 2.08–2.02 (m, 2H), 1.70–1.68 (m, 2H), 1.62–1.57 (m, 2H), 1.29–1.25 (m, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 199.1, 164.8 (d, *J* = 256.4 Hz), 159.6, 147.8, 144.4 (d, *J* = 1.9 Hz), 132.4, 127.8 (d, *J* = 16.2 Hz), 127.2 (d, *J* = 6.9 Hz), 124.6 (d, *J* = 10.2 Hz), 119.1, 116.3 (d, *J* = 25.1 Hz), 111.4, 97.7, 55.8, 55.1, 53.9 (2C), 40.9, 35.4, 32.3 (2C), 31.3; MS (m/z) $[M + H]^+$ 450.51–452.50; HRMS (m/z) calcd for $C_{22}H_{26}ClFN_3O_4$ $[M + H]^+$ 450.1596, found 450.1594.

1-(4-Amino-5-chloro-2-methoxyphenyl)-3-(1-(5-amino-2-fluorobenzyl)piperidin-4-yl)propan-1-one (5t)

Following procedure B. Yellow-brown oil (11%); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.64 (s, 1H), 6.83 (dd, *J* = 9.5 Hz, *J* = 8.8 Hz, 1H), 6.72 (dd, *J* = 6.2, 2.8 Hz, 1H), 6.65 (ddd, *J* = 8.7, 4.1, 2.8 Hz, 1H), 6.44 (s, 1H), 3.85 (s, 3H), 3.55 (d, *J* = 1.2 Hz, 2H), 2.98–2.95 (m, 2H), 2.91–2.87 (m, 2H), 2.16–2.11 (m, 2H), 1.73–1.71 (m, 2H), 1.59–1.52 (m, 2H), 1.33–1.26 (m, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 200.1, 161.7, 156.3 (d, *J* = 234.0 Hz), 151.7, 144.8, 132.9, 124.1 (d, *J* = 16.4 Hz), 119.7 (d, *J* = 3.3 Hz), 117.9, 117.3 (d, *J* = 7.7 Hz), 116.4 (d, *J* = 23.8 Hz), 111.6, 98.1, 56.3 (d, *J* = 1.3 Hz), 56.1, 54.4 (2C), 41.6, 36.4, 32.6 (3C); MS (m/z) $[M + H]^+$ 420.60–422.60; HRMS (m/z) calcd for $C_{22}H_{28}ClFN_3O_2$ $[M + H]^+$ 420.1854, found 420.1857.

1-(4-Amino-5-chloro-2-methoxyphenyl)-3-(1-(3-methylbenzyl)piperidin-4-yl)propan-1-one fumaric acid salt (10)

To a solution of 85 mg of compound **5k** (0.212 mmol) in 3 mL of iPrOH was added 25 mg of fumaric acid (0.212 mmol, 1 equivalent). The solution was refluxed for 2 h. The mixture was then concentrated *in vacuo*. The residue is triturated in Et_2O and then filtrated. Sixty milligram of desired compound were obtained as a beige solid (55%). Yellow solid; mp 185.4°C; ^{1}H NMR (400 MHz, CD_3OD) δ 7.56 (s, 1H), 7.28–7.16 (m, 4H), 6.60 (s, 2H), 6.35 (s, 1H), 4.11 (s, 2H), 3.75 (s, 3H), 3.32 (*br* d, *J* = 12.4 Hz, 2H), 2.88–2.81 (m, 4H), 2.28 (s, 3H), 1.86 (*br* d, *J* = 13.9 Hz, 2H), 1.55–1.52 (m, 3H), 1.38–1.32 (m, 2H); ^{13}C NMR (100 MHz, CD_3OD) δ 198.7, 169.8 (2C), 160.3, 150.4, 139.0, 134.8 (2C), 131.5, 130.4, 129.3, 128.8, 127.9, 116.3, 110.3, 96.6, 60.2, 54.7 (2C), 52.1, 48.2, 39.8, 33.1, 30.1, 28.9, 20.0 (2C). HRMS (m/z) calcd for $C_{23}H_{30}ClN_2O_2$ $[M + H]^+$ 401.1994, found 401.1996.

In vitro Biological Studies

Pharmacological Characterization of Drugs on Human 5-HT$_4$R

For competition studies, 2.5 μg of proteins (5-HT$_{4(b)}$ membrane preparations, HTS110M, Eurofins. Eurofins' 5-HT$_{4(b)}$ membrane preparations are crude membrane preparations made from their proprietary stable recombinant cell lines to ensure high-level of GPCR surface expression) were incubated in duplicate at 25°C for 60 min in the absence or the presence of 10^{-6} or 10^{-8}M of each drug (**9** was used as a reference standard) and 0.2 nM [^{3}H]-GR113808 (NET 1152, Perkin Elmer) in 25 mM Tris buffer (pH 7.4, 25°C). At the end of the incubation, homogenates were filtered through Whatman GF/C filters (Alpha Biotech) presoaked with 0.5% polyethylenimine using a Brandel cell harvester. Filters were subsequently washed three times with 1 mL of ice-cold 25 mM Tris buffer (pH 7.4, 4°C). Non-specific binding was evaluated in parallel in the presence of 30 μM serotonin.

For some of these compounds, affinity constants were calculated from five-point inhibition curves using the GraphPad Prism 6 software and expressed as Ki ± SD.

Pharmacological Characterization of Drugs on Human 5-HT$_6$R

Drugs were evaluated through their possibility to compete for the binding of [^{3}H]-LSD on membranes of HEK-293 cells transiently expressing the human 5-HT$_6$ receptors (ref. RBHS6M, Perkin Elmer). In brief, 4 μg of proteins were incubated at 37°C for 60 min in duplicate in the absence or the presence of 10^{-6} or 10^{-8}M of each drug (**2** was used as a reference

standard) and 2.5 nM [^{3}H]-LSD (ref. NET638250UC, Perkin Elmer), in 25 mM Tris-HCl buffer (pH 7.4) supplemented with 0.5 mM EDTA. At the end of the incubation, the homogenates were then filtered through Whatman GF/C filters and washed five times with ice-cold 25 mM Tris-HCl buffer. Non-specific binding was evaluated in the presence of 100 μM serotonin. Radioactivity associated to proteins was then quantified and expressed as the percentage of inhibition of the drugs under study.

For some of these compounds, affinity constants were calculated from five-point inhibition curves using the GraphPad Prism 6 software and expressed as K*i* ± SD.

Determination of cAMP Production

COS-7 cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% dialyzed fetal calf serum (dFCS) and antibiotics. Cells were transiently transfected by electroporation with plasmids encoding HA-tagged 5-HT_4R (100 ng/10^6 cells) or HA-tagged 5-HT_6R (70 ng/10^6 cells or 300 ng/10^6 cells), then seeded in 96-well plates (16,000 cells/well).

5-HT_4R-induced cAMP production: 24 h after transfection, cells were exposed to the indicated concentrations of 5-HT_4R ligands in the presence of 0.1 mM of the phosphodiesterase inhibitor RO-20-1724, at 37°C in 100 μL of HBS (20 mM HEPES; 150 mM NaCl; 4.2 mM KCl; 0.9 mM $CaCl_2$; 0.5 mM $MgCl_2$; 0.1% glucose; 0.1% BSA). After 10 min, cells were then lysed by addition of the same volume of Triton-X100 (0.1%).

Blockade of the 5-HT_6R-induced cAMP production: 24 h after transfection, cells were exposed to the indicated concentrations of 5-HT_6R ligands at 37°C in 50 μL of HBS. After 7 min, 5-HT (5.10^{-7}M final concentration) in the presence of RO-20-1724 (0.1 mM final concentration), in 50 μL HBS, was added to the wells. After 10 min at 37°C, cells were then lysed by addition of 100 μL of Triton-X100 (0.1%).

Reversion of the basal 5-HT_6R-induced cAMP production: the 5-HT_6R expression was augmented by transfection of higher quantities of cDNA (300 ng/10^6 cells) to increase the basal cAMP production linked to the constitutive activity of the receptor. Twenty-four hours after transfection, cells were exposed to 10^{-4}M of 5-HT_6R ligands in the presence of RO-20-1724 (0.1 mM final concentration), at 37°C in 100 μL of HBS. This experiment is performed in the absence of 5-HT. After 10 min at 37°C, cells were then lysed by addition of 100 μL of Triton-X100 (0.1%).

Quantification of cAMP production was performed by HTRF® by using the cAMP Dynamic kit (Cisbio Bioassays) according to the manufacturer's instructions.

In vitro Tests of AChE Biological Activity

Inhibitory capacity of compounds on AChE biological activity was evaluated through the use of the spectrometric method of Ellman et al. (1961). Acetylthiocholine iodide and 5,5-dithiobis-(2-nitrobenzoic) acid (DTNB) were purchased from Sigma Aldrich. AChE from human erythrocytes (buffered aqueous solution, ≥500 units/mg protein (BCA), Sigma Aldrich) was diluted in 20 mM HEPES buffer pH 8, 0.1% Triton X-100 such as to have enzyme solution with 0.25 unit/mL enzyme activity. In the procedure, 100 μL of 0.3 mM DTNB dissolved in phosphate buffer pH 7.4 was added into the 96-well plate followed by 50 μL of test compound solution and 50 μL of enzyme (0.05 U final). After 5 min of preincubation at 25°C, the reaction was then initiated by the injection of 50 μL of 10 mM acetylthiocholine iodide solution. The hydrolysis of acetylthiocholine was monitored by the formation of yellow 5-thio-2-nitrobenzoate anion as the result of the reaction of DTNB with thiocholine, released by the enzymatic hydrolysis of acetylthiocholine, at a wavelength of 412 nm using a 96-well microplate plate reader (BioTek, Synergy 2). Test compounds were dissolved in analytical grade DMSO. Donepezil (DPZ) was used as a reference standard. The rate of absorbance increase at 412 nm was followed every minute for 10 min. Assays were performed with a blank containing all components except acetylthiocholine, in order to account for non-enzymatic reaction. The reaction slopes were compared and the percent inhibition due to the presence of test compounds was calculated by the following expression: $100 - (v_i/v_0 \times 100)$ where v_i is the rate calculated in the presence of inhibitor and v_0 is the enzyme activity.

First screening of AChE activity was carried out at a 10^{-6}M concentration of compounds under study. For the compounds with significant inhibition (≥50%), IC_{50} values were determined graphically by plotting the % inhibition vs. the logarithm of six inhibitor concentrations in the assay solution using the GraphPad Prism 6 software.

In vivo Biological Studies

Animals

Adult male NMRI mice (3 months old, weighing 35–40 g) from Janvier labs (Le Genest-Saint-Isle, France) were used to perform experiments. Mice were housed by ten in standard polycarbonate cages in standard controlled conditions (22 ± 2°C, 55 ± 10% humidity) with a reversed 12 h light/dark cycle (light on at 7 pm). Food and water were available *ad libitum* in the home cage. All experiments were conducted (between 9 am and 3 pm) during the active-dark-phase of the cycle, were approved by the ethics committee of Normandy (CENOMEXA, n°5161) and were in agreement with the European Directives and French law on animal experimentation (personal authorization n° 14–17 for MB and 14–60 for TF).

CNS-Activity and Acute Toxicity Test

Behavioral and neurological changes induced by graded doses (1, 10, 100 mg/kg) of the tested derivatives were evaluated in mice, in groups of four, by a standardized observation technique at different times (30 min, 3 and 24 h) after intraperitoneal administration (Morpugo, 1971). Major changes of behavioral data (for example, hypo- or hyperactivity, ataxia, tremors, convulsion, etc...) were noted in comparison to the control group. The approximate DL_{50} of the compounds were also calculated through the quantification of mortality after 24 h. Amphetamine (2 mg/kg), chlorpromazine (10 mg/kg) were used as a stimulant and depressive references, respectively.

Locomotor Activity

Locomotion of mice was measured using an actimeter (Imetronic®) through infrared detection. Eight individual removable polycarbonate cages (21 cm length, 7 cm wide and 12 cm high), where each mouse was placed, are disposed in the actimeter. Locomotor activity was measured by recording the number of interruption of beams of the red light over a period of 30 min through an attached recording system to the actimeter. Compound **10** was tested at 1, 3 and 10 mg/kg. Amphetamine (2 mg/kg), chlorpromazine (3 mg/kg) were used as a stimulant and depressive references, respectively (Freret et al., 2012).

Spatial Working Memory

Anti-amnesic activity of tested compounds was evaluated by reversal of scopolamine (0.5 mg/kg)-induced deficit on spontaneous alternation behavior in the Y maze test (Hooper et al., 1996). The Y maze made of gray plastic consisted of three equally spaced arms (21 cm long, 7 cm wide with walls 15-cm high). The mouse was placed at the end of one of the arms and allowed to move freely through the maze during a 5 min session while the sequence of arm entries was recorded by an observer. An arm entry was scored when all four feet crossed into the arm. An alternation was defined as entries into all three arms on a consecutive occasion. The number of possible alternation is thus the total number of arm entries minus two; the percentage of alternation was calculated as (actual alternation/ possible alternation) × 100. Compound **10** was tested at 0.3, 1 and 3 mg/kg. DPZ (1 mg/kg) was tested as a clinical reference.

Pharmacological Treatments

Amphetamine, (+)-α-Methylphenethylamine hemisulfate, chlorpromazine hydrochloride, imipramine hydrochloride and scopolamine hydrobromide were purchased from Sigma (France). All those pharmacological compounds were dissolved in NaCl 0.9% as the vehicle were administered IP 30 min before tests, except scopolamine which was subcutaneously administered 20 min before spontaneous alternation test.

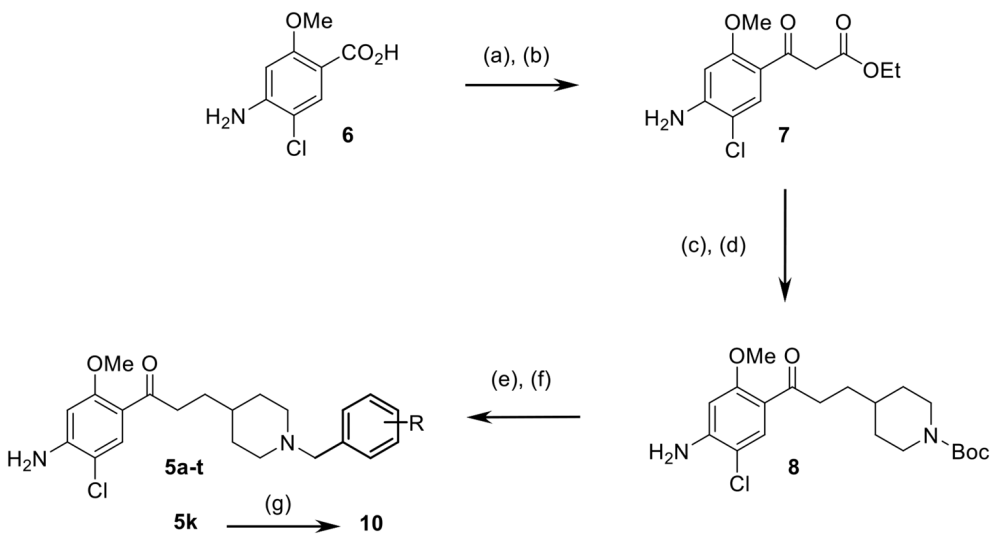
Statistical Analysis

Results were expressed as mean ± SD and were analyzed by one-way analysis of variance (ANOVA), with Statview® software. In case of significance, a SNK (Student-Newman-Keuls) *post hoc* test was realized. Additionally, for the spontaneous alternation test, the percentage of alternation was compared to a theoretical 50% value (random alternation) by an univariate *t*-test. Differences were considered as statistically significant if the *p*-value was strictly under 0.05.

RESULTS

Chemistry

The targeted compounds **5a–t** were obtained starting from 4-amino-5-chloro-2-methoxybenzoic acid (**6**) as reported for the synthesis of **5a** (**Scheme 1**; Rochais et al., 2015). The synthesis of a β-ketoester (**7**) was achieved in 62% yield using the carbonyldiimidazole (CDI) activation of the carboxylic acid group of **6**. A nucleophilic substitution, using *N*-Boc 4-(iodomethyl) piperidine at room temperature to avoid the risk of a double substitution, allowed the installation of the methylpiperidine moiety. The latter was immediately followed by a saponification–decarboxylation sequence using hydroalcoholic potassium hydroxide. The reaction yielded **8** in a 86% yield. Finally, the TFA-*N*-deprotection of **8**, immediately followed by another nucleophilic substitution with various benzyl bromides yielded compounds **5a–t**. The fumaric acid salt **10** was synthesized starting from **5k** and using fumaric acid in iPrOH.



SCHEME 1 | Synthetic pathways for access to compounds **5a–t**, **10**. Conditions and reagents: (a) CDI, THF; (b) $KO_2CCH_2CO_2Et$, $MgCl_2$, THF; (c) *N*-Boc 4-iodomethylpiperidine, K_2CO_3, DMF; (d) KOH, EtOH/H_2O; (e) TFA, 1,4-dioxan or DCM; (f) R-benzyl bromide, K_2CO_3, 1,4-dioxane or DMF; (g) fumaric acid, iPrOH.

TABLE 1 | (*h*)AChE inhibitory activity, (*h*)5-HT_6R and (*h*)5-HT_4R affinity for DPZ, **2**, **9** and compounds **5a–t** (% inhibition at 10^{-6}M, 10^{-8}M and 10^{-8} M, respectively).

Cmpd	R	(*h*)AChE	(*h*)5-HT_6R		(*h*)5-HT_4R	
		%10^{-6}M	%10^{-6}M	%10^{-8}M	%10^{-6}M	%10^{-8}M
DPZ	-	97	-	-	-	-
2	-	-	106	88	-	-
9	-	-	-	-	100	42
5a	H	98	29	0	102	41
5b	2-Cl	91	92	27	94	16
5c	2-Br	80	65	34	96	17
5d	2-F	96	68	21	94	10
5e	2-I	37	81	25	96	1
5f	2-Me	87	85	24	98	9
5g	2-OBn	33	18	0	70	7
5h	2-OH	94	24	0	98	10
5i	3-Br	44	77	7	94	5
5j	3-F	97	59	9	98	12
5k	3-Me	89	80	9	98	25
5l	3-OMe	35	78	6	94	9
5m	3-NH_2	93	74	31	100	35
5n	3-NO_2	64	51	0	90	3
5o	4-F	92	58	13	102	19
5p	4-OH	98	24	0	100	23
5q	4-Me	87	21	21	100	24
5r	2-Br;5-NHCOMe	13	35	7	96	6
5s	2-F;5-NO_2	63	18	5	62	4
5t	2-F;5-NH_2	79	65	3	98	18

In vitro Results

The biological evaluation of the synthesized compounds as potential inhibitors of human AChE was performed using the Ellman assay (Ellman et al., 1961), as well as their affinity for human 5-HT_6R and 5-HT_4R using a radioligand displacement assay (**Table 1**). In these tests, DPZ was used as a reference AChE inhibitor (AChEI), **2** as a 5-HT_6R ligand and **9** (RS67333; Eglen et al., 1995) as a 5-HT_4R ligand.

The results of these *in vitro* evaluations are reported in a 3-fold-entry figure (**Figure 3**).

Among the 20 tested compounds, 12 novel derivatives appear as moderate to potent AChEIs with %inhibition at 10^{-6}M $\geq$ 80%. Their IC_{50} values ranged from 450 (**5q**) to 6 nM (**5p**; **Table 2**). Some of these AChEI showed an additional affinity towards 5-HT_6R with %inhibition at 10^{-6}M $>$ 70% (**5b, f, k, m**) and K*i* ranging from 1,150 to 110 nM. Three derivatives (**5e, i, l**) displayed affinity for 5-HT_6R, while being devoid of inhibitory effect towards AChE. Finally, among the four dual compounds, **5k** and **5m** further exhibited an affinity towards 5-HT_4R with %inhibition at 10^{-8}M $\geq$ 25% and K*i* of 168 and 43 nM respectively. Three other compounds (**5o–q**) displayed good affinity towards 5-HT_4R but are devoid of such an affinity towards 5-HT_6R.

By virtue of its well-balanced *in vitro* activities towards the three targets, **5k** was selected for further investigation. According to the concept of MTDL and the synergy theoretically displayed by the association of activities into a sole compound, **5k** has been preferred to more potent compounds (e.g., **5m**) but with unbalanced levels of activity towards the three targets. This derivative has then been used as its fumaric acid salt **10**. The *in vitro* activities of **10** are depicted in **Table 3**.

The pharmacological profile of **10** was established towards (*h*)5-HT_4R and (*h*)5-HT_6R, respectively. It acts as a partial agonist towards (*h*)5-HT_4R, in a similar manner as RS67333 (**9**), and as an inverse agonist towards (*h*)5-HT_6R, in a similar manner as SB271046, used as a control 5-HT_6R antagonist, and idalopirdine (**2**; **Figure 4** and **Table 3**). Indeed, in our hands, these three compounds decreased cAMP production below the basal, in the absence of agonist (**Figure 4C**).

In vivo Results

Whatever the dose tested (1, 10 and 100 mg/kg), preliminary toxicological and pharmacological screening of **10** (**Table 4**) did not show any deleterious signs, suggesting thus a LD_{50} quite higher than 100 mg/kg.

None of the tested doses of **10** (1, 3 and 10 mg/kg) did neither modify spontaneous locomotor activity (**Figure 5**).

Moreover, an anti-amnesic effect was observed in the scopolamine-induced spontaneous alternation deficit test, after IP administration of **10** at doses starting from 0.3 mg/kg (**Figure 6**). The degree of effect was similar to that of the dual-targeted compound donecopride in the same dose range (Rochais et al., 2015).

DISCUSSION

Trying to establish SAR for MTDL is always quite challenging taking into consideration that the structural requirements can be divergent between the expected interactions with the different targets. Considering the three present targets, the SAR appeared similar between AChE and 5-HT_4R, but partially different from those observed for 5-HT_6R.

Indeed, with regard to AChE and 5-HT_4R activities, the best substituent of the piperidine moiety of compounds **5** is the unsubstituted benzyl group (**5a**). According to our previous work (Yahiaoui et al., 2016), the nature of the substitution of a phenyl group distant from the piperidine moiety could influence in a great manner the affinity of the ligands towards both 5-HT receptors. For the two targets, poly or bulky substituents (Br, I, OBn, OMe, NO_2), appear detrimental to the activities (**5c, g, i, l, n**) while a smaller one (OH, NH_2, F, Me) seems likely to relatively maintain the activity (**5h, p, m, d, j, o**), especially when present in para position (**5p**).

Conversely, the 5-HT_6R affinity appears linked to the substitution of the benzyl group within the studied scaffold, since the unsubstituted derivative **5a** was devoid of such an activity. Further, small substituents (OH, F) do not favor, this time, the affinity (**5d, h, j, o, p**), whatever their position on the phenyl ring, while bulkier ones (Cl, Br, I, Me, OMe) promote it, especially in *ortho* or *meta* positions (**5b, c, e, f, i, k, l**).

These contradictory requirements explain that **5k** with a methyl group in *meta* position displayed balanced activities towards the three targets and was chosen for further investigation under its fumarate form (**10**). Indeed, according to the MTDL concept (Cavalli et al., 2008),

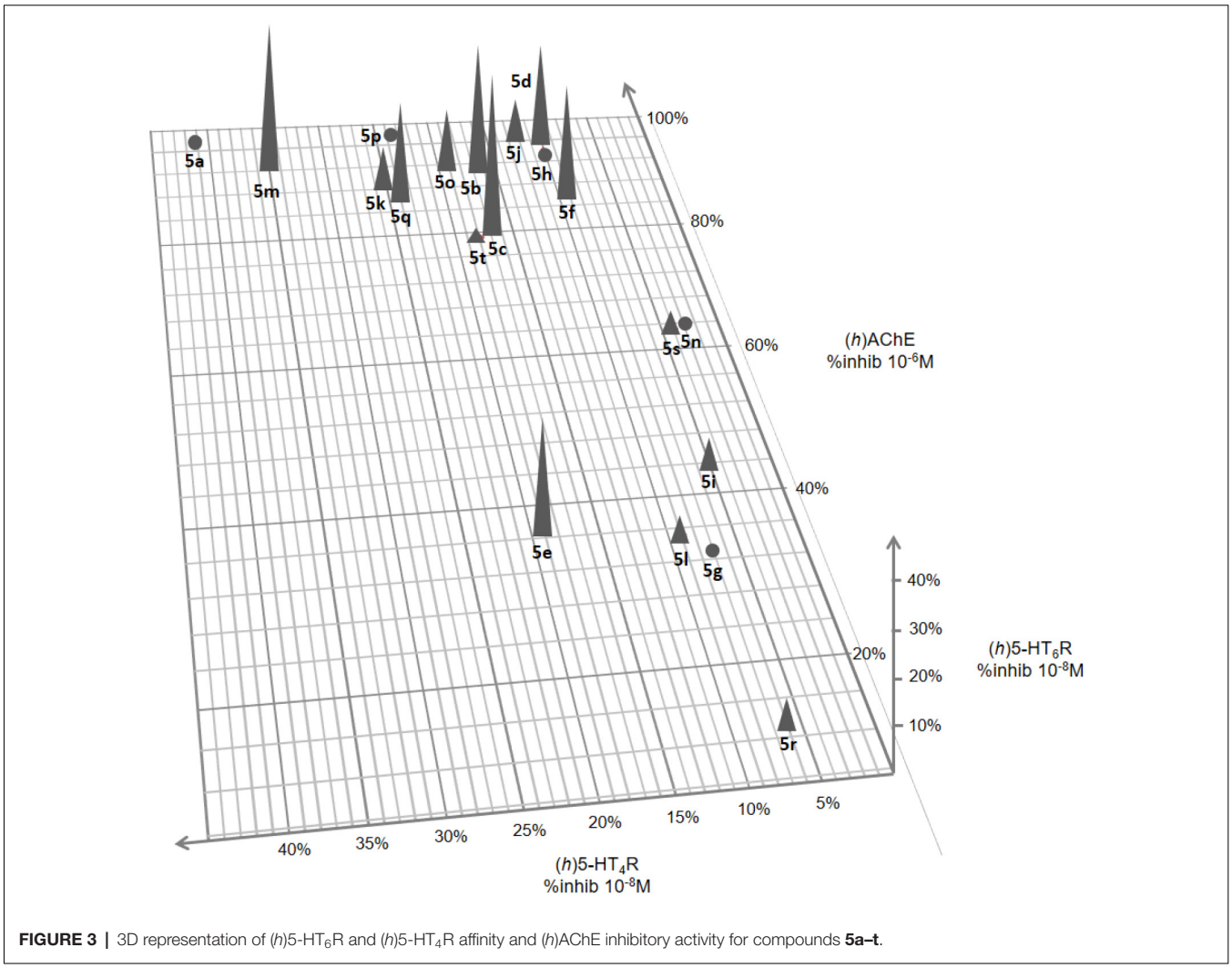


FIGURE 3 | 3D representation of (*h*)5-HT_6R and (*h*)5-HT_4R affinity and (*h*)AChE inhibitory activity for compounds **5a–t**.

TABLE 2 | (*h*)AChE inhibitory activity (IC_{50}) and (*h*)5-HT_6R and (*h*)5-HT_4R affinity (K*i*) for DPZ, **2**, **9**, **5b–f, h–m, o–q, t** and the fumarate **10**.

Cmpd	R	(*h*)AChE	(*h*)5-HT_6R	(*h*)5-HT_4R
		IC_{50} (nM) *n* = 2	K*i* (nM) *n* = 3	K*i* (nM) *n* = 3
DPZ	-	7 ± 1.5	ND	ND
2	-	ND	6.9 ± 1.2	ND
9	-	ND	ND	5.1 ± 0.5 (Irving et al., 2010)
5b	2-Cl	156 ± 71	110 ± 19	ND
5c	2-Br	317 ± 115	ND	ND
5d	2-F	101.6 ± 65.4	ND	ND
5e	2-I	ND	186 ± 71	ND
5f	2-Me	225.2 ± 57.3	270 ± 14	ND
5h	2-OH	57.4 ± 9.4	ND	ND
5i	3-Br	ND	169 ± 44	ND
5j	3-F	91 ± 53.8	ND	ND
5k	3-Me	161 ± 43	230 ± 49	168 ± 15
5l	3-OMe	ND	265 ± 64	ND
5m	3-NH_2	41 ± 5	1150 ± 559	42.5 ± 2.6
5o	4-F	78.4 ± 12.1	ND	ND
5p	4-OH	6.0 ± 0.4%	ND	ND
5q	4-Me	450.5 ± 89.5	ND	ND
5t	2-F;5-NH_2	152.7 ± 19.9	ND	ND
10	3-Me, fumarate salt	33.7 ± 1.7	219 ± 42	210 ± 43

ND, not determined.

TABLE 3 | (*h*)5-HT$_6$R and (*h*)5-HT$_{4(a)}$R pharmacological profile of compounds **2**, **9** and **10**.

	(*h*)5-HT$_6$R			(*h*)5-HT$_{4(a)}$R		
	Log(IC$_{50}$)	% control antagonist response	Profile	Log(EC$_{50}$)	% control agonist response	Profile
2	−6.2 ± 0.2 *n* = 2	101.2 ± 3.2 *n* = 2	Inverse agonist	-	-	-
9	-	-	-	−8.8 ± 0.2 *n* = 2	48.7 ± 5.2 *n* = 3*	Partial agonist
10	−5.0 ± 0.1 *n* = 2	93.4 ± 1.8 *n* = 2	Inverse agonist	−7.7 ± 0.0 *n* = 2	59.8 ± 2.5 *n* = 2	Partial agonist

*SB271046 was used as a control 5-HT$_6$R antagonist and 5-HT as control 5-HT$_4$R agonist. *Result reported from Lecoutey et al. (2014).*

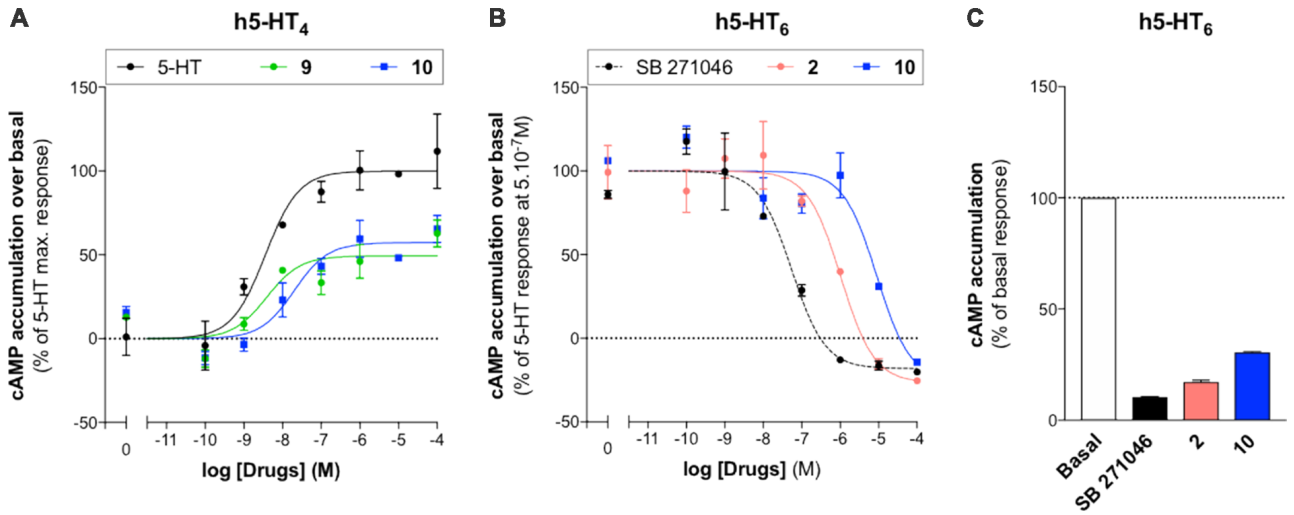


FIGURE 4 | Pharmacological profile of compounds **2**, **9** and **10**. Representative experiments illustrating agonist activities of **9** and **10** towards (*h*)5-HT$_{4(a)}$R **(A)** and antagonist activities of SB271046, **2** and **10** towards (*h*)5-HT$_6$R stimulated with 5.10^{-7}M of 5-HT [**B**; data are means ± standard error of the mean (SEM); *n* = 2]. **(C)** Inverse agonist activities of SB271046, **2** and **10** (10^{-4}M) towards (*h*)5-HT$_6$R in absence of 5-HT (means ± SEM of two independent experiments performed in duplicates).

TABLE 4 | Pharmacological and toxicological properties of **10**.

Compound	Doses (mg/kg)	LD$_{50}$ (mg/kg)	Symptoms (subtoxic doses)
10	1–10–100	>100	No symptoms
Amphetamine	2		Hyperactivity Exopthalmy Irritability
Chlorpromazine	10		Hypoactivity Ataxia Sleep

pleiotropic ligand could present moderate *in vitro* activities toward their targets that could, however, lead to potent and promising *in vivo* activities explained by their synergistic effect (Prati et al., 2015).

Of interest, compound **10**, idalopirdine and SB271046 were able to reverse the constitutive activity of the 5-HT$_6$ receptor, thus demonstrating an inverse agonist profile. Such a property is of high interest regarding this receptor as it is known to possess a high constitutive activity in the brain, exerting a tonic activity (Duhr et al., 2014; Deraredj Nadim et al., 2016). Regarding the 5-HT$_4$R, **10** demonstrated a partial agonist activity similar to the one of **9**, which had proven its efficiency to prevent amyloid production in a mouse model of AD (Hashimoto et al., 2012; Giannoni et al., 2013; Baranger et al., 2017).

The main result of the behavioral study concerns the anti-amnesic effect of **10** in a model of scopolamine-induced deficit of working memory from the dose of 0.3 mg/kg.

Although no pharmacokinetic data are available for compound **10** yet, this result indicates that it reaches the general circulation and crosses the blood-brain barrier upon IP administration. The anti-amnesic effect of compound **10** suggests that the co-modulation of the three targets could be a therapeutic strategy of interest in the field of memory impairments. The anti-amnesic properties of the compound **10**, together with the absence of any deleterious signs during the pharmacological screening (even when tested at high dose: 100 mg/kg), are arguing in favor of **10** as a new promising drug, with excellent general tolerance.

CONCLUSION

We have synthesized a variety of compounds possessing both *in vitro* and *in vivo* activities towards three therapeutic targets (5-HT$_4$R/5-HT$_6$R, AChE) in the potential treatment

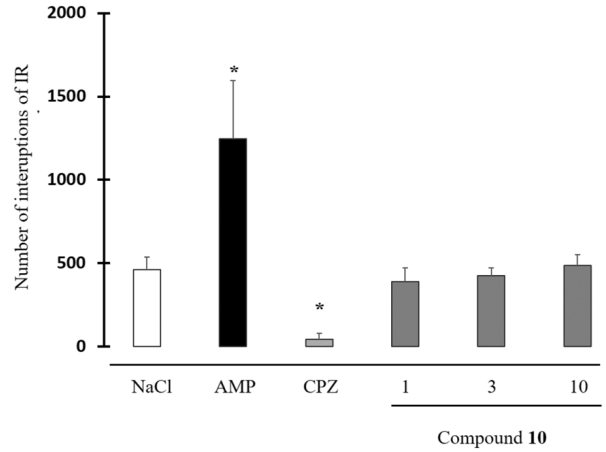


FIGURE 5 | Effect of compound **10** on spontaneous locomotor activity. Data are expressed as the mean ± standard deviation (n = 8). Drugs were administered intraperitoneally (IP) 30 min before the behavioral test. Compound **10**: 1–3–10 mg/kg; AMP: amphetamine 2 mg/kg; CPZ: chlorpromazine 3 mg/kg [*$p < 0.05$ NaCl, Student-Newman-Keuls (SNK) test].

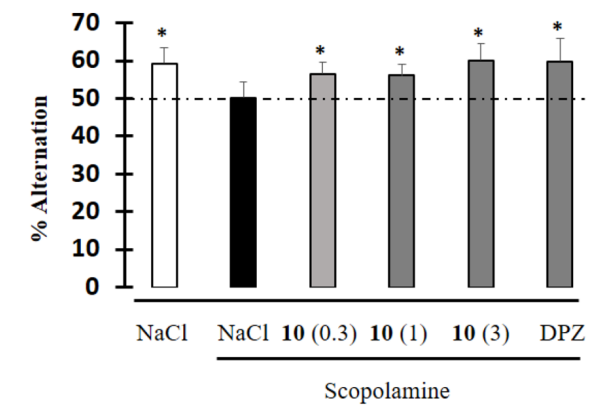


FIGURE 6 | Effect of compound **10** on scopolamine-induced impairment during the spontaneous alternation test. Data are expressed as the mean ± standard deviation (n = 8). Drugs were administered IP 30 min before the test, and scopolamine was administered SC 20 min before the test. Compound **10**: 0.3, 1, 3 mg/kg; DPZ: Donepezil; 1 mg/kg; Scopolamine: 0.5 mg/kg (*$p < 0.05$ vs. 50%; univariate t-test).

of AD. The use of these innovative MTDL which may exhibit a greater benefit than single-targeted drugs towards the neuropathological hallmarks of the disease and the associated behavioral deficits will require further studies in animal models and potential future clinical trials to demonstrate their efficacy.

ASSOCIATED CONTENT

Analytical data of final compounds. This material is available free of charge *via* the Internet at http://pubs.acs.org.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the **Supplementary Files**.

ETHICS STATEMENT

All experiments were conducted in agreement with the European Directives and French law on animal experimentation (personal authorization n° 14-17 for MB and 14-60 for TF).

AUTHOR CONTRIBUTIONS

The manuscript was written through contributions of SC, TF, MB, CR and PD. All authors have given approval to the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

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frontiers in Aging Neuroscience

BRIEF RESEARCH REPORT
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Taking Advantage of the Selectivity of Histone Deacetylases and Phosphodiesterase Inhibitors to Design Better Therapeutic Strategies to Treat Alzheimer's Disease

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The discouraging results with therapies for Alzheimer's disease (AD) in clinical trials, highlights the urgent need to adopt new approaches. Like other complex diseases, it is becoming clear that AD therapies should focus on the simultaneous modulation of several targets implicated in the disease. Recently, using reference compounds and the first-in class CM-414, we demonstrated that the simultaneous inhibition of histone deacetylases [class I histone deacetylases (HDACs) and HDAC6] and phosphodiesterase 5 (PDE5) has a synergistic therapeutic effect in AD models. To identify the best inhibitory balance of HDAC isoforms and PDEs that provides a safe and efficient therapy to combat AD, we tested the compound CM-695 in the Tg2576 mouse model of this disease. CM-695 selectively inhibits HDAC6 over class I HDAC isoforms, which largely overcomes the toxicity associated with HDAC class 1 inhibition. Furthermore, CM-695 inhibits PDE9, which is expressed strongly in the brain and has been proposed as a therapeutic target for AD. Chronic treatment of aged Tg2576 mice with CM-695 ameliorates memory impairment and diminishes brain Aβ, although its therapeutic effect was no longer apparent 4 weeks after the treatment was interrupted. An increase in the presence of 78-KDa glucose regulated protein (GRP78) and heat shock protein 70 (Hsp70) chaperones may underlie the therapeutic effect of CM-695. In summary, chronic treatment with CM-695 appears to reverse the AD phenotype in a safe and effective manner. Taking into account that AD is a multifactorial disorder, the multimodal action of these compounds and the different events they affect may open new avenues to combat AD.

Keywords: Alzheimer's disease, multitarget therapy, histone deacetylase, phosphodiesterase, memory

INTRODUCTION

Alzheimer's disease (AD) is a multifactorial neurodegenerative disorder for which no effective treatment has yet been found, despite the effort and resources invested to date. One reason for this failure may be that most of the efforts to develop therapies have been directed towards single pathways and mainly, the amyloid pathology. However, the complex nature of this disease means that multiple events are likely to be implicated in its progression and thus, effective therapies must modulate several targets, as is now being considered with state-of-the-art treatments for many cancers and AIDS. Moreover, the focus in AD is now shifting from the pathways that directly decrease the amyloid pathology to address those that dampen the tau pathology or that effect synaptogenesis (Cummings et al., 2018).

We recently validated the efficacy of a novel multitarget therapy for AD that focused on the concomitant inhibition of histone deacetylases (HDACs) and a phosphodiesterase 5 (PDE5). The approach was validated using reference compounds (tadalafil and vorinostat), and with a new drug and novel chemical entity (NCE), CM-414, which displays moderate class I HDAC inhibition, and more potent HDAC6 and PDE5 inhibition (Cuadrado-Tejedor et al., 2015, 2017; Rabal et al., 2016). We demonstrated that chronic treatment of Tg2576 mice (a well-studied model of AD) with CM-414 diminished the accumulation Aβ and pTau in the brain, reversing the decrease in dendritic spine density on hippocampal neurons and the cognitive deficits in these mice. These effects were at least in part produced by inducing the expression of genes related to synaptic transmission. Interestingly, we demonstrated that these therapeutic effects persisted 1 month after the completion of a 4-week treatment period (Cuadrado-Tejedor et al., 2017).

CM-414 was designed taking into account that class I HDACs and HDAC6 (class IIb) are the HDACs most likely to be involved in AD-memory related dysfunction (Ding et al., 2008; Guan et al., 2009; Mahady et al., 2019). The inhibition of class I HDACs, and particularly that of HDAC2, seems to be essential to restore memory by remodeling chromatin and enhancing gene expression (Guan et al., 2009; Singh and Thakur, 2018). However, the inhibition of HDAC class I isoforms has also been associated with cytotoxicity (Subramanian et al., 2010), precluding their chronic use. Recently, a new chemical series of HDAC1 and 2 inhibitors designed to inhibit the HDAC-CoREST co-repressor complex were seen to have lower toxicity, while maintaining the beneficial effects in terms of synaptic plasticity (Fuller et al., 2019). By contrast, the inhibition (or reduction) of HDAC6, a cytoplasmic HDAC isoform that regulates microtubule behavior and stability *via* α-tubulin acetylation (Hubbert et al., 2002), seems to promote tau and Aβ clearance, thereby ameliorating the memory deficits in AD models (Cook et al., 2012; Sung et al., 2013; Zhang et al., 2014). Furthermore, inhibiting HDAC6 rescues the reduced mitochondrial axonal transport and mitochondrial length in hippocampal neurons treated with Aβ (Kim et al., 2012), as well as in pluripotent stem cells (iPSCs) from Amyotrophic Lateral Sclerosis (ALS) patients (Guo et al., 2017). In fact, due to its safety profile, HDAC6 is currently being considered as one of the most promising epigenetic targets in AD. Given the above, and despite the fact that CM-414 acts as a symptomatic and disease-modifying agent in AD mice models (Cuadrado-Tejedor et al., 2017), it is possible that some toxicity may be associated with the inhibition of the class I HDAC1, precluding its use in the chronic treatment of AD patients. Thus, in order to improve the safety profile of CM-414, we synthesized a new compound, CM-695, with higher selectivity for HDAC6 over class I HDACs.

PDE9 is a cyclic guanosine monophosphate (cGMP) specific PDE and it is the PDE most strongly expressed in the brain (Andreeva et al., 2001). In fact, when we compared the expression of PDE5 and PDE9 in the mouse hippocampus, we found that PDE9 is expressed 10 times more strongly than PDE5 (**Supplementary Figure S1**). Interestingly, the expression of PDE5 and PDE9 were increased in the cortex of AD patients compared to age-matched control subjects. Accordingly, levels of cGMP were decreased in the cerebrospinal fluid (CSF) of AD patients compared to that of healthy control individuals (Ugarte et al., 2015). By restoring cGMP levels through PDE5 and 9 inhibition, intracellular signaling pathways that are important in memory and learning could be stimulated. For example, an activation of the cAMP-responsive element binding protein (CREB) transcription factor can be observed, a factor known to be crucial for synapse formation and memory consolidation (García-Osta et al., 2012; Heckman et al., 2017). Since PDE9 has the highest affinity for cGMP of all the PDEs (Singh and Patra, 2014), it becomes an attractive target to increase the GMP in the brain. It was recently proposed that PDE9 inhibitors provide more protection against Aβ42 than PDE4 and PDE5 inhibitors in an *in vitro* model of AD (Cameron et al., 2017). Nevertheless, when specific PDE9 inhibitors (PF-04447943 and BI-409306) have been tested to treat AD in Phase II clinical trials, they both failed to meet their AD efficacy endpoints relative to the placebo (Schwam et al., 2014; Frölich et al., 2019). As indicated above, the complexity of the AD pathology means it is possible that the inhibition of a single enzyme alone will not produce therapeutic benefits in patients. Accordingly, we designed a new first-in class dual activity compound CM-695, that targets HDAC6 and PDE9 for inhibition, and with acceptable brain permeability, for it's *in vivo* efficacy in Tg2576 mice.

MATERIALS AND METHODS

Biological Activity *in vitro* and *in vivo*

Cells of the human neuroblastoma SH-SY5Y cell line were plated in 6-well plates and incubated at 37°C in a humid atmosphere with 5% CO_2 until reaching a confluence of 80%–90%. They were cultivated in Eagle's medium modified by Dulbecco (DMEM, Gibco BRL, Grand Island, NY, USA) supplemented with 1% non-essential amino acids solution (Gibco BRL, Grand Island, NY, USA), penicillin/streptomycin 100 U/ml (Gibco BRL, Grand Island, NY, USA) and 10% fetal bovine serum (Gibco BRL, Grand Island, NY, USA). Cells were incubated with different concentrations of CM-695 for 2 h. After incubation, medium was removed, washed with PBS and cells were lysed in a buffer containing SDS 2%, Tris-HCl (10 mM, pH 7.4), protease

inhibitors (Complete Protease Inhibitor Cocktail, Roche) and phosphatase inhibitors (0.1 mM Na_3VO_4, 1 mM NaF). The homogenates were sonicated for 2 min and centrifuged at 14,000× *g* for 15 min.

Protein concentration was determined using the Pierce™ BCA Protein Assay kit (Thermo Fisher Scientific, Waltham, MA, USA). For western blot analysis of acetylated histone 3 at lysine 9 (AcH3K9), pCREB and acetylated tubulin, protein samples (15–20 μg) were mixed with 6× Laemmli sample buffer and resolved onto SDS-polyacrylamide gels and transferred to nitrocellulose membrane. Membranes were blocked for 1 h with 5% milk in TBS and incubated overnight with the corresponding primary antibody: rabbit monoclonal anti-AcH3K9, rabbit monoclonal anti-pCREB (Ser129; 1:1,000, Cell Signaling Technology, Danvers, MA, USA), mouse monoclonal anti-Acetylated tubulin, mouse monoclonal anti-β-actin (1:50,000, Sigma-Aldrich, St. Luis, MO, USA).

Animals and Treatments

Transgenic mice (Tg2576) between 16 and 18 months of age and female gender were used. This strain expresses the human 695-aa isoform of the amyloid precursor protein (APP) carrying the Swedish (K670N/M671L) familial AD mutation driven by a hamster prion promoter (Hsiao et al., 1996). Mice were on an inbred C57BL/6/SJL genetic background. The Tg2576 AD mice strain accumulates Aβ peptide exponentially, in the brain, between 7 and 12 months of age, showing a hippocampal damage from the age of 9–10 months (Chapman et al., 1999; Westerman et al., 2002).

Animals were bred at "Centro de Investigación Médica Aplicada" (CIMA) in Pamplona, Spain. Animals were housed 4–5 per cage with free access to food and water and maintained in a temperature controlled environment on a 12 h light-dark cycle. All procedures were carried out in agreement with the European and Spanish regulations (2010/63/EU; RD1201/2005), and the study was approved by the Ethical Committee for the Animal Experimentation of the University of Navarra.

Tg2576 mice were treated six times a week for 4 weeks. They were administered intraperitoneally with CM-695 (40 mg/kg n = 10) or vehicle (10% DMSO, 10% Tween-20 in saline solution). The preparation of drug was performed daily to avoid precipitation due to their hydrophobic nature. Behavioral and biochemical studies were performed comparing transgenic mice to age-and strain-matched transgenic negative littermates (WT). The behavioral tests were always carried out during light time (from 9 am to 14 pm), in order to minimize the possible influence of circadian rhythms.

Behavioral Studies

Behavioral studies were carried out during light time (from 9 am to 2 pm). Protocols were approved by the Ethical Committee of the University of Navarra (in accordance with the European and Spanish Royal Decree 1201/2005).

Fear Conditioning Test

To evaluate the effects of drugs on cognitive function a fear-conditioning (FC) paradigm was used after 2 weeks of treatment with CM-695 (n = 11) or vehicle (n = 10). The FC is an hippocampus-dependent test to measure long-term memory consolidation by assessing the association between two stimuli, one conditioned (context) and another unconditioned (an electric shock; Maren, 2008). The conditioning procedure was carried out in a StartFear system (Panlab S.L., Barcelona, Spain) as described previously with slight modifications (Ricobaraza et al., 2012). During training phase mice received two footshocks (0.3 mA, 2 s) separated by an interval of 30 s. After 24 h mice were returned to the conditioning chamber and freezing behavior was recorded during 2 min.

Morris Water Maze Test (MWM)

During the 3rd week of treatment, Tg2576 mice treated with CM-695 (n = 11) or vehicle (n = 10) and non-transgenic littermates (n = 10) underwent spatial reference learning and memory testing in the Morris water maze (MWM) test (Morris, 1984) as previously described (Westerman et al., 2002). In this case, the hidden-platform training (with all visible cues present) was conducted during nine consecutive days (four trials per day) and memory retention was analyzed with three probe trials at the beginning of days 4th, 7th and 9th. Four animals per groups were sacrificed for hippocampal gene expression analysis 24 h after the last probe trial and the remaining animals were maintained to perform a reversal phase of MWM after a washout period of 4-weeks. In this phase, the platform was placed in the opposite quadrant of the tank and the hidden platform training during five consecutive days (four trials per day) was performed. All cues remained in their original positions. Memory retention was analyzed in a probe at day 6. Mice were monitored by a camera mounted in the ceiling directly above the pool, and all trials were recorded using an HVS water maze program for subsequent analysis of escape latencies and percent time spent in each quadrant of the pool during probe trials (analysis program WaterMaze3, Actimetrics, Evanston, IL, USA). All experimental procedures were performed blind to groups. Animals were euthanized 24 h after the last probe.

Determination of Aβ Levels

$Aβ_{42}$ pool containing intracellular and membrane-associated $Aβ_{42}$ levels were measured in the parieto-temporal cortical extracts by using a sensitive sandwich ELISA kit (Invitrogen, Camarillo, CA, USA). Tissue was homogenized in a buffer containing SDS 2%, Tris-HCl (10 mM, pH 7.4), protease inhibitors (Complete Protease Inhibitor Cocktail, Roche) and phosphatase inhibitors (0.1 mM Na_3VO_4, 1 mM NaF). The homogenates were sonicated for 2 min and centrifuged at 100,000× *g* for 1 h. Aliquots of supernatant were directly diluted and loaded onto ELISA plates in duplicate. The assays were performed according to the manufacturer's instructions.

Affymetrix Microarray Hybridization and Data Analysis

The hippocampi were dissected and RNA was extracted with TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and purified

with the RNeasy Mini-kit (Qiagen, Hilden, Germany). RNA integrity was confirmed on Agilent RNA Nano LabChips (Agilent Technologies, Santa Clara, CA, USA). The sense cDNA was prepared from 300 ng of total RNA using the Ambion® WT Expression Kit. The sense strand cDNA was then fragmented and biotinylated with the Affymetrix GeneChip® WT Terminal Labeling Kit (PN 900671). Labeled sense cDNA was hybridized to the Affymetrix Mouse Gene 2.0 ST microarray according to the manufacturer protocols and using GeneChip® Hybridization, Wash and Stain Kit. Genechips were scanned with the Affymetrix GeneChip® Scanner 3,000. Microarray data files were submitted to the GEO (Gene Expression Omnibus) database and are available under accession number GSE128422.

Both background correction and normalization were done using RMA (Robust Multichip Average) algorithm (Irizarry et al., 2003). Then, a filtering process was performed to eliminate low expression probe sets. Applying the criterion of an expression value greater than 16 in at least three samples for each experimental condition (hippocampi from mice treated with vehicle or CM-695, with three biological replicates for condition), 22,191 probe sets were selected. R/Bioconductor (Gentleman) was used for preprocessing and statistical analysis.

First, we applied one of the most widely used methods to find out the probe sets that showed significant differential expression between experimental conditions, LIMMA (Linear Models for Microarray Data; Wettenhall and Smyth, 2004). Genes were selected as significant using p-value < 0.01 as threshold. Using False Discovery Rate (FDR) method to correct for multiple hypotheses testing no significant results was obtained.

Additional network and functional analyses were analyzed through the use of IPA (QIAGEN Inc.[1]).

Quantitative Real-Time PCR

The RNA was treated with DNase at 37°C for 30 min and reverse-transcribed into cDNA using SuperScript® III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). Quantitative real-time PCR (QRT-PCR) was performed to quantified gene expression. All the assays were done in triplicate using Power SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK) and the corresponding specific primers for heat shock protein family A (Hsp70) member 1 A/B (HSPA1A/B; Fw: 5′-AGCCTTCCAGAAGCAGAGC-3′; Rev: 5′-GGTCGTTGGCGATGATCT-3′), 78-KDa glucose regulated protein (GRP78; Fw: 5′-ACCAACTGCTGAATCTTTGGAAT-3′; Rev: 5′-GAGCTGTGCAGAAACTCCGGCG-3′) and for the internal control 36B4 (5′-AACATCTCCCCCTTCTCCTT-3′; 5′-GAAGGCCTTGACCTTTTCAG-3′). Real-time was carried out using an ABI Prism 7300 sequence detector (Applied Biosystems, Foster City, CA, USA) and data were analyzed using the Sequence Detection software v.3.0 (Applied Biosystems, Foster City, CA, USA). The relative gene expression was calculated with reference to the control group using the DDCT method (Livak and Schmittgen, 2001).

[1]https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis/

Dendritic Spine Density Measurement by Golgi-Cox Staining

A modified Golgi-Cox method (Glaser and Van der Loos, 1981) was used to analyze dendritic spine density. Half-brains were incubated in Golgi-Cox solution (1% potassium dichromate, 1% mercury chloride, 0.8% potassium chromate) for 48 h at RT (protected from light). The solution was then renewed and tissue was maintained there for another 3 weeks. Thereafter, brains were maintained in 90° ethanol for 30 min until being processed in 200 μm-thick coronal slices using a vibratome. The slices were incubated in 70° ethanol, reduced in 16% ammonia for 1 h and fixed in 1% sodium thiosulfate for 7 min. They were then dehydrated in an increasing alcohol graduation and mounted with DPX mountant (VWR International, Leuven, Belgium).

Spine density was determined in the secondary apical dendrites of the pyramidal cells located within the CA1 region of the hippocampus (Megías et al., 2001). Each selected neuron was captured using a Nikon Eclipse E600 light microscope and images were recorded with a digital camera (Nikon DXM 1200F) at a resolution of 1,000–1,500 dots per inch (dpi). For each mouse ($n = 3$), three dendrites of nine different neurons were used for the analysis.

Data Analysis and Statistical Procedures

The data were analyzed with SPSS for Windows, version 15.0 (SPSS, Chicago, IL, USA) and unless otherwise indicated, the data are expressed as means ± standard error of the mean (SEM). Normal distribution of data was checked by the Shapiro–Wilks test.

In the MWM, latencies to find the platform were examined by two-way repeated measures analysis of variance (ANOVA) test (genotype × trial) to compare the cognitive status in WT mice and Tg2576 mice. Likewise, the treatments effect in spatial memory was examined also by a two-way repeated measures ANOVA test (treatment × trial) followed by *post hoc* Scheffe's analysis. When two groups were compared, Student's t-test was used, whereas when more than two experimental groups were compared, one-way ANOVA followed by *post hoc* Scheffe's test was used.

RESULTS

Biological Activity

The functional activity of the chemical probe CM-695 against its targets (HDAC class I, PDE9 and HDAC6) was assessed *in vitro*, in SH-SY5Y neuroblastoma cells. These cells were exposed to CM-695 for 2 h at concentrations ranging from 1 nM to 1 μM in order to determine its effect on histone acetylation (lysine 9 of histone 3, H3K9 mark), CREB-Ser133 phosphorylation and α-tubulin acetylation by western blot analysis. There was a significant increase in α-tubulin acetylation (4.47 ± 1.36 fold change vs. control, $^*p < 0.05$, **Figure 1D**) but not of AcH3K9 (0.81 ± 0.33 fold change vs. control, **Figure 1B**) in SH-SY5Y cells exposed to CM-695 (100 nM), consistent with its selective inhibition of HDAC6 (IC50 = 40 nM) as opposed to class I HDACs (IC50 = 593 and 3530 nM for HDAC1 and HDAC2, respectively). However, CM-695 had a

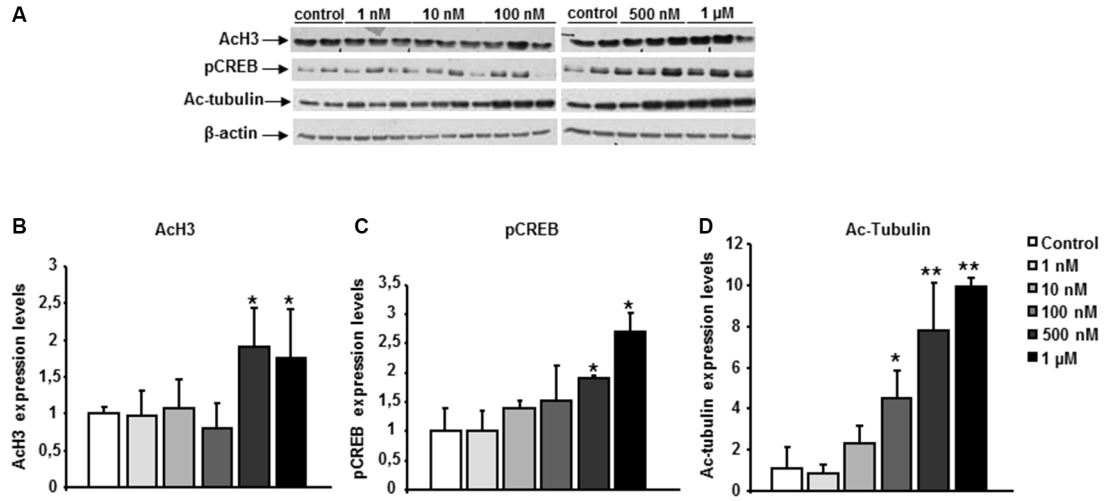

FIGURE 1 | CM-695 shows *in vitro* functional activities. **(A)** Representative bands of western blots showing histone 3 acetylation at lys 9 (AcH3K9), pCREB and Ac-Tubulin levels in SH-SY5Y cells treated with CM-695 at different concentrations (1 nM to 1 μM) for 2 h. **(B–D)** Histograms show the quantification of the immunochemically reactive bands in the western blot, $n = 3$ wells per condition, repeated in three different cultures. β-actin was used as a loading control. Data are represented as mean ± standard error of the mean (SEM) expressed as the fold change vs. vehicle; $^{*}p < 0.05$, $^{**}p < 0.01$.

significant effect on AcH3K9 at 500 nM (1.91 ± 0.56 fold change vs. control, $^{*}p < 0.05$, **Figures 1A,B**), although this effect was much stronger on tubulin acetylation (7.78 ± 2.36 fold change vs. control, $^{**}p < 0.01$, **Figures 1A,D**). In addition, at a concentration of 500 nM CM-695 significantly increased the levels of pCREB (1.91 ± 0.02 fold change vs, control, $^{*}p < 0.05$, **Figures 1A,C**), indicating this compound acts also against PDE9 (IC50 = 107 nM). These data confirmed the functional activity of this compound *in vitro* against its targets (HDAC6, PDE9 and HDAC class I).

CM-695 Ameliorates Memory Impairment in Aged Tg2576 Mice

Pharmacokinetic studies described for this compound indicated that CM-695 reached an acceptable brain concentration (around 100 nM/kg) 15 min after administering a dose of 40 mg/kg, concentrations that, according to the IC50, would ensure an effect on HDAC6 and PDE9. Furthermore, functional response in mouse brain was assessed *in vivo* in a group of animals to demonstrate the ability of the compound to inhibit HDAC and PDE9. Fifteen, 30 and 60 min after i.p. injection of 40 mg/Kg mice were sacrificed by cervical dislocation and their hippocampus were dissected. A western-blot was carried out to analyze pCREB and Ac-Tubulin in the hippocampus. As it shown in the **Supplementary Figure S2**, CM-695 increased pCREB levels at 15, 30 and 60 min. Regarding Ac-Tubulin, as basal levels of this protein are high in the brain of the animals, levels were slightly increased but no significant differences were appreciated. Likewise, a higher effect would probably be obtained with longer exposing times or in a chronic treatment. These data, demonstrated that CM-695 cross the BBB and reach the brain at a concentration which is enough to inhibit PDE9 (IC50 107 nm). Considering that HDAC6 IC50 is 40 nM and that a chronic treatment is achieved, an effect on Ac-Tubulin was assumed.

We analyzed the effects of administering CM-695 on the memory impairment in aged Tg2576 mice after a 2-week treatment (40 mg/kg, i.p. daily), assessing their performance in the fear conditioning test, a hippocampus-dependent learning task (**Supplementary Figure S3A**). Interestingly, the freezing response of Tg2576 animals was significantly ($^{*}p < 0.05$) stronger in those mice that received CM-695 (60.9% of freezing) than in those that received the vehicle alone (36.9% of freezing, **Figure 2A**). Moreover, 1 week later we evaluated the effects of CM-695 administration on learning in the MWM test (**Supplementary Figure S3B**). While the latency to find the platform was prolonged in Tg2576 mice relative to the WT mice, the escape latency was shorter when these Tg2576 mice received CM-695, although globally no significant differences were found between groups (**Figure 2B**). Interestingly, the Tg2576 mice remained significantly longer time in the target quadrant during the probe tests on days 4th (29.08 ± 4.6% vs. 18.91 ± 2.52%, $^{*}p < 0.05$), 7th (40.13 ± 5.50% vs. 24.61 ± 2.62%, $^{**}p < 0.01$) and 9th (32.18 ± 3.0% vs. 23.70 ± 2.45%, $^{*}p < 0.05$) when they received CM-695 (**Figure 2C**). To determine whether the effect of CM-695 persisted when the mice no longer received this compound, mice were re-trained in a reversal phase of the MWM test after a month wash-out, placing the platform in the opposite quadrant. The hidden platform training was carried out over 5 days (four trials per day) and it was followed by a memory retention test on day 6. No significant differences were found between the Tg2576 mice that received CM-695 or the vehicle alone in the hidden platform phase (**Figure 2D**) or in the probe trial (**Figure 2E**), indicating that none of the mice learned the platform location. Thus, it appears that the effect of CM-695 did not persist after the 4-week wash-out period.

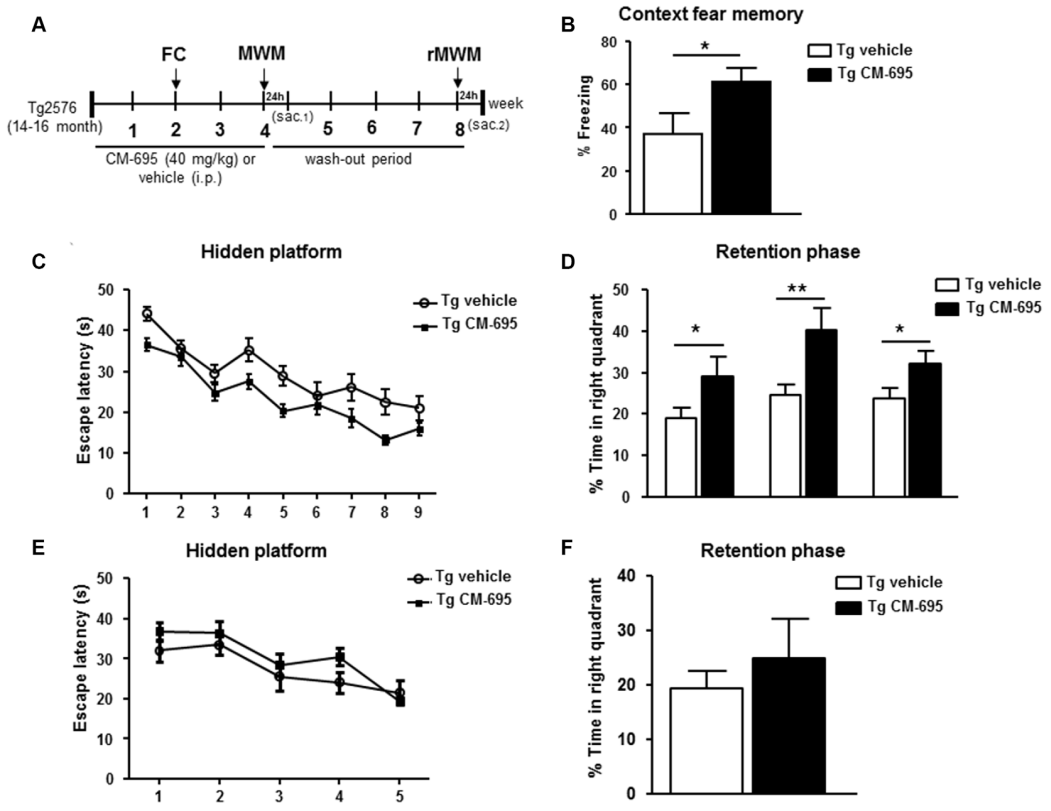

FIGURE 2 | Chronic treatment with CM-695 reversed learning deficits in aged Tg2576 mice. **(A)** Scheme showing timeline for treatment, behavioral tasks, and killing of mice. FC, fear conditioning; MWM, Morris water maze; rMWM, reversal MWM; Sac, sacrificed. **(B)** Freezing behavior from Tg2576 mice treated with vehicle (n = 10) or CM-695 (n = 11). Data represent the percentage of time of freezing during a 2 min test. **(C)** Escape latency of the hidden platform in the MWM test for the Tg2576 mice treated with vehicle (n = 10) or CM-695 (n = 11). **(D)** Percentage of time spent in correct quadrant during the probe test (days 4, 7, and 9). **(E)** Escape latency during the rMWM test for the Tg2576 mice treated with vehicle (n = 10) or CM-695 (n = 11) after the washout period. **(F)** Percentage of time spent in correct quadrant during the probe test after rMWM phase (day 6). In all figures results are expressed as mean ± SEM; $^{*}p < 0.05$, $^{**}p < 0.01$.

Taking all these results into account, it can be inferred that a chronic treatment with CM-695 ameliorated memory impairment in aged-Tg2576 mice although its effect was lost after a wash-out period of 4 weeks.

CM-695 Diminishes the Pathological Markers of AD in Aged-Tg2576 Mice

To determine the effects of CM-695 on amyloid pathology in aged-Tg2576 mice, ELISA was used to assess the $A\beta_{42}$ in parieto-temporal cortical extracts (see "Materials and Methods" section). The $A\beta_{42}$ levels were measured in two different groups of animals: one sacrificed at the end of the first MWM (after the 4th week of treatment, n = 4 per condition) and the other sacrificed at the end of the reversal-MWM (after the 4-week wash-out period in which the mice were not treated, n = 6–7 per condition). There was a significant decrease in $A\beta_{42}$ in the Tg2576 mice treated with CM-695 and sacrificed at the end of the treatment (7.70 ± 2.07, $^{**}p < 0.01$) relative to those that received the vehicle alone 17.8 ± 1.40, **Figure 3A**). Moreover, no $A\beta_{42}$ was detected in the WT littermates (data not shown). However, it was noteworthy that there was a significant decrease in the hippocampal $A\beta_{42}$ in the group of animals that was sacrificed after the 4-week wash-out period (13.33 ± 2.1, vs. 19.05 ± 2.69, $^{*}p < 0.05$, **Figure 3B**), although these differences (45% reduction) were not as strong as those observed prior to the wash-out period (56% reduction). These results suggest that a chronic treatment with CM-695 decreased amyloid pathology in elderly Tg2576 mice.

Given that Tg2576 mice display synaptic loss and dysfunction (Ricobaraza et al., 2012), we assessed whether the behavioral recovery induced by CM-695 was reflected by structural changes in dendritic spine density. Consistent with previous data, there was a significantly lower density of apical dendrites on CA1 pyramidal neurons in Tg2576 mice than in WT mice (**Supplementary Figure S4**). Taking into account the behavioral data obtained at the end of the treatment (**Figure 2**), we assumed that they could correlate with an increase in the density of dendritic spines in the treated animals, thus, we analyzed if the effect was maintained after 4 weeks without treatment as we observed for CM-414. However, when the mice were analyzed after the wash-out period, those that received CM-695 did not show any significant change in the density of spines on these neurons. It should be noted that there was a tendency to increase the density of spines that might account for the memory

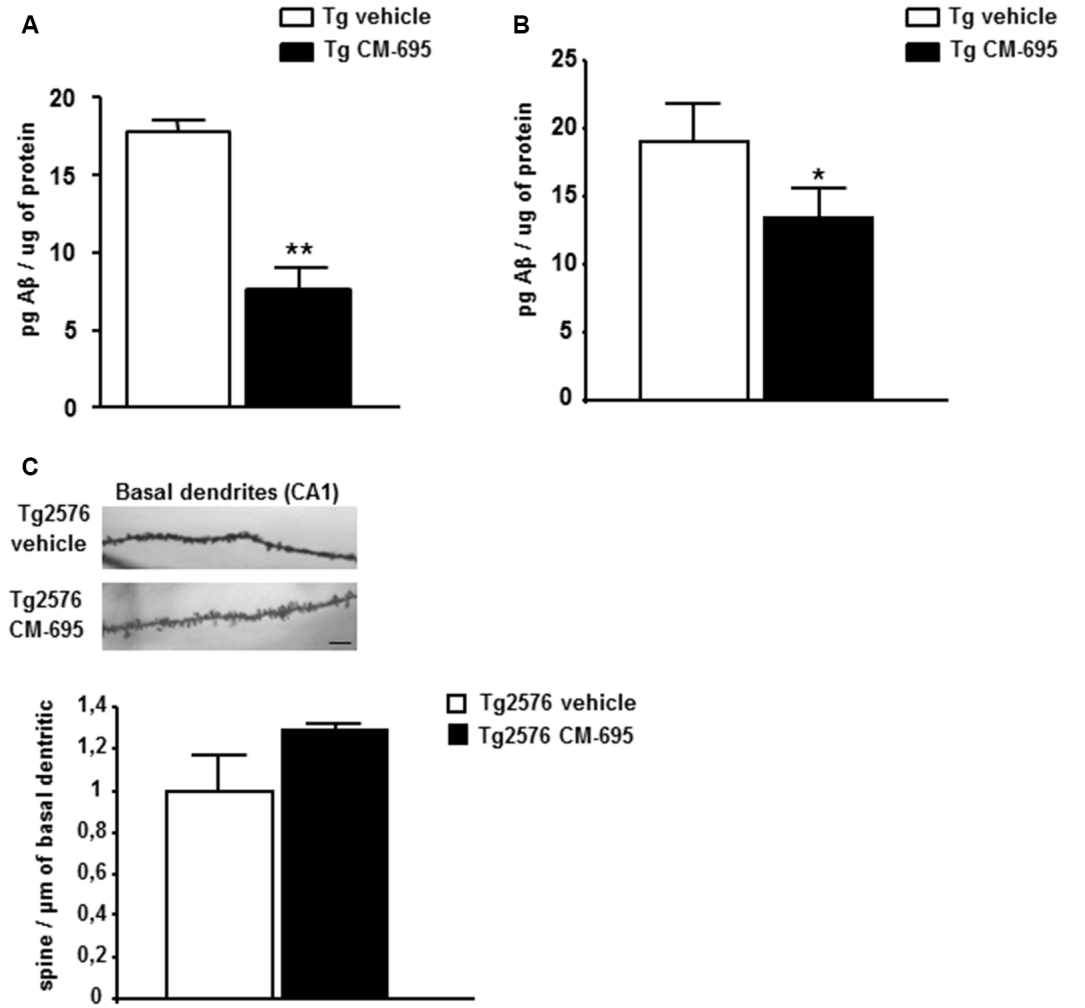

FIGURE 3 | Chronic treatment with CM-695 decreased amyloid levels but did not affect hippocampal dendritic spine density. $A\beta_{42}$ levels determined by ELISA in SDS hippocampal tissue extracts of Tg2576 mice treated with vehicle or CM-695 after 4 week of treatment ($n = 4$; **A**) or after a washout period of 4 weeks ($n = 6$–7). **(B)** $^{*}p < 0.05$, $^{**}p < 0.01$. **(C)** Representative Golgi staining images of the apical dendrites on CA1 hippocampal pyramidal neurons. Scale bar, 10 µm. The histograms represent the quantification of spine density of basal dendrites of hippocampal CA1 pyramidal neurons from Tg2576 mice treated with vehicle or CM-695 ($n = 34$–36 neurons from three animals per group).

improvement observed 4-weeks after treatment (**Figure 3C**). Nevertheless, after the wash-out period neither the effect on memory function nor the potential changes in dendritic spine density persisted.

To explore the mechanisms underlying the effect of CM-695 on amyloid pathology and on memory function, we analyzed the effects of CM-695 on gene expression in the hippocampus of Tg2576 mice compared to a group of transgenic animals receiving vehicle using Affymetrix microarrays. LIMMA was applied to find out the probe sets that showed significant differential expression between experimental conditions (Smyth, 2004). Genes were selected as significant using $p < 0.01$ as threshold. Differentially expressed genes were analyzed by using Ingenuity Pathways Analysis in order to gain information about the mechanistic approach of CM-695 therapeutic effect. Importantly, the Protein Ubiquitination Pathway (p-value = 6.76, E-05) and the Unfolded protein response (UPR, p-value = 3.1, E-05) were included among the top-ranked canonical pathways (**Figure 4A**) and more specifically, BIP (GRP78) and HSPA1A/B were among the genes overexpressed in the hippocampus of mice administered with CM-695 respect to the mice receiving vehicle (**Supplementary Figure S5**). In accordance, when differentially expressed genes were also categorized to diseases and biological functions "folding protein" (p-value and molecules) was significantly regulated (**Figure 4B**). Since chaperone activity plays a crucial role in proper protein folding activity, we analyzed the expression of GRP78 and HSPA1A/B by quantitative real time PCR. Accordingly to the results obtained in the array, a significant ($p < 0.01$) increase was observed in both GRP78 and HSPA1A/B mRNA levels in the hippocampus of CM-695 treated mice respect to mice receiving vehicle (**Figure 4C**). Next, we checked

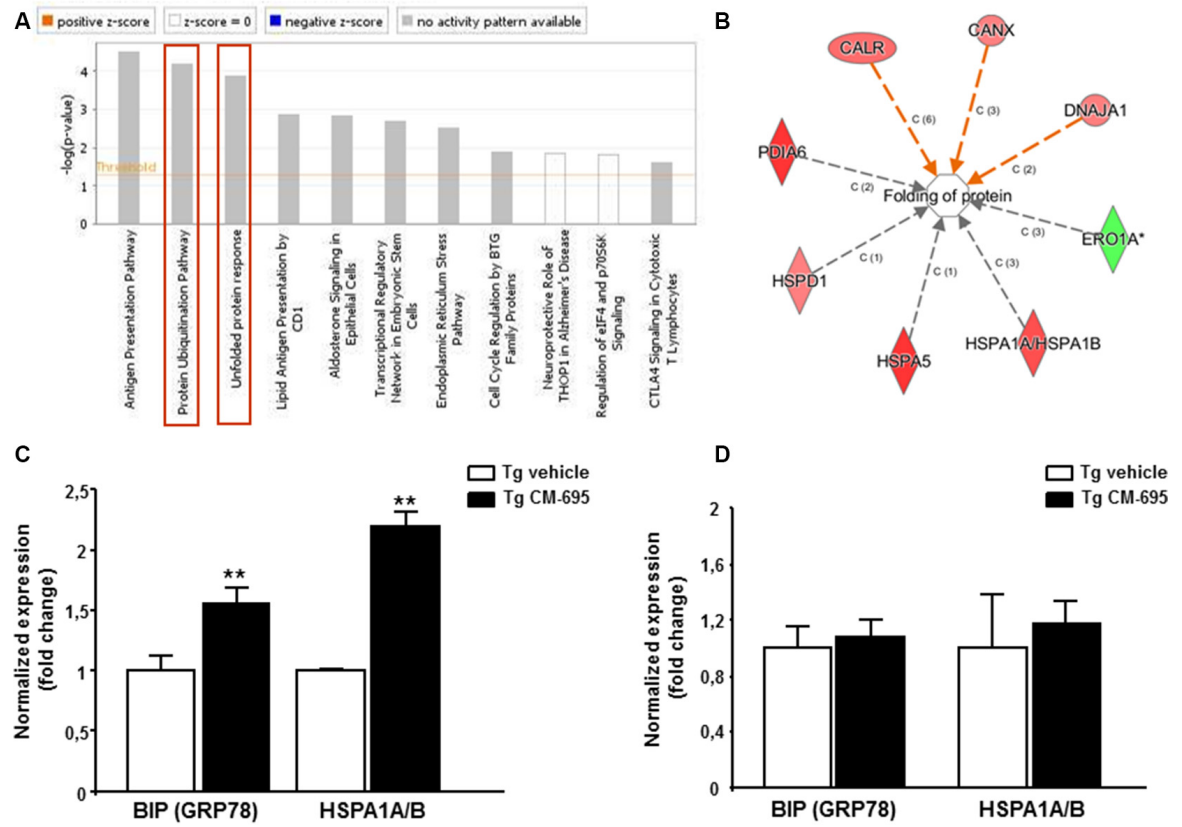

FIGURE 4 | Chronic treatment with CM-695 significantly increases chaperones: GRP78 and HSP70. **(A)** Ingenuity pathway analysis showing the most highly scoring canonical pathways (according to *p*-value). Horizontal orange line running through the bars is the threshold for *p*-value for these pathways. Color coding for positive and negative *z*-score and for pathways with no activity pattern available are shown in the figure. "Protein Ubiquitination Pathway" and the "Unfolded protein response" (remarked with a red box) were included among the top-ranked canonical pathways. **(B)** Network of differentially expressed genes categorized in Ingenuity by Disease and biological functions. **(C,D)** Quantitative RT-PCR (QRT-PCR) analysis of BIP (GRP78) and HSPA1A/B mRNA in the hippocampus of CM-695 treated mice vs. vehicle, 4 weeks after treatment (**C**, $n = 4$) and after the washout period respectively (**D**, $n = 6$–7). Bars represent the fold change (mean ± SEM) in gene expression normalized to vehicle-treated mice; $^{**}p < 0.01$.

if this effect in gene expression was maintained after the washout period. As depicted in **Figure 4D**, animals receiving CM-695 and sacrificed after a 4-weeks wash out period showed similar expression levels of GRP78 and HSPA1A/B to control group.

These results suggest that the increase in the levels of chaperones GRP78 and Hsp70, which are involved in protein folding, may underlie the improvement in AD symptoms observed after daily administration of the compound.

DISCUSSION

Given the high failure rate in AD drug development, with no new drug having been approved for this diseases since 2003 (Cummings et al., 2014), it is clearly necessary to change the way we approach the search for new AD targets to improve the results obtained in clinical trials. We have identified a NCE, CM-695, that has potential therapeutic effects in a well-established mouse model of AD. CM-695 is a potent HDAC6 and PDE9 inhibitor that, after chronic administration, ameliorates the cognitive impairment and amyloid pathology evident in aged-Tg2576 mice. An increase in the expression of the chaperones GRP78 and Hsp70, involved in protein folding, may underlie the improvement in AD symptoms observed after daily administration of the compound. The benefits obtained with this dual inhibitor are not maintained when the compound is no longer administered. Thus, CM-695 appears to be a safe and efficient disease-modifying agent for AD treatment, confirming that multi-target therapies may represent better options for the treatment of complex diseases.

HDACs are emerging targets for the treatment of AD (Yang et al., 2017). The inhibition of HDAC class I facilitates gene transcription and the formation of new synapses (Rumbaugh et al., 2015). The inhibition of HDAC class IIb (HDAC6), by targeting cytoplasmic proteins is involved in the clearance of misfolding proteins (Sung et al., 2013). The inhibition of both isoforms could be a promising and synergistic therapy to treat AD, which has been demonstrated by using pan-HDAC inhibitors. However, these compounds have unfavorable side effects when administered chronologically and at a dose

sufficient to reach the brain at appropriate concentrations. One strategy to reduce toxicity while maintaining effectiveness is to obtain new disease-modifying molecules maintaining HDAC inhibition combined with an additional function such as inhibition of PDEs, inhibition of glycogen synthase kinase 3β or antioxidant activity (De Simone and Milelli, 2019).

Based on the results obtained previously with different selectivity profiles of HDAC and PDE inhibitors (Rabal et al., 2016, 2018, 2019; Cuadrado-Tejedor et al., 2017; Sánchez-Arias et al., 2017), we designed a new series of PDE9 inhibitors that more specifically target HDAC6 over class I HDAC isoforms. This shift in specificity aimed to reduce the toxicity associated with class I inhibition which complicates the further therapeutic development of the new compound. Furthermore, since PDE inhibitors have pro-cognitive and neuroprotective effects, the inhibition of PDE9 was selected rather than PDE5 as its brain expression and affinity for cGMP is higher than that of other PDEs (**Supplementary Figure S1**, Andreeva et al., 2001; Singh and Patra, 2014). Among the new compounds designed, CM-695 was tested in AD model following the same guidelines used with CM-414 (Cuadrado-Tejedor et al., 2017), in order to compare the efficacy between the two compounds. The therapeutic effects obtained after chronic treatment with CM-695 were similar to those obtained with CM-414, except that the effect did not persist when the drug was no longer administered. Taking into account the differences in the inhibition profile of the two compounds, we confirm that the inhibition of class I HDACs, and more specifically HDAC2, plays an important role in maintaining memory function.

Interestingly, the effect of CM-695 on Aβ clearance is maintained, which may be mediated by the inhibition of HDAC6 (Boyault et al., 2007). One of the targets of HDAC6 is heat-shock protein 90 (Hsp90). As such, the inhibition of HDAC6 increases Hsp90 acetylation, releasing its client proteins like heat shock transcription factor 1 (HSF1), which in turn translocates to the nucleus and mediates the transcription of HSPA1A/B genes, and ultimately, Hsp70 (Wang et al., 2014). HSPA1A/B was among the genes overexpressed in the Affymetrix microarray analysis and validated by qRT-PCR in the hippocampus of mice administered CM-695. Significantly, a similar induction was induced by CM-695 in a model of thrombosis (Allende et al., 2017). Hsp70 fulfills a neuroprotective role in AD by decreasing the oligomerization and production of toxic Aβ isoforms, and by increasing its degradation (Magrané et al., 2004; Muchowski and Wacker, 2005; Kumar et al., 2007). Interestingly, Hsp70 upregulation by inhibiting Hsp90 was also proposed as a mechanism to normalize synaptic transmission in a transgenic model of tau aggregation (Thirstrup et al., 2016). These results suggest that the increase in Hsp70 expression observed at the end of treatment is at least partially responsible for the marked decrease in hippocampal Aβ levels observed. Together with PDE9 inhibition, this increase in Hsp70 may help restore learning ability. However, Hsp70 was no longer upregulated 4 weeks after the end of the treatment, causing a loss in Aβ clearance and a deterioration of the animals learning capacity. Considering the safe profile of CM-695, chronic treatment with no need for wash-out periods could be an option to consider for this NCE.

In conjunction, it seems that CM-695 is a safe and efficient disease-modifying agent to treat AD, confirming that multitarget therapies may provide good options to combat AD, as seen in other multifactorial diseases like cancer or AIDS.

DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

All procedures were carried out in agreement with the European and Spanish regulations (2010/63/EU; RD1201/2005), and the study was approved by the Ethical Committee for the Animal Experimentation of the University of Navarra.

AUTHOR CONTRIBUTIONS

AG-O, MC-T and JO conceived the general framework of this study, designed experiments and discuss results. MP-G, CG-M, DM and CG-B performed the *in vitro* functional assays in cell culture, treatments, behavioral experiments and biochemical assays. JS-A performed biochemical experiments related to CM-695 compound *in vitro*. VS performed the bioinformatic analysis of the microarrays. OR and JO designed and characterized CM-695. AG-O and MC-T wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnagi.2019.00149/full#supplementary-material

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REVIEW
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Decoding the Role of Platelets and Related MicroRNAs in Aging and Neurodegenerative Disorders

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Platelets are anucleate cells that circulate in blood and are essential components of the hemostatic system. During aging, platelet numbers decrease and their aggregation capacity is reduced. Platelet dysfunctions associated with aging can be linked to molecular alterations affecting several cellular systems that include cytoskeleton rearrangements, signal transduction, vesicular trafficking, and protein degradation. Age platelets may adopt a phenotype characterized by robust secretion of extracellular vesicles that could in turn account for about 70–90% of blood circulating vesicles. Interestingly these extracellular vesicles are loaded with messenger RNAs and microRNAs that may have a profound impact on protein physiology at the systems level. Age platelet dysfunction is also associated with accumulation of reactive oxygen species. Thereby understanding the mechanisms of aging in platelets as well as their age-dependent dysfunctions may be of interest when evaluating the contribution of aging to the onset of age-dependent pathologies, such as those affecting the nervous system. In this review we summarize the findings that link platelet dysfunctions to neurodegenerative diseases including Alzheimer's Disease, Parkinson's Disease, Multiple Sclerosis, Huntington's Disease, and Amyotrophic Lateral Sclerosis. We discuss the role of platelets as drivers of protein dysfunctions observed in these pathologies, their association with aging and the potential clinical significance of platelets, and related miRNAs, as peripheral biomarkers for diagnosis and prognosis of neurodegenerative diseases.

Keywords: platelets, microRNAs, aging, Alzheimer disease, Parkinson disease, multiple sclerosis, Huntington disease and amyotrophic lateral sclerosis

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INTRODUCTION

Endothelial cells, coagulation factors and platelets essentially form the hemostatic system in blood. Platelets derive from the cytoplasm of megakaryocytes by fragmentation in bone marrow and circulate for 7–10 days in blood as disk-shaped anucleate particles (Machlus et al., 2014). Endothelial cells prevent the interaction of platelets with coagulation factors to avoid thrombosis

under normal conditions. However, disruption of the endothelium leads to extracellular matrix exposition that ultimately triggers a response known as primary hemostasis to repair tissue damage (Born and Cross, 1963).

Upon tissue damage, the glycoprotein IIb/IIIa complex (GP IIb/IIIa) facilitates platelet aggregation platelet-to-platelet interactions; allowing their binding to fibrinogen or von Willebrand factor (vWF) that bonds adjacent platelets. Morphologically, activated platelets radically change their shape from disks to become spiny spheres (Holinstat, 2017).

Platelets have two important types of granules: dense granules and alpha granules. The dense granules are loaded with proaggregatory factors such as 5-hydroxytryptamine serotonin, calcium and adenosine 5′-diphosphate (ADP). In platelet activation, these granules release their content to the open canalicular system to then be expelled by the platelet (Johnson et al., 1966). On the other hand, the alpha granules have many hemostatic proteins for example growth factors, e.g., platelet-derived growth factor, vWF and fibrinogen.

Once activated, platelets can recruit other platelets to the activation site: the release of proaggregatory substances, e.g., ADP, Thrombin and Calcium and the synthesis of prostanglandin. Thromboxane A2 (TXA2) is locally synthesized from arachidonic acid. These two mechanisms act concertedly to consolidate the initial recruitment by promoting the participation of other platelets in this site (Gryglewski and Ramwell, 1980). Additionally, when the platelets are activated, the phospholipids located on the inner side of the lipid bilayer are moved to the outer face of this lipid bilayer. Now this negatively charged surface provides binding sites for co-factors and coagulation enzymes, enhancing blood clot formation secondary hemostasis (Bevers and Zwaal, 1983).

PLATELETS AND AGING

The effect of age on the function of platelets is not yet fully elucidated Changes in platelet numbers and function have broadly been related to aging (Jones, 2016). It has been reported that platelet count remains relatively stable in people under 60 but decreases after that age, diminishing by around 8% about 20,000 platelets/μl (Segal and Moliterno, 2006).

Interestingly, platelet decline occurs even in the presence of an increase in the content of hematopoietic stem cells in aging suggesting that some of their functions are impaired (Hartsock et al., 1965). Such apparent paradox may be explained by defects observed in progenitor cells cycle and changes in the activity of some enzymes such as helicase (Flach et al., 2014).

It is worth noting that the increase in platelet activation is a reflection of the surrounding environment, for example endothelial activation increases platelet function and may end in thrombosis (Tomaiuolo et al., 2017). This situation is increased with age, due to an alteration in the mitochondrial function, among which is the mTOR pathway and alterations in the levels of fatty acids and glucose (Houtkooper et al., 2011).

Changes in the redox tone associated to aging contribute to the activation of platelets, and could be related with an increased production of reactive oxygen species (ROS) (Donato et al., 2013). There is a strong association between ROS levels and platelet activation leading to increased thrombotic diseases, diabetes and metabolic syndrome (Violi and Pignatelli, 2014; Bonomini et al., 2015). The activity of NADPH oxidase and superoxide dismutase, two enzymes responsible for producing ROS and H2O2, is significantly elevated in aged mice platelets (Drummond et al., 2011). Increased ROS are responsible for activating signaling pathways such as p38 MAP kinase that predisposes platelets to further activation resulting in an increased prothrombotic state (Herkert et al., 2002; Dayal et al., 2013).

Concomitantly, aging-dependent reduction of glutathione peroxidase and nitric oxide synthase activities contributes to increased platelets activations (Alexandru et al., 2008; Dayal et al., 2013).

With regard to platelet function aggregation there are several studies that indicate that it is altered in an age-dependent manner (Bastyr et al., 1990). A positive correlation between age and the ADP levels and beta-TBG a indication of platelet secretion *in vivo* was found when analyzing platelets from 40 healthy individuals 22 to 62-year-old with no clinical evidence of atherosclerotic vascular disease (Bastyr et al., 1990). Gleerup and Winther (1995) also found that platelet aggregation was significantly increased in a middle-aged group 41–72 years old compared with a young-aged group 21–30 years old, furthermore, in this last group vigorous exercise caused platelet agreeability to decrease, which was not observed in the middle-aged group.

Additionally, different authors have shown many alterations in platelets with respect to aging such as variations in the activities of protein serine/threonine kinases, signal transducers, ubiquitin-protein ligases and GTPases as well as in vesicle transport and cytoskeletal organization (Simon et al., 2014; Jones, 2016). Age-related variations in the expression of specific platelet receptors and platelet activators have also been reported and exemplified by a decrease in the number of receptors for PGI2 potent inhibitor of platelet function and high levels of TXA2 activator of platelet function in older individuals (Simon et al., 2014). All these assays come to show that platelets decrease in number but become hyperreactive in older adults.

PLATELET MICRORNAs AND EXTRACELLULAR VESICLES IN AGING

The increase in platelet reactivity and aggregation observed in aging seems to be primarily driven by changes in platelet receptors and an increase in oxidative stress. However, differential regulation of gene expression by microRNAs (miRNAs) may also have an important role (Jones, 2016). miRNAs are small non-protein-coding RNAs involved in the post-transcriptional regulation of gene expression (Bartel, 2009). The functional miRNA molecule is the result of a maturation process that starts with the transcription of the miRNA gene into a primary miRNA transcript that is further processed by Drosha into a ∼70 nt hairpin miRNA precursor whose stem loop structure is recognized and cut by Dicer. The result of

Dicer cleavage is a miRNA duplex formed by two molecules of mature miRNA of ∼20 nt (Krol et al., 2010). One of these mature miRNAs is then incorporated into RISC (Argonaute-containing miRNA induced silencing complex) that guides the interaction between the miRNA and its target messenger RNA (mRNA) resulting in gene expression inhibition either by degradation of the mRNA or by repression of the transcript translation (Bartel, 2009). Each mature miRNA may regulate hundreds of genes presenting a particular and characteristic spectrum of target genes (Friedman et al., 2009). Similarly, mRNAs may be regulated by several miRNAs, which is consistent with the idea that miRNAs function as controllers of complex gene networks that virtually include all known biological processes (Shalgi et al., 2007; Friedman et al., 2009). Involvement of miRNAs in disease has been largely reported in a plethora of human disorders (Hrdlickova et al., 2014; Vijayan and Reddy, 2016; Liang et al., 2018; Vijayan et al., 2018) with most research in the field being focused on neurodegenerative disorders (Weinberg and Wood, 2009; Sonntag, 2010) and more intensely on cancer (Lu et al., 2005; Meltzer, 2005; Elghoroury et al., 2018).

Numerous miRNAs are known to participate in the development and production of megakaryocytes and, ultimately, platelets (Edelstein and Bray, 2011; Dahiya et al., 2015; Sunderland et al., 2017). Platelets have fully functional miRNA machinery and display their own repertoire of miRNAs (Landry et al., 2009; Edelstein and Bray, 2011). In this regard, microarray expression profiling revealed a diverse and relatively rich population of miRNAs in human platelets being the most abundant miR-142-3p, miR-223, let-7a/c/i/b, miR-185, miR-126, miR-103, miR-320, miR-30c/b, miR-130a and miR-26 (Landry et al., 2009). Another microarray expression study also reported miR-142-3p, miR-223, miR-126, and miR-26 as the most expressed miRNAs in platelets (Simon et al., 2014). Additionally, a high-throughput sequencing-based analysis of human platelet miRNAs partially agreed with these two previous studies and revealed that more than 75% of the platelets expressed miRNAs were mainly from only 5 miRNA families that include let-7 the most abundant, representing 48%, miR-25, miR-103, miR-140, and miR-199 (Plé et al., 2012). These studies lead to the idea that platelets may represent one of the richest sources of human miRNAs. In addition, some of these and other miRNAs have been found to be important for either platelet production, reactivity, aggregation, secretion or adhesion; particularly miR-21, miR-34a, miR-96, miR-126, miR-146a, miR-150, miR-155, miR-200b, and miR-223; the last being exceptionally relevant for the transition from megakaryocytes to platelets and for platelet functioning (Edelstein and Bray, 2011; Dahiya et al., 2015; Fuentes et al., 2015). miR-223 has also been found deregulated in many inflammatory conditions (Edelstein and Bray, 2011; Gatsiou et al., 2012; Dahiya et al., 2015; Fuentes et al., 2015) and has further been described as a neuroprotective molecule due to its capacity to control responses to neuronal injury by controlling the functional expression in the brain of the *N*-methyl-D-aspartate receptor subunit NR2B and the ionotropic AMPA glutamate receptor 2 GluR2 (Nagy et al., 2004).

Interestingly, several of these platelet miRNAs have been included in a group of miRNAs named geromiRs because of their involvement in aging-related processes (Ugalde et al., 2011). Of special interest are miR-146a and miR-155 that have been specifically associated with inflammatory processes mediating brain aging (Olivieri et al., 2013). Additionally, the expression of certain miRNAs in platelets is found to be coordinated with platelet reactivity and with pathological states that may be related to aging (Edelstein and Bray, 2011; Pienimaeki-Roemer et al., 2017; Sunderland et al., 2017). According to this, in a study performed on 154 healthy subjects, fifteen platelet miRNAs were found to be differentially controlled by age, eleven of these miRNAs decreased with older age and, conversely, their respective target mRNAs increased their expression (Simon et al., 2014).

Senescent and/or activated platelets are widely found in vascular and neurological disorders and are known to induce the liberation of platelet extracellular vesicles (EVs) (Aatonen et al., 2012; Pienimaeki-Roemer et al., 2015, 2017) making up about 70–90% of all circulating vesicles in the blood (Hunter et al., 2008; Flaumenhaft et al., 2009; Gatsiou et al., 2012; Dahiya et al., 2015). EVs are an heterogeneous group of particles that are enriched in miRNAs, which are largely protected from degradation when carried by EVs, and may exert important regulatory functions in platelet activation pathways associated with platelet hyperactivity as well as functioning as mediators in the communication between platelets and other cells (Edelstein and Bray, 2011; Gatsiou et al., 2012; Laffont et al., 2013; Pienimaeki-Roemer et al., 2017). This is illustrated by miR-223, one of the most abundant miRNAs found in platelet EVs, which was shown to be transferred from platelet EVs to endothelial cells regulating pro-apoptotic gene pathways (Laffont et al., 2013). Also several miRNAs enriched in platelet EVs have been shown to be deregulated in the blood and/or brain of patients diagnosed with a neurodegenerative disease (Pienimaeki-Roemer et al., 2017). It is the case, for example, of the geromiR miR-146, which is carried by platelet EVs and is deregulated in the blood of patients with Parkinson disease (PD) and in the blood, hippocampus and frontal cortex of patients with Alzheimer disease (AD) (**Table 1**). In this sense, increasing evidence suggests that senescence-associated EVs, which are EVs secreted by senescent cells, could be novel senescence-associated secretory phenotype factors with unique characteristics that could participate on tuning the phenotype of recipient cells, through their ability to function as intercellular communicators (Willeit et al., 2013; Kadota et al., 2018; Takasugi, 2018).

PLATELETS AND NEURODEGENERATIVE DISEASES

Even though the most important function of platelets is to prevent bleeding (Alarcon et al., 2015), they also play an important function in pathological conditions, as for example neurological and neurodegenerative diseases, including PD (Lim et al., 2009), Schizophrenia (Asor and Ben-Shachar, 2012), and AD (Ciabattoni et al., 2007). It is also important noting that platelets show high expression of several proteins associated with the development of AD, such as the APP amyloid

TABLE 1 | Platelet-related and Platelet-EVs-related miRNA families more frequently involved in neurodegenerative disorders.

miRNA	Disorder	Evidence for involvement of miRNAs in neurodegenerative disorders	References
miR-15a/b	AD	Down-regulated in the plasma of patients	Wang et al., 2011
	AD	Hyperphosphorylation of Tau protein through up-regulation of ERK kinases	Hébert et al., 2010
	MS	Potential informative biomarker to distinguish relapsing-remitting from progressive MS	Ebrahimkhani et al., 2017
	MS	Predicted regulation of *FGF2* and *KIF1B*	Fenoglio et al., 2012
	HD	Up-regulated in the frontal cortex of patients	Martí et al., 2010
miR-16	AD	Regulation of *APP*	Maciotta Rolandin et al., 2013
	PD	Up-regulated in the blood of levodopa treated patients	Margis et al., 2011
	MS	Up-regulated in the blood of patients	Keller et al., 2014
	HD	Up-regulated in the striatum and frontal cortex of HD patients	Martí et al., 2010
miR-20a/b	AD	Regulation of *APP*	Hébert et al., 2009; Maciotta Rolandin et al., 2013
	MS	Down-regulated in the blood of patients	Keller et al., 2014
	MS	Up-regulated in the plasma of patients	Martí et al., 2010
	HD	Up-regulated in the frontal cortex of patients	Siegel et al., 2012
miR-29a/b	AD	De-regulated in the brain of patients	Hébert et al., 2008
	PD	Down-regulated in the blood of patients	Margis et al., 2011
	PD	Up-regulated in the blood of levodopa treated patients. Predicted regulation of *PARK7*, *GPR37*, *CDC42*, *BACE1*, and *BCL2*	Maciotta Rolandin et al., 2013; Serafin et al., 2015
	HD	Up-regulated in the Brodmann's area 4 of patients	Johnson et al., 2008
	HD	Down-regulated in the Brodmann's area 4 of HD patients	Packer et al., 2008
miR-30b/c/e	AD	Up-regulated in the cerebrospinal fluid of patients	Cogswell et al., 2008
	AD	Up-regulated in circulating exosomes of patients	Tan et al., 2014; Cheng et al., 2015
	AD	Deregulated in several areas of the brain of patients	Cogswell et al., 2008
	PD	Up-regulated in the blood of levodopa treated patients. Predicted regulation of *LRRK2* and *BCL2*	Margis et al., 2011; Serafin et al., 2015
	MS	Potential informative biomarker to distinguish relapsing-remitting from progressive MS	Ebrahimkhani et al., 2017
miR-34a/b/c	AD	Up-regulated in blood mononuclear cells of patients	Schipper et al., 2007; Burgos et al., 2014; Kiko et al., 2014
	AD	Up-regulated in the hippocampus and frontal cortex of patients	Cogswell et al., 2008; Banzhaf-Strathmann et al., 2014; Müller et al., 2014
	AD	Up-regulated in the serum of patients	Bhatnagar et al., 2014
	AD	Affecting the clearance of Tau from the circulation through repressing *SIRT1*	Schonrock and Götz, 2012
	PD	Down-regulated in amygdala, frontal cortex and cerebellum patients. Regulation of *PARK7* and *PRKN*	Miñones-Moyano et al., 2011
	HD	Up-regulation in the plasma of patients	Gaughwin et al., 2011
miR-146a/b	AD	Up-regulated in the hippocampus and frontal cortex of patients	Cogswell et al., 2008; Müller et al., 2014
	AD	Down-regulated in the plasma of patients	Kiko et al., 2014
	AD	Deregulated in the cerebrospinal fluid of patients	Cogswell et al., 2008; Alexandrov et al., 2012
	AD	Regulation of *CFH* and *TLR4*	Pienimaeki-Roemer et al., 2017
	PD	Down-regulated in the blood of patients	Caggiu et al., 2018
	ALS	Up-regulated in the spinal cord of patients. Regulation of *NFL*	De Felice et al., 2012
	ALS	Up-regulated in CD14+ CD16− monocytes of patients	Butovsky et al., 2012
miR-155	AD	Regulation of *APP*	Maciotta Rolandin et al., 2013
	AD	Up-regulated in the cerebrospinal fluid of patients	Alexandrov et al., 2012
	PD	Up-regulated in the blood of patients. Promising as target for anti-inflammatory therapy	Caggiu et al., 2018
	MS	Up-regulated in the brain and plasma of patients. Regulation of *CD47*	Jagot and Davoust, 2016
	ALS	Up-regulated in the spinal cord of patients. Potential therapeutic target	Koval et al., 2013
	ALS	Up-regulated in CD14+ CD16− monocytes of patients	Butovsky et al., 2012

FGF2, fibroblast growth factor-2; KIF1B, Kinesin family member 1BAPP; APP, amyloid precursor protein; PARK7, protein deglycase DJ1; GPR37, G Protein-Coupled Receptor 37; CDC42, cell division control protein 42 homolog; BACE1,β-secretase; BCL2, apoptosis regulator; SIRT1, sirtuin; PRKN, parkin; CHF, complement factor H; TLR4, Toll-like receptor 4; NFL, low MW neurofilament; CD47, cluster of differentiation 47.

precursor protein (Bush et al., 1990) and tau protein (Mukaetova-Ladinska et al., 2013). Additionally, platelets express enzymes involved in protein modifications such as Glycogen synthase kinase 3 β (GSK-3β) (Li et al., 2008), α, β, and γ secretases (Smith et al., 2009). Of note, platelets have been compared with neurons because they have many biochemical similarities

(Talib et al., 2012), as it is the storage and release of neurotransmitters from platelets such as serotonin, glutamate and dopamine (Cupello et al., 2005; Rainesalo et al., 2005) and the expression of neuron-related proteins such as NMDA receptors (Kalev-Zylinska et al., 2014). Together this makes it interesting to consider the contribution of platelets to the hallmarks of neurodegeneration.

Neurodegenerative diseases affect cells primarily neurons in the central nervous system (CNS) including the brain, spinal cord, the optic and olfactory nerves but some times may also affect the peripheral system (PNS). Many of the neurodegenerative diseases with no cure are associated with different symptoms such as progressive degeneration and/or death of nerve cells producing a lot of problems related to movement, e.g., ataxias, and/or mental functioning, e.g., cognitive impairment and dementias. Dementias are responsible for the highest percentage of neurodegenerative diseases, contributing to AD, which represents around of the 60–70% of dementia cases (Selkoe, 2001).

PLATELETS AND ALZHEIMER'S DISEASE

Alzheimer disease is a chronic progressive neurodegenerative disorder characterized by memory decline and several alterations at the cognitive level. It is the most common cause of dementia in the elderly being calculated that 26.6 million people worldwide suffer from AD, and whose prevalence is estimated to quadruple by 2050 (Qiu et al., 2009).

There are several forms of AD, although the patients who develop clinical symptoms older than 65 years are the great majority (late onset AD, LOAD; 95%), the rest (5%) of patients have an earlier onset of the disease (early-onset AD) (Plagg and Humpel, 2015). The early onset AD is related to the existence of rare autosomal dominant forms of AD, which manifest as early onset AD, although most of these patients do not present a pattern of autosomal clear inheritance. However, genetic predisposition is very important, even in patients with late-onset AD, which estimated heritability is 60–80% (Gatz et al., 2006).

The two major hallmarks of the disease in patients with AD are the presence of senile plaques and neurofibrillary tangles in the brain, which are related to vascular dysfunction, inflammation and neurological damage including loss of synapses and glial and cholinergic degeneration (Polanco et al., 2018).

Another element that should be highlighted is the relationship between AD and oxidative stress, which has been shown to participate in the development of AD and vascular dementia (Luca et al., 2015).

Swerdlow was the first to associate the hypothesis of mitochondrial dysfunction with the early pathological events that occurred in AD (Swerdlow and Khan, 2004). He pointed out that the increase and accumulation of the amyloid β (Aβ) peptide produces an alteration in the mitochondria that leads to an increase in oxidative stress and neuroinflammation including apoptosis that leads to the development of AD (Hauptmann et al., 2006). Specifically, deposits of the Aβ peptide have been found in the mitochondria causing an alteration in the mitochondrial respiration since it affects the enzymatic complexes III and IV. This produces a decrease in the production of ATP and increase of the production of ROS (Rhein et al., 2009; Swerdlow et al., 2010). The above results in the opening of the mitochondrial permeability transition pore (mPTP), increasing oxidative stress and apoptosis, inducing the liberation of cytochrome C and producing damage and mutation of mitochondrial DNA (Hauptmann et al., 2006; Lakatos et al., 2010; Calkins et al., 2011). All these processes enhance neurodeneration (Manczak et al., 2011).

The activity of several enzymes that regulate oxidative stress such as cytochrome oxidase and mitochondrial pyruvate dehydrogenase is affected in the brain of AD patients resulting in a diminished barrier against oxidative stress (Swerdlow, 2011). *In vitro* tests have been able to demonstrate that the Aβ peptide could increase the levels of lipid peroxides and hydrogen peroxide and in this way it would relate to AD and vascular dementia (Gustaw-Rothenberg et al., 2010).

Even in cultures of hippocampal neurons the soluble Aβ peptide induced high levels of ROS producing a great synaptic damage and neuronal loss, in a way that could explain some of the toxicity mechanisms of the peptide (De Felice et al., 2007).

Moreover some research shows oxidative stress as responsible for the generation of this peptide, as an example transgenic mice over expressing the APP, and whose antioxidant system is altered, showed a significant increase in the deposit of this peptide in the brain (Li et al., 2004).

Also oxidative stress is capable of altering the intracellular location of the β-secretase the enzyme responsible for processing the β- APP, promoting the amyloidogenic processing of the APP, which consequently increases the Aβ peptide (Tan et al., 2013). In the brains of patients with AD characteristic effects of oxidative stress, i.e., oxidation of lipids, protein and DNA damage have been shown (Butterfield et al., 1999) and increase in ROS levels were observed (Christen, 2000).

There are studies that have demonstrated that the Aβ peptide 1–40 ($A\beta_{40}$) possesses a capacity to generate free radicals that are very important in AD pathogenesis, because in position 35 it possesses a methionine which is classified as an active redox amino acid vital in the neurotoxicity of peptide (Hensley et al., 1994). It has been demonstrated that by substituting methionine for a noreleucine exchange of a sulfide group for a methylene, the neurotoxic properties of this peptide are considerably reduced (Varadarajan et al., 1999).

It is also important to note that this peptide is capable of generating ROS in hippocampal (Varadarajan et al., 2000) and cortical synaptosomes (Kanski et al., 2002) and significantly increases the levels of carboxylated proteins in cortical synaptosomes (Ansari et al., 2006). These same results were corroborated in cortical synaptosomes from knockout mice in APOE, where it was shown that the $A\beta_{40}$ peptide produces oxidation and peroxidation of proteins and lipids (Keller et al., 2000; Lauderback et al., 2001).

Other qualities of this peptide that promote AD are that prior to neuronal damage the $A\beta_{40}$ peptide significantly produces a considerable reduction in the Na^+/K^+-ATPase activity in

hippocampal neurons of rats, which would favor neuronal death (Mark et al., 1995).

Glutamine synthase is another affected enzyme that loses its activity when incubated with this peptide in hippocampal neurons, which exacerbates the neurotoxic capabilities of $A\beta_{40}$ peptide (Aksenov et al., 1995; Harris et al., 1995). Similarly, a loss of function and activity of glutamine synthase has been observed in AD brains (Smith et al., 1991; Butterfield et al., 1997).

Platelets and Amyloid β-Peptide

The abnormal accumulation of the Aβ gives rise to senile plaques, its structure is in the form of β-plated sheet fibrils in cerebral arteries and capillaries nervous tissues. Cerebral amyloid angiopathy (CAA) is a disorder characterized by deposits of $A\beta_{40}$ in cerebral arteries and capillaries (Pezzini et al., 2009), with an estimated prevalence of 90–98% in AD patients. Also the CAA is present in 30% of individuals without dementia over 60 years old (Weller et al., 2009). This disease increases the risk of haemorrhagic stroke, dementia and contributes to neurodegeneration and thus to cognitive decline, being a key factor in the etiology of AD (Jellinger and Attems, 2007). It is very imperative to note that the brains of CAA patients show several alterations in cerebrovascular tissues like endothelial cell alteration, i.e., elevated levels of adhesion molecules: VCAM-1 (vascular cell adhesion protein 1), ICAM-1 (Intercellular Adhesion Molecule 1), *E*-selectin (Endothelial Leukocyte Adhesion Molecule-1), (Zuliani et al., 2008), inflammatory interleukin (IL-1β, IL-6, IL-8) and other molecules such as TNFα (tumor necrosis factor alpha), TGFβ (transforming growth factor beta), MCP-1 (monocyte chemoattractant protein-1) and matrix metalloproteases (Grammas, 2011). All these alterations lead to a proinflammatory state in the brain neuroinflammation, for example the IL-1β and TNFα increase the blood–brain barrier permeability and tight junctions (Steinman, 2013) generating neuronal degeneration, and memory dysfunction.

Aβ peptide is derivative from APP, which is a large type I transmembrane protein (Masters et al., 1985). APP is present in brain and in cells that circulate peripherally such as lymphocytes, monocytes and interestingly, it is also highly expressed in platelets (Bush et al., 1990). Many APP isoforms arise from alternative splicing but the three most common isoforms are APP695, APP751 and APP770, where APP695 is highly expressed in neurons while APP751 and APP770 are expressed in platelets (Li et al., 1999). Human platelets have high levels of APP and are thought to contribute to more than 90% of circulating APP (Li et al., 1994). Regulation of APP alternative splicing is therefore important to determine tissue specificity of APP isoforms, a process that is at least partially mediated by miRNAs (Smith et al., 2011). This has been proven specifically for the neural specific miRNA the miR-124 and its direct target PTBP1 (polypyrimidine tract binding protein 1) (Li et al., 1994; Makeyev et al., 2007). PTBP1 is highly implicated in the regulation of alternative splicing in the brain and its expression levels are tightly related to both, neuronal APP splicing and miR-124 expression (Li et al., 1994; Makeyev et al., 2007). The expression of miR-124 is down-regulated in patients with AD (Li et al., 1994; Lukiw, 2007), which could result in abnormal neuronal splicing of APP and, consequently, affect β-amyloid peptide production (Niwa et al., 2008; Smith et al., 2011). These findings provide new perspectives into the physiological and pathological role for miRNA-mediated regulation of APP in AD.

Amyloid precursor protein is post-translationally processed in two different ways depending on the secretases involved in its cleavage, one pathway leads to amyloid plaque formation amyloidogenic, while the other does not produce peptide aggregation in pathological deposits non-amyloidogenic (Selkoe and Hardy, 2016). In the non-amyloidogenic pathway, the extracellular domain of APP is cleaved by an enzyme called the α-secretase generating the soluble amyloid precursor protein α (sAPPα) and C-terminal fragment α (CTFα) retained in the membrane, where it is acted upon by the γ-secretase complex including different enzymes such as Anterior Pharynx defective 1, Nicastrin, Presenilin enhancer 2, Presenilin 1 and or Presenilin 2, generating two fragments that are the amyloid precursor protein intracellular domain (AICD) and a soluble N-terminal fragments p3 (Shoji et al., 1992; Chang and Suh, 2010). Meanwhile, APP is cut by β-secretase *BACE1* to produce a soluble amyloid precursor protein β (sAPPβ) and a C-terminal fragment β (CTFβ) also retained in the membrane, which is subsequently processed close to the N-terminal to generate CTFβ. Finally, the CTFβ is cleaved byγ-secretase complex producing the Aβ peptide that is larger than p3 and AICD (Zhang et al., 2012). The Aβ peptide may vary in size from 38 to 43 amino acids, depending on the cutting activity of theγ-secretase where it mainly produces two isoforms where the $A\beta_{40}$ is the most abundant ∼80–90%, followed by $A\beta_{42}$ ∼5–10%. This last isoform is more toxic and hydrophobic and is capable of being added in oligomers and fibrils to form the extracellular plaques that are deposited in the brain (Selkoe, 2001). The most important circulating peptide is $A\beta_{40}$ over 95%, and in AD contributes to the formation of perivascular amyloid plaques (Li et al., 1994; Chen et al., 1995; Herzig et al., 2004; Fryer and Holtzman, 2005).

Platelets express all the necessary enzymes for $A\beta_{40}$ production α, β, and γ-secretases and release all the segments of APP: sAPPα, sAPPβ and Aβ that may also be stored into alpha granules (Li et al., 1998). The processing of APP may occur at two different sites, either in the intracellular organelles secretory pathway or on the platelet surface (Li et al., 1998; Evin et al., 2003) being released as exocytosis products of the increase of intra-platelet calcium (Ca^{2+}) levels by two agonists: Thrombin and Collagen (Bush et al., 1990; Evin et al., 2003). It is worth to remark that there have been high levels thrombin in senile plaques in patients with AD (Akiyama et al., 1992). Increased protein kinase C (PKC) activity dependent on phosphatidylinositol 3-kinase (PI3K) has also been reported (Barry et al., 1999; Skovronsky et al., 2001) in platelets releasing $A\beta_{40}$ that ultimately leads to calpain activation and the consequent increase of Aβ secretion by the platelets (Chen et al., 2000; Getz, 2012). Indeed calpain activation is involved in p35 protein processing that leads to increased Cdk5 activation involved in AD (Contreras-Vallejos et al., 2012).

Aβ peptide released from activated human platelets contributes to vascular amyloid deposits, as previous studies have shown that the Aβ infiltration induces a cellular replacement

in the vasculature, specifically in media and adventitia layers, leading to thinner vessel wall damage (Mandybur, 1986). Additionally, a decrease in the expression of tight junction proteins claudin-1 and claudin-5 and increased matrix metalloproteases 2 and 9 production enhance vessel wall damage (Hartz et al., 2012). All of these changes induce blood vessel rupture resulting in a intracerebral hemorrhage with a decrease in blood flow and a suspension of oxygen supply to the brain, possibly inducing neuronal loss involved in dementia (Song et al., 2014). In addition, endothelial cells present in the vasculature express the receptor for advanced glycation end products (RAGE) that has been previously linked to neurodegeneration in a mechanism involving the exposure of endothelial cells to $A\beta_{40}$ peptide, increased levels of inflammatory cytokines (Interleukin-6, IL-6; Interleukin-1β, IL-1β; Monocyte Chemoattractant Protein-1, MCP-1 and of c-Jun N-terminal kinase/Activator protein 1, JNK-AP1) activation (Vukic et al., 2009). Platelets are also able to adhere to the vascular wall, leading to sustained platelet recruitment in these plaques and potentially to full vessel occlusion, producing increased platelet activation, increased $A\beta_{40}$ peptide secretion, development of CAA, dementia and, finally acceleration of the progression of AD (Canobbio et al., 2013; Gowert et al., 2014).

$A\beta_{40}$ peptide activates and promotes platelet adhesion and aggregation (Shen et al., 2008; Canobbio et al., 2014), mediated by different receptors such as CD36 and GPIbα, triggering several signal transduction pathways involving p38MAPK, COX1 and synthesis of TXA2, which ultimately increase Ca^{2+} levels, activates calpain and increases $A\beta_{40}$ peptide secretion (Herczenik et al., 2007). The thrombin receptor PAR1 could also have a role in the consequent activation of p38 MAPK and cytosolic phospholipase A2 (PLA2), and TXA2 formation (Shen et al., 2008).

Also $A\beta_{40}$ peptides may modify platelet shape change and granule release through activation of the small GTPase activation of the small GTPase RhoA and phosphorylation of its downstream effector, myosin light chain kinase, involving cytoskeletal reorganization (Canobbio et al., 2014). In platelets $A\beta_{40}$ peptides may also regulate phosphatidylserine exposure, production in platelets is also linked to increased $A\beta_{40}$ levels (Gowert et al., 2014). A correlation between increased ROS formation in AD platelets an increased oxidative stress in AD patients has been demonstrated (Cardoso et al., 2004).

The complex amyloidogenic pathway is also post-transcriptionally regulated by miRNAs at several levels (**Table 1** and **Supplementary Table S1**). Among the validated AD-related targets it is worth mentioning fibrinogen (*FGF*), a clotting protein that contributes to Aβ deposition and is regulated by miR-144-3p, miRNA found in platelet EVs (Cortes-Canteli et al., 2012). By the same token, *BACE1*, the enzyme responsible for β-secretase cleavage of APP, which is regulated by miR-9, miR-29a/b-1, miR-124, miR-195, miR-285, miR-298 among others. Finally, *APP* is also tightly regulated by some platelet-related miRNAs such as let-7i, miR-16, miR-20a, miR-101, miR-106a/b, and miR-155, and by other miRNAs formally miR-17, miR-147, miR-153, miR-323-3p, miR-644, and miR-655 (Maciotta Rolandin et al., 2013). *APP* is therefore fine-tuning regulation by miRNAs through three different means: directly, indirectly, and by regulation of its alternative splicing (Maciotta Rolandin et al., 2013).

Recently platelet activation has been related to an increase in the levels of inflammatory mediators, i.e., Chemokines (RANTES, PF4, MIP-1α), interleukins (IL-1β, IL-7, and IL-8), prostaglandins, CD40L and these proteins can perpetuate platelet activation (Thomas and Storey, 2015). The increased levels of all these inflammatory proteins are associated with AD (Leung et al., 2013). Therefore, uncontrolled platelets' activation could mediate a chronic inflammatory reaction associated with AD progression, favoring a feed-forward circle that increases inflammation and release of more $A\beta_{40}$ peptides.

During platelet activation the secretion of Aβ peptide considerably increases, for example Kucheryavykh et al. (2017) showed that during clot formation the release of Aβ peptide significantly increased 500 times in the clot formation site. The study also detected Aβ peptide around blood vessels and brain cortex, even determining the presence of Aβ peptide at a very close distance to the entorhinal cortex, this place is the principal zone affected by AD (Selkoe, 1991; Honig et al., 2003). This confirms that the circulating Aβ peptide could be deposited in different brain tissues and contributes to the development of AD. Different mechanisms exist by which the peptide could pass through of the blood–brain barrier BBB: (a) binding to different apolipoprotein such as apolipoprotein J (ApoJ) or clusterin, a heterodimeric glycoprotein that binds Aβ at a binding ratio of 1:1 and with and affinity constants of Kd = 2.0 nM (Shayo et al., 1997), (b) binding to apolipoprotein E (APOE) is a protein with 299 amino acids and transports lipoproteins, fat-soluble vitamins and cholesterol, in the nervous system, astrocytes and microglia (Garai et al., 2014), *APOE* is polymorphic, with three major alleles (*epsilon* 2, *APOE2*; *epsilon* 3, *APOE3*; and *epsilon* 4, *APOE4*). The presence of the *APOE4* is considered a risk factor for AD (Roses and Saunders, 1994). One factor that triggers cell death in the brains of AD patients is oxidative stress and platelets are an important source of oxidative stress (Marcourakis et al., 2008), Marcourakis showed an increase in thiobarbituric acid-reactive substances (TBARS) content and in the activities of Na, K-ATPase and nitric oxide synthase in patients carrying the *APOE4* allele in AD patients (Marcourakis et al., 2008). A decrease in the activity of cytochrome oxidase has been reported in neurons but also in platelets from AD patients (Hirai et al., 2001). Recently, it is reported that the *APOE4* allele inhibits the activity of cytochrome oxidase (Wilkins et al., 2017), confirming the association between APOE alleles and AD risk. Finally, Rosenberg et al. (Rosenberg et al., 1997) showed an altered processing of APP in platelets of AD patients carrying the *APOE4* allele, this alteration may contribute to chronic platelet activation in AD patients. Moreover, these data may relate to alterations in the Amyloid precursor protein processing that may occur in specific areas in the AD brain; (c) binding to RAGE, a multiligand receptor in the immunoglobulin superfamily, Mackic et al. (1998) showed that binding, endocytosis, and transcytosis of $A\beta_{40}$ peptide in brain microvascular was inhibited in 63% by anti-RAGE antibody and the inhibition of RAGE suppresses accumulation of Aβ peptide in brain parenchyma in a mouse transgenic model also showing a reduction in the expression

of proinflammatory cytokines, i.e., TNF-α in the brain and production of Endothelin-1 ET-1 (Deane et al., 2003). Binding to the low-density lipoprotein receptor-related protein 1 (LRP1), a multifunctional scavenger and endocytic receptor, a member of the LDL receptor family has been linked to AD and CAA that may regulate $A\beta_{40}$ peptide uptake. LRP1 was demonstrated to be substantially inhibited by anti-LRP-1 antibodies in mice (Deane et al., 2009) and two transporters the ABCB1 and ABCG2 members of the superfamily of ATP-binding cassette ABC transporters, Zhang et al. (2013) found that an injection of $A\beta_{40}$ peptide fluorescent in mice that was quickly cleared of circulation between 30 min and 2 h. and this quickly increased the fluorescence in the brain in KO mice compared with wild type animals. On the other hand, different studies showed decreased expressions of these transporters in elderly people (Wu et al., 2009; Chen et al., 2016).

Platelets and Neurofibrillar Tangles

One of the principal hallmarks of AD is the presence of neurofibrillary tangles mainly composed of hyperphosphorylated Tau cytoskeletal microtubule-associated protein (Iqbal et al., 2010). Recently this protein has been detected in the platelet proteome and the levels of oligomeric Tau species have been proposed as a novel and robust AD biomarker (Neumann et al., 2011) and correlate with the cognitive status in these patients (Farías et al., 2012) although these results need to be further validated. Platelets also express glycogen synthase kinase 3β (GSK3β), one of the many protein kinases involved in Tau hyperphosphorylation (Forlenza et al., 2011). A complete profile of Tau hyperphosphorylation in platelets may provide fundamental insights into the post-translational modifications of Tau, and may be a surrogate proxy of neuronal dysfunction.

Remarkably, recent findings support a significant role for miRNAs in the regulation of Tau at several levels. These associations include regulation of Tau splicing, and therefore the ratio between different Tau isoforms modulated by miR-132 (Smith et al., 2011), Tau post-transcriptional regulation by miR-219 (Hébert et al., 2010), hyperphosphorylation of the Tau protein through either up-regulation of ERK kinases by miR-15 (Hébert et al., 2010; Santa-Maria et al., 2015) or via activation of the cyclin-dependent kinase 5. The last is associated with over-expression of one the more abundant miRNAs in platelets, miR-26b (Absalon et al., 2013) (**Table 1**). Finally, miRNAs may also be affecting the clearance of the Tau protein from the circulation through the repression of SIRT1 by miR-9, and by the platelet-related miR-34 and miR-181c (Schonrock and Götz, 2012) (**Table 1** and **Supplementary Table S1**).

In agreement with miRNAs having a role in the pathophysiology of AD, evidence also indicates that miRNAs show abnormal expression in AD. As shown in **Table 1** and **Supplementary Table S1**, the expression levels of several brain-enriched miRNAs miR-9, miR-29a/b, miR-128, miR-134, miR-137, miR-146a, and miR-339 among others and platelet-related miRNAs miR-25, miR-29a/b, miR-30e, miR-34a/c, miR-103, miR-130a, miR-146a, and miR-200c, among others were found to be significantly deregulated in plasma, serum, cerebrospinal fluid and/or brain from AD patients (Schipper et al., 2007; Cogswell et al., 2008; Hébert et al., 2008; Alexandrov et al., 2012; Cheng et al., 2013; Bhatnagar et al., 2014; Burgos et al., 2014; Kumar et al., 2017). Interestingly, some of these miRNAs, such as miR-29 and miR-146a, are found in platelet EVs (Pienimaeki-Roemer et al., 2017) and could take part in the existing intercellular communication between the central nervous and the vascular systems.

PLATELETS AND PARKINSON'S DISEASE

Parkinson's disease is clinically characterized by a plethora of symptoms such as resting tremor, bradykinesia, rigidity, and postural imbalance. The pathological explanation underlying these traits is the selective death of dopaminergic neurons located in the substantia nigra (Lotharius and Brundin, 2002).

These neural losses are a consequence of the accumulation of abnormal aggregates of protein alpha synuclein, which is the major structural element in Lewy bodies that develop inside nerve cells (Popescu et al., 2004), but are also a product of the proteasomal system dysfunction, reduced mitochondrial enzymes activities and oxidative stress accompanying aging (Puspita et al., 2017).

Although inflammatory changes are thought to be mainly caused by neuronal destruction and a risk factor for PD, an increased concentration of the same neuroinflammatory markers mentioned above for AD, i.e., RANTES, MIP-1α, IL-1β, TNF-α have even been detected in PD (Reale et al., 2009).

Another characteristic of PD is the increased oxygen consumption and increased ROS production. ROS is produced by platelets under many conditions and can dramatically increase as a consequence of several circumstances such as inflammation. Increase in ROS levels may produce cellular lesions and damage that may eventually lead to cell death (Qiao et al., 2018), it is also is important to mention that ROS is related with platelet hyperactivation and platelet secretion (Carrim et al., 2015) producing a cycle that increases all the previously mentioned components related to neurodegenerative diseases.

According to several reports, changes in the ultrastructure, mitochondrial dysfunction, increased glutamate level and morphology of platelets have been observed in patients with PD (Keane et al., 2011). The mitochondria have a dual function because as they produce and are a ROS target, their deregulation also plays a critical role in PD pathogenesis; this organelle has many functions such as energy generation, calcium homeostasis and response to stress and cell death. Therefore, any damage related to their dysfunction leads to cellular damage and is related to neurodegeneration (Dias et al., 2013).

Mitochondrial dysfunction was first linked to PD upon the recognition that 1-methyl-4-phenyl-1, 2,3,6-tetrahydropyridine (MPTP) induced PD in a study on drug abusers (Perier and Vila, 2012). The MPTP is metabolized into 1-methyl-4-phenylpyridinium MPP+ by the monoamine oxidase B (MAO-B) produced in platelets, crosses the blood-brain barrier and inhibits Complex I of the mitochondrial electron transport chain producing neuronal degeneration (Lim et al., 2009).

Monoamine oxidases (MAO) are a family of enzymes belonging to the flavin-containing amine oxidoreductases (Danielczyk et al., 1988) that catalyze the oxidative of monoamines. In the mitochondria they are bound to the outer membrane in most cell types in the body.

Oxygen is frequently used to remove amines from several molecules by the action of MAO producing different groups such as aldehyde and ammonia. The enzymatic capacity of MAO degrades amine neurotransmitters, such as dopamine, norepinephrine, and serotonin (Shih et al., 1999). Two isoforms of MAO, A and B exist, while MAO-A is specialized on the oxidation of serotonin 5-hydroxytryptamine, 5-HT and norepinephrine (NE), MAO-B is specialized on the oxidation of phenylethylamine (PEA) (Shih et al., 1999). There are two forms that can oxidize dopamine (DA). Platelets also possess mitochondrial MAO-B, this enzyme mediates the toxicity of MPTP by catalyzing the formation of the MPP+ which produces PD (Steventon et al., 1989; Götz et al., 1998). In addition, some studies report high dopamine uptake in patients with PD (Roussakis et al., 2015).

miRNAs are crucial in the regulation of redox-signaling pathways associated with several pathological processes related to PD such as mitochondrial dysfunction, α-Synuclein (α-Syn) aggregation, and neuroinflammation (Hsu et al., 2000; Tansey et al., 2007; Subramaniam and Chesselet, 2013; Emde and Hornstein, 2014; Xie and Chen, 2016). As an example, the brain-enriched miRNAs miR-7 and miR-153 have important roles in the regulation of α-Syn expression prompted by mitochondrial ROS-mediated action, both miRNAs can synergistically down-regulate α-Syn expression (Junn et al., 2009; Thompson et al., 2016; Xie and Chen, 2016) and may be associated with the familial form of PD through deregulation of the leucine-rich repeat kinase 2 (*LRRK2*) gene (Gehrke et al., 2010). Of particular interest is miR-34, a brain-enriched geromiR that has been found down-regulated in the amygdala, cerebellum and frontal cortex from PD patients (Miñones-Moyano et al., 2011). In this work miR-34b and miR-34c were demonstrated to alter the mitochondrial function in neuronal cells through the inhibition of protein deglycase (*DJ1*, also known as *PARK7)*, a redox-sensitive protein which triggers activation of antioxidant defenses via the Nrf2/ARE system, and Parkin (*PRKN*), which, together with *PARK7*, are associated with familial forms of PD (Dodson and Guo, 2007). Down-regulation of miR-133b and miR-34b/c was also detected in mid-brain dopaminergic neurons of patients with PD (Kim et al., 2007; Miñones-Moyano et al., 2011). Interestingly, miR-34 had previously been shown to be an enhancer of megakaryocitopoiesis (Gatsiou et al., 2012). Moreover, as shown in **Table 1** and **Supplementary Table S1**, elevated blood expression of the platelet-related miRNAs miR-22-3p, miR-146a and miR-155 were found to be a possible PD-specific miRNA signature (Margis et al., 2011; Caggiu et al., 2018). According to miRNAs as potential biomarkers for PD, one of these studies identified three miRNAs carried by platelet EVs miR-16-2, miR-26a2 and miR-30a as able to differentiate levodopa treated patients compared to untreated patients with PD (Margis et al., 2011). In the same direction, a recent investigation also showed that three other platelet-related miRNAs miR-29a-3p, miR-30b-5p, and miR-103a-3p were over-expressed in PD patients treated with levodopa *versus* untreated PD patients (Serafin et al., 2015).

Potential target genes for these three last miRNAs comprise genes implicated in PD such as, *LRRK2* predicted target for miR-30b-5p and miR-103a-3p; *PARK7* predicted target for miR-29a-3p; the G Protein-Coupled Receptor 37 (*GPR37*) a modulator of the dopaminergic system predicted target for miR-29a-3p; the cell division control protein 42 homolog, *CDC42*, a candidate gene for PD involved in neural death and the antiapoptotic predicted target for miR-29a-3p and miR-103a-3p; and the apoptosis regulator (*BCL2*), predicted target for miR-29a-3p, miR-30b-5p, and miR-103a-3p, which down-regulated by the above stated miRNAs could at least partially responsible for the death of dopaminergic neurons (Serafin et al., 2015).

PLATELETS AND MULTIPLE SCLEROSIS

Multiple sclerosis (MS) is a neurodegenerative disease primarily related to damage in the brain and spinal cord CNS, but it may also affect the peripheral nervous system. In this disease the immune system attacks the protective sheath myelin that covers nerve fibbers and causes communication problems between the brain and the rest of the organism (Sheremata et al., 2008). Signs and symptoms depend on nerve damage and affected nerves, the patients may lose the ability to walk independently and cognitive impairment may also appear. There is no known cure for MS. However, treatment can help speed recovery from attacks, modify the state of many diseases and manage symptoms.

The principal damage is characterized by immune-mediated responses with microglial activation and cellular infiltration. These alterations are mainly associated with inflammation of white matter and lead to a progressive demyelination and the destruction of axons (Høglund and Maghazachi, 2014).

Oxidative stress in patients with MS is associated with an increase in myelin and axonal damage that may lead to the apparition of clinical symptoms (Ohl et al., 2016). Different studies related the presence of lesions in MS patients with the apparition of proteins of the coagulation cascade (Morel et al., 2015). There are a lot of ways in which platelets may contribute to the pathophysiology of MS. For example, platelets may modulate inflammation in relation to leukocytes interaction and the release several mediators, i.e., matrix metalloproteinases and chemokines (Sheremata et al., 2008; Horstman et al., 2010). Platelets could be essential for the production of IL1-α, this can activate the endothelium in the brain thus allowing the entry of white blood cells and producing cerebrovascular inflammation, which has a significant role in the production of brain injury in MS. It has been found that the platelets are abundant in the inflamed brain and spinal cord of subjects with MS (Horstman et al., 2010). Under normal conditions the BBB attends to prevent infiltration and adhesion of inflammatory cells into the brain, but the proinflammatory states or damage may allow that different cells to penetrate in the BBB. In this situation the platelets rapidly adhere to the endothelium cells, become activated and secrete bioactive mediators, in this way platelets may contribute to

BBB permeability and increases the neurovascular inflammation typical of MS (Morrell et al., 2014).

Platelets may also recognize specific glycolipid structures, i.e., sialated gangliosides in brain, again promoting neuroinflammation and then neurovascular damage. The platelets that penetrate the BBB may recognize sialated gangliosides within the lipid rafts and accumulate, releasing the above stated molecules and so could play an important role in neuronal damage in the induction and perpetuation of inflammation in the CNS (Wachowicz et al., 2016).

Another mechanism whereby platelet are related to CNS damage in MS patients is through the production of ROS (Wachowicz et al., 2016). These levels can dramatically increase under neuroinflammation producing lesions and damage to different cellular structures and potentially cell death. Oligodendrocytes are more sensitive to oxidative damage than astrocytes and microglia. The reactive species may activate the macrophages promoting the damage of the myelin sheath by the attacking to this structure.

In CNS activated platelets may represent an additional source of ROS and therefore could lead to an increase in oxidative stress, which may be at least partially related to the characteristic neuronal demyelination and tissue damage of MS.

Therefore, platelet activation could be a great consequence of the disease, perhaps secondary to an endothelial lesion. Many studies have reported a strong association between MS and the increase in platelet adhesiveness, which would be strongly associated with the activity of the disease. Importantly, platelets contain at least 300 proteins, many of them being involved in the regulation of inflammation, platelets participate in one of the most important pathological processes of MS, mainly a product of the activation of the immune system against the CNS myelin at the beginning.

Moreover, various miRNAs, primarily miR-155 and miR-326, are involved in the regulation of neuroinflammatory processes observed in MS have been associated with disease activity and duration (Zhang et al., 2014; Jagot and Davoust, 2016). Particularly the platelet-enriched geromiR miR-155, which is up-regulated in MS patients, down-regulates CD47 in astrocytes and oligodendrocytes and could contribute to MS-associated inflammation and neurodegeneration. Current reports also show that free circulating miRNAs, including at least four platelet-enriched miRNAs (miR-16, miR-20, miR-22, and miR-145), are deregulated in MS fluids such as plasma, serum, or cerebrospinal fluid (Siegel et al., 2012; Keller et al., 2013; Søndergaard et al., 2013) (**Supplementary Table S1**). A recent study aimed at the use of exosomal miRNA profiles as signatures in MS identified nine miRNAs as informative biomarkers to distinguish relapsing-remitting from progressive MS (**Table 1**), which includes two platelet-enriched miRNAs, miR-30b-5p and one of the major miRNA drivers of platelet production, miR-223 (Ebrahimkhani et al., 2017). Interestingly, miR-223 has also been identified as a potential MS biomarker across several independent blood-based miRNA studies (Fenoglio et al., 2016) and has been involved in the pathophysiology of MS by targeting the transcription factor *STAT5* and other inflammatory regulators implicated in MS such as heat shock protein 90 and E2 (Ebrahimkhani et al., 2017). In the same study other miRNAs were also found as promising candidate biomarkers for relapsing-remitting MS and progressive MS, including the platelet-related miRNAs miR-30b-5p, miR-223, and miR-15-5p, the last predicted as regulator of the fibroblast growth factor-2 gene (*FGF2*), a gene involved in demyelination and remyelination (Fenoglio et al., 2012).

PLATELETS AND OTHER NEURODEGENERATIVE DISEASES

Huntington's disease (HD) is a neurodegenerative genetic disorder originated by the expansion of the single tandem repeat CAG in the Hungtingtin gene (HTT) and it is characterized by the occurrence of abnormal involuntary movements, cognitive decline and psychiatric disorders such as depression (Arrasate and Finkbeiner, 2012).

The finding of hyperactive platelets is the principal characteristic in HD patients in response to many agonists such as epinephrine, dopamine, serotonin, adenosine diphosphate, arachidonic acid, and collagen (Muramatsu et al., 1982). This characteristic can be explained as the levels of NO are very diminished in platelets in HD patients, it is important to note that NO is a potent vasodilator and inhibitor of platelet activation (Carrizzo et al., 2014). Under this state of platelet hyperactivity there is an increase in the inflammatory components secreted by platelets that allow amplifying the proinflammatory state in patients with HD, a component that is related to HD development and prognostics.

Mutant Huntingtin represses the formation of P bodies through its interaction with Ago1 and Ago2, two proteins that are crucial for the biogenesis of miRNAs (Savas et al., 2008). Thus, deregulation of several miRNAs, miR-9, miR-16, miR-22, miR-29a/b, miR-132, miR-196, and miR-330 among others, has been reported in the brain of HD patients (Johnson et al., 2008; Packer et al., 2008; Martí et al., 2010). Efforts are nowadays focusing on identifying miRNAs whose expression in the blood could correlate with disease progression as is the case of the platelet-related miR-34b that is regarded as a reliable and promising HD biomarker prior to the beginning of symptoms (Gaughwin et al., 2011) and the case of the platelet-enriched miRNAs miR-22-5p, miR-30d-5p, and miR-223 (Díez-Planelles et al., 2016). Furthermore, a therapeutic role for some miRNAs, the most remarkable being miR-27 and miR-196a, has been suggested in HD (Packer et al., 2008; Maciotta Rolandin et al., 2013; Ban et al., 2017).

Another biological component that has been used as biomarkers is MAO. Some studies relate the neuronal damage with the high levels of MAO-A and MAO-B activity in brain and platelets and the HD progression (Markianos et al., 2004). The expression of this protein is regulated by the transcription factors in response to stress such as ischemia and inflammation (Gupta et al., 2015).

An increase in the mitochondrial-dependent apoptosis in platelets in HD patients has also been shown. Based on this evidence, it is justified that, on the whole, platelets play a significant role in HD (Ehinger et al., 2016).

Amyotrophic lateral sclerosis (ALS) is a disease characterized by a gradual degeneration of motor neurons and neuromuscular paralytic disorder that leads to respiratory failure and death (Kiernan et al., 2011). The main etiological factors responsible for ALS are found in the CNS and in peripheral tissues, for example skeletal muscle, liver, lymphocytes, platelets etc. In this sense, mitochondrial dysfunction and changes in the ultrastructure of ALS platelets such as alterations in permeability transition and in mitochondrial membrane potential (MMP) have been found related to ALS (Shrivastava et al., 2011).

Increased glutamine synthetase together with normal expression of excitatory amino acid transporter 2 responsible for over 90% of glutamate reuptake within the CNS in the platelets of ALS patients, involving glutamate excitotoxicity in the pathogenesis of ALS has also been reported (Bos et al., 2006). A significant decrease of serotonin, a molecule that controls motor neuron excitability and energy metabolism, has also been observed in the platelets of ALS patients (Dupuis et al., 2010). On the other hand, thrombospondin, a glycoprotein released from platelet alpha-granules, has found significantly increased in ALS patients suggesting stressing the potential of platelets as biological markers.

As for the previous neurodegenerative diseases, miRNAs also show up as promising biomarkers for ALS. Profiling of miRNAs in ALS patients has been performed including analysis of blood samples and peripheral tissues (Butovsky et al., 2012; De Felice et al., 2012; Campos-Melo et al., 2013; Koval et al., 2013; Takahashi et al., 2015). Among these studies Butovsky identified an inflammatory miRNA signature in CD14+CD16− monocytes from ALS patients. Deregulated miRNAs in ALS monocytes include miR-338-3p, a miRNA that has also been found significantly deregulated in blood, cerebrospinal fluid, serum, spinal cord, and brain from ALS patients (Shioya et al., 2010; De Felice et al., 2012). This last study identified, for the first time, specific disease-related changes in miRNAs as putative biomarkers for early diagnosis of the ALS. Differential miRNA expression in the spinal cord of ALS patients leads to the identification of up-regulation of two platelet-related miRNAs, miR-146 and miR-155, in ALS patients compared to healthy controls (Campos-Melo et al., 2013; Shaner et al., 2013) (**Table 1**). These two miRNAs are considered geromiRs and have been largely associated with inflammatory processes (Olivieri et al., 2013) with miR-155 being considered a promising therapeutic target for ALS. Moreover, miR-146 has also been reported to

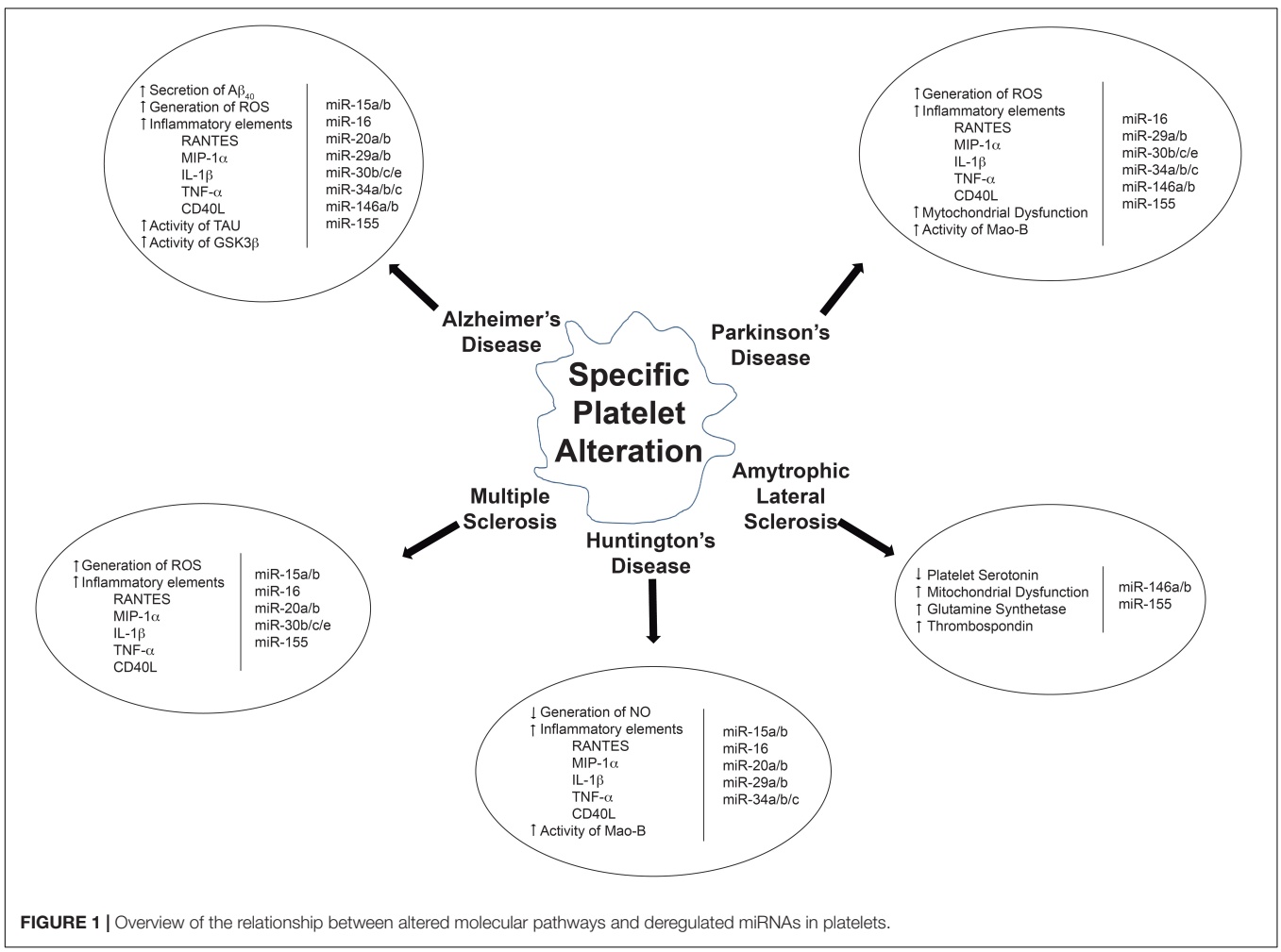

FIGURE 1 | Overview of the relationship between altered molecular pathways and deregulated miRNAs in platelets.

directly regulate the low MW neurofilament (*NFL*) mRNA, which may points toward the involvement of miR-146 in the repression of *NFL* observed in the spinal motor neurons of ALS patients (Campos-Melo et al., 2013).

CONCLUSION

From the elements of human blood, platelets are about one of the most important derivatives from megakaryocytes in the bone marrow. Platelets are crucial in the regulation of thrombosis and hemostasis as well as in vessel constriction, repair and clot retraction. In addition to their haemostatic function, platelets have an essential role during inflammatory processes and are an important source of proinflammatory molecules such as P-selectin, tissue factor, CD40L and metalloproteinase.

Overall, biochemical alterations in patients suffering from neurodegenerative disorders may not only occur in the brain, but also could affect blood vessels and blood cells. In this regard, vascular and metabolic disorders associated with aging are recognized risk factors for neurodegeneration.

Extracellular vesicles secreted by platelets may function as intercellular communicators, carrying pathologic neurological disease-related molecules, such as miRNAs, from the circulation into other organs and tissues such as the brain, reinforcing the existence of a molecular association between vascular and neurodegenerative disorders. It is worth noting that miR-34a/b/c, miR-146, and miR-155, three miRNAs with important roles in platelet production and function, have also been found to be repeatedly involved in the regulation of neurodegeneration-related processes and are being considered as potential geromiR biomarkers for numerous neurodegenerative disorders. According to this, platelets could represent a major source and vehicle of miRNAs, e.g., miR-223 that serve as secreted molecules acting on target cells other than the ones where they are produced.

Activated platelets are crucial in the development of major diseases like CNS diseases AD, PD, MS and others and also as potential biomarkers for neurological diseases, they are easy to obtain, manipulate and analyze (**Figure 1**). Platelets seem to be important executors of neuropathological diseases and therefore platelets might be regarded as novel therapeutic targets for neurodegeneration. Platelets could somehow reflect what is happening in the CNS along the course of neurodegenerative pathological states, and therefore could be promising biomarkers for early onset diagnosis of a pathological condition. Different from neurons, platelets are easy to work with and could be thus considered as a promising and effective tool on the study neurodegeneration. Therefore, platelets and their secreted molecules, including EVs and platelet miRNAs, remain as promising peripheral biomarkers in understanding the diagnosis and prognosis of neurodegenerative disorders.

AUTHOR CONTRIBUTIONS

YE-P, CG-B, EF, and IP wrote the original draft of the manuscript. YE-P and MA wrote, reviewed, and edited the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnagi.2019.00151/full#supplementary-material

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frontiers in Aging Neuroscience

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3D Reconstruction of the Neurovascular Unit Reveals Differential Loss of Cholinergic Innervation in the Cortex and Hippocampus of the Adult Mouse Brain

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Increasing evidence supports a role for cerebrovasculature dysfunction in the etiology of Alzheimer's disease (AD). Blood vessels in the brain are composed of a collection of cells and acellular material that comprise the neurovascular unit (NVU). The NVU in the hippocampus and cortex receives innervation from cholinergic neurons that originate in the basal forebrain. Death of these neurons and their nerve fibers is an early feature of AD. However, the effect of the loss of cholinergic innervation on the NVU is not well characterized. The purpose of this study was to evaluate the effect of the loss of cholinergic innervation of components of the NVU at capillaries, arteries and veins in the hippocampus and cortex. Adult male C57BL/6 mice received an intracerebroventricular injection of the immunotoxin p75NTR mu-saporin to induce the loss of cholinergic neurons. Quadruple labeling immunohistochemistry and 3D reconstruction were carried out to characterize specific points of contact between cholinergic fibers and collagen IV, smooth muscle cells and astrocyte endfeet. Innate differences were observed between vessels of the hippocampus and cortex of control mice, including a greater amount of cholinergic contact with perivascular astrocytes in hippocampal capillaries and a thicker basement membrane in hippocampal veins. Saporin treatment induced a loss of cholinergic innervation at the arterial basement membrane and smooth muscle cells of both the hippocampus and the cortex. In the cortex, there was an additional loss of innervation at the astrocytic endfeet. The current results suggest that cortical arteries are more strongly affected by cholinergic denervation than arteries in the hippocampus. This regional variation may have implications for the etiology of the vascular pathology that develops in AD.

Keywords: neurovascular unit, cholinergic, Alzheimer's disease, cerebral amyloid angiopathy, cortex, hippocampus

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INTRODUCTION

The loss of cholinergic neurons in the basal forebrain and the areas innervated by their fiber projections is a hallmark of Alzheimer's disease (AD; Whitehouse et al., 1982; Francis et al., 1999). Decreased cholinergic innervation of the hippocampus and cortex is associated with memory impairment (Damasio et al., 1985), decreased mini-mental state examination scores and behavioral changes (Perry, 1980; Tong and Hamel, 1999; Garcia-Alloza et al., 2005). Moreover, acetylcholinesterase inhibitors (AChEIs) are currently one of only two approved drugs for the treatment of AD (Hampel et al., 2018). Recent studies have demonstrated that loss of basal forebrain gray matter occurs before the onset of clinical symptoms (Schmitz and Nathan Spreng, 2016) and that administration of the AChEI Donepezil during the prodromal stage of AD prevented basal forebrain degeneration (Cavedo et al., 2017). This highlights the significance of cholinergic neurotransmission in AD.

Numerous experimental models have been used to mimic the loss of basal forebrain cholinergic neurons and their fiber projections. These include injection of ibotenic acid into the substantia innominata (Vaucher and Hamel, 1995), lesioning of the fimbria fornix (van der Staay et al., 1989) and electric pulse ablation of the medial septum (Scheiderer et al., 2006; Nelson et al., 2014). However, these models can result in widespread degeneration that may not specifically target cholinergic cell populations. The discovery that cholinergic neurons in the basal forebrain express the p75 neurotrophin receptor (NTR), while other populations of cholinergic neurons do not (Steininger et al., 1993), allowed for the development of targeted immunotoxins such as 192 IgG-saporin and its mouse analog, mu-p75-saporin. *In vivo* administration of mu-p75-saporin has been shown to selectively kill cholinergic neurons in the medial septum, horizontal and diagonal bands of Broca and nucleus basalis of Meynert and cause withdrawal of cholinergic projections in the cortex and hippocampus in mice (Berger-Sweeney et al., 2001; Hunter et al., 2004; Hamlin et al., 2013; Kerbler et al., 2013; Laursen et al., 2013; Ramos-Rodriguez et al., 2013). Although recent genetically-driven technologies such as optogenetics and designer receptor exclusively activated by designer drug (DREDD) have led to more targeted approaches to silence specific cholinergic populations (Hangya et al., 2015; Zhang et al., 2017), it is not clear if these techniques replicate the loss of cholinergic innervation that is seen in AD.

In addition to early loss of cholinergic neurons, increasing evidence suggests that alterations of the cerebrovasculature contribute to the etiology and/or progression of AD. In fact, vascular pathology has been suggested to be the earliest indicator of the development of AD (Jack et al., 2010; Iturria-Medina et al., 2016). The most common form of cerebrovascular pathology associated with AD is cerebral amyloid angiopathy (CAA). CAA is defined as the presence of β-amyloid (Aβ) deposits in the walls of cerebral blood vessels (Vinters, 1987) and is believed to develop due to an age-related failure of clearance of Aβ from the brain. CAA develops principally in cortical and leptomeningeal arteries, with additional capillary involvement in individuals carrying the apolipoprotein E4 (apoE4) allele (Thal et al., 2008). It is observed least frequently in veins. Topographically, CAA starts in blood vessels of neocortical areas (e.g., occipital and parietal lobe), while subcortical vessels (e.g., hippocampus, thalamus) are typically not affected until later stages of the disease (Thal et al., 2008; Vinters and Gilbert, 1983). The reasons underlying the development of CAA and its pattern of distribution are currently unknown.

Blood vessels in the brain are composed of endothelial cells, basement membrane proteins, pericytes, smooth muscle cells, astrocytes and neurons that are collectively referred to as the neurovascular unit (NVU; Iadecola, 2017). The NVU is also a target of cholinergic innervation, which can occur at multiple sites, including astrocytes, smooth muscle cells and endothelial cells (Vaucher and Hamel, 1995). This innervation is important for the maintenance of vascular tone and in mediating site-directed blood flow *via* neurovascular coupling (Hamel, 2006). Loss of cholinergic contact with blood vessels has been reported in the cortex of AD brains (Tong and Hamel, 1999) and in transgenic mouse models of AD (Kuznetsova and Schliebs, 2013; Michalski et al., 2017). However, most of these studies have been carried out using 2D images and have focused on specific brain regions and/or selected vessels. Thus, the impact of the loss of cholinergic innervation on the entire NVU is not well characterized.

In this study, the mu-p75-saporin saporin model was used to induce death of basal forebrain cholinergic neurons and their fiber projections. Quadruple labeling immunohistochemistry and 3D reconstruction were carried out to characterize specific points of contact between cholinergic fibers and various components of the NVU and to compare this pattern between the cortex and the hippocampus.

MATERIALS AND METHODS

Animals

Eight- to ten-week-old male C57BL/6 mice were obtained from The Open University (OU, Milton Keynes, UK) or the University of Southampton (Southampton, UK) and were kept on a 12 h light/dark cycle with access to food and water *ad libitum*. Experiments were carried out in compliance with guidelines of the Animal Welfare and Ethics Research Boards at the Open University and the University of Southampton and with approval from the Home Office (PPL 70/8507; PPL 30/3095).

Intracerebroventricular Injections

Mice were anesthetized under isoflurane gas and placed into a stereotaxic frame (Kopf instruments, CA, USA). Topical anesthetic (Cryogesic, Acorus Therapeutics Limited Chester, UK) was applied to the scalp before the head was shaved. A midline incision was made and the skull cleaned. A small burr hole was drilled over the left and right lateral ventricles and 0.5 μL of mu-saporin (0.596 μg/μL, Advanced Targeting Systems, San Diego, CA, USA; $n = 16$) or 0.9% saline ($n = 19$) was injected into each ventricle (coordinates from Bregma: AP = −0.4 mm, ML = 1.0 mm, DV = −2.3 mm) at a rate of 0.2 μl/min using a 32G Hamilton syringe. The needle was left *in situ* for 2 min after the injection to allow for

diffusion. Analgesia was administered intraperitoneally at the time of surgery (Carprieve, 5% w/v, 0.32 ml/kg, Norbrook, Northamptonshire, UK) and mice were able to self-administer sugar-free jelly (Hartley, Histon Sweet Spreads Limited, Leeds, UK) containing Carprofen (250 μg, Zoetis, London, UK) for 1 week post-surgery.

Tissue Processing

All mice were perfused intracardially with 0.01 M phosphate buffered saline (PBS, pH 7.4) 45 days after surgery. For Western blots, brains were immediately dissected and snap frozen on dry ice and stored at −80°C. For immunohistochemistry, mice were perfused with 4% paraformaldehyde, the brains were post-fixed overnight and left in 30% sucrose for 1 week. Brains were cryosectioned at 20 μm thickness and collected as free-floating coronal sections and stored in anti-freeze storage solution (30% glycerol, 30% ethylene glycol, 40% 0.01 M PBS) at −20°C.

Western Blotting

Tissues from control (n = 8) and saporin-treated mice (n = 6) were homogenized in Ripa lysis buffer [20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Igepal, 50 mM NaF, 1 mM $NaVO_3$] containing a protease inhibitor cocktail (Merck Millipore, Watford, UK), spun down (13,000 g, 10 mins, 4°C) and supernatants collected, aliquoted and frozen at −80°C until further use. Proteins (30 μg) were separated by gel electrophoresis on 4%–20% Tris-acetate gels (Fisher Scientific) and transferred onto a nitrocellulose membrane. Membranes were incubated overnight at 4°C with anti-choline acetyltransferase (ChAT, 1:500, Merck Millipore), stripped and re-probed with anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:50,000, Sigma-Aldrich, Dorset, UK) antibody to ensure equal protein loading. Two blots were replicated for each brain region. Immunoblots were quantified by densitometry using ImageJ software (NIH, MD, USA) and calculated as an optical density ratio of protein levels normalized to GAPDH levels.

Immunohistochemistry

For single-labeling immunohistochemistry of cholinergic cell bodies and fibers, tissue sections were washed in 0.01 M PBS, blocked with 15% normal donkey serum (NDS; Sigma-Aldrich) and incubated overnight with either anti-ChAT (1:100) or anti-laminin (1:350, Sigma-Aldrich), after pre-treatment with pepsin (1 mg/mL in 0.2 N HCl, 30 s at 37°C). The next day, sections were washed in PBS and incubated for 2 h at room temperature with anti-donkey AlexaFluor 488 (Fisher Scientific, Loughborough, UK). For quadruple labeling of the NVU, sections were treated with pepsin, incubated overnight with anti-ChAT (1:100), washed in PBS and incubated with anti-goat AlexaFluor 555. After washing in PBS, sections were incubated simultaneously with anti-collagen IV (1:100, Abcam, Cambridge, UK), anti-α smooth muscle actin (α-SMA)-FITC (1:350, Sigma-Aldrich) and anti-glial fibrillary protein (GFAP, 1:500, Abcam). Sections were then developed with anti-rabbit AlexaFluor 405 and anti-chicken AlexaFluor 633 (1:200, Fisher Scientific). All fluorescent sections were coverslipped using Mowiol® (Sigma-Aldrich) containing 0.1% v/v Citifluor (Citifluor Limited, London, UK) mounting media.

Image Acquisition and Analysis

Coronal brain sections were imaged with an SP5 Leica scanning laser confocal microscope. Low magnification images of the cortex and hippocampus were stitched together using ImageJ software (NIH, MD, USA). The density of neuronal cell bodies, fibers and blood vessels in each region of interest was quantified by calculating the percentage area covered by staining using ImageJ software. NVUs in the hippocampus and cortex were imaged using the ×100 oil immersion objective, using z stacks with ≤2 μm spacing between slices. Images were deconvolved and converted into Imaris-compatible files using AutoQuant X3 version X3.0.4 software (MediaCybernetics Inc., Rockville, MD, USA).

3D Reconstruction of the NVU

To quantify the parameters of each component of the NVU, deconvolved images were processed using Imaris software (Bitplane®) and surfaces were created for each component of the NVU. For each vessel, the following measurements were acquired: the total area of a selected surface (μm^2), the volume of a selected surface (μm^3), the length of the vessel imaged (μm) and the average diameter of the vessel (μm). The total area of contact between two selected surfaces (e.g., ChAT nerve fibers contacting collagen IV) was calculated using the Imaris Xtension "Surface to Surface Contact Area" (Imaris V8.31, ImarisXT Bitplane Inc created by Matthew J Gastinger, Bitplane). Only surfaces that made direct contact with each other (i.e., 0 μm apart) were quantified. Vessels were classified as capillaries if they were ≤10 μm in diameter, arteries were identified as having a diameter of >10 μm and positive for SMA, while veins were identified as having a diameter >10 μm but lacking SMA. A total of five capillaries, five arteries and three veins were quantified for each mouse (n = 7–11 control, n = 6–10 saporin) per brain region and the average values per mouse were used for statistical analysis.

Statistical Analysis

Data were tested for normality using the Kolmogorov-Smirnov test. For normally distributed data, comparisons between two groups were carried out using two-tailed Student's t-test. Where there were more than two groups, one-way or two-way repeated measures ANOVA was used followed by Sidak's *post hoc*. The ROUT test was used to identify and exclude any outliers. For data that were not normally distributed, the Mann-Whitney U test or Kruskal-Wallis test with Dunn's *post hoc* test was used. Data represents mean ± SEM and $p < 0.05$ was considered to be statistically significant. Analysis was carried out using GraphPad Prism software.

RESULTS

Cholinergic Loss in the Medial Septum, Hippocampus and Cortex Following Administration of Mu-Saporin

As shown in **Figure 1**, ChAT-positive cholinergic neurons were observed in the medial septum, diagonal band of Broca,

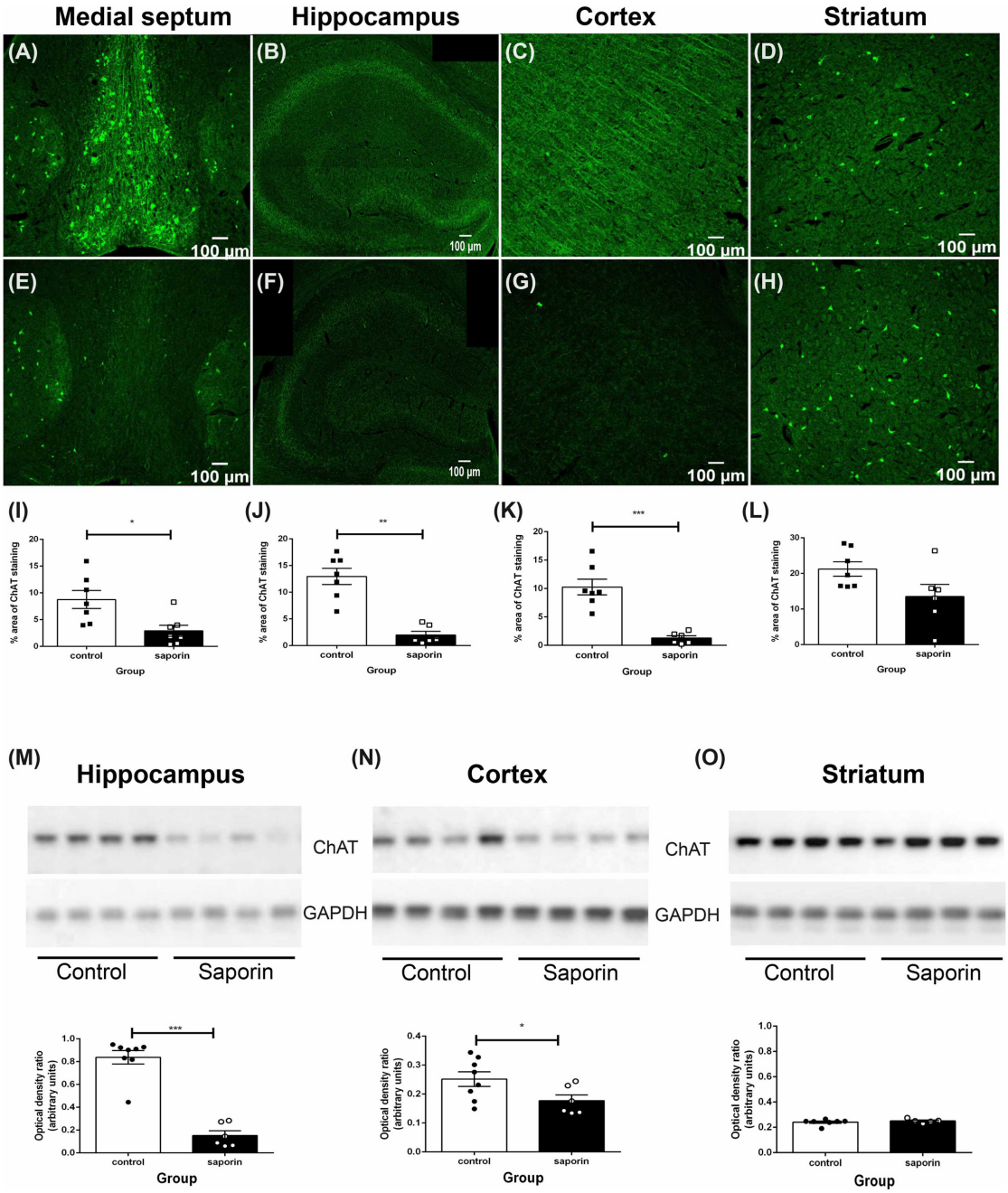

FIGURE 1 | Loss of cholinergic neurons and fiber projections following administration of mu-p75-saporin. **(A–H)** Photomicrographs of choline acetyltransferase (ChAT)-positive neurons and fibers in the medial septum **(A,E)**, hippocampus **(B,F)**, cortex **(C,G)** and striatum **(E,H)** in control **(A–D)** and saporin-treated **(E–H)** mice. **(I–L)** Quantification of the percent area covered by ChAT-positive staining in control and saporin-treated mice in the medial septum **(I)**, hippocampus **(J)** and cortex **(K)**. p75-negative cholinergic neurons in the striatum were not affected by saporin treatment **(L)**. **(M–O)** Western blotting confirmed a significant loss of ChAT protein expression in the hippocampus **(M)** and cortex **(N)** after saporin administration, while ChAT levels in the striatum did not differ between control and treated mice **(O)**. Data represent mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, two-tailed Student's *t*-test. Scale bar = 100 µm.

nucleus basalis of Meynert and in the striatum of control mice. Cholinergic fiber projections were also observed in the hippocampus (**Figure 1B**) and cortex (**Figure 1C**). Significantly less ChAT staining was detected in the medial septum of saporin-treated mice at 45 days post-surgery (**Figures 1E,I**). This was accompanied by a significant decrease in cholinergic nerve fiber density in the hippocampus (**Figures 1F,J**) and the cortex (**Figures 1G,K**). As expected, p75 NTR-negative neurons in the striatum were not affected by saporin treatment (**Figures 1H,L**). Western blotting confirmed a significant decrease in ChAT

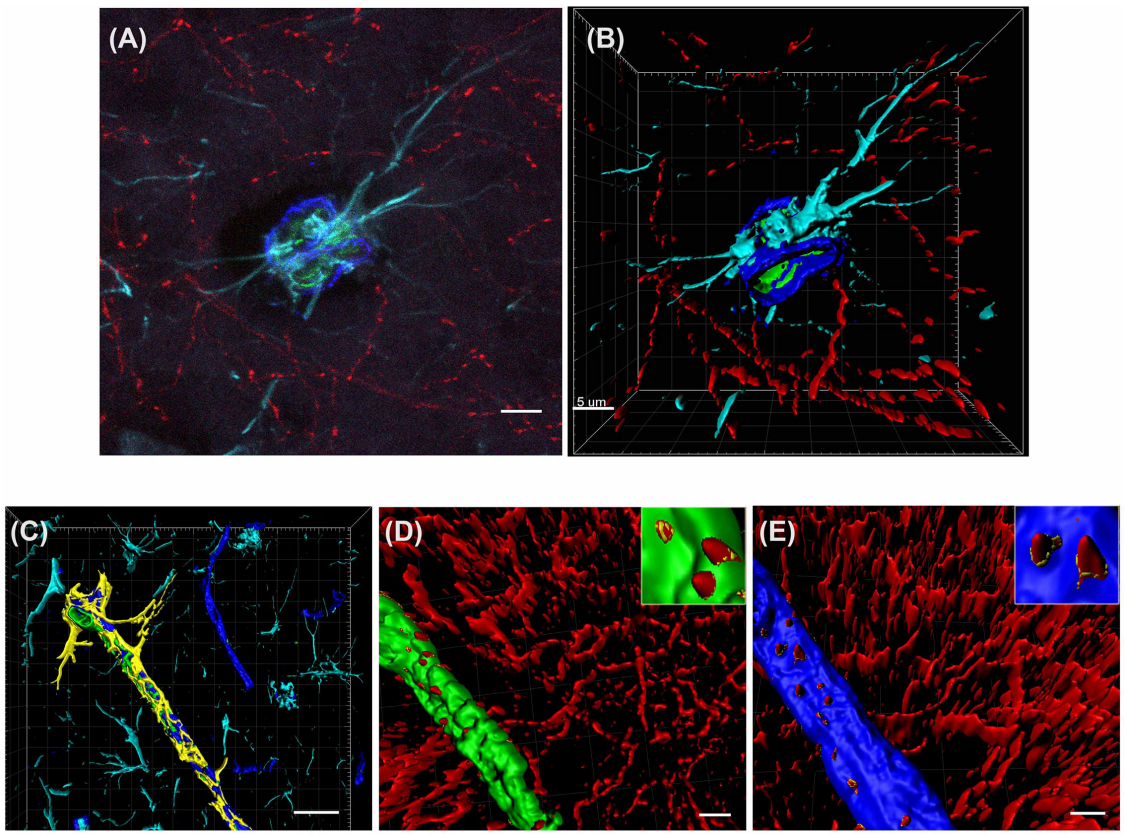

FIGURE 2 | 3D reconstruction of the neurovascular unit (NVU). **(A,B)** Photomicrograph **(A)** and 3D reconstruction **(B)** of an artery stained for collagen IV (dark blue), smooth muscle actin (SMA; green), astrocytes (turquoise) and cholinergic nerve fibers (red). **(C–E)** Yellow outlines indicate the surfaces created for each of the NVU components. Examples are shown for surface area contact between perivascular astrocytes and collagen IV **(C)**, contact of cholinergic nerve fibers to smooth muscle cells (**D**, yellow outlines in inset) and contact of cholinergic nerve fibers to collagen IV (**E**, yellow outlines in inset). Scale bar **(A,B,D,E)** = 5 μm, **(C)** = 20 μm.

protein levels in the hippocampus (**Figure 1M**) and cortex (**Figure 1N**) following saporin administration and no difference in ChAT expression between control and saporin-treated mice in the striatum (**Figure 1O**).

Characterization of Cholinergic Loss at the NVU in the Hippocampus and Cortex

Cholinergic nerve fibers are known to innervate blood vessels in the hippocampus and cortex (Vaucher and Hamel, 1995). To characterize the precise effects of saporin treatment at the NVU, quadruple-labeling immunohistochemistry was used to label cholinergic fibers and three components of the NVU—collagen IV-positive basement membranes, smooth muscle cells and astrocytes (**Figure 2A**). Confocal images of the vessels were then reconstructed using 3D modeling software and surfaces were created for each of the four proteins (**Figures 2B–E**). The surface area of ChAT contact with each component of the NVU (standardized to vessel length) was analyzed across capillaries, arteries/arterioles and veins/venules from control and saporin-treated mice.

At the basement membrane of vessels in the hippocampus, saporin treatment induced a decrease in the amount of contact between cholinergic nerve fibers and collagen IV at capillaries, arteries and veins (**Figures 3A–F**), although this decrease was only statistically significant at arteries (**Figure 3G**). In the cortex, cholinergic innervation of the basement membrane did not differ between control and saporin-treated mice at capillaries and veins but was significantly decreased at the arteries of saporin-treated mice (**Figures 3H–N**). No differences were noted between control and saporin-treated mice at any vessel type in the striatum (**Supplementary Figure S1A**).

Analysis of perivascular innervation at the smooth muscles of arteries found that there was a significant decrease in the surface area contact between ChAT and α-SMA in saporin-treated in both the hippocampus (**Figures 4A–C**) and the cortex (**Figures 4D–F**), while the striatum was not affected (**Supplementary Figure S1B**).

Quantification of ChAT contact with perivascular astrocytes in the hippocampus revealed no difference in the amount of contact with GFAP-positive astrocytes between control and saporin-treated mice in any vessel type (**Figures 5A–G**). By contrast, significantly less ChAT contact was observed at arteries in the cortex of saporin-treated mice compared to control animals (**Figures 5H–N**). No differences were

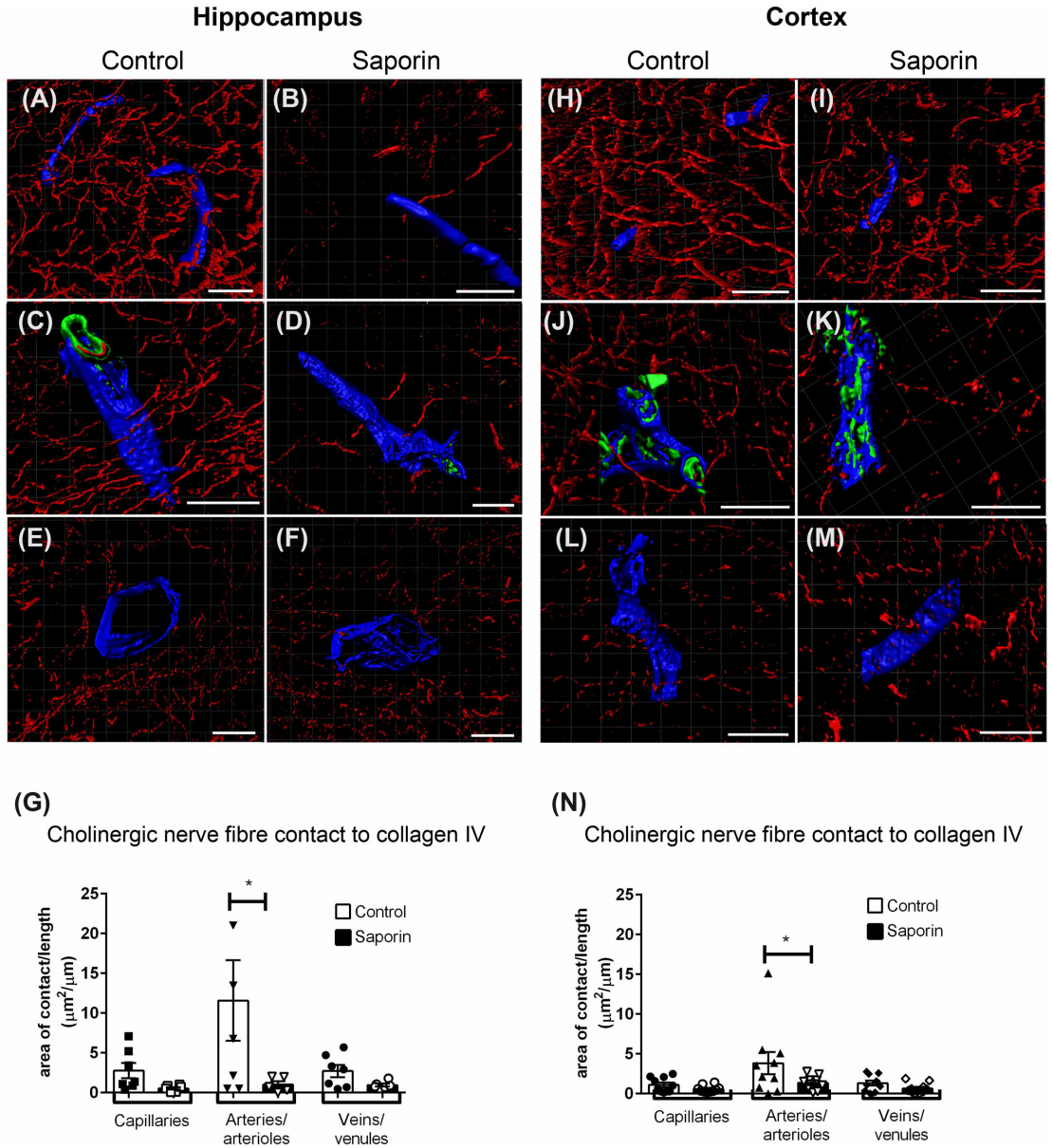

FIGURE 3 | Perivascular cholinergic contact at the basement membrane. **(A–M)** Representative images of the 3D reconstruction of the NVU in capillaries **(A,B,H,I)**, arteries **(C,D,J,K)** and veins **(E,F,L,M)** in the hippocampus **(A–F)** and cortex **(H–M)** of control **(A,C,E,H,J,L)** and saporin-treated mice **(B,D,F,I,K,M)**. ChAT-positive fibers are shown in red, collagen IV is shown in blue and SMA is shown in green. **(G,N)** Quantification of the surface area of contact between ChAT-positive fibers and collagen IV demonstrated a significant decrease in contact at the arteries of saporin-treated animals in both the hippocampus **(G)** and cortex **(N)**. Data represent mean ± SEM. $^*p < 0.05$, two-way ANOVA with Sidak's *post hoc* test. Scale bars = 20 μm.

observed between control and saporin mice at cortical capillaries or veins (**Figures 5H–N**) or between control and saporin-treated mice at any vessel type in the striatum (**Supplementary Figure S1C**).

To determine if saporin induced changes in blood vessel density, the percent area covered by laminin-positive capillaries and large-diameter vessels were quantified in the cortex and hippocampus of control and saporin-treated mice (**Figures 6A–E**). In control mice, the density of both capillaries and arteries/veins was significantly higher in the cortex compared to the hippocampus (**Figures 6A,B**). No differences in vessel density were noted between control and saporin-treated mice in either brain region (**Figure 6E**).

Regional Variation in Perivascular Innervation

To determine if there were regional differences in endogeneous and saporin-induced cholinergic innervation, measurements of

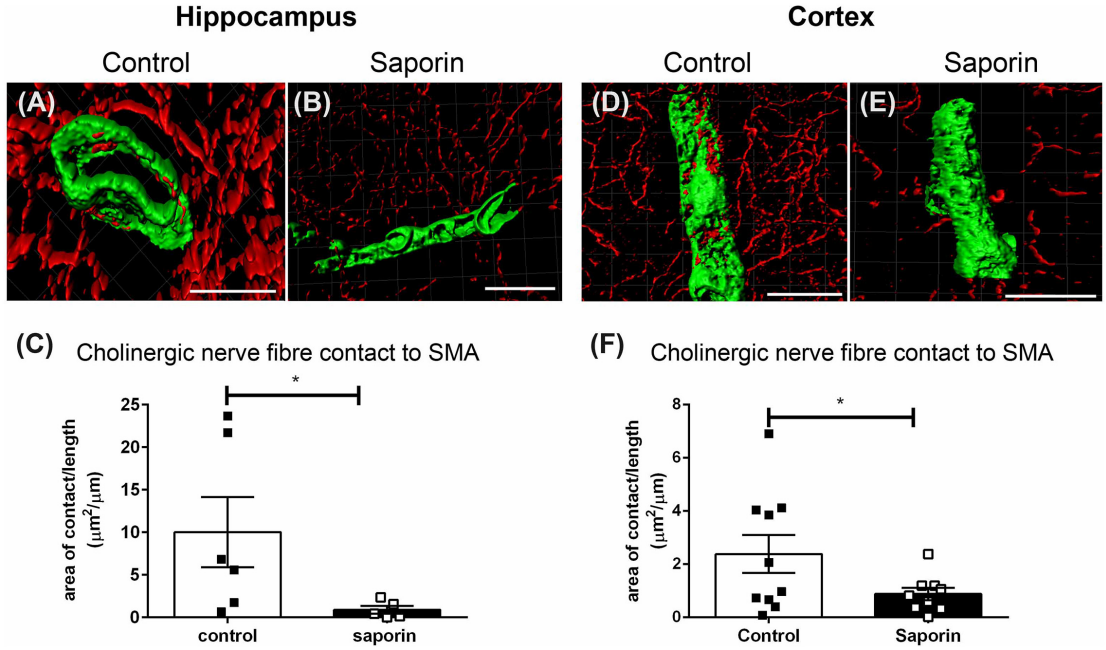

FIGURE 4 | Perivascular cholinergic contact at smooth muscle cells. **(A–E)** Representative images of the 3D reconstruction of the NVU in arteries in the hippocampus **(A,B)** and cortex **(D,E)** from control **(A,D)** and saporin-treated **(B,E)** mice. ChAT-positive nerve fibers are shown in red and smooth muscle cells are shown in green. **(C,F)** Quantification of the surface area of contact between ChAT-positive fibers and SMA demonstrated a significant decrease in contact in saporin-treated animals in both the hippocampus **(C)** and cortex **(F)**. Data represent mean ± SEM. $^*p < 0.05$, Mann-Whitney *U* test (hippocampus) and one-tailed Student's *t*-test (cortex). Scale bars = 20 μm.

ChAT contact with components of the NVU were compared between the hippocampus and cortex. Quantification of overall cholinergic nerve fiber density was found to be significantly higher in the hippocampus compared to the cortex in control mice (**Figure 7A**). Following saporin treatment, this difference was lost (**Figure 7B**). The degree of cholinergic innervation at the basement membrane did not differ between the hippocampus and cortex at capillaries, arteries or veins of control (**Figure 7C**) or saporin-treated mice (**Figure 7D**). ChAT innervation of smooth muscle cells also did not differ between arteries in the hippocampus and cortex in either control (**Figure 7E**) or saporin-treated mice (**Figure 7F**). However, perivascular astrocyte endfeet surrounding cortical capillaries in control mice received significantly less cholinergic input compared to astrocytes at the capillaries in the hippocampus (**Figure 7G**). Significantly less cholinergic input onto astrocyte endfeet was also noted at veins in the cortex of saporin-treated mice compared to hippocampal veins (**Figure 7H**).

Impact of Loss of Perivascular Innervation on Components of the NVU

To determine if loss of cholinergic innervation induced changes in components of the NVU, the volume of collagen IV and smooth muscle cells as well as the area of astrocyte endfoot coverage was evaluated in vessels of control and saporin-treated mice. Comparisons between the cortex and hippocampus were also carried out.

As shown in **Table 1**, within the hippocampus, the volume of collagen IV was significantly higher in veins compared to capillaries in control mice. This relationship was maintained following saporin treatment. However, collagen IV volumes did not differ between control and saporin-treated mice in any vessel type. In the cortex, the volume of collagen IV was highest in arteries compared to capillaries and veins in both control and saporin-treated mice (**Table 1**). Regional comparisons including the striatum revealed that the volume of collagen IV was significantly higher in veins in the hippocampus than veins in the cortex and striatum in both control mice and those treated with saporin.

Analysis of smooth muscle volume found no significant difference between control and saporin mice in any brain region (**Table 2**). Similarly, no significant differences in smooth muscle volume were noted between the cortex, hippocampus or striatum in either treatment group (**Table 2**).

Finally, analysis of perivascular astrocyte coverage of hippocampal vessels revealed a significantly higher amount of endfoot contact in veins compared to capillaries in control animals (**Table 3**). Following saporin administration, astrocyte coverage remained significantly higher in hippocampal veins compared to both capillaries. No differences in perivascular astrocyte coverage were noted between vessel types in the cortex in either control or saporin-treated mice (**Table 3**). Comparison between vessels of the hippocampus, cortex and striatum found that astrocyte contact was lowest in all the

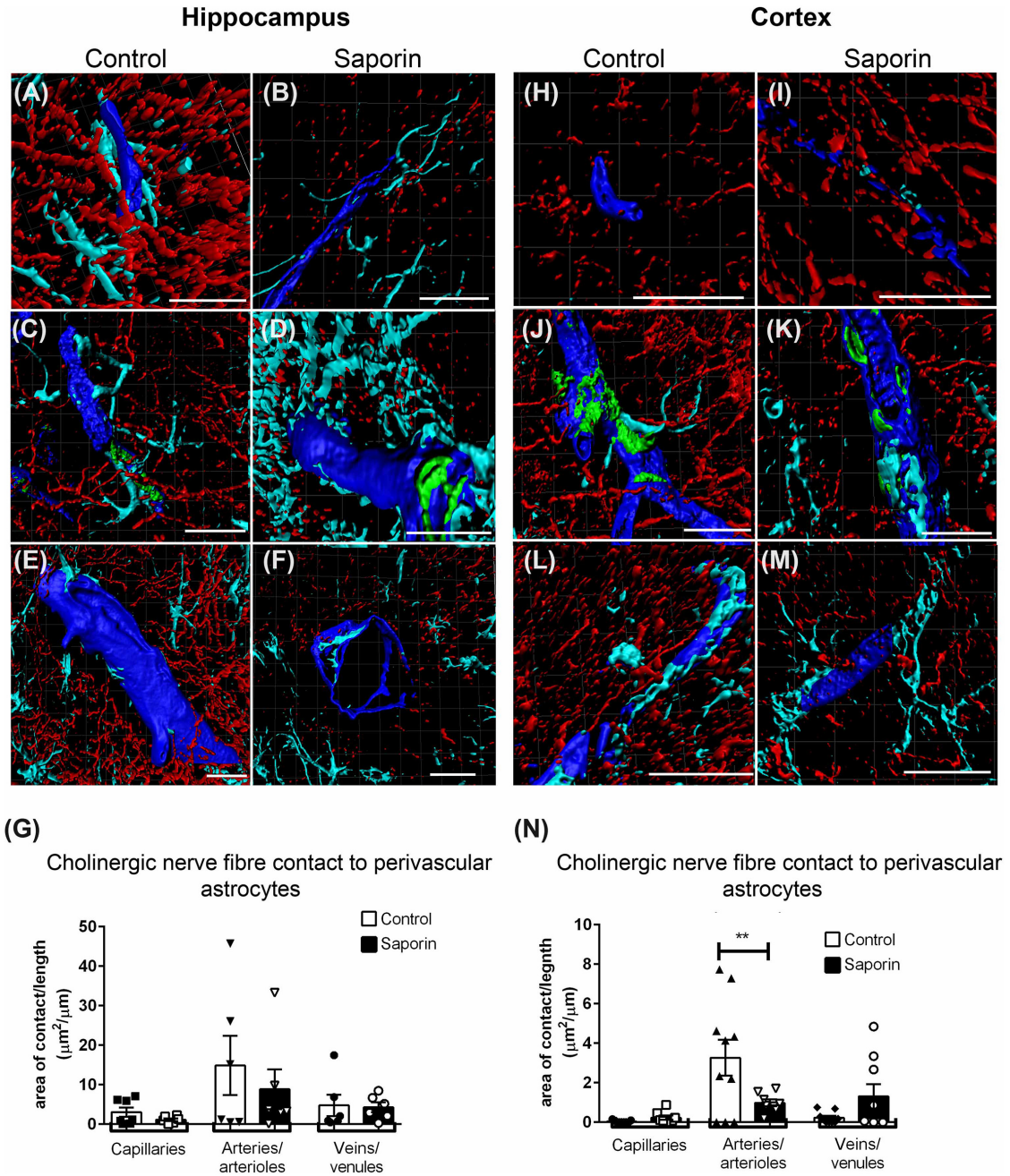

FIGURE 5 | Perivascular cholinergic contact at perivascular astrocytes. **(A–M)** Representative images of the 3D reconstruction of the NVU in capillaries **(A,B,H,I)**, arteries **(C,D,J,K)** and veins **(E,F,L,M)** in the hippocampus **(A–F)** and cortex **(H–M)** of control **(A,C,E,H,J,L)** and saporin-treated mice **(B,D,F,I,K,M)**. ChAT-positive fibers are shown in red, collagen IV is shown in blue, SMA is shown in green and glial fibrillary protein (GFAP) is shown in turquoise. **(G,N)** Quantification of the surface area of contact between ChAT-positive fibers and astrocyte endfeet found no differences between control and saporin-treated animals in any vessels of the hippocampus **(G)**, but a significant decrease in contact at the arteries of saporin-treated animals the cortex **(N)**. Data represent mean ± SEM. $^{**}p < 0.01$, two-way ANOVA with Sidak's *post hoc* test. Scale bars = 20 μm.

vessel types in the striatum. In addition, astrocyte coverage of veins in the hippocampus was approximately 10-fold higher compared to coverage of veins in the cortex and 100-fold higher than veins in the striatum in both control and saporin mice (**Table 3**).

DISCUSSION

Although the loss of cholinergic neurons in AD has been described extensively, the effect of this loss on the NVU is not well characterized. Moreover, previous studies that have

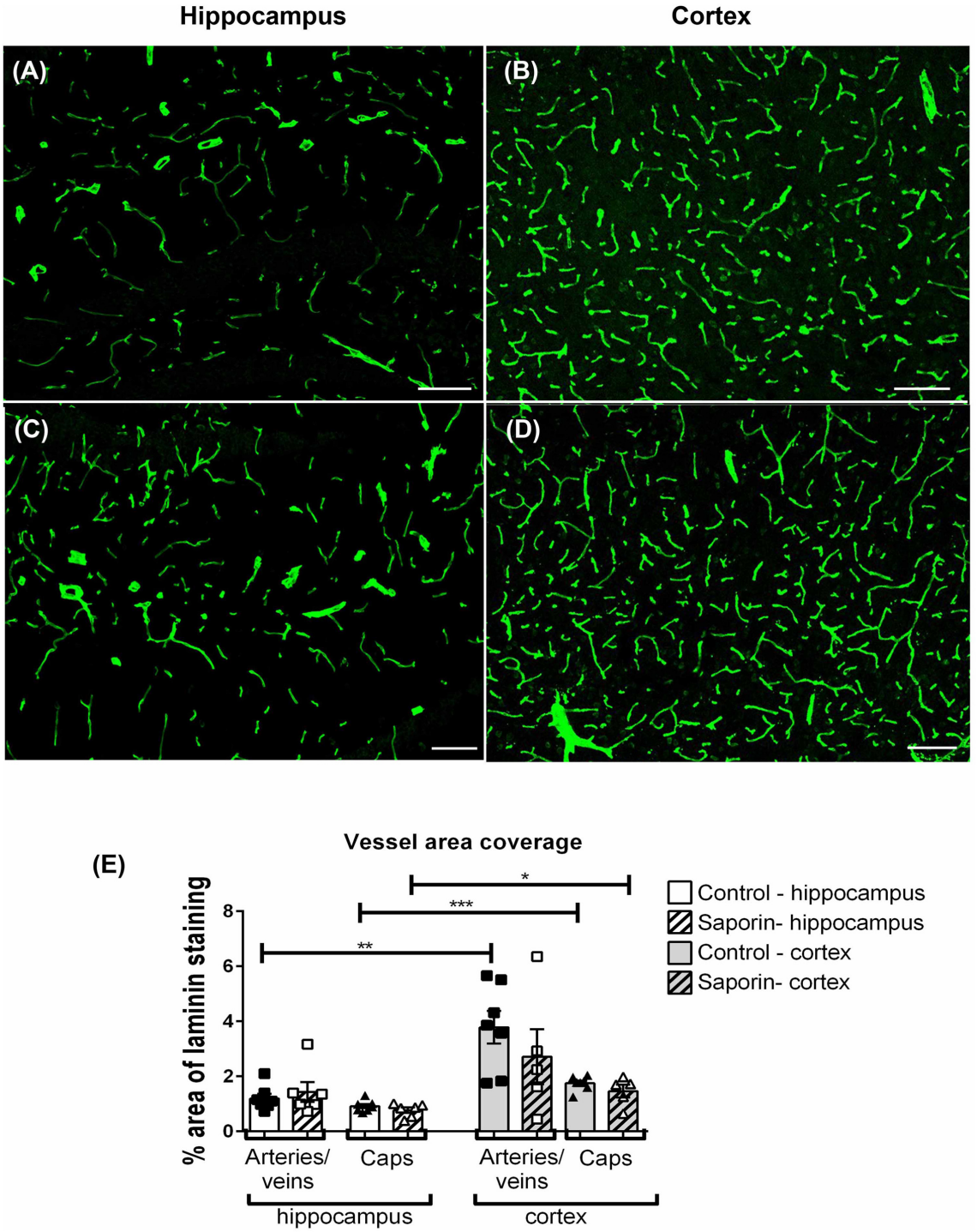

FIGURE 6 | Regional comparison of blood vessel density. **(A–D)** Photomicrographs of laminin-positive blood vessels in the hippocampus **(A,C)** and cortex **(B,D)** of control **(A,B)** and saporin-treated mice **(C,D)**. **(E)** Quantification of the density of capillaries (caps) and large-diameter vessels in the hippocampus and cortex in treatment groups. Data represent mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, two-tailed Student's *t*-test. Scale bar = 100 μm.

looked at perivascular innervation by cholinergic nerve fibers have largely been carried out using 2D images obtained from double or triple labeling immunohistochemistry or by immuno-EM (Itakura et al., 1977; Tong and Hamel, 1999; Kuznetsova and Schliebs, 2013). By combining quadruple labeling immunohistochemistry with 3D reconstruction, the

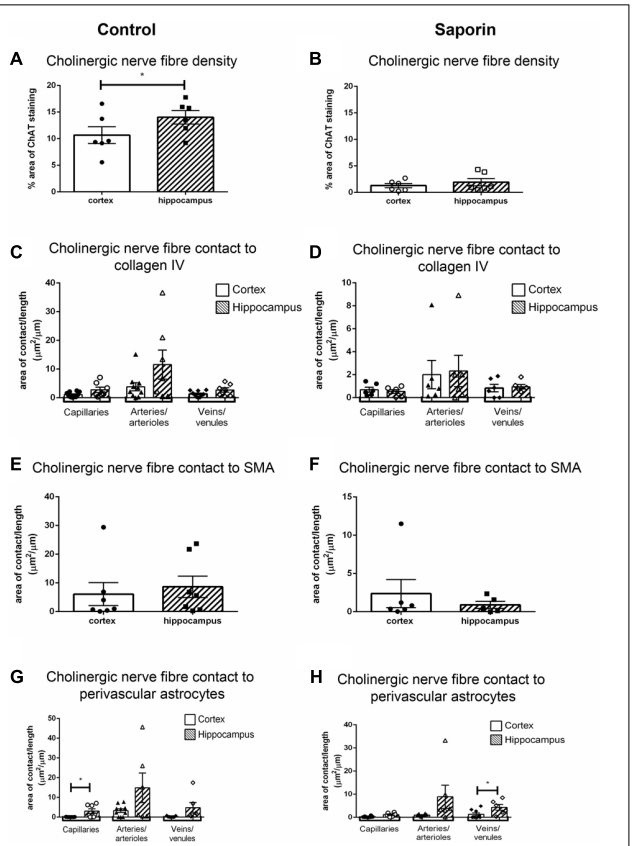

FIGURE 7 | Regional comparison of measures of the NVU. **(A,B)** Total density of ChAT-positive fibers in the cortex and hippocampus in control mice **(A)** and following administration of saporin **(B)**. **(C–H)** Quantification of area of contact between ChAT-positive fibers and collagen IV **(C,D)**, SMA **(E,F)** and astrocyte endfeet **(G,H)** in the cortex and hippocampus in control **(C,E,G)** and saporin-treated mice **(D,F,H)**. Data represent mean ± SEM. $*p < 0.05$, paired two-tailed Student's *t*-test **(A,B)**, Kruskal-Wallis with Dunn's *post hoc* test, **(C,D)**, Wilcoxon matched paired test **(E–H)**.

current study allowed not only for individual components of the NVU to be assessed under basal and pathological conditions but also for perivascular innervation to be quantified along multiple components of the NVU around the entire surface of the blood vessel.

We chose to use the saporin model of cholinergic denervation because of its well characterized ability to cause selective death of basal forebrain cholinergic neurons across many species, including mice, rats and non-human primates (Fine et al., 1997; Leanza, 1998; Lin et al., 1999; Berger-Sweeney et al., 2001; Lehmann et al., 2002; Birthelmer et al., 2003; Hawkes et al., 2005; Scheiderer et al., 2006; Ramos-Rodriguez et al., 2013). Such immunotoxin models are preferable to older lesioning models that can result in widespread, non-specific neuronal damage (van der Staay et al., 1989; Scheiderer et al., 2006; Nelson et al., 2014). On the other hand, the relatively rapid time course of death induced by saporin is unlikely to mimic the progressive loss of cholinergic neurons that is seen in AD. More refined methods have been developed to silence basal forebrain cholinergic neurons using optogenetics and DREDD

TABLE 1 | Regional comparison of volume of basement membrane across vessel type.

Basement membrane Mean volume/length (μm³/μm) ± SEM	Hippocampus			Cortex			Striatum		
	Capillaries	Arteries	Veins	Capillaries	Arteries	Veins	Capillaries	Arteries	Veins
Control	32.63 ± 7.9*	87.49 ± 22.6	148.9 ± 40.6*▲	18.82 ± 3.4▽	114.5 ± 40.3▽	42.68 ± 7.7▲	26.82 ± 3.6▽	106.7 ± 29.2▽	51.05 ± 6.7▲
Saporin	32.71 ± 5.4*	114.7 ± 22.2	153.8 ± 30.3*▲	21.26 ± 2.4	84.56 ± 11.5∞	37.38 ± 7.2▲∞	21.95 ± 1.6▽	73.46 ± 18.3▽	35.45 ± 2.8▲

*Data represents mean ± SEM. *$p < 0.05$ capillaries vs. veins; ▽$p < 0.05$ capillaries vs. arteries; ∞$p < 0.05$ arteries vs. veins (one-way ANOVA with Sidak's post hoc) ▲$p < 0.01$; hippocampus vs. cortex or striatum (two-way repeated measures ANOVA with Sidak's post hoc analysis).*

technologies (Shi et al., 2015; Chen et al., 2016). However, whether such techniques fully replicate the loss of cholinergic signaling, including withdrawal of trophic support and related inflammatory processes, that is observed in AD is not yet known. In the present study, administration of saporin led to a significant and specific loss of cholinergic neurons in the basal forebrain at 45 days post-surgery. The fiber projections from these neurons were also lost in the hippocampus and cortex. Other major populations of cholinergic neurons that do not express the p75NTR, including those in the striatum (Yeo et al., 1997) were not affected by saporin treatment.

Using 3D reconstruction of blood vessels, ChAT-positive fibers were found to innervate capillaries, arteries and veins in the hippocampus and cortex. As expected (Toribatake et al., 1997; Mulligan and MacVicar, 2004; Hamel, 2006; Hamilton et al., 2010; Chen et al., 2014), cholinergic innervation was observed at all levels of the NVU investigated, including the basement membranes, smooth muscle cells and perivascular astrocytes. The majority of the innervation was observed at arteries, in agreement with previous studies (Chédotal et al., 1994; Vaucher and Hamel, 1995; Luiten et al., 1996; Kuznetsova and Schliebs, 2013). Predominant targeting of arteries by cholinergic nerve fibers is perhaps unsurprising given the role of ACh in mediating neurovascular coupling (Hamel, 2006; Willis et al., 2006; Lecrux et al., 2017).

Increasing evidence suggests that there is heterogeneity of cells of the NVU, including pericytes and astrocytes, across both vessel type and brain regions (Shepro and Morel, 1993; Noumbissi et al., 2018). In the present study, we observed innate differences between vessels of the hippocampus and cortex. These included: (i) a significantly higher vessel density in the cortex; (ii) a higher overall ChAT fiber density in the hippocampus and more ChAT contact with perivascular astrocytes in hippocampal capillaries; (iii) thicker basement membrane in the veins of the hippocampus; and (iv) greater coverage of the basement membrane by astrocyte endfeet in hippocampal veins compared to veins in the cortex.

The observation that total cholinergic fiber density was higher in the hippocampus compared to the cortex is in agreement with previous reports (Kitt et al., 1994). However, given that vesssel density showed the opposite pattern, it is perhaps surprising that there was no difference in the amount of cholinergic innervation at blood vessels in the hippocampus vs. those in the cortex. It is possible that by only quantifying direct contact (e.g., 0 μm distance) between ChAT fibers and basement membrane or smooth muscle cells, we have underestimated the potential degree of cholinergic innervation at the NVU, which has been classified in previous studies to be within 3 μm from the basement membrane (Vaucher

TABLE 2 | Regional comparison of volume of smooth muscle cells in arteries.

SMA Volume/length ($\mu m^3/\mu m$) ± SEM	Hippocampus	Cortex	Striatum
Control	60.02 ± 10.6	50.75 ± 16.2	65.88 ± 19.7
Saporin	33.10 ± 7.4	39.54 ± 9.0	33.03 ± 5.7

Values represent mean ± SEM. No significant differences were noted (two-way repeated measures ANOVA with Sidak's post hoc test).

TABLE 3 | Area of astrocyte coverage of basement membrane.

Astrocyte endfoot coverage of basement membrane. Area of contact/length ($\mu m^2/\mu m$) ± SEM	Hippocampus			Cortex			Striatum		
	Capillaries	Arteries	Veins	Capillaries	Arteries	Veins	Capillaries	Arteries	Veins
Control	6.7 ± 2.1*	38.43 ± 8.8▲	81.84 ± 21.4*▲	2.23 ± 1.5	23.72 ± 13.8	10.24 ± 6.1▲	0.32 ± 0.2	0.357 ± 0.08▲	0.457 ± 0.2▲
Saporin	15.26 ± 3.5*	52.54 ± 12▲	84.35 ± 14.4*▲	4.22 ± 1.8	21.05 ± 6.6	8.57 ± 5.3▲	0.317 ± 0.2	0.105 ± 0.03▲	0.121 ± 0.06▲

*Values represent mean ± SEM. *$p < 0.05$ capillaries vs. veins (two-way repeated measures ANOVA with Sidak's post hoc) ▲$p < 0.05$; hippocampus vs. cortex or striatum (two-way repeated measures ANOVA with Sidak's post hoc).*

and Hamel, 1995). Our findings that cholinergic contact with perivascular astrocyte endfeet tended to be higher in the hippocampus across all vessel types and was significantly higher at hippocampal capillaries compared to cortical capillaries, suggest that there may be functional differences between cortical and hippocampal vessels in their responsiveness to cholinergic signaling.

Astrocyte coverage of vessels was also observed to be higher in the hippocampus than the cortex, although this was only significant at veins. This is in keeping with the reported distribution of parenchymal GFAP-positive astrocytes (Emsley and Macklis, 2006). This finding was likely related to the observed greater thickness of collagen IV, given that astrocytes and endothelial cells are the main sites of basement membrane production (Baeten and Akassoglou, 2011). Expression of collagen IV has been shown to be significantly upregulated in capillaries and arteries during normal and in AD (Kalaria and Pax, 1995; Farkas and Luiten, 2001; Christov et al., 2008; Magaki et al., 2018). Thickening of the basement membrane and alterations in basement membrane composition has been hypothesized to precede the development of CAA (Wyss-Coray et al., 2000). However, veins are the vessel type least likely to be affected by CAA and CAA develops more slowly in vessels in the hippocampus than those in the cortex (Thal et al., 2008). It may be that increased basement thickness makes the veins in the hippocampus less likely to be deformed by pressure changes and thus helps to ensure a consistent cerebral perfusion (Zócalo et al., 2013; Thorin-Trescases et al., 2018) and to maintain a driving force for clearance of solutes in the cerebral spinal fluid (CSF) and/or interstitial fluid (ISF). In addition, the walls of veins are important for the egress of leukocytes from the blood into the brain in neurodegenerative diseases and this process requires that leukocytes enter a perivenular space bounded by endothelial and glia limitans basement membranes (Owens et al., 2008; Engelhardt et al., 2016). The variation in the degree of collagen IV and astrocyte coverage may reflect regional variability in the neuroinflammatory properties of the veins in the hippocampus compared to the cortex.

Saporin treatment significantly reduced the amount of cholinergic contact with the basement membrane of arteries in both the cortex and hippocampus, while capillaries and veins were unaffected. This may reflect the proportional endogeneous degree of cholinergic innervation between vessel types, which was highest in arteries. Regional differences were also observed in the degree of cholinergic loss at the NVU. While saporin treatment induced a loss of cholinergic innervation at the basement membrane and smooth muscle cells of arteries in both the hippocampus and cortex, there was additional loss of cholinergic contact of astrocyte endfeet in cortical arteries. The reason for this variability is unknown. It may be that cholinergic supply of the cortical astrocytes is important for their function in the convective influx/glymphatic entry of CSF along the pial glial basement membranes (Albargothy et al., 2018). Recent 3D mapping studies have shown that the dendritic arbors of basal forebrain neurons that project to the cortex differ from those that project to the hippocampus in that single cortical dendrites innervate large areas of the neuropil (Wu et al., 2014; Li et al., 2018). It is possible that a similar pattern of innervation exists at blood vessels in the cortex such that the loss of one dendritic arbor affects multiple vessels. This may also be related to the lower endogeneous level of contact between cholinergic nerves and astrocytes in the cortex, which may make cortical vessels more susceptible than those in the hippocampus to loss of cholinergic innervation.

Each of the NVU components studied have been shown to play a role in mediating the clearance of Aβ from the brain. Cerebrovascular basement membranes act as conduits along which Aβ contained within CSF and ISF is removed from the brain (Iliff et al., 2012; Hawkes et al., 2013; Morris et al., 2014; Albargothy et al., 2018). Smooth muscle cells express low-density receptor related protein-1 (LRP-1) which mediates cellular uptake of Aβ and its transcytosis across the blood brain barrier (BBB; Kanekiyo et al., 2012). Moreover, localized contraction of smooth muscles has recently been proposed to generate the force that drives intramural periarterial drainage of Aβ (Aldea et al., 2019). Astrocytes contribute to the formation of the basement membrane and have also been shown to take up Aβ *via* LRP-1 (Basak et al., 2012). In addition, astrocytes are the main producers of apolipoprotein E, which chaperones Aβ across the BBB (Bell et al., 2007). Therefore, it is possible that the combined loss of cholinergic innervation at each of these components contributes to the increased susceptibility of cortical vessels to the development of CAA. Further studies are needed to investigate this putative relationship in human brain tissues.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Animal Welfare and Ethics Research Boards at the Open University and the University of Southampton. The protocol was approved by the Home Office (PPL 70/8507; PPL 30/3095).

AUTHOR CONTRIBUTIONS

SN performed the experiments and data analysis. RC, IR and CH planned the experimental design. SN and CH wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnagi.2019.00172/full#supplementary-material

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BRIEF RESEARCH REPORT
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The Synthetic Retinoid Acitretin Increases IL-6 in the Central Nervous System of Alzheimer Disease Model Mice and Human Patients

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These days, the important role of retinoids in adult brain functionality and homeostasis is well accepted and has been proven by genomic as well as non-genomic mechanisms. In the healthy brain, numerous biological processes, e.g., cell proliferation, neurogenesis, dendritic spine formation as well as modulation of the immune system, have been attributed to retinoid signaling. This, together with the finding that retinoid metabolism is impaired in Alzheimer's disease (AD), led to preclinical and early clinical testing of natural and synthetic retinoids as innovative pharmaceuticals with multifactorial properties. Acitretin, an aromatic retinoid, was found to exert an anti-amyloidogenic effect in mouse models for AD as well as in human patients by stimulating the alpha-secretase ADAM10. The lipophilic drug was already demonstrated to easily pass the blood brain barrier after i.p. administration and evoked increased nest building capability in the 5xFAD mouse model. Additionally, we analyzed the immune-modulatory capacity of acitretin *via* a multiplex array in the 5xFAD mouse model and evaluated some of our findings in human CSF derived from a pilot study using acitretin. Although several serum analytes did not display changes, Interleukin-6 (IL-6) was found to be significantly increased in both—mouse and human neural material. This demonstrates that acitretin exerts an immune stimulatory effect—besides the alpha-secretase induction—which could impact the alleviation of learning and memory disabilities observed in the mouse model.

Keywords: alpha-secretase, ADAM10, gp130, IL-6, IL-6R, inflammation, retinoic acid, vitamin A

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INTRODUCTION

In a meta-analysis, significantly lower vitamin A plasma levels were found in Alzheimer's disease (AD) patients (Lopes da Silva et al., 2014) and genetic linkages in regard to retinoic acid receptors as well as altered enzymatic function e.g., for retinaldehyde dehydrogenase (Connor and Sidell, 1997; Goodman and Pardee, 2003). Pre-clinical data support that retinoids might contribute to development and progression of AD: for example, vitamin A deficiency evoked in adult rodents cognitive impairment (Jiang et al., 2012; Hou et al., 2015) and a shift towards the amyloidogenic processing of the amyloid precursor protein (APP; Reinhardt et al., 2016). Additionally, marginal vitamin A deficiency (MVAD) that has also been correlated with cognitive decline in the

elderly, led to enhanced Aβ synthesis, plaque formation, and cognitive deficits in APP/PS1 mice (Zeng et al., 2017a). Moreover, rats fed on a MVAD diet with combinatory injection of Aβ showed cognitive impairment (Zeng et al., 2017b). This indicates that retinoic acid deficiency may lead to enhanced risk of developing AD and has given rise to the idea of using retinoids as therapeutics (Fahrenholz et al., 2010; Chakrabarti et al., 2016).

Severe unwanted side-effects from systemic retinoid-intake mainly comprise teratogenicity (Hunt, 1996), which should not be highly relevant in patients aged over 60 (beyond childbearing age). Nevertheless, retinoids can also lead to symptoms such as bone fractures (Green et al., 2016), which would contribute to frailty in the aged patients. Moreover, retinoids might also exert psychiatric side-effects such as those reported for isotretinoin application in acne-treatment and depression or aggression (Bremner et al., 2012), thereby further substantiating their importance for neuronal network stability. This makes it absolutely mandatory to monitor side-effects and identify further potential molecular targets of retinoid-treatment. We hypothesized that treatment with acitretin, a synthetic retinoid that already has proven anti-amyloidogenic effect in AD patients (Endres et al., 2014), would lead to modifications in peripheral and central immunity where retinoids have been identified as potent regulators (reviewed in Erkelens and Mebius, 2017). This can consequently also impact retinoic acid metabolism in a sort of vicious cycle: for example, pro-inflammatory activation can result in increased retinoic acid catabolism as demonstrated for primary mouse microglia (Hellmann-Regen et al., 2013). Therefore, we analyzed immunological markers in serum and brain samples from AD model mice treated with acitretin and evaluated the finding of elevated brain Interleukin-6 (IL-6) in human CSF samples.

MATERIALS AND METHODS

Animals

5xFAD mice (Jackson Laboratory; Oakley et al., 2006) stably cross-bred with C57Bl6/J mice and wild type littermates were used at an age of 30 weeks (all female, for housing conditions and genotyping see Brandscheid et al., 2017). All experimental procedures were carried out in accordance with the European Communities Council Directive regarding care and use of animals for experimental procedures and was approved by local authorities (LUA Rhineland-Palatinate; G14-1-087).

Treatment of Mice

Mice were injected intraperitonally [daily dosage: 10 mg acitretin (LGC Promochem) in corn oil (Sigma Aldrich)/kg] for 7 days including a 2-day break (see treatment schedule, **Figure 1A**).

Nesting Test

Nest building ability was assessed as modified from Reinhardt et al. (2018) and scored as follows: 0 = material unused; 1 = material used but not collected; 2 = material collected; 3 = nest with low walls; 4 = nest with walls as high as the mouse; 5 = walls higher than the mouse (full dome, newly introduced score level); 6 = closed dome.

ADAM10 Activity Assay

A fluorescent enzyme assay (Sensolite 520 ADAM10 Activity Assay, Anaspec) was used according to the manufacturer's recommendations. Brains were homogenized in ice-cold PBS supplemented with protease inhibitor cocktail (without EDTA, Roche). Homogenates were centrifuged at 3,000 *g* (3 min, 4°C); the resulting pellet was washed and resuspended with assay buffer. For each sample, a solvent control and a GM6001-treatment were measured at 480/520 (exc./em.) using the FluostarOmega (BMG; 1 measurement per minute). Usage of the metalloprotease inhibitor GM6001 ascertains control for unspecific signals obtained by the *in vitro* assay. Forty minutes from the linear range were used to calculate the fluorescence increase per minute. Specific RFU (relative fluorescence units) were calculated by subtracting the values from each GM6001-treatment sample from its corresponding solvent control.

APP Processing Product Quantitation

Brain samples were prepared as described before for APPs-alpha quantitation (Reinhardt et al., 2016). For APP full length, CTFs and Aβ, homogenates from activity assay were used. Twenty micrograms of protein were subjected to SDS polyacrylamide gel electrophoresis. Proteins were blotted onto nitrocellulose membrane and blocked with 0.2% I-Block (Thermo Fisher Scientific) solution including 0.05% Tween20. As primary antibodies 6E10 (Covance, Madison, WI, USA), 6687 (APP CT, Steiner et al., 2000), and anti-GAPDH (14C10, Cell Signaling, Danvers, MA, USA) were used in combination with respective secondary antibody coupled with horseradish peroxidase (Thermo Scientific, Karlsruhe, Germany). Signals were obtained by SuperSignal West Femto chemiluminescent substrate (Thermo Scientific, Karlsruhe, Germany) and exposure in a CCD-camera imaging system (Raytest, Straubenhardt, Germany). Densitometric analysis was performed by Aida Image Analyzer v4.26 (Raytest).

MSD Multiplex Array for Cytokine/Interleukin Serum Level Quantitation

The V-PLEX Proinflammatory Panel 1 Mouse Kit (Sulfo-tag antibodies, K15048D-1, Meso Scale Discovery) was used as recommended by the vendor with mouse serum diluted 1:2.75. IL-12p70, IL-2, and IL-4 from this multiplex assay were not detected in sufficient amounts (below LOD) to perform analysis.

Interleukin-6 Measurement

Brain homogenates were centrifuged for 10 min at 10,000 *g*, and at 4°C. The supernatant was used for IL-6 measurement following the manufacturer's protocol (IBL International, Hamburg, Germany). Values were normalized to protein content of the supernatant.

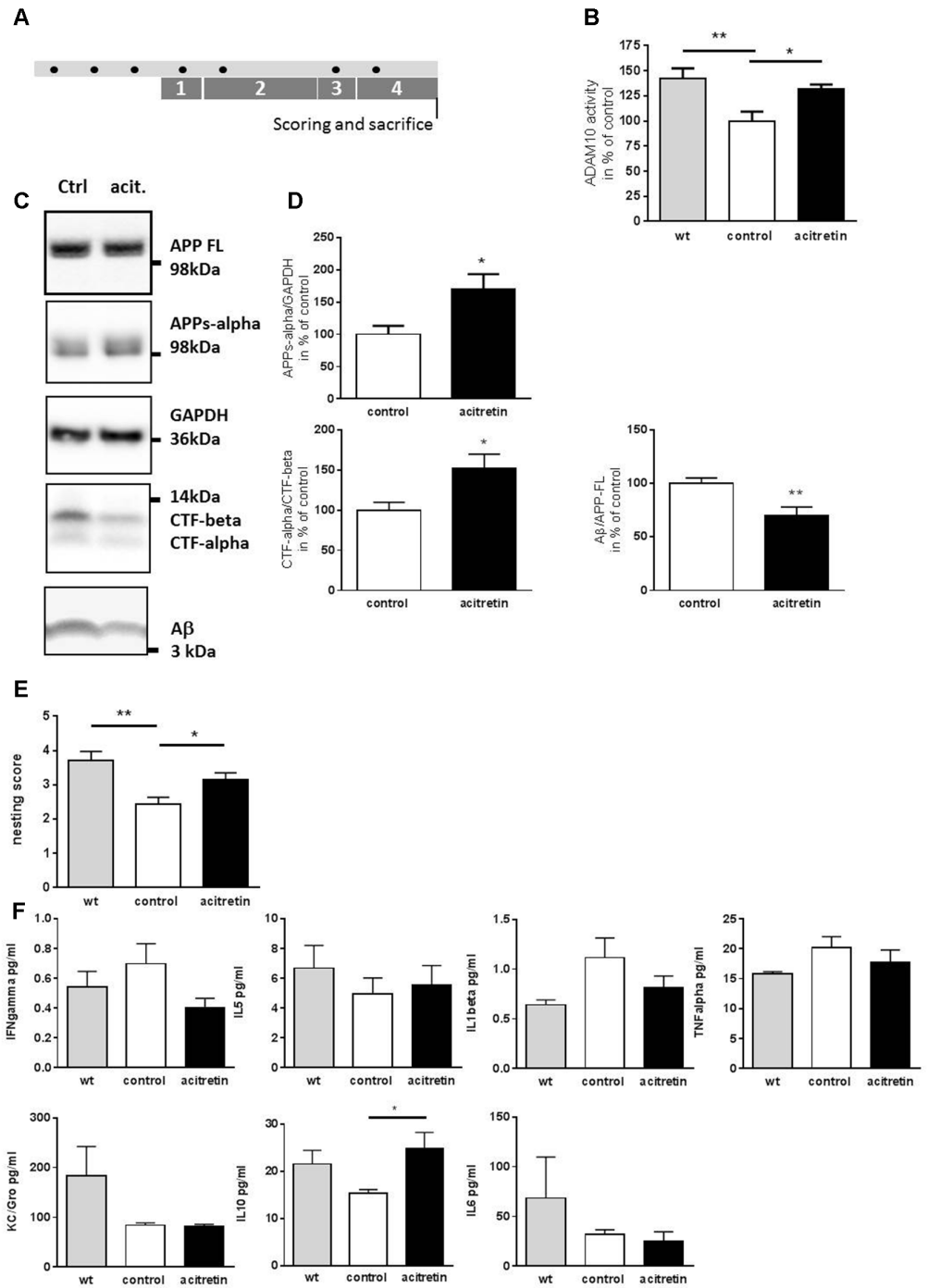

FIGURE 1 | Effect of acitretin on alpha-secretase activity, nest building ability, and immunological serum markers in 5xFAD mice. Mice were treated as depicted in the scheme (**A**, black dot: injection) with a daily dosage of acitretin or with corn oil as solvent-control. As a control, wild type littermates injected with corn-oil were included. In parallel, the nesting test was conducted (1, habituation to nesting material; 2, habituation to nesting material as the sole bedding; 3, depletion of nesting material; 4, nest-building). **(B)** ADAM10 catalytic activity was measured in brain homogenates using a FRET-dependent assay. Values obtained for control-treated animals were set to 100%, mean + SEM are presented (for each group: $n = 4$; Student's unpaired t-test; $^{*}p \leq 0.05$; $^{**}p \leq 0.01$). **(C,D)** Amyloid precursor protein (APP) processing products were measured by western blot and normalized to GAPDH (APPs-alpha) or to full length APP (Aβ). For CTFs, a ratio was calculated without further normalization. Values obtained for control-treated animals were set to 100%, mean + SEM are presented ($n = 3$–5 for control, $n = 4$–5 for acitretin; Student's unpaired t-test; $^{*}p \leq 0.05$). **(E)** Nests were scored following a rating scale (for each group: $n = 7$–8; Mann Whitney test; $^{*}p \leq 0.05$; $^{**}p \leq 0.01$). **(F)** Analysis of peripheral immune markers by multiplex analysis. Serum samples from $n = 4$–6 animals were analyzed (one way ANOVA; Sidaks multiple comparison test; $^{*}p \leq 0.05$).

Quantitation of IL-6R and gp130

Quantitation of IL-6R and gp130 was done *via* ELISA (R&D Systems) and normalized to the protein amount of the tissue lysate.

Clinical Study

The acitretin-treatment study has been described in detail in Endres et al. (2014). In brief, men as well as women aged over 50 years with mild to moderate dementia and a diagnosis of probable AD were randomized to either placebo or acitretin group. Thirty milligrams of acitretin were taken daily. CSF was collected at two time points: before start of treatment ("baseline") and after 30 days ("treatment"). IL-4 and -6 in CSF were analyzed by ELISA following the manufacturer's instructions (IBL International, Hamburg, Germany). The study is registered with ClinicalTrials.gov (NCT01078168). Patients provided written informed consent before enrolment.

Statistics

Testing of statistical significance was performed using one way ANOVA followed by appropriate post-test or by unpaired Student's *t*-test (Graph Pad Prism6, San Diego, CA, USA). In case of the nesting test (ordinal scale), Mann Whitney test was used.

RESULTS

Acitretin Activates ADAM10 in the Brain of 5xFAD Model Mice and Ameliorates Cognitive Deficits

In a previous study, a single stereotactic acitretin-injection was sufficient to balance APP processing towards the non-amyloidogenic pathway in APP/PS1 AD model mice (Tippmann et al., 2009). Here, we wanted to examine if peripheral (i.p.) injection in the 5xFAD mouse model also suffices to achieve this effect. We treated 30-week-old 5xFAD mice with acitretin over 10 days (treatment schedule shown in **Figure 1A**): first, ADAM10 activity within brain homogenates of 5xFAD mice was decreased by ca. 40% as compared to wild type littermates (**Figure 1B**). This deficiency could be partially restored by acitretin-administration (131% of control-treated 5xFAD, **Figure 1B**) and was accompanied by increased shedding of APPs-alpha, increased CTF-alpha/-beta ratio, and decreased amount of Aβ (**Figures 1C,D**). Moreover, acitretin-treatment was able to ameliorate cognitive impairment as demonstrated by nest building test (**Figure 1E**).

Acitretin Has Only Minor Effect on Peripheral Immune Markers but Increases Cerebral IL-6 Amount

No significant differences in serum interferon gamma, IL-5, KC/Gro, TNF alpha, and IL-1beta could be measured in acitretin-treated vs. control-injected animals (**Figure 1F**). Only anti-inflammatory IL-10 increased significantly, reaching similar levels as in wild type littermates. All-trans retinoic acid has been shown to suppress IL-6, an important cytokine of the aging brain (Godbout and Johnson, 2004) *via* ERK1/2 activation

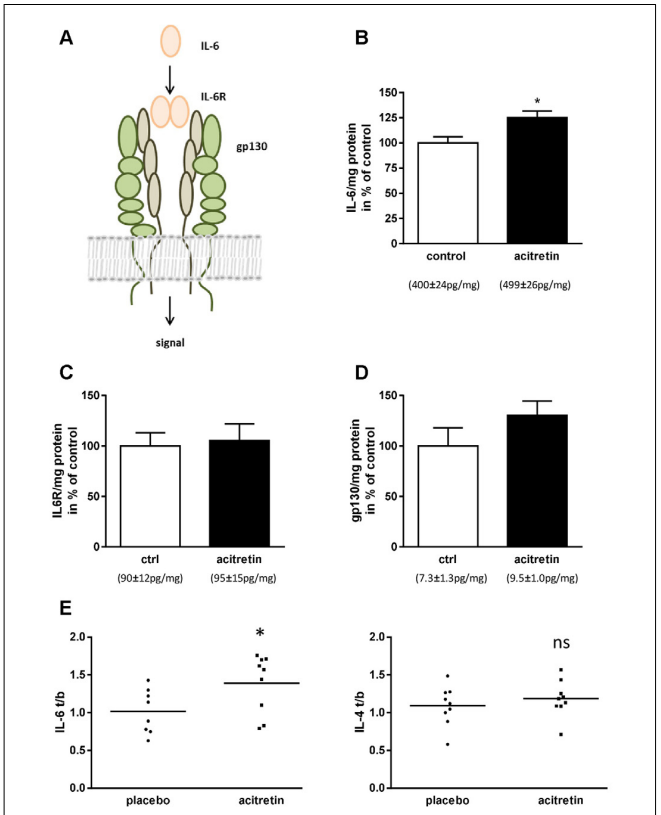

FIGURE 2 | Impact of acitretin-treatment on Interleukin-6 (IL-6) signaling in murine cortex and human CSF. IL-6 dimers bind to IL-6 receptor and, together with gp130, induce downstream classical signaling **(A)**. 5xFAD mice were treated as depicted in **Figure 1** and brain homogenates analyzed for IL-6 (**B**; means indicated), IL-6 receptor (IL-6R, **C**), and gp130 (**D**). $n = 5$ per group; Student's unpaired *t*-test; $^*p \leq 0.05$). IL-6 and -4 were measured before and after 30 days of acitretin-administration in CSF of Alzheimer patients (**E**, for details of the clinical study see Endres et al., 2014). To account for the inter-individual differences in the small sample size [$n = 8$ (IL-6) and 9 (IL-4) for placebo and $n = 9$ for acitretin], ratios were built [value after treatment/value at baseline (t/b); Student's unpaired *t*-test; ns, $p \geq 0.05$; $^*p \leq 0.05$].

(Kirchmeyer et al., 2008) and we could see non-significant reduction of IL-6 in the peripheral samples (32 vs. 25 pg/ml, **Figure 1F**).

We, therefore, assumed that IL-6 should also be unaltered or diminished in the brains of acitretin-treated mice. Astonishingly, we observed the contrary: IL-6 was increased to 125% of control animals (**Figure 2B**). IL-6 exerts its biological function *via* two molecules: IL-6 receptor (IL-6R) and gp130 (reviewed e.g., in Garbers and Rose-John, 2018, **Figure 2A**). Therefore, subsequently to IL-6 quantitation, we analyzed the amount of both receptors. Neither IL-6R nor gp130 was significantly changed on protein level (**Figures 2C,D**).

Acitretin-Treatment of Alzheimer Patients Increases CSF Levels of IL-6

In a formerly published clinical study, we demonstrated general feasibility of ADAM10-enhancement by acitretin in patients with

mild to moderate AD by measuring APPs-alpha in CSF (Endres et al., 2014). As representatives of anti- and pro-inflammatory markers, IL-4 and IL-6 were measured in CSF (**Figure 2E**). While IL-4 remained unaffected (placebo group: 1.09 ± 0.09; acitretin group: 1.19 ± 0.08; $p = 0.44$), IL-6 levels increased in the acitretin-group about 40% as compared to placebo-group (placebo group: 1.02 ± 0.10; acitretin group: 1.39 ± 0.13; $p = 0.04$).

DISCUSSION

Acitretin was able to increase non-amyloidogenic APP processing and AD-related impaired brain function in mice due to short-term treatment. This is in accordance with previous publications reporting on beneficial cognitive effects of retinoids in AD models (Takamura et al., 2017). In addition to its impact on secretase expression, retinoic acid has been shown to control inflammation e.g., in the central nervous system (Raverdeau et al., 2016). Almost all investigated peripheral immune molecules remained unaffected in the acitretin-treated animals despite IL-10. Interestingly, IL-10 has been found to be increased in brain samples derived from humans with intermediate probability of AD in the absence of dementia, designated as resilient (Barroeta-Espar et al., 2019). IL-10 measurements were not considered in the initial clinical study; therefore, the potential effect of acitretin on this IL in human patients still has to be analyzed. IL-6 has been suggested as a plasma biomarker for AD (e.g., Wu et al., 2015) and has also been reported to be repressed by retinoids (Kirchmeyer et al., 2008). However, we observed no change in peripheral IL-6 in mice but only increase in brain IL-6 in both, mice and human patients. In regard to AD, the impact of IL-6 has been found to be multi-faceted: the neurotoxic peptide Aβ induces inflammatory molecules such as IL-6 in glia cultures and in stereotactically injected animals (Forloni et al., 1997; Song et al., 2001). Additionally, the administration of IL-6 to cultured cortical neurons exacerbates toxicity of the peptide (Qiu and Gruol, 2003). However, *in vivo*, IL-6 showed beneficial effects in early stages of disease due to induction of plaque clearance (Chakrabarty et al., 2010) and IL-6-deficient mice showed enhanced neuronal vulnerability in a MPTP-induced Parkinson model (Bolin et al., 2002). The difficulties in deciphering the exact role of IL-6 might be due to the fact that IL-6 may stimulate a response in its target cell in two different manners—the classical signaling and the pathogenicity-driving trans-signaling pathway (Campbell et al., 2014). The first involves binding of IL-6 to the membrane-bound IL-6R, which initiates dimerization of gp130 and subsequent downstream signaling (reviewed in Rothaug et al., 2016). Alternatively, the IL-6 receptor may be shed by proteases such as ADAM10 (Garbers et al., 2011), form a soluble complex with IL-6, and stimulate cells that only express gp130 but not surface-bound IL-6R. Here, we observed an increase in brain IL-6 in mice (and humans) treated with the synthetic retinoid acitretin while IL-6R and gp130 were not significantly impacted. Reports on basal IL-6 secretion or production in brain of 5xFAD mice are somehow controversial (e.g., Ardestani et al., 2017; Mariani et al., 2017); however, another study using also MSD multiplex technique described levels of IL-6 in brain homogenates from these mice comparable to wild type animals at 7 months of age (Chen et al., 2016)—a similar age as for the animals used in our study. This indicates that the comparably small increase of IL-6 due to acitretin-administration may have functional relevance. As the measurement of IL-6R and gp130 was not able to distinguish between soluble and non-soluble forms, we cannot decide which pathway was triggered by acitretin-treatment. Nevertheless, as cognitive function of the mice increased, we could conclude that induction of IL-6 signaling due to acitretin-treatment was not detrimental for the model mice and, therefore, further supports its future therapeutic application in human patients.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Landesärztekammer Rheinland-Pfalz with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the ethics committee of the Landesärztekammer Rheinland-Pfalz. The trial was monitored by the Interdisciplinary Centre for Clinical Trials Mainz (IZKS, University Medical Centre, Mainz) and registered with ClinicalTrials.gov (NCT01078168). This study was, in regard to animal experiments, carried out in accordance with the recommendations of the European Communities Council Directive regarding care and use of animals for experimental procedures and was approved by local authorities (Landesuntersuchungsamt Rheinland-Pfalz; approval number G14-1-087).

AUTHOR CONTRIBUTIONS

MSG quantified CTFs and Aβ, helped with writing the manuscript, interpreted, and visualized the data. NS performed ELISAs, calculated and analyzed the results and conducted the nesting tests. SR-J and CG edited the manuscript and contributed to interpreting the results. CG performed IL-6R and gp130 measurements. AF conducted the clinical trial and edited the manuscript. KE initiated the study, interpreted results, and wrote the manuscript.

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REVIEW
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Multi-Loop Model of Alzheimer Disease: An Integrated Perspective on the Wnt/GSK3β, α-Synuclein, and Type 3 Diabetes Hypotheses

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As the prevalence of Alzheimer disease (AD) continues to rise unabated, new models have been put forth to improve our understanding of this devastating condition. Although individual models may have their merits, integrated models may prove more valuable. Indeed, the reliable failures of monotherapies for AD, and the ensuing surrender of major drug companies, suggests that an integrated perspective may be necessary if we are to invent multifaceted treatments that could ultimately prove more successful. In this review article, we discuss the Wnt/Glycogen Synthase Kinase 3β (GSK3β), α-synuclein, and type 3 diabetes hypotheses of AD, and their deep interconnection, in order to foster the integrative thinking that may be required to reach a solution for the coming neurological epidemic.

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INTRODUCTION

Alzheimer disease (AD) is among the most ominous of modern health epidemics. The current costs, both human and financial, are staggering and climbing at a precipitous rate. In the United States alone, 5.5 million adults live with AD, imposing an economic burden of $259 billion (Alzheimer's Association, 2017). Over the next three decades, the number of people living with AD is expected to triple to 13.8 million and the economic costs are projected to quadruple to $1.1 trillion, single-handedly crippling the United States health care system. AD is also the only disease on the list of the top 10 disease causes of death for which there is currently no effective treatment (Alzheimer's Association, 2017).

AD is not alone in its ascent. Other chronic diseases, particularly Parkinson's disease (PD), a neurodegenerative disorder associated with the build-up of α-synuclein protein and death of dopaminergic neurons, and type 2 diabetes mellitus (T2DM) are increasing in prevalence at similarly alarming rates (Boyle et al., 2010; Rocca, 2018). Although AD, PD, and T2DM share common risk factors, chief among these being age, there is more to their relationship. Evidence suggests that the pathophysiological mechanisms underlying AD, PD, and T2DM interact synergistically (Giasson et al., 2003; de la Monte and Wands, 2008; Duka et al., 2009; Wills et al., 2010; Gao et al., 2012; Gąssowska et al., 2014; Roberts et al., 2017; Yan et al., 2018).

In addition to the well-known amyloid cascade hypothesis of AD, other hypotheses have been proposed that include: (1) the Wnt/Glycogen Synthase Kinase 3β (GSK3β) hypothesis (Hooper et al., 2008; De Ferrari et al., 2014; Llorens-Martín et al., 2014), (2) the α-synuclein hypothesis

(Moussaud et al., 2014; Yan et al., 2018), and (3) the type 3 diabetes hypothesis (de la Monte and Wands, 2008). In this review article, we focus on the Wnt/GSK3β hypothesis, describing how it serves as a platform for a set of positive feedback loops that contribute to the pathogenesis of AD. In turn, we also discuss the α-synuclein and type 3 diabetes hypotheses, describing how they each constitute their own feedback loops and interact with the Wnt/GSK3β model.

WNT/GSK3β

Overview of Wnt-Signaling

Wnt-signaling refers to a set of highly conserved signal transduction pathways that are widely expressed throughout the body and that play a vital role both in neuronal development and in the maintenance of proper neuronal function in the adult human brain (Patapoutian and Reichardt, 2000; Oliva et al., 2013; Rosso and Inestrosa, 2013; Nusse and Clevers, 2017). In this article, we focus on the better-studied canonical Wnt-β-catenin-signaling pathway, leaving the topic of the two non-canonical Wnt-signaling pathways (the Wnt-planar cell polarity and Wnt-calcium pathways) for others to discuss in depth (Mudher et al., 2001; Gao et al., 2012; Oliva et al., 2013; Rosso and Inestrosa, 2013; Wan et al., 2014). Canonical Wnt-β-catenin-signaling (hereafter, referred to simply as Wnt-signaling) is initiated by the binding of Wnt ligands to the Wnt receptor pair, Low-Density Lipoprotein Receptor-Related Protein 6-Frizzled (LRP6-Fz). LRP6 then recruits Dishevelled (DVL), a scaffolding protein that sequesters GSK3β from the cytoplasm. The inhibition of GSK3β, a constitutively active kinase that targets the transcriptional cofactor β-catenin for proteasomal degradation, is central to Wnt-signaling. Simply put, Wnt-signaling inhibits GSK3β, permitting β-catenin to accumulate in the cytoplasm and translocate into the nucleus to mediate the transcription of genes, such as *BACE1* and *ADAM10* (elaborated upon below), involved in the pathogenesis of AD (Rosso and Inestrosa, 2013; **Figure 1A**).

Dysfunctional Wnt-Signaling Causes the Production of Aβ

Amyloid plaques, aggregates of the amyloid β (Aβ) peptide, are the primary pathological hallmark of AD. Aβ is formed by the sequential cleavage of amyloid precursor protein (APP) by β- and γ-secretase (**Figure 1B**). This amyloidogenic processing is in contrast to nonamyloidogenic processing, in which α-secretase replaces β-secretase and cleaves APP within the Aβ domain such that no Aβ is produced (Haass et al., 2012; **Figure 1A**). By altering α- and β-secretase gene expression, as well as by decreasing APP phosphorylation, Wnt-signaling shifts APP metabolism away from amyloidogenic processing and protects against Aβ neuropathology (Alvarez et al., 2004; Parr et al., 2012; Liu et al., 2014; De Ferrari et al., 2014; Llorens-Martín et al., 2014; Wan et al., 2014).

With respect to secretase gene expression, Wnt-signaling downregulates the sole β-secretase gene, *BACE1* (Haass et al., 2012; Parr et al., 2015), and upregulates the primary neuronal α-secretase gene, *ADAM10* (Haass et al., 2012; Wan et al., 2012). In APP-overexpressing mice, GSK3β inhibition has been shown to decrease *BACE1* expression and activity, thereby reducing amyloid plaque load (Ly et al., 2013). Furthermore, in cultured neurons, activating Wnt-signaling, by using Wnt ligands or overexpressing β-catenin, is sufficient to increase *ADAM10* expression (Wan et al., 2012) and decrease *BACE1* expression, again reducing Aβ levels (Parr et al., 2015). These data are consistent with a model in which dysfunctional Wnt-signaling in the AD brain causes GSK3β-mediated β-catenin depletion, which leads to a pathological decrease in the ratio of α-secretase to β-secretase expression (**Figure 1B**i) and, thus, to an increase in the amyloidogenic processing of APP to Aβ (Mudher et al., 2001; Chami et al., 2012; Wan et al., 2012; Ly et al., 2013; De Ferrari et al., 2014; Llorens-Martín et al., 2014; Wan et al., 2014; Golpich et al., 2015; Parr et al., 2015).

Wnt-signaling further suppresses amyloidogenic processing by inhibiting APP phosphorylation. Specifically, Wnt-signaling inhibits GSK3β, which otherwise phosphorylates APP on Thr668 (Saeki et al., 2011; Acevedo et al., 2014), contributing to the elevated p-Thr668 APP levels that are observed in the human AD brain (Lee et al., 2003; **Figure 1B**ii). The direct consequences of Thr668 phosphorylation are two-fold. First, p-Thr668 APP is a better substrate for β-secretase than unphosphorylated APP (Lee et al., 2003). Second, if the APP intracellular domain (AICD)—which contains Thr668 and is produced in conjunction with Aβ by γ-secretase-mediated cleavage—is phosphorylated, it can translocate into the nucleus to upregulate *GSK3β* gene expression (Chang et al., 2006; **Figure 1B**). In this way, dysfunctional Wnt-signaling permits the phosphorylation of APP by GSK3β, leading to both an increase in Aβ production and an increase in *GSK3β* expression, establishing a positive feedback loop.

As predicted by this model, inhibiting Wnt-signaling with the LRP6 inhibitor, Dicckopf-1 (DKK1), increases the amyloidogenic processing of APP and impairs learning and memory in mice (Killick et al., 2012; Parr et al., 2015; Marzo et al., 2016; Elliott et al., 2018; Sellers et al., 2018), whereas activating Wnt-signaling with different GSK3β inhibitors decreases *BACE1* expression, decreases APP phosphorylation, decreases Aβ production, prevents neurodegeneration, and reduces learning and memory (Ryder et al., 2003; Chang et al., 2006; Rockenstein et al., 2007; Fiorentini et al., 2010; Toledo and Inestrosa, 2010; Ly et al., 2013; Pan et al., 2018).

Aβ Causes Dysfunctional Wnt-Signaling

Aβ, in turn, can inhibit Wnt-signaling to establish another positive feedback loop. Treatment of rat neurons *in vitro* with Aβ induces the expression of DKK1 and increases GSK3β activity, thereby decreasing β-catenin levels and contributing to the death of neurons (Alvarez et al., 2004; Caricasole et al., 2004; Killick et al., 2012; Elliott et al., 2018; Sellers et al., 2018). Importantly, activation of Wnt-signaling via a variety of mechanisms—by treatment with Wnt ligands, neutralization of DKK1, or inhibition of GSK3β—appears sufficient to protect neurons against β-catenin depletion and, ultimately, death (Alvarez et al., 2004; Caricasole et al., 2004; Silva-Alvarez et al., 2013).

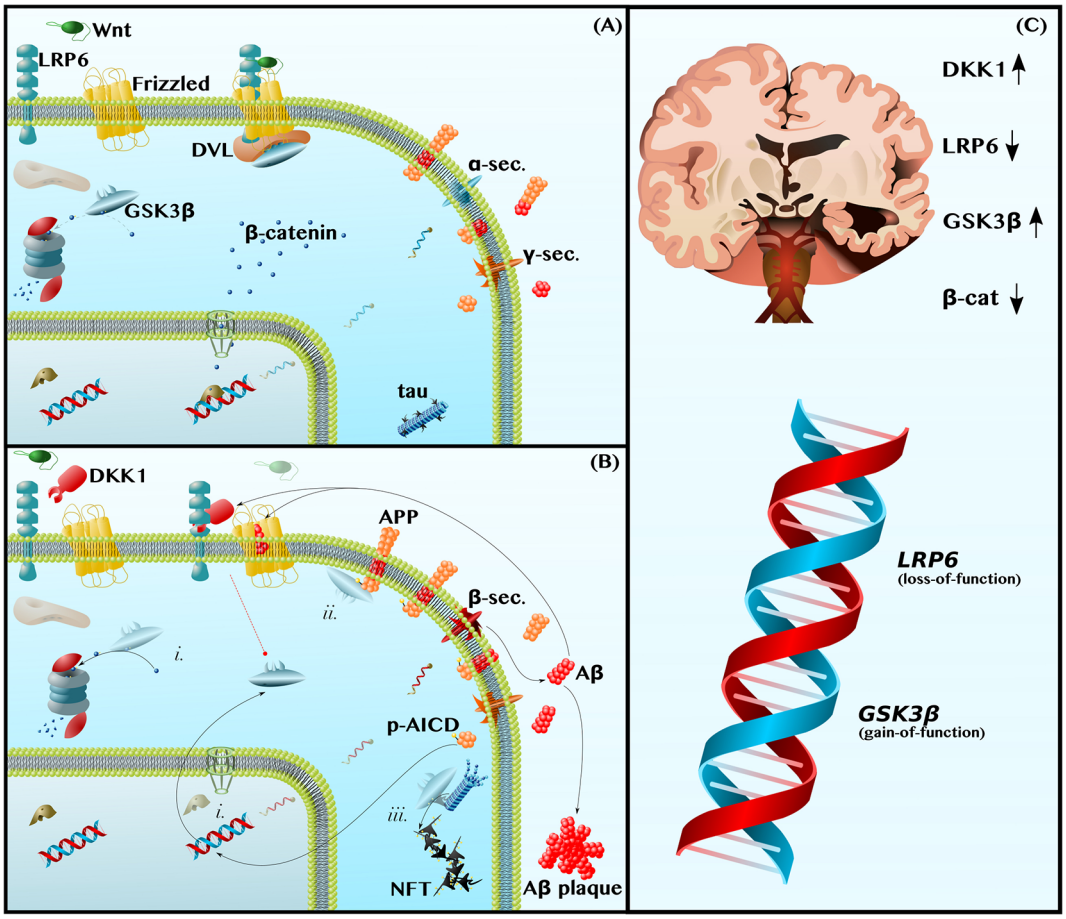

FIGURE 1 | Dysfunctions in canonical Wnt-Signaling contribute to the neuropathology of Alzheimer disease (AD). **(A)** Functional Wnt and Nonamyloidogenic Processing—Glycogen Synthase Kinase 3β (GSK3β) is a constitutively active kinase that phosphorylates and targets β-catenin for proteasomal degradation. The binding of Wnt ligands to LDL Receptor-Related Protein 6 (LRP6) and Frizzled induces the receptor pair to bind Dishevelled (DVL), a protein that serves as a docking platform for GSK3β. The sequestration of GSK3β by the Wnt receptor complex permits β-catenin to accumulate and translocate into the nucleus, where it binds transcription factors to induce gene expression. This includes promoting anti-amyloidogenic α-secretase expression (blue mRNA) and inhibiting pro-amyloidogenic β-secretase expression. **(B)** Dysfunctional Wnt, Amyloidogenic Processing, and Tau Hyperphosphorylation—The LRP6 antagonist, Dickkopf 1 (DKK1), prevents Wnt-induced GSK3β inhibition (broken red line). Thus, (i) GSK3β causes β-catenin depletion, contributing to a decrease in α-secretase expression and increase in β-secretase expression (red mRNA). In addition, (ii) GSK3β phosphorylates the intracellular domain of Amyloid Precursor Protein (APP), making APP a better substrate for β-secretase and further promoting amyloidogenic processing and the production of Amyloid β (Aβ) by β- and γ-secretase. Aβ inhibits Frizzled and induces DKK1 expression to feedback and prevent GSK3β inhibition (broken red line). Aβ also forms extracellular plaques. The leftover phosphorylated APP Intracellular Domain (p-AICD) induces GSK3β expression. Finally, (iii) GSK3β, also known as Tau Kinase I, phosphorylates tau, contributing to microtubule instability and to the formation of neurotoxic oligomers and phospho-tau (p-tau) Neurofibrillary Tangles (NFTs). **(C)** Human Neuropathological and Genetic Data are Consistent with the Wnt/GSK3β Model of AD—In the Alzheimer brain, as compared to the healthy aged brain, the levels and activities of Wnt-signaling components are indicative of pathway hypoactivity: DKK1 levels are elevated, LRP6 levels are reduced, GSK3β activity is high, and β-catenin is depleted. Furthermore, *LRP6* loss-of-function and *GSK3β* gain-of-function alleles are risk factors for AD.

Not only does Aβ indirectly inhibit the initiation of Wnt-signaling by increasing the expression of the LRP6 antagonist, DKK1 (Caricasole et al., 2004; Killick et al., 2012; Purro et al., 2012; Marzo et al., 2016; Elliott et al., 2018), but it also directly blocks the binding of Wnt ligands to the other half of the LRP6-Fz receptor pair. Using cultured mouse neurons, Magdesian et al. (2008) demonstrated that Aβ competitively inhibits the binding of Wnt ligands to Fz and, consequently, prevents β-catenin from translocating into the nucleus to induce Wnt target gene expression (**Figure 1B**). Aβ also increases GSK3β activity leading to neurodegeneration (Alvarez et al., 2004; Caricasole et al., 2004; Hooper et al., 2008; De Ferrari et al., 2014; Llorens-Martín et al., 2014; Wan et al., 2014). Importantly, interventions that either block the interaction between Aβ and the Wnt receptors, or those that circumvent the Aβ blockade and activate Wnt-signaling downstream of LRP6-Fz, protect neurons against Aβ toxicity (Alvarez et al., 2004; Caricasole et al., 2004; Hooper et al., 2008; Magdesian et al., 2008; De Ferrari et al., 2014; Llorens-Martín et al., 2014; Wan et al., 2014). Some examples are as follows: a synthetic soluble peptide homologous to Fz competitively inhibited

Aβ binding to Fz and, thereby, protected against β-catenin depletion (Magdesian et al., 2008); upstream activation of Wnt-signaling using competitive amounts of exogenous Wnt ligands (Wnt3a or Wnt7a) prevented Aβ-induced neuron apoptosis; downstream activation of Wnt-signaling using multiple different GSK3β inhibitors also prevented Aβ-induced neurodegeneration (Alvarez et al., 1999, 2004; Silva-Alvarez et al., 2013).

An LRP6 Deletion Model Supports the Wnt/GSK3β-Aβ Feedback Loop

An *LRP6* deletion mouse model provides further support for the hypothesis that dysfunctional Wnt-signaling and Aβ constitute two halves of a positive feedback loop. Liu and coworkers demonstrated that conditional deletion of *LRP6* in mouse neurons increased levels of β-secretase cleavage products and precipitated the formation of Aβ plaques, consistent with the notion that decreased Wnt-signaling promotes the formation of amyloid pathology. The neuropathological changes were associated with significant memory deficits, similar to those exhibited by more common mouse models of AD (Liu et al., 2014). Importantly, Aβ, in turn, decreased *LRP6* expression, thus validating the positive feedback loop model in which dysfunctional Wnt-signaling causes an increase in Aβ, and vice versa.

These mouse data paralleled those from human patients with AD. Liu et al. not only found (1) lower LRP6 and β-catenin levels in the post-mortem brains of AD patients relative to age-matched control brains (**Figure 1C**), but also (2) a negative correlation between LRP6 and Aβ levels in these brains and (3) a positive correlation between LRP6 levels and Mini-Mental State Examination (MMSE) scores, a test in which higher scores indicate better cognitive function (Liu et al., 2014). Thus, the level of Wnt-signaling dysfunction may predict the degree of neuropathology and cognitive impairment in AD patients.

Human Neuropathological and Genetic Data Support the Wnt/GSK3β Model

Not only are LRP6 levels reduced in the post-mortem brains of AD patients, but DKK1 levels are also elevated (Caricasole et al., 2004; Oliva et al., 2013; Wan et al., 2014). The simultaneous decrease in the Wnt receptor (LRP6) and increase in its inhibitor (DKK1) cooperatively downregulates Wnt-signaling and increases GSK3β activity in patients' brains (Leroy et al., 2007; Hooper et al., 2008; Oliva et al., 2013; Llorens-Martín et al., 2014; Wan et al., 2014; Lazzara and Kim, 2015). The genetic data concur. Specifically, a loss-of-function mutation in *LRP6* has been identified as a risk factor for AD (De Ferrari et al., 2007), as have gain-of-function mutations in the *GSK3β* gene (Schaffer et al., 2008; **Figure 1C**).

Furthermore, evidence suggests that the strongest known genetic risk factor for AD in humans, the *ApoE4* allele (Liu et al., 2013), may negatively impact Wnt-signaling. Similar to Aβ, the ApoE4 protein increases DKK1 expression, binds to the LRP6-Fz receptor complex, activates GSK3β, and promotes the amyloidogenic processing of APP (Kim et al., 1998; Cedazo-Mínguez et al., 2003; Caruso et al., 2006; Chami et al., 2012; De Ferrari et al., 2014; Wan et al., 2014; Theendakara et al., 2016). Therefore, there is a case to be made that ApoE4 either sparks the positive feedback loop between Wnt-signaling and Aβ, decreases the threshold for the establishment of the feedback loop, and/or accelerates the rate at which the loop spirals into life-altering disease.

GSK3β Links Aβ to p-tau

In addition to contributing to the build-up of amyloid plaques, the first of the two pathological hallmarks of AD, dysfunctional Wnt-signaling may also contribute to the development of the second hallmark of AD, phospho-tau (p-tau) Neurofibrillary Tangles (NFTs). GSK3β, alternatively known as Tau Kinase I, is thought to be the mechanistic link between Aβ and p-tau (Lucas et al., 2001; Leroy et al., 2007; Saeki et al., 2011; De Ferrari et al., 2014; Llorens-Martín et al., 2014). By inhibiting Wnt-signaling, Aβ increases GSK3β activity (Alvarez et al., 2004; Caricasole et al., 2004; Hooper et al., 2008; De Ferrari et al., 2014; Llorens-Martín et al., 2014; Wan et al., 2014). In turn, GSK3β phosphorylates tau on a set of residues known to be phosphorylated in AD (Lucas et al., 2001; Leroy et al., 2007; Saeki et al., 2011; De Ferrari et al., 2014; Llorens-Martín et al., 2014). This results in two events. First, tau dissociates from microtubules, disabling tau's physiological function as a microtubule-associated protein and thereby contributing to cytoskeleton instability [as an aside, it's worth noting that recent data suggest tau functions as more than just a microtubule-associated protein and that tau loss-of-function can contribute to a broader array of cellular defects than previously thought, including brain insulin resistance (Marciniak et al., 2017)]. Second, hyperphosphorylated tau aggregates into neurotoxic oligomers that exert further harmful effects on the cell, such as inducing mitochondrial dysfunction, oxidative stress, neuroinflammation, and apoptosis (Götz et al., 2013; Nilson et al., 2017; Shafiei et al., 2017; **Figure 1B**iii).

Experiments conducted in two different animal models of AD, GSK3β mice and APP mice, build a strong case for the serial connection amongst Aβ, GSK3β, and p-tau. First, conditional overexpression of *GSK3β* in the cortices and hippocampi of adult mice has been shown to reduce levels of nuclear β-catenin and increase levels of p-tau (Lucas et al., 2001). The GSK3β-induced increase in p-tau pathology is further associated with an increase in neuronal apoptosis and performance deficits in the Morris water maze test of spatial memory (Lucas et al., 2001; Hernández et al., 2002). Second, mice overexpressing APP have increased Aβ and p-tau loads, along with memory deficits. However, inhibition of GSK3β in these APP mice is sufficient to protect against p-tau pathology and against cognitive impairment (Rockenstein et al., 2007). The neuroprotective and anti-p-tau effects of GSK3β inhibition in the APP mouse model have been replicated by multiple independent groups (Fiorentini et al., 2010; Pan et al., 2018). In short, the two murine models suggest that GSK3β/Tau Kinase I, a central player in Wnt-signaling, links the Aβ and p-tau pathologies of AD.

α-SYNUCLEIN

Human Neuropathological and Genetic Data Suggest Overlapping Pathology Between AD and PD

Neither AD nor PD are monolithic disease entities; it is likely that each is composed of several subtypes that have yet to be effectively characterized. At least some of the putative AD subtypes overlap in pathology with those of PD, and vice versa. More than half of patients with AD present with Lewy bodies, aggregates of α-synuclein that are the PD equivalent of Aβ plaques (Moussaud et al., 2014; Yan et al., 2018). Furthermore, α-synuclein is a component of AD plaques themselves. In fact, the creatively named non-Aβ component (NAC) of plaques is a fragment of α-synuclein (Uéda et al., 1993; Jakes et al., 1994). Thus, α-synuclein lesions are present in the AD brain as distinct Lewy body structures and as part of amyloid plaques.

Complementarily, classic AD inclusions are observed in the PD brain. Specifically, in PD patients, p-tau tends to aggregate in the substantia nigra and other PD-associated brain regions (Kotzbauer et al., 2004; Wills et al., 2010; Moussaud et al., 2014; Yan et al., 2018). This presence of p-tau tangles also correlates with increased GSK3β activity, an observation that suggests GSK3β may be responsible for tau phosphorylation in PD, as it is in AD (Duka et al., 2009; Nagao and Hayashi, 2009; Wills et al., 2010; Golpich et al., 2015; Lazzara and Kim, 2015). An extension of this logic is that dysfunctional Wnt-signaling may be a convergence point for the world's two most common neurodegenerative disorders.

The genetic evidence also suggests that GSK3β, tau, and α-synuclein can synergistically interact in neurodegeneration. As in AD, polymorphisms in the genes that code for GSK3β and tau (*MAPT*) are risk factors for PD (Kwok et al., 2005; Goris et al., 2007; Schaffer et al., 2008; Moussaud et al., 2014; Golpich et al., 2015). Furthermore, there is a genetic interaction between *MAPT* and the α-synuclein gene (*SNCA*) in which the high-expression *MAPT* haplotype (H1) and a polymorphism in *SNCA* synergistically increase PD risk (Goris et al., 2007). Notably, in this study, only PD patients with the H1/H1 *MAPT* haplotype went on to develop PD with dementia, hinting that this may be an instance in which the pathology and symptoms of a PD subtype overlap with those more typical of AD (Goris et al., 2007).

More relevant to this review article, the *SNCA* gene also affects AD risk. Some *SNCA* polymorphisms double the risk of AD (Matsubara et al., 2001; Wang et al., 2016), whereas others decrease the risk of AD (Xia et al., 1996). With respect to the latter, a retrospective study conducted by Xia et al. (1996) showed that a particular allele in the *SNCA* promoter was enriched 4-fold in cognitively healthy *ApoE4* carriers as compared to *ApoE4* carriers with AD, suggesting that this *SNCA* polymorphism has a protective effect against the strongest known risk factor for AD. This interaction was dose-dependent as the presence of the *SNCA* allele decreased AD risk by 3-fold in *ApoE4* heterozygotes and by 10-fold in *ApoE4* homozygotes (Xia et al., 1996). The fact that *SNCA* mutations affect AD risk is consistent with the hypothesis that α-synuclein plays a role in the development of AD, at least in some instances.

α-Synuclein Induces Amyloid Pathology, Possibly in a Wnt/GSK3β-Dependent Manner, and Is in Positive Feedback With Aβ

Studies using cultured neurons have demonstrated that either exogenous treatment with α-synuclein or α-synuclein overexpression is sufficient to increase the production and secretion of Aβ (Majd et al., 2013; Roberts et al., 2017). One mechanism by which α-synuclein could increase Aβ levels is by activating GSK3β, as suggested by mouse experiments that show that α-synuclein overexpression increases GSK3β activity (Duka et al., 2009; Golpich et al., 2015). Exactly how α-synuclein activates GSK3β is a matter that requires further investigation; however, several lines of *in vitro* and mouse data imply that α-synuclein in neurons could induce GSK3β-activating ROS (Xu et al., 2002; Witt and Flower, 2006; Wakatsuki et al., 2011, 2015; Perfeito et al., 2017; **Figure 2A**i) and decrease the production neuroprotective canonical Wnt ligands by astrocytes (L'Episcopo et al., 2011, 2013, 2014; Okamoto et al., 2011; Lindström et al., 2017; Liu et al., 2018; **Figure 2A**ii; for an excellent review of the role of Wnt-signaling in neuron-microglia-astrocyte crosstalk in neurodegeneration, see L'Episcopo et al., 2018). Although the dominant mechanism by which α-synuclein induces GSK3β *in vivo* is unclear, the observation that intracranial injections of α-synuclein increase β-secretase and Aβ levels in mice (Roberts et al., 2017) is, at minimum, consistent with the model presented in **Figure 1B** and with the hypothesis that α-synuclein-induced Aβ production is mediated by the Wnt/GSK3β axis.

In turn, exogenous treatment with Aβ, even at concentrations as low as 1 μM, has been shown to increase α-synuclein levels in neurons (Majd et al., 2013). Although the mechanisms by which Aβ reciprocally induces α-synuclein likewise remains a gap in the literature, it is worth noting that upregulation of Wnt-signaling via β-catenin overexpression or GSK3β inhibition protects PD models from developing α-synuclein pathology and motor deficits (Yuan et al., 2015; Stephano et al., 2018). Furthermore, *in vitro*, *Drosophila*, mouse, and human data collectively suggest that GSK3β specifically phosphorylates Ser129 of α-synuclein (**Figure 2A**1), a post-translational modification predominant in Lewy bodies and in the PD brain that may enhance α-synuclein aggregation and/or neurotoxicity (Fujiwara et al., 2002; Chen and Feany, 2005; Anderson et al., 2006; Credle et al., 2015). GSK3β is also a known inhibitor of autophagy (Parr et al., 2012; Ren et al., 2016; Weikel et al., 2016), a ubiquitous cellular recycling process required for the effective clearance of excess α-synuclein (Vogiatzi et al., 2008; Sato et al., 2018; **Figure 2A**2). Therefore, it

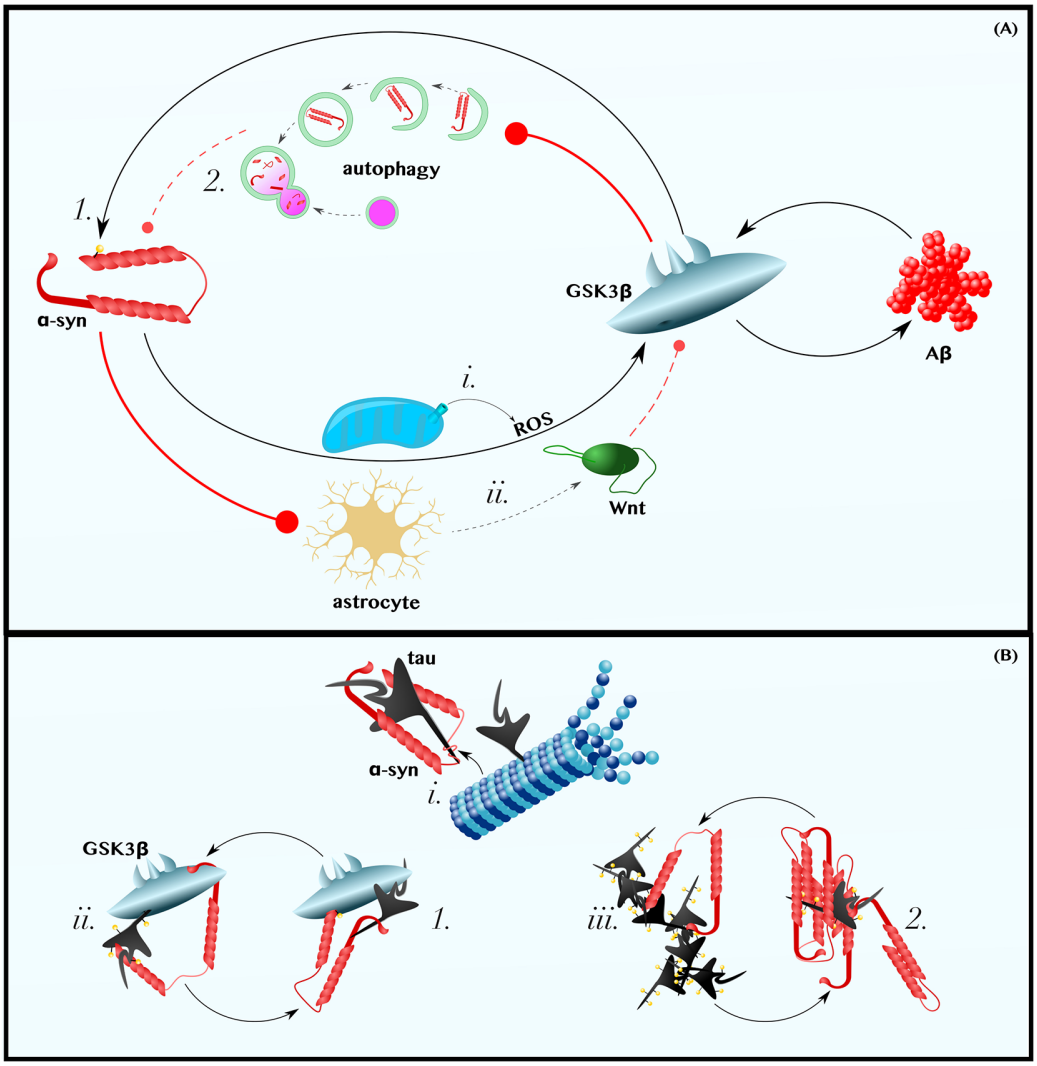

FIGURE 2 | α-Synuclein is in positive feedback with the Aβ and tau pathologies of AD. **(A)** α-Synuclein is in Positive Feedback with Aβ—α-synuclein may induce oxidative stress and promote astrocytic dysfunction. Thus, perhaps by (i) increasing the levels of cytoplasmic ROS and (ii) decreasing those of extracellular astrocyte-derived Wnt ligands, α-synuclein activates GSK3β and induces the production of Aβ (for a more comprehensive discussion about the role of Wnt-signaling in neuron-glia crosstalk in neurodegeneration, see L'Episcopo et al., 2018). In turn, Aβ activates GSK3β, which (1) phosphorylates α-synuclein on Ser129 and (2) may impair the autophagic clearance of α-synuclein. **(B)** α-Synuclein is in Positive Feedback with Tau—α-synuclein can (i) bind tau's microtubule-binding domain, causing tau to disassociate from microtubules, (ii) recruit GSK3β to tau and, thereby, promote tau hyperphosphorylation, and (iii) directly seed or chaperone the pathological aggregation of p-tau. Tau can reciprocate by (1) recruiting GSK3β to α-synuclein, thereby permitting pathogenic Ser129 phosphorylation, and by (2) promoting the aggregation of α-synuclein. Dashed and solid lines indicate regulatory mechanisms that are, respectively, impaired and enhanced in AD.

is plausible that Aβ-induced GSK3β activation (**Figure 1B**) completes an α-synuclein-Aβ feedback loop relevant in some cases of AD.

α-Synuclein, Directly and via GSK3β, Induces Tauopathy and Is in Positive Feedback with p-tau

α-synuclein and tau interact directly (Jensen et al., 1999; Yan et al., 2018). Specifically, α-synuclein binds tau within tau's microtubule-binding domain (Jensen et al., 1999). Even were this interaction not sufficient to cause tau to disassociate from microtubules, the binding of α-synuclein to tau induces the phosphorylation of tau on Ser262, a post-translational modification observed in the AD brain that causes tau to release from microtubules, contributing to cytoskeleton instability (Jensen et al., 1999). Subsequently, α-synuclein can serve as a necessary cofactor to help p-tau form oligomers and, eventually, tangles (Giasson et al., 2003; Cremades et al., 2012). Thus, as reviewed by Moussaud et al. (2014), there are at least three ways by which α-synuclein can instigate and aggravate tauopathy: by blocking the interaction between tau and microtubules, thereby interfering with tau's physiological function (**Figure 2B**i), by recruiting kinases that promote tau hyperphosphorylation

(**Figure 2B**ii), and by seeding or chaperoning the aggregation of tau into neurotoxic oligomers and fibrils (**Figure 2B**iii).

With regard to the kinase mechanism listed above, GSK3β/Tau Kinase I may play a particularly important role in the relationship between α-synuclein and tau. Not only does α-synuclein interact with tau, but both proteins also interact with, and are phosphorylated by, GSK3β (Duka et al., 2009; Credle et al., 2015). Thus, α-synuclein can recruit GSK3β to tau, leading to tau hyperphosphorylation (**Figure 2B**ii). As this model predicts, exogenous treatment of cultured cells with α-synuclein increased levels of p-tau, this phenomenon being blocked by the inhibition of GSK3β (Gąssowska et al., 2014). Similar findings have been produced in mice in which the overexpression of α-synuclein is sufficient to induce GSK3β-mediated p-tau pathology (Duka et al., 2009). Reflecting on the stimulatory effect of α-synuclein on GSK3β, as well as that of Aβ on GSK3β (**Figure 1B**), we can elaborate upon our model: GSK3β can be conceptualized as the convergence point of a Y-shaped cascade in which either Aβ or α-synuclein can activate and/or recruit GSK3β to induce tau pathology.

Similar to the mutualistic case of Aβ and α-synuclein, p-tau can promote α-synuclein pathology (Giasson et al., 2003; Badiola et al., 2011; Yan et al., 2018). Using multiple different cell models, Badiola et al. (2011) demonstrated that tau enhanced the aggregation of α-synuclein. In these experiments, tau overexpression also reduced cell viability in an α-synuclein-dependent manner (Badiola et al., 2011), perhaps by promoting the GSK3β-mediated neurotoxic phosphorylation of α-synuclein on Ser129 (Fujiwara et al., 2002; Chen and Feany, 2005; Anderson et al., 2006; Credle et al., 2015), and promoted the secretion of α-synuclein (Badiola et al., 2011). Thus, tau can complete an intracellular positive feedback loop with α-synuclein, possibly by facilitating the pathogenic phosphorylation of α-synuclein Ser129 by GSK3β (**Figure 2B**1) and/or by promoting α-synuclein's aggregation (**Figure 2B**2), and tau might also support the prionic cell-to-cell propagation of α-synuclein (not shown in **Figure 2**). Independent of the exact mechanisms, the relevance of tau on α-synuclein pathology and its attending symptoms has been demonstrated *in vivo*. In mice, the transgenic expression of tau enhances the formation of α-synuclein inclusions and the corresponding Parkinsonian phenotype (Giasson et al., 2003).

TYPE 3 DIABETES

Overview of Insulin Signaling and Its Role in the Brain

Several lines of evidence suggest that, in the central nervous system, insulin does much more than promote glucose uptake. Insulin is a neuromodulator, affecting the reuptake and production of particular neurotransmitters (Schulingkamp et al., 2000; Plum et al., 2005); insulin regulates food intake and reproduction by acting on the hypothalamus to alter endocrine system function (Plum et al., 2005); and, glucose transport into neurons is largely insulin-independent. Building upon this last key piece of evidence, neuron energy utilization also correlates poorly with the heterogeneous distribution of Insulin Receptor (IRs) throughout the brain, further suggesting that insulin's primary functions in the brain include more than glucose uptake (Schulingkamp et al., 2000). And, although IRs are also concentrated in the hypothalamus, olfactory bulb, and cerebellum, it's notable that IRs are particularly densely packed in the hippocampus and cerebral cortex, two brain regions important in learning and memory that are critically impacted by AD (Marks et al., 1990; Schulingkamp et al., 2000; Plum et al., 2005).

The insulin signaling cascade is initiated when insulin binds to the IR, a heterotetrameric receptor tyrosine kinase that autophosphorylates in order to recruit the adaptor protein IR Substrate (IRS). IRS subsequently recruits and activates Phosphoinositide 3-Kinase (PI3K), a lipid kinase that generates the second messenger Phosphatidylinositol (3,4,5)-trisphosphate (PIP_3). PIP_3 can diffuse along the membrane to activate Phosphoinositide-Dependent Kinase 1 (PDK1), which phosphorylates and activates the terminal kinase in the core of this cascade, AKT (De Meyts, 2000; **Figure 3A**).

AKT regulates an expansive set of pathways and processes, only some of which will be discussed in the following section. AKT (i) regulates translocation of GLUT3, the canonical neuronal glucose transporter, and of GLUT4, which is also essential in neurons (Ashrafi et al., 2017), to the plasma membrane (Grillo et al., 2009; Ferreira et al., 2011). At the axon terminal and post-synaptic density, the insulin-AKT pathway (ii) modulates catecholamine release and uptake, the trafficking of ion-gated channels, and the expression and localization of neurotransmitter receptors (Chiu et al., 2008; De Felice and Benedict, 2015). Finally, AKT (iii) is a potent GSK3β inhibitor (Zhou et al., 2014; **Figure 3B**). Each of these mechanisms will be discussed further in the following subsections.

Lack of Energetic Substrates as an Exacerbating Factor for AD

Even preclinically, patients with AD show widespread impairment in glucose metabolic rates (Willette et al., 2015), a deficiency associated with decreased levels of GLUT1 and GLUT3 (Liu et al., 2008), which import glucose across the blood-brain barrier and into neurons, respectively. As the brain can only use either glucose or ketones, and ketones are not normally available as a fuel, insulin resistance and the ensuing decrease in GLUT membrane expression (**Figure 3B**i) can decrease mitochondrial ATP production and all ATP-dependent maintenance processes that are critical to neuron survival (Fong et al., 2016; Blonz, 2017).

Animal models support the relevance of GLUT transporter underexpression in AD, as well as the potential involvement of dysfunctional Wnt-signaling in this process. For example, overexpression of GLUT3, which is regulated, in part, by AKT (Ferreira et al., 2011), helps rescue *Drosophila* from the morphological and behavioral features associated

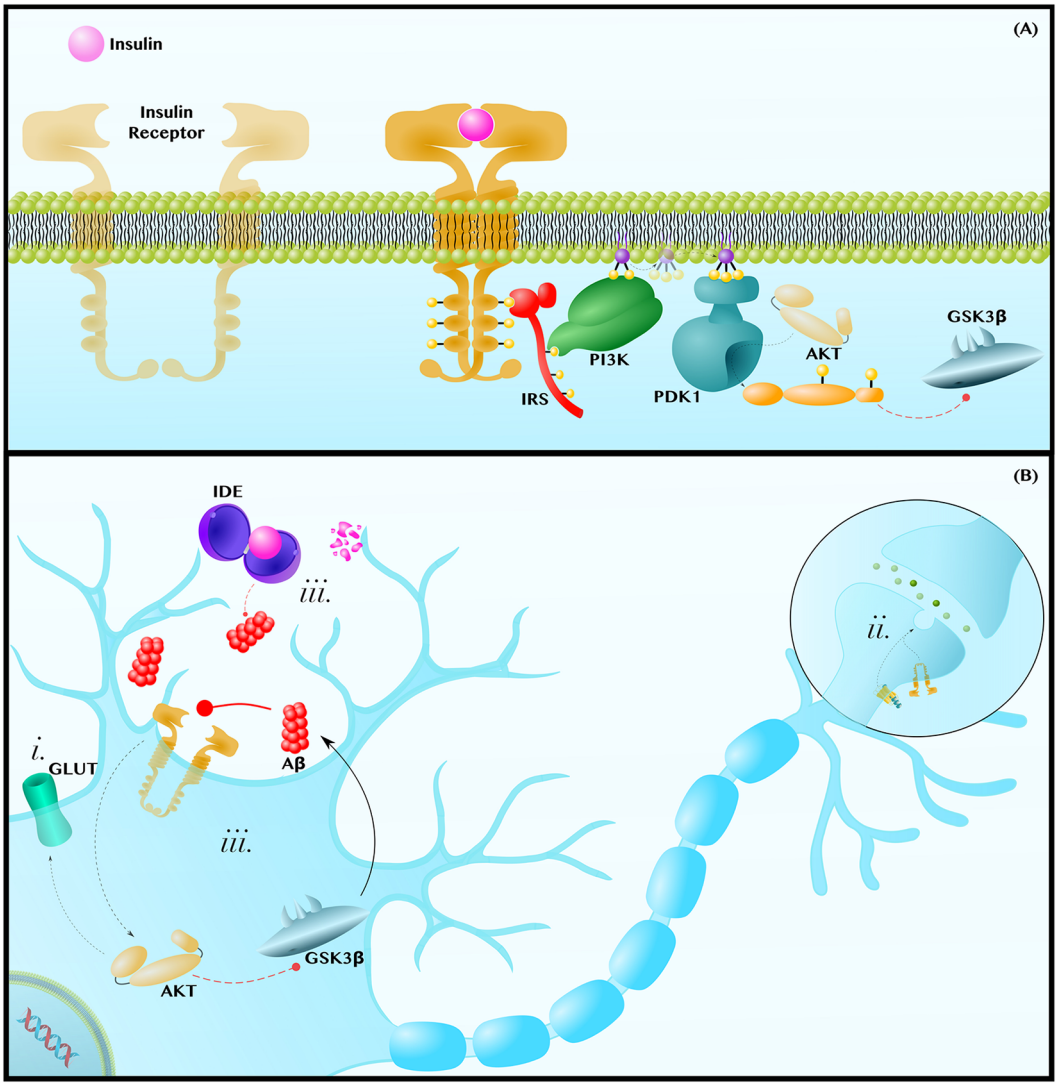

FIGURE 3 | Insulin resistance exacerbates the pathology of AD. **(A)** Insulin-AKT Pathway—Insulin binds to the Insulin Receptor (IR) tyrosine kinase, which autophosphorylates and binds the adaptor protein, Insulin Receptor Substrate (IRS). IRS recruits Phosphoinositide 3-Kinase (PI3K), which phosphorylates PIP_2 into PIP_3. PIP_3 diffuses along the membrane to activate Phosphoinositide-Dependent Kinase 1 (PDK1), which activates AKT. AKT phosphorylates many enzymes; this includes inhibiting GSK3β. **(B)** Insulin Resistance Contributes to Neuropathology—Insulin resistances (i) causes a decrease in AKT-mediated translocation of GLUT transporters to the membrane. This contributes to the decreased glucose metabolic rate and mitochondrial dysfunctions observed in AD and PD brains. Insulin-AKT signaling is critical in synaptic transmission, as is Wnt-signaling. Therefore, insulin resistance (ii) may synergize with dysfunctions in Wnt-signaling to decrease synaptic transmission and synapse integrity. Lastly, insulin resistance (iii) can contribute to hyperinsulinemia and the competitive inhibition of Insulin Degrading Enzyme (IDE), which also degrades Aβ. Since Aβ inhibits insulin-AKT signaling, either insulin or Aβ can establish a positive feedback loop in which Aβ inhibits insulin signaling to decrease AKT activity, increase GSK3β activity and, thus, further increase Aβ levels. Dashed and solid lines indicate regulatory mechanisms that are, respectively, impaired and enhanced in AD.

with Aβ toxicity (Niccoli et al., 2016). Furthermore, in a mouse model of AD, Nishida et al. (2017) demonstrated that decreased GLUT1 expression at the blood-brain barrier was associated with decreased cerebral blood flow, increased Aβ accumulation, and memory impairment. Interestingly, Wnt-signaling has been identified as necessary for GLUT1 expression at the blood-brain barrier (Daneman et al., 2009), and Pan et al. (2018) showed that inhibition of GSK3β in AD mice has precisely the opposite effects to those just described in that GSK3β inhibition increased cerebral blood flow, prevented Aβ accumulation, and rescued memory impairment. The complementary findings of the two mouse studies, in combination with the fact that Wnt ligands have been observed to increase AKT activity and neuronal glycolytic rate (Cisternas et al., 2016), hints at the possibility that dysfunctions in the insulin-AKT and Wnt-signaling pathways may cooperate to contribute to glucose metabolism deficiency in AD.

Insulin Resistance and Wnt-Signaling in Synaptic Dysfunction

As insulin regulates the release and reception of neurotransmitters, cerebral insulin resistance can contribute to a decrease in synaptic activity and density (Abbott et al., 1999; Chiu et al., 2008; Lee et al., 2011; De Felice and Benedict, 2015; **Figure 3B**ii). In *Xenopus* tadpoles, the expression of a dominant-negative IR decreased excitatory post-synaptic potentials and synaptic density (Chiu et al., 2008). Conversely, activation of the insulin-AKT axis, by pharmacologically stimulating AKT or PI3K, increased synaptic density and rescued aberrant synaptic plasticity in wildtype and AD rodents (Cuesto et al., 2011; Yi et al., 2018).

At the synapse, the effects of dysfunctional Wnt-signaling have been shown to be analogous to those of dysfunctional insulin-signaling. Specifically, blocking the initiation of Wnt-signaling with DKK1 induced synaptic loss in mice (Purro et al., 2012; Marzo et al., 2016). Furthermore, as with AKT activation (Yi et al., 2018), direct pharmacological activation of Wnt-signaling was sufficient to rescue aberrant synaptic plasticity (Purro et al., 2012; Marzo et al., 2016). This, along with the suggestion of crosstalk between the Wnt and AKT pathways (Palsgaard et al., 2012; Cisternas et al., 2016), raises the possibility that insulin resistance and dysfunctional Wnt-signaling may interact to induce synaptic dysfunction in cognitive decline.

Insulin Resistance and Aβ Can Establish a Wnt/GSK3β-Dependent Positive Feedback Loop

Insulin Degrading Enzyme (IDE) is a cytoplasmic and secreted enzyme that degrades both insulin and Aβ in the human brain (Qiu et al., 1998; Pérez et al., 2000). Accordingly, hyperinsulinemia, which is associated with an approximately two-fold increase in AD risk (Luchsinger et al., 2004), can competitively inhibit IDE-mediated Aβ degradation (Qiu et al., 1998; Pérez et al., 2000; Farris et al., 2003; Neth and Craft, 2017). In turn, Aβ can exacerbate hyperinsulinemia by inhibiting IDE and competing for IR binding (Pérez et al., 2000; Zhao et al., 2008; O'Neill, 2013).

But, even in those cases in which cerebral hyperinsulinemia does not initiate the accumulation of Aβ, a vicious cycle between Aβ and insulin-AKT signaling can arise once some degree of amyloid pathology has been established (**Figure 3B**iii). The De Felice group has shown that intracerebroventricular infusion of Aβ oligomers in monkeys disrupts insulin-AKT signaling in the hippocampus in a TNFα-dependent manner, leading to memory impairment (Lourenco et al., 2013). In this way, Aβ releases GSK3β from AKT-mediated inhibition and, reciprocally, GSK3β increases Aβ production via the mechanisms displayed in **Figure 1B**.

It is also notable that the De Felice group later showed that intracerebroventricular infusion of Aβ oligomers caused hypothalamic dysfunction and peripheral insulin resistance in mice, again in a TNFα-dependent manner. This latter finding, in conjunction with epidemiological data showing AD increases an individual's risk of developing T2DM, suggests yet another pathological feedback loop in which systemic insulin resistance increases Aβ production, leading to Aβ-mediated hypothalamic inflammation that further exacerbates systemic insulin resistance (Clarke et al., 2015).

The AKT Paradox

Obviously, **Figure 3** is a simplification of insulin resistance pathology in the AD brain. What is not as obvious is how it is a simplification. Not only are pathways and relationships among proteins necessarily omitted, but there is also a lack of consensus on the fundamental nature of key relationships. An important and illustrative example is that AKT may be either underactive or overactive in the post-mortem human AD brain (Rickle et al., 2004; Lee et al., 2009).

While this AKT paradox remains to be resolved, one hypothesis is that the opposite dysfunctions in AKT activity are time-dependent. For example, intracellular and extracellular Aβ may have different effects on AKT activity, with intracellular Aβ (not explicitly shown in **Figure 3B**) accumulating well before extracellular Aβ (Magrané et al., 2005). Intracellular Aβ can interfere with the interaction between PDK1 and AKT, contributing to a decrease in AKT activity and to disease progression (Magrané et al., 2005; Lee et al., 2009). However, as extracellular Aβ builds up later, a tipping point [possibly one that is neuron-specific and heterogenous across the brain (Rickle et al., 2004)] may be reached whereby Aβ binds to IRs and constitutively overstimulates AKT (Xie et al., 2002; Zhao et al., 2008; Chiang et al., 2010). Rather than being neuroprotective, this 180° flip may be pathogenic in other ways, including saturating pathway activity, such that the pathway is no longer responsive to insulin, and inducing mTOR1-mediated IRS inhibition, thus reinforcing insulin resistance (Zhao et al., 2008; Han et al., 2018). Moreover, Aβ binding to IRs causes a dramatic migration of IRs away from neurites to the soma (Zhao et al., 2008), impairing synaptic integrity and compounding spatial complexity on top of temporal complexity.

Evidently, the AKT paradox adds a major qualification to the model presented in **Figure 3B**, which we presented as is for the following two reasons: (1) decreased GLUT transporter expression, decreased synaptic integrity, and increased GSK3β activity have been more consistently observed in the AD brain (Leroy et al., 2007; Liu et al., 2008; Llorens-Martín et al., 2014; Wan et al., 2014) and (2) pharmacological activators of AKT have demonstrated therapeutic efficacy in *Drosophila* and mouse models of AD (Zhang et al., 2016; Yi et al., 2018), whereas the same cannot be said for AKT inhibitors. It is important to acknowledge the AKT paradox as a representative example of the nuance present within even a single model of AD. Appreciating this nuance will help us better appreciate the true complexity of AD that arises out of an interrelationship among the models.

AN INTEGRATED PERSPECTIVE AND CONCLUDING REMARKS

In this review article, we began by summarizing the cellular, animal, and human work that demonstrate dysfunctional

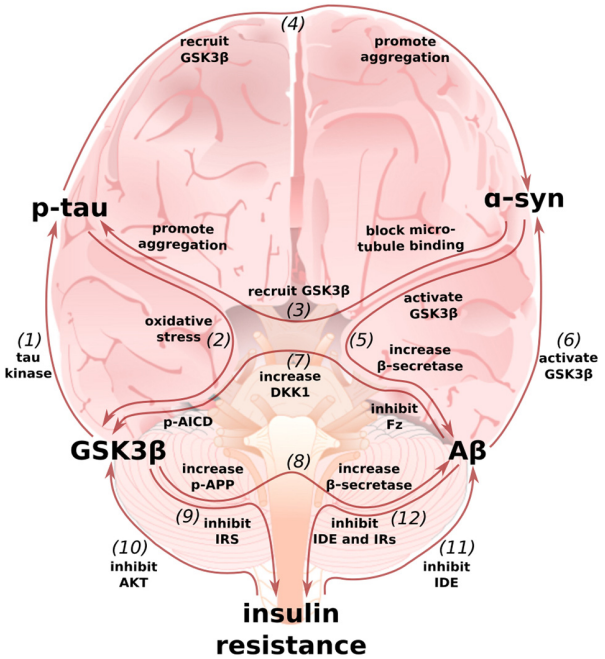

FIGURE 4 | Multi-loop model of AD: an integrated perspective on the Wnt/GSK3β, α-synuclein, and type 3 diabetes hypotheses. (1) GSK3β, also known as Tau Kinase 1, phosphorylates tau (De Ferrari et al., 2014). (2) In turn, p-tau may increase GSK3β activity by inducing oxidative stress (Cente et al., 2006; Feng et al., 2013; Liu et al., 2015). (3) α-synuclein can also contribute to tau pathology by binding to tau's microtubule binding domain (Jensen et al., 1999), recruiting GSK3β to tau, and helping to promote pathological p-tau aggregation (Gąssowska et al., 2014; Moussaud et al., 2014). (4) In reciprocation, tau can promote α-synuclein's phosphorylation by GSK3β and α-synuclein aggregation (Giasson et al., 2003; Credle et al., 2015). (5) In addition to facilitating tauopathy, α-synuclein can promote Aβ production by increasing GSK3β activity (Duka et al., 2009) and β-secretase levels (Roberts et al., 2017). (6) In turn, Aβ can increase α-synuclein levels (Majd et al., 2013), possibly by stimulating GSK3β (Yuan et al., 2015). (7) Aβ can simulate GSK3β activity by inducing the expression of DKK1 and by binding to and inhibiting Frizzled (Caricasole et al., 2004; Magdesian et al., 2008). p-AICD, a by-product of Aβ production, can increase *GSK3β* gene expression (Chang et al., 2006). (8) GSK3β phosphorylates APP to enable p-AICD production and to make APP a better substrate for β-secretase (Lee et al., 2003; Acevedo et al., 2014). GSK3β overactivity and Wnt-signaling underactivity also increase β-secretase levels, further promoting the generation of Aβ (Ly et al., 2013; Parr et al., 2015). (9) GSK3β can contribute to insulin resistance by phosphorylating and inhibiting IRS1 (Lee and Kim, 2007). (10) In turn, insulin-AKT pathway dysfunction can contribute to an increase in GSK3β activity (Magrané et al., 2005; Lee et al., 2009). (11) Because insulin and Aβ are both IDE substrates, hyperinsulinemia prevents Aβ degradation (Qiu et al., 1998; Pérez et al., 2000; Farris et al., 2003; Neth and Craft, 2017; Folch et al., 2018). (12) Aβ can then further exacerbate insulin resistance by preventing insulin degradation and by binding to IRs (Pérez et al., 2000; Zhao et al., 2008; O'Neill, 2013). The above figure shows only mechanisms whereby these models feedback on one another and not those additional mechanisms whereby they cooperate to intensify AD pathology, such as may be the case for glucose transporter expression and synaptic activity (Chiu et al., 2008; Daneman et al., 2009; Ferreira et al., 2011; Purro et al., 2012; Marzo et al., 2016).

Wnt-signaling can contribute to the development of AD and its two pathological hallmarks, Aβ plaques and p-tau tangles. We next described how the canonical PD-associated protein α-synuclein may be locked in pathological positive feedback loops with Aβ and tau. Finally, we discussed some of the mechanisms by which insulin resistance in the brain, "type 3 diabetes," may contribute to development and exacerbation of AD. Throughout each section, we attempted to highlight some of the ways in which each model interacts with the others. These interrelationships, summarized in **Figure 4**, make it clear that the pathology of AD is not a linear cascade, nor a simple feedback loop, but rather a network of cross-talking models and overlapping vicious cycles.

Given the cooperative and reinforced nature of this complex network, it is no surprise that the prototypical monotherapeutic approach to AD has reliably failed. Certainly, drugs that target key nodes within the network, such as GSK3β inhibitors (Noble et al., 2005; Parr et al., 2012; Licht-Murava et al., 2016) or AKT activators (Zhang et al., 2016; Yi et al., 2018), have shown promise in animal models, and this important work affords us valuable mechanistic insights. However, these pre-clinical successes generally have not translated into clinical success, at least not with the same degree of efficacy. This is likely because animal models harboring distinct AD-causing mutations and dysfunctions in particular linear pathways do not accurately recapitulate the complex pathologies underlying sporadic human AD. In brief, we are proposing that the single-target silver-bullet approach to AD drug discovery is doomed to fail and that we may only be able to treat or prevent AD by developing new multifaceted treatment options.

Further complicating matters, the initial movers of sporadic human AD are likely highly individual. As examples, only about half of AD patients present with Lewy Body/α-synuclein pathology (Yan et al., 2018) and there is evidence to suggest that diabetes may specifically predispose carriers of the *ApoE4* risk allele to develop AD (Zhao et al., 2017; Folch et al., 2018). If AD is, indeed, composed of many different subtypes, then even imagining AD as a network of reinforcing positive feedback loops, as we have done here, underestimates the pathology. We may not only need multifaceted treatment options, but personalized ones.

The cost of continuing to simplify AD pathology is a continuation in the rapidly rising prevalence of AD. It is, therefore, critical that the global biomedical community take steps towards thinking more comprehensively about the mechanisms underlying AD, for only by doing so can we hope to develop multifaceted, and perhaps one day individualized, therapies to prevent or treat this devastating disease and reverse the worldwide neurodegeneration epidemic.

AUTHOR CONTRIBUTIONS

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

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REVIEW
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Microglia in Alzheimer Disease: Well-Known Targets and New Opportunities

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Microglia are the resident macrophages of the central nervous system. They play key roles in brain development, and physiology during life and aging. Equipped with a variety of molecular sensors and through the various functions they can fulfill, they are critically involved in maintaining the brain's homeostasis. In Alzheimer disease (AD), microglia reaction was initially thought to be incidental and triggered by amyloid deposits and dystrophic neurites. However, recent genome-wide association studies have established that the majority of AD risk loci are found in or near genes that are highly and sometimes uniquely expressed in microglia. This leads to the concept of microglia being critically involved in the early steps of the disease and identified them as important potential therapeutic targets. Whether microglia reaction is beneficial, detrimental or both to AD progression is still unclear and the subject of intense debate. In this review, we are presenting a state-of-knowledge report intended to highlight the variety of microglial functions and pathways shown to be critically involved in AD progression. We first address both the acquisition of new functions and the alteration of their homeostatic roles by reactive microglia. Second, we propose a summary of new important parameters currently emerging in the field that need to be considered to identify relevant microglial targets. Finally, we discuss the many obstacles in designing efficient therapeutic strategies for AD and present innovative technologies that may foster our understanding of microglia roles in the pathology. Ultimately, this work aims to fly over various microglial functions to make a general and reliable report of the current knowledge regarding microglia's involvement in AD and of the new research opportunities in the field.

Keywords: Alzheimer disease, microglia, neuroinflammation, microglia diversity, purinergic signaling, sexual dimorphism, early stage, hiPSCs

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INTRODUCTION

Microglia cells are the main immunocompetent cells in the brain. They colonize the brain in the early prenatal period (Ginhoux et al., 2010), but contrary to other tissue resident macrophages, they remain secluded within the CNS throughout life and self-renew at slow pace (Ajami et al., 2007). Importantly, the CNS microenvironment significantly shapes the microglia's phenotype, endowing them with specific important homeostatic and supportive brain functions (Kierdorf and Prinz, 2017). Should the brain homeostasis be compromised, microglia change their phenotype and initiate a defense program. Thus, under pathological conditions, they adopt reactive states

characterized by multiple morphological and functional changes including but not limited to increased phagocytosis and increased expression of receptors, cytokines, chemokines and additional inflammation related molecules (Wolf et al., 2017).

Alzheimer's disease (AD) classical hallmarks include brain atrophy, extracellular amyloid-beta (Aβ) deposits, intracellular aggregated phosphorylated tau, dystrophic neurites, synapses and neurons loss (Bedner et al., 2015). The presence of reactive glial cells within the neuritic plaques was described by Alois Alzheimer himself (Alzheimer, 1907; Graeber et al., 1997) and further studies identified both reactive astrocytes and microglia in the vicinity of the Aβ deposits (Verkhratsky et al., 2016). Long considered as a consequence of the pathology, reactive glia and associated neuroinflammation are now regarded as playing key roles in both disease initiation and progression. Indeed, Human genetic studies identified over 25 genetic loci that robustly associate with AD risk (Hansen et al., 2018; Verheijen and Sleegers, 2018). Among them, most of the common (ApoE, Sp1l) or rare (Trem2, Cd33) genetic variants code for proteins that are preferentially or exclusively expressed in microglia. These findings strongly support a causal involvement of microglial cells in AD pathogenesis and generated a strong interest for studying these cells in AD. Yet, the roles of microglia in AD initiation and progression are unclear and heavily debated, with conflicting reports regarding their detrimental or protective contribution to the disease.

In the present review, we have summarized the main findings regarding the role of microglia in AD. Microglia reaction is known to be associated with the acquisition of many immune functions which are triggered by the activation of receptors designed to recognize danger or pathogen associated molecular patterns (DAMPs/PAMPs). Its role is to restore homeostasis and is also associated with the loss or the alteration of homeostatic functions which are important for brain physiological functioning. In the two first parts, we thus provide an overview of the microglial functions and pathways that are known to be altered during AD. We then highlight factors such as mouse models, sex, age whose influence may have been under-examined in assessing the contribution of microglial cells to the disease progression. Finally, we identified new research topics that are likely to foster our understanding of the roles of microglia to AD initiation and progression and may help design more targeted therapeutic strategies.

NEW FUNCTIONS FOR REACTIVE MICROGLIA IN AD

Neuroinflammation is a common feature of neurodegenerative diseases and inflammatory processes are thus among the most studied microglial functions in AD. Microglia, which represent the main immune cells of the brain, have been shown to play key roles in orchestrating this brain inflammation. In the following section, we are reporting the main microglial processes involved in neuroinflammation (**Figure 1**, top part). However, more detailed description of these processes can be found in recent reviews that are focusing on these specific points (Labzin et al., 2017; Nizami et al., 2019).

Inflammasomes Are Central Hubs for Cytokines Production

One of inflammation hallmarks is the release of cytokines. This secretion requires the activation of inducible multiproteic complexes called inflammasomes. Several inflammasomes have been characterized but the most important microglial contributor in pathologies is certainly the NLRP3. It is composed of the sensor protein NLRP3 and the adaptor protein apoptosis associated Speck-like protein (ASC), which contains a caspase recruitment domain. ASC can recruit and activate pro-caspase-1. When stimulated, the complex induces the cleavage of pro-caspase-1 into active caspase-1, which in turn cleaves pro-IL-1β and IL-18 triggering their release in the extracellular space (Martinon et al., 2002). NLRP3 activation pathway is not fully characterized, but the current view is that NLRP3 activation requires the occurrence of two independent but co-concomitant priming and activation signals (Próchnicki et al., 2016).

In the context of AD, IL-1β is known to elicit the secretion of NO and TNFα, promoting the formation of deleterious amyloid plaques and neuronal degeneration (Griffin et al., 1989). In accordance with these data, frontal cortex from AD patients exhibit an increase of caspase-1 which is correlated with an attenuation of Aβ peptide phagocytosis (Burguillos et al., 2011; Heneka et al., 2013). Likewise, genetic deletion of NLRP3 in mice with familiar AD associated mutation reduces the level of IL-1β and Aβ deposits, and correlates with positive impacts on synaptic dysfunction and cognitive performances (Heneka et al., 2013).

Other members of the caspase family are also involved in AD pathology. Interestingly, the activity of caspase-3 prematurely increases in hippocampal neurons in an AD mouse model (D'Amelio et al., 2011). Similarly, in samples from AD patients, caspase-8 and -3 are upregulated in cortical microglia (Burguillos et al., 2011). Microglial caspase-3 around amyloid plaques also present a cytosolic location, suggesting a non-apoptotic role of caspases in AD (Burguillos et al., 2011). Such evidence has also been described in others degenerative models such as brain ischemia, in which reactive astrocytes and microglia express cytoplasmic non-apoptotic caspase-3 (Wagner et al., 2011). This non-nuclear localization could be involved in cytoplasmic rearrangement and modifications of cell populations surrounding lesions. Although it is undeniable that caspase signaling is crucial in the development of AD, the delay of activation and the pathway downstream of each of them need to be clarified.

The Complement System, the Microglial Way of Eating Synapses?

The complement pathway is part of the innate immune system and mediates the recognition and elimination of pathogens and cellular debris. It is involved in many physiological and pathological functions throughout life, including activity-dependent synapse elimination during

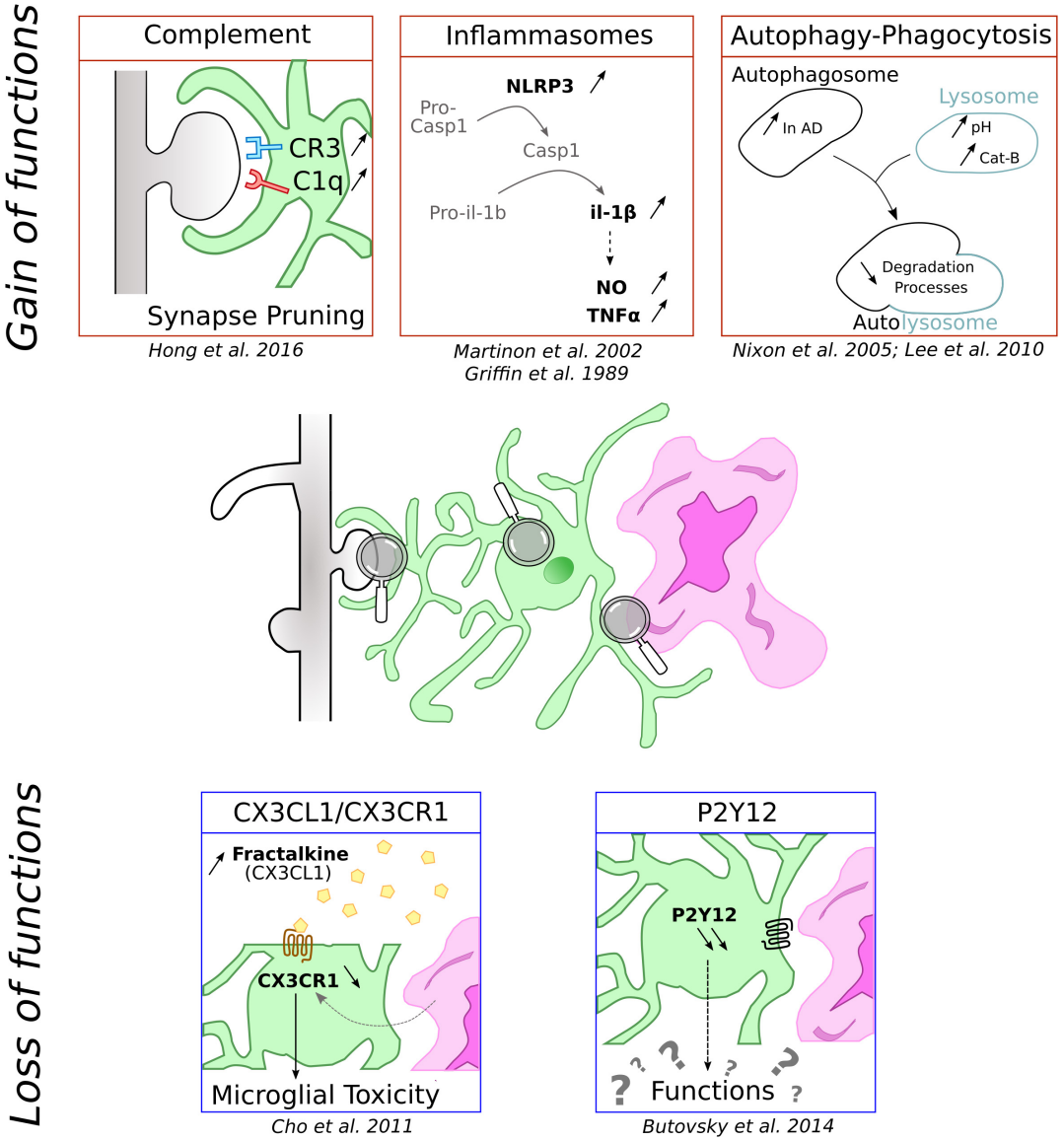

FIGURE 1 | Schematic representation of functions microglial cells can loose and gain in context of AD. Microglia is represented in green associated to amyloid-β deposit in purple and dendritic spines in gray.

development (Schafer et al., 2012). In AD, reactivation of this pathway by Aβ deposits has been associated with synapse loss.

The complement cascade is composed of a large panel of mediators including C1q and C3 complex proteins which can be activated by three different pathways, all of which are capable of triggering phagocytosis (Stephan et al., 2012). In the CNS, complement proteins are expressed in neurons and glial cells, although microglia and astrocytes are the major sources of complement. Particularly, microglia expressed high level of C1q and CR3 (Veerhuis et al., 2011) and microglial CR3 have been shown to be crucial in synapse pruning during development (Schafer et al., 2012).

In AD context, patients showed elevated CSF concentrations of C3 and CR1, pointing out an alteration of complement system in the pathology (Daborg et al., 2012). The complement has been associated with Aβ but it is not clear whether it is protective or detrimental. Indeed, some studies reported that complement inhibition or deficiency results in accelerated amyloid pathology (Wyss-Coray et al., 2002; Maier et al., 2008) and that C3 along with CR3 contribute to Aβ phagocytosis (Fu et al., 2012). On the other hand, other studies pointed out that elimination and modulation of microglial CR3 decrease Aβ level (Czirr et al., 2017) and C3 antagonist ameliorates plaque load (Lian et al., 2016). Further studies are thus required to better understand the role of complement in Aβ pathology.

As AD is marked by an important synapse loss, questions have been raised on whether the complement could mediate such synapse elimination. Fonseca et al. (2004) demonstrated

that C1q deficiency in an AD mouse model partly restores synapse integrity pointing out a role of the complement system in AD. More recently, works from Hong et al. demonstrated that microglial C3/CR3 mediates synapse elimination when challenged with oligomeric Aβ (Hong et al., 2016). More specifically, they found that C1q is upregulated into synapse early in J20 mice and that Aβ oligomers increase C1q and microglia phagocytic activity. This resulted in synapse elimination by microglia, a process which is lost in CR3 KO mice. This study proposes a model in which C1q and oligomer Aβ would activate the complement cascade to drive synapse elimination through microglial CR3.

Eat Me and Eat Myself

Autophagy and phagocytosis are cellular degradation processes, necessary to degrade additional or damaged particles in lysosomes. These processes, ensured by a large enzymatic degradation system, are dysregulated during aging and are of particular importance during AD, as shown with the autophagy failure and the increase of autophagosomes in AD patients (Nixon et al., 2005). Moreover, lysosomal acidification and autophagy are disrupted by Alzheimer-related PS1 mutation (Lee et al., 2010). Numerous studies demonstrate that microglial Aβ phagocytosis contribute to degeneration by triggering NLRP3 and lysosomal cathepsin-B that subsequently results in maturation and release of IL-1β (Halle et al., 2008). Cellular degradation processes could thus, by differently modulating the inflammasome, present opposite effects. It could be protective in normal physiological states and during the premature state of the pathology, and detrimental during chronic and late phases of diseases.

LOSS OF HOMEOSTATIC FUNCTIONS IN REACTIVE MICROGLIA

Although the majority of the studies concentrate on the microglial reactivity-acquired functions and assess their contribution to neurodegeneration, loss of key homeostatic functions may also be detrimental to neuronal functions and may contribute to the detrimental effects of microglia reaction. In the following part, we are reviewing few key microglial functions that are compromised in AD (**Figure 1**, bottom part).

Consequences of CX3CL1/CX3CR1 Signaling Loss in AD

In brain, the CX3CR1 receptor is predominantly expressed in microglia. Its ligand is the secreted soluble form of fractalkine, also named CX3CL1, and is constitutively expressed by neurons. CX3CL1 exerts an inhibitory signal, maintaining microglia in a resting state. CX3CL1-CX3CR1 is a critical signaling pathway during development as shown by the delay of glutamatergic synapse maturation (Paolicelli et al., 2011; Hoshiko et al., 2012) and functional consequences in adult synapses (Basilico et al., 2019) in CX3CR1$^{-/-}$ mice. Age is also a decisive factor in the regulation of CX3CR1 expression as LPS challenge is responsible for a more pronounced impairment of CX3CR1 expression in aged compared to young rats (Lyons et al., 2009).

The roles of CX3CL1/CX3CR1 communication during neuroinflammation are still subject to debate since CX3CR1 deletion effects differ depending on the challenge. In CX3CR1$^{-/-}$ mice and in both PD and ALS models, Cardona et al. (2006) demonstrated an extensive neuronal loss due to an alteration of cytokines production. CX3CR1 decrease is also observed in AD models. In neurodegenerative conditions, this disruption is associated with a strong microglial toxicity and an aggravation of the pathology (Keren-Shaul et al., 2017). The involvement of the CX3CL1/CX3CR1 signaling pathway in AD is confirmed by an increase in the plasmatic concentration of CX3CL1 in AD and MCI patients compared to healthy control subjects (Kim et al., 2008). However, the role of this pathway might be more complex as CX3CR1 deletion was shown to prevent neuronal loss in 3 × Tg AD mice (Fuhrmann et al., 2010) but worsen cellular and behavioral deficits in hAPP-J20 mice (Cho et al., 2011).

All these data point out to critical roles for CX3CL1–CX3CR1 signaling during neurodegenerative diseases, including AD, but also demonstrate that its complex spectrum of responses may depend on the genetic model of the disease.

The Yet Unresolved Role of P2Y12 Down-Regulation in AD

In physiological conditions, P2Y12 receptor is involved in chemotaxis and mice lacking this receptor showed altered microglia migration and polarization (Haynes et al., 2006). *P2ry12* was identified as a unique microglia gene in the CNS (Butovsky et al., 2014). It is one of the most highly expressed genes in microglia and is downregulated in reactive microglia (Haynes et al., 2006). Consequently, *P2ry12* gene expression levels have been proposed to be a marker of the homeostatic microglial signature (Butovsky et al., 2014). In agreement, in AD transgenic mouse model, microglia located at proximity of amyloid plaques do not express P2Y12R whereas the receptor is observed in plaque-distant ones (Butovsky et al., 2014; Jay et al., 2015). Similar findings have also been reported in human AD patients (Sanchez-Mejias et al., 2016; Mildner et al., 2017). However, so far, the consequences of this down-regulation for microglia functions are unknown and merit further attention.

MOST STUDIED MICROGLIAL MOLECULAR TARGETS IN AD

In the past years, genome-wide association studies (GWAS) identified over 25 genetic loci that robustly associate with risk of late onset Alzheimer disease (LOAD); many of these, relate to neuroinflammation and are preferentially or exclusively expressed in microglial cells, implicating microglia

reaction as not only a consequence of Alzheimer's but likely also a cause. In this section, we are reviewing the current knowledge regarding the roles of the most studied genes in this context.

APOE: Beyond Aβ Modulation, a Microglia-Function Modifier

The ε4 isoform of the Apolipoprotein E (APOE) represents a common genetic variant associated with AD and is the most significant known risk factor. APOE is an apolipoprotein implicated in cholesterol and lipid transfer between cells. In the brain, it is produced mainly by astrocytes, but also by microglia and to a lesser extent by neurons. In humans, APOE is found in three main isoforms: ε2, ε3, and ε4. The APOE-ε4 isoform represents the most significant risk factor for LOAD: the presence of one APOE-ε4 copy increases the risk of developing LOAD by three-fold whereas two APOE-ε4 copies lead to a nine-fold increase (Corder et al., 1993). On the opposite, individuals carrying the rare ε2 variant are less likely to develop AD and the most common APOE-ε3 isoform is thought to be neutral (Serrano-Pozo et al., 2015). How APOE isoforms affect the onset and development of AD remains unclear. Based on the early-described interaction between APOE and Aβ, studies mainly focused on the effects of APOE on amyloid load and oligomerization, which was shown to be isoforms-dependent (Strittmatter et al., 1993; Naslund et al., 1995; Hashimoto et al., 2012). Thus, APOE-ε4 patients have more Aβ plaques and oligomers than ε3 carriers (Schmechel et al., 1993; Tiraboschi et al., 2004; Hashimoto et al., 2012; Koffie et al., 2012), and mouse models expressing human APOE isoforms mimics the isoform-dependent modifications on Aβ deposits (Fagan et al., 2002; Hudry et al., 2013; Zhao et al., 2014). It was also reported that APOE can also influence Aβ clearance (DeMattos et al., 2004; Castellano et al., 2011), however, APOE-knockdown mice develop less Aβ deposits (Holtzman et al., 1999; Fagan et al., 2002).

Little is known regarding the microglial role of APOE. Early post-mortem studies in humans found a higher number of reactive microglia in APOE-ε4 carriers compared to APOE-ε3 (Egensperger et al., 1998), and recently Minett et al. (2016) found that APOE-ε4 allele was strongly associated with reactive microglia. Studies using mouse models expressing human isoforms also showed increased microgliosis in APOE-ε4 expressing mice compared to APOE-ε3 (Belinson and Michaelson, 2009; Rodriguez et al., 2014). Overall, these studies highlight a relation between APOE isoforms and reactive microglia. Evidences have also been pointing out a role of APOE in inflammatory processes (Lynch et al., 2001; Thangavel et al., 2017). Thus, APOE is up-regulated in disease-associated microglia (DAMs) and modulates transcription of homeostatic and inflammatory factors (Krasemann et al., 2017). Moreover, APOE modifies inflammatory response in an isoform manner; APOE-ε4 expressing mice releasing more pro-inflammatory cytokines than APOE-ε3 (Guo et al., 2004; Vitek et al., 2009; Zhu et al., 2012). However, the concept of APOE triggering more pro-inflammatory response has been challenged since APOE in the presence of Aβ can induce a decrease in pro-inflammatory cytokine release (Guo et al., 2004), indicating a more complex link between APOE, Aβ and glial cells. Overall, APOE is undoubtedly implicated in LOAD through Aβ aggregation and clearance but also by modulating microglia activation and cytokine release. However, additional studies are needed to explore these processes.

TREM2, the Well-Known Risk Factor With Unclarified Roles

Aside from APOE, the other major well-studied gene associated with LOAD is the Triggering receptor expressed on myeloid cells 2 (TREM2). Indeed, GWAS studies identified several rare Trem2 gene variants associated with LOAD (Guerreiro et al., 2013; Jin et al., 2014). Among them, the rs75932628 variant, which causes the loss-of-function R47H mutation, showed significant association with AD and suggests a protective role for TREM2 activation pathway in AD (Guerreiro et al., 2013; Jonsson et al., 2013). TREM2 is a cell surface receptor expressed in myeloid cells, including microglia (Schmid et al., 2002; Kiialainen et al., 2005) and was shown to modulate inflammation (Piccio et al., 2007), phagocytosis (Takahashi et al., 2005) and chemokine secretion (Bouchon et al., 2002; Sieber et al., 2013). How TREM2 affect AD is currently not well understood but TREM2 is increased in APP/PS1 hippocampus and cortices (Jiang et al., 2014) and its expression increases with age (Jay et al., 2015). Moreover, TREM2 expression is increased in plaque-associated microglia (Frank et al., 2008; Guerreiro et al., 2013), and modulating TREM2 expression can reprogram microglia response (Krasemann et al., 2017; Lee C.Y.D. et al., 2018). In particular, Keren-Shaul et al. demonstrated that TREM2 is required for a complete activation of DAMs (Keren-Shaul et al., 2017), pointing out a major role of TREM2 in those cells.

Most of the studies on TREM2 in AD relate to microglia-mediated Aβ phagocytosis, however, they do not all agree on whether it has beneficial or detrimental effects. Indeed, Ulrich et al. (2014) found no change in amyloid burden in 3-month-old TREM2-heterozygous APP-21 mice whereas Jay et al. (2015) showed reduced 6E10 staining in 4-month-old TREM2 deficient APP/PS1 mice. On the opposite, Wang et al. (2015) suggested using 8-month-old 5xFAD mice, that TREM2 deficiency would be detrimental as it increases hippocampal Aβ peptide. Similarly, Jiang et al. (2014) showed, *in vitro*, that TREM2 facilitates Aβ 1-42 phagocytosis and, *in vivo*, that TREM2 overexpression reduces plaque density in the cortex and hippocampus of APP/PS1 mice. Altogether, these different studies suggest a complex role of TREM2 on Aβ and suggest an age- or stage-dependent effect.

Contradictory results have also been found in TREM2-dependent inflammatory response. Jay et al. (2015) showed that TREM2 deficiency in APP/PS1 mice reduces the pro-inflammatory response. On the contrary, in *in vitro* studies, TREM2 overexpression was shown to reduce - while TREM2 deficiency increases - pro-inflammatory cytokine production (Takahashi et al., 2005; Jiang et al., 2014). Therefore, further studies are required to clarify how TREM2 influences inflammatory response.

Despite discrepancies found in TREM2 implication in inflammatory response and Aβ deposition, it is of agreement that TREM2 deficiency causes a decrease in Aβ-associated microglia (Jay et al., 2015; Wang et al., 2015; Zhao et al., 2018). Altogether, those studies show TREM2 implication in AD by modulating inflammatory processes and Aβ deposition. Moreover, several studies found that Aβ binds to TREM2 and that this interaction can modulate microglia functions such as proliferation, Aβ degradation and inflammatory response (Zhong et al., 2018). Interestingly, a link between TREM2 and APOE has recently been highlighted suggesting that TREM2 modulates APOE expression and signaling (Krasemann et al., 2017; Parhizkar et al., 2019).

Many studies aim to decipher the role of TREM2 in AD. However, current studies mainly used TREM2 overexpression or knockout although it might be relevant to also study TREM2 variants.

Other AD Risk Factors

Alzheimer disease-related GWAS have identified many other microglia genes as potential risk factors in AD. This includes genes such as *Cd33*, *Cr1*, or *Abca7*, which have been implicated in phagocytosis (Villegas-Llerena et al., 2016). Additionally, CD33 was shown to inhibit Aβ clearance whereas CR1 is part of the complement pathway that helps eliminating Aβ plaques. Various SNPs of CD33 have been found: the rs3826656 and rs3865444 variants are associated with LOAD whereas the rs3865444 variant confers protection (McQuade and Blurton-Jones, 2019). Overall, more than 25 microglia genes have been highlighted by GWAS studies (Verheijen and Sleegers, 2018). Their contribution to AD pathogenesis is yet unknown and merit scientists' attention as they tend to be more common genetic variants.

EMERGING MICROGLIAL TARGETS IN AD

Emerging microglial targets are highlighted in **Figure 2**.

Purinergic Signaling

Of the many mechanisms implicated in microglial functions, purinergic signaling is one worth mentioning. Indeed, microglial purinergic receptors are known to modulate several processes including phagocytosis, chemotaxis and cytokine release (Abbracchio and Ceruti, 2007; Calovi et al., 2019). Purinergic receptors are divided into two super families: P1 receptors respond to adenosine whereas P2 respond to ADP and ATP. Purinergic receptors are widely expressed in several CNS cell types including microglia (Butovsky et al., 2014), and both ionotropic P2X and metabotropic P2Y receptors have been implicated in neurological diseases including AD (Burnstock, 2016).

Of the G protein-coupled P2Y receptors, P2Y6R, 12, and 13 are the three most investigated subtypes expressed in microglia (Calovi et al., 2019). In AD, the most studied is P2Y12 receptor which plays important role in homeostatic microglia and whose role is described above (see section "Loss of Homeostatic Functions in Reactive Microglia"). As for the other P2YRs, P2Y6R might be implicated in amyloid phagocytosis as Wendt et al. (2017) demonstrate an impaired P2Y6R-dependent phagocytosis mechanism in 9-month-old 5xFAD mice. Although P2Y2R expression in microglia seems very low (Calovi et al., 2019), studies have also investigate its implication in AD. *In vitro*, Aβ treatment of primary microglia induces an increase of P2Y2R expression and Aβ-treated $P2Y2R^{-/-}$ microglia showed loss of motility and altered ATP/UDP-triggered Aβ uptake (Kim et al., 2012) suggesting that P2Y2R up-regulation enhances microglia-dependent Aβ degradation. Interestingly, P2Y2R expression is up-regulated in 10 weeks old TgCRND8 AD mouse models and P2Y2R heterozygous mice showed accelerated pathology compared to wild-type mice (Ajit et al., 2014). In contradiction with data from the TgCRND8 mice but supporting the idea of P2Y2R displaying protective effect, data demonstrate a downregulation of P2Y2R in parietal cortex of AD patients (Lai et al., 2008). Discrepancies between mice and human P2Y2R expression regulation may be explained by the stage at which the studies were conducted. Altogether, these results suggest a protective role of P2Y2R signaling in the context of AD.

The other major P2 receptors are the ATP-gated ion channels P2X receptors. There are seven P2XR subunits whose assembly in trimers forms cation-permeable channels that are widely expressed in the CNS. To date, P2X4R and P2X7R are the only P2XRs pertinently characterized in microglia. P2X7 is the most studied in AD context because of its well-established pro-inflammatory role, being a key step in the NLRP3 inflammasome activation leading to IL-1β release (Bhattacharya and Biber, 2016; Adinolfi et al., 2018). P2X7R is up-regulated in AD patient brains (McLarnon et al., 2006; Martin et al., 2018) and administration of Aβ peptide in rat hippocampus increases P2X7R expression (McLarnon et al., 2006). Moreover, downregulating or blocking P2X7R decreases pro-inflammatory cytokines release in microglia cell cultures stimulated by ATP and Aβ (Ni et al., 2013). On the other hand, Martin et al. (2018) demonstrate that P2X7R deficiency ameliorates cognitive functions and reduces amyloid load without altering IL-1β level. This set of data suggest a pro-inflammatory role of P2X7R in AD. Lastly, the C489T polymorphism of P2X7R, which causes a loss of function, was found to be less frequent in AD patients, supporting a potential contribution of P2X7R in AD pathogenesis (Sanz et al., 2014).

Another highly expressed P2X receptor in reactive microglia is P2X4R. P2X4R are highly up-regulated upon microglia activation and have been implicated in neurological disorders and inflammatory processes (Suurväli et al., 2017). Although P2X4R is decreased in AD brain patients and neuronal P2X4R seems to modulate Aβ-induced neuronal death (Varma et al., 2009), no work has yet been performed on microglial P2X4R in AD.

The third main purinergic receptor family is that of adenosine P1Rs, which comprises A1, A2a, A2B, and A3 receptors. P1R are G protein-coupled receptors abundantly expressed in the CNS and all P1 subtypes are expressed in microglia. A1 and A2a receptor expressions have been found to be dysregulated in post-mortem tissues from AD patients (Erb et al., 2018), thus suggesting that P1 receptors might be implicated in AD. However, to date, not much is

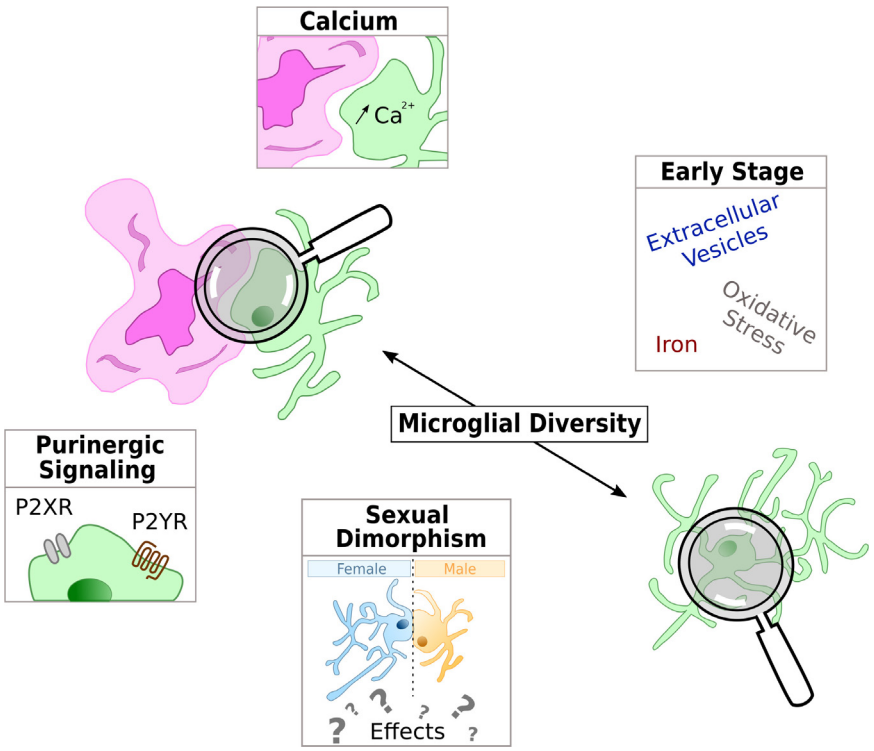

FIGURE 2 | Schematic representation of the current hot topics and emerging microglial targets in AD physiopathology studied in this review. Microglia is represented in green and either with a reactive shape associated to amyloid-β deposit in purple or in the parenchyma with an homeostatic shape, away from Aβ.

known regarding the contribution of microglial P1 signaling in the context of AD.

Overall, those few studies suggest the implication of microglia purinergic receptors in AD through different processes such as inflammation or phagocytosis. However, since purinergic receptors are expressed in all neural cell types incriminated in AD, more specific tools such as cell-specific KO models are required to decipher the roles of microglial purinergic signaling.

Microglial Calcium Signaling, a Poorly Studied Messenger in AD

Calcium signaling is a key second messenger in almost all cell type and is essential for the normal CNS functioning. Many microglia functions are mediated by Ca^{2+} (McLarnon, 2005; Färber and Kettenmann, 2006). In particular, microglia reaction is accompanied with intracellular calcium increase, a process that is required to induce cytokines and chemokines release (Hoffmann et al., 2003).

Calcium signaling dysregulation in AD has been widely studied in neurons (Tong et al., 2018), but very little is known about calcium signaling in microglia in the context of AD. In an early study, Combs et al. (1999) demonstrated that stimulation of cultured microglia with $A\beta_{25-35}$ peptide results in a transient increase in intracellular $[Ca^{2+}]$. This calcium is released from intracellular store and leads to microglia activation and neurotoxic factors production. More recently, McLarnon et al. showed that cultured microglia from AD patients have higher basal Ca^{2+} level but lower amplitude and longer-lasting ATP-induced calcium response with respect to that measured in microglia from non-demented individuals, thus suggesting that calcium signaling is impaired in microglia from AD patients (McLarnon et al., 2005).

Altered P2YR-dependent calcium signaling has been observed in plaque-associated microglia in AD mouse models whereas plaque-distant microglia showed similar Ca^{2+} activity than matched controls (Brawek et al., 2014). As calcium mediates many microglial functions, it might be interesting to further investigate microglia calcium signaling in the context of AD. Particularly since some purinergic receptors are likely implicated in AD, it might be worth studying whether calcium signaling dysregulation involves purinergic signaling impairment.

MICROGLIA IN ALZHEIMER DISEASE: CURRENT HOT TOPICS

The involvement of microglia in AD is a relatively new area of research, but it is growing at a fast pace. In addition to the pathways described above, new areas of investigation are emerging or revisited based on our current knowledge of microglial functions. In the following part, we highlight few current hot topics (**Figure 2**). These new areas of research will help to increase our understanding of AD pathogenesis, and may,

on a longer term, help to better stratify the patients and to design tailored therapeutic strategies.

Sexual Dimorphism, a Key Factor to Design Efficient Therapeutic Strategies

The impact of sexual dimorphism in biology is a hot topic, and AD research makes no exception. Indeed, AD prevalence is two-fold higher in women (Hebert et al., 2013), and women suffering from AD display specific cognitive alterations, biomarker patterns or risk factors susceptibility (Ferretti et al., 2018). This sex effect was, at first, thought to be due to higher longevity but recent reports show that it is clearly not the only explanation (Viña and Lloret, 2010). The involvement of sex is still a subject of intense debate and biological mechanisms involved in human pathology are still controversial. Because most AD pre-clinical studies use either males or females, but rarely both, the cellular and molecular mechanisms involved in AD sexual dimorphism are still poorly understood. Yet, the few studies comparing the influence of sexual dimorphism in AD mouse models, all agree on a sooner precocious and stronger AD phenotype in females (**Table 1**). Sex-based effects are observed in different AD mouse models, both at behavioral and histological levels. Globally, cognitive alterations in females appear at younger ages and remain stronger than in males (Clinton et al., 2007; Carroll et al., 2010; Gallagher et al., 2013; Yang et al., 2018). Whatever brain areas, models and ages, females display more amyloid plaques and higher amount of soluble Aβ (Wang et al., 2003; Carroll et al., 2010; Gallagher et al., 2013; Ben Haim et al., 2015; Janus et al., 2015; Jiao et al., 2016; Yang et al., 2018). Furthermore, in late stages, Tau phosphorylation levels and number of Phospho-Tau positive cells are higher in females (Clinton et al., 2007). Synaptic and neuronal degeneration processes seem also to be stronger in aged females (Jiao et al., 2016). Relative to neuroinflammation, glial cells from old female AD mice present stronger reactive states compared to males and are associated with higher levels of pro-inflammatory factors (Jiao et al., 2016; Yang et al., 2018). While sex effects are clearly established in both AD mice and patients, it remains unknown whether these modifications are due to systemic/hormonal effects or whether it also exists at the cellular level.

A growing number of studies highlights the impact of sexual dimorphism on microglia shape and functions (**Table 2**). Many sex mediated effects on microglia have been demonstrated. They seem to be highly dependent on age and brain region: (1) in both mice and rats, adult males show higher microglia densities (Guneykaya et al., 2018; Perkins et al., 2018); (2) adult females microglia also display higher proportion of cells with long and thick processes while male microglia have larger soma (Schwarz et al., 2012; Guneykaya et al., 2018; Weinhard et al., 2018). These differences may indicate that adult female microglia are in a more homeostatic state while males display a more reactive state. Functional differences are also observed, with microglia from male brain displaying higher migration rates while female's present stronger phagocytic activity related to higher expression of phagocytosis associated genes (Nelson et al., 2017; Yanguas-Casás et al., 2018). RNA sequencing also revealed a more protective transcriptomic profile for female microglia while male microglia displayed a more inflammatory phenotype (Villa et al., 2018). A recent study also reports a sex impact on microglial electrical properties, suggesting that sex may affect microglia secretory profile, a property that can directly influence inflammatory response abilities (Guneykaya et al., 2018). It was initially thought that microglia sex differences depend from circulating sexual hormones (Nissen, 2017), but this view was recently challenged as it was shown that female microglia retain their protective properties when transplanted in males brain (Villa et al., 2018).

Thus, although the molecular pathways involved in this microglial sexualization remain largely unknown, the current data suggest that, at least in adulthood, female microglia are in a more homeostatic and protective state. In the context of AD, it could be speculated that female microglia committed to homeostatic functions would need longer time exposure or stronger stimuli to polarize their shape and functions to answer correctly to harmful stimuli. Additionally, when they finally react to Aβ, they will change their local environment from a low- to a high-inflammatory environment. This drastic change could lead to more harmful effects on microglia themselves but also on all other surrounding cells and may explain why female are more likely to develop the pathology.

Although the effect of sex on microglial functions has been implicated in various CNS pathologies (Sorge et al., 2015; Charriaut-Marlangue et al., 2018; Jullienne et al., 2018; Mapplebeck et al., 2018; Thion et al., 2018), further studies are needed to understand the impact of microglial sexual dimorphism in AD initiation and progression and to which extend it relies to sex disbalance in AD. Whether sexual dimorphism mechanisms are similar in human and animal models also need to be clarified.

Microglial Diversity Is Both a Challenge and an Opportunity

Microglia cell diversity has been recognized since their first description by Pio del Rio-Hortega in 1919 (Sierra et al., 2016), who reported the existence of different morphological microglia phenotypes in the human brain. He also established that microglia morphology is considerably altered in disease conditions, introducing the concept that microglia tightly adapt to their local environment. More recent functional, morphological, immunohistochemical, and medium throughput analyses of microglia in pathological conditions also pointed out to the existence of a diversity of reactive phenotypes (Hanisch and Ketternmann, 2007; Ransohoff and Perry, 2009; Kierdorf and Prinz, 2017). In this respect, several semi-automated tools are now available to enable the identification of phenotypically distinct microglial cell subpopulations (Wagner et al., 2013; Verdonk et al., 2016; Salamanca et al., 2019).

In the last decade, the development of high throughput transcriptomic approaches combined with improved cell purification techniques helped refine our understanding of the molecular diversity of microglia both in physiological (Grabert et al., 2016) and pathophysiological conditions (Hirbec et al.,

TABLE 1 | Impact of sexual dimorphism on AD pathophyiology in various mice models.

AD mice model	Age	Brain area	Studied parameter (readout)	Sex influence	References
Behavior studies					
3xTg	6–9 mo	–	MWM learning deficit	♀ only	Clinton et al., 2007
	15mo	–	MWM learning deficit	♀ = ♂	Clinton et al., 2007
	2–6–9–15mo	–	NOR	♀ = ♂	Clinton et al., 2007
	12mo	–	MWM retention task	♀ < ♂	Yang et al., 2018
	12–14mo	–	Spontaneous alternation	♀ < ♂	Carroll et al., 2010
Amyloid beta cascade					
3xTg	2–6–12–15mo	Brain homogenate	Aβ 40/42 soluble and insoluble fractions (ELISA)	♀ = ♂	Clinton et al., 2007
	6–8mo	PFCx	Plaques density (Aβ antibody)	♀ > ♂	Carroll et al., 2010
	12mo	SB, CA1, PFCx	Plaques density (Aβ antibody)	♀ > ♂	Carroll et al., 2010
	12mo	HC	Aβ positive area (6E10 staining)	♀ > ♂	Yang et al., 2018
APP(swe)/PS1(de9)	4–12–17mo	HC	Aβ 40/42 (ELISA)	♀ > ♂	Wang et al., 2003
	12–17mo	HC	Plaque area and density (W0–2 staining)	♀ > ♂	Wang et al., 2003
	?	HC, Cx	Plaque density (Congo Red staining)	♀ > ♂	Gallagher et al., 2013
	?	Brain homogenate	Insoluble Aβ42 (ELISA)	♀ > ♂	Gallagher et al., 2013
	?	Brain homogenate	Soluble Aβ42 (ELISA)	♀ = ♂	Gallagher et al., 2013
	?	Brain homogenate	*Ide* (mRNA expression)	♂ > ♀	Gallagher et al., 2013
	?	Brain homogenate	*Bace-1* (mRNA expression)	♀ > ♂	Gallagher et al., 2013
	12mo	OB, HC, NC	Plaque area and density (Congo red compared to Thioflavin-S and 6E10 staining)	♀ > ♂	Jiao et al., 2016
	12mo	Brain homogenate	Aβ 40/42 (Western Blot and ELISA)	♀ > ♂	Jiao et al., 2016
	18mo	HC, Cx	Plaque area (Campbell-Switzer Silver staining)	♀ > ♂	Janus et al., 2015
Tau load					
3xTg	6mo	HC	Tau positive region (HT7 antibody)	♀ = ♂	Clinton et al., 2007
	12mo	HC	Number of P-Tau positive neurons and P-Tau area (pT231-Tau antibody)	♀ > ♂	Yang et al., 2018
APPswe/PS1de9	12mo	CA1, CA3, NC	P-Tau positive area (IHC) and P-Tau quantity (WB)	♀ > ♂	Jiao et al., 2016
Inflammation					
3xTg	12mo	HC	IBA1 and GFAP positive area	♀ > ♂	Yang et al., 2018
APPswe/PS1de9	12mo	Brain homogenate	TNF, IFN and Il-1β protein levels (ELISA)	♀ > ♂	Jiao et al., 2016
	12mo	OB, HC, NC	IBA1 and GFAP positive area	♀ > ♂	Jiao et al., 2016
	12mo	HC, NC	Caspase-3 positive neurons	♀ > ♂	Jiao et al., 2016
Neurodegeneration					
APPswe/PS1de9	12mo	CA1, CA3, NC	Number of neurons	♂ > ♀	Jiao et al., 2016
	12mo	Brain homogenate	Synaptic proteins expression level	♂ > ♀	Jiao et al., 2016
	10mo	Whole Brain	White matter, axonal and myelinated fibers volumes	♀ > ♂	Zhou et al., 2018
Comorbidity					
APPswe/PS1de9	12mo	Whole Brain	Microhemorrage number	♀ > ♂	Jiao et al., 2016

This table is a non exhaustive compilation of the literature concerning AD sexual dimorphism. NC for Neocortex, PFCx for PreFrontalCortex, HC for Hippocampus, SB for Subiculum, OB for Olfactory Bulb, MWM for Morris Water Maze, IDE for Insulin Degrading Enzyme, ? for missing data.

2017; Holtman et al., 2017; Sousa et al., 2017; Dubbelaar et al., 2018). They established that microglia cannot be categorized in a discrete number of physiological and pathological states. However, the breakthrough in the appreciation of the microglia diversity arose from the emergence of single-cell high throughput approaches. In particular single-cell RNAseq (scRNA-seq)

TABLE 2 | Impact of sexual dimorphism on microglial shape and function.

Parameter	Read out	Brain area/cellular model	Age	Sex influence	References
Microglia density					
Microglia density	IBA1 positive cells	Hippocampus	P21	♂ > ♀	Guneykaya et al., 2018
		Amygdala	P21	♀ > ♂	
		Cx, HC, and Amygdala	13w	♂ > ♀	
		HC, Amygdala, and Striatum	3mo	♂ > ♀	Perkins et al., 2018
Shape					
Microglial compartment volume	IBA1 and CD68 staining	CA1	P8	♀ > ♂	Weinhard et al., 2018
			P28	♂ > ♀	
	Soma size	Cx, HC, and Amygdala	13w	♂ > ♀	Guneykaya et al., 2018
Microglia ramification complexicity	IBA1 positive cells: Ameboid	CA3, Dentate Gyrus, and Amygdala	P0	♀ > ♂	Schwarz et al., 2012
		CA1, CA3, Dentate Gyrus, Parietal Cortex, and Amygdala	P4	♂ > ♀	
	IBA1 positive cells: Ramified (thick and long processes)	CA3, Dentate Gyrus, Parietal Cortex, and Amygdala	P30 and P60	♀ > ♂	
Function					
Cell migration	Transwell migration	Primary microglia culture	P0 to P2	♂ > ♀	Yanguas-Casás et al., 2018
Phagocytosis	Phagocytic cup (CD68+, Dapi+)	HC	Neo-natal	♀ > ♂	Nelson et al., 2017
	Latex beads	Acute cortical and hippocampal slices	13w	♀ = ♂	Guneykaya et al., 2018
	Fluorescent beads	Primary microglia culture	P0 to P2	♀ > ♂	Yanguas-Casás et al., 2018
Electrophysiological properties	Baseline inward and outward conductance	Microglia located in layers 2–6 of the somatosensory cortex in acute slices	N/A	♂ > ♀	Guneykaya et al., 2018
	Resting membrane potential		N/A	♂ > ♀	
Inflammatory profile (protein level)	Il-10	Cx and HC	P60	♀ = ♂	Schwarz et al., 2012
	Il-1b	Cx and HC	P60	♀ > ♂	
Inflammatory profile. (gene expression)	Il-10, Il1r1, Il1f1 genes	Cx and HC	P0, P4, P60	♀ > ♂	Schwarz et al., 2012
	RNASeq	Isolated microglia	3mo	♀: protective ♂: Inflammatory	Villa et al., 2018

This table is a non exhaustive compilation of the literature concerning microglial sexual dimorphism. Cx for Cortex, HC for Hippocampus, N/A for missing data.

enables investigating the diversity at cellular resolution and allows a detailed examination of cell states diversity and changes that are reflective of those *in vivo* (Macosko et al., 2015).

Two very recent scRNA-seq studies established the spatial and temporal diversity of microglia during the mouse development, and in either neurodegenerative or inflammatory conditions (Hammond et al., 2019; Masuda et al., 2019). These studies, together with more focused previous ones, (Keren-Shaul et al., 2017; Friedman et al., 2018) established that different microglia subpopulations co-exist at a given physiological or pathological state. Such heterogeneity within the microglia cell population represents both a challenge and an opportunity: the existence of distinct subpopulations supports the design of specific treatments targeting specific subpopulations with the aims of either promoting the beneficial subpopulations and/or hampering the deleterious ones.

What about microglia diversity in AD? It is known for years that AD brains exhibit at least two very distinct morphological microglia phenotypes: cells associated to the amyloid plaques display a "reactive"/amoeboid-like phenotype whereas those present in the rest of the parenchyma have a homeostatic-like morphology (Krabbe et al., 2013). As a mark of the importance of this diversity, it was shown that transcripts up-regulated in microglia isolated from AD mice were more highly expressed

in tissues isolated in the vicinity of amyloid plaques compared to those distant from plaques (Orre et al., 2014). This suggested that microglia associated to plaques activate specific signaling pathways and functions. Using scRNA-seq of sorted 5xFAD mouse brain immune cells, Keren-Shaul et al. (2017) identified three distinct subtypes of microglia, including DAMs whose relative abundance increases with disease progression and which are preferentially located around amyloid plaques. The translational relevance of the DAMs was confirmed in human postmortem brains in which specific DAM markers are expressed in a subset of microglia in AD patients, but not in control subjects. More recently, in the knock-in APP^{NLFG} AD mouse model (Saito et al., 2014; Sala Frigerio et al., 2019) identified activated- and interferon response microglia (ARM and IRM, respectively). ARMs partly overlap with DAMs, but by applying cell trajectory inference methods, these last authors demonstrated that ARMs were not solely present in disease conditions and are part of the normal brain aging process. However, many unresolved questions remain regarding the functional significance of this diversity. In particular, when it arises during AD progression and whether it is already present at the prodromal stages of the disease. With progress of both technology and analytic methods, it is now feasible to get a deeper understanding of the microglia diversity across AD progression. Identification of specific, potentially small in size, microglia subpopulations may have important implications for translational applications. Identification of factors released by these subpopulations and that can diffuse to the CSF may lead to the identification of new biomarkers. Additionally, identification of specific subpopulations will help discover new pathways and functions that contribute to the disease progression and may lead to the development of more specific functional positron emission tomography (PET) tracers. Deciphering, whether these subpopulations have beneficial, neutral or detrimental effects on brain cells, including neurons, will open the way for the design innovative disease-modifying therapeutic strategies.

Studying AD at Early Stages to Identify Early Biomarkers

After years of unsuccessful clinical trials, scientists are now realizing that AD is a pathology we may want to prevent (or stop) rather than to cure. This is calling for the identification of early diagnostic biomarkers, which are sorely lacking to help prodromal diagnostic, or design innovative disease-modifying therapeutic strategies. Although AD early stages are still less studied, increasing number of studies are now focusing on the prodromal phases to unravel early dysregulations.

Because one of microglia's main role is to sense their environment and to react to danger, they are likely to play key roles in initial brain response to AD-driven changes. In agreement, a recent PET and magnetic resonance imaging (MRI) based study showed that AD patients have a first microglial activation peak before they display any other hallmarks of the pathology, suggesting that microglial dysfunction is critically involved in AD initiation (Fan et al., 2017). The involvement of microglia in AD initiation stages raises the question of which microglial targets could be modulated at an early stage to slow down the progression of the disease.

Extracellular vesicles (EVs) are a family of small membrane vesicles, which includes exosomes and microvesicles (MVs), transporting various types of molecules. In the recent years, EVs have emerged as a new mean of communication between cells. Microglia abilities to secrete EVs and MVs are part of their essential inflammatory functions. In the Aβ phagocytosis process, overloaded microglia can release Aβ-containing small secretory vesicles (Joshi et al., 2014), as well as Tau or P-Tau through exosomes (Saman et al., 2012; Asai et al., 2015). Accordingly, the levels of myeloid MVs detected in the CSF of MCI or early stage AD patients, is correlated with the extent of their white matter damage (Agosta et al., 2014). Microglial EVs may thus be regarded as a possible mean to spread the pathology during early stages. Interestingly, because they are secreted and can access first the CSF compartment and then the blood, microglial EVs may represent valuable diagnosis tools (Trotta et al., 2018).

Oxidative stress is known as a necessary but potentially harmful process. In response to fibrillary Aβ, reactive microglia produce free radicals, notably superoxide via the NADPH oxidase (NOX) (Harrigan et al., 2008). Moreover, NOX activity is increased in MCI patients compared to controls (Bruce-Keller et al., 2010). These findings open the way for using NOX activity as the marker of early microglia reaction in AD. Interfering with NOX activity through specific compounds may also provide a mean to slow down the pathology (Dumont and Beal, 2011).

Iron is an important metal implicated in vital biological processes. However, intracellular iron overload can lead to neuronal degeneration (Zhang et al., 2014). Microglia are able to interact, transport and metabolize iron. When they secrete pro-inflammatory factors, microglia can stimulate neuronal iron uptake leading to an increase in neurodegeneration (Zhang et al., 2014). Interestingly, patients with specific iron overload disorder are subject to earlier onset of AD, suggesting that iron may be involved in the initiation steps of the pathology. Moreover, iron chelation seems to be beneficial to AD patients. Further work is needed to understand the early implication of microglial iron regulations in AD (Nnah and Wessling-Resnick, 2018).

BARRIERS TO DISCOVERIES

Although fundamental knowledge is of importance, the ultimate goal of characterizing cells functions and dysfunctions in physiological and pathological conditions is to design efficient therapeutic strategies with clinical benefit. Understanding the complexity of Alzheimer's disease is one of the XXIth century challenges. To do so we mainly rely on animal models that mimic the symptoms of the pathology (amyloid deposits, hyperphosphorylated tau, cognitive alterations, etc.). However, so far, the results generated from these models have generally failed to translate to clinic, and only symptomatic and poorly efficient treatments exist (Ransohoff, 2018). In the two following paragraphs, we explore two important factors that may have been under-estimated in AD pre-clinical research: the relevance of current AD mouse models and the immunological differences

between mice and humans. These remarks do not only apply to the study of microglia' roles in AD, but are important issues to consider for the design of any preclinical studies.

Are the Current Mouse Models Tailored to Understand AD and Identify Drug Targets?

Most of the animal models are genetically engineered to overexpress human protein mutations also leading to an overexpression of their respective by-products, thus generating potential confounding factors (Sasaguri et al., 2017). In addition, most AD models are based on familial mutations whereas early-onset familial AD is estimated to account for only 3.5% of total AD cases (Harvey et al., 2003). In addition, some of these models are based on a combination of mutations that were never found in patients. Because it is the first hallmark of AD, most of the models focused on the amyloidogenic pathway (Sasaguri et al., 2017). However, it is clear that, in humans, there is a concomitant effect of Aβ and P-Tau. To solve this issue, the 3x-Tg model is combining Aβ and Tau associated features. However, it only poorly represents the pathology in term of kinetic of appearance of the symptoms as cognitive deficits appear way too early and before any amyloid accumulation or Tau dysregulation (AlzForum[1]). To solve these issues, new mouse-based or human-based models are created (see section "New opportunities in AD research").

To study microglia impact on AD, new models have been created by combining microglial dedicated tools with AD models (Zhou et al., 2018). One of the most commonly used models is the $CX3CR1^{+/eGFP}$ x APP/PS1 strain, in which an allele of the fractalkine receptor CX3CR1 is replaced by a GFP allowing to track microglial cells. However, to our knowledge, the impact of the CX3CR1 haplo-deficiency on AD development has not yet been thoroughly investigated. Finally, AD studies are generally conducted in old mice, when the pathology symptoms and hallmarks are well established. This stage of the pathology is probably too advanced for the identification of efficient disease modifying drugs.

Human and Mice Are Not Immunologically Similar

In the field of inflammation, the comprehensive study by Seok et al. (2013) revealed that gene dysregulation in mouse models of severe human inflammatory conditions (endotoxemia, burns, and trauma) do not correlate with human genomic changes. This raises the question of the relevance of mouse models to study the role of immune cells in diseases, including brain disease. Several factors known to be of importance for immune functions differ between rodents and human studies. Most often, rodent models and studies use inbred strains whereas human genetic backgrounds are much more diverse. Additionally, humans are exposed to multiple diseases whereas research mouse models are raised in tightly controlled environment (Davis, 2012).

Functional studies investigated the response of human microglia to either endogenous (i.e., M-CSF) or exogenous (i.e., LPS) stimuli (Melief et al., 2012; Smith et al., 2013). For the most part, they show comparable results with mouse analyses, thus agreeing with two comprehensive transcriptomic studies which compared gene expression profiles in human and mouse microglia and concluded that, overall, gene expression is very similar in the two species (Galatro et al., 2017; Gosselin et al., 2017). Of interest to brain diseases, a good correlation between human and mouse microglia in response to neurodegeneration was also observed by other authors (Holtman et al., 2015; Keren-Shaul et al., 2017; Krasemann et al., 2017).

However, notwithstanding their global resemblance, significant number of genes are differentially expressed in mouse and human microglia. In particular, specific immune genes are only expressed in human samples (Gosselin et al., 2017). In addition, differences in the relative expression of lineage- and signal-dependent transcription factors between mice and humans were observed (Holtman et al., 2017). In line, previous studies identified several molecular pathways and signaling functions that significantly differ between mouse and human microglia. This includes proliferation, response to TGFβ1, Siglecs signaling, nitric oxide (NO) production, and response to certain drugs such as valproic acid (VPA) (Smith and Dragunow, 2014).

Of relevance to AD, Siglec-3 (CD33) that has been identified as a risk factor for AD (Bertram et al., 2008) displays substantial species differences in expression patterns and ligand recognition (Lajaunias et al., 2005). Additionally, age-related changes of immune and cognitive functions may not be correctly modeled in rodents, whose life expectancy is far less compared to humans. Accordingly, gene expression changes in aged human brain are significantly different from those in the mouse brain (Loerch et al., 2008; Bishop et al., 2010). In agreement, Galatro et al. (2017) revealed that there is a limited overlap in age-related changes in human and mouse microglia, highlighting that data obtained in aged mice should be extrapolated to the human situation with caution. Translation of results from mice to humans is also hampered by the lack of tools to precisely characterize microglial reactivity in patients: TSPO binding is so far the only way to study microglia reaction in a clinical context.

Although methods to purify, culture and experiment on human microglia have been established, access to human samples that meet the requirements of high-quality studies is limited. AD mouse models are thus undoubtedly useful to decipher the roles of specific microglial functions and signaling pathways. However, the species differences highlighted above should prompt scientists to confirm the results they obtained in mouse models on human samples.

NEW OPPORTUNITIES IN AD RESEARCH

While current models to study AD have multiple limitations, scientists are trying to generate new models with limited pitfalls. In this last part, we are reviewing new approaches currently

[1] https://www.alzforum.org/

developed. These approaches are expected to improve our knowledge on microglial functions and help bridge the gap between *in vitro* studies, rodent models and the human disease.

Promising Animal Models to Overcome Identified Pitfalls

Although mouse models present significant pitfalls, they are attractive models in preclinical studies. Indeed, they are small, easy to raise, mammals with reasonable life expectancy. They reproduce well in captivity and can be engineered and/or humanized in a relatively easy way. Furthermore, with the exponential development of a wide repertoire of mouse lines (Model Organism Development and Evaluation for Late-Onset Alzheimer's Disease[2]), it is possible for scientists to explore new hypotheses by combining different mouse lines. In this context and to overcome the problems associated with APP overexpression, Drs Saito and Saido developed new knock-in AD mouse models, namely the APP^{NLF} and APP^{NLFG} mice (Saito et al., 2014). In these models, mutated APP is expressed from its endogenous promoter, leading to a kinetic of appearance of the symptoms which is more comparable with human pathology (Sakakibara et al., 2018). These models are not thoroughly characterized yet and still excludes Tau-associated dysfunctions. However, in the APP^{NLF} model, the delay before Aβ deposits appearance supports that it may represent an interesting model to study the prodromal phase of the disease.

Other models, not based on genetic modifications, have also emerged in the last decades. Compared to humans, dogs share an almost identical enzymatic machinery to process APP. Moreover, they may naturally develop an age-related cognitive dysfunction that reproduces several aspects of AD (Schmidt et al., 2015). With its high life span, the canine model can be useful specifically to study pathways involved in Aβ deposition and clearance in the early phases of the disease (Sarasa and Pesini, 2009). To get closer to human pathology, several primate models are currently used to study AD. Depending on their phylogenetic distance to humans, they neither display the same kinetic nor kind of neurological alterations (Heuer et al., 2012). Among primates, *Microcebus Murinus* also known as the gray mouse lemur, is particularly interesting (Bons et al., 2006). Indeed, they are small animals, just over the size of a mouse. They can be raised in cohorts of several individuals, and can reproduce in captivity. Along aging some individuals naturally develop an AD like pathology with Aβ plaques (Mestre-Francés et al., 2000), associated with specific transcriptomic remodeling (Abdel Rassoul et al., 2010). Histological features also seem to be associated with cognitive alterations (Trouche et al., 2010). Although we know that, at least in the spinal cord, the gray mouse lemur microglial shape and distribution is closer to human compared to mouse (Le Corre et al., 2018), microglia are still poorly studied in this model. Further studies are needed to investigate the involvement of microglia in this age-related AD like pathology.

Although, both dog and primate models represent interesting models, they are not so easy to use. For example, there are only few breeding centers for *Microcebus Murinus* worldwide. Those animals also need more housing space and their use is subjected to drastic ethic rules. Moreover, many of the research tools are designed on mouse genome/proteins limiting the use of these peculiar models.

hiPSCs-Derived Microglia: An Emerging Tool to Decipher the Role of Microglia in AD

To overcome species issues, microglial cell lines of human origin can be useful. They allow to study specific biological functions or functional pathways; however, they cannot reproduce the complexities of cells. This particularly true for cells which, like microglia, are highly adapted to their environment. Bone marrow-derived or blood monocytes-derived macrophages represent another source of human microglia-like cells. However, because these cells are of fundamentally different embryonic origin (Hoeffel and Ginhoux, 2015), they are likely poor models of adult microglia. Adult primary microglia can be harvested from dissociated brain area, but this process generally lead to low yields and can only sparsely be obtained for human samples. Moreover, isolation processes and culturing methods impact on gene expression pattern thus calling for caution in interpreting results based on these approaches (Gosselin et al., 2017).

An alternative to those issues is the development of cell reprograming methods, which offer the possibility to generate human pluripotent cell lines (hiPSCs) from healthy individuals but also from patients with specific diseases (Sullivan and Young-Pearse, 2017). Human iPSCs-derived neural cells can be produced in consistent yields. In the context of AD, iPSC-derived neurons issued from either familial AD (FAD) patients carrying mutations found in AD or sporadic AD (SAD) patients recapitulate some of the main hallmarks of the disease (Poon et al., 2017). Similarly, astrocytes derived from AD-patient iPSC show significant morphological and functional alterations, including up-regulation of inflammatory cytokines (Chandrasekaran et al., 2016; Auboyer et al., 2019).

Human pluripotent cell lines have recently been successfully differentiated into microglia (Muffat et al., 2016; Abud et al., 2017; Douvaras et al., 2017; Haenseler et al., 2017; Takata et al., 2017; Pocock and Piers, 2018). Although there is yet no consensus protocol to obtain hiPSCs-derived microglia, all protocols follow similar steps based on this cell type ontogeny. Based on such protocol, Garcia-Reitboeck et al. (2018) recently described microglial dysfunctions in hiPSCs-derived microglia from TREM2 T66M and W50C carriers, thus outlining the interest of such iPSCs approaches to decipher the role of microglia functions and dysfunctions in AD. Deciphering to which extend hiPSCs microglia derived from different patients (i.e., different SADs and/or different FAD mutations) share the same molecular and/or functional characteristics will help better understand the heterogeneity of AD, and may open the way toward the use of hiPSCs to implement personalized treatment in AD.

An important emerging concept is that, because microglia are CNS resident macrophages highly adapted to the CNS environment, a two-steps protocol is required to obtain

[2]https://model-ad.org

hiPSCs-derived microglia with genuine microglial functions. In this scheme, the first step is to obtain iPSCs-derived macrophages progenitors and second to grow them in a conditioned environment, i.e., in the presence of other neural cells (Lee C.Z.W. et al., 2018). Under such experimental settings, hiPSCs-derived microglia exhibit phenotype, gene expression profile and functional properties close to brain-isolated microglia (Pandya et al., 2017; Takata et al., 2017).

Maintenance of tissue architecture is an important aspect of *in vivo* studies. Interestingly, hiPSCs derived cells can be grown in 3D cultures to model the cytoarchitecture and the connectivity of the brain while allowing *in vitro* manipulation and experimentation. Such 3D or organoids models open the way for studying human microglia under conditions that are close to physiological and physio-pathological context. Although specific pitfalls exist with such approaches, including the need to develop isogenic controls to alleviate the risk of cofounding factors due to difference in genetic backgrounds between patients and controls, there is little doubt that these approaches will be instrumental to decipher the role of microglia in the progression of AD.

CONCLUDING REMARKS

Glial cells, including microglia, have long been suspected to play a role in Alzheimer's disease but only because of their ability to react to neuronal dysfunctions (e.g., Amyloid and Tau aggregates). This neurocentric view, which considered glial cells as secondary, has been challenged recently by the results of genetic association studies identifying genetic loci associated with risk of Alzheimer's that are associated to genes preferentially or exclusively expressed in glial cells (Verheijen and Sleegers, 2018). This has refined our view of how Alzheimer disease initiates and progresses, and introduced new concepts and ideas for Alzheimer's pathophysiological mechanisms, both at the molecular and cellular levels.

Because of their abilities to sense and react to their environment, reactive microglia are likely playing key early roles in the disease progression and may lead to the identification of early biomarkers. Because they can drive functional changes in astrocytes (Liddelow and Barres, 2017) and crosstalk with non-neuronal immune cells (Dionisio-Santos et al., 2019), they also represent attractive drug targets to stop or limit the disease progression.

As reported here, the exact contribution of the different reactive microglia subtypes to AD is currently unclear and the subject of intense researches. Over the recent years, several technological breakthroughs have been achieved, allowing scientists to address new challenging questions. These technical developments now allow studying microglia roles with medium or high throughput flows, and perform fine analysis of their functions in preserved environments. A better understanding of the contribution of microglia cells to AD initiation and progression is expected to renew the interest of big pharma to re-invest in the field and will pave the way toward better designed strategies.

Many factors need to be considered, including sex, age, species, molecular diversity, health status, communication with the periphery, etc., to fully decipher the role of microglia in AD. These are undoubtedly challenging but also very exciting fields of research, which hold the promise of defining innovative therapeutic strategies and reduce the socio-economic burden of this devastating disease.

AUTHOR CONTRIBUTIONS

A-LH, JH, LU, and HH designed and drafted the sections of the manuscript. A-LH prepared the tables and the figures.

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REVIEW
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Accelerated Ovarian Failure as a Unique Model to Study Peri-Menopause Influence on Alzheimer's Disease

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Despite decades of extensive research efforts, efficacious therapies for Alzheimer's disease (AD) are lacking. The multi-factorial nature of AD neuropathology and symptomatology has taught us that a single therapeutic approach will most likely not fit all. Women constitute ∼70% of the affected AD population, and pathology and rate of symptoms progression are 2–3 times higher in women than men. Epidemiological data suggest that menopausal estrogen loss may be causative of the more severe symptoms observed in AD women, however, results from clinical trials employing estrogen replacement therapy are inconsistent. AD pathological hallmarks—amyloid β (Aβ), neurofibrillary tangles (NFTs), and chronic gliosis—are laid down during a 20-year prodromal period before clinical symptoms appear, which coincides with the menopause transition (peri-menopause) in women (∼45–54-years-old). Peri-menopause is marked by widely fluctuating estrogen levels resulting in periods of irregular hormone-receptor interactions. Recent studies showed that peri-menopausal women have increased indicators of AD phenotype (brain Aβ deposition and hypometabolism), and peri-menopausal women who used hormone replacement therapy (HRT) had a reduced AD risk. This suggests that neuroendocrine changes during peri-menopause may be a trigger that increases risk of AD in women. Studies on sex differences have been performed in several AD rodent models over the years. However, it has been challenging to study the menopause influence on AD due to lack of optimal models that mimic the human process. Recently, the rodent model of accelerated ovarian failure (AOF) was developed, which uniquely recapitulates human menopause, including a transitional peri-AOF period with irregular estrogen fluctuations and a post-AOF stage with low estrogen levels. This model has proven useful in hypertension and cognition studies with wild type animals. This review article will highlight the molecular mechanisms by which peri-menopause may influence the female brain vulnerability to AD and AD risk factors, such as hypertension and apolipoprotein E (APOE) genotype. Studies on these biological mechanisms together with the use of the AOF model have the potential to shed light on key molecular pathways underlying AD pathogenesis for the development of precision medicine approaches that take sex and hormonal status into account.

Keywords: Alzheimer's disease, menopause, peri-menopause, accelerated ovarian failure, ovariectomy (OVX), reproductive senescence

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INTRODUCTION

Alzheimer's disease (AD) is an irreversible, progressive neurodegenerative disorder and a leading cause of mortality (Alzheimer's Association, 2016; Scheltens et al., 2016). AD accounts for approximately 60%–80% of dementia cases in older adults with an average age at onset of around 65 years of age (Alzheimer's Association, 2016). Symptoms are characterized by memory loss and impairment in other non-memory aspects of cognitive function such as word-finding, vision/spatial issues, and impaired reasoning or judgment. This loss of cognitive functioning along with loss of behavioral abilities are extremely debilitating for AD patients and dramatically interfere with their daily life (Scheltens et al., 2016).

Neuropathologically, AD is characterized by progressive deposition of parenchymal senile plaques comprised of amyloid-β (Aβ) protein, intracellular neurofibrillary tangles (NFTs) of abnormal phosphorylated tau, and chronic gliosis primarily in the hippocampus and neocortex (Price et al., 1991; Braak and Braak, 1997; Montine et al., 2012; Au et al., 2016; Scheltens et al., 2016; Lane et al., 2018), brain areas critical for learning and memory. This leads to synaptic damage, cognitive impairment, and neurodegeneration (Canter et al., 2016; Scheltens et al., 2016).

Cause(s) of AD are still unknown but several gene mutations, as well as other genetic and modifiable risk factors, have been identified. Age is the major risk factor for sporadic AD. As the number of aging Americans rapidly increases, so does the incidence of AD. An estimated 47 million people are currently living with AD worldwide, with 5.8 million people only in the US. This number is projected to triple by 2,050 to over 16 million (Alzheimer's Association, 2016; Deb et al., 2017; Nebel et al., 2018). The ongoing demographic changes as well as the lack of treatments and preventive strategies will contribute not only to the rising of AD prevalence, but also to the increasing medical costs associated with the disease, which will exceed $1 trillion (Alzheimer's Association, 2016; Deb et al., 2017; Nebel et al., 2018).

Although remarkable research advancements were made in the past decade, the complexity of the cellular mechanisms underlying AD pathogenesis and neuropathology as well as the heterogeneity of symptoms among AD patients caused many clinical therapeutic trials to fail. Understanding the multi-factorial nature of AD pathogenesis and pathology is a crucial step to develop efficacious prevention and treatment strategies, and to optimize care and reduce high costs associated with the disease.

Women constitute nearly 70% of the affected AD population (Fisher et al., 2018), ∼3.5 million Americans aged 65 or older. AD prevalence is 2–3 times higher in post-menopausal women than men, even after controlling for lifespan (Alzheimer's Association, 2016; Laws et al., 2016; Scheltens et al., 2016; Fisher et al., 2018). Furthermore, women present with a faster pathology progression and greater memory impairment than men (Alzheimer's Association, 2016; Laws et al., 2016; Fisher et al., 2018). Despite the established vulnerability, the biological mechanisms underlying the increased risk of AD in women are largely unknown. Clinical data suggest that loss of estrogen at menopause may be a main factor influencing susceptibility to AD, but results from clinical trials employing estrogen replacement therapy in post-menopausal women are inconsistent (Alzheimer's Association, 2016; Fisher et al., 2018). Nevertheless, results from recent clinical studies have suggested that hormone replacement therapy (HRT) initiated during peri-menopause may lower the risk of dementia and have cognitive benefits (Henderson et al., 2005; Whitmer et al., 2011; Shao et al., 2012).

Pre-clinical rodent models have proven to be important for understanding the wide range of estrogen effects that occur during human menopause. Rodent models have been constructed to replicate different elements of human menopause. These include natural aging, ovariectomy (OVX) and hormone replacement, and genetic models (Marques-Lopes et al., 2018). Although the current rodent models of menopause have and will continue to inform our understanding of the role of both estrogen and HRT in menopause, they fail to adequately recapitulate the human menopause process. The intact aging model fails to achieve very low estrogen levels, and the OVX model lacks a stage mimicking peri-menopause. Among the pre-clinical models, the innovative accelerated ovarian failure (AOF) using the chemical 4-vinylcyclohexene diepoxide (VCD), uniquely recapitulates hormonal changes that occur during human menopause, including estrous acyclicity and fluctuation (as in human peri-menopause), followed by undetectable, estrogen levels (as in human post-menopause). These model also allow for the dissociation of the effects of hormone levels from the effects of aging in young animals (reviewed in Van Kempen et al., 2011).

Over the last 20 years, striking advancements have been made in our understanding of AD sex dimorphism. The overarching goal of this review article is to highlight recent advancements in understanding the molecular mechanisms by which menopause may influence the female brain vulnerability to AD in view of the rodent models of menopause available. Studies on these biological mechanisms in combination with the use of the AOF model have the potential to shed light on key signaling pathways with the potential to improve diagnosis, prevention or stabilization of risk factors, and clinical outcomes.

THE HUMAN MENOPAUSE PROCESS

Menopause, or cessation of menstrual cycling, is a uniquely human process and marks the beginning of women's reproductive senescence (Walker and Herndon, 2008; Alberts et al., 2013). The majority of women enter menopause *via* a gradual and irreversible process (peri-menopause) of reduction in ovarian function and decline in estrogen levels followed by a decrease in estrogen receptor (ER) expression over several years. Duration of peri-menopause is ∼5 years, between ages of 45 and 54, which is followed by amenorrhea and then post-menopause (Practice Committee of the American Society for Reproductive Medicine, 2008; Butler and Santoro, 2011; Harlow et al., 2012). Peri-menopause is marked by irregular

estrous cycles and fluctuations in ovarian hormones, but not loss of estrogen levels which are typical of post-menopause (Harsh et al., 2007). This period of irregular hormone-receptor imbalance contributes to the physiological and psychological symptoms associated with menopause (Morrison et al., 2006; Practice Committee of the American Society for Reproductive Medicine, 2008). Although clinically menopause is primarily defined as reproductive senescence, biological changes occur that significantly alter brain function which is reflected by a range of neurological symptoms including depression, insomnia, hypertension, and cognitive dysfunction (Morrison et al., 2006; Practice Committee of the American Society for Reproductive Medicine, 2008).

With current age of menopause transition around 50 years and life expectancy getting close to 80 years, it is estimated that by 2,060 20%–30% of women in the US will be in post-menopause, meaning that a women will spend approximately 1/3 of her life in post-menopause (U.S. Census Bureau, 2017). Mounting evidences indicate that menopause constitutes a risk factor for developing AD and newly published work suggests that peri-menopause, as a neurological transition state, may be the key time for the female brain susceptibility to the disease (Brinton et al., 2015).

MENOPAUSE INFLUENCE ON CLINICAL PRESENTATION OF ALZHEIMER'S DISEASE

There is epidemiological, clinical, and biological evidence that sex dimorphism influences the onset, progression, and clinical manifestation of AD (Mazure and Swendsen, 2016; Fisher et al., 2018; Nebel et al., 2018). It is recognized that aging is the major risk factor for AD (Mielke et al., 2014; Alzheimer's Association, 2016). Although, women live longer than men, they carry an increased life-long risk of being diagnosed with AD even after adjusting for age and lifespan (Barron and Pike, 2012; Vest and Pike, 2013; Alzheimer's Association, 2016). AD women tend to exhibit a broader spectrum of dementia-related behavioral symptoms and experience greater cognitive deterioration than men in the progression of the disease (Schmidt et al., 2008; Chapman et al., 2011; Mazure and Swendsen, 2016; Nebel et al., 2018). Although men with AD have a shorter survival time (Burns et al., 1991; Todd et al., 2013; Kua et al., 2014; Wattmo et al., 2014a,b), a meta-analysis of neurocognitive data from 15 published studies revealed that women with AD showed a consistent worse mental deterioration than men with the disease, even when at the same stage of the condition (Irvine et al., 2012). Moreover, AD pathology appears more likely to be clinically expressed as dementia in women than in men (Barnes et al., 2005; Lin and Doraiswamy, 2014).

There are multiple biological hypotheses by which female sex may affect AD. As recently reviewed by the Society for Women's Health Research Interdisciplinary Network on AD, these could be represented by: (1) genetic factors that have a stronger effect in women [i.e., apolipoprotein E (APOE) genotype]; (2) risk factors that differentially affect men and women (i.e., hypertension); or (3) biological events that are uniquely experienced by women (i.e., menopause, pregnancy; Nebel et al., 2018).

Clinical data suggest that neurological consequences of menopause may trigger more severe symptoms and pathology observed in women with AD compared to men (Barnes et al., 2005; Hua et al., 2010; Skup et al., 2011; Hall et al., 2012; Holland et al., 2013; Lin et al., 2015; Ball and Chen, 2016; Filon et al., 2016; Sundermann et al., 2016a,b, 2017; Jack et al., 2017; Koran et al., 2017; Fisher et al., 2018; Buckley et al., 2019; reviewed in Li and Singh, 2014; McCarrey and Resnick, 2015; Georgakis et al., 2016; Hampel et al., 2018). In support of this, early surgical menopause was associated in women with a 2-fold increase in dementia risk, faster rate of cognitive decline, and more AD pathology (Bove et al., 2014; Fisher et al., 2018). Randomized clinical trials and observational studies found menopause, and particularly the peri-menopause phase, associated with decline in memory function, and increase risk of mild cognitive impairment and dementia (Joffe et al., 2006; Morrison et al., 2006; Greendale et al., 2009; Epperson et al., 2013). Findings from two longitudinal studies reported that peri-menopause was associated with decreased cognitive performance compared to not only pre-menopause, but also post-menopause (Greendale et al., 2009; Epperson et al., 2013), suggesting that the memory impairment was temporary during menopause transition. Recent cross-sectional studies, however, found impairment in verbal memory also in post-menopausal women (Jacobs et al., 2016, 2017; Rentz et al., 2017).

AD pathological hallmarks—Aβ plaques, NFTs, chronic gliosis, and neurodegeneration (Hampel et al., 2018)—are laid down during a prodromal period beginning ∼20 years before clinical symptoms appear, which coincides with the time of peri-menopause in women (Jack et al., 2013; Dubois et al., 2016).

Clinical findings suggest that neurological changes during peri-menopause increase the female brain vulnerability to AD. Accordingly, two recent amyloid-PET and magnetic resonance imaging (MRI) studies on cognitively normal women reported increased indicators of AD phenotype—Aβ deposition in frontal and temporal cortex, hypometabolism, and reduced brain volume in AD-vulnerable regions—starting at peri-menopause (Mosconi et al., 2017b) compared to pre-menopause and male sex (Mosconi et al., 2017a,b). This suggests that irregular estrogen fluctuations and neuroendocrine changes unique to peri-menopause, rather than the loss of estrogen at post-menopause, may be the triggering events that increase the susceptibility to AD risk accelerating AD neuropathology and cognitive decline in women.

Observational data link the use of HRT with lower AD risk in women, but clinical trials employing HRT have produced many inconsistent results, largely due to certain caveats such as hormone formulation, timing of therapy, and dose or route of hormone administration as well as studies limited to post-menopausal women (McCarrey and Resnick, 2015; Georgakis et al., 2016).

Ovaries produce and secrete several types of estrogens, including estrone (E1), 17α- and 17β-estradiol (E2), and estriol (E3). 17β-estradiol is the most abundant and potent female gonadal hormone based on binding activity to ERs

(Folmar et al., 2002; Blaustein, 2008; Koebele and Bimonte-Nelson, 2015). Most human studies, but not all, have used the HRT formulation consisting in Conjugated Equine Estrogens (CEE; Hersh et al., 2004), which upon absorption and metabolism are primarily converted into 17β-estradiol and equilin (Sitruk-Ware, 2002; Bhavnani, 2003).

The initial results from controlled clinical trials, including the Women's Health Initiative (WHI), WHI Memory Study (WHIMS), and WHI Study of Cognitive Aging (WHISCA) found that HRT in women may lead to no, or even adverse, effects on cognition and AD risk (Shumaker et al., 2003, 2004; Maki and Henderson, 2012, 2016; Gurney et al., 2014; Hampel et al., 2018). Administration of CEE and progestin medroxy-progesterone acetate after a prolonged period of hypogonadism or menopause diminishes the neuroprotective effect of HRT and enhances neuroinflammation (Shumaker et al., 2003, 2004). Data from the recent WHI publication on the effect of HRT on mortality reported lower risk of dying from AD and dementia for women receiving estrogen, but not estrogen in combination with progestin (Manson et al., 2017). To date, there is no evidence to support that HRT initiated during early post-menopause confer cognitive benefit, but it appears to be safe for cognitive function (Espeland et al., 2013; Gleason et al., 2015; Georgakis et al., 2016; Henderson et al., 2016). However, recent studies have shown that HRT initiated during peri-menopause lowers the risk of AD and has cognitive benefits, especially to hippocampal-mediated memory processes (Henderson et al., 2005; Whitmer et al., 2011; Shao et al., 2012). In the Cache County Memory Study, peri-menopausal women who used HRT had a reduced risk of AD later in life (Zandi et al., 2002; Shao et al., 2012). Data from the WHIMS-Young study and the Kronos Early Estrogen Prevention Study (KEEPS), which only enrolled early menopausal women, support the beneficial effect of HRT on cognitive function early in the menopause transition process (McCarrey and Resnick, 2015). Therefore, the "Window of opportunity" hypothesis was formulated that the beneficial effect of estrogen on cognition and AD depends on women's age and stage of menopause, and HRT started during post-menopause when a new hormone-receptor equilibrium is already achieved may disturb the established balance (Resnick and Henderson, 2002; Alzheimer's Association, 2016; Pines, 2016; Pike, 2017; Fisher et al., 2018). Similarly, the "Healthy Cell Bias of estrogen action" hypothesis has been proposed (Brinton, 2008; Gillies and McArthur, 2010). The healthy cell bias postulates that, as cognitive health declines over women's lifetime, so are the benefits of HRT treatment on the brain. Specifically, the effects of HRT on cognitive function may progress from beneficial to neutral or deleterious over time. In this scenario, estrogen is neuroprotective if neurons are healthy at time of HRT administration. In contrast, estrogen exposure negatively affects neurological functions if neuronal health is compromised. This may explain the initial results from the different WHI studies where HRT administered later in life (and after menopause) had neutral or adverse effects on cognition. Since it is more likely that neurons during peri-menopause are healthier than at post-menopause, the window of opportunity and healthy cell bias hypotheses are inter-related. Yet, the critical cellular and molecular mechanisms underlying the influence of peri-menopause are still awaiting a clear understanding.

MOLECULAR MECHANISMS FOR THE EFFECT OF PERI-MENOPAUSE ON AD BRAIN

Estrogens exert their physiological and pathological actions mainly through nuclear estrogen receptors (nERs—ERα, ERβ), and membrane estrogen receptors (mERs) which include ERα, ERβ, G-protein coupled receptor 30 (GPER1 aka GPER30), ER-X, Gq-coupled membrane receptor (Gq-mER; Milner et al., 2001, 2005; McEwen et al., 2012; Hara et al., 2015; Korol and Pisani, 2015; McEwen and Milner, 2017). Each brain region possesses a specific ERs gene expression profile, and activity of each ER is differentially regulated by estrogen, which may account for the sex brain dimorphism (Waters et al., 2009; Mitterling et al., 2010; Li and Singh, 2014). Acting *via* genomic and non-genomic signaling pathways, estrogen has long been known to regulate brain function in both males and females *via* multiple mechanisms of action (Cui et al., 2013). For a review of estrogen actions on the brain, see Spencer et al. (2008); Gillies and McArthur (2010); Luine (2014); Frick (2015); Koebele and Bimonte-Nelson (2017) and McEwen and Milner (2017). In the interest of space, this review article will only discuss biological mechanisms that are related to menopause and AD. For a review of studies on sex differences in mouse models of AD, see Gillies and McArthur (2010); Dubal et al. (2012) and Li et al. (2014).

Changes in estrogen levels during menopause have been associated with mitochondrial dysfunction, synaptic decay, and neuroinflammation. Localization of ERs directly within mitochondria suggests that estrogen may regulate **mitochondrial activity** *via* genomic mechanism, but also affect its function directly by modulating mitochondrial respiration and mitochondrial DNA transcription (Klinge, 2017). This is particularly relevant in the brain as mitochondrial dysfunction plays a role in aging and neurodegenerative diseases, specifically in AD (Barja, 2004; Cantuti-Castelvetri et al., 2005; Kujoth et al., 2007). In the 3xTgAD mouse model of AD, Dr. Brinton lab showed that there is a significant correlation between decreased mitochondrial and glucose metabolism and Aβ load in the hippocampus of aged or ovariectomized OVX females with respect to intact females (Brinton, 2008; Yao et al., 2010, 2012; Ding et al., 2013a,b). However, the hypometabolism in the hippocampus of aged and OVX females was observed across all ages, and in both 3xTgAD and non-transgenic mice suggesting that these mechanisms may influence AD, but are not exclusive to it (Yao et al., 2012; Ding et al., 2013a). Accordingly, pre-clinical and human brain imaging studies showed that Aβ and tau pathology precede metabolic changes and cognitive deficits in AD (Landau et al., 2012; Jack et al., 2013), supporting the idea that mechanisms other than hypometabolism may act during menopause to accelerate the initiation of AD.

Another possible mechanism for menopause effect on AD may be through the estrogen modulation of **neuronal excitability and synaptic plasticity** (Spencer et al., 2008; Spencer-Segal et al., 2012; Baudry et al., 2013; Arevalo et al., 2015; Hara et al., 2015; Waters et al., 2015), which are major mechanisms underlying learning and mnemonic processes. Indeed, high levels of 17β-estradiol during the proestrus phase of the estrous cycle increases hippocampal excitability, long term potentiation (LTP), and remodels dendritic spines in female rodents (Woolley and McEwen, 1992; McEwen et al., 2001; Spencer et al., 2008; Mukai et al., 2010; Broestl et al., 2018). This suggests that irregular estrogen fluctuations during peri-menopause and the following decline in estrogen levels may profoundly impact neuronal activity in females. The effects of menopause transition on neuronal activity may also be related to the estrogen action on neurotrophins, including brain-derived neurotrophic factor (BDNF; Spencer et al., 2008, 2010; Spencer-Segal et al., 2011; Wei et al., 2017), which is an important regulator of synaptic plasticity in the brain (Fisher et al., 2018). BDNF is neuroprotective against Aβ-induced cell death, and its levels are reduced in hippocampus and serum of individuals with AD (Laske et al., 2006a,b; Arancibia et al., 2008; Tapia-Arancibia et al., 2008; Nagahara et al., 2009; Nagahara and Tuszynski, 2011). Interestingly, recent studies on carriers of the BDNF single-nucleotide polymorphism Val66Met showed an increase in AD risk in women but not in men, and that women respond differently to estrogen in hippocampal-related working memory tasks compared to non-carrier controls (Wei et al., 2017, 2018; Fisher et al., 2018). Serum levels of BDNF significantly decrease in menopause women and during aging. In rodent models, aging and OVX induce a significant reduction in hippocampal BDNF expression which is ameliorated by HRT.

Other than affecting neurophysiology in higher cognitive brain regions—hippocampus, frontal cortex, basal forebrain, and striatum—estrogen also influences the neuropathology of AD including seeding of parenchymal Aβ plaques, intracellular NFTs, and chronic inflammation (Brinton, 2008; Hirata-Fukae et al., 2008; Lee et al., 2014; Au et al., 2016; McEwen and Milner, 2017; Merlo et al., 2017; Fisher et al., 2018).

Amyloid precursor protein (APP) is processed by two competing pathways, the non-amyloidogenic pathway *via* α-secretase, and the amyloidogenic *via* β-secretase (BACE1), which produces the **toxic β-APPs and Aβ40/Aβ42 peptides** (Baranello et al., 2015). Estrogen can reduce Aβ deposits by favoring the non-amyloidogenic pathway and reducing BACE1 levels, promoting Aβ glial phagocytosis, and regulating the major enzymes involved in Aβ degradation (Jaffe et al., 1994; Xu et al., 1998, 2006; Li et al., 2000; Manthey et al., 2001; Levin-Allerhand et al., 2002; Joffe et al., 2006; Yao et al., 2007; Liang et al., 2010; Zhao et al., 2011; Lee et al., 2014). In hippocampus and prefrontal cortex (PFC) of AD patients, estrogen loss exacerbates deposition of Aβ and NFTs, which induces synaptic dysfunction (Pike, 2017). Following OVX, levels of Aβ peptide and Aβ plaque burden are increased in different transgenic mouse models of AD in females compared to males, and are rescued by estradiol treatment (Callahan et al., 2001; Levin-Allerhand et al., 2002; Zheng et al., 2002; Carroll et al., 2007, 2010). In the 3xTgAD mouse model there is a significant correlation between decreased mitochondrial and glucose metabolism and Aβ load in the hippocampus of aged or OVX females with respect to intact females (Yao et al., 2010, 2012; Ding et al., 2013a; Ding et al., 2013b).

Deposition of NTFs **containing hyperphosphorylated tau protein** may also be an early event during the prodromal phase of AD, which correlates significantly with cognitive symptoms later in life (Nelson et al., 2009; Jack et al., 2013; Wang and Mandelkow, 2016). In the presence of brain tau pathology, women have an increased risk of AD compared to men (Barnes et al., 2005). In mouse models overexpressing mutant tau, females show higher levels of tau pathology and more severe cognitive deficits compared to males (Asuni et al., 2007; Yue et al., 2011; Buccarello et al., 2017), although the animal's ages spanned from young to old (5–15 months). This discrepancy may be explained by the mouse models used or by the concurrence of estrogen-independent mechanisms. Most pre-clinical studies agree that estradiol reduces levels of hyperphosphorylated tau *via* ERs, though opposing roles for ERα and ERβ were reported (Merlo et al., 2017).

Several lines of evidence support the pivotal role of **chronic inflammation** in the initiation and progression of AD (Frautschy et al., 1998; Benzing et al., 1999; Parachikova et al., 2007; Kraft et al., 2013; Christensen and Pike, 2015; Heneka et al., 2015; Matarin et al., 2015; Au et al., 2016; Song et al., 2016; Villa et al., 2016; Wang et al., 2016; Czirr et al., 2017; Jevtic et al., 2017; Liu et al., 2017). An increase in density of resting microglia precedes Aβ plaque formation, and increased activated microglia is observed around Aβ plaques in the brain of transgenic models of AD (Heneka et al., 2015; Wang et al., 2015; Manocha et al., 2016; Jevtic et al., 2017). Inflammation is another risk factor for AD that varies by sex (Hanamsagar and Bilbo, 2016). The expression of genes related to inflammation increases with age more in the forebrain of menopausal women than men and pre-menopausal women (Sárvári et al., 2012; Christensen and Pike, 2015). Acting *via* ERα and ERβ on glial cells, estrogen regulates glial response to neurotoxins and promotes Aβ removal (Li et al., 2000; Blurton-Jones and Tuszynski, 2001; Au et al., 2016; Villa et al., 2016; Merlo et al., 2017). At menopause, estrogen level fluctuations affect conversion of microglia phenotype from resting state to reactive state (Sárvári et al., 2012). In rodents, aging or OVX induce changes in ERα and ERβ levels on astrocytes which leads to impaired neurotrophic responses to estrogen (Rozovsky et al., 2002; McAsey et al., 2006; Morgan and Finch, 2015). Accordingly, reactive astrogliosis and microglia reactivity marker genes are up-regulated in OVX female mice (Sárvári et al., 2012).

Furthermore, besides directly influencing brain structures regulating cognitive function, hormonal and neurological changes during menopause transition may indirectly influence other risk factors that differentially affect men and women, such as APOE genotype and hypertension (see also paragraph on

the use of VCD mouse model to study hypertension; Nebel et al., 2018). For instance, the most well-established genetic risk factors for late onset AD is the presence of a common allele ε4 in the **APOE gene** (Corder et al., 1993; Coon et al., 2007), which encodes for a lipid-binding protein crucial in triglycerides and cholesterol transport to neurons (Puglielli et al., 2003; Bu, 2009; Leduc et al., 2010). APOE4 genotype increases risk of developing AD over the other alleles APOE2 and APOE3 by 5–10 years (Noguchi et al., 1993; Ossenkoppele et al., 2015; Fisher et al., 2018), and was reported to increase Aβ deposition and oligomer formation, as well as phosphorylated tau within neurons (Riedel et al., 2016; Fisher et al., 2018). The APOE4 allele shows sex-dependent effects whereby it is a stronger risk factor for AD in female than male carriers of same age (Farrer et al., 1997; Mortensen and Hogh, 2001; Beydoun et al., 2012; Altmann et al., 2014; Neu et al., 2017). APOE4 women also show a sharper decline in cognitive function and have a greater Aβ brain pathology than APOE4 men (Mortensen and Hogh, 2001; Corder et al., 2004; Beydoun et al., 2012; Fisher et al., 2018). *In vitro* and *in vivo*, activation of ERα up-regulates levels of ApoE mRNA and protein, whereas selective ER agonists down-regulate ApoE mRNA and protein levels in rat hippocampal neurons (Wang et al., 2006). Altogether, this may suggest that HRT could constitute a possible approach to provide therapeutic benefits and/or reduce the risk of developing AD in APOE4 carriers. However, studies on the use of estrogen formulations have provided inconclusive results thus far highlighting a complex interaction between estrogen, APOE and AD (Depypere et al., 2016; Riedel et al., 2016).

In conclusion, there is enough evidence to support that widely fluctuating changes in estrogen-ER response network may underlie the increased susceptibility of female brains to AD conferred by menopause transition. In many women, the brain compensates for these changes during peri-menopause. However, it is likely that in the presence of other AD risk factors for some women this adaptive compensation is diminished, which gives rise to the increased the susceptibility to AD pathogenesis.

RODENT MODELS OF MENOPAUSE

Despite continuous advances in understanding AD pathophysiology, it has been challenging to systematically evaluate the biological mechanisms underlying the influence of menopause on AD in the human population, and clinical research continues to have many inconsistent results and unresolved issues. Pre-clinical rodent models of menopause have proven useful to start teasing out the neurological changes that during menopause may increase the female brain vulnerability to AD. The disparity between animal study results and epidemiological and clinical data has highlighted the need to address the strengths and weaknesses of current animal models of menopause and their use to predict therapeutic outcomes.

The most employed models, ovary-intact aging (reproductive senescence) and ovariectomy present several drawbacks that limit our understanding of the neurological underpinning of menopause transition. These models will be discussed in the following paragraphs in view of their application in AD research and compared to the more recently developed model of AOF (**Figure 1**).

Reproductive Senescence

The ovary is the main site of female sex hormone production. The mammalian ovary has two major functions: (1) differentiation, maturation, and release of oocytes for fertilization (McGee and Hsueh, 2000); and (2) production and sequential secretion of the hormones estrogen, progesterone, and inhibin A and B that regulate the hypothalamus–pituitary–gonadal axis in a repetitive process of follicle development, ovulation, corpus luteum formation and regression during the menstrual/estrous cycle (Peters et al., 1975; Hirshfield, 1991). At birth, the mammalian ovary carries a finite number of immature oocyte-containing follicles, which represent a limited pool of germ cells for reproduction. Following follicular maturation, oocytes are released during ovulation to be fertilized. Most follicles do not reach maturity and go through cell death named atresia. Physiologically, menopause occurs following depletion of all follicles in the ovary (McGee and Hsueh, 2000).

Women have a menstrual cycle, whereas female rodents have an estrous cycle which occurs every 4–5 days and consists of proestrus, estrous, metestrus, diestrus phases that involve similar hormonal fluctuations seen in the human cycle (Goldman et al., 2007). Similar to women, rodents experience natural hormonal fluctuations that emerge at middle age (9–12 months of age) which involves irregular cycles with prolonged diestrus phases and possible absence of ovulation (Maffucci and Gore, 2006; Downs and Wise, 2009; Finch, 2014). Around 1 year of age, differently from the human menopause, rodents transition into a state of extended estrous phases (estropause) with cessation of reproductive cycles. Eventually, at about 16–18 months of age, rodents transition into an acyclic, anestrous state involving persistent estrous and complete halt of ovulation (Chakraborty and Gore, 2004; Maffucci and Gore, 2006). Stages of pseudopregnancy with irregular ovulation and dysregulation of estrogen levels are also common during the rodent estropause. Interestingly, the ovary also produces testosterone. However, levels of ovarian testosterone are not impacted by menopause transition, unlike estrogens and progestins, but declines slowly with age (Burger, 2002; Davis and Wahlin-Jacobsen, 2015). Research conducted over the past 20 years in the ovary-intact aging model supports the notion that female brain vulnerability to AD is associated with loss of ovarian hormones after menopause. For instance, several groups reported an increase in Aβ deposition in the brains of 1-year-old, and older, females compared to age-matched males and young females in rodent models of AD (Zheng et al., 2002; Wang et al., 2003). Using the aging model, the Brinton group has provided gene expression and proteomic evidence of an early shift in mitochondrial and lipid metabolism in the hippocampus of females tested staring at ∼9 months of age compared to age-matched males and young females in the 3xTgAD mouse model (Yao et al., 2009; Ding et al., 2013a; Zhao et al., 2016). However, this does not rule out the possibility of an influence of the aging process itself and high estrogen levels in the elderly rodent systems used.

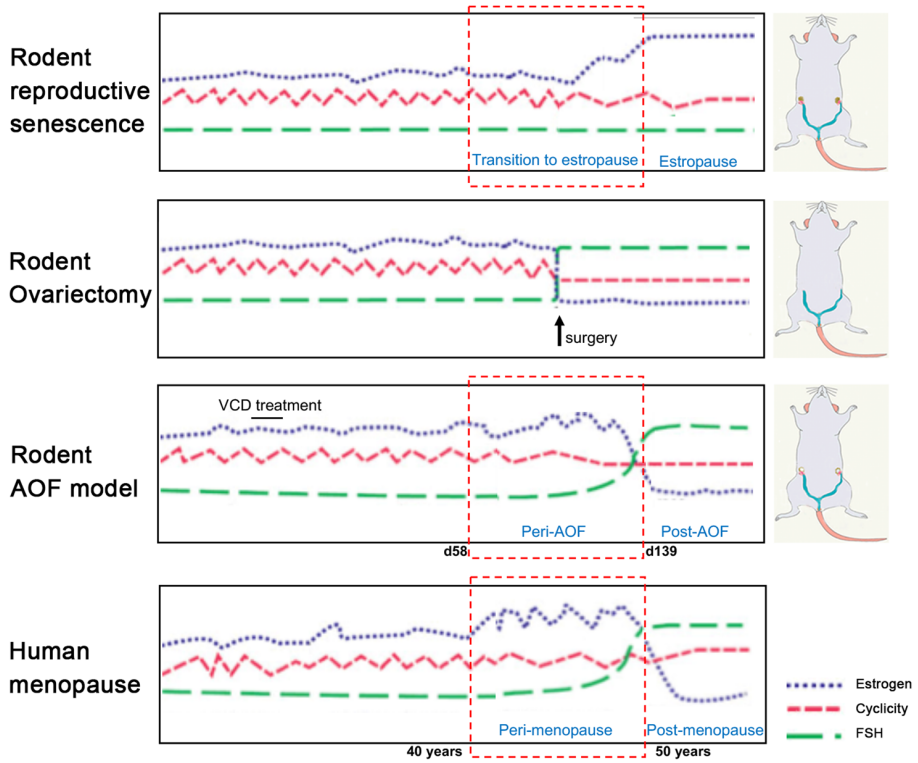

FIGURE 1 | Rodent models of menopause. (On the left) Schematic illustration of hormonal levels and cyclicity in the different rodent models of menopause in comparison with the human menopause process. (On the right) Schematic drawings of the models. In the senescence model, ovaries are preserved, and animals have regular estrous cycles in adulthood before transitioning to estropause in middle-age. In the ovariectomy (OVX) model, ovaries are surgically removed and there is complete and abrupt loss of ovarian hormones. In the innovative vinylcyclohexene diepoxide (VCD) model, ovaries are preserved, and ovarian follicles are depleted inducing a progressive transition to ovarian failure with gradual hormonal changes similarly to the human menopause process. Image was adapted with permission from Jackson lab (www.jax.org).

In conclusion, the rodent reproductive senescence model is characterized by multiple features found in the human menopause including: (1) retention of ovarian tissue; (2) irregular cycling and steroid hormone fluctuations; and (3) irregular fertility. However, because rodents do not exhibit significant depletion in the pool of ovarian follicles at the onset of reproductive senescence, circulating ovarian hormone levels do not present a drastic reduction (Dubal et al., 2012). This rodent model of aging contrasts from the human menopause, where levels of estrogens are very low or undetectable (Chakraborty and Gore, 2004).

Ovariectomy Model

Ovariectomy, or bilateral surgical removal of the ovaries, is a well-established model of menopause (Olson and Bruce, 1986; Maffucci and Gore, 2006). In this model, rodents can be OVXed at different ages that correlate with different life stages. For example, females may be OVXed at 2–6 months of age with regular estrous cycles, 10 months at the beginning of the acyclicity, and at 18 months of age at the beginning of persistent estrous. This is an ideal model to study the effect of ovarian hormones deficit and influence of exogenous hormonal treatments on the brain. Experimental interventions may occur either at the time of OVX or commence once 17β-estradiol has reached a low to non-detectable level in the plasma, which typically occurs within 1–2 weeks (Marques-Lopes et al., 2018).

Using this model, many laboratories have studied the effect of estrogen loss, and 17β-estradiol and other hormonal formulations, on cognitive function in normal wild type and AD rodents (Diaz Brinton, 2012; Li et al., 2014; Koebele and Bimonte-Nelson, 2016). For instance, administration of 17β-estradiol to OVX females have shown to increase performance in spatial memory behavioral tasks (Koebele and Bimonte-Nelson, 2016; Koebele et al., 2017). As mentioned above, in the 3xTgAD mouse model of AD, there is a significant correlation between decreased mitochondrial and glucose metabolism and Aβ load in the hippocampus of OVX females with respect to intact females (Yao et al., 2009, 2012; Ding et al., 2013a,b). The shift in energy metabolism was associated with an increase in Aβ deposition in OVX females compared to males and ameliorated by treatment with 17β-estradiol (Callahan et al., 2001; Levin-Allerhand and Smith, 2002; Zheng et al., 2002; Yue et al., 2005; Yao et al., 2012; Ding et al., 2013b; Lee et al., 2014).

In the OVX model, some shortcomings may influence the interpretation of data: (1) age of mice at OVX; (2) dose,

duration, and mode of administration of hormonal treatment; and (3) differential animal hormonal responsiveness depending of time elapsed since OVX (Gibbs, 1997; Rapp et al., 2003; Maffucci and Gore, 2006; Scharfman et al., 2007). For instance, several studies have addressed these issues and support that estrogen sensitivity decreases when greater time has lapsed since OVX or with increasing age making it difficult to compare across experiments (Morrison et al., 2006; Smith et al., 2010; Koebele et al., 2017).

While the OVX model provides insights into the role of estrogen and other gonadal hormones separately from aging as a confounding factor, this has two drawbacks. First, in addition to estrogens, OVX depletes other hormones that have important roles in menopause and can impact brain function (Maffucci and Gore, 2006; Rocca et al., 2011). Second, since the majority of menopausal women have intact ovaries, the abrupt loss of gonadal hormones models surgical menopause, but not natural transitional menopause. This does not allow to shed light on the progressive changes in estrogen responsiveness that occur when the natural cycle of circulating estrogens are disrupted at peri-menopause (Nejat and Chervenak, 2010; Shuster et al., 2010; Van Kempen et al., 2011). In support of this, Koebele and Bimonte-Nelson (2017) have shown that in rats, the model of ovarian hormone loss influences spatial memory performance and response to estrogen therapy, supporting the importance of studying surgical and transitional menopause independently (Acosta et al., 2009, 2010).

The Accelerated Ovarian Failure (AOF) Model

Until recently, aging and OVX have been the primary rodent models of menopause to examine effects of ovarian hormone loss on cognition and AD in females (Koebele and Bimonte-Nelson, 2016). Although these models have furthered our understanding of estrogen actions on the central nervous system, as reported above, the rodent aging and OVX do not fully recapitulate the transitional events of human menopause (Van Kempen et al., 2011). The lack of an adequate model that includes a progressive transition through menopause has hindered studies on how irregular hormonal changes during peri-menopause impact the female brain susceptibility to AD, and the identification of the molecular mechanisms underlying this phenomenon that can be targeted to prevent or delay the disease. Lack of an optimal model that could also dissociate the effect of menopause from aging has limited the studies on the timing of hormonal replacement therapies and how these can influence disease outcomes.

In early 2000s, the Hoyer group developed the innovative model of AOF that successfully replicates the human peri- and post-menopause stages, including irregular estrous fluctuations at peri-AOF (Mayer et al., 2004, 2005; Williams, 2005; Van Kempen et al., 2011, 2014), and acyclicity at post-AOF stage with very low estrogen levels, and follicle-stimulating hormone (FSH) and luteinizing hormone (LH) levels raising accordingly (Mayer et al., 2002; Van Kempen et al., 2011; Brooks et al., 2016; Marques-Lopes et al., 2018). In this model, 15-day administration of low doses of the 4-VCD (130–160 mg/Kg in 0.5% dimethyl sulfoxide in sesame oil; i.p.) induces selective depletion of ovarian primary and primordial follicles (Lohff et al., 2006; Acosta et al., 2009; Van Kempen et al., 2011, 2014; Brooks et al., 2016; Koebele and Bimonte-Nelson, 2016). For a mechanistic review, see Hoyer and Sipes (1996, 2007) and Hoyer et al. (2001). Following VCD treatment, mature follicles deplete with normal estrous cycles (Flaws et al., 1994; Mayer et al., 2002; Lohff et al., 2006) resulting in a progressive transition to acyclicity and ovarian failure (Mayer et al., 2004). In addition to reducing confounds associated with surgical manipulations, the AOF model maintains the presence of ovarian tissue and presents a peri-AOF stage which importantly parallels human peri-menopause (Mayer et al., 2004; Rivera et al., 2009). Another advantage of this model is that it allows for the longitudinal study of the effects of hormone levels dissociated from the effects of aging in young animals (Danilovich and Ram Sairam, 2006; Koebele and Bimonte-Nelson, 2016). The drawback of this model is that VCD is toxic at higher doses (National Toxicology Program, 1986). However, in a laboratory setting, administration of low doses of VCD does not negatively affect peripheral tissues, including liver and kidney function and organ weights (Devine et al., 2001; Hoyer et al., 2001; Mayer et al., 2005; Haas et al., 2007; Sahambi et al., 2008; Wright et al., 2008; Frye et al., 2012; Van Kempen et al., 2014), and does not appear to cross the blood–brain barrier (Van Kempen et al., 2011, 2014). Work from the Milner group established that this model is suitable for longitudinal studies (Van Kempen et al., 2014), and does not have any direct effects on induction of inflammation markers in brain areas inside (e.g., hippocampus and hypothalamus) and outside (e.g., circumventricular organs) the blood–brain barrier (Van Kempen et al., 2014). Based on the assessment of ovarian follicle depletion and responses of plasticity markers in brain areas known to be estrogen responsive, timepoints for pre-AOF (∼25 days after first injection), peri-AOF (>58 days post-injection), and post-AOF (>139 days post-injection) stages have been established corresponding to the human pre-, peri-, and post-menopause, respectively (Mayer et al., 2004; Lohff et al., 2006; Van Kempen et al., 2011, 2014; Marques-Lopes et al., 2018; **Figure 2**). Length of peri-AOF period and beginning of post-AOF can be manipulated by varying the duration of VCD treatment (Brooks et al., 2016).

Several laboratories have implemented this model to study cognition, cardiovascular disease, insulin resistance, atherosclerosis, bone loss, anxiety (Van Kempen et al., 2014), and hypertension (see next paragraph). A fairly recent summary of VCD doses and organs evaluated in studies applying the AOF model can be found in Van Kempen et al. (2011) and Marques-Lopes et al. (2018). A single study using the AOF has been reported in Tg2576 mouse model of AD. Golub et al. (2008) treated female mice with VCD at 60–75 days of age to induce AOF, and found no significant improvement of estrogen replacement in cognitive performance or Aβ plaque load in the hippocampus and anterior cortex of post-AOF females. The study was affected by low power due to small sample size and measured the effect of estrogen only on late post-AOF mice at 15 months

of age, thus missing the opportunity to quantify the influence of peri-AOF on AD phenotype and introducing aging as a variable during data collection. Therefore, the influence of peri-AOF on neuropathology and phenotype in AD model has not been tested and cannot be ruled out. Nevertheless, the AOF models has been used to study cognitive changes in both mice and rats, which suggested a need for caution in extrapolating data on cognitive function from OVX models to a VCD model, and demonstrated the importance of studying transitional and surgical menopause independently (Acosta et al., 2009, 2010; Koebele and Bimonte-Nelson, 2017; Koebele et al., 2017).

In summary, studies employing VCD-treated animals highlight that this model of AOF is extremely useful for modeling biological mechanisms and disease states associated with peri- and post-menopause, and that studies employing the VCD model will be particularly valuable to validate the most promising findings generated in the aged and OVX models of AD.

Lessons From Hypertension Studies as an Illustration of Rodent AOF Model Utilization

Although works employing the AOF in AD animals are lacking, this model has been used to study sex dimorphism in other diseases and in risk factors of AD, such as the study of the influence of menopause transition on chronic elevated blood pressure (hypertension; Van Kempen et al., 2011; Iadecola, 2014; Wiesmann et al., 2017; Marques-Lopes et al., 2018). Given that some AD risk factors, like hypertension, are modifiable, determining the extent to which sex differences contribute to differential vulnerability may present opportunities for the development of more targeted and efficacious therapies. A large body of literature supports the deleterious role of hypertension in midlife as a risk factor for AD in both men and women (Feldstein, 2012; Davey, 2014a,b; Iadecola, 2014; Iadecola et al., 2016; Kehoe, 2018). There is both clinical and pre-clinical evidence that hypertension induces cerebrovascular damage that causes the reduction of Aβ removal from the brain, and activates chronic inflammation, factors that accelerate the neuropathology and cognitive impairment in AD (Zlokovic, 2011; Carnevale et al., 2012; Shah et al., 2012; Attems and Jellinger, 2014; Kruyer et al., 2015; Faraco et al., 2016).

Sex differences in blood pressure control have been extensively reported (Lima et al., 2012; Sandberg and Ji, 2012). Men with hypertension seem to have a higher risk of developing AD than pre-menopausal women, but this relationship is inverted during the menopause transition and postmenopausal women have a higher risk of AD than age-matched men (Burt et al., 1995a,b; Zanchetti et al., 2005; Yanes and Reckelhoff, 2011; Lima et al., 2012). Notably, estrogen influence on blood pressure involves regulation of a number of brain regions including the ventrolateral medulla, nucleus of solitary tract, and paraventricular nucleus of the hypothalamus (McEwen et al., 2012). Within this circuitry, there are many potential sites by which estrogen can interact with renin-angiotensin system and molecular signaling pathways critical for regulation of brain cardiovascular circuits (McEwen et al., 2012; Maranon et al., 2014; McEwen and Milner, 2017).

It is now established that young female mice are protected from slow pressor Angiotensin II (AngII)-induced hypertension compared to age-matched males (Girouard et al., 2009; Xue et al., 2009, 2013, 2014; Marques-Lopes et al., 2014, 2015, 2017; Van Kempen et al., 2015). An increasing number of studies using either the senescence or OVX models have shown that in "post-menopause-like" state females are more susceptible to AngII-induced hypertension to a magnitude similar to that observed in males (Tiwari et al., 2009; Capone et al., 2012; Sandberg and Ji, 2012; Coleman et al., 2013; Marques-Lopes et al., 2014, 2015). These studies provide a first indication that estrogen may play a critical role in protection against hypertension. Interestingly, by using the AOF model, work from Dr. Milner group showed for the first time that peri-AOF is the critical time for the susceptibility to AngII-induced hypertension (Marques-Lopes et al., 2014, 2017; Van Kempen et al., 2016). While the data obtained employing the aging and OVX models only suggested that estrogen plays a role in hypertension in rodents, in the studies by the Milner group, the use of the AOF model uniquely allowed the testing of the hypothesis that irregular estrogen fluctuations during AOF transition, rather than loss of estrogen at post-AOF, may be responsible for the observed increase in susceptibility to AngII-induced hypertension. Furthermore, it was shown that estradiol activation of ERβ in the paraventricular nucleus of the hypothalamus (PVN) attenuates the glutamate-induced increase in blood pressure (Gingerich and Krukoff, 2006). To better understand the link between estrogen and hypertension, Milner et al. studied the changes in subcellular localization of the glutamate N-Methyl-D-aspartate (NMDA)

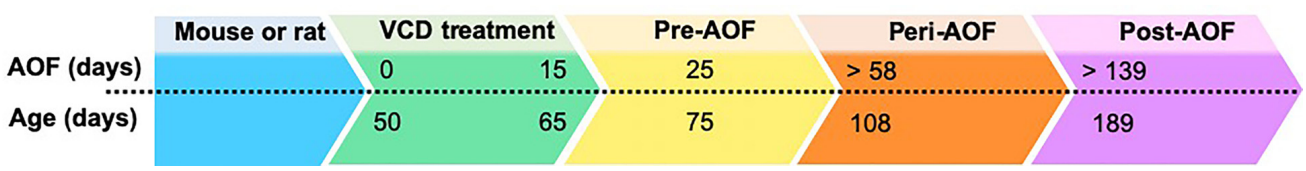

FIGURE 2 | Accelerated ovarian failure (AOF) model timeline. AOF stages can be calculated in days following initiation of VCD treatment (15 days total). Days above the dotted line indicate time after start of VCD treatment, while days below the line indicate the animal chronological age. Pre-, peri, and post-AOF stages are indicated by the arrowhead boxes.

receptor subunit GluN1 in ERβ-positive neurons of the PVN following slow pressor AngII treatment in males and females using both the senescence and AOF models (Marques-Lopes et al., 2014, 2017). In both models, AngII-treated young females, males, and peri-AOF females show decreased total density of GluN1 in ERβ dendrites of PVN, whereas aged females and post-AOF females had an increase in total density. However, in males and peri-AOF females, plasmalemmal affiliation of GluN1 was increased, while it was unchanged in post-AOF females. As for the studies by Acosta et al. (2009, 2010) on the influence of the menopause model used in cognitive tasks, these findings indicate that distinct neurobiological processes underlie AngII-induced hypertension in aging and AOF, which may arise from the differences in estrogen levels between aged and AOF female mice.

CONCLUSION

The multi-factorial nature of AD neuropathology and symptomatology has taught us that a single therapeutic approach will most likely not fit all. Studying the effects of sex differences and menopause will lead to the development of novel targeted precision medicine approaches that take sex and hormonal status into account.

Although substantial advances in medicine and research for AD have been made over the past years, the degree to which human studies can understand the neurological mechanisms of menopause in AD is limited. Rodent models of menopause have proven very useful to start dissecting the key molecular mechanisms underlying the influence of menopause transition on AD. In particular, the innovative model of AOF can provide a valuable approach to the study of physiological changes that more closely parallel to the ones of human menopause. The advantages of the AOF as a model of transitional menopause make it an ideal choice for studies of menopause and HRT in mouse AD models.

AUTHOR CONTRIBUTIONS

RM performed literature search, prepared and reviewed the manuscript.

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A Pilot Study Investigating Changes in the Human Plasma and Urine NAD+ Metabolome During a 6 Hour Intravenous Infusion of NAD+

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Accumulating evidence suggests that active maintenance of optimal levels of the essential pyridine nucleotide, nicotinamide adenine dinucleotide (NAD+) is beneficial in conditions of either increased NAD+ turnover or inadequate synthesis, including Alzheimer's disease and other neurodegenerative disorders and the aging process. While studies have documented the efficacy of some NAD+ precursors such as nicotinamide riboside (NR) in raising plasma NAD+, no data are currently available on the fate of directly infused NAD+ in a human cohort. This study, therefore, documented changes in plasma and urine levels of NAD+ and its metabolites during and after a 6 h 3 μmol/min NAD+ intravenous (IV) infusion. Surprisingly, no change in plasma (NAD+) or metabolites [nicotinamide, methylnicotinamide, adenosine phosphoribose ribose (ADPR) and nicotinamide mononucleotide (NMN)] were observed until after 2 h. Increased urinary excretion of methylnicotinamide and NAD+ were detected at 6 h, however, no significant rise in urinary nicotinamide was observed. This study revealed for the first time that: (i) at an infusion rate of 3 μmol/min NAD+ is rapidly and completely removed from the plasma for at least the first 2 h; (ii) the profile of metabolites is consistent with NAD+ glycohydrolase and NAD+ pyrophosphatase activity; and (iii) urinary excretion products arising from an NAD+ infusion include NAD+ itself and methyl nicotinamide (meNAM) but not NAM.

Keywords: NAD+, nicotinamide (NAM), ADP ribose, methyl nicotinamide, nicotinamide mononucleotide

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INTRODUCTION

The parent pyridine nucleotide, nicotinamide adenine dinucleotide (NAD+) is present in all cells of the body and is essential for cell viability and function.

As a cofactor responsible for electron transport into the respiratory chain, NAD+ and its redox couple NADH are central to energy (ATP) production in the mitochondria *via* oxidative

phosphorylation. The phosphorylated metabolite of NAD+, NADP+ with its redox couple NADPH also provides the reducing power to drive a number of anabolic reactions, including cholesterol and nucleic acid synthesis, elongation of fatty acids and regeneration of glutathione (GSH), one of the body's main antioxidants. Overall this family of pyridine nucleotides, [NAD(P)(H)], contributes to the redox exchange of over 400 enzyme reactions. Importantly, when acting as a redox couple NAD+ is not consumed. However, NAD+ also serves as a substrate for a number of other important metabolic processes and is therefore consumed as a consequence of their chemical reactions, potentially depleting the tissue of NAD+. Included in this number are reactions driven by the poly adenosine phosphoribose-ribose (ADPR) family of enzymes (PARP 1–17) controlling DNA repair and nuclear stability, epigenetic control enzymes (Sirt1–7), intercellular immune communication (CD38/CD157) and neuronal regeneration (SARM1; Essuman et al., 2017). The potential exists therefore for the disruption of cellular metabolism at multiple levels in conditions where NAD+ consumption by these enzymes exceeds NAD+ supply or synthesis.

The clinical importance of maintaining cellular NAD^+ levels was established early in the last century with the finding that pellagra, a disease characterized by diarrhea, dermatitis, dementia and death, could be cured with foods containing the NAD^+ precursor niacin (also known as vitamin B_3; Goldberger, 1914). Although pellagra is rare in developed countries, cellular concentrations of NAD^+ have been shown to decrease under conditions of increased oxidative damage such as occur during aging (Braidy et al., 2011; Massudi et al., 2012; Guest et al., 2014). Altered levels of NAD^+ have been found to accompany several disorders associated with increased oxidative/free radical damage including diabetes (Wu et al., 2016), heart disease (Pillai et al., 2005), age-related vascular dysfunction (Csiszar et al., 2019), ischemic brain injury (Ying and Xiong, 2010), misfolded neuronal proteins (Zhou et al., 2015) and Alzheimer's dementia (Abeti and Duchen, 2012).

In addition to the pathological hallmarks of Aβ plaques and neurofibrillary tau tangles, oxidative damage is a consistent finding in Alzheimer's disease and is widely recognized as an early event in the pathogenic process, even preceding Aβ deposition (Su et al., 2008). While the reactive oxygen species (ROS) causing this damage likely originate from multiple sources, dysfunctional mitochondria and availability of redox active metals such as Fe++ and Cu+ are thought to play dominant roles (Zhua et al., 2007).

A major consequence of cellular oxidative stress, in the brain and elsewhere, are single or double stranded breaks to the DNA. In response to DNA damage, PARP1 hydrolyzes NAD+ to produce polymers of ADP-ribose (Ying, 2013). We showed previously that NAD+ levels were inversely correlated to measures of oxidative stress in human tissue (Massudi et al., 2012) and in the rat brain (Braidy et al., 2014). Thus, while reduced levels of NAD+ in the brain of living Alzheimer's sufferers awaits confirmation by non-invasive techniques the consistent findings of oxidative damage in the post mortem brain strongly supports the view that accelerated NAD+ turnover and depletion contribute to the neurological dysfunction in this disease. As interventions targeted at restoring NAD+ have been shown in animal models to support healthy aging and improve metabolic function (Yoshino et al., 2011; Mills et al., 2016) and dementia (Long et al., 2015), strategies to raise NAD+ levels in the human are being actively explored.

The most direct method of increasing NAD+ levels is through intravenous (IV) administration. Though data from experimental research is minimal, the significant clinical benefit of IV NAD+ infusion in alcohol withdrawal has been previously reported (O'Holleran, 1961; Mestayer, 2019). Surprisingly, while the oral administration of NAD+ precursors such as nicotinamide riboside (NR) or nicotinamide mononucleotide (NMN) are being enthusiastically investigated for their impact on NAD+ levels (Yoshino et al., 2011, 2018; Mills et al., 2016; Airhart et al., 2017), the metabolic fate and pharmacokinetic properties of IV NAD+ administration are yet to be reported in humans. This study, therefore, presents for the first time the changes in concentrations of NAD+ and its metabolites during an IV infusion of NAD+ in a cohort of healthy male participants.

MATERIALS AND METHODS

Participants

Eleven (Test n = 8, Control n = 3) male participants aged 30–55 years were recruited *via* advertisements on radio, TV and social media outlets. All participants had a BMI less than 30 kg/m^2 (Test average BMI = 27.5 ± 2.5 kg/m^2; Control average BMI = 24.6 ± 6.5 kg/m^2), were not diabetic, smoked less than 1 cigarette and consumed less than 2 standard alcoholic drinks per day. Individuals taking lipid lowering or anti-inflammatory drugs, who had a history of liver or renal failure or recently experienced a microbial infection, trauma or any other significant or untreated medical disorders were excluded from the study. Subjects received monetary compensation for their time and participation, regardless of whether they completed the experiment. Participants were not permitted to use natural health products containing NAD+, NR or Nicotinamide within 14 days prior to and during the course of the study.

Diet Standardization

On the day prior to the NAD+ IV infusion participants consumed an identical niacin-reduced diet and drank only water.

No food was consumed on the day of the study until after the final 8 h blood/urine sample had been collected. Participants were encouraged to drink water to remain normally hydrated. No other beverage types were permitted.

To ensure adequate energy intake was maintained for participants during the 6 h infusion the IV solution also contained 0.1% dextrose, providing approximately 2,000 calories over the 6 h infusion period.

Infusion Protocol

Participants were randomized to either the Test (n = 8) or Control (n = 3) group. Participants in the Test group were intravenously administered 750 mg NAD+ in normal saline

(Archway Apothecary, Covington, LA, USA) over a 6 h period (infusion rate = ∼2 mg/min ≡ 3 μmoles/min). This dosage of NAD+ was derived empirically and reflects a common dosing regimen in clinics (e.g., Springfield Wellness Clinic, Springfield, LA, USA) which regularly provide IV NAD+ infusions in clinical practice. Participants in the Control group were intravenously administered normal saline over a 6 h period.

Clinical administration and supervision of the IV infusions and sample collection were carried out at the Springfield Wellness Center, Springfield, LA, USA.

Blood Sample Collection

Baseline (TO) blood samples were collected on all participants after an overnight 12 h fast timed to occur immediately prior to the start of the infusion. Additional samples were then collected at 30, 60, 120 (2 h), 360 (6 h) and 480 (8 h) minutes after the start of the infusion.

Whole blood was collected *via* standard venepuncture (opposite arm to infusion site) into a 5 mL non-gel heparinized tube. Immediately after the collection, the blood was centrifuged at 4°C for 10 min at 1,409× *g*.

The plasma and red blood cells fractions were immediately separated and distributed into 5 × 500 μL aliquots each. All aliquots were then immediately frozen and stored at −80°C until analysis.

Urine Sample Collection

After the collection of a baseline midstream urine sample, participants were asked to void all urine into the receptacle(s) provided at 30 min, 2 h, 6 h and 8 h after initiation of the NAD+ infusion. If participants needed to pass urine intermittently between these time points they were asked to do so into the next successive receptacle. All samples were aliquoted and stored at −80°C immediately upon receipt.

Analytical Method

Chromatographic separation of NAD+ and related metabolites and MS detection Liquid chromatography coupled to tandem mass spectrometry (LC/MS/MS) was carried out using a Sciex QTRAP 5500 mass spectrometer (Sciex, Redwood City, CA, USA) as previously described (Clement et al., 2018). Briefly, 100 μL of human plasma or urine was extracted in 400 μL of ice-cold methanol, centrifuged at 4°C for 10 min, and filtered through 3 kDa membrane cartridges. Sample extracts were dried under vacuum, reconstituted in 200 μL of 100 mM NH4OAc buffer and transferred into 200 μL glass vials and capped before LC/MS/ MS analysis. Standards and samples (20 μL) were injected onto a Phenomenex NH_2 column (150 mm· 2 mm· 3 mm) as previously described. A binary solvent gradient consisting of 5 mM NH4OAc pH 9.5 adjusted with ammonia (mobile phase A) and acetonitrile (mobile phase B) with a flow rate of 250 μL/min was used. Initial solvent composition at injection was 25% A, followed by a 2-min gradient to 45% A and a fast gradient ramp to 80% A (0.1 min) that was maintained for 5.9 min, A was increased again to 95% (2 min), held for 13 min, and then reverted to initial conditions (0.1 min) for equilibration, with a total run time of 30 min. The column flow was directed into the MS detector. Calibration curves of individual metabolites were constructed using the peak area ratios (peak area of the metabolite divided by peak area of the selected IS) of each calibrator vs. its concentration.

Internal standards consisted of 2H2NAM (for NAM, methylNAM and ADPR) and $^{13}C_5$;-Cyclic AMP (for NMN and NAD+). Note that as isotopic labels are not commercially available for all NAD metabolites, a closely related molecule (structural analog) can also be used (Yamada et al., 2006) provided that it is deemed of similar stability and ionization efficiency during analysis. The internal standards selected in this study have been previously optimized for the related metabolite (Bustamante et al., 2017).

Safety

The safety of IV NAD+ was assessed using liver function tests and clinical observation of any adverse events. Liver function tests consisted of serum, total bilirubin (bili), alkaline phosphatase (ALP), alanine aminotransferase (ALT), gamma glutamyl transferase (GGT), lactate dehydrogenase (LD) and aspartate aminotransferase (AST).

Statistical Analysis

Statistical analysis was completed using SPSS version 24 and GraphPad Prism version 8 for windows. An unweighted means, two-way ANOVA with Bonferroni's multiple comparison *post hoc* test was used to determine if the mean concentration for analysts tested were different over the 8 h period and between Test and Control groups. The Wilcoxon Signed Ranks test was used to determine if differences in mean liver function test concentrations were significant between baseline and 8 h time points. Differences were considered statistically significant when $p < 0.05$.

Ethics

This study was carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. Ethical approval was obtained from the William Carey University Institutional Review Board, Hattiesburg, MS (Protocol #2017-12). Informed consent was obtained from all participants.

RESULTS

Safety

No adverse events were observed during the 6 h infusion with either placebo (saline) or test (NAD+) cohorts.

A significant decrease in activity (1.3, 57, 3.6 units/L) for the liver function enzymes GGT, LD and AST, respectively was observed at 8 h after initiation of the NAD+ infusion (**Table 1**). No significant change in activity for any liver function marker was apparent at 8 h in the placebo (saline) treated samples, however low sample number may reduce discrimination sensitivity. A significant increase of 2.75 μmoles/L in plasma bilirubin was also observed. However, none of the changes were considered clinically significant.

TABLE 1 | The Wilcoxon Signed Ranks test was used to determine if differences in mean liver function test concentrations were significant between baseline and 8 h time points.

	Reference range	Group	Time 0 h Mean (SD)	Time 8 h Mean (SD)	*p* (T0 vs. T8)
Albumin (g/L)	36–47	Test	46.0 (2.5)	45.5 (2.9)	ns
		Control	44.3 (5.6)	42.0 (5.7)	ns
Total protein (g/L)	64–83	Test	72.1 (4.3)	72.0 (4.5)	ns
		Control	76.3 (5.9)	73.7 (4.5)	ns
Total bilirubin (μmol/L)	4–20	Test	9.3 (4.0)	12.0 (3.0)	≤0.05
		Control	13.7 (5.5)	13.3 (4.0)	ns
ALP (units/L)	35–110	Test	77.1 (23.9)	75.9 (21.9)	ns
		Control	67.7 (4.0)	62.7 (2.1)	ns
ALT (units/L)	5–40	Test	40.8 (16.7)	38.9 (15.1)	ns
		Control	18.7 (4.9)	17.7 (4.0)	ns
GGT (units/L)	5–50	Test	25.1 (17.4)	23.8 (16.9)	≤0.05
		Control	23.3 (7.6)	21.7 (7.2)	ns
LD (units/L)	20–250	Test	256.8 (66.3)	198.8 (78.0)	≤0.05
		Control	133.3 (12.7)	146.3 (43.5)	ns
AST (units/L)	10–40	Test	29.6 (6.7)	26.0 (6.7)	≤0.05
		Control	22.0 (6.1)	21.7 (9.0)	ns

Differences were considered statistically significant when $p < 0.05$.

Plasma

A continuous infusion of NAD+ at a rate of 3 μmoles/min resulted in a significant (398%) increase in plasma NAD+ levels only at the 6 h time point (i.e., end of infusion) relative to baseline ($p < 0.0001$). This was significantly different from the 6 h saline-treated control ($p < 0.001$).

NAD+ levels remained elevated at 8 h (i.e., 2 h post-infusion) relative to baseline and saline treated control samples.

Plasma NAD+ levels did not change significantly from baseline across the 8 h assessment period in saline treated control samples (**Figure 1A**).

Similar to the changes observed for NAD+, plasma levels of the NAD+ metabolite nicotinamide (NAM) increased significantly by 409% at the end of the NAD+ infusion (i.e., 6 h) relative to baseline ($p < 0.0001$). This was likewise significantly different to the 6 h saline treated control ($p < 0.001$).

At the 8 h time point (i.e., 2 h after end of infusion), NAM levels between the control and treated groups were no longer significantly different ($p > 0.05$).

NAM levels for saline treated control samples did not change significantly across the 8 h time period ($p > 0.05$, **Figure 1B**).

Consistent with the changes observed for NAM, plasma levels of the NAD+ metabolite ADPR increased significantly by 393% at the end of the NAD+ infusion (i.e., 6 h) relative to baseline ($p < 0.0001$). This was significantly different to the 6 h saline treated control ($p < 0.0001$).

At the 8 h time point (i.e., 2 h after the end of infusion), ADPR levels remained 305% above baseline ($p < 0.0001$). However, this was not significantly greater than ADPR levels in the saline treated control samples at the same time point ($p > 0.05$).

ADPR levels for saline treated control samples did not change significantly across the 8 h time period ($p > 0.05$, **Figure 1C**).

A Spearman's correlation analysis between group averages across the 8 h time points for the two NAD+ catabolic metabolites, NAM and ADPR, produced a correlation coefficient of 1.00 ($p < 0.001$).

Again, consistent with the changes observed for NAM, plasma levels of the NAM metabolite, methyl-nicotinamide (meNAM) increased significantly to 350% at the end of the NAD+ infusion (i.e., 6 h) relative to both baseline ($p < 0.0001$) and the 6 h saline treated control ($p < 0.01$).

At the 8 h time point (i.e., 2 h after end of infusion) meNAM levels remained 393% above baseline ($p < 0.0001$) and significantly greater than the saline treated control samples at the same time point ($p < 0.05$).

meNAM levels for saline treated control samples did not change significantly across the 8 h time period ($p > 0.05$, **Figure 1D**).

Plasma levels of NMN, a metabolite of NAM *via* the anabolic salvage pathway, was significantly elevated (472%) only at the 8 h time point (i.e., 2 h after end of infusion, $p < 0.05$).

NMN levels for saline treated control samples did not change significantly across the 8 h time period ($p > 0.05$, **Figure 1E**).

Urine

The continuous IV infusion of NAD+ at a rate of 3 μmoles/min resulted in a significant (538%) increase in the urine NAD+ excretion rate at the 6 h time point (i.e., end of infusion) relative to that excreted at 30 min ($p < 0.001$). This was also significantly different to the amount of urine NAD+ excreted at 6 h by the saline treated controls ($p < 0.05$).

The amount of NAD+ excreted in urine decreased by 43% ($p < 0.05$) at 8 h (i.e., 2 h post-infusion) relative to the peak excretion at the 6 h time point.

The urine excretion rate for NAD+ did not change significantly across the 8 h assessment period in saline treated control samples (**Figure 2A**).

The excretion rate of the NAD+ metabolite NAM did not change significantly across the 8 h test period and was not different to the observed NAM excretion rate for the saline treated controls (**Figure 2B**).

The urine excretion rate of the NAM metabolite meNAM was significantly increased (403%) at the 6 h time point (i.e., end

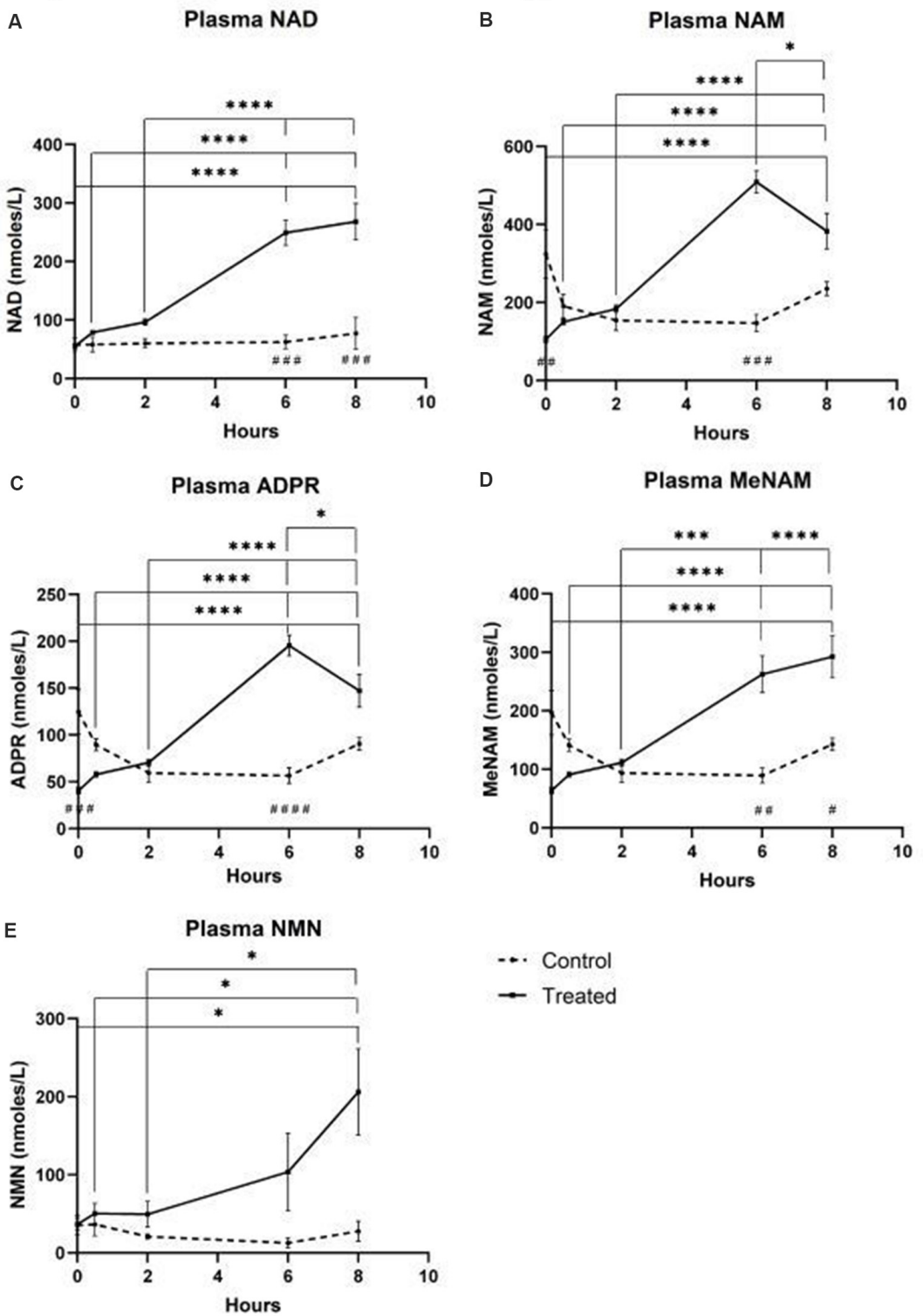

FIGURE 1 | Changes in plasma nicotinamide adenine dinucleotide (NAD+) and metabolites over 8 h [1st 6 h consisting of a continuous (3 μmoles/min) NAD+ IV infusion]. **(A)** NAD+, **(B)** Nicotinamide (NAM), **(C)** adenosine phosphoribose (ADPR), **(D)** methyl nicotinamide (meNAM), **(E)** nicotinamide mononucleotide (NMN). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$, as indicated. Two-way ANOVA with Bonferroni's multiple comparison *post hoc* test was used to determine if the mean concentration for analysts tested were different over the 8 h period and between Test ($n = 8$) and Control ($n = 3$) groups.

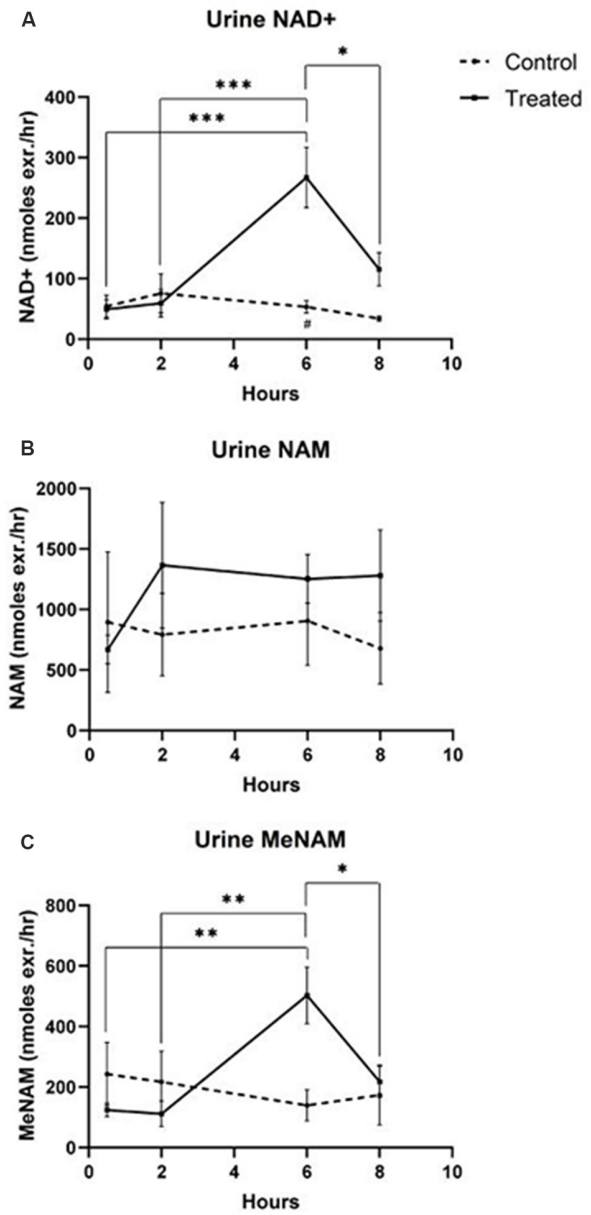

FIGURE 2 | Changes in urine NAD+ and metabolites over 8 h (1st 6 h consisting of a continuous, 3 μmoles/min, NAD+ IV infusion). **(A)** NAD+, **(B)** NAM, **(C)** meNAM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, as indicated. Two-way ANOVA with Bonferroni's multiple comparison *post hoc* test was used to determine if the mean concentration for analysts tested were different over the 8 h period and between Test ($n = 8$) and Control ($n = 3$) groups.

of infusion) relative to that observed at 30 min ($p < 0.01$). The amount of meNAM excreted in urine decreased by 43% ($p < 0.05$) at 8 h (i.e., 2 h post-infusion) relative to the peak excretion at the 6 h time point.

The urine excretion rate for meNAM did not change significantly across the 8 h assessment period in saline treated controls (**Figure 2C**).

DISCUSSION

A growing interest in NAD+ based therapies including NAD+ infusions has highlighted the need for a clearer understanding of the fate of NAD+ and its metabolites following IV administration. The current study documents changes in levels of NAD+ and key metabolites in both plasma and urine over 8 h using a typical clinical dosing regimen of 750 mg NAD+ administered IV over a 6 h period.

Importantly infusion of NAD+ did not produce any observable adverse events in the test cohort but rather reduced plasma activities of enzymes indicative of hepatic stress such as the intrahepatic LD and AST and the post-hepatic (bile duct) enzyme GGT suggesting that the integrity of both intra hepatic and post-hepatic tissue was enhanced even within the relatively short 8 h time frame. The rise in bilirubin, a red cell degradation product, at 8 h may reflect either a very small increase in red cell turnover, as can occur due to infusion induced hemeolysis, or reduced heme metabolism (**Table 1**). However, given the very low magnitude of this change, this was not considered clinically relevant.

As expected, in saline-treated (i.e., control) participants, plasma levels of NAD+ and metabolites NAM and ADPR and the NAM metabolites, NMN and meNAM remained essentially unchanged across the 8 h period. However, an apparent decrease in NAM, meNAM and ADPR were observed between 30 min and 6 h, most likely due to a saline dilution effect. Consistent with this notion, values for these analytes were seen to return to baseline levels at 8 h (i.e., 2 h after the end of the saline infusion).

Unexpectedly however in NAD+ infused participants, plasma NAD+ levels failed to rise until after the 2 h time point reaching a maximum of ∼400% above baseline for NAD+ and metabolites NAM, meNAM and ADPR (398%, 409%, 393%, respectively) only at the 6 h time point (**Figures 1A–E**). This was internally consistent with the peak of urinary excretion for both NAD+ and meNAM also occurring at 6 h before rapidly decreasing after the end of the infusion (**Figures 2A–C**).

NAD+ was infused at a constant rate of 3 μmoles/min. Therefore 90 μmoles of NAD+ was being infused directly into the vascular compartment every 30 min delivering a total of 1,080 μmoles by the end of the infusion at 6 h. Intravascular mixing from any point of infusion occurs within ∼2 min; assuming an average blood volume of 5, the added NAD+, in the absence of significant metabolism or absorption, would correspond to an additional rise in (NAD+) of at least 18 μM, every 30 min across the 6 h of infusion. While an increase of this magnitude is well within this study's analytical detection limits no increase in either NAD+ or its metabolites, were observed until after 2 h (i.e., at the 6 h time point) in either plasma or urine. This unexpected observation indicates a rapid, and for at least the first 2 h, complete tissue uptake and/or metabolism of NAD+ and/or its metabolites.

A number of enzymes can achieve effective NAD+ catabolism including the sirtuins (SIRTs 1–7) the adenosine diphosphate (ADP)–ribose transferases (ARTs) and poly(ADP-ribose) polymerases (PARPs 1–17) and the cyclic ADP-ribose (cADPR) synthases (CD38, CD157). Extracellular NAD+

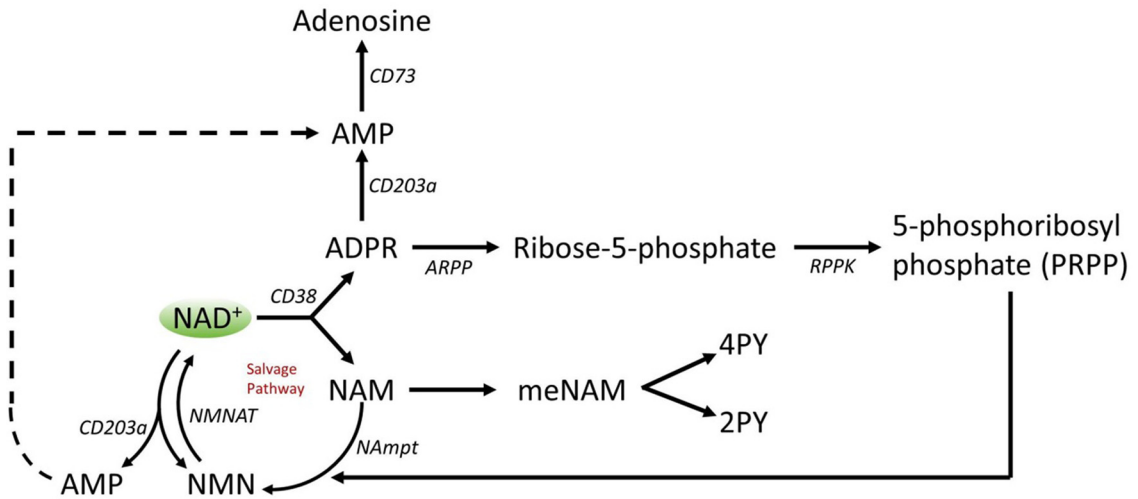

FIGURE 3 | Potential extracellular catabolism of exogenously supplied NAD+ through activity of ectoenzymes CD38 (ADP-ribose (ADPR) synthase), CD203a (NAD+ pyrophosphatase), CD 73 (5′-nucleotidase), CD157-ADP-ribosyl cyclase 2. Abbreviations: NAM, nicotinamide; NMN, nicotinamide mononucleotide; ADPR, adenosine diphosphate riboside; meNAM, methyl nicotinamide; 4PY, N-methyl-4-pyridone-3-carboxamide; 2PY, methyl-2-pyridone-5-carboxamide; ARPP, ADP ribose pyrophosphatase; RPPK, ribosyl pyrophosphokinase; NAmpt, nicotinamide phosphoribosyltransferrase; NMNAT, nicotinamide mononucleotideadenyltransferase; CX-43, connexin 43.

pyrophosphatases present in human serum can also degrade NAD+ to AMP and NMN (Schmidt-Brauns et al., 2001). The cell-surface protein CD73 also converts NMN into NR, which easily crosses cell membranes for potential resynthesis to NAD+. Importantly the NAD+ catabolizing glycohydrolases, CD38 and CD157, ecto-nucleotide pyrophosphatase (CD203a) and the NMN catabolizer CD73 are ectoenzymes found on a wide variety of cells including lymphoid, granulocytic, neuronal and endothelial cells (Wei et al., 2014) and plasma soluble NAD glycohydrolases may also be present (**Figure 3**; Funaro et al., 2009). The parallel rise of plasma NAM and ADPR (correlation coefficient of 1.000, $p < 0.001$, data not shown) strongly suggests that at least by 6 h a major fate of NAD+ is metabolism through cleavage of the glycosidic ADPribose–nicotinamide linkage to NAM and ADPR, by-products symptomatic of NAD glycohydrolase (e.g., CD38) activity. This is consistent with evidence by others where CD38, in particular, has been shown to have a primary role in controlling extracellular NAD+ levels (Wei et al., 2014). Adult human erythrocytes are CD38 positive and express high levels of NAD+ glycohydrolase activity, cleaving exogenous NAD+ to supply erythrocytes with ADP ribose that can be efficiently taken up into the cell (Kim et al., 1993; Albeniz et al., 2004). The absence of any rise in either NAD+ or metabolites, in plasma or urine, until after the first 2 h of the infusion indicates that NAD+ and/or its metabolites are trafficked out of the extracellular vascular space and efficiently sequestered into tissue or extravascular compartments as NAD+ and/or its metabolites during this time period.

Though PARP and sirtuins are involved in NAD+ catabolism, as intracellular enzymes with representatives in cytoplasm, nucleus and mitochondria they are unlikely to directly impact extracellular NAD+ levels. However, these enzymes may be expected to respond to changes in intracellular concentrations of NAD+, NAM and NR that may arise from exogenously supplied NAD+. **Figure 4** summarizes schematically the possible fates of exogenous NAD+.

While significant capacity exists for the rapid degradation of the exogenously supplied IV NAD+ into constituent metabolites, it is also worth noting that uptake of extracellular NAD+ may also be occurring. As NAD+, has an overall negative charge it is unable to cross cellular membranes passively and therefore must be actively transported across the membrane. That this occurs has been shown by a number of researchers who have reported that exogenous NAD+ applied to a variety of human cell types does indeed result in a significant elevation of intracellular NAD+ (Ying et al., 2003; Zhu et al., 2005; Billington et al., 2008; Pittelli et al., 2011; Felici et al., 2013). While the mechanism(s) involved are not yet fully characterized, Alano et al. (2010) reported that exogenous NAD+ could enter neurons through P2X7 gated channels and others have consistently observed the transport of NAD+ across membranes by connexin 43 (CX43) hemichannels, even at concentrations as low as 250 pM (Billington et al., 2008). As connexins have a wide distribution in human tissue and CX43 appears to be the most ubiquitous connexin in many cell types the potential for rapid uptake of NAD+ cannot be discounted.

Thus cellular uptake and/or metabolism of NAD+ and metabolites NAM and ADPR and secondary metabolites meNAM and NMN appeared to proceed at pace with the 3 μmoles/min NAD+ infusion for at least the first 2 h.

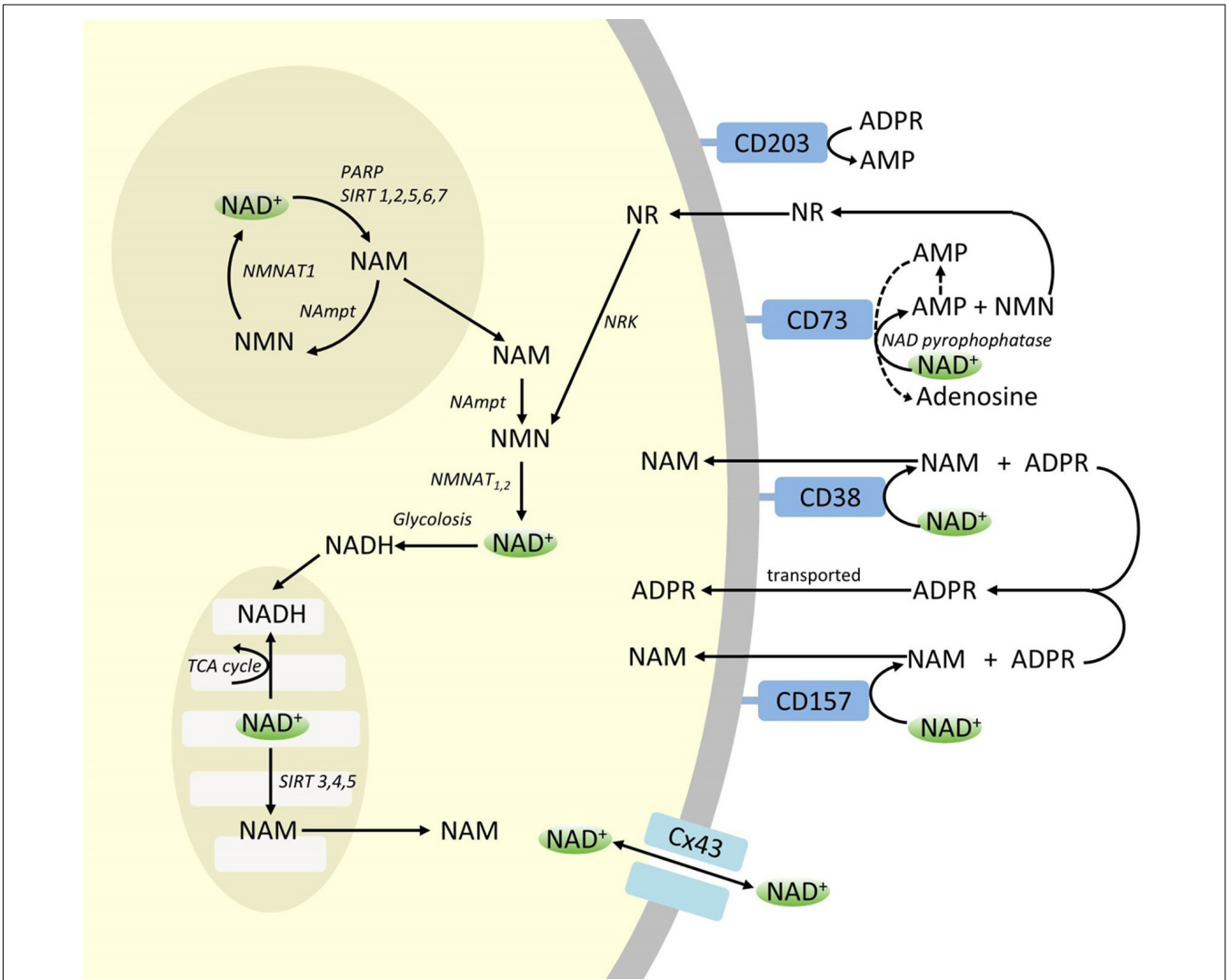

FIGURE 4 | Potenital Intra and extracellular fate of exogenously supplied NAD+. Abbreviations: CD38 [cyclic ADP-ribose (cADPR) synthase], CD203a (NAD+ pyrophosphatase), CD 73 (5′-nucleotidase). NAM, nicotinamide; NMN, nicotinamide mononucleotide; ADPR, adenosine diphosphate riboside; meNAM, methyl nicotinamide; 4PY, N-methyl-4-pyridone-3-carboxamide; 2PY, methyl-2-pyridone-5-carboxamide; ARPP, ADP ribose pyrophosphatase; RPPK, ribosyl pyrophosphokinase; NAmpt, nicotinamide phosphoribosyltransferrase; NMNAT, nicotinamide mononucleotideadenyltransferase; PARP [poly ADPribose polymerase; SIRT [sirtuin]; NRK [nicotinamide riboside kinase].

Either NAD+ and/or the primary metabolites tested are being efficiently sequestered during this first 2–6 h period or secondary metabolites are also being formed. ADPR can be recycled to produce NAD+ from NAM (**Figure 4**) or further metabolized *via* ectoenzymes such as CD203a (NAD+ pyrophosphatase) to produce AMP which can be further rapidly metabolized to adenosine by CD73 a 5′-nucleotidase (Bogan and Brenner, 2010; Horenstein et al., 2016; Morandi et al., 2018). In support of this hypothesis, an increase in blood adenosine levels has been recognized by others as a consequence of extracellular NAD+ infusion (Szczepańska-Konkel et al., 2003). NAM can also be metabolized to NMN *via* the salvage pathway before resynthesis to NAD+ (**Figures 3**, **4**) or acted on by hepatic methyltransferases to produce N1-methylnicotinamide which may either be excreted directly or further converted to N-methyl-2-pyridone-5-carboxamide (2PY, +99% of meNAM) and N-methyl-4-pyridone-3-carboxamide (4PY, ∼0.25% of meNAM) before excretion (Shibata and Matsuo, 1989; Okamoto et al., 2003).

It is evident that at the dosing regimen used the mechanisms involved in the metabolism and sequestering of NAD+ and metabolites have reached saturation sometime after 2 h resulting in a significant elevation of plasma NAD+ and accumulation of all metabolites tested (NMA, ADPR, meNAM and NMN) at 6 h. As mentioned previously these data support the view that one major pathway for NAD+ metabolism under these conditions is the cleavage of the glycosidic bond, by ectoenzymes such as CD38 to produce NAM and ADPR. However, the rise in plasma

NMN after 2 h also suggests that exogenous NAD+ is likely acted on by extracellular NAD+ pyrophosphatases, elevating plasma NMN and releasing AMP as an additional metabolite.

In summary, this study revealed for the first time that: (a) at a flow rate of 3 μmole/min all exogenously infused NAD+ was rapidly and completely removed from the plasma for at least the first 2 h; (b) the increase in metabolic bi-products analyzed is consistent with NAD+ glycohydrolases and NAD+ pyrophosphatase activity; and (c) the urinary excretion products arising from NAD+ infusion include native NAD+ and meNAM but not NAM.

While the findings of this investigation are novel and go some way to advancing our understanding of the timed fate of exogenous NAD+ in humans, limitations were identified. To improve overall metabolic reckoning future studies should investigate changes in red cell NAD+ and metabolites and urinary excretion of the secondary meNAM metabolites, 2PY and 4PY. In addition, it will likely be useful to assess the impact of exogenous NAD+ on changes in purine metabolism which should include at least assessment of plasma and red cell AMP and adenosine. The use of animal models may also help to clarify the relative involvement of the various metabolic pathways through the use of suitable pharmacological inhibitors and tissue sampling.

In conclusion, this study was able to reveal for the first time some very useful, previously unknown, information about the fate of exogenous IV NAD+ in humans including, the overall safety and tolerability of an IV NAD+ infusion at a rate of 3 μmoles/min, the rapid sequestering of NAD+ from the plasma, the likely contribution of both NAD+ glycohydrolase and NAD+ pyrophosphate activity in the metabolism of NAD+ and the apparent efficient renal tubular reabsorption of NAM. However additional research is required to fully reveal the complex metabolic fate of this important molecule. Further, characterization of these changes will help progress the development and improvement of NAD+ based treatment regimens for disorders which are likely to benefit from increased NAD+ availability including conditions requiring increased cellular regeneration and repair such as Alzheimer's and other neurodegenerative dementias.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by William Carey University Institutional Review Board, Hattiesburg, MS (Protocol #2017-12). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

RG participated in study design and oversight, critical discussion and manuscript authorship. JBer participated in study design, critical discussion, data collection, statistical analysis, manuscript drafting and manuscript review. RM participated in study design, critical discussion, clinical supervision and manuscript review. NB participated in biochemical analysis and manuscript review. JW participated in study design, critical discussion and manuscript review. JBen participated in data collection, and manuscript review. SB participated in study design and manuscript review. All authors have reviewed, read and approved the final version of the manuscript, and agree with the order of presentation of the authors.

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frontiers in Aging Neuroscience

REVIEW
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Emerging Alternative Proteinases in APP Metabolism and Alzheimer's Disease Pathogenesis: A Focus on MT1-MMP and MT5-MMP

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Processing of amyloid beta precursor protein (APP) into amyloid-beta peptide (Aβ) by β-secretase and γ-secretase complex is at the heart of the pathogenesis of Alzheimer's disease (AD). Targeting this proteolytic pathway effectively reduces/prevents pathology and cognitive decline in preclinical experimental models of the disease, but therapeutic strategies based on secretase activity modifying drugs have so far failed in clinical trials. Although this may raise some doubts on the relevance of β- and γ-secretases as targets, new APP-cleaving enzymes, including meprin-β, legumain (δ-secretase), rhomboid-like protein-4 (RHBDL4), caspases and membrane-type matrix metalloproteinases (MT-MMPs/η-secretases) have confirmed that APP processing remains a solid mechanism in AD pathophysiology. This review will discuss recent findings on the roles of all these proteinases in the nervous system, and in particular on the roles of MT-MMPs, which are at the crossroads of pathological events involving not only amyloidogenesis, but also inflammation and synaptic dysfunctions. Assessing the potential of these emerging proteinases in the Alzheimer's field opens up new research prospects to improve our knowledge of fundamental mechanisms of the disease and help us establish new therapeutic strategies.

Keywords: amyloid precursor protein, matrix metalloproteinases, eta-secretase, meprin-beta, legumain, rhomboid-like protein-4, caspase, neurodegenerative disease

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Abbreviations: 5xFAD: transgenic mouse model of AD bearing 3 familial mutations on human *APP* and 2 on *PSEN1* genes; AD: Alzheimer's disease; ADAM: a disintegrin and metalloproteinase; AICD: APP intracellular domain; AMPA: α-amino-3-hydroxy-5-methyl-4- isoxazolepropionic acid; APLP1/2: amyloid precursor like protein 1/2; APOE: apolipoprotein E; APP: amyloid-beta precursor protein; APP-IP: APP-derived inhibitor peptide; Aβ: amyloid-beta peptide; BACE-1: beta-site APP cleaving enzyme 1; C99/C83: APP-CTF of 99/83 amino acids; CSF: cerebrospinal fluid; CST3: cystatin C encoding gene; CXCL12: C-X-C motif chemokine ligand 12; DR6: death receptor 6; ECM: extracellular matrix; GABA: gamma-aminobutyric acid; GluA1/A2: glutamate A1/A2; GPI: glycosylphosphatidyl inositol; HEKswe: Human Embryonic Kidney cells 293 stably expressing *APP* with the familial Swedish mutation; IL-1β: interleukin-1 beta; IL-8: interleukin-8; iPS: induced pluripotent stem cells; LOAD: late onset Alzheimer's disease; LTP: long-term potentiation; mEPSCs: mini excitatory postsynaptic currents; mIPSCs: mini inhibitory postsynaptic currents; MMP: matrix metalloproteinase; MT-MMP: membrane-type matrix metalloproteinase; N/CTF: N-terminal or C-terminal APP fragments generated by APP-cleaving enzymes; NFT: neurofibrillary tangles; NMDA: *N*-methyl-D-aspartate; PARL: presenilin associated rhomboid like; PP2A: protein phosphatase 2; PS1/2: presenilin 1 and 2; PSD95: postsynaptic density protein 95; RHBDL4: rhomboid-like protein-4; sAPPα/β: soluble APPα/β; SDF1α: stromal cell-derived factor 1; SLPI: secretory leukocyte proteinase inhibitor; TIMP: tissue inhibitor of MMPs; TMD: transmembrane domain; TNF-α: tumor necrosis factor alpha; VEGF: vascular endothelial growth factor.

ALZHEIMER'S DISEASE, A PROTEOLYTIC PROBLEM

Alzheimer's disease (AD) is the most common type of neurodegenerative disorder for which only a few drugs have shown transient and moderate anti-symptomatic effects, but there is no treatment that slows down or prevents the progression of the disease. A minority of AD cases find their cause in deterministic genetic mutations in three genes: *PSEN1*, *PSEN2* and *APP*, encoding, respectively, aspartyl proteinase presenilin 1 and 2 (PS1/PS2) and amyloid precursor protein (APP; Van Cauwenberghe et al., 2016). These mutations account for the so-called "familial forms" of the disease. The overwhelming majority of AD cases (∼95%) are sporadic forms of unknown etiology. Despite controversies over the causes of AD, it is still recognized that brain accumulation of the amyloid peptide-β (Aβ) plays a central role in the pathogenic process (Selkoe and Hardy, 2016). Aβ results from the proteolysis of APP, a type I transmembrane protein targeted first at the plasma membrane, then rapidly endocytosed to endosomes to be metabolized to Aβ or subsequently sent to the lysosomal compartment for degradation (Wang X. et al., 2017; Van Acker et al., 2019). Endosomes are thought to be the main locus of Aβ production, which is ensured by canonical β- and γ-secretases (Vassar et al., 1999).

β-site APP cleaving enzyme 1 (BACE-1) is the main β-secretase. This type I transmembrane protein of the aspartyl proteinase family needs an acidic environment (optimum pH 4.5) to be enzymatically active (Saric et al., 2013). BACE-1 cleaves numerous substrates, which confers this enzyme a wide spectrum of physiological and pathological activities (Kuhn et al., 2012; Zhou et al., 2012; Vassar et al., 2014), but it is indisputably its ability to process APP that has attracted much attention, especially in relation to AD. As illustrated in **Figure 1**, BACE-1 cleaves APP between Met_{671} and Asp_{672} to generate the soluble APP-β fragment (sAPPβ) and its complementary C-terminal counterpart of 99 amino acids termed β-CTF or C99. This cleavage signals the first emblematic proteolytic step to the production of Aβ, considered to be one of the main driving forces in AD pathogenesis. BACE-1 can also catalyze amyloidolytic processing by cleavage between Tyr_{10} and Glu_{11} of the Aβ sequence (Huse et al., 2002; Liu et al., 2002; Kimura et al., 2016), the so-called β'-cleavage site (**Figure 1**). Interestingly, β'-cleavage is favored in the neuroprotective Icelandic APP mutation while the canonic β-cleavage at Asp_1 is reduced (Kimura et al., 2016). However, a causal effect between β'-cleavage and neuroprotection is not straight forward as $Aβ_{11-40}$ is found in insoluble Aβ pools of post-mortem AD brains (Huse et al., 2002). More recently, it has been shown that BACE-1 can also cleave $Aβ_{40}$ or $Aβ_{42}$ to generate a C-terminal truncated $Aβ_{34}$ form, which appears to be a new biomarker of Aβ clearance in AD as it is noticeably increased in mild cognitive impaired patients along with strong BACE-1 activity (Liebsch et al., 2019). Together, these data confirm early studies (Fluhrer et al., 2003; Shi et al., 2003), placing BACE-1 as a prominent Aβ-generating, but also as an occasional Aβ-"degrading" enzyme under some circumstances. Physiologically relevant Aβ degradation has been mainly attributed to 4 metalloproteinases: neprilysin, insulin degrading enzyme, endothelin converting enzyme and angiotensin converting enzyme, the regulation and functions of which have been extensively reviewed elsewhere (De Strooper, 2010; Nalivaeva et al., 2012). Matrix metalloproteinases (MMPs), including MMP-2, MMP-3, MMP-7 and MMP-9 also cleave within the Aβ sequence (Rivera et al., 2019) and these cleavages have been compared to those of membrane-type MMPs (MT-MMPs) in **Figure 1**.

The γ-secretase complex is formed by a PS1 or PS2 catalytic subunit and 3 partner proteins, Aph-1, pen-2 and nicastrin (Haass et al., 2012; Rajendran and Annaert, 2012; Masters et al., 2015; Selkoe and Hardy, 2016). Presenilins are acidic proteinases [optimum pH 6.3, (Campbell et al., 2003)] that belong to the family of seven transmembrane domain proteins. Like BACE-1, γ-secretase targets many substrates in addition to APP, with the consequent impact on a vast array of physiological and pathological processes (Haapasalo and Kovacs, 2011). The subcellular location and substrate specificity of γ-secretase may vary depending on the presence of PS1 or PS2 in the complex. While the complex containing PS1 is widely distributed in the cell, a single acidic-dileucine sorting motif present in PS2 directs the γ-secretase complex to late endosomes/lysosomes (Sannerud et al., 2016). γ-secretase performs regulated intramembrane proteolysis to process C99 and release Aβ and the remaining APP intracellular domain (AICD). The latter can be translocated in the nucleus and engages in transcriptional activities, which are to some extent still controversial in the AD field (reviewed in Pardossi-Piquard and Checler, 2012) (**Table 1**).

Along the secretory pathway, APP can also undergo α-secretase cleavage when reaching the plasma membrane. This is performed by a disintegrin and metalloproteinases (ADAMs) of the metzincin superfamily of metalloproteinases (Rivera et al., 2010; Paschkowsky et al., 2019), in a constitutive (ADAM10) or regulated manner (ADAM17; also known as TACE for TNF-α converting enzyme) (Lammich et al., 1999; Jorissen et al., 2010; Kuhn et al., 2010). ADAMs cleave APP in the middle of the Aβ sequence, thereby preventing its generation and releasing at the same time a soluble APP fragment (sAPPα) of ∼110 kDa. In cultured neurons from rodents and humans, sAPPα has shown anti-excitotoxic properties through a mechanism involving the reduction of Ca^{2+} cytotoxic influx (Mattson et al., 1993). These data linking sAPPα to neuroprotection were later confirmed by studies showing that moderate neuronal overexpression of ADAM10 in transgenic AD mice favored an increase in sAPPα levels and a decrease in Aβ, concomitant with a reduction of deficits in long-term potentiation (LTP) and learning (Postina et al., 2004). Likewise, sAPPα could rescue LTP in acute hippocampal slices from mice lacking both APP and its family homolog APP-like protein 2 (APLP2) (Hick et al., 2015), and prevented memory deficits after lentiviral overexpression in the APPswe/PS1dE9 AD mouse model (Tan et al., 2018). While sAPPα release is usually considered as the result of physiological APP processing, Aβ oligomers may be responsible for sAPPα release by cultured neurons and also *in vivo*, which could be considered as a neuroprotective cellular response to the amyloid challenge (Rose et al., 2018). This idea is all

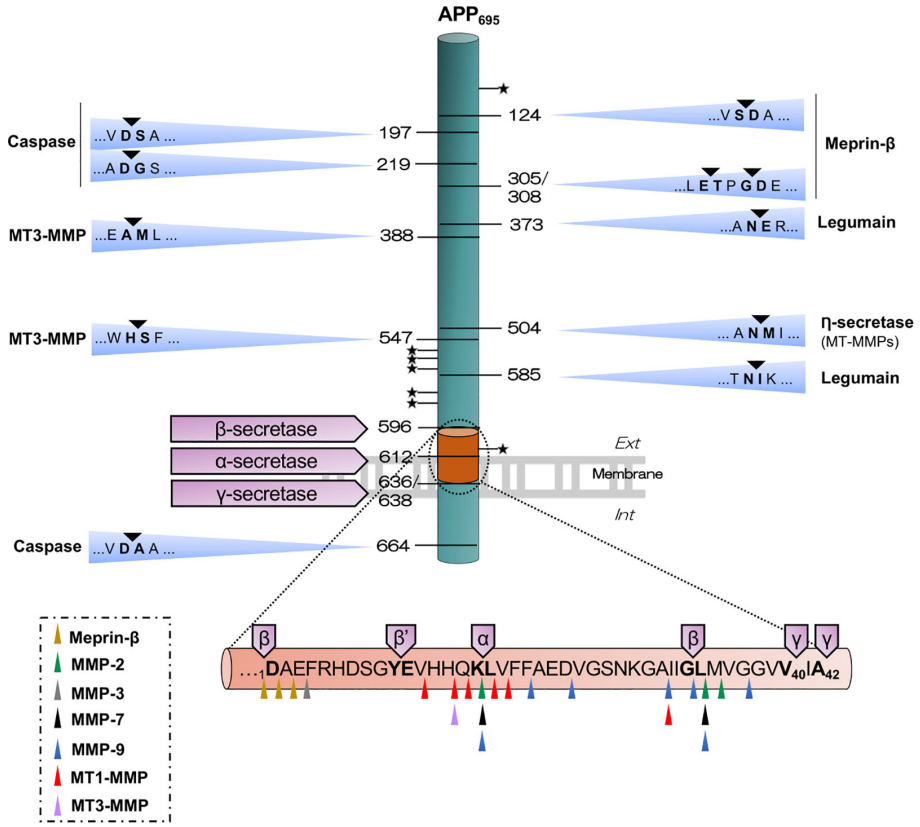

FIGURE 1 | Illustrative outline of the different APP cleavage sites. The scheme shows the canonical (β-, α- and γ-secretases) and non-canonical APP-cleavages that result in the different APP fragments. Meprin-β cleaves APP in five sites, possibly generating soluble APP fragments of different size, sAPPβ or sAPPβ-like (sAPPβ with one or two additional amino acids) and its complementary C-terminal β-CTF or β-CTF-like (β-CTF with one or two amino acids less), which is further processed by γ-secretase, thus producing $A\beta_{1-X}$, $A\beta_{2-X}$ or $A\beta_{3-X}$. Legumain cleaves in two sites, giving rise to two soluble fragments and one C-terminal transmembrane fragment than can be further processed by the canonical enzymes. Caspase can cleave in the N-terminal domain, generating truncated APP-species, and within the intracytoplasmic domain, generating an APP-Ncas fragment and a C-terminal residual peptide of 31 amino acids. RHBDL4 cleavages within the ectodomain at several sites are not yet identified (represented with a star), generating different N- and C-terminal fragments. Cleavage by η-secretase generates a soluble fragment (sAPPη/sAPP95) and a paired transmembrane product η-CTF/CTF-30 that can be further processed by β- or α-secretase to release Aη-β and Aη-α, respectively. MT3-MMP can cleave APP in 4 sites, including the η-secretase site and also within the Aβ sequence. The scheme below represents the Aβ sequence with multiple cleavage sites for various soluble MMPs and MT1-MMP, for meprin-β and for canonical secretases. All the residues are termed using APP_{695} numbering.

the more interesting in the context of data indicating that sAPPα interacts with BACE-1 and inhibits Aβ production (Obregon et al., 2012), in clear contrast with sAPPβ, which fails to interact with BACE-1 because the truncation of 16 aminoacids at the carboxy end (sequence between the α- and β-cleavage sites) is sufficient to impose significant structural changes compared to sAPPα (Peters-Libeu et al., 2015). Recent studies have highlighted new mechanisms by which sAPPα could influence synaptic function through the modulation of the GABA and glutamate neurotransmitter systems. Thus, sAPPα has been shown to act as a ligand of $GABA_BR1a$ - a metabotropic receptor for GABA neurotransmitter-, which results in modulation of hippocampal synaptic plasticity and neurotransmission *in vivo* by decreasing the release of synaptic vesicles (Rice et al., 2019). In addition, sAPPα stimulates trafficking of GluA2-lacking AMPA and NMDA receptors to the synapse, as well as *de novo* protein synthesis of GluA1 protein, thereby providing mechanistic ground for promotion of LTP and synaptic plasticity (Mockett et al., 2019). Consistent with the aforementioned anti-amyloidogenic properties of sAPPα, its complementary C-terminal fragment of 83 amino acids (C83) can also inhibit Aβ generation by interfering with C99 processing by γ-secretase in HEK cells (Tian et al., 2010). The idea that there is cross-regulation between amyloidogenic and non-amyloidogenic pathways is further supported by the fact that Aβ inhibits ADAM10 in mouse primary neuronal cultures, thus favoring endogenous APP processing by β-secretase (Spilman et al., 2016). Since activation and inhibition of ADAMs may be a determining factor for AD pathology, the regulatory potential of the tissue inhibitors of metalloproteinases (TIMPs) should be considered. TIMPs are mainly known as inhibitors of MMPs (see section below), but they also modulate ADAMs activity with low Ki values of 0.5 and 0.1 nM for TIMP-1 and TIMP-3, respectively (Rapti et al., 2008; Baranger et al., 2014). One of our studies shows that TIMP-1 expression is highly upregulated in the brain of transgenic 5xFAD mice at early stages of the pathology and remains durably high, likely matching the progression of neuroinflammation and gliosis (Py et al., 2014). In

TABLE 1 | Representative of single or combined cleavages of APP by proteinases and known functions of generated fragments.

Proteinase	APP fragment	Functions	References
Single cleavage			
β-secretase	sAPPβ	Modulator of GABAergic transmission	Vassar et al., 2014*; Rice et al., 2019
	β-CTF/C99	Cell toxicity	
		Impairs synaptic transmission	
		Behavior impairment	
α-secretase	sAPPα	Neurotrophic, neuroprotective, neurogenic, neuronal plasticity and memory enhancer	Tian et al., 2010; Obregon et al., 2012; Peters-Libeu et al., 2015; Muller et al., 2017*; Mockett et al., 2019; Rice et al., 2019
		BACE-1 inhibitor	
		Modulator of GABAergic transmission	
	α-CTF/C83	Modulator of APP cleavage by γ-secretase	
Meprin-β	$sAPP_{1-124}$	Unknown functions	Jefferson et al., 2011; Bien et al., 2012; Becker-Pauly and Pietrzik, 2016*
	$sAPP_{1-305/308}$	No cytotoxicity	
	sAPPβ/β-CTF	Same functions as those mentioned above	
	sAPPβ#/β-CTF#	Unknown functions	
Legumain	APP_{1-373}	Axonal fragmentation and neuronal death	Basurto-Islas et al., 2013, 2018; Zhang et al., 2014, 2015
	$APP_{586-695}$	Better substrate for BACE-1, thus increasing Aβ production	
RHBDL4	N-terminal fragments	Unknown functions	Paschkowsky et al., 2016, 2018
	C-terminal fragments	Decrease Aβ production	
Caspases	APP-NCas (APPΔ31)	Unknown functions	Gervais et al., 1999; Bertrand et al., 2001; Madeira et al., 2005
	C31	Neurotoxic	
		Promotes neuronal apoptosis	
η-secretase (MT-MMPs)	sAPP95 (sAPPη)	Binds $GABA_BR1a$	Py et al., 2014; Willem et al., 2015; Baranger et al., 2016a, 2017a; Paumier et al., 2019
		Boosts endosomal APP trafficking and Aβ production	
	η-CTF	Associated with distrophic neurites close to amyloid plaques	
Combined cleavage			
β-secretase +γ-secretase	Aβ	Stimulates synaptic vesicle release and transmission	Puzzo et al., 2008, 2011; Abramov et al., 2009; Soscia et al., 2010; Benilova et al., 2012*; Mucke and Selkoe, 2012*; Cline et al., 2018*; Hong et al., 2018; Moir et al., 2018*; Dominy et al., 2019
		Promotes neurogenesis, neurite outgrowth and cell proliferation	
		Antimicrobial	
		Inhibitor of α-secretase	
		Its accumulation triggers toxicity, mitochondrial dysfunction, oxidative stress and inhibits LTP	
	AICD	Transcriptional regulator, modulates cell death programs and intracellular homeostasis of Ca^{2+}, stabilizes microtubule structure, regulates synaptic plasticity	Pardossi-Piquard and Checler, 2012*
Meprin-β + γ-secretase	Aβ	Same functions as those mentioned above	Jefferson et al., 2011; Bien et al., 2012; Scharfenberg et al., 2019*
	$Aβ_{2-X}$, $Aβ_{3-X}$	Prone to aggregate and to deposit in Aβ clusters	
η-secretase + β-secretase	Aη-β	Unknown functions	Willem et al., 2015
		No neurotoxicity	
η-secretase + α-secretase	Aη-α	Decreases neuronal activity and LTP	
caspase + γ-secretase	Jcasp	Inhibits APP function	Gervais et al., 1999; Bertrand et al., 2001; Madeira et al., 2005
		Induces neuronal apoptosis	
		Decreases basal synaptic transmission and rises synaptic frequency	
α-secretase + caspases + γ-secretase	p3	Unknown functions	Gervais et al., 1999; Bertrand et al., 2001; Madeira et al., 2005

**The asterisk indicates review articles. #The hash indicates alternative cleavages for meprin-β close to β-secretase site, thus generating a sAPPβ with one or two additional amino acids and its complementary C-terminal β-CTF with one or two less amino acids.*

the diseased brain, reactive astrocytes are the main cellular source of TIMP-1 (Rivera et al., 1997, 2002; Pagenstecher et al., 1998), which in turn promotes astrocyte proliferation (Ogier et al., 2005; Hernandez-Guillamon et al., 2009), suggesting altogether that TIMP-1 could contribute to AD neuroinflammation and/or downregulate ADAM10 activity. As for TIMP-3, one study in

neuroblastoma cells reported that this inhibitor not only reduces α-cleavage of APP but also the cell surface levels of ADAM10 and APP, which leads to increased endocytosis and β-secretase cleavage. The potential relevance of TIMP-3 in promoting AD pathogenesis is reinforced in this study by the elevated neuronal immunostaining found in 3xTg transgenic mouse and by increased levels in AD brain homogenates (Hoe et al., 2007). However, another study in 5xFAD mice, showed mild variations of TIMP-3 mRNA levels at different ages, compared to wild type (Py et al., 2014). Clearly, studies are lacking to confirm or refute the idea that a regulatory action of TIMPs on ADAMs could impact on the progression of the disease.

The precise neurotoxicity mechanisms of Aβ are still poorly understood, probably reflecting a combination of events triggered by its accumulation, including stimulation of Tau hyperphosphorylation and subsequent formation of neurofibrillary tangles (NFTs), promotion of inflammation and also synaptic dysfunctions (Benilova et al., 2012; Mucke and Selkoe, 2012; Selkoe and Hardy, 2016; Cline et al., 2018). Monomeric Aβ can be assembled into oligomers, protofibrils or fibrils, the latter being the main constituent of amyloid plaques. Although oligomers are increasingly considered to be the most toxic Aβ assemblage, recent data suggest that only a limited fraction of highly diffusible small oligomers have adverse effects, for instance on the disruption of synaptic activity (Hong et al., 2018). Missing relevant Aβ structures as possible therapeutic targets might be one of the reasons why anti-Aβ strategies developed to fight AD have not yet demonstrated significant clinical benefits (Panza et al., 2019). In the case of therapies based on β- and γ-secretase inhibitors, a possible cause of clinical failure could be the difficulty of inhibiting these proteinases without causing side effects resulting from non-targeted proteolytic inhibition on their many physiological substrates (Karran et al., 2011; Vassar et al., 2014; De Strooper and Chavez Gutierrez, 2015; Barao et al., 2016; Ohno, 2016; Yan, 2017). Besides Aβ, other potential neurotoxic APP metabolites generated with the contribution of BACE-1 activity were initially reported. This was the case of a 35 kDa sAPP NTF fragment (1-286) and sAPPβ whose binding to death receptor 6 (DR6) induced axonal pruning and neuronal apoptosis mediated by caspase activation (Nikolaev et al., 2009). The implication of this 35 kDa fragment was later refuted; instead sAPPβ was reported to induce axonal pruning but not neuronal death, and to interact with DR6 through the more C-terminal E2 domain placed above the β-cleavage site (Olsen et al., 2014). Furthermore, the involvement of DR6 in Alzheimer's neurodegeneration could not be proved in two transgenic mouse models of AD deficient for DR6 (Kallop et al., 2014). The roles of C-terminal APP fragments resulting from the proteolytic activity of canonical secretases have also been investigated. Thus, many studies using genetic or pharmacological approaches to increase C99 levels in animal models consistently demonstrate the pathogenic properties of C99 linked with AD phenotypes (Neve et al., 1996; Oster-Granite et al., 1996; Song et al., 1998; Matsumoto et al., 2002; Lauritzen et al., 2012, 2016; Bourgeois et al., 2018). It is noteworthy that C99 accumulates in the brains of AD patients (Pera et al., 2013; Kim et al., 2016) and shows a better correlation than Aβ with the degree of vulnerability to neurodegeneration (Pulina et al., 2019). The other C-terminal APP fragment that has focused a great deal of research activity in recent years is the AICD. Despite controversial data has been often reported on its physiological and pathological functions, AICD has gained consensus as signaling molecule that can translocate to the nucleus and regulate the expression of manifold genes, some of them involved in AD pathogenic mechanisms (Kim et al., 2003; Chang et al., 2006), but also in cytoskeleton dynamics, cell cycle control or synaptic plasticity. A recent review has identified nearly 40 genes targeted by the AICD in relation with these and other processes (Bukhari et al., 2017). These genes will need to be tested for their possible contribution to signaling pathways that depend on APP and their consequent impact on brain pathology and physiology.

Other APP fragments generated by emerging APP processing enzymes are currently being investigated to determine their potential physiological or pathological actions and they will be discussed below.

INSUFFICIENTLY EXPLORED TERRITORIES

The idea that APP metabolites other than Aβ could actually impact on AD pathology is gaining momentum. However, the assessment of their physiological functions should also be carried out in parallel in order to have a more accurate picture of the pathophysiological scenario. The same holds true for Aβ, which remains the main driving force behind AD pathology, but whose physiological activities are still poorly understood and certainly insufficiently studied. Thus, for example, a few studies indicate that Aβ has antimicrobial activities, leading some authors to propose that it could act as an innate immunity protein in the brain (Soscia et al., 2010; Moir et al., 2018; Dominy et al., 2019). In addition, Aβ has been shown to be a positive modulator of synaptic vesicle release (Abramov et al., 2009) and synaptic transmission (Puzzo et al., 2008, 2011). In fact, picomolar concentrations of Aβ are sufficient to stimulate nicotinic receptors and subsequently LTP and learning and memory in healthy rodents (Puzzo et al., 2008, 2012; Morley et al., 2010). To what extent the failure of current anti-Aβ therapies could be due to interference with these (or other unknown) physiological roles of Aβ, remains an open question.

Despite the importance of APP proteolysis and the resulting fragments in the context of AD, APP role as a cell adhesion protein or receptor also deserves consideration insofar as the alteration of these functions may indeed contribute to the disease process. Accordingly, it is proposed that APP contributes to cell adhesion responsible for maintaining the structure of synapses and neural circuits through its interactions with extracellular matrix and adhesion molecules, but also through its own dimerization at the cell surface (see van der Kant and Goldstein, 2015; Montagna et al., 2017; Muller et al., 2017; Sosa et al., 2017), which is regulated, *inter*

alia, by APP binding with Cu^{2+} and Zn^{2+} (Wild et al., 2017). Disruptions of physiological functions of APP could cause synaptic dysfunctions that occur in the early phases of AD, but it could also alter the subcellular location of APP and thus the degree of pathogenicity of proteolytic fragments depending on whether they are mainly generated in endosomes (e.g., C99, Aβ) or in the cell membrane (e.g., sAPPα). APP has also been described as a functional cellular receptor for Aβ to the point that Aβ oligomers isolated from AD brains require APP expression to induce synaptotoxic effects on mice hippocampi (Wang Z. et al., 2017). By the same time, it was shown that Aβ oligomers and also Tau could cause APP-dependent impairment of LTP and spatial learning in mice (Puzzo et al., 2017). Another work highlights the potential of full length APP as functional receptor for the regulation of cholesterol/lipoprotein metabolism and Aβ clearance (Fong et al., 2018). In this case, human astrocytes derived from induced pluripotent stem cells (iPS) deficient for APP exhibited reduced levels of intracellular cholesterol, as well as a reduced ability to endocyte apolipoprotein E (APOE) and Aβ, which are major processes disturbed in AD and possible cause of deficient Aβ clearance. Similar deficits have been observed in astrocytes expressing *APP* harboring the Swedish familial mutation, characterized by reduced canonical levels of APP due to exacerbated cleavage of APP by β-secretase. It is interesting to note that the inhibition of β-secretase reversed these effects, thereby highlighting the potential benefits of such inhibition specifically in astrocytes (Fong et al., 2018). These few examples illustrate the importance of canonical APP as a regulator of tissue homeostasis and the extent to which subtle alterations in its biology could trigger or accompany pathological processes. Further research will be needed to increase our knowledge of the biology of APP and thus determine whether maintaining APP levels could be a therapeutic strategy of interest.

The complexity of interactions between APP and canonical secretases results in multifunctional derivatives of APP (Chen et al., 2015; Liu et al., 2019). The emergence of proteinases with new APP transformation activities and new fragments does not precisely simplify this scenario, but may be useful to broaden the spectrum of mechanisms to be targeted in a therapeutic context. We will focus our attention in the following section on a subfamily of MMPs, the MT-MMPs, but will also discuss recent findings linking meprin-β, legumain, rhomboid-like protein-4 (RHBDL4) and caspases to APP/Aβ metabolism (**Figure 1** and **Table 1**).

SOME EMERGING APP PROCESSING PROTEINASES

Meprin-β

Meprin-β is a type I transmembrane protein of the astacin group of the metzincin superfamily of metalloproteinases (Villa et al., 2003). The enzyme is naturally inhibited by fetuin-A (or alpha2-Heremans-Schmid glycoprotein) and fetuin-B, but not by cystatin C, a closely related inhibitor (Hedrich et al., 2010; Karmilin et al., 2019). Meprin-β activity has been linked to the processing of a variety of substrates, including inflammatory cytokines, and cell adhesion and extracellular matrix molecules in different organs. Overall, this proteinase exerts control on inflammatory/immune and cell migration processes. In the nervous system, meprin-β has been described as a new alternative APP processing enzyme, which links its activity to AD (reviewed in Scharfenberg et al., 2019). In this context, several studies have reported increased mRNA and protein levels of meprin-β in AD patients compared to age-matched healthy individuals (Bien et al., 2012; Schlenzig et al., 2018). Produced by brain neurons, meprin-β exists in plasma membrane-bound form or in soluble form after shedding by ADAM10 or ADAM17. Using a synthetic peptide mimicking APP/Aβ sequence, it was shown *in vitro* that meprin-β was able to cleave APP at the β-cleavage site and after the first and second amino acids of the Aβ sequence, suggesting that meprin-β can behave as a β-secretase. This was further supported by the fact that meprin-β expression in BACE-1/2 knockout fibroblasts was sufficient to generate Aβ (Bien et al., 2012). In addition, meprin-β can cleave APP near the N-terminal end to generate truncated fragments of 11 and 20 kDa (N-APP), as demonstrated by terminal amine isotopic labeling of substrates. N-APP were detected in mice brains as well as in the brains of healthy individuals and patients with AD, but not in meprin-β knockout mice, validating the role of meprin-β in the physiological processing of APP (Jefferson et al., 2011). These APP fragments did not induce toxicity in neuronal cultures and therefore further work will be needed to elucidate their physiological functions. Unlike BACE-1, meprin-β cleaves APP on the plasma membrane before it undergoes endocytosis, and preferentially generates $A\beta_{1-40}$ and a N-terminally truncated Aβ ($A\beta_{2-X}$), which is more prone to aggregation than $A\beta_{1-40}$. This is of interest, as truncated Aβ species may promote the formation of highly toxic Aβ oligomers. Also important, $A\beta_{2-X}$ can only be generated by membrane-bound meprin-β and not by the soluble form after ADAM10/17-mediated shedding (Bien et al., 2012). An additional regulatory interplay between meprin-β and α-secretase has been identified; meprin-β deletion in mice correlates with higher levels of sAPPα, thereby suggesting *in vivo* competition with α-secretase for membrane-bound APP (Schonherr et al., 2016). It has to be noted that *APP* mutations proximal to the β-cleavage site, like the Swedish ($K_{670}N$-$M_{671}L$) or the "protective" Icelandic ($A_{673}T$), prevent the generation of $A\beta_{2-X}$ by meprin-β as opposed to the lack of effect of distal mutations (i.e., London ($V_{717}I$), highlighting the importance for meprin-β of aminoacid composition around the β-cleavage site (Schonherr et al., 2016). Overall, meprin-β bears great interest as alternative APP processing enzyme with amyloidogenic features. It remains to be determined to which extent the relatively small amount of $A\beta_{2-X}$ generated in AD compared to Aβ42 provides meprin-β with a relevant pathogenic role in AD. Further investigations will also be necessary to assess the possible impact of meprin-β as a regulatory factor

of neuroinflammatory processes operating in AD and other brain disorders.

Legumain

Legumain, also known as asparagine endopeptidase, is a soluble cysteine proteinase mainly found in endo-lysosomal compartments that provide the optimal functional pH of 6. Its proteolytic activity is inhibited by cystatin C and closely related cystatins E/M and F (Chen et al., 1997; Alvarez-Fernandez et al., 1999). The enzyme was later renamed δ-secretase (Zhang et al., 2015) to highlight its role in APP cleavage at Asn_{586} that generates the δ-CTF first identified in the nineties (Simons et al., 1996; Scharfenberg et al., 2019). As lysosomal dysfunction is a transversal pathogenic mechanism, legumain has been involved in numerous pathological settings, including atherosclerosis, osteoporosis, cancer, ischemic stroke, and neurodegenerative diseases (Lunde et al., 2019). Legumain has been described in relation with AD as a modulator of Tau phosphorylation (Basurto-Islas et al., 2013), and as a Tau- or APP-cleaving enzyme (Zhang et al., 2014, 2015). Under acidic conditions (e.g., brain ischemia, hypoxia, or AD), legumain can translocate from neuronal lysosomes into the cytoplasm, where it cleaves I_2^{PP2A} (also known as SET). This cleavage generates two fragments, I_2^{NTF} and I_2^{CTF} that inhibit protein phosphatase 2A (PP2A), a key phosphatase that limits Tau hyperphosphorylation both *in vitro* (Basurto-Islas et al., 2013) and *in vivo* (Basurto-Islas et al., 2018). In total, legumain activity promotes the hyperphosphorylation of Tau protein, which is a major hallmark of AD pathogenesis.

In addition to controlling Tau phosphorylation, legumain generates neurotoxic fragments after cleavage of Tau and APP. The brain of AD patients show increased levels of the Tau_{1-368} fragment compared to healthy individuals, in correlation with increased levels of legumain activity (Zhang et al., 2014). This study showed that Tau_{1-368} generated by legumain inhibits microtubule polymerization *in vitro* and promotes apoptosis in rat cultured neurons. Consistent with these *in vitro* findings, the deletion of legumain in the Tau P301S transgenic mouse model of AD prevents synaptic dysfunction and improves learning and memory. Furthermore, mice virally infected with uncleavable Tau mutant showed reduced pathological and behavioral defects as compared with mice infected with Tau P301S. Of note, antibodies specifically raised against the Tau_{1-368} neoepitope were found in AD brains and absent in legumain knockout mice, thus validating *in vivo* legumain-mediated Tau processing (Zhang et al., 2014). The same group also demonstrated that legumain can cleave APP at positions 373 and 585 (APP_{695} numbering), generating 2 fragments with distinctive cytotoxicity (Zhang et al., 2015). While APP_{1-373} triggers axonal fragmentation and neuronal death in primary cultured neurons, the $APP_{586-695}$ fragment appears to be a better pro-amyloidogenic substrate for BACE-1 than full-length APP and the other legumain-derived C-terminal fragment ($APP_{374-695}$). In support of these *in vitro* data, legumain deficiency in the 5xFAD transgenic mouse model of AD (Oakley et al., 2006) causes a drop of $Aβ_{40}$, $Aβ_{42}$ and amyloid plaque burden, while it increases spine density, and prevents deficits in LTP and learning and memory (Zhang et al., 2015). The first step for pharmacological validation of legumain in AD was obtained using a chemical inhibitor, which reduced the formation of neurotoxic Tau fragments and Aβ accumulation in P301S and 5xFAD mouse models, respectively. In addition, legumain inhibition reduced synaptic loss, improved LTP, prevented microglial activation and reduced the levels of inflammatory mediators TNF-α and IL-1β (Zhang et al., 2017).

Rhomboid-Like Protein-4

There are five active intramembrane proteinases Rhomboids in humans, RHBDL1-4 and PARL. RHBDL1-4 are located in the secretory pathway, while PARL is found in mitochondria. This family of serine proteinases is involved in many cellular processes, such as inflammatory signaling, cell migration, proliferation and mitochondria homeostasis (reviewed in Dusterhoft et al., 2017; Paschkowsky et al., 2019). Recently, Paschkowsky et al. (2016) showed in HEK cells expressing APP_{695} that RHBDL4 but not RHDBL1, 2 and 3, cleaves at multiple sites within the ectodomain of APP (**Figure 1**) and APLP1 and APLP2, generating a N-terminal fragment of ∼70 kDa and different C-terminal fragments whose functions are still unknown. The use of specific inhibitors for a wide spectrum of proteinases, including BACE-1, α- and γ-secretase, aspartyl and cysteine proteinases, and metalloproteinases did not modify the APP fragmentation profile generated by RHBDL4, suggesting a specific action of this proteinase. Moreover, it was shown that RHBDL4 activity decreases the levels of $Aβ_{38}$, $Aβ_{40}$ and $Aβ_{42}$. RHBDL4 is mainly located in the endoplasmic reticulum, which contains low levels of membrane cholesterol. Interestingly, RHBDL4-mediated cleavage of APP is negatively regulated by cholesterol when it binds to two transmembrane domains of the proteinase, but not to APP. Thus, it is possible that lowering cholesterol could increase RHBDL4 activity on full-length APP, with the consequent generation of CTFs and the decrease in Aβ generation (Paschkowsky et al., 2018). Overall, these data underline the unexpected important role of RHBDL4, which is not a classical secretase, in the metabolism of APP/Aβ. Nevertheless, much remains to be done to validate most of the referred *in vitro* observations in *in vivo* models with relevance for AD pathogenesis.

Caspases

Caspases are pleiotropic cysteine proteinases that specifically cleave target proteins after an aspartic acid residue (Hyman and Yuan, 2012). Several studies have demonstrated in a variety of culture cell systems the ability of caspase-3, -6 and -8 to cleave APP, mainly between amino acids V_{664}-D_{665} (APP_{695} numbering) within the intracytoplasmic domain (Gervais et al., 1999; LeBlanc et al., 1999; Pellegrini et al., 1999; Weidemann et al., 1999). Cleavage of APP by caspases generates a membrane bound N-terminal fragment, named APP-Ncas, and an intracellular C-terminal peptide of 31 amino acids, named APP-Ccas or C31. When γ-secretase processes C99 into Aβ, the remaining AICD is further cleaved by caspase to generate C31 and JCasp fragments, both of which promote neuronal apoptosis (Bertrand et al., 2001; Madeira et al., 2005). For this reason, it was suggested that the cytotoxicity of C99 might actually result

from subsequent formation of C31 by caspases (Lu et al., 2000). It is also noteworthy that AD brains show elevated levels of caspases and that APP fragments resulting from caspase cleavage colocalize with amyloid plaques (Gervais et al., 1999; Lu et al., 2000). Eventually, caspase-mediated APP cleavage stimulates Aβ production in B103 and NT2 cells (Gervais et al., 1999). However, other authors have challenged these data in a study where caspase removed the internalization signal (YENPTY) located in the extremity of the AICD, thereby leading to impaired APP internalization and Aβ production in B103 cells (Soriano et al., 2001). These results were recently confirmed using CRISPR/Cas9 editing in human iPS to remove the last 36 amino acids of APP containing the internalization motif. The expression of this C-terminal truncated APP in human iPS-derived neurons prevented the production sAPPβ and Aβ, while stimulating α-secretase cleavage. In addition, it was shown that cultured hippocampal neurons with truncated *APP* gene accumulate APP on the plasma membrane and present reduced colocalization of BACE-1 and APP in endosomes, confirming a key role of the YENPTY sequence in APP internalization and Aβ production by endosomes (Sun et al., 2019).

Caspases have also been proposed as a possible molecular link between amyloid and Tau pathologies in AD, since Aβ simulation of neuronal apoptosis is accompanied by an increase of Tau cleavage by caspases at Asp_{421}. This is consistent with the observation that C-terminal truncated forms of Tau show increased polymerization capacity *in vitro* and are associated with NFTs formation in the brain of AD patients (Gamblin et al., 2003; Rissman et al., 2004).

MT-MMPs

MT-MMPs are new players in the field of AD, mainly because of their ability to regulate APP metabolism and therefore amyloidogenesis. In the next sections, we will focus on two MT-MMPs, MT1-MMP and MT5-MMP, which have been associated with the pathophysiological mechanisms that support AD. More generally, the involvement of other MMPs in AD and other neurodegenerative disorders has been discussed elsewhere (Rivera et al., 2019).

Structure of MT1-MMP and MT5-MMP

MMPs constitute a multigenic family of 24 endopeptidases that belong to the metzincin superfamily of metalloproteinases. MMPs show pleiotropic regulatory actions in many processes in the nervous system, including axonal growth (Pastrana et al., 2006; Ould-yahoui et al., 2009; Trivedi et al., 2019), neurogenesis (Wojcik et al., 2009), learning and memory (Beroun et al., 2019), glial reactivity and inflammation (Chopra et al., 2019; Montaner et al., 2019; Muri et al., 2019), cell migration (Ould-Yahoui et al., 2013) or neuronal death (Gu et al., 2002; Jourquin et al., 2003). The many aspects of MMP functions and action mechanisms in the nervous system have been extensively discussed earlier (Rivera et al., 2010, 2019; Baranger et al., 2014).

MMPs are mostly secreted proteinases, but 6 transmembrane proteins form the so-called subfamily of MT-MMPs: MT1-MMP (MMP-14), MT2-MMP (MMP-15), MT3-MMP (MMP-16), MT4-MMP (MMP-17), MT5-MMP (MMP-24) and MT6-MMP (MMP-25). All MT-MMPs share closely related structural features, described in detail elsewhere (Itoh, 2015). These include a signal peptide that targets the MT-MMP to the endoplasmic reticulum, where it is proteolytically excised, and then a pro-domain bearing a conserved cysteine that interacts with the catalytic domain and maintains the enzyme as an inactive zymogen. The pro-peptide can be separated from the rest of the molecule by redox-mediated chemical reactions or after proteolytic cleavage by serine endoproteinase furin. This mechanism, called "cysteine switch," implies the dissociation between the Cys residue of the pro-peptide and the Zn^{2+} of the catalytic domain, which leads to enzymatic activation. The catalytic domain is highly conserved domain across MT-MMPs (*de facto*, across MMPs) and is linked by a hinge region to the hemopexin domain, which has a more variable sequence, thus conferring certain specificity to the binding of substrates and endogenous TIMPs. MT-MMPs are linked to the plasma membrane either by a glycosylphosphatidyl inositol (GPI) bond (MT2-MMP and MT6-MMP) or by a transmembrane domain (MT1-, MT2-, MT3- and MT5-MMP), followed in this case by an intracytoplasmic domain that can control MT-MMP cell traffic and its proteolytic activity (Uekita et al., 2001; Wang et al., 2004; Sakamoto and Seiki, 2009). Analysis of the amino acid sequence reveals a variable percentage of sequence identity between human MT1- and MT5-MMP, ordered from N- to C-terminus as follows: pro-domain (44%), catalytic (72%), hinge (41%), hemopexin (66%), stem part (17%), transmembrane (37%) and intracytoplasmic (20%). Despite the high degree of sequence identity between MT1- and MT5-MMP, human and mouse MT5-MMP carry unique dibasic motifs in their stem regions, which are recognized by proteinases from the furin proprotein convertase family. Proteolytic cleavage by furin gives MT5-MMP singular biochemical properties, in particular an ephemeral presence on the plasma membrane since it is released into the medium in soluble form (Pei, 1999; Wang and Pei, 2001).

Physiological Functions of MT1- and MT5-MMP

MT1-MMP

MT1-MMP is ubiquitously distributed in the body. In the nervous system it is mainly expressed by microglia, astrocytes and neurons (Liao and Van Nostrand, 2010; Langenfurth et al., 2014; Py et al., 2014; Itoh, 2015). As most MMPs, MT1-MMP degrade extracellular matrix (ECM) proteins, in particular type I and III collagen, fibronectin, laminin, vitronectin and proteoglycans (Ohuchi et al., 1997). MT1-MMP catalyzes the conversion of pro-MMP-2 into the active MMP-2 form through a mechanism involving one of its endogenous inhibitors (see below) (Sato et al., 1996; Llano et al., 1999; Pei, 1999; Baranger et al., 2014). MT1-MMP is the most studied of the MT-MMPs and the progressive identification of new substrates beyond ECM has led to an expansion of the biological functions of the proteinase. MT1-MMP has an impact on inflammatory processes by modulating the action of cytokines, chemokines, proteinase inhibitors or receptors. For example, MT1-MMP can activate pro-TNF-α into active TNF-α, and inactivate CXCL12 (SDF1α), as well as complement protein C3, secretory leukocyte proteinase inhibitor (SLPI), IL-8 and other chemokines. Cellular receptors and membrane proteins such as DR6, the VEGF receptor neuropilin,

the prion protein or a ligand of Notch receptor Delta-like I, are also processed by MT1-MMP (Overall et al., 2004; Tam et al., 2004; Jin et al., 2011; Starr et al., 2012; Kojima et al., 2014; Itoh, 2015). MT1-MMP knockout mice are not viable beyond one month of age, suggesting an important role of this proteinase in development (Holmbeck et al., 1999). In this context, it is now well documented that MT1-MMP contributes to cell migration including monocytes/macrophages, endothelial cells or neural stem cells (Galvez et al., 2001; Matias-Roman et al., 2005; Ould-Yahoui et al., 2013). It is noteworthy that in some cases the stimulation of migration or chemotaxis by MT1-MMP does not depend on its catalytic activity, but rather on the properties of its cytoplasmic tail. Thus, proteolytic inhibition of MT1-MMP does not affect migration of isolated macrophages on matrigel invasion tests. In addition, the expression of catalytically active and inactive forms of MT1-MMP in MT1-MMP knockout macrophages rescue chemotaxis properties, while a mutant form lacking the cytoplasmic tail does not (Sakamoto and Seiki, 2009). Recently, Aguirre and collaborators elegantly demonstrated that MT1-MMP is crucial in the inflammatory response upon intraperitoneal LPS injection in wild type and MT1-MMP knockout pups (5–8 days post-natal). Under these experimental conditions, they observed that the absence of MT1-MMP reduces life span, which is associated with lung enlargement and higher neutrophil recruitment (Aguirre et al., 2017). The effects of MT1-MMP appear to be related to MMP-2 activation, as lower concentrations of active MMP-2 are found in MT1-MMP knockout pups, associated with higher levels of MMP-2 substrate S100A9 (Aguirre et al., 2017). The latter is an inflammatory protein (Vogl et al., 2012) also implicated in AD pathogenesis (Chang et al., 2012). Taken together, these results clearly reflect the multifaceted mode of action of MT1-MMP, with functions that depend on proteolysis, but also on protein-protein interactions mediated by the C-terminal intracytoplasmic tail. It remains to be demonstrated whether this functional diversity is limited to certain cell types or organs, in particular with regard to the role of MT1-MMP in the nervous system.

MT5-MMP

To date, fewer substrates have been described for MT5-MMP, compared to MT1-MMP (reviewed in Itoh, 2015). MT5-MMP preferentially cleaves proteoglycans and to a lesser extent fibronectin, but not type I collagen or laminin (Wang et al., 1999). MT5-MMP expression is mainly confined to the nervous system, with levels in rodents reaching their maximum before birth and remaining high into adulthood in areas like the hippocampus or the cerebellum (Pei, 1999; Jaworski, 2000; Hayashita-Kinoh et al., 2001; Sekine-Aizawa et al., 2001; Warren et al., 2012). Such a distribution is consistent with the proposed role of MT5-MMP in brain development and more broadly in neural plasticity. Similarly, MT5-MMP has been reported to promote axonal growth (Hayashita-Kinoh et al., 2001) and to control the activation of adult neural stem cells under physiological and regenerative conditions by a mechanism that requires the cleavage of N-cadherin (Porlan et al., 2014), one of the non-ECM substrates of MT5-MMP. Despite the presumed developmental role of MT5-MMP, knockout mice are viable and have no apparent phenotypes, indicating that there is functional redundancy with other proteinases in physiological conditions. However, phenotypes linked to MT5-MMP deficiency become evident when mice are subjected to stressful conditions. This is for instance the case after sciatic nerve injury, where MT5-MMP deletion prevents aberrant sprouting of nociceptive Aβ-fibers and the resulting mechanical allodynia (Komori et al., 2004). The implication of MT5-MMP in post-lesion axonal regeneration has also been suggested in the context of concerted work with ADAM10 in reactive astrocytes to control post-lesion synaptic remodeling (Warren et al., 2012). We will see later that the combined action of MT5-MMP and ADAM10 has also been proposed in AD, with different functional implications. Deletion of MT5-MMP also revealed a reduction in hyperalgesia after intraplantar injections of IL-1β or TNF-α in a model of thermal pain, indicating that MT5-MMP plays role in the inflammatory pathways driven by these cytokines. The proposed mechanism associates the absence of MT5-MMP with deficient cleavage of its substrate N-cadherin, a cell adhesion molecule involved in synapse architecture. N-cadherin cleavage is required to ensure communication between mast cells and sensory neurons for the transmission of nociceptive stimuli (Folgueras et al., 2009). It is therefore concluded that MT5-MMP is capable of regulating the neuroinflammatory process (Baranger et al., 2016a).

The implication of MT5-MMP in synaptic processes has been suggested by the discovery that the proteinase interacts with the AMPA binding protein and the glutamate receptor-interacting protein. Both are postsynaptic density-95/Discs large/zona occludens-1 (PDZ) protein containing domains. The interactions between these PDZ proteins and MT5-MMP are mediated by the last three carboxyl terminus amino acids of the proteinase, thus facilitating its targeting to synapses and the cleavage of N-cadherin (Monea et al., 2006). In addition, it has been reported that MT5-MMP could act in synergy with γ-secretase to affect synaptic protein levels of GluA2 and PSD95, eventually resulting in a decrease in synaptic transmission (Restituito et al., 2011); however, functional evidence of such interaction is still lacking.

MT5-MMP processing and metabolism are tightly controlled; before reaching the plasma membrane, MT5-MMP can be cleaved by a furin-like activity in its stem part, leading to generation of a catalytically active soluble form of the enzyme that lacks the C-terminal part (Wang and Pei, 2001). Once on the membrane, MT5-MMP can be internalized in the endosomes, where it interacts with the PDZ Mint3 protein, allowing proteinase to be recycled into the membrane (Wang et al., 2004). Despite having the structure of a membrane protein, MT5-MMP can mostly be found in intracellular and extracellular compartments due to its shedding and cellular routing.

TIMPs

As with the other members of the MMP family, TIMPs control the catalytic activities of MT1- and MT5-MMP. Of the four TIMPs, TIMP-2, -3 and -4 inhibit MT1-MMP, while MT5-MMP is only targeted by TIMP-2. Both MT-MMPs can activate MMP-2 through a process that requires the formation of a tripartite

molecular complex formed by two MT-MMPs, a TIMP-2 and a pro-MMP-2; the N-terminal domain of TIMP-2 interacts with MT1-MMP on the plasma membrane, while its C-terminal domain binds to the hemopexin domain of pro-MMP-2, then the pro-peptide of pro-MMP-2 is readily removed by an adjacent MT1-MMP eventually resulting in MMP-2 activation (Strongin et al., 1995). It should be noted that TIMP-1, which targets soluble MMPs and ADAM10, is a very poor inhibitor of MT-MMPs (reviewed in Baranger et al., 2014).

MT1-MMP and MT5-MMP Contribute to Alzheimer's Pathogenesis

MT1-MMP

Higashi and Miyazaki were the first to show that MT1-MMP could cleave APP between residues Asn_{579} and Met_{580} ($VLAN_{579}$-M_{580}ISEPR) of APP_{770} after being activated by concanavalin A in the HT1080 fibrosarcoma cell line (Higashi and Miyazaki, 2003b). Three years later, Ahmad and collaborators showed that MT1-, MT3-, and MT5-MMP, but not MT2-, MT4- and MT6-MMP were able to cleave APP_{770} when co-expressed in HEK293 cells (Ahmad et al., 2006). Mass spectrometry analysis for MT3-MMP cleavage-sites revealed the same $VLAN_{579}$-M_{580}ISEPR site previously identified for MT1-MMP and 3 other cleavage sites at Ala_{463}-Met_{464}, His_{622}-Ser_{623} and His_{685}-Gln_{686} (Higashi and Miyazaki, 2003b), the latter being located in the Aβ sequence, just upstream of the α-cleavage site (**Figure 1**). Despite this, Aβ levels remained stable in HEK293 cells expressing MT3-MMP, probably indicating that MT3-MMP processes APP in cell compartments that do not influence amyloidogenesis. Although the APP cleavage sites for MT1-MMP and MT5-MMP were not specifically identified in the Ahmad study, the analysis of the APP degradation profile by western blot suggested that both MT-MMPs should cleave APP at the same location as MT3-MMP (Ahmad et al., 2006). Unlike MT3-MMP, the functional interaction between MT1-MMP and Aβ was shown when MT1-MMP overexpressed in COS cells degraded exogenous Aβ. In addition, the recombinant catalytic domain of MT1-MMP could degrade amyloid plaques when incubated *ex vivo* on brain slices of transgenic AD Tg2576 mice (Liao and Van Nostrand, 2010). In this way, MT1-MMP joins the list of metalloproteinases with Aβ-degrading, activity, such as MMP-2, MMP-3, MMP-7 and MMP-9 (Roher et al., 1994; Backstrom et al., 1996; Deb et al., 2003; White et al., 2006; Yan et al., 2006; Yin et al., 2006; Hernandez-Guillamon et al., 2010, 2015; Liao and Van Nostrand, 2010; Py et al., 2014; Brkic et al., 2015; Taniguchi et al., 2017; Rivera et al., 2019) (**Figure 1**). In complement of these results, we have recently reported that MT1-MMP can also prominently increase Aβ levels in the HEK293 cell model of amyloidogenesis, which stably expresses *APP* with the familial Swedish mutation (HEKswe) (Paumier et al., 2019). Transiently expressed MT1-MMP in HEKswe interacts with APP and induces the release of a soluble APP fragment of ∼95 kDa (sAPP95), distinct of sAPPα or sAPPβ generated by α- and β-secretase, respectively. The complementary transmembrane fragment of ∼30 kDa (CTF-30) and C99 are also dramatically increased and their levels highly correlated, suggesting that CTF-30 may be the precursor of C99 (Paumier et al., 2019). It is also noteworthy that MT1-MMP content is strongly upregulated in the cortex and hippocampus of 6-month old 5xFAD mice (Py et al., 2014). This mouse model mimics the symptoms and pathology of AD, including exacerbated amyloidosis, neuroinflammation and synaptotoxicity (Oakley et al., 2006), deficits in LTP (Crouzin et al., 2013) and cognition (Girard et al., 2013, 2014; Giannoni et al., 2016; Baranger et al., 2017a,b), and eventually neuronal death (Oakley et al., 2006; Jawhar et al., 2012; Eimer and Vassar, 2013). MT1-MMP upregulation in 5xFAD mice timely and spatially correlates with the increase in C99 levels (Py et al., 2014), thus providing indirect *in vivo* support for the pro-amyloidogenic features of this MMP described in HEKswe cells (Paumier et al., 2019).

Although functional interactions between MT1-MMP and APP/Aβ are not fully understood, it has been shown that the release of sAPP95 by MT1-MMP does not involve BACE-1, but it may involve MMP-2. It is estimated that about 50% of the sAPP95 released after MT1-MMP expression in HEKswe cells results from concerted action with MMP-2, MT1-MMP being in any case the limiting factor in this enzymatic tandem (Paumier et al., 2019). A plausible scenario would be that activation of MMP-2 by MT1-MMP (Sato et al., 1996) allows MMP-2 access to membrane substrates (i.e., APP), which would not otherwise be accessible for this soluble MMP. MMP-2, mainly considered as a soluble Aβ-degrading proteinase (Yin et al., 2006), could thus extend its range of action to the pericellular environment thanks to membrane docking provided by MT1-MMP. Another interesting feature linking MT1-MMP, MMP-2 and APP is that MT1-MMP processing of APP releases a soluble APP fragment (likely sAPP95) lacking a potent inhibitor of MMP-2 activity (Miyazaki et al., 1993). In this study, purified soluble forms of APP could efficiently inhibit MMP-2 degradation of gelatin as well as Aβ cleavage between K_{16}-L_{17} (α-cleaving site) (Miyazaki et al., 1993). Years later, the authors identified a decapeptide sequence ISYGNDALMP, called APP-derived peptide inhibitor (APP-IP), located 6 amino acids downstream the cleavage site (N_{504}-M_{505}ISEPRISYGNDALMP) shared by MT1-MMP and MT3-MMP (and also MT5-MMP, see below). APP-IP shows low nanomolar range potency toward MMP-2 (IC_{50} value of 30 nM) and is a weaker inhibitor for MT1-MMP, MMP-3, -7 and -9, with IC_{50} values between 2 and 10 μM. Although sAPPα contains the decapeptide, it appears to be a weaker MMP-2 inhibitor than APP-IP (Higashi and Miyazaki, 2003a,b, 2008).

The mechanism by which MT1-MMP could promote amyloidogenesis is still elusive. Nevertheless, reminiscent of legumain, MT1-MMP cleavage upstream of the β-cleavage site could facilitate β-secretase cleavage of APP and thus the subsequent production of C99 and Aβ. Not exclusively, MT1-MMP could favor amyloidogenic β-secretase processing by promoting APP sorting into BACE-1-enriched early endosomes (Paumier et al., 2019). Most interestingly, it has been proposed that MT1-MMP could also function as a surrogate β-secretase when BACE-1 is inhibited; this is supported by experiments in HEKswe cells where MT1-MMP is able to restore the basal levels of Aβ, almost abolished in the presence of BACE-1 inhibitor IV (or C3) (Paumier et al., 2019). It should be noted that a β-cleavage site for MT1-MMP has not yet been identified. MT1-MMP

replacing BACE-1 to maintain physiological levels of Aβ could be relevant in the event of therapeutic inhibition of BACE-1 if it is assumed that reducing Aβ levels below a physiological threshold could have adverse consequences.

Overall, these data highlight potential new mechanisms in the control of APP processing involving functional interactions of α- and β-secretases with MT1-MMP and MMP-2. It is now time to develop *in vivo* experimental models where genetic (i.e., conditional MT1-MMP knockout mice in transgenic AD mouse) and/or pharmacological (i.e., neutralizing antibodies) manipulation of MT1-MMP activity should further confirm the pathophysiological relevance of the aforementioned *in vitro* data. This includes a functional assessment of the apparent dual activity of MT1-MMP as an amyloidolytic and amyloidogenic enzyme, as well as the *in vivo* validation of APP cleavage and the resulting NTFs and CTFs.

MT5-MMP

MT5-MMP was detected in neurons and around senile plaques in the brains of AD patients (Sekine-Aizawa et al., 2001) and it was later suggested to cleave APP, yielding a fragmentation profile similar to that of MT1- and MT3-MMP (Ahmad et al., 2006). More recently, two independent studies highlighted in the same time the involvement of MT5-MMP on APP processing and the subsequent functional consequences (Willem et al., 2015; Baranger et al., 2016b). Willem and collaborators identified by mass spectrometry the $VLAN_{504}$-M_{505}ISEPR (APP_{695} numbering) as a cleavage site for MT5-MMP on APP, which was named η-cleavage site (Willem et al., 2015). This site was previously identified for MT1-MMP (Higashi and Miyazaki, 2003b) and MT3-MMP (Ahmad et al., 2006). Intriguingly, the Willem's study did not identify MT1-MMP as cleaving at this $VLAN_{504}$-M_{505}ISEPR/η-site and it was concluded that only MT5-MMP could act as a η-secretase *in vivo* (Willem et al., 2015). η-secretase cleavage of APP generates sAPPη and a residual transmembrane C-terminal APP fragment-η (η-CTF), which accumulates in dystrophic neurites around amyloid plaques in APP/PS1 mice. Using specific antibodies against the neoepitopes of the α-, β- and η-sites, it was observed that BACE-1 inhibition promoted the release of a peptide Aη-α (for amyloid eta-alpha) resulting from the combined action of MT5-MMP and α-secretase. Recombinant Aη-α was able to impair LTP when incubated on primary rat neuronal cultures. In addition, the inhibition of ADAM10 promoted the concerted action of MT5-MMP and BACE-1 to release a shorter peptide, Aη-β, unexpectedly innocuous (Willem et al., 2015). Counter-intuitively, ADAM10 is presented in this work as conveying neurotoxic actions by contributing to the generation of Aη-α together with MT5-MMP. Moreover, the fact that Aη-α levels are exacerbated upon BACE-1 inhibition may raise concerns of possible side effects of therapeutic anti-BACE-1 strategies. Aη-γ peptides, potentially generated by a combined action of MT5-MMP and γ-secretase, were not detected, probably revealing a lack of functional interaction between η- and γ-secretase as it is the case with BACE-1, ADAM10 and γ-secretase (Chen et al., 2015; Liu et al., 2019). Indeed, a recent study has shown that MT5-MMP does not co-purify in the high molecular weight complex formed by BACE-1 and γ-secretase. Replacing the transmembrane domain (TMD) of BACE-1 by that of MT5-MMP did not prevent BACE-1 complex with γ-secretase and Aβ production, suggesting that motifs other than TMD are involved in the formation of the BACE-1/γ-secretase interactions (Liu et al., 2019). Aη-α and Aη-β peptides were both detected in human cerebrospinal fluid (CSF) of healthy and AD patients, but no differences in their levels were observed between groups (Willem et al., 2015). Another study identified a ∼25 kDa η-CTF in CSF, but in this case the peptide content was higher in AD patients with a *PSEN1* familial mutation, in patients with sporadic AD and in Down Syndrome individuals with Alzheimer's type dementia, compared to age-matched non-demented controls, raising the possibility that η-CTF could be a new disease biomarker (Garcia-Ayllon et al., 2017).

By the same time, our group demonstrated that MT5-MMP induced the production of a soluble APP fragment of sAPP95 in HEKswe, concomitant with elevated levels of C99 and $Aβ_{40}$ (Baranger et al., 2016b). Although the exact nature of sAPP95 produced upon MT1-MMP and MT5-MMP expression needs to be established, it likely corresponds to what was defined as sAPPη by others (Willem et al., 2015). The function of sAPP95/sAPPη is not yet known, but a recent study showed that it binds $GABA_BR1a$, which also acts as a receptor for sAPPα and sAPPβ, resulting in a reduced probability of presynaptic release (Rice et al., 2019). These findings link MT5-MMP (as well as α- and β-secretases) with the regulation of a major inhibitory neurotransmitter system coupled to Ca^{2+} and K^+ channels, and expands the range of possible mechanisms by which APP proteolysis may influence changes in synaptic activity occurring at early stages of the disease. Gaining insight into the functional interplay between proteinases and neurotransmission will require precise assessment of the functional specificities of 3 APP NTFs generated by 3 different enzymes sharing a common receptor.

Similarly to MT1-MMP, we found that MT5-MMP could interact with APP and promote its endosomal sorting, thereby providing mechanistic support for MT5-MMP pro-amyloidogenic activity (Baranger et al., 2017a). Moreover, to evaluate the genuine role of MT5-MMP *in vivo*, we generated a bigenic mouse model by crossing 5xFAD mice (Oakley et al., 2006) and MT5-$MMP^{-/-}$ ($MT5^{-/-}$) mice (Komori et al., 2004). Compared to 5xFAD, 5xFAD/$MT5^{-/-}$ mice showed significantly lower levels of sAPP95, validating our *in vitro* observations and APP being an *in vivo* physiological substrate of MT5-MMP (Baranger et al., 2016b). Most important, 5xFAD/$MT5^{-/-}$ mice showed strikingly lower levels of Aβ (soluble $Aβ_{40}$ and $Aβ_{42}$, oligomers and plaques) and C99 at prodromal-like stages of the pathology (Baranger et al., 2016b, 2017a). Moreover, decreased APP processing and amyloidogenesis upon MT5-MMP deficiency were concomitant with fewer reactive microglial cells and astrocytes around amyloid plaques, as well as reduced levels of IL-1β and TNF-α (Baranger et al., 2016b, 2017a). From a functional standpoint, the absence of MT5-MMP prevented deficits in LTP and spatial and working memory observed in 5xFAD mice (Baranger et al., 2016b, 2017a). Most interestingly, some of these beneficial effects were also observed at advanced stages of the pathology in 16-month-old 5xFAD/$MT5^{-/-}$ mice,

which presented a better preservation of neuronal networks and synaptic integrity compared to age-matched 5xFAD control mice (Baranger et al., 2016b).

Mechanistically, it is tempting to speculate that MT5-MMP could share with MT1-MMP and legumain proteolytic actions by which cleavage of APP upstream of the β-cleavage site would facilitate the processing of APP by β-secretase, especially in endosomes. Two sets of findings support such hypothesis: (1) bigenic 5xFAD/legumain$^{-/-}$ and 5xFAD/MT5$^{-/-}$ mice both show significant reductions in Aβ burden and glial reactivity, as well as prevention of LTP and learning and memory deficits (Zhang et al., 2015; Baranger et al., 2016b, 2017a), reminiscent of observations made in bigenic 5xFAD/BACE-1$^{-/-}$ or 5xFAD/BACE-1$^{+/-}$ mice (Ohno et al., 2007; Kimura et al., 2010) and (2) MT5-MMP can be internalized in endosomes (Wang et al., 2004; Baranger et al., 2017a) and thus promote endosomal APP sorting (Baranger et al., 2017a). In such hypothetical model, the first cleavage by MT5-MMP at the η-site could have place on the membrane and be followed by joint internalization with the APP transmembrane breakdown product (CTF-30/η-CTF). It is noteworthy that MT5-MMP trafficking involves interactions between the last three residues at carboxyl terminus and Mint3, a protein that contains two type III PDZ domains (Wang et al., 2004). Remarkably, Mint3 can also function as an adaptor protein to promote APP export from the Golgi complex (Caster and Kahn, 2013). These observations highlight Mint3 as a potential molecular bridge between MT5-MMP and APP, which could facilitate their interactions in the context of traffic-based mechanisms for the control C99/Aβ production. This is consistent with the increasing importance granted to traffic regulation in the amyloidogenic process, but also as means of exchanging relevant biofactors between cells. In this vein, η-CTF was detected in extracellular vesicles released by N2a neuroblastoma cells expressing APP Swedish. The vesicles were uptaken by neurons, suggesting the possibility that η-CTF could function as a vector for intercellular pathogenic propagation (Laulagnier et al., 2018).

SECRETASE INHIBITORS/MODULATORS CAN BE LINKED TO AD

An indirect way to assess the physiological impact of the new APP processing enzymes is through the study of their endogenous inhibitors, assuming that the control of proteolytic activities is an important component of the final proteolytic balance. Such an evaluation is not an easy task because the inhibitory selectivity is not always completely elucidated, as is often the case with metalloproteinases, which have a highly conserved catalytic site. In general, the available literature on proteinase inhibitors and AD remains essentially correlative and the link between both is to date inconclusive or, at best, speculative. In this context, the levels of fetuin-A, an inhibitor of meprin-β, were found to be reduced in CSF of AD patients compared to healthy individuals (Puchades et al., 2003). In addition, a polymorphism in the promotor sequence of the fetuin-A encoding gene has been associated with late onset AD (LOAD) (Geroldi et al., 2005), and lower plasma levels of fetuin-A correlated with severe to mild cognitive impairment (Smith et al., 2011). Likewise, a polymorphism in the cystatin C gene (CST3) was significantly associated with LOAD patients, while lower levels of this legumain inhibitor were found in their plasma (Chuo et al., 2007). Other studies have shown that overexpression of cystatin C in brains of APP-transgenic mice reduces amyloid plaque formation (Kaeser et al., 2007). A potential protective role of cystatin C in AD could be related to its ability to bind soluble Aβ and thus prevent Aβ deposition (Mi et al., 2007). A clear link between high cholesterol levels and the pathogenesis of AD has not yet been confirmed, but patients taking statins, a cholesterol-lowering drug, are still less likely to develop the disease (Eckert et al., 2005; Fonseca et al., 2010; Wood et al., 2014). In this context, it has been shown that high cholesterol levels inhibit RHBDL4 activity, which has been correlated with an increase in the formation of Aβ (Paschkowsky et al., 2018). For TIMP-2, the main inhibitor of MT-MMPs, clinical data are rather inconsistent. For example, one study showed no change in plasma TIMP-2 concentrations in patients with AD compared to healthy individuals (Lorenzl et al., 2003b), while in the CSF, two independent studies showed no difference (Mroczko et al., 2014) or increased (Lorenzl et al., 2003a) TIMP-2 levels in AD patients. More recently, Duits and collaborators reported decreases in TIMP-2 (and also TIMP-1) in the CSF of AD patients with microbleeds (Duits et al., 2015). Pre-clinical work has reported TIMP-2 as a possible anti-aging factor as it promotes neuronal plasticity and hippocampal-dependent cognition in aging mice (Castellano et al., 2017). In this study, the authors used parabiosis experiments to demonstrate that plasma from young donor mice (3 months) enriched with TIMP-2 reversed cognitive deficits in elderly mice (18 months). The confirmation that TIMP-2 was responsible for the observed effects was obtained in experiments where the plasma of young TIMP-2 knockout mice failed to rescue the aged phenotype. It is noteworthy that the levels of many immune and trophic factors were upregulated in plasma from TIMP-2 knockout mice, indicating a possible regulatory role of TIMP-2 on molecules that influence the functional cross talk between the CNS and peripheral tissues. It is therefore tempting to speculate on TIMP-2 as a pleiotropic "rejuvenating" factor with anti-MMP activities and, as shown earlier, with anti-mitotic and neural cell-differentiation promoting effects independent of MMP-inhibitory activity (Perez-Martinez and Jaworski, 2005). The idea of a "global" anti-aging factor is particularly appealing because aging is by far the main risk factor for AD and other degenerative disorders of nervous and non-nervous tissues.

Overall, the available partial data on proteinase inhibitors highlight the need for more functional studies. These should confirm their potential in modulating proteolytic balance or non-proteinase-dependent actions as a means of deciphering new AD pathophysiological pathways and developing new therapeutic strategies.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

The importance of APP proteolysis in AD mechanisms is not in doubt. The emergence of new APP cleaving enzymes significantly

broadens the scope of study and makes evident the need for a comprehensive understanding of the complex molecular processes that encompasses APP proteolysis and the subsequent range of beneficial/pathological effects. Gaining insight in such processes will depend on our ability to determine the nature and functions of new APP fragments generated by these enzymes in a given cell type, subcellular compartment or stage of the disease, in particular at early stages where they could affect neurotransmitter release, synaptic function or incipient neuroinflammation. APP fragments can also be a new source of inspiration for developing therapeutic approaches based on blocking or promoting their formation. This may intrinsically require specific modulation of a given proteolytic cleavage, which turns out to be extremely difficult when the targeted proteinase has many substrates. This is the case for BACE-1 or γ-secretase, with dozens of substrates identified to date (Haapasalo and Kovacs, 2011; Kuhn et al., 2012; Zhou et al., 2012; Vassar et al., 2014). The list of substrates for most of the emerging proteinases discussed here is still relatively short and could therefore offer a therapeutic advantage in this respect. Time will tell whether the number of substrates will increase as new studies are conducted. Alternatively, APP proteolytic fragments may become full-fledged targets and their functions inhibited or potentiated, thus making it unnecessary to specifically inhibit the proteolytic processes that produce them. This option logically relies on a thorough knowledge of the biological functions of these fragments but also of the structural APP features. An additional alternative to modulating proteinase catalytic activity is to gain insight into the trafficking of APP and its metabolites. Mounting evidence indicates that the way these molecules circulate in the cell (or between cells) affects their functions and their availability for proteolytic processing. Similarly, understanding the cellular routing of proteinases may also be crucial to better target them. Beyond proteolytic activity, proteinases can also exert non-proteolytic actions through domains of interaction with other proteins; the question arises of the importance that these phenomena could have in the pathogenesis of AD and the new avenues they open to targeting domains other than the catalytic domain. This idea works as a mirror when it comes to the implications of proteinase inhibitors (e.g., TIMPs), which are known to control cell cycle, cell migration or cell fate also independent of anti-proteinase activities (Baranger et al., 2014). Since inhibitors are usually circulating proteins, they could favor communication between the CNS and peripheral systems (e.g., cardiovascular and immune systems), a process on which brain function and aging may depend.

In summary, this review highlights APP as a central molecule in the pathophysiology of AD and as a common substrate for MT-MMPs and other emerging proteinases. However, in the context of a more holistic approach to the disease that goes beyond APP/Aβ, we anticipate that in the coming years proteolytic and non-proteolytic interaction models will be established between proteinases, inhibitors and their partner proteins to continue to reveal the diversity of pathogenic pathways in AD and to learn how better control them.

AUTHOR CONTRIBUTIONS

All the authors discussed, wrote and approved the manuscript. KB and SR supervised the writing of the manuscript.

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Is AD a Stress-Related Disorder? Focus on the HPA Axis and Its Promising Therapeutic Targets

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Alzheimer's disease (AD) is a progressive neurodegenerative disorder that has important health and economic impacts in the elderly. Despite a better understanding of the molecular mechanisms leading to the appearance of major pathological hallmarks (*senile plaques and neurofibrillary tangles*), effective treatments are still lacking. Sporadic AD forms (*98% of all cases*) are multifactorial, and a panoply of risk factors have been identified. While the major risk factor is aging, growing evidence suggests that chronic stress or stress-related disorders increase the probability to develop AD. An early dysregulation of the hypothalamic-pituitary-adrenal axis (HPA axis or stress axis) has been observed in patients. The direct consequence of such perturbation is an oversecretion of glucocorticoids (GC) associated with an impairment of its receptors (glucocorticoid receptors, GR). These steroids hormones easily penetrate the brain and act in synergy with excitatory amino acids. An overexposure could be highly toxic in limbic structures (*prefrontal cortex and hippocampus*) and contribute in the cognitive decline occurring in AD. GC and GR dysregulations seem to be involved in lots of functions disturbed in AD and a vicious cycle appears, where AD induces HPA axis dysregulation, which in turn potentiates the pathology. This review article presents some preclinical and clinical studies focusing on the HPA axis hormones and their receptors to fight AD. Due to its primordial role in the maintenance of homeostasis, the HPA axis appears as a key-actor in the etiology of AD and a prime target to tackle AD by offering multiple angles of action.

Keywords: Alzheimer's disease, glucococorticoids, stress-related disorder, CRH (corticotropin-releasing hormone), HPA axis (hypothalamus-pituitary-adrenal), AVP (arginine vasopressin), 11β hydroxysteroid dehydrogenase

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GENERAL ASPECTS

Sporadic Alzheimer's disease (AD; *98% cases*) is a progressive neurodegenerative pathology, and its complexity could be explained by a wide range of risk factors. Growing evidence suggests that lifetime events such as chronic stress or stress-related disorders, like major depression disorder (MDD) or anxiety, may increase the probability to develop AD (Heininger, 2000; Blennow et al., 2006; Querfurth and LaFerla, 2010; Canet et al., 2019). In patients, an early dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis was observed (Hartmann et al., 1997; Csernansky et al., 2006). The direct consequence of such perturbation is a glucocorticoids (GC)

over-secretion associated with GC receptors (GR) signaling impairment. These steroid hormones easily penetrate the brain tissue and act in synergy with excitatory amino acids. A GC overexposure is highly toxic in limbic structures (McEwen, 2008), especially in prefrontal cortex and hippocampus, which could participate to the cognitive decline occurring in AD. Furthermore, GC and GR dysregulations are involved in lots of functions disturbed in AD: i.e., dysregulation of the amyloid precursor protein (APP) processing, Tau phosphorylation, neuroinflammation, oxidative stress and excitotoxicity (Sapolsky, 1996; McEwen, 2008; Bengoetxea et al., 2016). Thus, a vicious cycle between AD and the HPA axis seems to occur, where AD induces the dysregulation of the HPA axis, which in turn potentiates the pathology (Brureau et al., 2013; Pineau et al., 2016; Canet et al., 2019).

This article reviews some preclinical and clinical studies focusing on the HPA axis hormones and receptors as potential targets for AD. Promising results are obtained by targeting corticotropin releasing hormone (CRH), arginine vasopressin (AVP) and GR, or by inhibiting the 11β-hydroxysteroid dehydrogenase-1 (11β-HSD1), involved in GC synthesis. Finally, this review intends to show that the HPA axis, due to its essential role in the maintenance of homeostasis, could be a key factor in the etiology of AD and a prime target to tackle AD by offering multiple druggable opportunities.

THE HPA AXIS

The HPA axis is required to provide appropriate adaptation to external or internal challenges called stress, which initiates a cascade of hormonal processes (**Figure 1A**). This cascade starts in the hypothalamic paraventricular nucleus (PVN) with a release of CRH and AVP at the median eminence level. AVP acts in synergy with CRH to induce pituitary adrenocorticotropin (ACTH) release in blood (Whitnall et al., 1987; Mouri et al., 1993; Raff, 1993; Torner et al., 2017). Then, ACTH triggers the adrenal cortex to release GC (cortisol in human, corticosterone in rodents). These hormones act widely throughout the body and brain to mobilize energy resources in order to fight stress and maintain homeostasis (Carroll et al., 2011). To avoid an overactivation of the HPA axis, GC exert an inhibitory feedback at all stages of the axis (**Figure 1A**; Tasker and Herman, 2011). These steroid hormones bind to low affinity GR and high affinity mineralocorticoid receptors (MR; Reul and de Kloet, 1985). These nuclear receptors are necessary for normal cellular activity and crucial for many central nervous system functions, including learning and memory (Roozendaal, 2000). The GC circadian regulation is under the control of MR, while under stress conditions, GC rise significantly causing substantial activation of GR (Reul and de Kloet, 1985; Thomas, 2015).

It is interesting to note that AVP and CRH are also locally synthesized within the brain, where they are important neuromodulators involved in the central organization of various brain-mediated stress responses (Buijs, 1990; Tilders et al., 1993).

In human, chronic stress and HPA axis dysregulation with chronic GC hypersecretion appear to exert detrimental effects in normal aging and AD (Hartmann et al., 1997; Lupien et al., 1998; Notarianni, 2013; Givalois, 2014; Canet et al., 2018), but also in Parkinson's disease (Wu et al., 2016), Cushing's syndrome (Kroon et al., 2018), and MDD (Canet et al., 2018). A prolonged exposure to GC appears to damage particularly limbic structures (*hippocampus and prefrontal cortex*) by inducing a state of vulnerability in these neurons through the disruption of various cellular mechanisms (Sapolsky, 1996; McEwen, 2008). This review will present several current strategies in AD, aiming to restore HPA axis function and to limit GC production and toxicity.

HPA AXIS: MULTIPLE PROMISING TARGETS FOR AD TREATMENT

Targeting CRH Receptors In AD

CRH acts *via* G-protein-coupled receptor type 1 or 2 (CRH-R1 and CRH-R2) with widespread brain expression to orchestrate the stress response (Bale and Vale, 2004). In AD patients, investigators reported a reduction of immunoreactive cells for CRH in the cortex, associated with an increase of postsynaptic CRH receptors density (Whitehouse et al., 1987; Pomara et al., 1989; Behan et al., 1995). Besides clinical evidence, it was shown that CRH and CRH-R1 play critical roles in the regulation of stress-induced neuropathogenesis and behavioral deficits in mice models of AD (Dong et al., 2008; Carroll et al., 2011; Zhang et al., 2016; **Table 1**). CRH overexpressing mice display increased Tau hyperphosphorylation and aggregation in the hippocampus (Campbell et al., 2014). In Tg2576-AD mice, chronic isolation stress increases the expression of CRH-R1 in cortex and hippocampus (Dong et al., 2008). These authors also showed that chronic administration of antalarmin (*a CRH-R1 antagonist*) significantly decreased plasma corticosterone levels, tissue $A\beta_{1-42}$ levels and Aβ plaques deposition in the brain, and blocked the effects of isolation stress on anxiety levels and memory (Dong et al., 2014). A few years later, a 3 month-treatment in food with CRH-R1 antagonists (*Antalarmin and R121919*) was shown to prevent stress-induced behavioral changes (*anxiety and memory*) and synaptic loss in aged rats, perhaps by reversing HPA axis dysfunction (Dong et al., 2018). Moreover, in another mouse model of AD (*APP/PS1*) a 5 month-treatment with R121919 prevented the onset of cognitive impairment, reduced cellular and synaptic deficits and Aβ levels (Zhang et al., 2016). Similarly, pre-treatment with another CRH-R1 antagonist (*NBI27914*) in PS19-AD mice (*P301S mutation*) before restraint/isolation stress prevented Tau hyperphosphorylation and aggregation, neurodegeneration and fear-memory impairment (Carroll et al., 2011).

Such molecules have already been used in clinical trials for mood disorders (Zorrilla and Koob, 2004, 2010) and MDD (Nielsen, 2006) for many years, with disappointing results. However, all these preclinical studies highlight a promising therapeutic potential of CRH-R1 antagonists as treatments for neurodegenerative disorders such as AD. New CRH-R1 antagonists are in development to improve their bioavailability and safety profiles (Spierling and Zorrilla, 2017).

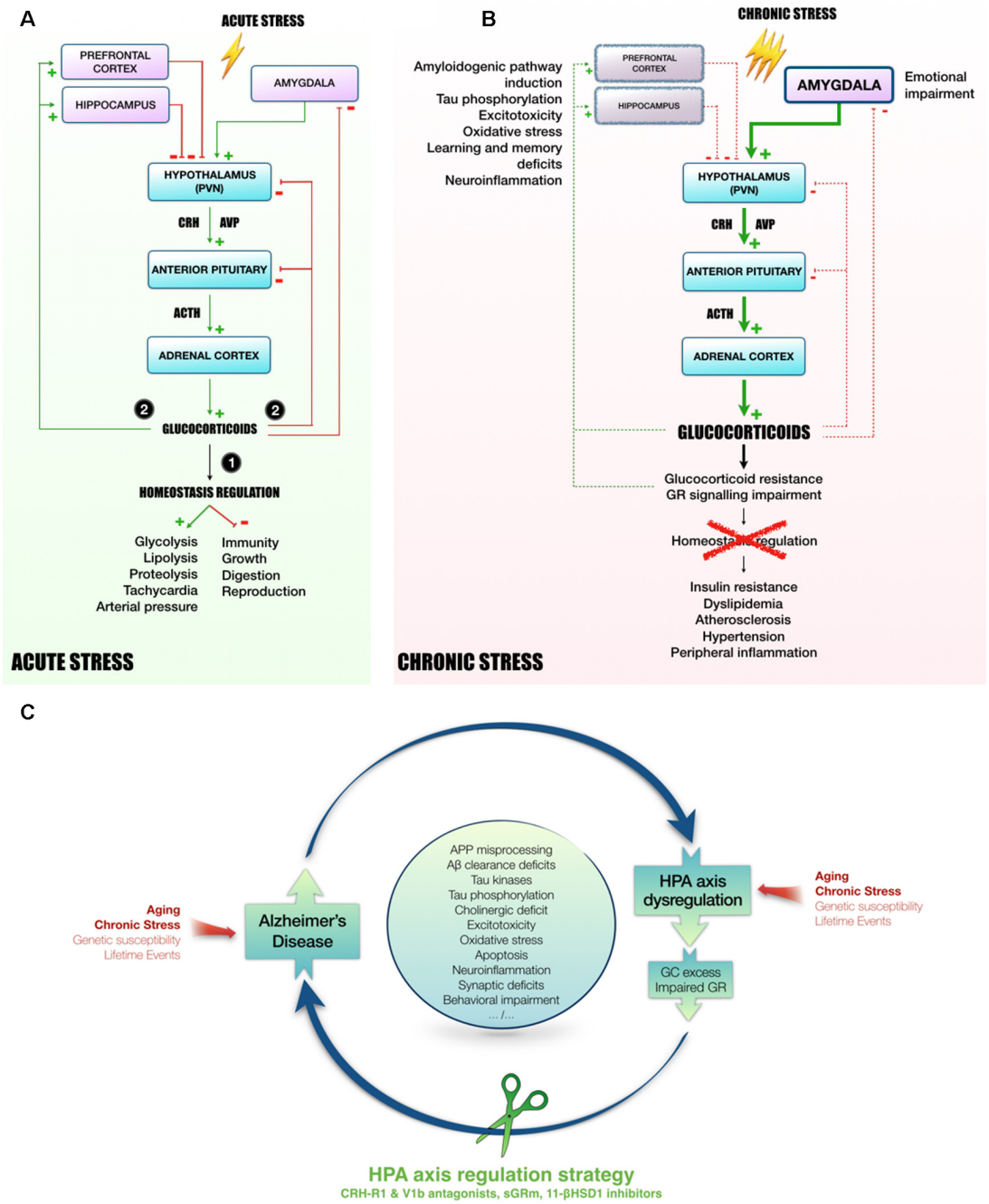

FIGURE 1 | Mechanisms linking HPA axis dysregulation and AD. Following acute stress **(A)**, hypothalamic PVN releases CRH and AVP in the blood portal of median eminence. In response to CRH and AVP, corticotropic cells of anterior pituitary release ACTH in the peripheral circulation to induce GC secretion in blood by adrenal cortex. Succinctly, (1) GC mobilize energy resources and increase cardiovascular function to fight stress. Besides, GC inhibit unnecessary functions in the early phase of stress response, such as immunity, growth, digestion and reproduction. Then, (2) to avoid runaway of the system, GC exert an inhibitory feedback at all stages of HPA axis (hypothalamus and pituitary). In addition, as they easily penetrate in the brain, GC also act on several regions involved in the control of HPA axis activity, such as hippocampus and prefrontal cortex (*tonic inhibition*) or amygdala (*tonic stimulation*, Canet et al., 2018). However, chronic stress leads to a sustained activation of HPA axis and could induce stress-related disorders, as for instance MMD and AD (**B**, Canet et al., 2018). In this context, GC over-secretion is associated with GC resistance and GR signaling impairment (Chrousos et al., 1993). Homeostasis maintenance is compromised, leading to insulin resistance, dyslipidemia, atherosclerosis, hypertension and a massive peripheral inflammation (Vitellius et al., 2018; Maslov et al., 2019). In limbic structures (*hippocampus, prefrontal cortex*), it was shown that GC overexposure induces hippocampal and cortical atrophy (McEwen, 2008) and amygdala hypertrophy (Vyas et al., 2003, 2004), that could be related to learning and memory deficits, emotional impairment, excitotoxicity, neuroinflammation and oxidative stress (Sapolsky, 1996;

(*Continued*)

FIGURE 1 | Continued
McEwen, 2008; Bengoetxea et al., 2016). In the AD context, high levels of GC, and the dysregulation of the HPA axis activity observed in patients (Hartmann et al., 1997; Swanwick et al., 1998), seems to be particularly involved in the induction of amyloidogenic pathway and the abnormal phosphorylation of Tau (Green et al., 2006; Pineau et al., 2016; Sotiropoulos and Sousa, 2016; Vyas et al., 2016; Canet et al., 2019). Thus, it appears that the rise of circulating GC increases AD pathology, resulting in a vicious cycle by which pathology induces HPA axis dysregulation, GC overexposure and GR signaling impairment, which in turn potentiates the pathology. Due to its primordial role in the maintenance of homeostasis, targeting HPA axis offers multiple angles of action to break this vicious cycle and pave the way to new therapeutic strategies **(C)**. Abbreviations: 11-βHSD1, 11β-hydroxysteroid dehydrogenase-1; Aβ, amyloid-β protein; APP, amyloid precursor protein; ACTH, adrenocorticotropin; AD, Alzheimer's disease; AVP, arginine-vasopressin; CRH, corticotropin releasing hormone; CRH-R1, CRH receptor type 1; GC, glucocorticoids; GR, glucocorticoid receptors; HPA axis, Hypothalamic-pituitary adrenal axis; MDD, Major depressive disorder; PVN, paraventricular nucleus; sGRm, Selective GR modulator; V1b, Arginine-vasopressin receptor sub-type 1b.

Targeting AVP Receptors In AD

Hypothalamic neurons of PVN and supra-optic nucleus are the major source of AVP (Swaab, 1998). This hormone is especially involved in the regulation of water, electrolyte balance and in the stress response (Swaab, 1995; Twist et al., 2000). AVP is also implicated in many central processes including learning and memory (de Wied et al., 1993; Caldwell et al., 2008; Lee et al., 2009), anxiety (Caldwell et al., 2008; Lee et al., 2009), and processing of social information (Cilz et al., 2018). The effects of AVP are mediated by different receptor subtypes: V1a, V1b, and V2, which are G-protein coupled receptors (Cilz et al., 2018). However, the limbic action of AVP seems to be mediated by the V1b subtype, enriched in the hippocampus (Young et al., 2006). In rodents, its pharmacological activation enhances excitatory post-synaptic currents (EPSCs) that are absent in $V1b^{-/-}$ mice. V1b potentiation of EPSCs is dependent of N-methyl-D-aspartate (NMDA) receptors activation and intracellular Ca^{2+} signaling (Pagani et al., 2015; Cilz et al., 2018).

In a depressive and/or anxiety context, the therapeutic interest of V1b antagonists has been known for many years (Griebel et al., 2003). This target is particularly studied in pathologies associated with HPA axis dysregulation (Griebel et al., 2003; Katz et al., 2016; **Table 1**). The first selective and orally active V1b antagonist developed was SSR149415. In rodent models of anxiety and depression, SSR149415 produced a clear-cut anxiolytic-like activity in traumatic stress models (*social defeat paradigm*) and antidepressant-like effects (Griebel et al., 2002). More recently, in the same paradigm, other V1b antagonists (*TASP0390325 and TASP0233278*) elicited antidepressant-like effects (Iijima et al., 2014). These findings evidenced that V1b receptor blockade is particularly interesting for the treatment of affective and emotional disorders. For instance, several clinical trials are in progress with the V1b antagonist ABT-436, which is in phase 1b for depression treatment (Katz et al., 2016, 2017), and in phase 2 for alcohol dependence treatment (Ryan et al., 2017).

Thus, while evidences about a direct involvement of hypothalamic AVP in AD patients are not yet available (Goudsmit et al., 1990; Lucassen et al., 1994; Ishunina et al., 2002), many studies showed a crucial role played by AVP in stress and stress-related disorders such as MDD or anxiety. These findings are interesting as MDD could be a prodromal feature of AD and dementia (Herbert and Lucassen, 2016; Ishijima et al., 2017; Canet et al., 2018), justifying further investigations.

Targeting GC Synthesis In AD

11β-HSD1 is a key enzyme that mediates the intracellular conversion of inactive cortisone to cortisol in humans (*11β-dehydrocorticosterone to corticosterone in rodent*), the active form of GC, thus amplifying steroid action. An haplotype in the 11β-HSD1 gene that increases 6-fold the risk to develop AD was reported (de Quervain et al., 2004).

Lowering GC exposure in the brain *via* intracellular inhibition of 11β-HSD1 has emerged as a therapeutic strategy to treat cognitive impairment in AD (**Table 1**). Pharmacological inhibition of 11β-HSD1 (*UE1961*) in aged mice improved spatial memory performance (Sooy et al., 2010). More recently, authors also demonstrated using another inhibitor (*UE2316*), a reduction of Aβ plaques in the cortex of old Tg2576-AD mice associated with an increase of IDE levels and memory improvements (Sooy et al., 2015). Moreover, in aged rodents, modulation of 11β-HSD1 by genetic knockdown or pharmacological inhibition improved memory. In Molher's study, the authors tested an acute treatment with two novels inhibitors (*A-918446 and A-801195, Abbott Laboratories*). These compounds were able to improve memory consolidation, short-term memory, and recall in inhibitory avoidance (Mohler et al., 2011).

It is also known that aged mice develop elevated plasma corticosterone levels that correlate with learning deficits in the water-maze. This poor performance in a long-term memory task can be ameliorated by an 11β-HSD1 knockout, implicating lower intraneuronal corticosterone levels (Yau et al., 2001, 2015). In senescence-accelerated SAMP8 mice, pre-treatment with metyrapone, which inhibits the 11β-hydroxylase, completely normalized corticosterone levels and restored spatial memory (Iinuma et al., 2008).

These promising data led to a clinical trial with a novel brain-penetrant 11β-HSD1 inhibitor (UE2343, Xanamem™) for AD treatment. The Phase 1 clinical studies showed that UE2343 is safe, well tolerated and is able to moderately penetrate the brain (Webster et al., 2017). In addition, it is interesting to note that treatment of cognitively impaired elderly male patients with carbenoxolone (*a non-selective 11β-HSD1 inhibitor*) improved verbal fluency and memory (Sandeep et al., 2004).

Targeting GR In AD

The ubiquitous expression of GR and their involvement in a broad range of biological processes make them a prime target in many diseases. On the other hand, the global inhibition or activation of these receptors can lead to many potential side effects. For instance, it was demonstrated that a blockade of brain GR with Mifepristone (RU486, the reference non-selective GR antagonist) impaired spatial learning and memory in rodent (Roozendaal and McGaugh, 1997; Oitzl et al., 1998). A more recent study showed that central or peripheral administration of RU486, immediately following memory reactivation, induced

TABLE 1 | Preclinical and clinical studies targeting corticotropin releasing hormone (CRH), arginine vasopressin (AVP), glucocorticoids (GC) or glucocorticoid receptors (GR).

	Context/model	Molecular, cellular & behavioral impacts	Reference
CRH receptors inhibition			
CRH overexpression	C57/B16 mice	Increase Tau phosphorylation and aggregation.	Campbell et al. (2014)
Antalarmin and R121919 (CRH-R1 antagonists)	Aged rats	Prevention of stress-induced memory deficits and anxiety; Prevention of stress-induced synapse loss and HPA axis dysfunction.	Dong et al. (2018)
R121919 (CRH-R1 antagonist)	APP/PS1 mice	Prevention of the onset of cognitive impairment; Reduction of cellular and synaptic deficits; Decrease of Aβ and C-terminal fragment levels.	Zhang et al. (2016)
NBI 27914 (CRH-R1 antagonist)	PS19 mice	Prevention of stress-induced Tau hyperphosphorylation and aggregation, neurodegeneration and fear memory impairment.	Carroll et al. (2011)
Antalarmin (CRH-R1 antagonist)	Tg2576-AD mice	Decrease level of plasma $A\beta_{1-42}$ and Aβ plaque deposits; Decrease level of plasma corticosterone; Improve memory and anxiety behavior.	Dong et al. (2014)
	Primary hippocampal culture	Inhibition of $A\beta_{1-42}$ levels and PKA expression after a CRH treatment.	
AVP receptors inhibition			
SSR149415 (V1b antagonist)	Anxiety/depression rodent models	Anxiolytic-like activity in models involving traumatic stress exposure; Antidepressant-like effects in FST.	Griebel et al. (2002)
TASP0390325 and TASP0233278 (Vl b antagonists)	Depression rodent models	Antidepressant-like effects in the FST; Reduction of the hyperemotionality after olfactory bulbectomy.	Iijima et al. (2014)
ABT-436 (V1b antagonist)	Human (MDD subjects)	Phase 1b in clinical trial for MDD; Reduction of HPA axis hyperactivity; Favorable symptoms changes.	Katz et al. (2017)
ABT-436 (V1b antagonist)	Human (alcohol dependence)	Phase 2 in clinical trial for alcohol-dependence; Increase of alcohol abstinence; Reduction of alcohol outcomes for subjects with higher baseline levels of stress.	Ryan et al. (2017)
GR modulation			
CORT108297 (sGRm)	3xTg-AD mice	Reduction of APP C-terminal fragments and p25 levels.	Baglietto-Vargas et al. (2013)
	Wistar rats	Attenuation of electroconvulsive shock-induced retrograde amnesia.	Andrade et al. (2012)
	Sprague–Dawley rats	Reduction of neuroendocrine stress responses and immobility in the FST.	Solomon et al. (2014)
	$oA\beta_{25-35}$ rat	Reverse $oA\beta_{25-35}$-induced neuroinflammatory and apoptotic processes, cognitive and synaptic deficits, and APP misprocessing.	Pineau et al. (2016)
CORT113176 (sGRm)	Wobbler mice	Reduction of neurodegeneration and neuroinflammation.	Meyer et al. (2014)
	$oA\beta_{25-35}$ rat	Reverse $oA\beta_{25-35}$-induced neuroinflammatory and apoptotic processes, cognitive and synaptic deficits, and APP misprocessing.	Pineau et al. (2016)
GC synthesis inhibition			
UE1961 (11β-HSD1 inhibitor)	Aged mice	Improvement of short-term memory.	Sooy et al. (2010)
UE2316 (11β-HSD1 inhibitor)	Tg2576-AD mice	Reduction of Aβ plaques in cortex; Increase of IDE levels; Memory improvements.	Sooy et al. (2015)
A-918446 (11β-HSD1 inhibitor)	Aged rodents	Improvement of memory consolidation and recall in inhibitory avoidance; Increase of CREB phosphorylation.	Mohler et al. (2011)
A-801195 (11β-HSD1 inhibitor)		Improvement of short-term memory	
11β-HSD1 knock-out	Aged mice	Prevention of intra-neuronal corticosterone increase; Improvement of long term memory (watermaze).	Yau et al. (2001)
Metyrapone (1lβ-hydroxylase inhibitor)	SAMP8 mice	Prevention of stress-induced corticosterone elevation, spatial memory deficits and hippocampal neurons loss.	Iinuma et al. (2008)
UE2343 (11β-HSD1 inhibitor)	Human	Phase 1 of clinical trial: compound safe, well tolerated and able to penetrate the brain (healthy subjects).	Webster et al. (2017)
Carbenoxolone (11β-HSD1 inhibitor)	Aging human	Improvement of verbal memory and fluency.	Sandeep et al. (2004)

a deficit in post-retrieval long-term memory, and memory did not re-emerge after a footshock reminder (Nikzad et al., 2011). Indeed, GC, GR and associated signaling pathways are crucial for memory consolidation. Acute stress and GR activation were shown to enhance memory consolidation and to inhibit working memory at the same time (Barsegyan et al., 2010). Many factors should be taken into consideration to understand and predict these GC contradictory effects. Depending on the brain area and on acute vs. chronic stress conditions, GC/GR can exert opposite effects (Roozendaal, 2002; McEwen, 2008; Barsegyan et al., 2010; **Figure 1**).

It is also important to mention the key role of GR in inflammatory processes. GR are necessary to maintain homeostasis since they control the expression of a large part of anti- and pro-inflammatory genes (Barnes, 1998; Van Bogaert et al., 2011; Coutinho and Chapman, 2011). An antagonist can prevent a crucial action of GR and lead to severe side effects associated with aberrant inflammatory processes. For example,

it was shown that RU486 blocked the anti-inflammatory effects of exercise in a murine model of allergen-induced pulmonary inflammation (Pastva et al., 2005).

In AD, it was reported that patients treated with prednisone (*a GC used for its anti-inflammatory properties*) presented a more pronounced behavioral decline compared to the placebo-treated cohort (Aisen, 2000). Similarly, it was shown that GC administration (*dexamethasone*) in 3xTg-AD mice increases Aβ pathology and subsequent Tau accumulation and hyperphosphorylation (Green et al., 2006). It results inexorably in a vicious cycle whereby the pathology increases the secretion of GC, which further enhances the pathology (Baglietto-Vargas et al., 2013; Brureau et al., 2013; Pineau et al., 2016; Canet et al., 2018, 2019). All these findings tend to prove that in AD, but also in all pathologies implying GC, both GR agonists or antagonists should be used with caution.

Recently, in a search to abrogate potential side effects of GR antagonism, new compounds were developed and act as highly selective GR modulators (sGRm; Clark et al., 2008; Beaudry et al., 2014; Hunt et al., 2015; Pineau et al., 2016; Meijer et al., 2018; Viho et al., 2019). The use of sGRm is an attractive approach to separate wanted from unwanted outcomes. Different sGRm were developed and vary substantially in their biological activities. These molecules have the ability to act as agonist, as well as antagonist depending on the tissue or gene targets, but also on the physiological context. However, one thing to keep in mind is that the prediction of exact sGRm action is really challenging considering the numerous factors to take into account. Meijer et al. (2018) recently stated about the complexity of sGRm mechanisms: "*even if we know the coactivators that will be recruited by a sGRm-GR complex, in most cases it is unknown which signaling pathways are involved in which transcriptional process. Given the large number of coactivators and their highly gene- and tissue-specific regulation, such analyses are very time consuming, if informative*".

In a direct application, sGRm offered promising results (**Table 1**). One of them, and the first to be developed by Corcept Therapeutics (Menlo Park, San Mateo, CA, USA), is CORT108297. It was first described as an antagonist (Belanoff et al., 2010), but its modulatory properties were discovered few years later (Zalachoras et al., 2013). In fact, the authors surprisingly observed that CORT108297 induced a unique interaction profile between GR and its coregulators compared with the full agonist dexamethasone and the non-selective antagonist, RU486. This atypical profile could explain the paradoxical effects of CORT108297 according to the brain region (Zalachoras et al., 2013; Viho et al., 2019). In rats, CORT108297 attenuates electroconvulsive shock-induced retrograde amnesia (Andrade et al., 2012). This molecule also presents antidepressant properties and reduces neuroendocrine stress responses and immobility in the forced-swimming test (FST), alike imipramine, a classic treatment for MDD (Solomon et al., 2014). By contrast, treatment with another member of this family, CORT118335, which has the particularity of being a GR modulator but also a MR antagonist, did not affect immobility in the FST (Nguyen et al., 2018), confirming a differential specificity and efficacy of each molecule.

In the 3xTg-AD mice, a 21 days treatment with CORT108297 reduced APP C-terminal fragments and p25 levels (Baglietto-Vargas et al., 2013), which is the activator of cyclin dependent kinase 5 (Cdk5), involved in AD pathophysiology (Patrick et al., 1999). In an acute rat model of AD (the $oA\beta_{25-35}$ model), we tested the therapeutic potential of CORT108297 and CORT113176 (Pineau et al., 2016). CORT113176 is also a sGRm, but displays a better affinity for GR than CORT108297 (Beaudry et al., 2014; Pineau et al., 2016). In this acute model, we previously evidenced a strong, long-lasting activation of the HPA axis, associated with a modification of GR and MR expression in brain regions involved in the control of GC secretion (hippocampus, amygdala and hypothalamus; Brureau et al., 2013). We found that both compounds (CORT113176 at a lower dose than CORT108297) were able to reverse neuroinflammatory and apoptotic processes, cognitive and synaptic deficits, and APP misprocessing (Pineau et al., 2016). To confirm the interest of sGRm, we compared these compounds with RU486. Interestingly, we observed that this non-selective antagonist only partially reversed previously observed pathological impairments (Pineau et al., 2016). In the same line of evidence, CORT113176 showed promising effects in a mouse model of amyotrophic lateral sclerosis by reducing neuronal injury and neuroinflammation in the spinal cord (Meyer et al., 2014). Thus, even if these compounds seemed to exhibit predominantly antagonistic actions in limbic structures in pathological conditions (Andrade et al., 2012; Atucha et al., 2015; Pineau et al., 2016), they could avoid side effects of GR blockade by some agonistic properties.

CONCLUSIONS

If we postulate that AD is a stress-related disorder, targeting HPA axis hormones and receptors could be an attractive approach and pave the way for new therapeutic strategies. Indeed, due to its primordial role in the maintenance of homeostasis, the HPA axis seems to be a key-actor in the etiology of AD and a prime target to tackle the pathology by offering multiple angles of action. Several strategies aiming to restore a normal functionality and activity of the HPA axis are in development with promising results. Some of these molecules are already in clinical studies, not necessarily in an AD context. Since some of them already obtained a safe and tolerated profile, testing them in AD patients could be a really encouraging challenge in the near future. Finally, this review wishes also to alert about the risk in prescribing GC-based therapies in the elderly or in early AD patients.

AUTHOR CONTRIBUTIONS

GC, CH, CZ, NC, CD and LG equally contributed to the definition of the scope and to writing of the manuscript.

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Non-specific Detection of a Major Western Blotting Band in Human Brain Homogenates by a Multitude of Amyloid Precursor Protein Antibodies

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The use of human post-mortem brain material is of great value when investigating which pathological mechanisms occur in human brain, and to avoid translational problems which have for example been evident when translating animal research into Alzheimer disease (AD) clinical trials. The amyloid β (Aβ)-peptide, its amyloid precursor protein (APP) and the intermediate APP-c-terminal fragments (APP-CTFs) are all important players in AD pathogenesis. In order to elucidate which APP CTF that are the most common in brain tissue of different species and developmental stages, and whether there are any differences in these fragments between AD and control brain, we investigated the occurrence of these fragments using different APP c-terminal antibodies. We noticed that whereas the conventional APP-CTFα and CTFβ fragments were most prominent in rat and mouse brain tissue, the major western blotting band detected in human, macaque and guinea pig was of approximately 20 kDa in size, possibly corresponding to the newly discovered APP-CTFη. However, this band was also intensely stained with a total protein stain, as well as by several other antibodies. The staining intensity of the 20 kDa band by the APP antibodies varied considerably between samples and correlated with the staining intensity of this band by the total protein stain. This could potentially be due to non-specific binding of the antibodies to another protein of this size. In-gel digestion and mass spectrometry confirmed that small amounts of APP were present in this band, but many other proteins were identified as well. The major hit of the mass spectrometry analysis was myelin basic protein (MBP) and a myelin removal protocol removed proportionally more of the 20 kDa APP band than the full-length APP and APP-CTFα/β bands.

Abbreviations: Aβ, amyloid β-peptide; AD, Alzheimer disease; ADAM10, a disintegrin and metalloproteinase 10; AICD, amyloid precursor protein intracellular domain; APP, amyloid precursor protein; BA9, Brodmann area 9; BACE1, β-site amyloid precursor protein cleaving enzyme 1; CSF, cerebrospinal fluid; CTF, C-terminal fragment; DTT, Dithiothreitol; EDTA, Ethylenediaminetetraacetic acid; FL-APP, full length APP; GC, Vitamin D binding protein; H, homogenate; HRP, horseradish peroxidase; KEAP1, Kelch-like ECH-associated protein 1; PMD, post-mortem delay; PVDF, polyvinylidene difluoride; RIPA, radio immunoprecipitation assay; sAPP, soluble amyloid precursor protein; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

However, the signal could not be immunodepleted with an MBP antibody. In summary, we report on a potentially non-specific western blotting band of approximately 20 kDa and call for precaution when analyzing proteins of this size in human brain tissue.

Keywords: amyloid precursor protein, human brain, western blotting, cross-reactivity, η-secretase

INTRODUCTION

Alzheimer disease (AD) is the most common form of dementia and causes extensive suffering for millions of patients and their relatives. Unfortunately, the only medication available gives just subtle symptomatic relief, and a large number of clinical trials of disease-modifying drugs have failed in the last decade. This is partially due to problems with translation, i.e., compounds that work well in mouse models fail in clinical trials. Thus, it is of utmost importance to study the alterations in human AD brain.

The synaptotoxic amyloid β-peptide (Aβ) plays a critical role in the pathophysiology of AD (Selkoe, 2011; Prince et al., 2013). Aβ is generated by the cleavage of the 695–770 amino acid long amyloid precursor protein (APP) by first β-secretase and then the γ-secretase complex. After the first cleavage by β-site APP cleaving enzyme 1 (BACE1), the main β-secretase of neurons, the 99 or 89 amino acid long c-terminal fragment (CTFβ) remains in the membrane while the soluble APPβ (sAPPβ) is released. The CTFβ is further cleaved by the protein complex γ-secretase, generating Aβ and the APP intracellular domain (AICD; Selkoe, 2011). In a separate, non-amyloidogenic pathway, APP is cleaved by the α-secretase a disintegrin and metalloproteinase 10 (ADAM10) instead of BACE1. This cleavage produces the 83 amino acids long CTFα which can also be cleaved by the γ-secretase complex, thus precluding Aβ production since the α-cleavage site is situated within the Aβ sequence (Postina et al., 2004).

APP can also be cleaved by a number of other proteases, such as caspases (Galvan et al., 2002), aspargine endopeptidase (δ-secretase; Zhang et al., 2015), the recently identified η-secretase (Wang et al., 2015; Willem et al., 2015), or other unknown proteases (Nikolaev et al., 2009), to produce toxic fragments and/or influence further processing. The situation is further complicated by the fact that also CTFβ and the fragment derived from sequential cleavage by η- and α-secretase (Aη-α) have been shown to be neurotoxic and may therefore be involved in the pathology of AD (Oster-Granite et al., 1996; McPhie et al., 1997; Bittner et al., 2009; Jiang et al., 2010; Lauritzen et al., 2012; Willem et al., 2015). However, which of these fragments that are most abundant in human brain, and whether the processing is the same in different species, or at different developmental stages, has to our knowledge not been carefully investigated.

Here, we studied the occurrence of different APP-derived fragments in human as well as in other species including macaque, guinea pig, rat and mouse brain using western blotting. Using several APP-antibodies we detected a major band of approximately 20 kDa in adult human, macaque and guinea pig brain. However, more careful investigation using total protein stain, mass spectrometry and immunoprecipitation indicated that this band was unspecific and was also recognized by several other antibodies.

MATERIALS AND METHODS

Human Post-mortem Material

The use of human brain material in this study was conformed to the Declaration of Helsinki and approved by the regional ethical review board of Stockholm (2015/1803-31/2 and 2007/1477-3). Brain tissues from frontal cortex Brodmann area 9 (BA9) of 10 sporadic AD and 10 control cases were obtained from Brains for Dementia Research, London, UK, while the human brain tissue (mixed cortex) used for the experiments with the different antibodies, immunoprecipitation and different species was obtained from the Brain Bank at Karolinska Institutet, Stockholm, Sweden. All patients were clinically and pathologically diagnosed and the characteristics of the patients are described in **Table 1**. Cortical brain tissue from human fetuses, post-conception age 7–11 weeks, was obtained from clinical routine abortions through the Developmental Tissue Bank at Karolinska Institutet. All participants or their consultees (for participants that lacked capacity) gave informed consent to the donations and all procedures were approved by ethical review boards.

Animals

Male Wistar rats (Charles River) were killed by carbon dioxide treatment while female C57BL/6 mice (bred at Karolinska Institutet) were killed by cervical dislocation. The animals used in this study were handled according to the Karolinska Institutet guidelines, Swedish national guidelines and current European Law (Directive 2010/63/EU). The use of rat brains was approved by the animal research ethical committee of southern Stockholm (S21-14) while the use of mouse brain was approved by Linköping ethical committee (ID156). Brain lysates from guinea pig and macaque was purchased from Novus Biologicals who ensure that the animals have been handled according to the United States Department of Agriculture (USDA) animal welfare act as well as the National Institutes of Health (NIH), Office of Laboratory Animal Welfare (OLAW) and the Public Health Service (PHS) policy on humane care and use of laboratory animals. No experiments were performed on live animals.

TABLE 1 | Characteristics of cases for post-mortem human brain samples Brodmann area 9 (BA9) obtained from the Brains for Dementia Research.

	Age (years)	Gender	PMD (h)
Sporadic AD (*n* = 10)	88.7 ± 6.6	70% F	34.0 ± 23.3
Control (*n* = 10)	82.3 ± 7.4	70% F	35.7 ± 20.2

F, female; PMD, post-mortem delay.

Brain Homogenization

Brain homogenates derived from different sources were homogenized in slightly different buffers and with slightly different protocols. For all samples except the ones mentioned specifically below the following method was used: homogenization of the cortical tissue from human, rat and mouse was carried out in four volumes of cold homogenization buffer [20 mM HEPES, 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), pH 7.0] with Complete Protease Inhibitor Cocktail (Roche) by eight strokes at 800 rpm using a mechanical glass-teflon homogenizer. Brain tissues from human and mouse embryo were then sonicated. AD and control samples from the Brains for Dementia Research were homogenized in 50 mM Tris-HCl, 5 mM EGTA, 10 mM EDTA and Sigma protease inhibitor cocktail. The guinea pig and macaque homogenates were delivered as ready lysates from Novus Biologicals. Protein determination was done by Pierce™ BCA Protein Assay Kit (Thermo Fisher). The brain homogenates were stored at −80°C.

Antibodies

All primary antibodies used in the study are described in **Table 2** and the epitopes of the APP antibodies are delineated in **Figure 1A**. The secondary antibodies IRDye 800CW donkey anti-mouse IgG and IRDye 680RD donkey anti-rabbit IgG were purchased from LI-COR®. For the in-gel digestion, we instead used a horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (GE-Healthcare).

SDS-PAGE and Western Blotting

Equal amounts of protein (30–40 μg depending on experiment) from each sample were denatured in either 4× of protein sample loading buffer (LI-COR) containing 2-mercaptoethanol, or 4× NuPAGE LDS sample buffer containing dithiothreitol (DTT), or 2× Laemmli buffer and boiled at 95°C for 5 min. Samples and 7 μl of the Chameleon duo pre-stained protein ladder (LI-COR) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 4%–12% polyacrylamide bis-tris gels (Invitrogen) or 16% polyacrylamide tricine gels (Invitrogen) and transferred to nitrocellulose membranes (GE Healthcare). As a reference, a recombinant C99-FLAG peptide (a gift from Dainippon Sumitomo Pharma) was loaded. After transfer, nitrocellulose membranes were blocked in Odyssey blocking buffer (TBS) for up to 1 h at room temperature, followed by incubation with primary antibodies at 4°C overnight. Membranes were washed with TBS-T and then incubated with fluorescently labeled IRDye secondary antibodies (LI-COR) for 1 h at room temperature. After TBS-T washes, membranes were washed in TBS for one last time, and digital fluorescent visualization of signals was detected at 700 and/or 800 nm channels using the Odyssey® CLx Imaging System (LI-COR). All primary and secondary antibodies were diluted in Odyssey blocking buffer (TBS). In some experiments, membranes were stained with a total protein stain [REVERT™ Total Protein Stain (LI-COR)] especially for normalization of the signal (**Figures 3A,B**). In order not to interfere with the signal that could arise from antibodies, this step was done right after transfer. Once membranes were incubated with total protein stain and the signal was immediately detected at the 700 nm channel. After removal of the stain using reversal buffer, the membrane was incubated with Odyssey blocking buffer (TBS) and incubation with primary and secondary antibodies was done as mentioned above. Quantitation of protein (20 kDa band and total protein) was done using Image Studio Lite v5.2 (LI-COR). In order to assure linearity of the signals three different concentrations of a standard sample (A1) was loaded on all gels and a standard curve was created which was used for quantification. Statistical analysis of the comparison of the levels of the 20 kDa band in AD and control was performed using Student's *t*-test.

For the in-gel digestion experiment, equal amounts of samples were loaded onto the 4%–12% polyacrylamide bis-tris gels (Invitrogen) and transferred to polyvinylidene difluoride (PVDF) membranes (GE Healthcare). Following primary antibody incubation, membranes were incubated with secondary antibodies coupled to HRP (GE-Healthcare). SuperSignal West Pico enhanced chemiluminescent reagent (Pierce) was then used to visualize the signals which were exposed to film (GE-Healthcare).

In-Gel Digestion and Mass Spectrometry

Protein bands were excised manually from a bis-tris 4%–12% gel, using western blotting as a location reference, and in-gel digested using a MassPREP robotic protein-handling system (Waters). Gel pieces were destained twice with 100 μl 50 mM ammonium bicarbonate (Ambic) containing 50% acetonitrile at 40°C for 10 min. Proteins were reduced with 10 mM DTT in 100 mM Ambic for 30 min at 40°C and alkylated with 55 mM iodoacetamide in 100 mM Ambic for 20 min at 40°C followed by

TABLE 2 | List of primary antibodies.

Antibody name	Company and product number	Dilution (WB)	Final concentration (μg/ml)
Anti-amyloid beta precursor protein (Y188)	Abcam (ab32136)	1:5,000	0.08
Anti-APP c-terminal fragment (C1/6.1)	BioLegend (802801)	1:5,000	0.2
Anti-amyloid beta (6E10)	BioLegend (803001)	1:1,000	1
Anti-amyloid beta precursor protein (A8717)	Sigma (A8717)	1:2,000	5
Anti-beta amyloid (7N22)	Invitrogen (AHB0272)	1:5,000	0.1
Anti-amyloid beta precursor protein (9478)	Gift from Dr. Willem	1:500	4
Anti-myelin basic protein (MBP)	Abcam (ab216668)	1:500	0.1
Anti-kelch-like ECH-associated protein 1 (KEAP1)	Protein Tech (10503-2-AP)	1:5,000	0.07
Anti-vitamin D-binding protein (GC)	AbFrontier (LF-MA0147)	1:20,000	0.025

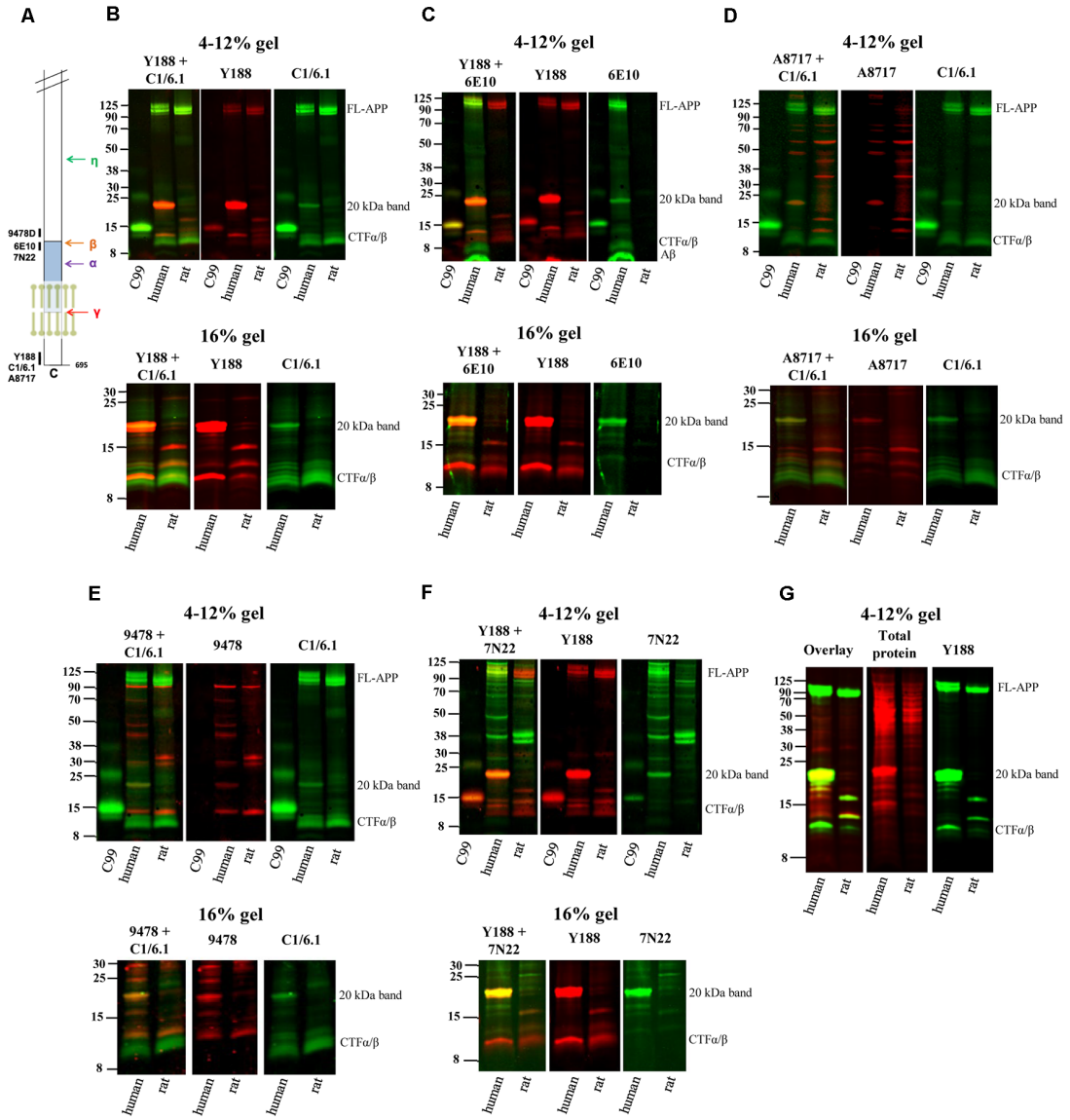

FIGURE 1 | Elucidating the pattern of bands detected by different amyloid precursor protein (APP) antibodies in rat and human brain. **(A)** The APP protein, indicating the epitopes of the antibodies used in the study. **(B–F)** Equal amounts of homogenates of human Alzheimer disease (AD) and adult rat brain were loaded on 4%–12% (upper panels) or 16% (lower panels) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and subjected to western blotting using the Odyssey digital fluorescent system with detection by primary antibodies **(B)** Y188 and C1/6.1, **(C)** Y188 and 6E10, **(D)** A8717 and C1/6.1, **(E)** 9478 and C1/6.1 or **(F)** Y188 and 7N22. A recombinant C99-FLAG peptide was loaded on the 4%–12% gels to indicate the approximate position of c-terminal fragments (CTF)-β (minus the 1 kDa FLAG-tag). The figures are representative figures and four additional rat brain samples and five additional human brain samples showed a similar pattern using the Y188 and C1/6.1 antibodies (see also **Figure 5**). **(G)** Equal amounts of homogenates of human AD and adult rat brain were loaded on a 4%–12% SDS-PAGE gel and subjected to western blotting using the Odyssey digital fluorescent system with detection by primary antibody Y188 together with a total protein stain. A major band was detected around 20 kDa by all antibodies as well as by the total protein stain.

in-gel digestion with 0.3 μg Trypsin (Sequence grade, Promega) in 50 mM Ambic for 5 h at 40°C. The tryptic peptides were extracted with 1% formic acid in 2% acetonitrile, followed by 50% acetonitrile twice. The liquid was evaporated to dryness and the peptides were separated on an EASY-spray column connected to an EASY-nLC 1000 system (Thermo Scientific). The peptides were eluted in a 60 min gradient (from 5% to 26% of buffer B (2% acetonitrile, 0.1% formic acid) in 55 min and up to 95% of buffer B in 5 min) at a flow rate of 300 nL/min and analyzed on a Fusion Orbitrap mass spectrometer (Thermo Scientific). The spectra were analyzed using the Mascot search engine v.2.4 (Matrix Science Limited).

Immunoprecipitation

For immunoprecipitation with the Y188 and A8717 antibodies, antibodies or a rabbit IgG (used as a negative control) were

bound to PureProteome™ Protein A/G mix magnetic beads (Millipore). Brain homogenates from AD cortex were solubilized in 1× RIPA lysis buffer without NP-40 (150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris/HCl, pH 8.0) containing Complete Protease Inhibitor Cocktail (Roche). The sample was precleared with magnetic beads for 2 h at 4°C in order to remove any potential non-specific binding. Twenty microliter of sample was collected as input for western blotting, whereas the remaining sample was incubated with the antibody coupled beads overnight at 4°C. After immunoprecipitation, 20 μl of sample was collected to detect unbound proteins. Precipitated proteins were washed with PBS and eluted in 20 μl of 1× NuPAGE LDS sample buffer (Invitrogen) and boiled at 95°C for 5 min. The eluates were transferred to new microcentrifuge tubes and subjected to western blotting as described above.

For myelin basic protein (MBP) immunodepletion, brain homogenate from AD cortex was solubilized in 1× RIPA lysis buffer as mentioned above, and the sample was precleared with magnetic beads for 30 min at room temperature in order to remove any potential non-specific binding. Twenty microliter of sample was collected as inputs before and after pre-absorption with beads, respectively. The remaining sample was incubated with the MBP antibody for 1 h at room temperature to create antibody-antigen complex, which was followed by incubation with the magnetic beads for 30 min at room temperature. After immunoprecipitation, 20 μl of the unbound sample was collected and the sample bound to the beads was washed three times in 1× RIPA. Precipitated proteins were eluted in 40 μl of 1× Protein Sample Loading buffer (LI-COR) and boiled at 95°C for 5 min. The eluates were transferred to new microcentrifuge tubes and subjected to western blotting as described above.

Myelin Removal

Myelin Removal Beads II (Miltenyi Biotec) was used according to the manufacturer's protocol with some modifications. The protocol builds on binding of myelin to a specific but confidential antibody coupled to the beads. Sixty microliter beads were added to 500 μl of AD brain homogenate in 0.5% BSA in PBS (1.5 μg/μl) and incubated for 15 min, rotating at room temperature. The sample was then further diluted 5.5 times with PBS/BSA and centrifuged at 100,000× *g*, 30 min and resuspended in 1 ml of PBS/BSA in order to first wash and then concentrate the membranes. The sample was then passed through a magnetic LS column and the flow-through (myelin-depleted sample) was collected.

RESULTS

A Multitude of APP Antibodies Labels a 20 kDa Predominant Band in Human Brain

Using different APP antibodies, we analyzed APP fragments in human AD and rat brain homogenates after separation on BisTris 4%–12% polyacrylamide gels or tricine 16% polyacrylamide gels. The use of the LI-COR detection system, which allows simultaneous detection with two different antibodies, enabled us to visualize whether the same band was stained with the different antibodies. Surprisingly, we found that in human brain, a predominant band of approximately 20 kDa was detected by all antibodies tested, including Y188 (**Figures 1B,C,F**), C1/6.1 (**Figures 1B,D,E**), 6E10 (**Figure 1C**), A8717 (**Figure 1D**), 9478 (**Figure 1E**) and 7N22 (**Figure 1F**). From now on, we will refer to this band as the 20 kDa band although the molecular weight varies slightly depending on gel systems and molecular weight markers, and the exact size has not been determined. In contrast to human brain, the 20 kDa band was not detected in rat brain homogenates by any of the antibodies. The 20 kDa band corresponds to the expected weight of the proposed APP-CTFη, generated by η-secretase cleavage at N504 in the APP695 sequence (Willem et al., 2015). When using the Y188 antibody and 16% gels, which effectively separate proteins of lower molecular weight, we observed that the 20 kDa band is actually a double band (**Figures 1B,C,F**) in agreement with the double CTF-η band detected by Wang et al. (2015).

ADAM10 cleavage of APP gives rise to the 83 amino acids long CTFα, while CTFβ is either 99 or 89 amino acids long depending on where APP is cleaved by BACE1. In addition, post-translational modifications of APP, such as phosphorylation and glycosylation may give rise to even more CTF bands using western blotting. Thus, several bands between 8 and 14 kDa were detected, most clearly demonstrated with the C1/6.1 antibody. Using this antibody, the staining intensity for the CTFα and -β bands were similar between human and rat homogenates (**Figures 1B,D,E**). The 9478 antibody is directed to the N-terminal part of the CTFη sequence and is not expected to detect CTFα or -β. Instead, the band of approximately 12 kDa detected by 9478 could be the proposed Aα-η peptide (**Figure 1E**). A major human-specific band of around 11 kDa was detected with the Y188 antibody. On 16% gels, this band migrates as one of the lower conventional CTF bands (**Figures 1B,C,F**, lower panels). Since this band does not overlap with bands detected by 6E10, we can conclude that it does not include the first eight amino acids in the Aβ sequence and is thus not identical to C99. Y188 also detected a major 15 kDa fragment in rat brain, possibly reflecting CTFδ (Zhang et al., 2015).

In addition, the expected immature (non-glycosylated) and mature (glycosylated) forms of full-length APP [FL-APP, approximately 100 kDa (Weidemann et al., 1989)] were clearly detected with the Y188, C1/6.1 and 6E10 antibodies (**Figures 1B–F**), whereas the A8717 did not detect these bands (**Figure 1D**), 9478 detected a band of slightly smaller molecular weight (**Figure 1E**) and 7N22 only detected the lower FL-APP band in human brain (**Figure 1F**). In general, the A8717, 9478 and 7N22 antibodies detected a multitude of bands of unexpected molecular weight, possibly due to non-specific or cross-reactive binding. The staining intensity of FL-APP and APP-CTFα and -β were similar between human and rat when detected with the Y188 or C1/6.1 antibodies. Since 6E10 is a human-specific antibody that recognizes the first amino acids in the APP-CTFβ sequence, no bands were detected in rat brain homogenate using this antibody and the lower CTFα/β bands, corresponding to CTFα were not detected (**Figure 1D**).

Peculiarly, the ratio between the different western blotting bands varied between the different antibodies. Whereas the 20 kDa band gave rise to the strongest signal of all APP-derived

fragments in human brain using the Y188 or 6E10 antibodies (**Figures 1B,C,F**), the signal from this band was about as intense as the signal from the other CTFs when using the C1/6.1 antibody (**Figures 1B,D,E**). However, the 20 kDa band was always stronger in human than in rat brain samples. We also noticed that a band stained intensively with a total protein stain, overlapped with the 20 kDa band (**Figure 1G**).

In summary, using six different APP antibodies (Y188, C1/6.1, 6E10, A8717, 9478 and 7N22), we detected a 20 kDa band in human AD brain homogenate which was hardly detectable in rat brain homogenate. In the subsequent experiments, we chose to focus on the combination of Y188 and C1/6.1 antibodies. It should also be noted that although all experiments in **Figure 1** are performed using the same human brain homogenate, the 20 kDa band was present in almost all human brain samples tested (see **Figure 5**) while being non-detectable or present in very low levels in five additional rat brain samples (data not shown). The presence of the 20 kDa band was not dependent on sample treatment, since it was present independently on which loading buffer that was used (data not shown) or if the samples were treated with RIPA buffer.

The 20 kDa Band Is Also Present in Guinea Pig and Macaque Brain but Not in Mouse Brain or Human Embryonic Brain

Since the staining pattern of the different bands detected by APP antibodies was so different in human and rat brain (**Figure 1**), we decided to examine these bands in brain homogenates from a few more animal species. We found that the 20 kDa band was present also in brain homogenates from guinea pig, and to less extent in macaque, but not in mouse brain homogenates (**Figure 2A**). However, a faint band slightly above the prominent 20 kDa band was detected by C1/6.1 in all species. Again, the intensity ratios between 20 kDa band and the CTFα and -β bands were different depending on whether the Y188 or the C1/6.1 antibody was used for detection (**Figure 2A**).

To investigate whether the presence of the 20 kDa band is developmentally regulated, we next compared the expression pattern of APP-CTFs in brain homogenates prepared from embryo and adult (**Figure 2B**). Interestingly, the pattern of CTFs in brain homogenates from human fetus was clearly different from that in human adult brain, with undetectable levels of the 20 kDa band (**Figure 2B**). This pattern was further confirmed in three additional human fetus brain homogenates (data not shown). The pattern of CTFs in brain homogenates from mouse embryo was similar to that from adult mouse (**Figure 2B**).

In summary, the 20 kDa was present in adult brain from human, macaque and guinea pig, but was not present at detectable levels in adult brain from rat or mouse, nor in human fetal or mouse embryonic brain.

Large Variation in Staining Intensity of the 20 kDa Band in Human Brain Samples

In order to determine whether there were any differences in the levels of the 20 kDa band between AD and control brain, we loaded equal amounts of homogenate from BA9 of the frontal cortex from 10 sporadic AD and 10 control subjects on 16% SDS-PAGE gels. Using both the Y188 and the C1/6.1 antibody for detection, we found that the 20 kDa was present in the majority of the brain homogenates from AD and control subjects (**Figure 3**, representative figure). The levels of the 20 kDa band varied considerably between individuals, and no significant differences could be detected between AD and control subjects. In addition, no obvious correlations could be found between levels of the 20 kDa band and parameters such as gender, age, post-mortem delay or storage time (data not shown). In order to normalize the levels to the total protein content, we also performed a total protein stain of the membrane. As in our initial experiments shown in **Figure 1G**, we noticed that a band that was intensively stained with the total protein stain overlapped in size with the 20 kDa band detected by the APP antibodies. Furthermore, the staining intensity of this band by the total protein stain correlated well with the intensity of the 20 kDa APP band.

Thus, the 20 kDa band was present in all human brain homogenates studied but that the levels varied considerably between samples and no significant differences in the levels of this CTF between AD and control cases. In addition, the

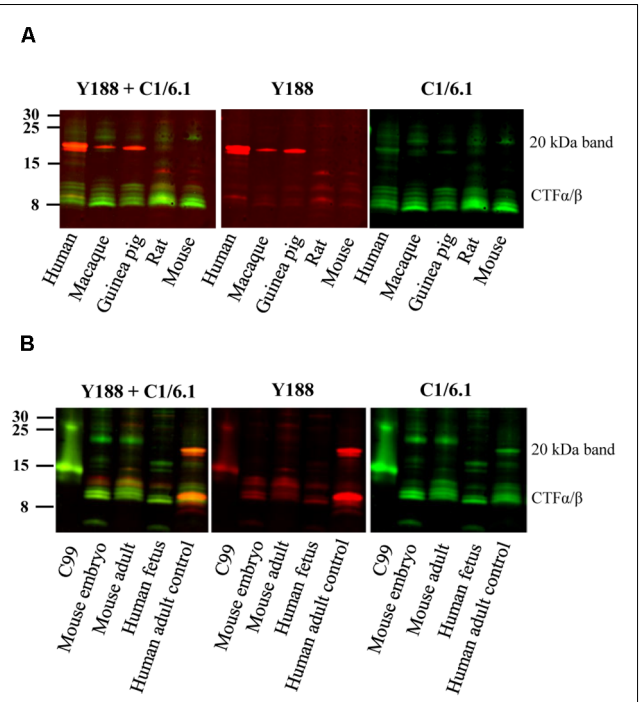

FIGURE 2 | Bands detected by APP antibodies in different species and developmental stages. **(A)** Equal amounts of human AD, macaque, guinea pig, rat and mouse brain homogenates were loaded on a 16% gel and subjected to western blotting using the Y188 and the C1/6.1 antibodies. The major 20 kDa band is detected in human, macaque and guinea pig brain, but not in rat or mouse brain. **(B)** Equal amounts of brain homogenates from mouse embryo, adult mouse, human embryo and human adult control were loaded on a 16% gel. The Y188 and C1/6.1 antibodies were used for detection. The 20 kDa band was only detected in adult human brain whereas only lower molecular CTFs were present in human fetal brain. The pattern of CTFs was similar in embryonic and adult mouse brain. Panel **(B)** is a representative figure and the results were confirmed using an additional three human and four mouse embryonic brain samples.

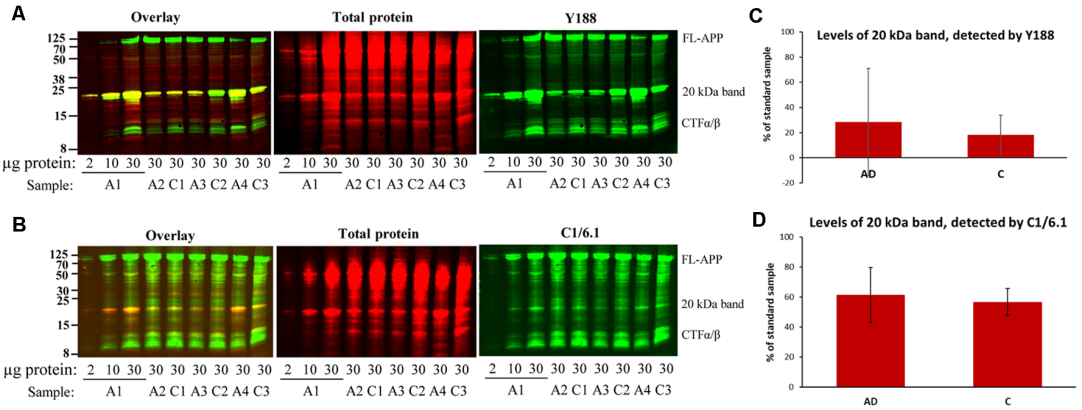

FIGURE 3 | Bands detected by APP antibodies in human AD and control brain homogenates. Equal amounts of homogenates form AD and control brain were loaded on 16% gels. In order to assure linearity of the signals, three different concentrations of a standard sample (A1) was loaded on all gels and a standard curve was created which was used for quantification. The gels were subjected to SDS-PAGE and western blotting using **(A)** Y188 antibody together with a total protein stain or **(B)** C1/6.1 antibody together with a total protein stain. Representative gels are shown with age- and gender-matched pairs of AD and control cases loaded beside each other. **(C,D)** Densitometric analysis of the 20 kDa band using the Y188 **(C)** or the C1/6.1 antibody **(D)**. All values were normalized to the total protein stain and expressed as % of the standard sample. The major 20 kDa APP band varies considerably between cases and correlates with the size and intensity of a major total protein stained band. No difference in the levels of the 20 kDa band could be detected between AD and control brain. A, AD; C; control.

band overlapped in both size and intensity with a major total protein band.

Mass Spectrometry Confirms That the 20 kDa Band Contain APP but That Many Other Proteins Are Present in This Band as Well

The overlap of the 20 kDa APP band with a major total protein band raised our concern whether the antibodies studied cross-react or bind non-specifically to constituents of this band. In order to further elucidate the identity of this band, we therefore loaded human brain homogenate on a gel and cut out the bands corresponding to the size of full-length APP (as a positive control) and the 20 kDa band (as indicated by detection by western blotting using the Y188 antibody of parallel lanes of the same gel). The gel pieces were destained and alkylated, and in-gel digestion was performed. The digested band corresponding to full-length APP gave rise to a number of spectra of high quality whereof one corresponded to the LVFFAEDVGSNK peptide (Aβ 17–28, **Figure 4**, upper panel). This peptide was also identified in the digested 20 kDa band with a similar spectrum and retention time (**Figure 4**, lower panel), showing that this band indeed is partially derived from APP. However, the score of the APP-derived peptide was low and the digested gel piece also contained a large number of other proteins (**Supplementary File S1**). The protein with the highest score was MBP for which several of the major isoforms have the correct size of the digested band (17–21.5 kDa[1]). Other major hits included glutathione S-transferase P as well as different isoforms of tubulin beta and alpha. The peptides used for identifying the different tubulin isoforms were to a large degree overlapping.

[1]www.uniprot.org/uniprot/P02686

The 20 kDa Band Cannot be Efficiently Immunoprecipitated From Human Brain Using APP Antibodies and Is Depleted by Myelin Removal Beads but Not by Immunodepletion With an MBP Antibody

Next, we tested whether the 20 kDa band could be immunoprecipitated using the Y188 and the A8717 antibodies. To our surprise, immunoprecipitation of human AD brain

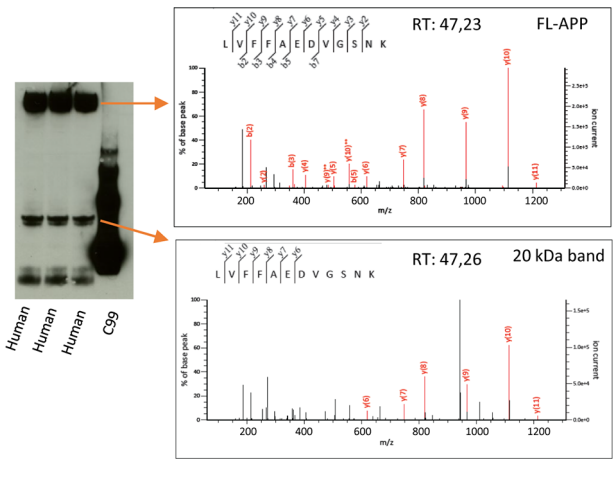

FIGURE 4 | Mass spectrometry analysis of the 20 kDa band. Human AD brain homogenate was loaded on a 4%–12% gel and bands corresponding to the size of FL-APP or the 20 kDa band were cut out, in-gel digested with trypsin and subjected to liquid chromatography tandem mass spectrometry. The digested bands of both FL-APP (upper panel) and the 20 kDa band (lower panel) gave rise to spectra corresponding to the peptide LVFFAEDVGSNK of the APP sequence. Many other proteins were identified in the 20 kDa band to a higher score (**Supplementary File S1**).

homogenates with these antibodies immunoprecipitated only low amounts of the 20 kDa band and not significantly more than the IgG control (**Figure 5A**). In contrast, FL-APP, as well as the lower molecular weight APP-CTFs detected by the C1/6.1 antibody were very efficiently immunoprecipitated using either of the antibodies (**Figure 5A**). The large variation of the intensity of the 20 kDa APP-reactive band in human samples, and the correlation with the intensity of the same band visualized with a general protein stain, could potentially be explained by different amounts of white matter in these samples. In line with this, the levels of the 20 kDa APP band were undetectable in early human fetal stages (**Figure 2B**), where myelin is absent (Dubois et al., 2014). These observations, combined with the fact that MBP was the most abundant protein in the 20 kDa band, made us suspect that the labeling of the band was due to non-specific binding to myelin and/or cross-reaction of antibodies to the MBP protein. We, therefore, co-stained the immunoblots with the C1/6.1 and MBP antibodies. Several MBP isoforms between 14–21 kDa have been identified (Barbarese et al., 1977) and we detected several bands using the MBP antibody (**Figure 5B**). A major MBP-reactive band overlapped perfectly with the 20 kDa band detected with the C1/6.1 antibody (**Figure 5B**).

We also performed a myelin removal protocol using Myelin removal beads from Miltenyi Biotec. This product is developed to remove myelin from single-cell suspensions, not from tissue homogenates, and we experienced large loss of material during the procedure. However, proportionally more of the 20 kDa band was lost compared to the APP-FL and APP-CTFα and -β bands (**Figure 5C**). Thus, although we cannot be sure that this is due to the removal of myelin, the proteins represented by the 20 kDa band appear to bind more to the myelin removal beads (specifically or non-specifically) than the other APP bands do. An indication that myelin indeed was removed by this protocol was that both of the major MBP bands were also depleted. The intensity of the staining of the 20 kDa band with a total protein stain was also decreased after myelin depletion (**Figure 5C**).

To further elucidate whether the detection of the 20 kDa band by APP antibodies is due to cross-reactivity to MBP, we immunodepleted the homogenates from MBP using an MBP antibody. However, whereas an approximately 15 kDa band detected by the MBP antibody was efficiently depleted from the homogenates, and detected in the immunoprecipitates,

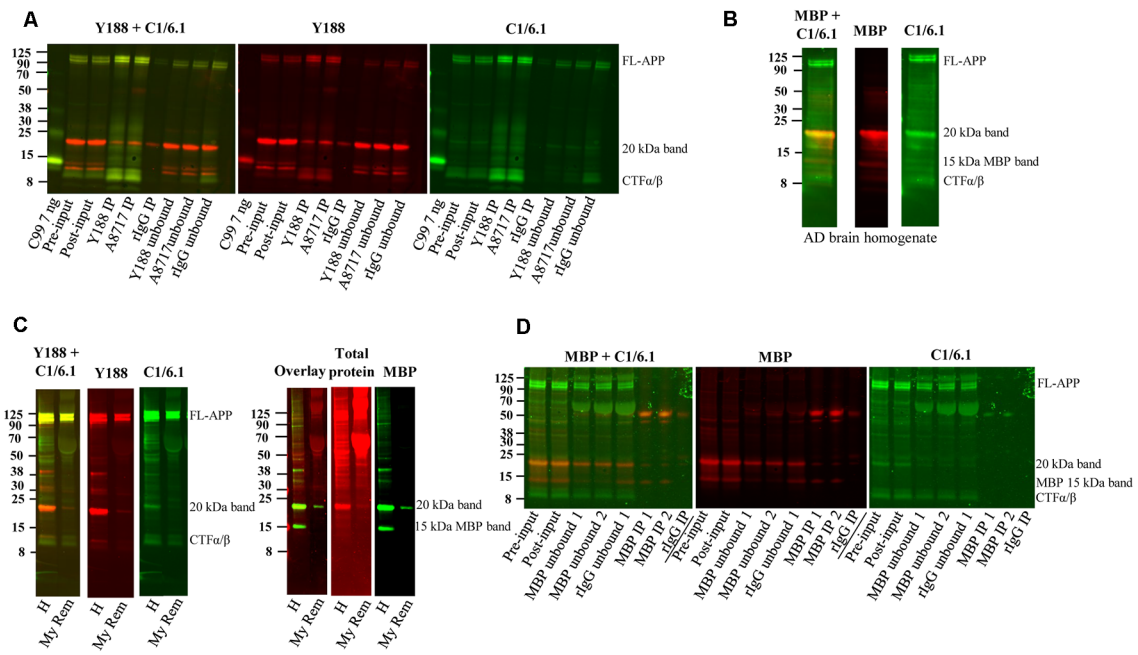

FIGURE 5 | Immunoprecipitation of APP and depletion of myelin and myelin basic protein (MBP). **(A)** Western blot of immunoprecipitation of human AD brain homogenate using the Y188, the A8717 antibody or a rabbit immunoglobulin antibody (rIgG) as negative control. A recombinant C99-FLAG peptide was loaded as a size indicator. The 20 kDa band could not be immunoprecipitated to a higher level with the specific antibodies Y188 and A8717 than with the control rIgG, and the majority of the 20 kDa remained in the unbound fraction. On the contrary, full length (FL) APP and some of the lower molecular weight CTFs, were successfully immunoprecipitated. Fifteen percentage of the brain homogenate was collected before immunoprecipitation and loaded as input. This is a representative figure of eight experiments. **(B)** Western blotting of a human brain AD homogenate using the MBP and the C1/6.1 antibody shows overlap of the signal in the 20 kDa band. **(C)** A human brain AD homogenate was subjected to myelin removal beads (My Rem) or kept on ice (H), loaded on a gel and subjected to western blotting using Y188, C1/6.1 and MBP antibodies as well as a total protein stain. The loaded My Rem sample corresponded to 25 times more of the input than the loaded H sample. The 20 kDa band and the 15 kDa MBP band was depleted proportionally more than the FL-APP and APP-CTFα/β bands. **(D)** Immunodepletion of a human AD brain using an MBP antibody was performed using 2.5 μg (IP 1) or 5 μg (IP 2) MBP antibody or a rabbit IgG (rIgG) as negative control. Five percentage of input before (pre-input) and after (post-input) pre-clearing with beads only, as well of immunodepleted samples were loaded. Whereas the 15 kDa MBP band was efficiently and specifically depleted, the 20 kDa band detected by C1/6.1 was not depleted more by the MBP antibody than by the rIgG.

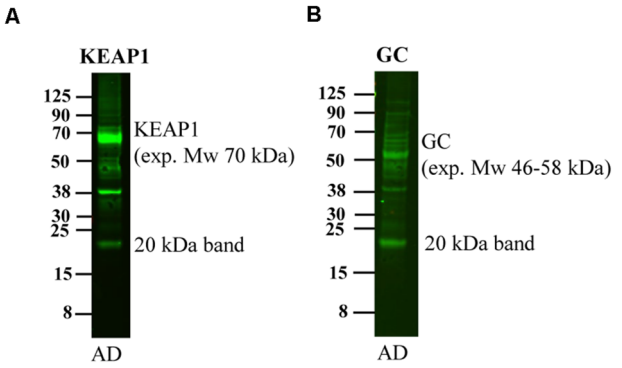

FIGURE 6 | Detection of the 20 kDa band by other antibodies. An AD human brain homogenate was loaded on a 4%–12% gel and subjected to western blotting using antibodies directed to **(A)** Keap1 and **(B)** GC. These antibodies detect bands of their expected molecular weight of 70 and 46–58 kDa, respectively, but also detect a band of approximately 20 kDa.

the 20 kDa band detected by the C1/6.1 antibody was not specifically immunodepleted or immunoprecipitated using the MBP antibody. The 20 kDa band (as detected either by C1/6.1 or the MBP antibody) was, to a certain degree, immunodepleted both by the MBP antibody and the negative control rabbit IgG. In contrast, no differences in staining intensity could be observed before and after pre-clearing of the homogenates with beads only (**Figure 5D**).

Thus, both APP antibodies and the MBP antibody detects a 20 kDa band in human brain but the same antibodies cannot specifically immunoprecipitate this band, indicating that the detection is non-specific also for MBP. However, the protein(s) responsible for this staining can, to a certain degree, bind to any antibody-bead complexes (also negative control rabbit IgG) or to myelin removal beads but not to the magnetic beads alone.

Several Other Antibodies Also Recognize a 20 kDa Band in Human Brain

Upon running western blotting experiments of human brain homogenates for other proteins in our laboratory, we noted that also these antibodies detected a band of approximately 20 kDa although the expected molecular weight was of a different size. These antibodies include Kelch-like ECH-associated protein 1 (Keap1, expected molecular weight 70 kDa, **Figure 6A**), and Vitamin D-binding protein (GC, expected molecular weight 46–58 kDa, **Figure 6B**). Thus, a multitude of antibodies, including APP antibodies, an MBP and other antibodies recognize this 20 kDa band, strongly indicating that the binding is at least partially non-specific.

DISCUSSION

Using western blotting to characterize the different APP-CTFs in brain homogenates, we here demonstrate that a band of approximately 20 kDa is abundant in human brain but not in rat or mouse brain. We performed a detailed investigation of this band in brain tissue from different species and developmental stages, AD patients and healthy controls, using a range of antibodies, mass spectrometry and immunoprecipitation.

Several studies have shown that bands that are detected by APP antibodies and migrate between 20 kDa and 30 kDa on SDS-PAGE gels are present in human brain (Estus et al., 1992; Haass et al., 1992; Tamaoka et al., 1992), but the identity of these bands has not been thoroughly investigated. However, recently Wang et al. (2015) identified an APP-band of similar size in human cell-lines and named it APP-CTFη. This band was further characterized by Willem et al. (2015) who also showed that subsequent cleavage of APP-CTFη by α-secretase resulted in a synaptotoxic Aη-α peptide. The detection of the APP-CTFη in mouse brain was specific since it was not detected in APP knock-out mice (Willem et al., 2015).

The 20 kDa band that we detect in human, guinea pig and macaque brain was much more prominent than any bands of similar size in mouse or rat brain, although we could detect a weak band of slightly higher molecular weight in mouse brain homogenates using the C1/6.1 antibody. In addition, the 20 kDa band was not present in human embryonic brain. Given the similarity in size between the 20 kDa band and the APP-CTFη band, we investigated whether the 20 kDa band could indeed be APP-CTFη. If so, the APP-CTFη levels would be much higher in human, guinea pig or macaque brain than in mouse or rat brain and thus reveal important species and developmental differences in APP processing. However, several facts indicate that the detection of the binding of APP antibodies to the 20 kDa band is partially non-specific:

(1) The binding of the Y188 and C1/6.1 to the 20 kDa band correlates both in molecular weight and intensity with a major band in a total protein stain. The total protein stain of this band is not likely to be due to staining of APP since APP was only detected with a low score in mass spectrometry analysis of this band. It should be noted, however, that APP is present in this band as well, albeit at low levels.
(2) The pattern of bands varied considerably depending on which APP antibody that was used. For example, the Y188 antibody binds proportionally more of the 20 kDa band than the APP-CTFα/β and APP-FL bands, despite the fact that the epitope of the antibodies should be present in all these fragments.
(3) Several other antibodies also detect the 20 kDa band although their expected molecular weight is not 20 kDa.
(4) It was not possible to specifically immunoprecipitate the 20 kDa band either with APP antibodies or with an MBP antibody even though other APP and MBP bands were efficiently immunoprecipitated.
(5) The 20 kDa band was proportionally more depleted than other APP fragments using a myelin removal protocol. Although this protocol is not designed for brain homogenates and we thus cannot exclude that proteins were non-specifically depleted, MBP was also depleted to a higher degree than APP-FL and APP-CTFα/β.

To conclude, these results indicate a non-specific binding of a multitude of antibodies to a 20 kDa band that also correlates to a band stained by a total protein stain. Regarding the proportional

differences in binding capacity of different APP antibodies to the 20 kDa band compared to other APP fragments, as well as the inability to immunoprecipitate the 20 kDa band, we cannot totally rule out that this is due to a conformational change in the proposed APP-CTFη but we find this explanation quite unlikely.

The large variation of intensity of the 20 kDa band (detected by APP antibodies and a total protein stain) between different human brain samples could possibly be due to variation in white matter content. This note, together with the selective depletion of the 20 kDa band using a myelin removal protocol and the fact that MBP was the top hit in the MS analysis of the band, speak for that the binding was due to myelin. Using immunohistochemistry of human brain sections, the Y188 antibody also stained axonal fibers, more strongly than the C1/6.1 antibody (Jordá-Siquer et al. unpublished results), in line with the more prominent detection of the 20 kDa band with the Y188 than with the C1/6.1 antibody, further indicating binding to myelin. In addition, a previous study has shown non-specific immunohistochemical labeling of IgMs to myelin in human brain sections (Perentes and Rubinstein, 1986).

We could not, however, specifically immunodeplete the 20 kDa band detected by the C1/6.1 antibody using an MBP antibody, suggesting that the detection of this band is due to non-specific binding to a component in the 20 kDa band rather than cross-reactivity of these antibodies to MBP. The fact that we could non-specifically immunodeplete the C1/6.1-reactive 20 kDa band using a control rabbit IgG, as well as the detection of this band by a multitude of antibodies, further supports this conclusion. The myelin-removal beads technology also builds on immunodepletion but we cannot conclude whether this depletion is specific or not.

Although the major 20 kDa band, which is most prominently stained band by the Y188 antibody, is most probably to a large degree non-specific, we still detected an APP peptide in this band using in-gel digestion and mass spectrometry. Although the score of this peptide was low, it showed a similar spectrum and identical retention time as a peptide from the in-gel-digested FL-APP band, indicating that an APP-derived fragment of approximately 20 kDa indeed exists. In line with this, Willem et al. (2015) could detect the APP-CTFη in wild-type but not in APP knock-out mice, which strongly indicate the presence of a specific APP fragment of approximately 20 kDa in mouse brain. The levels of APP-CTFη in mouse brain and N2a cells were generally low but were increased upon BACE1 inhibition (Willem et al., 2015). In line with this, using the C1/6.1 antibody, we could detect a weak band migrating slightly above the major 20 kDa band in human brain. The fact that Willem et al. (2015) can detect low levels of APP-CTFη also using the Y188 antibody, whereas we cannot, is most probably due to slight differences in the detection limit between our protocols. Thus, this upper band might be a specific APP band, but in human brain this signal is hidden by the major potentially non-specific 20 kDa band. This fact makes it difficult to interpret results intended to rule out differences in levels of this band in different species, developmental stages or between AD and control brain.

García-Ayllón et al. (2017) reported on a 25 kDa fragment, presumably corresponding to APP-CTFη with increased levels in cerebrospinal fluid (CSF) of AD and Down syndrome patients compared to healthy controls. Since we do not know whether the major band detected by the total protein stain is present in CSF, we cannot determine whether the detection of the APP fragment in CSF is specific. In contrast to our results, García-Ayllón et al. (2017) succeeded in immunoprecipitating the 25 kDa band using Y188 or A8717 antibodies, which supports that the antibody binding to the band they detect is specific.

In summary, our results strongly indicate non-specific binding to a 20 kDa band in human brain by a multitude of APP and other antibodies. We call for precaution when analyzing proteins of a similar size in human brain. However, APP is present in this 20 kDa band and there is good evidence that a specific APP band of a similar size is present in mouse brain and possibly also in CSF.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the regional ethical review board of Stockholm (Regionala etikprövningsnämnden i Stockholm). The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by the animal research ethical committee of southern Stockholm (Stockholm södra djurförsöksetiska nämnd) and Linköping ethical committee (Linköpings djurförsöksetiska nämnd).

AUTHOR CONTRIBUTIONS

JL, SF and HH planned the experiments. JL and HH performed the major part of the experiments. TK, TJ-S and MS performed the analysis of the different human brain samples. ES provided the human fetal tissue. MK performed the western blotting with Keap1 and GC antibodies. HH, JL, SF, LT, GB, ES and BW analyzed the data and contributed to the interpretation of the data. JL, SF and HH wrote the manuscript and all authors proof-read the manuscript.

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SUPPLEMENTARY MATERIAL

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Neuroinflammation and the Gut Microbiota: Possible Alternative Therapeutic Targets to Counteract Alzheimer's Disease?

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Alzheimer's disease (AD) is a complex, multi-factorial disease affecting various brain systems. This complexity implies that successful therapies must be directed against several core neuropathological targets rather than single ones. The scientific community has made great efforts to identify the right AD targets beside the historic amyloid-β (Aβ). Neuroinflammation is re-emerging as determinant in the neuropathological process of AD. A new theory, still in its infancy, highlights the role of gut microbiota (GM) in the control of brain development, but also in the onset and progression of neurodegenerative diseases. Bidirectional communication between the central and the enteric nervous systems, called gut-brain axes, is largely influenced by GM and the immune system is a potential key mediator of this interaction. Growing evidence points to the role of GM in the maturation and activation of host microglia and peripheral immune cells. Several recent studies have found abnormalities in GM (dysbiosis) in AD populations. These observations raise the intriguing question whether and how GM dysbiosis could contribute to AD development through action on the immune system and whether, in a therapeutic prospective, the development of strategies preserving a healthy GM might become a valuable approach to prevent AD. Here, we review the evidence from animal models and humans of the role of GM in neuroinflammation and AD.

Keywords: Alzhimer's disease, neuroinflammation, gut microbiota, immune cells, therapy

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INTRODUCTION

Alzheimer's disease (AD) remains the most spread form of dementia afflicting 45 million people worldwide and continuously challenging the scientific community in the hard task of identifying a therapy. AD pathogenesis starts around 15–20 years before the clinical symptoms become detectable. Within this time frame, the patient's brain accumulates multiple-system damages including synaptic and mitochondrial alterations, vessel damage, chronic neuroinflammation, cognitive dysfunctions and neuronal cell death. amyloid-β (Aβ) extracellular plaques and intracellular neurofibrillary tangles enriched of hyperphosphorylated Tau protein are the two main histological brain lesions. Aβ is still held to be the main culprit, especially in its soluble oligomeric form. Aβ oligomers are indeed considered as the most neurotoxic species and the best correlates of disease severity (Forloni et al., 2016). However, Aβ can no longer be considered the sole target because of the multiple failures in anti-Aβ trials (Panza et al., 2019). More likely, the complex pathological condition of AD conceivably calls for alternative targets and multi-target therapies.

Based on these considerations, this review aims to highlight two therapeutic targets, which are attracting much attention in the fight against AD: neuroinflammation and the gut microbiota (GM). The former has strongly re-emerged as crucial in the neuropathogenic process of AD, whereas the latter, though still in its infancy, is attracting interest as a promising new alternative target. Both systems intimately interact in physiology and pathology.

NEUROINFLAMMATION AND AD

A large body of evidence has accumulated in the last few years on the vital role of neuroinflammation in the pathogenetic process of AD. In physiological conditions glial cells are determinants in the regulation of brain development, neuronal activity and survival. Microglia patrol the brain microenvironment guaranteeing its defense from exogenous pathogens or endogenous dangers. In response to bacterial and viral infections or brain damage, microglia are rapidly activated and phagocytize pathogens, including Aβ, and damaged neurons. With elimination of harmful stimulus, neuroinflammation is resolved and microglia return to a resting state. In AD, neuroinflammation is chronic and resolution is not achieved. This implies that microglia constantly release pro-inflammatory cytokines, favoring neuronal cell death (Heneka et al., 2015). Indeed, many of the inflammatory mediators, such as pro-inflammatory cytokines, chemokines as well as factors of the complement system are produced locally and elevated in the brain of AD patients (Rogers et al., 1992; McGeer and McGeer, 2002). The most representative cytokines of AD are IL1β, TNFα and IL6, all up-regulated in AD tissues and prominently associated with AD lesions (Griffin et al., 1989; Dickson et al., 1993). It was recently demonstrated that neurodegeneration very likely involves astrocytes which, by taking on a microglia-induced A1 pro-inflammatory phenotype, would promote neuronal cell death, with TNFα as the most prominent mediator (Fiala and Veerhuis, 2010; Liddelow et al., 2017). In addition, activated microglia loses their phagocytic properties, thus reducing the degree of Aβ phagocytosis, and consequently promoting its accumulation (Krabbe et al., 2013). These findings are supported by the discovery of a relation between an increase in AD risk and mutations in genes encoding immune receptors such as TREM2, myeloid cell surface antigen CD33 and CR1 (Balducci and Forloni, 2018). This was compelling since they are all expressed on myeloid cells, thus demonstrating that alterations in microglial biology are linked to AD pathogenesis and an increased risk of its development. Of note, a series of transcriptomic and proteomic analysis of inflammatory cells might provide biomarkers for preclinical detection as well as insights on progression from MCI to AD condition (Fiala and Veerhuis, 2010; Wes et al., 2016; Rangaraju et al., 2018; Bonham et al., 2019).

A close relation has also been described between primed microglia and cognitive dysfunction. In healthy tissue, microglia have a ramified morphology and the prolongations constantly survey synaptic activity. Phagocytic microglia have an important role in synaptic pruning and refinement in the developing nervous system (Weinhard et al., 2018). The most intriguing mechanism explaining memory dysfunction in AD implies that Aβ oligomers, the most toxic species, foster microglial activation which then excessively engulf and eliminate synapses through C1q and C3 complement factors (Hong et al., 2016). We too have reported that Aβ oligomer-mediated memory impairment is closely associated with glial activation (Balducci et al., 2017).

New evidence is now shedding light on a dangerous dialogue between central immune cells and the gut, potentially leading to AD.

MICROBIOTA-GUT-BRAIN AXES

Constant communication between the central and enteric nervous systems is required to maintain body homeostasis. This complex interplay, the "Gut-brain-axis," is mediated by neural, endocrine and immune signals (Carabotti et al., 2015). GM, the dense population of bacteria, viruses, fungi, and protozoa inhabiting the human gut, is now recognized as an important part of this interaction and the new term Microbiota-Gut-Brain axis, has been introduced. Recent progress in high-throughput analyses has permitted to study more in-depth the microbial composition and appreciate its complexity (Rooks and Garrett, 2016).

Every person has a distinct and widely variable GM, with some common features emerging only at higher level of organization (Tremaroli and Bäckhed, 2012). This dynamic system is subject to re-modeling in response to aging, environmental perturbations, changes in lifestyle and diet and it is therefore prone to maladaptive modifications (Santoro et al., 2018). Substantial shifts in human GM composition have been observed in CNS disorders such as depression, anxiety, autism (Finegold et al., 2012; Liang et al., 2018) and neurodegeneration (Fung et al., 2017).

GUT MICROBIOTA IN CNS PHYSIOLOGY

Germ free (GF) and antibiotic-treated rodents provided the necessary tool to study the impact of intestinal microbes on CNS development and physiology.

A pioneering study used GF mice, which are generated and raised in sterile conditions, to investigate the influence of GM on hypothalamic-pituitary-adrenal (HPA) response to stress. The HPA response was significantly higher in GF relative to mice raised with normal GM. The introduction of the complex microbiota at an early stage (up to 9 weeks old), could partially reverse this enhanced HPA response to stress. GF mice also had lower brain-derived neurotrophic factor (BDNF) expression, which is important for neuronal growth and synaptic plasticity, in the cortex and hippocampus (Sudo et al., 2004).

Subsequent studies showed that the absence of complex microbiota has profound effects on adult behavior and CNS development and that the timing and duration of exposure to microorganisms are critical. GF condition altered spatial, working and reference memory, increased motor activity and reduced anxiety (Diaz Heijtz et al., 2011; Gareau et al., 2011). It also impaired hippocampal development

and morphology, increased dorsal hippocampal neurogenesis and BBB permeability, and significantly altered levels of noradrenaline, dopamine and serotonin (Braniste et al., 2014; Luczynski et al., 2016; Sharon et al., 2016; Lin et al., 2018).

SYMBIOTIC RELATIONSHIP BETWEEN THE IMMUNE SYSTEM AND GM

The microbial ecosystem co-evolved with our immune system over millennia and host-specific antimicrobial peptides and pattern recognition receptors evolved not only to protect against pathogens but also to promote resident beneficial microbes (Bosch, 2014). The immune system closely controls the GM composition and distribution (Sigal and Meyer, 2016), while the microbial symbionts regulate immune system maturation and function (Belkaid and Hand, 2014). Commensal GM can profoundly affect both innate and adaptive immune systems. Several studies have confirmed the interaction between microbiota and various immune cell populations: peripheral T cells, myeloid cells and mast cells (Round and Mazmanian, 2009; Kamada et al., 2013; Forsythe, 2016).

Khosravi et al. (2014) provided evidence that GM influences the development of the immune system by regulating hematopoiesis of primary immune cells. They showed that GF mice have lower proportions and less differentiation potential of myeloid cell progenitors of both yolk sac and bone marrow origin. This could help explain the widespread effects of GM on the immune system, microglia included.

GM-MICROGLIA INTERACTION

Mounting evidence from animal studies demonstrates that GM regulates microglial maturation and function. Microglia from GF or antibiotic-treated mice had an immature profile and impaired immune response (Erny et al., 2015). The absence of gut flora altered microglia mRNA profiles and downregulated several microglial genes involved in cell activation, pathogen recognition and host defense. The microglia transcription and survival factors SFPI1 and CSF1R, normally downregulated in mature adult microglia, were upregulated in GF mice (Kierdorf and Prinz, 2013). Matcovitch-Natan et al. (2016) examined the transcriptional profiles of different microglial development stages, showing that the genes related to the adult phase of microglial maturation and immune response are dysregulated in GF mice.

Products derived from bacterial metabolism such as short-chain fatty acids (SCFAs) were identified as key mediators of GM-microglia interaction. These molecules are able to translocate from colonic mucosa to systemic circulation (Schönfeld and Wojtczak, 2016), cross the BBB and affect CNS function (Borre et al., 2014). A SCFA supplement in drinking water of GF mice for 4 weeks restored many aspects of the immature microglial morphology, re-established microglial density and normalized CSF1R surface expression (Erny et al., 2015).

From the therapeutic perspective, it is important to highlight that the GM-microglia interaction is highly dynamic as many of the defects observed in microglia of GF mice could be partially restored by recolonization with conventional GM or SCFA supplementation.

GM ALTERATIONS IN AD

An association between gut dysbiosis and neurodegeneration is mostly supported by pre-clinical studies, while the clinical data are still limited. The most consistent clinical evidence of deviation from the healthy microbial composition in a neurodegenerative condition derives from studies of Parkinson's disease (PD) patients (Keshavarzian et al., 2015; Scheperjans et al., 2015; Hopfner et al., 2017). Only few studies have investigated GM populations in AD patients. Microbial diversity was reduced in AD patient feces compared to age- and sex-matched controls. At the phylum level, there was a decrease in the numbers of Firmicutes and Actinobacteria and an increase in abundance of Bacteroidetes. The relative abundance of bacterial genera correlated with the levels of cerebrospinal fluid biomarkers of AD (Vogt et al., 2017). A recent study examined the link between selected bacterial taxa and brain amyloidosis in patients with cognitive impairment. Amyloid deposition was associated with an increased stool content of the pro-inflammatory taxa *Escherichia/Shigella* and low anti-inflammatory taxon *Eubacterium rectale*. These changes correlated well with a peripheral inflammatory state (Cattaneo et al., 2017). A few human studies have also linked dysbioses of oro-nasal cavity microbiota with neurodegeneration (Kamer et al., 2008; Cockburn et al., 2012).

In AD animal models was found a significant shift in the composition of GM, and microbial manipulations could affect disease outcomes as summarized in **Figure 1** (Brandscheid et al., 2017; Harach et al., 2017; Shen et al., 2017). A combination of broad-spectrum antibiotics or GF condition in AD mice reduced Aβ plaques and attenuated plaque-surrounding glial reactivity and the levels of circulating cytokines and chemokines (Minter et al., 2016). Conversely, re-introduction of conventionally raised AD mice gut flora in GF mice increased Aβ pathology (Harach et al., 2017).

HOW CHANGES IN MICROBIAL COMPOSITION COULD BE RELEVANT TO AD

A plethora of hypotheses have been advanced to explain possible mechanisms linking GM alteration to neurodegenerative processes, many of them involving neuroinflammation as a common driving force.

The GM is a major source of the bacterial surface lipopolysaccharide (LPS) and other pro-inflammatory molecules and endotoxins. AD patient's brains contain more frequently pathogenic bacteria and LPS compared to controls (Itzhaki et al., 2004; Fox et al., 2019). LPS was found in the hippocampus and cortex and at higher concentrations in plasma than in healthy controls (Zhao et al., 2017; Kowalski and Mulak, 2019). In addition, LPS co-localizes with $A\beta_{1-40/42}$ in amyloid plaques and

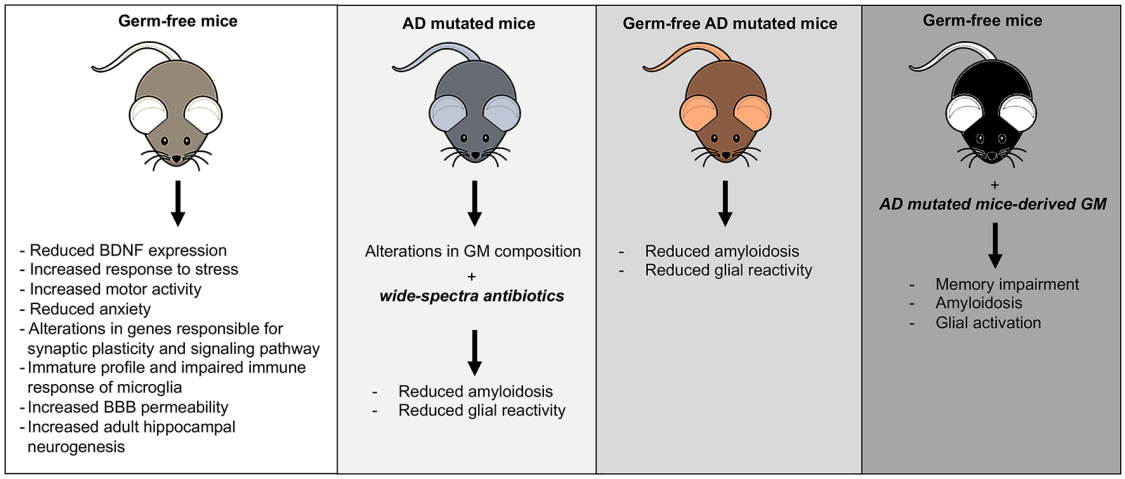

FIGURE 1 | Mouse models of gut microbiota (GM) manipulation. The figure illustrates the typical mouse phenotypes resulting from various GM manipulations.

around blood vessels (Zhan et al., 2016). Bacterial LPS can bind to microglial receptors (TLR2, TLR4 and CD14) and trigger an inflammatory response. In a recent study LPS was able to strongly activate the NF-kB (p50/p60) complex, an important initiator for neuroinflammatory processes occurring in AD (Lukiw, 2016; Lin et al., 2018).

The most common form of AD typically affects elderly people and aging is associated with significant changes in GM composition. These age-related changes are mainly due to modifications in life-style, diet and a chronic low-grade inflammatory state called "inflammaging" (Santoro et al., 2018). GM in the elderly is reduced in diversity and stability and is less resistant to environmental perturbations such as stress and antibiotics (Biagi et al., 2010). Therefore, it is more vulnerable to the invasion of opportunistic species and to clinically important changes in microbial composition. It has been shown that implantation of aged GM in young GF mice induced inflammaging. In addition, the aged GM promoted small intestine inflammation in implanted GF mice and weakened the intestinal barrier making possible the infiltration of inflammatory bacterial components into the circulation. An increase in systemic T cell activation was also observed (Fransen et al., 2017). In humans, there is an age-related decline in immune system function (Fulop et al., 2018), that could make gut dysbiosis more relevant in triggering low-grade systemic inflammation.

A recent study in mice lacking PINK1$^{-/-}$, which has a key role in adaptive immunity by repressing presentation of mitochondrial antigens, suggests that specific deficits in the immune system function could make intestinal infection a risk factor for neurodegeneration. In these mice, gut infection triggered an autoimmune mechanism involving the mitochondria specific CD8^{+} cells, which are toxic for both peripheral and central neurons. These events led to the degeneration of dopaminergic neurons and motor symptoms typical of PD (Matheoud et al., 2019).

In animal models, dysbiosis increases gut permeability and promotes inflammation and macrophage dysfunction (Thevaranjan et al., 2018). There is evidence of BBB damage and accumulation of blood-derived products in AD brains (Kowalski and Mulak, 2019). The passage of harmful agents from the gut to the brain is still not adequately explained, but compromised the integrity of epithelial barriers might play a role (Sochocka et al., 2019).

Several gut bacterial species such as *E. coli, Salmonella* and *Citrobacter* produce Aβ (O'Toole et al., 2000; Zhou et al., 2012). Amyloids are common structural components of the extracellular matrix in which bacterial cells stay close to each other. Exposure to microbial amyloids might trigger amyloid misfolding in the brain. Increasing evidence supports the idea that the formation and propagation of Aβ seeds is a prion-like mechanism (Walker et al., 2016). However, it is still not clear how bacterial amyloids could gain access to the brain. One possibility is uptake through specialized epithelial cells of the mucosa-associated lymphoid tissues, then physical interaction between enteric nervous system fibers and parasympathetic neurons of vagusnerve where they could reach the CNS *via* retrograde axonal transport (Kujala et al., 2011; Friedland and Chapman, 2017). The key study supporting the hypotheses that the spread of misfolded proteins from the gut to the brain could occur *via* the vagus nerve comes from the context of α-synuclein propagation in PD (Ulusoy et al., 2015; Breen et al., 2019; Santos et al., 2019).

Soscia et al., in 2010 noticed some interesting similarities in biophysical properties of Aβ and a family of biomolecules called "antimicrobial peptides" (AMPs). AMPs are potent broad spectrum antibiotics and modulators of immune system in the brain and other immune-privileged tissues. Dysregulation of these molecules can lead to neurotoxicity and chronic inflammation (Soscia et al., 2010). This study, followed by few others, confirmed the antimicrobial properties of Aβ and proposed its possible physiological role in brain's innate immune

response to microbes. They advanced the hypotheses that brain infiltration of gut bacteria or their components might stimulate Aβ production and deposition (White et al., 2014; Kumar et al., 2016; Eimer et al., 2018).

Figure 2 summarizes the possible pathological events increasing the risk of AD as a consequence of GM dysbiosis.

THERAPEUTIC POTENTIAL OF MICROBIOTA IN NEUROINFLAMMATION AND AD

At present, the existing data on the mechanisms linking GM to neurodegeneration, mostly based on animal studies, are still not sufficient to provide directions for the development of GM-based therapeutic strategies.

Attempts to use probiotics were made mostly in animal models, although there is no evidence that this approach can lead to long-term alterations in GM composition (Akbari et al., 2016; Musa et al., 2017; Plaza-Díaz et al., 2017; Kowalski and Mulak, 2019). In one clinical trial, PD patients were given antibiotics to treat small intestinal bacterial overgrowth, with improvements in gastrointestinal symptoms and motor fluctuations (Fasano et al., 2013). Another strategy for modifying GM composition is fecal transplant, already successful for treating infections of *Clostridium difficile* (Xu et al., 2015), but there are only limited attempts to use it outside gastrointestinal diseases (Evrensel and Ceylan, 2016).

Pre-clinical evidence suggests that microbial metabolism products such as SCFAs could be signaling molecules used by gut microbes to act on the CNS (Erny et al., 2015). Their interaction with the immune system and anti-inflammatory properties make them interesting therapeutic candidates for neurodegenerative disorders. Several strategies for delivery of SCFAs are summarized in Gill et al. (2018).

To date, dietary and lifestyle modifications are the most effective way to produce long-term changes in GM. Some healthy dietary patterns such as Mediterranean, Japanese or FINGER (Finish Geriatric intervention study) diet can positively influence the rates of cognitive decline (Pistollato et al., 2018; Wahl et al., 2019) and also induce significant changes in GM composition.

Among the most significant examples of nutritional intervention with neuroprotective and age-delaying potential is caloric restriction (CR) which can be obtained by reducing the daily caloric intake or by intermittent fasting (Fontana, 2018). CR delays the onset of neurodegeneration in rodents and prevents several hallmarks of brain aging in non-human primates and humans (Pani, 2015). The possible underlying mechanisms are numerous and reviewed in Zullo et al. (2018). Briefly, CR increases levels of neuroprotective factors while decreasing oxidative stress, inflammation, and activity of pro-apoptotic factors (Maalouf et al., 2009). At a molecular level, CR acts on nutrient-sensing pathways through different mechanisms. Notably, it can activate the SIRT1 enzyme (a member of

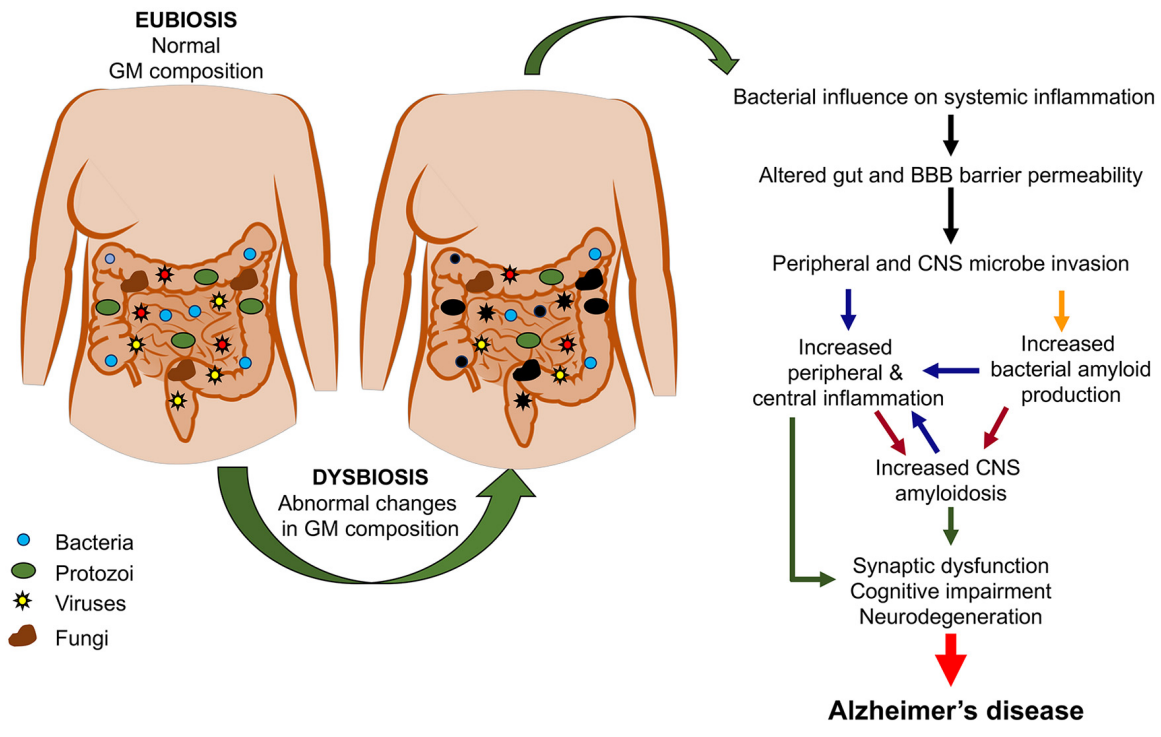

FIGURE 2 | From gut dysbiosis to Alzheimer's disease (AD). The figure depicts the possible pathological events associated with gut dysbiosis leading to both peripheral and central pathological events which would increase the risk of AD.

the sirtuin family that regulates gene expression) which downregulates the mammalian target of rapamycin (mTOR), thus suppressing NF-kB-dependent neuroinflammation and inducing autophagy as neuronal self-defense mechanism (Maalouf et al., 2009; Shirooie et al., 2018).

Fasting can induce rapid adaptations in GM composition favoring growth of beneficial and anti-inflammatory microbial phylotypes and lead to significant changes in the SCFA biosynthesis (Zhang et al., 2013; Tanca et al., 2018). GM interacts with several mechanisms of metabolic response to nutrient deprivation. For instance, SIRT1 activation regulates, the GM resulting in lower intestinal inflammation during aging (Wellman et al., 2017). Some substances such as resveratrol, a natural phenol found in grapes and red wine can activate, in alternative to CR, the sirtuine pathway (Kelly, 2010) and positively influence GM. The interaction between resveratrol and GM is bidirectional as gut microbes affect also resveratrol bioavailability (Hu et al., 2019).

CR shares some common metabolic effects with the ketogenic diet (KD) which is high in fat, moderate in proteins and very low in carbohydrate. KD is already used to treat patients with drug resistant epilepsy (Stafstrom and Rho, 2012) and in a few studies have revealed the potential to reduce symptoms of neurodegeneration (Wodarek, 2019). Remarkably, a study using mouse models of drug-resistant epilepsy showed that the KD anti-seizure properties were mediated by microbiota. Depletion of GM with a high dose antibiotic treatment abolished the KD beneficial effects (Olson et al., 2018).

Among other modifiable factors, exercise is considered to promote diversity and enhance beneficial metabolic functions of microbial species in the gut and improve cognitive performance (Ticinesi et al., 2019).

A preventive therapy based on changes in diet and levels of physical activity seems to be the most promising approach for delaying cognitive decline and improving metabolic, neuroendocrine and vascular abnormalities that often precede and likely significantly contribute to cognitive deterioration (Sohn, 2018). A successful preventive strategy must recognize that GM is an important mediator of the effects of diet and exercise on cognitive decline, aging and inflammation. However, additional studies are needed to understand if dietary interventions, such as CR, could be safely recommended to elderly population, which is already at risk of malnutrition and sarcopenia (Sieber, 2019).

One of the present difficulties in tailoring a GM-based therapy is its inter-individual variability in composition and metabolism, but with the rapid advancement in research and diagnostic technologies a new type of personalized medicine might well become possible.

GM AND AD DEVELOPMENT: MAIN EXPERIMENTAL LIMITATIONS

Although the study of microbiota-gut-brain axis in recent years has flourished, there are still many obstacles. For instance, a lack of well-defined methodological standards make it hard to compare studies and numerous confounding factors including diet, drugs and concomitant pathologies must be carefully considered in the analysis (Marizzoni et al., 2017). One of the key questions that need to be addressed is whether changes in GM are cause or secondary effects of the disease. At present, GF and antibiotic-treated rodents remain the best available tools for transitioning from observational studies to understanding the cause-effect directionality. However, the translational value of studies of human microbiota in rodent models is limited by obvious differences in diet and microbiota composition.

AUTHOR CONTRIBUTIONS

MC and CB wrote the manuscript. GF revised the manuscript.

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REVIEW
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Sex and Gender Driven Modifiers of Alzheimer's: The Role for Estrogenic Control Across Age, Race, Medical, and Lifestyle Risks

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Research indicates that after advanced age, the major risk factor for late-onset Alzheimer's disease (AD) is female sex. Out of every three AD patients, two are females with postmenopausal women contributing to over 60% of all those affected. Sex- and gender-related differences in AD have been widely researched and several emerging lines of evidence point to different vulnerabilities that contribute to dementia risk. Among those being considered, it is becoming widely accepted that gonadal steroids contribute to the gender disparity in AD, as evidenced by the "estrogen hypothesis." This posits that sex hormones, 17β-estradiol in particular, exert a neuroprotective effect by shielding females' brains from disease development. This theory is further supported by recent findings that the onset of menopause is associated with the emergence of AD-related brain changes in women in contrast to men of the same age. In this review, we discuss genetic, medical, societal, and lifestyle risk factors known to increase AD risk differently between the genders, with a focus on the role of hormonal changes, particularly declines in 17β-estradiol during the menopause transition (MT) as key underlying mechanisms.

Keywords: Alzheimer's disease, estrogen hypothesis, sex differences, gender differences, menopause transition, risk factors

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INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disease marked by impairments in memory, attention, language, and daily living activities (Alzheimer's Association, 2017). While AD currently impacts 5.7 million Americans regardless of ethnic and cultural backgrounds (Alzheimer's Association, 2017), the prevalence is expected to triple by 2050, with nearly 14 million patients affected. Similar trends have been reported worldwide with a projected 130 million patients in the next 30 years.

Alzheimer's disease is an extremely debilitating condition currently falling within the top 10 causes of death across the world. This causes a severe fiscal burden on health services since AD is an extremely financially costly neurological disease to manage (Nichols et al., 2019). Addressing the

economic and social costs of AD is increasing in urgency as the Baby Boomer generation ages and life expectancy increases. Recent studies estimate that, from 2010 to 2050, annual costs will increase from $307 billion to $1.5 trillion in the United States alone (Zissimopoulos et al., 2015). Medical advances that delay disease onset for 5 years or longer would result in a 41% lower prevalence and 40% lower cost of AD in 2050 (Zissimopoulos et al., 2015).

To date, there has been a lack of therapeutics to prevent, delay, or reverse late-onset AD, resulting in a host of unsuccessful clinical trials. Research efforts over the past decade have prioritized therapeutic strategies that aim to remove beta-amyloid (Aβ) and tau pathology or prevent their accumulation, with limited success (Andrieu et al., 2015). Therefore, there exists an urgent and unmet need to develop novel strategies to prevent dementia, or at the very least delay its onset, or slow down progression. Several reasons underlie these past failures; among the most far-reaching are the stage at which therapeutic interventions are initiated, and the sex differences in the underlying mechanisms leading to AD.

It has become widely accepted that the pathophysiological mechanisms of AD begin decades before the emergence of clinically detectable symptoms and contribute to a 15–20 year's prodromal or "preclinical" disease stage starting in midlife (Sperling et al., 2014). Failure to develop successful disease-modifying therapies may be because the majority of interventions have been tested in cohorts with clinically manifest disease and thus substantial synaptic and neuronal damage. Initiating therapies during the preclinical phase of AD will likely yield greater chances of success, a recognition that has effectively paved the way for primary and secondary AD prevention trials (Andrieu et al., 2015).

There is also emerging evidence that several medical, environmental, and lifestyle risk factors that lead to AD development are modifiable (Livingston et al., 2017). At least one out of three AD dementia cases can be linked to medical factors such as cardiovascular conditions, obesity, diabetes, and lifestyle factors such as physical activity, diet, social engagement, and educational attainment (Norton et al., 2014). Until disease-modifying treatment becomes available, risk reduction interventions could still drastically reduce the future burden of AD at the population level (Isaacson et al., 2018).

In this context, it is being widely accepted that many of the above AD risk factors show gender effects, with female sex being more severely impacted (Ferretti et al., 2018; Nebel et al., 2018; Scheyer et al., 2018). It has long been known that, after advanced age, female sex is the major risk factor for AD (Farrer et al., 1997). Currently, two-thirds of AD patients are females. Postmenopausal women comprise over 60% of those patients (Brookmeyer et al., 1998). Increasing effort has thus been devoted to identifying sex-specific differences in disease etiology, manifestation, and progression as a crucial step toward gender-based disease prevention. Among putative biological mechanisms, it is becoming widely accepted that gonadal steroids contribute to the gender disparity in AD, as evidenced by the "estrogen hypothesis" presented herein. This posits that female sex hormones, 17β-estradiol in particular, exert a neuroprotective effect by buffering females' brains against disease development. Hormonal changes in the years leading up to and after menopause are linked to the emergence of AD-related brain changes in females in contrast to males of the same age. In this review, we provide a comprehensive review of genetic, medical, societal, and lifestyle risk factors known to increase AD risk differently between the genders, with a focus on the role of gonadal hormones as key underlying mechanisms.

THE ESTROGEN HYPOTHESIS

It is has long been proposed that gonadal steroids contribute to gender differences in AD. Several reproductive hormones and their interactions may be implicated, including estrogen, progesterone, luteinizing hormone, and follicle stimulating hormones. All these so-called female hormones naturally fluctuate over endogenous hormonal cycles. Nonetheless, this review will focus primarily on estrogen since considerable evidence from molecular, animal, and clinical studies indicates that, of all gonadal hormones, estrogen may be particularly involved in the pathophysiology of AD-dementia in women. The "estrogen hypothesis" postulates that estrogen plays a protective role against AD-dementia, while that estrogen dysfunction seems to exacerbate, or perhaps precipitate the AD process in women.

Even though it is present in both sexes, estrogen is often considered the primary female sex hormone. Reference to estrogen broadly refers to numerous compounds such as estrone (E_1), estradiol (E_2), and estriol (E_3). The primary circulating estrogen during a woman's reproductive years is 17β-estradiol, which is also the strongest form. For the purposes of this review, estrogen refers to 17β-estradiol, the endogenous form. 17β-estradiol plays a role in the formation of secondary sex characteristics in females and reproduction in males, and has peripheral effects in the liver and bone in both sexes (Cui et al., 2013). While it is primarily central to the ovaries for menstrual cycle coordination in women, it is also made by non-endocrine tissues, such as fat, breasts, and the brain (McEwen et al., 1997).

Estrogen affects several areas of the brain, thereby influencing cognitive function, affect, and behavior (Fink et al., 1996; Dumitriu et al., 2010; Brinton et al., 2015). Several lines of research have demonstrated that estrogen is a vital signaling molecule within the brain (Brinton, 2008; Rettberg et al., 2014). It can not only go through the blood–brain barrier but the brain also produces estrogen endogenously from cholesterol (Balthazart and Ball, 2006; Rettberg et al., 2014). Estrogen utilizes a network of receptors and signaling pathways to initiate and regulate molecular and genomic responses required for survival at the level of the cells, genes, organs, and ultimately, the whole body (**Figure 1**; Rettberg et al., 2014). Estrogen receptors (ERs) are expressed by both sexes and are found on both neurons and glial cells throughout the brain (Rettberg et al., 2014). These receptors are conserved evolutionarily, with homologs present in all vertebrates. There are three types of ERs that have been discovered, to date: estrogen receptor 1 (ESR1 or ERα), estrogen receptor 2 (ESR2 or ERβ), and G-protein coupled estrogen

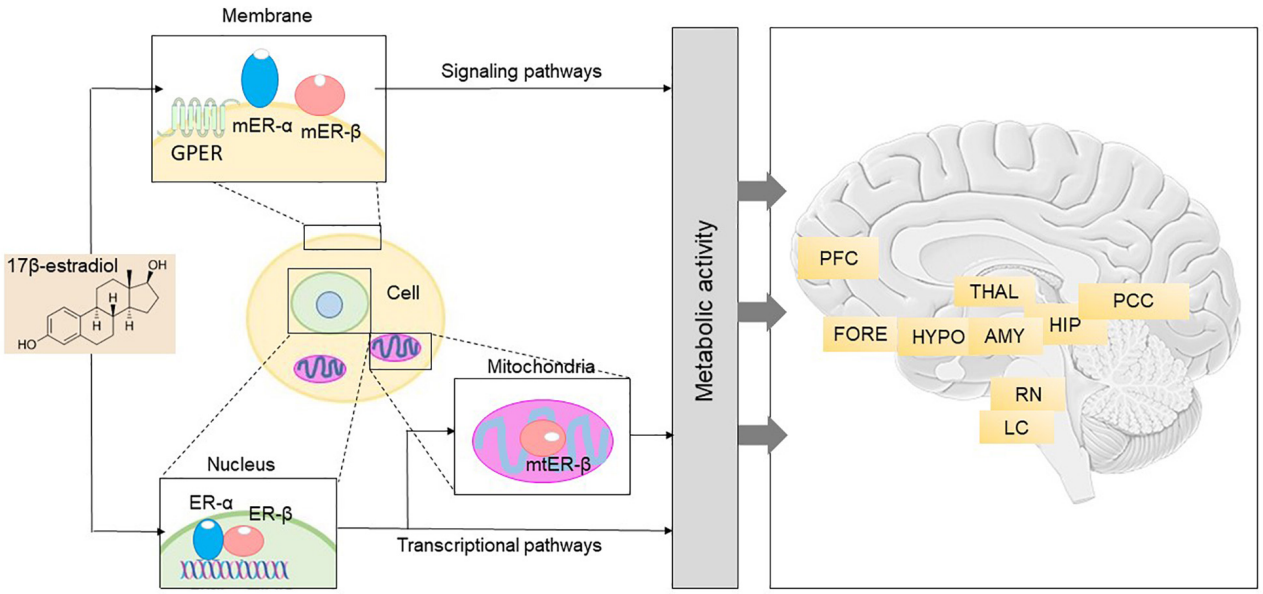

FIGURE 1 | Brain 17β-estradiol receptor network and anatomical distribution (Adapted with permission from Brinton et al., 2015). Right: 17β-estradiol receptor network in the brain includes different pathways. The binding of estrogen and consequent activation of the membrane and trans-membrane receptors, mER-α, mER-β, and GPER, contributes to initiation of signaling networks that mediate early and intermediate gene expression response. Binding of estrogen to ER-α and ER-β, the nuclear estrogen receptors, leads to initiation of transcriptional pathways that also regulate late response gene expression. Activation and translocation of ER-β to the mitochondria has been implicated in expression of mitochondrial genes. Furthermore, estrogen can modulate transcriptional gene expression via epigenetic regulation. This integrated network of receptors enables coordination of a broad spectrum of cellular elements, which ultimately results in generation of energy to fuel neurological function. ER, estrogen receptor; GPER, G-protein coupled estrogen receptor 1; mER, membrane estrogen receptor; mtER, mitochondrial estrogen receptor. Left: Anatomical basis for the neurological symptoms that can emerge during the menopause transition. Nuclear, membrane-associated, and mitochondrial estrogen receptors are distributed within each of the neural circuits depicted and can be present in both neurons and glial cells. Dysregulation of estrogen signaling and transcriptional pathways, either through changes in estrogen concentration or through modifications of estrogen receptor activity, impacts neurological function in those areas. AMY, amygdala; FORE, basal forebrain; HIP, hippocampus; HYPO, hypothalamus; LC, locus coeruleus; PCC, posterior cingulate cortex; PFC, prefrontal cortex; RN, raphe nucleus; THAL, thalamus.

receptor 1 (GPER) (Brinton et al., 2015). Binding of estrogen to these receptors activates several signaling pathways and cellular processes via both genomic and non-genomic processes (Brinton et al., 2015).

Of importance to the brain aging process, estrogen has known neuroprotective properties through its effects on spinogenesis, protecting the brain from age-related and toxic insults. Research using female rats in the early 1990s demonstrated that the density of dendritic spines on the CA1 region neurons of the hippocampus shifts over the ovarian cycle period (Gould et al., 1990) and that surgical oophorectomy, the removal of one or both ovaries, contributes to a 30% loss in spine density that can be recovered neurons by estrogen replacement (Woolley et al., 1990). This estrogen-led spinogenesis is followed by an equal increase in synapses (Woolley and McEwen, 1992) pointing to potential integration of the new spines into the hippocampal network.

Estrogen is also fundamental in metabolic regulation of the brain and body (Brinton et al., 2015). For instance, it regulates glucose transport, aerobic glycolysis, and mitochondrial function to generate ATP in the brain (Rettberg et al., 2014). In animal models, oophorectomy causes a significant reduction in multiple brain glucose transporters, including GLUT-1, GLUT-3, and GLUT-4 (Brinton, 2009). Loss of ovarian hormones with reproductive aging leads to a significant reduction in brain glucose activity, which could be attributed to decreased neuronal glucose transporter expression, compromised hexokinase activity, inactivation of the pyruvate dehydrogenase complex (PDC), and eventually a functionally significant decrease in mitochondrial bioenergetic function (Ding et al., 2013; Rettberg et al., 2014). In addition to facilitating glucose transport, estrogen also promotes neuronal aerobic glycolysis and potentiates mitochondrial bioenergetics through its positive effects on pyruvate dehydrogenase (PDH), aconitase, and ATP synthase (Nilsen et al., 2007; Rettberg et al., 2014).

Estrogen has also been shown to protect DNA against damage induced by hydrogen peroxide (H_2O_2) and arachidonic acid by increasing expression of a multitude of antioxidant enzymes, such as glutaredoxin, peroxiredoxin 5, and MnSOD (Nilsen et al., 2007; Rettberg et al., 2014). This estrogen-induced increase in antioxidants subsequently leads to a decrease in free radicals and oxidative damage to mitochondrial DNA and is potentially thought to contribute to the longer life span of women compared to men (Vina et al., 2006).

Overall, these studies highlight the role of estrogen in brain aging and neurodegenerative diseases such as AD. More research is warranted to understand the effect of aging on brain estrogen

activity, especially in the context of ERα and ERβ expression and signaling. So far, data suggest that in different parts of the brain, decreased ERα responsiveness may mediate cognitive decline and dementia risk (Yaffe et al., 2009). Although ERβ is at least partially receptive to E_2 during aging, it may be unable to compensate for the lack of ERα (Foster, 2012). With aging, there is also an increase in particular ERα splice variants in some parts of the brain, especially the hippocampus, that cause most of the available ERα to be non-functional (Ishunina et al., 2007). Interestingly, research has shown that elderly women are more likely to have greater expression of ERα splice variants than elderly men (Foster, 2012; Rettberg et al., 2014). In addition to splice variants, there are numerous ERα polymorphisms that increase AD risk specifically in women, particularly when linked to the APOE ε4 allele (Ryan et al., 2014; Brinton, 2017) which is a major AD genetic risk factor (discussed below).

Further evidence for the "estrogen hypothesis" comes from studies that have implicated the menopause transition (MT) with the emergence of AD-related brain changes in women at risk for developing AD (Brinton et al., 2015). The MT is associated with neurological symptoms such as disturbances of estrogen-regulated thermoregulation, sleep, onset of depression, and cognitive changes and ultimately results in reproductive senescence (Brinton et al., 2015; **Figure 1**). In the brain, ERs are widely found in the hypothalamic preoptic nucleus which serves as the primary thermoregulatory center; the suprachiasmatic nucleus of the hypothalamus which plays a central role in sleep and circadian rhythms regulation; the 5-hydroxy-tryptaminergic neurons of the raphe nucleus involved in affect and mood; and neurons in the locus coeruleus responsible for arousal and anxiety (McEwen et al., 1997; Brinton, 2017). In brain regions that are important for thinking, learning, and memory, ERs are present in the prefrontal cortex, medial temporal regions such as hippocampus and amygdala, and in the posterior cingulate and retrosplenial cortex (Shughrue et al., 1997; McEwen et al., 2012). It has been proposed that during menopause, decline in circulating estrogen is coincident with decline in brain bioenergetics and shift toward a metabolically compromised phenotype in these brain regions (Rettberg et al., 2014). Inadequate or absent compensatory bioenergetic adaptations to lack of estrogenic activation would then trigger not only the signature symptoms of menopause (hot flashes, night sweats), insomnia, and depressive mood symptoms, but also cognitive changes, thereby increasing risk of late-onset AD in postmenopausal women (Zhao et al., 2016; Bacon et al., 2019).

As later described in more detail, epidemiological data found an increased risk of dementia in women who underwent either unilateral or bilateral oophorectomy (surgical removal of the ovaries) before the onset of natural menopause (Rocca et al., 2007). These findings have been confirmed and extended to include hysterectomy with and without oophorectomy (Phung et al., 2010). Additionally, brain imaging studies of women undergoing natural menopause provided evidence that the MT is related to a greater risk for AD-brain changes in middle-aged peri- and postmenopausal women compared to men of similar age (Mosconi et al., 2017b, 2018). Further, the MT leads to an increased risk of depression, cardiovascular disease (CVD), type 2 diabetes mellitus (T2DM), and metabolic syndrome (MetS) in women (Pucci et al., 2017), as well as a compromised response to head injuries – all of which serve as AD risk factors (Biessels et al., 2006; Livingston et al., 2017). Women exhibit increased vulnerability to a variety of environmental and lifestyle AD risk factors like physical inactivity, an imbalanced diet, disrupted sleep, and chronic stress. These findings highlight the higher susceptibility of women to AD and propose a potential window of opportunity for the implementation of AD risk reduction strategies.

In the end, several lines of research provide support to the hypothesis that repeated hormone influxes in women confer protection against brain aging, while the onset of menopause may exacerbate an existing AD predisposition (Paganini-Hill and Henderson, 1994; Rocca et al., 2007, 2014; Fisher et al., 2018). An increasing number of studies have investigated estrogen therapy to potentially treat AD, as well as for CVD risk reduction in women (Mulnard et al., 2000; Resnick and Henderson, 2002; Maki, 2013). As reviewed below, earlier studies generally showed lack of benefits and even a potential harmful effect, whereas recent re-examinations indicate that the efficacy of estrogen in sustaining neurological health and function depends on several key factors, such as the time of initiation of estrogen therapy, the functioning of the brain at the time of therapy initiation, and the forms of hormones used (Brinton, 2004). Additionally, both pharmacological and non-pharmacological therapies aimed at supporting hormonal levels in aging women may help attenuate the impact of modifiable AD risk factors on the brain.

The aim of this review is to offer an updated review of the literature with respect to female-specific risk factors for AD (summarized in **Table 1**), and to put forth the estrogen hypothesis as a unifying mechanism of estrogen action on pre-existing and environmental risk. As previously discussed by Nebel et al. (2018), sex points to differences in biology such as chromosomal or hormonal factors, whereas gender refers to differences in the impact of psychosocial, cultural, and environmental influences on biological factors between men and women. Both sex- and gender-related risk factors are included below.

Female Sex

As mentioned above, female sex is a major risk factor for late-onset AD (Farrer et al., 1997). Approximately two-thirds of the more than 5 million Americans affected with AD are women and two-thirds of the more than 15 million Americans caring for someone with AD are women. As such, women find themselves at the epicenter of the impending AD epidemic. As grave a concern as breast cancer is to women's health, women in their 1960s are almost two times more likely to develop AD over the rest of their lives as they are to develop breast cancer (Alzheimer's Association, 2017). A woman in her 1960s has an estimated lifetime risk of one in six for developing Alzheimer's whereas the risk is 1 in 11 for a man of the same age (Alzheimer's Association, 2017).

TABLE 1 | Sex- and gender-related AD risk factors.

		Risk factor	Effect on AD risk
Sex differences	Genetic risks	APOE epsilon 4 allele	F > M
		Race (black, hispanic)	F > M
	Medical risks	Cardiovascular disease: Microvascular pathology (e.g., coronary microvascular obstruction and endothelial inflammation) Myocardial infarction Stroke (aneurysms)	F > M F > M after menopause F > M after menopause
		Type 2 diabetes; insulin resistance; prediabetes	F > M after menopause
		Depression	F > M after menopause
		Traumatic brain injury; concussions	F > M
		Chronic inflammation	F > M
		Systemic infection	F > M
Female sex-specific	Hormonal risks	Female sex	F only
		Thyroid disease (hyperthyroidism; hypothyroidism)	F > M
		Pregnancy (preeclampsia, gestational diabetes, post-partum depression)	F only
		Menopause (natural menopause; surgically induced menopause)	F only
Gender differences	Lifestyle risks	Educational attainment	May affect F > M
		Occupation	May affect F > M
		Intellectual activity	May affect F > M
		Physical activity	F > M
		Diet	May affect F > M
		Sleep	F > M after menopause
		Stress	F > M after menopause
		Caregiver burden	F > M
		Marital status	M > F

F, female, M, male.

The prevailing view holds women's greater longevity relative to men, which makes women more likely to reach the ages of greater risk, as the main reason for the increased AD prevalence (Seshadri et al., 1997; Hebert et al., 2001). Studies in support of the increased longevity view have focused on estimates of incidence as well as prevalence. While it is well established that the prevalence of AD (i.e., the number of affected patients) is higher in females than in males, it is still not known whether incidence, i.e., the number of people who develop AD during a particular time period, also differs. The few studies investigating this issue found that in Europe and other areas, women also develop AD at a higher rate than men, especially at older ages (Fratiglioni et al., 1997; Ott et al., 1998; Ruitenberg et al., 2001; Prince et al., 2016). However, in the United States, the incidence seems to be similar across both genders (Edland et al., 2002; Miech et al., 2002; Chêne et al., 2015). It is important to recognize that if men and women are developing AD at the same rate, but prevalence is ultimately higher in women, then the higher disease prevalence in women might indeed be attributed to their longer survival rates.

Research on prevalence and incidence rates of mild cognitive impairment (MCI), an intermediary stage between cognitive changes associated with normal aging and dementia, has provided mixed results (Mielke et al., 2014). Some studies report that men have a higher prevalence of MCI (Koivisto et al., 1995; Ganguli et al., 2004) whereas others indicate greater prevalence in women (Larrieu et al., 2002; Di Carlo et al., 2007). In terms of incidence, women tend to show an increased MCI incidence at older ages (Mielke et al., 2014), while men consistently exhibit a higher incidence of the non-amnestic MCI, which is believed to be prodromal for other dementias, such as vascular dementia (Caracciolo et al., 2008; Roberts et al., 2012).

While longevity is an important issue to consider, emerging evidence suggests that there are unique biological reasons for the increased AD prevalence in women beyond longevity alone. These biological, as well as social and lifestyle underpinnings contribute to differences in brain changes, progression, and symptom manifestation in AD between the genders (Mielke et al., 2014; Ferretti et al., 2018; Scheyer et al., 2018).

In fact, the longevity hypothesis does not take into account some important facts. First, the average life expectancy in the United States is currently 82 years for females and a little over 77 years for males, a difference of less than 5 years (Riedel et al., 2016). As male survival rates have been steadily increasing, studies in Europe anticipate the longevity gap to be less than 2 years by 2030 (Bennett et al., 2015). Second, statistical models have shown that women exhibit a twofold higher incidence and lifetime AD risk even after accounting for gender-dependent mortality rates, age at death, and differences in lifespan (Vina and Lloret, 2010; Carter et al., 2012).

Further, there are well-documented differences in brain anatomy, function, and age-related brain changes between men and women (Carter et al., 2012). Recent studies found that women tend to accumulate greater tangle burden than do men with the same brain Aβ levels, but with no difference in lifetime AD risk (Buckley et al., 2018), suggesting an earlier onset of AD pathophysiology. These observations are consistent with brain imaging findings of earlier emergence of AD-related brain

changes in middle-aged women compared to age-matched men (Mosconi et al., 2017b, 2018). Women also exhibit greater rates of neuropathological decline after an AD diagnosis, as evidenced by increased hippocampal atrophy and neurofibrillary tangles compared to men (Barnes et al., 2005). In keeping with this, while women score generally higher on cognitive performance tests than men (Rentz et al., 2017), female AD patients exhibit a faster rate of cognitive decline and loss of independence in comparison to male patients at the same level of dementia severity (Mielke et al., 2014). Collectively, these data suggest an earlier start of AD pathogenesis in women, which might be masked by the female advantage in cognitive performance, resulting in females being diagnosed at a later stage than their male counterparts. Additionally, it highlights the importance of considering gender specific cut-offs in neuropsychological measures designed to detect AD-related cognitive impairments. Sex-adjusted cutoffs in the interpretation of verbal memory test results have led to improved diagnostic accuracy for both women and men (Sundermann et al., 2017a).

Furthermore, female sex may be associated with AD pathology seen in other conditions, like dementia with Lewy bodies, in which AD pathology occurs in a subset of patients. In one large study, while a composite AD biomarker profile was detected in 25% of all subjects, it was more frequent in women and was associated with worse cognitive performance (Van Steenoven et al., 2016). Given all these differences, further work into understanding sex differences in AD is an important step toward gender-based disease prevention.

GENETIC RISK FACTORS

APOE Genotype

The APOE gene is currently the strongest genetic risk factor for late-onset AD (Harold et al., 2009). APOE codes for the Apolipoprotein E protein, an important cholesterol carrier that primarily coordinates transport of lipids in the brain. It consists of three major alleles: ε2, ε3, and ε4. APOE isoforms coordinate Aβ accumulation and removal in the brain, and play distinct roles in glucose metabolism, neuronal signaling, neuro inflammation, and mitochondrial function (Liu et al., 2013).

Individuals with the ε4 allele are at a higher AD risk compared to those with the more common ε3 allele, whereas the ε2 allele has been associated with decreased risk (Farrer et al., 1997). The ε4 allele is also associated with an earlier age onset in a gene dose-dependent manner (Corder et al., 1993). The frequency of AD and mean age at clinical onset for the different isoforms are as follows: 91% and 68 years of age in ε4 homozygotes, 47% and 76 years of age in ε4 heterozygotes, and 20% and 84 years in ε4 non-carriers (Corder et al., 1993). The ε4 allele is also related to an increased risk for cerebral amyloid angiopathy and age-related cognitive decline during normal aging (Liu et al., 2013).

Sex differences in the effects of the ε4 allele have been well documented, with female carriers being more likely than male carriers to develop AD (Farrer et al., 1997; Kim et al., 2009; Altmann et al., 2014; Ungar et al., 2014). AD risk increases nearly 4- and 10-fold in women with one and two ε4 alleles, whereas men exhibit essentially no increased risk with one ε4 allele and a fourfold increased risk with two ε4 alleles (Farrer et al., 1997; Kim et al., 2009). A recent longitudinal study demonstrated that the conversion risk from normal aging to MCI or AD and from MCI to AD conferred by the ε4 allele is also significantly greater in women compared to men (Altmann et al., 2014). However, a recent meta-analysis examining the relationship between APOE genotype and AD-dementia risk between men and women found no significant sex differences, except for a slightly increased risk for ε3/ε4 female carriers compared to male carriers within the ages of 65 and 75 (Neu et al., 2017).

Clearer evidence for negative associations of APOE ε4 genotype with female sex comes from biomarker studies showing that, among MCI patients, female ε4 carriers had significantly greater levels of CSF tau protein than male ε4 carriers (Altmann et al., 2014; Hohman et al., 2018). Among dementia-free individuals, female carriers exhibited greater brain hypometabolism, hippocampal volume reduction, and cortical thinning compared to male carriers (Altmann et al., 2014; Sampedro et al., 2015) Even in the absence of dementia, APOE ε4 significantly increases brain Aβ deposition and atrophy, and decreases brain connectivity in the default mode network much more effectively in women than in men (Fleisher et al., 2005; Damoiseaux et al., 2012; Mosconi et al., 2017b).

Given these findings, a greater comprehension of the APOE ε4 allele's interaction with sex can have potential implications for AD treatment. To date, the few studies examining this issue have provided conflicting information (Berkowitz et al., 2018). A research study investigating the efficacy of Tacrine, an FDA-approved cholinesterase inhibitor for AD treatment, found that female ε2/ε3 carriers showed greater improvements compared to female ε4 carriers (Farlow et al., 1998). In contrast, men did not differ in their treatment responses based on APOE genotype (Farlow et al., 1998). Another study assessing the efficacy of anticholinesterase therapy showed that female ε4 carriers derived the greatest cognitive benefit compared to non-carriers (Macgowan et al., 1998). A study examining the efficacy of intranasal insulin on cognitive function found that ε4 negative males demonstrated improvements but female non-carriers did not derive any benefits (Claxton et al., 2013). Recent clinical trials of Aβ immunotherapy demonstrate that treatment was more effective in individuals with the ε4 genotype compared to non-carriers (Salloway et al., 2014), though the data were not broken down by sex. More work is needed to systematically examine the differential response to pharmacological interventions by sex and APOE genotype.

It is unclear why the APOE gene confers different risk in women, but some research suggests that it could be due to its interaction with estrogen (Yaffe et al., 2000; Kang and Grodstein, 2012). Studies in mice exhibited that APOE expression in different brain regions varied with the female reproductive cycle stages (Struble et al., 2003), consistent with the hypothesis that estradiol might induce APOE expression in the brain, as had already been demonstrated for APOE in blood (Srivastava et al., 1997). Moreover, trophic effects of estradiol on neurite growth in cultured mouse cerebral cortical neurons are reported to be highly dependent on APOE expression (Horsburgh et al., 2002).

Responses to estradiol are also in part dependent on APOE status: whereas estradiol is neurotrophic in the presence of human APOE ε2 or ε3, the ε4 variant does not support this response (Nathan et al., 2004). In keeping with these findings, several lines of evidence indicate differential effects of estrogen replacement therapy dependent on APOE status, with ε4 positive women exhibiting worse rates of cognitive decline compared to non-carriers (Yaffe et al., 2000; Kang and Grodstein, 2012). Interestingly, a recent study found that transdermal estrogen therapy was associated with reduced Aβ deposition in postmenopausal women, particularly in ε4 carriers (Kantarci et al., 2016). In contrast, oral doses of conjugated equine estrogens (CEEs) was not associated with lower Aβ deposition. These results highlight the interaction of the APOE ε4 allele with estrogen and provide support for a biologically medicated relationship between APOE, estrogen use, and cognitive impairments.

Race

In general, older Hispanics and African Americans are at a higher AD risk in comparison to older whites (Alzheimer's Association, 2017). Differences in various health, lifestyle, and socioeconomic factors likely contribute to their higher AD risk (Alzheimer's Association, 2017). These include a greater prevalence of CVD, T2DM, hypertension, and early life adversity (Lines et al., 2014), as well as lower rates of education and physical activity (Glymour and Manly, 2008).

African American women in particular are twice more likely as white women to develop AD, strokes, and other forms of dementia (Alzheimer's Association, 2002). Likewise, women of Hispanic origin have a one and a half times greater risk for developing dementia, as well as CVD and T2DM than those who are white (Alzheimer's Association, 2017). This is of particular concern because in addition to a rapidly growing aging population, the United States is also becoming increasingly diverse. African Americans currently comprise 14.6% of the United States population and it is estimated that, by the year 2060, Hispanics who are currently the largest minority group will comprise over 28% of the United States population.

Additionally, the caregiving burden among women within these communities is especially high (Nebel et al., 2018). For instance, in some studies, Hispanic and African-American caregivers were more depressed and reported worse physical health than their white counterparts (Napoles et al., 2010). While data on minority groups remain limited, there is an ongoing effort to produce high-quality data on large numbers of racial and ethnic minorities to better understand and treat possible AD-related risk factors.

MEDICAL RISK FACTORS

Cardiovascular Disease

Cardiovascular disease, including coronary heart disease, stroke, atrial fibrillation, and heart failure, is the leading cause of death worldwide and a major risk factor for AD (Hall et al., 2013; de Bruijn and Ikram, 2014). The association between CVD and AD has been attributed to shared modifiable risk factors such as hypertension, obesity, diabetes mellitus, high cholesterol, and smoking (de Bruijn and Ikram, 2014). Several studies point to alterations in brain gray matter volume, increases in white matter lesions, and subcortical damage related to CVD as factors that could potentially increase AD-related neurodegeneration risk (Hajjar et al., 2011).

Historically, CVD has been viewed as a typically "male" disease. The Framingham Heart study found that CVD related mortality and morbidity was two times higher in men than in women aged 50 and younger (Kannel et al., 1976). However, even though CVD risk increases with age in both genders, it shows a steeper increase in risk in women after the age of 50 coinciding with the loss of estrogens occurring during and after menopause (Möller-Leimkühler, 2007). Furthermore, coronary artery disease (CAD) is more prevalent in young females who underwent oophorectomy compared to those with intact ovaries (Parker et al., 2009).

Several studies have documented the protective role of estrogen in CVD via its role in regulating LDL-cholesterol levels (Mendelsohn and Karas, 1999; Iorga et al., 2017; Lagranha et al., 2018). During menopause, both natural and surgically induced, women experience an increase in LDL cholesterol levels. After age 50, LDL levels tend to increase at an average rate of 0.05 mmol/L per year in women aged 40–60 whereas they generally plateau in men (Johnson et al., 1993). This postmenopause induced increase in LDL levels could be explained by declining estradiol levels that result in a downregulation of the activity of LDL receptors in the liver. This, in turn, leads to a reduction in the clearance of LDL from blood serum levels (Pilote et al., 2007). Furthermore, estradiol's interaction with ERα, ERβ, and GPER present in adult cardiomyocytes (Grohé et al., 1997; Ropero et al., 2006) exerts a protective role by increasing angiogenesis (new blood vessels formation from older vessels), improving mitochondrial activity and reducing oxidative stress and fibrosis (Iorga et al., 2017).

Sex differences in terms of CVD risk and underlying pathology have also begun to emerge.

Hypertension, a major risk factor for cognitive decline and a leading cause of cardiovascular morbidity, also increases significantly in women after menopause (Blacher et al., 2019). A meta-analysis found that for every 10 mmHg increase in systolic blood pressure, there was a 25% and 15% increase in CVD risk for women and men, respectively (Wei et al., 2017). Sex differences in terms of CVD treatment have also been documented. For example, statins may be less effective at lowering cholesterol in women compared to men (Assmann et al., 2006; Santos et al., 2009), although the complex relationship between statin exposure and sex-dependent risk reduction is complex and still remains to be understood (Zissimopoulos et al., 2017). Additionally, some clinical trials found that angiotensin receptor blockers improve survival rates in men, but not in women with hypertension or CVD (Fletcher et al., 1988; Rabi et al., 2008). The renin–angiotensin system is no an intense focus of research, given its potential association with risk of Alzheimer's (Kehoe, 2018) and interaction of estrogen with this system (O'Hagan et al., 2012). Overall, hypertension seems to develop differently in women and men, and to respond

differently to medications. The new guidelines by the American Heart Association for hypertension treatment will hopefully lead to better management of this risk factor in the future (Brook and Rajagopalan, 2018).

Stroke has also been associated with an increased AD risk and earlier age of onset for dementia (Honig et al., 2003). Sex differences in terms of the underlying causes of stroke have been documented. The two major types of strokes are ischemic (caused by a blood clot that blocks a vessel in the brain) and hemorrhagic (caused either by a brain aneurysm burst or a weakened blood vessel leak). Hemorrhagic stroke is the lesser common of the two but often results in death. Aneurysmal subarachnoid hemorrhage (aSAH) is higher in women than in men (De Marchis et al., 2017), possibly as the result of female specific factors such as repeated childbirths and hormonal changes. Pregnancy-induced hypertension and vascular tension during delivery may lead to the formation of aneurysms. Several studies have shown that the increased aSAH prevalence in women occurs after the age of 50, coinciding with postmenopausal-related estrogen declines (Kongable et al., 1996; Hamdan et al., 2014). However, a systematic review found that the role of hormone replacement therapy on the manifestation of aSAH is currently unclear (Feigin et al., 2005).

Finally, although sex differences in CAD have not been investigated adequately, there is some research indicating that women may be more prone to cardiac ischemia due to coronary microvascular obstruction than men (Jones et al., 2012). Women are also more affected by microvascular endothelial inflammation, a condition that contributes to heart failure (Jones et al., 2012). Compared to men, women who have experienced a myocardial infarction have a higher death rate, particularly evident in postmenopausal women, and experience more complications post-MRI such as stroke, congestive heart failure, cardiogenic shock, and depression (Shirato and Swan, 2010).

Diabetes

Diabetes mellitus, a common condition characterized by dysregulation of insulin and glucose levels, increases risk for incident AD, MCI, and cognitive impairment (Biessels et al., 2006; Li et al., 2016) that posits a greater risk in women than men (Den Ruijter et al., 2015). For instance, women with type 1 diabetes mellitus (T1DM) exhibit a two times higher risk of cardiovascular events compared to men with T1DM (Huxley et al., 2015). This increased CVD risk has been associated with significantly worse cardiac risk profiles, poorer diabetes management, and treatment options in women (Humphries et al., 2017).

Type 2 diabetes mellitus is also linked to an increased CVD (Juutilainen et al., 2004) and AD risk in women, especially after menopause. The prevalence of T2DM increases with age in a sex-specific manner (Wild et al., 2004). The Study of Women's Health Across the Nation (SWAN) found that declining estrogen levels resulted in a 47% greater T2DM risk during the MT (Park et al., 2017). The length of the reproductive lifetime, defined by age at last period and at menarche, has also been linked to women's increased T2DM risk. The Women's Health Initiative (WHI) showed that women with a reproductive lifetime of less than 30 years exhibited a nearly 40% increased T2DM risk than women with a lifetime reproductive span of 36–40 years (LeBlanc et al., 2017).

This menopausal-related increase in T2DM risk could be explained by biochemical and metabolic changes that take place during the MT (Slopien et al., 2018). For instance, it is linked to an increase in fat deposition (especially in the abdominal region), reduction in lean body mass, and decline in overall energy expenditure (Lovejoy et al., 2008; Leeners et al., 2017). The increased visceral fat accumulation leads to the development of insulin resistance (IR) and the MetS, which play a major role in the development of T2DM (Westphal, 2008). This finding is in accordance with previous data from experimental studies showing that reduced estrogen levels and decreased ERα activity is associated with IR development (Bryzgalova et al., 2006; Riant et al., 2009). Furthermore, T2DM and IR have been associated with atrophy of medial temporal regions such as the hippocampus and amygdala, which are particularly rich in ERs (den Heijer et al., 2003; Convit, 2005; Brinton et al., 2015). These results provide further support to T2DM as a risk factor for AD via dysfunction of insulin signaling.

Depression

Depression falls among the most common mental disorders in the elderly and is strongly linked to a higher risk for cognitive decline in both genders (Yaffe et al., 1999; Wilson et al., 2002; Barnes et al., 2006; Verdelho et al., 2013). However, women are two times more likely than men to experience depression (Albert, 2015). Studies have shown a rapid increase in depression rates starting at puberty and continuing through adulthood in women (Piccinelli and Wilkinson, 2000). Vulnerabilities to mood disorders in women tend to coincide with hormonal fluctuations experienced during and after pregnancy, as well as at the MT, suggesting a link between sex hormones and depression (Steiner et al., 2003). For instance, women undergoing the MT experience a two- to threefold increase in major depressive disorder rates (Gordon et al., 2015). It has been well documented that during the perimenopause period, women are two to three times more likely than men to experience a first episode of depression (Nemeroff, 2007).

The association among sex, depression, and AD risk needs to be more carefully considered. The data in terms of depressive symptoms and cognition stratified by sex have been mixed. Some studies demonstrate a stronger inverse relationship among depression and cognitive function in women, whereas other studies exhibit a stronger association in men (Sundermann et al., 2017b). Furthermore, men with mild depressive symptoms exhibit an increased risk of amnestic MCI, while women with moderate or severe symptoms exhibit a higher AD risk (Sundermann et al., 2017b). This suggests that symptoms might have to meet a higher severity threshold to increase clinical risk conversion in women compared to men.

Traumatic Brain Injury

Several studies suggest a link between traumatic brain injury (TBI) and an increased AD risk (Mortimer et al., 1991; Fleminger et al., 2003). Emerging evidence indicates that even mild TBI is

linked to cortical thinning in AD-sensitive areas and reduced memory performance in patients at risk for AD (Hayes et al., 2017). Moreover, a history of TBI has been associated with AD neuropathology as evidenced by increased accumulation of Aβ and tau protein in patients with a history of TBI (Uryu et al., 2007). TBI is also associated with chronic brain inflammation which has been shown to further accelerate AD disease progression (Perry et al., 2007; Podcasy and Epperson, 2016).

Some studies have highlighted sex-based differences in the context of recovery from TBI from sports-related head injuries. Female athletes are at a significantly higher risk of poorer outcomes, greater symptom severity, and lower recovery rate following mild TBI and concussions compared to their male counterparts (Broshek et al., 2005; Bazarian et al., 2010). A recent MRI study focused on soccer related heading impacts found a sex-based association between heading and brain microstructure (Rubin et al., 2018). In response to similar levels of heading, females had a fivefold greater volume of affected white matter than men, demonstrating a higher burden of microstructural consequences.

The neuroprotective effects of estrogen in the context of recovery from TBI have been demonstrated in preclinical studies (Brotfain et al., 2016). Estrogen administration pre- and post-TBI is associated with increased neuronal survival, significant reductions in apoptosis, and improvements in functional outcomes (Soustiel et al., 2005; Day et al., 2013; Naderi et al., 2015). Estrogen is believed to be neuroprotective by increasing blood flow to ischemic regions after brain injuries, promoting antioxidant activity, and boosting the activity of astrocytes and microglia which provide neurons with metabolic support and elevate the immune response, respectively (Brotfain et al., 2016). Data from human studies show that mild TBI can potentially damage the anterior pituitary gland (Kelly et al., 2006; Klose et al., 2007), which is responsible for producing FSH and LH. This reduction could significantly disrupt the production and circulation of endogenous estrogen levels (Davis et al., 2006). The decline of estrogen associated with menopause could potentially explain the poorer outcomes exhibited by females post-TBI compared to males.

Infections and Chronic Inflammation

Systemic infections and related inflammation may potentially lead to a worsening of AD symptoms and increase the progression of AD-related neurodegeneration (Perry et al., 2007). A retrospective study found that the occurrence of two or more infections within a 4-year time period was linked to an almost twofold greater risk of developing AD in men and women (Dunn et al., 2005). Following infections and injury, there is a heightened response of microglia and macrophages that lead to an increased inflammatory response.

Emerging evidence suggests that chronic inflammation in the brain may be central to AD pathogenesis and that this may be triggered through Aβ accumulation (Wyss-Coray, 2006). Postmortem brains examination of people with AD show increased expression of inflammatory mediators and complement factors, clusters of activated microglia, and cytokines in and near Aβ plaques (Hashioka et al., 2008; Minett et al., 2016). Although there is limited evidence that inflammation is a possible cause of late-onset AD, research on mouse models suggests that activation of inflammatory pathways is potent drivers of the disease (Wyss-Coray and Mucke, 2002). For instance, specific receptors on microglia and monocyte/macrophages are involved in determining whether Aβ clearance is carried out through non-inflammatory phagocytosis or via pro-inflammatory cytokine generation (Heneka et al., 2015). Further, gene expression related to inflammation in brain is increased in aging, and this effect is heightened in patients with AD (Villegas-Llerena et al., 2016). Some epidemiological studies also link anti-inflammatory drugs usage with reduced risk for the disorder, although results are not always consistent (Wyss-Coray, 2006).

Sex differences in terms of response and prevalence to infections and inflammation have been documented, with females experiencing greater disease severity and worse outcomes than males, especially in the presence of reduced estradiol levels (Klein et al., 2010). For instance, women are at a greater risk for chronic inflammatory conditions such as lupus, rheumatic arthritis, and multiple sclerosis, especially after menopause (Straub and Schradin, 2016). Additionally, preclinical studies demonstrate that the presence of influenza infection was associated with reduced reproductive functions in females (Robinson et al., 2011). Furthermore, females treated with estradiol or an ERα receptor agonist had improved survival rates compared to females with either low levels or no estradiol (Robinson et al., 2011).

Overall, these findings suggest that sex differences in microglia activity in response to fluctuating hormone levels may lead to increased inflammatory responses, which may in turn increase women's vulnerability to AD related neurodegeneration in later life stages (Peterson et al., 2015; Hanamsagar and Bilbo, 2016).

HORMONAL RISK FACTORS

Thyroid Disease

Thyroid function is routinely screened for in the clinical assessment of AD because thyroid dysfunction can cause symptoms that mimic those of dementia (Tan and Vasan, 2009). Thyroid complications arise from an imbalance of triiodothyronine (T3) and thyroxine (T4) hormones, which regulate metabolism and vital functions. Hypothyroidism and hyperthyroidism result from an under and over production of T3 and T4 hormones, respectively. Among other potential causes, Graves' disease and Hashimoto's disease (two autoimmune conditions) are the most common causes of hyper- and hypothyroidism.

It is widely reported that women are more likely to experience thyroid problems than men (del Ghianda et al., 2014). One in eight women is expected to be affected by thyroid problems throughout their lifetimes. Some evidence shows that thyroid hormones can interfere with menstrual cycles and cause problems during pregnancy (discussed below) by reducing the clearance of estradiol and acting synergistically with

FSH to increase the production of progesterone (Yen, 1986; Cecconi et al., 1999).

Pregnancy

Pregnancy and childbirth are characterized by obvious fluctuations in hormonal regulation that causes wide-ranged metabolic changes. Sometimes these can lead to a higher occurrence of IR and dyslipidemia, with a greater risk of future diabetes and obesity, all of which could potentially exacerbate AD risk later in life (Cohen et al., 2006). There are mixed results on whether pregnancy increases AD risk later in life. Some studies report that a higher number of pregnancies are indeed linked to a higher risk and an earlier age of AD onset (Sobów and Kloszewska, 2003; Colucci et al., 2006). For instance, one study estimated that women who had at least three pregnancies had a threefold greater risk of developing AD (Colucci et al., 2006). The number of children born is also linked to increased neuropathological lesions of AD in women (Beeri et al., 2009). However, a recent study reported the opposite trend, with a higher number of pregnancies linked to a lower AD risk in later life (Fox et al., 2018).

Even though the data on pregnancy have been mixed, pregnancy-related conditions such as gestational diabetes and preeclampsia (pregnancy-related hypertension) can worsen CVD risk, and therefore risk of dementia (Garovic et al., 2010). Further, hypertension due to pregnancy and vascular tension during delivery can potentially lead to aneurysms formation, which can contribute to an increased risk of stroke later in life.

Menopause

As mentioned throughout the article, the MT is the only known female-specific risk factor for AD to date (Brinton et al., 2015). The effects of MT on AD risk have been highlighted by neuroimaging studies demonstrating a link between menopausal changes and emergence of AD pathology in midlife (Mosconi et al., 2017a, 2018; Scheyer et al., 2018; **Figure 2**). Among cognitively intact participants, postmenopausal and perimenopausal women exhibit higher AD-burden, as reflected by reduced glucose metabolism, increased Aβ deposition, gray matter volume loss (atrophy), and white matter volume loss than premenopausal women and age-matched men (Mosconi et al., 2017b). Furthermore, a 3-year longitudinal study demonstrated that postmenopausal and perimenopausal women exhibited higher rates of AD biomarker progression, as evidenced by greater rates of metabolic declines and Aβ accumulation (Mosconi et al., 2018). These data point to the MT overlapping with the time course of preclinical AD. This is also supported by studies showing that estrogen depletion following oophorectomy is linked to an increased AD risk by up to 70% (Rocca et al., 2007, 2014; Phung et al., 2010).

Altogether, research provides support to the idea of the MT as an "optimal window of opportunity" for AD preventative interventions in women. The "critical window hypothesis," also known as "the timing hypothesis" or "the critical period hypothesis," says that the impact of hormonal replacement therapy (HRT) depends on the timing of treatment onset with respect to age and/or menopause onset, with benefits pertaining to early initiation (Maki, 2013).

This is in stark contrast with the historical blanket use of high-dose HRT for treatment of menopause symptoms in postmenopausal women, which was common practice from the 1960s through 2003. In 2003, the primary results from the WHI study, a pivotal study investigating the effect of HRT on women's health, were published. The WHI had two arms, one for hysterectomized women where the active treatment was estrogen-alone therapy (ET), and the other for postmenopausal women with a uterus where the active treatment was estrogen-plus-progestin therapy (EPT). Both trials were interrupted as early results showed a higher risk of CAD, stroke, and blood clots, with the EPT arm of the study also showing an increased risk of cancer (Rossouw et al., 2002; Anderson et al., 2004).

Further, the WHI included an additional arm, the WHI Memory Study (WHIMS), which investigated the outcome of HRT on dementia risk (Shumaker et al., 2003). In order to test whether HRT was effective in dementia prevention, the trial focused on postmenopausal women who were aged 65 or older at the time of enrollment. From a public health standpoint, it was thought that those women had the most to gain from the intervention since they were the most vulnerable to developing AD, as well as other conditions like CVD that could further increase AD risk. In the EPT arm, with a sample size of 4,532 women, there was a doubling of the risk of all-cause dementia with active treatment compared to placebo after an average follow-up of 4 years (Shumaker et al., 2003). The ET arm, with a sample size of 2,947 hysterectomized women followed over an average of 5 years, reported no significant impact of ET on dementia risk (Marder and Sano, 2000). These findings were in striking contrast to previous observational studies reporting a reduced risk of AD among women who had used HRT compared to those who had not (Zandi et al., 2002) as well as with smaller clinical trials showing no effects of HRT in AD patients (Mulnard et al., 2000; Wang et al., 2000).

It is important to note several limitations pertaining to the WHI trials. First, the treatment administered was in the form of CEE tablets rather than 17 beta estradiol, with or without continuous medroxyprogesterone acetate, depending on the subject's hysterectomy status (Rossouw et al., 2002). This might not have produced the same effects as oral or transdermal administration of estrogen or progesterone. Additionally, participants were already postmenopausal, generally older than 65 at the time of enrollment (therefore several years into menopause), possibly with pre-existing cardiovascular conditions. This raises the question of whether the results are applicable to younger post or peri-menopausal women. More work is needed to better examine effects of HRT dose, formulation, and mode of delivery on women's brain health, especially for younger women without pre-existing conditions.

Recent re-examination of results from the WHIMS indicates that treatment risks and benefits associated with HRT largely depend on three main factors: the patient's chronological age, endocrine age (years to/from menopause), and hysterectomy status. Re-examination of the WHI data 18 years after they were interrupted reported that women who initiated HRT

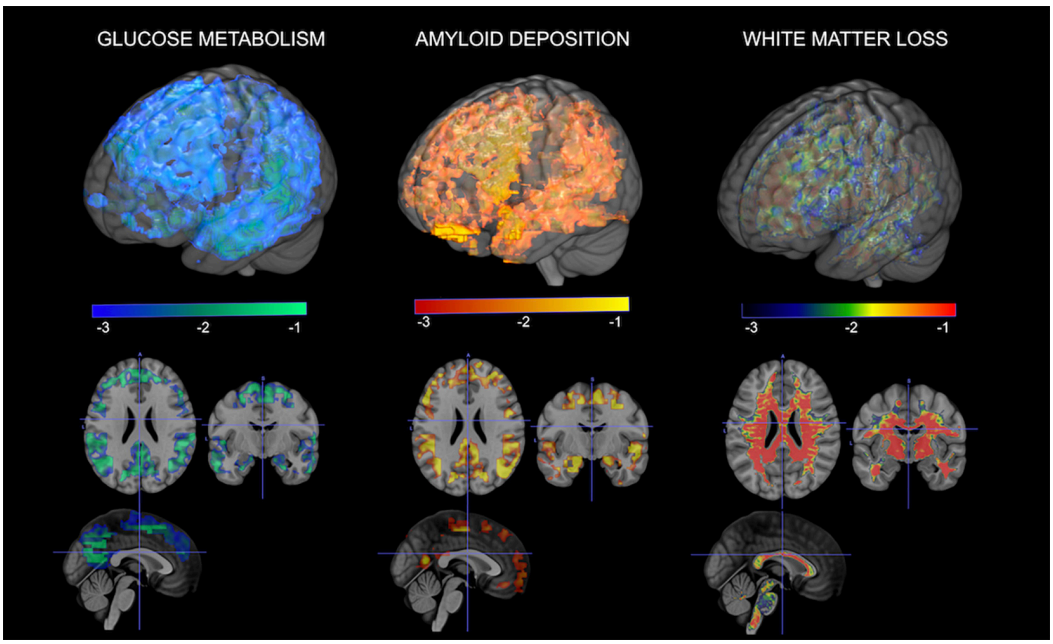

FIGURE 2 | Multi-modality brain imaging of the menopausal transition. From left to right: 3D statistical parametric maps (SPMs) depicting areas of brain hypometabolism, increased amyloid-beta deposition, and white matter loss in peri- and postmenopausal women relative to age-matched men. Corresponding *Z* scores are displayed using a color coded scale at $p < 0.001$.

before the age of 60 or within 10 years after menopause had a lower mortality rate than placebo (Salpeter et al., 2004; Manson et al., 2017). Further, the Early versus Late Intervention Trial with Estradiol (ELITE) conducted with more than 600 postmenopausal women provided evidence that HRT reduced the progression of subclinical atherosclerosis when therapy was initiated right after menopause onset (Hodis et al., 2016), which has been associated with a 30% reduced number of heart attacks and cardiac deaths (Salpeter et al., 2009).

It is possible that early initiation of estrogen therapy may also provide protection against dementia later in life. Results on this topic have been mixed. On the one hand, meta-analysis of 18 studies demonstrated that among younger, 50–59-year-old women, those who used HRT had a 30–44% reduction in AD risk compared to those who did not use HRT (LeBlanc et al., 2001; Maki, 2013), although these data need to be verified in formal clinical trials. In contrast, two recent randomized clinical trials – the ELITE study mentioned above and the Kronos Early Estrogen Prevention Study (KEEPS) – showed no cognitive improvements in women who started HRT within 6 years of menopause, but also no adverse effects of HRT (Gleason et al., 2015; Henderson et al., 2016; Miller et al., 2019). As both trials focused on women who were several years past menopause, more work is needed to systematically look at HRT effects in younger women, especially those of perimenopausal age.

More persuasive evidence that HRT has value for dementia prevention comes from studies of hysterectomized women, particularly those who had their ovaries removed (Rocca et al., 2007). A recent epidemiological study of 1,884 women showed that those who initiated ET within 5 years of surgery and continued until the natural age at menopause had a lower AD risk compared to those who did not take the drug (Bove et al., 2014). Additionally, randomized clinical trials of younger hysterectomized women showed that ET therapy had general beneficial effects on memory performance (Maki, 2013).

Taken together, the majority of studies suggest that, for women with a uterus, EPT therapy initiated within 5 years of menopause onset or in the perimenopausal period may lower AD risk, whereas initiating therapy more than 5 years postmenopause may have the opposite effect. For women without a uterus, ET therapy started as close as possible after surgery and continuing until the natural age of menopause may offset the negative effects of the surgeries and also reduce AD risk (Rocca et al., 2012). The value of initiating ET after the natural age at menopause is unclear.

LIFESTYLE FACTORS

As previously discussed by Nebel et al. (2018), in medical research, the term "sex" refers to biological differences such as chromosomal or hormonal factors, whereas "gender" refers to differences in the impact of psychosocial, cultural, and environmental influences on biological factors between men and women. Gender-related risk factors for AD are discussed below.

Educational Attainment, Occupation, and Intellectual Activity

Low levels of educational achievement and occupation are associated with an increased AD risk in both genders (Katzman, 1993; Stern et al., 1994; Karp et al., 2004). A possible explanation

for this relationship lies in the idea of "cognitive reserve," the brain's ability to effectively utilize cognitive networks to allow individuals to normally perform cognitive activities despite sustaining pathological brain abnormalities such as increases in Aβ and tau levels (Stern, 2012). Higher education levels and cognitively stimulating occupations build more cognitive reserve. Likewise, several systematic reviews demonstrate that participation in cognitively stimulating activities is linked to a lower dementia risk (Stern and Munn, 2010; Wang et al., 2012; Fallahpour et al., 2016).

Historically, women had limited access to educational opportunities compared to men, which may have put them at a disadvantage in terms of their cognitive reserve build-up. This is especially relevant for people currently aged 70 years or older, who are at the greatest risk for AD. These findings suggest that lower educational achievement in women compared to men born early on in the 20th century could potentially play a role in women's increased AD prevalence. However, according to the recent census, women had a higher educational attainment in the United States compared to men (Ryan and Siebens, 2012). There has been a significant change in occupational engagement with women taking on higher level positions and other roles that used to be men's prerogatives. Hopefully these changing trends may help reduce the prevalence of Alzheimer's in women in the future.

Although studies that examined gender differences between cognitively engaging tasks and dementia risk are scarce, a recent study demonstrated that higher engagement in intellectual-cultural activities such as reading, radio and TV, and partaking in social and cultural activities were associated with improved verbal abilities in women, whereas higher engagement in self-improvement activities (playing sports, clubs and organizations, studies, outdoor activities) were associated with improved cognitive function in men (Hassing, 2017). Despite the fact that women generally partake in more cognitive activities such as reading, arts and crafts, and social activities, the effect of these activities on cognitive reserve may be weaker than that of educational and occupational attainment (Mielke et al., 2014; Vemuri and Lesnick, in press).

Physical Activity

Low levels of physical activity are associated with a higher risk of dementia and greater cognitive decline among older adults (Groot et al., 2016; Tan et al., 2016; Willey et al., 2016). Physical activity can improve cognition through indirect effects on modifiable risk factors such as hypertension, obesity, IR, or via direct effects on neuronal activity (Van Praag, 2009; Livingston et al., 2017). Increased physical activity has been shown to promote the formation, survival, and synaptic plasticity of new neurons in the hippocampus (Van Praag et al., 1999; Farmer et al., 2004; Van Praag, 2008), and increase the production of brain-derived neurotrophic factor (BDNF) which play an important role in the formation, growth, and plasticity of neurons (Mulnard et al., 2000).

Neuroimaging studies have also shown the beneficial effects of physical activity on brain structure and function (Hillman et al., 2008). Individuals in a 3-month fitness training program showed increases in blood flow to the hippocampus, which was linked to improvements on memory and verbal learning tasks (Pereira et al., 2007). Cross-sectional MRI studies report that increased fitness activity was related to larger anterior matter, prefrontal and temporal gray matter volumes (Colcombe et al., 2004, 2006; Marks et al., 2007; Gordon et al., 2008). An fMRI study showed that individuals who underwent an aerobic fitness training program exhibited increased activity in the middle frontal gyrus, superior parietal cortex, and significant improvements in cognitive performance (Colcombe et al., 2004).

Despite the well-known link between exercise and improved brain function, a gender gap exists in terms of physical activity engagement. For instance, women tend to engage in less physical activities than men (Troiano et al., 2008; Edwards and Sackett, 2016). Research suggests that women's societal roles can play a role, as parenthood and marital status may hinder women's participation in physical activities (Verhoef et al., 1993). The prevalence of higher physical inactivity among women is concerning due to its association with T2DM, CVD, obesity, and hypertension (Barnes et al., 2005).

However, the relationship between long-term exercise and reduced cognitive impairment and AD dementia risk is more pronounced in women than men (Laurin et al., 2001). For example, a meta-analysis examining the relationship between fitness training levels and cognition in older adults showed that fitness-related benefits on cognition were greater in women compared to men (Colcombe and Kramer, 2003). A study conducted with over 9,000 women found that, although exercising in the teenage years was particularly brain-protective in the long term, being physically active reduced risk of cognitive impairment no matter their age (Middleton et al., 2010). Elderly women with greater physical activity exhibit a reduced risk for AD dementia and are less likely to experience cognitive decline compared to women with lower physical activity levels (Yaffe et al., 2001). Importantly, exercise has been shown to ameliorate cognitive deficits even in women with a diagnosis of cognitive impairment and dementia (Eggermont et al., 2006; Hogervorst et al., 2012).

Diet

Specific dietary patterns like the Mediterranean (MeDi) diet and the Mediterranean-DASH Intervention for Neurodegenerative Delay (MIND) diet have been associated with a reduced risk of dementia in both genders (Scarmeas et al., 2006, 2018; Mosconi et al., 2014; Morris et al., 2015). Further, higher MeDi adherence is associated with a lower risk of AD biomarker abnormalities such as hypometabolism and Aβ deposition in AD-sensitive brain regions already in midlife (Mosconi et al., 2014; Berti et al., 2018).

Both these dietary patterns focus on consumption of vegetables, fruit, whole grains, and legumes, with moderate amounts of fish and poultry, and limited amounts of dairy, red meat, and alcohol. Although results are not always consistent, several observational studies have shown that a MeDi-style diet is particularly protective for women, conferring a lower risk of AD, CVD, and diabetes (Gu et al., 2010; Gu and Scarmeas, 2011; Kaczmarczyk et al., 2012). A study conducted exclusively in elderly women showed that higher MeDi adherence was also moderately related to better cognition and verbal memory

(Samieri et al., 2013; Berendsen et al., 2018). A recent study investigating the effect of a coconut oil enriched MeDi diet in mild to moderate AD patients found that women derived greater cognitive benefits than men (de la Rubia Ortí et al., 2018).

Higher whole grains and legumes consumption have also been related to reduced CVD risk, T2DM, obesity, and MetS in women (Rietjens et al., 2017) which could be attributed to the fact that they contain high concentrations of phytoestrogens. Phytoestrogens are polyphenols which have similar molecular structure to endogenous estrogen. Evidence suggests that phytoestrogens exert their beneficial effects on female physiology via binding to ERs, activating epigenetic mechanisms, and increasing antioxidant activity (Kuiper et al., 1998; Casanova et al., 1999; Jungbauer and Medjakovic, 2014; Remely et al., 2015). Furthermore, some preclinical studies have shown beneficial effects of phytoestrogens on cognitive function and AD (Um et al., 2009; Giridharan et al., 2011; Hu et al., 2012; Jeong et al., 2013). Perhaps as a result, increased intake of legumes and fish has been associated with a delayed menopause onset, whereas refined carbs, sugar, and processed foods resulted in an earlier onset of menopause and reduced ovulatory fertility (Nagel et al., 2005).

Other nutrients have also been associated with improved health outcomes in women, especially those past menopause. The Nurses' Health Study conducted on over 75,000 women showed that replacement of saturated and trans-unsaturated fat for carbohydrates significantly reduced the risk of heart attack and stroke later in life (Hu et al., 1997). For each 5% energy intake increase from saturated fat, compared to the same energy intake from carbohydrates, a 17% increase in coronary disease risk was observed. Many other studies have shown that diets that favor carbohydrates, especially those with low glycemic load and high fiber content, also reduce T2DM risk (Liu et al., 2000; Schulze et al., 2004) and breast cancer in women (Monroe et al., 2007). Further, replacement of 5% of energy from saturated fat with energy from unsaturated fats was estimated to reduce CVD risk in women by 42%, while replacement of 2% of energy from trans-fat with energy from un-hydrogenated, unsaturated fats would reduce risk by 53% (Hu et al., 1997). These data suggest that a similar nutritional pattern might be protective against AD in women as well, given that higher intakes of saturated fat and trans-fat have been linked to an almost doubled risk of dementia, whereas higher intake of unsaturated fat has been linked to a reduced risk (Morris et al., 2004; Okereke et al., 2012; Morris and Tangney, 2014).

Sleep

Poor sleep quality and circadian rhythm disruptions have been associated with an increased AD risk in the elderly (Ju et al., 2013, 2014; Spira et al., 2014). Preclinical and human studies have confirmed the beneficial effects of sleep via increases in cerebral blood flow and the clearing of Aβ plaques by microglial cells and astrocytes (Mangold et al., 1955; Xie et al., 2013). Sleep deprivation leads to an increase in Aβ plaques accumulation (Shokri-Kojori et al., 2018). Subjective measures such as self-reports of sleep deterioration have also been linked to greater Aβ burden (Sprecher et al., 2015).

When compared to men, women are generally at a greater risk for insomnia and experience greater age-dependent sleep quality deterioration, especially after menopause (Madrid-Valero et al., 2017; Auer et al., 2018). Sleep apnea, a condition marked by recurring interruption of breathing during sleep, has been linked to cognitive decline and AD risk (Ancoli-Israel et al., 2008). Even though sleep apnea affects more men than women, its incidence significantly increases after menopause in women (Bixler et al., 1998). Declining levels of estrogen and progesterone are thought to contribute to these findings. The Nurses' Health Study II showed that women who underwent surgical menopause, which results in a shorter lifetime exposure to estrogen, had a 26% higher risk of experiencing obstructive sleep apnea (OSA) (Huang et al., 2018). Even though the exact underlying biological mechanisms remain unclear, few studies have suggested that estrogen contributes to OSA risk by acting on upper airway dilatory pathways to coordinate ventilation (Popovic and White, 1998; Pillar et al., 2000). This sex-specific increase is concerning because women experiencing sleep disturbances are more prone to metabolic and cardiovascular dysfunction and mood disorders such as depression, previously established AD risk factors (Mong et al., 2011).

Historically, women have been widely underrepresented in sleep studies which means that our current understanding of sleep-related disorders mostly comes from research conducted in men (Mong et al., 2011). This imbalance has significant implications for the efficacy of treatment interventions since strategies catered to men might not be effective or applicable to women.

Stress

Cortisol dysregulation associated with repeated activation of the hypothalamic pituitary adrenal (HPA) axis in response to chronic stress is commonly found in patients with AD (Giubilei et al., 2001). It has been linked to memory impairments, cognitive decline, as well as brain atrophy (Huang et al., 2009; Rothman and Mattson, 2010; Brureau et al., 2013).

Sex-related differences in the HPA axis reactivity to early childhood and chronic stress have been previously reported. In response to early childhood trauma, women exhibit a significantly lower cortisol response compared to men later on in life (DeSantis et al., 2011). Moreover, this blunted HPA axis response occurs in a dose-dependent manner. The gender differences with regard to chronic stress could potentially be explained by the activity of gonadal hormones (Stephens et al., 2016). An fMRI study demonstrated that brain circuitry activation patterns in response to stress in menis more similar to women in the early follicular phase of the menstrual cycle, during which estrogen and progesterone are low, compared to women in the late follicular/midcycle phase, during which estrogen is high and progesterone is still relatively low (Goldstein et al., 2010). Women demonstrated lower stress response circuitry activation compared to men, with differences being particularly evident as they progressed through their menstrual cycles. This finding implies that hormonal changes specifically estrogen or progesterone could potentially account for these activation differences. Additionally, it raises the question of whether stress

impacts brain aging and neurodegeneration differently in women and men. A recent imaging study conducted in cognitively normal middle-aged adults demonstrated that increased cortisol levels were linked to brain volume reductions and impaired memory, with the brain shrinkage being only evident in women (Echouffo-Tcheugui et al., 2018), which further highlights the link between hormonal changes and stress reactivity in sex differences (Goldstein et al., 2010; Bale and Epperson, 2015).

Caregiver Burden

Research indicates that caregiving demands can severely tax the caregivers' health and physical abilities, while compromising their immune response to stress, a condition known as "caregiver burden." Caregiver burden has been associated with increased stress, sleep disturbances, depression, difficulties in social functioning, and declines in cognitive function (Alzheimer's Association, 2017). At the same time, the stress associated with caregiving can worsen existing chronic health conditions (Navaie-Waliser et al., 2002), with higher rates of cholesterol, blood pressure, and obesity (Anderson et al., 2010). This has been associated with a greater risk of heart disease, stroke, and premature mortality, particularly under conditions of high strain (Schulz and Beach, 1999). Moreover, perhaps due to all the reasons above, caregivers are at a greater risk for developing AD themselves (Dassel et al., 2017). Approximately two-thirds of caregivers for AD dementia are women (Alzheimer's Association, 2017).

In keeping with the notion that women's reaction to stress is stronger than men's, female caregivers report twice as more caregiver burden than their male counterparts. A study examining biological and emotional responses among spousal caregivers of patients with AD found that men reported significantly lower levels of stress, depression, subjective caregiver burden, and anxiety than women (Thompson et al., 2004). Additionally, men reported higher levels of mental health functioning, sense of coherence, and social and physical well-being. The gender differences could be partly due to the fact that women tend to devote longer hours and perform a higher number of caregiving tasks than men (Pinquart and Sörensen, 2003).

Marital Status

Longitudinal studies have demonstrated that unmarried or single individuals are at an increased risk for cognitive decline, MCI, and AD (Helmer et al., 1999; Sundström et al., 2014). Currently, marital status is the only AD risk factor that affects men more than women. While single people tend to have twice the risk of developing AD versus people with partners (Håkansson et al., 2009), non-cohabiting men are at a greater risk of experiencing cognitive impairment later in life compared with non-cohabitating women (Håkansson et al., 2009). Divorced men also exhibit a higher AD risk compared to divorced women (Sundström et al., 2014). Interestingly, this difference was reduced after adjusting for socioeconomic (e.g., education and income) and demographic characteristics (e.g., age) suggesting that these factors could reduce risk in both genders. For instance, it has been shown that widowed women have a greater tendency be more socially active compared to widowed men which might reduce the negative effects of widowhood (Dykstra and de Jong Gierveld, 2004).

CONCLUSION

In conclusion, AD is a neurodegenerative disorder that has shown strong sex and gender differences in several aspects of the disease, including a faster onset of AD pathology and disease progression after diagnosis, and different risk factors that may account for the increased female prevalence of AD. This review article focused on the research dedicated to understanding the effects of estradiol in terms of gender and sex differences in AD, and the negative effects of MT as a tipping point for middle-aged women. The research findings presented range from studies on molecular mechanisms and preclinical models that clearly highlight estradiol's interactions with a number of signaling and transcriptional pathways involved in cognition and memory, to neuroimaging studies that visualized AD-related brain changes during the MT. Recent clinical trials and re-examinations of existing data lend support to the use of HRT as a possible risk reduction intervention in women at risk for AD, though more work is needed to examine this. Future research studies examining the underlying mechanism of estradiol's neuroprotective action in AD are warranted.

In order to address the growing AD epidemic, the field is shifting toward early detection and primary and secondary prevention efforts (Isaacson et al., 2018). It is crucial that these prevention and clinical trials take into account sex differences in AD biomarkers, disease progression, and gender differences with respect to modifiable risk factors to aid in the development of therapeutics for both men and women. Historically, women have been underrepresented in studies elucidating the underlying mechanisms of AD which has significantly impeded our understanding of gender differences (Mazure and Jones, 2015). Women still remain underrepresented in clinical trials of CVD, a known AD risk factor (Shen and Melloni, 2014). Future AD research studies should actively aim to increase women's overall participation and analyze the influence of sex or gender on health outcomes. A better understanding of sex and gender differences is crucial toward the development of individualized AD risk reduction strategies and treatments.

AUTHOR CONTRIBUTIONS

LM and AR discussed the concepts and wrote the manuscript. HJ, HH, RI, NS, TS, OE, CH, and RB reviewed the literature and provided critical revision of the manuscript for important intellectual content.

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REVIEW
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Peripheral Routes to Neurodegeneration: Passing Through the Blood–Brain Barrier

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A bidirectional crosstalk between peripheral players of immunity and the central nervous system (CNS) exists. Hence, blood–brain barrier (BBB) breakdown is emerging as a participant mechanism of dysregulated peripheral–CNS interplay, promoting diseases. Here, we examine the implication of BBB damage in neurodegeneration, linking it to peripheral brain-directed autoantibodies and gut–brain axis mechanisms. As BBB breakdown is a factor contributing to, or even anticipating, neuronal dysfunction(s), we here identify contemporary pharmacological strategies that could be exploited to repair the BBB in disease conditions. Developing neurovascular, add on, therapeutic strategies may lead to a more efficacious pre-clinical to clinical transition with the goal of curbing the progression of neurodegeneration.

Keywords: blood–brain barrier, neurodegeneration, peripheral immunity, traumatic brain injury, status epilepticus, autoantibodies, gut–brain axis, inflammation

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BRAIN BARRIERS' PATHS, LEAKS, AND NEURODEGENERATION

The term neurodegenerative describes a progressive deterioration of the central nervous system (CNS) that is frequently associated with abnormal accumulation of proteins. Importantly, neurofibrillary tau-protein tangles are not only a major sign of Alzheimer's disease (AD) but are reported in temporal lobe epilepsy and post-traumatic encephalopathies (Tai et al., 2016). Among the emerging disease mechanisms, a peripheral–CNS pathological interplay is proposed to contribute to the neurodegenerative process (Marchi et al., 2014; Engelhardt et al., 2017; Fung et al., 2017; Pavlov and Tracey, 2017; Prinz and Priller, 2017; Le Page et al., 2018). Accordingly, harmful events occurring at the cerebrovascular interface are being examined as key determinants partaking to or even preceding neurodegeneration (Zlokovic, 2011; Nation et al., 2019; Sweeney et al., 2019). At the cerebrovasculature, specialized endothelial cells, mural cells, and astroglia constructs (Abbott et al., 2010; Giannoni et al., 2018; Sweeney et al., 2019) provide physical and biological properties governing the homeostatic–immune interactions between peripheral blood cells, or molecules, and brain neuroglia. The physiological parenchymal milieu composition ensures a healthy neuronal transmission, attainable because of the tightness of the blood–brain barrier (BBB; Zlokovic, 2008; Giannoni et al., 2018; Nation et al., 2019). At the pial arterial and venous level, the cerebrovasculature is permissive to blood cells or molecules, while it becomes impermeable at the arteriole–capillary level where barriers' properties are fully established (Abbott et al., 2010).

BBB vessels also contribute to cerebrospinal and interstitial fluid movements and the elimination of waste products from the interstitial and perivascular spaces (Noé and Marchi, 2019).

It is increasingly recognized that a BBB pathological imprint can provoke a brain pro-inflammatory disequilibrium sufficient to modify neuronal activity in the long term (Marchi et al., 2007, 2014; Nation et al., 2019). Vascular-dependent mechanisms of neurodegeneration can rapidly elicit as a consequence of peripheral infections, head trauma, ischemic stroke, or status epilepticus (**Figure 1**; Nation et al., 2019; Sweeney et al., 2019). These are risk factors for the development of long-term neurodegenerative sequelae and encephalopathies (e.g., post-concussion or head trauma-related chronic traumatic encephalopathy, CTE), cerebral amyloid angiopathy (CAA), AD, and epilepsy. Under conditions of increased BBB permeability, an aberrant bidirectional exchange between the neurovascular unit and the peripheral blood occurs, compounding to neurodegenerative modifications (**Figure 1**; Marchi et al., 2014; Engelhardt et al., 2017; Fung et al., 2017; Pavlov and Tracey, 2017; Prinz and Priller, 2017; Le Page et al., 2018). Completing a vicious cycle, beta-amyloid deposition in the brain can provoke capillaries dysfunction (Thomas et al., 1996; Zhang et al., 1997; Iadecola et al., 1999; Deane et al., 2003, 2012; Nortley et al., 2019). As an example, reactive oxygen species and endothelin-1 production were proposed to elicit vasoconstriction at pericyte locations (Nortley et al., 2019). A question remains regarding whether the endothelin-1 mechanism can directly drive neurodegeneration.

AUTOANTIBODIES AND NEURODEGENERATION: BAD, GOOD, OR NIL?

The communication between the peripheral blood and the brain occurs at preferential cerebrovascular sites (Zlokovic, 2011; Noé and Marchi, 2019), e.g., at post-capillary venules or pial vessels, and by a system of lymphatic vessels draining the cerebrospinal and interstitial fluids to cervical lymph nodes (Aspelund et al., 2015; Louveau et al., 2015a,b, 2018). At the intravascular level, moving leukocytes shape a peripheral–brain immune dialog where endothelium activation or permeability, perivascular immune cell homing, and brain entry of immune soluble factors prompt and sustain neuroglia inflammation [**Figure 1**; see Engelhardt et al. (2017) and Ransohoff (2016) for fundamental aspects of endothelial–leukocyte adhesion]. The implication of the cerebrovascular interface to innate and adaptive modalities of immunity is central (Schwartz and Shechter, 2010; Sommer et al., 2017). Adaptive immunity to the brain requires T- and B-cell stimulation at extra-CNS lymphatic organs and by professional antigen-presenting cells (Janeway et al., 2001), thus implying the existence of a peripheral–brain immune dialog, e.g., via the CNS vascular and lymphatic routes (Noé and Marchi, 2019).

A question exists on whether neurodegeneration may result from autoimmune-like processes (**Table 1**). Contingent to a prolonged or recurrent BBB permeability, specific antigens could exit the brain to reach the bloodstream, mounting a peripheral humoral response. Newly formed brain-directed autoantibodies could be neuropathological upon their entry into the brain across a continuously damaged BBB (Levin et al., 2010). Importantly, autoantibodies and autoreactive T cells were reported in the cerebrospinal fluid (CSF), sera, as well as in the brain of AD patients and experimental models of disease (**Table 1**; Kronimus et al., 2016; Wu and Li, 2016). Anti-Aβ antibodies (Ig type G) correlated with scores of dementia (Wilson et al., 2009). Intrathecal antibodies against tau filaments were reported in AD patients (Mruthinti et al., 2004) and were proposed as contributors of disease progression (Bartos et al., 2012). Anti-tau autoantibodies are not specific to AD as they are increased in patients suffering from other neurodegenerative diseases, e.g., multiple sclerosis (Fialová et al., 2011).

The significance of peripheral autoantibodies as biomarkers of neurodegenerative conditions also remains to be established. Autoantibodies against the glutamate receptor *N*-methyl-D-aspartate receptor (NMDAR) were detected in plasma of AD patients (Davydova et al., 2007). Levels of antibodies were shown to correlate with clinical severity, as patients affected by moderate and severe dementia presented a twofold autoantibody increase compared with patients suffering from mild dementia (Davydova et al., 2007). The presence of autoantibodies against 5-HT was also reported (Myagkova et al., 2001), with levels increasing during the mild phase of the disease, subsequently reaching a plateau (Myagkova et al., 2001). Similar findings were reported for autoantibodies directed against the receptor for advanced glycation end products (Wilson et al., 2009). In a transgenic model of AD, autoantibodies against the sphingolipid ceramide correlated with amyloid plaque increase (Posse de Chaves and Sipione, 2010; Dinkins et al., 2015). Autoantibodies against ATP synthase (Vacirca et al., 2012), α(1)-adrenergic, and the β(2)-adrenergic receptors were also reported (Karczewski et al., 2012). Autoantibodies against the α(1)-adrenergic and the β(2)-adrenergic receptors may contribute to vascular lesions and increased plaque formation in AD patients (Karczewski et al., 2012).

Importantly, not all autoantibodies are harmful. Brain-reactive natural autoantibodies (NAbs) are protective (Britschgi et al., 2009; Kellner et al., 2009; Dodel et al., 2011; Bach and Dodel, 2012). NAbs are mostly IgM and are spontaneously produced. NAbs are polyreactive with low affinity for self-antigens (Casali and Schettino, 1996). Physiologically, NAbs facilitate phagocytosis of apoptotic cells, inhibit inflammatory pathways, and have a role in maintaining immune tolerance (Elkon and Silverman, 2012). NAbs to Aβ can inhibit plaque aggregation, block Aβ toxicity, and catalyze Aβ clearance (Lindhagen-Persson et al., 2010). Immunotherapies using specific, or aspecific, autoantibodies were tested. Bapineuzumab is the humanized form of a monoclonal anti-Aβ antibody targeting the N-terminus of Aβ. In phase II trials, Bapineuzumab administration reduced Aβ plaques in AD brains (Salloway et al., 2009; Rinne et al., 2010) and was associated with decreased total and phospho-tau levels in the CSF (Asuni et al., 2007). Bapineuzumab was, however, discontinued after a phase III trial and showed no beneficial effects on cognitive or functional outcomes (U.S. National Library of Medicine, 2019a,b). Aducanumab

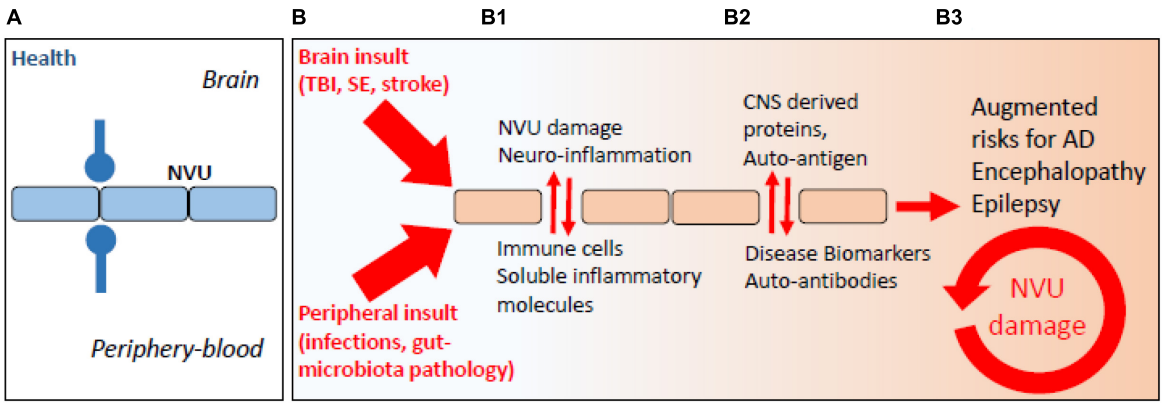

FIGURE 1 | The periphery–brain interplay and CNS disease: the neurovascular pathological imprint. **(A)** Proper peripheral–brain segregation under healthy conditions (neurovascular unit, NVU; *blue lines*). **(B)** Pathological insult(s) elicited in the periphery or in the brain (traumatic brain injury, TBI; status epilepticus, SE) converge to NVU damage (e.g., BBB permeability) and neuro-inflammation, leading to temporary or prolonged loss of brain homeostatic control (**B1**, *red arrows*). **(B2)** Under conditions of BBB permeability, concentration gradients favor brain-derived proteins to extravasate into the peripheral blood. Under this condition, a peripheral auto-immune reaction may mount leading to the production of autoantibodies, possibly re-entering into the CNS if BBB damage endures **(B3)**.

(BIIB037) is a human monoclonal antibody selectively targeting aggregated Aβ (oligomers and fibrils) (Sevigny et al., 2016). An Aducanumab phase III trial was terminated as endpoints were not meet. The analysis of a larger data set is ongoing. Tau immunotherapies are also being developed, attenuating or preventing functional impairment in experimental models, as reviewed in Sigurdsson (2018).

AUTOANTIBODIES AND POST-TRAUMATIC ENCEPHALOPATHY

Resulting from repeated head trauma and BBB damage, chronic traumatic encephalopathy (CTE) presents with accumulation of neurofibrillary tau-protein tangles. In TBI subjects, blood and CSF autoantibodies were suggested as etiological components or as possible biomarkers of neurodegeneration (Raad et al., 2014; Kobeissy, 2015; **Table 1**). Anti-glial fibrillary acidic protein (GFAP) fragments were found in the sera of TBI patients (Zhang et al., 2014). Serum autoantibodies against S100B were reported in American football players when repeated sub-concussive events were associated with BBB damage (Marchi et al., 2013). Autoantibodies against the neuronal α7-subunit of the acetylcholine receptor (Sorokina et al., 2011) as well as AMPA and NMDA receptors (Goryunova et al., 2007) were detected in TBI subjects, while IgG autoantibodies to neurons and basal lamina were reported in rat serum following experimental head trauma (Rudehill et al., 2006). Autoantibodies to the pituitary gland were reported in TBI subjects 3 years after the trauma (Tanriverdi et al., 2008, 2010). Damage to the pituitary gland is distinctive of the TBI pathology with 20–50% of patients showing some degrees of pituitary dysfunction, which affects growth hormone production (Aimaretti et al., 2005; Tanriverdi et al., 2006). An association between anti-pituitary autoantibodies and pituitary dysfunction was reported in patients suffering from mild TBI, including repetitive concussions (Tanriverdi et al., 2010).

Autoreactive antibodies have been proposed for the treatment of TBI sequelae. The presence of hyper-phosphorylated tau accumulating in neurofibrillary tangles is a characteristic of CTE (Omalu et al., 2010). Even if phospho-tau is detectable only at low levels acutely after TBI (Smith et al., 2003; Blennow et al., 2012; Goldstein et al., 2012; Mannix et al., 2013), a specific form of phospho-tau can be produced in response to TBI (*cis* P-tau) (Kondo et al., 2015). This protein spreads throughout the brain, harming cells and leading to post-traumatic neurodegeneration and dementia. In two animal models of TBI, administration of a monoclonal antibody discriminating between the *cis* and the *trans* forms of the protein and blocking *cis* P-tau prevented the onset of tauopathy and cortical atrophy. These accumulating evidence supports the possible involvement of autoantibodies in post-TBI neurodegenerative conditions, perhaps providing new disease biomarkers and therapeutic entry points.

THE GUT–BRAIN AXIS AND NEURODEGENERATION: IS THERE A BARRIER IMPLICATION?

Here, we discuss a specific framework where alterations of the gut microbiota (GM) could impact BBB permeability, promote neuro-inflammation, and favor neurodegenerative modifications (**Figure 2**; Braniste et al., 2014; Cerovic et al., 2019; Parker et al., 2019; Wang et al., 2019). Bacteria, viruses, parasites, and non-pathogenic fungi constitute the intestinal microbiota. These complex communities of microbes colonizing the gastrointestinal tract are major players in health. Modern life and diets have progressively induced changes in the composition of the GM, perhaps for the worse, as this can contribute to chronic illnesses (Lozupone et al., 2012; Myles, 2014; Kumar and Forster, 2017; Shanahan et al., 2017; Cryan et al., 2019; Pagliai et al., 2019; Reza et al., 2019). Intestinal microbes can influence brain function through a continuous dialog involving the immune,

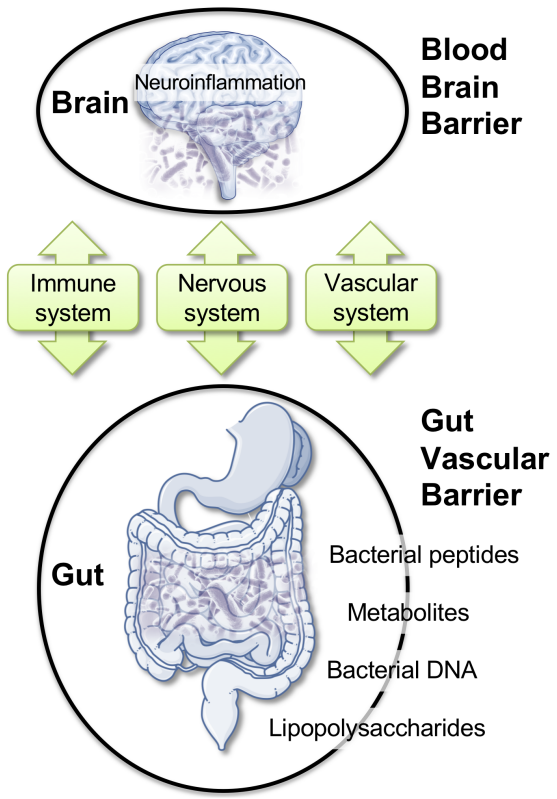

FIGURE 2 | Gut–brain axis: communication routes and physiological barriers. A double, peripheral, and brain homeostatic control is performed by the intestinal–epithelial and blood–brain barriers under healthy conditions. Rupture of one barrier (e.g., gut) may impact the other (e.g., brain), with the blood stream and the immune system being the facilitators or the arbitrators of the pathological spread and neuro-inflammation.

the vascular, and the nervous systems (**Figure 2**; Schroeder and Bäckhed, 2016; Cox and Weiner, 2018; Butler et al., 2019; Cryan et al., 2019). Modifications in the composition of the GM was reported in brain disorders, such as autism (Adams et al., 2011; Kang et al., 2019), depression (Kelly et al., 2016; Zheng et al., 2016), Parkinson's disease (Scheperjans et al., 2015; Sampson et al., 2016), and AD (Cattaneo et al., 2017; Vogt et al., 2017; Zhuang et al., 2018). Intriguingly, the extent of the amyloid pathology in AD mice appears to be dependent of the microbial status, which is specific to the animal housing facility. APP/PS1 mice bred in a germ-free facility displays decreased amyloid plaque number compared to mice housed in non-germ-free conditions (Harach et al., 2017). Moreover, the administration of broad-spectrum, combinatorial antibiotics to APP/PS1 mice, either during the peri-natal or the adult stage, reduced brain Aβ deposition (Minter et al., 2016, 2017).

Existing reports support the hypothesis of a possible infectious origin of AD. Aβ was proposed as an antimicrobial peptide (Soscia et al., 2010; Moir et al., 2018) responding to pathogens (Kumar et al., 2016; Eimer et al., 2018). Infectious agents, such as *Chlamydia pneumonia*, *Proprionibacterium acne*, *Helicobacter pylori*, *Porphyromonas gingivalis*, or *spirochetes*, are associated with AD (Kornhuber, 1996; Balin et al., 1998; Kountouras et al., 2006; Miklossy, 2011; Poole et al., 2015). A microbial hypothesis is supported by evidence describing the presence of viruses, such as Herpes simplex virus type I, in the brains of AD patients (Lin et al., 2002; Alonso et al., 2014; Itzhaki et al., 2016).

Within the complex interplay between the gut microbiome and the CNS, a role for brain barriers and neuroinflammation is becoming important (Braniste et al., 2014; Cerovic et al., 2019; Parker et al., 2019; Wang et al., 2019). The impact of the gut microbiome composition on CNS health was reported (Amedei and Boem, 2018; Chu et al., 2019; Sherwin et al., 2019; Virtue et al., 2019). Recent work demonstrated that GM composition controls BBB development and permeability in mice (Braniste et al., 2014). In AD, increased gut permeability due to GM dysbiosis was reported during prolonged stress. In this condition, molecules that are normally secluded in the intestine, e.g., inflammatory mediators, bacteria, or bacterial-derived agents, could leak out and reach the peripheral blood. Bacterial DNA, metabolites, or proteins circulating in the blood stream could, in turn, modify BBB permeability (Braniste et al., 2014; Myles, 2014; Kumar and Forster, 2017; Cerovic et al., 2019; Parker et al., 2019; Wang et al., 2019). Existing reports indicated bacterial DNA in human blood with a possibility for brain access (Lelouvier et al., 2016; Païssé et al., 2016; Schierwagen et al., 2018). Brain entry of *P. gingivalis*, a bacterium associated with periodontal disease, has been described (Dominy et al., 2019). Gingipain inhibitors reduced the bacterial load and the bacteria-induced neuro-inflammation in a mouse model (Dominy et al., 2019). Among Spirochetes, *Borrelia burgdorferi* is a strain associated with Lyme dementia that could enter the brain. In humans, this specific strain can form biofilms similar to senile plaques. Aβ and bacterial DNA appear as important constituents of these biofilms, suggesting that amyloid plaques may originate in association with or from the spirochetal colonies (Allen, 2016; Miklossy, 2016).

These examples highlight the need of tightly regulated intestinal and brain barriers (Rahman et al., 2018). In AD, a dysbiotic GM may enhance gut permeability and alter BBB integrity, allowing the access of infectious agents or associated molecules into the brain (Martin et al., 2018). Significantly, intestinal and brain barriers are reactive to analogous pro-inflammatory triggers. Circulating inflammatory cytokines IL-17, interferon-gamma (IFN-γ), and the small intestine epithelium protein zonulin can damage the intestinal–epithelia and BBBs (Rahman et al., 2018).

GUT MICROBIOTA AND AUTOANTIBODIES: INITIAL CLUES

Hypotheses linking modifications of the GM and production of autoantibodies are emerging (Petta et al., 2018). Some evidence supports the concept that specific dietary components may affect B-cell maturation and activity, ultimately leading to the formation of autoantibodies (Petta et al., 2018). Obesity was associated with a systemic pro-inflammatory state, characterized by changes in the frequency of B-cell

TABLE 1 | Autoantibodies reported in neurodegenerative disease and post-TBI.

Autoantibodies	Neuro-pathology	Stage	Model investigated	Sample	Observed effects	Isotype	References
Anti-neuronal antibody	TBI	Moderate acute TBI	AM	Serum	–	IgG	Rudehill et al., 2006
Anti-neurofilament	AD	Moderate forms of AD	H	Serum, CSF	–	IgG. IgM	Bartos et al., 2012
Anti-Aβ	AD	Mild to severe forms of AD, early and late onset	H	Serum, CSF	Suggested to favor Aβ clearance; correlation with global scores of dementia	IgG {IgG2}, Nab	Myagkova et al., 2001; Bell et al., 2010; Daneman et al., 2010; Schwartz and Shechter, 2010; Armulik et al., 2017; Kisler et al., 2017; Rustenhoven et al., 2017; Sommer et al., 2017; Montagne et al., 2018; Nikolakopoulou et al., 2019
Anti-Tau	AD, TBI	Mid to severe forms of AD	H	Serum, CSF, tissue	Levels correlated with reduced Plaque burden	IgG, Nab	Du et al., 2001; Weksler et al., 2002; Mruthinti et al., 2004; Brettschneider et al., 2005; Rosenmann et al., 2006; Gruden et al., 2007; Gustaw et al., 2008; Britschgi et al., 2009; Wilson et al., 2009; Maftei et al., 2013; Qu et al., 2014
Anti-AMPA receptor	AD, TBI	Moderate to severe AD Mild and repetitive concussion in children	H	Serum	Levels increased in moderate and severe dementia	–	Goryunova et al., 2007
Anti-NMDA receptor	AD, TBI	Moderate to severe AD and dementia, mild and repetitive concussion	H	Serum	Relationship between autoantibody titers and aging	IgG	Goryunova et al., 2007; Busse et al., 2014
Anti-acetyl choline receptor	TBI	TBI to different severity in children	H	Serum	Levels correlate with trauma severity	–	Sorokina et al., 2011
Anti-Dopamine	AD	Mid to severe forms of AD	H	Serum	Match to moderate to severe dementia progression	IgG	Myagkova et al., 2001; Gruden et al., 2007
Anti-5-HT	AD	Mild to severe forms of AD	H	Serum	Levels increased during mild dementia	–	Myagkova et al., 2001
Anti-GFAP	AD, TBI	Pre-senile and senile forms of AD, senile vascular dementia	H	Serum	Relationship between autoantibody titers and aging suggested as a maker of BBB damage	IgG	Tanaka et al., 1989; Gruden et al., 2007

(Continued)

TABLE 1 | Continued

Autoantibodies	Neuro-pathology	Stage	Species model investigated	Sample	Observed effects	Isotype	References
Anti-S100β	AD, TBI	Mild to severe AD, senile vascular dementia, repealed acute sub-concussion	H	Serum	Match to moderate–severe dementia progression; relationship between autoantibody titers and aging	IgG	Mecocci et al., 1995; Gruden et al., 2007; Marchi et al., 2013
Anti-microglia	AD	Mid to severe forms of AD	H	CSF	–	IgG	McRae et al., 2007
Anti-phospholipid	AD, TBI	Mid cognitive impairment to advanced AD, severe TBI	H	Serum, CSF	Levels correlate with erythrocytes and proteins in CSF	IgG	McIntyre et al., 2007; McIntyre et al., 2015
Anti-ceramide	AD	Chronic pathology in TG mice	AM	Serum	Levels correlate with plaque formation	IgG	Dinkins et al., 2015
Anti-RAGE	AD	Mild cognitive impairment to severe AD	H	Serum	Relationship with anti-Aβ levels; correlation with global scores of dementia	IgG	Mruthinti et al., 2004; Wilson et al., 2009
Anti-ATP synthase	AD	Mild to severe AD	H	Serum	Induced the inhibition of ATP synthesis	IgG	Vacirca et al., 2012
Anti-pituitary	TBI	Mild to severe TBI, acute and long-term	H	Serum	Association between antibody positivity and hypopituitarism due to head trauma	IgG	Tanriverdi et al., 2008; Tanriverdi et al., 2010; Pani et al., 2019
Anti-adrenergic receptors	AD	Mild to moderate dementia	H	Serum	Suggested contribution to vascular lesions and increased plaque formation	IgG	Karczewski et al., 2012

AM, data derives from animal models only.

TABLE 2 | Available molecules exerting BBB repairing and anti-inflammatory effects.

	Category	Mechanism(s) of action	Reported effects	*In vivo* /*in vitro* models	Clinical trials	References
Losartan	Antihypertensive	Angiotensin II antagonist	Improves barrier function	Rats	Antihypertensive drug	Kucuk et al., 2002; Kaya et al., 2003; Hong et al., 2019
Ripamycin	Immunosuppressant	mTOR antagonist	Improves barrier function, promotes claudin-5	Mice	Prevention of transplant rejection	Van Skike et al., 2018
Anakinra	Interleukin-1 receptor antagonist	Human interleukin-1 receptor antagonist (IL-IRa)	Decreases inflammation	Rats	Anti-inflammatory drug currently used against rheumatoid arthritis cryopyrin-associated periodic syndromes (CAPS) and Still's disease.	Kenney-Jung et al., 2016; Van Skike et al., 2018
IPW	TGFβR1 kinase inhibitor	Inhibition of TGFβR signaling	Reduces brain hyperexcitability, indirect BBB repair	Mice	NA	Senatorov et al., 2019
3K3A-APC	Activated protein C (APC)	BACE-1 inhibition, activation of protease-activated receptor 1	Cytoprotective properties, neovascularization, neurogenesis	Mice	In clinical trial for ischemic stroke (RHAPSODY)	Thiyagarajan et al., 2008; Zhong et al., 2009; Wang et al., 2016; Sinha et al., 2018; Lazic et al., 2019; Lyden et al., 2019
PDGF-BB	PDGFRb agonist	Increased expression of p-Smad2/3	Ameliorates BBB function	*In vivo*	NA	Arango-Lievano et al., 2018
Imatinib	Kinase inhibitor	Inhibition of PDGFR signaling	anti-inflammatory?	Mice	Precursor cell lymphoblastic leukemia–lymphoma, dermatofibrosarcoma	Su et al., 2015; Klement et al., 2019
Dexamethasone	Glucocorticoid	Decreased JMJD3 gene expression, suppression of MMP-2, MMP-3, and MMP-9 gene activation	Preserves tight junctions integrity	*In vitro* BBB model	Inflammatory conditions	Hue et al., 2015; Na et al., 2017
Annexin-A1 (ANXA1)	Glucocorticoid anti-inflammatory effector	Binding to FRP2 receptor, inhibition of phospholipase-2	Restores cell polarity, cytoskeleton integrity, and paracellular permeability	*In vitro* BBB model, Anxa−/− mice	NA	Cristante et al., 2013; Purvis et al., 2019; Zub et al., 2019
VEGF	Vascular endothelial growth factor	Prevention of Aβ-induced apoptosis	Improves vascular functions	Mice	NA	Religa et al., 2013
Tetramethylpyrazine	Cardiovascular	Blocking JAK/STAT signaling	Reduces BBB damage	Rats	NA	Gong et al., 2019
S-nitrosoglutathione	Nitric oxide donor	Suppression of MMP-9 activity	Prevents BBB damage	Mice	NA	Aggarwal et al., 2015
Cannabidiol	Analgesic, anti-inflammatory, antineoplastic	Activation of PPARγ and 5-HT_{1A} receptors	Prevents BBB damage	*In vitro* BBB model	NA	Hind et al., 2016

subpopulation [e.g., reduction of the anti-inflammatory IL-10^{+} regulatory B cell (Nishimura et al., 2013)] and by an increase in autoantibody levels (Kosaraju et al., 2017). Diets rich in polyunsaturated fatty acid are associated with the suppression of pro-inflammatory responses and a reduction of circulating autoantibodies (Pestka et al., 2014; Tomasdottir et al., 2014). Dietary components impact the composition of the gastrointestinal bacterial populations:

consumption of prebiotics increases the intestinal levels of *Bifidobacterium* and *Lactobacillus* (Singh et al., 2017), with a possible link to B-cell differentiation, maturation, and activation (Ouwehand et al., 2002). Diet can impact autoantibody production, directly by promoting pro-inflammatory conditions and indirectly by altering the GM. In experimental autoimmune encephalomyelitis (EAE) it was demonstrated that the commensal microbiota composition is a pivotal factor for disease development (Lee et al., 2011) and that modifying the GM impacts the levels of T and B cells or the levels of circulating autoantibodies (Ochoa-Repáraz et al., 2009, 2010).

BBB REPAIRING PHARMACOLOGY: AVAILABLE OPTIONS

The multi-level implication of BBB damage in neurodegenerative disorders has prompted the quest for pharmacological repairing strategies, either directed at the endothelium or by indirect targeting of the cellular players of peripheral and neuro-inflammation. Currently tested drugs are either repurposed or new (**Table 2**). Examples include losartan, an anti-hypertensive molecule acting as an angiotensin II antagonist. Losartan was shown to reduce BBB permeability in a rat model of hypertension (Kucuk et al., 2002; Kaya et al., 2003) and following pilocarpine-induced status epilepticus (Hong et al., 2019). BBB protection by losartan depends on angiotensin receptor type 1 (AT1) blockade. Another drug is rapamycin, a specific inhibitor of the mammalian target of rapamycin (mTOR) pathway. Rapamycin improved cerebrovascular and cognitive function in a mouse model of AD (Van Skike et al., 2018). Inhibition of mTOR preserved BBB integrity through the upregulation of tight junction proteins and downregulation of matrix metalloproteinase-9. A third option is anakinra, which is the recombinant form of the human IL-1 receptor antagonist (IL1-Ra) that inhibits IL-1α and IL-1β binding to the IL-1 receptor type 1. As inflammation comprises BBB dysfunction, the inhibition of IL-1 as proposed is a strategy enabling cerebrovascular protection (Marchi et al., 2009, 2011; Vezzani et al., 2011; Kenney-Jung et al., 2016). Recent strategies include the development of IL-1Ra molecules fused with a cell-penetrating peptide to enhance brain access (Zhang et al., 2017). After transient middle cerebral artery occlusion in rats, IL-1Ra-PEP reduced neuro-inflammation and ischemia (Zhang et al., 2017). The fourth option is IPW-5371, a small molecule blocking the transforming growth factor β receptor (TGFβR) signaling. In a recent study (Senatorov et al., 2019), IPW reduced hyperexcitability in a mouse model, protecting BBB functions. The activated protein C (APC) therapeutic analog 3K3A-APC is a fifth option. This compound has BBB and neuro-protective properties (Thiyagarajan et al., 2008; Zhong et al., 2009; Wang et al., 2016; Sinha et al., 2018; Lazic et al., 2019; Lyden et al., 2019) and it is in clinical trial for stroke treatment (Lyden et al., 2019). Next is platelet-derived growth factor subunits BB (PDGF-BB). Following an acute vascular insult, activation of the PDGF receptor beta (PDGFRβ) by PDGF-BB is beneficial, protecting the endothelium–pericyte structures. The latter was reported in mouse models of status epilepticus (Arango-Lievano et al., 2018) and cerebral ischemia (Marushima et al., 2019). Conversely, in chronic disease settings (e.g., AD, epilepsy, etc.), activation of PDGFRβ may participate to inflammation (Rustenhoven et al., 2017; Klement et al., 2019). Under this circumstance, blocking PDGFRβ signaling by using the tyrosine kinase inhibitor Imatinib could represent an anti-inflammatory strategy (Rustenhoven et al., 2017; Klement et al., 2019). In general, reducing PDGFRβ signaling could lead to contrasting effects, e.g., pericyte deficiency and BBB breakdown (Bell et al., 2010; Daneman et al., 2010; Armulik et al., 2017; Kisler et al., 2017; Montagne et al., 2018; Nikolakopoulou et al., 2019) or anti-inflammatory (Rustenhoven et al., 2017; Klement et al., 2019), depending on disease stage (acute vs. chronic). Another option, Dexamethasone, is a glucocorticoid effective in the formation and maintenance of endothelial tight junctions (Hue et al., 2015; Na et al., 2017). Dexamethasone was proposed to decrease the expression of the Jumonji Domain Containing 3 gene (JMJD3) and metallo-proteinases (MMP-2, MMP-3, and MMP-9). Finally, there is the vascular endothelial growth factor (VEGF). Amyloid accumulation is associated with endothelial apoptosis (Religa et al., 2013) in Alzheimer's patients as well as in mouse models. In AD mice, VEGF administration rescued memory deficits by preventing Aβ-induced vascular apoptosis (Religa et al., 2013). See **Table 2** for complete drug listing, mechanisms and bibliography.

PERSPECTIVES AND CHALLENGES

The importance of cerebrovascular dysfunction in neurodegenerative disorders is twofold: BBB damage is pathophysiological and it allows a diagnostic window, the latter by exploiting specific proteins that shed from the damaged or vascular wall cells to appear into accessible fluids, e.g., blood or CSF. For instance, by dosing soluble PDGFRβ in CSF and by using dynamic contrast-enhanced magnetic resonance imaging, a recent study demonstrated BBB breakdown as an early biomarker of human cognitive dysfunction (Montagne et al., 2015; Nation et al., 2019).

Tackling the complex neurodegenerative puzzle requires a continuous sharpening of pharmacological tools. This is important because no efficacious disease-modifying strategy is available to meaningfully delay or prevent disease progression. The problematics here presented may stem from semantic habits as the term *neuro-* indicates, for most, neurons only. Revisiting nomenclature(s) may benefit, if not legitimize, holistic, and neurovascular approaches to CNS disorders since it is evident that considering neuronal circuits insulated from the influence of glio-vascular cells is excessively reductionist.

AUTHOR CONTRIBUTIONS

NM planned, drafted, and corrected most of the manuscript, including figures and tables. FN wrote the parts on auto-immunity and created the table. SC was responsible for the

section "The Gut-Brain Axis and Neurodegeneration: Is There a Barriers' Implication?". PG contributed to the section on BBB drugs and to the table, and also contributed to the sections "Gut Microbiota and Autoantibodies Production: Initial Clues" and "References."

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