THE ROLE OF GLIA IN ALZHEIMER'S DISEASE

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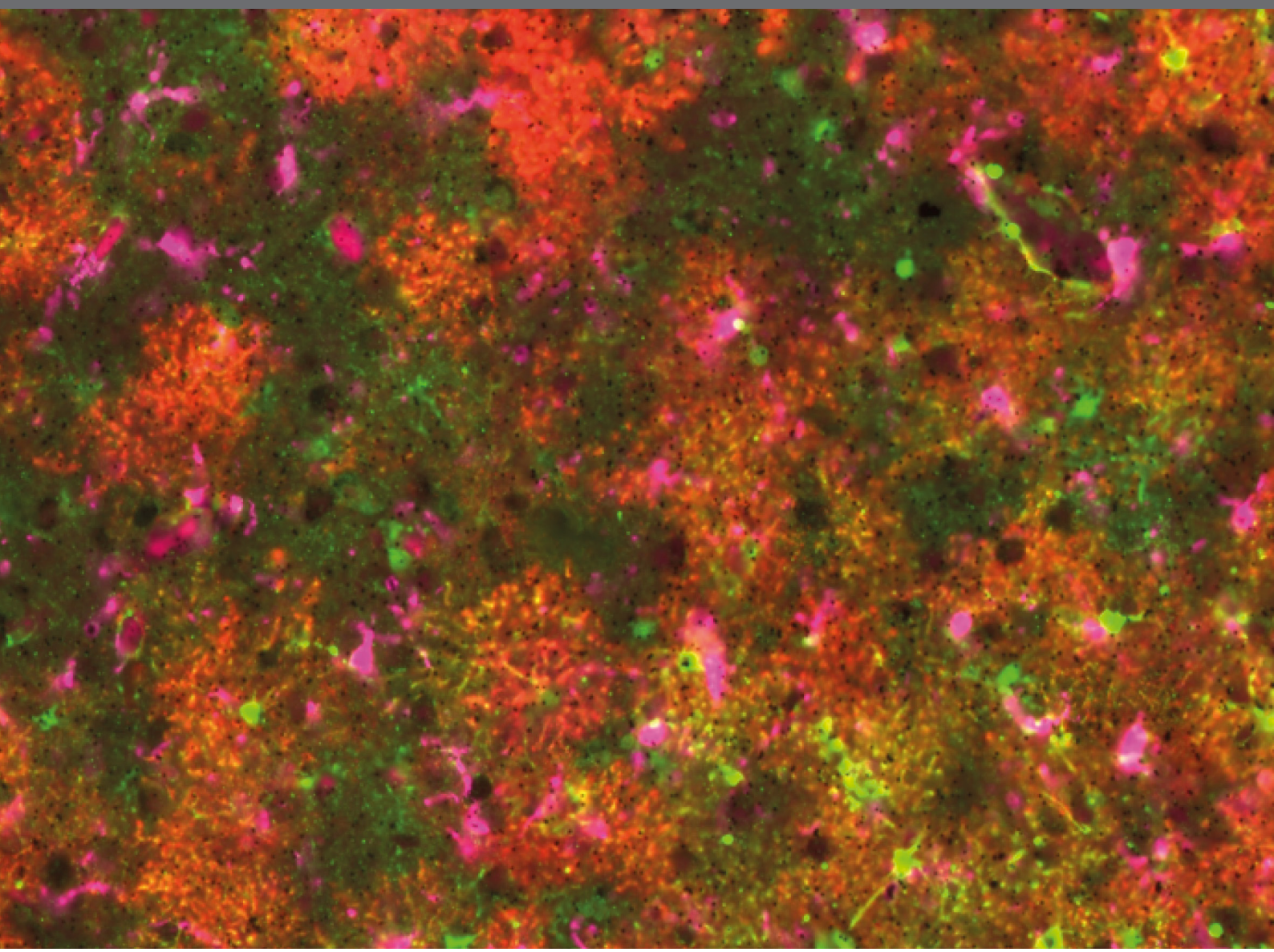

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THE ROLE OF GLIA IN ALZHEIMER'S DISEASE

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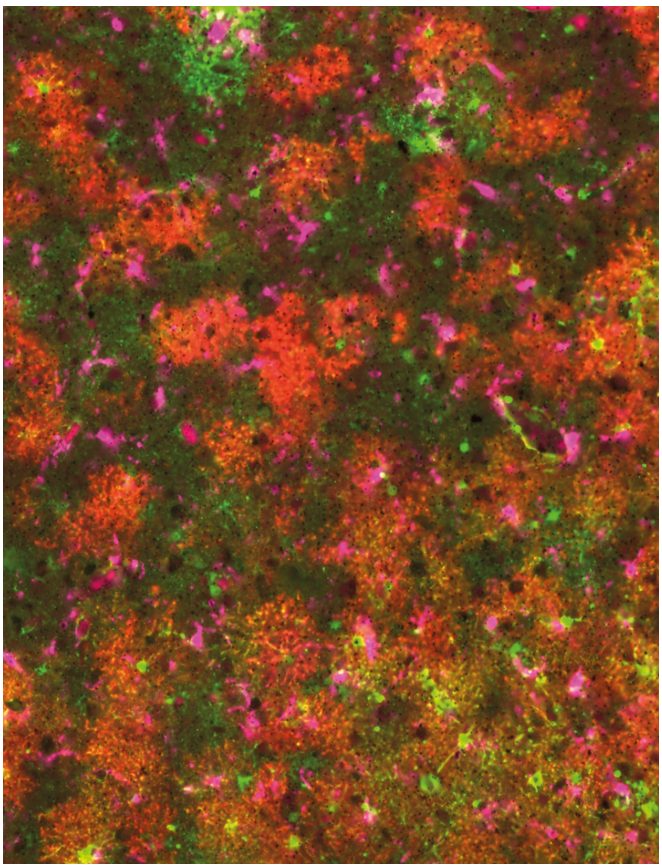

Triple fluorescent immunostaining for EAAT2/GLT-1 (red, astrocytes), EAAT1/GLAST (green, astrocytes) and Tmem119 (magenta, microglia) in the human temporal neocortex (Brodmann Area 38).

We believe that the role of glia is the next frontier to be explored in Alzheimer's disease research. This eBook is an update on both the current knowledge of astrocytes and microglia involvement in Alzheimer's disease pathophysiology, and some of the techniques available to study them.

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EDITORIAL
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Editorial: The Role of Glia in Alzheimer's Disease

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Keywords: Alzheimer's disease, amyloid plaques, astrocytes, chemokines/chemokine receptors, glia, microglia, neurofibrillary tangles (NFTs), neuroinflammation

Editorial on the Research Topic

The Role of Glia in Alzheimer's Disease

For decades, Alzheimer's disease (AD) research has focused on the two pathological hallmarks of the disease: amyloid plaques and neurofibrillary tangles. Reactive astrocytes and activated microglia decorating amyloid plaques are other long known pathological features of the AD brain (1–4), yet only recently has the role of glia in AD gained momentum as a research topic (5). This growing interest in glia is primarily fueled by the GWAS discovery of several risk loci in genes related to the innate immune system (6), and by the recent involvement of microglia and astrocytes in synaptic pruning and the modulation of synaptic activity in physiologic conditions (7–10). Indeed, reactive glia has been correlated with both clinical expression and progression of cognitive decline in AD (1, 11). In the 10 articles that form this Frontiers Research Topic, now edited as an eBook, the readers will find an update on some of the most crucial aspects of astrocyte and microglia involvement in AD pathophysiology, as well as some of the most novel and useful tools to study both glial cell types in the context of AD.

We start with a comprehensive review on the role of reactive astrocytes in the disease, highlighting the heterogeneity and complexity of astrocytes in the healthy brain, the molecular signaling pathways involved in astrocyte reaction in AD, the phenotypic changes exhibited by reactive astrocytes in the AD brain, and the consequences of this astrocyte reaction with respect to plaques, tangles, neurons, and synapses (Perez-Nievas and Serrano-Pozo). Next, Garcia-Esparcia et al. compare the astrocyte reaction present in AD and dementia with Lewy bodies (DLB) brains by quantifying both protein and mRNA levels of several astrocyte markers such as glial fibrillary acidic protein (GFAP), excitatory amino acid transporter 2 (EAAT2/GLT-1), and aldehyde dehydrogenase 1 L1 (ALDH1L1). They observed a non-significant reduction of EAAT2/GLT-1 protein levels and a normal EAAT2/GLT-1 immunoreactivity around plaques, suggesting limited consequences of astrocyte reaction for glutamate transport in AD.

The Frontiers Research Topic/eBook switches then gears to focus on the role of microglia in AD. Navarro et al. summarize their recent findings comparing microglia from the hippocampus of APP-overexpressing transgenic mice and human AD brains (12). They postulate that, while microglia becomes uniformly activated and pro-inflammatory in the hippocampus of mouse models of amyloid plaque deposition, a subset of microglia from the human AD hippocampus might be dysfunctional and exhibit an attenuated inflammatory response, and even degenerate due to the toxicity mediated by soluble tau oligomers. Zhou et al. review the physiology of triggering receptor expressed on myeloid cell 2 (TREM2) and its implication in amyloid plaque and tangle formation from studies on *Trem2* deficient AD mouse models. They also review

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their recent finding that TREM2 enhances microglial metabolism through the mammalian target of rapamycin (mTOR) pathway (13), suggesting that the AD-linked *TREM2* variants (14, 15) can modulate AD pathogenesis through an aberrant microglial metabolism. Guedes et al. review the contributions of microglial and monocyte chemokines and their receptors (CCL2/CCR2, CX3CL1/CX3CR1, CCL5/CCR5, CXCL10/CXCR3, and CXCL1/CXCR2) to amyloid and tau pathologies. Thei et al. contribute with a review of the ion channels expressed by homeostatic microglia, their potential disruption in activated microglia in AD, and how human inducible pluripotent stem cell (hiPSC)-derived microglia could be a better tool than primary microglial cultures to elucidate the role of these ion channels. And, finally, Chun et al. summarize their experience with novel *in vitro* approaches to study glia, including microfluidic devices with human microglia exposed to Aβ to investigate microglial chemotaxis (16), and a 3D organotypic AD brain model (17) consisting of culturing neurons, microglia and astrocytes from immortalized human AD neural progenitor cells or hiPSC-derived neural progenitor cells in a 3D microfluidic platform.

Lastly, the Frontiers Research Topic/eBook deals with the imaging methods available to study reactive glia. Edison et al. review the literature on PET imaging of reactive glia in both human AD subjects and AD mouse models. PET radiotracers targeting the translocator protein of 18 KDa (TSPO) have been widely used for almost two decades to image activated microglia *in vivo* (18), whereas [^{11}C]deuterium-L-deprenyl ([^{11}C]DED)—an irreversible inhibitor of monoamine oxidase B—has recently been introduced to image reactive astrocytes (19), which are known to up-regulate this enzyme (20). Hierro-Bujalance et al. provide an update on the methodology of intravital multiphoton microscopy and its applications to image microglia *in vivo* in AD mouse models. Examples of key observations using this technique include the microglia chemotaxis toward amyloid plaques after these are formed, its limited role in controlling plaque growth, and its activation and participation in plaque clearance upon treatment with anti-Aβ antibodies. Kelly et al. address the current applications of intravital multiphoton microscopy to image astrocytes *in vivo* in AD mouse models. These include, among others, the topical use of the dye sulforhodamine-101 (SR-101) to study astrocyte morphology and distribution (21), and the virally-mediated expression of genetically-encoded calcium indicators to track astrocyte calcium dynamics as a functional read-out (e.g., calcium waves at both intracellular and network scales) (22). Practical examples of these functional studies include the investigation of spontaneous calcium transients as a function of proximity to amyloid plaques and cerebral amyloid angiopathy (CAA)-affected vessels, and the examination of evoked calcium transients in paradigms of functional hyperemia.

In summary, although acknowledging that the topic of glial cells in AD is a rapidly evolving field, we believe that the present Frontiers Research Topic/eBook will provide the interested readers with the most recent developments on the role of reactive astrocytes and activated microglia to AD pathophysiology, and the latest technical advances to study and image these glial cells *in vitro* and *in vivo* in AD patients and mouse models.

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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Deciphering the Astrocyte Reaction in Alzheimer's Disease

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Reactive astrocytes were identified as a component of *senile* amyloid plaques in the cortex of Alzheimer's disease (AD) patients several decades ago. However, their role in AD pathophysiology has remained elusive ever since, in part owing to the extrapolation of the literature from primary astrocyte cultures and acute brain injury models to a chronic neurodegenerative scenario. Recent accumulating evidence supports the idea that reactive astrocytes in AD acquire neurotoxic properties, likely due to both a gain of toxic function and a loss of their neurotrophic effects. However, the diversity and complexity of this glial cell is only beginning to be unveiled, anticipating that astrocyte reaction might be heterogeneous as well. Herein we review the evidence from mouse models of AD and human neuropathological studies and attempt to decipher the main conundrums that astrocytes pose to our understanding of AD development and progression. We discuss the morphological features that characterize astrocyte reaction in the AD brain, the consequences of astrocyte reaction for both astrocyte biology and AD pathological hallmarks, and the molecular pathways that have been implicated in this reaction.

Keywords: Alzheimer's disease, amyloid plaques, astrocytes, glia, microglia, neurofibrillary tangles

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INTRODUCTION

The term "glia" was first coined by Virchow to refer to the non-neuronal cells that form the "glue" of the brain (Virchow, 1858). Different types of glial cells were distinguished in the early years, including astroglia (van Lenhossék, 1895), microglia (del Río-Hortega and Penfield, 1892) and oligodendroglia (del Río-Hortega, 1921).

Astroglia or astrocytes were named after their stellate shape under the microscope. Soon after the development of appropriate staining methods, it became apparent that both acute (i.e., traumatic brain or spinal cord injury, stroke) and chronic (epilepsy, neurodegenerative diseases) insults to the central nervous system (CNS) are associated with a dramatic change in astrocyte morphology. In these conditions astrocytes appear hypertrophic and overexpress two intermediate-filament proteins of their cytoskeleton: glial fibrillar acidic protein (GFAP) and vimentin. These two characteristics qualify the astrocytes as "reactive", as opposed to "resting" non-reactive astrocytes, which are not truly quiescent but exert many of the functions listed below. We prefer the terms astrocytic "reaction" or "response" over "astrocytosis" or "astrogliosis", because the suffix *–osis* implies a pathological state of astrocytes or astroglia, which at present is not well characterized.

In this review article, we will first summarize the current knowledge about the physiological roles of astrocytes. A review of the morphological and molecular basis of astrocyte reaction in

Alzheimer's disease (AD) will follow next, with special emphasis in its consequences for AD pathophysiology. We will highlight the main ongoing scientific controversies and the remaining areas of uncertainty.

ASTROCYTES IN THE HEALTHY BRAIN

Morphological and Molecular Heterogeneity of Astrocytes

Traditionally astrocytes have been classified in protoplasmic and fibrous. Protoplasmic astrocytes are cortical astrocytes, with no or minimal GFAP immunoreactivity in normal conditions, and with a "bushy" appearance owing to the profuse ramification of their processes in fine prolongations or leaflets, which reach the pre-and post-synaptic elements of the neurons (**Figure 1A**). By contrast, fibrous astrocytes are GFAP-immunoreactive astrocytes found in the white matter along the myelinated axons (**Figure 1B**). Research interest has focused by far on protoplasmic cortical astrocytes, in detriment of fibrous white matter astrocytes. Subpial interlaminar astrocytes are a third kind of astrocyte (**Figure 1C**). These are exclusively found in the most superficial layer of the cortex of primates and are characteristic for the flat shape of their soma and their long perpendicular processes towards the layers III and IV of the cortex (Colombo et al., 2000; Oberheim et al., 2009).

However, the morphological, functional and molecular heterogeneity of astrocytes is much broader and just starting to emerge (Sosunov et al., 2014; Chai et al., 2017; John Lin et al., 2017). For example, using the extracellular matrix receptor CD44 as pan-astrocytic marker, cortical and hippocampal human astrocytes have been recently classified in CD44+ astrocytes with and without long processes. CD44+ astrocytes with long processes are distinct from protoplasmic astrocytes in that they are located in the subpial layer (interlaminar astrocytes), deep cortical layers and hippocampus, and express high levels of GFAP and S100β and low levels of glutamine synthetase and glutamate transporters. CD44+ astrocytes without long processes are variable in shape and number and display a mixed phenotype between protoplasmic and

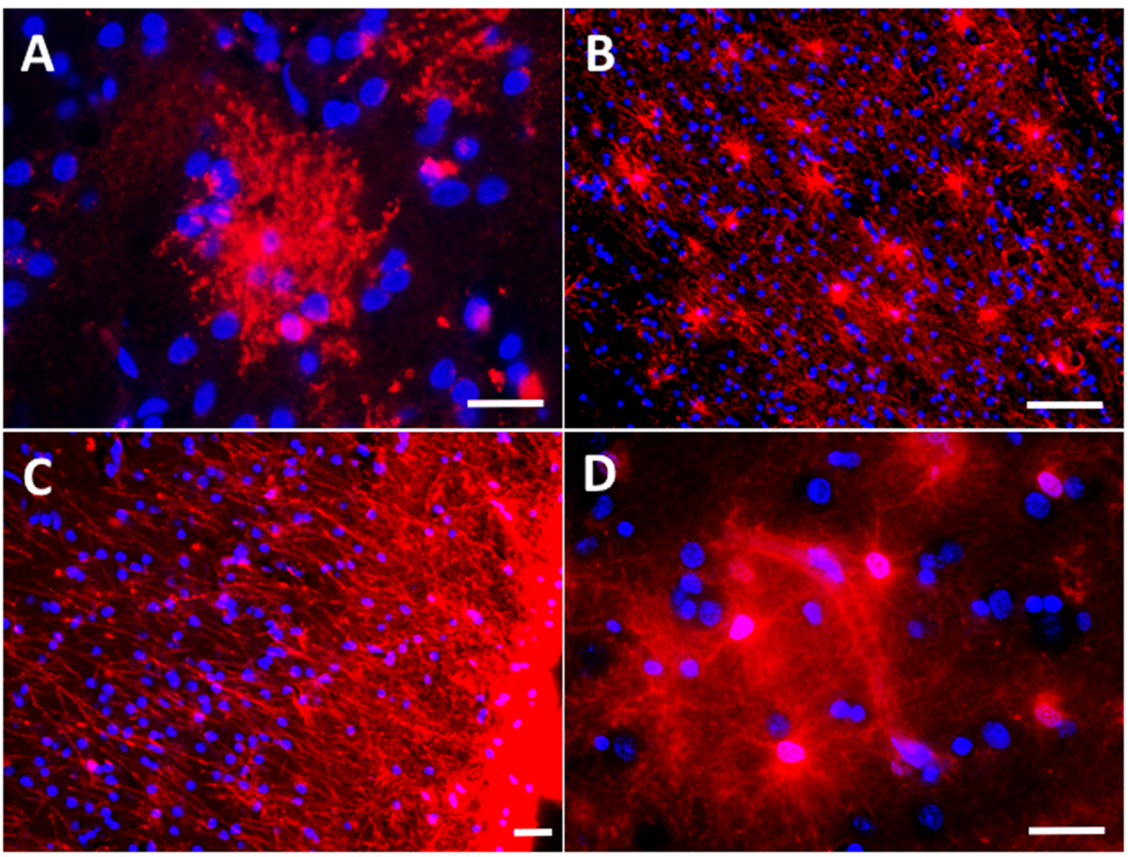

FIGURE 1 | Astrocyte morphological features in the normal brain. **(A)** Protoplasmic astrocyte in layer II of the occipital neocortex with its typical bushy appearance depicted with immunohistochemistry for the glutamate transporter GLT-1/EAAT2. **(B)** Fibrous astrocytes in the white matter of the temporal lobe are rich in glial fibrillary acidic protein (GFAP). **(C)** Subpial interlaminar astrocytes in the frontal association neocortex of a healthy control individual form a palisade of processes which extend towards deep layers perpendicularly to the cortical surface and are GFAP-immunoreactive. **(D)** Perivascular astrocytes with their vascular endfeet wrapping a capillary vessel, here shown with immunohistochemistry for aldehyde dehydrogenase 1 L1 (constitutively present in all astrocytes). Scale bars: 10 μm in **(A,C,D)**; 20 μm in **(B)**.

fibrous astrocytes (Sosunov et al., 2014). A regional network-specific specialization of astrocytes has recently been unveiled. For example, mouse striatal and hippocampal astrocytes differ in some morphological aspects (higher astrocyte-neuron ratio in striatal, shorter astrocyte-synapse distance in hippocampal), electrophysiological properties (calcium spontaneous and evoked responses in hippocampal astrocytes), and transcriptomic and proteomic signature (Chai et al., 2017). Up to five distinct subpopulations of astrocytes have been distinguished in the mouse CNS, with variable proportions depending on the CNS region, each characterized by a characteristic transcriptomic program, and a different migration, proliferative and synaptogenic potential during development (John Lin et al., 2017).

Physiological Roles of Astrocytes

A prolific research in the last two decades has expanded the role of astrocytes from a mere structural function to critical functions in CNS development, modulation of synaptic activity and glutamate homeostasis, blood-brain barrier (BBB) formation and neurovascular coupling, and inflammatory response. The basis of this astrocyte functional specialization remains largely unraveled, but it is plausible that specific subpopulations of astrocytes with distinct morphology and molecular equipment carry out different functions in different neural circuits (Chai et al., 2017).

Neurodevelopment

The refinement of the neural circuits during CNS development requires axon growth and synapse formation as well as pruning of redundant unnecessary synapses and axons. Recently, both microglia and astrocytes have been implicated in these processes. Astrocytes have been shown to promote the formation of excitatory synapses during CNS development (Allen et al., 2012) and to engulf and eliminate both excitatory and inhibitory synapses during prenatal development, and also in the adult brain (Chung et al., 2013).

Synaptic Function

Approximately 60% of excitatory synapses in the CA1 region of the rat hippocampus are tripartite, that is, have an astrocyte leaflet next to the presynaptic bouton and the postsynaptic dendritic spine (Ventura and Harris, 1999). Both cortical and hippocampal astrocytes are distributed in essentially non-overlapping domains (so called "islands") with very little inter-digitation between their fine processes, a phenomenon termed "tiling" (Bushong et al., 2002; Halassa et al., 2007). In the mouse cortex, each astrocyte wraps an average of four neurons and up to 600 dendrites from different neurons (Halassa et al., 2007). The intimate relationship between astrocytes and neurons and the ratio astrocyte-neuron enable astrocytes to coordinate synaptic networks.

Astrocytes can modulate synaptic transmission and plasticity mainly by the re-uptake of glutamate from the synaptic cleft through their membrane glutamate transporters GLT-1 (also called excitatory amino acid transporter 2 or EAAT2; **Figure 1A**) and GLAST (also called excitatory amino acid transporter 1 or EAAT1). GLT-1 is more abundant than GLAST. Of note, the degree of astrocytic coverage of synapses is thought to be changing and this dynamic process can also impact the concentration and time course of glutamate at the synaptic cleft. If the coverage is reduced or there is a down-regulation of glutamate transporters, the resulting increased glutamate concentration for a prolonged time can cause either its spillover and activation of extra-synaptic neuronal NMDA receptors, leading to neuronal excitotoxicity, or its binding to presynaptic neuronal metabotropic glutamate receptors (mGluRs class III) leading to inhibition of glutamate release from the presynaptic neuron (Oliet et al., 2001; **Figure 2A**).

In addition, several groups have proposed that astrocytes can release small molecules that act as modulators of synaptic activity, a phenomenon they termed *gliotransmission*. The main purported gliotransmitters are adenosine triphosphate (ATP) and D-serine. During long term potentiation (LTP), astrocytes would release ATP to the synaptic cleft, which would be then rapidly hydrolyzed to adenosine by extracellular ectonucleotidases. Adenosine would bind A1 adenosine receptors in neurons to suppress excitatory transmission in neighboring non-stimulated pathways, a function named *heterosynaptic depression* (Pascual et al., 2005). On the other hand, astrocytes would synthesize and release D-serine, which would bind the glycine site in the NMDA receptor of post-synaptic neuron, a binding that is necessary for the opening of the NMDA receptor gate upon glutamate binding and for LTP (Henneberger et al., 2010). Extracellular D-serine is at least as abundant as glycine but an up to three times more potent ligand of the glycine site. To produce D-serine, astrocytes would uptake L-serine from the extracellular space and convert it with the cytosolic enzyme serine racemase using pyridoxal 5'-phosphate as cofactor, whereas D-serine degradation is carried out by the also cytosolic enzyme D-amino acid oxidase.

Recent studies, however, have challenged the concept of gliotransmission and a heated debate is currently ongoing (Fiacco and McCarthy, 2018; Savtchouk and Volterra, 2018). The gliotransmission body of evidence was largely built upon observations of *in vitro* studies using primary astrocyte cultures as well as *in vivo* studies using a dominant-negative SNARE (dnSNARE) mouse model under the GFAP promoter to suppress synaptic-like vesicle release specifically in astrocytes (Pascual et al., 2005). The observation that this promoter is leaky to neurons, that is, the dnSNARE transgene was largely expressed by neurons rather than astrocytes, has essentially invalidated many of the above reports (Fujita et al., 2014; Sloan and Barres, 2014). Similarly, the notion that D-serine is produced and released by astrocytes has recently been challenged (Wolosker et al., 2016). Using BAC-transgenic mice expressing enhanced green fluorescent protein (eGFP) under the serine racemase promoter (*Srr*), Ehmsen et al. (2013) elegantly showed that serine racemase is expressed almost exclusively in neurons. In any case, D-serine levels have been reported to be unchanged in soluble cortical extracts from AD subjects compared to aged controls (Chouinard et al., 1993; Nagata et al., 1995).

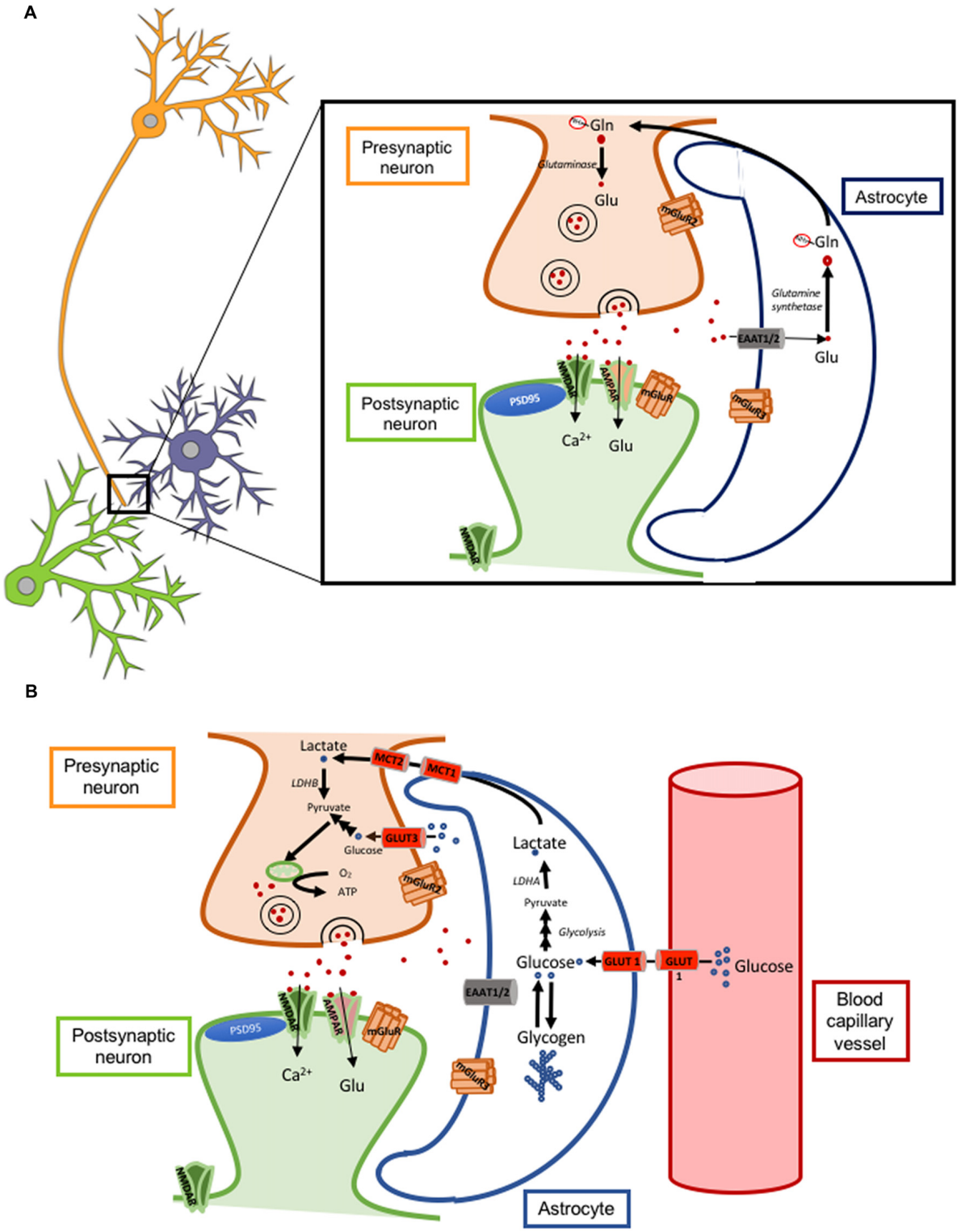

FIGURE 2 | Astrocyte-neuron glutamate-glutamine cycle and lactate shuttle. **(A)** Cartoon illustrating a tripartite excitatory synapse with the glutamate-glutamine cycle. **(B)** Cartoon illustrating a tripartite excitatory synapse and a glioneurovascular unit with the lactate shuttle. Abbreviations: AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; ATP, adenosine triphosphate; EAAT, excitatory amino acid transporter; Gln-NH3, glutamine; Glu, glutamate; GLUT, glucose transporter; LDH, lactate dehydrogenase; MCT, monocarboxylate transporter; mGluR, metabotropic glutamate receptor; NMDAR, N-methyl-D-aspartate receptor.

Neurovascular Unit

The astrocytic endfeet are a structural part of the BBB together with the endothelial cells, basement membrane and pericytes (**Figure 1D**). Together with pericytes (Hall et al., 2014), astrocytes are thought to help coordinate blood flow with neuronal activity, a concept known as *neurovascular coupling* (Mishra et al., 2016). In addition, astrocytes control water flux between the brain and the bloodstream through surface water channels called aquaporins (AQ), especially AQ1 (also expressed in choroid plexus) and AQ4 (also expressed in ependymal cells). Moreover, astrocytes have recently been involved in the paravascular clearance of toxic solutes through the expression of AQ4 in the astrocyte perivascular endfeet. The discovery of this mechanism was possible by tracking small fluorescent tracers injected in the subarachnoid space of living mice with multiphoton microscopy through a craniotomy. This novel drainage system, proposed by Nedeegaard and termed *glymphatic pathway*, posits that the arterial pulse wave within the brain determines the rapid movement of cerebrospinal fluid (CSF) from the subarachnoid cortical space into the paravascular space of the penetrating arteries, and from them to the capillary beds and the interstitial fluid (ISF), where an exchange of toxic solutes would take place. Efflux of these solutes would then occur though the paravenous spaces. Sleep, anesthesia, exercise, body posture—supine and specially lateral positions—facilitate this glymphatic transport, whereas sleep deprivation and prone position reduce its rate (Xie et al., 2013; Lee et al., 2015; He et al., 2017; von Holstein-Rathlou et al., 2018). This clearance system has been reported to largely rely on the expression of AQ4 in the astrocyte perivascular endfeet, because it is severely impaired in AQ4 knock-out mice (Iliff et al., 2012, 2013). The implications of these findings in AD pathophysiology will be discussed in detail below.

Energy Metabolism

Glucose is the main source of energy for the brain. Glucose utilization has been traditionally correlated with neuronal activity and, since the mid 1980s, the radiotracer 18-fluorodeoxyglucose ([^{18}F]-FDG) has been used for PET imaging of neuronal activity and for the diagnosis of AD, where there is a typical symmetric bilateral temporo-parietal hypometabolism. However, a recent study with micro-PET in rats has elegantly demonstrated that astrocytes also actively uptake glucose and contribute, at least to some extent, to the brain [^{18}F]-FDG PET signal (Zimmer et al., 2017). Of note, the uptake of glucose by astrocytes is coupled with the uptake of glutamate, because blocking the glutamate transporter GLT-1 prevents glucose utilization and aerobic glycolysis in astrocytes (Zimmer et al., 2017). Astrocytes store the glucose in the form of glycogen and contribute to fuel neurons by supplying lactate in certain situations such as hypoglycemia and ischemia, a phenomenon often referred to as *lactate shuttle* (Mächler et al., 2016; **Figure 2B**). Of note, lactate has been reported to be necessary for LTP and memory formation by supplying energy to the neurons (Suzuki et al., 2011).

MORPHOLOGICAL FEATURES OF ASTROCYTE REACTION IN ALZHEIMER'S DISEASE

Reactive Astrocytes Associate With Alzheimer's Pathological Hallmarks

AD is the most common neurodegenerative disease and the most common cause of dementia. Pathologically, AD is defined by the presence of two core lesions: amyloid plaques and neurofibrillary tangles (NFTs). Amyloid plaques are extracellular deposits of the *amyloid* β *peptide* (Aβ), which is a normal by-product resulting from the sequential cleavage of the transmembrane protein amyloid β precursor protein (AβPP) by the aspartyl-proteases β- and γ-secretases. NFTs are intracellular inclusions of the microtubule associated protein *tau*, which in the AD brain is aberrantly hyperphosphorylated and misfolded. Besides the development of these lesions, AD is associated with the disappearance of synapses, dendritic branches and neurons (Serrano-Pozo et al., 2011a). The first descriptions of the existence of prominent cortical astrocytic and microglial reactions in the AD brain date from the late 1980s (Beach and McGeer, 1988; Beach et al., 1989; Itagaki et al., 1989), but amyloid plaques, NFTs, and their effects on neurons and synapses have monopolized researchers' interest for a long time, and the role of non-neuronal cells such as microglia and astrocytes is only recently gaining scientific momentum.

While reactive astrocytes are not exclusive of AD, the relationship between reactive astrocytes and AD pathological hallmarks, particularly amyloid plaques, is the most intimate in any neurodegenerative disease. In fact, senile amyloid plaques are defined among other features by the presence of a cluster of reactive astrocytes that penetrate and embrace amyloid deposits with their processes, fragmenting and isolating plaques from the surrounding neuropil (Itagaki et al., 1989; **Figures 3A,B**), and reactive astrocytes follow the laminar distribution of amyloid plaques in the association cortex (Beach and McGeer, 1988). Postmortem quantitative neuropathological studies have shown that the number of reactive astrocytes in the vicinity of amyloid plaques increases as the disease advances (Pike et al., 1995; Vehmas et al., 2003) and is independent of plaque size and apolipoprotein E (*APOE*) genotype (**Figures 3C–F**).

The association between reactive astrocytes and NFTs—the other core pathological lesion of the disease—has received much less attention, not only in AD but also in all the other tauopathies such as Pick's disease, progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), argyrophilic grain disease (AGD), or chronic traumatic encephalopathy (CTE). However, immunohistochemical and electron microscopy studies have shown that reactive astrocytes can also penetrate with their processes the extracellular "ghost" NFTs present in the midst of the neuropil in advanced AD (Ikeda et al., 1992a,b; **Figures 4A–D**). Therefore, these end-stage NFTs can exhibit both tau and GFAP immunoreactivities

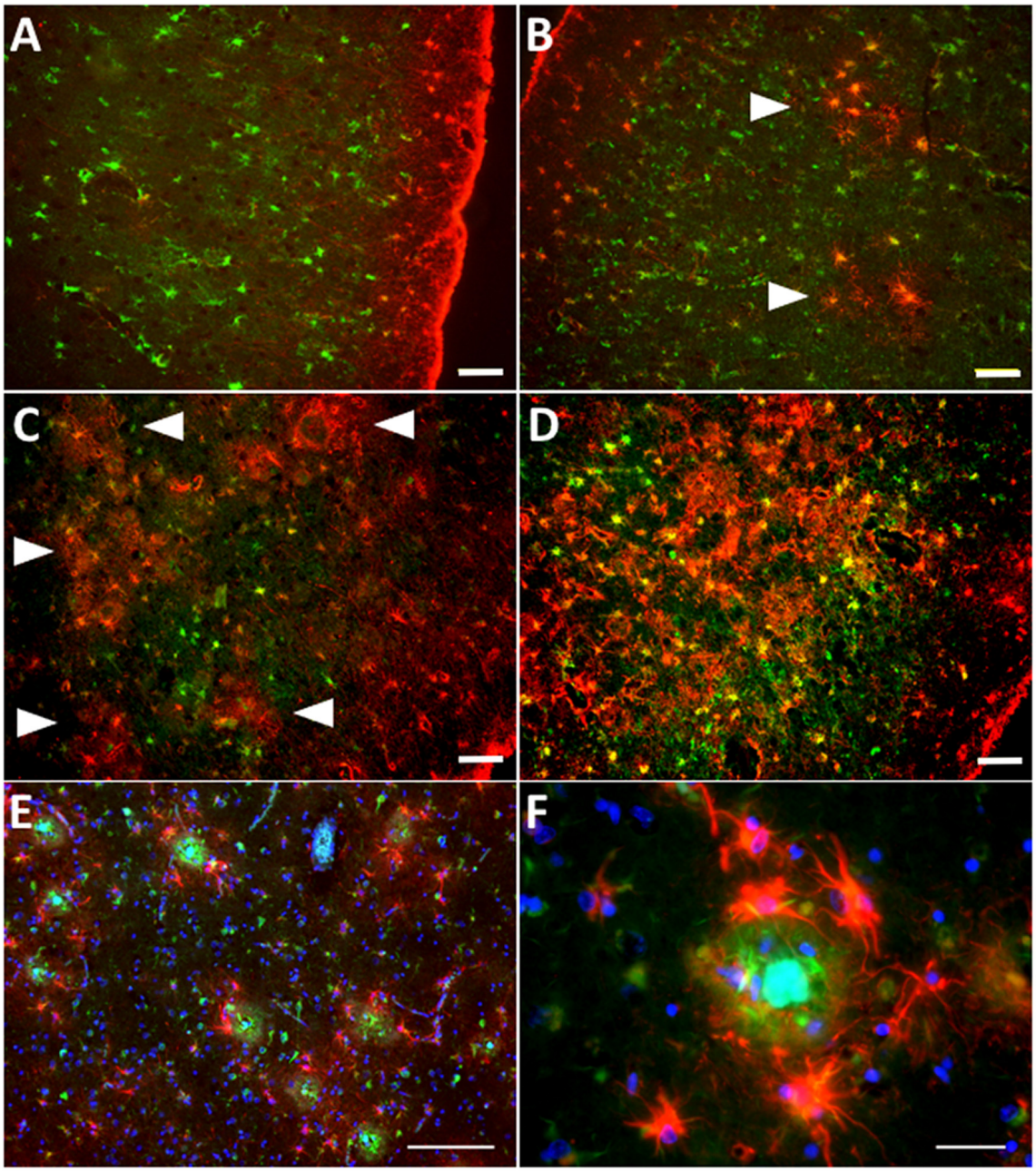

FIGURE 3 | Astrocyte reaction to amyloid plaques in the Alzheimer's brain. **(A–D)** Double fluorescent immunohistochemistry for GFAP (red) and the enzyme glutamine synthetase (green) showing the progression of astrocyte reaction (defined by GFAP immunoreactivity) in the temporal association neocortex (Brodmann's area 38) of a healthy control subject without Alzheimer's disease (AD) changes **(A)**, another healthy control individual with sparse neuritic plaques **(B)**, and two patients with an AD diagnosis **(C,D)**. Arrowheads point to increasingly numerous clusters of GFAP-immunoreactive astrocytes, which become confluent in advanced stages of the disease. **(E)** Clusters of GFAP-immunoreactive astrocytes (red) surrounding dense-core amyloid plaques (Thioflavine-S positive, green) in the temporal association neocortex (Brodmann's area 38) of an AD patient. **(F)** GFAP-immunoreactive astrocytes penetrate and surround dense-core amyloid plaques with their processes. Scale bars: 50 μm in **(A–D)**, 100 μm in **(E)**, and 10 μm in **(F)**.

(Probst et al., 1982; Irwin et al., 2012). Postmortem quantitative neuropathological studies have shown that this spatial association between reactive astrocytes and NFTs also parallels the progression of the disease (Simpson et al., 2010).

Migration or Just Reorientation of Processes?

When astrocytes respond to an experimental acute injury and become reactive, they occupy the same neuropil volume and do not lose their even and non-overlapping distribution within

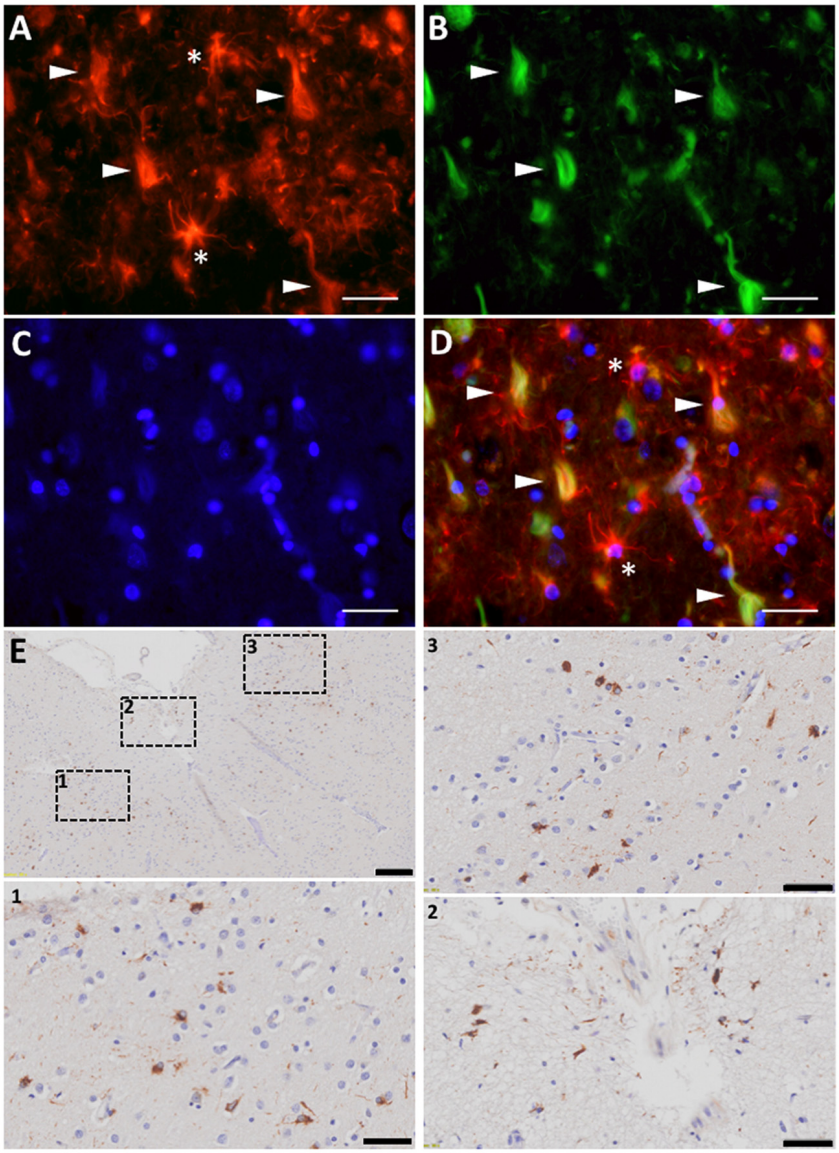

FIGURE 4 | Astrocyte reaction to neurofibrillary tangles (NFTs) in the Alzheimer's brain. **(A–D)** Fluorescent immunohistochemical staining for GFAP (**A**, red) with Thioflavine-S (**B**, green) and DAPI (**C**, blue) staining in the temporal association neocortex (Brodmann's Area 38) of an individual with a diagnosis of AD. Reactive (GFAP+) astrocytes surround and penetrate extracellular "ghost" NFTs, so that these late-stage extracellular NFTs become GFAP-immunoreactive (**D**, merge). **(E)** Peroxidase-DAB immunohistochemical staining for total tau showing thorn-shaped tau-immunoreactive astrocytes in the subpial (inset 2) and mid layers (insets 1 and 3) of the frontal association cortex (Brodmann's areas 8/9) of a subject with a diagnosis with AD. Scale bars: 10 μm in **(A–D)**, 200 μm in **(E)**, and 50 μm in insets 1–3.

the neuropil despite the hypertrophy and increased number of their main processes (Wilhelmsson et al., 2006). Whether reactive astrocytes migrate towards amyloid plaques or just reorient their processes towards them has not been investigated in detail. This distinction is important because the physiologic distribution of astrocytes in non-overlapping domains described above is thought to be crucial for their homeostatic and synaptic functions.

Several studies provide indirect evidence that astrocytes direct their processes but do not substantially move their cell bodies towards the plaques. The observation that the number of reactive astrocytes does not correlate with plaque size argues against their chemotactic migration towards plaques and in favor of a reaction of local "resting" or homeostatic astrocytes. A more recent statistical physics-based spatial analysis of astrocytes imaged *in vivo* in the cortex of APPswe/PS1dE9 and wild-type littermates confirmed that plaques in these transgenic mice do not alter the spatial distribution of astrocytes much (Galea et al., 2015). By contrast, homeostatic microglial cells are known to be highly dynamic and motile surveillants of the brain

(Nimmerjahn et al., 2005), activated microglial cells have been shown to migrate towards dense-core amyloid plaques in mice *in vivo* (Meyer-Luehmann et al., 2008), and the number of activated microglial cells in the vicinity of plaques does correlate with plaque size, supporting a chemotactic effect (Serrano-Pozo et al., 2013b).

The reorientation of astrocyte processes towards amyloid plaques is dependent on the intermediate filaments of the cytoskeleton because depletion of GFAP and vimentin from astrocytes in a mouse model of AD causes a failure of astrocytes to penetrate within plaques and a decrease in astrocyte-plaque overlap (Kraft et al., 2013; Kamphuis et al., 2015). This phenomenon is so prominent that the palisade of long interlaminar astroglial processes, characteristic of the supragranular layers of the isocortex of primates, is severely disrupted or even virtually absent in AD and Down's syndrome patients with AD neuropathological changes (Colombo et al., 2000, 2002, 2005). Importantly, these interlaminar astrocytes are likely major contributors to the microcolumnar functional organization of neurons in the isocortex (perpendicular to the pial surface), which is disrupted in AD, with thinner microcolumns and wider spaces between them (Buldyrev et al., 2000).

The up-regulation of GFAP in reactive astrocytes can be so dramatic in advanced AD that the GFAP protein can deposit in the astrocyte primary processes forming eosinophilic elongated structures called Rosenthal fibers (Wegiel and Wisniewski, 1994). These Rosenthal fibers are very much like those that define Alexander's disease, a rare, usually childhood-onset, progressive leukodystrophy that causes spastic quadriparesis, seizures and intellectual decline. Interestingly, Alexander's disease is due to mutations in the *GFAP* gene that result in decreased solubility and subsequent deposition of the GFAP protein within the cytoplasm of the white matter fibrous astrocytes (Hsiao et al., 2005; Hagemann et al., 2006). The insolubility of GFAP in advanced AD and Alexander's disease could have deleterious effects in astrocyte biology by both overwhelming their protein degradation systems, autophagy (Tang et al., 2008) and proteasome (Tang et al., 2010; Orre et al., 2013), and restricting their range of migration and process motion.

Proliferation or Just Phenotypic Change?

Because reactive astrocytes are conventionally identified by their GFAP immunoreactivity and GFAP expression in non-reactive (resting) astrocytes is often below the detection level of immunohistochemistry, it is not uncommon to misinterpret the enhanced GFAP immunoreactivity seen in the AD brain as evidence of astrocyte proliferation. A few studies have found increased expression of cell division markers (i.e., cyclins, PCNA, Ki67) within astrocytes in the brain of AD subjects (Nagy et al., 1997; Wharton et al., 2005).

Despite these studies, there is more substantial and stronger evidence against proliferation of astrocytes or glial progenitors in AD. Experiments injecting BrdU in plaque-bearing mice have definitively demonstrated that only microglia exhibits significant proliferation, particularly in the proximity of plaques (Bondolfi et al., 2002; Kamphuis et al., 2012; Luccarini et al., 2012; Sirko et al., 2013). The same conclusion was achieved by a recent human postmortem study using the proliferative marker PCNA, Aβ and Iba1 or GFAP triple immunohistochemistry (Marlatt et al., 2014). Similar numbers of GFAP-positive astrocytes were found in wild-type and 3xTg AD mice hippocampus, where >80% astrocytes are GFAP-immunoreactive (Olabarria et al., 2010). Last, the number of cortical astrocytes was found to be similar between healthy and AD brains in two stereology-based quantitative human postmortem studies: one using morphological identification of astrocytes in Nissl-stained sections (Pelvig et al., 2003), and another using double fluorescent immunohistochemistry with GFAP and aldehyde dehydrogenase 1 isoform L1 (ALDH1L1) or glutamine synthetase as pan-astrocytic constitutive markers (Serrano-Pozo et al., 2013a).

Immortal or Senescent and Mortal?

Cell death is the other side of the coin with respect to cell proliferation. The finding of a similar number of astrocytes in AD and normal brains and the lack of correlation between total astrocyte number and AD progression could have two interpretations: (1) there is no significant astrocyte proliferation or death in either the normal or the AD brain; and (2) there is significant proliferation and death but both rates are similar. Several postmortem studies using terminal transferase-mediated dUTP-biotin nick end labeling (TUNEL) and caspase 3 activated immunohistochemistry (Smale et al., 1995; Li et al., 1997; Kobayashi et al., 2004) have reported apoptotic astrocytes, but other authors have not found astrocyte apoptosis (Sugaya et al., 1997). These studies are likely confounded by the cause of death and the potential pro-apoptotic effects of the agonal period immediately before death. The finding of increased CSF levels of astrocytic markers in AD patients with respect to healthy control subjects, including GFAP (Jesse et al., 2009; Ishiki et al., 2016) and glutamine synthetase (Gunnersen and Haley, 1992; Tumani et al., 1999), argues in favor of some astrocyte cell death.

Another possibility is that astrocytes reach a senescent state in the AD brain. Morphologically, senescent-looking astrocytes have been characterized in the hippocampus and entorhinal cortex of 3xTg AD mice as astrocytes that are located far from amyloid plaques (>50 μm), with atrophied cell somas, and simplified processes (Olabarria et al., 2010; Yeh et al., 2011). However, other authors have reported that the size of astrocyte somas far from amyloid plaques (>60 μm) in an APP/PS1 transgenic mouse model is comparable to that of wild-type mice (Brawek et al., 2018). Another more dramatic morphological change that may resemble astrocyte senescence is the so-called *clasmatodendrosis*. Clasmatodendritic astrocytes are characteristic for perikaryal swelling and accumulation of lysosomes and vacuoles, condensed chromatin leading to pyknotic nuclei, and fragmented or beaded processes. Recently, astrocytes expressing green-fluorescent protein (GFP) under the GFAP promoter have been reported to be sensitive to clasmatodendrosis in a plaque-bearing AD mouse model (Daschil and Humpel, 2016). However, it should be noted that this phenomenon has been described mostly in the fibrous astrocytes from periventricular and deep white matter

lesions of individuals with vascular dementia and/or AD, rather than in cortical astrocytes. Moreover, the development of clasmatodendrosis has been attributed to ischemia, hypoxia, local disruption of the local BBB and acidification of the local environment, rather than to the toxicity of AD pathological hallmarks (Tomimoto et al., 1997; Sahlas et al., 2002; Chen et al., 2016). $p16^{INK4a}$ and matrix metalloproteinase 1 (MMP1) have been proposed as molecular markers defining senescent astrocytes (Bhat et al., 2012).

FUNCTIONAL CONSEQUENCES OF ASTROCYTE REACTION IN ALZHEIMER'S DISEASE

As with the morphological aspects, considerable controversy surrounds the topic of the functional consequences of astrocyte reaction in AD. Conceptually, AD-associated astrocyte reaction could entail a loss of the normal functions of the astrocyte (i.e., modulation of synaptic function, BBB integrity and function), a gain of a new toxic function (i.e., inflammation), or both.

Plaque Formation or Plaque Clearance?

Whether reactive astrocytes contribute to plaque formation and maturation or to Aβ clearance and plaque growth restriction is still debated. Multiple immunohistochemical and immunoelectron microscopy postmortem studies on the AD brain have revealed that astrocytes and, to a lesser extent, microglia, contain granules of non-fibrillar Aβ, presumably engulfed from extracellular diffuse amyloid deposits (Akiyama et al., 1996, 1999; Funato et al., 1998; Yamaguchi et al., 1998; Thal et al., 2000; Oide et al., 2006). However, this observation has been also interpreted as evidence for an active participation of reactive astrocytes in plaque formation (Nagele et al., 2003), rather than clearance. A feed-forward mechanism has been proposed by which reactive astrocytes could up-regulate BACE1 and promote the amyloidogenic processing of AβPP in response to noxious stimuli such as inflammation (Zhao et al., 2011) or ischemia (Hartlage-Rübsamen et al., 2003). It is noteworthy that Aβ-containing astrocytes have been found preferentially near fleecy N-terminally truncated Aβ deposits in the human entorhinal cortex (Thal et al., 2000) and that astrocytes have been implicated in the generation and secretion of N-terminally truncated Aβ in a BACE1-independent manner (Oberstein et al., 2015).

On the other hand, *ex vivo* and *in vivo* experiments applying adult mouse or human astrocytes on the brain of plaque-bearing mice have confirmed that astrocytes uptake and clear Aβ, with diffuse deposits over larger fibrillar aggregates being preferentially removed (Koistinaho et al., 2004; Pihlaja et al., 2008, 2011; Nielsen et al., 2009, 2010), These experimental studies are in agreement with a postmortem immunohistochemical study that has confirmed the presence of oligomeric protofibrillar forms of Aβ within reactive astrocytes using conformation-specific antibodies (Lasagna-Reeves and Kayed, 2011). Two main mechanisms have been proposed: secretion of Aβ-degrading enzymes and phagocytosis (uptake and lysosomal degradation).

The phagocytosis of Aβ by astrocytes requires the participation of APOE because APOE null astrocytes are not efficient at amyloid plaque removal (Koistinaho et al., 2004). Using intracortical microinjection of fluorescently-labeled Aβ and *in vivo* multiphoton microscopy through a craniotomy in 5xFAD mice—characterized by a fast amyloid plaque deposition, it has been shown that the phagocytic potential of astrocytes declines with aging (Iram et al., 2016). Interestingly, in these mice the complement factor C1q can facilitate Aβ phagocytosis by astrocytes (Iram et al., 2016). Enhancing lysosomal biogenesis in astrocytes through viral delivery of the transcription factor EB (TFEB) results in increased Aβ phagocytosis and degradation in APP/PS1 mice (Xiao et al., 2014).

Postmortem immunohistochemical studies have revealed that astrocytes express some of the known Aβ-degrading enzymes. For example, endothelin-converting enzyme-2 (ECE-2) is up-regulated in AD astrocytes, although also in neurons and some microglial cells (Palmer et al., 2009). MMP3 is present in plaques and in white matter fibrous astrocytes in the AD brain (Yoshiyama et al., 2000; but see also, Baig et al., 2008). Astrocyte deficiency of lipoprotein-related protein 1 (LRP1), an apoE receptor, reduces the secretion of Aβ-degrading enzymes such as matrix metalloproteases (MMPs) 6 and 9 and insulin degrading enzyme (IDE) and accelerates amyloid plaque deposition (Liu et al., 2017). Of note, IDE expression has been shown to increase in reactive astrocytes around plaques in the Tg2576 AD mouse model (Leal et al., 2006).

It should be noted that both amyloid plaque burden and plaque size distribution remain relatively stable throughout the disease progression, indicating that there is no significant net plaque growth and clearance, that is, the clearance mechanisms might just enable to neutralize growth but not to effectively eliminate plaques (Hyman et al., 1995; Serrano-Pozo et al., 2011b, 2012). Although depletion of GFAP and vimentin from astrocytes led to a failure of penetration into amyloid plaques in a mouse model of AD, it is uncertain whether plaque growth and amyloid deposition subsequently accelerate because the two existing studies showed conflicting results (Kraft et al., 2013; Kamphuis et al., 2015). Therefore, although there is no proportionality between plaque size and number of surrounding reactive astrocytes (Serrano-Pozo et al., 2013b), it is plausible that reactive astrocytes form an effective physical barrier around the plaques and limit their growth.

Interestingly, besides Aβ, astrocytes can accumulate other proteins associated with neurodegeneration such as tau and α-synuclein. The presence of tau-immunoreactive "tufted" astrocytes pathologically defines PSP, whereas the finding of tau-immunoreactive "astrocytic plaques" defines the pathological diagnosis of CBD. An aging-related tau astrogliopathy (ARTAG) has recently been described with "thorn-shaped" and "granular fuzzy" tau-positive astrocytes in the medial temporal lobe and other brain regions (Kovacs et al., 2016; Liu et al., 2016). These thorn-shaped astrocytes have also been identified in the brain of individuals with AD (Lace et al., 2012; López-González et al., 2013; **Figure 4E**). In fact, classic electron microscopy studies in the 1990s already described the presence of paired helical filaments

of tau in astrocytes in AD (Ikeda et al., 1992a; Yamazaki et al., 1995; Arima et al., 1998). Of note, although NFTs in AD are composed of similar amounts of three repeat (3R) and four repeat (4R)-tau, astrocyte tau immunoreactivity in AD, ARTAG, and the classic tauopathies PSP and CBD is predominantly 4R-tau, not 3R. Whether astrocytes have a predilection for up-taking 4R-tau over 3R-tau, or whether they degrade 3R-tau more readily than 4R-tau, remains unknown. Similarly to tau, α-synuclein accumulation has been found within astrocytes in the basal forebrain of subjects with sporadic Parkinson's disease (PD; Braak et al., 2007). Although it is possible that astrocytes express low levels of these genes and up-regulate their expression in these neurodegenerative diseases, it is more plausible that they take up misfolded forms of these proteins from the ISF, since both tau and α-synuclein are thought to be released by neurons at the synapse and to propagate from neuron to neuron trans-synaptically (Luk et al., 2012; de Calignon et al., 2012). Whether and how astrocyte reaction impacts the prion-like propagation of these proteins, and the consequences of these astrocyte proteinopathies for the astrocyte biology remain to be investigated.

Neuroprotection or Neurotoxicity?

A simplistic view of reactive astrocytes as neuroprotective in AD alludes to the formation of a scar-like physical barrier between the amyloid plaques and the surrounding neuropil. Reactive astrocytes around amyloid plaques may, thus, limit the collateral damage from diffusible soluble Aβ oligomeric species by isolating the reservoirs of these especially neurotoxic Aβ species. For example, in a quantitative neuropathological study from the population-based Medical Research Council-Cognitive Function and Aging Study (MRC-CFAS), the number of diffuse and compact plaques lacking astrocyte reaction in layer VI of the cingulate cortex was independently associated with worse cognition (lower MMSE score; Mathur et al., 2015). The attenuation of astrocyte reaction by depletion of GFAP and vimentin led to a multiplication of plaque-associated dystrophic neurites, also suggesting a net neuroprotective effect of reactive astrocytes in AD (Kraft et al., 2013). On the other hand, reactive astrocytes around neuritic plaques have recently been shown to engulf plaque-associated dystrophic neurites of APPPS1 mice and AD patients and this phagocytic function has been deemed neuroprotective (Gomez-Arboledas et al., 2018). These observations contrast with other studies supporting the idea that astrocyte reaction contributes to neurodegeneration in dementia due to AD. Astrocytes isolated from aged 5xFAD offer much less neurotrophic support when co-cultured with neonatal neurons as compared with astrocytes from wildtype mice (Iram et al., 2016). Postmortem studies comparing subjects with dementia due to AD and cognitively intact subjects with high levels of amyloid plaques and NFTs (so called "high pathology control", "asymptomatic AD", or "mismatch AD" cases) have shown that the latter group lacks the prominent microglial and astrocyte responses typical of the AD brain, and have lower or normal levels of inflammatory cytokines and preserved neuron number and synaptic density (Lue et al., 1996; Perez-Nievas et al., 2013).

An unbiased way to approach this question is to study the transcriptomic profile of astrocytes from the AD brain compared to the normal aging brain. It should be noted that a substantial region-dependent shift in the astrocyte transcriptome has been reported with aging in both mouse and human normal brains (Soreq et al., 2017; Boisvert et al., 2018). Specifically, aged murine astrocytes down-regulate cholesterol synthesis and up-regulate synaptic elimination and immune pathways, whereas homeostatic and glutamate neurotransmission genes do not appear to change much with aging (Boisvert et al., 2018). Several human postmortem studies using laser capture microdissection (LCM) of astrocytes in brains from AD patients and non-demented healthy controls have investigated the transcriptomic changes that occur in AD astrocytes. Simpson et al. (2011) used GFAP to identify and capture astrocytes by LCM and compared the transcriptomic profile between subjects with an advanced Braak stage of NFTs (V–VI) and subjects with low Braak stages (I–II). They observed a dysregulation of genes associated with the actin cytoskeleton, proliferation, apoptosis, and ubiquitin-mediated proteolysis at low Braak stages, that contrasted with an altered regulation of intracellular signaling pathways, including insulin, phosphatidylinositol 3-kinase (PI3K)/Akt, and mitogen-activated protein kinase (MAPK) pathways at high levels of AD pathology. In another more recent LCM-based transcriptomic study using ALDH1L1 rather than GFAP as astrocytic marker, the authors found an up-regulation of genes encoding both astrocytic immune response and mitochondrial machinery in the posterior cingulate gyrus—an area of abundant and early Aβ deposition—of AD patients compared to healthy subjects (Sekar et al., 2015). The advantage of using ALDH1L1 instead of GFAP is that non-reactive "resting" GFAP-negative astrocytes are better represented in the healthy control group, allowing for the detection of more subtle differences in gene expression between health and disease.

Although meritorious, the results of human postmortem transcriptomic studies should be taken with caution because they can be affected by several confounders including the effects on gene expression of the cause of death and the agonal period prior to death (i.e., hypoxia, ischemia, sepsis), the postmortem interval, and the common presence of mixed pathologies such as Lewy bodies and cerebrovascular disease. Moreover, it is not easy to dissect the effect of amyloid plaques and NFTs on astrocyte gene expression. By contrast, transcriptomic studies in transgenic mice are devoid of these confounders. Orre et al. (2014) performed a transcriptional analysis on acutely isolated astrocytes from the cortex of aged controls and APPswe/PS1dE9 AD mice using GLT-1 as astrocytic marker for fluorescently-assisted cell sorting (FACS). These mice develop amyloid plaques similar to human AD plaques, but not NFTs. In this transgenic AD mouse model, astrocytes exhibited a proinflammatory immune phenotype and a reduced expression of neuronal support genes and genes involved in neuronal communication. Based on the

distinct transcriptomic profiles observed in mouse models of acute brain injury, such as the lipopolysaccharide (LPS) model of neuroinflammation and the stroke model of middle cerebral artery occlusion, Barres proposed a classification of astrocytes in neurotoxic (A1) and neuroprotective (A2; Zamanian et al., 2012). These authors recently showed that, *in vitro*, A1 astrocytes lose their ability to promote neuronal survival, neurite outgrowth, synapse formation and phagocytosis, leading to neuron and oligodendrocyte death, and postulated that A1 astrocytes can be identified by the expression of complement fraction 3 (C3) and are abundant in many human neurological diseases such as AD, amyotrophic lateral sclerosis, Huntington's disease, and multiple sclerosis (Liddelow et al., 2017). Following this classification, an age-dependent neurotoxic transcriptomic signature (A1) has recently been reported in astrocytes isolated from a tauopathy mouse model under the human *APOE*ε4 knock-in (KI) background, as compared with the *APOE*ε3 KI background (Shi et al., 2017).

Thus, taken together, astrocyte transcriptomic studies from human AD brain and AD mouse models support the idea that astrocyte reaction in AD involves a gain of neurotoxic function and loss of neuroprotective function of astrocytes.

Effects on Synaptic Function

There is growing evidence indicating that astrocyte reaction impairs the normal function of astrocytes as modulators of neuronal synaptic transmission. Most studies have focused on glutamatergic transmission and have attributed reactive astrocytes a crucial participation in glutamate-mediated neuronal excitotoxicity.

Using *in vivo* multiphoton microscopy through a craniotomy and the glutamate-sensitive probe iGluSnFR delivered with an intracortical injection of an adeno-associated viral vector, Hefendehl et al. (2016) have recently reported that the microenvironment surrounding plaques in an APPPS1 mouse model has chronically elevated glutamate concentrations, and that neurons in the immediate vicinity of plaques do not appropriately respond to stimuli, such as hindlimb pinch in the somatosensory cortex or visual stimuli in the visual cortex, as judged by the *in vivo* imaging analysis of calcium transients and glutamate concentration. This increased peri-plaque glutamate concentration correlated with a reduced expression of GLT-1/EAAT2 in the reactive (GFAP+) astrocytes surrounding plaques, and was partially corrected by the intravenous administration of the antibiotic ceftriaxone, which is known to up-regulate the expression of GLT1-/EAAT2 by astrocytes (Hefendehl et al., 2016). Of note, soluble Aβ oligomers reduce the expression of the glutamate transporter GLT-1/EAAT2 in astrocyte cultures through a mechanism involving calcineurin (CN)/nuclear factor of activated T cells (NFAT) pathway (Abdul et al., 2009) and oxidative stress (Scimemi et al., 2013). This reduction of GLT-1/EAAT2 expression by astrocytes parallels the progression of AD pathology in the human brain (Simpson et al., 2010). Recently, it has been proposed that preservation of GLT-1/EAAT2 expression in GFAP+ reactive astrocytes could be a mechanism of resilience against AD neuropathological changes (Kobayashi et al., 2018). Other authors have shown a decreased solubility of GLT-1/EAAT2 in the brain of patients with AD, which could potentially impair the re-uptake of synaptic glutamate (Woltjer et al., 2010).

A second proposed mechanism for astrocyte-mediated neurotoxicity is the downregulation of glutamine synthetase expression level or activity, leading to a subsequent reduction in the astrocyte capacity to detoxify neuronal glutamate to glutamine. Indeed, acute viral-induced astrocyte reaction can induce neuronal glutamate excitotoxicity via down-regulation of the astrocyte cytosolic enzyme glutamine synthetase (Ortinski et al., 2010) and glutamine synthetase levels have been reported to be reduced in the 3xTg mouse prefrontal cortex (Olabarria et al., 2011; Kulijewicz-Nawrot et al., 2013). However, whether this is also the case in the human AD brain remains unclear (Le Prince et al., 1995; Tumani et al., 1999; Serrano-Pozo et al., 2013a). Notwithstanding these conflicting reports, glutamine synthetase catalytic activity has been shown to be sensitive to oxidation and may be impaired by oxidative damage in AD (Smith et al., 1991; Hensley et al., 1994, 1995). Perhaps as compensatory mechanism against glutamate excitotoxicity, glutamine synthetase can be up-regulated in groups of cortical pyramidal neurons in AD brains, although not selectively in the vicinity of plaques (Robinson, 2000; Serrano-Pozo et al., 2013a). The common denominator of all these mechanisms is thought to be an increase in extracellular glutamate levels leading to excessive activation of extra-synaptic NMDA receptors and, thereby, neuronal excitotoxicity (Li et al., 2011). This common mechanism could explain the finding of calcium overload (Kuchibhotla et al., 2008) and hyperactive neurons (Busche et al., 2008, 2012) found in the proximity of plaques using multiphoton calcium imaging in live APP/PS1 mice, although the latter finding was attributed to a decreased gabaergic inhibition instead of an excess of glutamate.

A third alternative novel mechanism proposed for astrocyte-mediated neurotoxicity is an enhancement of gabaergic tone, rather than excessive glutamatergic transmission or reduced gabaergic inhibition. Reactive astrocytes from plaque-bearing AD mouse models have been shown to induce neuronal tonic inhibition via increase in GABA release to the synaptic cleft (Mitew et al., 2013; Jo et al., 2014; Wu et al., 2014). A recent detailed histological study has described a transient increase in GABA immunoreactivity of astrocytes in middle aged (but not young or very old) APP/PS1 transgenic mice, specifically in reactive astrocytes surrounding dense-core plaques (Brawek et al., 2018). This mechanism could explain the co-existence of an abnormally high proportion of silent neurons in multiphoton calcium imaging studies on living APP/PS1 mice (Busche et al., 2008, 2012).

Effects on Blood Brain Barrier Integrity and Function

Besides forming amyloid plaques in the brain parenchyma, Aβ can deposit in the wall of small cortical and leptomeningeal vessels and capillaries, a condition called cerebral amyloid

angiopathy (CAA). CAA can occur without concomitant AD pathology, but more commonly is present in up to 90% of individuals with a postmortem diagnosis of AD, usually with a mild degree (Serrano-Pozo et al., 2011a). In addition to the possibility of causing lobar intracerebral and focal subarachnoid hemorrhages due to vessel rupture, CAA can cause brain hypoperfusion and subsequent ischemia, and independently contributes to AD-related cognitive decline (Neuropathology Group of the Medical Research Council Cognitive Function and Ageing Study, 2001; Pfeifer et al., 2002; Arvanitakis et al., 2011; Serrano-Pozo et al., 2013c). Moreover, an increased CSF/plasma albumin ratio, indicating increased BBB permeability, has been repeatedly shown in AD and other dementias (Skoog et al., 1998; Bowman et al., 2007; Skillbäck et al., 2017).

Since astrocytes are part of the BBB, the contribution of astrocyte reaction to BBB disruption and amyloid plaque and CAA accumulation are gaining increasing research interest. Does astrocyte reaction affect BBB integrity in AD and CAA? Indeed, a number of astrocyte abnormalities have been described in the neurovascular unit of mouse models of AD and CAA and human AD brains including: (1) swelling and detachment of astrocyte endfeet; (2) altered secretion of proteins that are part of the basement membrane or the extracellular matrix; (3) reduction of both endothelial and astrocytic glucose transporter 1 (Glut1); (4) reduced astrocytic expression of monocarboxylate transporter 1 (MCT1) resulting in a decreased lactate release; and (5) loss of potassium and water channels (AQ; Wilcock et al., 2009; Hawkes et al., 2011; Merlini et al., 2011). Furthermore, astrocyte-dependent cerebral vasoreactivity was reported to be impaired in amyloid-laden vessels in a mouse model of CAA (Kimbrough et al., 2015).

In addition, it is possible that astrocyte reaction impairs the clearance of Aβ at the BBB. Weller et al. (2008) proposed that Aβ is cleared from the ISF through a perivascular drainage from capillaries to cortical and leptomeningeal arteries through the basement membranes. They proposed that the suction effect after the arterial pulse wave drives this perivascular drainage and that the stiffening of the arterial walls with aging and vascular risk factors could attenuate this arterial pulsatility and favor Aβ accumulation in capillaries and arteries in the form of CAA and in the brain parenchyma in the form of amyloid plaques (Weller et al., 2008; Hawkes et al., 2011, 2014; Arbel-Ornath et al., 2013). In 2012, Nedergaard redefined this hypothesis by proposing that toxic solutes including Aβ are cleared from the ISF through a paravascular drainage system that involves astrocytes and would serve as the brain lymphatic system, hence the term *glymphatic* (Iliff et al., 2012, 2013). Specifically, the glymphatic system hypothesis postulates that there is an inflow of CSF from the subarachnoid space to the paravascular space of penetrating arteries, between the basement membrane of the endothelium and the smooth muscle cell layer; then, low molecular weight solutes including Aβ pass to the ISF through the astrocyte end-feet of the BBB and efflux from the brain parenchyma through the paravenous space by a convective bulk-flow driven by the arterial pulse wave and the arterio-venous pressure gradient, rather than simple diffusion. Noteworthy, sleep has been shown to potentiate this transport system (Xie et al., 2013), and sleep deprivation has been shown to enhance Aβ deposition (Kang et al., 2009). This glymphatic drainage system would be largely dependent on the expression of AQ4 in the astrocyte perivascular endfeet, because genetic deletion of AQ4 severely impairs this glymphatic system, disrupts the paravascular flow of Aβ (Iliff et al., 2012), and leads to an increased amyloid burden in the form of both plaques and CAA, and further cognitive impairment in APP/PS1 mice (Xu et al., 2015). In addition, aging has been associated with a decreased perivascular localization of AQ4 and, thus, impaired paravascular clearance of endogenous Aβ in wild-type mice (Kress et al., 2014).

However, these studies have been recently disputed. Using computational models and similar multiphoton microscopy experiments in wild-type and AQ4 null living mice and rats, the Verkman lab has refuted both the hypothesis that arterial pulse-driven convective bulk flow rather than simple diffusion is responsible for the vast part of solute movement through the brain parenchyma, as well as the involvement of AQ4 in solute transport in the rodent brain (Jin et al., 2016; Smith et al., 2017). Another recent study applied computational modeling to 3D-reconstruction of electron microscopy images of the neuropil and concluded than diffusion rather than bulk-flow accounts for the transport of interstitial solutes (Holter et al., 2017). Other computational modeling studies have tried to explain cerebral metabolite clearance by proposing that there is a network of astrocytes connected through AQ4 channels that serve as sites of low resistance to bulk flow, and that the arterial pulsation determines a fast para-arterial transport through dispersion (the combined effect of local mixing and diffusion in the para-arterial space), rather than bulk flow (Asgari et al., 2015, 2016). Studies on AQ in the human AD brain have also yielded conflicting results. Aquaporin 1 (AQ1) immunoreactivity has been shown to increase in close proximity to amyloid plaques and CAA in some studies (Misawa et al., 2008; Hoshi et al., 2012), whereas other authors found no difference in AQ1 immunoreactivity between AD and healthy control subjects, reported AQ1 to be expressed primarily in the white matter rather than the cortical astrocytes, and observed a decrease in expression with aging (Moftakhar et al., 2010). Furthermore, in another study, the researchers only found significantly increased levels of AQ1 by Western blot at early stages of the disease (Braak II; Pérez et al., 2007). With regards to aquaporin 4 (AQ4), no significant differences in protein levels were found between AD and healthy control subjects by Western blot (Pérez et al., 2007), but an enhanced immunoreactivity associated with amyloid plaques and CAA has been reported by other authors (Moftakhar et al., 2010; Hoshi et al., 2012), suggesting the possibility of a redistribution of AQ4 within astrocytes in AD. More recently, in a population-based clinic-pathological study, Zeppenfeld et al. (2017) reported an increased global AQ4 immunoreactivity in AD patients compared to aged and young healthy controls, associated with a loss of perivascular localization that correlated inversely with an increased plaque burden and a higher Braak NFT stage, which seemingly reconciles the human data with the mouse data above.

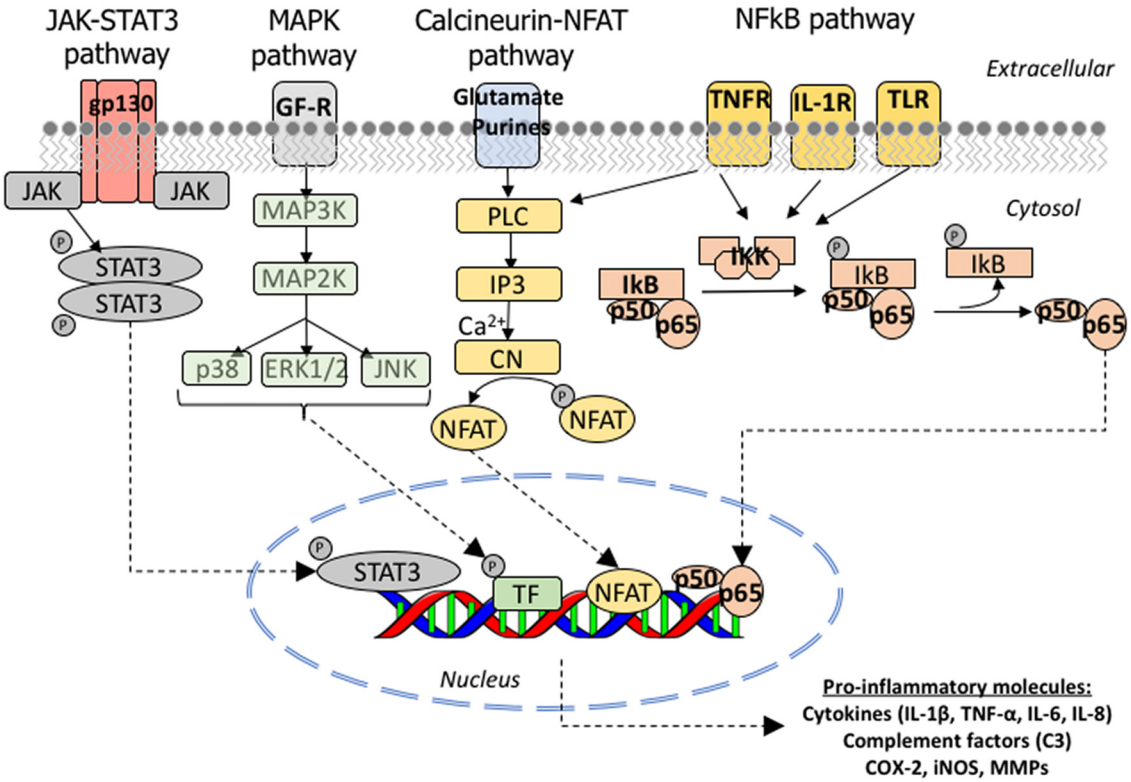

FIGURE 5 | Molecular signaling pathways involved in astrocyte reaction in AD. Cartoon illustrating the JAK/STAT3, MAPK, CN/NFAT and NFκB signaling cascades. *JAK/STAT3 pathway*: the JAKs (JAK 1, 2, 3 and Tyk2) are tyrosine-kinases associated with the intracellular domain of cytokine receptors. The binding of a cytokine ligand to its receptor triggers a dimerization or oligomerization of the associated JAK and this leads to its transphosphorylation and activation. The activated JAK then phosphorylates key tyrosine residues in the cytokine receptor, which leads to the recruitment of STAT (STAT1, 2, 3, 4, 5A, 5B or 6) and a single tyrosine phosphorylation at the Y of its C-terminus. Once phosphorylated, STAT forms homo or heterodimers and is imported to the nucleus, where it complexes with other transcription factors, notably NFkB, to bind its target genes. *MAPK pathway*: the p38 mitogen-activating protein kinase (p38 MAPK) is a family of serine-threonine kinases of which p38α is the most studied due to its ubiquitous expression at high levels. p38α phosphorylates multiple substrates including transcription factors, DNA and RNA binding proteins, other serine-threonine kinases (including GSK3β), and cell cycle and pro-apoptotic proteins. *Calcineurin/NFAT pathway*: the phosphatase function of CN is activated upon the binding of calcium and calmodulin to its regulatory subunit. Once activated, CN removes several phosphate residues from the N-terminus of the transcription factors NFATc (nuclear factor of activated T cells) proteins, named NFATc1 to 4, and exposes their nuclear localization motif, leading to their rapid entry into the nucleus. NFATc targets are largely genes encoding cytokines, growth factors and their receptors, and cell adhesion proteins, as well as many microRNAs. *NFκB pathway*: NFκB is a heterodimeric transcription factor composed of the subunits p65/RelA and p50, which belong to the Rel family of proteins. It is normally located in the cytoplasm in an inactive form due to its binding to the inhibitor IκB, which masks its nuclear localization signal located in the p65 subunit. Activation of IKK complex by certain extracellular signals can dissociate NFκB and IκB by promoting the phosphorylation of the IκB-α, which is a necessary step for IκB-α ubiquitination and subsequent degradation by the proteasome. Once free from IκB, NFκB can translocate to the nucleus and bind to the 10 base pair consensus-sequence GGGACTTTCC GGGRNNYYCC in the promoter of its target genes (NFκB response elements). Abbreviations: CN, calcineurin; COX, cyclo-oxygenase; ERK, extracellular signal-regulated kinases; IL, interleukin; IL1-R, interleukin 1 receptor; iNOS, inducible nitric oxide synthetase; IP3, inositol-3-phosphate; JAK, Janus kinase; MAPK, mitogen-activated protein kinase; MMPs, matrix metalloproteases; NFAT, nuclear factor of activated T cells; PL3, phospholipase 3; STAT, signal transducer and activator of transcription; TLR, toll-like receptor; TNF-α, tumor necrosis factor α; TNF-R, tumor necrosis factor receptor.

SIGNALING PATHWAYS INVOLVED IN ASTROCYTE REACTION IN ALZHEIMER'S DISEASE

To date, *in vivo* and postmortem evidence has implicated four main signaling pathways in the astrocyte reaction in AD: the Janus kinase (JAK)/STAT3, the calcium/CN/NFAT, the NFκB and the MAPK pathways. **Figure 5** depicts these molecular cascades that transmit information from extracellular signals to target genes in the nucleus. Other pathways probably involved in astrocyte reaction but with less *in vivo* supporting evidence are calpain and caspase activation.

JAK/STAT3 Pathway

The JAK-STAT (signal transducer and activator of transcription) is a signaling pathway activated by extracellular cytokines (Mertens and Darnell, 2007). The levels of STAT1 protein were shown to be increased in both the cytosolic and particulated fractions from the cortex of AD patients compared to healthy subjects (Kitamura et al., 1997).

Recently, STAT3 immunoreactivity was shown to be increased in the nucleus of GFAP- and vimentin-immunoreactive astrocytes in the APPswe/PS1dE9 and 3xTg mouse models of AD. Lentiviral expression of suppressor of cytokine signaling protein 3 (SOCS3)—a negative regulator of this pathway, which is upregulated by STAT and switches off the activity of JAK—specifically in astrocytes led not only to a decrease in nuclear STAT3 immunoreactivity but also to a reduction of GFAP immunoreactivity, implicating the JAK/STAT3 pathway in astrocytic reaction (Ben Haim et al., 2015).

Calcium/Calcineurin/NFAT Pathway

The calcium/CN/NFAT pathway links cytosolic calcium levels to gene expression (Crabtree and Schreiber, 2009). CN has been reported to play a central role in AD pathophysiology both in neuronal and astrocytic phenotypes. In neurons, CN excessive activation has deleterious consequences, both functional and morphological. Soluble Aβ oligomers bind to NMDA receptors and increase cytosolic calcium, leading to CN activation and enhanced long term depression (LTD), which contributes to memory impairment. The morphological substrate of CN overactivation is the triad of synaptic loss, dystrophic neurites, and neuron loss (Wu et al., 2010). Inhibiting CN/NFAT pathway in APP/PS1 mice, either by treatment with the CN inhibitor immunosuppressant drug tacrolimus (also called FK-506; Rozkalne et al., 2011) or through the viral-mediated neuronal expression of the specific inhibitor peptide VIVIT, restored this phenotype (Hudry et al., 2012).

Soluble Aβ oligomers also determine a cytosolic calcium overload and CN activation in astrocytes *in vitro*, which leads to an overexpression of GFAP and, subsequently, a reactive phenotype characterized by the release of inflammatory cytokines and the down-regulation of the glutamate transporter GLT-1/EAAT2 (Norris et al., 2005; Abdul et al., 2009). *In vivo* studies also support CN overactivation in astrocytes. Resting calcium level and frequency and synchrony of calcium transients are increased in reactive astrocytes from APP/PS1 mice (Kuchibhotla et al., 2009; Delekate et al., 2014). CN immunoreactivity is increased in hippocampal astrocytes of aged wild-type mice and APP/PS1 mice (Norris et al., 2005). A high activity proteolytic fragment of CN with 45–48 KDa molecular weight range is highly expressed by astrocytes surrounding plaques in human postmortem brain sections (Pleiss et al., 2016). In the human AD hippocampus, CN-Aα and NFAT3 have been shown to increasingly translocate to the astrocyte nucleus as the disease advances, whereas NFAT1 has been shown to translocate to the nucleus mainly in the stage of mild cognitive impairment (Abdul et al., 2009). Inhibition of CN/NFAT pathway in hippocampal astrocytes through the selective viral-mediated expression of VIVIT in astrocytes improved cognitive and synaptic function, reduced glial activation, lowered Aβ levels, increased GLT-1 expression, and reduced glutamate-mediated neuronal excitotoxicity in plaque-bearing AD mice (Furman et al., 2012; Sompol et al., 2017).

NFκB Pathway

The NFκB pathway is activated by membrane receptor signals from Toll-like receptors, TNFα receptor, T-cell receptor and B-cell receptor. Among NFκB target genes are cytokines (Il-1, IL-6, TNFα, COX-2), complement proteins (i.e., C3), major histocompatibility complex (MHC) class 1 and 2, β-2 microglobulin and APOE (Hayden et al., 2006). Of note, complement fraction C3 has been proposed as a specific marker of a subtype of reactive GFAP-positive astrocytes that are neurotoxic (so-called A1 astrocytes; Liddelow et al., 2017).

Among the stimuli that can trigger this pathway are $A\beta_{1-40}$ and $A\beta_{1-42}$, S100β protein and reactive oxygen species (ROS). *In vitro* experiments in primary culture of rat cortical astrocytes have shown that Aβ at nM or μM concentrations can activate NFκB leading to an upregulation of pro-inflammatory cytokines IL-1 and IL-6 (Bales et al., 1998) and inducible nitric oxide synthetase (iNOS; Akama et al., 1998). The astrocytic secreted S100β protein can also up-regulate iNOS in cultured astrocytes in an autocrine fashion through a NFκB-dependent mechanism (Lam et al., 2001).

Importantly, suppression of NFκB in the APPswe/PS1dE9 ameliorated astrocytic reaction. However, intriguingly, the level of the NFκB inhibitor IκB-α was normal in 3xTg AD mice compared to wild-type littermates, arguing against NFκB activation in this mouse model (Ben Haim et al., 2015). The levels of NFκB p65 protein were shown to be increased in the cortex from AD patients compared to healthy subjects (Kitamura et al., 1997). However, neuropathological studies in AD brains addressing the cell type responsible for this increase have revealed that NFκB immunoreactivity is increased in the cytoplasm and nucleus of neurons, especially those surrounding amyloid plaques or bearing a NFT (Terai et al., 1996; Kaltschmidt et al., 1997; Ferrer et al., 1998). According to these studies the astrocytic NFκB immunoreactivity is either undetectable (Ferrer et al., 1998), only present in the cytoplasm of cortical layer I astrocytes and to a similar extent than in control brains (Terai et al., 1996), or only present in reactive astrocytes around diffuse plaques (Kaltschmidt et al., 1997).

MAPK Pathway

The mitogen-activating protein kinase (MAPK) pathway is represented by three main families of kinases, all of which are activated by extracellular signals: the p38 MAPK, the c-Jun kinase (JNK), and the extracellular signal-regulated kinases (ERK 1 and 2).

The p38 MAPK together with Jun kinase (JNK) are known as the stress kinases. p38α is activated by very diverse extracellular stimuli including UV light, heat and osmotic shock, oxidative stress, cytokines, chemokines, hormones and growth factors and is thought to have a key role in the cell response to many extracellular threats, leaning the fate of the cell towards survival vs. apoptosis. Inhibition of the p38 MAPK pathway in astrocytes has anti-inflammatory effects in astrocytes *in vitro* (Da Silva et al., 1997; Bhat et al., 2012).

The ERKs are essentially activated by trophic factors and are thought to play an important role in synaptic plasticity. Importantly, a postmortem immunohistochemical

study revealed that this pathway is upregulated in the AD brain specifically in GFAP+ reactive astrocytes, but only in the white matter and only at early AD stages corresponding to mild dementia. In moderate and advanced AD dementia, the astrocytic expression was equivalent to non-demented control individuals and predominated in cortical pyramidal neurons, their axons, and plaque-associated dystrophic neurites (Webster et al., 2006). The authors speculated with a protective role of this signaling pathway in astrocytes against early neuronal and synaptic damage.

APOLIPOPROTEIN E GENOTYPE AND ASTROCYTE REACTION

The *APOE*ε4 allele remains the strongest genetic risk factor for the development of AD. Compared to the most common genotype in the general population ε3/ε3, carrying one copy of the ε4 allele increases the risk of developing AD ∼2–3 times, whereas homozygous (ε4/ε4) individuals have a ∼8–12-fold higher risk. Moreover, the ε4 allele anticipates the onset of AD in a dose-dependent manner (Corder et al., 1993). By contrast, the ε2 allele is protective against the development of AD (Corder et al., 1994; Serrano-Pozo et al., 2015). The main normal function of the APOE is the transport of cholesterol within the brain in the form of high-density lipoprotein (HDL) particles. However, apoE4 has been shown to favor Aβ accumulation by promoting its aggregation in the form of amyloid plaques (Hyman et al., 1995) and soluble Aβ oligomers (Hashimoto et al., 2012), and by reducing Aβ clearance (Castellano et al., 2011). Remarkably, although large clinico-pathological studies have established that the *APOE*ε4 allele does not increase either the burden or the Braak stages of NFTs independently of amyloid plaques (Serrano-Pozo et al., 2015; Farfel et al., 2016), apoE4 has recently been shown to promote tau pathology and neurodegeneration in a mouse model of tauopathy (Shi et al., 2017).

Because, together with microglia, astrocytes are the main source of apoE within the brain, whether the *APOE* genotype has any influence on the astrocyte reaction found in the AD brain is a research topic of growing interest. Using stereology-based quantitative methods in postmortem human AD brain specimens, *APOE*ε4 carriers and non-carriers did not differ significantly in either the number of total, resting (GFAP-negative), or reactive (GFAP-positive) astrocytes (Serrano-Pozo et al., 2013a), the progression of astrocyte reaction along the clinical course of the disease (Serrano-Pozo et al., 2011b), or the proximity of the association between reactive astrocytes and plaques (Serrano-Pozo et al., 2016), but see also (Mathur et al., 2015). However, it is still possible that reactive astrocytes behave differently in different *APOE* genetic backgrounds. For example, *APOE*ε4 KI mice show increased numbers of reactive astrocytes and activated microglia, increased levels of pro-inflammatory cytokines, and decreased levels of synaptic markers after administration of LPS, compared with *APOE*ε3 and *APOE*ε2 KI mice, suggesting that *APOE*ε4 astrocytes may be more susceptible to react to pro-inflammatory stimuli (Zhu et al., 2012). Indeed, E4FAD and P301S/E4 mice (corresponding to the 5xFAD mouse model of brain β-amiloidosis and the P301S tau transgenic mouse of tauopathy under a human *APOE*ε4 KI background, respectively) have a hyperactivated microglia and increased levels of proinflammatory cytokines as compared to the *APOE*ε3 and *APOE*ε2 KI double transgenic mice (Rodriguez et al., 2014; Shi et al., 2017). On the other hand, human inducible pluripotent stem cell (hiPSC)-derived astrocytes from *APOE*ε4/ε4 human subjects exhibit a loss of neurotrophic function with respect to neurons and synapses *in vitro* (Zhao et al., 2017). In another study, *APOE*ε4 astrocytes showed decreased potential to phagocytose synapses, whereas this ability was enhanced in *APOE*ε2 astrocytes compared to *APOE*ε3. However, *APOE*ε4 KI mice had an increased proportion of C1q-tagged synapses (and *APOE*ε2 KI mice a decreased proportion) compared to *APOE*ε3 KI mice, which would make them vulnerable to elimination by microglia (Chung et al., 2016).

CROSS-TALK BETWEEN ASTROCYTES AND MICROGLIA

Although astrocytes and microglia share the consideration of glial cells, microglial cells are thought to derive from mesenchymal cells of the yolk sac. Microglia are the innate immune cells of the brain, likewise macrophages in other organs. As such, they are highly dynamic cells that continuously survey the brain tissue in normal conditions, migrate to areas of injury (Nimmerjahn et al., 2005), and phagocytose virus, bacteria and neuronal debris (Fuhrmann et al., 2010). There are multiple lines of evidence supporting the existence of a cross-talk between astrocytes and microglia in AD. First, like astrocytes, activated microglia decorate dense-core amyloid (senile) plaques (Itagaki et al., 1989; Serrano-Pozo et al., 2013b, 2016), where they establish an intimate relationship with astrocytes (Bouvier et al., 2016). Second, there is a strong correlation between the number of activated microglial cells and that of reactive astrocytes, and both parallel disease progression (Serrano-Pozo et al., 2011b). Third, the attenuation of astrocyte reaction around plaques observed in APP/PS1: *GFAP/Vimentin* double knock out mice is associated with increased numbers of activated microglia around plaques (Kraft et al., 2013). Fourth, the "paracrine" secretory function of microglial cells can change astrocyte phenotype and vice versa. Inflammatory cytokines secreted by microglia (i.e., TNFα, IL-1, C1q) can transform neuroprotective resting astrocytes (A2) into neurotoxic (A1). Conversely, astrocytes can release C3 complement fraction via NFκB in response to oligomeric Aβ and C3 can in turn activate microglia through its C3R receptor (Lian et al., 2016). Fifth, microglial processes can be present in excitatory synapses together with astrocyte processes and pre- and post-synaptic neuronal elements, forming a "quadripartite" synapse. Activated microglia can cause direct synaptotoxicity through the secretion of C1q (Hong et al., 2016). Sixth, astrocytes appear to influence the degree of microglial reactivity in mouse models of brain β-amyloidosis (Rodriguez et al., 2014) and tauopathy (Shi

et al., 2017) in an apoE isoform-dependent fashion (E4 > E3 > E2). Thus, it is likely that reactive astrocytes and activated microglia do not "play solo", but act in a concerted fashion.

BIOMARKERS OF ASTROCYTE REACTION

Since astrocyte reaction appears to be linked to the neurodegenerative process in AD, it follows that imaging and CSF biomarkers of astrocyte reaction could be useful to improve the accuracy of the clinical diagnosis of AD dementia and monitor and predict the progression of the disease.

With regards to CSF biomarkers, initially a candidate-based approach led researchers to measure the levels of classic astrocyte markers in CSF such as GFAP, S100β, and glutamine synthetase. Compared to healthy controls, GFAP levels have been found to be higher in the CSF of AD, dementia with Lewy bodies (DLB), frontotemporal lobar degeneration (FTLD) and Creutzdfeldt-Jakob disease (CJD) patients, whereas S100β was found to be elevated in CJD, but not in AD. Reports on elevated CSF levels of glutamine synthetase in AD are conflicting (Gunnersen and Haley, 1992; Tumani et al., 1999; Timmer et al., 2015). However, it is a "non-*a priori* hypothesis" proteomic approach that has rendered the most promising CSF biomarker of astrocyte reaction to date: chitinase 3 protein-like 1 (also called YKL-40). This CSF biomarker has been shown to predict progression from normal cognition to MCI and from MCI to AD dementia (Craig-Schapiro et al., 2010). Subsequent studies confirmed a correlation between CSF YKL-40 levels and biomarkers of neurodegeneration, such as CSF total and phospho-tau levels and cortical thickness, at the earliest stages of AD (Antonell et al., 2014; Alcolea et al., 2015). However, elevated CSF YKL-40 levels are not specific of AD; the highest levels appear to occur in sporadic CJD patients, followed by AD and tauopathies (PSP, CBD and Pick's disease), whereas patients with vascular dementia and with Parkinson disease dementia or DLB have been reported to have normal levels (Llorens et al., 2017). Importantly, YKL-40 is expressed by GFAP-immunoreactive astrocytes near plaques and CAA-laden vessels (Craig-Schapiro et al., 2010; Llorens et al., 2017), but also in white matter fibrous astrocytes and in random cortical protoplasmic and perivascular astrocytes (Llorens et al., 2017). A recent postmortem quantitative neuropathological study in AD and other tauopathies has described that YKL-40 is only expressed by a subset of GFAP-immunoreactive astrocytes and that there is a positive correlation between YKL-40 and tau immunoreactivities, in agreement with the correlations found in CSF (Querol-Vilaseca et al., 2017).

The development of PET radiotracers specific for astrocyte reaction has proven to be more challenging than for activated microglia. While PET radioligands of the translocator protein 18 kDa (TSPO), also called peripheral benzodiazepine receptor (PBR), have been used to depict microglial activation *in vivo* for almost two decades (Cagnin et al., 2001), no such established radiotracer exists for reactive astrocytes. However, it should be noted that TSPO is not only up-regulated in activated microglial cells but also in reactive astrocytes, and that it is also expressed by endothelial and vascular smooth muscle cells (Cosenza-Nashat et al., 2009). Recently, [^{11}C]-deuterium-L-deprenyl ([^{11}C]-DED), a modified inhibitor of the monoamino oxidase B (MAO-B) enzyme, has been proposed as a PET imaging biomarker of astrocyte reaction (Carter et al., 2012; Schöll et al., 2015; Rodriguez-Vieitez et al., 2016). Indeed, MAO-B was shown to be up-regulated in GFAP-immunoreactive astrocytes many years ago (Nakamura et al., 1990; Jossan et al., 1991). However, the specificity of this radiotracer requires further validation because MAO-B is also expressed by neurons and the contribution of each cell type to the radiotracer uptake remains to be clarified.

The results of astrocyte-specific transcriptomic studies in the human AD brain and AD mouse models will inform the development of new CSF and PET imaging biomarkers specific of astrocyte reaction (Zamanian et al., 2012; Orre et al., 2014; Liddelow et al., 2017).

CONCLUSION

In summary, recent evidence directly implicates astrocytes in the pathophysiology of AD and supports the idea that astrocyte reaction against amyloid plaques and NFTs leads to a loss of their neurotrophic potential and a gain of neurotoxic properties. Large gaps of knowledge remain regarding the molecular pathways involved in this reaction and its functional consequences for both astrocytes themselves, and neurons and their synapses. Future research should also address the implication of each AD pathological hallmark in astrocyte reaction, and further characterize the cross-talk between astrocytes and microglia and the influence of the *APOE* genotype. Technological advances will help answer some of these questions, including some new *in vitro* tools (human induced pluripotent stem cells (hiPSCs)-derived astrocytes, 3D-cultures, brain organoids, protocols of isolation and purification of astrocytes from the adult mouse and human brains), recently developed unbiased and high-throughput molecular biology techniques (i.e., single-cell RNAseq), new mouse models and gene delivery approaches for astrocyte-specific manipulations, and more specific CSF biomarkers and PET radiotracers for *in vivo* studies of astrocyte reaction in the human brain.

AUTHOR CONTRIBUTIONS

AS-P and BGP-N designed this review outline, performed literature review, prepared the figures and wrote the manuscript.

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Glutamate Transporter GLT1 Expression in Alzheimer Disease and Dementia With Lewy Bodies

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Glutamate transporter solute carrier family 1, member 2 (GLT1/EAAT2), a major modulator of glutamate homeostasis in astrocytes, is assessed in post-mortem human brain samples of frontal cortex area 8 in advanced stages of Alzheimer disease (AD) and terminal stages of dementia with Lewy bodies (DLB) in order to gain understanding of astrogliopathy in diseases manifested by dementia. Glial fibrillary acidic protein (GFAP) mRNA expression is significantly increased in AD but not in DLB, whereas *GLT1*, vesicular glutamate transporter 1 (*vGLUT1*) and aldehyde dehydrogenase 1 family member 1 (*ALDH1L1*) are not modified in AD and DLB when compared with controls. GLT1 protein levels are not altered in AD and DLB but GFAP and ALDH1L1 are significantly increased in AD, and GFAP in DLB. As a result, a non-significant decrease in the ratio between GLT1 and GFAP, and between GLT1 and ALDH1L1, is found in both AD and DLB. Double-labeling immunofluorescence and confocal microscopy revealed no visible reduction of GLT1 immunoreactivity in relation to β-amyloid plaques in AD. These data suggest a subtle imbalance between GLT1, and GFAP and ALDH1L1 expression, with limited consequences in glutamate transport.

Keywords: Alzheimer disease, dementia with Lewy bodies, glutamate transporter 1, EAAT2, vGLUT1, glial fibrillary acidic protein, ALDH1L1

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INTRODUCTION

Alzheimer disease (AD), the main cause of dementia in old age, is characterized by β-amyloid deposition forming plaques and amyloid angiopathy, and hyperphosylated tau in neurons with neurofibrillary tangles (NFTs) and pre-tangles, dystrophic neurites of senile plaques, and neuropil threads (Duyckaerts and Dickson, 2011; Ferrer, 2012; Braak and Del Tredici, 2015). NFTs increase in number and distribution from stages I-II to stages V-VI with generalized involvement of the neocortex (Braak and Braak, 1991). Amyloid plaque burden also increases with disease progression from stages A (low density of plaques in the isocortex), B (medium density of plaques in the associative isocortex) and C (high density of plaques in motor and sensory isocortex) although there are important individual variations not related to NFTs (Braak and Braak, 1999).

β-amyloid deposition has been categorized into phase 1 involving the neocortex, phase 2 with additional involvement of the allocortex, phase 3 involving the diencephalic nuclei, striatum, and cholinergic nuclei of the basal forebrain, phase 4 involving several nuclei of the brain stem, and phase 5 with cerebellar involvement (Thal et al., 2002).

Dementia with Lewy bodies (DLB), the second most common neurodegenerative dementia in the elderly, is pathologically characterized by Lewy bodies and Lewy neurites, composed of abnormal α-synuclein in the brainstem, limbic system and cortical areas (McKeith et al., 2004; Fujishiro et al., 2008; Ince, 2011).

Glutamate transporter solute carrier family 1, member 2 (GLT1/EAAT2) expressed in astrocytes regulates glutamate levels at the synapse and plays a cardinal role in preventing excitotoxic neuronal damage in certain neurodegenerative diseases (Nedergaard et al., 2002; Maragakis and Rothstein, 2006). Expression levels of GLT1 and glutamate homeostasis in prefrontal cortex in AD are controversial as they are reported to be decreased in some studies (Masliah et al., 1996) and preserved in others (Kulijewicz-Nawrot et al., 2013). An inverse relation between increased Glial fibrillary acidic protein (GFAP) and reduced GLT1 is described in AD with disease progression as defined by Braak stage of NFT pathology (Simpson et al., 2010, 2011). Indirect data point also to functional alterations of glutamate transporters in AD as a result of oxidative damage (Lauderback et al., 2001), altered solubility (Woltjer et al., 2010), and splice variants (Scott et al., 2011). Altered mRNA and/or protein expression of glutamate transporters have been reported in transgenic models of AD (Masliah et al., 2000; Cassano et al., 2012). Astrocytes in transgenic mice expressing mutant A53T α-synuclein have reduced expression of GLT1 (Gu et al., 2010) but there is no information, as far as we know, regarding GLT1 expression in DLB.

The present study analyses GLT1 mRNA and protein expression in frontal cortex in AD and DLB in a series of post-mortem human brains in order to learn about the possible implication of this astrocytic glutamate transporter in the pathogenesis of these diseases.

MATERIALS AND METHODS

Human Cases

Brain tissue was obtained from the Institute of Neuropathology HUB-ICO-IDIBELL Biobank and the Hospital Clinic-IDIBAPS Biobank following the guidelines of Spanish legislation on this matter (Real Decreto de Biobancos 1716/2011) and approval of the local ethics committees. Processing of brain tissue has been detailed elsewhere (Ferrer, 2014). One hemisphere was immediately cut in coronal sections, 1 cm thick, and selected areas of the encephalon were rapidly dissected, frozen on metal plates over dry ice, placed in individual air-tight plastic bags, and stored at −80°C until use for biochemical studies. The other hemisphere was fixed by immersion in 4% buffered formalin for 3 weeks for neuropathological studies as detailed elsewhere (Ferrer, 2014). Neuropathological diagnosis of AD was categorized following Braak stages of NFT pathology adapted to paraffin sections (Braak and Braak, 1991; Braak et al., 2006) and Thal phases of β-amyloid plaques (Thal et al., 2002). DLB was diagnosed following accepted neuropathological criteria (Braak et al., 2003; McKeith et al., 2004; Alafuzoff et al., 2009). Middle-aged control cases (MA) had not suffered from neurological or psychiatric diseases, infections of the nervous system, brain neoplasms, systemic and central immune diseases, or metabolic diseases (including metabolic syndrome), and did not have abnormalities in the neuropathological examination excepting stages I–II of NFT pathology and phases 1–2 of β-amyloid plaques. Cases with associated pathologies such as vascular diseases (excepting mild atherosclerosis and arteriolosclerosis), TDP-43 proteinopathy, metabolic syndrome and hypoxia were excluded from the present study.

MA cases were 22 men and 17 women (n = 39, mean age 61.8 ± 14.0 years). AD cases, categorized as stages V–VI of NFT and phases 3–4 of β-amyloid, were 9 men and 11 women (n = 20, mean age 80.5 ± 6.9 years). DLB, categorized as stages 5–6 of Parkinson's disease-related pathology, Thal phase 3–4, and NFT stages III–IV, were 8 men and 1 woman (n = 9, mean age 76.4 ± 5.7). The post-mortem delay was between 3 h and 9 h 35 min in MA, 2 h 30 min and 17 h 30 min in AD, and 5 h and 9 h in DLB cases.

RNA Purification

Purification of RNA from right frontal cortex area 8 of human frozen brain was carried out using RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany) following the protocol provided by the manufacturer combined with DNase digestion to avoid extraction and later amplification of genomic DNA. The concentration of each sample was obtained from A260 measurements with a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). RNA integrity was tested using the Agilent 2100 BioAnalyzer (Agilent, Santa Clara, CA, USA). Values of RNA integrity number (RIN) were between 6.4 and 8.4 in MA, between 6.4 and 9.1 in AD, and between 5.2 and 7 in DLB. Bivariate analyses were carried out to detect association of our variables with potential confounding factors (age, post-mortem delay and RIN) using Spearman or Pearson correlations for quantitative variables (data not shown). Statistical analysis was performed with GraphPad Prism version 5.01. post-mortem delay had no effect on RIN values in MA, AD and DLB cases.

Retrotranscription Reaction

Retrotranscription reaction of RNA samples was carried out with the High-Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA, USA) following the guidelines provided by the supplier, and using Gene Amp® 9700 PCR System thermocycler (Applied Biosystems). A parallel reaction for one RNA sample was processed in the absence of reverse transcriptase to rule out DNA contamination.

Real Time PCR

Real Time quantitative PCR (RT-qPCR) assays were conducted in duplicate on 1000 ng of cDNA samples obtained from

the retro-transcription reaction, diluted 1:20 in 384-well optical plates (Kisker Biotech, Steinfurt, Germany) utilizing the ABI Prism 7900 HT Sequence Detection System (Applied Biosystems). Parallel amplification reactions were carried out using 20× TaqMan Gene Expression Assays and 2× TaqMan Universal PCR Master Mix (Applied Biosystems). Parallel assays for each sample were carried out using β-glucuronidase (*GUS-B*), X-prolyl aminopeptidase (aminopeptidase P) 1 (*XPNPEP1*) and alanyl-transfer RNA synthase (*AARS*) probes for normalization. The selection of these housekeeping genes was based on previous data showing low vulnerability in the brain of several human neurodegenerative diseases (Barrachina et al., 2006; Durrenberger et al., 2012). The reactions were performed using the following parameters: 50°C for 2 min, 95°C for 10 min, 40 cycles at 95°C for 15 s and 60°C for 1 min. TaqMan PCR data were captured using the Sequence Detection Software (SDS version 2.2, Applied Biosystems). Subsequently, threshold cycle (CT) data for each sample were analyzed with the double-delta CT (ΔΔCT) method. First, delta CT (ΔCT) values were calculated as the normalized CT values for each target gene in relation to the mean values of *GUS*-β, *XPNPEP1* and *AARS*. Second, ΔΔCT values were obtained with the ΔCT of each sample minus the mean ΔCT of the population of control samples (calibrator samples). The fold-change was determined using the equation $2^{-\Delta\Delta CT}$. Genes analyzed with the corresponding abbreviations and TaqMan probes used in the study are shown in **Table 1**. Pearson's correlation coefficient was used to assess a possible linear association between two continuous quantitative variables. To determine the relationship between gene expression and RIN values according to pathologic variables, we used the analysis of covariance. No significant correlations were found testing gender with diagnosis, post-mortem delay and RIN values. Taking into account that each group of pathological cases (AD and DLB) were processed in parallel with its respective group of controls, but not both pathologies in the same RT-qPCR plate, statistical *t*-test was chosen to compare sample groups instead of One-way ANOVA. Thus, statistical *t*-test was performed to analyze each pathological group with its respective control. Significant *p*-value: $^{**}p < 0.01$.

Gel Electrophoresis and Western Blotting From Total Homogenate

Tissue was processed as reported elsewhere (Garcia-Esparcia et al., 2015). A total of 0.1 g of frozen tissue from frontal cortex area 8 of all MA, AD and DLB cases was lysed with a glass homogenizer in Mila lysis buffer (0.5 M Tris at pH 7.4 containing 0.5 methylenediaminetetraacetic acid at pH 8.0, 5M NaCl, 0.5% Na deoxycholic acid, 0.5% Non-idet P-40, 1 mM phenylmethylsulfonyl fluoride, bi-distilled water, and protease and phosphatase inhibitor cocktails (Roche Molecular Systems, Pleasanton, CA, USA)), and then centrifuged for 15 min at 13,000 rpm at 4°C (Ultracentrifuge Beckman with 70Ti rotor, CA, USA). Protein concentration was measured with Smartspect™ plus spectrophotometer (Bio-Rad, CA, USA) using the Bradford method (Merck, Darmstadt, Germany). Samples containing 15 μg of protein and the standard Precision Plus Protein™ Dual Color (Bio-Rad) were loaded onto 10% acrylamide gels. Proteins were separated with sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to nitrocellulose membranes using the Trans-BlotTurbo transfer system (Bio-Rad) at 200 mA/membrane for 60 min. Non-specific bindings were blocked by incubation with 5% milk in Tris-buffered saline (TBS) containing 0.1% Tween for 1 h at room temperature. After washing, the membranes were incubated at 4°C overnight with appropriate primary antibodies. Anti-GLT1 (Ab1783, Merck Millipore, Darmstadt, Germany) and anti-ALDH1L1 (ab56777, Abcam, Cambridge, UK) were used at a dilution of 1:500 in TBS containing 3% albumin and 0.1% Tween; both antibodies were detected after 1 h incubation with the appropriate HRP-conjugated secondary antibody (1:2000, Dako, Glostrup, Denmark), and the immune complexes were revealed with a chemiluminescence reagent (ECL, Amersham, GE Healthcare, Buckinghamshire, UK). Membranes were washed and incubated for 1 h with anti-β-actin antibody (1:30,000, A5316; Sigma-Aldrich, St. Louis, MO, USA), and blotted to control protein loading. After the immune complexes were revealed, membranes were stripped and blocked again by incubation with 5% milk in TBS containing 0.1% Tween for 1 h at room temperature. Once membranes were washed, they were incubated at 4°C overnight with rabbit anti-human GFAP (1:30,000, Z0334, Dako, Glostrup, Denmark) and detected after 1 h of incubation with anti-rabbit HRP-conjugated secondary antibody (1:2000, Dako, Glostrup, Denmark), and the immune complexes were revealed with ECL chemiluminescence reagent. Quantification of western blot optical densities (O.D.) was performed using *ImageJ* software (Bethesda, MD, USA), and analyzed with GraphPad Prism version 5.01 software (La Jolla, CA, USA) and Statgraphics

TABLE 1 | Taqman probes used in the present study including housekeeping genes (*GUS-B*, *XPNPEP1* and *AARS*), glutamate transporter 1 (*GLT1*), vesicular glutamate transporter 1 (*vGLUT1*), aldehyde dehydrogenase 1 family member 1 (*ALDH1L1*) and glial fibrillary acidic protein (*GFAP*).

Gene	Full name	Reference
Housekeeping genes		
GUS-B	β-glucuronidase	Hs00939627_m1
XPNPEP1	X-prolylaminopeptidase (aminopeptidase P) 1	Hs00958026_m1
AARS	Alanyl-tRNA synthetase	Hs00609836_m1
Genes analyzed		
GLT1	Glutamate transporter 1	Hs01102423_m1
vGLUT1	Vesicular glutamate transporter 1	Hs00220404_m1
ALDH1L1	Aldehyde dehydrogenase 1 family member 1	Hs01003842_m1
GFAP	Glial fibrillary acidic protein	Hs00909233_m1

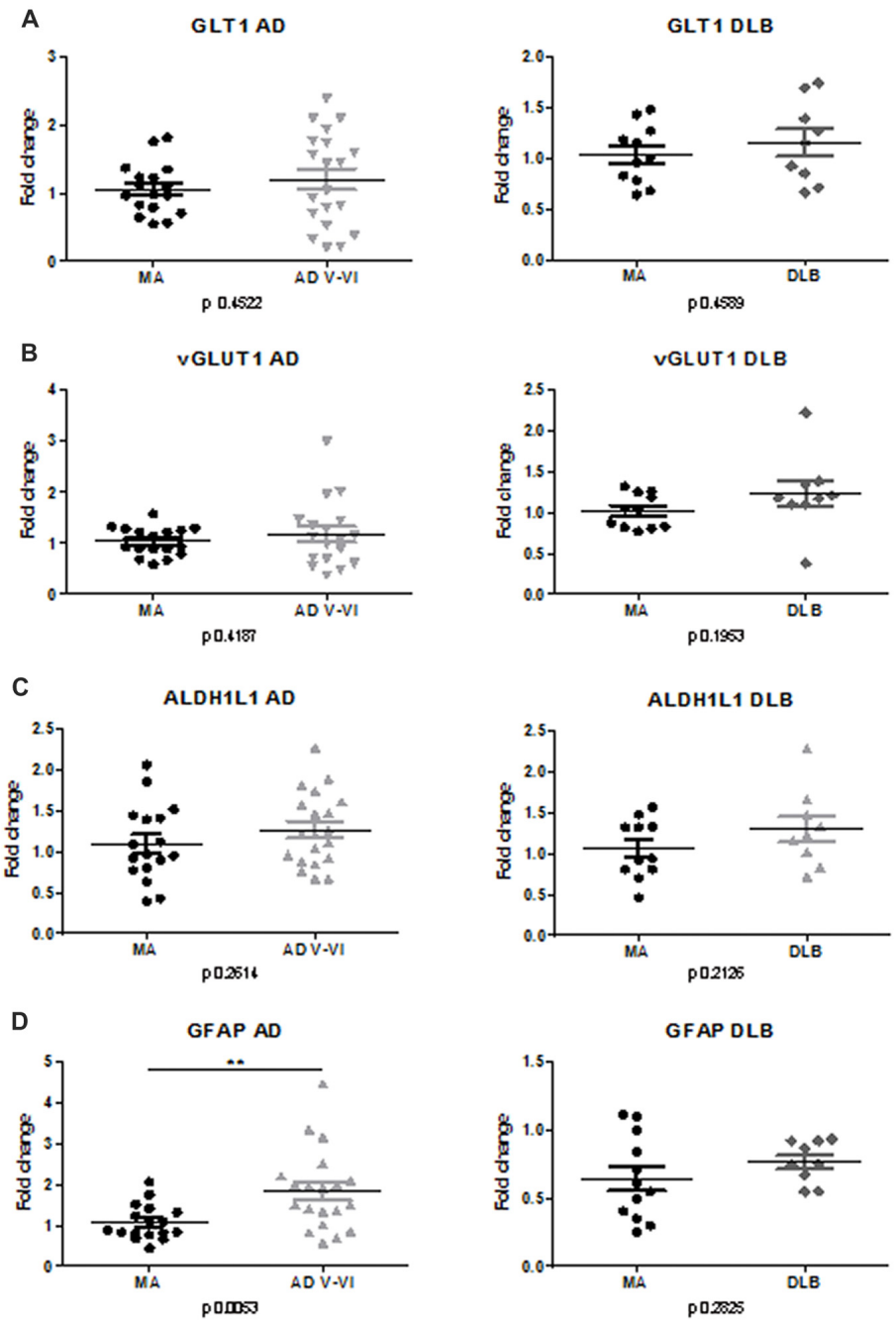

FIGURE 1 | mRNA expression of *GLT1*, *vGLUT1*, Glial fibrillary acidic protein (GFAP) and *ALDH1L1* in frontal cortex area 8 in Alzheimer disease (AD) and dementia with Lewy bodies (DLB). Mean expression of housekeeping genes *GUS-B*, *XPNPEP1* and *AARS* was used to normalize samples. Variations are represented as Fold-change. No significant differences are observed regarding GLT1 **(A)**, *vGLUT1* **(B)**, *ALDH1L1* **(C)** expression in AD and DLB compared with middle-aged control cases (MA). **(D)** In contrast, significant increase in *GFAP* mRNA is found in AD but not in DLB. Statistical *t*-test was performed to compare each group. *p* values are depicted in every analysis. Significant *p*-value stated at $^{**}p < 0.01$.

Statistical Analysis and Data Visualization Software version 5.1 (Warrenton, VA, USA). Statistical *t*-test was performed to analyze each group with its respective control; significant *p*-values were established as: $^{*}p < 0.05$ and $^{**}p < 0.01$.

Double-Labeling Immunofluorescence and Confocal Microscopy

For double-labeling immunofluorescence and confocal microscopy, de-waxed sections, 4 μm thick, of frontal cortex

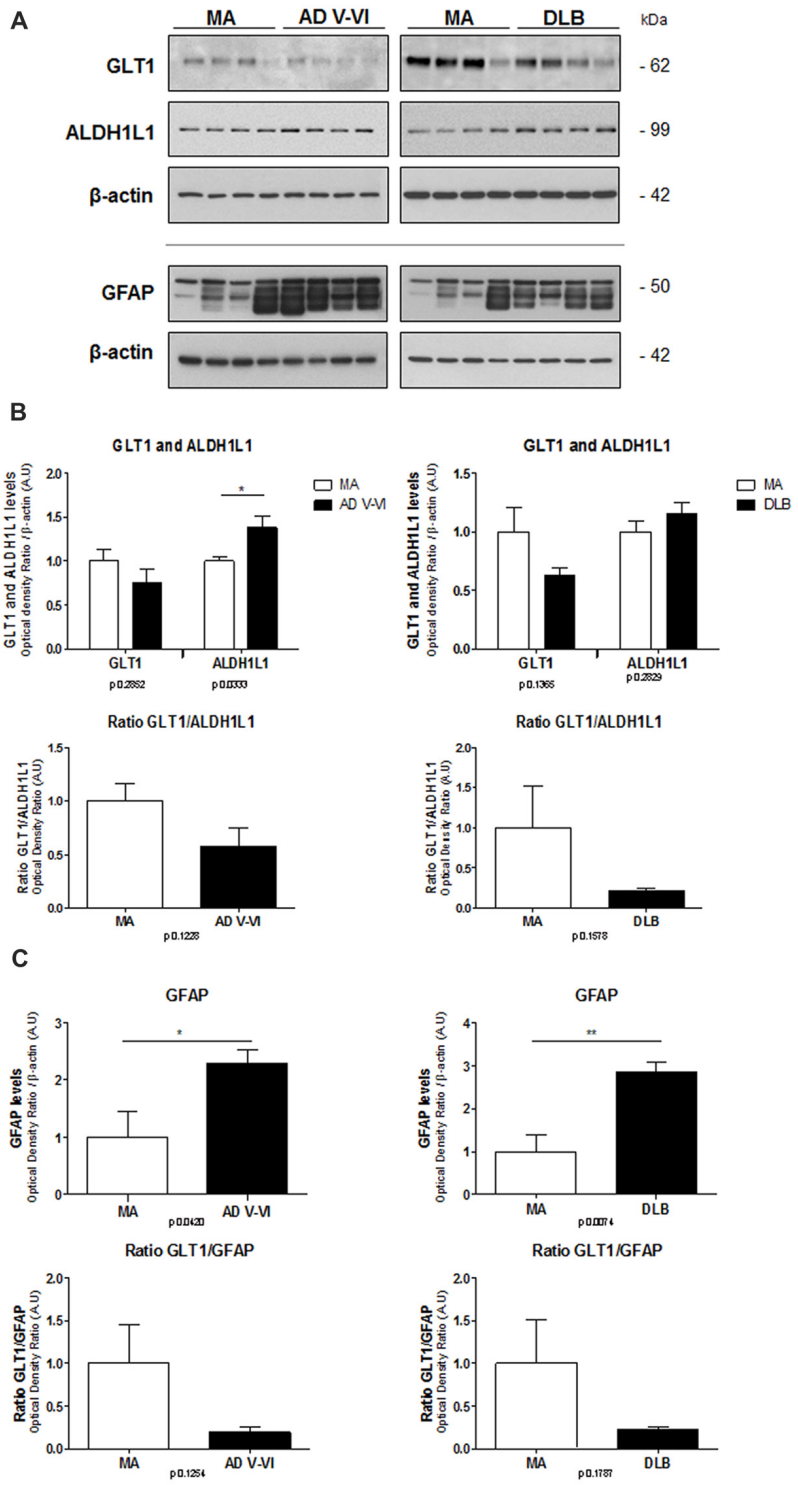

FIGURE 2 | Protein expression levels of GLT1, ALDH1L1 and GFAP in frontal cortex in AD and DLB compared with MA. β-actin is used as control of protein loading **(A)**. No significant differences in the levels of GLT1 are found in AD and DLB compared with MA. However, significantly increased protein levels of ALDH1L1 are noted in AD, but not in DLB compared with MA. No significant differences, but just a trend to decrease, are found in the ratio between GLT1 and ALDH1L1 **(B)**. Significant increase in GFAP levels is found in AD and DLB when compared with MA cases. As a result, the ratio between GLT1 and GFAP is markedly reduced, although without statistical significance, in AD and DLB **(C)**. Statistical *t*-test: $^{*}p < 0.05$, $^{**}p < 0.01$. Ratios of optical densities between GLT1 and GFAP, and GLT1 and ALDH1L1 in MA are ascribed as value 1.

area 8 were stained with a saturated solution of Sudan black B (Merck Millipore, Darmstadt, Germany) to block the autofluorescence of lipofuscin granules present in cell bodies (15 min), and then rinsed in 70% ethanol and washed in distilled water. The sections were boiled in citrate buffer to enhance antigenicity and blocked for 30 min at room temperature with 10% fetal bovine serum diluted in PBS. The sections were incubated at 4°C overnight with combinations of primary antibodies GLT1 (1:500, Merck Millipore, Darmstadt, Germany), GFAP (1:400, Dako, Glostrup, Denmark) and β-amyloid (1:50, Sigma, St. Louis, MO, USA). After washing, the sections were incubated with Alexa488 or Alexa546 (1:400, Molecular Probes, Waltham, MA, USA) fluorescence secondary antibodies against the corresponding host species. Nuclei were stained with DRAQ5^{TM} (1:2000, Biostatus, Leicestershire, UK). After washing, the sections were mounted in Immuno-Fluore mounting medium (ICN Biomedicals, Irvine, CA, USA), sealed, and dried overnight. Sections were examined with a Leica TCS-SL confocal microscope (Wetzlar, Germany).

RESULTS

GLT1, *vGLUT1*, *ALDH1L1* and *GFAP* mRNA Expression in AD and DLB

No differences in the expression levels of *GLT1* were detected in frontal cortex area 8 in AD and DLB when compared with MA cases (**Figure 1A**).

No differences in the expression levels of *vGLUT1* were detected in frontal cortex area 8 in AD and DLB when compared with MA (**Figure 1B**).

No differences in the expression of *ALDH1L1* were found in frontal cortex area 8 in AD and DLB compared with MA (**Figure 1C**).

Significant differences in GFAP mRNA expression ($p < 0.01$) were seen between AD and MA cases. Yet no differences were observed between DLB and MA (**Figure 1D**).

GLT1, ALDH1L1 and GFAP Protein Levels in AD and DLB

Protein levels of GLT1, ALDH1L1 and GFAP were assessed with gel electrophoresis and western blotting using β-actin as control of protein loading (**Figure 2A**).

GLT1 protein levels showed a tendency to decrease in AD and DLB without statistical significance. In contrast, ALDH1L1 protein levels were significantly increased in AD ($p < 0.05$) but not in DLB. As a result, the ratio between GLT1 and ALDH1L1 showed a trend to decrease without statistical significance in AD and DLB (**Figure 2B**).

GFAP protein levels were significantly increased in AD and DLB ($p < 0.05$ and $p < 0.01$, respectively). The ratio between optical densities of GLT1 and GFAP in MA cases was considered as ratio 1. The ratio between optical densities of GLT1 and GFAP showed a non-significant decrease in AD and DLB when compared with MA (**Figure 2C**).

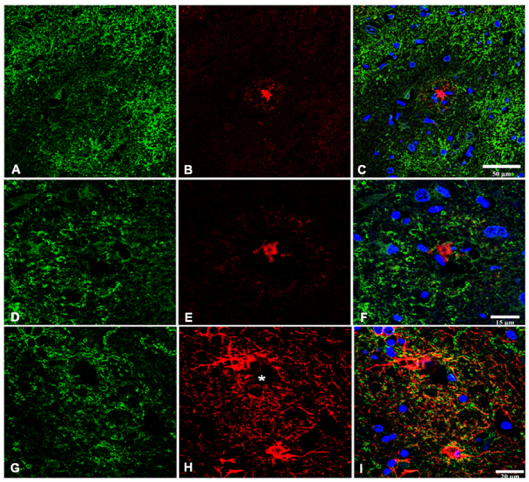

FIGURE 3 | Double-labeling immunofluorescence and confocal microscopy of GLT1 (green), β-amyloid (red; **B,C,E,F**), and GFAP (red; **H,I**) in frontal cortex area 8 in AD. GLT1 immunoreactivity is preserved in the vicinity of β-amyloid plaques **(A–F)**. Astrocytes surrounding small β-amyloid deposit (asterisk) show preserved GLT1 immunoreactivity at the cell membrane **(G–I)**. Paraffin sections, nuclei (blue) are stained with DRAQ5^{TM}; **(A–C)**, bar = 50 μm; **(D–F)**, bar = 15 μm; **(G–I)**, bar = 20 μm.

Double-Labeling Immunofluorescence and Confocal Microscopy

Double-labeling immunofluorescence and confocal microscopy disclosed preservation of GLT1 immunofluorescence surrounding β-amyloid plaques (**Figures 3A–F**). At higher magnification, astrocytes showed GLT1 immunofluorescence at the cell membrane (**Figures 3G–I**).

DISCUSSION

Present observations show preserved *GLT1* mRNA expression in cerebral cortex area 8 in AD and when compared with MA individuals. A tendency of GLT1 protein levels to decrease, with no statistical significance, is found by western blotting in AD and DLB. Moreover, GLT1 immunoreactivity localizes equally in reactive astrocytes in the vicinity of β-amyloid plaques and in astrocytes with no apparent association with plaques. This would suggest no modifications of GLT1 expression in AD. In contrast, significant increase of GFAP mRNA and protein expression occurs in AD and DLB. Therefore, GLT1 protein levels do not parallel GFAP protein levels in frontal cortex in AD and DLB. This observation is in line with previous reports indicating an inverse relation between increased GFAP expression and reduced GLT1 expression with disease progression in AD (Simpson et al., 2010, 2011). This is further demonstrated by analyzing the ratio between GLT1 and GFAP protein levels. A decreased ratio, although not significant, occurs in AD and DLB, thus suggesting an imbalance between GFAP and GLT1 expression in astrocytes in these diseases. However, this interpretation must be taken with caution as not all astrocytes express GFAP and other astrocyte markers

may not be expressed in GFAP-immunoreactive astrocytes (Kimmelberg, 2004; Sofroniew and Vinters, 2010; Oberheim et al., 2012).

ALDH1L1 has been used as a marker of total astrocytes (Ferrer, 2017). No significant differences in *ALDH1L1* mRNA expression are seen in AD and DLB when compared with MA. However, significant increase in ALDH1L1 protein levels is found in AD but not in DLB, thus indicating an increase in the number of astrocytes in AD when compared with MA. The ratio between ALDH1L1 and GLT1 is decreased, although not significantly, in AD and DLB, thus suggesting an imbalance between the total number of astrocytes and GLT1 protein levels.

These subtle differences do not necessarily indicate primary alterations in the production of GLT1 in astrocytes, but rather secondary adaptations to neuronal dysfunction. GLT1 expression depends on the number of functional synapses under physiological and pathological conditions (Kugler and Schleyer, 2004; Genoud et al., 2006; Yang et al., 2009). Since synaptic connectivity is reduced in frontal cortex in AD and DLB (Jellinger, 2000; Selkoe, 2002; Hashimoto and Masliah, 2003; Gong and Lippa, 2010; Schulz-Schaeffer, 2010; Ferrer, 2012; de Wilde et al., 2016; Santpere et al., 2017), the possibility of reduced GLT1 expression secondary to decreased synaptic activity must not be overlooked in AD and DLB.

AUTHOR CONTRIBUTIONS

PG-E and DD-L carried out biochemical study of AD and DLB. MA, BT-E and MC performed immunohistochemistry. FL helped in the design of the study. IF selected the cases, designed and supervised the study and wrote the manuscript, which was circulated among the authors for approval.

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frontiers in Aging Neuroscience

MINI REVIEW
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Microglia in Alzheimer's Disease: Activated, Dysfunctional or Degenerative

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Microglial activation has been considered a crucial player in the pathological process of multiple human neurodegenerative diseases. In some of these pathologies, such as Amyotrophic Lateral Sclerosis or Multiple Sclerosis, the immune system and microglial cells (as part of the cerebral immunity) play a central role. In other degenerative processes, such as Alzheimer's disease (AD), the role of microglia is far to be elucidated. In this "mini-review" article, we briefly highlight our recent data comparing the microglial response between amyloidogenic transgenic models, such as APP/PS1 and AD patients. Since the AD pathology could display regional heterogeneity, we focus our work at the hippocampal formation. In APP based models a prominent microglial response is triggered around amyloid-beta (Aβ) plaques. These strongly activated microglial cells could drive the AD pathology and, in consequence, could be implicated in the neurodegenerative process observed in models. On the contrary, the microglial response in human samples is, at least, partial or attenuated. This patent difference could simply reflect the lower and probably slower Aβ production observed in human hippocampal samples, in comparison with models, or could reflect the consequence of a chronic long-standing microglial activation. Beside this differential response, we also observed microglial degeneration in Braak V–VI individuals that, indeed, could compromise their normal role of surveying the brain environment and respond to the damage. This microglial degeneration, particularly relevant at the dentate gyrus, might be mediated by the accumulation of toxic soluble phospho-tau species. The consequences of this probably deficient immunological protection, observed in AD patients, are unknown.

Keywords: Alzheimer disease, microglia, APP models, inflamation, Abeta plaques

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INTRODUCTION

Alzheimer's disease (AD) is characterized by complex molecular and cellular alterations, including the development of extracellular amyloid-beta (Aβ) deposits, intracellular aggregated phosphorylated tau, dystrophic neurites, loss of synapses and neurons, and a prominent gliosis. The reactive gliosis involves alterations in morphology and function of microglia and astrocytes

(for recent reviews Heneka et al., 2015; Heppner et al., 2015; Calsolaro and Edison, 2016; Ransohoff, 2016; Cuello, 2017). In fact, the so-called neuroinflammative process is certainly a critical factor in the pathogenesis of multiple neurological disorders, such as inflammatory autoimmune diseases (e.g., multiple sclerosis). This inflammatory response is also clearly associated to the development of AD and microglia have recently emerged as crucial players in the pathogenesis of the sporadic forms (Heneka et al., 2015; Calsolaro and Edison, 2016; Ransohoff, 2016; Sarlus and Heneka, 2017) though it is still unclear whether a detrimental or protective but insufficient function contributes to disease. It has long been recognized that Aβ neuritic plaques are surrounded by activated microglial cells that could contribute to Aβ phagocytosis and/or compaction (Hickman et al., 2008; Frenkel et al., 2013). In fact, chronic microglial activation could improve the AD pathology reducing the Aβ levels in APP-based models (Michaud et al., 2013). However, the inflammatory response has also been associated with neurotoxic detrimental effects mediated by the release of proinflammatory cytokines/chemokines and neurotoxins (Kettenmann et al., 2011; Heneka et al., 2015; Heppner et al., 2015; Calsolaro and Edison, 2016). The implication of microglia in the AD pathology is also reinforced by human genome-wide association studies (GWAS). These GWAS analysis have identified multiple polymorphisms associated with the microglial immune response (Guerreiro et al., 2013; Cuyvers and Sleegers, 2016; Hansen et al., 2018). Within the different AD-associated genes, the microglial triggering receptor expressed in myeloid cells 2 (TREM2) gene seems to perform a pivotal role in the AD-associated immune response (Ulrich et al., 2017; Yeh et al., 2017; Hansen et al., 2018). TREM2 is a lipid and lipoprotein sensor that, through its adapter molecule DAP12, supports reactive microgliosis (Wang et al., 2015; Yeh et al., 2016). Furthermore, it has been recently demonstrated that TREM2, interacting with ApoE (the major genetic risk factor for AD), regulates the transcriptional activation of microglial cells (Krasemann et al., 2017). However, the role of TREM2-mediated microglial activation, or even the function of the microglial cells in the pathology of AD, is not elucidated (Ulrich et al., 2017).

Concerning to AD patients, the neuroinflammatory response is probably not exclusively detrimental or beneficial. An excessive microglial reaction could indeed be detrimental for the surrounding neurons or neuronal elements. However, the contrary, a deficient microglial response could also induce a similar detrimental effect on neurons. For instance, the absence of microglial response due to null mutations in TREM2 produces Nasu-Hakola disease, a rare neurodegenerative disease (Dardiotis et al., 2017). Therefore, the implication of the microglial response on the development of a neurodegenerative disease, such as AD, could be due to either an excessive or, on the contrary, a deficient microglial activation. In consequence, restoring microglial function may be a therapeutic option for AD treatment.

The achievement of effective therapies for AD requires modeling very specific aspects of the human pathology in the mouse models. Most of our knowledge about microglial activation in AD is so far based in studies using APP-based transgenic mice which robustly recapitulate the amyloid pathology but fail to develop neurofibrillary pathology. The failure of translating anti-inflammatory strategies into the clinical practice could be explained by a complex and differential glial response in AD models and patients. Here we provide an overview of the microglial response in amyloidogenic models and human *post-mortem* hippocampus with the aim of developing more predictive valuable animal models and gain success in clinical trials using neuroinflammatory targets.

DECODING MICROGLIAL ACTIVATION IN MOUSE MODELS AND HUMAN BRAINS

APP-based transgenic mice displayed a considerable and progressive extracellular Aβ accumulation in hippocampus and cerebral cortex (Sasaguri et al., 2017). We and others (Jimenez et al., 2008; Heneka et al., 2015) have previously described that this extensive Aβ accumulation was accompanied by intense local microglial activation (see **Figures 1A,B**). More recently, the molecular signature of the microglial activation has been broadly studied using single-cell RNA-sequencing (Keren-Shaul et al., 2017). Using this powerful approach, genes associated to the microglial activation have been identified. In fact, these studies have clearly probed that microglial cells are activated and express a particular genetic program called "disease associated microglia (DAM)". This DAM program includes the expression of multiple genes (such as Csf1, Clec7a, Igf-1), and some others associated to AD, like TREM2 or ApoE. The same active microglia down-regulate the "homeostatic" genes, such as Cx3cr1, P2ry12 or Tmem119 (Keren-Shaul et al., 2017). To investigate the microglial activation in our APP/PS1 model, we have directly determined the change in expression of some of these DAM and homeostatic genes from hippocampal samples. To briefly summarized our data, the change in the expression of microglial genes was displayed graphically as heat-maps (**Figure 1A**) by calculating the z-score between controls (wild-type mice, WT) and APP/PS1 samples (9- to 12-month-old; $n = 10$ per genotype). As expected, the levels of all these genes classified as part of the DAM program were noticeably and significantly ($p < 0.05$, t-test) induced. Furthermore, these activated microglial cells were located surrounding, and probably isolating, the Aβ plaques (**Figures 1B,b1,b2**). Therefore, as proposed previously, this microglial activation could exert a protective role in the AD pathology (Jimenez et al., 2008; Yeh et al., 2016; Yuan et al., 2016; Zhao et al., 2017). In this sense, it has been proposed that DAM phenotype could be neuroprotective (Keren-Shaul et al., 2017), although this role is actually under debate.

Concerning to the homeostatic genes, we have determined the expression of three of them, such as CX3CR1, P2ry12 and Tmem119 (see **Figure 1A**). As shown, in the APP/PS1 model, the expression of these particular genes was either not altered (P2ry12, Tmem119) or exhibited a small but significant

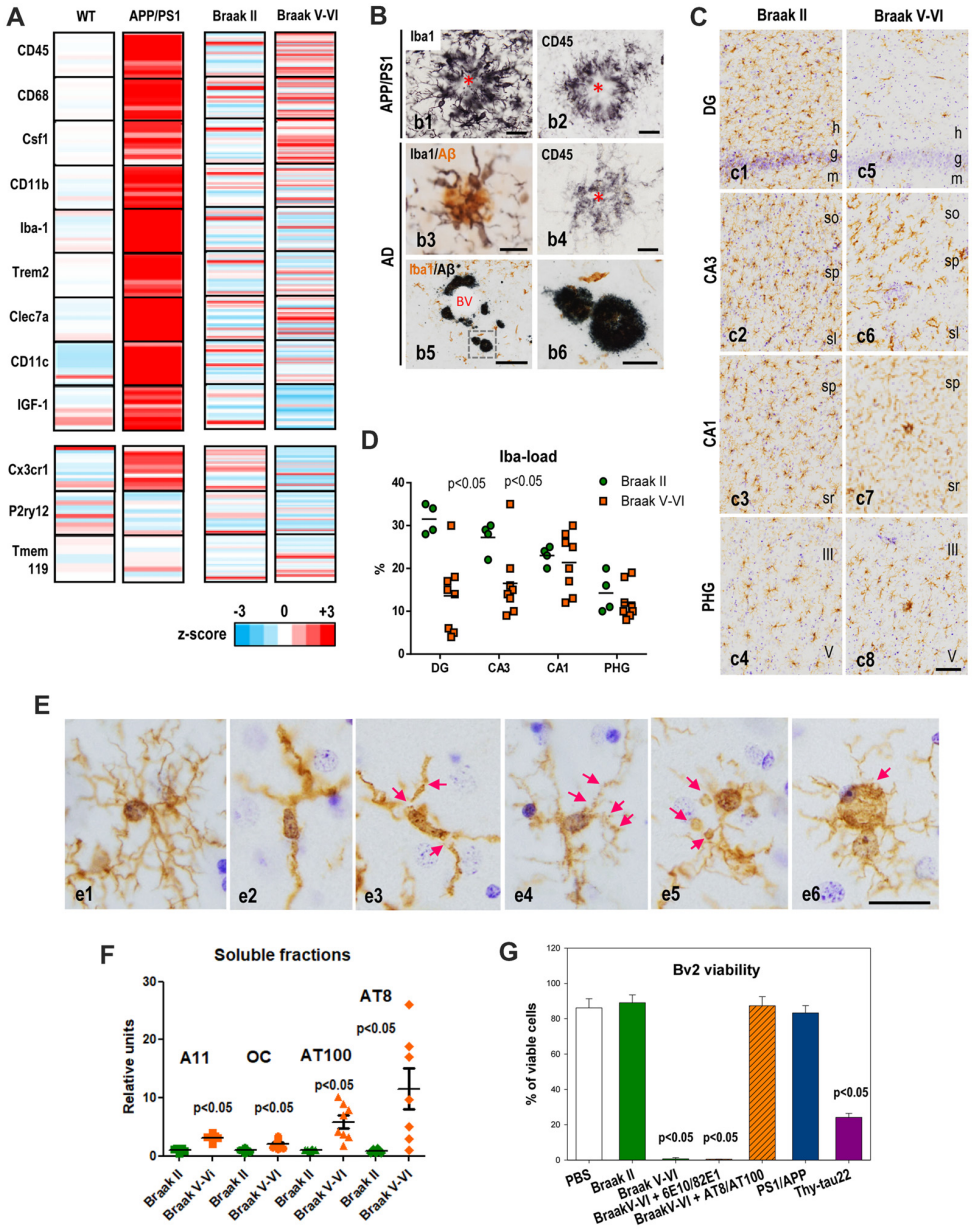

FIGURE 1 | Microglial activation in APP/PS1 and microglial pathology in Alzheimer's disease (AD) hippocampus. **(A)** Differential expression of selected microglial genes from hippocampus of 9- to 12-month-old APP/PS1 mice (age-matched wild-type (WT) as controls; $n = 10$ per genotype) and post-mortem human Braak V–VI samples ($n = 28$; age-matched non-demented BraaK II individuals as controls, $n = 21$) determined by qPCR analysis. Data were graphically plotted as Z-score and represented the deviation in the expression of a gene over the mean expression of this particular gene in the control populations (WT or Braak II). Each bar represented an individual murine or human sample. High expression is marked by the red color spectrum, low expression by blue colors. **(B)** Immunohistochemical detection of microglial cells around amyloid plaques in 12-month old APP/PS1 mice **(b1,b2)**, human AD brains staged as Braak IV **(b3,b4)** or Braak V–VI **(b5,b6)**. Amyloid plaques are surrounded by abundant activated Iba1-positive **(b1)** and CD45-positive **(b2)** microglia in the transgenic mice. Human plaques also displayed Iba1-positive and CD45-positive associated microglia at Braak IV stage, however the presence of amyloid deposits devoid of Iba1-positive microglia was often present in the hippocampus of Braak V–VI cases. Asterisks indicate amyloid plaques. BV, blood vessel. Scale bars **(b1–b4)** 20 μm; **(b)** 200 μm; **(b6)** 50 μm. **(C,D)** The Iba1-load quantitative analysis revealed a regional pattern with DG>CA3>CA1>parahippocampal gyrus (PHG) in Braak II (**C**,**c1–4**,**D**, green circles). In Braak V–VI individuals a significant ($p < 0.05$, Mann Whitney test) reduction of the microglial load was evidenced in the DG and CA3 regions (**D**, orange squares). **(E)** Compared to Braak II microglial cells (**e1** shows a non-activated healthy Iba1-positive microglial cell), Braak V–VI cases exhibit microglial cells with degenerative morphological features including deramification **(e2)**, fragmentation **(e3)**, beadings and spheroidal swellings **(e4,e5)** and dystrophies **(e6)** of the processes. h, hilar region; g, granular layer; m, molecular layer; so, stratum oriens; sp, stratum pyramidale; sl, stratum lucidum; sr, stratum radiatum. Scale bars, **(c1–8)** 100 μm; **(e1–6)** 20 μm. **(F)** Differential accumulation of soluble Aβ and phospho-tau forms in the hippocampus of human Braak II and Braak V–VI brains. Quantitative data from dot blots or western blots of soluble S1 fractions (extracellular/cytosolic) isolated from Braak II ($n = 8$) or Braak V–VI ($n = 8$) individuals. The relative abundance

(*Continued*)

FIGURE 1 | Continued
of A11 or OC positive Abeta oligomers, AT8 or AT100 phospho-tau proteins was determined by densitometry analysis. Significance was determined by Mann Whitney test. **(G)** Soluble phospho-tau toxicity on BV2 microglial cells. Microglia were incubated with S1 fractions isolated from Braak II, V–VI samples or 18-month-old APP/PS1 or 12-month-old Thy1-tau22 models. The toxicity was analyzed by flow cytometry and the percent of viable cells was showed. The Aβ or phospho-tau was immunodepleted from the Braak V–VI S1 fractions using 6E10 plus 82E1 or AT8 plus AT100 antibodies, respectively. The data are shown as the mean ± SD of six different experiments. Significance (indicated in the figure) was determined by ANOVA and Tukey *post hoc* test. Data were taken from Jimenez et al. (2008, 2014); Moreno-Gonzalez et al. (2009); Sanchez-Mejias et al. (2016); Baglietto-Vargas et al. (2017) and Gutierrez and Vitorica (2018).

($p < 0.05$) increase (CX3CR1), compared with age-matched WT mice. As mentioned above, since the expression of these particular genes decreased upon "activation" (Keren-Shaul et al., 2017; Krasemann et al., 2017) these data were somehow conflictive. In fact, we have corroborated by *in vitro* experiments using primary microglial cells the decrease in these homeostatic genes after stimulation with either LPS or oligomeric Aβ (oAβ). Thus, the increase observed in some of these homeostatic genes could also reflect the proliferation of microglial cells in these models (Baglietto-Vargas et al., 2017). Microglia are self-renewal cells that, upon activation, could proliferate to develop an immune response (Askew et al., 2017; Tay et al., 2017). In fact, we also observed an increase in the expression of the mitotic marker Ki67 paralleled by increase in the BrdU incorporation on microglial cells (Baglietto-Vargas et al., 2017). Similarly, Füger et al. (2017) have reported a three-fold increase of the microglial proliferation in a different APP/PS1 model.

On the other hand, it has been classically assumed that the microglial response in AD patients could be similar to that observed in the amyloidogenic transgenic mice. Thus, we have also analyzed the microglial response in human hippocampus from non-demented Braak II controls ($n = 21$) and age-matched AD cases staged as Braak V–VI ($n = 28$). The expression of the different genes was calculated as z-score and showed individually in the graph (**Figure 1A**). Using this graphic approach, it is clear that the microglial reaction in AD samples differs obviously from that observed in the amyloidogenic model. An attenuated, rather than amplified, microglial response, was identified in the hippocampus of AD patients. As shown in **Figure 1A**, only few DAM genes were significantly (Mann Whitney test, $p < 0.05$) induced, such as CD45, CD68 and Csf1 in Braak V–VI samples, as compared with age-matched control individuals. Furthermore, this induction was clearly weakened as compared with APP/PS1 models. The apparently incomplete-activated microglial cells were principally surrounding the extracellular amyloid deposits (**Figures 1B,b3,b4**). On the other hand, the expression of homeostatic genes, such as Cx3cr1 and P2ry12, seemed to be significantly decreased in AD patients. We do not know whether this decrease in the expression reflects the partial microglial activation or, on the contrary, reflects a change in the microglial number.

Although microglial activation has been observed in cerebral regions with early and abundant extracellular Aβ deposits (Serrano-Pozo et al., 2013; Hamelin et al., 2016), our data clearly show that the microglial activation observed in the hippocampus of APP-based models undoubtedly differs both qualitative and quantitative from that observed in AD patients. This clear difference could reflect a different kinetic and/or magnitude of the Aβ accumulation. It is possible that the microglial response to the fast and extensive Aβ accumulation in transgenic models may resemble an acute response rather than a chronic and insidious pathology as observed in patients. On the other hand, and restricted to human samples, in Braak V–VI hippocampus we also observed neuritic plaques not surrounded by Iba1-positive microglial cells (**Figures 1B,b5,b6**). These "nude" amyloid plaques were not diffuse unreactive plaques. Thus, these data demonstrated the existence of substantial differences in the microglial response between APP-models and AD cases, at least at the hippocampal formation.

MICROGLIAL DETERIORATION IN THE HIPPOCAMPUS OF AD PATIENTS

As we pointed above, the microglial response in AD samples is quite dissimilar from that observed in APP-models. Furthermore, the existence of Aβ plaques with no surrounding/isolating Iba1-positive microglia led us to speculate with the existence of a pathological microglial process in AD patients. Thus, we analyzed using immunohistochemical staining the microglial response in AD cases (Braak V–VI) compared to controls (Braak II; **Figure 1C**). Using these experimental approaches, we first realized the existence of a regional microglial compartmentalization within the hippocampal formation. In Braak II control samples the parenchymal distribution of microglia cells, determined as Iba1-load, was significantly higher at the hilar region and CA3 than in CA1 or parahippocampal gyrus (PHG; **Figures 1C,c1–4,D**, green circles). The reasons and implications of this particular heterogeneity were actually unknown; however, it could reflect a different role or different immune-requirements associated to the particular connections between DG and CA3 regions. On the other hand (**Figures 1C,c5–8,D**), there was a clear and consistent (seven out of eight individuals tested) reduction in the microglial load (Iba1-immunopositive area) in Braak V–VI hippocampus. This reduction was not homogeneous for all regions analyzed. In fact, it was restricted to the hilar region of the DG and the CA3 subfield of the hippocampal formation. Other regions, such as CA1 and PHG were less affected, or unaffected, in the Braak V–VI samples. These data could indicate the existence of a possible microglial degenerative process restricted to DG and CA3 subfields. Thus, we focused our attention on the hilar region of the DG and further analyzed this putative microglial degeneration by determining: (1) the numerical density (cells/mm^3) of Iba1-positive cells; (2) the parenchymal area covered by each microglial cell (microglial domain); and (3) the spatial distribution pattern of microglia

(spatial covered). Interestingly, the numerical density of Iba1-positive cells was reduced in approximately 50% of the AD cases (Sanchez-Mejias et al., 2016). On the other hand, all tested samples showed clear modifications of the microglial morphology (**Figures 1C,c1–4**). In Braak II samples, microglial cells displayed a healthy morphology with highly ramified processes and were arranged with a regular spatial distribution covering, and probably protecting, most parenchymal space. This scenario was totally different in pathological Braak V–VI individuals. We observed a high and consistent reduction in both the microglial domain (area of surveillance of an individual cell) and in the spatial coverage of the hilar region of the DG (Sanchez-Mejias et al., 2016). In consequence, in AD hippocampus most of the parenchymal space of the hilar region was devoid on microglial protection.

This decrease was due to the existence of a clear pathological morphology of the microglial cells. As mentioned above, in Braak II samples, microglia showed a healthy morphology (**Figures 1E,e1**). On the contrary, Braak V–VI microglial cells displayed a shortened and less branched processes that usually were deformed, displaying cytoplasmic abnormalities (including spheroids) and even fragmentation (cytorrhexis; **Figures 1E,e2–6**). This microglial pathology in AD and even in Down syndrome brains has been previously reported (Lopes et al., 2008; Streit et al., 2009, 2014; Xue and Streit, 2011), however, it has never been detected in APP-based models (**Figure 1**; Jimenez et al., 2008; Heneka et al., 2015). This remarkable discrepancy between models and patients could also explain the patent differences in the expression of the DAM phenotype markers. As also mentioned, we also noted the existence of amyloid plaques without surrounding Iba1-positive microglial cells. If the microglial activation around Aβ plaques were indeed protective and constituted a preventive response by compacting plaques and isolating the putative toxic Aβ oligomers from the neuronal environment (Yuan et al., 2016), the existence of "nude" Aβ plaques in AD hippocampus could indeed increase the toxicity of these extracellular aggregates and drive neuronal damage and death.

SOLUBLE PHOSPHO-TAU IS RESPONSIBLE FOR THE MICROGLIAL DEGENERATION IN AD BRAINS

So far, we have demonstrated the existence of an evident pathological process in AD hippocampus affecting the microglial cells. To address which was the potential microglial toxic factor(s) in AD brains we examined whether soluble factors, such as oAβ or phosphorylated tau (phospho-tau) in Braak V–VI samples were the toxic agents (Sanchez-Mejias et al., 2016). In this sense, our previous work demonstrated that the hippocampus of APP-based models accumulated, in an age-dependent mode, soluble oAβ (Torres et al., 2012; Trujillo-Estrada et al., 2013; Jimenez et al., 2014). These soluble oAβ increased in aged APP-models and induced microglial activation in the inter-plaque areas (Jimenez et al., 2008). However, though the soluble oAβ also increased in Braak V–VI samples (tested using A11 or OC antibodies in dot-blots, **Figure 1F**), these oligomeric species were very scarce in Braak V–VI hippocampus (Jimenez et al., 2014). This apparent contradiction could be explained by the distinct development of AD pathology (lesions, course) between amyloidogenic models and AD samples. Models presented a high extracellular Aβ accumulation in both cortical areas and hippocampal complex since early ages (Trujillo-Estrada et al., 2014). However, human AD hippocampus, along with the entorhinal cortex, is one of the first regions to be affected by tau pathology and only, in very advanced stages, amyloid plaques are also present. In fact, the soluble fractions extracted from Braak V–VI hippocampus displayed relatively high content in AT100 and AT8 positive phospho-tau (**Figure 1F**) and relatively low oAβ levels.

We tested *in vitro*, using Bv2 cells or primary microglial cultures, whether soluble fractions (S1) derived from AD hippocampus were toxic (**Figure 1G**). The S1 fractions form Braak V–VI samples produced a clear reduction on the number of viable microglia, either Bv2 or primary cultures. This toxic effect was avoided by immunodepletion using a combination of AT8 plus AT100 antibodies whereas the same approach using 6E10 plus 82E1 monoclonal antibodies for Aβ produced absolutely no effect (**Figure 1G**). Thus, soluble phospho-tau seemed to be toxic for microglial cells *in vitro*. We further confirmed these results using soluble fractions extracted from aged APP/PS1 (18-month-old) or Thy-Tau22 (12-month-old) models. As expected, S1 fractions enriched in Aβ (APP/PS1; Jimenez et al., 2008) produced no effect whereas those containing phospho-tau (tau22) were toxic (**Figure 1G**). All these data demonstrated that, at least *in vitro*, soluble phospho-tau reduced the viability of microglial cells. This hypothesis was also supported by *in vivo* colocalization of dystrophic microglial cells with phospho-tau positive neuronal structures (Sanchez-Mejias et al., 2016).

In sum, our work highlights relevant differences in the hippocampal inflammatory response between APP-transgenic mice and AD patients regarding microglial gene expression, morphology and survival (see **Figure 2**). In APP-models, the high and probably fast accumulation of extracellular Aβ produces a prominent microglial response. The activated microglial cells are predominantly located surrounding Aβ plaques, although active microglia are also identified in inter-plaque regions. While the role of the activated microglia could be neuroprotective, the strong microglial activation in APP models could also drive the AD pathology (Maphis et al., 2015; Olmos-Alonso et al., 2016; Spangenberg et al., 2016) and, in consequence, it could be implicated in the neurodegenerative process observed in APP-models. On the contrary, the microglial response in AD hippocampus is really mild. This patent difference could simple reflect the lower and probably slower Aβ production observed in human hippocampal samples, in comparison with models. However, and more relevant, in Braak V–VI samples there is a prominent

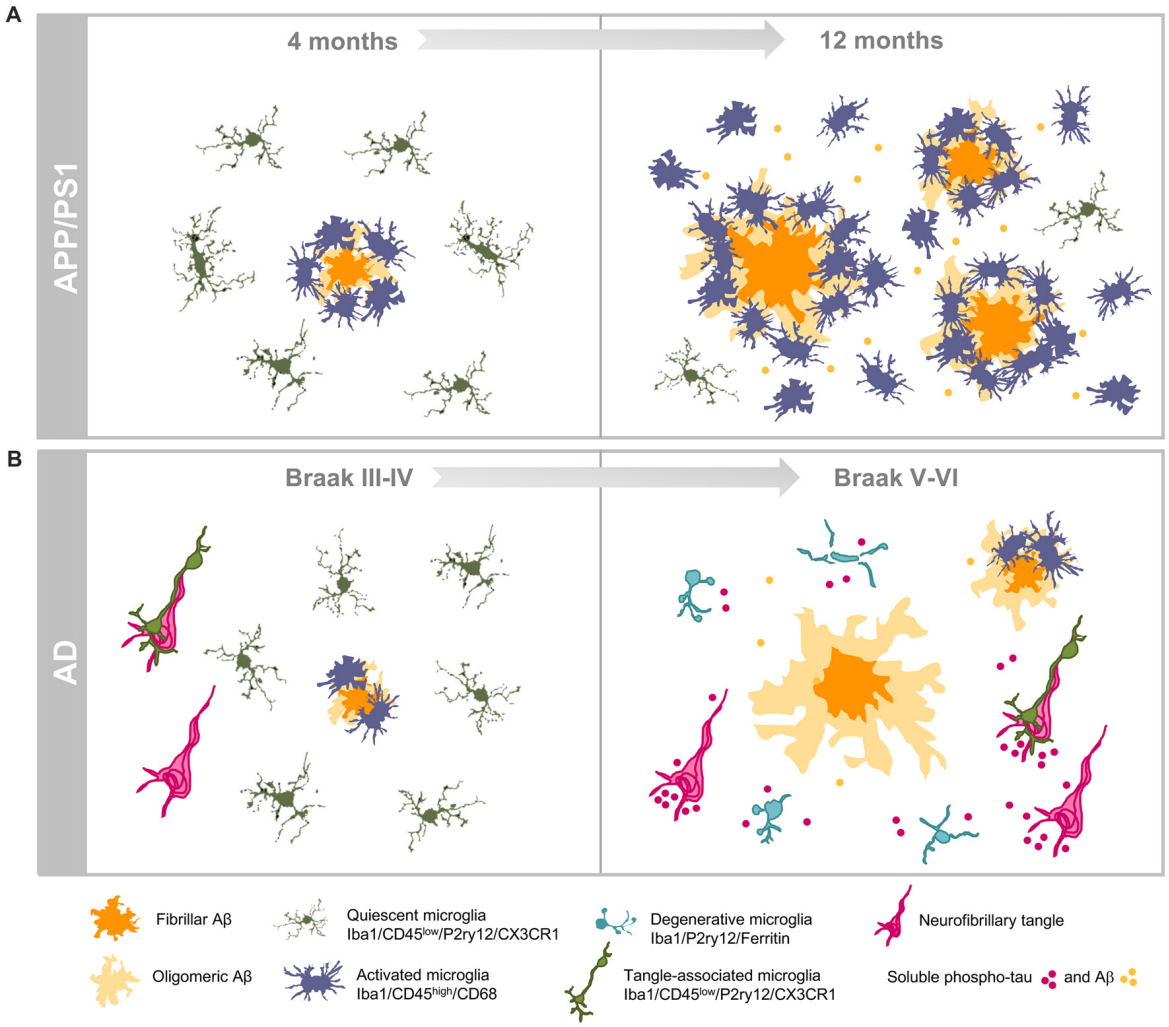

FIGURE 2 | Dissimilar progressions of hippocampal microglial response in APP/PS1 mice and human brains. **(A)** Illustrate the age-dependent microglial activation in the amyloidogenic model from 4 (left) to 12 (right) months of age. Activated microglia characterized by morphological changes (hypertrophy of cell body and shortened processes) selectively cluster around amyloid plaques at early ages. Increased amyloid load with age is associated with expansion of the microglial activation. The age-dependent accumulation of soluble Aβ oligomers induces the activation of the interplaque microglia in aged transgenic mice. **(B)** Illustrate the microglial response during the course of AD pathology in the hippocampus of human brains. Amyloid pathology is a late event in the hippocampus while tau pathology shows a very early onset. In Braak III–IV hippocampus, some amyloid plaques are present and they are surrounded by activated microglia, however in late stage AD (Braak V–VI) microglial-bare plaques are usually found. The high content of phospho-tau forms (intra- and/or extracellular) induces the degeneration of microglial cells in human Braak V–VI hippocampus.

microglial degenerative process that, indeed, could compromise their normal role of surveying the brain environment and respond to the damage. This microglial pathology, particularly relevant at the dentate gyrus of the hippocampal formation, might be mediated by the accumulation of toxic soluble phospho-tau species. The consequences of this probably deficient immunological protection due to the microglial degeneration, observed in AD patients, are unknown. Considering that microglial cells are implicated in multiple beneficial functions, such as Aβ phagocytosis, senile plaque compaction and limitation of Aβ toxicity and eliminating damaged neuron or neuronal debris (Yeh et al., 2016; Yuan et al., 2016; Ulland et al., 2017), a deficient microglial response could indeed aggravate the progression of AD pathology. In this sense, deficiencies on key genes for the microglial survival and/or proliferation (such as CSF1R or TREM2) are associated with rare hereditary neurodegenerative diseases, such as adult-onset leukoencephalopathy with axonal spheroids or Nasu-Hakola disease (respectively) (Paloneva et al., 2000, 2002; Chitu et al., 2016). In both diseases, the microglial response and, more relevant, the microglial survival seems to be compromised (Nataf et al., 2005; Satoh et al., 2011; Konno et al., 2014; Tada et al., 2016). On the other hand, missense mutations in TREM2 (such as R47H TREM2) produce an increases risk for late-onset AD (Guerreiro et al., 2013; Korvatska et al., 2015; Yeh et al., 2016) and a reduction

in the number of microglial cells surrounding Aβ plaques in models. This decrease produced less compact and higher toxic plaques (Yeh et al., 2016; Yuan et al., 2016; Ulland et al., 2017). Therefore, a deficient rather than an exacerbated microglial response could be implicated in the development of sporadic AD.

AUTHOR CONTRIBUTIONS

JV and AG: drafting of the manuscript. ES-M, VN, JCD: design of figures. VN, ES-M, SJ, CM-C, RS-V, MV, JCD, AG and JV: critical revision of the manuscript for important intellectual content. All authors approved the manuscript in its final form.

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TREM2-Dependent Effects on Microglia in Alzheimer's Disease

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Alzheimer's disease (AD) is a late-onset dementia characterized by the deposition of amyloid plaques and formation of neurofibrillary tangles (NFTs) which lead to neuronal loss and cognitive deficits. Abnormal protein aggregates in the AD brain are also associated with reactive microglia and astrocytes. Whether this glial response is beneficial or detrimental in AD pathology is under debate. Microglia are the resident innate immune cells in the central nervous system (CNS) that survey the surrounding environment. Genome-wide association studies (GWAS) have identified the R47H variant of triggering receptor expressed on myeloid cell 2 (TREM2) as a risk factor for late-onset AD (LOAD) with an odds ratio of 4.5. TREM2 is an immunoreceptor primarily present on microglia in the CNS that binds to polyanionic molecules. The transmembrane domain of TREM2 signals through DAP12, an adaptor protein that contains an immunoreceptor tyrosine-based activation motif (ITAM), which mediates TREM2 signaling and promotes microglial activation and survival. In mouse models of AD, *Trem2* haplodeficiency and deficiency lead to reduced microglial clustering around amyloid β (Aβ) plaques, suggesting TREM2 is required for plaque-associated microglial responses. Recently, TREM2 has been shown to enhance microglial metabolism through the mammalian target of rapamycin (mTOR) pathway. Although aberrant metabolism has long been associated with AD, not much was known regarding how metabolism in microglia might affect disease progression. In this review, we discuss the role of TREM2 and metabolism in AD pathology, highlighting how TREM2-mediated microglial metabolism modulates AD pathogenesis.

Keywords: Alzheimer's disease, microglia, TREM2, metabolism, autophagy

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ALZHEIMER'S DISEASE AND GENETIC RISK FACTORS

Alzheimer's disease (AD), the most common form of dementia, is a progressive neurodegenerative disorder clinically distinguished by loss of memory and deficits in cognitive functions. Histologically, the hallmarks of AD are aggregation and accumulation of extracellular β amyloid (Aβ) plaques and intracellular tau protein neurofibrillary tangles (NFTs), which results in extensive neuronal death (Holtzman et al., 2011). Plaque forming Aβ peptides are derived from amyloid precursor proteins (APP) that are sequentially cleaved by β-secretase and γ-secretase (Holtzman et al., 2011). On the other hand, hyperphosphorylated, aggregated microtubule-binding protein tau dissociates from microtubules and forms NFT. Accumulation of Aβ plaques precedes tau-mediated neuronal dysfunction and cognitive decline in both autosomal dominant and late-onset AD (LOAD) patients (Jack et al., 2010; Bateman et al., 2012). Whether tau pathology is independent or

downstream of Aβ remains elusive. Abnormal protein aggregates in the AD brain are also associated with a glial response, which includes activation and recruitment of microglia and astrocytes to amyloid plaques (Sastre et al., 2006; Ransohoff, 2016). The impact of AD-associated gliosis remains a topic of extensive research.

Aging is the greatest risk factor for sporadic AD. Besides that, genetic, epigenetic, and environmental factors all contribute to the complexity of AD. Mutations in *APP*, presenilin 1 (*PSEN1*), and presenilin 2 (*PSEN2*) cause a rare form of AD that occurs in an autosomal dominant fashion (Bertram et al., 2010). Patients with familial AD develop symptoms as early as their 20 s or 30 s. Mutations in *APP, PSEN1* and *PSEN2* cause increased total Aβ level or an increased ratio of Aβ42 to Aβ40, leading to familial AD (Takasugi et al., 2003). The genetics of the more common LOAD is more complex. Many genetic risk factors have been implicated in increasing susceptibility for LOAD, among which apolipoprotein E (*APOE*) confers the highest odds ratio. One copy of the ε4 allele of *APOE* increases the risk of AD by ∼4 fold (Strittmatter et al., 1993), and individuals carrying two alleles of *APOE*4 have a risk of developing AD 12-fold higher than individuals with two copies of *APOE*3. In contrast, the *APOE2* allele is neuroprotective and reduces risk of AD by 50% compared with *APOE*3 (Bertram et al., 2007). Genome-wide association studies (GWAS) have identified additional genes associated with AD. Multiple variants associated with an increased risk of developing LOAD are in genes related to immune functions, including triggering receptor expressed on myeloid cells 2 *(TREM2), CD33, CR1, EPHA1* and *ABCA7* (Hollingworth et al., 2011; Naj et al., 2011; Lambert et al., 2013). Notably, multiple human heterozygous rare variants in TREM2 were found with high risks of LOAD (Guerreiro et al., 2013; Jonsson et al., 2013). The most common variant within TREM2, rs75932628, encoding an arginine to histidine at position 47 (R47H) that imparts a partial loss of function, increases the risk for developing LOAD by 4-fold (Guerreiro et al., 2013; Jonsson et al., 2013). Other TREM2 variants, including R62H, D87N, T96K, E151K, H157Y and L211P, have been associated with LOAD, although their functional effects vary and the impacts on TREM2 signaling require further investigations (Guerreiro et al., 2013; Jin et al., 2014; Song et al., 2017). Altogether, these genetic studies highlighted the important role of microglia in regulating AD progression.

MICROGLIA AND AD

Microglia are the resident innate immune cells in the central nervous system (CNS) that account for ∼10%–15% of cells. Microglia are yolk sac-derived and represent a self-renewing population that requires colony-stimulating factor 1 receptor (CSF1R) signaling for development and survival (Ginhoux et al., 2010; Wang et al., 2012; Elmore et al., 2014). Besides their function in brain immunosurveillance, microglia play an important role in brain development and synaptic plasticity by constantly surveying the surroundings. In steady state, microglia engulf synapses through the complement pathway, which is essential for synaptic connectivity and normal brain development (Stevens et al., 2007; Schafer et al., 2012; Hong et al., 2016). Cognitively, mice depleted of microglia show defective learning and memory formation abilities (Parkhurst et al., 2013).

The exact role microglia play in AD is not completely clear. *In vitro*, Aβ oligomers can induce the production of proinflammatory cytokines such as interleukin 1 beta (IL-1β) and tumor necrosis factor alpha (TNFα) in microglia primed by LPS or IFNγ, as well as trigger reactive oxygen species (ROS) and nitric oxide (NO) production (Meda et al., 1995; Parajuli et al., 2013), leading to the hypothesis that microglia are neurotoxic and contribute to a chronic neuroinflammatory environment in neurodegeneration. Consistent with the idea, knockout of the NLRP3 inflammasome pathway in APP/PS1 mice skews microglia to anti-inflammatory states and protects the mice from memory loss (Heneka et al., 2013). Additionally, Aβ deposition induces inflammasome-dependent ASC specks formation in microglia, which in turn seed Aβ oligomers and aggregates and increase Aβ pathology in a feed forward loop. This seeding is absent in ASC-deficient mice (Venegas et al., 2017), suggesting that microglia facilitate plaque formation and play a detrimental role in AD pathology. In contrast to these findings, several studies support a neuroprotective role of microglia. One striking feature of microglia in both AD mouse models and AD patients is that they cluster around plaques (Ulrich et al., 2014; Condello et al., 2015; Jay et al., 2015; Wang et al., 2015; Yuan et al., 2016), providing a protective barrier between neurons and Aβ and thus preventing neuronal dystrophy. Furthermore, primary microglia cultures are able to phagocytose Aβ complexed with apolipoproteins (Yeh et al., 2016). However, in transiently microglia depleted AD mice, the overall Aβ level is not affected, compared to wild-type (WT) AD controls (Spangenberg et al., 2016), suggesting microglia's dispensable role in Aβ phagocytosis, which might be compensated by astrocytes.

Single-cell RNA sequencing analysis has allowed finer characterization of disease-associated microglia (DAM; also defined as microglial neurodegenerative phenotype (MGnD)), which localize to plaques in AD mouse models and are also found in other neurodegenerative models, namely amyotrophic lateral sclerosis (ALS) and experimental autoimmune encephalomyelitis (EAE; Keren-Shaul et al., 2017; Krasemann et al., 2017). DAM downregulate homeostatic microglial genes such as *P2ry12*, *Tmem119* and *Cx3cr1*, while inducing the expression of several AD associated activation markers, such as *Apoe*, *Tyrobp* and *Trem2* (Keren-Shaul et al., 2017; Krasemann et al., 2017). Keren-Shaul et al. (2017) proposed that TREM2 is required for induction of fully activated DAM, which is preceded by an intermediate state of microglial activation initiated in a TREM2-independent manner. On the other hand, Krasemann et al. (2017) showed that induction of MGnD can be initiated by phagocytosis of apoptotic neurons and is mediated by TREM2-induced expression of ApoE and miR-155. TREM2 is a critical regulator of DAM activation yet the exact role of TREM2 in this process needs further investigations.

FUNCTIONS OF TREM2

TREM2 is an immunoglobin (Ig) superfamily receptor present on various cells of the myeloid lineage including CNS microglia, bone osteoclasts, alveolar and peritoneal macrophages (Colonna and Wang, 2016). TREM2 consists of an extracellular V-type Ig-like domain, a transmembrane domain, a stalk region that connects the two and a short cytoplasmic tail. TREM2 binds to polyanionic molecules such as bacterial lipopolysaccharide (LPS; Daws et al., 2003), phospholipids (Wang et al., 2015), lipoproteins such as HDL and LDL (Song et al., 2017), which form complexes with APOE and APOJ (Atagi et al., 2015; Yeh et al., 2016) and apoptotic neurons, and signals through DAP12 (TYROBP; **Figure 1**). DAP12 is an adaptor protein that contains immunoreceptor tyrosine-based activation motifs (ITAMs), which function as docking sites for protein kinases. Upon TREM2 ligand binding, the ITAMs of DAP12 get phosphorylated and recruit spleen tyrosine kinase (SYK), which initiates protein tyrosine kinase phosphorylation, phosphoinositide 3-kinase (PI3K) activation, efflux of Ca^{2+} and mitogen-activated protein kinase (MAPK) activation. One report showed that DAP10, an adaptor closely related to DAP12, is required for the recruitment of the p85 subunit of PI3K to DAP12 (Peng et al., 2010; **Figure 1**). The triggering of kinase cascades by TREM2 activation promotes microglial survival, proliferation and leads to rearrangement of actin cytoskeleton. Lack of *Trem2* impairs proliferation of osteoclast precursors (Otero et al., 2012) and *Trem2*$^{-/-}$ microglia or macrophages are less viable under stress (Wang et al., 2015; Wu et al., 2015). *In vitro*, several studies have suggested that TREM2 may be a phagocytic receptor that mediates phagocytosis of apoptotic neurons (Takahashi et al., 2005; N'Diaye et al., 2009) or lipidated Aβ (Yeh et al., 2016). TREM2 mutations Y38C and T66M that reduce cell surface TREM2 expression impair phagocytosis (Kleinberger et al., 2014). Migration of microglia towards injected apoptotic neurons was also attenuated in *Trem2*$^{-/-}$ mice (Mazaheri et al., 2017). In addition, TREM2 also modulates inflammation. In *Trem2*$^{-/-}$ or *Dap12*$^{-/-}$ macrophages stimulated with low amounts of toll-like receptor (TLR) agonists, the level of inflammatory cytokines produced, such as TNFα and IL-6, is significantly increased (Hamerman et al., 2006; Turnbull et al., 2006), indicating TREM2 modulates TLR-mediated inflammatory responses but only in response to low levels of TLR stimulation. Furthermore, Aβ binds to TREM2 and activates TREM2 signaling pathway (Lessard et al., 2018; Zhao et al., 2018). Whether TREM2 binding to Aβ affects inflammatory responses in microglia needs further studies, since very few changes in cytokine production after Aβ stimulation alone were detected (Zhao et al., 2018).

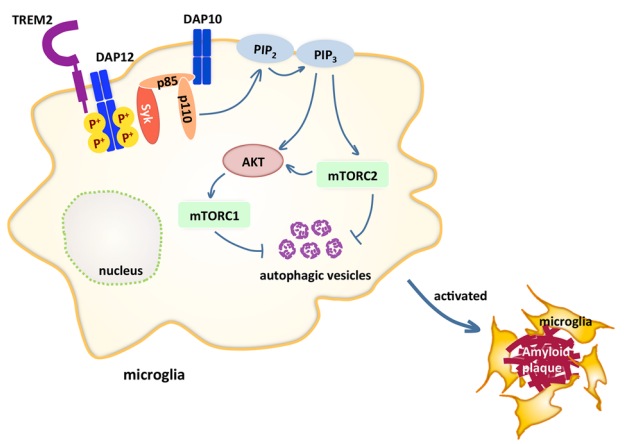

FIGURE 1 | Triggering receptor expressed on myeloid cells 2 (TREM2) maintains microglial metabolism through the mammalian target of rapamycin (mTOR) pathway in Alzheimer's disease (AD). TREM2 pairs with DAP12 through charge interactions in the transmembrane domain. Upon TREM2 ligand binding, DAP12 gets phosphorylated and recruits spleen tyrosine kinase (SYK), which initiates a cascade of signaling events, including phosphoinositide 3-kinase (PI3K) activation, which is composed of p85 and p110. The recruitment of p85 requires DAP10. One of the PI3K downstream targets AKT then activates mammalian target of rapamycin complex 1 (mTORC1) and mTORC2, which inhibits autophagy. These signaling events maintain microglia at high energy states so that in AD models, microglia are able to cluster around amyloid plaques.

Membrane bound full-length TREM2 undergoes sequential protease cleavages by a disintegrin and metalloproteases (ADAMs) and γ-secretase, which produces soluble TREM2 (sTREM2; Wunderlich et al., 2013; Kleinberger et al., 2014). In AD patients, sTREM2 levels increase in the cerebrospinal fluid (CSF) and correlate with the levels of tau in CSF (Suárez-Calvet et al., 2016), indicating sTREM2 as a biomarker for AD. It is possible that sTREM2 acts as a decoy receptor that inhibits full length membrane-bound TREM2 from binding to its ligands. Recently, two groups suggested sTREM2 increases cell viability (Wu et al., 2015; Zhong et al., 2017). However, a receptor responsible for activating downstream survival signals was not identified and thus the physiological function of sTREM2 remains elusive.

TREM2 AND AD PATHOLOGY

The importance of TREM2 in the CNS was first highlighted by the discovery of Nasu-Hakola disease (NHD), a rare autosomal recessive disorder presented with an early onset dementia. Homozygous loss-of-function mutations in either TREM2 (such as Q33X, Y38C and T66M) or DAP12 lead to NHD and FTD-like syndrome (Bianchin et al., 2006; Guerreiro et al., 2013). More recent studies have associated TREM2 with increased risk of several neurodegenerative diseases, including AD (Guerreiro et al., 2013; Jonsson et al., 2013), Parkinson's Disease (Rayaprolu et al., 2013), frontotemporal dementia (Thelen et al., 2014) and ALS (Cady et al., 2014).

Mechanisms of TREM2-related neurodegeneration have been intensively investigated. Many studies have shown that microglia in mouse models and patients with AD upregulate TREM2 expression (Frank et al., 2008; Lue et al., 2015; Wang et al., 2015), suggesting that upregulation of TREM2 may be associated with AD progression. Moreover, TREM2 plays a prominent role in driving microgliosis in AD mouse models and patients. In AD mice expressing WT *Trem2*, microglia

cluster around plaques, providing a barrier to the surrounding neurons (Ulrich et al., 2014; Jay et al., 2015; Wang et al., 2015, 2016; Yuan et al., 2016). On the contrary, the number of plaque-associated microglia is significantly reduced in *Trem2* deficient AD mice, and the effect is *Trem2* gene dose-dependent (Jay et al., 2015; Wang et al., 2015). In both transgenic mice expressing the human TREM2 R47H or patients with the R47H variant, a similar reduction in microgliosis is observed (Yuan et al., 2016; Song et al., 2018), supporting that R47H is a loss-of-function mutation. Consistently, a larger number of dystrophic neurites accumulate around plaques in $Trem2^{-/-}$ 5xFAD mice, compared to WT *Trem2* 5xFAD. The observations of plaque-associated microglia are consistent with the identification of DAM (Keren-Shaul et al., 2017; Krasemann et al., 2017). As DAM are phagocytic, one would hypothesize that in $Trem2^{-/-}$ AD mice, lack of DAM would lead to increased Aβ burden. However, observations on plaque load in *Trem2*-deficiency AD mice are inconsistent (Ulrich et al., 2014; Jay et al., 2015; Wang et al., 2015), which is likely due to different mouse models used or timing at which the analyses were done (Jay et al., 2017). Despite discrepancies on plaque load, changes in plaque morphology have been reported by several groups. In $Trem2^{-/-}$ 5xFAD mice, amyloid plaques appear with loosely packed cores and more diffuse structures extending outward (Wang et al., 2016; Yuan et al., 2016), which is also observed in R47H carriers (Yuan et al., 2016), suggesting that TREM2-mediated microglia-plaque interaction may be critical for compacting amyloid fibrils. Whether the changes in plaque structure are relevant to the role of TREM2 in AD is uncertain, since diffuse non-fibrillar plaques also occur in cognitively normal individuals (Morris et al., 2014). More studies are needed to investigate if compositions of plaques are different. Besides, whether the increased neuritic dystrophy in *Trem2* deficient mice is due to impaired clearance of damaged neurites by $Trem2^{-/-}$ microglia or the result of elevated damage by loosely compacted plaques also remains unclear. Collectively, these studies indicate that TREM2 signaling promotes microglial responses to Aβ in AD.

The effects of TREM2 on tau-driven AD models have not been intensely investigated. Recently, two groups that studied tau models showed inconsistent results. In the PS19 tau transgenic mice that express a human tau with the P301S mutation, lack of *Trem2* leads to less brain atrophy but no change in tau phosphorylation or aggregation. These tau mice lacking *Trem2* show reduced microgliosis and decreased microglial activation at 9-month old (Leyns et al., 2017). In contrast, in another study using a mouse model expressing the full-length human tau gene, *Trem2* deficiency resulted in elevated hyperphosphorylation and aggregation of tau (Bemiller et al., 2017). As with Aβ models, controversy on effects of TREM2 in tau pathology remains. The relationship of Aβ and tau and how it is affected by TREM2 are interesting topics for future studies.

TREM2 AND METABOLISM

Metabolic dysfunctions have long been associated with AD, while most studies focused on neuronal metabolisms. Our group and others have recently linked defective microglial function to metabolism in dementia. Kleinberger et al. (2017) demonstrated that cerebral metabolic rates of glucose slowed down as shown by reduced FDG-uPET signal in a Trem2 T66M knock-in mouse model of NHD. The reduced glucose usage could be due to defective microglial function that impairs the metabolic states of the brain, hinting that dysfunctional TREM2 might alter the brain metabolism and thus promote pathogenesis (Kleinberger et al., 2017).

In another study, it was shown that *Trem2*-deficient bone marrow-derived macrophages exhibit a defective energetic and anabolic state, which is further exacerbated by stress, such as CSF1 reduction (Ulland et al., 2017). This metabolic defect is a result of reduced mammalian target of rapamycin (mTOR) signaling, which is ameliorated by activation of Dectin-1, a receptor that activates downstream PI3K and mTOR independent of TREM2, or cyclocreatine, a creatine analog that restores ATP level. Impaired mTOR signaling was also observed in microglia sorted from $Trem2^{-/-}$ 5xFAD mice. These results suggest that TREM2 maintains microglia at high metabolic states through enhanced activation of the mTOR pathway (**Figure 1**). Furthermore, increased autophagy is detected in *Trem2*-deficient microglia and in AD patients carrying one allele of the R47H or R62H variant of TREM2, suggesting microglia attempt to compensate the mTOR defects with autophagy as a survival mechanism.

AUTOPHAGY AND AD

Autophagy is a self-eating process where cells clear misfolded proteins and damaged organelles as a housekeeping mechanism and as a survival mechanism during stress and starvation. The formation of an autophagophore, an isolation membrane that initiates autophagy and becomes the autophagosome later, requires the ULK complex and the class III PI-3 kinase (PI3K) complex composed of Vps34, Beclin-1, p150 and ATG14. The ULK complex is inactivated by mammalian target of rapamycin complex 1 (mTORC1) and positively regulated by AMP-activated protein kinase (AMPK). Activation of ULK and PI3K complexes recruit additional autophagy related (ATG) proteins to drive autophagosome nucleation. Then the ATG12-ATG5-ATG16 complex is recruited to facilitate lipidation of microtubule-associated protein 1 light chain 3 (LC3), resulting in the conversion of LC3-I to LC3-II, as the isolation membrane expands to form the autophagosome (Kabeya et al., 2000). In mammalian cells, only LC3-II remains on autophagosome membranes until after their fusion with lysosomes, allowing it to be a marker for autophagy (Mizushima et al., 2010).

Autophagy has been associated with AD. The levels of autophagy protein Beclin 1 are decreased in AD patients but not in AD mouse models (Pickford et al., 2008), pointing to the possibility that Beclin 1 reduction occurs upstream of amyloid pathology. Genetic ablation of one copy of *Beclin1* reduces autophagy in cultured primary neurons and increases Aβ deposition and neuronal loss in an AD model. On the other hand,

an increase of CD68 immunoreactivity, an activation marker, with the absence of change in Iba1 marks microglial activation in *Beclin* $1^{+/-}$ AD mice without affecting microglial number (Pickford et al., 2008). These findings indicate that neuronal autophagy plays a protective role in AD progression. In line with this, overexpression of another autophagy gene, p62, by adeno-associated virus (AAV) infection in neurons improves cognitive functions and reduces Aβ pathology in an autophagy-mediated manner (Caccamo et al., 2017). Pharmaceutically, feeding mice with rapamycin, an inhibitor of the mTOR pathway, induces autophagy in two mouse models of AD but not in non-AD littermate controls, suggesting high Aβ level is a trigger of autophagy. This rapamycin-mediated mTOR inhibition leads to a reduction of Aβ42 level in the hippocampus and an amelioration in memory deficits by increasing autophagy (Caccamo et al., 2010; Spilman et al., 2010). Although all these studies suggest a protective function of autophagy, whether this effect is only mediated by neurons or if glia also play a role is unclear.

Rather, our study demonstrated a role of TREM2 in attenuating microglial autophagy (Ulland et al., 2017). Compared to control, microglia lacking TREM2 contain more autophagic vesicles as shown by electron microscopy. Increased autophagy in *Trem2*-deficient microglia is a result of reduced mTOR signaling in response to a low metabolic state of microglia and this increase in autophagy is not sufficient to rescue microglia from dying under stress, such as neuroinflammation. Interestingly, dietary supplementation with cyclocreatine rescues *Trem2*-deficient microglia from autophagy and partially corrects the defect in microglial clustering in *Trem2*$^{-/-}$ 5xFAD. These results suggest that TREM2 sustains microglial metabolism thus allowing them to function properly. This study links TREM2 to metabolic states of microglia and allows us to revisit the role of TREM2 and microglia in AD pathology.

FUTURE PERSPECTIVES

The role of microglia in neurodegeneration has been hotly debated while no concluding agreement has been made. Such an agreement is difficult to achieve as different groups using similar models show different results, perhaps due to microbiota. Notably, studies on germ-free mice in AD models are currently being conducted. As TREM2 is required to turn homeostatic microglia into activated microglia, whether these activated microglia acquire a unique metabolic state is an intriguing topic. It remains unclear what exact role TREM2 plays. Does it sense changes in environment or just provide a tonic signal to keep microglia ready to go? Further studies are needed to distinguish the two possibilities. Defining the ligands of TREM2 *in vivo* and how it is activated will help answer the question. Microglia can be a double-edged sword. Deciphering the role of DAM will advance our therapeutic approaches in neurodegenerative diseases.

AUTHOR CONTRIBUTIONS

YZ drafted the manuscript. TU and MC reviewed and edited the manuscript.

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Roles of Microglial and Monocyte Chemokines and Their Receptors in Regulating Alzheimer's Disease-Associated Amyloid-β and Tau Pathologies

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Chemokines and their receptors have been shown to affect amyloid-β (Aβ) and tau pathologies in mouse models of Alzheimer's disease (AD) by regulating microglia and monocyte-associated neuroinflammation, microglial movement and monocyte recruitment into the brain. These cells in turn can promote and mediate Aβ phagocytosis and degradation and tau phosphorylation. In this review we discuss published work in this field in mouse models of AD and review what is known about the contributions of microglial and monocyte chemokines and their receptors to amyloid and tau pathologies. We focus on the roles of the chemokine/chemokine receptor pairs CCL2/CCR2, CX3CL1/CX3CR1, CCL5/CCR5, CXCL10/CXCR3 and CXCL1/CXCR2, highlighting important knowledge gaps in this field. A full understanding of the functions of chemokines and their receptors in AD may guide the development of novel immunotherapies for this devastating disease.

Keywords: Alzheimer's disease, amyloid-β peptide, protein tau, microglia, monocytes, chemokine receptors, chemokines, neuroinflammation

INTRODUCTION

Neuroinflammation is an important contributor to Alzheimer's disease (AD) pathogenesis and progression (1, 2). Indeed, several inflammatory mediators such as tumor necrosis factor (TNF) and interleukin-1 (IL-1) are elevated in the brains of AD patients and mouse models of AD (3, 4). In addition, several variants in immune genes such as TREM2, CD33, and CR1 that regulate the inflammatory response have been identified by Genome-Wide Association Studies (GWAS) as genetic risk factors for AD (5–9). Microglia and recruited peripheral blood monocytes are the principal innate immune cells involved in the pathogenesis of AD and extensive evidence indicate that their functions are in part regulated by chemokines and their receptors (1).

BETA AMYLOID DEPOSITION AND TAU HYPERPHOSPHORYLATION ARE HALLMARKS OF AD

Clinically, AD is associated with dementia and progressive cognitive decline. Pathologically, AD is characterized by the presence of extracellular senile plaques and intraneuronal neurofibrillary tangles (NFTs). These protein deposits contain aggregates of the amyloid-β (Aβ) peptide and hyperphosphorylated microtubule-associated protein tau (p-τ), respectively. The processes of Aβ production and tau hyperphosphorylation involve different pathways. Aβ accumulation is the result of tandem cleavage of the amyloid precursor protein (APP) by secretases, whereas tau hyperphosphorylation results from the activity of several kinases. However, increasing evidence indicate that these abnormal protein deposits influence each other and have an additive effect on disease progression and that Aβ deposition appears to regulate tau pathology (10, 11). Indeed, in experimental animals, Aβ injection in the brain of P301L mice, a model for tau pathology, increases formation of NFTs (12). Furthermore, breeding mice expressing 5 early onset familial AD mutations (5XFAD) with mice expressing the tau P301S mutation (PS19) results in a ∼10-fold aggravated tauopathy (13). In contrast, APP-KO mice subjected to subtoxic doses of soluble oligomeric forms of tau protein do not exhibit tau-induced defects in spatial/associative memory and its electrophysiological surrogate long-term potentiation (LTP), suggesting that tau pathology is dependent on APP expression (14). Aβ accumulation appears to promote tau hyperphosphorylation via activation of glycogen synthase kinase-3β (GSK-3β) (15).

The effects of Aβ deposition on tau pathology and neurodegeneration may not be necessarily due only to fibrillar Aβ deposits in senile plaques. In fact, cognitive deficits start before these plaques are visible and it has been proposed that the spectrum of Aβ species, including intraneuronal Aβ, soluble Aβ and Aβ oligomers also contribute to synaptic disruption and tau hyperphosphorylation. Regardless of the exact species of Aβ involved in this process, the co-localization of Aβ and p-τ in synaptic sites (16) suggest that the deposition of these protein aggregates and their contribution to neuronal loss is affected by their interactions. In this regard, strategies for treatment of AD should consider both Aβ and p-τ aggregates and how they influence each other (17).

THE MONONUCLEAR PHAGOCYTE SYSTEM IN ALZHEIMER'S DISEASE

There is strong supporting evidence that the inflammatory response in AD is in part driven by the interaction of Aβ with mononuclear phagocytes, including microglia and recruited peripheral blood monocytes (1, 18, 19). Multiple forms of Aβ accumulate in the AD brain before the development of visible senile plaques and formation of NFT. Since these forms of Aβ can interact with microglia and/or monocytes and lead to an inflammatory response, several groups have suggested that neuroinflammation is an early event in Aβ and tau pathologies (20–22), preceding the accumulation of larger visible protein deposits. The mononuclear phagocyte system has been extensively studied in the context of neuroinflammation in AD. However, the contribution of different cells types of this system, such as microglia and monocytes to AD pathogenesis is only beginning to be understood.

Microglia are the principal resident sentinels in the brain and can rapidly sense changes in their environment such as Aβ deposition, using a set of genes termed the sensome (23). Subsequent to sensing Aβ, the interaction of microglia with Aβ is a double-edged-sword. On one hand, microglia can phagocyte and clear Aβ deposits thereby limiting the progression of AD pathology (24, 25). In support of this pathway, reduced expression of Aβ-binding receptors in microglia from aged mice is associated with reduced Aβ phagocytosis and clearance and increased Aβ accumulation and disease progression (4). In contrast, the continuous interaction of microglia with Aβ induces the activation of the inflammasome pathway and produce several inflammatory mediators and neurotoxins, thereby contributing to the progression of AD in several ways (2, 19, 26–30). It is possible that persistent Aβ-induced microglial activation could also contribute to Aβ deposition, since continuous production of neurotoxic factors by microglia can induce neuronal apoptosis and consequent release of intraneuronal Aβ into the extracellular space. In addition, inflammatory cytokines as IL-1β, INF-γ, and TNF-α have been shown to upregulate β-secretase expression thereby increasing Aβ production (31). Such inflammatory cytokines also affect LTP in the hippocampus (32), suggesting that they can influence synaptic plasticity, which is essential for memory formation. In support of this, during microglia activation, their branches disappear, giving place to an amoeboid morphology which may limit their ability to refine and sustain synapses.

In contrast to what we know about microglia-Aβ interactions, less is known about microglia and NFTs. Microglia appear to promote tau propagation and contribute to the spreading of tau pathology in the brain (33, 34). Microglial activation also induces tau phosphorylation and aggregation (35). Some anti-inflammatory drugs have been shown to reduce p-τ in P301S and 3xTg AD mice (22, 36), but whether the mechanism is microglia-dependent remains to be determined. Published reports also suggest that injured neurons exhibiting tau hyperphosphorylation modulate microglia-mediated neuroinflammation (37). While these studies are compelling, they do not provide evidence of direct activation of microglia by p-τ. However, they suggest that neuroinflammation is closely linked to tau pathology.

In addition to studies on microglia function in AD, monocytes can infiltrate the AD brain and, although their role in AD progression is unclear at this time, it has been proposed that they have both detrimental and beneficial effects depending on the stage of disease development (38–40). Monocytes infiltrate the AD brain and clear perivascular Aβ (39, 41, 42). In support of this, stimulating monocyte infiltration by peripheral

challenging with macrophage colony-stimulating factor (M-CSF) and lipopolysaccharide (LPS) or blocking immunosuppressive transforming growth factor beta (TFG-β) can attenuate AD pathology (43). While these studies suggest a protective role for monocytes in AD, monocytes, similar to microglia, can become activated and promote neuroinflammation and neurotoxicity (44).

In contrast to the studies supporting a role of monocytes in AD, a recent report using parabiosis experiments and staining for CD11b and CD45 suggests that monocytes do not infiltrate the brain in APP/PS1 and 5XFAD mouse models of amyloidosis (45). Interestingly, the same group also showed the presence of monocytes in the brain of 5XFAD mice when single cell RNAseq was performed on myeloid cells and more cell specific markers were analyzed (46). Additional mouse and human studies using single cell RNASeq, flow cytometry with multiple microglia and monocyte markers and *in situ* hybridization will help clarify this apparent discrepancy.

CHEMOKINES ARE ESSENTIAL FOR THE ACCUMULATION OF MONONUCLEAR PHAGOCYTES IN THE ALZHEIMER'S DISEASE BRAIN

Chemokines are chemotactic cytokines which mediate immune cells migration to sites of inflammation. Initially designated with specific protein names, chemokines are now classified based on the number of amino acids between two cysteine residues: α-chemokines, with the first cysteine residues separated by one amino acid (CXC); β-chemokines, with adjacent cysteine residues (CC); lymphotactin, with only two cysteines, and fractalkine (CX3CL1) in which the first two cysteine residues are separated by three amino acids (47, 48). These small chemoattractant proteins bind to chemokine receptors classified in the same manner. Different chemokine receptors are expressed on different immune cells. Monocytes, for example, express CCR1, CCR2, CCR5, CCR8, CXCR4, and some CX3CR1 (49). In contrast, microglia express very high levels of CX3CR1 and CCR5 and, to a lesser extent CXCR4, CXCR3, and CXCR2 (23).

Since chemokines mediate the infiltration of peripheral monocytes into the inflamed central nervous system (50), we proposed that the same occurs in AD (47, 48). The association between chemokines and AD is supported by studies showing that CCL2 and CCL5 expression are increased in the AD brain (51, 52). *In vitro*, microglia express CCL2 when incubated with Aβ (19) and neurons from AD brains have been shown to upregulate CCL5 expression (53). Moreover, CCL5 is essential to Aβ induced-microglia chemotaxis (54, 55). Similarly, the CX3CR1 ligand CX3CL1 is highly expressed on neurons. These studies suggest that chemokines are important in AD and possibly mediate the infiltration of peripheral monocytes into the AD brain and/or the accumulation of microglia at sites of Aβ deposition. Additional insights into the roles of chemokines, their receptors and mononuclear phagocytes in AD progression come from animal models where the function of chemokine receptors have been challenged.

CCL2/CCR2 AXIS

When combined with Aβ and p-τ levels, CCL2 expression in the brain and cerebrospinal fluid (CSF) is a reliable predictor of AD severity (51, 56). In support of these observations, the CCL2 receptor, CCR2, was the first chemokine receptor shown to be associated with AD. We found that, in Tg2576 AD mice, CCR2 deficiency accelerates early disease progression by impairing the accumulation of mononuclear phagocytes (39). APP-*CCR2*$^{-/-}$ mice exhibited higher Aβ levels and reduced CD11b^{+} cell recruitment into the brain. Importantly, these mice showed higher mortality in a *CCR2* gene dosage-dependent manner. Subsequent studies showed that the decrease in perivascular Aβ observed in our study was due to the lack of blood monocytes accumulation at these sites and possibly their infiltration in the brain parenchyma (39). These findings were corroborated by the finding that CCR2 deficiency worsened memory deficits and increased soluble Aβ in APP/PS1 mice (57). This study also showed that lack of CCR2 stimulated the expression of TGF-β receptors and CX3CR1 in plaque-associated microglia, implicating another chemokine receptor in AD pathology. The progressive cognitive decline in these mice was associated with a decrease in the numbers of CX3CR1^{low}Ly6-C^{high}CCR2^{+}Gr1^{+} circulating inflammatory monocytes (58) and restoring CCR2 expression in bone marrow cells reestablished memory capacities and decreased soluble Aβ accumulation (58). Altogether, these reports show that CCR2^{+} monocytes can be protective in AD.

We propose that CCR2 promotes the recruitment of monocytes initially from the bone marrow into the blood, then from the blood across the blood-brain barrier (BBB), to the perivascular space, then from the perivascular space to sites of Aβ deposition in the parenchyma where these cells can potentially clear Aβ by phagocytosis (**Figure 1**). In support of these findings, we recently showed that the CCL2/CCR2 axis was impaired in blood-derived monocytes from AD patients, causing a deficit in cell migration (59).

CX3CL1/CX3CR1 AXIS

Another chemokine receptor thoroughly studied in AD is CX3CR1. This receptor is highly expressed in microglia and CX3CR1-GFP *knock-in* mice (where GFP replaced one CX3CR1 allele) have been used to specifically study, *in vivo*, the role of microglia in AD and other brain diseases. In physiological conditions, disruption of CX3CR1 function affects cognitive functions in a IL-1β-dependent manner (60) and exacerbates LPS-induced inflammation (61), suggesting that CX3CR1 maintains microglial homeostasis, being essential for their function in synaptic support and limiting their activation. In contrast to its clear role in maintaining microglial homeostatic functions under physiological conditions, dysregulation of the CX3CL1/CX3CR1 axis in AD mouse models can have both neuroprotective and neurotoxic effects depending on the mouse model used. CX3CR1 deficiency in three different AD mouse models—APP/PS1, R1.40 and CRND8—reduced amyloid deposits and enhanced Aβ phagocytic ability by microglia (62, 63). These effects were associated with decreased

Receptor	CCR2		CX3CR1		CCR5		CXCR3		CXCR2	
Chemokine (Source)	CCL2 (Microglia, Astrocytes)		CX3CL1 (Neurons)		CCL3, CCL4, CCL5 (Microglia)		CXCL10 (Astrocytes)		CXCL1 (Astrocytes, neurons)	
Monocyte	Highly Expressed		Moderate expression on a subset of monocytes		Expressed		Expressed		Expressed	
	Aβ: Bone Marrow ⇩ Blood ⇩ BBB ⇩ Aβ Deposit	Tau: Unknown role	Aβ: Unknown role	Tau: Unknown role	Aβ: Recruitment to Aβ sites	Tau: Unknown role	Aβ: Unknown role	Tau: Unknown role	Aβ: Unknown role	Tau: Unknown role
Microglia	low level of Expression		Highly Expressed		Highly Expressed		Expressed		Expressed	
	Aβ: Unknown role	Tau: Unknown role	Aβ: Deficiency Promotes Aβ Clearance	Tau: Deficiency worsens Tau Pathology	Aβ: Recruitment to Aβ sites	Tau: Unknown role	Aβ: Deficiency Promotes Aβ Clearance	Tau: Unknown role	Aβ: Possibly Recruitment to Aβ sites	Tau: Unknown role

FIGURE 1 | Major chemokine receptors expressed on monocytes and microglia and their possible roles in Aβ and Tau pathologies.

microglia activation and TNF levels and increased IL-1β levels. Similarly, following injection of $A\beta_{1-40}$ fibrils in the hippocampus, downregulation of CX3CR1 with siRNAs also suppressed microglial activation, increasing synaptic strength and cognitive functions (64). On the other hand, CX3CR1 deficiency in hAPP mice worsened behavioral deficits associated with cytokine production independent of plaques deposition (65). Although these mice do not express tau mutations, the effect of CX3CR1 disruption was associated with enhanced tau pathology. Moreover, hTau mice (which exhibit p-τ) lacking CX3CR1 exhibited enhanced tau phosphorylation and aggregation associated with microglial activation, as well as behavioral impairments (35, 66). Analyzing these studies, it is interesting to observe that the neuroprotective role of CX3CR1 is always associated with tau and not Aβ. In support of these findings, overexpression of soluble CX3CL1 in mice with Tau but not Aβ pathology led to substantial improvements (67). Interestingly, CX3CR1 deletion in 3xTg mice, which have both Aβ and tau pathologies, prevented neuronal loss, suggesting that the effect of CX3CR1 deficiency on Aβ pathology may be more dominant than its effect on tau pathology (68). It is possible, however, that CX3CR1 is involved in the killing of neurons with intracellular tau deposits and the subsequent release of tau (68) (**Figure 1**). Additional studies with AD models that exhibit both Aβ and tau pathologies are needed to definitively clarify the role of CX3CR1 in AD.

CCL5/CCR5 AXIS

$CCR5^{+}$ reactive microglia are found associated with Aβ deposits in AD patients (69). $CCR5^{-/-}$ mice have higher levels of Aβ, C99 [a product that results from APP cleavage by β-secretase 1 (BACE1)] and BACE1 itself compared to normal mice (70). These levels were associated with astrocyte activation and CCR2 overexpression leading to cognitive impairments. Also, following injection of $A\beta_{1-40}$ into the lateral ventricle of $CCR5^{-/-}$ mice, a reduced activation of microglia and astrocytes was observed compared to wild-type mice (71). Interestingly, CCR5 appears to be essential for the transendothelial migration of T cells across the BBB in the hippocampus of rats injected with Aβ (72), suggesting that CCR5 may be essential for the infiltration of non-phagocytic peripheral immune cells to the AD brain. Since CCR5 is also expressed on a subset of monocytes and on microglia, it is possible that it plays an additive role to CCR2 in mediating monocyte movement across the BBB, as well as in the movement of microglia toward sites of Aβ deposition in the parenchyma (**Figure 1**).

In addition to CCL5, we have shown that microglia and macrophages stimulated with Aβ, *in vitro*, show increased mRNA levels for the chemokines CCL3 and CCL4 also known as MIP1α and MIP1β respectively (19). These two chemokines are also upregulated in adult human microglia isolated from post-mortem brains and stimulated with Aβ (73) and in plaque-associated microglia in AD patients (74). The exact roles of these chemokines in AD is not clear. However, both chemokines are ligands for CCR5, suggesting possible complementary roles to CCL5 in the accumulation of T cells, monocytes or microglia in the AD brain. To date, there are no published reports describing the effects of CCL3, CCL4, or CCL5 deficiency on Aβ deposition or tau pathology in a transgenic Aβ or tau model. Such studies will help clarify the role of CCR5 ligands in AD development and progression, an important current knowledge gap in the field.

CXCL10/CXCR3 AXIS

CXCL10 levels in CSF are significantly increased in patients with amnestic mild cognitive impairment (MCI) and patients with mild AD, but not in patients with severe AD (75). Unlike its

receptor CXCR3, which is constitutively expressed on microglia and neurons, CXCL10 is expressed in a subpopulation of astrocytes in the normal brain and is markedly elevated in astrocytes in AD (76, 77). Peripheral injection of LPS strongly induces CXCL10 the brain of rats, as well as in cultured astrocytes and microglia (78), indicating its involvement in the response to inflammatory stimuli. Although CXCL10 was found to co-localize with Aβ plaques in APP mice (79), its function in regulating Aβ pathology remains unclear. A recent study using CXCR3-deficient AD mice showed that deletion of CXCR3 significantly reduced plaque burden and Aβ levels in APP/PS1 mice with morphological evidence for microglial activation, but reduced plaque association. This study suggests a possible role in the recruitment of microglia to Aβ deposits (80). CXCR3 deficiency also increased microglial uptake of Aβ, reduced concentrations of proinflammatory cytokines and attenuated behavioral deficits in APP/PS1 mice (80), suggesting an important role for CXCL10/CXCR3 signaling in mediating Aβ-induced pathology in mouse models. The role of CX3CR1 in tau pathology is not clear and needs to be investigated (**Figure 1**).

CXCL1/CXCR2 AXIS

CXCR2 is expressed at low levels on microglia (23). CXCR2 deficiency results in reduction of Aβ with concurrent increases of γ-secretase substrates in the APP/PS1 mice (81). Whether microglia play a direct role in this process is not clear and requires a more detailed analysis of CXCR2- deficient APP/PS1 mice. Insights into a possible microglial role on the effect of CXCR2 depletion came from studies performed in rats injected with Aβ. Administration of a competitive CXCR2 antagonist to Aβ-injected rats significantly reduced expression of CXCR2 and microgliosis (82). Similar studies in mouse models of AD need to be done to further validate these findings.

CHEMOKINES RECEPTORS AS TARGETS FOR AD THERAPY

Based on the above discussion, it may be possible, in the future, to consider chemokine receptors as potential targets for the treatment of AD. One could envision upregulating CCR2 expression to increase influx of monocytes into the AD brain, therefore leading to increased removal of Aβ deposits and reduced Aβ burden. However, it will be difficult to devise such a strategy for CX3CR1 and CCR5, until the effects of deleting these receptors on both Aβ- and tau-associated pathologies are defined in animal models that exhibit both features. Importantly, one must consider that these receptors can display different functions in different stages of AD progression.

CONCLUSION

The work summarized in this review suggests that chemokines and their receptors are important for AD pathogenesis and in the development of the two main pathological hallmarks of AD—Aβ deposition and tau hyperphosphorylation. These receptors are associated with innate and adaptive responses regulating microglia and peripheral immune cells activation, and are essential for the infiltration of immune cells to the AD brain and movement of recruited monocytes and microglia toward sites of Aβ deposition. These studies clearly point to the importance of the neuroinflammatory component of AD as an active process which contributes to AD development and suggest that the presence of immune cells in the AD brain is tightly regulated during different stages of AD progression, constituting a double-edged sword that may lead to neuroprotective or neurotoxic outcomes. However, in spite of the significant amount of knowledge gained so far, there are important knowledge gaps that limit our understanding of the roles of chemokines and their receptors in AD. Additional studies to explore how these receptors and their ligands influence Aβ deposition, tau hyperphosphorylation, microglia and monocyte accumulation, the overall inflammatory response and neuronal degeneration are needed. A full understanding of the roles of chemokines and their receptors in AD may guide the development of multiple novel immunotherapies targeted to various stages of the disease.

AUTHOR CONTRIBUTIONS

JRG and JE wrote the initial version of the manuscript, then all authors contributed to writing and editing the submitted manuscript.

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REVIEW
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Microglia in Alzheimer's Disease: A Role for Ion Channels

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Alzheimer's disease is the most common form of dementia, it is estimated to affect over 40 million people worldwide. Classically, the disease has been characterized by the neuropathological hallmarks of aggregated extracellular amyloid-β and intracellular paired helical filaments of hyperphosphorylated tau. A wealth of evidence indicates a pivotal role for the innate immune system, such as microglia, and inflammation in the pathology of Alzheimer's disease. The over production and aggregation of Alzheimer's associated proteins results in chronic inflammation and disrupts microglial clearance of these depositions. Despite being non-excitable, microglia express a diverse array of ion channels which shape their physiological functions. In support of this, there is a growing body of evidence pointing to the involvement of microglial ion channels contributing to neurodegenerative diseases such as Alzheimer's disease. In this review, we discuss the evidence for an array of microglia ion channels and their importance in modulating microglial homeostasis and how this process could be disrupted in Alzheimer's disease. One promising avenue for assessing the role that microglia play in the initiation and progression of Alzheimer's disease is through using induced pluripotent stem cell derived microglia. Here, we examine what is already understood in terms of the molecular underpinnings of inflammation in Alzheimer's disease, and the utility that inducible pluripotent stem cell derived microglia may have to advance this knowledge. We outline the variability that occurs between the use of animal and human models with regards to the importance of microglial ion channels in generating a relevant functional model of brain inflammation. Overcoming these hurdles will be pivotal in order to develop new drug targets and progress our understanding of the pathological mechanisms involved in Alzheimer's disease.

Keywords: microglia, Alzheimer's disease, ion channel, stem cells, iPSCs

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INTRODUCTION

Alzheimer's disease (AD) is the most prevalent neurodegenerative disorder and accounts for approximately 60–80% of all dementia cases worldwide (Alzheimer'sstatistics, 2016). Initial studies focussed on trying to identify a genetic basis to the disease (Gatz et al., 2006). Although some AD cases are caused by defined mutations in one of three genes (APP, PSEN1 and PSEN2) these account for fewer than 10% of all cases and occur before 65 years of age. The majority of cases are sporadic, have no defined etiology and occurs at or after a mean age of 65. Our understanding has progressed through evidence obtained from large cohort studies identifying genetic variants which are associated with and potentially result in the late onset form of AD (LOAD). These

genome wide association studies (GWAS) have demonstrated that LOAD is a multifactorial disease with many different genes and single nucleotide polymorphisms contributing to disease onset (Gatz et al., 2006). The most strongly associated gene with LOAD is Apolipoprotein E (APOE), which encodes a polymorphic glycoprotein that is involved in cholesterol and other lipid transport (Poirier, 2005) alongside tissue repair (Huang, 2010) and neuronal growth (Nathan et al., 1994). There are three isoforms of APOE, ε2, ε3, and ε4 that all correspond to cysteine to arginine substitutions at the amino acid positions 112 and 158 (Zlokovic, 2013). The ε4 variant confers increased risk of developing LOAD, and each additional copy of the ε4 allele lowers the mean age of onset (Corder et al., 1993). Neurodegenerative diseases such as AD were traditionally considered to be ''neurocentric," however recent findings are challenging this view, implicating glia as primary targets. GWAS studies reveal there have been a number of single nucleotide polymorphisms that are associated with AD which reside in genes involved in microglial biology. These include common variants such as CR1 (complement receptor 1), CD33 (sialic acid binding Ig-like lectin 3), CLU (clusterin), ABCA7 (ATP-binding cassette, sub family A, member 7), MS4A (membrane-spanning 4-domain family, subfamily A) and EPHA1 (ephrin type-A receptor 1) (Bertram et al., 2008; Harold et al., 2009; Hollingworth et al., 2011; Naj et al., 2011; Lambert et al., 2013; Zhang et al., 2013), and also more rare coding variants in genes such as TREM2 (triggering receptor expressed on myeloid cells 2) (Guerreiro et al., 2013; Jonsson et al., 2013). TREM2 is a cell surface receptor of the immunoglobulin superfamily that is expressed on microglia (reviewed by Colonna and Wang, 2016). Several variants within TREM2 appear to significantly increase the risk of developing AD (Jin et al., 2014; Song et al., 2017), in particular rs75932628, an SNP that confers an arginine to histidine change at amino acid 47 (R47H) (Guerreiro et al., 2013; Jonsson et al., 2013). Although TREM2 polymorphisms are associated with a risk of late-onset AD (Guerreiro et al., 2013), their role in neurodegenerative diseases is controversial. Indeed, recent evidence proposes that the TREM2-APOE pathway induces a microglia phenotypic switch from a homeostatic to neurodegenerative phenotype (Krasemann et al., 2017). One of the main functions of TREM2 is regulating microglial phagocytosis (Hsieh et al., 2009), and as a ligand for TREM2 in microglia, APOE binds to dead neurons and increases Trem2-mediated phagocytosis (Atagi et al., 2015). Interestingly, Kleinberger et al. (2014) showed that missense mutations in TREM2 resulted in impaired phagocytic activity with a reduced level of soluble TREM2 in cerebrospinal fluid (CSF) of AD patients. Indeed TREM2 deficiency has been shown to alter microglial function in both primary microglial cultures and in mouse models of AD where a decrease in plaque-associated microglia are observed alongside an increase in apoptosis of both resting and activated microglia and reduced phagocytosis (Ulrich et al., 2014; Jay et al., 2015, 2017). These findings suggest that the role of TREM2 in modulating inflammation may be more complex than previously appreciated and may be dependent on the cell type in which it is expressed and the inflammatory context in which it is studied. For a more in depth discussion we refer the reader to the following very comprehensive review articles (Colonna and Wang, 2016; Ulrich et al., 2017; Li and Zhang, 2018).

Microglia are thought to regulate the degree of Aβ deposition by phagocytosis with potentially protective impact on AD progression (Lee and Landreth, 2010). One striking feature of the behavior of microglia in the AD brain is their marked clustering around fibrillar Aβ deposits and they adopt a polarized morphology with hypertrophic processes extending toward plaques (Condello et al., 2015). This aids as a protective physical barrier mechanism through which the Aβ fibrils cannot extend, promoting the formation of highly compact plaque micro regions that have minimal affinity for soluble $A\beta_{1-42}$ (Condello et al., 2015; Yuan et al., 2016). Conversely, areas not covered by microglia processes display "hotspots" with very high soluble $A\beta_{1-42}$ affinity, leading to markedly concentrated protofibrillar Aβ42 plaque regions (Condello et al., 2015). These "hotspots" are neurotoxic given that adjacent axons develop a greater extent of dystrophy compared to those covered by microglia (Yuan et al., 2016).

On the other hand, most studies in TREM2- deficient AD-like mice have shown reduced number of microglia around Aβ plaques (Jay et al., 2015; Wang et al., 2015). Similar reports suggest that in R47H human mutants, microglial processes were also unable to form a robust barrier, resulting in a decreased Aβ fibril compaction (Yuan et al., 2016). With the decrease in microglial number, there are less compact Aβ fibrils and a higher ratio of $A\beta_{1-42}$ plaques (Yuan et al., 2016; Ulland et al., 2017), therefore a deficient rather than an exacerbated microglial response could give rise to the development of sporadic AD. Once activated by pathological triggers, like neuronal death or protein aggregates, microglia extend their processes to the site of injury, migrate to the lesion and initiate an innate immune response (Heneka et al., 2015). Mounting evidence from polymorphisms linking microglial dysfunction to AD could have a causal role in disease onset and progression and are not just a consequence of neuropathological hallmarks that are characteristic of AD.

THE INNATE IMMUNE SYSTEM IN AD

Of increasing interest is the involvement of the innate immune system in AD, particularly the role of microglia. Microglia are the resident immune cells in the brain and spinal cord, and play important roles in neurodevelopment, immune surveillance, disease and homeostasis (Nayak et al., 2014). Unlike neurons and other glial cell types, microglia are of haematopoietic lineage, arise early during development (Hutchins et al., 1990), and are derived from erythromyeloid progenitors (EMPs) in the yolk sac (Ginhoux et al., 2010).

Microglia can exist in several morphological/phenotypic states depending on the environment they are in or the factors they are stimulated by. From a highly processed state, the microglia become more amoeboid with increased numbers of intracellular vesicles in preparation for engulfment of foreign particles. These differential states have been termed accordingly as "classical activation," "alternative activation," and "acquired deactivation"

(Colton, 2009; Colton and Wilcock, 2010). Previous studies defined these states as separate from one another, a profiling index of M1 or M2 phenotyping suggesting a pro- or anti-inflammatory state respectively. More recently it has become more apparent that this is derived from the idea that microglia are central macrophages and so must follow by the same "kill or cure" switch seen in these cell types. However, microglia can exist in multiple phases with the same cell producing markers of both pro- and anti- inflammatory components depending on stimulus. Usage of M1/M2 profile terminology fails to capture the heterogeneity of microglia which is a vital to their local and global physiological responses (Mosser et al., 2017).

Classical activation, considered to be pro-inflammatory, is stimulated by IFN-γ and is associated with the production of cytokines such as TNF-α and IL-1β and nitric oxide production (Li et al., 2004; Block et al., 2007). On the other hand, alternative activation, is defined by the release of anti-inflammatory cytokines IL-4 and IL-13 and arginase 2. This results in gene expression to promote tissue repair and extracellular matrix reconstruction (Ponomarev et al., 2007; Colton, 2009). Acquired deactivation, is mainly seen in the presence of apoptotic cells and is characterized by the release of IL-10, TGF-β, IL-6, and CSF1 and the production of scavenger receptors (Sawada et al., 1999; Colton, 2009; Colton and Wilcock, 2010; Saijo and Glass, 2011). Microglial phagocytosis relies on specific receptors expressed on the cell surface and their downstream signaling pathways to instigate engulfment of harmful particulates (**Figure 1**).

Microglia mediate the innate immune response of the brain and are involved in the phagocytosis and clearance of debris, pathogens, and toxins. Their dysfunction and increased Aβ accumulation is universal to AD patients and not just those with familial APP mutations. This suggests that Aβ build-up is due to poor clearance and not APP proteolysis. Microglia will secrete both pro- and anti-inflammatory factors, which can either be beneficial or detrimental in neurodegenerative diseases. Here exists extensive literature showing that inflammation is integral to AD progression, facilitating Aβ deposition, neuronal loss and cognitive deficits. Brains from AD patients and those from murine models of Aβ pathology uniformly display high expression of pro-inflammatory cyto- and chemokines including TNFα, IFNγ, IL-1β, and IL-6 (Zheng et al., 2016). IL-1β and TNFα can impair neuronal function by suppression of long-term potentiation of synaptic transmission (LTP) (Rowan et al., 2007). Multiple interactions as well as elevated expression of additional cytokines/chemokines and innate immune receptors favor a pro-inflammatory activation state in AD.

Accumulating evidence demonstrates that inflammasomes, which cleave precursors of interleukin-1β (IL-1β) and IL-18 to generate their active forms, play an important role in the inflammatory response in the CNS and in AD pathogenesis. The inflammasome is an inducible, high molecular weight, protein complex consisting of the antigen sensor protein NLRP3, adaptor protein ASC, and pro-caspase 1 (Heneka et al., 2015). The complexing of these three components results in cleavage of caspase 1 and instigates a cascade of pro-inflammatory cytokine activation of the IL-1b family. In murine mutants where APP/PS1 was crossed with NLRP3-/- mice, a decrease in cC1 and IL-1β is observed (Heneka et al., 2013).

Conversely an anti-inflammatory profile of microglia also contributes to Aβ pathology. In murine models where IL-10 was either knocked down or knocked out in the APP/PS1 model, a decrease in Aβ load, increases phagocytosis and reduces microglial APOE expression was observed (Chakrabarty et al., 2015). Further studies showed that this was due to preventing downstream pathways involving Jak1/Stat3 and consequential transcription factor activity (Guillot-Sestier et al., 2015). Additionally, primary microglia treated with fibrillar $A\beta_{1-42}$ and recombinant IL-10 showed that fibrillar $A\beta_{1-42}$ is prevented from inducing a pro-inflammatory response of cytokine release including CCL5, CXC10, and TNFα, suggesting a push to an anti-inflammatory profile (Chakrabarty et al., 2015).

Therefore, it is pertinent to think that the Aβ activates microglia and results in an innate immune response. Indeed, it has been shown that exposure of microglia to fibrillar Aβ by CD36, a class B scavenger receptor (Coraci et al., 2002), causes the formation of a heterodimer of the TLR4 and TLR6 through NF-κB signaling (Stewart et al., 2010). However, on deletion of MyD88, an adaptor protein essential for downstream TLR signaling, there was a significant decrease in both Aβ load and microglial activation in APP/PS1 mice (Lim et al., 2011). Despite this the MyD88 deletion only resulted in minor improvements in cognitive functions (Lim et al., 2012).

Microglial activation by Aβ does not necessarily only occur after Aβ deposition but can also occur before plaques are even formed. Maezawa and colleagues have shown that nanomolar concentrations of Aβ oligomers activated microglia and that they required another scavenger receptor, SR-A, and the Ca^{2+}-activated potassium channel KCa3.1 (Maezawa et al., 2011). Another group has also shown microglial activation precedes Aβ aggregation in APP[V717I] transgenic mice and that this coincides with increased BACE1 activation (Heneka et al., 2005).

Intracellular neurofibrillary tangles of hyperphosphorylated tau are another pathological hallmark of AD. However, the exact mechanisms which lead to the hyperphosphorylation of tau are still unclear. Previously, it has been demonstrated that neuro-inflammation positively correlates with tau aggregation, hyperphosphorylation and neurodegeneration in several models (Sheng et al., 1997; Sheffield et al., 2000; Bellucci et al., 2004, 2011; Ikeda et al., 2005; Yoshiyama et al., 2007).

Microglial activation also precedes tau pathology in the P301S tauopathy model (Yoshiyama et al., 2007). In the triple transgenic model of AD, lipopolysaccharide administration significantly increased tau phosphorylation through toll like receptor 4 signaling (Kitazawa et al., 2005). Interestingly, one paper has demonstrated that microglia may be involved in the propagation of tau pathology through non-synaptic transmission in mammals (Asai et al., 2015). Asai et al. (2015) used two different tau mouse models to show that tau propagation is mediated through microglia which phagocytose tau-positive neurons or synapses and secrete tau protein in exosomes, efficiently transmitting tau to neurons. They also demonstrated that this propagation is sensitive to microglial depletion and inhibition of nSMase2 activity. On the other

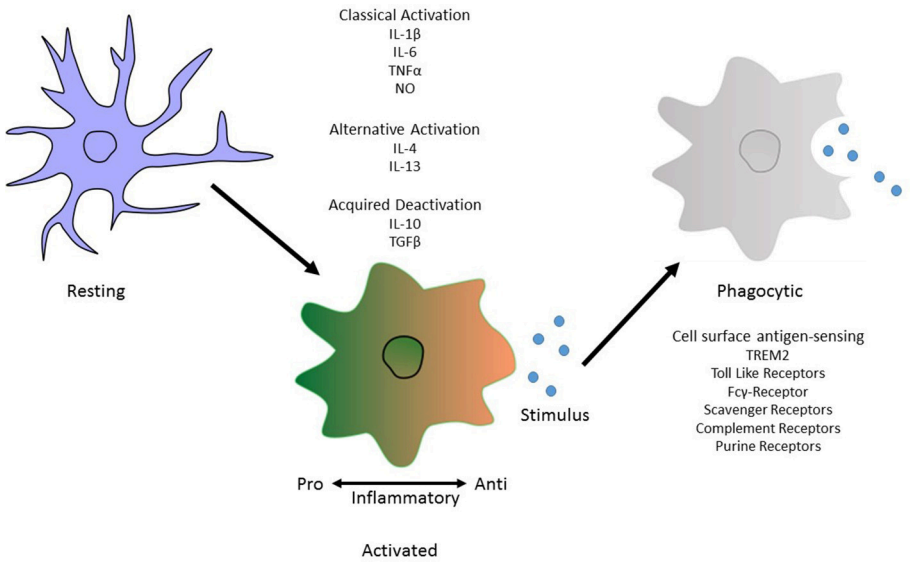

FIGURE 1 | Morphological phenotype of Microglia. An illustration depicting the different phenotypic states. These are "classical activation," "alternative activation," and "acquired deactivation." Classical activation, otherwise considered to be the M1 phenotype and so is pro-inflammatory, is stimulated by IFN-γ and is associated with the production of cytokines such as TNF-α and IL-1β. Subsequently the alternative activation, or M2 phenotype, defined by the release of anti-inflammatory cytokines IL-4 and IL-13. The third activation phenotype, acquired deactivation, is thought to be a subtype of the M2 phenotype, releases IL-10 and TGF-β. Activation does not inclusively mean a phagocytic phenotype in microglia. For this to occur, antigen sensing receptors are made available on the cell surface to allow pathogen recognition. In Alzheimer's disease, the best known of these is TREM2 but others include Toll-like receptors and members of the complement system.

hand, significant ablation of microglia in a mouse model of amyloidopathy indicated that Aβ formation, maintenance and associated neuritic dystrophy was not depended on microglia (Grathwohl et al., 2009). Interestingly, (Krabbe et al., 2013) reported that Aβ may directly affect microglial function. This *in vivo* study detected a significant inverse correlation between Aβ plaque burden and microglial phagocytic activity (Krabbe et al., 2013). They found that microglial dysfunction develops early during AD in an Aβ-dependent fashion and can be restored by interventional anti-Aβ approaches, such as Aβ vaccination (Krabbe et al., 2013).

MICROGLIA PHYSIOLOGY AND ION CHANNELS

Studies have highlighted the importance of microglia in brain ionic homeostasis (Annunziato et al., 2013; Szalay et al., 2016; Shibata and Suzuki, 2017). For example, depletion of microglia results in the loss of potassium chloride induced neuronal depolarisation (Szalay et al., 2016) and the microglia KCa3.1 channel has been proposed as a valid therapeutic target for modulating cortical spreading depression (Shibata and Suzuki, 2017). Therefore ion channels and transporters, regulating ionic flux, are essential regulators of a variety of microglial functions, including proliferation, morphological changes, migration, cytokine release and reactive oxygen species production (Schilling and Eder, 2015). Ion channel expression in microglial cells is tightly regulated, with the expression of most ion channel types noticeably depending on the cells' functional state (Eder, 1998, 2005, 2010; Kettenmann et al., 2011). Despite being non-excitable cells, the plethora of voltage-gated ion channels present in microglia suggests they play a prominent role in both physiological as well as pathological states. Brain inflammation is a characteristic of AD and numerous studies have demonstrated that microglia can directly interact with neurons to induce inflammation (Hashioka et al., 2012). Due to this interaction, the study of microglial ion channels may shed light on brain inflammation seen in neurodegenerative diseases such as AD (Silei et al., 1999). In this review, we have summarized the most prominent ion channels involved in microglial cells which may contribute to AD pathology, as demonstrated in **Figure 2**.

POTASSIUM CHANNELS

Potassium channels are present in all cells within the body and have many diverse functions. In particular, they are capable of regulating cell excitability and influence action potential waveform. To identify therapeutic targets to modulate microglial activation, numerous studies are addressing the contributions of several K^+ channels. Based on both their structural and functional properties, K^+ channels have been subdivided into specific families. They have transmembrane helices (TMs) spanning the lipid bilayer (Kuang et al., 2015). The largest of these consist of K^+ channels that are activated by membrane depolarisation, with subsequent families consisting of channels that are activated by altered intracellular Ca^{2+} ions and others that are constitutively active. Based on the structure and function,

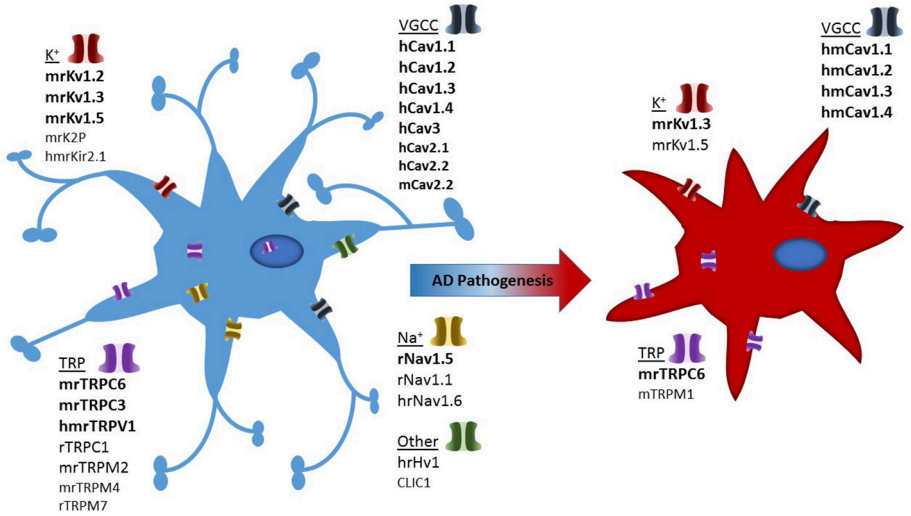

FIGURE 2 | Illustration depicting presence of ion channels observed in microglial models and which of these have confirmed activity in Alzheimer's disease associated microglia. Strength of evidence is depicted in bold to un-bold text. In addition each channel is pre-fixed with the species in which they have been investigated: h, human; m, mouse; r, rat.

the channels are categorized into three major classes: the voltage-gated (Kv) (six TMs), inwardly rectifying (Kir) (two TMs), and tandem pore domain (K2P) (four TMs) channels (Kuang et al., 2015). K^+ channels are particularly important in microglia since their activation can induce membrane hyperpolarisations, which are essential for driving Ca^{2+} influx through inward rectifying Ca^{2+}-Release-Activated-Ca^{2+} channels (CRAC) (Kraft, 2015; Nguyen et al., 2017a) ATP-activated P2X receptors (Burnstock, 2015) and other Ca^{2+}-permeable cation channels (Kettenmann et al., 2011).

Voltage-Gated Potassium Channels

Kv channels form an exceedingly diverse group, their structure consists of six TMs, of which the first four helices (S1–S4) form the voltage sensor domain (VSD) (Jiang Y. et al., 2003; Long et al., 2007). The last two helices (S5–S6, corresponding to the outer and inner helices in KcsA, respectively) form the pore-forming domain. The VSD senses the membrane potential alteration, and is followed by a conformational change that is coupled to gate the pore-forming domain (Long et al., 2005). In more general terms Kv currents can be classified into showing A-type (inactivating) or delayed rectifier behavior (non-inactivating). The Kv channels present in microglia to date have been summarized in **Table 1** and mainly comprises of delayed rectifier Kv channels.

Kv1.2, Kv1.3, and Kv1.5 transcripts and protein have been detected in both primary rat and mouse microglia (Kotecha and Schlichter, 1999; Khanna et al., 2001; Fordyce et al., 2005; Pannasch et al., 2006; Li et al., 2011). Microglia are widely distributed throughout the brain; however some regions express much higher levels than others (Lawson et al., 1990). The hippocampus, an area particularly affected by AD, is rich with microglia and is especially sensitive to cerebrovascular insults which have been shown to rapidly activate microglia (Wu and Ling, 1998). The reasons for the highly variable expression of Kv channels and the role this plays in non-excitable cells such as microglia are not well understood. It is now known that microglia in culture can express different proteins when compared to microglia *in situ* in brain slices or *in vivo* (Boucsein et al., 2003; Butovsky et al., 2014; Yamasaki et al., 2014; Gosselin et al., 2017). Earlier studies mostly used cultured microglia from enzymatically dissociated tissue, thus removing cell– cell contacts and key secretory products such as growth factors affecting Kv channel expression itself (Kettenmann et al., 1990; Ganter et al., 1992; Draheim et al., 1999). *In vitro* studies are currently the only way to stimulate microglia in isolation in order to elucidate similarities and differences in how different species respond (Lam et al., 2017).

It is becoming more apparent that altered expression of Kv channels could trigger the mechanisms underlying microglial polarity and could characterize these microglial states (Saijo and Glass, 2011; Maezawa et al., 2012). In a study on freshly isolated microglial cells, Kotecha and Schlichter (1999) found both Kv1.3 and Kv1.5, the former being associated with proliferating cells and the latter with non-proliferating cells. This shift in microglial activation also results in changes in the physiological properties of the cells (Kotecha and Schlichter, 1999). Resting microglia express Kv1.5 channels and upon activation and proliferation they upregulate Kv1.3 and down-regulate Kv1.5 channels (Pannasch et al., 2006). Kv1.3 channels migrate to the cell surface while Kv1.5 channels are internalized, making Kv1.3 channels not only functionally relevant but highly susceptible to pharmacological manipulation through selective channel blockers. As we have highlighted, majority of microglial studies use animal models, in particular rodents. Lam et al. (2017) found distinct variability between the different rodent models in expressing different Kv channels. It is also apparent that Kv channel expression of microglial cells in brain slices from juvenile mice (P5-P9) differs to some extent from that of cells

TABLE 1 | Microglia ion channels and their functionality after cell activation.

Channel type	Subunit	Expression and modulation	Proposed functions	Models	References
Voltage-gated Calcium Channels (VGCC)	N-type $Ca_V2.2$ Possible L-type (Cav1.X)	Very low expression levels at both protein and mRNA level	Cav2.2 control chemokine release from microglia Cav1.2 and 1.3 activity results in proliferation, increased intracellular Ca^{2+} with Aβ, microglial survival and pro-inflammatory action	Rat, mouse and Human primary cultures BV2 cell lines	Silei et al., 1999 Hashioka et al., 2012 Nicoletti et al., 2017 Espinosa-Parrilla et al., 2015
Inwardly rectifying potassium current (K_{ir})	$K_{ir}2.1$	Generally expressed in activated microglia; very low densities in resting microglia Modulated by intracellular factors, such as, G-proteins, intracellular calcium, pH and protein kinase C (PKC) LPS reduces or enhances current density in cultured murine microglia.	Sets a negative resting membrane potential (RMP)	Rat, mouse and primary and secondary cultures BV2 cell line Adult mouse brain slices	Schilling et al., 2000 Eder et al., 1999 Draheim et al., 1999 Boucsein et al., 2003 Kettenmann et al., 1993, 2011; Nörenberg et al., 1994a; Fischer et al., 1995; Chung et al., 1999; Franchini et al., 2004
Delayed outwardly rectifying potassium current (K_{dr})	$K_V1.3$ $K_V1.5$ To a lesser extent $K_V1.1$ $K_V1.2$	Transiently up-regulated upon stimulation with LPS interferon-β, TNF-α, TGF-β, GM-CSF, pH, Aβ, prostaglandin E2 receptor activation. Hypoxic insults increase Kv1.2 expression in adult microglial cells	Re-establishing a negative RMP during membrane oscillations Both $K_v1.3$ and $K_v1.5$ play a role in proliferation and ROS production $K_v1.3$ is involved the respiratory burst $K_v1.5$ is involved in NO release $K_V1.1$ and $K_V1.2$ involved LPS-induced release of TNF-α, interleukin-1β, endothelins and NO	Rat and mouse primary cultures Human brain slices and primary cultures	Nörenberg et al., 1994a; Schilling et al., 2000; Fordyce et al., 2005 Jou et al., 1998; Kotecha and Schlichter, 1999; Khanna et al., 2001 Pannasch et al., 2006; Eder, 1998 Chung et al., 1999 Eder and Heinemann, 1996
Ca^{2+}-dependent potassium channels (KCa)	Large conductance: KCa1.1 Small conductance: KCa2.1 KCa2.2 KCa2.3 KCa3.1	KCa2.3 channels are predominantly expressed in both cultures and healthy striatum tissue; LPS treatment or ischemic insult increased the level of expression.	Blocking KCa2.2/KCa2.3 with CyPPA reduces cytokine release Migration was inhibited when KCa1.1 was blocked with charybdotoxin and clotrimazole Small conductance channels are linked to NO release, MAPK signaling and respiratory burst Inhibition of KCa2.3 affected microglial activation and reduced microglial neurotoxicity.	Calf, rat, mouse, human, adult, and new born rat primary cultures and brain slices	Walz et al., 1993 McLarnon et al., 1997; McLarnon et al., 2001; Bordey and Spencer, 2003 Khanna et al., 2001 Spranger et al., 1998 Papavlassopoulos et al., 2006; Stock et al., 2006; Kaushal et al., 2007
Two-Pore Domain Potassium Channel (K2P)	K2P13.1 (THIK-1)	High RNA expression in resting microglia Age-dependent decrease of mRNA	Maintaining RMP. Mediates release of the pro-inflammatory cytokine interleukin-1β from activated microglia Regulates microglial ramification	Rat hippocampal brain slices and primary cultures	Madry et al., 2018

(Continued)

TABLE 1 | Continued

Channel type	Subunit	Expression and modulation	Proposed functions	Models	References
Voltage-gated Na^+ channels	$Na_V1.1$, $Na_V1.5$ $Na_V1.6$	$Na_V1.6$ expression correlates with the transition between resting and activated microglia in EAE Protein and mRNA for $Na_V1.6$ were detected in EAE mice spinal cord and optic nerve, as well as in MS-affected human spinal cord.	Phagocytosis and cell volume regulation Treatment of LPS-activated microglia with TTX or phenytoin reduced IL-α1, IL-1β, and TNF-α secretion Decreased motility responses to ATP Phenytoin reduced microglial activation in EAE model	Rat primary cultures	Craner et al., 2005 Korotzer and Cotman, 1992 Nörenberg et al., 1994b Schmidtmayer et al., 1994 Eder, 2005 Black et al., 2009
Transient Receptor Potential channels (TRP)	TRPC1, 3, 6 TRPM2, 4 TRPV1, 2 and to a lesser extent other TRPs	Low expression of RNA in resting microglia. Transiently up-regulated upon stimulation with LPS, ATP, or BDNF	Activation of TRPs contributes to microglial Ca^{2+} signaling and are linked to IL-6 release, NO, TNFα associated initiation of microglial cells death Contribute to the regulation of transcription factors' function, including NFkB, NRF2, and AP-1, cytokine production, cell proliferation, activation, apoptosis and oxidative stress	Rat and mouse primary cultures BV-2 cell lines Human primary cultures	Sun et al., 2014 Mizoguchi et al., 2014 Liu et al., 2017 Huang et al., 2017 Sappington and Calkins, 2008 Schilling and Eder, 2011
Proton channels	H_V1 voltage-activated H^+ channels	Unknown	Single-channel conductance fS range High sensitivity to extracellular pH; associated with generation of respiratory burst Disruption of cytoskeleton leads to a 50% reduction in H^+ current amplitude Cell swelling potentiates H^+ currents.	Mouse, rat and human primary cultures	Eder et al., 1995; Klee et al., 1998, 1999 Tian et al., 2016 Wu et al., 2012
Chloride channels	CLIC-1	mRNA highly expressed in mammalian microglia channel protein relocation from cytosol to membrane after Aβ	Morphological changes based on Cl^- responses to stretch	Rat and bovine primary cultures	Visentin et al., 1995; Schlichter et al., 1996 McLarnon et al., 1995 Skaper et al., 2013 Milton et al., 2008 Paradisi et al., 2008

in adult mice (Boucsein et al., 2003; Schilling and Eder, 2007; Menteyne et al., 2009; Arnoux et al., 2013, 2014). The passive membrane properties and Kv channel expression of microglial cells undergo substantial changes upon aging (Schilling and Eder, 2015). In comparison with microglia of young adult mice, microglial cells of aged mice are characterized by more negative resting membrane potentials, decreased input resistances and upregulated expression of inward rectifier and outward rectifier Kv channels. Interestingly, the outward rectifier Kv channel current is strongly age-dependent both *in vitro* and *in vivo* (Schilling and Eder, 2015). It is clear from the literature that the way in which we study Kv channel physiology in microglia varies dramatically and depends on the methodology used (Lam et al., 2017).

Further complications include the potential for strain differences in rodents (Becker, 2016), and genetic polymorphisms and epigenetic changes in humans (Boche and Nicoll, 2010). There is considerable debate as to how closely mouse models resemble human responses in inflammatory diseases (Seok et al., 2013; Takao and Miyakawa, 2015). A better understanding of microglial K^+ channel regulation and expression patterns in neurodegenerative states could also yield targets for drug development using K^+ channel blockers.

Voltage-Gated Potassium Channels and AD

Microglia are the key inflammatory cells in AD that mediate neuro-inflammation, and Kv channels are key regulators of microglial function, in particular Kv1.3 (Rangaraju et al., 2015). In animal models of AD, Aβ-induced priming of microglial NADPH oxidative activity depends on Kv1.3 channels, however the exact mechanisms that contribute to this priming is still poorly explored (Kotecha and Schlichter, 1999; Schilling and Eder, 2011). It is thought that the activity of Kv channels lead to membrane hyperpolarization, this Kv1.3 channel-induced membrane hyperpolarisation could enhance Ca^{2+} influx through Transient receptor potential (TRP) channels (see section Calcium Channels; Schilling and Eder, 2011) aiding in the translocation of PKC and therefore leading to NADPH oxidative priming. Franciosi et al. (2006) demonstrated that the broad spectrum Kv channel inhibitor 4-aminopyridine (4-AP) suppressed microglial activation *in vivo* and reduced microglia-induced neuronal death (Franciosi et al., 2006). This inhibition using 4-AP, which also blocks Kv1.3 channels, could attribute to inhibition of microglial priming and subsequent reduction of microglial ROS production, supporting a role for Kv1.3 channels as a therapeutic target in AD (Schilling and Eder, 2011). More recently immunohistochemistry experiments on human brain cortices revealed the presence of Kv1.3 channels in cortical microglia at levels higher than non-AD controls (Rangaraju et al., 2015). This particular study also revealed a "plaque-like" pattern of Kv1.3, suggesting that it may be possible for Aβ to interact with Kv1.3. Interestingly, $Aβ_{1-42}$ oligomers, but not soluble Aβ, accelerate the activation and inactivation kinetics of Kv1.3 channels in lipid bilayers without altering channel conductance (Lioudyno et al., 2012). It is possible that altered channel conductance of Kv1.3 channels could affect calcium fluxes in neurons and microglia, however the relevance of this potential Aβ-Kv1.3-interaction remains to be clarified. Another study by Chung et al. (2001) also confirmed that Aβ was capable of upregulating Kv1.3 as well as the Kv1.5 channel current density. More recently, low levels of soluble oligomeric Aβ have been reported to upregulate primary cultured microglial activity as well as Kv1.3 at transcript and as protein levels (Maezawa et al., 2017). Electrophysiological studies using whole-cell patch clamp also revealed enhanced outward rectifier current, characteristic of homotetrameric Kv1.3 channels. Pharmacological characterization revealed that the currents were sensitive to the Kv1.3 specific blockers ShK-186 (Tarcha et al., 2012), margatoxin (Garcia-Calvo et al., 1993) and the selective Kv1.3 blocker PAP-1 [5-(4-phenoxybutoxy) psoralen (Schmitz et al., 2005). Oligomeric Aβ further induced a significant increase in Kv1.3 current density compared to unstimulated microglia (Maezawa et al., 2017). Following long-term treatment of an APP/PS1 mouse model, the selective Kv1.3 blocker PAP-1 mitigated some key AD-like phenotypes such as reducing Aβ deposition as well as restoring hippocampal synaptic plasticity. The observation that pharmacological targeting of Kv1.3 channels in microglia with the selective inhibitor PAP-1 supports PAP-1 as a promising potential for neuro-immunomodulation therapy and the treatment of neurodegenerative diseases such as AD.

The age-dependent changes in microglial Kv1.3 noted in 5xFAD mice followed a similar trend—initially an age-dependent increase, then a substantial decrease between 10 and 15 months of age. We suspect that these changes in K^+ channel expression form part of the age-related changes in microglial function, documented by several lines of investigation, such as altered responses to Aβ aggregates or downregulation of "sensome" genes (Hickman et al., 2008; Cameron et al., 2012; Heneka et al., 2013; Hickman and El Khoury, 2013; Johansson et al., 2015) ever, this downregulation is not reflected in a human study in which Kv1.3 expression remains robust in microglia, particularly in the later stages of AD (Rangaraju et al., 2015). More recently transcriptomic data from Rangaraju et al. (2018), revealed that Kv1.3 plays a distinct role in disease-associated-microglia in the 5XFAD mouse model (Rangaraju et al., 2018). It is pertinent to say that the evidence presented here from the existing human and rodent studies, show Kv1.3 could be a therapeutic target even at the late stage of the disease. Similar to what we have previously discussed, it appears that the current transgenic models of AD do not replicate the patterns of microglia activation in human AD. Many potential treatments identified in rodents have failed in human clinical trials. To narrow this translational gap, it is essential to investigate and acknowledge species similarities and differences. With the promises of stem cell therapy and use of iPSCs to model diseases in a dish, pharmacological manipulation on a more directly available human source may reveal further species differences.

Other Potassium Channels

Recent evidence has suggested that two-pore domain K^+ (K2P) channels may play a role in microglia physiology (Madry et al., 2018). Functional investigations provide data to support the involvement of THIK-1 in the cytokine release of microglia *in situ*. This study revealed two functionally and

mechanistically distinct modes of microglial motility. THIK-1 regulates microglial ramification, surveillance and interleukin-1β release (Madry et al., 2018). This is the first study of its kind to implicate K2P channels in microglia physiology. Future work will provide a better understanding of its role *in vivo* as well as neuro-inflammatory responses. The impairment of motility of microglial processes that occurs in some pathological conditions, e.g., in models of Alzheimer's disease with Aβ plaque deposition (Koenigsknecht-Talboo et al., 2008; Krabbe et al., 2013; Condello et al., 2015) raises the question of whether the dependence of surveillance on THIK-1 activity can be employed therapeutically for the treatment of AD (Madry et al., 2018). Currently there has been no direct experimental evidence linking THIK-1 to AD.

Another important K^+ channel that has been shown to play a key role in microglia activation by modulating Ca^{2+} signaling and membrane potential is calcium-activated KCa3.1 (also known as IK1, SK4 or KCNN4) channels (Maezawa et al., 2012). This channel is predominantly expressed in microglia and has been a potential target for both industry and academia as a potential drug target for AD (as reviewed by Maezawa et al., 2012).

The strong inwardly rectifying K^+ (Kir) channel belong to a family of K^+ channels that have only two membrane-spanning domains and are responsible for stabilization of the resting membrane potential (V_{rest}) near to the K^+ equilibrium potential (E_K) (Kettenmann et al., 1990; Tsai et al., 2013). Blocking Kir channels depolarizes the cell and decreases the driving force for inwardly transported Ca^{2+} in microglia. In a study by (Tsai et al., 2013), addition of the AD drug memantine suppressed Kir as well as depolarized the membrane potential of BV-2 cells. This block of Kir2.1 channels could represent one of the important mechanisms underlying its actions on the functional activities of microglial cells. It remains unclear what the *in vivo* function of Kir are, an area showing significant promise for AD.

Interestingly, in the transgenic mouse model of AD (5xFAD) (Wendt et al., 2017) reported that the impairment in phagocytic function of microglia was due to altered purinergic signaling. They found evidence of altered physiological phenotype only of microglia in 5xFAD mice that were located close to Aβ plaques (Wendt et al., 2017). Supporting the idea that functional and pathological alterations of microglia in AD may be a consequence of their association with Aβ plaques. Their detailed study on the 5xFAD model revealed an initial induction of Kir current, followed by subsequent activation of outwardly rectifying currents at a later age. Therefore the induction of Kir current could be considered a first response followed up with outward K^+ current developing at a later stage of microglial activation, similar to their previous studies (Boucsein et al., 2000; Kettenmann et al., 2011). This data supports the fact that microglia can undergo chronic changes in physiological properties in a disease model over a prolonged period. It appears from the literature that Kv1.3, KCa3.1, and Kir 2.1 inhibitors seem to constitute relatively general anti-inflammatory effects and it could therefore be useful to preferentially target detrimental pro-inflammatory microglia functions associated with neuro-inflammation, such as AD (Nguyen et al., 2017b). A more recent study investigated the effects of Aβ plaque-dependent morphological and electrophysiological heterogeneity of microglia in the AD mouse model, TgCRND8. Plescher et al. (2018) revealed increased K^+ currents in plaque-associated but not plaque distant microglia. They believe that this electrophysiological heterogeneity is likely to reflect the different functional states of the microglia in TgCRND8 (Plescher et al., 2018). Their finding that outwardly rectifying currents (Kv 1.3) were confined to a subset of plaque associated microglial cells emphasizes the potential of specific ion channel inhibitors to target only specific (i.e., detrimental) subtypes of microglia in AD (Plescher et al., 2018).

VOLTAGE-GATED SODIUM CHANNELS

Sodium voltage channels (NaV) are formed of one pore-α-subunit associated with one/more β-subunits. The α-subunit acts as the "voltage sensor" being activated by changes in membrane potential (Payandeh et al., 2011). The β-subunits have multiple roles, from modulating channel gating and regulating channel expression, to interacting with the cytoskeleton and the extracellular matrix, as cell adhesion molecules (Brackenbury and Isom, 2008). It is now known that there are nine pore forming α-subunits of sodium channels, Nav1.1-Nav1.9, encoded by genes SCN1A-SCN11A (Catterall et al., 2005), which associate with one or more non-pore-forming β- subunits encoded by SCN1B-SCN4B (Brackenbury and Isom, 2011). In addition to being expressed in cells capable of generating action potentials, sodium channels have also been identified in cells that have not traditionally been considered to be electrically excitable ("non-excitable cells"), leading to speculation as to their functional role (Pappalardo et al., 2016). Sodium channels contribute to multiple, varied cellular functions in these cells including phagocytosis (Carrithers et al., 2007), migration (Kis-Toth et al., 2011), and proliferation (Wu et al., 2006). Voltage-gated sodium channels have been documented in immune cells such as macrophages (Schmidtmayer et al., 1994; Carrithers et al., 2007, 2009, 2011; Black et al., 2013).

Patch-clamp recordings have since confirmed the expression of functional sodium channels in microglia (Korotzer and Cotman, 1992; Nicholson and Randall, 2009; Persson et al., 2014). A number of voltage-gated ion channels have been identified in microglia, in particularly, voltage-gated Na^+ channels isoforms (VGSC): Nav1.1, Nav1.5, and Nav1.6 (Craner et al., 2005; Black and Waxman, 2012).

In vitro, microglia derived from mixed glial cultures from neonatal rats, exhibit immunolabeling for Nav1.1, Nav1.5, and Nav1.6, which is most prominent, while Nav1.2, Nav1.3, Nav1.7, Nav1.8, and Nav1.9 are not detectable above background levels (Black et al., 2009). Whole-cell voltage clamp experiments on cultured rat microglia revealed that, depolarization-induced sodium currents were elicited and then completely blocked by 0.3 μM TTX, consistent with the presence of functional TTX-S sodium channels (Persson et al., 2014). Similarly, microglia within normal CNS tissues exhibit low levels of Nav1.6 immunolabeling *in situ* (Black and Waxman, 2012).

There is a handful of electrophysiological studies of cultures of human microglia derived from native tissue which reports the presence of Na^+ currents (Nörenberg et al., 1994b; Nicholson and Randall, 2009), however, these are not observed in every laboratory (McLarnon et al., 1997). Na^+ currents have also been reported in rat microglia (Korotzer and Cotman, 1992). A study in mice provides evidence that Nav1.6, plays a central role in the infiltration and phagocytosis of microglia in experimental autoimmune encephalomyelitis. Furthermore, the same channel is reported to be up-regulated in macrophages and microglia in the lesions of multiple sclerosis patients (Craner et al., 2005). To date there is no direct evidence for the involvement of microglial VGSC in AD. This same group, however, also report the presence of Nav1.1 and Nav1.5 in cultured rat microglia and demonstrate their function in many key microglial processes (Black et al., 2009). Although Aβ is a known activator for microglia, treatment of the human microglial cell line with Aβ (12 h, 10 μM) there was no significant change in Na^+ current or Nav1.5 expression (Nicholson and Randall, 2009). Although there is clear involvement for VGSC in microglial function its role in AD remain less well defined. This could be due to a number of different contributing factors such as species variation, individual laboratory protocols, as well as non-standardized preparation of exogenous Aβ and Aβ species selection.

TRANSIENT RECEPTOR POTENTIAL CHANNELS

Transient receptor potential (TRP) channels are non-selective, non-voltage gated cation channels, ubiquitously expressed in mammalian cells. The TRP gene was initially discovered in Drosophilla where mutant gene expressing animals showed impaired vision due to dysregulated Ca^{2+} influx into photoreceptor cells. TRP channels play important physiological role in cells by their regulation of temperature, chemoception, mechanoception, and nocioception. There are 30 known members of the mammalian superfamily, which can be divided up into six subfamilies, based on amino acid sequence homology. These are: TRPA (Ankyrin); TRPC (Canonical); TRPM (Melastatin); TRPML (Mucolipin); TRPP (Polycystin); and TRPV (Vanilloid). TRP channels are tetramers made of monomeric subunits that include a six trans-membrane (TM) domain with a pore-forming loop between TM 5 and 6. In addition, their C- and N-termini are intracellular. Functionally, they act by changing cytoplasmic free Ca^{2+} concentrations via Ca^{2+} permeable pore or by modulating ionic movement via changes to the membrane potential. Microglia are evidenced to express some TRP subfamily members, including those of the TRPC, TRPM, and TRPV families.

TRPA

The smallest of the TRP subfamilies. Its only mammalian member is TRPA1, a mechano- and chemo-sensor. Its name is derived from the 14 N-terminal ankyrin repeats. To date there is no evidence that it is present in microglia, although it's silencing in dorsal root ganglion results in reduced microglia activation following hyperalgesia (Meotti et al., 2017). Similarly, there is no evidence of the presence of TRPML nor TRPP channels being expressed in nor influencing function of microglia.

TRPC

The TRPC subfamily consists of seven homologs (C1-7), with TRPC2 being exclusively expressed in mouse. TRPC members share a structural motif in the COOH-terminal tail, TRP box, located close to the intracellular border of TM6. In addition, they contain three or four N-terminal ankyrin repeats.

TRPC channels are activated via the stimulation of GPCRs and receptor tyrosine kinases, leading to phospholipase C, inositol 1,4,5-triphosphate, and diacylglycerol production. This stimulation results in a biphasic Ca^{2+} release with a first phase ER release, followed by sustained Ca^{2+} influx across the membrane. TRPC channels are known mostly as store operated Ca^{2+} entry (SOCE) mediators.

In microglia, all seven members have shown RNA expression in *in vitro* cell line models, although only C1 and C3 have been reported *in vivo*. TRPC1 is a non-selective Na^+/Ca^{2+} permeable channel with known function in cell survival and proliferation. Their expression is commonly on organelle membranes such as ER and intracellular vesicles. TRPC1 negatively regulates the ORAI1 Ca^{2+} channel resulting in suppression of NKkB, JNK and ERK1/2 signaling from microglia (Sun et al., 2014).

TRPC3 is widely expressed in the CNS where it has modulation via the growth factor BDNF to induce axonal guidance, neuronal survival, and postsynaptic glutamate transmission. In microglia, pre-treatment with BDNF inhibits NO and TNF-α upregulation, via sustained Ca^{2+} influx through upregulated TRPC3 channels at the plasma membrane. Effects were reversed using the siRNA against TRPC3 (Mizoguchi et al., 2014).

TRPM

The TRPM subfamily has eight mammalian members. Unlike TRPA/C there are no N-terminus anykin repeats, instead having functional protein domains, in addition to the TRP box, in the C-terminus. TRPM's are non-selective cation channels with a verity of cellular functions including temperature sensing, osmolarity, redox, Mg^{2+} homeostasis, proliferation, and cell death. These channels can be subdivided further into four groups: M1/3; M4/5; M6/7; M2/8. M1, 2, 4, and 7 have all been reported as present in microglia.

TRPM1 was the first to be cloned, in 1998 (Harteneck, 2005), however its function and activation remains unknown. TRPM1 has a high capacitance for splice variance, similarly so with TRPM3-with whom M1 shares strong sequence homology. In murine models of AD (5XFAD/MHCII+) high levels of Aβ plaque burden correlated to an increase in TRPM1 gene expression compared to age matched control animals (Yin et al., 2017).

M2 contains an adapted adenosine 5′-diphosphoribose ribose (ADPR)-recognizing Nudix box domain at its c-terminus. It is a redox modulator, activated by reactive oxygen species,

ADP ribose, NAD^+ and Ca^{2+}. M2 will mediate the release of lysosomal Zn^{2+} stores in response to reactive oxygen species, leading to increased cytosolic Zn^{2+} levels, leading to regulation of cell motility and actin remodeling. Additionally, Ca^{2+} influx via TRPM2 leads to increased intracellular insulin release in pancreatic β-cells (Uchida et al., 2011). A number of studies, both *in vitro* and *in vivo* confirm TRPM2 expression and activity in microglia.

In a TRPM2 KO mouse model, microglia show an abolishment of Ca^{2+} influx after LPS or IFNγ stimulation. Activation by these stimuli results in Pyk2-mediated activation of p38 MAPK and JNK signaling as well as an increase in nitric oxide production (Miyake et al., 2014). Similarly studies of MCAO-induced hypoxia in TRPM2 KO mice saw reduced MG activation, reduced cytokine expression and increased brain volume after damage (Huang et al., 2017). Lastly, TRPM2 channels are functionally expressed in the murine microglia cell line BV2. Here these channels have been shown to be involved in LPC-induced p38 MAPK phosphorylation. LPC-induced intracellular Ca^{2+} increase and inward currents dependent on TRPM2 channels (Jeong et al., 2017).

TRPM4 are non-selective cation channels with a greater affinity for Na^{2+} over Ca^{2+}. TRPM4 are activated by increased intracellular Ca^{2+} due to changes in cell membrane potential, ATP, PKC-dependent phosphorylation and calmodulin (CaM) binding to the channels C-terminal CaM domain (Nilius et al., 2005). Functional channels were detected in the mouse primary microglia, both quiescent and active. Here they are thought to mediate membrane depolarisation, in correlation to Ca^{2+} influx (Beck et al., 2008). Sulfonylurea receptor 1 activates TRPM4 channels in mouse primary microglia. Receptor binding regulates NOS and NO transcription on microglia activation via LPS action at TLR4 (Kurland et al., 2016).

TRPM7, like TRPM6, is a channel-enzyme. It is Mg^{2+}, Zn^{2+}, and Ca^{2+} permeable with a strong outward rectifying current-voltage relationship. In addition to its ionic pore, it contains a tyrosine kinase domain on its N-terminal. Activity at both pore region and kinase domain are implemented to be involved in the channels activity. For example, in rat brain microglia there is a strong increase of intracellular Mg^{2+} via the channel, however the currents generated were kinase activity-dependant and not due to pore, nor cell, activation (Jiang X. et al., 2003). TRPM7 also plays a role in cell motility. Migration and invasion of M1 (pro-apoptotic) microglia was observed in rat primary and MLS-9 microglia after priming with LPS (Siddiqui et al., 2014). In addition, flow cytometry and Ca^{2+} imaging studies in neonatal mouse microglia saw an increase in intracellular Ca^{2+} with cell activation by Polyl:C. Increased Ca^{2+} led to a correlated increase in TNFα and P38, in a TRPM7-dependent manner.

TRPV

The final sub-family are the vanilloids, the largest (1-6) and most in depth studies of the TRP channel families. All TRPVs are highly selective to Ca^{2+}. The most well-known is TRPV1 for its actions as a thermosensor (temperatures >43C). V1 mediates heat response and inflammation in addition to nociceptive responses to capsaicin, the main "heat" compound of chili peppers. In addition, application of compounds with a pH <5.9 will shift the temperature gated threshold of these channels to 20–23 C. Heat-mediated activation is shared quality with other TRPV members, specifically 2, 3, and 4. However, these channels are insensitive to capsaicin and pH. V5 and V6 are not thermosensors but have enhanced selectivity to Ca^{2+} over other monovalent cations. Lastly, all TRPV channels are functionally regulated by their insertion, or retention to the plasma membrane.

TRPV1 has a high protein expression in microglia, with the majority of these channels showing co-localisation to organelles including the golgi, ER, lysosomes, and mitochondria. Interestingly, at resting state there is very little expression at the plasma membrane (Miyake et al., 2015). In a model of rat spinal cord injury, activation of TRPV1 channels, via I.V injection of capsaicin, gave increased expression of SOD1 and pro-inflammatory cytokines from spinal microglia (Talbot et al., 2012). Similar influence on pro-inflammatory markers were observed in retinal microglia where activation of TRPV1 resulted in increased IL-6 and NFkB expression (Sappington and Calkins, 2008). Expression of TRPV1 protein and function was confirmed in HMO6 human microglial cell line. Application of capsaicin resulted in increased intracellular Ca^{2+}, and subsequently cytochrome C and cleaved caspase 3 release (Kim et al., 2006). Together this suggests a strong role of TRPV1 in the pro-inflammatory profile of microglia.

Little is known about the other TRPV channels in microglia, although an RNA-based analysis by Raboune et al. (2014). showed upregulation of TRPV1-4 in BV2 cells following cell activation by N-acyl amide.

Microglial TRP Channels in AD

Aβ accumulation, one of the major hallmarks of AD, commonly results in excitotoxicity and cell death via the disruption of normal Ca^{2+} homeostasis and release of pro-inflammatory factors such as ROS, NO, and cytokine release. The previous section highlights the role of TRP channels in intracellular Ca^{2+} regulation as well as differentially switching the phenotype of microglia between M1 (pro-apoptotic) and M2 (pro-survival). Despite this there is little research into glial TRP channel activity in AD, with most of the focus being on neuronal responses.

Aβ treatment of BV-2 cells gave an upregulation of protein and mRNA for TRPC6 that is dependent on NFkB activity. When these cells had TRPC6 knocked down via siRNA, the condition media was neuroprotective to cultured hippocampal cells compared to sham BV2 cells. Neuronal influence of TRPC6 activates via an upregulation of COX2 downstream (Liu et al., 2017). By using familial AD mouse models- APP23 and 5XFAD, plaque associated microglia from these animals were homogenized and run through flow cytometry to observe upregulated genes. From these TRPM1 was pulled out, however its role in AD remains unclear (Yin et al., 2017). BV2 cells treated with either fibrillary or soluble Aβ saw high levels of ROS which was attenuated with simultaneous application of TRPV1 via I-RTX (Schilling and Eder, 2011).

CALCIUM CHANNELS

Plasma membrane calcium channels are subdivided into three main groups according to their manner of activation; the voltage-gated calcium channels (VGCCs), the store-operating calcium channels (SOCs) and the receptor-operated calcium channels (ROCs). VGCCs specifically, play a vital role in maintaining calcium homeostasis, with important roles in cellular processes such as neurotransmission, control of gene expression, hormone secretion and cell apoptosis (Ertel et al., 2000; Valerie et al., 2013). Therefore, developing therapeutics that target these channels may be of benefit in treating various diseases of the CNS, such as AD. Structurally VGCCs consists of the α1 pore-forming subunit consisting of four transmembrane domains, the cytoplasmic β subunit, the peripheral α2δ and occasionally the γ accessory subunit (Ertel et al., 2000). VGCCs are divided into subfamilies according to their pore-forming subunit; the high voltage-activated channels known as Cav1 (Cav1.1-1.4) and Cav2 (Cav2.1-2.3), and the low voltage-activated Cav3 channels (Cav3.1-3.3) (Ertel et al., 2000).

Voltage-Gated Calcium Channels

To date, evidence suggesting the existence of microglial VGCCs and their involvement in AD is limited. Although numerous studies, mainly via electrophysiology and Fura-2 calcium imaging, have proven that various agents such as Aβ, ATP, and K^+, cause an increase in intracellular Ca^{2+}, the mechanism by which this phenomenon occurs is still under debate (Korotzer et al., 1995; McLarnon et al., 1999; Valerie et al., 2013). Thus, there is no clear indication of the existence of microglial VGCCs or whether the increase in intracellular Ca^{2+} is due to other factors such as ion exchange transporters or opening of intracellular stores (Korotzer et al., 1995; McLarnon et al., 1999; Valerie et al., 2013).

The majority of human studies, have investigated the presence and functionality of VGCCs in human glioblastoma cell lines, consisting of a mixed culture of glial cells, including astrocytes and microglia. Therefore, a major limitation of human *in vitro* studies, is that identifying VGCCs in glioblastoma cells does not necessarily indicate the presence of these channels in microglia. For instance, Valerie et al. (2013), demonstrated that pharmacological inhibition via the calcium channel blocker (CCB) mibefradil, or siRNA-induced downregulation of the Cav3 channel (T-type current) in human glioblastoma cell lines, led to cell apoptosis. Additionally, Nicoletti et al. (2017) demonstrated that Cav2.1 and Cav2.2 are involved in glial proliferation, through using of pharmacological tools (Nicoletti et al., 2017). Furthermore, via the use of an Iba-1 antibody, a marker of inflammation, and immunohistochemistry, in an *in vivo* rodent glioblastoma model (GL261 glioma cells), it was revealed that the degree of Iba-1 positive microglia had increased following N-type inhibition. This highlights a role of microglial VGCCs not only in cell proliferation and microglial survival, but also in inducing their pro-inflammatory action (Nicoletti et al., 2017). Evidence from human glial cells, demonstrates that VGCCs are expressed in human microglia, and that microglia VGCCs may also have a role in neurotoxicity (Hashioka et al., 2012). Prior to 48-h treatment with LPS and IFN-γ to induce inflammation, primary human microglial cells were treated with the L-type blocker nimodipine, significantly reducing neuronal toxicity induced by the microglia (Hashioka et al., 2012). In contrast to other studies, Hashioka et al. (2012) provided more conclusive evidence in indicating the presence of microglial VGCCs due to the use of primary human microglia and not a cell line consisting of a mixed glial population. A 1999 study demonstrated a more direct involvement of microglia VGCCs with progression of AD by investigating how $A\beta_{25-35}$ alters Ca^{2+} signaling in human microglia (Silei et al., 1999). Incubation with Aβ caused an increase in microglia proliferation and additionally an increase in intracellular Ca^{2+} levels (Silei et al., 1999). As no significant increase in microglial intracellular Ca^{2+} levels were observed when microglia were incubated in Ca^{2+} -free media, it was suggested that this change was due to VGCC-mediated Ca^{2+} influx (Silei et al., 1999). This was verified via co-incubation of microglia with Aβ and the CCBs verapamil, nifedipine and diltiazem which lead to a half-reduction in intracellular levels (Silei et al., 1999). Moreover, incubation of peptide-treated microglia with nifedipine not only lead to a reduction in intracellular Ca^{2+}, but also significantly prevented the increase in microglia proliferation induced by the peptide. Therefore, this study proposes that Aβ has the ability to increase microglia number and also induce their activation and consequently inflammatory action, through a VGCC manner (Silei et al., 1999).

In contrast to human studies, the majority of studies using animal models, have not provided conclusive evidence to indicate the existence and activity of VGCCs in microglia (Toescu et al., 1998; Silei et al., 1999).

A possible explanation for this, could be that microglial VGCC expression and activity is species-dependent. For instance, studies have shown that rodent microglia can express very low levels of VGCC activity which may even remain undetected (Toescu et al., 1998). Toescu et al. (1998), demonstrated that adding ATP to microglia isolated from murine cortex lead to a significant increase in intracellular Ca^{2+} levels. In contrast, KCl induced microglial depolarisation, did not lead to an increase in intracellular Ca^{2+} thus it was proposed that increased Ca^{2+} levels involved VGCC independent pathway (Toescu et al., 1998). Prolonged elevation in intracellular Ca^{2+} levels can activate pathways involved in regulation of gene expression such as the Ca^{2+}-calmodulin pathway, and therefore altered Ca^{2+} signaling in microglia may occur as a pathway for microglia activation and may even induce the progression of various pathological conditions such as AD (Toescu et al., 1998).

Although the majority of animal model studies have not definitively proven the existence of the channels in microglia, a few were able to provide some evidence indicating their existence. In a study carried out in 2014 by Saeugusa and Tanabe, where rodent lines were created where expression of Cav2.2 was suppressed, they indicated dynamic modulation of microglia Cav2.2 in regulation of pain related behavior. (Saegusa and Tanabe, 2014). Saeugusa and Tanabe also highlight neuronal

and microglial crosstalk, in controlling response to pathology (Saegusa and Tanabe, 2014). A more recent study investigated how microglial activation, verified by immunostaining and morphological changes, alters the activity of the L-type currents in an *in vivo* animal model for neurodegeneration, and in the *in vitro* BV2 cell line (Espinosa-Parrilla et al., 2015). Comparison of microglia before and after LPS and IFNγ stimulation revealed differences as seen via immunostaining and molecular approaches such as western blotting and PCR (Espinosa-Parrilla et al., 2015). Additionally, as depolarisation of LPS/IFNγ treated microglia demonstrated changes in intracellular Ca^{2+} by treatment with either nifedipine or Bay K8644 (agonist), it was suggested that VGCCs, may form part of the mechanism involved in the activation of microglia, inducing their pro-inflammatory action (Espinosa-Parrilla et al., 2015).

To summarize, even though human microglia studies have proposed the existence of functional VGCCs, the majority of the studies were carried out in mixed glial cell lines. Additionally, animal studies have either demonstrated very low expression of VGCCs in microglia or were not able to prove their existence, either at a functional or expression level (protein and mRNA). Thus, due to the limited and contradicting evidence on human and rodent microglial VGCC existence, the use of human induced pluripotent stem cells (iPSCs) may allow a more effective study of microglial ion channel role in neuro-inflammation observed in neurodegenerative diseases such as AD.

CHLORIDE CHANNELS

Chloride channels are a diverse superfamily of channels proteins, incorporating the volume regulated chloride channels, the ClC proteins, Ca^{2+} activated chloride channels, CFTR and maxi chloride channels (Alexander et al., 2017). Studies have identified Cl^- channels in rat (Visentin et al., 1995; Schlichter et al., 1996), bovine (McLarnon et al., 1995) and human microglia (McLarnon et al., 1997). These have mainly been based on pharmacological studies using a range of Cl^- channel blockers (e.g. nifluemic acid). Pharmacological modulation of Cl- channels indicates a role for in the regulation of microglia process outgrowth (Hines et al., 2009). However, the lack of specific pharmacological tools has hindered our progress in identifying specific channel entities, and indeed their contribution to microglia physiology. This is backed up with a lack of experimental evidence as to the molecular identity of the channels that have been suggested to be responsible for experimental observations. While the molecular identity remains to be resolved, evidence indicates that is a similar fashion to Cl^- currents within lymphocytes, microglia Cl^- conductance are responsive to stretch (Lewis et al., 1993; Steinert and Grissmer, 1997). Interestingly CLIC1 an intracellular chloride channel has received some attention with relation to amyloid pathology (Novarino et al., 2004; Milton et al., 2008; Paradisi et al., 2008). This suggests a role for modulation of chloride conductances in microglial generation of reactive oxygen species, but robust evidence for this is lacking in relevant *in situ* models of microglia.

VOLTAGE GATED PROTON CHANNELS (HV1)

Voltage-gated proton channels (H_v1; Alexander et al., 2017) reportedly consist of 4 proton sensitive transmembrane domains which are sensitive to both membrane depolarisation and transmembrane pH gradient (DeCoursey, 2008; Capasso et al., 2011). There is widespread expression of these channels within the central nervous system, highlighting both regional and cellular variation (Eder et al., 1995; McLarnon et al., 1997). Functional evidence comes from both studies carried out on murine microglia (Eder et al., 1995; Klee et al., 1998, 1999), rat (Visentin et al., 1995), and human microglia (McLarnon et al., 1997). There is also evidence to link Hv1 to both microglia polarity and brain responses to stroke (Wu et al., 2012; Tian et al., 2016). This could be pertinent given the link between hypoxia and Alzheimer's disease (Peers et al., 2007). However, one drawback from these studies is the use of culture preparations. This is pertinent given that work on brain slices was unable to detect any H^+ conductance *in situ* (De Simoni et al., 2008). This again raises the question about membrane properties in cultured preparations in contrast to *in situ* set ups. In addition there are questions around the physiological role of these channels when present in microglia (Eder and Decoursey, 2001) It is well established that microglial reactive oxygen species contribute to neuronal cell death in AD (see review Block et al., 2007). This process likely involved the build-up of protons within microglia, which will impact on the flux through Hv1 channels. However, a direct demonstration of the involvement of H_V1 in this process is lacking. There is greater evidence to indicate the involvement of other channels (e.g., Kv1.3) which are discussed elsewhere in this article.

MODELING MICROGLIAL INVOLVEMENT

To fully understand a disease and its etiology it is necessary that extensive modeling takes place. By tradition this has been through the use of a number of different model systems including both animal (murine) models and primary patient cell lines. Currently in AD research there is a large focus on the use of animal models, particularly transgenic mice (McGowan et al., 2006), as a lot is understood about their genetics and the availability of well-characterized genetic manipulation techniques in this organism. Not only this, mice are more closely phylo-genetically related to humans than other model systems such as *Caenorhabiditis elegans* or *Drosophila melanogaster*. The genetic similarities between humans and mice means that they have utility in studying the familial aspect of AD by using transgenic mice that contain mutations in the APP and PSEN genes. There are over 100 different transgenic mouse models available to study the familial aspect of AD, with some models containing five different mutations in the APP and PSEN genes (Oakley et al., 2006).

As it is widely accepted that Aβ plaques and neurofibrillary tangles cause neuro-inflammation, models which overexpress mutant human versions of APP have been shown to present

microglial activation (Bornemann et al., 2001; Wright et al., 2013). In addition to this it was shown that there were significant increases in CD38-positive microglia before Aβ deposition which also correlated with neuronal cell death in the CA1 region of the hippocampus (Wright et al., 2013). The inflammatory processes of in another APP mouse model, Tg2576, were investigated by looking at individual microglial cells using *in vivo* multiphoton imaging. Meyer-Luehmann and his colleagues showed that Aβ plaques can form within days and once formed it only takes 1–2 days before microglial cells begin to aggregate around the depositions. Alongside this accumulation of microglia is accompanied by changes to neurite morphology (Meyer-Luehmann et al., 2008). Whilst these have their advantages, they also have a number of limitations. These types of models do not accurately recapitulate human pathology as they do not develop the robust tauopathy or neuronal cell death that is seen in human disease without the addition of extra transgenes such as tau (Ribé et al., 2005).

The triple-transgenic model of AD, which contains the APP_{SWE}, Presenilin-1 ($PSEN_{M146V}$) and tau mutations (tau_{P301L}) offers the advantage that they develop Aβ plaques, tau tangles, synaptic dysfunction and LTP deficits which all manifest in an age-related manner (Oddo et al., 2003). Janelsins et al. demonstrated that this model shows a 14.8 fold increase of TNF-α and 10.8 fold increase in MCP-1 mRNA in 6 month old triple transgenic mice when compared to 2 month old mice. However these increases were only seen in the entorhinal cortex and could not be replicated in the hippocampus, suggesting that different cell types or environments may be responsible for the differential transcript levels and inflammatory responses in these disease relevant brain regions (Janelsins et al., 2005).

Mouse models containing just tau mutations have also been investigated in terms of neuro-inflammatory response and they too also display microglial changes (Wes et al., 2014; Cook et al., 2015). For example, the P301S tau model whose neurons develop bundles of hyperphosphorylated tau also have significant increases in inflammatory molecules such as IL-1β and COX-2 within the tau-positive neurons. Alongside this they also demonstrated that there were activated microglia throughout the brain and spinal cord, but that these microglia could be predominantly found surrounding the tau-positive neurons (Bellucci et al., 2004). Interestingly, this microglial activation was shown to begin before neurofibrillary tangle formation, but could be ameliorated using an immunosuppressive drug, FK506, early in life increasing life span and attenuating tau pathology (Yoshiyama et al., 2007). One important thing to bear in mind that mutations in tau do not cause AD but instead cause frontotemporal dementia. So whilst these models can provide useful information about how mutations in tau can cause cellular dysfunction and neurodegeneration they do not completely replicate AD in terms of other pathological markers (Wolfe, 2012).

Whilst proven useful for modeling autosomal disease, such as the familial form of AD, as previously mentioned, these murine models do not accurately recapitulate AD. A more promising avenue for modeling complex diseases, such as sporadic AD, is through the use of stem cell technology. Embryonic stem cells (ESCs) are derived from the inner cell mass, or blastocyst, of an embryo and can differentiate into any cell in the body (Evans and Kaufman, 1981). Despite their many potential uses, the ethical issues surrounding the use of embryo-derived cells are numerous. However, recent advances in stem cell technology have meant that it is now possible to derive stem cells from differentiated adult cells/tissue. Takahashi et al. showed it was possible to use ectopic transcription factors to induce pluripotency and ESC properties (Takahashi and Yamanaka, 2006). These transcription factors were known for being important in the long term maintenance of ES cell phenotype (Oct3/4 and Sox2) and pluripotency (c-myc and Klf4) (Takahashi and Yamanaka, 2006). These iPSCs are almost identical to ESCs in terms of their characteristics. They are able to differentiate into any cell type in the body, have infinite potential to grow, share the same morphology and have the same expression pattern of genes (Yamanaka, 2009); making them a potentially very powerful tool for complex disease research.

Primary microglia cultures are often used to study neuro-inflammation, they can be derived from rat or mouse brain before birth or early on in development. In addition, human microglia cultures have also been established from fetal brain (McLarnon et al., 1997). One method of generating these cells was developed by Giulian and Baker (1986) and involving a specific process of adhesion and agitation. These cells are often used as they show similarities to microglial cells *in vitro*, however, the process of extraction and culture itself alters microglial phenotype (Caldeira et al., 2014). Given the degree of variability in ion channel distribution during development and aging (Harry, 2013), using this type of model for investigating neurodegeneration is less than ideal. Another method to study microglia is through the use of retroviral-immortalized cell lines, such as the mouse and rat microglial cell lines N9 and BV-2 respectively (Righi et al., 1989; Blasi et al., 1990). These cell lines offer an advantage in the fact that they are fast to grow, and large numbers of cells can be generated quickly. However, as they have been immortalized using oncogenes which means they differ from primary microglia as they have increased adhesion and proliferation and can vary in terms of their morphology (Horvath et al., 2008).

Until recently, being able to generate iPSC-derived microglia has been elusive, with previous attempts being met with skepticism as the microglia were made from induced hematopoietic stem cells (HSCs). HSCs have the potential to give rise to other cell types such as blood derived macrophages and as already stated microglia arise from EMPs. In order to generate EMPs from the iPSCs, Muffat and his colleagues developed a serum free media that contains high levels of IL-34 and colony stimulating factor 1 (CSF1) (Muffat and Li, 2016). These conditions were chosen as the media mimics the brain cerebrospinal fluid and the factors have been shown to be necessary for microglia differentiation and maintenance. Under these conditions they found that the cells soon formed rope-like structures that when plated onto low adherence plates gave rise to highly adherent pluripotent stem cell–derived microglia-like cells (pMGLs). These cells express many of the markers that would be expected from microglia, such as TMEM119, P2RY12/13, HEXB and GPR34. Alongside this they

are also highly phagocytic and gene transcriptomic analysis demonstrated they resemble human primary fetal and adult microglia (Muffat and Li, 2016). This is not the only protocol that has been published which describes the derivation of microglia from iPSCs, subsequently there have been four more protocols released. In fact the next two papers described the generation of iPSC-derived microglia going through a hematopoietic progenitor cell (HPC) stage. Both methodologies use defined media systems that contain a number of growth factors including IL-3, BMP4 and L-ascorbic acid to generate HPCs (Abud et al., 2017; Pandya et al., 2017). Both protocols take about 10 days to generate HPCs at which point they were checked for markers of the hematopoietic lineage such as CD43 before differentiating for a further 2 weeks using another media to form induced microglia like cells (iMGLs). One way in which these protocols differ is that Pandya et al. (2017) co-culture the HPCs with astrocytes to enhance microglial differentiation. This is not the only protocol that uses co-culture to generate iPSC-derived microglia, a paper released by Walter Haenseler also uses co-culture but neuronal microglial co-culture instead (Haenseler et al., 2017).

Whilst iPSC-based models offer a number of advantages to modeling complex diseases there are a number of limitations that should also be considered. Firstly, the cells which are derived from iPSCs have been found to display the functional and epigenetic signatures of fetal neurons and do not maintain the features, such as telomere length and mitochondrial metabolism, of the cells from which they were originally derived (Lapasset et al., 2011). One of the current major stumbling blocks for iPSC research (control and those derived from patients with specific neurodegenerative disorders), is the lack of standard culturing or differentiation methods (Wen et al., 2016). Resulting in the unavailability of established protocols to generate entirely pure populations of a specific cell type, therefore making cross lab comparisons particularly difficult. However, more recently the availability of human tissue as well as iPSCs have provided new opportunities for academic and industry-based researchers to identify optimal cell types and culture conditions to efficiently generate stable, defined and reproducible cell types for their specific research–with limited variability. Whilst this may not be an issue for some studies, when trying to investigate diseases of aging such as AD it could pose more problems as cells may not show age related phenotypes or degeneration. One way in which it may be possible to overcome this is through maintain and aging the cells in culture for as long as possible.

One of the challenges to date has been modeling sporadic AD in both rodent and human models of disease, with familial AD mutations accounting for only 5–10% of all AD cases (Kim et al., 2017). Excitingly, Lin et al. (2018) describes the first experiments in which CRISPR/Cas9 technology has been used to generate isogenic APOE4 iPSC-derived microglia. In this study the APOE4-like microglia exhibited altered morphology correlating to the reduced Aβ phagocytosis seen in rodent models. They found that consistently converting APOE4 to APOE3 in brain cell types from sporadic AD iPSCs was sufficient to diminish multiple AD-related pathologies (Lin et al., 2018).

TABLE 2 | Comparison of multiple transcriptome studies of regulated microglial genes, relating to ion channels, in models of aging or Alzheimer's disease.

Species	Sample type	Potassium channels	Sodium channels	TRP channels	Calcium channels	Others	Reference
Human	iPSC	KCNA5, KCNK13, KCNN4	SCN5A	TRPM2, TRPM4, TRPM8, TRPV1, TRPV2	CACNA1S	HVCN1, CLIC1	Haenseler et al., 2017
Human	Biopsy primary microglia culture	KCNK13, KCNN1, KCNN4	Not determined	TRPC1, TRPC2, TRPM2, TRPM3, TRPM4, TRPM7, TRPMV1, TRPV2, TRPV4	Not determined	HVCN1, CLIC1	Gosselin et al., 2017
Human	Purified from post-mortem dorsal lateral pre-frontal cortex	KCNN4	Not determined	TRPM2, TRPV2	Not determined	CLIC1	Olah et al., 2018
Human	Purified from post-mortem dorsal lateral pre-frontal cortex	KCNJ2, KCNK13, KCNN4	Not determined	TRPM2, TRPM7, TRPV1, TRPV2	CACNA1A, CACNA1D	HVCN1, CLIC1	Olah et al., 2018
Human	Purified from post-mortem right parietal cortex	KCNK13, KCNN4	Not determined	TRPC2, TRPV2, TRPV4	Not determined	CLIC1	Galatro et al., 2017
Human	Purified from post-mortem right parietal cortex	KCNN4	Not determined	Not determined	CACNA1F	Not determined	Galatro et al., 2017
Mouse	Primary microglia culture	KCNA3, KCNK13, KCNN4	Not determined	TRPM4	CACNA1A, CACNA1D	Not determined	Gosselin et al., 2017
Mouse	Collated Meta-Analysis	KCNA1, KCNA2, KCNN1, KCNN3	Not determined	TRPA1, TRPC1, TRPC3, TRPC4, TRPC6, TRPC7, TRPM3, TRPM8, TRPV1, TRPV6	Not determined	HVCN1	Olah et al., 2018
Rat	Primary microglia culture	KCNK13, KCNN4	Not determined	TRPC4, TRPC6, TRPM2, TRPM4, TRPV1	Not determined	CLIC1	Bohlen et al., 2017

Regulation threshold was set at a 3-fold change over all studies. Anything below this is referred to as not determined.

They also showed that in their iPSC-derived microglia, TREM2 was positively correlated to the APOE4 genotype. This data is consistent with reports showing increased levels of soluble TREM2 in cerebrospinal fluid of AD patients (Heslegrave et al., 2016). Similarly, protocols for microglia differentiated from patients carrying missense mutations in TREM2 (that are causal for frontotemporal dementia-like syndrome and Nasu-Hakola disease). These studies found subtle effects on microglia biology, consistent with the adult onset of disease in individuals with these mutations (Brownjohn et al., 2018). These particular studies establish a reference for human cell-type-specific changes associated with the risk of developing AD, providing critical insight into potential treatments for sporadic AD.

As more is understood about the developmental origin and unique identity of microglia, recent studies have attempted to circumvent this issue by deriving microglia from iPSCs in order to study human and cell-type-specific biology and disease (Muffat et al., 2016; Abud et al., 2017; Douvaras et al., 2017; Haenseler et al., 2017; Pandya et al., 2017; Takata et al., 2017; Brownjohn et al., 2018; Lin et al., 2018). At the whole-transcriptome level, microglia generated by the methods reported here most closely resemble cultured primary microglia (Brownjohn et al., 2018). Due to a lack of unique surface markers, it has historically been difficult to distinguish microglia from other macrophages and cells of myeloid lineage. It is only recently that a distinct transcriptomic profile of microglia has emerged (Hickman et al., 2013; Butovsky et al., 2014; Holtman et al., 2015; Bennett et al., 2016; Gosselin et al., 2017; Keren-Shaul et al., 2017; Krasemann et al., 2017). In this review we have highlighted the similarities between rodent and human microglia transcriptomics and have identified key ion channels prominent in human iPSC-derived microglia, some of which we have already been highlighted earlier in this review as prominent targets associated with AD (**Table 2**) including KCNK13, KCNN4, TRPV2, HVCN1, and CLIC1. Indeed, the ion channels found from iPSC-derived microglia to date mirror those found in aged-human tissue (Olah et al., 2018).

Finally, the characterization of the electrophysiological properties of neurons derived from iPSCs are extremely limited and even fewer reports on the functional properties of iPSC-derived glia (microglia and astrocytes). However, with the development of standardized methods and differentiation protocols and, importantly, broader *functional* characterization of the complex collection of ion channels and receptors expressed in defined glial and neuronal subtypes from iPSCs, their significance in drug discovery and neuroscience will become increasingly valuable.

CONCLUDING REMARKS

Microglial research has expanded dramatically in the last 5 years, this combined with the lack of new therapeutic options for treating complex neurological conditions highlights the potential of these cells to provide a viable alternative. For this to be realized a clearer picture of human microglial physiology needs to be established. The development of iPSC technology has been a great advance in these efforts, but robust protocols are still in their infancy (Douvaras et al., 2017; Haenseler et al., 2017; Brownjohn et al., 2018). With microglia being dependent *in situ* environments the need to generate more complex 3D models is even greater. While the development of 3D scaffolds continues at pace (Saliba et al., 2018), some initial research indicates the possibility of 3D microglia cultures (Cho et al., 2018). The challenge will now be to incorporate the diverse range of cells into these cultures with the ability to provided measurable outcomes (e.g. electrophysiology). Establishing robust and reproducible protocols will also allow us to progress into addressing the role of microglia in pathological states. This is vital if we are to achieve a therapeutic purpose for targeting microglia ion channels.

The role of ion channels is extensive within the central nervous system, however as non-excitable cells microglia channels often get overlooked. Here we have examined the microglia ion channel landscape and the evidence that supports the involvement in Alzheimer's disease pathogenesis. While there is still work to be done as highlighted above, this review indicates that microglia ion channels play a pivotal role in their physiology and can contribute to the fight against dementia.

AUTHOR CONTRIBUTIONS

TK and MD contributed to the initial design and conception of the review. LT, JI, EK, MD, and TK wrote individual sections of the review. TK wrote the first draft of the manuscript. LT and JI prepared figures. TK and LT outlined the tables and compiled final version of manuscript. All authors approved, read and revised final version before submission.

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REVIEW
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Elucidating the Interactive Roles of Glia in Alzheimer's Disease Using Established and Newly Developed Experimental Models

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Alzheimer's disease (AD) is an irreversible neurodegenerative illness and the exact etiology of the disease remains unknown. It is characterized by long preclinical and prodromal phases with pathological features including an accumulation of amyloid-beta (Aβ) peptides into extracellular Aβ plaques in the brain parenchyma and the formation of intracellular neurofibrillary tangles (NFTs) within neurons as a result of abnormal phosphorylation of microtubule-associated tau proteins. In addition, prominent activation of innate immune cells is also observed and/or followed by marked neuroinflammation. While such neuroinflammatory responses may function in a neuroprotective manner by clearing neurotoxic factors, they can also be neurotoxic by contributing to neurodegeneration *via* elevated levels of proinflammatory mediators and oxidative stress, and altered levels of neurotransmitters, that underlie pathological symptoms including synaptic and cognitive impairment, neuronal death, reduced memory, and neocortex and hippocampus malfunctions. Glial cells, particularly activated microglia and reactive astrocytes, appear to play critical and interactive roles in such dichotomous responses. Accumulating evidences clearly point to their critical involvement in the prevention, initiation, and progression, of neurodegenerative diseases, including AD. Here, we review recent findings on the roles of astrocyte-microglial interactions in neurodegeneration in the context of AD and discuss newly developed *in vitro* and *in vivo* experimental models that will enable more detailed analysis of glial interplay. An increased understanding of the roles of glia and the development of new exploratory tools are likely to be crucial for the development of new interventions for early stage AD prevention and cures.

Keywords: neuroinflammation, Alzheimer's disease, astrogliosis, microgliosis, animal models, brain-on-a-chip, astrogliosis-microgliosis axis

INTRODUCTION

Neurotoxic Glial Activation Exacerbates AD Dementia

Many researchers have genetically modified human AD genes in mice and rats to overexpress Aβ peptides and/or tau proteins to mimic Aβ plaques and/or NFTs, which are features of human AD brain pathology. These animal models have, therefore, been widely used to test potential AD therapies, but more than 20 agents that have shown promise in these models have failed in clinical trials (1, 2), raising suggestions that amyloid and tau may need to be targeted decades before clinical symptoms appear, and causing some to even question the validity of the amyloid and tau hypothesis. In addition to Aβ plaque and NFT deposition, it has been recently recognized that brain inflammation involving glial cell activation is a prominent feature of AD. For example, increased inflammatory mediator expression has been reported in postmortem brains of AD patients (3, 4), and epidemiological studies have linked the use of anti-inflammatory drugs with a reduced risk for this disorder (5, 6). It is known that the degree of glial cell activation and their interplay correlates with the extent of brain atrophy and cognitive impairment (7, 8). It is, therefore, reasonable to suggest that glial neuroinflammatory responses, and those of microglia and astrocyte in particular, exacerbate the neurodegeneration associated with AD. In this review, we will discuss the available evidence that supports such a hypothesis.

Reactive Astrocytes and Activated Microglia in Neuroinflammation

Astrocytes are the most abundant glial cells in the brain and are responsible for brain homeostasis. In pathological conditions, reactive astrocytes are ubiquitously detected throughout the central nervous system (CNS). Reactive astrocytes are identified by increased expression of intermediate filament proteins such as glial fibrillary acidic protein (GFAP) and vimentin. The astrocyte reactivity can be categorized as mild/moderate or severe. In brain injury model, the mild/moderate reactive astrocytes show hypertrophy having ramified processes without proliferation, whereas severe reactive astrocytes have proliferating potential with severe hypertrophy (9). Recently, Liddelow et al. categorized reactive astrocytes into A1 and A2 in conditions of lipopolysaccharide (LPS) treatment and middle cerebral artery occlusion (MCAO), and characterized these astrocytes as neurotoxic or neuroprotective, respectively. They identified that the A1 astrocytes were triggered by activated microglia through secreting tumor necrosis factor (TNF), interleukin-1α (IL-1α) and complement component 1q (C1q) and lost many neuroprotective functions of astrocytes (10).

Microglia, resident myeloid cells in a CNS, continually survey their microenvironments in normal and diseased brains while providing immune surveillance and activation in response to infection, non-infectious diseases, and injury (11–13). Although microglial hyper-activation or dysfunction is a potential mechanism leading to neurodegenerative and neuroinflammatory diseases, the roles of microglia are still under debate (14). There have also been attempts to understand the heterogeneity of activated microglia, as M1 and M2. M1 microglia are classically activated microglia, which produce inflammatory cytokines and reactive oxygen species (ROS) (**Figure 1**), whereas M2 microglia are in a state of alternative activation that show an anti-inflammatory phenotype (15). However, these categorizations remain under consideration because microglial activation shows more complex variations in phenotypes (16).

Neuroinflammatory Aspects of Animal Models of AD and Their Limitations

The most widely employed transgenic animal models for AD display substantial reactive gliosis that includes activated astrocytes and microglia (17, 18). These cellular responses are detected before the appearance of Aβ plaques and NFT pathology (19, 20). P301S tau transgenic mice are a model for tauopathy and exhibit not only aggregated tau, but also the production of inflammatory cytokines including IL-1β and glial activation around tau-positive neuronal cells (21). Such microglial activation precedes NFT formation and appears in 3-months old P301S tau transgenic animals (22). Similarly, in the APP/PS1 mice model, the reactive astrocytes near Aβ plaques look increasingly hypertrophied as AD develops. In addition to glial activation, these mice show enhanced levels of gliotransmitters such as gamma-aminobutyric acid (GABA) (23), more ROS, elevated production of cytokines including tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), IL-1β, IL-1α, chemoattractant protein-1, and greater expression of the inflammatory mediators cyclooxygenase-2 (COX-2) and C1q (17, 24) (**Figure 1**). Importantly, the degree of inflammatory cell activation and cytokine production correlates with disease progression and severity in mouse models of AD (23, 25).

To investigate the causal relationship between such neuroinflammatory responses and AD pathology, genetic and pharmacological manipulation of inflammatory components including IL-12, IL-23, TNF-α, prostaglandin E2, and cluster of differentiation (CD) 40 ligand (CD40L) has been performed in animal models of AD. In these studies, inflammatory factor inhibition has been found to decrease Aβ plaque loads (26–28). However, the role of neuroinflammation in other aspects of AD pathology, such as neuronal death or cognitive decline, remains elusive. Additionally, most animal models of AD that show amyloidogenesis fail to exhibit the tauopathy, brain atrophy, and neuronal death, that are common features in AD patients (29, 30). To overcome these limitations, researchers have made triple transgenic mice (3XTg-AD) harboring a tau transgene (tau_{P301L}) in addition to APP_{SWE} and presenilin-1 ($PS1_{M146V}$) mutations (31), or developed promoters exclusively expressing transgenes in neurons. Some of these mice show neuronal death and brain atrophy in specific brain regions while showing progressive Aβ plaque and NFT formation (32, 33). However, such tau transgenic mice have limitations for the study of AD due to the various isoforms of the tau gene that are possible. The tau gene generates six isoforms by alternative splicing, which are divided depending on the combination of exon 2/3 and exon 10. Tau transgenic mice that overexpress the longest form of the tau gene and/or modulate tau phosphorylation through

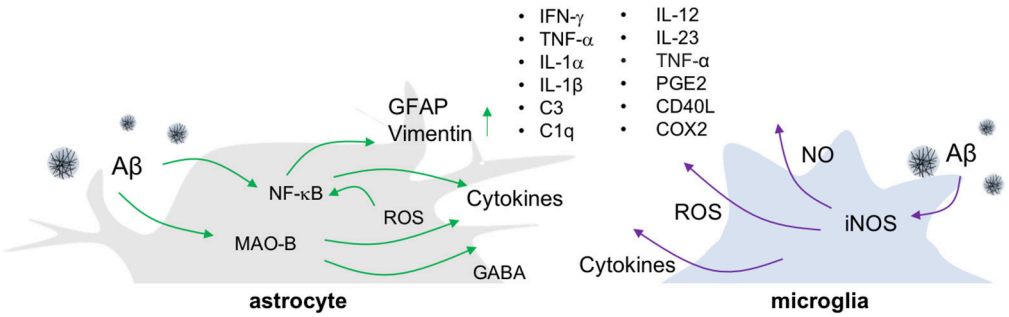

FIGURE 1 | Release of inflammatory molecules from activated glial cells in AD. In an Aβ-overproducing animal model of AD, inflammatory molecules such as cytokines, ROS/RNS and gliotransmitters are released from reactive astrocytes and activated microglia.

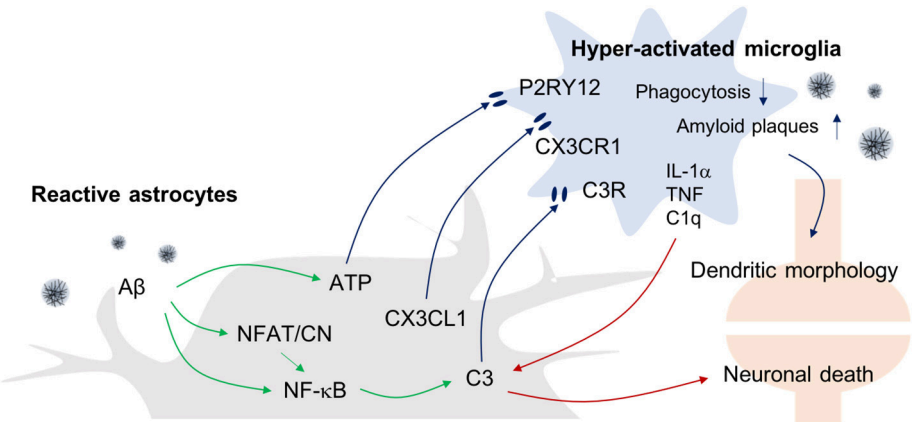

FIGURE 2 | Astrogliosis-Microgliosis Axis (AMA) in AD. Schematic showing the crosstalk between reactive astrocytes and hyper-activated microglia in AD. It has been reported that reactive astrocytes release ATP, CX3CL1, and C3 to activate microglia, whereas the activated microglia release inflammatory molecules such as IL-1α, TNF-α, and C1q to increase C3 expression in astrocytes, which consequently causes an increase in AD pathology indices such as Aβ plaques and neuronal death.

glycogen synthase kinase 3 beta (GSK-3β) paradoxically do not exhibit NFT.

THE ASTROGLIOSIS-MICROGLIOSIS AXIS (AMA) CONTRIBUTES TO AD PROGRESSION

Functional studies of astrogliosis and microgliosis and their relationship with AD have been performed in various experimental conditions. However, the relationship between gliosis in AD pathogenesis is complex and remains unclear despite the fact that resolving the timeline of AD pathology is essential to defining the cellular and molecular mechanisms underlying AD pathogenesis. For example, an increase in Aβ levels and astrocyte activation are found to occur early, even before the mild cognitive impairment (MCI) phase and reach a plateau when clinical symptoms appear (3, 34). In contrast, microglial activation, tau pathology, and neuronal death occur later in the disease and correlate with the severity of clinical symptoms (3). Below, we summarize the available evidence for astrocyte and microglia crosstalk during AD pathogenesis (**Figure 2**).

From Astrogliosis to Microgliosis

Under physiological conditions, astrocytes modulate the status of microglial activation. In the presence of astrocytes, Aβ toxin-induced microglial responses such as reactive morphological changes, inducible nitric oxide synthase (iNOS) induction, and decreased reductive metabolism, are attenuated (35). Astrocyte-derived transforming growth factor (TGF)-β1 deactivates microglial cells and abolishes neurotoxicity (36) and its modulatory effect involves the activation of the Smad3 pathway, which is down regulated in AD patients (37), and the activation of mitogen-activated protein kinase (MAPK)-extracellular-signal-regulated kinase (ERK) pathways (38) that also appear to be neuro-protective (39). Dynamic regulation of Smad, phosphoinositide 3-kinase (PI3K), and MAPK pathways, which are associated with TGF-activity in addition to inflammatory cytokine-mediated effects, is an important

component in the control of cell integrity and inflammatory responses. The abolition or decreases in the level of Smad3, a major effector pathway for anti-inflammatory responses, would therefore modify the regulatory feedback signals that result from inflammation.

However, reactive astrocytes under pathological conditions elicit microglial activation *via* several mechanisms that subsequently lead to AD pathology. When the reactivity of astrocytes is attenuated by blocking the inflammatory calcineurin/nuclear factor of activated T-cells (NFAT) signaling pathway in the presence of Aβ, microglial activation is significantly reduced, indicating that reactive astrocytes utilize that pathway to direct microglial activation. Moreover, such reactive astrocyte attenuation and diminished numbers of activated microglia was associated with reduced amyloid levels and improved cognitive and synaptic functions in APP/PS1 mice (40). These findings therefore suggest that microglial activation underlies the deleterious effects of reactive astrocytes in AD progression.

With regard to the possible mechanisms linking astrogliosis to microglial activation, it has been reported that C3 released from Aβ-treated astrocytes can upregulate C3a receptor (C3aR) expression by microglia (41). Importantly, nuclear factor of kappa-light-chain-enhancer of activated B cells (NF-κB) hyperactivation due to inhibitor of κB kinase (IKK) knockout (KO) results in complement expression by astrocytes because C3 protein secretion is driven by NF-κB activation in these cells, and such responses worsen Aβ-associated pathology, with reduced numbers of synapses and shortened dendritic lengths, and impaired synaptic functions, due to reduced microglial Aβ phagocytosis in AD mouse models (42, 43). Conversely, C3aR antagonists reduce Aβ plaques formation and attenuate microgliosis. Together, these data suggest that astrocytic activation in response to Aβ leads to microgliosis *via* the C3-C3aR pathway.

In addition, reactive astrocyte-mediated increases in the levels of synaptically localized C1q may be responsible for the microglial activation that results in age-dependent cognitive dysfunctions (14). In support of this idea, Bialas et al. showed that severe neuroinflammation might be a cause of autoimmune diseases such as lupus (44). Using live-cell imaging approaches in a mouse model of interferon over-expression, hyperactivated microglia were seen to ingest synaptic debris from neurons resulting in a reduced synaptic network.

In addition to complement components, other evidence has shown that extracellular ATP released from astrocytes can activate microglia *via* purinergic receptors. ATP is known to be released by N-methyl-D-aspartate (NMDA)-sensitive neurons (45), damaged astrocytes, and leaky blood vessels (46). ATP recruits and activates microglia to sites of injury *via* the P2RY12 purinergic receptor leading to synapse remodeling (47). Finally, astrocytes can also express the chemokine C-X3-C motif chemokine ligand 1 (CX3CL1) in inflammatory conditions and microglia express its receptor, C-X3-C motif chemokine receptor 1 (CX3CR1). Interestingly, it has been reported that CX3CR1-deficiency increases the functional connectivity of neural circuits while decreasing the number of microglia. However, the mechanism underlying this effect remains unclear (48, 49).

From Microgliosis to Astrogliosis

Recently, there have been attempts to categorize and characterize reactive astrocytes into distinct A1 and A2 phenotypes in the brain. It is suggested that the A1 phenotype represents reactive astrocytes that are induced by systemic LPS injection and whose function is detrimental to neurons, whereas A2 reactive astrocytes act in a protective manner and are induced in conditions such as the MCAO stroke model (10). Interestingly, in this study, the formation of A1 reactive astrocytes appeared to be dependent on the activation of microglia as colony stimulating factor 1 receptor (Csf1r) KO mice that lack this cell type fail to form this astrocytic phenotype following LPS challenge. This led these investigators to suggest that detrimental A1-type reactive astrocytes are induced secondary to the release of proinflammatory factors such as IL-1α, TNF-α, and C1q by microglia. Interestingly, C3 has been used as a marker for A1-type reactive astrocytes, and this complement component is ubiquitously expressed in AD brain astrocytes. However, it remains to be seen whether the microglia-induced detrimental reactive astrocytes seen following systemic LPS-treatment model are replicated in AD models.

ESTABLISHED AND NEW EXPERIMENTAL MODELS TO STUDY THE ROLE OF GLIOSIS IN AD

Appropriate and controllable models are required if we are to determine the role of reactive astrocytes and activated microglia in neurodegenerative diseases such as AD, and define the interplay between these cell types. Below, we describe currently employed models to evaluate and manipulate astrocyte and microglial functions, and discuss newly developed models that may prove useful in establishing the role of gliosis in AD.

Experimental Animal Models to Modulate Astrocyte Reactivity in AD

MAO-B-Modulating Reactive Astrocyte Models

Monoamine oxidase B (MAO-B) has been implicated in the pathogenesis of AD due to the increased expression of this molecule in astrocytes in the brains of AD patients (50) and adjacent to Aβ plaques in animal model of AD (23). The activation of MAO-B results in the aberrant production and release of GABA by reactive astrocytes, leading to reduced spike probability of granule cells *via* presynaptic GABA receptors (23). In addition, MAO-B produces hydrogen peroxide, a type of ROS, further contributing to AD pathology. As such, a reduction in ROS-induced oxidative stress *via* MAO-B activity inhibition would be expected to delay the progression of the disease. For these reasons, MAO-B inhibitors have been used to pharmacologically block astrogliosis in experimental models of AD. The MAO-B inhibitor selegiline has been reported to reduce the reactivity of astrocytes in APP/PS1 mice and this effect is associated with reductions in memory impairment (23).

Interestingly, transgenic animals that conditionally overexpress MAO-B in GFAP-positive astrocytes demonstrate elevated astrocyte reactivity that is associated with increased ROS formation and neuronal loss, a phenotype that is reversed by treatment with the MAO-B inhibitor sembragiline (51). Clearly, further study of the therapeutic effects of MAO-B inhibitors on astrogliosis in AD patients is warranted.

Virus-mediated Reactive Astrocytes Models: HSV, AAV, and Adenovirus

Viruses have been implicated in the etiology of AD through the induction of acute and chronic diseases in the CNS (52, 53). Recently, Eimer et al. suggested that herpes simplex virus-1 (HSV-1) infection aggravates Aβ deposition and AD progression by protective amyloidosis (54). Reactive astrocytes have been suggested to be one of the key cellular mediators of virus-induced CNS pathology. In the CNS, it has been reported that HSV-1 can induce astrocyte activation (55) and these cells have been shown to take up Zika virus *via* the astrocytic protein AXL (56). Recently, it was reported that HSV-1 causes the activation of GSK-3, which subsequently phosphorylates amyloid precursor protein (APP) (57). However, the mechanisms underlying virus-induced reactive astrogliosis are still unclear and the high degree of association between such infections and AD indicate that virus-mediated experimental models are required to further investigate this link.

Previously, AAV virus has been used to induce reactive astrocytes *in vivo* and the AAV2/5-gfp104-eGFP virus has been shown to increase the number of reactive astrocytes in a titer-dependent manner (58). In this model, authors found that reactive astrocytes downregulate the expression level of glutamate synthetase, impair inhibitory neurotransmission, and affect network hyperexcitability. Similarly, Woo et al. showed that the adenovirus, Adeno-GFAP-GFP, induces astrocyte reactivity in the dentate gyrus region of the mouse hippocampus (59), with astrocytes in this region becoming hypertrophied following virus-injection compared to astrocytes in uninfected mice. These models could, therefore, be readily employed to induce focal reactive astrogliosis in specific brain regions of interest. However, it must be noted that the use of such models will require caution to distinguish the effects of reactive astrogliosis from virally-induced inflammatory responses.

GFAP- and Vimentin-Modulated Reactive Astrocyte AD Models

Because astrocyte reactivity is characterized by hypertrophied processes and increased expression of intermediate filaments such as GFAP and vimentin, the role of astrocyte intermediate filaments in AD has been investigated by crossing mice genetically deficient in GFAP and vimentin with APP transgenic mice. While the validity of these models remains controversial, studies using these models has shown that GFAP and vimentin KO in APP/PS1 mice is associated with an almost two-fold increase in Aβ plaque formation at 8 and 12-months of age. In these triple transgenic mice, APP processing and soluble and interstitial fluid Aβ levels were unchanged, which suggests that Aβ degradation, rather than Aβ generation, is affected by the deletion of astrocyte intermediate filaments (IF). Astrocytes in GFAP and vimentin deficient animals showed marked alterations in their morphology near Aβ plaques, with little process hypertrophy and lacking contact with adjacent Aβ plaques. Moreover, these mice showed a marked increase of neurite dystrophy. Such findings indicate that activation-associated changes in astrocyte morphology limit Aβ plaque growth and attenuate plaque-related dystrophic neurites (60, 61). However, caution is needed when interpreting these studies, as intermediate filament KO appears to result in additional effects that include the absence of endothelin B receptor protein expression increases in GFAP/vimentin KO mice that normally occur in reactive astrocytes (62).

Animal Models That Feature the Modulation of Inflammatory Signaling Pathways in Reactive Astrocytes

Astrocyte reactivity can be blocked by genetic manipulation using AAV-GFAP-VIVIT in which VIVIT is a synthetic peptide that disrupts the physical interaction between calcineurin and NFAT. It interferes with NFAT activation in astrocytes and consequently reduces cytokine release and neuroinflammation. *In vivo* application of this tool to APP/PS1 mice significantly reduces AD pathology, reducing amyloid levels and improving cognitive functions, indicating that reactive astrocytes act in a deleterious manner (40). This is supported by *in vitro* studies that also showed astrocyte VIVIT expression ameliorates the neurotoxic effects of activated astrocytes on neighboring neurons (63, 64). Another strategy to block astrocyte reactivity is to knockout/knockdown IKKβ expression or overexpress its dominant negative form in these cells (65–67). Such approaches inhibit the inflammatory NF-κB pathway in reactive astrocytes. Finally, deletion of aquaporin 4 (AQP4) has also been employed to block astrocytic functions, and deletion of this channel has been shown to exacerbate brain Aβ accumulation and memory deficits in APP/PS1 mice (68). However, it is unclear from the limited number of studies employing these models whether and how the reactivity of reactive astrocytes contributes to AD pathogenesis.

Experimental Animal Models to Modulate Microglia Activation in AD

Microglial hyper-activation is believed to be neurotoxic and, therefore, blocking microglial activation is predicted to attenuate disease progression. Pharmacological and genetic manipulation of microglial activation in animal models of AD have been performed to determine the contribution of microglia activation to AD pathogenesis. Asraf et al. reported that blocking microglial activation through Captopril, which inhibits angiotensin-converting enzyme (ACE) and blocks the formation of angiotensin II (Ang II), decreased LPS-induced NO release and regulated iNOS, TNF-α, and IL-10 in BV2 microglia cells. This tool was also applied to an *in vivo* AD mouse model, 5X FAD. Intranasal treatment with Captopril for 2-months ameliorated microglial activation and decreased Aβ burden, indicating that microglial activation exacerbates AD pathology (69). On the other hand, Manocha et al. reported

that inhibition or deletion of NFAT 2c isoform, which is the most highly expressed gene in microglial culture, modulated microglial activation and blocked the release of cytokines from microglia. When NFATc2 KO mice were crossed with APP/PS1 mice, cytokine levels and microgliosis were reduced. However, there was no effect on plaque load (70). On the other hand, phagocytic roles of microglial triggering receptor expressed on myeloid cells 2 (TREM2) have been reported in AD and in mice. TREM2 deficiency results in a reduced microglial inflammatory response (71). By crossing TREM2 deficient mice crossed with AD mouse models, researchers have shown that TREM2-deficient microglia are ineffective at either clustering around and/or removing fibrillary Aβ (72, 73). These studies suggest that microglial activation exacerbates AD pathogenesis.

Novel *in vitro* Culture Models of Human Glia in AD

A Microfluidic Chip to Monitor Microglial Responses to Disease-Related Soluble Cues

In vivo studies of microglial migration in AD have been hampered by the complexity of the effects of Aβ on microglia due to the multiple forms of Aβ and heterogeneous microglial activation (74). Aβ peptides form deposits of insoluble forms of this molecule surrounded by a mixture of soluble oligomeric Aβ (75), accumulated microglia, and dystrophic neurites (76). Activated microglia can take on a variety of morphologies that include rounded, ramified, rod-like, and amoeboid forms that are followed by motile activation, and these changes complicate visual tracking of individual cells. Previous microfluidic attempts to study rat microglial migration in the presence of short-lived damaged axons (77) did not establish long-lasting chemoattractant gradients, and failed to conclusively differentiate gradual microglial accumulation from heterogeneous activation and random navigation (78, 79). More recently, we have developed a novel microfluidic chemotaxis platform to study the regulated and stimuli-selective microglial motility (80). To understand the specific role of Aβ in microglial accumulation, we generated soluble Aβ (sAβ) gradients that lasted a week, patterned insoluble surface-bound Aβ (bAβ) to mimic the Aβ signature in AD brains, and were able to isolate human microglia responding to Aβ in this platform. We evaluated the Aβ sensitivity of primary human microglia isolated from human fetal brain (HMG 030, Clonexpress, Inc.) and adult human microglial cell lines (T0251, ABM Inc.) and characterized their responses. In addition, we were able to monitor single cell changes in microglial morphology in real time in response to soluble Aβ that were associated with directional migration. We found that soluble monomeric and oligomeric Aβ can act as a microglial chemoattractant at a broad range of concentrations from picomolar to nanomolar that correspond to levels in normal and AD brains, respectively. Importantly, we were also able to discern co-localization of microglia to insoluble Aβ in this model that was similar to Aβ plaques seen in AD.

A Microfluidic Model to Assess Crosstalk Between Central and Peripheral Immunity in AD

The accumulation of immune cells in the brain parenchyma is a critical step in the progression of neuroinflammatory diseases including AD. While the mechanisms underlying central immunity activation and Aβ clearance are well studied in the context of AD pathogenesis, the mechanisms responsible for the recruitment of peripheral immune cells from the blood stream to CNS disease sites are less clear. Peripheral immune cells including T and B lymphocytes, monocytes, and neutrophils, have been identified in the brains of human AD patients and corresponding animal models (81–83). Among these immune cells, neutrophils are of great interest because they are key effector cells in many inflammatory responses, and they show a remarkable ability to migrate within and through blood vessels (84, 85). To better assess the potential for neutrophil recruitment and activation in AD, we have reconstituted an AD microenvironment in a microfluidic model that includes the induction of cytokine/chemokine production by human microglial cells stimulated with Aβ, and employed this model to investigate the recruitment of human neutrophil in the context of innate-peripheral immunity crosstalk (86). In this model, we observed that Aβ stimulated microglial cells induce the robust recruitment of human neutrophils concomitant with the release of mediators including IL-6, IL-8, chemokine ligand (CCL) 2, CCL3/4, and CCL5. We subsequently confirmed a role for IL-6, IL-8, and CCL2 in neutrophil recruitment with the demonstration that such responses were attenuated by the presence of neutralizing antibodies against these factors. Interestingly, the recruited neutrophils in this system prompted the release of additional inflammatory mediators such as macrophage migration inhibitory factor (MIF) and IL-2. As such, this microfluidic system shows great promise for the study of chemotactic crosstalk between resident CNS cells and circulating leukocytes in AD and may prove useful in determining the therapeutic potential of targeting neutrophil neuroinflammatory activity to limit AD pathogenesis.

In vitro Generation of iPSC-Derived Microglia-like Cell

Haenseler et al. developed microglia-like cells by co-culturing iPSC-derived macrophages with iPSC-derived cortical neurons and demonstrated the expression of major microglia-specific markers and neurodegenerative disease-relevant genes (87). In transcriptome analyses, the microglia isolated using CD11β show upregulation of the six key microglia-specific genes: MER proto-oncogene, tyrosine kinase (MERTK), G-protein coupled receptor 34 (GPR34), protein S (PROS1), C1QA, growth arrest-specific 6 (GAS6), and P2RY12 in relevant homeostatic pathways and downregulation of antimicrobial pathways. Also, microglia expressed neurodegeneration-involving genes: fermitin family member 2 (FERMT2), TREM2, apolipoprotein E (APOE), and ubiquitin C-terminal hydrolase L1 (UCHL1), AD genes: APP, phosphatidylinositol binding clathrin assembly protein (PICALM), and CD33, PD genes: Parkinson disease 15 (PARK15), PTEN-induced putative kinase 1 (PINK1), synuclein alpha (SNCA), and protein deglycase (DJ-1), motor neurone disease (MND) genes: C9orf72, TAR DNA-binding protein

(TARDBP), and superoxide dismutase 1 (SOD1). Co-cultured microglia showed various alterations including changes in microglia-related protein expression and morphogenesis, and elevated motility and phagocytosis.

Further to this, Abud et al. generated human iPSC-derived microglia-like cells (iMGLs) with a two-step culturing protocol. Whole-transcriptome analysis demonstrated high similarity to cultured adult and fetal human microglia by expressing microglial genes: P2RY12, GPR34, C1Q, Cdk5, and Abl enzyme substrate 1 (CABLES1), basic helix-loop-helix family, member e41 (BHLHE41), TREM2, PROS1, APOE, solute carrier organic anion transporter family member 2B1 (SLCO2B1), solute carrier family 7 member 8 (SLC7A8), peroxisome proliferator activated receptor delta (PPARD), and crystallin beta B1 (CRYBB1) (88). Functional assessments revealed that iMGLs secrete eight different cytokines and chemokines, including TNF-α, CCL2, CCL4, and CXCL10 in response to IFN-γ or IL-1β, migrate along an ADP gradient, produce calcium transients initiated via P2RY12, and exhibit robust C1q/CR3-mediated phagocytosis. In particular, they showed that iMGLs can internalize fluorescently labeled fibrillar Aβ and tau oligomers. In addition to genes involved in AD pathology, iMGLs express other neurodegenerative disease-relevant genes, including APP, PSEN1/2, huntingtin (HTT), progranulin (GRN), TARDBP, leucine-rich repeat kinase 2 (LRRK2), C9orf72, SOD1, valosin-containing protein (VCP), and FUS, which are correlated with amyotrophic lateral sclerosis (ALS), Huntington's disease (HD), frontal temporal dementia (FTD), and dementia with Lewy bodies (DLB), which supports the potential for these cells in the study of a variety of neurological diseases.

A 3D Organotypic Model of the Human AD Brain

We have recently developed a new 3D organotypic human cell AD brain model by tri-culturing human AD neurons, astrocytes, and adult microglial cells in a 3D microfluidic platform (3D hNeuroGliAD) (89). This model replicates key characteristic features of AD including accumulation of Aβ, phosphorylated tau (pTau) accumulation, and AD neuron and astrocyte damage associated with microglial inflammatory responses. A central chamber was loaded with immortalized AD human neural progenitor cells (hNPCs), or iPSC-derived human AD NPCs provided by Drs. Tanzi and Kim (90), suspended in a growth factor-reduced Matrigel 150 μm in height, and differentiated into human AD neurons and astrocytes on the chip, while an angular chamber was loaded with human adult microglia. The central and angular chambers were linked by migration channels that formed soluble factor gradients from the central chamber and served as mechanical barriers to spontaneously activated microglia.

Our model provided representative AD signatures that included pathological accumulation of Aβ and pTau, NFT-like structure formations inside neurons, and IFN-γ production by astrocytes. Microglial morphological changes and migration toward the central chamber began at 48 h following cell seeding with the microglial cells elongating as the length of their somata (cell bodies) increased disproportionally. Furthermore, microglia exposed to soluble AD cues from the cultured AD neurons and astrocytes showed an up-regulation in the expression of activation markers including CD11b. This 3D AD brain model was associated with significant increases in the release of several key chemokines including CCL2 (2.1-fold), CCL5 (26-fold), CXCL10 (2.6-fold), and CXCL12 (1.2-fold), and inflammatory cytokines such as IL-6 (2.2-fold), IL-8 (2.7-fold), and TNF-α (1.3-fold), by microglia as compared to controls. We also observed the unique production of the leukocyte growth factors, granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF) in our AD model that was in contrast to the expression of anti-inflammatory markers including IL-1RA, IL-10, and TGF-β, that was very low or undetectable.

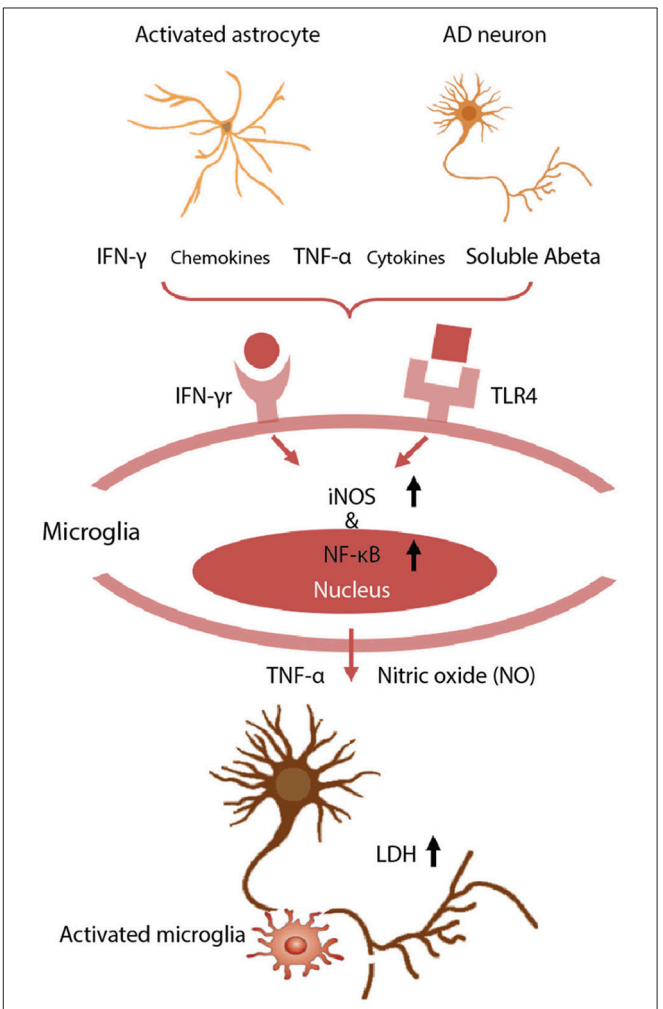

FIGURE 3 | Neurotoxic glia interactions mediated by TLR4 and IFN-γ receptor. Schematic of detrimental astrocyte-microglia activation in a 3D human AD model. The combination of Aβ derived from AD neurons and inflammatory cytokines from reactive astrocytes are detected by microglia *via* TLR4 and IFN-γ receptors. Then, the microglia subsequently activate the expression of iNOS and NF-κB, and release TNF-α and cytotoxic NO, leading to neurodegeneration. Neuronal damage was assessed using a LDH assay to quantify membrane damage. Reproduced/adapted from reference [(89)].

We have observed significant neuronal loss in this human tri-culture 3D model at 9 weeks that corresponds to a late AD stage (**Figure 3**) (89). The mechanisms underlying such neuronal damage in our 3D AD brain model were investigated by examining the astrogliosis-microgliosis axis using glial cell monocultures. We found that Aβ derived from AD neurons and IFN-γ produced by reactive astrocytes combined to activate microglia *via* toll like receptor 4 (TLR4) and IFN-γ receptors, respectively, and these cells subsequently expressed iNOS, and released TNF-α and cytotoxic nitric oxide (NO), leading to neuronal damage. In contrast, exposure to IFN-γ alone did not trigger TNF-α or NO release by microglia. NO levels were increased by 9.1-fold in 3D AD brain models cocultured with microglia, but this mediator was not detectable in the absence of microglia or models of early stage AD due to a lack of IFN-γ production. Blocking microglial TLR4 receptors, either with a neutralizing anti-TLR4 antibody or a TLR4 antagonist, decreased the levels of TNF-α, iNOS, and NO release, indicating that enhanced iNOS expression and concomitant NO release is mediated by a TLR4 dependent mechanism. Interestingly, the release of lactate dehydrogenase (LDH), a biochemical marker of cell death, was significantly decreased in the presence of TLR4 antagonist, LPS-RS, in our 3D AD brain model. These data indicate that microglia in a 3D AD brain model induce neuronal loss through an IFN-γ and Aβ dependent mechanism, which could have critical implications for future AD therapy drug screening efforts (87).

FUTURE PROSPECTS

Recent clinical trial failures of AD drugs that target Aβ have led researchers to consider alternative molecular targets that involve neuroinflammation, in particular, the activation of microglia and astrocytes. A major function of astrocytes and microglia appears to be the degradation of toxic molecules such as Aβ and hyperphosphorylated tau. However, it is unclear how this degradative function results in reactivated or hyper-activated astrocytes and microglia. While the appearance of reactive astrocytes and activated microglia precedes the symptomatic and neurodegenerative stages of AD, it is still unclear whether astrogliosis precedes microgliosis or vice versa. Because microglia are far more mobile than astrocytes, it is possible that astrocytes that are normally in contact with neurons, especially at the synaptic junctions, sense danger and alert nearby microglia to sites of injury and/or amyloid deposits. Therefore, reactive astrocytes might be the initial trigger for the cascade of events that leads to neuroinflammation and neurodegeneration. The alternative is that microglia, whose principle function is immune surveillance, are the first to detect danger and instruct nearby astrocytes, *via* released cytokines and other inflammatory mediators, to recruit more microglia in a feed-forward manner that leads to neuroinflammation and neurodegeneration. This question is extremely difficult to address with current *in vivo* and *in vitro* experimental models due to a number of limitations that we have discussed. However, some of the newly developed reactive astrocyte models may prove useful in addressing such questions. Furthermore, our newly developed multicellular human AD *in vitro* models have great potential as tools to define the molecular and cellular mechanisms underlying reactive astrogliosis and microglial hyper-activation and their role in AD pathogenesis. For example, recently identified reactive astrocyte markers, such as MAO-B, and the microglial marker, TREM2, could be investigated at the molecular and cellular level using these recently developed *in vivo* and *in vitro* AD models (91). While glial responses might be intended to be protective for individual cells and/or the brain tissue as a whole, the accumulated cellular defensive responses could result in the chronic brain alterations associated with AD. As such, defining the cellular mechanisms underlying these responses could help to understand the controversial roles of glial activation during the progression of AD pathology. Moreover, an examination of the events responsible for astrogliosis and microgliosis will provide mechanistic insights into disease progression, and may provide novel diagnostic markers or even therapeutic interventions at the very earliest stages of AD. Considering the recent discoveries implicating astrogliosis and microgliosis in AD pathology, and an array of newly developed models to pursue such avenues of investigation, there is reason for optimism for the treatment of AD.

AUTHOR CONTRIBUTIONS

HeC, IM, CL, and HaC reviewed literature, outlined, wrote the manuscript, and prepared figures. All authors read and edited the manuscript extensively.

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In vivo Imaging of Glial Activation in Alzheimer's Disease

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Alzheimer's disease (AD) is characterized by memory loss and decline of cognitive function, associated with progressive neurodegeneration. While neuropathological processes like amyloid plaques and tau neurofibrillary tangles have been linked to neuronal death in AD, the precise role of glial activation on disease progression is still debated. It was suggested that neuroinflammation could occur well ahead of amyloid deposition and may be responsible for clearing amyloid, having a neuroprotective effect; however, later in the disease, glial activation could become deleterious, contributing to neuronal toxicity. Recent genetic and preclinical studies suggest that the different activation states of microglia and astrocytes are complex, not as polarized as previously thought, and that the heterogeneity in their phenotype can switch during disease progression. In the last few years, novel imaging techniques e.g., new radiotracers for assessing glia activation using positron emission tomography and advanced magnetic resonance imaging technologies have emerged, allowing the correlation of neuro-inflammatory markers with cognitive decline, brain function and brain pathology *in vivo*. Here we review all new imaging technology in AD patients and animal models that has the potential to serve for early diagnosis of the disease, to monitor disease progression and to test the efficacy and the most effective time window for potential anti-inflammatory treatments.

Keywords: inflammation, TSPO, Alzheimer's disease, positron emission tomography (PET), microglia, astrocyte, imaging

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INTRODUCTION

Neuroinflammation is the term used to denote the response of the central nervous system to harmful stimuli such as protein aggregation, pathogens, and any other insult to the brain. While timely initiated and resolved, the inflammatory response is necessary reaction to noxious stimuli and hence protective, sustained and/or disproportionate (neuro)inflammation will likely contribute, exacerbate or induce tissue damage and thereby aggravate disease pathology (1). The nature of the inflammatory process is therefore complex and dynamic and changes along different stages of the disease, involving phenotypic alterations in all cells present within the CNS including neurons, microglia, astrocytes, and other inflammatory cells.

Microglia, act as part of the innate immune system, are constantly scanning and surveying the local microenvironment for signals of infection and injury [for a review see (2)]. Amyloid-β has been reported to "prime" or activate microglial cells (3). Different activation states were described in the past, so called "M1" or classically activated microglia or "M2" alternatively activated (2). The classically activated or pro-inflammatory phenotype has been associated with disease aggravation.

However, this classification has been recently challenged by single-cells transcriptomics, which suggests that the gene expression profile progressively switches with the disease and that this may even depend on how close they are to the amyloid plaques (4).

Astrocytes, are key mediators of many essential processes in the CNS. As for microglia, astrocytes have been classified into two distinct reactive states, A1 (inflammatory) and A2 (ischemic) states (5), although this classification seems to be over-simplistic. Accumulation of hypertrophic reactive astrocytes around senile plaques has been observed in post-mortem human tissue from AD patients [Reviewed by (6)] and in animal models of the disease (7). It is worth noting that astrocytes and microglia communicate with each other and this cross talk is important in promoting glial activation (8, 9).

In order to follow-up changes in microglial and astrocytic activation *in vivo*, radioactive tracers for positron emission and single-photon emission computed tomography (PET and SPECT) have been developed in the past decades. In this review, we will analyze different *in vivo* imaging techniques that allow the visualization of changes in neuroinflammation in animals and humans.

IN VIVO IMAGING OF MICROGLIA ACTIVATION

PET Imaging With TSPO Ligands in AD Patients

Following activation, microglia proliferate, and express a series of genes for pro-inflammatory cytokines and certain receptors on their surface, including the 18 kDa translocator protein (TSPO). TSPO, primarily but not exclusively expressed in the mitochondrial membrane of microglia, was previously identified as the peripheral-type benzodiazepine receptor (PBR) (10). Besides microglia, TSPO has been detected in other types of gliosis as well, such as in astrocytoma (11), and is generally expressed in highly proliferating cells. While the exact function of TSPO still remains to be elucidated, initially its role was associated with the transport of cholesterol, with the TSPO complex being a rate-limiting step in the synthesis of steroid hormones (12). Initial attempts to create TSPO knockout mice reported a non-viable phenotype [described in (13)]. However, the successful development of conditional TSPO knockout mice suggested that TSPO might not be a crucial part of steroid hormone synthesis, e.g., testosterone production (14). In addition, the mitochondrial expression-paradigm was challenged by reports of TSPO expression in other subcellular locations (15), e.g., nuclear/perinuclear-located TSPO, where is believed to play a part in cell proliferation (16). Moreover, plasma membrane bound TSPO has been observed as well, for instance in mature human red blood cells, lacking mitochondria (17, 18).

In the mammalian brain, the expression of TSPO turned out to be very low, compared to other tissues (19). Only the olfactory bulb and non-parenchymal regions, such as the ependymal and choroid plexus, showed higher TSPO densities in comparison with most gray and white matter structures (20, 21). However, under conditions of local inflammatory responses, e.g., caused by a multitude of brain injuries, neoplasms and infections, TSPO appears to be upregulated. This effect was quickly recognized and made TSPO a potentially ideal and sensitive biomarker of brain injury (10, 11, 22–25). Therefore, PET tracers for TSPO were developed in the past decade as markers for microglia activation and neuroinflammation in AD (10, 26) (see **Table 1**).

The first and probably most widely used TSPO radiotracers, with over thousand publications, are the antagonist PK11195 [1-(2-chlorophenyl)-N-methyl-N-(1-methylpropyl)-3-isoquinoline carboxamide] and the agonist Ro5-4864 (63). Initial studies with PK11195 were conducted more than two decades ago, with numerous subsequent papers demonstrating upregulation in different neurodegenerative diseases and in neuroinflammatory conditions (64–67). Unsurprisingly, increased TSPO expression was reported by autoradiography in a wide range of brain regions of post-mortem samples from AD patients, including hippocampus, frontal, temporal, and parietal cortices (22, 68, 69).

Cagnin et al. published the first PET study using [^{11}C]PK11195 in 2001, demonstrating an increase of tracer uptake in AD cases (28). While subsequent reports generally have shown increased tracer binding in AD brains, some publications found no differences between Alzheimer's subjects and healthy individuals (29, 33, 35). In spite of these conflicting results, it is generally accepted that there is increased microglial activation in AD. Recent studies have extended this to patients with Mild cognitive impairment (MCI), showing that glia activation can precede clinical AD (70).

Recent reports have evaluated the relationship between amyloid load and neuroinflammation, suggesting that microglial activation is associated with amyloid load. Interestingly, using [^{11}C]PK11195 and the amyloid tracer [^{11}C]PIB, one study did not show any correlation between the binding of these tracers, while another one suggested a negative correlation between amyloid-β and TSPO density (31, 33). The reason behind these different outcomes could be related to the limitations of the current amyloid ligands; while we are able to image amyloid plaque deposition using amyloid imaging agents, other forms of amyloid, such as β-amyloid oligomers, which may be contributing to the microglial activation, are not currently detectable by PET (36, 71).

However, rigorous quantification of TSPO density using [^{11}C]PK11195 has been confronted by limitations of the ligand, including its modest binding affinity, high non-specific binding and elevated lipophilicity, generating a low signal-to-noise ratio (72). This has led to the development of numerous "second-generation" TSPO ligands [^{11}C]AC-5216, [^{18}F]PBR111, both ^{11}C and ^{18}F radiolabeled derivatives of PBR06 and PBR28, [^{18}F]FEPPA, [^{18}F]DPA-714 and the SPECT tracer [^{123}I]CLINDE (46, 48, 64, 73–79). **Figure 1** illustrates an example of PET imaging with [^{11}C]-PBR28 showing increased binding in an AD patient.

However, while affinity and nonspecific binding properties were usually found to be improved as compared to PK11195, it quickly became apparent that these new TSPO tracers are affected by genetic variability of TSPO binding site induced by the rs6971 single-nucleotide polymorphism (80), resulting in high-, mixed

TABLE 1 | *In vivo* imaging studies in patients with Alzheimer's disease or Mild Cognitive Impairment (MC) with the primary purpose of investigating neuroinflammatory changes through radiolabeled tracers.

Radiotracer	Subjects used	Characteristics	References
18 kDa TRANSLOCATOR PROTEIN (TSPO)			
[^{11}C]PK11195	8 probable AD, "normal" elderly controls	No differences between controls and AD patients	(27)
	8 AD, 1 MCI, 15 HC	Increased tracer uptake in most brain regions	(28)
	13 AD, 14 HC	Increased tracer uptake in cortices, striatum and other regions, but not in hippocampus; positive correlation of tracer binding and cognitive scores, but not with [^{11}C]PIB amyloid load	(29)
	10 AD, 10 HC	Higher average BP in AD subjects in neocortex and cerebellum; vascular binding different between AD and controls	(30)
	6 AD, 6 MCI, 6 HC	No differences between controls and MCI/AD patients	(31)
	22 AD, 14 MCI; 24 HC	Increased tracer binding in the frontal cortex of MCI subjects, coinciding with higher [^{11}C]PIB amyloid load in the frontal cortex; no correlation between tracer binding and amyloid load or cognitive scores; tracer binding in AD patients not compared to MCI and controls	(32)
	11 AD, 4 HC	Increased tracer binding in cortices and cingulate but not in hippocampus; negative correlation of tracer BP and [^{11}C]PIB amyloid load	(33)
	8 AD, 9 MCI, 17 HC	Comparison of different modeling approaches	(34)
	19 AD,10 MCI; 21 HC	No differences between groups in ROI analysis but small significantly increased clusters in voxel-wise comparison; no correlation of BP_{ND} and cognitive function	(35)
	8 AD, 15 HC	Increases in tracer binding in AD patients in several cortical regions, hippocampus and striatum with further mean increases at 16 months later; tracer binding coincided with increased [^{11}C]PIB amyloid load and decreased [^{18}F]FDG glucose metabolism	(36)
	9 PD dementia, 8 AD, 8 HC	Negative correlation between hippocampal volume and tracer binding	(37)
	8 MCI, 8 AD, 14 HC	MCI and AD patients showed an increased tracer binding as compared to control but reductions in tracer binding 14 months later	(38)
	42 MCI; 15 HC	85% of MCI patients showed increased tracer uptake in cortical regions; positive correlation of tracer uptake and [^{11}C]PIB amyloid load in cortical regions; [^{11}C]PIB positive MCI patients showed higher tracer binding; correlation of tracer binding with some cognitive scores	(39)
	16 PSP, 9 probable AD, 7 MCI; Part of the NIMROD study	Increased tracer uptake in most neocortical regions and putamen of AD patients; tracer binding correlates with cognitive scores	(40)
[^{123}I] iodo-PK11195 (SPECT)	10 AD, 9 HC	Significant increase in tracer uptake in frontal and right mesotemporal cortices; significant negative correlation between cognitive scores and tracer uptake in several brain regions	(41)
[^{11}C] vinpocetine	6 AD, 12 HC	No difference between AD and controls; Study limited by poor modeling and use of %SUV used as main outcome measure	(42)
[^{11}C] DPA-713	7 AD, 12 healthy elderly and 12 healthy young subjects	Comparison with [^{11}C]PK11195 binding; BP_{ND} levels significantly elevated in AD, but not for [^{11}C](R)PK11195; Correlation of [^{11}C]DPA-713 BP_{ND} levels and cognitive scores, again not observed for [^{11}C]PK11195; no rs6971 genotyping	(43)
[^{18}F] DPA-714	10 AD, 6 HC	No difference between AD and controls; no rs6971 genotyping	(44)
	9 probable-AD, 6 HC	significant differences in frontal and medial temporal lobes between subject groups in BP_{ND}; no correlation between age, cognitive scores and disease duration; no rs6971 genotyping	(45)
	64 AD, 32 HC	rs6971 genotyping; higher tracer uptake in high and mixed affinity binders with AD compared to controls using volumes of interest and voxel-wise comparison, especially at the prodromal stage; tracer binding correlated with cognitive scores	(46)
[^{11}C] DAA1106			
	10 AD and 10 HC	Mean BP increased in AD patients in all measured regions, significant in dorsal/medial prefrontal cortex, anterior cingulate cortex, striatum and cerebellum; no rs6971 genotyping	(47)
	10 MCI, 10 AD and 10 HC	Increased BP diffusely in MCI compared to control but no AD; No difference between AD and aMCI; no rs6971 genotyping	(48)
[^{18}F] FEDAA1106	9 AD, 7 HC	No difference between AD and controls; no rs6971 genotyping;	(49)

(Continued)

TABLE 1 | Continued

Radiotracer	Subjects used	Characteristics	References
[^{11}F] FEPPA	21 probable AD, 21 HC	rs6971 genotyping; adjusted tracer binding significantly higher in AD in various gray and white matter areas; tracer binding was positively correlated with cognitive and sensory impairments	(50)
[^{11}F] FEMPA	10 AD, 7 HC	rs6971 determined; increased V_T in the medial temporal cortex of high- and mixed affinity AD patients; High-affinity binding AD patients showed significantly higher tracer binding most investigated brain regions; correlation with cognitive scores	(51)
[^{11}C] PBR28	10 MCI, 19 AD, 13 HC	rs6971 genotyping; No difference between MCI and healthy controls; AD patients showed widespread increases in cortical but not subcortical tracer binding as compared to MCI and controls, largely independent of genotype; tracer binding was significantly negatively correlated with several cognitive scores, gray matter volume and [^{11}C]PIB amyloid burden	(52)
	9 MCI, 6 AD and 7 HC	Association of higher [^{11}C]PBR28 SUV with poly (ADPribose) polymerase 1 polymorphisms in subjects at risk for AD	(53)
	11 MCI, 25 AD and 21 HC	All patients [^{11}C]PIB positive and rs6971 genotyped; tracer V_T higher temporal and parietal cortex in MCI/AD; cerebellum a suitable pseudo-reference region not significantly different among the three groups in cerebellum	(54)
	14 probable MCI/AD, 8 HC	[^{11}C]PIB positive patients (rs6971 genotyped) had higher tracer binding in cortical regions and hippocampus and showed a 2.5–7.7% annual increase in follow-up scans; tracer binding was correlated with disease progression, measured as worsening of cognitive scores	(55)
	11 PCA, 11 AD, 15 HC	Higher tracer retention of PCA patients in occipital, posterior parietal, and temporal regions, AD patients with increased tracer binding in inferior and medial temporal cortex; tracer binding correlated with cortical volume loss and reduced glucose metabolism	(56)
	21 AD and 15 HC	BP_{ND} can be estimated without arterial input function and shows similar effect sizes in AD patients compared to arterial input function derived data in AD patients	(57)
MONOAMINE OXIDASE B - [^{11}C]DEUTERIUM-L-DEPRENYL			
[^{11}C]deuterium-L-deprenyl	9 AD, 11 HC	Significantly increased tracer retention after blood-flow corrections in frontal, parietal and temporal cortex of AD patients, No difference in sensorimotor, occipital cortex and subcortical regions; significant correlation of tracer binding and [^{11}C]PIB amyloid load	(58)
	8 MCI, 7 AD, 14 HC	Significant increase in tracer binding in bilateral frontal and parietal cortices in MCI and AD patients; no change in subcortical regions; no correlation of the tracer binding with [^{11}C]PIB amyloid load or [^{18}F]FDG glucose metabolism	(59)
	17 MCI, 8 AD	Significant correlation between tracer binding and [^{11}C]PIB amyloid burden in AD; MCI [^{11}C]PIB positive patients show higher tracer binding in parahippocampus	(60)
	17 MCI, 8 AD, 11 HC	Tracer binding found to be independent of brain perfusion and able to discriminate between groups	(61)
CALCIUM INFLUX USING ^{57}CO SPECT			
^{57}Co (SPECT)	6 probable AD, 5 vascular/fronto-temporal dementia	No specific uptake patterns in probable AD patients as compared to other dementia patients	(62)

AD, Alzheimer's disease; HC, Healthy controls; MCI, Mild Cognitive Impairment; PCA, posterior cortical trophy; PD, Parkinson's disease; PSP, progressive supranuclear palsy.

and low-affinity binders. This polymorphism restricts studies with these tracers to high- and mixed-affinity binders. Recently, "third-generation" TSPO tracers, such as GE-180 and ER176 (81, 82) were developed and aimed at allowing TSPO quantification regardless of rs6971 genotype, however with mixed success and no published data in AD patients. Additional considerations to take into account when using these ligands *in vivo* are the different modeling approaches and reference regions (83), along with other methodological issues reviewed by Donat et al. (84).

In order to follow up changes of TSPO over time, longitudinal studies in AD have recently been published. Two studies (36, 38) revealed reductions in [^{11}C]PK11195 binding in MCI patients with different amyloidosis status, whereas increased binding was found in diagnosed AD patients. A moderate increase of [^{11}C]PBR28 uptake in 14 patients with AD was associated with worsening clinical symptoms (55). The most recent longitudinal study with [^{18}F]DPA-714 demonstrated that prodromal and demented AD patients display an initially higher TSPO density as compared to controls. However, when classifying patients into slow and fast decliners according to functional (Clinical Dementia Rating change) or cognitive (Mini-Mental State Examination score decline) outcomes, it was shown that slow decliners show a higher initial [^{18}F]DPA-714 binding than fast decliners, suggesting that higher initial [^{18}F]DPA-714 binding is associated with better clinical prognosis (85).

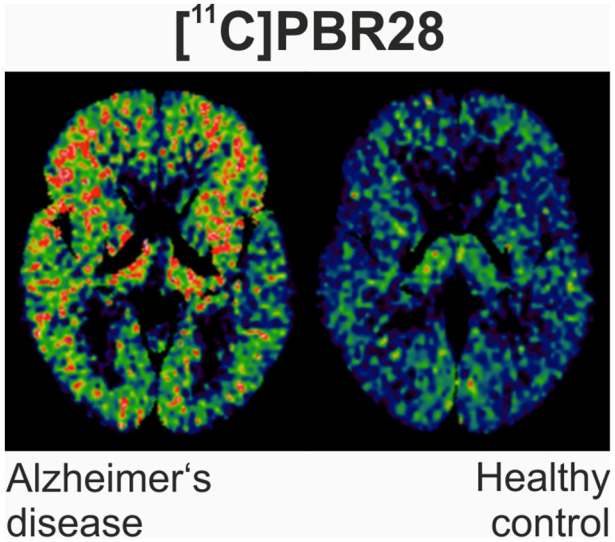

FIGURE 1 | [^{11}C]PBR28 binding is significantly increased in different cortical regions in an Alzheimer's disease subject (MMSE of 22/30) compared to healthy control (MMSE 30/30).

PET Imaging in Animal Models of AD

The first studies carried out in animal models of amyloidosis demonstrated a significant age-dependent increase in the specific binding of [^{3}H]PK11195 in the TASTPM model (APPswxPS1M146V) by autoradiography (8), in agreement with age-dependent increases in CD68 immunoreactivity co-localized with Aβ deposits. However, reports on [^{11}C]PK11195 PET imaging in mouse models of amyloidosis have exposed conflicting results, depending on the model used and age of the animals. A higher [^{11}C]PK11195 uptake was shown in the brains of older APP/PS1 mice when compared with age-matched controls (68, 86). Surprisingly, [^{11}C]PK11195 binding in younger transgenic APP/PS1 mice was not different from their controls, even though immunostaining revealed activated microglia in close proximity of amyloid deposits. Similar to human data, it is likely that different modeling approaches and reference regions may contribute to the seemingly conflicting *in vivo* findings.

Our own recent autoradiographic and PET data provided evidence of an increased binding of [^{3}H]PBR28 in the brain of the aggressive 5XFAD mouse model, compared with wild-type controls, which coincided with the strongly increased immunoreactivity of the microglial marker Iba1 in the same brain areas (87). These results provided support for the suitability of PBR28 as a tool for monitoring of (micro)-glial activation. [^{3}H]PBR28 binding was significantly higher in female animals and positively correlated between Aβ plaque load and tracer binding. In addition, using [^{11}C]PBR28 in healthy rats, *in vitro* brain autoradiography showed a 19% increase of binding in aged (19.6 months) as compared in young rats (4 months) (88).

Besides PBR28, other new generation tracers have exhibited similar patterns in animal models of AD. Increases in TSPO density were reported from 10 months old Thy1-hAPPLond/Swe (APPL/S) mice compared with wild-type controls, using *ex vivo* autoradiography with [^{18}F]PBR06, but this increase was only observed in older mice, at 16 months of age by PET (89). Similar findings were published by Liu et al. (90), who performed [^{18}F]GE180 PET in young and old wild-types (WTs) and APP/PS1dE9 transgenic mice, showing higher uptake in transgenic and WT mice at 24 months of age but not in young 4 months old transgenics (90). In a different study, [^{18}F]GE180 uptake was slightly increased in PS2APP mice at 5 mo and markedly elevated at 16 mo. Over this age range, there was a highly positive correlation between TSPO PET uptake, amyloid load and likewise with tracers for brain metabolism (91). However, a recent study in APP23 mice showed that the increased rate of (micro)glia activation detected with [^{18}F]GE-180 appears to be of less magnitude than the elevation in amyloidosis detected with [^{11}C]PIB over time. In fact, [^{18}F]GE-180 binding seems to plateau at an earlier stage of pathogenesis, whereas amyloidosis continues to increase. These results suggest that TSPO might be a good marker for early pathogenesis detection, but not for tracking long-term disease progression (92).

These tracers have also served to assess and monitor the efficacy of anti-inflammatory treatments. LM11A-31 is a p75 neurotrophin receptor ligand that was shown to reduce the hyperphosphorylation and misfolding of tau, decrease neurite degeneration, and attenuate microglial activation. LM11A-31-treated APPL/S mice displayed significantly lower [^{18}F]GE-180 binding in cortex and hippocampus of as compared to vehicle-treated animals, corresponding to decreased TSPO and Iba1 staining (93).

As AD is characterized by substantial aggregation of hyperphosphorylated tau, second-generation TSPO ligands have also been employed in transgenic models of tau pathology, such as the PS19 mice. Here, uptake of [^{11}C]AC-5216 was found linearly proportional to the phospho-tau immunolabelling (94).

While TSPO is the most widely recognized biomarker of neuroinflammations, other targets have been explored in recent years. Radiolabeled ketoprofen methyl ester, [^{11}C]-KTP-Me is a highly selective tracer for the cyclooxygenase-1 (COX-1). In APP transgenic mice, [^{11}C]-KTP-Me uptake was significantly increased in the brain of 16 to 24 mo old mice in comparison to their age-matched controls, coinciding with the histopathologic appearance of abundant Aβ plaques and activated microglia. Furthermore, [^{11}C]-KTP-Me accumulation was observed in the frontal cortex and hippocampus, whereby COX-1-expressing activated microglia appeared surrounding Aβ plaques, indicating neuroinflammation that originated with Aβ deposition (95). Another currently investigated alternative to TSPO ligands are tracers for the cannabinoid 2 receptor, such as [^{11}C]Sch225336 and [^{11}C]A-836339 (96–98) and tracers for the purinergic receptors P2Y12 and P2X7 (99).

Magnetic Resonance Spectroscopy (MRS)

Magnetic resonance spectroscopy (MRS) is a new technique that can provide information about several relevant metabolites for neuroinflammation and neurodegeneration. Recently, chemical exchange saturation transfer (CEST), as a novel molecular MR imaging approach, has been developed, which uses proton exchange as a means of enhancing the contrast of

specific molecules in the body (100). Endogenous CEST compounds include hydroxyl (OH), amine (NH2), and amide groups (NH). In the last few years, several studies have explored the possibility of imaging neuroinflammatory and neurodegeneration biomarkers *in vivo* with CEST, such as CEST imaging of myo-inositol (101), glutamate (102) and glucose (103) in AD mouse models.

IN VIVO IMAGING OF ASTROCYTES

PET Imaging of Astrocytes in Humans

The best-known tracer for astrocytes so far is [^{11}C]deuterium-L-deprenyl [[^{11}C]DED], which is an irreversible monoamine oxidase B (MAO-B) inhibitor. This is based on previous findings showing that astrocytes express elevated levels of MAO-B during their activation. The ligand has been therefore employed as biomarker of astrocytosis in pathologies such as AD (59) and amyotrophic lateral sclerosis (ALS) (104). Increased [^{11}C]DED binding throughout the brain was detected in MCI [^{11}C]PIB-positive patients compared with controls and MCI [^{11}C]PIB-negative and AD patients (59). In autosomal dominant AD carriers, astrocytosis measured by [^{11}C]DED was found initially high and then declining, contrasting with the increasing amyloid-β plaque load during disease progression, suggesting astrocyte activation is implicated in the early stages of AD pathology (61).

In the last years, new ligands for the potential imaging of astrocytes have been developed, including those for type-2 imidazoline receptors (I2Rs), which were found to be expressed primarily in astrocytes. These receptors were described for the first time in the 90's and the first studies performing *in vitro* binding with [^{3}H]idazoxan in postmortem cortical membranes showed increased density in AD patients (105). Later on, work carried out with the I2R PET tracer [^{11}C]FTIMD reported specific binding to these receptors in rat and monkey brains, but exhibiting a relative low binding specificity (106, 107). More recently, [^{11}C]BU99008 (2-(4,5-Dihydro-1H-imidazol-2-yl)-1- methyl-1H-indole) was developed as a more potent PET ligand for I2Rs imaging (108, 109), displaying relatively high binding specificity and brain penetration in the porcine and rhesus monkey brain (108, 110). There are ongoing human PET imaging trials in Alzheimer's and healthy control patients at the moment using [^{11}C]BU99008 and the preliminary results have shown good brain delivery, reversible kinetics, heterogeneous distribution specific binding signal consistent with I2BS distribution and good test-retest reliability (111).

Imaging of Astrocytes in Models of AD

PET Imaging for Astrocytes

Imaging studies with [^{11}C]DED carried out in transgenic APP Swedish (APPswe) mice and wild–type animals at different ages, have demonstrated that tracer uptake was significantly higher at 6 months than at 18–24 months in APPswe mice, preceding Aβ deposition (112). However, no differences in [^{3}H]-L-deprenyl obtained by autoradiography were observed between WT and APPswe mice across different ages. Furthermore, staining of the astrocyte marker GFAP was increased in older transgenic APPSwe mice as compared to younger mice (112), raising questions about the specificity of this ligand as marker for astrocytes.

In vivo Bioluminescence Imaging (BPI)

Bioluminescence describes the light produced by the enzymatic reaction of a luciferase with its substrate (luciferin) and the emitted light is detected with a camera. The technique allows for fast acquisition times so that subjects can be imaged quickly, serially over time and with minimal distress. The Prusiner's lab developed *in vivo* bioluminescence imaging and quantitative determination of inflammation in a model of prion related neurodegenerative disease (113). Additionally, bigenic mice overexpressing APP and GFAP-Luc were reported to show an age-dependent increase in signal that was corresponded to major areas of Aβ deposition. Bioluminescence signals began to increase in 7-mo-old Tg(CRND8:Gfap-luc) mice and at 14-mo-old in Tg(APP23:Gfap-luc) mice (114).

Gfap-luciferase reporter mice have also been crossed-bred with hTau40AT/C57BL/6N mice. *In vivo* bioluminescence imaging (BLI) showed activation of astrocytes in response to aggregation of Tau, from 5 months of age compared with wild-type animals (115).

CONCLUSIONS AND FUTURE PERSPECTIVES

The development of new neuroinflammation tracers in the last decade has allowed characterizing the pattern of glia activation in AD patients, showing that it occurs ahead of amyloid deposition, correlates in many cases with amyloid plaque density and allows limited predictions of disease progression. The longitudinal studies have shown that this glial activation, as detected by PET, fluctuates during disease progression. Although reports in animal models of AD have helped confirming the specificity of TSPO tracers for microglia, the situation is not the same for tracers for astrocytes and more research needs to be done regarding this aspect. In addition, new tracers able to differentiate between the potential M1 and M2 microglial phenotypes would be advantageous in identifying their function *in vivo* (116).

Future studies should include imaging in patients after intervention with anti-inflammatory drugs; however so far, there are no reports in that aspect in AD cases. Therefore, imaging studies are key to test the efficacy and the most effective time window for potential anti-inflammatory treatments.

AUTHOR CONTRIBUTIONS

PE and MS designed this review outline, PE performed most of the literature review on TSPO imaging in humans, CD edited and the manuscript, performed the literature review for the table and MS wrote the rest of the manuscript.

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METHODS
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In Vivo Imaging of Microglia With Multiphoton Microscopy

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Neuroimaging has become an unparalleled tool to understand the central nervous system (CNS) anatomy, physiology and neurological diseases. While an altered immune function and microglia hyperactivation are common neuropathological features for many CNS disorders and neurodegenerative diseases, direct assessment of the role of microglial cells remains a challenging task. Non-invasive neuroimaging techniques, including magnetic resonance imaging (MRI), positron emission tomography (PET) and single positron emission computed tomography (SPECT) are widely used for human clinical applications, and a variety of ligands are available to detect neuroinflammation. In animal models, intravital imaging has been largely used, and minimally invasive multiphoton microcopy (MPM) provides high resolution detection of single microglia cells, longitudinally, in living brain. In this study, we review *in vivo* real-time MPM approaches to assess microglia in preclinical studies, including individual cell responses in surveillance, support, protection and restoration of brain tissue integrity, synapse formation, homeostasis, as well as in different pathological situations. We focus on *in vivo* studies that assess the role of microglia in mouse models of Alzheimer's disease (AD), analyzing microglial motility and recruitment, as well as the role of microglia in anti-amyloid-β treatment, as a key therapeutic approach to treat AD. Altogether, MPM provides a high contrast and high spatial resolution approach to follow microglia chronically *in vivo* in complex models, supporting MPM as a powerful tool for deep intravital tissue imaging.

Keywords: multiphoton microscopy, Alzheimer's disease, microglia, amyloid-beta, immunotherapy

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NEUROIMAGING TECHNIQUES: AN OVERVIEW

In the last few decades there have been dramatic advances in clinical neuroimaging. These approaches serve as methods of diagnosis and prognosis, and provide the ability to monitor the natural history of patients and the progress of pharmacological treatments (Garcia-Alloza and Bacskai, 2004; Atri, 2016; Vegting et al., 2016). However, the techniques have also been adapted to follow chronically animal models of disease (Kang and McGavern, 2009) as well as to test new therapeutic alternatives. An ideal neuroimaging technique would provide spatial resolution to allow subcellular morphological and physiological studies, as well as high temporal resolution and sensitivity. Labeled ligands and tracers used should also be characterized to the extent that the metabolism of the compound does not interfere with the sensitivity or specificity of the ligand while being able to cross the blood-brain barrier. Also, the half-life of radioactive compounds must be long enough to allow their quantification and provide a high signal-to-noise ratio.

Among these techniques, positron emission tomography (PET) and single positron emission computed tomography (SPECT) are regularly used to allow three-dimensional, non-invasive imaging of the brain *in vivo*. Both PET and SPECT are very sensitive noninvasive *in vivo* imaging techniques, that allow the detection of neurotransmitters, neuroreceptors or transporters in the picomolar range. The spatial resolution for PET is relatively limited and by working with detector widths that balance spatial resolution and manufacturing limitations, the spatial resolution can reach ∼1.0 mm for pre-clinical PET and ∼3.0 mm for clinical PET (Moses, 2011). More typically, however, the spatial resolution of pre-clinical PET scanners is about 1–2 mm and about 4–6 mm for clinical PET scanners (Khalil et al., 2011; **Table 1**). PET tracers for inflammation have most recently focused on labeling the cannabinoid receptor type 2, cyclooxygenase-2, or reactive oxygen species (Janssen et al., 2018). However, the translocator protein (TSPO) 18 KDa, a mitochondrial molecule that gets upregulated when microglia is activated (Airas et al., 2018), is the most commonly used marker for microglia activation and inflammation in PET studies (Owen et al., 2017).

Preclinical SPECT resolution may also reach <1 mm and about 8–12 mm in the case of clinical SPECT (Khalil et al., 2011). In these cases, molecules labeled with positron emitting radionucleotides, such as [^{15}O], [^{11}C], [^{18}F] have been widely used. As the radioactive isotopes decay, the resulting emission of detected γ-rays are used to image and measure biochemical processes *in vivo*. However, the short half-lives of [^{15}O] or [^{11}C] require the on-site presence of a cyclotron to produce the radioisotopes, limiting their widespread use, particularly in more rural areas. Techniques employing tomographic reconstruction are used to generate three-dimensional images and it is possible to measure metabolic processes, perform receptor-binding studies, and explore brain pathophysiology as well as drug treatment responses.

Spin properties of atomic nuclei with an odd number of protons or neutrons, such as [^{1}H], [^{13}C], or [^{31}P], are used in magnetic resonance imaging (MRI). MRI usually refers to the representation of the spatial distribution of [^{1}H] from water and fat molecules in the sample, allowing the anatomical identification of the areas under study as well as providing information about abnormalities in different pathologies (Dijkhuizen and Nicolay, 2003; Vargas et al., 2018). MRI resolution may reach ∼10 μm in high magnetic field scanners (Jasanoff, 2007), but typical resolutions in clinical scanners are in the range of hundreds of microns (**Table 1**). Despite this advantage in spatial resolution, MRI is much less sensitive than PET or SPECT. The sensitivity requires high concentrations of the imaged molecule, in the high micromolar to low millimolar range, to be detected (Caravan, 2009). The administration of exogenous contrast media allows the measurement of biological processes in some occasions, although the applications are limited.

Classical intravital microscopy (IVM) has long evolved in the last decades and provides microscopic resolution to follow and analyze physiological and pathological processes in live animals (Pittet and Weissleder, 2011). Confocal IVM can follow cellular events up to 200 μm deep, however resolution is compromised at deeper depths due to of out-of-focus emission light and scattering of in-focus emission light (Taqueti and Jaffer, 2013), with general resolution in the tens of microns. This approach has been largely used to assess cellular processes including cell development, migration or death (Pittet and Weissleder, 2011), vascular events and immune system responses, (Aulakh, 2018; De Giovanni and Iannacone, 2018; Russo et al., 2018), including microglia (Bayerl et al., 2016), among others. Recent approaches have allowed the application of IVM to cancer diagnosis and tumor vessel characterization in patients (Gabriel et al., 2018). Also, novel advances make it possible to work with more fluorescent channels, longer imaging times and larger depths in animal models (but still limited to <250 um; for review see Pittet and Weissleder, 2011). While extremely useful in animal studies, it has been the development of multiphoton microscopy (MPM) that has significantly improved the possibility of deep (upto 1 mm), chronic *in vivo* imaging at the subcellular level.

MPM offers very high spatial resolution, in the range of micrometers, and very fast imaging acquisition. Optical imaging precludes the need for radioactive ligands used in PET and SPECT and the large number of fluorescent ligands allows extremely diverse structural and functional readouts. The main disadvantages are that the approach is invasive and the fact that only a limited portion of the brain can be assessed, restricting its use to animal imaging. Nevertheless, this is a very powerful approach for animal studies. MPM has been largely used as a reference technique to explore the central nervous system (CNS) morphology and function in preclinical studies that include neural network activity, synaptic development, brain damage, immune system responses and the role of microglia, progressive pathology or cellular responses in different pathological situations.

PRINCIPLES AND ADVANTAGES OF MULTIPHOTON MICROSCOPY

MPM is based on the probability that two or more low energy photons interact nearly simultaneously with a fluorescent

TABLE 1 | *In vivo* neuroimaging techniques.

Technique	Preclinical spatial resolution	Clinical spatial resolution	Tracers/Labelers
Positron emission tomography (PET)	∼1.0 mm	∼3.0 mm	Radionuclei
Single positron emission computed tomography (SPECT)	<1 mm	8–12 mm	Radionuclei
Magnetic resonance imaging (MRI)	∼10 μm	>100 μm	Magnetic nuclei
Intravital microscopy (IVM)	∼10 μm	N.A.	Fluorophores
Multiphoton microscopy (MPM)	<1 μm	N.A.	Fluorophores

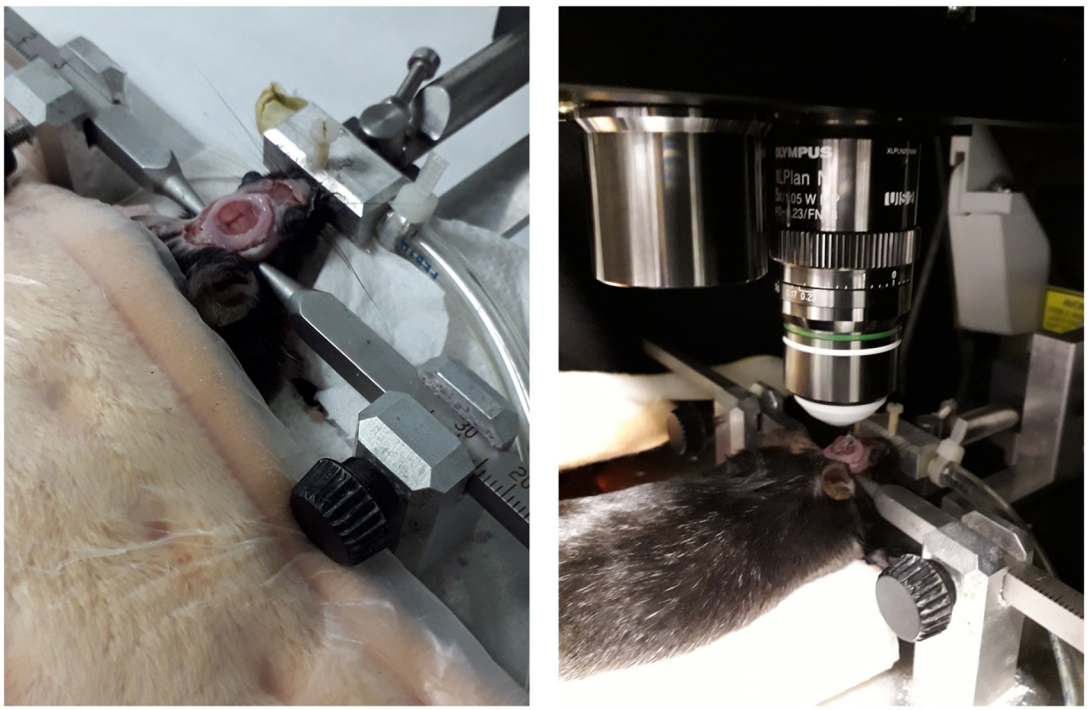

FIGURE 1 | Representative images of cranial window implantation and imaging setup for real time *in vivo* brain imaging with minimally invasive multiphoton microcopy (MPM).

molecule. This induces an electronic transition comparable to the absorption of one photon with double the energy. Then, a single photon is emitted by the excited fluorophore (Denk et al., 1990). By reaching <1 μm spatial resolution, MPM allows cellular and subcellular discrimination without suffering from the slow image acquisition of MRI and PET.

MPM offers advantages over other modes of fluorescence or confocal fluorescence, that have been previously reviewed (Oheim et al., 2006; Svoboda and Yasuda, 2006). Briefly, MPM uses low energy, near infrared light, with wavelengths above 700 nm, reducing phototoxicity and tissue damage, as excitation is limited to the plane of focus. This allows chronic *in vivo* imaging over long periods, without significantly damaging imaged areas. Absorption and scattering are limited when compared to UV or visible light, so excitation penetrates deeper into the sample, and the loss that occurs can be compensated, at least partially, by optimizing signal collection with efficient photomultiplier tubes. Since MPM fluorescence is limited to the point of focus of the objective, out of focus fluorescence is greatly reduced. Common MPM imaging depths reach ∼500 μm and different approaches have been developed to gain even deeper access (∼1 mm). Additionally, gradient index lenses allow the possibility of acquiring images of high quality a few centimeters from the object plane, with modest tissue damage (Levene et al., 2004; Velasco and Levene, 2014; Moretti et al., 2016). However, the surrounding sites are likely to be damaged, making this approach much more invasive. Another possibility to further increase the depth of imaging in highly scattering environments, such as brain tissue, is the use of longer wavelengths. By using a spectral excitation window of 1700 nm, subcortical structures within an intact mouse brain can be reached (Horton et al., 2013). These approaches open the door to image deeper structures of the mouse brain, including the corpus callosum or even the hippocampus. Also, other dense structures such as the kidney, skin or muscle might be imaged (Miller et al., 2017). Additionally, the multiphoton absorption spectrum is much broader than the single photon absorption spectrum. Multiphoton excitation at a single wavelength stimulates multiple fluorophores without requiring multiple lasers or other illumination sources required for multi-color fluorescence imaging. Therefore, simultaneous assessment of different probes is feasible due to the large variety of fluorophores available that allows true multiplexing. In order to fully explore this option, sophisticated fluorescent sensors to monitor oxidative stress (Xie et al., 2013; Wagener et al., 2016; Galvan et al., 2017), oxygen distribution (Gagnon et al., 2016), calcium homeostasis (Kuchibhotla et al., 2014; Eikermann-Haerter et al., 2015; Arbel-Ornath et al., 2017; Bai et al., 2017), chloride concentration assayed by Clomeleon imaging (Dzhala et al., 2010) or potassium sensors (Sui et al., 2015) have been developed to observe cellular and subcellular structures and activity *in vivo*. Moreover, cells also produce autofluorophores, such as porphyrins, NAD(P)H, flavin, lipofuscin, melanin or collagen, allowing the imaging of cellular and metabolic processes using intrinsic signals. Therefore, MPM has revealed unanticipated new principles and

mechanisms after imaging and reimaging at both acute and chronic time points (Akassoglou et al., 2017). Additionally, the fact that MPM provides high contrast and high spatial resolution suggests that it has promise as a tool for specialized intravital deep tissue imaging in certain clinical studies (Wang et al., 2010).

TECHNICAL APPROACHES FOR BRAIN *IN VIVO* MULTIPHOTON IMAGING

MPM has been used to study many biomedical problems, including applications in urology (Katz et al., 2014), tumor assessment (Muensterer et al., 2017), infectious diseases (Belperron et al., 2018) and cardiovascular research (Wu et al., 2017), among others. However, MPM has been most widely used in neuroscience studies for pathophysiological assessment and follow-up of the brain. Insights of the CNS provided by MPM were not previously possible with classical histological endpoint studies. Minimally invasive MPM provides high spatial resolution for imaging glial cells, vascular structures and single neurons, as well as spines and subcellular components in the intact brain. Moreover, MPM allows functional measures of brain physiological processes, as well as dynamic responses to injury and disease in real time (Akassoglou et al., 2017).

A craniotomy is required to directly access the brain. The so-called "open skull" technique requires the permanent removal of a circular portion of skull, with or without altering the dura, and the placement of a cover glass (Arbel-Ornath et al., 2017; Askoxylakis et al., 2017; **Figure 1**). While this is the most common approach, it is also possible to image the brain through a "thin skull" preparation (Marker et al., 2010). In this case, the skull of living animals is thinned down to ∼15 μm (Christie et al., 2001; Allegra Mascaro et al., 2010; Isshiki and Okabe, 2014). This approach is slightly more challenging technically, but it does not compromise intracranial pressure, even in the short term. Whereas the open skull window technique is more invasive, it also allows higher flexibility, with larger imaged areas of the brain and longer imaging periods (Isshiki and Okabe, 2014). However, while the resolution of both cranial window preparations is comparable in the superficial layer of the neocortex, it has been reported that at points deeper than 50 μm, the thin skull windows suffer from lower imaging quality (Isshiki and Okabe, 2014), making open skull more appropriate when nanosurgery approaches, such as neuronal ablation or individual blood vessel disruption (Allegra Mascaro et al., 2010; Garcia-Alloza et al., 2011) are required. Also, open cranial windows allow larger fields of view, that can be chronically imaged in the long term, while thin skull preparations require bone thinning at every experimental session. Repeated skull thinning may induce variations in imaging quality between sessions, limiting the number of successful reimaging sessions (Holtmaat et al., 2009).

As an evolving technique, other types of windows have been developed in the last few years to accommodate new experimental necessities, such as cerebellar implantation and imaging (Askoxylakis et al., 2017). Also, transparent, silicone-based polydimethylsiloxane membranes have been used as coverslips. These membranes are not only transparent but they allow access to the brain with microelectrodes (Heo et al., 2016), so electrophysiology studies can be performed simultaneously. Also, removable and replaceable windows have been developed for chronic widefield imaging in awake head-fixed mice (Goldey et al., 2014), making this technique particularly useful for the study of chronic diseases or local pharmacological treatments. Both thin skull and open window preparations have been carefully reviewed before (Allegra Mascaro et al., 2010; Holtmaat et al., 2009; Mostany and Portera-Cailliau, 2008), and new technical approaches have been described to overcome some of the difficulties associated with the techniques (Goldey et al., 2014; Heo et al., 2016).

Cranial Window Implantation

Cranial window surgeries have been performed with slightly different approaches and detailed protocols have been previously described (Mostany and Portera-Cailliau, 2008). Briefly, before the commencement of the procedure, the stereotaxic frame and all surgical surfaces are disinfected. Surgical material including forceps, scissors, drill and glass coverslips are sterilized in a micro bead sterilizer. Small pieces (∼1.5 mm^2) of gel foam are soaked in sterile saline to be used during the surgical procedure. Animals are anesthetized with isoflurane: ∼3% for induction, in an induction chamber, and ∼1% for the surgery while continuously monitoring animal reflexes. Body temperature is maintained with a water recirculating blanket during the entire surgery-imaging procedure, and until the animal is fully awake. To begin the surgical procedure, the sedated animal is placed in a stereotaxic frame and eye ointment is applied to prevent dry eyes. The hair is trimmed in between the eyes from the neck to the eyes and the area is swapped with cotton tips dipped in iodopovidone and ethanol alternatively, for three times, to disinfect the skin surface. Local anesthesia (lidocaine) is subcutaneously injected and anti-inflammatory drugs (corticoids or non-steroid anti-inflammatory drugs) can also be administered before commencing the surgery. With a scalpel and scissors the skin is opened and the muscles and periosteum are carefully removed to guarantee access to the cranium. Initial drilling is performed to mark the cranium area between Bregma and Lambda to be removed (∼6 mm in diameter). The skull is thinned in a circular pattern by careful drilling until it is almost detached. At this moment the area is removed with fine forceps and wet gel foam is gently applied to the brain surface to limit swelling and bleeding. If dura matter needs to be removed, fine forceps can be used to pull it gently from the craniotomy borders and leave it on the midline without damaging the leptomeningeal vessels. The coverslip (8 mm in diameter) is dipped in sterile saline, placed on top of the craniotomy and attached to the cranium with dental cement. Before finishing the procedure, the animal receives subcutaneous opioids (buprenorphine), and acetaminophen is administered in the drinking water for the next three consecutive days. Animals can then be imaged acutely, or allowed recover from the surgery for several days

or weeks. A well-performed surgery leads to windows that allow longitudinal imaging for up to 1 year or even longer periods.

APPROACHES FOR *IN VIVO* MULTIPHOTON MICROGLIA IMAGING IN PHYSIOLOGICAL AND PATHOLOGICAL CONDITIONS

Microglia constitute ∼5%–12% of all cells in the mouse brain, and while their density varies among brain regions, they are prevalent in the gray matter (Brawek and Garaschuk, 2017). At the simplest level, microglia are the immune cells of the brain. They have a distinct developmental origin that differentiates them from other myeloid cells in the CNS (Tvrdik and Kalani, 2017). Microglia play relevant roles in the support, surveillance, protection and restoration of tissue damaged in the CNS (Davalos et al., 2005; Neumann et al., 2009; Schafer et al., 2012). However, microglia have also been implicated in neurogenesis and synapse formation and pruning (Akerblom et al., 2013), giving them a much more complex role in brain homeostasis and function.

Immunolabeling microglia in *ex vivo* or *postmortem* brain tissues provides only a snapshot of complex dynamic processes, while *in vivo* imaging of microglia allows the study of these cells in their environment over time and their implication in physiological and pathological situations of the CNS (Venneti et al., 2009). Multiple and complex techniques have been developed such as confocal microscopy in zebra fish embryos, PET in larger animal models and humans, or multiphoton imaging (Venneti et al., 2009). In the latter approach, the development of fluorescent engineered probes and mice has allowed chronic *in vivo* monitoring not only of synaptic, dendritic and neuronal alterations (Li and Murphy, 2008; Garcia-Alloza et al., 2011; Bai et al., 2017) or brain vascular events (O'Herron et al., 2016; Taylor et al., 2016), but also glial activity (Galea et al., 2015; Füger et al., 2017; Lind et al., 2018; Stobart et al., 2018).

While *in vivo* imaging is technically challenging to visualize and follow microglia, many efforts have been directed towards faithfully monitoring these cells by MPM in real time. Some approaches to visualize microglia have included the use of commercially available plant lectins conjugated with fluorophores, such as conjugated Tomato lectin or Isolectin IB4 (Brawek and Garaschuk, 2017). However, labeled volumes are limited, endothelial cells surrounding blood vessels are also labeled and the quality of labeling depends on the use of the specific plant lectin. Also, a major limitation is that longitudinal imaging is not possible since the requirement for intraparenchymal injection of the fluorophore limits the approach to acute experiments (Brawek and Garaschuk, 2017). To overcome this restriction, the delivery of viral vectors, inducing stable expression of fluorescent proteins in microglia have been used, such as microRNA-9-regulated vectors. Degradation of the transgene messenger RNA in microglia is induced by the incorporation of complementary microRNA-9 target sites into the transgene cassette (Brawek and Garaschuk, 2017; **Table 2**). Since rodent microglia lack miR-9 expression, microRNA-9-regulated expression of GFP in the brain parenchyma can be used to label these cells. However, NeuN colabeling has also shown the existence of neurons, without miR-9 activity, that express GFP (Akerblom et al., 2013), indicating that miR-9 activity is not entirely selective. Also, the fact that the strength of the GFP expression varies depending on the microRNA-9 activity may limit the applicability of this tool in disease models. On the other hand, recombinant adeno-associated viruses (rAAV) have also been tested to express GFP in microglial cells (**Table 2**). Multiple rAAV have led to efficient gene expression from neurons, oligodendrocytes, and astrocytes when injected into the brain, however rAAV-mediated genetic targeting of cells of myeloid lineage, and specifically microglia, remains challenging. Recent approaches with capsid-modified adeno-associated virus six vectors and microglia-specific promoters (scF4/80 and scCD8) have reported selective GFP expression in primary mixed neuroglial cultures and *in vivo* after intracerebroventricular injections in wildtype mice. However, since aberrant activation of microglia may induce pathological conditions, manipulating microglia function could result in disease modification in such intractable diseases (Rosario et al., 2016).

Even though some limitations still apply, microglial expression of the chemokine fractalkine receptor, CX3CR, has allowed the generation of knockin CX3CR1-GFP (Jung et al., 2000) or -EGFP (Nimmerjahn et al., 2005) mice and conditional models (Parkhurst et al., 2013; Yona et al., 2013) as the most widely used approach to visualize microglia *in vivo* and in real time. The microglia are brightly labeled in these mice, allowing *in vivo* visualization of the microglial cell morphology, including very fine processes (Brawek and Garaschuk, 2017), allowing the assessment of microglia morphology and function *in vivo* (Bennett et al., 2016). While extremely useful, it is important to bear in mind that in these mice the CX3CR1 gene is replaced

TABLE 2 | Multiphoton *in vivo* tools for microglia imaging.

Labeling approach	Options for multiphoton *in vivo* imaging
Histochemical labeling	Plant lectin conjugated with fluorophores (tomato lectin, isolectin IB4) (Brawek and Garaschuk, 2017)
Genetic labeling	Knock in CX3CR1-GFP mice (Jung et al., 2000) CX3CR1-EGFP mice (Nimmerjahn et al., 2005) CD11b-CreERT2;R26-tdTomato mice mice (Füger et al., 2017) CD11b-CreERT2;R26-tdTomato; Iba1-eGFP mice (Füger et al., 2017)
Viral labeling	Viral vectors: microRNA-9-regulated vector (Brawek and Garaschuk, 2017) Recombinant adeno-associated viruses (rAAV) (Rosario et al., 2016)
Calcium indicators	Oregon Green BAPTA-1 GCaMP2 (retroviral vector) (Seifert et al., 2011) Twitch-2B (lentiviral vector) (Brawek et al., 2017) GCaMP5G mice (genetically encoded) (Gee et al., 2014).

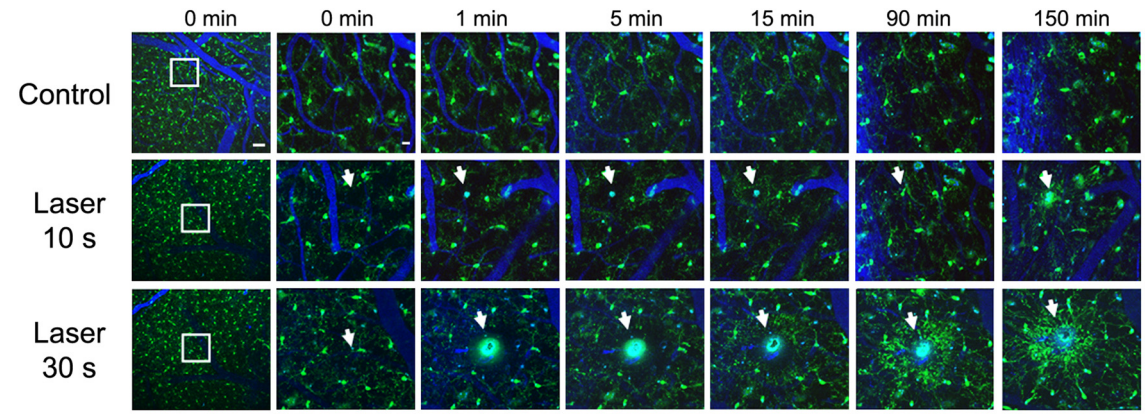

FIGURE 2 | Real time *in vivo* multiphoton imaging of microglia in the cerebral cortex from CX3CR1-GFP mice. Representative images of sequential follow up of a control mouse and two mice after parenchyma laser ablation for 10 and 30 s. Same regions are imaged before the lesion, immediately after, 1, 5, 15, 90 and 150 min afterwards. Microglia processes start going towards the lesion site as soon as 1 min after laser ablation and the processes continue extending towards the lesion 150 min later. Vessels (blue) are filled with Texas Red dextran 70 KD, microglia (GFP, green) and white arrows point to laser ablation sites. Scale bar 50 μm and insets 12 μm.

by GFP, generating a partially functional KO of the receptor. Therefore, these mice can be used to study the receptor function, however, the partial CX3CR1 KO might also induce functional alterations (Brawek and Garaschuk, 2017).

Imaging Microglia With Multiphoton Microscopy in Physiological Conditions

CX3CR1-EGFP mice have allowed the characterization of cortical microglia using MPM (Nimmerjahn et al., 2005; **Table 2**). Microglia are characterized by a ramified morphology in a resting state. *In vivo* multiphoton imaging studies have revealed that even in this resting state, microglia show filopodia-like protrusions that are highly motile and of variable shape (Nimmerjahn et al., 2005). Microglia also radially extend and retract their processes at an average rate of 2.2 ± 0.2 μm/min, to survey and assess their environment, while they continuously interact with other surrounding elements (Nimmerjahn et al., 2005). A recent study described fundamental concepts of microglial function and lifespan directly, by *in vivo* MPM imaging (Füger et al., 2017). Alternative approaches to label individual microglia include triple-transgenic mice that also carried an Iba1-eGFP transgene or double-transgenic CD11b-CreERT2;R26-tdTomato mice (**Table 2**). Microglia were followed chronically for 15.5 months in young (3 months old) and old (10 months old) mice. Median microglial lifetime span was estimated to be 29 months in young mice and 22 months is older animals, matching approximately the mean age difference of the two groups of mice imaged. These data support that microglial proliferation in the mouse neocortex appears to be a rather deactivated process and that about half of the microglia persist until the end of the 26–28-month mean lifespan of C57BL/6 mice. This extreme longevity may explain how stimulation of microglia early in life might be crucial for long-term changes in human brain function and the risk of neurodegenerative diseases (Füger et al., 2017). Further assessment of microglial proliferation in young animals revealed that the number of cells that were lost or appeared was similar over the 6-month period when mice were imaged biweekly. Since new tdTomato-expressing cells were reported to appear in the proximity of an existing tdTomato-expressing microglial cell after an increase in cell body volume, Füger et al. (2017) speculate that these are the mother cells resulting in the newly generated microglia. Newly generated microglia cells then move away and extend their ramified processes interconnecting with the existing microglial networks (Füger et al., 2017). On the other hand, previous *in vivo* multiphoton studies have also shown that a small population of circulating CX3CR1-GFP cells may infiltrate the brain parenchyma through a compromised blood-brain barrier, induced by ischemic stroke. It has been reported that these cells do not proliferate and are morphologically distinguishable from resident microglia, suggesting that infiltration and proliferation are two independent events, with different kinetics (Li et al., 2013). On the other hand, chronic multiphoton *in vivo* studies in CX3CR1-GFP have reported that microglia are fast and continuously remodeling. Askew et al. (2017) show that the number of microglia is maintained until aging due to temporal and spatial coupling of proliferation and apoptosis, due to the fact that many more cells proliferate in the close proximity of a dying cell.

Imaging Microglia With Multiphoton Microscopy in Pathological Conditions

While the concept of resting and activated microglia might be ambiguous, since different phenotypic and morphological stages of microglia are represented *in vivo* (Madore et al., 2013), previous studies have analyzed the acute effect of different lesions on microglial responses. *In vivo* multiphoton imaging studies have revealed that microglia extend their processes towards an injured area, depending on the severity of the lesion, in an ATP-dependent fashion (Davalos et al.,

2005). **Figure 2** shows real time *in vivo* imaging of how parenchymal laser ablation triggers microglia processes towards the lesion area in CX3CR1-GFP mice, and this effect seems to depend on the severity of the lesion. Likewise, laser disruption of blood-brain barrier induces a prompt microglia response, that includes a change from undirected to directed movement of nearby microglial processes toward the injured area (Nimmerjahn et al., 2005). Also, in response to an injury, such as an ischemic stroke, Aβ deposition (Lull and Block, 2010) or probe implantation (Kozai et al., 2012), it has been reported that microglia change into an ameboid morphology and activate several markers such as CD68 and MHCII (Kettenmann et al., 2011; Lartey et al., 2014). Specifically, after electrode implantation, microglial cells adjacent to the probe focus their processes toward the probe while cells distant to the probe maintain ramified morphology. Moreover, microglia cell body displacement toward the probe is also detected (Kozai et al., 2012).

Once microglia are activated, an immune cascade begins, including the production of many cytokines, chemokines and reactive oxygen and nitrogen species as well as phagocytosis of dead cells (Dheen et al., 2007; Jin et al., 2010). The role of microglia in synaptic function has also been analyzed by MPM *in vivo*, and previous studies have shown that the physiological and pathological state of the local brain environment determinates the associated response of microglial processes and synapses (Wake et al., 2009). It appears that microglial processes and neuronal synapses make intimate but transient connections, that are dependant on the functional status of the synapse. In this sense, it has been suggested that microglia may contribute to the increased turnover of synaptic connections observed in injured areas after long-lasting microglial contact, (Wake et al., 2009). On the other hand, recent studies, focusing on the close relationship between microglia and synapses, have suggested that the role of microglia at this level might be more complex than mere phagocytosis. Rapid microglial trogocytosys of presynaptic components is observed, as well as the induction of postsynaptic spine head filopodia, suggesting a facilitator role of microglia in synaptic remodeling and maturation (Weinhard et al., 2018). It has also been reported that selective microglia elimination deregulates neuronal network activity after stroke (Szalay et al., 2016).

Other multiphoton *in vivo* approaches have been directed to study the role of microglia in multiple sclerosis. In an animal model, Davalos et al. (2012) have shown that microglia movement is triggered by the plasma protein fibrinogen before neurological signs of multiple sclerosis commence in the spinal cord, and that microglia clusters around vessels facilitate local axonal damage as the disease progresses. Following these approaches, other *in vivo* studies have focused on exploring therapeutic agents that lower microglial activation and hence neuroinflammation (Bok et al., 2015). Also, real time *in vivo* microglia assessment has been used to further study other CNS diseases such as glioblastoma multiforme models, where activated microglia were found to contact glioma cells shortly after tumor seeding (Resende et al., 2016).

As described above, some studies have assessed microglia structure and motility *in vivo* with real time imaging, and since microglia are non-excitable cells, they depend on changes in the intracellular concentration of calcium to communicate with other cells. However, calcium indicators for microglia have faced some challenges when used *in vivo* (Brawek and Garaschuk, 2017). First approaches have used single-cell electroporation of individual cortical microglial cells with the calcium indicator Oregon Green BAPTA-1 to analyze calcium signals. While the majority of microglial cells presented no spontaneous calcium transients at rest and during strong neuronal activity, they respond with large, generalized calcium transients to an individual damaged neuron (Eichhoff et al., 2011). Alternative approaches have included the use of retroviruses encoding the calcium sensor GCaMP2, in the cortex. However, since retroviruses only infect dividing cells, to stimulate microglial proliferation, the authors used stab wound injury (Seifert et al., 2011). Other studies have included the development of a mouse line that expresses the single-wavelength calcium indicator, GCaMP5G, and the red fluorescent protein tdTomato (Gee et al., 2014). This reporter shows strong expression in different cell types, including microglia and astrocytes (Tvrdik and Kalani, 2017), showing that calcium activity decreases at later stages of inflammation, especially after microglia acquire ameboid morphology in response to LPS challenge. Likewise, calcium responses to laser lesions remain low for at least 1 month after a single LPS administration (Tvrdik and Kalani, 2017). Recently Brawek et al. (2017) have also reported the use of lentiviral vectors driving expression of the calcium reporter Twitch-2B in microglia as a feasible approach to analyze calcium signaling with some selectivity in cortical microglial cells. All of these promising tools will help to evaluate microglial immunomodulation in physiological and pathological brain conditions.

IN VIVO IMAGING OF MICROGLIA IN ALZHEIMER'S DISEASE MODELS

Senile plaques, neurofibrillary tangles and synaptic and neuronal loss are the classical neuropathological features of Alzheimer's disease (AD; Serrano-Pozo et al., 2011). Also, microglia play a significant part in CNS diseases and specifically in AD. Microglia have a dual role in AD and it has been described that they can be protective and promote phagocytosis, degradation and ultimately clearance of amyloid-β (Aβ) with disease progression. On the other hand, microglia become dysfunctional, their ability to clear Aβ is affected and they release neurotoxins and produce pro-inflammatory cytokines that contribute to Aβ production and deposition (Hickman and El Khoury, 2014). Altogether, it seems that the microglia role in clearing Aβ is compromised while their inflammatory activity in AD is increased (Sarlus and Heneka, 2017). Microglia clusters around senile plaques are observed in AD patients and animal models, and it has been suggested that the brain can compensate for Aβ toxic effects of up to a limited level (Baron et al.,

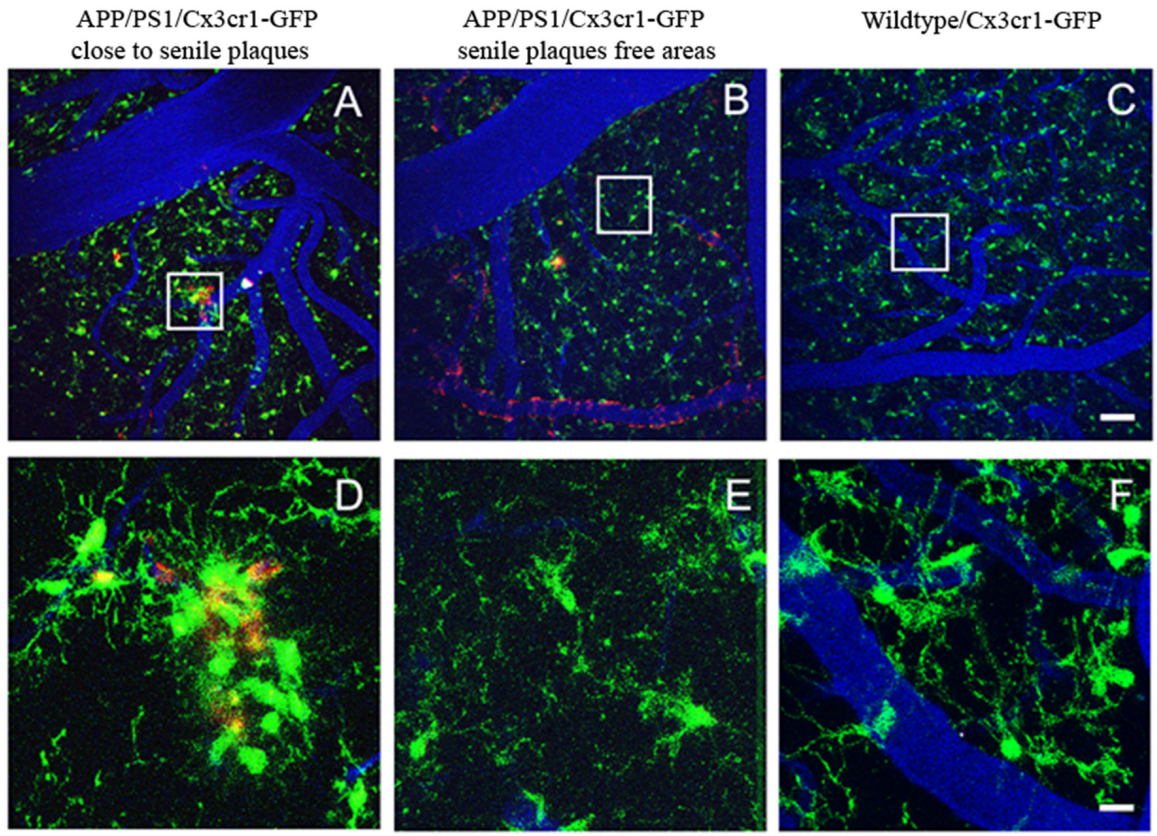

FIGURE 3 | Real time *in vivo* multiphoton imaging of microglia in the cerebral cortex from APP/PS1×CX3CR1-GFP mice. Representative images of microglia clusters around senile plaques **(A,D)**, in areas free from senile plaques **(B,E)**, and in a wildtype mouse **(C,F)**. Vessels (blue) are filled with Texas Red dextran 70 KD, senile plaques are labeled with methoxy-XO4 (red), and microglia express GFP (green). The scale bar is 50 μm and for the insets 10 μm.

2007) in which microglia may play a relevant role. On the other hand, microglia impairment seems to increase amyloid burden (El Khoury et al., 2007) and alter plaque structure with significantly greater neuritic damage (Wang et al., 2016). Also, the positive role of microglial activation in removing Aβ can be compromised by the concomitant effect of an increased secretion of proinflammatory compounds that might be toxic for nearby neurons.

In the last few years, a rare functional variant (R47H) in triggering receptor expressed on myeloid cells (TREM) two gene, encoding TREM2 protein, has been reported to increase susceptibility to late-onset AD through impaired containment of the inflammatory processes (Guerreiro et al., 2013; Jiang et al., 2013; Jonsson et al., 2013). TREM2 can regulate the inflammatory response of myeloid cells and their phagocytic ability. It has also been reported that TREM2 expression is positively correlated with amyloid deposition in individuals with AD and upregulated around plaques in AD models. Furthermore, an overall decrease in microgliosis surrounding Aβ plaques is observed in TREM2 haploinsufficient and TREM2 deficient mice (Ulrich and Holtzman, 2016). TREM2 enables microglia to circumscribe and interfere with Aβ plaque structure, limiting neuronal damage and ultimately protecting from AD (Wang et al., 2016). In this context, microglia accumulation is likely neuroprotective, helping to contain the spread of plaques and shield the rest of the brain from the synaptotoxic Aβ oligomers (Hong and Stevens, 2017). However, in TREM2 deficient mice Aβ plaques are not fully enclosed by microglia and are associated with significantly greater neuritic damage (Wang et al., 2016). Additional efforts are required to define the role of TREM2 in health and disease.

Moreover, microglia have been implicated in synaptic pruning in the developing brain and it still needs to be determined whether normal pruning could be activated and mediate synaptic loss in the AD brain before senile plaque deposition commences. In this sense, microglia engulf synaptic elements, that are internalized into lysosomal compartments in a manner similar to developmental synaptic pruning, when challenged with oligomeric Aβ (Hong et al., 2016). However oligomeric Aβ does not increase synaptic engulfment in microglia lacking complement receptor 3 (CR3), showing that CR3 is necessary for oligomeric Aβ-dependent engulfment of synapses by microglia (Hong et al., 2016). Therefore, the therapeutic objective would be to reduce the neurodegenerative phenotypes of microglia, implicated in secreting pro-inflammatory cytokines, without affecting the

beneficial role of microglia implicated in amyloid clearance. Despite the relevant role that microglia play in AD, only a handful of studies have analyzed these cells using *in vivo* MPM. Most of this work has focused on the role that microglia plays in reducing or exacerbating amyloid pathology (Meyer-Luehmann et al., 2008; Venneti et al., 2009) or the implication of microglial cells in the clearing process after anti-Aβ treatments (Koenigsknecht-Talboo et al., 2008; Garcia-Alloza et al., 2013).

In Vivo Study of Microglia Dynamics in AD Models

While it has been reported that activated microglia accumulate Aβ in lysosomes, preceding neuronal death (Baik et al., 2016) and secondarily contributing to plaque formation, the majority of the *in vivo* multiphoton studies support that microglia recruitment follows plaque formation, and not the other way around (Bolmont et al., 2008; Meyer-Luehmann et al., 2008). Initial studies in AD mice (PDAPP) crossed with CX3CR1-EGFP mice (PDAPPxCX3CR1-EGFP) provided for the first-time the temporal relationship between amyloid deposits, microglial recruitment and the time-course of activation *in vivo* (Meyer-Luehmann et al., 2008). PDAPPxCX3CR1-EGFP mice were imaged before and after plaque formation. The study described in detail how senile plaques deposit in hours, and that microglia subsequently cluster around these newly formed deposits. Microglia were detected at the site of plaque formation within 1–2 days of a new plaque's appearance, and while existing microglia remained stable, new microglia were dynamically recruited to the new plaques. On the other hand, none of the new plaques appeared immediately close to resident microglia, suggesting that microglia do not provide the nidus to form a new plaque. It was also implied that microglia do not successfully clear plaques, unless further activated, suggesting that they restrict senile plaque growth instead, and contribute to the steady state of plaque size after initial formation (Meyer-Luehmann et al., 2008). Other studies have shown similar results, and chronic *in vivo* imaging of microglia clusters did not predict the deposition of new senile plaques (Garcia-Alloza et al., 2013), as can be observed in **Figure 3**. Comparable observations have also been reported in other AD models (APP/PS1×Iba-1-GFP mice) after long-term *in vivo* microglia imaging (Bolmont et al., 2008). In depth analysis of microglia dynamics showed that the number of microglia increases over a month, independent of the volume of senile plaques. While larger plaques were surrounded by larger microglia, the average microglia size appeared to be stable over time.

Although individual microglia surrounding amyloid plaques remained stable for a month, new microglia were also observed. It was also shown that resting microglia first send processes and then migrate to contact a plaque (Bolmont et al., 2008). Likewise, Hefendehl et al. (2011) showed a local increase in the number of microglia cells surrounding amyloid plaques in APP/PS1×Iba1-GFP mice after long-term *in vivo* imaging, for up to 25 weeks. These authors also reported a limited role of the imaging or surgery processes on microglia morphology or number, whereas a relevant effect of pathology-related changes were reported, as previously described (Bolmont et al., 2008).

Surprisingly, it has been shown that laser ablation in 5XFAD/CX3CR1-GFP mice may induce methoxy-X04-positive deposits 2 weeks later in the damaged area, suggesting that microglial activation by laser irradiation promotes plaque formation *in vivo* (Baik et al., 2016). Mixed AD models have also been used to assess neuron-microglia interaction (Fuhrmann et al., 2010). The observed neuronal loss in layer III from APP/PS1/tau/CX3CR1-GFP/YFP-H was rescued by knockout of the chemokine receptor CX3CR1, suggesting that microglia are necessary for neuron elimination. The density of microglia was significantly increased over time around the lost neurons, within a range of 100 μm, and the total turnover rate of fine processes in individual microglial cells was reduced. It appears that microglia are recruited to the neuron with increased migration velocity before neuronal elimination. The authors suggest that this finding might result from the increased number of microglia around disappearing neurons in APP/PS1/tau/CX3CR1-GFP/YFP-H mice, indicating that maintenance of brain tissue is carried out by other cells (Fuhrmann et al., 2010). Since CX3CR1 knockouts did not change the levels of Aβ in the transgenic mouse models of AD, it appears that the phagocytic activity of microglia was not altered or was not involved. Additionally, the *in vivo* follow up of microglia in PDAPPxCX3CR1-GFP, before (3.5 months old) and after (14–17 months old) amyloid deposition demonstrated that young mice extend and retract their processes significantly more than microglial cells in older mice. The microglial cells in the immediate vicinity of amyloid pathology in older mice, as well as in areas distant from pathology, were stable and showed significantly less process movement when compared to young mice (Koenigsknecht-Talboo et al., 2008). These observations are in agreement with previous studies showing that the expression of receptors and enzymes involved in Aβ removal by microglial cells is progressively downregulated in AD mice (Hickman et al., 2008), supporting the idea that microglia at later stages of AD become dysfunctional and less efficient at removing and degrading Aβ (Hickman et al., 2008; Wake et al., 2009).

Recently, Füger et al. (2017) have also followed the natural history of microglia in an AD mouse model (CD11b-CreERT2;R26-tdTomato;APPPS1 mice). These authors reported a higher microglia cell loss in AD mice (∼20%) when compared with wildtype mice (∼13%) at 10 months of age. They also showed that cell division of non-plaque-associated microglia was over three times more common than microglia loss in APP/PS1 animals. Moreover, in the APP/PS1 mice, newly formed microglia were reported to move toward nearby amyloid plaques. Since the rates of plaque-associated microglia disappearance and proliferation were similar, the authors postulated that the increase in the numbers of microglia surrounding plaques, results from the proliferation of microglia in plaque-free areas. However, it cannot be ruled out that plaque-associated tdTomato-positive cells are derived, at least partially, from peripheral myeloid cells. Altogether, it

seems that microglial motility and recruitment, assessed by multiphoton imaging, are affected in different ways, with results depending on specific amyloid depositing transgenic mouse models.

In Vivo Microglia Response to Aβ Immunotherapy

Anti-Aβ immunotherapy triggered an increased interest in the role of microglia as the mediators of neuroinflammation in the AD brain. In this regard, a comprehensive analysis of the underlying mechanisms of antibody-mediated clearance and microglia activation could improve immunotherapy treatments for AD, while avoiding negative inflammatory side effects (Garcia-Alloza et al., 2013). The direct effect of anti-Aβ antibodies on amyloid deposition and clearance has been followed in detail *in vivo* and in real time with MPM (Bacskai et al., 2002; Prada et al., 2007). It has been reported that direct antibody administration increases the microglial response (Krabbe et al., 2013). However, it seems that activating or inhibiting microglia *per se* has a limited role in eliminating senile plaques (Garcia-Alloza et al., 2007). Additionally, *in vivo* studies using Fab2 fragments of the anti-Aβ 3D6 antibody, lacking the microglial activating Fc portion, demonstrated that these are as effective in eliminating Aβ as complete antibodies, indicating that clearance of amyloid deposits *in vivo* may involve a non-Fc-mediated disruption of plaque structure (Bacskai et al., 2002). While acute peripheral administration of 3D6 antibody does not seem to affect microglia dynamics in PDAPPxCX3CR1-GFP mice, antibody treated mice presented significantly more cells, with twice as many processes (Koenigsknecht-Talboo et al., 2008) and this effect was particularly striking around senile plaques. However, the effect seems to be limited to older PDAPP mice (14–17 months of age), while no differences were observed in younger mice (3.5 months old), suggesting that the microglial response is only detected when aggregated Aβ is present, at later stages of the disease.

Other studies have also reported that long-term peripheral administration of anti-Aβ treatment not only reduces senile plaque load, but also restores microglial phagocytic capacity in APP/PS1 mice in the hippocampus, suggesting that restoring microglia activity might provide an attractive therapeutic approach even at advanced stages of AD (Krabbe et al., 2013). It has also been shown that direct administration of anti-Aβ antibodies to APP/PS1/CX3CR1-GFP mice increases microglia size and the number of processes in the close proximity to senile plaques within 1 week. A tendency towards increasing the numbers of cells located in the immediate surround of the senile plaques was also observed (Garcia-Alloza et al., 2013). The fact that senile plaques were not cleared in untreated mice, even though extensive microglia numbers were detected around amyloid deposits, supports the idea that mechanisms, both dependent and independent of microglia, may act in the immunotherapy mediated clearance of Aβ plaque (Garcia-Alloza et al., 2013). Together, it seems that senile plaques are a triggering factor to form microglia clusters and support the idea that while microglia do not seem to successfully clear plaques by themselves, they might be activated by anti-Aβ antibodies and contribute to Aβ clearance. However, the underlying mechanisms remain to be completely elucidated.

POTENTIAL CAVEATS OF *IN VIVO* IMAGING OF MICROGLIA

Chronic real time *in vivo* microglia imaging provides a powerful tool to help untangle the role of microglia in physiological homeostasis and in neurodegenerative diseases, specifically in AD. With this idea in mind, engineered animal models have been developed to follow morphological changes and dynamics of microglia. Even though functional imaging data are highly desirable, these tools are still limited (Tvrdik and Kalani, 2017). On the other hand, taking into account the critical role of microglia in neuroinflammation, the surgical approach for multiphoton *in vivo* imaging may induce microglia activation itself, and this caveat must be acknowledged. However, previous studies have reported a very limited effect of the surgeries in the performance of microglia (Garcia-Alloza et al., 2013; Füger et al., 2017). Additionally, the high spatial resolution and limited penetration of the MPM technique precludes whole brain imaging, even in mice. Apart from that, significant training and expertise to successfully perform the surgeries, with limited trauma, and the ability to image and reimage the brains of mice requires a significant investment.

Whereas microglia can be classically identified by post-mortem histological methods, histological studies only provide a snapshot in time, obscuring potentially important dynamic processes (Koshinaga et al., 2000; Petersen and Dailey, 2004). Non-invasive neuroimaging techniques such as MRI and PET, with direct clinical applications (Donat et al., 2017), have also faced some technical difficulties and limitations, apart from those inherent to the techniques that were previously discussed. *Ex vivo* histological-MRI approaches have reported hypointensities corresponding to iron deposits, largely associated with activated microglia (Ali et al., 2015; Bulk et al., 2018), however, to our knowledge no *in vivo* studies have been reported, supporting the difficulty to directly assess *in vivo* microglia. PET approaches have included radiolabeled ketoprofen, a selective cyclooxygenase-1 inhibitor, associated with Aβ deposits in animal models (Shukuri et al., 2016) although with limited binding affinity and specificity in human studies (Ohnishi et al., 2016). Different efforts have also been directed to label TSPO, which becomes over-expressed upon activation of microglial cells, revealing increased inflammation that overlaps with Aβ deposition in mild cognitive impairment patients (Parbo et al., 2017). However, translocator protein radioligands present some limitations related to affinity and patients need to be classified as high, mixed and low affinity binders (Hamelin et al., 2016; Knezevic and Mizrahi, 2018). Also, PET does not permit the visualization of microglia at the molecular and cellular levels, nor the ability to obtain the precise timing of their dynamic changes (Kozai et al., 2012), making it hard to detect whether the neuroinflammatory progress occurs early on or later during disease, which is a primary aim of the study of microglia *in vivo*.

An additional challenge in intravital optical imaging is compensating for motion artifacts, particularly with small processes of individual cells, and particularly in awake behaving animals. Even under anesthesia, tissue motion may significantly impair imaging acquisition and resolution. The two major sources of physiological movements are the respiratory and the heart cycles (Vinegoni et al., 2014). Also, common anesthetics may interfere with brain hemodynamics and cellular activity. Therefore, new approaches for imaging the brain in unanesthetized, awake mice, with head fixed systems have been steadily improving (Kuchibhotla et al., 2008; Kuhn et al., 2008). Despite these advances, motion artifacts may need to be further addressed afterwards by image processing (Greenberg and Kerr, 2009; Vinegoni et al., 2014).

In summary, *in vivo* multiphoton imaging is a powerful approach to assess the role of microglia in AD. It allows structural and functional imaging of the living brain, with subcellular resolution, over time. Future studies exploiting this technique should be able to clearly delineate the normal and pathophysiological role of neuroinflammation in the brain, increasing our understanding of the cellular and molecular changes during progression of disease.

ETHICS STATEMENT

All studies were conducted with approved protocols from the Massachusetts General Hospital Animal Care and Use Committee, and in compliance with NIH guidelines for the use of experimental animals or approved by the Animal Care and Use Committee of the University of Cadiz, in accordance with the Guidelines for Care and Use of experimental animals (European Commission Directive 2010/63/UE and Spanish Royal Decree53/2013).

AUTHOR CONTRIBUTIONS

CH-B drafted part of the manuscript and reviewed it. BB and MG-A designed, drafted and reviewed the manuscript. All authors contributed to and have approved the final manuscript.

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In Vivo Two Photon Imaging of Astrocytic Structure and Function in Alzheimer's Disease

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The physiological function of the neurovascular unit is critically dependent upon the complex structure and functions of astrocytes for optimal preservation of cerebral homeostasis. While it has been shown that astrocytes exhibit aberrant changes in both structure and function in transgenic murine models of Alzheimer's disease (AD), it is not fully understood how this altered phenotype contributes to the pathogenesis of AD or whether this alteration predicts a therapeutic target in AD. The mechanisms underlying the spatiotemporal relationship between astrocytes, neurons and the vasculature in their orchestrated regulation of local cerebral flow in active brain regions has not been fully elucidated in brain physiology and in AD. As there is an incredible urgency to identify therapeutic targets that are well-tolerated and efficacious in protecting the brain against the pathological impact of AD, here we use the current body of literature to evaluate the hypothesis that pathological changes in astrocytes are central to the pathogenesis of AD. We also examine the current tools available to assess astrocytic calcium signaling in the living murine brain as it has an important role in the complex interaction between astrocytes, neurons and the vasculature. Furthermore, we discuss the altered function of astrocytes in their interaction with neurons in the preservation of glutamate homeostasis and additionally address the role of astrocytes at the vascular interface and their contribution to functional hyperemia within the living murine brain in health and in AD.

Keywords: Alzheimer's disease, astrocytes, two-photon microscopy, *in vivo* imaging, calcium

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INTRODUCTION

Recent global estimates indicate that over 46 million individuals are living with dementia (Prince, 2017) and current clinical therapies are unable to slow progressive neurodegeneration. It is expected that by the year 2050, over 131 million people will have dementia (Prince, 2017). Alzheimer's disease (AD) is the most prevalent form of dementia and aging is the strongest risk factor and driver of disease progression (De Strooper and Karran, 2016). Synaptic dysfunction is an important neuropathological correlate of cognitive impairment in individuals with AD (Terry et al., 1991; DeKosky et al., 1996; Serrano-Pozo et al., 2011; Selkoe and Hardy, 2016) but causative mechanisms remain elusive. An unbiased stereological analyses of a large cohort of human AD brain tissues asserted that the number of *reactive* astrocytes increased linearly with increasing

disease severity and amyloid plaque burden reached a plateau early in the pathogenesis of AD (Serrano-Pozo et al., 2011). *Reactive* astrocytes undergo a complex cascade of morphological alterations that include hypertrophy and the upregulation of cytoskeleton proteins such as glial fibrillary acidic protein (GFAP) and vimentin, which may interfere with the multitude of complex physiological homeostatic functions conducted by astrocytes and contribute to the evolution of AD. Indeed, position emission tomography (PET) imaging studies using the tracer ^{11}C-deuterium-L-deprenyl, directed towards the monoamine oxidase B enzyme, reported that *reactive* astrocytes are present in patients at early stages of AD, particularly those patients with Mild Cognitive Impairment (MCI) (Carter et al., 2012) and that *reactive* astrocytes precede amyloid plaques in a mouse model of AD (Rodriguez-Vieitez et al., 2015).

IN VIVO IMAGING OF ASTROCYTES WITHIN THE INTACT MURINE BRAIN

The normal morphology of astrocytes within the murine brain consists of a central core, occupying approximately 25 percent of the volume of the entire cell, which can range from 3000–35,000 μm^3 (Bindocci et al., 2017). The perivascular endfoot processes occupy only 3.5 percent of cellular volume and contribute to a continuous lining on the abluminal surface of cerebral vessels (McCaslin et al., 2011). Perivascular endfoot processes express a rich complement of membrane molecules that include potassium and water channels that are important for ionic and osmotic regulation (Yang et al., 2011). The remaining cellular volume is occupied by approximately 3–9 processes radiating from the soma, each with an estimated length ranging between 5–64 μm, in addition to a peripheral region densely populated by very fine and optically unresolvable processes (Bindocci et al., 2017). Astrocytes have been studied extensively *in vitro*, thus providing a useful proxy for astrocytes *in vivo* (Cahoy et al., 2008), however astrocytes exist within artificial experimental conditions *in vitro* and may express immune system genes not expressed by astrocytes *in vivo* (Cahoy et al., 2008). Therefore, experiments testing hypotheses pertaining to the structure and function of astrocytes in health and in AD should aim to preserve the intricate interrelations between astrocytes, neurons and the vasculature by examining astrocytic signaling *in vivo*, which permits discrimination between physiological and pharmacological effects (Khakh and McCarthy, 2015).

Two photon excitation laser scanning microscopy is especially well suited to the *in vivo* study of the morphology and function of fluorescently labeled astrocytes, throughout hundreds of microns of intact cortex within rodent brain (Denk et al., 1990; Svoboda and Yasuda, 2006). Two photon microscopy offers a tight laser focus of highly localized excitation in contrast to using either wide-field or confocal microscopy, in which a scattering induced loss of excitation photons can greatly reduce imaging depth (Svoboda and Yasuda, 2006). Furthermore, mice can be trained to become habituated to head-restraint so that they are awake during two-photon imaging, thus circumventing the need for using general anesthesia (Shih et al., 2012a).

The *in vivo* imaging of astrocytes within the living mouse brain by two-photon microscopy requires an aseptic surgical implantation of a chronic cranial window in which a portion of skull bone (0.8–12 mm in diameter; Holtmaat et al., 2009) is replaced with a coverslip, that can be tightly sealed using dental cement. The degree of surgical skill elicited during preparation of the cranial window will largely determine the quality and duration of optical clarity of the window, which has been shown to be suited for repeated imaging for at least 6 months (Hefendehl et al., 2011). Alternatively, a small area of murine skull bone (2 mm in diameter; Shih et al., 2012b) can be thinned using a microsurgical blade to a final thickness of approximately 10–20 μm (Holtmaat et al., 2009; Drew et al., 2010; Shih et al., 2012b). Additional skull polishing, cryanoacrylate adhesive and a coverslip can help stabilize the weakened skull bone and prolong optical access to thinned skull preparations, permitting repeated two-photon microscopic examination of astrocytes within the living murine cortex for up to 3 months, without need for repeated surgery (Shih et al., 2012b). The chronic cranial window and thinned skull preparation are each technically challenging but both procedures, once mastered, can be conducted without inducing astrogliosis (Drew et al., 2010; Hefendehl et al., 2011; Zhang et al., 2015).

IN VIVO IMAGING OF ASTROCYTIC CALCIUM DYNAMICS IN AD MOUSE MODELS

The physiological function of astrocytes is intricately coupled with a multitude of spontaneous oscillations in the concentration of intracellular calcium ions that have considerable spatial and temporal heterogeneity throughout the astrocyte (see **Supplementary Figure S1**; Bazargani and Attwell, 2016; Bindocci et al., 2017). It remains incompletely understood how the spontaneous calcium activity of astrocytes is decoded into local and global neurovascular function in brain health and in AD (Bazargani and Attwell, 2016; Bindocci et al., 2017). Fluorescent calcium indicators such as fura-2, fluo-3, fluo-4 and Oregon Green 488 BAPTA-1 (OGB-1) permit observation of the complex and asynchronous calcium signaling occurring within and between murine astrocytes *in vitro* (Cornell-Bell et al., 1990; Glaum et al., 1990; Jensen and Chiu, 1990; Charles et al., 1991), *in situ* (Dani et al., 1992) and *in vivo* (Hirase et al., 2004; Kuchibhotla et al., 2009). In particular, the acetoxymethyl ester form of OGB-1 (OGB-1AM) can be delivered by pressurized ejection from a micropipette at a depth of approximately 100–200 μm below the murine cortical surface (Stosiek et al., 2003). Concomitant use of the fluorescent dye, sulforhodamine 101 (SR-101) provides a method for discriminating astrocytes from other cells within the living mouse cortex because the multi-cell bolus loading of calcium sensitive dyes results

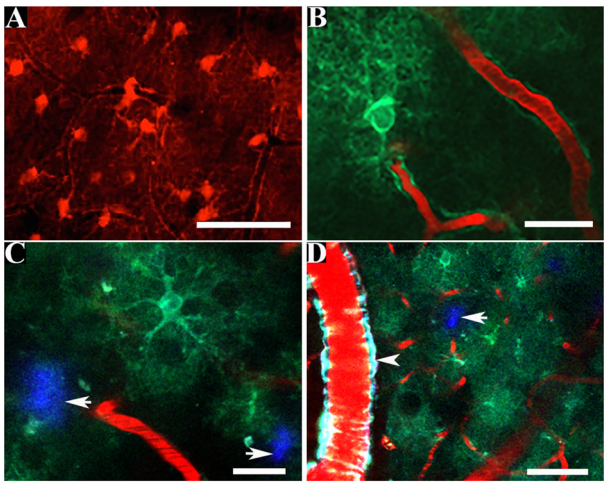

FIGURE 1 | *In vivo* multiphoton imaging of astrocytic structure within the living mouse brain. Astrocytes fluorescently labeled by topical application of sulforhodamine 101 (SR-101) reveal astrocytic somas, proximal processes and perivascular endfoot processes **(A)** whereas the intracortical injection of a virally transduced genetically encoded calcium indicator (GECI) can be used to reveal the finer astrocytic processes *in vivo* **(B)**. GECIs can be transduced by astrocytes within the amyloid plaque containing brain of a murine model of Alzheimer's disease **(C,D)**. Amyloid plaques are fluorescently labeled by methoxy-X04 (white arrows; **C,D**). The presence of a vessel affected by cerebral amyloid angiopathy (CAA) (positive for methoxy-X04) is clearly visible (white arrowhead; **D**). Scale bar = 50 μm **(A,D)**, 20 μm **(B,C)**.

in a universal labeling of all cells (Nimmerjahn et al., 2004). SR-101 can be delivered to astrocytes within the living brain of mice under anesthesia by either carefully perforating the dura, ensuring minimal damage to cortical blood vessels, or by intracortical injection using a micropipette (Nimmerjahn and Helmchen, 2012). The SR-101 dye is actively taken up by astrocytes and diffusional spread occurs through the astrocytic syncytium via astrocytic gap junctions, permitting *in vivo* imaging over several hours (see **Figure 1A**). Experimental protocols requiring chronic *in vivo* imaging of astrocytes over days or weeks can be technically challenging considering the requirement to deliver SR-101 into each mouse brain prior to each imaging session (Nimmerjahn et al., 2004; Nimmerjahn and Helmchen, 2012), which involves the re-opening of the cranial window.

The topical application of SR-101 onto the brain of 5–9 months old APPswe/PS1dE9 (APP/PS1) transgenic AD mice (Jankowsky et al., 2001) with subsequent *in vivo* imaging by two-photon microscopy revealed that astrocytes retain their exclusive and highly organized domains in the presence of amyloid plaques, thus supporting the hypothesis that amyloid plaques do not exert chemotactic effects on astrocytes within the living murine brain (Galea et al., 2015). Additionally, a concomitant multi-cell bolus loading of OGB-1AM with a subsequent intracortical injection of SR-101 following surgical implantation of a cranial window provided optical access to measure calcium activity in astrocytes within the somatosensory cortex of the living brain of APP/PS1 transgenic and age-matched wild-type mice (Kuchibhotla et al., 2009). This study demonstrated that in the living brain of 6–8 months old APP/PS1 transgenic mice, which progressively accumulates amyloid plaques from 4–5 months of age (Jankowsky et al., 2001; Garcia-Alloza et al., 2006), there is a >80% increase in spontaneous intracellular calcium activity within astrocytes when compared to age-matched wild-type mice (Kuchibhotla et al., 2009). Additionally, astrocytes within amyloid plaque containing brain regions in the intact brain of APP/PS1 transgenic mice exhibit spontaneous intercellular propagating calcium waves that occur independently of neuronal activity (Kuchibhotla et al., 2009). *Reactive* astrocytes associated with amyloid plaques exhibit an increased expression of the purinergic P2Y1 receptor (P2Y1R; Delekate et al., 2014) and transgenic mice that overexpress the P2Y1R exhibit spontaneous intercellular propagating calcium waves with increased amplitude that are independent of local neuronal activity (Shigetomi et al., 2018). These findings suggest that astrocytes spatially associated with amyloid plaques may propagate global astrocytic calcium waves that are at least partly mediated by purinergic signaling and occur independently of synaptic transmission.

The finer perisynaptic processes of astrocytes (see **Figure 1B**) are the predominant intracellular sites of initiation for the spontaneous astrocytic calcium waves and are thus important for interrogating the interrelationship between astrocytic calcium signaling and synaptic function in health and in the course of AD (Shigetomi et al., 2018; see **Figure 1C**). A Sholl morphological analyses of astrocytes bulk loaded with the commonly used calcium sensitive dyes reported that the detection of astrocytic calcium activity was limited to the soma and proximal processes to a radial distance of approximately 20 μm, with no detection of the calcium dynamics in the finer processes (Reeves et al., 2011). Genetically encoded calcium indicators (GECIs), such as the Fluorescence Resonance Energy Transfer (FRET)-based Cameleons (Miyawaki et al., 1997; Nagai et al., 2004) and the *single* wavelength GCaMP series (Nakai et al., 2001) offer a uniform, stable and specific fluorescent labeling of astrocytes and their processes to a radial distance of approximately 50 μm, thus resolving the finer perisynaptic processes (Shigetomi et al., 2013).

Cameleon (yellow cameleon; yc2.60 or yc3.60) consist of a calmodulin moiety fused to a M13 calmodulin binding peptide and two fluorescent proteins (cyan-yellow FRET pair) (Miyawaki et al., 1997). The binding of free calcium to calmodulin results in an intramolecular rearrangement which aids the energy transfer between both fluorescent proteins leading to a change in fluorescence ratio (Pérez Koldenkova and Nagai, 2013). These GECIs are known as ratiometric because the ratio between the fluorescence emission intensities of both fluorescent proteins reflects the concentration of free cytosolic calcium (Pérez Koldenkova and Nagai, 2013). The GCaMP series (GCaMP3, GCaMP5, GCaMP6) comprise an intensiometric chimeric construct consisting of a circularly permutated green fluorescent protein that exhibits an altered emission fluorescence intensity upon the binding of free calcium (Nakai et al.,

2001). Thus, they are useful for measuring dynamic changes in intracellular calcium but not for quantitative measures of concentration. The astrocytic-specific expression of GECIs can be achieved by the transduction of an adeno-associated virus of the 2/5 serotype with a promoter such as gfaABC$_1$D (Shigetomi et al., 2013) that can be injected into the brain region of interest of anesthetized mice (Jiang et al., 2014). GECIs can also be targeted to the astrocytic plasma membrane by an additional membrane-tethering domain thus increasing exposure to subcellular calcium dynamics resulting in the detection of a significantly greater number of calcium signals within astrocytic somas and processes when compared to the bulk loading of Fluo-4 AM (Shigetomi et al., 2010, 2013).

IN VIVO IMAGING OF ASTROCYTES IN FUNCTIONAL HYPEREMIA

Physiological brain function is heavily dependent upon a continuous and uninterrupted blood supply that can adapt to meet the metabolic needs of active brain regions (Girouard and Iadecola, 2006). This complex phenomenon, termed functional hyperemia, involves a sophisticated spatiotemporally correlated interplay between neurons, astrocytes and local hemodynamic responses, mediated by smooth muscle cells and endothelial cells (Zonta et al., 2003; Girouard and Iadecola, 2006; MacVicar and Newman, 2015; Tarantini et al., 2017). Impaired functional hyperemia is evident in individuals with AD at mild severity (Janik et al., 2016) and in transgenic AD mice (Niwa et al., 2000; Park et al., 2004). Therefore, deciphering the functional relationship between neurons, astrocytes and the vasculature is critical to further understanding of the pathogenesis of AD. Acute brain slices have provided useful demonstration of evoked synaptic release of glutamate which triggers propagating oscillations of intracellular calcium ions in astrocytic endfeet with temporally correlated release of vasoactive metabolites that elicit hemodynamic responses (Zonta et al., 2003; Mulligan and MacVicar, 2004; Bazargani and Attwell, 2016). However, acute brain slices are devoid of intraluminal flow required for removing vasoactive agents and for preserving myogenic tone, thus the complexities underlying the role of astrocytes in functional hyperemia may best be resolved *in vivo* using two photon microscopy, independently of the use of anesthesia (Zonta et al., 2003; Girouard and Iadecola, 2006; Takano et al., 2006; Bazargani and Attwell, 2016).

Sensory stimulation of the living murine barrel cortex *in vivo* evokes calcium signaling in neurons and astrocytes with instantaneous hemodynamic responses that correlate with elevated calcium activity in astrocytic somas and perivascular endfeet but not within parenchymal astrocytic processes or neuronal somas (Lind et al., 2013). Independent and isolated calcium responses in astrocytic perivascular endfeet, elicited by sensory stimulation in mouse brain *in vivo*, was most closely associated with the vasodilatory hemodynamic responses to neuronal activity (Lind et al., 2018). Additionally, evoked perivascular endfeet calcium responses *in vivo* was similar at penetrating arterioles and capillaries and the temporal dynamics were similar when labeled with either OGB-1AM/SR-101 or the GECI GCaMP6f (Lind et al., 2018). An *in vivo* examination of the spatiotemporal oscillations in calcium signaling in astrocytic perivascular endfeet concomitantly with changes in cerebral vascular diameters and/or cerebral blood flow requires a robust fluorescent labeling of perivascular endfeet that can be difficult to achieve when using the bulk loading of calcium sensitive indicators, such as OGB-1AM (Delekate et al., 2014).

IN VIVO IMAGING OF GLUTAMATE DYNAMICS IN AD

Glutamatergic signaling dysfunction is a significant pathological component within the human AD brain (Jacob et al., 2007) and in transgenic murine models of AD (Masliah et al., 2000; Hefendehl et al., 2016). Optimal physiological glutamatergic signaling is mediated by the interplay between neurons and astrocytes thus protecting post-synaptic glutamate receptors from chronic overstimulation, which can lead to neuronal injury and death. The tightly controlled calcium-dependent release of glutamate from neurons activates post-synaptic N-methyl-D-aspartate (NMDA) and alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) glutamate receptors to increase intracellular calcium and sodium and evoke action potentials (Lin et al., 2012). Astrocytes predominantly clear glutamate via functional excitatory amino-acid transporters (EAATs) and convert glutamate to glutamine for neurons which convert glutamine to glutamate for storage and release from synaptic vesicles (Lin et al., 2012). Significant gene and protein alterations of the NMDA and AMPA glutamate receptors and EAAT1 and EAAT2 glutamate transporters was detected in the hippocampus and frontal cortex of human AD brain tissues (Jacob et al., 2007). Immunohistochemical analyses of human AD brain tissues reported loss of astrocytic EAAT receptor expression in close proximity to amyloid plaques (Jacob et al., 2007) with a trend for decreased immunoreactivity of EAAT2 with increasing Braak stage, which was inversely correlated with increasing GFAP immunoreactivity in astrocytes with disease duration (Simpson et al., 2010).

Spontaneous or sensory evoked local glutamate dynamics within the intact murine brain can be evaluated using two-photon imaging of the genetically encoded intensity-based glutamate sensing fluorescent reporter (iGluSnFR; Marvin et al., 2013; Hefendehl et al., 2016), which has greater temporal resolution than the microdialysis technique (Marvin et al., 2013; Cifuentes Castro et al., 2014). Multiphoton microscopic examination of the iGluSnFR within the intact brain of APP/PS1 transgenic mice (Radde et al., 2006) reported that the plaque microenvironment had aberrant spontaneous fluctuations in the local glutamate concentration, reduced glutamate

clearance following sensory stimulation and loss of the murine glutamate receptor-1 (GLT-1/EAAT2; Hefendehl et al., 2016). The selective deletion of GLT-1 in murine astrocytes resulted in spontaneous seizures and premature death, which was not evident following the selective deletion of GLT-1 in neurons (Petr et al., 2015). The administration of the beta-lactam antibiotic, ceftriaxone to APP/PS1 mice partly restored GLT-1 expression and improved the glutamatergic activity *in vivo* (Hefendehl et al., 2016). This suggests that aberrant astrocytic function contributes to the toxic microenvironment of amyloid plaques in AD and may be amenable to pharmacological intervention.

FUTURE PERSPECTIVES

In 1909, Santiago Ramón y Cajal proposed that the physiological role of glia was incompletely understood because physiologists did not have the necessary tools to examine glial function (Somjen, 1988; Barres et al., 1990). The advent of new tools for the *in vivo* imaging of astrocytes within the intact murine brain may accelerate the generation of new hypotheses for elucidating the roles of astrocytes within normal physiology and in the pathogenesis of AD. The segmental accumulation of amyloid peptide in the walls of cerebral arteries and capillaries is termed cerebral amyloid angiopathy (CAA; see **Figure 1D**) and is associated with intracerebral hemorrhages and degenerative morphological changes to amyloid peptide laden vessels (Biffi and Greenberg, 2011). Post-mortem confirmed AD brain tissues exhibit CAA in >90% of cases (Kalaria and Ballard, 1999; Jellinger, 2002) and astrocytic perivascular endfeet that are spatially associated with vessels moderately affected by CAA exhibit reduced expression of the bidirectional water channel, aquaporin-4 (AQP4), in the medial temporal lobe of human AD brain tissues (Wilcock et al., 2009). Transmission electron microscopic examination of brain sections from the Tg-ArcSwe transgenic mouse model of AD (Lord et al., 2006) showed close spatial association between astrocytic perivascular endfeet and amyloid peptide on cerebral vessels, which disrupts the close interaction between astrocytic endfeet and endothelium (Yang et al., 2011). Correspondingly, immunohistochemical analysis of brain sections from the arcAβ transgenic mouse model of AD (Knobloch et al., 2007) showed the retraction and loss of astrocytic perivascular endfoot processes at amyloid peptide laden vessels and parenchymal amyloid plaques at early stages of pathology (Merlini et al., 2011). The development of new probes to specifically target AQP4 *in vivo* would permit the discrimination of perivascular endfeet from astrocytic processes *in vivo*. Additionally, the combined use of a specific fluorescent probe for AQP4 with the systemic administration of methoxy-X04 (Klunk et al., 2002) may permit a longitudinal examination of the spatial association between the morphology of astrocytic perivascular endfeet and CAA within the intact brain of murine models of AD. The development of fluorescent probes to target astrocytic cytoskeletal proteins such as GFAP and/or vimentin would permit the *reactive* phenotype of astrocytes and their spatial association with amyloid plaques and CAA to be examined longitudinally within murine models of AD and should prove to be powerful. The *reactive* astrocytic phenotype is heterogeneous and the nature of the cerebral insult can determine whether astrocytes adopt a neurotoxic *reactive* phenotype (termed A1) or adopt the A2 reactive subtype, which may promote neuronal health (Zamanian et al., 2012; Liddelow et al., 2017). The development of fluorescent labeling tools capable of discriminating between *reactive* subtypes may provide an excellent opportunity to assess the *reactive* phenotype in astrocytes in AD murine models so that the pharmacological effects of modulating astrocytes may be assessed longitudinally. A previous *in vivo* two-photon microscopy study demonstrated that the hyperactive calcium signaling phenotype exhibited by astrocytes within the cortex of APP/PS1 mice is amenable to pharmacological modulation (Delekate et al., 2014). The frequency of astrocytes exhibiting hyperactive calcium signaling was reduced following topical application of antagonists of purinergic signaling and increased by elevating the concentration of ADP nucleotides within the cortex of anesthetized APP/PS1 mice (Delekate et al., 2014). Additionally, an *in vivo* two photon microscopy study that examined the effects of chronic antagonism of the purinergic signaling within the intact brain of APP/PS1 mice reported a reduction in the fraction of hyperactive astrocytes associated with preservation of synaptic structure and function (Reichenbach et al., 2018). These original research findings collectively support the current relevance of using an *in vivo* two-photon imaging-based interrogation of astrocytes in AD which enables the testing of candidate therapies directed towards protecting the integrity of the neurovascular unit against the pathological impact of AD.

AUTHOR CONTRIBUTIONS

PK, EH, SH and BB prepared the manuscript and figures.

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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.2018.00219/full#supplementary-material

FIGURE S1 | *In vivo* multiphoton imaging of the spatiotemporal intracellular astrocytic calcium dynamics within the living brain of a mouse model of Alzheimer's disease (AD). The astrocyte expresses the virally transduced genetically encoded calcium indicator (GECI) YC3.6 delivered by intracortical injection of the AAV followed by surgical implantation of a cranial window. The 10-min time course video demonstrates spontaneous oscillations in calcium throughout an astrocyte with the ratiometric changes in calcium concentration pseudo colored from blues to red.

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