PRIMARY GLIAL AND IMMUNE CELL PATHOLOGY IN NEURODEGENERATIVE DISEASES

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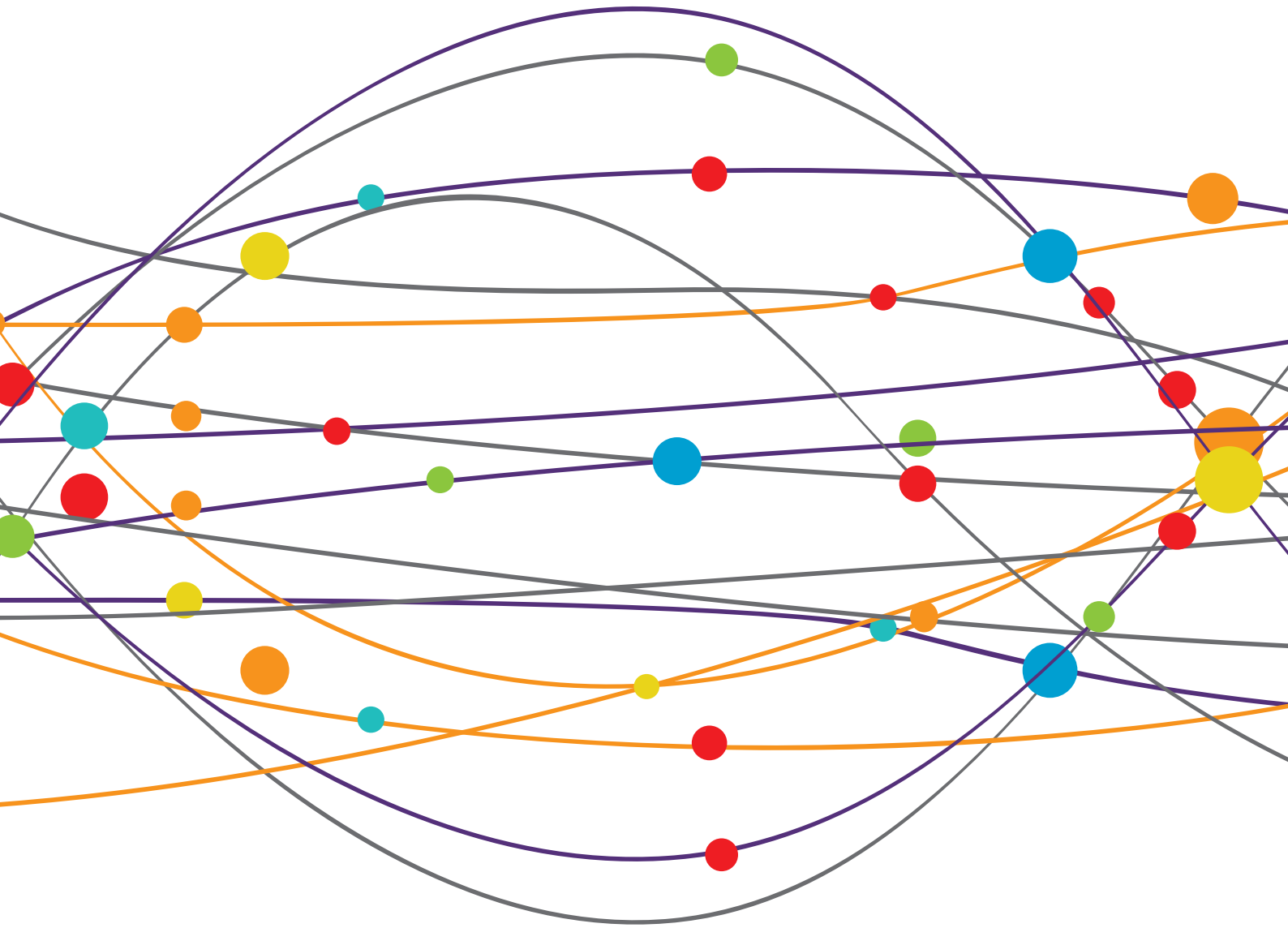

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PRIMARY GLIAL AND IMMUNE CELL PATHOLOGY IN NEURODEGENERATIVE DISEASES

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Table of Contents

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EDITORIAL
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Editorial: Primary Glial and Immune Cell Pathology in Neurodegenerative Diseases

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Editorial on the Research Topic

Primary Glial and Immune Cell Pathology in Neurodegenerative Diseases

Non-neuronal cells in the brain have been proposed as key modulators of neuronal network function in health and in neurological disease. Until recently, the involvement of glial cells—including astrocytes, microglial cells, oligodendrocytes and their progenitors—and infiltrating immune cells in neurodegenerative diseases has been merely viewed as a secondary adaptive response to disease-specific neuronal pathology. It emerges that glial and other non-neuronal cells can also be directly affected by neurodegenerative cues, worsening neuronal dysfunction (1, 2). This has highlighted new potential avenues in targeting disease pathways in a broad spectrum of neurodegenerative conditions, which would require teasing apart primary pathology from secondary processes in multiple cell types.

In this issue we brought together some of the leaders in glial cell biology and neurodegenerative disease research to cover recent advances in pathomechanistic studies informing novel experimental treatment strategies in six review articles.

The Research Topic starts with two articles discussing the emerging role of astrocytes in neurodegeneration. One of the most argued, and therapeutically relevant issues, is the extent to which morphological, gene expression and signaling changes characterize a primary cell-autonomous astrocyte pathology or a secondary adaptive or "reactive" response to damage. There is a consensus in the field to steer away from the less reliable phenotypic classifications and rather assess the function of various astrocytic states in individual disease models to help reveal potential treatment targets (3). What complicates this assessment is the growing evidence that astrocytes already represent a large and heterogenous cell population (4, 5). Monterey et al. review the "many faces" of astrocytes, which can represent both a regional cell diversity and a broad functional spectrum in disease with multiple targetable elements. They discuss recent advances in sequencing technologies, and how they can be used to distinguish pathological cell states and signaling disturbances in Alzheimer's disease (AD). The article by García-Bermúdez et al. emphasizes that glaucoma, a common eye disease that damages the optic nerve and the retina, shares glia-mediated mechanisms with many neurodegenerative diseases, such as AD. The concept of common pathological processes between the eye and the brain is also supported by observations of retinal changes in AD patients, forming the basis of an emerging ophthalmological diagnostic opportunity for neurodegenerative disorders (6). Furthermore, the authors provide a broad overview on retinal glia-ganglion cell interactions as potential therapeutic targets. Altogether,

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these articles highlight the importance of distinguishing homoeostatic and detrimental astrocyte responses for identifying potentially targetable pathological signaling cascades.

Another major advance concerns microglial transcriptomic and inflammatory signaling changes in neurodegenerative pathologies. Similar to astrocytes, microglia may adapt to neuronal injuries by regaining their homeostatic function but can rapidly escalate the expression of inflammatory mediators as part of the innate immune response. How the adaptive immune response and other risk factors trigger this process in neurodegeneration have been an exciting topic in research over the last decade (7, 8). A minireview by Candlish and Hefendehl highlight the recent significant advances in this field. In particular, they overview mechanisms that govern the transition of microglia into various subtypes during the neurodegenerative process. A particularly interesting angle is the discussion about the risks that lifestyle factors and aging processes impose on cell phenotype changes. Nitsch et al. then provides a detailed overview on the contribution of microglial signaling to AD pathology with a specific focus on interleukin-23 (IL-23). This paper sheds light on mechanisms by which p40, an IL-23 subunit can be released by microglial cells upon exposure to amyloid-beta (Aβ), a central molecule in AD pathogenesis. One of the key messages of this review is that p40 appears to establish a new link between Aβ and neuroinflammation, possibly via Th17 cells, astrocytes and microglia. Although the identity of effector cells and pathways induced by IL-23 require further elucidation, blocking or neutralizing antibodies for IL-23 may provide promises in reducing cerebral amyloid load or soluble Aβ species (9), which may attract therapeutic interests. Since anti-inflammatory or current antibody treatment approaches have yet to improve the clinical outcome in neurodegeneration, for instance in AD patients (10, 11), the articles in this special issue well serve the purpose of highlighting potential target options for more effective strategies.

The final two articles wrap up the recently revealed aspects of interactions between astrocytes, microglia, peripheral immune cells, and their effect on neuronal networks. Pietrowski et al. reviews the growing evidence of purinergic signaling and its breakdown between glial cells and neurons. This is of particular relevance to non-cell autonomous pathomechanisms in neurodegeneration (1, 7), which can worsen neuronal network function, leading to cognitive or motor decline. This paper also brings up the issue of emerging major transcriptional and functional differences between human and mouse astrocytes and microglia (12, 13), including expression of genes that are a pre-requisite for their interactions with neurons. Considering these potential differences is crucial when interpreting results in mouse disease models that do not entirely recapitulate the human pathobiological phenotype (14). Another timely issue related to glial cell communication concerns immune cells, a topic which has emerged onto the central stage of neurodegeneration research. Copas et al. put this into an interesting perspective. They describe how the genetic risk in Parkinson's Disease (PD) may affect glial cells and conspire with peripheral infections during lifetime, predisposing to a chronic neuroinflammatory response. The authors follow us through the ways infiltrating T cells could play a central role, triggered by antigen-presenting microglia. They argue that this may also lead to altered astrocytic inflammatory responses, and consequently contribute to the loss of dopaminergic neurons. The broad overview of the above disease-related pathways illuminates the role of infection and peripheral immune activation as important risk factors in neurodegenerative diseases.

CONCLUSIONS

Overall, the review articles in this issue remind us of the multiple cell-types that are primarily involved in disease, and also focus on those cell populations that are not innate in the brain. The discussions highlight the need for systems biology approaches to distinguish initiating molecular disturbances that can be obscured by secondary homeostatic responses in many cell populations. Recent examples have already shown us how new technologies and platforms, such as single cell or spatial transcriptomics and human stem cell-based or brain organoids could resolve the above problem (15–17). We anticipate that the emerging data demonstrating human-specific differences in pathogenesis will transform translational science and personalized treatment strategies in this decade.

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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Glial Cells in Glaucoma: Friends, Foes, and Potential Therapeutic Targets

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Glaucoma is the second leading cause of blindness worldwide, affecting ∼80 million people by 2020 (1, 2). The condition is characterized by a progressive loss of retinal ganglion cells (RGCs) and their axons accompanied by visual field loss. The underlying pathophysiology of glaucoma remains elusive. Glaucoma is recognized as a multifactorial disease, and lowering intraocular pressure (IOP) is the only treatment that has been shown to slow the progression of the condition. However, a significant number of glaucoma patients continue to go blind despite intraocular pressure-lowering treatment (2). Thus, the need for alternative treatment strategies is indisputable. Accumulating evidence suggests that glial cells play a significant role in supporting RGC function and that glial dysfunction may contribute to optic nerve disease. Here, we review recent advances in understanding the role of glial cells in the pathophysiology of glaucoma. A particular focus is on the dynamic and essential interactions between glial cells and RGCs and potential therapeutic approaches to glaucoma by targeting glial cells.

Keywords: Glaucoma, glia, retinal ganglion cells, Müller glial cells, microglia, astrocytes, oligodendrocytes, retinal glia interactions

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INTRODUCTION

Glaucoma is a group of eye diseases that can cause vision loss and blindness. The number of people with glaucoma is increasing due to the age-related nature of the disease (3). Hence, it is estimated that more than 120 million people worldwide will suffer from glaucoma in 2040 (4). Glaucoma is characterized by progressive degeneration of retinal ganglion cells (RGCs) and is often asymptomatic until its advanced stages when vision loss is irreversible (5). Glaucoma can be classified as either primary or secondary, with secondary glaucoma attributable to known pathologies or medications. Glaucoma may be further classified as either open-angle or angle-closure according to the anatomy of the aqueous outflow pathway (2, 6). In all subtypes of glaucoma, the inner retinal degeneration, especially the gradual loss of RGCs, is a hallmark (7). RGCs are the output neurons of the retina, and their axons transfer visual information from the retina to the brain (8). RGC dysfunction and death lead to vision impairment and ultimately to blindness. To date, no approved treatments for glaucoma directly target RGCs. Instead, the only available treatments are indirectly protective for RGCs by lowering the intraocular pressure (IOP).

It is, therefore, crucial to identify cellular mechanisms for the prevention of RGC degeneration, the repair of dysfunctional cells, and the promotion of axonal regeneration to limit the projected burden of vision impairment and blindness from glaucoma (9, 10). Although the most investigated risk factors for glaucoma progression include IOP, age, genetic background, thinner corneal thickness, and vascular dysregulation (11), other disease mechanisms such as oxidative stress, mitochondrial dysfunction, excitotoxicity, and immunological processes may contribute to the pathophysiology of the disease (2, 12, 13). In this context, accumulating evidence suggests that glial cells in the retina and optic nerve may play important roles in the pathogenesis of RGCs (14–16). However, despite having been studied for more than a century, there are substantial aspects of the interrelationship between glial cells and RGCs that are still to be elucidated (17, 18).

Emerging literature emphasizes the roles of glia in both the maintenance of the retina and in the pathogenesis of glaucoma (15, 19). Although Müller glia, astrocytes, oligodendrocytes, and microglia have different developmental origins, they are now known to share many functions. Although some functions are subserved simultaneously by different glia, others are performed by specific glial subtypes (14, 19–22). Similarly, attention has turned to the complex interactions between retinal glia and neurons and the centrality of these interactions to retinal homeostasis (14, 19, 23, 24). Likewise, it is evident that the glial response to injury stimuli can further perpetuate RGC damage (17, 23, 25, 26). Despite these important advances in our understanding of the interactions between glia and retinal neurons in health and in the context of glaucoma, there is still much to be learned.

GLIAL CELLS OF THE RETINA ARE NOT JUST SUPPORT CELLS

Glial cells are named after the Greek word for glue, as it was thought that their function was simply to bind the neurons in the central nervous system (CNS) together (27). It is now understood that glial cells play a range of diverse and complex functions beyond the provision of structural support to neurons. Two basic types of glial cells are found in the human retina: macroglia and microglia. Retinal macroglia are comprised of Müller glia and astrocytes. Macroglia maintain retinal homeostasis by regulating ion exchange, glucose, and neurotransmitter transport (14). Microglia respond to retinal injury and are important in the maintenance of neuronal networks and the mediation of neuroinflammation (14, 28–30). In the optic nerve, oligodendrocytes, another type of macroglia, and astrocytes provide essential support to RGC axons as they travel to the brain (31). Accumulating evidence suggests that both types of glial cells are interacting with the retinal and optic nerves, and are important contributors to the pathophysiological processes leading to glaucomatous RGC loss (14, 15, 17, 23, 32–34).

A PARTNERSHIP BETWEEN MÜLLER GLIA AND RETINAL GANGLION CELLS

Müller glia are found throughout the retina, with processes extending from the outer limiting membrane to the inner limiting membrane and surrounding the RGCs. Their unique morphology and distribution are related to the role of Müller glia as mediators of the transport of molecules between RGCs and the vitreous humor, retinal vessels, and the subretinal space (15) (**Figure 1**). Müller glia have multiple functions and are symbiotically associated with RGCs.

Glutamate Clearance to Avoid Neurotoxicity

An essential role of Müller glia is their ability to rapidly remove excess glutamate from the extracellular space by amino acid transporters (excitatory amino acid transporters), keeping it at low concentrations to avoid excitotoxicity (18, 24, 35, 36). Glutamate is converted to glutamine *via* the glial-specific enzyme glutamine synthetase. Glutamine subsequently serves as the precursor for glutamate in neurons. In patients with glaucoma and some animal models of the disease, an augmentation of glutamine expression in Müller glia has been shown, indicating an enhanced activation of the glutamate–glutamine cycle (37–39). In addition, it is thought that increased glutamine levels in Müller glia might be due to reduced glutamine requirement in damaged RGCs (37). In addition to removing excess glutamate from the synapse, Müller glia can also use glutamate as a metabolic substrate (16, 18, 26) (**Figure 2**).

Glial Cell Line-Derived Neurotrophic Factor in Glutamate Homeostasis

Another crucial Müller glia feature is the ability to release neurotrophic factors. In this context, studies have shown that ischemia-induced glial cell activation results in the release of glial cell line-derived neurotrophic factor (GDNF), which increases glutamate uptake, thereby potentially facilitating neuroprotection by reducing glutamate-induced excitotoxicity (37, 40). The potential neuroprotective role of GDNF has been supported in a rat ocular hypertension model, where an intravitreal injection of GDNF-containing microspheres was shown to increase RGC survival while reducing glia cell activation (41). The neuroprotective effect of GDNF has furthermore been associated with reduced activation of the L-glutamate receptor, *N*-methyl-D-aspartate receptor (NMDAR) (42), by receptor desensitization and downregulation in both neocortical neurons and astrocytes through activation of mitogen-activated protein kinase (MAPK) (43, 44). However, there are conflicting results regarding the effect of GNDF on glutamate homeostasis, with one study suggesting that GDNF pre-treatment can increase neuronal cell death *via* upregulation of glutamate transporters with a consequent increased excitotoxic concentration of glutamate (45).

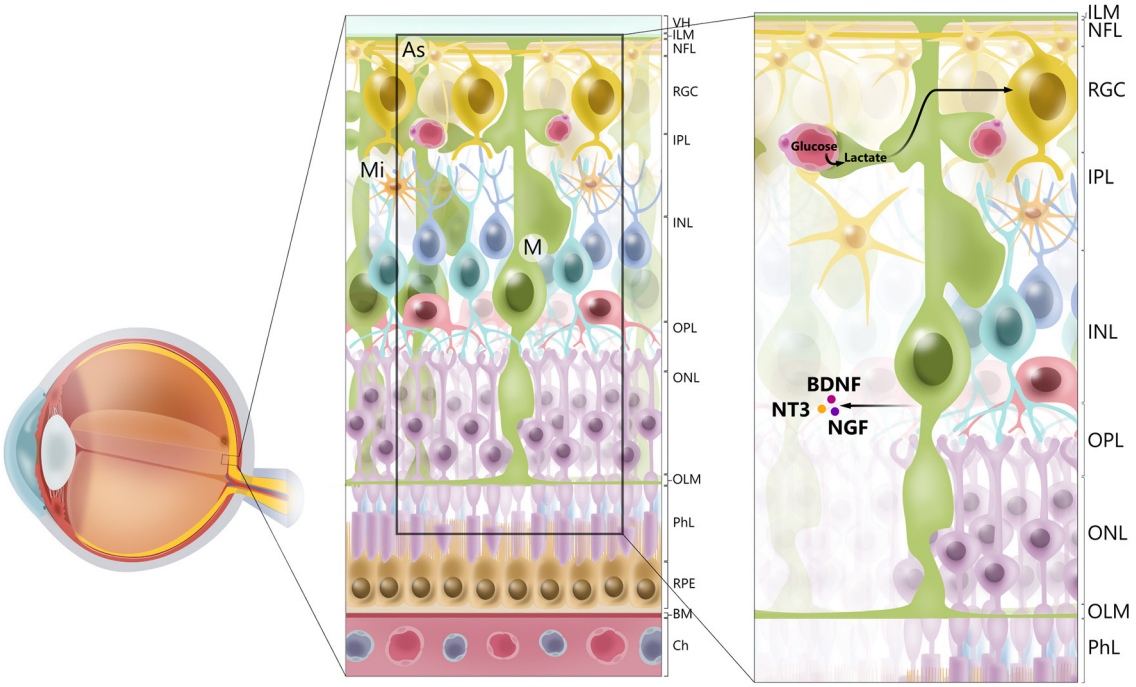

FIGURE 1 | Cellular architecture of mammalian retina. Retina consists of 10 layers. Starting with layer furthest away from vitreous humor (VH), layers of retina are *retinal* pigment epithelium (RPE), photoreceptor layer (PhL), outer limiting membrane (OLM), outer nuclear layer (ONL), outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL), retinal ganglion cell layer (RGC), retinal nerve fiber layer (RGC axons) (NFL), and inner limiting membrane (ILM). Main types of glial cells found in mammalian retina are macroglia [Müller glia (MG) and astrocytes (As), and microglia (Mi)]. Glial cells serve to maintain retinal homeostasis. As illustrated, Müller glia traverses retina, providing structural support to neurons, mediating transport of molecules between retinal vessels and RGCs, and secreting neurotrophic factors, such as BDNF, NT3, and NGF. Furthermore, processes of Müller glia comprise outer and inner limiting membranes of retina. Müller glia, end-feet of astrocytes and vascular endothelial cells, and basal membrane constitute blood–retinal barrier. BM. basal membrane; Ch, choriocapilaris.

N-Methyl-D-Aspartate Receptor Activation Is Crucial in Retinal Homeostasis

In general, safeguards against glutamate excitotoxicity have been proposed to be important treatment targets to prevent retinal neurodegeneration. In particular, NMDAR activation has been extensively studied and found to be essential for retinal homeostasis but, at the same time, to cause neurodegeneration when overactivated (46). To activate NMDARs, D-serine, or glycine along with glutamate are required. D-serine is a physiological coagonist of the NMDA subtype of glutamate receptor (47). The enzyme serine racemase has been shown to catalyze the conversion of L-serine to D-serine in rats and mouse Müller glia and in cortical astrocytes (46, 48). Furthermore, D-serine has been shown to play an important regulatory role in NMDAR response to light-evoked activity in retinal neurons (49). D-serine and serine racemase are mainly localized in Müller glia and retinal astrocytes (48), and in this regard, glia dysregulation of D-serine metabolism has been associated with retinal neurodegeneration, including glaucoma (47, 50).

Glycolysis Is the Energetic Support of Müller Glia

Bioenergetic support is central to retinal homeostasis, as the retina is one of the most metabolically active tissues in the body (51). Although retinal neurons are highly dependent on mitochondrial phosphorylation to produce adenosine triphosphate (ATP), aerobic glycolysis has been shown to be the major provider of ATP in Müller glia (52). It is still unclear why glucose is not completely oxidized under aerobic glycolysis conditions in Müller glia. Some studies have suggested that the absence of the aspartate glutamate carrier (AGC) in Müller glia may explain the predominance of glycolysis in these cells (53, 54). Thus, AGC is a major component of the malate-aspartate shuttle (MAS) that translocates electrons produced during glycolysis to mitochondria to oxidize glucose (54). Despite some studies claiming that oxidative phosphorylation is less likely in Müller glia, we and others have observed that Müller glia switch primarily to mitochondrial respiration under glucose-deprived conditions (26, 55–58), whereas in the presence of sufficient intracellular glucose levels, Müller glia mainly rely on a combination of aerobic and anaerobic glycolysis (59, 60).

Lactate Can Act as a Primary Energy Source

The predominant glycolysis during normal conditions results in the aerobic conversion of glucose into lactate, which is thought to be shuttled to the RGCs. The shuttling of lactate

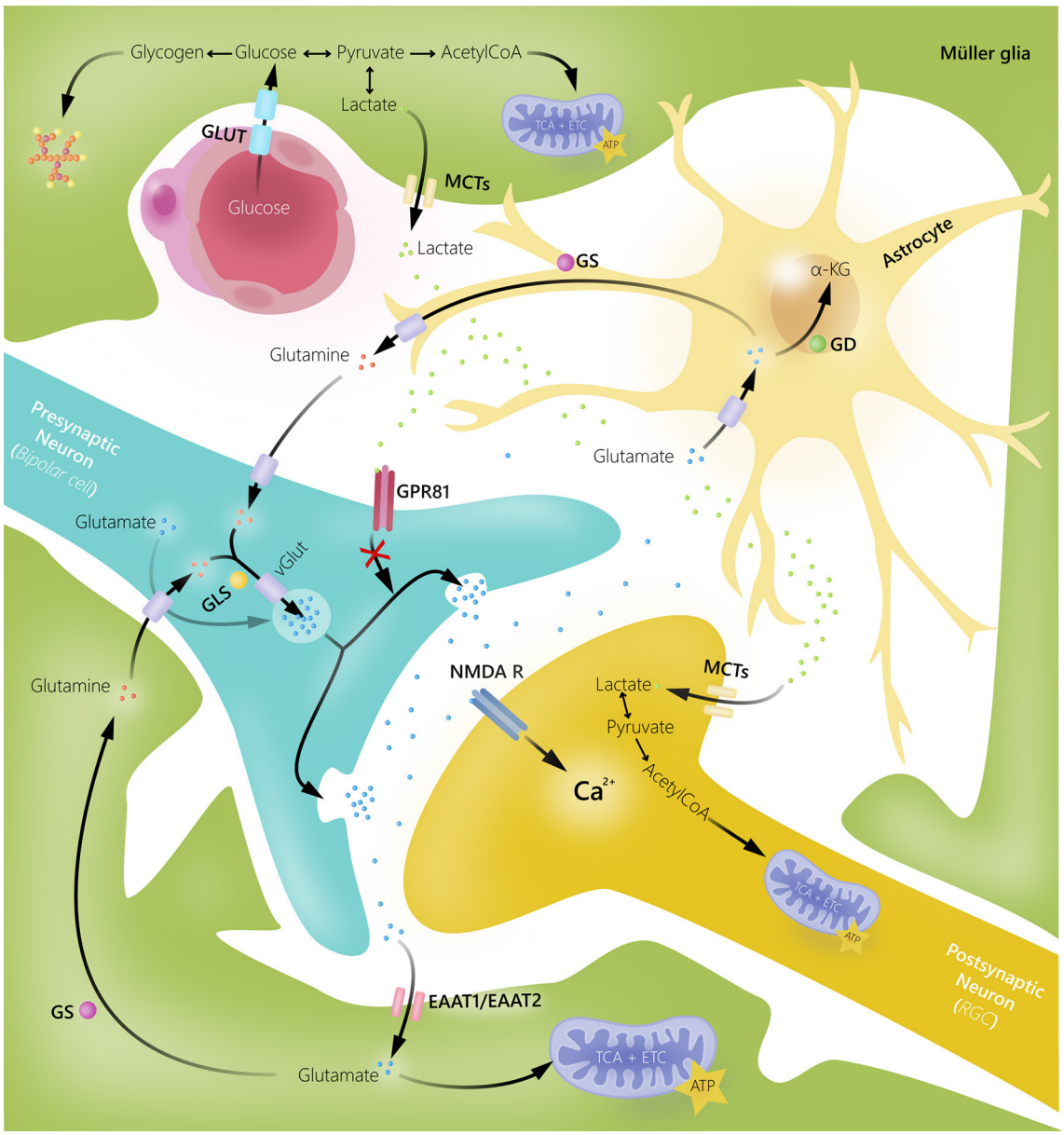

FIGURE 2 | Glial cell and neuronal interactions in human retina. Müller glia and astrocytes take up excess extracellular glutamate to prevent glutamate-induced excitotoxic damage of retinal ganglion cells (RGC). Once glutamate is transported into glial cells, it is converted into glutamine by glutamine synthetase (GS). Glutamine can then be released by glial cells, taken up by neurons, converted into glutamate by glutaminase (GLS), and reused in synaptic neurotransmission. Glutamate can also be converted into α-ketoglutarate by glutamate dehydrogenase (GD) and used as an energy substrate. Müller glia supply bipolar cells and RGCs with energy substrates in form of lactate. Additionally, lactate released by Müller glia may function as a signaling molecule for G-protein coupled receptor 81 (GPR81) to inhibit glutamate release. EAAT, excitatory amino acid transporter; GLUT, glucose transporter; MCTs, monocarboxylate transporters; NMDAR, *N*-methyl-D-aspartate receptor.

from glia to retinal neurons was originally hypothesized by Pellerin et al. (61). Their hypothesis predicted that uptake of glutamate would trigger glycolytic production of lactate, which in turn would be released and taken up by the surrounding neurons to fuel oxidative metabolism (62). It is clear that the lactate shuttle alone cannot explain the complex partnership between Müller glia and RGCs. Although lactate is highly produced in Müller glia, the overall role of lactate is likely to be greater in the retina compared with other tissues. Hence, the content of lactate is much higher in the retina compared with other tissues (16), and studies have reported a preference for lactate as a primary energy source in both Müller glia and RGCs compared with glucose (58, 63, 64).

It is clear that lactate is at the crossroads between glycolytic and oxidative energy metabolism and that more studies are

necessary to understand further the roles of lactate in retinal homeostasis and pathology (65).

Mitochondrial Dysfunction Is Associated With Glaucoma

Nevertheless, lactate metabolism is tightly associated with mitochondrial function, and disturbances in such have been identified as important to numerous retinal and optic nerve diseases, including glaucoma (52, 66, 67). Thus, age-related impairments of mitochondrial function may exacerbate these diseases (67–70). Mitochondrial genetic variations have been associated with primary open-angle glaucoma (POAG) in large genetic studies (71). In particular, genes involved in mitochondrial lipid and carbohydrate metabolism pathways have been implicated in the pathogenesis of POAG and normal-tension glaucoma (72). In line with these findings, metabolomic studies have also identified dysfunctional carbohydrate metabolism in POAG (73). In parallel, Müller glia seems to be vulnerable to the effects of metabolic stress and mitochondrial dysfunction (52, 54, 59, 74, 75). Because many of the essential functions of Müller glia are energy-dependent, mitochondrial dysfunction leaves these cells vulnerable during glucose restriction (52, 59).

Retinal Diseases Cause Müller Gliosis and Nitric Oxide Production

As Müller glia span the entire depth of the neural retina, they are vulnerable to most forms of retinal injury. Accordingly, Müller gliosis, characterized by increased expression of the glial fibrillary acidic protein and activation of extracellular signal-regulated kinases, occurs in a wide range of retinal diseases (23, 76–78). Immediately after injury, Müller gliosis may be neuroprotective due to the production and release of antioxidants and trophic factors, including expression of ciliary body-derived neurotrophic factor (CNTF) (79). In contrast, later-stage gliosis has been associated with cell death and the establishment of a glial scar that inhibits neuronal regeneration (15, 80, 81). A particular pathological event during gliosis is the accumulation of nitric oxide (NO). Thus, NO has been shown to cause intracellular damage by inhibiting mitochondrial function, lowering ATP, and *via* direct damage to DNA. Furthermore, in a rat experimental glaucoma model, NO synthase (NOS) levels were found to be increased at the optic nerve head in response to IOP increase, resulting in increased NO levels (82). Despite the substantial literature on the neurotoxic effects of NO, NO also plays an important role in regulating retinal vascular tone to match neuronal activity (83). Thus, although NO contributes to neurovascular coupling and retinal homeostasis, it may become injurious in excess and in the context of retinal injury.

Overall, there is a growing body of evidence indicating that Müller glia is essential for RGC survival and that Müller glial dysfunction and stress are important factors in the pathogenesis of glaucoma (26, 80).

ASTROCYTES AND THEIR ROLE IN RETINAL GANGLION CELL HOMEOSTASIS AND GLAUCOMA

Retinal astrocytes, also called astroglia, link neurons to blood vessels and are located almost exclusively in the retinal nerve fiber layer (84). They have been found to provide structural and physiological support to optic nerve head axons (85) and modulate remodeling of the extracellular matrix in response to IOP elevation (86, 87). During retinal injury or in response to elevated IOP, astrocytes are activated, followed by morphological changes, such as cell body hypertrophy and loss of thick processes (17, 87). In addition, astrocyte processes have been shown to lose their parallel orientation and distribution once axons are lost (88).

Astrocytes Have Several and Different Functions in the Retina

Retinal astrocytes supply bioenergetic substrates to RGCs *via* the glutamate/glutamine cycle and *via* the transport of lactate and pyruvate (88, 89). It is thought that astrocytes account for more than 70% of the mitochondria in the optic nerve head (90, 91). In this context, astrocytes have been shown to engulf and degrade dysfunctional axonal mitochondria, a process known as transmitophagy (92, 93). Moreover, astrocytes have been shown to remove ions and recycle neurotransmitters from the extracellular space (28). During various pathological events, including elevated IOP or simply during aging, astrocytes undergo gliosis, a process of neurochemical and morphological remodeling (90, 94). Astroglial activation in glaucoma has been shown to increase the expression of many factors, including endothelin-1 (ET-1), tumor necrosis factor-α (TNF-α), oxidative stress molecules, and trophic factors, e.g., CNTF, with varied neuroprotective and harmful properties (17, 25, 32, 95, 96).

Activated Astrocytes Play a Role in Glaucoma

In the course of progressive glaucoma, reactive gliosis and inflammation may potentially promote regeneration and remodeling in the optic nerve (90, 94). Within this era, IOP-induced mechanical stress has been shown to upregulate epidermal growth factor receptor after activation of astrocytes and finally leading to a neurodegenerative response with the upregulation of TNF-α, matrix metalloproteinases (MMPs), and endothelin and nitric oxide synthase-2 (NOS-2) (97–100). Upregulated expression of the phagocytosis-related gene Mac-2 (101) and ET-1 have also been described in experimental glaucoma as well as in the plasma and aqueous humor of glaucoma patients, indicating an association between these molecules and phagocytic degeneration of myelin in the optic nerve head transition zone in glaucoma patients (14, 92).

Astrocytes Are Involved in Retinal Homeostasis

Overall, there is considerable evidence that astrocytes are essential for the maintenance of retinal homeostasis by clearing debris from RGC axons, supporting RGCs with energy substrates, and finally by removing excess neurotransmitters from the synaptic cleft (89, 95, 102). In contrast, activated astrocytes may have detrimental properties such as secretion of neurotoxic molecules and induction of inflammatory responses (14, 17, 95, 103, 104). Despite the lower abundance of astrocytes compared with Müller glia in the retina, the two macroglia subtypes have multiple overlapping functions but also separate properties that, in most cases, remain unknown. Future studies are highly needed to investigate the individual and the similar functions of both astrocytes and Müller glia. Moreover, their potential partnership is also important to understand. Current knowledge of such a partnership is discussed later.

OLIGODENDROCYTE AND THEIR ESSENTIAL SUPPORT OF RETINAL GANGLION CELL AXONS

Oligodendrocytes myelinate axons in the CNS (31), significantly reducing the energy requirements for the propagation of action potential and further protecting against various cytotoxic and excitotoxic factors (105, 106). A single oligodendrocyte can produce numerous myelin segments on multiple axons (107). Myelinating oligodendrocytes express neurotransmitter receptors, ion channels, transporters, and gap junctions (108). RGC axons remain unmyelinated until they reach the retrolaminar portion of the optic nerve. At this point, the axons are ensheathed by and supported by oligodendrocytes (31).

Oligodendrocytes Support Metabolic Transport

Oligodendrocytes are great examples of metabolic coupling between glia, which furnishes essential metabolic substrates to RGC axons (108, 109). Thus, oligodendrocytes have been shown to shuttle lactate to axons, thereby promoting axonal function and survival (107, 110). Both lactate and pyruvate are transported *via* monocarboxylate transporters (MCTs). MCT-1 transports lactate out of the oligodendrocyte membrane, whereas MCT-2 transporters are located on the RGC-axons and transport lactate into the RGC-axon (111). Astrocytes are also involved in lactate transport, as glucose is taken up in astrocyte processes from the blood vessels and metabolized to lactate/pyruvate, which is then transported to the oligodendroctyes via gap junctions and subsequently to the axons (107).

Poor Myelination Is Involved in Neurodegeneration

An experimental link to the potential importance of impaired bioenergetic supply to axons in glaucoma has been provided. Thus, Rindholm et al. performed a study on mutant and transgenic mice with deficient proteolipid protein, a principal component of myelin, and showed that these mice had axonal degeneration, whereas the action potential propagation remained intact. Rindholm et al. further investigated the impact of myelin basic protein deficiency and reported that these mice lacked both compacted myelin and action potential propagation in the absence of axonal degeneration (112–114). Overall, these studies indicated that oligodendrocytes provide axons with support for survival and action potential propagation and that a dysbalance in oligodendrocyte myelination can be detrimental for RGC axon function and survival (112).

Mitochondrial Dysfunction Is Associated With Glaucoma

Mitochondrial dysfunction has also been shown to play crucial roles in the homeostasis of oligodendrocytes. Thus, mitochondria are essential for the development of myelin sheaths and the development of carbon skeletons and lipid metabolism (115). Oxygen starvation and glucose deprivation have furthermore been shown to inhibit myelin development, and added together, multiple stressors have been found to have a detrimental impact on oligodendrocytes and thus associated with retinal neurodegeneration, such as seen in glaucoma (116).

In humans, optic nerve oligodendrocytes from glaucoma patients have been found to have smaller mitochondria compared with age-matched controls (116), supporting findings in the DBA/2J mouse model of glaucoma in which bioenergetic impairment was associated with axonal degeneration (114). Overall, there is some evidence for the involvement of oligodendrocytes in the pathogenesis of axonal dysfunction and loss. However, future studies are needed to investigate further how these glial cells predispose to axonal metabolic compromise and loss in glaucoma (117).

MICROGLIA AND THEIR IMPACT ON RETINAL GANGLION CELL SURVIVAL AND FUNCTION

Microglia are innate immune cells of the CNS. Activation of microglia may be triggered by multiple events (20), such as ATP release from nerve terminals, activated immune cells or damaged cells (118), neurotransmitter accumulation (119), the release of growth factors or cytokines (120), and changes in ion homeostasis (121). Activation of microglia is highly regulated. Hence, beneficial activation of microglia leads to the secretion of anti-inflammatory cytokines, such as interleukin (IL)-10 vs interleukin-10 (IL-10) that inhibits the production of pro-inflammatory cytokines by microglia (122–124). In contrast, marked activation of microglia in the setting of major insults results in the release of pro-inflammatory cytokines and cytotoxic agents, such as TNF-α, IL-1β, IL-6, inducible NOS, and NO, which in turn kill potential pathogens (125, 126) (**Table 1**).

Activated microglia migrate toward sites of injury due to the expression of β-integrin CD11α (126) in a process mediated by the transcription factor nuclear factor-kappa-light-chain-enhancer of activated B cells (2). In addition, microglia support myelination, oligodendrogenesis, and neurogenesis, as well as stimulate synaptic formation and maturation (144).

TABLE 1 | *Effector molecules of activated microglia*.

Growth factors	References
Basic fibroblast growth factor	(127, 128)
Transforming growth factor α	(129, 130)
Transforming growth factor β	(129)
Cytokines	
Interleukin-1α	(129, 131)
Interleukin-1β	(132, 133)
Interleukin-3	(130)
Interleukin-6	(134, 135)
Interleukin-10	(136, 137)
Tumor necrosis factor α	(132, 138)
Complement factors	
C1, C2, C4	(129, 139)
Free radicals	
Superoxide anions	(138, 140)
Nitrix oxide	(141, 142)

Adapted from Minghetti et al. (143).

Activated Microglia and Glaucoma

Substantial evidence has shown microglial involvement in several eye diseases, including glaucoma (34, 145–148). In glaucoma, activated microglia have been described in clusters around blood vessels in the injured optic nerve and choriocapillaris, indicating an innate immune activation (149, 150). Dual roles for activated microglia have been proposed in glaucoma. On the one hand, activated microglia can phagocytose degenerated or dying RGCs, thereby maintaining a retinal environment free of toxic molecules (33), as well as the ability to secrete neurotrophic factors, such as brain-derived neurotrophic factor (BDNF) and CNTF (151), which provide neuroprotection and possibly promote neuroregeneration (95). On the other hand, chronic microglial activation leads to the release of neurotoxic and pro-inflammatory molecules, as mentioned earlier (28). In rodent glaucoma models, where mice were exposed to either hypoxic damage or ocular hypertension, a release of TNF-α and IL-1β from activated microglia accompanied by apoptosis of RGCs was found, supporting the involvement of microglia in glaucomatous neurodegeneration (152, 153). In addition, in an experimental rat model of glaucoma, microglial activation was shown to increase inducible NOS expression, NO production, and RGC injury (154). Activated microglia have furthermore been shown to release reactive oxygen species and prostaglandin E_2, which predispose to RGC apoptosis (155). In glaucoma, microglial cell activation at the optic nerve head has been shown to be associated with altered cellular morphology, protein expression, and antigen-presentation (149, 156). Moreover, damage-associated molecular patterns (DAMPs), released by RGCs or by astroglia in the optic nerve head, which can trigger an inflammatory response, have been shown in response to elevated IOP (32). Finally, microglial activation has been correlated with axonal degeneration in an experimental glaucoma model (33).

Microglia, Friend, or Foe?

As with macroglia, significant research has suggested that microglia can be considered either friend and foe depending on the degree of activation and context (21, 77, 143, 157, 158). One mechanism by which microglial activation is controlled is *via* a series of cell surface receptors (33). Thus, microglia are usually activated only when necessary to minimize safety damage to neighboring cells (159). Consequently, damage to RGCs may occur as observed in glaucoma when microglial homeostasis is disrupted. The expression of several inhibitory receptors decreases with age. Examples of such receptors are CX3CR1 and CD200. Ligand binding to CX3CR1 is crucial for the elimination of damaged or dying cells, whereas ligand binding to CD200 receptors leads to modulation of activated microglia during chronic as well as acute inflammation (138, 159). Other receptors vary with sex and age, such as the purinergic receptors P2 that bind to ATP to mediate intercellular communication (159). In a mice model of glaucoma, it has been demonstrated that a deficient activation of CX3CR1 enhances microglia activation and leads to neurotoxic loss of RGCs (34).

Emerging evidence exists on the impact of autophagy in microglia activation (160). In this context, autophagy modulation is thought to regulate microglia phagocytosis and inflammatory response (160, 161). Autophagy can either be considered as pro-inflammatory or anti-inflammatory depending on the acute or chronic stage of the injury (161). Potentially, the balance between autophagy and microglia can be regulated by pharmacological inhibition of, e.g., 3-methyladenine. Finally, studies indicate that some humans are genetically impaired of basal autophagy, which can impact retinal homeostasis and potentially promote retinal neurodegeneration, hereunder glaucoma (162–164). Future studies are needed to investigate further the partnership between autophagy and microglia as well as to elucidate further the interaction between microglia as well as other glia subtypes and RGCs.

RETINAL GLIAL INTERACTIONS

Emerging evidence has identified the importance of cross talk between retinal glial cells in health and disease (14, 165–167) (**Figure 3**). In general, glia interactions attempt to maintain retinal homeostasis and regulate each other's activity. However, glia interactions can also create imbalance and thus contribute to retinal neurodegeneration.

An example of a glia interaction is the modulation of T cell response due to microglia antigen presentation (165), which in turn regulate the inflammatory cytokine levels, including TNF-α and IL-1β, followed by astrocyte activation (28, 168). Furthermore, microglial migration and immune cell recruitment have been correlated with Müller glial activation (14), indicating tight coordination of retinal immune responses (169). In line with this, activated microglia secrete TNF-α, CCL2 (MCP-1), MCP-3, MIP-1α, MIP-1β, and CCL5 (RANTES), which mediate activation and recruitment of additional microglia, amplifying the inflammatory response (84, 165, 170). In addition, microglia-derived IL-1β has been shown to upregulate the expression of

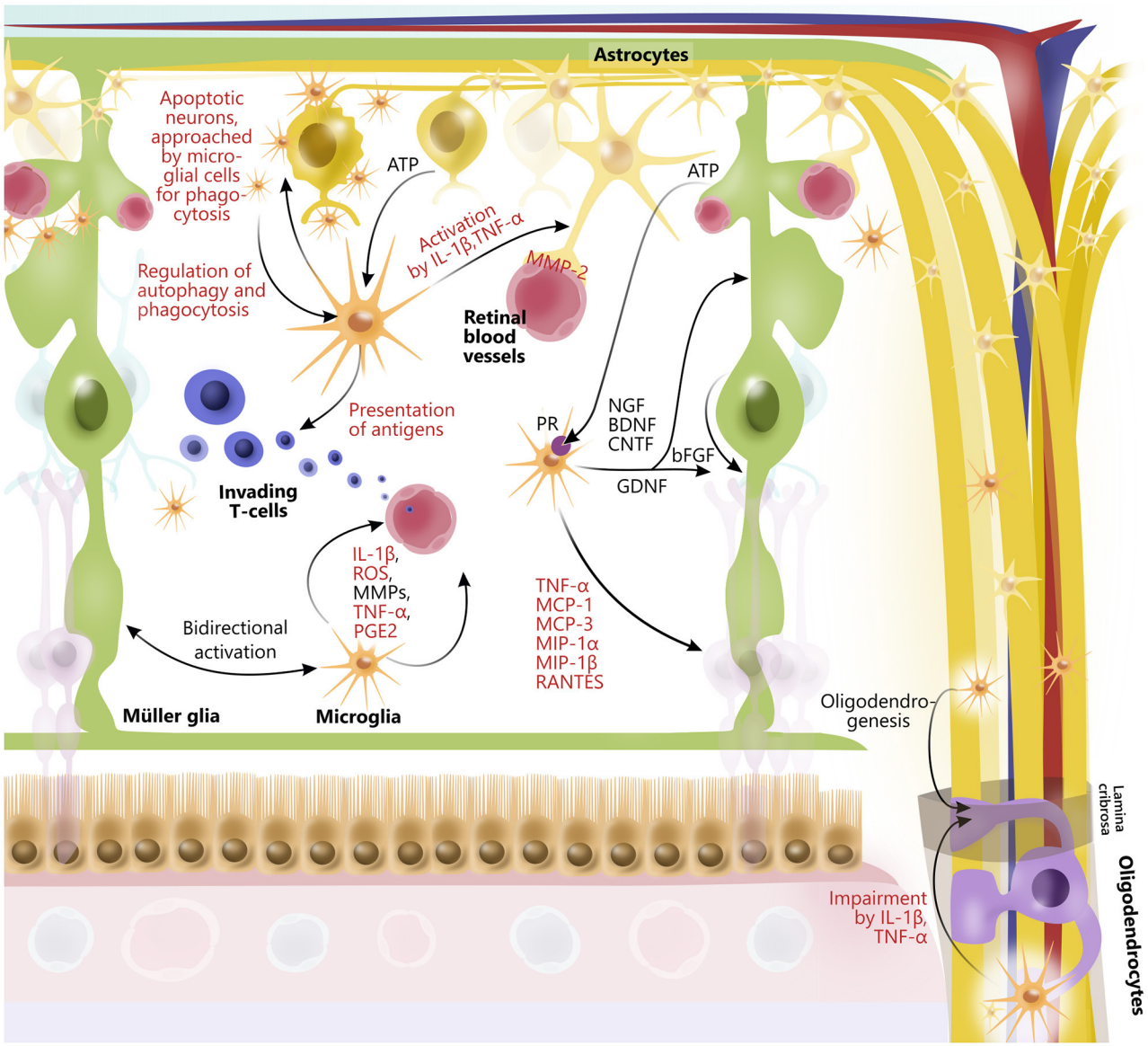

FIGURE 3 | Glial interactions in retina and optic nerve. In retina, astrocytes, Müller glia, oligodendrocytes, and microglia widely interact to maintain retinal homeostasis by release of trophic factors and cytokines, ATP-exchange, phagocytosis of neuronal debris, antigen presentation, and by promoting activity of each other. Furthermore, retinal glia cells are interactively involved in maintaining retinal vessels as well as blood–retinal barrier, for example, through interactions between microglia and astrocytes. Some glial interactions also impair function of other glial cells. In particular, microglia *via* release of cytokines (IL-1β and TNF-alpha) affect function of oligodendrocytes, which myelinate axons of retinal ganglion cells in optic nerve. However, microglia can also contribute to oligodendrogenesis. Although glial interactions attempt to maintain retinal homeostasis, they can also promote retinal neurodegeneration. Prodegenerative factors released from glial cells to interact with one another are highlighted in red.

Ccl2, Cxcl1, and Cxcl10 in Müller glia, which has been associated with retinal neurodegeneration (171). Another example of microglia and Müller glia interaction is the secretion of microglia-derived nerve growth factor (NGF), BDNF, and CNTF from microglia that in themselves protect RGCs (172) but also modulate the production of basic fibroblast growth factor and GDNF in Müller glia, conferring neuroprotection (167, 173). Both astrocytes, microglia, oligodendrocytes, and neurons secrete MMPs (174). Under normal conditions, astrocytes and microglia express MMP-2 (gelatinase A) in the foot processes near blood vessels (175). Upon astrocyte and microglial activation, MMP-2 is, however, increased, thereby causing increased permeability of the blood–retinal barrier (176), angiogenesis, and glial scar formation (177). Activated microglia also express MMP-3 (stromelysin-1), which in turn activates proMMP-9 (178). MMP-9 has been found to be elevated when there is an increase in the blood–retinal barrier permeability. In line with this, an MMP-9 increase has been shown in diabetic rat retinas when glucose

levels rise (176). MMP-9 has also been implicated in myelin basic protein degradation, and it has therefore been suggested that MMP-9 is associated with demyelination and axonal injury (178–180). Finally, both the effects of MMP-9 and MMP-3 have been shown to be enhanced by TNF-α and IL-1, further indicating complex interactions between molecules secreted by different retinal glia (181–183).

Glia–glia interactions have also been shown between oligodendrocytes and microglia as well as astrocytes. In this context, studies have shown that microglial activation can cause astrogliosis and apoptosis of oligodendrocytes *via* TNF-α and IL-1β, causing an increase in MMP production in astrocytes and microglia as well as a possible induction of oxidative stress, inflammation, and glutamate toxicity (183–185).

Although there is currently relatively sparse literature on glia–glia interactions, it can be expected that this interaction will prove to be very relevant for both the understanding of retinal homeostasis and for neurodegenerative diseases such as glaucoma (186).

THERAPEUTIC APPROACHES

Targeting Retinal and Optic Nerve Glial Cells to Treat Glaucoma

Glial cells in the retina and the optic nerve play an important role in supporting RGCs and their axons, and because glial cell dysfunction has been implicated in glaucoma, it is conceivable that future treatments for glaucoma may target glial cells.

Targeting Müller Glia and Astrocytes

Modulation of Glutamate via N-Methyl-D-Aspartate Receptors

RGC damage leads to elevated glutamate levels, causing NMDARs to be overstimulated with a consequent increase in Ca^{2+} influx and excitotoxicity (187). Impaired clearance of glutamate by Müller glia is involved in the pathogenesis of both glaucoma and diabetic retinopathy (37, 188, 189). Amidation and oxidation are the two routes of glutamate disposal. In a cultured rat retinal Müller cell line, treatment with hydrocortisone was shown to increase the amidation of glutamate to glutamine, whereas the addition of branched-chain keto acids was found to enhance oxidation of glutamate, suggesting that intracellular levels of glutamate play a role in the removal of extracellular glutamate (35). In a rodent model of ocular hypertension, the use of memantine nanoparticles, a non-competitive NMDAR antagonist, conferred RGCs neuroprotection (190). Unfortunately, oral memantine did not show any significant prevention of glaucoma progression when tested in a clinical trial (191). Another modulator of glutamate-induced toxicity, brimonidine, has been proposed to slow the rate of glaucoma progression in glaucoma patients in an additive manner other than its IOP-lowering effect (192). In a rodent model, brimonidine was shown to modulate glutamate uptake by glial cells after induction of ocular hypertension, suggesting neuroprotection through modulation of macroglia (193).

Neurotrophin Administration

Neurotrophins are important in the development, differentiation, and survival of RGCs. Many of these neurotrophins are produced by glial cells during normal conditions. In this context, BDNF has been shown to protect RGCs in mice with ocular hypertension (194). Furthermore, it has been shown that GDNF combined with BDNF convey synergic protective effects (195). Finally, glial cells are important producers of neurotrophins, including NGF, BDNF, CNTF, neurotrophin-3, and neurotrophin-4/5, all having potential as neuroprotective properties (196). Among the mentioned neurotrophins, more have already been tested in preclinical settings. An example is mature BDNF, which has been targeted to secretory vesicles within RGCs by adeno-associated virus gene therapy, increasing BDNF production and long-term BDNF receptor expression in a mouse model of optic nerve damage and in a rat model of chronic IOP, which provides neuroprotection against RGCs (197). In addition, increased release of CNTF by Müller glia has been shown to provide endogenous neuroprotection of RGCs after both ischemias and in response to the induced ocular hypertension (194). Recently, an encapsulated cell technology has allowed a controlled, continuous, and prolonged administration of CNTF in animal models that provide photoreceptor protection (198). In general, the administration of exogenous neurotrophins or the augmentation of endogenous production has been shown to have a protective effect on RGCs in several experimental models, highlighting this as a potential therapeutic strategy for glaucoma (199). The efficacy of such treatments may, however, decrease over time as treatment with chronic neurotrophin administration can lead to downregulation of the relevant receptors (197).

Targeting Astrocytes and Astrocyte Activation

Astrocyte activation may be an important factor in the pathogenesis of glaucoma. In this context, inhibition of astrocyte activation has been shown to increase neuronal survival in experimental glaucoma models *via* modulation of a tyrosine kinase inhibitor of epidermal growth factor receptor (200) or blocking of endothelin-1 (201). Similarly, the neuroprotective effects of calcium channel blockers and endothelin blockers in humans with glaucoma are thought to act *via* this mechanism (202).

In addition to the previously mentioned involvement of TNF-α in microglia activation, TNF-α has also been shown to mediate both astrocyte, Müller glia, and oligodendrocyte activation followed by RGC death (203–205). In a rodent glaucoma model, this detrimental effect of TNF-α was demonstrated after intravitreal TNF-α injections and reversed by an antibody neutralizing TNF-α activity or by deleting the genes encoding TNF-α or its receptor, TNF-R2 (204). Future studies are needed to define further the potential role of TNF-α inhibitors as a treatment target for neuroprotection (206).

Nutrition may affect retinal homeostasis and, in particular, mitochondrial function. Hence, a ketogenic diet was shown to increase mitochondrial respiration, hereunder mitochondrial respiration in astrocytes (207, 208). In addition, the ketogenic diet has been reported to restore monocarboxylate transporters to boost the antioxidant response followed by preservation of RGC

function and structure without affecting glycogen stores (117). Finally, a low-carbohydrate diet has been shown to reduce the risk of POAG in a US cohort (209). In summary, nutrition and ketogenic diets may increase the resistance toward glaucomatous neurodegeneration. Future studies are, however, necessary to further investigate such potential.

Targeting Oligodendrocytes

Inhibition of Inflammatory Mediators

Oligodendrocyte degeneration has been correlated to TNF-α release followed by increased IOP and optic nerve damage. In this context, suppression of TNF-α, with an anti-TNF-α blocking antibody or the deletion of the gene encoding TNF-α, was shown to elicit neuroprotective effects in the optic nerve and RGCs in a mouse model of glaucoma (204).

Inhibiting Lingo-1

LINGO-1 is a leucine-rich repeat, expressed on oligodendrocytes and neurons. It negatively regulates differentiation and myelination, neuronal survival, and axonal regeneration. In glaucoma models and human CNS diseases, LINGO-1 expression has been found to be upregulated (210). LINGO-1 has furthermore been shown to have negative regulatory functions in axonal regeneration, neuronal survival, and oligodendrocyte differentiation as well as myelination. In addition, LINGO-1 was found to be increased in models of spinal cord injury and glaucoma (211). The LINGO-1 monoclonal antibody, BIIB033, has shown promise as a neuroprotective and neuroregenerative strategy in clinical studies, but continued evaluations are needed to confirm this promising effect in glaucoma patients (211).

Targeting Microglia

Inhibition of Microglial Activation

Microglia have increasingly been identified as targets in glaucoma neuroprotection and neuroregeneration (22, 28, 212, 213). The blockade of the microglial adenosine A_{2A} receptor has been shown to protect RGCs from elevated IOP in murine glaucoma models by controlling the microglial activation and inhibiting reactive oxygen species (214). Minocycline has also been shown to inhibit microglial activation and upregulate pro-survival genes in experimental glaucoma (215, 216). Although minocycline has proven oral safety and has been found to cross the blood–brain barrier, long-term randomized control trials with the necessary high doses of minocycline are needed (217).

Modulation of Microglial Activation

In addition to the harmful effects of microglia, the activation of these cells can also benefit the healthy eye, where such activation, e.g., acute inflammation, has been shown to protect against neuronal damage (22). Microglia activation is regulated by several inhibitory pathways, such as the ligand fractalkine (FXN or Cx3cl1) in neurons and the receptors Cx3cr1 and CD200R in microglia (218). The loss of Cx3xl1 signaling has been shown to exacerbate dysfunctional axon transport in RGCs, increased CCR2+ macrophages infiltration (219), and upregulation of NOS-2 in myeloid cells from DBA/2J mice (219).

CD200 is expressed in the vascular endothelium of the retina, photoreceptors, and RGCs (34) and interacts with CD200R to modulate microglial activation (220). Breakdown of CD200-CD200R is involved in RGC loss in experimental glaucoma (221). Thus, in a rat model with optic nerve crush, the agonist of CD200R known as CD200Fc was shown to increase CD200R expression and inhibit CD200 expression, thereby assisting in the neuroprotection of RGCs (222, 223). Overall, microglial activation may be beneficial for RGC function and survival, and numerous studies emphasize the dual roles of microglia activation. However, more studies are needed to further understand such potential beneficial functions of microglia in glaucoma.

Inhibition of Inflammatory Mediators

In advanced glaucoma, inflammatory mediators, including TNF-α, are increased. Inhibition or genetic deletion of TNF-α reduces the activation of microglia (32, 224), and the blockade of TNF-α signaling has been shown to protect RGCs in an experimental glaucoma mouse model (204, 225).

The Fas ligand (FasL), a member of the TNF protein family, links microglia activation and the induction of apoptosis of RGCs through the Fas receptor. In the eye, FasL can be expressed as the membrane-bound pro-apoptotic and pro-inflammatory protein (mFasL) or as the soluble, non-apoptotic, and non-inflammatory form sFasL (226). Previous studies have shown that FasL is constitutively expressed in ocular tissue, where the ligand helps maintain the immune-privileged state of the eye and helps prevent neoangiogenesis. However, FasL has also been shown to play an important role in retinal neurotoxicity. In this context, FasL has been shown to accelerate RGC death in an experimental glaucoma model (227). In line with this, the peptide inhibitor of the Fas receptor, ONL1204, has been found to halt mFasL activation by inhibition of microglial activation, inflammation, and apoptosis of RGCs in a mice model (228). In contrast to the neurotoxic effects of FasL, FasL administration has been shown to protect RGCs from cell death (227). Overall, the contradictive roles of FasL and mFasL require more studies to investigate the potential roles of FasL/mFasL modulation in future neuroprotective treatments.

Reducing Oxidative Stress

Orally administered docosahexaenoic acid and intravitreal injection of polysialic acid has been shown to reduce microglial activation by decreasing oxidative stress and inflammation in rodent models of glaucoma and experimental glaucoma models (146). Moreover, the natural resin mixture, propolis, produced by honeybees, has been shown to reduce neuroinflammatory responses and reduce oxidative stress in microglia cell cultures by inhibiting nuclear factor-κB when cultures were exposed to hypoxia (229).

Macrophage Pro-Inflammatory Cytokines

Glial cell activation triggers macrophage infiltration and the release of pro-inflammatory cytokines that further

activate glial cells. Such pro-inflammatory cytokines have been found to be upregulated in both the blood and in the aqueous humor of patients with POAG (133, 230). Accordingly, treatments targeting macrophage-derived pro-inflammatory cytokines, such as IL-1β, may be used in the future treatment of glaucoma (133, 194).

CONCLUSION

Glial cells play complex and multifactorial roles in glaucoma. Advances in our understanding of the nature and regulation of these various roles in health and disease have enabled the identification of novel therapeutic strategies to protect RGCs in glaucoma. Therapies targeting glial cells or those emulating the protective effects of these cells on RGCs will likely go hand-in-hand with conventional therapies to lower IOP and with emerging approaches that aim to augment neuronal bioenergetic resilience and promote axonal repair.

AUTHOR CONTRIBUTIONS

MG-B has search the literature and written the first draft. MK has been main responsible for the process. ZM has designed the figures. KM, PW, and KF have all been participating in critical commenting and proof reading. All authors have contributed to the writing process.

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MINI REVIEW
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Microglia-Derived Interleukin 23: A Crucial Cytokine in Alzheimer's Disease?

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Neuronal cell death, amyloid β plaque formation and development of neurofibrillary tangles are among the characteristics of Alzheimer's disease (AD). In addition to neurodegeneration, inflammatory processes such as activation of microglia and astrocytes are crucial in the pathogenesis and progression of AD. Cytokines are essential immune mediators of the immune response in AD. Recent data suggest a role of interleukin 23 (IL-23) and its p40 subunit in the pathogenesis of AD and corresponding animal models, in particular concerning microglia activation and amyloid β plaque formation. Moreover, in animal models, the injection of anti-p40 antibodies resulted in reduced amyloid β plaque formation and improved cognitive performance. Here, we discuss the pathomechanism of IL-23 mediated inflammation and its role in AD.

Keywords: interleukin 23, microglia, neuroinflammation, antibody therapy, Alzheimer's disease

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INTRODUCTION

Alzheimer's disease (AD) is the most common form of dementia and affects millions of people worldwide (1). Despite the immense progress in AD research over the recent years, many aspects of the underlying pathomechanism remain elusive and new approaches for effective therapies are needed. Besides neurodegeneration, the disease-accelerating role of neuroinflammation has become a focus of research in AD. Since the central role of interleukin 23 (IL-23) in neuroinflammation, especially in multiple sclerosis (MS), has become clear, several interesting studies about AD and IL-23 have been published. Therefore, this review provides an overview of the current data on IL-23 mediated neuroinflammation in AD, open aspects for further research and possible therapeutic approaches.

IL-23

Cytokines are peptide hormones that act as messengers of the immune system and modulate the immune response in an autocrine, paracrine, or endocrine manner (2). They regulate activation or inhibition of immune cells, control their differentiation, proliferation and chemotaxis. In particular, the successful application of antibodies modulating the cytokine function for the treatment of a variety of diseases has further advanced the research in the field of cytokines and cytokine-inhibiting therapies.

A subgroup of cytokines are the interleukins. IL-23 consists of a unique p19 and a common p40 subunit, which is shared by the structurally related IL-12 (3). It appears that the heterodimeric molecule is the bioactive cytokine and both subunits, p19/p40 for IL-23 and p35/p40 for IL-12 must be co-expressed in the same cell to generate the bioactive form. However, some data also show an effector function of p40 alone as discussed below. Since the first study demonstrated that IL-23 and not the structurally similar IL-12 is the central cytokine contributing to the pathogenesis of autoimmune diseases (4, 5), the importance of IL-23 in neuroinflammation has been further deciphered in many preclinical and clinical studies. IL-23 is primarily secreted by antigen presenting cells (APC) like dendritic cells, macrophages, and B cells (3, 6, 7). The local production of IL-23 in the CNS has been demonstrated for astrocytes and infiltrating macrophages under inflammatory conditions (5, 8). In addition, some studies demonstrated the secretion of the IL-12/IL-23 subunit p40 by microglia (9, 10), while others provided evidence that microglia secrete the bioactive cytokine IL-23 upon activation (11–13).

Figure 1 summarizes how IL-23 activates the immune system. Best known responders to IL-23 stimulation are the CD4 T helper subset T helper 17 (Th17) cells, a distinct subpopulation of $\gamma\delta$ T cells, subsets of natural killer T cells, and innate lymphoid cells (14). IL-23 binds to its specific receptor complex, which consists of a unique IL-23 receptor subunit and a IL12β1 subunit. $\gamma\delta$ T cells express the IL-23 receptor constitutively, but naive CD4+ cells lack the IL-23 receptor. CD4+ cells are therefore first activated by other cytokines such as the transforming growth factor (TGFβ), IL-6 or IL-21, then differentiate into Th17 cells and express the IL-23 receptor (15–17).

By binding to the IL-23 receptor, IL-23 leads to a conformational change of the receptor, which promotes the phosphorylation of Janus kinase 2 (JAK2) and tyrosine kinase 2 (Tyk2) leading to phosphorylation of signal transducer and activator of transcription (STAT) factors with STAT3 primarily activated (18). The activated STAT protein enters the nucleus to exert its biological effects. This leads to the secretion of the Th17 cell characteristic cytokines IL-17A, IL-17F and several other proinflammatory cytokines like IL-22, the granulocyte-macrophage colony-stimulating factor (GM-CSF) or the tumor necrosis factor (TNFα) (14).

Among the different cell subtypes expressing the IL-23 receptor, Th17 cells could be established as key players in neuroinflammation, particularly in MS (19, 20). Beside these well-known responders of lymphocytic origin, expression of the IL-23 receptor on macrophages/monocytes, microglia, and dendritic cells was described (21, 22), enabling these cells to

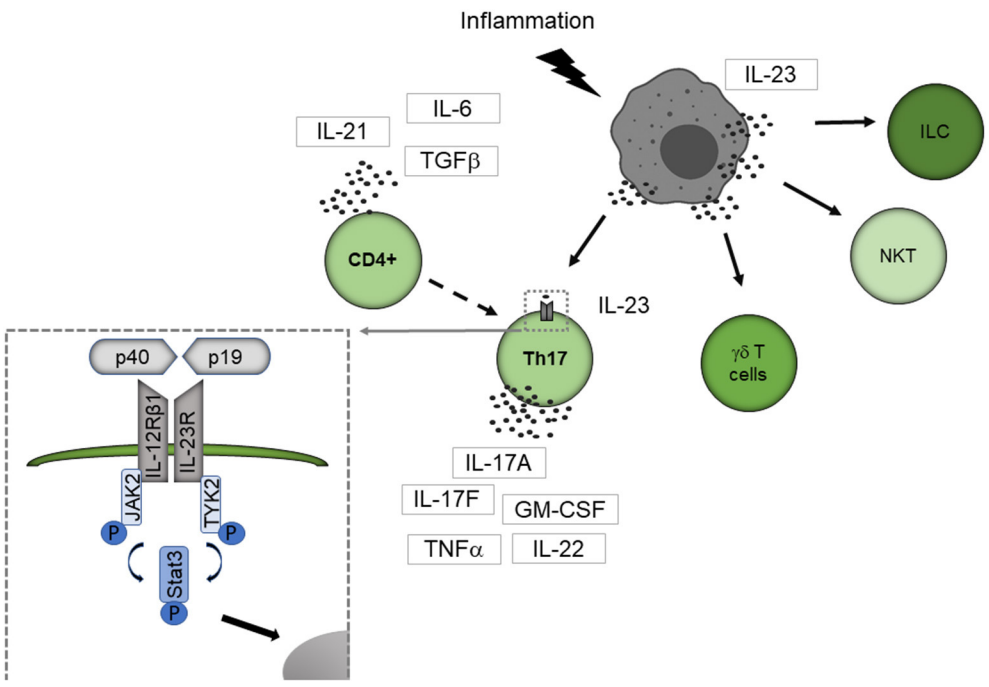

FIGURE 1 | IL-23 mediated activation of the immune system. IL-23 is primarily secreted by APC like dendritic cells, macrophages, and B cells. Best known responders to IL-23 stimulation are Th17 cells, a distinct subpopulation of $\gamma\delta$ T cells, subsets of natural killer T cells, and innate lymphoid cells. IL-23 binds to its specific receptor complex, which consists of a unique IL-23 receptor subunit and a IL12β1 subunit. $\gamma\delta$ T cells express the IL-23 receptor constitutively, but naive CD4+ cells lack the IL-23 receptor. CD4+ cells are therefore first activated by other cytokines such as TGF-β, IL-6, or IL-21, then differentiate into Th17 cells and express the IL-23 receptor. By binding to the IL-23 receptor, IL-23 leads conformational change of the receptor that promotes the phosphorylation of JAK2 and Tyk2 leading to phosphorylation of STAT factors. Thereby STAT3 is primarily activated. The activated STAT protein enters the nucleus to exert its biological effects. ILC, innate lymphoid cell; NKT, natural killer cell.

directly interact with IL-23. Thereby, IL-23 enhances the cytokine production of IL-23 receptor expressing myeloid cells (21, 22).

IL-23 AND NEUROINFLAMMATION

The crucial role of IL-23 in the pathogenesis of a vast variety of autoimmune diseases like inflammatory bowel diseases, rheumatoid arthritis, and psoriasis has been clearly demonstrated [(23–27), summarized in **Table 1**]. IL-23 is also important in the atherogenesis and progression of atherosclerotic plaques (28, 29).

Numerous studies could demonstrate the significance of IL-23 in neuroinflammation as well. Most of the data on IL-23 and neuroinflammation have been derived from studies on the pathogenesis of MS and corresponding animal models (38). The central role of IL-23 in the development of MS is beyond doubt. IL-23 is increased in serum, cerebrospinal fluid (CSF), and lesional tissue of MS patients (13–15). Animal models emphasize the non-redundant role of IL-23 in MS, as an experimental autoimmune encephalitis cannot be induced in mice lacking IL-23 or the receptor complex (30–32). Nevertheless, there are also studies suggesting IL-23 is critical in stroke patients, especially in the postischemic inflammatory phase (33, 34). Furthermore, the IL-23 signaling pathway is part of the defense mechanism in viral, bacterial, and fungal infections of the CNS (35–37). To further investigate how IL-23 mediates neuroinflammation in different animal models, we have recently established a mouse model with astrocyte-specific expression of IL-23 revealing unexpectedly, a spontaneous B cell accumulation in the cerebellum (39).

With the increasing number of studies demonstrating the importance of IL-23 in neuroinflammation, several studies have also investigated the influence of IL-23 on AD. Therefore, this review will give an overview of the role of IL-23 in AD.

TABLE 1 | Role of IL-23 in the pathogenesis of different diseases.

Disease	IL-23 effect	References
Inflammatory bowel diseases	Pathogenic	(24, 25)
Rheumatoid arthritis	Pathogenic	(25, 27)
Psoriasis	Pathogenic	(23–26)
Atherosclerosis	Pathogenic	(28, 29)
MS	Pathogenic	(13–15, 30–32)
Stroke/postischemic inflammation	Pathogenic	(33, 34)
Infections	Protective	(35–37)

AD AND NEUROINFLAMMATION

Characteristics of AD are amyloid β (Aβ) plaques, which are extracellular deposits of Aβ derived from the β-amyloid precursor protein (APP), neurofibrillary tangles composed of hyperphosphorylated tau and neuronal cell death (40). In addition to neurodegeneration, neuroinflammation is crucial in the pathogenesis and progression of AD (40). Although it is not clear to what extent neuroinflammation contributes to the pathogenesis of AD, it is generally acknowledged that the immune system influences the disease progression.

Neuroinflammation in AD is mainly promoted by CNS-resident cells like microglia and astrocytes. Microglia are CNS-resident cells of myeloid origin with immune-modulating and phagocytic capabilities (41). Activation of the innate immune system in AD seems to follow Aβ deposition. However, studies of patients with mild cognitive impairment demonstrate neuroinflammation even in the early phase (42, 43).

Aβ plaques are surrounded by reactive astrocytes and activated microglial cells (44, 45). The role of microglia in AD initiation and progression are debated, with conflicting reports regarding their detrimental or protective function (46). Microglia and astrocytes can remove Aβ by uptake and degradation or extracellularly degrade Aβ by enzyme secretion (40). However, they also lead to increased Aβ levels and contribute to tissue reaction and destruction in AD especially during disease progression (47). In addition, activation of microglia and complement-dependent pathways mediates synapse loss in AD (48). The state of activation appears to determine whether microglia have a protective or detrimental role in AD (49). Microglial mediated neuroinflammation is increased in AD while microglial-mediated Aβ clearance mechanisms are diminished (41).

Microglia and astrocytes are the major source of proinflammatory cytokines as essential regulators of the immune response in AD (50). IL-1, IL-6, IL-12, IL-23, GM-CSF, TNF-, C-X-C motif chemokine ligand 10 (CXCL10) are detectable or upregulated in animal models of AD, in the brain or CSF from AD patients (47, 51). APP/presenilin 1 (APP/PS1) mice, a well-established mouse model to study amyloid pathology in AD, show reduced plaque burden and Aβ levels if genetically deficient for CXCR3, the receptor for CXCL10 (52). The proinflammatory cytokine milieu in the AD brain contributes directly or indirectly to neuronal damage. Aβ stimulation results in secretion of proinflammatory cytokines, which trigger neuronal hyperexcitability and synaptic dysfunction (40). Moreover, cytokines stimulate the secretion of inducible nitric oxide synthase in microglia and astrocytes, which is toxic to neurons at high concentrations (40).

IL-23 AND AD

As the relevance of IL-23 in neuroinflammation, particularly in MS, has become evident, the question of how IL-23 affects inflammatory processes in AD has arisen. Therefore, several descriptive and experimental studies addressed the impact of IL-12/23 p40 and IL-23 signaling on AD in recent years.

Single nucleotide polymorphisms in the IL-12/23 subunit p40 (rs3212227) (53) and IL-23 receptor polymorphisms are associated with AD in a northern Han Chinese population (54).

AD patients show higher peripheral levels of IL-23 (55) and the concentration of the subunit p40 was identified as a serum marker for the prediction of the Aβ load in an AD cohort (56). A plasma multianalyte profiling study of patients with mild cognitive impairment and AD demonstrated an association of plasma p40 levels with abnormal cognitive performance (57).

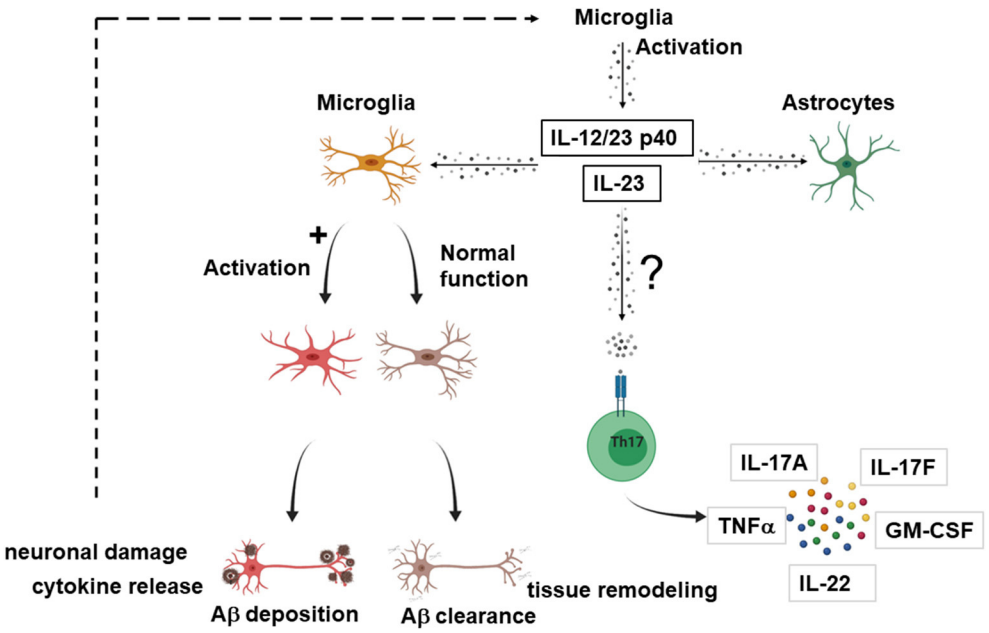

FIGURE 2 | Possible effector cells in IL-23 mediated neuroinflammation in AD. Suggested model how IL-23 and IL-12/23 p40 drives neuroinflammation in AD. Upon activation, microglia secrete IL-23 or/and IL-12/23 p40. IL-23 and IL-12/23 p40 lead to a shift of microglia from Aβ clearance and tissue remodeling toward an activated state with production of proinflammatory cytokines, inhibition of the clearance of A$\gamma\delta$ neuronal damage, which in turn could further enhance the IL-23 secretion. IL-23 and IL-12/23 p40 could also lead to activation of astrocytes. Nevertheless, it cannot be excluded that the effector cells for IL-23 signaling are partly T cells, as described for other neuroinflammatory diseases. The figure was generated with BioRender.com.

In contrast, a smaller study demonstrated reduced p40 concentration in CSF in patients with cognitive impairment including AD in early phases of the disease with a median Mini-Mental State Examination (MMSE) score of 23 (58). However, this study additionally found CSF IL-12/23 p40 concentrations correlated positively with CSF concentrations of Aβ1-42 and phosphorylated tau protein but not MMSE score in the total study population including patients with mild cognitive impairment, AD, and other dementia forms. But in AD patients CSF IL-12/23 p40 only correlated positively with CSF P-Tau (58). The most extensive study concerning the role of IL-23 in AD was performed by vom Berg et al. (59). They found increased expression of p40 in microglia in APP/PS1 mice and increased p40 in cerebrospinal fluid of AD patients. The cognitive performance measured with the MMSE score correlated in this study negatively with CSF IL-12/23 p40 levels. However, it should be noted that the number of AD patients studied was small ($n = 7$). Furthermore, p40 appears to contribute to the extent of cerebral plaque formation and activation of microglia in the mouse model. Cytokine-knockout (p19, p35, and p40) APP/PS1 mice showed reduced microglial activation and disease severity along with diminished accumulation of Aβ in young and older mice (59). Thereby, loss of the common IL-12/23 p40 subunit did show the greatest impact on Aβ plaque burden, whereas deficiency of the unique subunits IL-23 p19 or IL-12 p35 results in a similar but weaker reduction. Moreover, experiments with bone marrow-chimeric mice indicated that microglial cell-derived IL-12/23 p40, but not peripheral myeloid cell-derived IL-12/23 p40 is involved in the extent of in Aβ plaque load. This finding further illustrates the central role of microglia in mediating p40-related effects on Aβ burden (59). The p40 production by microglia was associated with the *de novo* expression of the activation marker CD11c indicating modulation of microglia activity when producing p40 (59). In addition, after injection of anti-p40 antibodies before or after the onset of amyloid accumulation, the mice showed reduced Aβ formation and improved cognitive performance (59). Thus, both female and male mice were used for this study and a gender bias cannot be ruled out. Another study examined the gender-specific effect in mice lacking IL12p40. Eede et al. found that IL12p40 deficiency reduces Aβ plaque burden in male APP23 mice, while female mice had a significant reduction in soluble Aβ1-40 without changes in Aβ plaque burden (60). Furthermore, plasma and brain cytokine levels are altered differently in female vs. male APP23 mice lacking IL12p40.

Which Cells Mediate the Effects of IL-23 in AD?

While the number of leukocyte subpopulations known to respond to IL-23 is growing (14), the effector cells upon IL-23 signaling in the context of AD remain elusive. Although the actions of IL-23 in other neuroinflammatory processes like MS are mediated *via* Th17 cells (19, 20), in AD, IL-23 and IL-12/23 p40 might act through novel mechanisms independent from T cells. **Figure 2** provides a proposed mechanism how IL-23 drives neuroinflammation in AD.

Vom Berg et al. provided a hypothesis involving cells of the innate immunity as effectors in the p40 driven signaling pathway (59). One possible scenario covers the sustained Aβ-driven release of p40 by microglia that binds to the IL12Rβ1 receptor on adjacent astrocytes in a paracrine manner. As a second scenario, autocrine activation of microglia by binding of p40 to the IL12Rβ1 receptor on microglia themselves is suggested, which promotes AD pathology.

However, it should be noted that the p40 subunit alone is in generally not considered a bioactive form. It appears that both subunits, p40 and p19 for IL-23, p40 and p35 for IL-12, must be co-expressed in the same cell to generate the bioactive form (IL-12 or IL-23). But several studies in fact show that p40 alone can act as a messenger substance (61). The p40 homodimer is capable of inducing the expression of immune factors in microglia *via* the IL-12Rβ1 (62).

Further data are needed to elucidate if neuroinflammation in AD is driven by IL-12/23 p40, IL-23 or even partly by IL-12. Since p40 is a subunit of both IL-23 and IL-12, this is difficult to determine from the data. The results of the APP/PS1 mouse model with deletion of the unique subunits IL-23 p19 or IL-12 p35, which results in a weaker but similar reduction of the plaque load, speak against the fact that the effect is mediated by p40 alone (59).

A study analyzing Aβ clearance in IL-23-treated microglia further enlightened, which effector cells might respond to p40 or IL-23. In a human macrophage cell line Aβ42 incubation increased the expression of IL-17, IL-18, IL-23, whereas the same cytokines impaired Aβ clearance by macrophages or microglia (63). The inhibitory effects of IL-18 were blocked by IL-23 or IL-17 neutralizing antibodies while the inhibitory effects of IL-23 were blocked by IL-17 neutralizing antibodies pointing to an interaction of IL-17, IL-18, and IL-23 and microglia for the Aβ clearance.

In another mouse model, the senescence-accelerated mouse prone-8 model (SAMP8), Tan et al. screened the cerebral expression of IL-12/23 in 3-, 7-, and 11-month-old mice and demonstrated that these cytokine levels in the brain were upregulated during aging (64). By *in vivo* infusion of non-viral small interfering RNA (siRNA) to knock down the common IL-12/23 subunit p40 in the brain, they demonstrated that these p40-deficient mice had significantly diminished cerebral Aβ42 levels, reduced synaptic and neuronal loss, and reversed cognitive impairments. In addition, treatment of the SAMP8 mice with a neutralizing p40-specific antibody also ameliorated AD-associated pathology and cognitive deficits.

Several studies have demonstrated that microglia can express both neurotoxic and neurotrophic factors (65). Tan et al. hypothesized that the beneficial changes by treatment of SAMP8 mice with a neutralizing p40-specific antibody might be derived from a shift of microglia from an activated state with release of proinflammatory cytokines and inhibition of the clearance of Aβ toward an increased Aβ clearance and enhanced tissue remodeling.

Nevertheless, it cannot be excluded that the effector cells for p40 or IL-23 signaling are partly T cells, as described for other neuroinflammatory diseases. In a mouse models of AD, Aβ vaccination results in a reduction in amyloid burden concomitant with decreased expression of the IL-12Rβ1 receptor by T cells, the receptor subunit binding to p40 (66). Th17 cell-mediated neuroinflammation is involved in neurodegeneration of a rat AD model (67). Furthermore, activation of Th17 cells in AD patients has been demonstrated by Saresella et al. (68) and Th17 cells, which infiltrated into AD brain parenchyma, participate in neuroinflammation and neurodegeneration of AD by release of proinflammatory cytokines and by direct action on neurons *via* the Fas/FasL apoptotic pathway (67). The role of IL-23 and IL-17a, as the signature cytokine of Th17 cells, was also reviewed by Mohammadi Shahrokhi et al. (69), which identified IL-17a as a main inducer of neuroinflammation in AD. In contrast, Saksida et al. identified IL-17 as a rather protective factor. The central finding of the study was a lower production of IL-17 in gut-associated lymphoid tissue cells of aged 5xFAD mice probably due to impaired post-transcriptional stabilization of the IL-17 mRNA mediated by miR-155 (70). The decreased IL-17 level could impair the homeostasis of the immune system in the gut-associated lymphoid tissue, but could also contribute to inappropriate Aβ clearance in gut-associated lymphoid tissue and CNS. Another review speculates about the beneficial use of anti-IL-17A and anti-IL-23 antibody in AD by interfering with neutrophil infiltration and thereby suggets another possible effector cell in IL-23-mediated neuroinflammation in AD (71).

A shift from the Th17 cell/regulatory T cell balance favoring the proinflammatory Th17 side is suspected to contribute to exacerbation of autoimmune disorders (72). Since regulatory T cells delay disease progression in AD pathology (73), the role of regulatory T cells in IL-23 and AD should also be enlightened in further studies.

However, it is important to take into account that most data on the functional role of IL-23 in AD have been generated from mouse models, and in particular from the APP/PS1 model, which develops Aβ plaques by 6-8 months, but no tau pathology and does not cover all aspects of AD pathology (74). The effect of IL-23 especially on tau pathology is certainly worthwhile to investigate further.

Nevertheless, the current knowledge of the role of IL-23 and especially the IL-12/23 common subunit p40 are promising. The signaling pathways and effector cells involved in IL-23 and IL-12/23 p40 mediated immune response in AD should be enlightened in further studies, particularly in the clinical context.

IL-23 an Attractive Therapeutic Target in AD?

Novel therapeutic options such as a variety of antibody therapies have led to significant progress in the treatment of many neurological diseases in recent years. However, the treatment of AD remains inadequate despite the immense progress in therapeutic options, so that the development of new therapeutic approaches remains a central aspect in AD research. Modulating the function of IL-23 appears to be an interesting target for AD although the precise signaling pathways and corresponding effector cells are not completely characterized. The beneficial results of the anti-IL-23 in preclinical studies

could be transferable to the patient. While antibody therapies that interfere with the IL-23 are firmly established in the therapeutic concept for diseases such as psoriasis, spondylitis ankylosans, or inflammatory intestine illness (75–77), they are yet not established for the treatment of inflammatory CNS diseases. Administration of the p40 antibody ustekinumab was not successful in clinical trials in MS patients (78). Similarly, the anti-p40 antibody briakinumab showed only a slight benefit in terms of imaging progress and clinical relapse rate (79). Nevertheless, clinical trials blocking IL-23 and investigating whether it results in reduced neuroinflammation, reduced plaque burden, and improved cognitive impairment appear worthwhile. Considering the large number of patients already receiving an approved anti-IL23 therapy, studies of whether anti-IL23 therapy can prevent the development of AD would also be interesting.

These studies should be in particular feasible, as the yet approved anti-IL23 antibody therapies for autoimmune diseases showed a favorable risk profile in terms of safety of use, especially with regard to more severe infections or malignancies (75, 78–80).

DISCUSSION

Taken together, the currently available studies underline the impact of the proinflammatory cytokines of IL-23 and its subunit p40 in the pathogenesis of AD. In addition to clinical data showing the association of single nucleotide polymorphisms, IL-23 levels and AD, preclinical data demonstrate that IL-23 plays a crucial role in neuroinflammation, plaque formation in AD models and identify anti-IL-23 therapy as a promising new therapeutic approach. The data suggest that the aspects of IL-23 mediated neuroinflammation in AD remain an interesting research field and further data will enlighten the significance and signaling pathways of IL-23 in AD.

AUTHOR CONTRIBUTIONS

All authors prepared, corrected, and modified the manuscript.

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Microglia Phenotypes Converge in Aging and Neurodegenerative Disease

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Microglia, the primary immune cells of the central nervous system, hold a multitude of tasks in order to ensure brain homeostasis and are one of the best predictors of biological age on a cellular level. We and others have shown that these long-lived cells undergo an aging process that impedes their ability to perform some of the most vital homeostatic functions such as immune surveillance, acute injury response, and clearance of debris. Microglia have been described as gradually transitioning from a homeostatic state to an activated state in response to various insults, as well as aging. However, microglia show diverse responses to presented stimuli in the form of acute injury or chronic disease. This complexity is potentially further compounded by the distinct alterations that globally occur in the aging process. In this review, we discuss factors that may contribute to microglial aging, as well as transcriptional microglia alterations that occur in old age. We then compare these distinct phenotypic changes with microglial phenotype in neurodegenerative disease.

Keywords: microglia, aging, neurodegeneration, alzheimer's disease, senescence

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INTRODUCTION

Microglia originate from hematopoietic progenitor cells found in the yolk sac and, upon entering the brain, gradually adapt a homeostatic microglial phenotype (1). Homeostatic microglia feature a distinct ramified morphology and were first identified by del Rio-Hortega (2). As the primary immune cells of the brain, microglia are mostly associated with acute or chronic responses to injury. In response to these stimuli, microglia display morphological and biochemical changes that have often been grouped under the term "activation." These changes can entail a variety of downstream effects including cytokine and chemokine production, enhanced phagocytosis, proliferation, and migration. Historically, based on *in vitro* experiments, this rather generalized diversion from the homeostatic cell state has led to a differentiation into two microglial groups: M1 (proinflammatory) and M2 (neuroprotective). This relatively oversimplified classification (3) of microglial reactivity is now being refined by single-cell resolution techniques that show diverse transcriptional states that can be adapted by microglia in either a gradual or acute manner. Intriguingly, we and others have shown that microglia are long-lived cells that undergo an aging process on a cellular level, altering their surveillance capacity and injury response time, and also influence neurodegenerative diseases (4–7) [reviewed in (8, 9)]. These rather slow and gradual alterations are contrasted by rapid changes brought on by acute damage. Local signals in the microglial microenvironment drive acute as well as gradual changes, leading to broad alterations in gene transcription, cell morphology, phagocytotic activity, and proliferation status (10–12). Over decades of research, a wide variety of terms have

been used to describe microglial cell states (13). As research has advanced, new distinctions in microglial phenotypes have been identified based on expression of particular genes and, most recently, their transcriptome signature. In this review, we aim to uncover potential similarities in microglial phenotypes in advanced age and neurodegenerative disease (**Figure 1A**) (12, 15, 16).

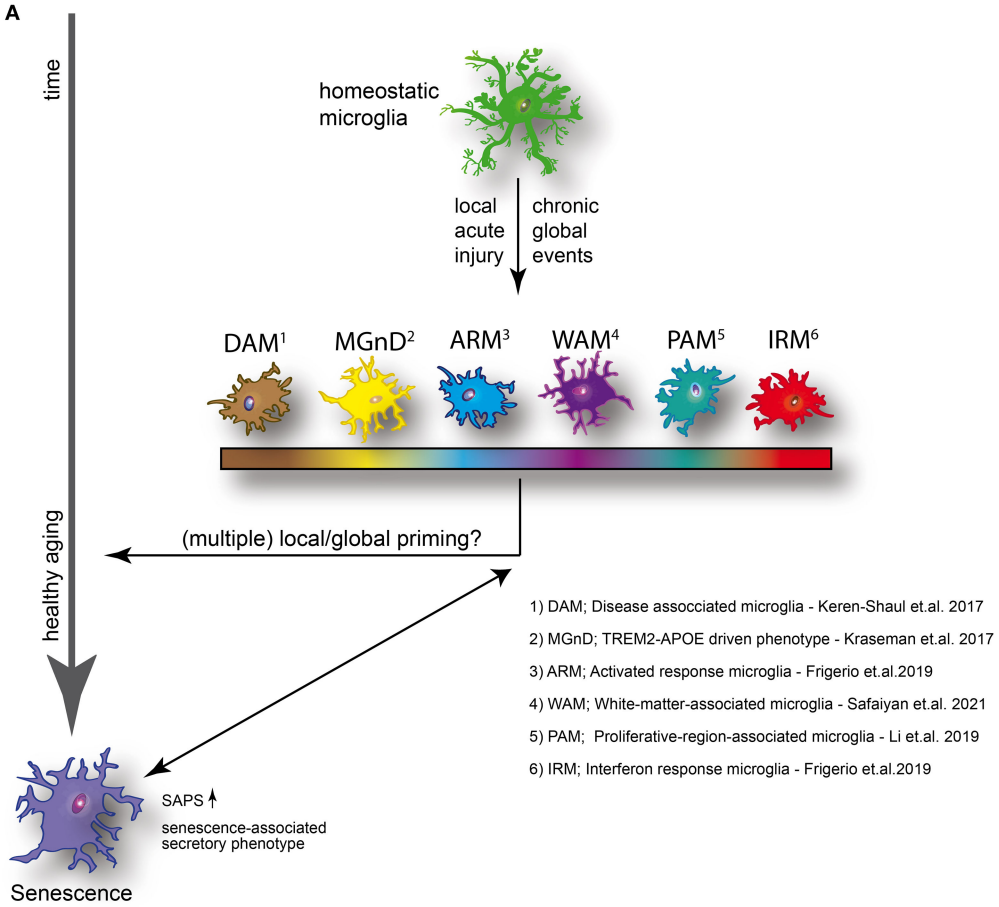
MICROGLIAL AGING: A SUMMATION OF FACTORS ACCUMULATED THROUGHOUT LIFE?

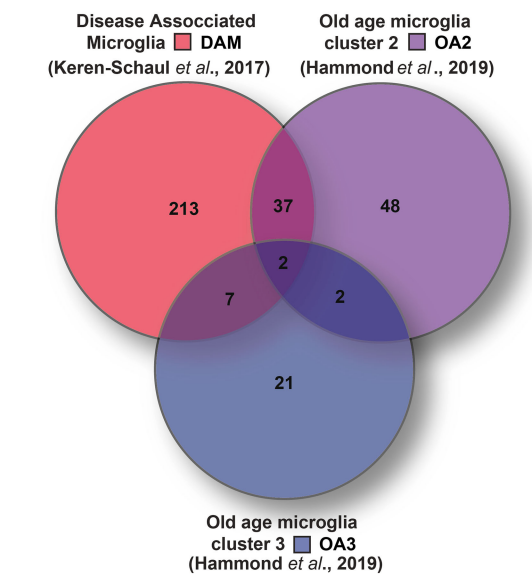
It recently was shown that not only cells of the adaptive immune system but also those of the innate immune system can display memory effects (5). The priming step of the immune cells, induced by a primary insult, can result in an enhanced or dampened subsequent injury response (17–19). *In vivo* tracking of individual microglial cells showed that microglia can revert to a homeostatic morphology post-injury (20–22). However, even if the morphological homeostatic phenotype is reestablished, epigenetic modification may render the cells altered from their homeostatic state. As long-lived cells, microglia are very likely to be primed by signals in their microenvironment, hence memory effects might add up during the cell's life span. This poses the question if these rather individualized priming steps might be part of the cellular aging process or if they have the potential of adversely affecting healthy aging. Furthermore, as some acute injuries such as microlesions are likely to only affect surrounding microglial cells, the priming impact might be locally restricted.

Individual local events could result in the generation of distinct spatially restricted transcriptional and phenotypic alteration as microglial phenotypes are partially regulated through membrane-bound pattern recognition receptors (PRRs), which depend on molecules released by cells in their microenvironment. In a local ischemic event, disease-associated molecular patterns are passively released from dying cells (23), which may lead to a transient activation with a priming effect, whereas the deposition of amyloid β (Aβ) in the parenchyma [a hallmark of Alzheimer disease (AD), but can also be found in aged brains on a lesser scale] may lead to a different chronic disease–associated microglial phenotype, which not only depends on PRRs, as microglia also possess a wide variety of receptors to detect other types of molecules such as hormones and neurotransmitters (23). Therefore, a multitude of factors exist that could potentially affect local and global microglial behavior both in a short- or long-term fashion. A recent study demonstrated the importance of the local milieu within the brain in this regard by transiently depleting microglia using the colony-stimulating factor 1 receptor antagonist PLX5622 (24) in aged mice (25). The authors hypothesized that withdrawal of the drug would result in the replenishment of "young" unprimed microglia (25). Conversely, they reported that the transcriptomic alterations in old age were only partially reversed, and the replenished microglia responded to lipopolysaccharide (LPS) with an exaggerated proinflammatory response, typical of primed microglia. Further *in vitro* experiments confirmed that media conditioned by 24-h cultivation of brain slices from aged, but not young adult, mice were sufficient to trigger an exacerbated response to LPS in neonatal microglia, elegantly demonstrating the importance of the milieu in which microglia are resident (24).

When focusing on the healthy aging process of microglia, they have been described as dystrophic or senescent [reviewed in (26)]. Historically, senescence is characterized by arrested growth caused by oxidative stress as well as elevated DNA damage. Age-related changes in the secretory profile were described to coin the term *senescence-associated secretory phenotype*, classifying a particular cell state in the aging brain (27, 28). The term *dystrophic*, on the other hand, was created by the observation of changes in microglial morphology in brain sections from elderly humans and potentially includes all visually altered microglia (20). Among other features, this phenotype includes the beading of microglial processes, which are held together by thin channels (29), and was proposed to signify microglial senescence (30). Previous studies addressing the question if one of the described phenotypes is purely age-related are controversial; some have found dystrophic microglia in aged humans without any underlying neurodegenerative disorders (30–32), whereas others gathered evidence suggesting that dystrophic microglia are associated with a variety of diseases including, e.g., AD (29, 31–34), Huntington disease (14), and multiple sclerosis (35). However, recently some light has been shed on the question whether these two terms are describing the same or two different phenotypes. To address this issue, Shahidehpour et al. (36) conducted stereological analysis of microglia in human brain tissue spanning the age of 10-90 years. The analysis revealed an increased number of dystrophic microglia with age, which, however, was much greater when neurodegenerative pathology was present as well (36). They hence conclude that aging itself is only associated with a minor increase in dystrophic microglia (36). It is thus possible that the disease event that generated an activated microglia phenotype, potentially early in life, has a priming effect on cellular aging, leading to an increase of dystrophic microglia in old age (**Figure 1A**).

Also, the opposite assumption is valid; the overall cellular aging process is likely causative for poor local injury responses, resulting in an ineffective healing process that in turn might again increase the amount of dystrophic/senescent microglia. One example is the finding that population RNAseq of murine microglia has identified a consistent age-dependent increase in genes associated with a low-grade inflammatory response (37), which might be causative for a poor local injury response by microglia to additional acute insults. We have found the injury response time to a local laser lesion of aged microglia (∼2.4-year-old mice) to be reduced by ∼50%. Additionally, while microglial process end-tips in young and adult mice showed an increase in local diameters after a microlaser lesion, the aged animals displayed significantly less morphological changes upon lesioning, as the process end-tips already were found to be enlarged prior to the insult (4). Further supporting a gradual overall drift into a low-grade inflammatory state





C

Genes mutually expressed in DAM and OA2

Fth1	*Adamts1*
Mt1	*Ctsz*
Atf3	*Pld3*
Gusb	*Cxcl16*
Glipr1	*Ctsb*
Cd9	*Bcl2a1b*
Gnas	*Flt1*
Aplp2	*Cd63*
Cd52	*Ccl6*
Cadm1	*Mif*
Dpp7	*Lgals3*
Fxyd5	*Cd83*
Ch25h	*Apoe*
Lyz2	*Capg*
Il1b	*Spp1*
Cox6a	*2Lpl*
Ccl3	*Csf1*
Ccl4	*Fam20c*
Cst7	*H2-K1*
H2-D1	

D

Genes mutually expressed in DAM and OA3

Pyhin1
Xaf1
Lgals3bp
Ifi204
Ifit1
Ifi27l2a
H2-Q7
H2-K1
H2-D1

FIGURE 1 | (A) Homeostatic microglia over the life span of the animal encounter both spatially restricted and global injuries that likely contribute to transcriptomic and functional alterations in microglia, potentially resulting in the adaption of specific disease- or injury-associated microglia phenotypes and conceivably contributing to premature cellular aging. **(B)** Venn diagram illustrating the number of enriched (1.5-fold or higher) mutually and exclusively expressed genes between DAM (identified in

(Continued)

FIGURE 1 | a mouse model of AD) (14) and two distinct clusters identified in aged wild-type mice [old age (OA) 2 and OA3] (7). **(C)** DAM and OA2 feature 39 mutually expressed genes suggesting the activation of common transcriptional programs in both neurodegenerative disease and a minority of cells in healthy aging. **(D)** Conversely, only nine genes were mutually expressed between DAM and OA3 suggesting that the OA3 population may be distinct from both DAM and OA2 microglia. Venn diagram generated using Venny 2.1 (https://bioinfogp.cnb.csic.es/tools/venny/).

during aging, Minhas et al. recently put forward evidence suggesting that a change in the metabolic state of macrophages (in the brain and periphery), signified by a reduction of the two main metabolic pathways (glycolysis and oxidative phosphorylation), is affecting brain health as acute energy demands, e.g., in order to support macrophage activation, cannot be met any longer (38). More specifically, prostaglandin E_2 (PGE_2), a proinflammatory signaling protein, which is known to increase not only during aging but also in AD, was investigated. The inhibition of PGE_2 was shown to lead to brain rejuvenation, reducing inflammatory levels in the aged brain (38). These findings are of particular interest as they support other reports that microglia can be influenced by stimulation of peripheral immune cells (5) and that their responsiveness can be (partially) restored even in the aged brain. With the detailed mechanism leading to the reported reduction in metabolism still being unknown, a future challenge is to unravel this pathway to explore possible therapeutic interventions, aiding in a wide range of diseases, as well as aging itself.

TRANSCRIPTOMIC ALTERATIONS IN MICROGLIA IN OLD AGE AND NEURODEGENERATIVE DISEASE

In the effort to characterize microglia phenotype in disease, scRNAseq has become a powerful weapon and has facilitated even greater insight into the transcriptomic alterations of microglia in diverse conditions. At present, however, comparing transcriptomic data from different studies comes with inherent challenges. Variability can be introduced at many stages such as dissociation, gating for cell sorting, the scRNAseq procedure, and data analysis. Technical limitations can further result in discrepant data. For example, it was recently revealed that the technical limitations of single-nucleus (sn)RNAseq (a theoretically useful approach for postmortem human tissue as it is compatible with frozen tissue) may have resulted in data that lead inadvertently to the overstating of differences between murine and human microglial transcriptomes (16). Thrupp et al. found that many transcripts associated with microglial activation are concentrated in the cytosol as opposed to the nuclei, resulting in many transcripts remaining undetected with snRNAseq (16). Another pertinent issue is that despite recent technical advances in scRNAseq, correlating transcriptional profiles to mechanistic data remains a persistent bottleneck. Spatially resolved scRNAseq, especially at a single-cell resolution, would provide an unparalleled advantage in correlating morphological observations to distinct transcriptome signatures. At present, however, these limitations greatly hinder correlating microglial phenotypes (as evident by morphology or behavior *in vivo*) with specific gene-expression profiles.

Despite these difficulties, highly valuable data have been collected in several high-impact studies. Hammond et al. (12) identified two distinct microglia clusters using scRNAseq that, in the absence of overt pathology, were expanded in aged (∼1.5 years old) mice. One cluster (entitled OA2) was found to up-regulate the chemokines *Ccl3* and *Ccl4* along with interleukin 1 beta (*Il1b*), indicative of a shift to a proinflammatory phenotype during aging (12). The other emerging cluster in aged animals was found to be enriched in several interferon-response genes [*Ifitm3*, *Rtp4*, and *Oasl2* (entitled OA3)] (12). This shift toward expression of interferon-response genes is highly interesting, given that recent research has demonstrated that interferon signaling in a mouse model of AD triggers microglial activation, neuroinflammation, and synaptic loss in response to nucleic acid containing Aβ plaques (39).

Sala Frigerio et al. (40) also identified two microglial clusters using scRNAseq that were expanded in aged mice. One cluster, entitled activated response microglia (ARM), increased from around ∼3% in 3-month-old mice to ∼12% of total microglia in 21-month-old mice (40). ARM microglia were found to up-regulate histocompatibility complex class II genes (*Cd74*, *H2-Ab1*, and *H2-Aa*) and proinflammatory genes *Cst7*, *Clec7a*, and *Itgax* (encoding CD11c). Notably, with the exception of *Itgax* and *Clec7a*, these genes were also found to be up-regulated in OA2 microglia (12). Another microglia cluster was also identified, dubbed interferon-response microglia (IRM) (40). This cluster was found to up-regulate *Ifit3*, *Ifitm3*, *Irf7*, and *Oasl2*, consistent with OA3 microglia (12). Furthermore, using semisupervised pseudotime analysis, the authors found that homeostatic microglia in old age can transition into either IRM or ARM. Taken together, these data suggest that in old age, microglia transition into one of two mutually exclusive states, one characterized by up-regulation of interferon-response genes and the other characterized by a shift to a proinflammatory state. However, as previously mentioned, it remains challenging to ascertain the impact that transcriptional alterations have on microglial phenotype *in vivo*.

Microglia play complex roles in neurodegenerative disease (41), often being beneficial in some respects, while pathogenic in others. Taking AD as an example, microglia phagocytose Aβ (42, 43) (although this becomes attenuated with aging/Aβ plaque load) and encircle Aβ plaques, prohibiting the spread of [comparably more toxic (44)] soluble amyloid species into the surrounding brain parenchyma (45). Consistent with this, ablation of microglia after amyloid deposition results in increased LAMP1 immunoreactivity surrounding Aβ plaques (46); indicative of dystrophic neurites (47). While these findings

suggest that microglia play a beneficial role in AD, microglia themselves contribute to synaptic and neuronal loss in AD (48). It was also shown that abolishing microglia after Aβ plaques are well-established in the brain fails to provide any beneficial effects (49) in the APPPS1 AD mouse model. Furthermore, if microglia are abolished prior to amyloid deposition, Aβ plaques fail to develop in the 5XFAD AD mouse model (46) (although Aβ accumulates on blood vessels resulting in cerebral amyloid angiopathy, a risk factor for hemorrhagic stroke). Taken together, because of the complex matter at hand, it is hard to determine whether microglia provide a net beneficial or detrimental role in AD, and a simple binary answer is not likely.

Despite the potential for global or spatially restricted alterations in transcriptome signature in aging and pathology, recent publications seem to suggest a common—or at least highly similar—transcriptional program (50, 51) in diseased states [i.e., disease-associated microglia (DAM) (52), microglia neurogenerative phenotype (53), ARM (40), and, most recently, white matter–associated microglia (54), a recently identified microglial phenotype that expands during aging and in neurodegenerative disease]. Massively parallel single-cell analysis with chromatin profiling on immune (CD45+) cells from 5XFAD mouse brains (a well-established AD mouse model) revealed two unique microglial clusters (52). Intriguingly, these clusters expressed genes implicated in lipid metabolism and phagocytosis (52). By analyzing mice from the age of 1-8 months (as Aβ deposition is advancing), the authors uncovered an age-dependent shift from a homeostatic phenotype to a DAM phenotype with one of the two clusters identified as being a transitory stage (defined as stage 1) (52). DAM are characterized by the up-regulation of *Itgax*, *Trem2*, *Axl*, *Cst7*, *Ctsl*, *Lpl*, *Cd9*, *Csf1*, *Ccl6*, *Clec7a*, *Lilrb4*, and *Timp2* (52). Notably, *Itgax* in particular was found in every cell featuring a DAM transcriptome signature (52). In addition, they down-regulate *Cx3cr1*, *P2ry12*, and *Tmem119* (52), genes typically expressed in homeostatic microglia (12). The authors further identified that DAM are in close association with Aβ plaques and contain phagocytosed Aβ (52). Intriguingly, the authors identified that a transition from stage 1 DAM to stage 2 DAM was dependent on triggering receptor expressed on myeloid cells 2 (*Trem2*) (52); homozygous loss-of-function mutations in this gene are known to cause autosomal recessive early-onset dementia [Nasu–Hakola disease and frontotemporal dementia (FTD)–like disease] (55–57). TREM2 acts as a receptor for apolipoprotein E, Aβ, and high- and low-density lipoprotein and has been identified as crucial for triggering microglial phagocytosis, proliferation, and inflammation (58). Importantly, loss-of-function mutations in *Trem2* have also been implicated in diverse neurodegenerative diseases including AD (59), amyotrophic lateral sclerosis (60), Parkinson disease (61), and FTD (62). Consistent with this, DAM transcriptomes have now been identified in diverse models of neurodegenerative disease. Based on these elegant findings, it is tempting to speculate that the DAM phenotype represents a pan-neurodegenerative disease response.

GRADUAL ACCUMULATION OF DAM DURING AGING

Even though we and others have shown morphological and functional changes of microglial cells in aged mice, only a rather small subpopulation show a transcriptomic profile consistent with that of DAM. By comparing (**Figure 1B**) the top up-regulated genes (greater than 1.5-fold change) between DAM (52) and the small clusters of transcriptionally distinct cells from aged mice (OA2 and OA3) (12), a set of 39 mutually expressed genes can be identified between DAM and OA2 (**Figure 1C**). Conversely, only nine genes were mutually expressed between DAM and OA3 (**Figure 1D**). The amount of overlap in genes up-regulated in DAM and OA2 appears to support the hypothesis that a common transcriptional program is activated in both DAM and aged microglia, but only in a minority of the cell population in healthy aging. However, in various disease models, the cell population displaying transcriptomic signatures consistent with DAM is much larger, suggesting that pathological insults during the animal's life span will heavily expand the DAM population, which has a potential impact on cellular aging. Consistent with this, the ARM phenotype described by Frigerio et al. (consistent with DAM) eventually becomes the majority population of microglia at 12 months in an AD mouse model (App^{NL-G-F}) (40). IRM (consistent with OA3) conversely seemed to increase with age more rapidly in App^{NL-G-F} mice than wild-type mice but ultimately also represented only a minority of cells ($< \sim$5%) similar to wild-type mice. Ferretin expression has been identified as a marker for dystrophic microglia (36) and senescence (26). Shahidehpour and colleagues (36) found ferretin-expressing dystrophic cells to be present, but again to a very small extent, in healthy aged humans. Conversely, the number of dystrophic microglia was significantly increased in patients suffering from neurodegenerative disorders. Consistent with the data of Shahidehpour et al. (36), ferritin is expressed in the OA2 subpopulation (12), which again is minimal in the absence of overt pathology, yet abundant in neurodegenerative disease conditions (DAM) (52). Taken together, the data suggests a disease-induced increase of cellular aging hallmarks.

CONCLUSIONS

Microglia with transcriptomic signatures consistent with that found in neurodegenerative diseases represent only a minority of microglia in healthy aging. It remains unclear what this subset of microglia contributes toward overall microglial dysfunction or, conversely, if they might have a beneficial impact. With regard to microglia featuring a neurodegenerative disease-associated transcriptome signature, it is possible that neurodegenerative disease causes advanced cellular aging, or conversely, advanced cellular aging may be a contributing factor to neurodegenerative disease (63). Further studies will be required to interrogate the roles of these interesting microglial populations in old age. In addition, it seems reasonable to speculate that data gathered from mice at the end of the mouse life span ($\sim$2.5 years)

would be particularly valuable given the advanced ages reached by humans in contemporary society. scRNAseq studies to date seem to suggest that, despite the many factors that could potentially influence microglial phenotypes in either a global or spatially restricted manner, microglia in aged mice appear to consist of homeostatic microglia, neurodegenerative disease-like microglia, and IRM. The lack of apparent heterogeneity could conceivably be in part due to the artificial conditions that laboratory rodents reside in. This is an important caveat that should be considered when attempting to extrapolate observations from laboratory rodents to humans. With highly individualized lifestyles, disease backgrounds, and environmental factors, microglia in humans are likely to be primed more diversely and extensively given human longevity in comparison to laboratory mice. Hence, much remains to be discovered that could potentially bring valuable mechanistic insights into both aging and neurodegenerative disease.

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REVIEW
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Glial Purinergic Signaling in Neurodegeneration

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Purinergic signaling regulates neuronal and glial cell functions in the healthy CNS. In neurodegenerative diseases, purinergic signaling becomes dysregulated and can affect disease-associated phenotypes of glial cells. In this review, we discuss how cell-specific expression patterns of purinergic signaling components change in neurodegeneration and how dysregulated glial purinergic signaling and crosstalk may contribute to disease pathophysiology, thus bearing promising potential for the development of new therapeutical options for neurodegenerative diseases.

Keywords: Alzheimer, neurodegeneration, ATP, adenosine, oligodendrocyte, microglia, astrocyte, purine

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INTRODUCTION

Neurodegenerative diseases are characterized by a progressive loss of structure and function of the CNS. An estimated 50 million patients worldwide are currently affected by neurodegenerative diseases and it has been projected that this number will rise to 131 million patients by 2050 (1). Many of these diseases are idiopathic/polygenic proteinopathies, which are characterized by accumulation and/or aggregation of proteins such as TAU, amyloid-β or α-synuclein (α-SYN) in the CNS and are multifactorial, with both genetic and environmental risk factors. The most common ones are the sporadic forms of Alzheimer's disease (AD; TAU/amyloid-β (Aβ) aggregates), Parkinson's disease (PD; α-SYN aggregates), amyotrophic lateral sclerosis (ALS; TAR DNA binding protein 43 (TDP-43)/FUS RNA binding protein (FUS) aggregates), Lewy body dementia (α-SYN aggregates) and frontotemporal lobar degeneration (FTLD; TAU/TDP-43/FUS aggregates) (2).

Despite decades of research, no causal treatment is available for any of these diseases. One promising approach to help overcome the lack of therapeutical options is to shift the focus from a linear neuron-centered view on how neurodegenerative diseases develop to a broader and integrative view in which interactions between all cell types in the brain are considered. This paradigm shift has led to an increased interest in the role of glial cells in neurodegeneration.

Indeed, there is strong evidence that changes in glial cells, microglia, astrocytes and oligodendrocytes, are causally involved in neurodegenerative diseases. Around half of the known genetic risk factors in sporadic AD are glial genes related to immune function such as *TREM2*, *CD33* or *CR1* (3, 4). Furthermore, recent data have highlighted phenotypic changes of glial cells over the disease course with the discovery of disease-associated microglial (DAM) and disease-associated astrocytic markers (DAA) (5, 6). Accordingly, new therapeutic strategies targeting glial signaling pathways are currently being tested in pre-clinical intervention studies, for example, treatments modulating the NLRP3 inflammasome (7), astrocytic nuclear factor kappa-β (8) and TREM2-mediated signaling (9), among others.

The targeting of purinergic signaling—i.e., signaling pathways mediated by extracellular nucleotides and nucleosides such as ATP or adenosine—belongs to these novel emerging therapeutic strategies (10). All CNS cell types, including glial cells, express purinergic receptors and purinergic signaling influences key CNS functions [for review see Agostinho et al. (11)] such as synaptic transmission, proliferation, maturation and neuroinflammation (12) that are altered in neurodegeneration. Thus, in the present manuscript, we discuss the current knowledge on purinergic signaling in glial cells and its potential relevance in neurodegenerative disease. We provide a comprehensive overview and cell-specific expression tables based on available transcriptomic data of purinergic genes in glial cells in neurodegeneration and link this data with data from functional studies. Finally, we discuss glial purinergic signaling as a potential target for future therapeutic intervention.

THE COMPONENTS OF PURINERGIC SIGNALING

Purines are a family of small molecules involved in DNA/RNA structure and key cellular processes such as cell metabolism, intracellular signaling and extracellular signaling. Geoffrey Burnstock coined the term of "purinergic signaling" for the latter in 1972 (13), in which he referred to cell signaling pathways that are activated by engagement of nucleosides and nucleotides with specific cell receptors. ATP, the main energy storage of the cell, can be hydrolyzed in the extracellular space through specific enzymes called ectonucleotidases into ADP, AMP and adenosine. These include ectonucleoside triphosphate diphosphohydrolases (E-NTPDases), ectonucleotide pyrophosphatase/ phosphodiesterases (ENPPs), alkaline phosphatases and ecto-5′-nucleotidase (*NT5E*/CD73) (14). Adenosine can also be produced through the S-adenosyl-L-homocysteine pathways and degraded by adenosine deaminase and adenosine kinase. Furthermore, ATP and its derivatives can be transported between cell compartments and released extracellularly through different transporters like the equilibrative nucleoside transporters (ENTs) or concentrative nucleoside transporters (CNTs), but also through channels such as Connexin-43 (CX43), Connexin-32 (CX32) or Pannexin-1 (PANX1), secretion involving vesicular nucleotide transporter (*SLC17A9*/VNUT) or ultimately by membrane rupture during cellular injury, allowing them to trigger purinergic receptor signaling (15, 16).

Purinergic receptors are subclassified into two large families: P1 receptors that are activated by nucleosides, and P2 receptors that are activated by nucleotides. P2 receptors are further subdivided into ionotropic P2X receptors and metabotropic P2Y receptors (17, 18). So-called P0 receptors have recently been defined and constitute adenine receptors (19, 20). The P1 receptor family consists of four receptors (adenosine A1, A2A, A2B and A3 receptor), the P2X family of seven receptors (P2X1-P2X7) and the P2Y family of eight receptors (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11-P2Y14).

GLIAL PURINERGIC SIGNALING AND CROSSTALK UNDER PHYSIOLOGICAL CONDITIONS

Purinergic signaling controls important physiological processes in the healthy CNS, such as synaptic transmission, cell proliferation and innate immune response (11, 12). ATP and adenosine are produced and released upon neuronal or glial activation and initiate various cellular pathways corresponding to the activation of P2X, P2Y and adenosine receptors. Neurons, microglia, astrocytes and oligodendrocytes express a unique repertoire of purinergic receptors (**Figure 1**, left), which due to receptor-specific intracellular downstream signaling cascades lead to specific responses in each CNS cell type. Many previous studies have contributed to our understanding of the purinergic signaling repertoires in microglia (21–24), astrocytes (25–27) and oligodendrocytes (28, 29). However, a full overview of the expression patterns of all components of purinergic signaling in the main mouse and human CNS cells has only become available through the seminal work of the laboratory of Ben Barres. The group created an unbiased RNA sequencing database of purified specific CNS cells from human and mouse cortex (30–32). Using this database, we here assembled expression data of purinergic genes from neurons, microglia, astrocytes and oligodendrocytes. With the caveat of missing validation on the protein level, possible cell impurities, region-specific differences or age-dependent modifications, this makes it possible to compare expression patterns of purinergic genes in these CNS cell types in human and mouse (**Figure 2**).

According to this data, microglia are the cells with the highest expression and largest number of expressed purinergic receptors (in particular *ADORA3*, *P2RX12*, *P2RY13*, and at lower levels *P2RX7* and *P2RY14* genes) and ectonucleotidases (in particular *ENTPD1* gene also known as CD39) in the human cortex. There are significant differences in murine microglial gene expression of purinergic components compared to human. For example, *Adora1*, *P2rx4* and *Entpd2* show higher expression levels in mouse. Additionally, genes encoding ENTs (ENT1, 2 and 3, encoded by *Slc29a1, Slc29a2* and *Slc29a3*, correspondingly) are expressed at higher levels in mouse microglia, suggesting that nucleoside transport processes may differ between humans and mice. Similarly, cell-specific expression of some ectonucleotidases, including *Entpd1* in microglia are considerably different in human and mouse (**Figure 2**).

Human astrocytes, despite displaying–in some cases considerably–lower expression values than microglia, also express a variety of purinergic receptors (*ADORA2B*, *P2RY1*, *P2RY12*) and ectonucleotidases (*ENTPD1, ENPP5* and *NT5E* also known as CD73). Similar patterns can be found in mouse astrocytes (**Figure 2**).

Lastly, human oligodendrocytes express *ADORA3*, *P2RX7*, *P2RY12*, *P2RY13,* and *NT5E*. Interestingly, ectonucleotide pyrophosphatases/phosphodiesterases (ENPPs, namely *Enpp2*, *Enpp4*, *Enpp5,* and *Enpp6*) in oligodendrocytes show a higher expression in mouse (**Figure 2**).

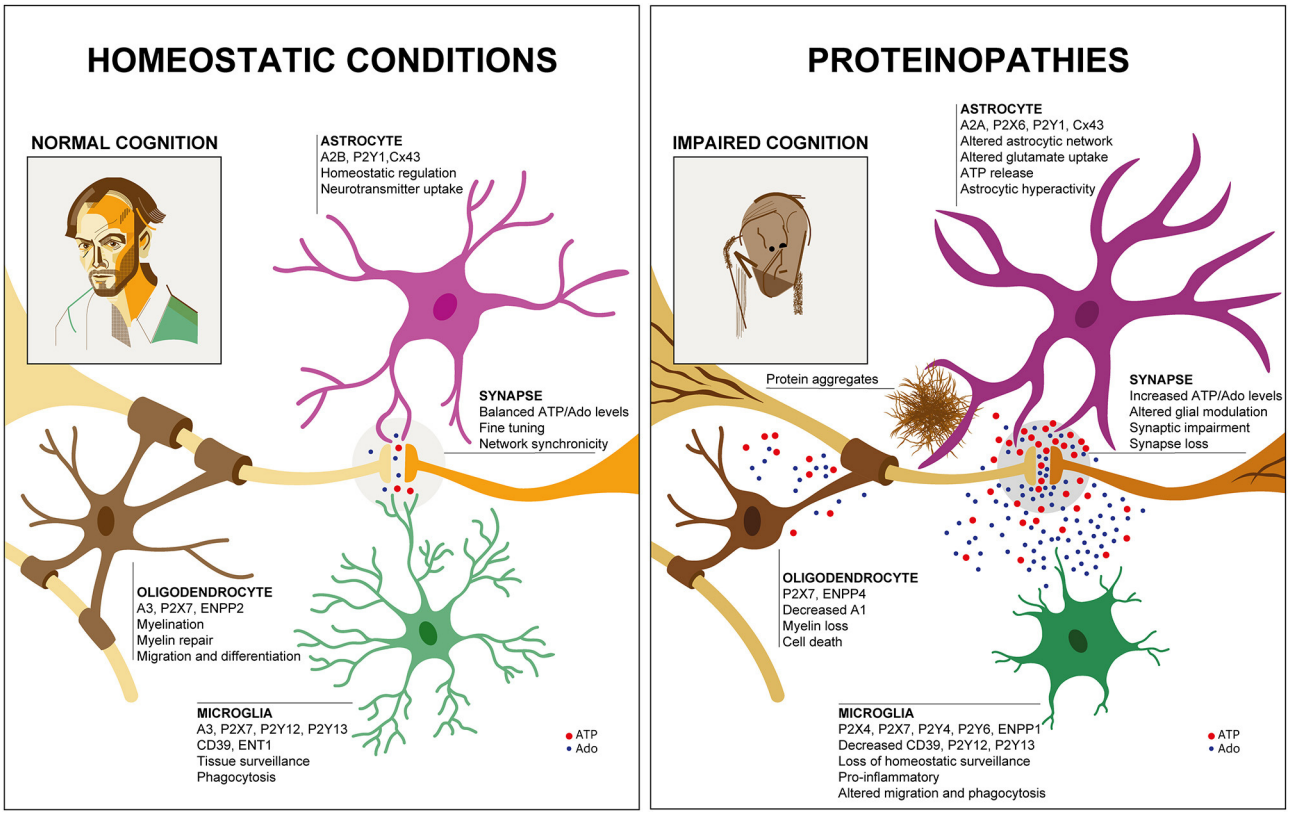

FIGURE 1 | Glial purinergic signaling under homeostatic conditions and in proteinopathies. Under healthy conditions **(Left)**, cell-specific glial purinergic signaling contributes to fine-tuning of synapse function and thus, normal cognitive abilities. In proteinopathies **(Right)**, accumulation of protein aggregates leads to glial phenotype changes that are associated with altered expression of purinergic signaling components and altered ATP and adenosine (Ado) levels. This impacts synaptic function and ultimately results in synapse loss, contributing to impaired cognition. Two self-portraits are shown to illustrate the cognitive status in health and disease, similar to the famous work of William Utermohlen, who continued to create self-portraits after being diagnosed with AD.

Although some of the data largely overlaps with previous findings in rodent and human CNS tissue, this cell type-specific gene expression table allows to better appreciate the considerable inter-species similarities–and differences–between human and mouse. It also shows the large spectrum of purinergic gene expression patterns in the different CNS cell types.

How is this cell type-specific purinergic signaling repertoire linked to glial cell function? Although some aspects have remained ill-defined, especially regarding the role of purinergic enzymes and transporters, a lot is known about how purinergic receptor signaling shapes glial cell function in the healthy brain [**Figure 1** left; for a recent detailed review see Agostinho et al. (11)].

For example, different P1 and P2 receptors such as A3, P2Y12, P2Y13, P2X4 control the motility of the ramified and dynamic cell processes of microglia, the tissue-resident immune cells of the brain (33–36). This motility of cell processes is important for surveillance and chemotactic cell process movement toward localized brain damage. Furthermore, phagocytosis and release of inflammatory molecules, both major effector functions of microglia, are modulated by purinergic signaling pathways, involving P2X4, P2X7, and P2Y6 (37–40). In addition, there is evidence that microglial contribution to synaptic pruning and synaptic function is at least partly controlled by purinergic mechanisms (41). It has also been shown that innate immune responses of microglia are positively modulated by ATP through P2X7 receptors (42) and negatively by adenosine through A2A receptors (43).

Astrocytes are involved in a wide variety of functions in the CNS, such as metabolic support, synaptic function, neuronal and synaptic maturation and blood brain barrier permeability (44–47). The relevance of astrocytic P2 receptor signaling in some of these cell functions has been implicated in a number of previous studies. More specifically, P2Y1 mediates calcium signaling in astrocytes, which is critical for modulating synapse function and blood-brain barrier maintenance (48). Neurotransmitter recapture is also modulated by astrocytic A2A receptor-mediated signaling, allowing for a reduced uptake of glutamate and greater uptake of GABA, reinforcing synaptic activation (49, 50).

Oligodendrocytes synthesize myelin sheaths, which insulate CNS axons, enabling a rapid action potential propagation. This process occurs life-long as oligodendrocytes perform myelin remodeling. Additionally, oligodendrocytes contribute to the metabolic support of axons through a wide variety of transporters (51). Oligodendrocyte progenitor cells (OPC, also called NG2 cells as they express the NG2 chondroitin sulfate proteoglycan)

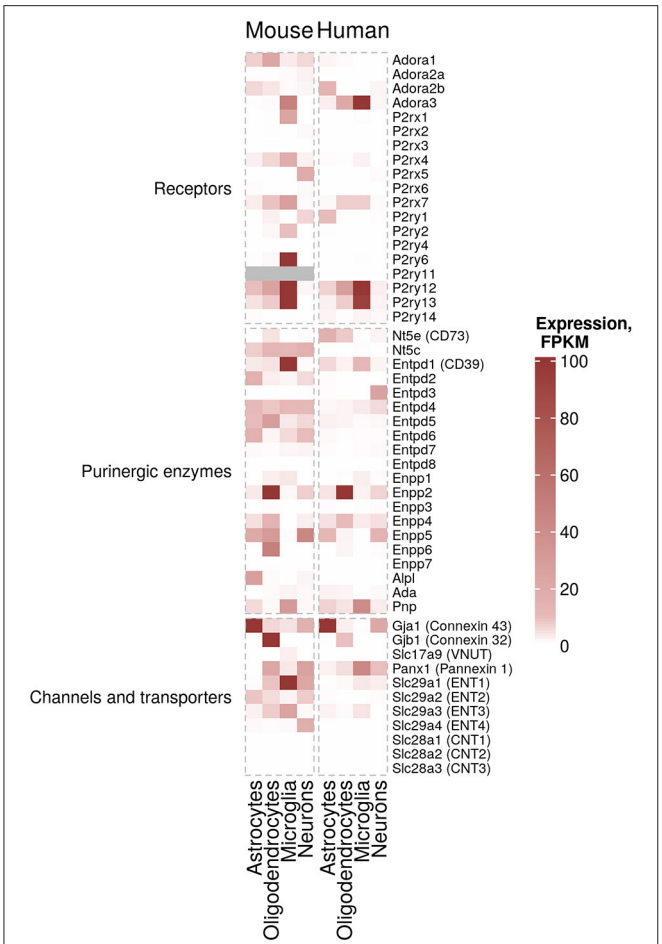

FIGURE 2 | Cell-specific expression of purinergic genes. Estimated expression levels FPKM (Fragments Per Kilobase Million) of genes involved in purinergic signaling pathways in microglia, myelinating oligodendrocytes, mature astrocytes and neurons was obtained from existing data sets from Zhang et al. (30) (mouse data) and Zhang et al. (31) (human data) (30, 31). FPKM values of 100 and above are shown in the same color for visualization purposes.

can differentiate into new mature oligodendrocytes. As for microglia and astrocytes, there is evidence that purinergic signaling controls a number of cell functions in oligodendrocytes. Adenosine and ATP/ADP have been shown to contribute to proliferation, migration and maturation of oligodendrocytes through P1 (A1 and A2A) and P2 receptors (P2X7 and P2Y1).

Apart from its importance in shaping cell functions of particular glial cells, purinergic signaling plays a key role in neuro-glial interactions (11). Neurons and glial cells reside in spatial proximity to each other, which is particularly important at the synaptic site. This proximity enables an efficient inter-cellular crosstalk through purinergic signaling, based on ATP/adenosine release, ectonucleotidase activity and receptor stimulation, affecting the neighboring cells in an autocrine and paracrine manner.

For example, ATP and adenosine released by synaptic stimulation trigger calcium wave signaling in the astrocytic syncytium through P2Y receptors, which in response decrease the activation of neighboring synapses through adenosine A1 receptor, a phenomenon called "heterosynaptic depression" (52, 53). Moreover, neuronal ATP release at the synapse recruits microglial cell processes, allowing cleavage of ATP into adenosine through microglial CD39 and ubiquitous CD73, leading to dampening of synapse activity upon A1 receptor stimulation (41).

Furthermore, ectonucleotidases such as CD73 are required for adenosine A2A receptor potentiation of synapses, and such mechanism could be similar in a glial context (54). For instance, *ENTPD1* (CD39) and *NT5E* (CD73) are expressed in all CNS cell types in the human CNS, albeit in case of CD73 at very low and in case of CD39 at high levels in microglia compared to the other cell types (31). This suggests that local degradation of ATP and ADP and production of adenosine may also occur in a cell-autonomous manner.

GLIAL PURINERGIC SIGNALING AND CROSSTALK IN NEURODEGENERATION

Since purinergic signaling is a multi-cellular, dynamic and complex signaling system, some of its aspects in neurodegenerative diseases have been difficult to evaluate and will require more specific quantitative tools in the future.

For instance, one major question that has remained unresolved due to the lack of specific tools with sufficient temporal and spatial resolution, is how the spatio-temporal kinetics of ligand availability and purinergic receptor activation is regulated in neurodegeneration (55). However, it has recently been shown that purinergic metabolites are strongly modified in AD. Adenosine was shown to be the most affected purine, increasing in temporal and parietal cortices of AD brains (56). Similarly, increased levels of adenosine are detectable in the CSF of ALS patients (57).

Another question that has not been sufficiently addressed until recently has been whether and how expression of purinergic signaling components is altered in glial cells in neurodegeneration. To get a better understanding of disease-associated changes, we took advantage of the increasing number of unbiased transcriptomic studies on human and murine glial cells in neurodegeneration. These studies did not have the primary goal to investigate differential purinergic gene expression in glial cells. However, they now constitute valuable resources to detect potential patterns of purinergic transcriptomic response in microglia, astrocytes and oligodendrocytes in neurodegeneration. To assemble the available data in a comprehensive manner, we searched for studies from transgenic AD, PD, tauopathy and ALS mouse models and human AD, PD, tauopathy and ALS patients, in which transcriptomic analyses on single cells or nuclei and bulk-sorted glial populations were performed. Among the available studies that mainly covered AD and ALS, we selected the data sets, in which at least one component of purinergic signaling was significantly dysregulated. We thus included 18 data sets from 14 studies on microglia (5, 58–68), nine data sets from eight studies on astrocytes (61, 68–74) and 4 data sets from

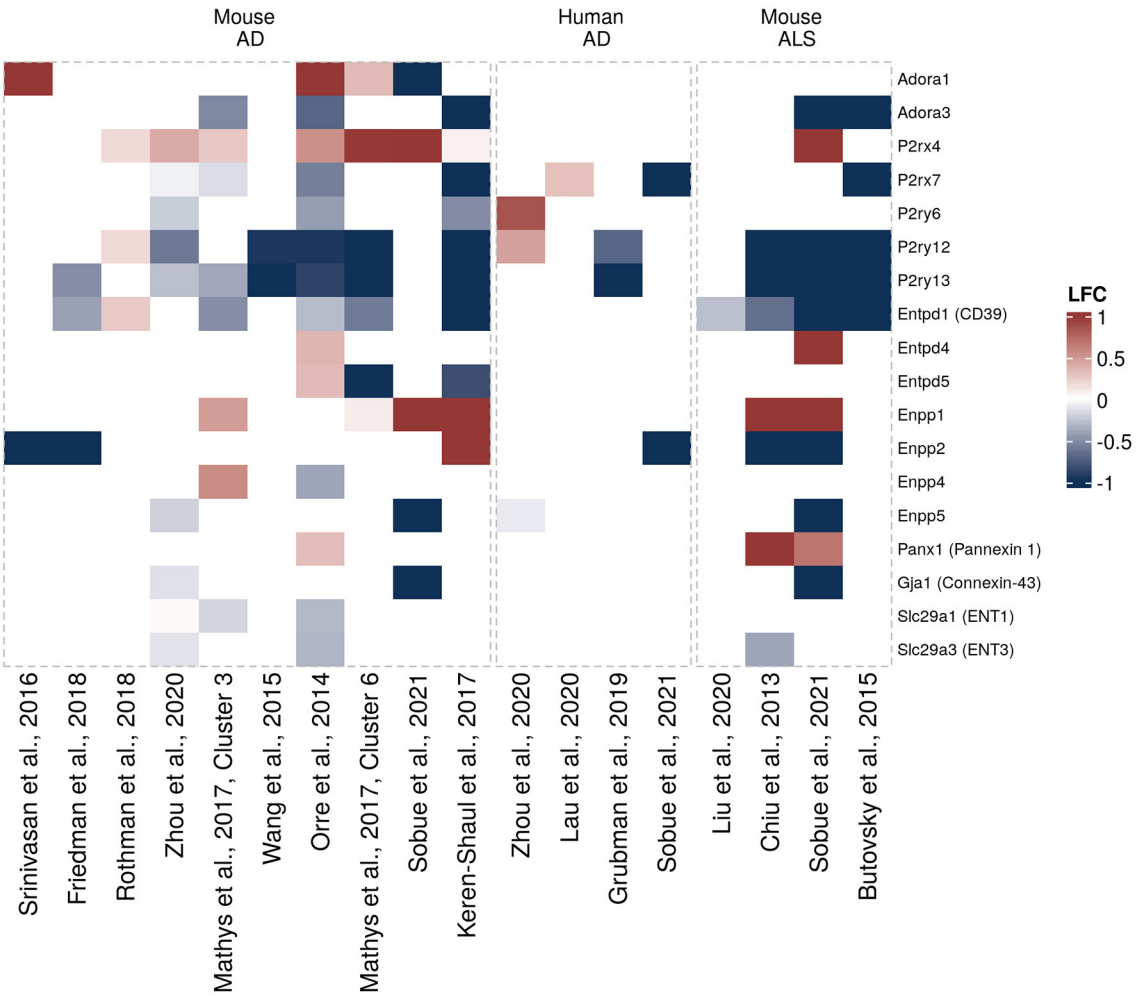

FIGURE 3 | Differentially expressed purinergic genes in microglia in neurodegeneration. Heatmap shows Log2 fold changes in expression of genes involved in purinergic signaling pathways between disease (AD or ALS, or relevant mouse models) and control samples. LFC values above 1 and below −1 are shown in the same color intensity for visualization purposes. Studies were selected according to the following criteria: transcriptomic analysis was performed on single cells or nuclei and allows disease/control comparisons for selected cell types, or on bulk-sorted glial populations; differential expression analysis results were provided by authors as supplementary material and were accessible. In total, 18 data sets from 14 studies were included for microglia [Orre et al. (69) (mouse, AD model, bulk), Chiu et al. (63) (mouse, ALS model, bulk), Wang et al. (64) (mouse, AD model, bulk), Srinivasan et al. (65) (mouse, AD model, bulk), Friedman et al. (66) (mouse, AD model, bulk), Rothman et al. (58) (mouse, AD model, bulk), Mathys et al. (72) (mouse, AD model, single-cell, two disease-associated clusters), Keren-Shaul et al., (5) (mouse, AD model, single-cell, DAM cluster), Zhou et al. (68) (two data sets, mouse AD model and human AD patients, single-nuclei), Liu et al. (59) (mouse, ALS model, single-cell), Lau et al. (61) (human, ALS, single-nuclei), Grubman et al. (74) (human, AD, single-nuclei), Butovsky et al. (75) (mouse, ALS model, bulk), Sobue et al. (60) (three data sets, mouse AD and ALS models, and human AD patients, bulk)] (5, 58–68).

three studies on oligodendrocytes (61, 68, 72) and used log2 fold change expression values between diseased and control samples to visualize shifts of gene expression involved in purinergic signaling pathways for each cell type (**Figures 3–5**).

Using this data and functional studies on glial purinergic signaling, we will discuss in the following sections what is known about purinergic signaling in microglia, astrocytes and oligodendrocytes in neurodegeneration.

It should be noted, however, that glial populations in neurodegeneration are spatially and temporally heterogeneous, depending on disease stage and proximity to specific pathological hallmarks. This heterogeneity is well-described for microglial populations in AD, in which "early" and "late" subsets of disease-associated microglia occur on both gene and protein expression levels (5, 76, 77). Moreover, single-cell transcriptomic studies have shown divergent but co-existent microglial subsets with different directions of gene expression changes (67, 76, 78). Therefore, it is likely that functionally and spatially distinct glial subsets co-exist within disease-associated reactive populations of glial cells [disease-associated microglia (DAM, (5)), disease-associated astrocytes (DAA, (73)) and pathology-associated populations of oligodendrocytes (67, 79)]. This may explain some of the discrepancies between purinergic gene expression changes and evidence from functional studies.

Microglia

Microglia are centrally involved in the pathophysiology of neurodegenerative diseases. Microglia not only drive inflammation in neurodegenerative diseases, but, as the main phagocytes in the CNS, are important for clearing protein aggregates, debris and apoptotic cells (80–86). Furthermore, the elaborate cellular morphology and process motility that enable efficient tissue surveillance are altered in neurodegeneration and this capability is therefore strongly affected (87–90). Some of these changes in microglial cellular functions may be caused by alterations in microglial purinergic signaling upon neurodegeneration (**Figure 1**, right).

When interrogating the microglia purinergic transcriptomic data from AD patients and AD mouse models we summarized here, a microglial response pattern emerges, in which *Adora1* and *P2rx4* are upregulated and *Adora3, P2rx7, P2ry6, P2ry12,* and *P2ry13* downregulated (**Figure 3**). There are also changes in ectonucleotidase expression, particularly in *Enpp1* (upregulated) and *Entpd1* (downregulated) that are consistent between several data sets in mice. However, the majority of differentially expressed genes is only detected as differentially expressed in a few studies. Some inconsistencies in expression changes of some genes including upregulation of *P2RY6* and *P2RY12* in Zhou et al. (68), or upregulation of *P2ry12* and *Entpd1* in Rothman et al. (58), may be explained by differences in the experimental approaches (58, 68). A number of ALS-related changes in gene expression are similar to gene expression patterns from AD studies, including *P2ry12* and *P2ry13* (91) that are downregulated both in murine AD and ALS mouse models. Transcriptomic data sets for glial cells in Parkinson's disease are still largely missing, both from mouse models and human brain tissue. However, there was no clear association between PD risk genes and microglia or astrocyte populations in a recent study, in which single nuclei from substantia nigra and cortex were extracted and analyzed, suggesting that microglial purinergic alterations may play a less prominent role in PD than in AD (92).

In contrast to these transcriptomic data, numerous experimental studies have shown that microglial P2X7 protein levels are elevated in human AD brains, in multiple rodent amyloidosis models (93–98), in the PD rat model 6-OHDA as well as in the spinal cord of ALS patients and SOD1 mutant mice that serve as ALS animal models (99–101).

These findings are functionally important since enhanced activity of P2X7 drives cellular inflammation in several neurodegenerative pathologies. On a molecular level, Aβ-induced ATP release activates P2X7, which in turn results in ROS production (98, 102–104) as well as activation of the inflammasome and subsequent release of the cytokine IL-1β (82, 105–108). Similarly, SOD1G93A and TDP-43Q331K in ALS and α-synuclein in PD, contribute to oxidative stress and inflammation via microglial P2X7 activation (83, 109–111). On a functional level, it was demonstrated that inhibition of P2X7 decreases migration of microglia *in vitro*, whereas phagocytosis is enhanced *in vivo* in the J20 AD model (93) making it an interesting target. The functional role of P2X7 in the pathogenesis of ALS is less clear than in AD. On the one hand, SOD1 mutant microglia show reduced ATP degradation and enhanced ATP sensitivity *in vitro* (100). On the other hand, the onset of clinical symptoms seemed to be accelerated and disease progression exacerbated (112) in P2X7-deficient SOD1 mutant mice. This indicates a complex scenario, which has implications for potential treatment strategies, as the time point of P2RX7 counteraction seems to play a key role. Microglia seem to play a dual role in ALS, acting beneficially at early stages of the disease, while exacerbating disease pathology at later stages (113). Taken together, P2RX7 activation and subsequent inflammatory

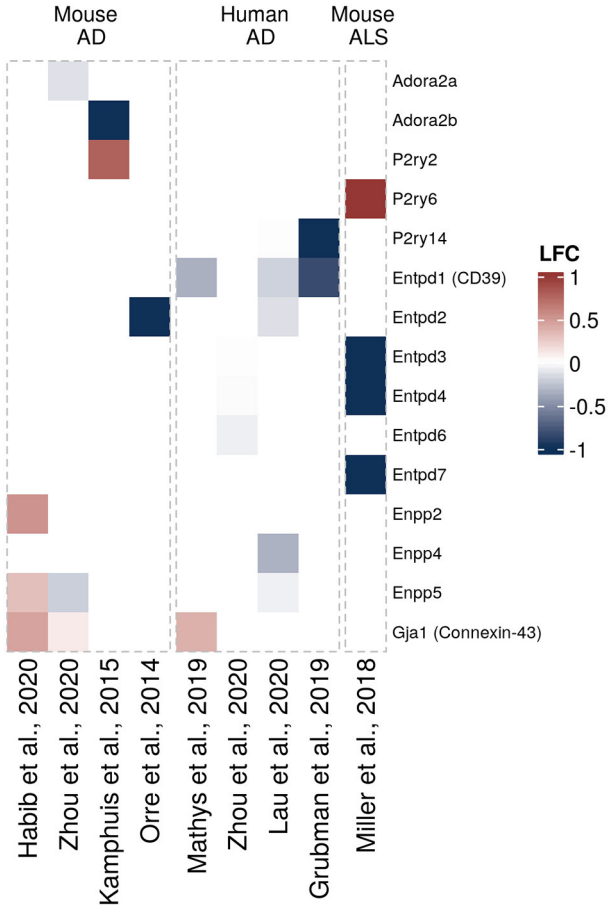

FIGURE 4 | Differentially expressed purinergic genes in astrocytes in neurodegeneration. Heatmap shows Log2 fold changes in expression of genes involved in purinergic signaling pathways between disease (AD or ALS, or relevant mouse models) and control samples. LFC values above 1 and below −1 are shown in the same color intensity for visualization purposes. Studies were selected according to the following criteria: transcriptomic analysis was performed on single cells or nuclei and allows disease/control comparisons for selected cell types, or on bulk-sorted glial populations; differential expression analysis results were provided by authors as supplementary material and were accessible. In total, nine data sets from eight studies were included for astrocytes [Orre et al. (69) (mouse, AD model, bulk), Kamphuis et al. (70) (mouse, AD model, bulk), Miller et al. (71) (mouse, ALS model, bulk), Mathys et al. (72) (human, AD, single-nuclei), Zhou et al. (68) (two data sets, mouse AD model and human AD patients, single-nuclei), Habib et al. (73) (mouse, AD model, single-nuclei), Lau et al. (61) (human, ALS, single-nuclei), Grubman et al. (74) (human, AD, single-nuclei)] (61, 68–74).

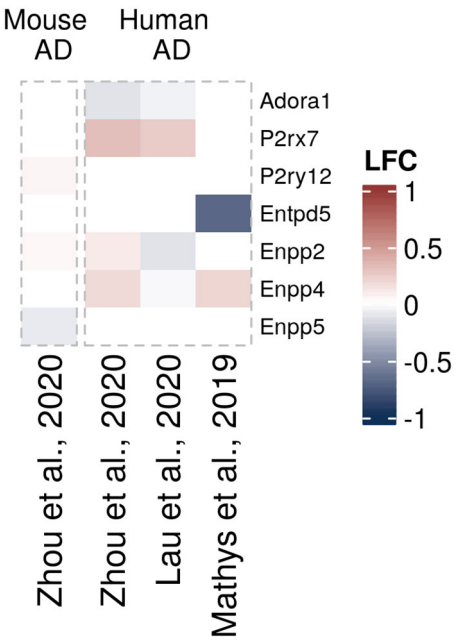

FIGURE 5 | Differentially expressed purinergic genes in oligodendrocytes in neurodegeneration. Heatmap shows Log2 fold changes in expression of genes involved in purinergic signaling pathways between disease (AD or relevant mouse models) and control samples. LFC values above 1 and below −1 are shown in the same color intensity for visualization purposes. Studies were selected according to the following criteria: transcriptomic analysis was performed on single cells or nuclei and allows disease/control comparisons for selected cell types, or on bulk-sorted glial populations; differential expression analysis results were provided by authors as supplementary material and were accessible. In total, four data sets from three studies were included for oligodendrocytes [Mathys et al. (72) (human, AD, single-nuclei), Zhou et al. (68) (two datasets, mouse AD model and human AD patients, single-nuclei), Lau et al. (61) (human, ALS, single-nuclei)] (61, 68, 72).

response represent a common purinergic dysregulation in proteinopathies such as AD, PD and ALS. However, further research needs to be conducted, especially in the fields of ALS and PD research.

As mentioned earlier, microglial morphology, motility and chemotaxis are regulated by the interplay of multiple different purinergic receptors including P1 and P2 receptors. The adenosine A1 and A3 receptors both play a role in microglial migration (33, 114). A1 is involved in morphological changes of microglia (115) and A3 contributes to the regulation of process extension (33). Consequently, the transcriptomic changes of *Adora1* (A1) and *Adora3* (A3) that can be detected in the transcriptomic studies in neurodegeneration (**Figure 3**) may contribute to alterations in microglial morphology, although functional studies are currently missing. In contrast to *Adora1* and *Adora3*, expression of *Adora2a* (encoding A2A) is barely detectable under normal conditions (**Figure 2**). Nevertheless, A2A has been shown to mediate microglia process retraction (116), to induce *Ptgs2* (Cyclooxygenase 2) expression (117) and proliferation of microglia (118). Interestingly, A2A was found to be increased in microglia in AD when using immunolabelling (119). Consequently, this upregulation could contribute to increased microglia cell number and decreased morphology around Aβ plaques. However, microglia-specific studies investigating the effects of P1 receptors in neurodegeneration are limited and further investigations will be required.

P2Y12 and P2Y13, encoded by *P2ry12* and *P2ry13,* are the main P2 receptors that regulate microglial surveillance motility and chemotaxis. More specifically, P2Y13 regulates microglial morphology and surveillance (35), whereas P2Y12 mediates directed motility in microglia, process extension and microglia migration toward stimuli including ATP release (34, 41, 88). *P2ry12* and *P2ry13* are strongly downregulated in the transcriptomic studies summarized here (**Figure 3**), a finding that has been confirmed in mouse and human tissue at the RNA and protein levels (74, 75, 91, 120). Given the functional role of these two receptors, a strong decrease of P2Y13 may contribute to a loss of microglial surveillance function in neurodegeneration that normally supports tissue integrity and downregulation of P2Y12 may affect microglial directed migration, including migration to the plaque site in AD. Furthermore, P2Y12 is important to balance hyperexcitability of neurons, most likely through the involvement of P2Y12 in cell process extension toward the synaptic site (41) and P2Y12 deletion exacerbates experimentally induced epileptic events in mice (121). It is thus tempting to speculate that a reduction of P2Y12 in microglia in AD may contribute to the increased occurrence of epileptic events in AD patients (122).

In addition, brain-derived neurotrophic factor (BDNF) serves, among other functions, as a signal to regulate neuronal activity, since BDNF-mediated disruption of chloride gradient and following disturbance of chloride-dependent GABAergic inhibitory function was reported (123–125). Exocytosis of BDNF is mediated by ATP-evoked P2X4 activation (123, 126–130) and regulated by A2A (118). Decrease of inhibitory signals favors increased neuronal activity and hyperexcitability, eventually causing epileptic seizures (131–134). Both of the mentioned purinergic receptors, P2X4 and A2A, that regulate BDNF release are upregulated in AD [**Figure 3**, (119)]. The upregulation and following release of BDNF may therefore also contribute to hyperexcitability and epileptic events in neurodegeneration.

Besides, P2X4 is involved in the chemotactic response of microglia, as pharmacological inhibition and downregulation of P2X4 lead to impaired chemotaxis *in vitro* (36). The transcriptomic studies we summarize here provide evidence that *P2rx4* is upregulated in different murine AD models (**Figure 3**), implicating that it could play a functional role in altering microglial chemotaxis in AD.

In summary, a number of purinergic receptors that regulate microglial morphology, motility and migration are dysregulated in neurodegeneration and may thus constitute pathways that contribute to impairment of these microglial motility functions. It will be important to validate this assumption in functional studies in the future.

Another important microglial cell function that is affected in neurodegeneration is the phagocytic capacity (87), which among many other mechanisms, is regulated by the UDP-sensitive

purinergic receptor P2Y6. Interestingly, it has been shown that the ion channel function of P2X4 is inhibited by P2Y6 upregulation. This suggests an interesting mechanism, in which P2RY6 would be key to switch from migratory to phagocytic behavior of microglia (135). Because *P2ry6* is downregulated in several murine AD models (**Figure 3**), this could mean that UDP-sensitive phagocytosis may be decreased in neurodegeneration. This could also be relevant for a process called "phagoptosis," i.e., the uptake of live neurons by microglia involving P2Y6 (136). Inhibition of P2Y6 prevents neuronal loss induced by low levels of Aβ, suggesting that microglial phagocytosis could be responsible for neuronal loss (136). However, the absence of P2Y6-dependent inhibition of *ex vivo* phagocytosis in 5xFAD mice indicates that in AD, purinergic receptors other than P2Y6 may regulate microglial phagocytosis (137). Unlike in AD models, *P2RY6* was upregulated in a PD model and in human PD brains, implying an opposite functional impact of P2Y6 in PD (101, 138).

In addition to P2Y6, there is also evidence that P2Y2 contributes to phagocytosis functions in microglia. Specifically, Aβ-induced microglial ATP release enhances phagocytosis of Aβ via P2Y2 activation in cultured cells (103, 139). *In vivo* data confirmed that heterozygous deletion of P2Y2 enhances β-amyloid plaque burden, indicating that downregulation of P2Y2 could contribute to AD pathology (140, 141). Apart from phagocytosis, pinocytosis induced by ATP or UTP is another mechanism that contributes to Aβ uptake by microglia. This process can be accelerated by yet another purinergic receptor, namely P2Y4 (142).

Altogether, since purinergic signaling regulates phagocytosis, a key function of microglia allowing clearance of pathologic protein aggregates, dysregulation of these purinergic pathways is likely to contribute to neurodegenerative disease pathology.

In light of the importance of purinergic signaling for the regulation of many microglia functions, the balance of extracellular purinergic ligands such as ATP, ADP, AMP and adenosine gains in significance. The concentration of extracellular purinergic elements is regulated by purinergic enzymes and transporters. Ectonucleotidases, namely CD39 and CD73, through ATP degradation and adenosine production, modulate microglial migration, microglia morphology and process elongation (114, 143, 144) and reduce phagocytosis (145). It is thus conceivable that downregulation and therefore loss of CD39 function, as observed in the spinal cord of ALS subjects (75) and in transcriptomic data from SOD1 mutant mice and murine amyloidosis models (**Figure 3**), could have detrimental consequences. Additionally, *Enpp1* in turn is upregulated, while microglial ENT1 and ENT3 are downregulated (**Figure 3**). This indicates that the regulation of extracellular ligands by ectonucleotidases and by cross-membrane transport may become disturbed in neurodegeneration, although the exact impact on microglia themselves and other CNS cells will require further investigation.

Astrocytes

Astrocytes become reactive upon neurodegeneration and display morphological, functional, and molecular changes. It has recently been re-confirmed in a consensus statement that reactive astrocytes should not be subclassified into binary categories but defined at multiple levels using morphological, molecular and functional parameters and considering temporal and spatial aspects (6). Interestingly, the presence of reactive astrocytes correlates with the cognitive status in AD (146, 147). This may be due to the fact that astrocytes are involved in a wide variety of functions in the CNS and during neurodegeneration lose some of these properties (**Figure 1**, right), such as their ability to maintain the integrity of the blood brain barrier, contribute to gliotransmitter release and glutamate uptake. This loss of homeostatic defensive function, which may correlate with astroglial atrophy or "asthenia," has been attributed to contribute to the propagation of cognitive decline in AD (148). At the same time, astrocytes gain potentially toxic functions and release pro-inflammatory molecules.

Based on the transcriptomic data we assembled here (**Figure 2**), it appears clear that mouse and human astrocytes show distinct expression patterns. *P2ry2*, *Enpp2*, *Enpp5*, and *Gja1* (encoding CX43) are upregulated in AD mouse models, although not consistently across the different studies, while only *Gja1* was found upregulated in human astrocytes in AD (**Figure 4**). Additionally, *Adora2a*, *Adora2b*, *P2y14*, *Entpd1* (encoding CD39), *Entpd2,* and *Enpp4* are downregulated in AD models or human AD. Furthermore, different sets of purinergic genes were found upregulated (*P2ry6*) or downregulated (*Entpd3*, *Entpd4*, *Entpd7*) in an ALS mouse model.

Functionally, the P2Y1 receptor signaling pathway is one of the major purinergic signaling pathways that may contribute to altered astrocyte properties in neurodegeneration, especially in AD. P2Y1 mediates calcium waves in astrocytes, which are linked to gliotransmitter release and synchronization of astrocytic syncytium, which is critical for synaptic plasticity (149–151). Unlike in the transcriptomic studies we summarized here, previous studies showed that astrocytic P2Y1 receptor expression is elevated in AD mouse models and human AD brain, especially in close vicinity to amyloid plaques (151, 152). This spatially restricted expression pattern may explain some of the discrepancies to the transcriptome data. P2Y1 activation has been linked to cognitive decline by enhancing astrocyte hyperactivity (153) and mediating astroglial network defects (151). Acute pharmacological inhibition of P2Y1 allows normalization of such defects (151), highlighting the therapeutical potential of targeting P2Y1 in AD. In particular, P2Y1 inhibitor injected through chronic ICV infusion reduced dystrophic neurite burden, improved astroglial function and long-term potentiation in an AD animal model (152).

Another study that shows discrepancies to the transcriptomic analyses shown here, found increased levels of CD39L1 (encoded by *ENTPD2*) at Braak stage III-IV in AD (154). Since CD39L1 modulates ATP metabolism and is particularly expressed in astrocytes (25), increased CD39L expression would reduce local ATP levels and thus dampen P2Y1-mediated astrocytic hyperactivity in CNS tissue with AD pathology, while its reduced expression, as suggested by the transcriptomic analyses, would have the opposite effect. Further studies are required to resolve these potential effects.

In agreement with the upregulation of *Gja1*/Connexin-43 (CX43) in astrocytes found in the transcriptomic analyses from AD mouse models and human AD brains we summarized here (**Figure 4**), Gja1 was detected among the astrocytic genes dysregulated in the proximity of amyloid plaques (155). CX43 contributes to ATP release (156). These effects seem to be mediated by amyloid pathology, as amyloid exposure triggers increased expression of CX43, both *in vitro* and in AD models (157–159). Deletion of astroglial CX43 in an AD mouse model was able to reduce astrocyte reactivity, ATP release, plaque-associated neuronal damage and improved synaptic function (160). In both, an *in vitro* and a mouse model of PD, astroglial CX43 was increased following exposure of rotenone, a neurotoxic substance (161, 162). Interestingly, CX43 activity is downstream of P2X7 and contributes to ATP release from astrocytes (163). Using a CX43 inhibitor, reduced α-synuclein deposits and attenuated neuroinflammation in a PD rat model were observed (164), highlighting the therapeutic potential of CX43 in several neurodegenerative diseases. CX43 and PANX1 are downregulated in an ALS mouse model (SOD1 mutant) at pre-symptomatic stage, while being upregulated at symptomatic stage (165), which again suggests that disease-dependent activation stages should be taken into account when designing therapeutic intervention.

Finally, despite weak expression in astrocytes under physiological conditions, adenosine A2A receptor was shown to be increased in astrocytes in both human AD brains (166) and amyloid models (49, 166, 167), although this finding is not supported by the transcriptomic data assembled here (**Figure 4**). Astrocytic A2A receptor is known for its regulation of glutamate and GABA uptake (49, 50) but also its *in vitro* effect on astrocyte gene regulation (168). Blocking A2A-mediated signaling was sufficient to mitigate memory defects in animal models (166).

Further investigations will be needed in the future to better understand whether A2A is clearly dysregulated in astrocytes or astrocytic subpopulations and whether astrocyte-specific A2A directly impacts astrocyte function in neurodegeneration.

Oligodendrocytes

In neurodegenerative diseases, oligodendrocytes become progressively damaged from various causes, such as neuroinflammation, direct effects from protein aggregates or oxidative stress, leading to myelin loss, disruption of energy transfer to neurons, and ultimately cell death (169). Regarding oligodendrocytes in neurodegenerative diseases, we could only find few transcriptomic data sets with significant differentially expressed purinergic genes. Of those, *P2rx7* and *Enpp4* genes appear to be upregulated in AD mouse models, and *Adora1* downregulated (61, 68, 72) (**Figure 5**).

All members of the oligodendrocyte lineage are very sensitive to adenosine and ATP released by neurons or other glial cells as they are able to migrate, differentiate and proliferate upon activation of A1, A2A and P2Y1 receptors through calcium signaling (29, 170–172). In AD, impaired repair of myelin is observed, which was postulated to contribute to disease initiation (173). During neurodegenerative conditions, increased ATP release induces activation of P2X7 in oligodendrocytes, which could serve as an early sensor of neuronal damage, promoting OPC migration *in vivo* (174). Despite several studies pointing out P2X7 implication in different demyelinating context as well as oligodendrocyte death, no experimental data was found in proteinopathies (175–178).

Also, the involvement of oligodendrocyte-specific connexins such as connexin-29 (CX29 or *Gjc3*), connexin-32 (CX32 or *Gjb1*) or connexin-47 (CX47 or *Gjc2*) in a manner similar to CX43 in astrocytes cannot be ruled out (179). A study showed a downregulation of oligodendrocytic CX47 in an AD model, contrasting with the increase of CX43 observed in astrocytes (180). The authors stipulate that such modification could favor astrocyte-astrocyte connection at the expense of astrocyte-oligodendrocyte communication, contributing to oligodendrocyte function impairment. Additionally, CX32 was suggested to be increased in PD and correlated with increased alpha-synuclein uptake (181). Thus, consequences of ATP release from oligodendrocyte connexins and pannexins in neurodegeneration remain to be investigated.

Overall, proliferation and maturation of oligodendrocyte lineage are strongly influenced by purinergic signaling. As such, an imbalanced purinergic signaling upon neurodegeneration could contribute to myelin loss. Noteworthy, most studies on oligodendrocytic purinergic signaling have been conducted on multiple sclerosis and other inflammatory diseases [for review see Welsh and Kucenas (182)]. The lack of data on the present topic should be addressed in the future as P2X7 was found significantly upregulated in oligodendrocytes in AD (61), which could imply a deleterious role in disease progression.

Purinergic Signaling Crosstalk in Neurodegeneration

Given the importance of purinergic signaling for inter-glial and neuro-glial communication, alterations in cell-specific glial purinergic signaling in neurodegeneration will inevitably lead to disruptions in purinergic inter-cellular crosstalk (**Figure 1**, right).

For example, although adenosine has been reported to be the main purine metabolite that is elevated in neurodegeneration, at least in AD (56), it cannot be ruled out that opposite changes may take place in a more restricted spatial or temporal manner. For instance, CD39, a critical enzyme for the extracellular hydrolysis of ATP, is downregulated in microglia in AD (**Figure 3**) (41), especially in the vicinity of amyloid-beta plaques. This could have two immediate consequences: firstly, a local lack of adenosine production, which would reduce adenosine receptor activation, and secondly, high local levels of ATP, which could trigger microglial P2X7 receptor. In line with this, global CD39 deletion was shown to increase ATP and decrease adenosine in the CSF (183). Furthermore, global and microglia-specific *Entpd1*/CD39 deletion is associated with neuronal hyperactivity and increased vulnerability to epileptic events, mediated by a decreased activation of the inhibitory neuronal adenosine A1 receptor (41, 183). The reduced expression of astrocytic *ENTPD1*/CD39 in human AD cases (**Figure 4**), could further amplify these events. Additionally, a local ATP increase would trigger other P2 receptors such as astrocytic P2Y1, which would further impair

neuronal synchronicity through astrocytic hyperactivation (151, 152). Adding further complexity, the ectonucleotidase ENPP1 is increased in microglia in AD (**Figure 3**). Whether this can–at least partially–compensate for decreased CD39-mediated ATPase activity and whether this affects astrocytic and oligodendrocyte functions has not been investigated. Hopefully, future studies will be able to answer these questions.

Furthermore, there is evidence that neuro-glial communication is affected by upregulation of glial purinergic receptors in neurodegeneration. For instance, microglial upregulated P2X7 (184–186) leads to an exacerbation of a pro-inflammatory microglial phenotype and increased release of proinflammatory molecules, which disrupt synaptic communication (187–189). Pro-inflammatory molecules like chemokines or reactive oxygen species also affect oligodendrocytes and astrocytes, causing oligodendrocyte cell death (190–192) and astrocytic reactivity (6), leading to further cell and network impairment. P2X7 is also upregulated in oligodendrocytes and has been implicated in oligodendrocytic cell death, which would critically alter inter-neuron communication and neuronal metabolic support. Furthermore, increased astrocytic P2Y1 in neurodegeneration, which is associated with astrocyte hyperexcitability, network synchronicity loss and increased ATP/glutamate release (152, 193) could in turn reinforce P2X7 activation in microglia and oligodendrocytes. Some studies have also reported increased A2A receptor expression in astrocytes, which has been shown to reduce glutamate intake and increase GABA intake (49, 50), thus favoring an excitotoxicity state, which is detrimental for synaptic function and reinforces reactive phenotypes of microglia and astrocytes.

In summary, increased ligand availability (ATP and adenosine) together with a distinct set of activated glial purinergic signaling pathways (P2X7, P2Y1, A2A) and loss of homeostatic neuronal and glial purinergic signaling pathways (A1, P2Y12, P2Y13) during neurodegeneration may alter the balance of purinergic signaling homeostasis. This in turn could lead to more cellular damage (excitotoxicity, neuroinflammation, myelin loss), loss of homeostatic functions (reduced energy delivery, reduced trophic factors, impaired synchronicity between networks) and promote protein aggregation (reduced phagocytosis of protein aggregates), thus ultimately favoring the progression of disease pathology in neurodegenerative diseases.

THERAPEUTICAL PERSPECTIVES

Recent advances have highlighted the beneficial effect of targeting purinergic signaling in neurodegenerative diseases, notably with the authorization of istradefylline, a selective antagonist of A2A receptors, in the co-treatment of Parkinson's disease in the USA in 2019 (194), following the approval in Japan in 2013. These first steps show that targeting purinergic signaling can be safe for use in neurodegenerative diseases and help to slow disease progression. Additionally, several drugs targeting purinergic signaling have gone into clinical trial in order to treat inflammatory diseases like rheumatoid arthritis, that are beyond the scope of this article [for review see Antonioli et al. (195)]. Noteworthy, proteinopathies are often associated with chronic low-grade neuroinflammation, reinforcing the potential benefits of using drugs targeting purinergic signaling (196).

P2X7 is one of the most evident targets of glial purinergic signaling in neurodegeneration, being upregulated in both microglia and oligodendrocytes at the protein levels (61). It was postulated that P2X7 is also expressed in neurons, but this was disproved by unbiased studies and the use of specific tools (197). Indeed, activation of P2X7 requires high level of ATP, which is found in neurodegeneration (198). It has been hypothesized that P2X7 acts as an early sensor, which represents a prerequisite for glial response to insults. However, chronic activation of P2X7 results in cell death, contributing to disease progression. Blocking P2X7 mitigates amyloid burden in AD models and improves synaptic plasticity, integrity and memory (94, 108, 199). Additionally, increased motor neuron survival and decreased microgliosis and inflammatory markers were shown after P2X7 inhibition at late pre-onset in SOD1 mutant mice (110). Furthermore, using P2X7- or P2Y6-selective antagonists, BBG or MRS2578 respectively, in an animal model of PD, neuroprotection and a reduced microglia reactive phenotype were observed (101, 200).

Adenosine receptors also represent suitable targets for glial modulation. Administration of the A2A receptor antagonist preladenant partly decreased *ex vivo* hyperactive motility around Aβ plaques in the 5xFAD amyloidosis model (89). In addition, preladenant restored microglial process extension toward tissue damage in the MPTP-induced PD model (201). Several studies have shown increased astrocytic A2A in pathological context such as AD, which suggests an abnormal function (166, 167). Additionally, A2A has also been studied as a therapeutical target in neuroinflammatory conditions involving myelin loss and T cell activation (202). Altogether, these results suggest that adenosine receptors like A2A could be targeted therapeutically to improve the disease-associated phenotype of glial cells in proteinopathies.

Apart from purinergic receptors, several studies have highlighted elevated astrocytic CX43 in neurodegenerative diseases, leading to increased ATP release. Together with other nucleotide transporter such as pannexin-1, they could be targets of choice in order to decrease ATP release and aberrant purinergic signaling in neurodegeneration (203).

CONCLUSION

As outlined here, purinergic signaling in neurodegeneration is not only altered in neurons, but in all CNS cell types, including glial cells. This highlights the potential to target purinergic signaling in a multi-cellular fashion. However, to develop this as a valuable strategy in the future, many functional aspects of purinergic signaling in glial cells need to be further elucidated. In particular, purinergic signaling in astrocytes and oligodendrocytes have remained ill-defined, as well as purinergic signaling pathways in proteinopathies involving FUS or TDP-43 aggregates. Furthermore, novel tools are needed that help to better define the cell-specific and the spatio-temporal aspect

of purinergic signaling in neurodegeneration. These challenges will need to be faced in the future to better understand this fascinating system, as within it potentially lies the hopes of tomorrow's treatments.

AUTHOR CONTRIBUTIONS

MP, AG, SK, AH, and KC wrote the manuscript. MP, AG, SK, DB, AH, and KC reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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The Pathogenesis of Parkinson's Disease: A Complex Interplay Between Astrocytes, Microglia, and T Lymphocytes?

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Parkinson's disease (PD), the second most common neurodegenerative disease, is characterised by the motor symptoms of bradykinesia, rigidity and resting tremor and non-motor symptoms of sleep disturbances, constipation, and depression. Pathological hallmarks include neuroinflammation, degeneration of dopaminergic neurons in the substantia nigra pars compacta, and accumulation of misfolded α-synuclein proteins as intra-cytoplasmic Lewy bodies and neurites. Microglia and astrocytes are essential to maintaining homeostasis within the central nervous system (CNS), including providing protection through the process of gliosis. However, dysregulation of glial cells results in disruption of homeostasis leading to a chronic pro-inflammatory, deleterious environment, implicated in numerous CNS diseases. Recent evidence has demonstrated a role for peripheral immune cells, in particular T lymphocytes in the pathogenesis of PD. These cells infiltrate the CNS, and accumulate in the substantia nigra, where they secrete pro-inflammatory cytokines, stimulate surrounding immune cells, and induce dopaminergic neuronal cell death. Indeed, a greater understanding of the integrated network of communication that exists between glial cells and peripheral immune cells may increase our understanding of disease pathogenesis and hence provide novel therapeutic approaches.

Keywords: Parkinson's disease, astrocyte, microglia, T lymphocytes, Th17 cell, neuroinflammation

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INTRODUCTION

Parkinson's disease (PD) is the second most common neurodegenerative disease affecting 1–2% of the population over the age of 65 (1) and it is estimated that the number of cases will exceed 12 million individuals in 2040 (2). PD is characterised by the degeneration of dopamine neurons in the substantia nigra (SN) of the midbrain, with concomitant loss of their axons that project to the striatum along the nigrostriatal pathway. This results in loss of the neurotransmitter dopamine which leads to the primary motor symptoms of PD, which were first described by James Parkinson in 1817 as a heterogeneous manifestation (3). These include bradykinesia, ataxia, tremor, rigidity, and postural instability which present themselves clinically once the levels of striatal dopamine

decrease by 70% (4). Another key pathological feature of PD is the presence of protein inclusions known as Lewy bodies (5). The protein α-synuclein (α-syn) is a major component of Lewy bodies (6) and its mutant forms can cause familial PD (7–10).

In the central nervous system (CNS), the continuous interactions of neurons, glia, and the microenvironment are key for the maintenance of neural homeostasis and failures in this homeostatic state leads to neurodegenerative conditions such as PD. In recent years the role of inflammatory processes in the death of dopamine neurons has come to the fore and is now considered vital to this process (11–13). Gliosis is a typical pathological feature of many neurodegenerative diseases and emerging evidence indicates that sustained activation of microglia and astrocytes is central to dopaminergic degeneration in PD (14). Indeed, activation of microglia in people with PD was described for the first time in 1988 (15), and this phenomenon has also been described in animal models (16, 17). Wilson et al. have recently described a PET ligand ^{11}C-BU99088 which is expressed on reactive astroglia, and as such, was reflective of astroglial pathology in people with PD and demonstrated a role for astroglia in the initiation and progression of PD (18). Astrocyte reactivity was also detected in the SN of patients with this condition [for review see (19)] and post-mortem studies of nigral tissue homogenates revealed multiple alterations in biochemical parameters attributed to astrocyte dysfunction including a global reduction of glutathione levels, mitochondrial damage, and accumulation of extracellular toxins (20). In addition, clinical and basic research have revealed a role for both the innate and adaptive immune system in PD [for review see (21, 22)]. In fact, the available data suggests that in PD there is a response not only by glial cells but also by peripheral immune cells, suggesting that an interplay between these cell types contributes to pathophysiology. We will review the role of astrocytes and microglia in PD, taking into account the emerging role of peripheral immune cells and its implications for disease pathogenesis.

ASTROCYTES AND THEIR ROLE IN PARKINSON'S DISEASE

Astrocytes are the most abundant non-neuronal cell type in the vertebrate nervous system (23) with the critical task of maintaining structural support and homeostasis. Their roles include provision of metabolic support, encapsulation of neuronal synapses (24), promotion of synaptogenesis (25), and control of the permeability of the blood brain barrier (BBB) (26). Classically they are recognised for their ability to secrete a number of neurotrophic factors such as glial derived neurotrophic factor (GDNF) and mesencephalic astrocyte derived neurotrophic factor (MANF); both of which have been shown to offer a degree of neuroprotection to dopamine neurons both *in vitro* and *in vivo* (27–29). However, in addition to this there is a growing appreciation of the role astrocytes play in neuroinflammation (30) in many neurodegenerative conditions including PD. Recently it has been shown that astrocytes become reactive in response to activated microglial secreted signals such as IL-1α, TNF-α, and C1q and as such adopt a pro-inflammatory phenotype (31, 32). Indeed, this astrocyte phenotype has been shown to exist in the post-mortem brain tissue of people with PD (31). Furthermore, astrocytes can also adopt a pro-inflammatory phenotype by endocytosis of α-syn released by neurons; they secrete cytokines, IL-1α, IL-1β, and IL-6 which are correlated to α-syn load (33). Moreover, α-syn accumulation in human astrocytes *in vitro* resulted in severe cellular stress which included mitochondrial, lysosomal, and endoplasmic reticulum deficiencies (34, 35). Indeed, these astrocytes responded to this stress by sending out nanotubes, which behaved like tunnels, and enabled the transfer of intracellular α-syn inclusions to nearby cells, indicating that astrocytes are critically important in the pathogenesis of PD (35). Interestingly, Yun et al., demonstrated that NLY101, a GLP-1R agonist is neuroprotective in the pre-formed α-syn fibril model of PD. It acts to prevent astrocyte stimulation by activated microglia and in so doing protects dopamine neurons and prevents behavioural deficits (36). Taken together, these studies would suggest that astrocyte dysfunction is a very strong contributor to the pathogenesis of PD.

Impact of Parkinson's Disease Related Genetics on Astrocyte Function

There are monogenic mutations identified in 20 genes that have been implicated in the pathogenesis of PD (37). Interestingly, a study by Zhang et al. (38) compared the transcriptome of human astrocytes to neurons, and found upregulation of some of these monogenic mutations in astrocytes was to a similar level and sometimes higher than that of neurons [for review see (39)]. This would strongly support the potential contribution of astrocytes to the pathogenesis of these familial forms of PD. Altered levels of these genes lead to many changes in astrocyte function including impaired glutamate uptake, liposomal homeostasis, lysosomal, and mitochondrial dysfunction and inflammatory response (**Table 1**).

DJ-1/PARK7

Some of these genes have been studied to a greater extent than others with respect to their roles in astrocyte biology. DJ-1, encoded by the *PARK7* gene, causes early onset autosomal recessive PD (54) and is probably the most extensively studied. Mullett and Hinkle utilised neuron-astrocyte co-cultures to demonstrate that siRNA knockdown of DJ-1 in mouse astrocytes impairs their ability to protect against neurotoxins such as rotenone relative to wild type control astrocytes (55). In addition, studies using DJ-1 knockout mice astrocytes from postnatal day 1 cerebral cortices have also shown that loss of this gene can cause alterations in cholesterol levels and glutamate uptake via regulation of the expression of flotillin-1 and caveolin-1 (40). Furthermore, Choi et al. demonstrated using astrocytes from DJ-1 knockout mice that its deficiency in astrocytes reduced expression of prostaglandin D2 synthase and subsequent secretion of prostaglandin D2 demonstrating that DJ-1 is involved in the regulation of the anti-inflammatory role of astrocytes through prostaglandin D2 synthase expression (41). Moreover, a study from the Kahle laboratory found that DJ-1 knockout mice astrocytes produced 10 times more

TABLE 1 | The role of genes that are causative in Parkinson's disease pathogenesis and their implications in astrocytes.

Gene/Protein	Model used	Main findings	References
Park 7/DJ-1	DJ-1 KO mice	• Alteration in cholesterol levels • Alterations in membrane fluidity and in lipid raft dependent endocytosis • Altered glutamate uptake capacity	(40)
Park 7/DJ-1	DJ-1 KO mice	• Regulates anti-inflammatory role of astrocytes through Prostaglandin D2 synthase expression	(41)
PARK2/Parkin	Parkin KO mice	• KO astrocytes exhibit exaggerated ER stress, JNK activation cytokine release and reduced neurotrophic factors	(42)
PINK1/PINK1	PINK1 KO mice	• Reduced astrocyte differentiation, increased p38 activation	(43)
PINK1/PINK1	PINK1 KO mice astrocytes	• Increased levels of iNOS, NO, TNF-α, and IL-1β	(44)
PINK1/PINK1	PINK1 KO rat pups	• PINK1 phosphorylation of ubiquitin is predominately in astrocytes	(45)
SNCA/α-syn	Human *SNCA* over-expressing primary foetal astrocytes	• α-syn changed expression of GF production and secretion, e.g., EGF, PDGF, VEGF, and their receptors • α-syn changed expression of IGF related proteins	(46)
SNCA/α-syn	A53T WT α-syn in mouse astrocytes	• Disrupted glutamate uptake • Increased neuronal cell death by overexpressing astrocytes	(47)
LRRK2/LRRK2	*G2019S- LRRK2*-Tg het mice astrocytes + α-syn	• Increased ER stress proteins • Increased cell death with α-syn • Mitochondrial dysfunction with α-syn	(48)
GBA1/GCase	Mice *GBA1 D409V* knockin astrocytes	• Defects in lysosomes • Defects in TLR4-dependent cytokine release	(49)
LRRK2/LRRK2	Human iPSC *LRRK2-G2019S* - astrocytes and neurons	• Impaired autophagy in astrocytes • PD astrocytes accumulate and transfer α-syn to healthy dopamine neurons	(50)
LRRK2/LRRK2	Human iPSC *LRRK2-G2019S*—MB astrocytes	• Downregulation of MMP2 and TGFβ	(51)
LRRK2/LRRK2	Human iPSC *LRRK2-G2019S*—astrocytes one patient also with *GBA N370S*	• Increased expression of α-syn, • Altered metabolism • Disrupted Ca^{2+} homeostasis • Increased cytokine release following inflammatory stimulation	(52)
GBA1/GCase	Human iPSC-derived astrocytes from GD1 with genotype *N370S/N370S* or *N370S/c.84insG*	• Abnormal α-syn accumulation due to Impaired lysosomal cathepsin activity • Increased inflammatory response	(53)

LRRK2, leucine rich repeat kinase 2; GCase, β-Glucocerebrosidase; α-syn, alpha-synuclein; iPSC, pluripotent stem cells; MMP2, matrix metalloproteinase 2; PINK1, PTEN-induced putative kinase-1; iNOS, inducible nitric oxide synthase; MB, midbrain; GF, growth factor; EGF, epidermal growth factor; PDGF, platelet derived growth factor; VEGF, vascular endothelial growth factor; IGF, Insulin like growth factor; GD, Gaucher disease.

nitric oxide (NO) than littermate controls when treated with lipopolysaccharide (LPS), a TLR4 agonist, and interestingly lentiviral reintroduction of DJ-1 restored the response to LPS (56). Taken together, these studies demonstrate that DJ-1 is an important regulator of the pro-inflammatory response and that its knockout in astrocytes deregulates inflammatory associated damage.

PARK2 and PINK1

Both *PARK2* and *PINK1* are expressed at similar levels in both astrocytes and neurons (38, 39, 57). Interestingly, astrocytes deficient in Parkin, encoded by the *PARK2* gene, demonstrated a stress induced increase in NOD2 expression; a receptor which integrates ER stress and inflammation and these astrocytes exhibited increased cytokine release and decreased secretion of neurotrophic factors (42). Parkin has also been shown to be involved in the response of astrocytes to an inflammatory signal; activation with TNF-α results in Parkin upregulation whereas activation with IL-1β results in Parkin downregulation (58). *PINK1* expression, which encodes the protein PTEN-induced putative kinase 1 (PINK1), is a loss of function mutation that is associated with early onset PD (59). Its expression increases during embryonic development and it was also shown to affect the development of GFAP-positive astrocytes (43). Indeed when PINK1 is knocked out in astrocytes, this causes them to exhibit reduced differentiation (43), reduced neurotrophic factor release, endoplasmic reticulum (ER) stress, JNK activation (42), and generate elevated levels of iNOS, NO, TNF-α, and IL-1β via NF-κB signalling (44). Interestingly, the steady state levels of PINK1 protein are very low even in cells that express PINK1, because PINK1 is normally targeted for degradation after mitochondrial import by a process that is dependent on mitochondrial membrane potential (60). While the high penetrance of PINK1 mutations establish its critical function for maintaining neurons the activity of PINK1 in primary neurons has been difficult to detect. However, Barodia et al. determined the levels of PINK1 in neurons, astrocytes, microglia, and oligodendrocyte progenitor cells

(OPCs) cultured from wild type and PINK1 knockout rat pups and showed that PINK1-dependent ubiquitin phosphorylation is predominately in astrocytes suggesting that the contribution of astrocyte dysfunction to PD pathogenesis warrants further investigation (45).

SNCA

SNCA encodes for the protein α-syn and missense mutations as well as duplication or triplication of this gene have been shown to lead to the development of rare hereditary forms of PD [for review see (61)]. Interestingly, one such mutation of the *SNCA* gene is also associated with an increase in α-syn aggregation in astrocytes; Gu et al. demonstrated that when *A53T* α-syn was selectively expressed in murine astrocytes it led to disrupted glutamate uptake and increased neuronal death (47). Indeed, *SNCA* is one of the PD associated genes that is expressed at higher levels in neurons than in astrocytes (38, 57). In 1997, Spillantini et al. reported that α-syn was the major component of Lewy bodies in the brains of people with idiopathic PD (6) and inclusions of α-syn have been found in astrocytes as well as neurons in post-mortem brains of people with PD (62, 63). Interestingly, it has been shown that neuronal α-syn can be directly transferred to astrocytes through sequential exocytosis and endocytosis (33, 64). In addition, overexpression of human α-syn in human primary foetal astrocytes resulted in significant changes in the profile of growth factor expression and release. The most remarkable changes were in epidermal growth factor (EGF), platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF), and their receptors as well as in insulin like growth factor (IGF) related proteins (46). Further analysis, using bioinformatics, revealed possible interactions between α-syn and EGFR and GDNF (46).

LRRK2 and GBA

LRRK2 encodes the protein leucine-rich repeat kinase 2 (LRRK2) and is causative for a dominantly inherited form of PD (65). Mutations in *LRRK2* have been recognised as genetic risk factors for both familial and idiopathic forms of PD (66). When the pathogenic mutation in *LRRK2*, *LRRK2-G2019S*, was expressed in mice it resulted in increased ER stress and cell death in astrocytes that was exacerbated by the addition of α-syn (48). *GBA* encodes an enzyme important in glycolipid metabolism, beta-glucocerebrosidase (GCase). The effects of a mutation in GCase encoded by the *GBA1* gene in astrocytes has been studied in mice with knockin of *GBA D490V*. This resulted in defects in lysosomes and in TLR4-dependent release of cytokines (49).

It is noteworthy that the vast majority of the studies carried out to date on the possible implications of the expression of PD related genes in astrocytes have been conducted in rodents. However, in the last 2 years a small number of studies have begun to emerge in the literature where human induced pluripotent stem cells (iPSC) carrying PD related mutations have been differentiated into astrocytes. It is not surprising that the majority of these are studying the *LRRK2-G2019S* mutation as this is the most common PD related mutation (67). A study by di Domenico et al. has shown that there is impaired autophagy in astrocytes with a *LRRK2-G2019S* mutation and that they can accumulate and transfer α-syn to healthy dopamine neurons (50). Another study patterned regional midbrain astrocytes from iPSC containing familial *LRRK2-G2019S* mutation and from healthy controls. RNAseq analysis revealed downregulation of genes such as *TGFβ1* and *MMP2* (51). TGFβ1 has previously been shown to inhibit the inflammatory microglial response in a rat model of PD (68), and MMP2 has been shown to be capable of degrading aggregates of α-syn (69). This suggests that these PD astrocytes have a reduced neuroprotective capacity and so may contribute to pathology (51). Further evidence for an increased expression of α-syn in astrocytes has been provided by Sonninen et al. using astrocytes differentiated from iPSC also with *LRRK2-G2019S* mutation. These astrocytes exhibited altered metabolism and increased cytokine release following inflammatory stimulation (52). iPSC-derived astrocytes with two different *GBA1* mutations (*N370S/N370S* and *N370S/c.84insG*) have also demonstrated abnormal α-syn accumulation due to impaired lysosomal cathepsin activity and had an enhanced inflammatory response (53). Taken together these studies are consistent with the role for both *LRRK2* and *GBA1* mutations in accumulation of α-syn and an increased inflammatory response in astrocytes as contributors to PD pathology.

Other Parkinson's Disease Risk Genes

In addition to the six PD risk genes mentioned above there are a number of other risk genes in which there is very high confidence that they are actual PD genes; these are *PLA2G6*, *ATP13A2*, *FBXO7*, and *VPS35* [for review see (37)]. Interestingly, the levels of *PLA2G6, ATP13A2*, and *FBXO7* gene expression has been shown to be the same in neurons as in astrocytes but that of *VPS35* is greater in astrocytes than in neurons (38, 39, 57). *PLA2G6* encodes Ca^{2+}-independent phospholipase A2 (iPLA2), an enzyme responsible for catalysing the release of fatty acids from phospholipids. A recent study by Strokin and Reiser in 2017 demonstrated that astrocytes from mice with a mutation in the *Pla2g6* gene, that were treated with Ru360 (a blocker of mitochondrial Ca^{2+} uniporter), or with rotenone, had a reduced rate of glutamate-induced Ca^{2+} influx, which was ∼2-fold lower than in wild type controls (70). The *ATP13A2* gene encodes a transmembrane lysosomal P5-type ATPase and its missense or truncation mutation leads to lysosomal dysfunction (71). A study using primary astrocytes from the mouse midbrain with a deficiency in *ATP13A2* demonstrated intense inflammation, which exacerbated dopamine neuron damage following MPP^+ exposure (71). Furthermore, this same study showed that astrocytes lacking *ATP13A2* had increased lysosomal membrane permeabilisation and cathepsin B release, which in turn led to the activation of the NLRP3 inflammasome. This led to increased production of IL-1β and suggested that there is a direct link between the astrocyte lysosome and neuroinflammation in PD (71). Lysosomal degradation was also shown using human iPSC, from healthy controls or from patients carrying a mutation in lysosomal *ATP13A2*, which were differentiated into midbrain dopamine neurons and astrocytes (72). This study showed that astrocytes rapidly internalised α-syn and when they were co-cultured with neurons, this led to a decreased accumulation of α-syn in neurons and as a consequence diminished interneuronal transfer of α-syn. Interestingly, loss of this protective function of astrocytes was seen with *ATP13A2* deficiency, suggesting that

this gene function in astrocytes, contributes partially to PD pathology (72).

Mutations in the *FBXO7* (*PARK15*) gene have been implicated in a juvenile form of PD. When this gene was deleted in tyrosine hydroxylase neurons this resulted in motor deficits, similar to the phenotype of *PARK15* patients (73). Vingill et al. also showed that the loss of *FBXO7* affected the assembly of proteosomes leading to reduced proteosome activity. This is in keeping with dysfunction of the ubiquitin proteosome system being central to neurodegeneration (74, 75). *VPS35* (*PARK17*) has recently come to the fore as a cause of late-onset familial PD (76). How this gene contributes to human PD is unclear however it has been suggested that *VPS35* mutations lead to mitochondrial dysfunction (77), and impaired lysosomal and autophagy function [for review see (76)], all of which contribute to PD pathogenesis. To date the potential effects that mutations in either *FBXO7* or *VPS35* might have on astrocyte function remain unknown; further experiments would be required to elucidate this.

MICROGLIA AND THEIR ROLE IN PARKINSON'S DISEASE

Microglia are the resident immune cells of the CNS. They originate in the yolk sac where they develop from early myeloid precursor cells. During embryonic development, primitive microglia migrate into the developing neural tube where they proliferate and populate the CNS (78). Due to the BBB, microglia lead a relatively sheltered existence compared to peripheral macrophages, although their functions remain the same. Their role is to continuously survey the microenvironment and respond to both physiological and pathological changes. In their capacity as the first line of defence in the CNS, they identify and remove unwanted material such as cellular debris.

The physiological roles of microglia include synaptic pruning and phagocytosis of apoptotic cells (79, 80). They may also aid synapse formation by releasing neurotrophic factors (81, 82). However, these cells do not escape the disruption that occurs during PD; they become activated and assume an inflammatory phenotype. Their expression of pattern-recognition receptors (PRR) allows them to respond to the presence of pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) in the microenvironment thereby resulting in microglial activation. Sustained pro-inflammatory activation of microglia, i.e., microgliosis, has been implicated in the pathogenesis of many neurodegenerative disorders including PD. These activated microglia assume an amoeboid morphology, coupled with increased phagocytic capacity. They are also highly reactive and are associated with expression of inflammatory molecules, such as pro-inflammatory mediators and reactive species, in addition to receptors for antigen recognition such as TLR2 (2). Their physiological cellular processes are also thought to be disrupted as a result, further contributing to disease pathogenesis.

While the clinical characteristics of PD have been well-defined, the aetiology of idiopathic PD is still unknown and under investigation. Among the potential causes of PD which are being considered are inflammation and microglia, whose role in PD was first posited in 1988 (15). There is evidence of microgliosis in the SN of PD patients as a result of post-mortem studies where significant CD68 and Iba1 immunoreactivity was detected. Since Iba1 immunoreactivity was co-localised with TLR2 it is thought that this receptor may play a key role in microgliosis (83). This localised microgliosis is also present in the MPTP mouse model of PD (84), the A53T α-syn transgenic mouse model of PD following administration of LPS (85) and the L-dopa-induced dyskinesia rat model of PD (86). Blocking microglial activation with a combination of matrix metalloproteinase inhibitor 1-DNJ plus ibuprofen is protective against dopamine neuronal loss (87) suggesting that microglia are key contributors to PD pathology.

The Impact of Parkinson's Disease Genes on Microglial Function

PD-associated genes are expressed by microglia, not just neurons and astrocytes. The products of these gene mutations are thought to affect the functioning of these cells (14), and can exacerbate microgliosis (**Table 2**). The dominant PD-risk genes *SNCA* and *LRRK2* promote neuroinflammation via activation of microglia and inflammatory signalling pathways such as NF-κB. Besides cell activation, PD-associated genes also disrupt other microglial processes including mitochondrial respiration and autophagy (99). Since autophagy is involved in regulating microglia inflammatory status (100, 101), its disruption has been reported to play a critical role in inflammation (102) and may also affect some of the key functions of microglia including phagocytosis (101). Furthermore, autophagy failure has been shown to promote intercellular propagation of α-syn (103) which in turn drives microglial activation and neuroinflammation. Mitochondrial dysfunction is another pathological feature in PD, as such the mitochondrial toxin MPTP, is commonly used to induce PD pathology in rodents in order to model disease pathology. Furthermore, pesticides paraquat and rotenone can also be used to induce parkinsonism via disruption of the respiratory chain in mitochondria (104). A number of genes associated with increased PD risk are linked to mitochondrial homeostasis. Among these are *SNCA*, *PARK2*, *PINK1*, *PARK7*, and *LRRK2* (104) (**Table 2**).

DJ-1/PARK7

DJ-1 is a sensor for oxidative stress which localises to the mitochondria when the cell is exposed to oxidative stress (90). DJ-1 mutations which lead to protein deficiency have been linked to PD, with 1% of hereditary cases of PD linked to this gene (88). DJ-1 deficient microglia demonstrate impaired uptake and degradation of α-syn, as well as impaired autophagy (88), and increased sensitivity to pro-inflammatory signals such as LPS (105, 106). This impairment of microglia function combined with increased sensitivity to inflammatory signals may leave PD patients with mutant DJ-1 prone to neuroinflammation. Monoamine oxidase is an enzyme which breaks down amine neurotransmitters such as dopamine. Monoamine oxidase inhibitors have been applied in combination therapy for PD and interestingly one of these, Rasagiline, has been shown to reduce

TABLE 2 | The role of microglia and Parkinson's disease risk genes in Parkinson's disease pathology.

Gene/Protein	Model used	Main findings	References
Park7/DJ-1	DJ-1 knockdown microglia	• DJ-1 deficient microglia have impaired uptake of α-syn • DJ-1 deficient microglia exhibit impaired autophagy which in turn affects α-syn clearance • DJ-1 deficient microglia show increased inflammatory response to α-syn	(88)
Park7/DJ-1	DJ-1 knockout mice	• DJ-1 KO enhanced expression of ICAM-1, IFN-γ, IL-1β, IL-17 and I-TAC, and enhanced dopamine neuron loss in response to LPS • DJ-1 deficiency sensitises microglia to release IFN-γ and I-TAC via enhanced NF-κB signalling	(89)
Park7/DJ-1	N9 murine microglia cells + DJ-1 shRNA	• DJ-1 deficiency increases mitochondrial and MAO activity, and reduces migration in microglia • DJ-1 deficient microglia have increased ROS, NO, IL-6, and IL-1β production and secretion following LPS insult • DJ-1 microglia have increased neurotoxicity	(90)
PINK1/PINK1	Primary glia cultures	• PINK1-deficiency in microglia causes increased NO production and inflammatory gene expression following LPS/IFN-γ stimulation • PINK1-deficiency reduces IL-10 expression in primary microglia	(44)
PARK2/Parkin	BV2, N9 and primary microglia	• Parkin knockdown exacerbates pro-inflammatory response to LPS via over-activation of JNK and NF-κB pathways • Parkin silencing attenuates progression of necroptosis	(91)
PARK2/Parkin	*Park2* KO microglia	• PARK2-deficiency has a priming effect on microglia leading to enhanced activation and NLRP3 induction following LPS exposure; due to loss of A20 negative feedback regulation	(92)
SNCA/α-syn	α-syn PFF mouse model	• MHCII immunoreactive microglia co-localise with α-syn in SN • SN microglia exhibit reactive morphology	(93)
SNCA/α-syn	Primary mouse microglia + A53T mutant	• Intensity of microglia activation is dependent on the type of α-syn • A53T promotes ROS production in microglia • A53T induces STAT1 phosphorylation and activation of MAP kinases activation, and induces microglia reactivity via NF-κB, AP-1, and Nrf2 pathways	(94)
SNCA/α-syn	BV2 cells and primary microglia	• A53T mutant α-syn promotes PHOX activation in BV2 and primary microglia • α-syn is recognised by the P2X7 receptor which is necessary for α-syninduced PHOX activation via the PI3K/AKT pathway	(95)
LRRK2/LRRK2	LRRK2 KO mice + LPS + paraquat	• LRRK2 KO prevents microglia activation and TH^+ neuron loss following intranigral injection of LPS and paraquat. Motor function is preserved. Mechanistic involvement of WAVE2 is proposed	(96)
LRRK2/LRRK2	Human iPSC-derived microglia	• IFN-γ increased LRRK2 expression in microglia • LRRK2 regulates function in microglia; *LRRK2-G2019S* mutant microglia had greater phagocytic capacity and decreased cytokine secretion • LRRK2 KO have defective glycolytic shift following stimulation with LPS	(97)
LRRK2/LRRK2	Human embryonic microglia	• Manganese increases LRRK2 expression and kinase activity • LRRK2 inhibition attenuates manganese-induced apoptosis, oxidative stress, TNF-α production, and MAPK signalling	(98)

KO, knockout; I-TAC, interferon-inducible T-cell alpha chemoattractant; MAO, monoamine oxidase; ROS, reactive oxygen species; NO, nitric oxide; LPS, lipopolysaccharide; JNK, c-Jun N-terminal kinase; NLRP3, NOD-, LRR- and pyrin domain-containing protein 3; PFF, pre-formed fibrils; PHOX, nicotinamide adenine dinucleotide phosphate oxidase; P2X7, purinergic receptor; PI3K/AKT, phosphoinositide 3-kinase/protein kinase B.

the pro-inflammatory phenotype in microglia and subsequently reduce neurotoxicity in a DJ-1 knockout model (90). This suggests that Rasagiline may be particularly successful in the treatment of PD patients with *PARK7* mutations.

SNCA

Encoded by the *SNCA* gene, α-syn is the main component of Lewy bodies in PD. Physiological roles of α-syn include synaptic vesicle trafficking and formation of the SNARE complex for exocytosis of synaptic vesicle contents into the synapse (14, 107). In PD, excess and/or mutant α-syn aggregates form fibrils. These neurotoxic fibrils act as an endogenous DAMP causing activation of microglia via TLR2 (2, 94) resulting in the activation of NF-κB and MAPK pathways and the production and release of pro-inflammatory mediators such as TNF-α, IL-6, CCL5, and IL-1β (2, 36). Interestingly, microglia activation in response to α-syn has been demonstrated to be mutant specific (94) (**Table 2**). While α-syn and Lewy bodies are characteristic of PD, it is possible that patients with *SNCA* mutations are vulnerable to enhanced levels of microgliosis and neuroinflammation. α-syn is now thought to aggregate in microglia as well as in neurons. α-syn binding to surface FcγRIIB receptors on microglia inhibits phagocytosis (108), furthermore α-syn accumulation within microglia can disrupt phagocytosis and lead to activation of microglia (109). TLR2 has been identified as a receptor for α-syn, capable of transporting secreted α-syn into the cytoplasm of microglia (110). Numerous studies have demonstrated a

role for α-syn in the activation of microglia, via TLR2 (111–113) and TLR4 (114). Besides TLR2 and TLR4, uptake of α-syn by microglia is facilitated by FYN kinase and CD36 (115). Internalised α-syn can act as a priming signal for the NLRP3 inflammasome, and also disrupts mitochondria function leading to the production of ROS which are capable of activating the NLRP3 inflammasome (115).

LRRK2

LRRK2 encodes the leucine-rich repeat serine/threonine-protein kinase 2. *LRRK2* is an incompletely penetrant gene associated with increased PD risk (116, 117). The *LRRK2-G2019S* mutation has a variable penetrance and is associated with both sporadic and familial PD. There is evidence that LRRK2 is capable of regulating macrophage and microglial motility and phagocytosis (118), mediated by RAB10 (119, 120). WAVE2, a novel interacting partner with LRRK2, regulates branched actin and the Rac1 effector molecule thereby promoting actin polymerisation and cytoskeletal reorganisation. This rearrangement of the cytoskeleton is crucial for the phagocytotic functions of myeloid cells (121). WAVE2 is proposed to be regulated by LRRK2, therefore LRRK2 deficiency can compromise this protein and subsequently reduce the phagocytic capacity of microglia (96, 121) (**Table 2**). Mutated LRRK2 is observed to alter mitochondrial morphology and increase mitochondrial fission in microglia. This was demonstrated via increased levels of Drp1, a mitochondrial fission marker, CD68, a microglia activation marker and TNF-α in LRRK2 mutant mice (122). Furthermore, inhibition of LRRK2 has been shown to attenuate microglial inflammatory response to TLR4 stimulation (123), and activation of microglia by extracellular α-syn (124, 125).

Other Parkinson's Disease Risk Genes

Besides the main culprits, there are many other genes associated with PD which are proposed to affect microglia function. *GBA* encodes the lysosomal hydrolase GCase; an incompletely penetrant gene associated with increased PD risk. There is limited literature pertaining to GBA microglia mutants in the PD context, the majority of papers discuss Gaucher disease. For example, in zebrafish, GBA knockout leads to early microglial activation, reduced motor activity, loss of dopaminergic neurons, and ubiquitin inclusions (126). Furthermore, GBA mutations can result in accumulation of glucosylceramide and complement activation which in turn drive inflammation (127).

Recent studies have revealed that autophagy dysfunction can be closely linked with PD pathogenesis. ATG5 (autophagy-related protein 5) is an essential component of the autophagosome, and deficiency results in impaired autophagy. A number of ATG5 variants have been found in the PD patient population (99, 128).

PINK1 and *PARK2* have been linked to autosomal recessive forms of PD (116). PINK1 deficiency in microglia has been linked to increased production and secretion of pro-inflammatory mediators, coupled with a reduction in the production and secretion of anti-inflammatory factors thereby favouring a reactive phenotype in microglia (44). The same phenotype is also associated with *PARK2* (**Table 2**). Deficiency in the associated protein Parkin, achieved via knockdown in microglial cells, has a priming effect and results in an enhanced response to the pro-inflammatory stimulus LPS (91, 92). PD patients with these genes may have greater levels of microgliosis as a result.

There is a clear role of microglia in the pathogenesis of PD and the major PD risk genes are a driving force behind this. However, it is becoming clear that microglia are not the only immune cells involved in PD. It is very well-established that the BBB is compromised in PD therefore allowing immune cells from the periphery to infiltrate the CNS.

AN EMERGING ROLE FOR THE PERIPHERAL IMMUNE SYSTEM IN PARKINSON'S DISEASE

Evidence of an important role for inflammation in the pathogenesis of PD is emerging and the data suggests that peripheral immune cells may contribute to this inflammation. In this section of the review we cover the latest studies demonstrating a role for the peripheral immune system and in particular T cells in PD. By way of introduction however a brief explanation of innate and adaptive immunity is outlined below.

The immune system is divided into the innate and adaptive arms which cooperate to defend against infection, however when dysregulated, immune responses can be important contributors to diseases. The innate immune system represents the body's first line of defence against invading pathogens, it is comprised of numerous cell types which carry out functions such as phagocytosis and antigen presentation. Innate immune cells include dendritic cells, macrophages and also microglia, which can be activated via PRR recognising not only PAMPS but also endogenous DAMPS including α-syn (129). On the other hand, adaptive immunity consisting of B and T lymphocytes provides a highly specific, targeted response capable of dealing with a variety of different intracellular or extracellular infections. T cells, which are either $CD4^+$ or $CD8^+$ are initially naïve until their T cell receptor (TCR) recognises its specific antigen presented by antigen presenting cells via MHC molecules (130). In general, endogenous antigens such as those from viruses are presented via MHCI to $CD8^+$ T cells whereas exogenous antigens are presented via MHCII to $CD4^+$ T cells (130). All cells in the body express MHCI and can activate $CD8^+$ T cells whereas only professional antigen presenting cells including dendritic cells, macrophages, B cells and microglial cells express MHCII and have the capacity to activate CD4 T cells. Once initially activated in the secondary lymphoid organs, naïve T cells differentiate into effector cells with specific functionalities tailored to the infection. $CD4^+$ T cells differentiate into one of the T-helper subtypes; Th1, Th2, Th17 cells which produce various cytokines and provide help to B cells (130). $CD8^+$ T cells differentiate into cytotoxic T lymphocytes which induce apoptosis of infected cells without affecting adjacent healthy cells. Naïve B cells, once activated by their specific antigen and with help from T helper cells, differentiate into plasma cells, producing antibodies which specifically target the antigen and promote clearance via phagocytosis (130). Once activated and differentiated in the lymphoid organs T and B cells traffic to the tissues where they become reactivated upon encounter of an

antigen and carry out their effector functions (130). Thus, the immune system has evolved to deal effectively with infection, however various genetic and other factors can conspire to result in inappropriate or chronic inflammation in response to altered proteins or self-antigens that manifests in autoimmune and inflammatory disease (131). Neuroinflammation associated with disorders such as Alzheimer's disease and PD attracts peripheral immune cells to the CNS, and the disruption of the BBB which is a pathological feature of these diseases means that there is a pathway for these cells to enter the brain parenchyma (132–134).

A Potential Role for T Cells in Parkinson's Disease

In the last decade there has been mounting evidence of a role for T cells in the pathogenesis of PD (**Table 3**). Despite their important role as part of the adaptive immune system there is little evidence of the involvement of B cells in PD. Brochard et al., identified both $CD8^+$ and $CD4^+$ T cells but not B cells or natural killer cells in the post-mortem brain tissue of PD patients (138). The presence of both $CD8^+$ and $CD4^+$ T cells was also evident in the MPTP and α-syn overexpressing mouse models of PD (138, 147). However, Theodore et al., observed both infiltrating B and T cells following injection with α-syn overexpressing adeno-associated viral vector (AAV) into the SN of mice (151). The differences with respect to the possible role of B cells in these studies may be attributed to the study model and human PD tissue vs. animal model, however more research is required before a definitive conclusion can be drawn.

Disruption of the BBB is commonly observed in PD pathogenesis, presenting an opportunity for peripheral immune cells to infiltrate into the brain. Infiltration of T cells from the periphery into the CNS has been observed in multiple PD studies (132, 144). Liu et al., demonstrated using MPTP treatment that BBB disruption increases the frequency of $CD4^+$ T cells in the SN of mice (144). The infiltration of $CD4^+$ T cells reduced the number of TH^+ neurons, decreased dopamine levels and increased the production of IL-1β and TNF-α. Importantly, Brochard et al., observing both $CD8^+$ and $CD4^+$ T cell infiltrates, determined using $CD4^{-/-}$ and $CD8^{-/-}$ mice that $CD4^+$ T cells, rather than $CD8^+$ T cells, were responsible for the neurodegeneration associated with their infiltration (138). As discussed above, the accumulation of misfolded α-syn protein contributes to dysregulation and inflammation in PD (152). Studies have demonstrated that T cell infiltration is associated with α-syn overexpression (147, 151), additionally the knockout of lymphocytes reduced α-syn protein aggregates in this PD model (147). These studies indicate a role for T cells in PD, however given that T cells are activated in an antigen-specific manner via MHC molecules, it is essential to understand the antigen specificity of T cells involved in PD and the possible contribution of different MHC haplotypes.

The Role of MHC and Antigen Presentation in Parkinson's Disease

In addition to the evidence above showing the presence of T cells in the SN of PD patients and mouse models, a role for T cells is also implied by the identification of specific MHC haplotypes and non-coding SNPs in MHC genes as risk factors for PD. Such MHC associations are a common feature of autoimmune diseases driven by auto-reactive T or B cells and are thought to result from preferential presentation of dominant self-epitopes by particular MHC molecules to auto-reactive T cells that have escaped thymic selection. Interestingly, Sulzer et al., demonstrated significantly enhanced T cell responses to immunodominant α-syn peptides in PD patients, relative to healthy controls (150). Furthermore, an immunodominant peptide was shown to bind with high affinity to the DRB1*15:01 and DRB5*01:01 alleles which were part of the MHC haplotype previously associated with PD (150). In a subsequent study, Lindestam Arlehamn et al., determined in a single longitudinal case study that this α-syn-specific T cell response was elevated prior to diagnosis and decreased thereafter (142). In the studies above, not all PD patients exhibited α-syn-specific T cell responses and responses were also observed in some healthy controls, indicating that in both PD and healthy controls these auto-reactive T cells escaped deletion during thymic selection. However, such auto-reactive T cells only become pathogenic if peripheral tolerance mechanisms are overcome and they become activated in the secondary lymphoid organs upon recognition of α-syn peptides presented via MHC molecules on activated dendritic cells (**Figure 1**). Tolerance can be overcome when self-antigens become post-translationally modified and then appear as neoantigens to which the immune system is not yet tolerised. Indeed, T cell responses to both native and post-translationally modified α-syn were observed in PD patients (150). The α-syn that activates naïve T cells in PD could either be peripherally derived or have drained from the CNS as suggested by the proposed body-first vs. brain-first subtypes, respectively (153). In addition, activation of the antigen presenting cell via PRR is required in order to induce the necessary co-stimulation signals required for naïve T cell activation and the source of this signal is unclear but could possibly be provided via DAMPs including α-syn.

Mitochondrial Antigen Presentation in Parkinson's Disease

Recent studies in a mouse model have identified an intriguing link between the PD-associated genes *PINK1* and *PARK2* and antigen presentation. *PARK2* and *PINK1*, which cause autosomal recessive forms of PD (116), maintain mitochondrial homeostasis through mitophagy (110). Loss of function mutations in the *PINK1* gene (a mitochondrial kinase) lead to disturbances in mitophagy, and mitochondrial fusion and fission (154). PINK1 deficiency is associated with increased ROS production, oxidative stress, abnormal mitochondrial function, and altered morphology (44). Mitochondrial dysfunction can result in the release of numerous pro-inflammatory factors such as mitochondrial DNA, RNA, ATP, and cytochrome c (110). The presence of ROS and DNA from damaged mitochondria can act as danger signals for activation of the NLRP3 inflammasome which is involved in neuroinflammation in PD (2, 155, 156). Mitochondrial dysfunction has been shown to over-activate the NLRP3 inflammasome in microglia resulting in dopaminergic neuron cell death (157). In its capacity as an activator of ubiquitin PINK1 cooperates with Parkin a ubiquitin

TABLE 3 | Evidence for the role of T lymphocytes in Parkinson's disease.

Specimens	Study groups	Main findings	References
Human PBMCs and plasma	PD patient and HS	• Decreased levels of Tregs observed in PD patients compared to controls • No significant difference in Th1, Th2, and Th17 levels between patients and control, however serum levels of IL-17A were decreased in PD patients • Serum levels of pro-inflammatory cytokines TNF-α, IL-1β, IL-6, and GM-CSF not significantly different between groups	(135)
Human whole blood	PD patient and HS	• Overall lymphocyte numbers were reduced • $CD4^+$ T cell levels were reduced, $CD8^+$ T cells increased • Treg cells were significantly reduced • IL-4 producing cells were significantly reduced, IFN-γ/IL-4 ratio was significantly increased	(136)
Human whole blood Rodent *in vivo*	PD patient and HS $MPTP^+$, $MPTP^-$ 6-$OHDA^+$, 6 $^-$OHDA-	• Decreased lymphocyte numbers, both B and T cells were reduced • $CD4^+$ T cells decreased, $CD8^+$ T cells were consistent • Significant increase in activated $CD4^+$ T cells and reduction in naïve and memory $CD4^+$ T cells • MPTP, but not 6-OHDA treatment, induced activation of $CD4^+$ T cells	(137)
Human post-mortem tissue Rodent *in vivo*	PD patient and HS $MPTP^+$, $MPTP^-$ $Tcrb^{-/-}$, $Rag1^{-/-}$, $CD4^{-/-}$, $CD8^{-/-}$	• Post-mortem tissue of PD patients and MPTP-mouse model demonstrates infiltration of $CD8^+$ and $CD4^+$ T cells in PD • MHCI expression was observed on dopamine neurons of the SN in PD post-mortem samples • MPTP-induced neurodegeneration decreased in the absence of T cells and cell death was attenuated by a lack of $CD4^+$ T cells not $CD8^+$ T cell	(138)
Human post-mortem tissue and hESC	PD patient and HS	• Microglia conditioned media from α-syn and neuromelanin activated microglia cause expression of MHCI in Vm-neurons • Vm-neurons are capable of inducing proliferation of cytotoxic T lymphocytes which in turn cause neuronal cell death	(139)
Rodent *in vivo*, *in vitro* primary microglia and $CD4^+$ T cells	AAV2-Syn or AAV2-GFP and WT or $MHCII^{-/-}$	• Overexpression of α-syn causes increased expression of MHCII on microglia • Knockout of MHCII attenuates α-syn-induced microglial activation in the SN pars compacta and dopaminergic cell loss	(140)
Human whole blood and isolated PBMCs	PD patient and HS	• PD patients demonstrate lower absolute counts but not frequency of Th17 cells and Tregs • PHA stimulation caused greater increase in IFN-γ and TNF-α in PD patients than HS, however no difference in IL-17A was observed and IL-10 was increased in HS but not in PD patients relative to non-stimulated cells • Co-culture of Teff and Treg cells caused ∼80% reduction of IFN-γ and TNF-α in HS but only ∼20% in PD patients	(141)
Human PBMCs	PD patient, AD patient and HS	• Increased α-syn specific T-cell reactivity prior to PD diagnosis declining post-diagnosis • Increased T cell reactivity in response to α-syn in PD patients compared to AD patients and HS	(142)
Rodent, *in vivo*, *in vitro* neurons and T cells	$MPTP^+$ and $MPTP^-$	• $MPTP^+$ mice demonstrate BBB disruption and infiltration of Th17 cells in SN • IL-17, IL-1β, TNF-α, iNOS, IL-22, and IFN-γ increase in the SN of $MPTP^+$ mice, BDNF and GDNF decrease • Co-culture of Th17 cells with Vm-neurons causes increased TNF-α and IL-1β, and induces neuronal cell death via LFA-1/ICAM-1	(143)
Rodent, *in vivo*, *in vitro* microglia, neurons and Th17 cells	$MPTP^+$ and $MPTP^-$	• MPTP caused BBB disfunction and increased IL-17A in SN only • Teff cells increase the frequency of $CD4^+$ T cells, reduce TH^+ cell numbers in the SN, decrease dopamine levels in the striatum and increase IL-1β and TNF-α levels in MPTP mice • Knockout of IL-17A alleviates these effects • IL-17A-induced neuronal cell death does not occur in the absence of microglia • Silencing IL-17A receptor on microglia prevents IL-17-induced cell death	(144)
Rodent, *in vivo, in vitro* Treg, Teff and microglia	$MPTP^+$ and $MPTP^-$	• Adoptive transfer of Treg attenuates MPTP-induced microglial activation and neuronal cell loss • Adoptive transfer of Treg increase neurotrophic factors; BDNF and GDNF	(145)
Rodent, *in vivo*, *in vitro* $CD4^+$ T cells	$MPTP^+$ and $MPTP^-$	• α-syn induced Th1/Th17 cell phenotypes from naïve T cells • Adoptive transfer of α-syn stimulated Th1 and Th17 cells caused neuronal death in the SN, and increases cell death observed in MPTP model	(146)
Rodent, *in vivo*	$WTS^+/Rag2^+$, $WTS^+/Rag2^-$, WTS^+/Rag^- and $WTS^-/Rag2^+$	• Increased levels of insoluble α-syn in Rag^+ mice compared to Rag^- mice • $CD4^+$ and $CD8^+$ T cells observed in the brain of WTS^+/Rag^+ mice but not in WTS^+/Rag^- or WTS^-	

(Continued)

TABLE 3 | Continued

Specimens	Study groups	Main findings	References
		• M1 phenotype prominent in WTS$^+$/Rag$^-$ mice compared with an M2 phenotype in WTS$^+$/Rag$^-$ mice which demonstrate increased phagocytosis of α-syn	(147)
Human iPSC and T cells	PD patient and HS	• Increased frequencies of IL-17 producing CD4$^+$ T cells in PD patients, no significant difference in IFN-γ or IL-4 producing cells • Co-culture of iPSC-midbrain neurons with Th17 cells/IL-17 increased neuronal cell death and levels of IL-17, IL-1β, TNF-α, and IL-6 in PD cells • Neuronal cell death in PD co-cultures occurred via IL-17/IL-17 receptor signalling, and potential activation of the NF-κB signalling pathway and preventing IL-17/IL-17 receptor interaction attenuated this	(148)
Human whole blood	PD patient and HS	• Reduced levels of B and T lymphocytes in PD patients	(149)
Human isolated PBMCs	PD patient and HS	• α-syn peptides presented by both MHCI and MHCII induce T cell proliferation in PD patients • T cells mainly either IFN-γ or IL-5 producing	(150)
Rodent, *in vivo*	AAV2-Syn or AAV2-GFP	• α-syn overexpression caused increased expression of CD68, IgG deposition and increased ICAM-1, IL-6, IL-1α, and TNF-α levels • COX-2 remained unchanged and iNOS expression decreased at a later time point • Increased levels of CD3$^+$ and CD45R$^+$ cells following α-syn overexpression	(151)

6-OHDA, 6-hydroxydopamine; Teff, T effector cells; Vm, ventral midbrain; HS, healthy subjects; PHA, phytohemagglutinin; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; Rag, recombination activation gene; Tcrb, T cell receptor beta; PBMC, peripheral blood mononuclear cell; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase 2; AAV2, adeno-associated viral vector, serotype 2; WTS, human wild type α-syn overexpressing.

ligase (158, 159). In addition to these established roles in maintaining mitochondrial homeostasis, PINK1 and Parkin have more recently been shown to play a role in suppressing immune responses by actively inhibiting the presentation of self-mitochondrial antigens via MHCI (160). Mitochondrial derived vesicles can arise by budding from the mitochondria and then fuse with lysosomes, processes that are dependent on the proteins SXN9 and RAB7 GTPase. Mitochondrial antigens are then processed and presented via the MHCI pathway to activate autoreactive CD8$^+$ T cells. PINK1 and Parkin however inhibited the activity of SXN9/RAB7 and thereby mitochondrial antigen presentation (160). These data suggest that familial PD associated with loss of function of PINK1/Parkin could involve an autoimmune component, whereby the presentation of self-mitochondrial antigens is enhanced in the absence of PINK1 or Parkin resulting in the activation of autoreactive CD8$^+$ T cells. These mechanisms were demonstrated in dendritic cells, macrophages, and fibroblasts; however, a key outstanding question is whether lack of PINK1/Parkin exerts similar effects on mitochondrial antigen presentation in astrocytes, microglia and neurons. If so, then naïve CD8$^+$ T cells could be initially activated in the periphery by dendritic cells, traffic to the CNS and cross the BBB where they would kill dopaminergic neurons upon recognition of their cognate mitochondrial antigen presented via MHCI. Furthermore, it is also unknown whether the absence of PINK1/Parkin could result in mitochondrial antigen presentation via MHCII to activate CD4 T cells. This is an important question given that neurodegeneration was shown to be dependent on CD4 T cells in a mouse model of PD (138). Interestingly mitochondrial antigen presentation was shown to be enhanced by an inflammatory insult and indeed in a follow up study, Matheoud et al. demonstrated that intestinal infection in PINK1 deficient mice promoted mitochondrial antigen presentation, the activation of CD8$^+$ T cells specific for mitochondrial antigens and motor impairment that was reversed by L-DOPA (161). Given that loss of function of PINK1 or Parkin results in symptoms of PD in humans but not mice (162), this suggests that it is a combination of the genetic mutation together with an inflammatory insult that leads to PD in individuals with mutations in *PINK1/PARK2*. Importantly, a role for the gut-brain axis in PD is implicated by these findings and supported by a study that showed that the gut microbiome was required to induce disease in an α-syn mouse model of PD. Furthermore, transfer of microbiota from PD patients into α-syn overexpressing mice resulted in exacerbation of disease symptoms when compared with transfer of microbiota from healthy controls (163). These studies suggest that dysbiosis could be responsible for triggering or exacerbating disease at least in some familial cases of PD and raise the interesting possibility of preventative or therapeutic targeting of the microbiome.

In summary, the evidence from post-mortem and MHC association studies in PD patients together with that from murine studies, suggests involvement of T cells in the pathogenesis of PD. However, there are still many unanswered questions and more extensive research is required. For example, to translate the autoimmune hypothesis of PD described above it will be important to identify mitochondrial antigen-specific T cells in PD patients with *PINK1/PARK2* mutations. The possible role of different T cell subtypes in PD is discussed below.

Th1 Cells in Parkinson's Disease

Th1 cells differentiate under the influence of cytokines IFN-γ and IL-12 released by antigen presenting cells, in combination with activation of the T-bet transcription factor. The main cytokines released by Th1 cells are IFN-γ and TNF-α, these cells are important in activation of B cells and increasing phagocytosis of microbes (130). PD studies have observed both increased frequencies of IFN-γ-producing Th1 cells in circulating blood from PD patients (136, 141), and no significant difference in Th1 levels (135). Kustrimovic et al., discovered decreased levels of the

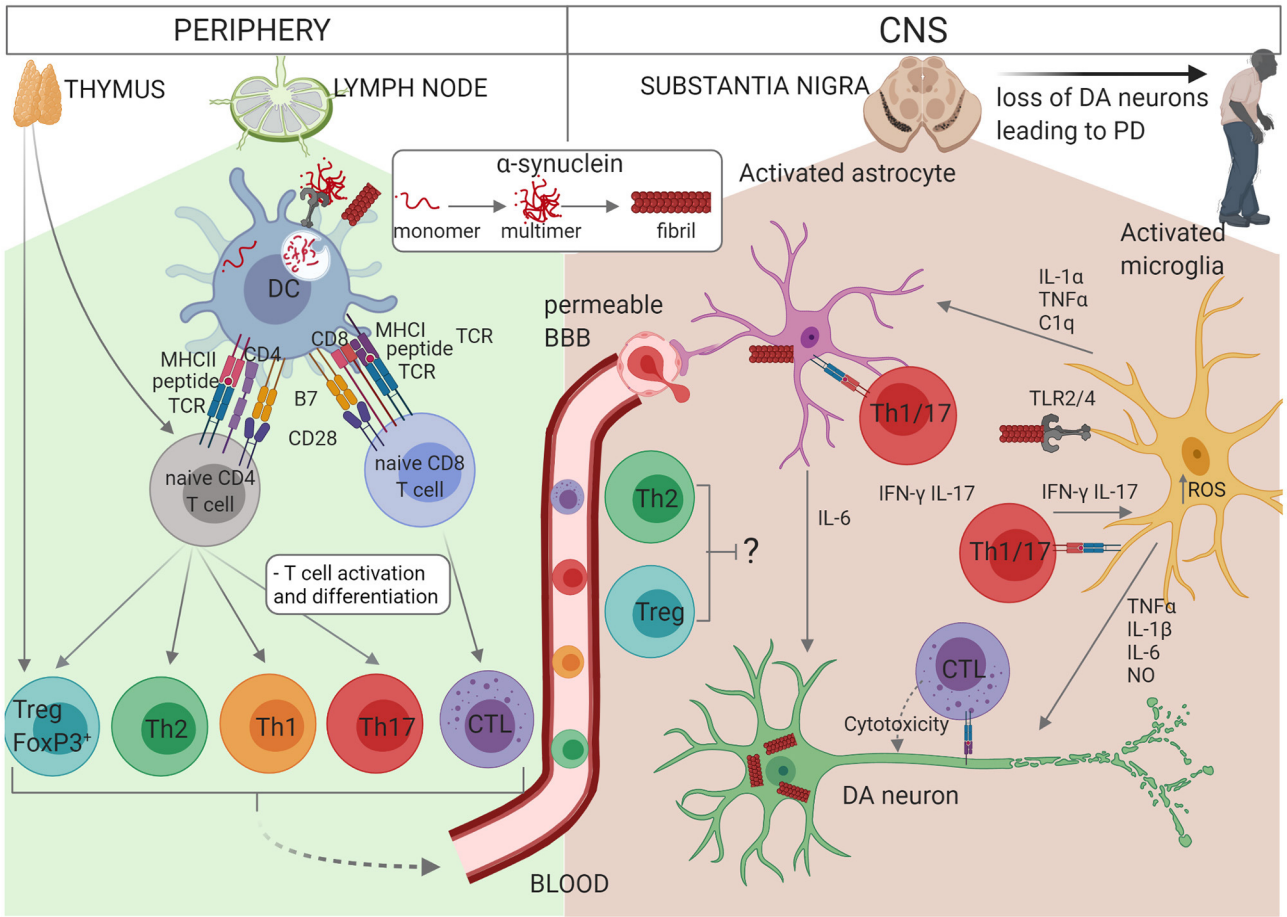

FIGURE 1 | Schematic representation of the interactions between glial cells and immune cells in PD. Self-reactive α-syn-specific naïve T cells may have escaped thymic selection, or alternatively post-translationally modified α-syn may be recognised by naïve T cells as a neoantigen. In individuals with *PINK1/PARK2* mutations, mitochondrial antigen presentation may also result in T cell activation. Autoreactive $CD4^+$ or $CD8^+$ T cells circulate through the lymph nodes where they may become activated by a dendritic cell (DC) presenting α-syn antigen via MHCII or MHCI, respectively. α-syn or other DAMPs activate the DC via PRR to express co-stimulatory molecules (B7) and cytokines which drive the proliferation and differentiation of effector T cell subsets. $CD4^+$ T cells differentiate into Th1, Th2, Th17, or Treg cells depending on the cytokine milieu and $CD8^+$ T cell differentiate into cytotoxic lymphocytes (CTL). Effector T cells traffic via the blood and extravasate through a permeable BBB into the CNS, where they re-encounter their α-syn antigens presented via MHCI on neurons or MHCII on astrocytes or microglia. Th1 and Th17 cells produce pro-inflammatory cytokines IFN-γ and IL-17 which contribute to the activation of astrocytes and microglia in synergy with other cytokines such as TNF-α, and CTL induce apoptosis in DA neurons. On the other hand, Treg and Th2 cells may protect against neuroinflammation. The accumulation of modified or aggregated α-syn is thought to be a key initiator of PD. α-syn accumulates within DA neurons and can also be secreted where it activates astrocytes and microglia. Activation of TLR2 or TLR4 by α-syn together with increased intracellular ROS activates the inflammasome. Activated microglia secrete TNF-α, IL-1β, IL-6, and NO which promote DA neuron degeneration. IL-1α, TNF-α, and C1q from activated microglia also activate astrocytes which in turn secrete IL-6, IL-1α, IL-1β, and NO. In addition, activated astrocytes exhibit decreased release of protective neurotrophic factors and impaired glutamate uptake. Thus, T cells, astrocytes and microglia cooperate to perpetuate neuroinflammation and loss of DA neurons in PD.

T-bet encoding gene, *TBX21* (141, 164) in PD patients compared with controls. However, naive $CD4^+$ T cells treated with α-syn polarised to a Th1 or Th17 phenotype causing cell death of TH^+ neurons in the SN and exacerbated MPTP-induced cell death (146). The evidence above suggests pathogenic potential for Th1 cells in PD, however the evidence is also sparse and conflicting, which clearly highlights the need for further research in this area.

Th17 Cells in Parkinson's Disease

A large amount of research exists into the role of Th17 cells in PD. TGF-β, IL-6, IL-1, and IL-23 along with the RORγt transcription factor are important in inducing differentiation of Th17 cells (130, 165). The signature cytokine produced by Th17 cells is IL-17. Upon binding to its receptor, which is widely expressed on epithelial and other cell types, it induces the release of chemo-attractants such as CXCL1 to recruit additional immune cells, particularly neutrophils. IL-17 also induces the secretion of anti-microbial peptides such as S100A8/A9 (166). Sommer et al., observed increased frequencies of IL-17 producing $CD4^+$ T cells in the peripheral blood of PD patients compared with control subjects (148) and infiltration of Th17 cells into the SN has been demonstrated in an MPTP mouse model of PD (143). In contrast however, a study using peripheral blood mononuclear cells (PBMCs) isolated from PD patients and healthy subjects

discovered lower absolute counts but not frequency of Th17 cells in PD (141). A separate study observed no difference in the levels of Th17 cells between PD patients and controls, with reduced IL-17A levels in PD patients (135, 141).

Although the conflicting studies above indicate that further work is required to determine whether enrichment of peripheral Th17 cells is a consistent finding in PD, there is experimental evidence to suggest a pathogenic role for Th17 cells in the context of PD. Stimulation of Th17 cells with α-syn was found to cause neuronal cell death in the SN in an MPTP mouse model of PD (146). Furthermore, co-culture of MPTP-treated neurons with Th17 cells further exacerbated neuronal cell death and increased IL-1α and TNF-α levels observed with MPTP-treatment alone (143, 146). Liu et al., determined that these effects were mediated via lymphocyte function-associated antigen 1 (LFA-1) and intracellular adhesion molecule-1 (ICAM-1) interactions and ablation of either ICAM-1 or LFA-1 attenuated the dopaminergic cell death observed in this model (143).

Importantly, Sommer et al., demonstrated that the co-culture of autologous iPSC-midbrain neurons and Th17 cells led to increased neuronal cell death in cells derived from PD patients compared with controls and this effect was not observed with non-autologous cell cultures (148). This suggested that antigens were presented in an MHC-restricted manner by PD neurons to Th17 cells which then induced neuronal death. It was determined that either IL-17 or the IL-17 receptor signalling and a potential activation of NF-κB signalling was responsible for the neuronal cell death observed in the co-cultures. Furthermore, attenuation of neuronal cell death in the PD co-cultures was achieved by blocking the IL-17 or the IL-17 receptor. Taken together the above studies indicate a role for Th17 cells in the pathogenesis of PD. Although much of the research to date has been undertaken in animal models, future studies like that of Sommer et al., could utilise human-based models of PD, and patient-derived models to determine whether these interactions translate to the human disease phenotype.

Th2 Cells in Parkinson's Disease

Th2 cells differentiate from naïve T cells under the influence of IL-4, and the activation of the GATA3 transcription factor. These cells produce IL-4, IL-5, and IL-13 (130). IL-4 activates IgE production causing mast cell degranulation, releasing histamines, and other pro-inflammatory cytokines. Additionally, IL-4 as well as IL-13 increase mucous production and IL-5 activates eosinophils, releasing various pro-inflammatory and cytotoxic proteins (167). In the context of neuroinflammation unlike inflammatory Th1 and Th17 cells, Th2 cells are usually considered to be anti-inflammatory and protective. Alvarez-Luquin et al., demonstrated no significant difference in Th2 cell counts in PD patients compared with controls, however there was a significant increase in IL-13 observed (135). In contrast, studies have observed lower absolute numbers and frequency of Th2 cells compared with the healthy subjects (136, 141), however, increased GATA3 levels were identified (141). Sulzer et al., observed that α-syn peptides activated mainly IFN-γ-producing Th1 and IL-5-producing Th2 cells. As anti-inflammatory, protective immune cells Th2 cells have the potential to alter the progression of PD pathology. However, as observed with Th1 and Th17 cells the existing evidence is often contradictory, and therefore inconclusive, indicating the necessity for further research into this area before any concrete conclusions can be drawn.

T Cell Regulation in Parkinson's Disease

It is well-established that an overactive, aberrant immune response is rooted in the pathogenesis of numerous inflammatory diseases. Therefore, proper functioning of the regulatory mechanisms is key to preventing pathology. CTLA-4 is a crucial regulator of T cell activation, upon activation T cells upregulate the expression of CTLA-4, its role is to preferentially bind the co-stimulatory B7 in place of CD28, having an inhibitory, rather than stimulatory effect (130, 168, 169). Importantly, Cook et al., discovered a reduction in CTLA-4 expression on the surface of T cells from PD patients following stimulation (169), indicating the possibility of a potentially unregulated T cell response occurring in PD.

Regulatory (Treg) cells play a key role in constraining effector T cells and prevent excessive inflammation and autoimmunity. Treg cells are either directly generated early in life in the thymus during T cell development or differentiate in the periphery from naïve $CD4^+$ T cells under the influence of TGF-β (130, 165) and they express the transcription factor FOXP3 which is necessary for their function. These cells produce IL-10 and TGF-β, which are important anti-inflammatory cytokines crucial to the regulation of the immune response and can also suppress effector T cells via various other mechanisms including via CTLA-4 (130). Reduced absolute numbers but not frequency of Treg cells have been observed in PD patients (135, 136, 141). Kustrimovic et al., also observed increased mRNA levels of FOXP3. Interestingly, T effector cells co-cultured with Treg cells only reduced IFN-γ and TNF-α by ∼20% in PD patients, compared to an ∼80% reduction observed in healthy controls, suggesting that Treg suppression in PD may be impaired (141). Reynolds et al., demonstrated a neuroprotective role for Treg cells in the MPTP mouse model of PD. The Treg cells were adoptively transferred into MPTP treated mice, a reduction in neuronal cell death, microglial activation, and increased production of both BDNF and GDNF were observed (145). Thus, Treg cells are likely to be protective in the context of PD and although there are some discrepancies, these data suggest that Treg cells may be numerically and or functionally impaired in PD and this may contribute to neuroinflammation.

The literature supporting a role for T cells in PD is growing, although there still remains evidence which contradicts this, as numerous studies have observed reduced levels of circulating T cells in the blood of PD patients compared with healthy subjects (136, 137, 141, 149). Similarly, most studies on T cell subsets presented contrasting findings. The discrepancies between studies may be explained in part by different methodologies; some studies have identified Th subsets via their production of signature cytokines whereas others used a combination of chemokine receptors. Similarly, the markers used to identify Treg cells were not always comparable between studies and identification of Treg cells is notoriously difficult as some of

the markers used can also be induced on non-Treg cells during inflammation. For this reason, a full panel of markers including CD4, CD25, CD127, and FOXP3 is required to most reliably identify Treg cells. Finally, it is worth noting that alterations in the frequency of peripheral T cell subsets may not reflect changes occurring in the inflamed tissues and a reduction observed in the periphery may be due to trafficking to the tissue. Thus, in summary, this area requires a greater amount of research before the precise role of these cells can be elucidated.

This review has outlined evidence demonstrating the role of microglia, astrocytes and even peripheral immune cells in PD pathogenesis. Logically, this leads to the question of whether there is potential interplay between these infiltrating peripheral immune cells and the resident microglia and astrocytes of the CNS in PD.

THE INTERPLAY BETWEEN GLIA AND PERIPHERAL IMMUNE CELLS IN PARKINSON'S DISEASE

Glial cells are crucial to the maintenance of homeostasis within the CNS and the disruption of this is linked to numerous CNS diseases. There appears to be little evidence available outlining potential interactions between peripheral immune cells and glia in PD, however the research into this area is increasing and is summarised in **Figure 1**. A recent study demonstrated antigen presenting capabilities in astrocytes. MHCII expressing astrocytes were identified in close proximity to $CD4^+$ T cells in the post-mortem brain tissue of PD patients and cultured human astrocytes exposed to pre-formed fibrils of α-syn expressed the T cell co-stimulatory structures, CD80, CD86, and CD40 (170), suggesting the capacity to activate $CD4^+$ T cells. Although Rostami et al., determined that cultured human microglia demonstrated poor antigen presenting capabilities, Harms et al., determined in mice that microglia exposed to α-syn increased their expression of MHCII, became activated and induced proliferation of $CD4^+$ T cells. Furthermore, the knockout of MHCII prevented microglial activation and dopaminergic cell loss in these mice (140).

ICAM-1 is present on immune cells and its binding to LFA-1 is key to the migration, extravasation and even activation of immune cells (171). Prajeeth et al., discovered that it is possible for Th1 and Th17 cells to induce reactive, pro-inflammatory astrocytes which microglia migrate toward and increase their phagocytic ability. In addition to this, the T cell activated astrocytes enhanced the infiltration of Th17 cells (172). In other neurodegenerative diseases, such as Alzheimer's disease, astrocytes expressing ICAM-1 have been observed in close proximity to LFA-1 positive microglia (173). Miklossey et al., discovered the occurrence of these key immune interactions in post-mortem brain tissue from PD patients. ICAM-1 expression by astrocytes in the SN of PD patients was observed, and co-localised to these areas were LFA-1 positive microglia and LFA-1 positive leukocytes (174), highlighting the presence of interactions between glia and immune cells in PD.

The ability of these immune cells to infiltrate into the brain in PD is demonstrated in numerous studies. BBB disruption in PD has been observed in both post-mortem studies and animal models of PD (132, 144) and is linked to increased infiltration of peripheral immune cells (144). Once the immune cells enter the brain parenchyma their functions include the release of various cytokines, which are capable of activating glial cells and causing neuronal cell death (175). Liu et al., observed increased levels of IL-17A following BBB disruption in the MPTP mouse model of PD. Addition of IL-17A to co-cultures of microglia and neurons resulted in microglial activation, TH^+ neuronal cell death and a decrease in dopamine levels and the inhibition of the IL-17A receptor on microglia was sufficient to attenuate these effects (144). The synergistic and additive effect of IL-17 with factors commonly secreted by astrocytes and microglia, such as IL-6, IL-1β, and TNF-α has been an area of great interest and numerous studies have demonstrated this phenomenon (176–180). The studies discussed in this review support the idea that inflammation is a key factor in the pathogenesis of PD and may be driven or compounded by T cells. However, there are important outstanding questions regarding the specificity of T cells involved in PD and whether auto-reactive T cells are involved only in certain subsets of PD patients such as those with genetic mutation, or whether they are more widely involved in sporadic PD. The role of the adaptive immune system in PD is a novel area of research and as such requires deeper investigation before a complete picture can be drawn of the role of these cells in PD pathogenesis and their therapeutic potential can be fully realised.

To aid in this, novel human-based models such as patient-derived iPSC and 3D culture systems should be utilised to determine if these findings from animal models translate to the human disease. As well as this, more patient studies will be required to determine what is occurring physiologically within PD patients and if this corresponds to what is observed within animal and *in vitro* human models.

IMPLICATIONS FOR THERAPEUTIC TARGETING

Our increased understanding of the critical role the immune system plays in PD has stimulated research into the viable therapeutic targets it presents; so as to allow determination of their potential as disease modifying therapies. As mentioned earlier, Yun et al., demonstrated that targeting the GLP-1 receptor with NLY01 prevented conversion of astrocytes to a pro-inflammatory phenotype by activated microglia and inevitably reduced neuronal cell death (36). As a long-acting GLP-1 receptor agonist, with the ability to cross the BBB, NLY01 is currently undergoing phase 2 clinical trials to investigate its efficacy in early PD (NCT04232969). Although results have yet to be published for NLY01, another GLP-1 receptor agonist previously FDA approved for treatment of type 2 diabetes mellitus, Exenatide, is currently in phase 3 clinical trials (NCT04154072) having successfully completed early phase 1 and 2 trials (181).

A second newly emerging PD therapeutic target is that of monoclonal antibodies, specifically those targeting α-syn. PRX002/RG7935, an IgG 1 monoclonal antibody targeting aggregated α-syn, has demonstrated safety and tolerability

in an initial phase 1 clinical trial and in a further phase 1b trial of multiple ascending-doses with a reduction in free serum α-syn observed (182). Phase 2 clinical trials are currently ongoing (NCT6868). Furthermore, BIIB054 another IgG 1 monoclonal antibody targeting α-syn has concluded phase 1 clinical trials (183) and is currently undergoing phase 2 (NCT03318523). However, these therapies involve regular injection with monoclonal antibodies to maintain an immune response, otherwise known as passive immunisation, another therapeutic avenue is the use of vaccines, or active immunisation in the treatment of PD. PD01A is an anti-α-syn vaccine, which maintains a higher affinity for aggregated rather than monomeric forms of α-syn. The results for the phase 1 clinical trial of this vaccine in PD patients demonstrated that it is safe and well-tolerated among treatment groups (184). Although these two strategies involve the use of antibodies to target the specified antigen, vaccination brings with it the added benefit of long-term immunisation however, passive immunisation enables treatment to cease if adverse side effects occur.

A phase 2 clinical trial is currently ongoing which investigates the use of the immunosuppressant drug, Azathioprine for treatment of PD. Azathioprine is an FDA approved, purine analogue and is used to treat numerous diseases, including multiple sclerosis. It acts by reducing B and T cell proliferation via nucleic acid synthesis inhibition (185). However, an important consideration for this therapy is the fact that it is an immunosuppressant, which exposes the patient to increased risk of infection and further illness. Another immunomodulatory therapy, Sargramostim, is an FDA approved GM-CSF that stimulates myeloid cell production and induces Treg cells. The phase 1 clinical trial demonstrated increased numbers and functionality of Treg cells while maintaining levels of T effector cells (186). Potentially enabling increased regulation of the immune response in PD.

As mentioned above, the gut-brain axis is being increasingly researched and studies have demonstrated a potential role for this pathway in PD. Importantly, gut microbiome alterations have been observed in PD patients (187) and faecal microbiome transplantation has been investigated as a therapeutic intervention for PD. A case report (188) and a preliminary study (187) have demonstrated potential benefits for this as a therapeutic strategy in the treatment of PD. Additionally, a clinical trial (NCT03808389) is ongoing in hopes that restoration of microbiome homeostasis will improve symptoms in PD patients.

These trials represent many varied and promising approaches targeting the immune system as a means of PD therapy which together may lead to future disease-modifying therapeutic strategies for the treatment of PD.

FUTURE DIRECTIONS TO INCREASE OUR UNDERSTANDING OF NEURO-IMMUNE CROSS TALK

The crosstalk between the brain and the immune system in PD is clearly complex with many questions remaining to be answered. Some of these many interesting questions include the following: (1) Is the increase or reduction of various T cell subsets in the blood of PD patients reflective of the situation in the CNS, or could for example a reduction in the blood vs. healthy controls merely indicate reciprocal trafficking to the CNS? (2) What role might the different PD associated genetic mutations have on crosstalk between glial and immune cells and how that crosstalk affects dopamine neuronal survival? (3) Is the peripheral activation of α-syn reactive T cells a primary event, or is it secondary to the aggregation/alteration/mutation of α-syn or other PD associated proteins in the CNS which are then released into the periphery to activate antigen specific naïve T cells? (4) Does T cell involvement only occur in a subset of PD patients who are genetically predisposed to T cell involvement as a result of their MHC haplotype, and are certain mutated peptides preferentially presented by particular MHC haplotypes? (5) What role might PD associated genetic mutations have on mitochondrial antigen presentation and could carriers of *PINK1* or *PARK2* mutations present with an autoimmune type of PD? (6) How might all of the queries above be influenced by PD medications?

The development of new research tools in recent years, including animal and various stem cell models, has already allowed a better understanding of astrocyte and microglial function and as these methods continue to improve, we can probe more deeply into how these cells communicate with one another and also begin to answer the questions posed above. Such tools include rapid and more cost-effective sequencing at single cells resolution at various stages of the progression of pathology (189). Others that have also become available are iPSC-derived neurons, astrocytes, and microglia (190), cerebral organoids (191), cerebral organoids containing microglia (192), CRISPR screening, and high content imaging (193). The major advantage offered by iPSC is that they allow the study of the known PD associated genetic mutations where genes are expressed at native levels without any forced genetic manipulation. To gain a better understanding of the mechanisms involved in glial and immune cell crosstalk will require a deeper knowledge of the proteome of T cell subsets, astrocytes and microglia and integration with multi-omic datasets. Research will also need to focus on functional studies to gain a better understanding of dysfunctional pathways (189) that may allow development of novel therapeutic targets and the continuing development of animal models will be critical to development of our increased understanding in this area. Many exciting discoveries have been made in this field in recent years; more are sure to follow.

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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The Many Faces of Astrocytes in Alzheimer's Disease

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Alzheimer's disease (AD) is a progressive neurodegenerative disease and is the most common cause of dementia in an aging population. The majority of research effort has focused on the role of neurons in neurodegeneration and current therapies have limited ability to slow disease progression. Recently more attention has been given to the role of astrocytes in the process of neurodegeneration. Specifically, reactive astrocytes have both advantageous and adverse effects during neurodegeneration. The ability to isolate and depict astrocyte phenotype has been challenging. However, with the recent development of single-cell sequencing technologies researchers are provided with the resource to delineate specific biomarkers associated with reactive astrocytes in AD. In this review, we will focus on the role of astrocytes in normal conditions and the pathological development of AD. We will further review recent developments in the understanding of astrocyte heterogeneity and associated biomarkers. A better understanding of astrocyte contributions and phenotypic changes in AD can ultimately lead to more effective therapeutic targets.

Keywords: neurodegeneration, Alzheimer's disease, biomarkers, reactive astrocyte, heterogeneity, single-cell sequencing, neuroinflammation

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INTRODUCTION

Alzheimer's disease (AD) is the most common form of dementia worldwide and was first described over 100 years ago by Alois Alzheimer. Alzheimer's disease is a prominent disease throughout the world with a significant impact on the health care system, estimated at nearly $500 billion annually (1). Currently, the FDA has approved few drugs for AD, which aim to improve quality of life but do not change or slow disease progression (2).

At this time the pathophysiological mechanisms of AD are not fully understood, and current therapeutic interventions are limited in efficacy. The pathological hallmark of the disease is the deposition of beta-amyloid (β-amyloid) plaques and the resulting formation of neurofibrillary tangles composed of hyperphosphorylated tau protein (3). Due to the location of these pathological markers within neurons, neurons have been the target of research. Ramon y Cajal eloquently demonstrated these pathological hallmarks decades earlier (4). Interestingly, Ramon y Cajal also noted reactive hypertrophic astrocytes that surrounded senile plaques and blood vessels with amyloid deposits in post-mortem AD patients (4). Thus, astrocytic changes due to neurodegeneration are not a new discovery. However, there has been minimal advancement in understanding the role of astrocytes in the development of AD. This lack of progress was likely due to insufficient technology and methods. Due to new innovative technologies, there is an increasing focus on elucidating the physiological changes within astrocytes during AD progression.

The astrocyte is a prevalent cell type within the central nervous system (CNS). They have diverse and vital functions within the CNS including contributions to synaptogenesis, ion homeostasis, neurotransmitter buffering, the blood brain barrier (BBB), and inter/intracellular communication (5). Furthermore, astrocytes are a heterogeneous group of cells with diverse phenotypes and functions specific to their origin regionally (5, 6). Currently, significant effort has been dedicated to investigating the distinct functions of astrocytes as it relates to neurodegenerative disease (5, 7).

This review will examine the current understanding of the roles of reactive astrocytes and potential astrocytic biomarkers unique to AD. We will further explore new technologies such as single-cell sequencing and its potential effectiveness in deciphering the phenotypic changes astrocytes undergo in the context of AD. Finally, we will examine how these technologies can help to dissect astrocyte states or subtypes during AD progression.

ALZHEIMER'S DISEASE PATHOLOGY

Alzheimer's disease is an irreversible brain disorder that slowly destroys memory and thinking skills. Two forms of AD exist, familial, and sporadic. Familial AD accounts for <5% of cases and is associated with three subtypes defined by unique genetic mutations (8). The first unique genetic mutation involves the amyloid-beta precursor protein (APP) gene, which controls the formation of the amyloid precursor protein. The APP role is not fully understood, but it is suspected that it helps direct the migration of neurons during early development (9). Mutations cluster around the γ-secretase cleavage site of APP, resulting in longer and more fibrillogenic β-amyloid (10). Two other genes implicated in familial AD are presenilin1 (*PSEN1*), and presenelin2 (*PSEN2*) (11). These genes encode for subunits of a complex of gamma (γ)-secretase, which is involved in the proteolysis and processing of APP. The sporadic or late-onset (>65 years old) of AD lacks a complete explanation for its development. However, there is a host of risk factors associated with the onset of the disease. For example, there is a genetic association of carriers of the Apolipoprotein E4 (*APOE4)* allele, Clusterin, and mutations in triggering receptor expressed on myeloid cells 2 (*TREM2*) (12, 13). Other risk factors for the development of sporadic AD are associated with both environmental and modifiable lifestyle factors (14, 15).

The gold standard of pathologic diagnosis of AD includes extracellular amyloid plaques and intracellular neurofibrillary tangles. Amyloid plaques aggregate within the isocortex and are found in all six cortical layers (16). β-amyloid deposition and plaque formation are accompanied by reactive astrogliosis and microglial activation (17). It has been shown in post-mortem specimens that neurofibrillary tangles were densely associated with those areas of the brain most affected by the disease, such as the hippocampus (18). The number of these tangles is correlated with severity of symptoms (19). Tau protein is a microtubule-associated protein (MAP) which aggregates into neurofibrillary tangles. It is necessary for the function and development of the nervous system and regulation of the normal function of neurons (20). In AD, tau aggregation secondary to post translational changes such as hyperphosphorylation, truncation, glycation, glycosylation, nitration, and ubiquitination results in the formation of neurofibrillary tangles in neuronal cytoplasm (20). For example, in AD, hyperphosphorylation of tau protein is produced by glycogen-synthase-kinase 3β, cyclin-dependent kinase 5 (CDK5), mitogen-activated protein kinase (MAPKs), Fyn, and many others (20). In addition, decreased phosphatases (which dephosphorylate tau) have been found in AD post-mortem specimens. A major phosphatase implicated in AD is protein phosphatase 2 (PP2A). Protein phosphatase 2 inhibition has been shown to increase tau hyperphosphorylation and has been demonstrated to be reduced in AD human brain specimens (20, 21). Thus, the imbalance of kinases and phosphatases together results in hyperphosphorylated tau and progression in AD.

Currently, the approved treatment for AD is directed at controlling symptoms. Further investigation is underway to evaluate possible disease modifying agents to attempt to slow the progression of the disease. Continued research efforts are required to clarify the pathological progression of AD and thus provide new targets for therapeutic development.

ROLE OF NORMAL ASTROCYTES

Astrocytes are specialized glial cells and have important roles within the CNS. They are essential to allow the brain to function as an organ and computational structure. Astrocytes have long been postulated and expanded upon since they were histologically depicted by Ramon y Cajal and his contemporaries (22). Initially it was believed that the astrocytes' role within the CNS was structural support for neurons. However, over 100 years ago, Ramon y Cajal found morphological heterogeneity of astrocytes (22). He described nine different morphological subtypes of astrocytes, which led to the development of multiple theories of the vital function of astrocytes. Unfortunately, due to the lack of technology these theories were left largely unproven and forgotten over the next century. More recently new methodologies have revealed that astrocytes execute a variety of essential functions including contributions to the BBB, synaptogenesis, ion homeostasis, neurotransmitter buffering, and the secretion of neuroactive agents (5) (**Figure 1**).

The understanding of the functional roles in cellular physiology first begins with understanding unique morphological characteristics. Typical protoplasmic astrocytes demonstrate a characteristic spongiform morphology (22). These astrocytes are ubiquitous throughout the gray matter. The astrocyte soma has numerous major branches with multiple secondary and tertiary branches that ultimately form interactions with other neurons and several synapses (23). Astrocytes regulate these synapses by secreting neurotransmitters to target pre and post synaptic sites and modulating function of adjacent neurons and astrocytes (24). This led to the development of the tripartite synapse, which is composed of an astrocyte and two neurons as a functional unit (24). The numbers of synapses an astrocyte interacts with

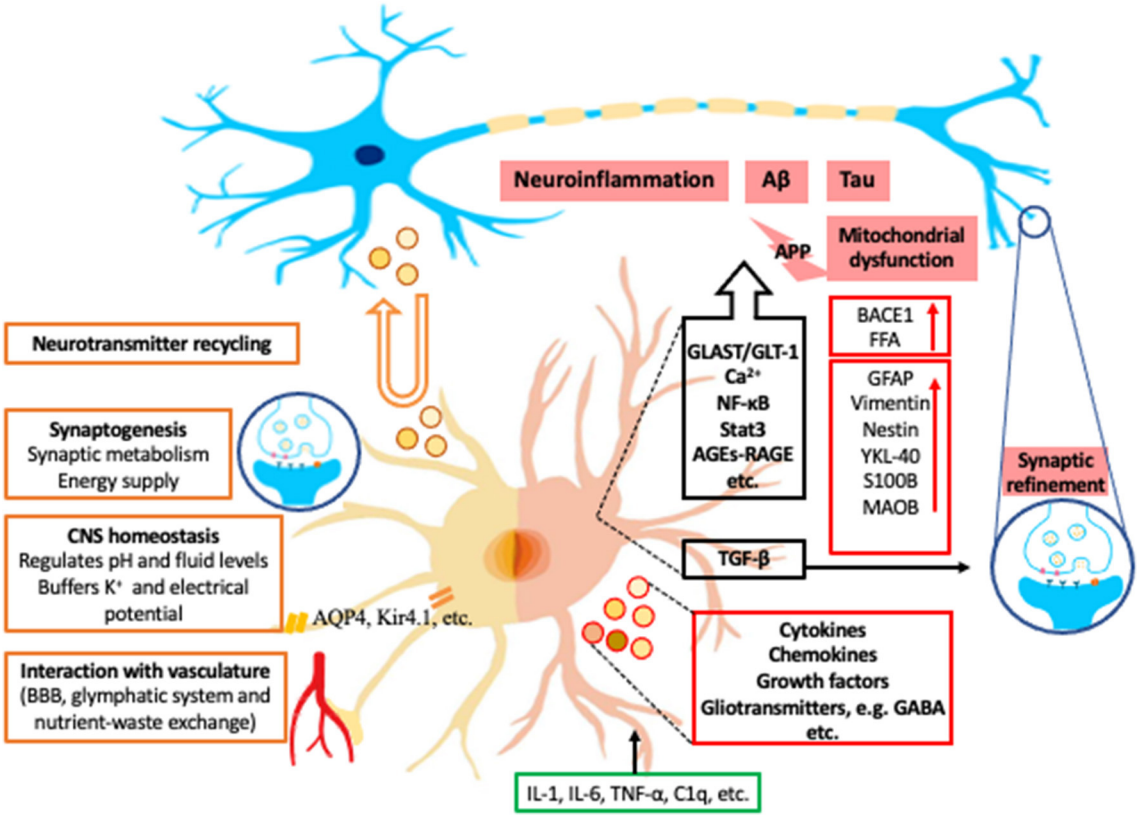

FIGURE 1 | Examples of roles of astrocytes in normal condition and in AD. The left half of astrocyte (yellow) depicts normal astrocyte functions, including neurotransmitter recycling (U-turn arrow and brown-outlined circles), synaptogenesis, central nerve system (CNS) homeostasis, and interaction with vasculature. The right half of astrocyte (Pink) depicts a reactive astrocyte in Alzheimer's Disease (AD). The black box contains some examples of the astrocytic signaling pathways related to AD. Green box indicates examples of inducing factors of reactive astrocytes from microglia. Red boxes are the representative intracellular and secretory molecules (red-outlined circles) and astrocytic makers expressed by reactive astrocytes in AD. Blue circles with icons are synapses. K^+, potassium; AQP4, aquaporin-4; Kir4.1, inwardly rectifying K^+ channel subunit 4.1; APP, amyloid-beta precursor protein; BACE1, β-site APP-cleaving enzyme 1; FFA, free fatty acid; GFAP, glial fibrillary acidic protein; YKL-40, chitinase-3 protein like-1; S100B, S100 calcium-binding protein B; MAOB, monoamine-oxidase-b; GLAST, glutamate aspartate transporter; GLT-1, glutamate transporter 1; Ca^{2+}, calcium; NF-κB, nuclear factor- kappa B; Stat3, signal transducer and activator of transcription 3; AGEs, advance glycation end-products; RAGE, Receptor for advanced glycation end products; TGF-β, transforming growth factor beta; GABA, gamma-aminobutyric acid; IL, interleukin; TNF-α, tumor necrosis factor alpha; C1q, complement component 1q.

are variable between circuits, brain regions and species. For example, a single astrocyte in the dorsolateral striatum can interact with 50,000 synapses while in the hippocampus stratum radiatum interactions can exceed 100,000 (5). This suggests that the morphological diversity of astrocytes is also related to their location within the CNS (6). The mechanism controlling the morphology of astrocytes remains ill-defined. More recently studies have proven that the loss of connexins and neuroligins alters astrocyte morphology, influencing synapse formation (25, 26). For example, Stogsdill et al. found that the morphological complexity of astrocytes relies on direct contact with neurons mediated by astrocyte neuroligin/neurexin interactions (26). Moreover, changes in astrocyte morphology in response to a pathological insult are ubiquitously noted in variety CNS diseases (27).

As stated, astrocytes are a mainstay in the development of the synapse. At a synaptic level, astrocytes have a plethora of roles to maintain normal synaptic activity. Astrocytes have known roles in synaptic metabolism and are implicated in glycogen processing and storage (**Figure 1**). Astrocytes can synthesize glycogen, provide glycolytic components for neurons during periods of high demand, and remove free radicals (28). Additionally, when neurons have further energy requirements, glycogen stores can be metabolized to form lactate which is transported from astrocytes to neurons via various monocarboxylate transporters (MCTs) (29).

A new waste clearance system formed by normal astrocytes has been discovered, deemed the glymphatic system (30). In this system, perivascular channels promote the efficient elimination of soluble proteins and metabolites, including β-amyloid, from the CNS. Astrocytes directly contact the CNS vasculature via their end feet and vasodilate or constrict to accommodate nutrient-waste exchange for neurons based on activity (5). For example, astrocytes highly express aquaporin-4 (AQP4) at the end foot processes and in turn can regulate extracellular matrix as well as cell membrane potential (**Figure 1**) (31). Aquaporin-4 is also vital

in the clearance and exchange of solutes in a water dependent manner between the cerebral spinal fluid (CSF) and interstitial fluid (31). Glymphatic dysfunction has been demonstrated in animal models of traumatic brain injury, AD, and ischemic disease, most likely related to dysfunction of AQP4 (32).

Moreover, CNS homeostasis is maintained by astrocytes by regulating pH and fluid levels in the brain, buffering potassium, and recycling neurotransmitters (**Figure 1**). For example, astrocytes possess a Kir4.1 potassium channel (33). In conjunction this allows astrocytes to control action potential firing (**Figure 1**) (34).

Overall, the roles of astrocytes are diverse and fundamentally important in the CNS. Therefore, any disruption regarding the normal roles of astrocytes can result in morphological and functional changes that result in pathological consequences. We will further review astrocyte function and the impact on AD.

ASTROCYTES IN ALZHEIMER'S DISEASE

Within the CNS astrocytes have a vital role in protecting and repairing neuronal damage (35, 36). Astrocytes respond to inflammatory substances and undergo a process known as reactive astrogliosis (34, 37). Astrocytes become reactive in response to multiple pathological conditions including acute injuries and progressive disorders such as tumor and AD (37). For example, Das et al. completed a meta-analysis of published astrocyte transcriptomic datasets in both acute and chronic neurodegenerative models, which displayed differing genetic astrocyte signatures (38). Specifically, in acute models, astrocytes were found to have upregulated expression for genes involved in protein synthesis, protein degradation, and antioxidant defense, whereas downregulated expression were noted for genes regulating chromatin structure and transcriptional repressors. Conversely, in chronic models, astrocytes were found to have upregulated expression for genes associated with extracellular matrix proteins and downregulated expression for genes associated with glycogenolysis, immune modulation, and antioxidant defense. Thus, astrocytes have unique genetic signatures in response to acute and chronic neurodegeneration.

Inflammation plays a prominent role in the development of AD (17). Reactive astrocytes release molecules including cytokines, chemokines, growth factors, and gliotransmitters (39). Astrocytes also release axon growth-promoting factors essential for axon growth and synaptic formation and maturation in response to injury (34, 40). Additionally, astrocytes increase neuronal viability and mitochondrial biogenesis, protecting neural cells from oxidative stress and inflammation induced by amyloid peptides (41).

The central hypothesis regarding the etiology of AD is that β-amyloid and neurofibrillary tangles produce acute inflammation which activates microglia, the primary inflammatory cells of the CNS, to release inflammatory mediators. This chronic inflammation results in neuronal dystrophy and ultimately leads to the clinical symptoms of AD. More recently, the role of astrocytes in the neuroinflammatory process has been closely evaluated (42, 43). For example, Orre et al., identified differentially expressed genes (DEGs) (807 upregulated and 571 downregulated genes) in AD astrocytes in APPswe/PS1dE9 double transgenic mouse model compared with WT mice (42). These up-regulated genes were enriched in inflammatory response, such as "defense response" and "positive regulation of immune response" and down-regulated genes were enriched in the regulation of synaptic transmission, neurogenesis, and brain and neuron development. Studies demonstrate that reactive astrocytes are induced by activated microglia that release IL-1alpha (IL-1α), IL-1beta (IL-1β), IL-6, tumor necrosis factor-α (TNF-α), and complement component 1q (C1q) (44–46). Furthermore, these cytokines can activate β-secretase and γ-secretase activity, cleaving APP, and stimulating β-amyloid formation by astrocytes, thereby supplementing neuronal β-amyloid production (47). For instance, Zhao et al. (48) demonstrated that primary astrocytes taken from mice and treated with a combination of INF-γ and TNF-α or IL-1β induced the secretion of β-amyloid. β-site APP-cleaving enzyme (BACE1) is an enzyme that works in conjunction with γ-secretase in cleaving APP to form β-amyloid. Previously it was thought that only neurons expressed BACE1, thus being the only cell capable of producing β-amyloid (35). Recent studies on post-mortem AD human brains have exhibited that astrocytes express BACE1 levels high enough to secrete β-amyloid (49). The mouse models that overexpress APP with the Swedish mutation (which is a mutation adjacent to the beta-secretase cutting site in the APP gene), displayed increased expression of BACE1 in correlation with elevated β-amyloid in reactive astrocytes, but BACE1 was not detectable by staining in resting astrocytes in the same mouse model (50). Therefore, inflammatory stimulation of astrocytes can induce BACE1 and in turn secrete more β-amyloid resulting in the progression of AD.

Another important protein associated with astrocytes and AD severity is S100B. During fetal development, it functions as a neurotrophic agent (51). S100B has been shown to induce astrocytes to become reactive in transgenic mice that overexpress *S100b* (52). Further studies have proven that cells, particularly astrocytes, that are S100B positive were located in higher concentrations around neuritic plaques in post-mortem AD brains (19). Specifically, there was a high concentration of these cells in areas of the brain known to be affected severely by AD, such as the hippocampus. The antiprotozoal medication pentamidine, which directly blocks S100B activity, has been studied in an AD mouse model (53). Pentamidine reduced GFAP, S100B and the receptor for advanced glycation end products (RAGE) protein expression, which are implicated in the neuroinflammatory response of astrocytes (53). Cirillo et al. also displayed the neuroprotective effect of pentamidine in CA1 pyramidal neurons (53). Thus, S100B is an important inflammatory regulator of astrocytes involved in phenotypic changes and progression of AD pathology.

Astrocytes are the primary source of cholesterol and lipid production and metabolism, and aberrant cholesterol processing has been implicated in AD development (54, 55). ATP-binding-cassette transporter 1 (ABCA1) is expressed on astrocytes and important in the lipidation of APOE. When cholesterol is abundant, neurons produce β-amyloid to suppress the expression

of the ABCA1, which results in increased deposition of β-amyloid (55). Increased free fatty acids have been suggested as a risk factor in the development of AD and high fat diets in animal models resulted in the accumulation of β-amyloid and plaque formation (54, 55). Ceramide, a metabolite of fatty acids, is increased in AD post-mortem brains and β-amyloid production (55). Furthermore, elevated ceramide levels have been shown to induce astrocytes to produce inflammatory cytokines; in turn this activated BACE1 activity and thus β-amyloid production in neurons (55). Therefore, cholesterol and fatty acids prompts astrocyte inflammatory response and further progression of AD.

Additionally, astrocytes recycle glutamate and GABA into glutamine, via glutamine synthetase (56). The glutamine from astrocytes is then used by neurons to produce more glutamate and glutathione (57), thus, providing additional nutrients and protection against reactive oxygen species. Glutamate Transporter (GLT-1) and Glutamate Aspartate Transporter (GLAST) are responsible for 90% of astrocytic glutamate uptake in the brain and are ubiquitous marker for astrocytes (57). In post-mortem AD brains and animal models mRNA of both *Glt-1* and *Glast* are reduced (**Figure 1**) (58). Astrocyte glutamatergic dysfunction, specifically GLT-1, is associated with the microenvironment of β-amyloid plaques in animal models. For example, when a mouse model lacking one allele of *Glt-1* is crossed with mice expressing mutations in APP and PS1, it accelerated memory impairment and increased β-amyloid (59). Additionally, β-amyloid oligomers and preplaque β-amyloid species have been demonstrated to decrease GLT-1 and GLAST in cultured astrocytes (60, 61). Thus, aberrant glutamate transport results in the disruption in the clearance of excitatory neurotransmitters and increased levels of β-amyloid and tau from astrocytes (62).

Aberrant gliotransmitter released by reactive astrocytes has been suggested as a possible role in AD symptomology, specifically memory loss (63). GABA is a major inhibitory neurotransmitter within the CNS. GABA is metabolized within astrocytes by GABA transaminase to succinate, entering the Krebs cycle, and used for energy production (64). Jo et al. displayed *in vivo* that reactive astrocytes produce GABA via MAOB and release GABA through the bestrophin-1 channel (63). GABA and MAOB content has been noted to be elevated in AD patients and mouse models (63, 64). The excessive GABA produced and released by reactive astrocytes results in activation of neuronal GABA receptors, which results in inhibition of glutamate release, and suppresses astrocytes' pro-inflammatory response (64). However, other studies have demonstrated decreased GABA levels in multiple areas of the brain in post-mortem AD samples (65). Although there are inconsistencies in how GABA influences AD progression, it is clear that GABA dysfunction within astrocytes is involved in AD pathogenesis.

Cellular senescence has been considered as a primary inducing factor of age-associated neurodegenerative disorders, and astrocytes can undergo stress-induced premature senescence (66). Recently, astrocytes have been shown to have decreased normal physiological function and increased secretion of senescence-associated secretory phenotype (SASP) factors in AD, which contribute to β-amyloid accumulation, tau hyperphosphorylation, and neurofibrillary tangle deposition (66). Senescent astrocytes share many similar phenotypes to reactive astrocytes, and it has been suggested that prior studies that focused on reactive astrocytes may have been focusing on senescent astrocytes (66). However, the topic of cellular senescence and its involvement in the development of neurodegenerative disease is controversial at this time (37). In order to verify if senescent astrocytes become reactive in the development of neurodegenerative disease, a significant amount of investigation remains. Specifically, defining molecular markers of normal aging astrocytes over multiple brain regions and compare with reactive astrocytes in neurodegenerative disease will be required (37).

SIGNALING CASCADES ASSOCIATED WITH ASTROCYTES IN AD

There are many molecules and signaling pathways that have been implicated in astrocytes in AD. We review some examples as the following. Astrocyte calcium regulation is regulated by a diverse set of stimuli that can alter intracellular levels. The pathological accumulation of β-amyloid results in inflammatory facilitators, such as bradykinin, to increase intracellular calcium via nicotinic receptors and the P13K-Akt pathway in cultured astrocytes (67, 68). Additionally, β-amyloid has the unique ability to interact with multiple astrocyte cell surface receptors, such as P2Y1, nicotinic receptors, and glutamate metabotropic mGlut receptor, increasing intracellular calcium (**Figure 1**) (69). Furthermore, Chiarini et al. (70) showed β-amyloid can bind to the calcium sensing receptor (CaSR) in human astrocytes, activating intracellular signaling, which resulted in the production and release of phosphorylated tau. Overall, there is sound evidence that calcium dysregulation is involved in the progression of AD. However, the receptors involved need further investigation to determine their diverse function and ability to be developed as a therapeutic target.

Another signaling cascade important in astrogliosis in AD is nuclear factor-kappa B (NF-κB). Nuclear factor-kappa B is a common transcription factor present in almost all cell types and has a critical function in numerous cellular processes. In the CNS, NF-κB requires strict control to ensure normal neuronal development and function (71). Abnormal NF-κB activation has been previously reported in multiple neurodegenerative diseases, including AD (72). Studies in rat models and post-mortem AD brains have shown an association of NF-κB with β-amyloid (73, 74). Specifically, NF-κB has been shown to have increased activity in neurons, astrocytes, and microglia due to exposure to β-amyloid. This activation results in induction of target genes in reactive astrocytes which induces astrocytes' morphological and functional changes. Nuclear factor-kappa B activation in reactive astrocytes is associated with elevated mitochondrial oxidative metabolism, limiting the supply of pyruvate substrate for neurons (75). The increased production of inflammatory substrates also influences neurons by inducing neuronal oxidative stress and apoptosis (72). The inhibition of NF-κB activation in AD mouse models has been demonstrated to slow the AD pathology and

improve neuronal survival and cognition, implicating that the use of NF-κB antagonists could provide therapeutic benefit (76, 77).

Signal transducer and activator of transcription 3 (STAT3) is a transcription factor that is activated through phosphorylation by Janus Kinases (JAK) in response to cytokines, growth factors, and intracellular mediators and has been implicated in the activation of astrocytes (78, 79). Ben Haim et al. showed that STAT3 is activated in reactive astrocytes of several murine and primate AD and Huntington's disease models (79). Conversely, two studies have been completed in AD mouse models with *Stat3* inactivation in astrocytes (78, 80). Ceyzeriat et al. demonstrated that inhibition of STAT3 *in vivo* resulted in reducing amyloid deposition, restoring synaptic deficits, and improved spatial learning (80). Similarly, Reichenbach et al. showed *Stat3* inactivation in astrocytes reduced plaque deposition and improved memory. However, it was also demonstrated that there was a reduction in pro-inflammatory cytokine activation (78). Altogether, these studies provide strong evidence of the potential for targeting STAT3 in astrocytes to slow the progression of AD (37). However, further investigation is required to determine the time point in which STAT3 activation in astrocytes results in pathological consequences.

Receptor for advanced glycation end products is a multi-ligand receptor of the immunoglobulin superfamily of cell surface molecules. They bind advance glycation end-products (AGEs) which are non-functioning glycated proteins or lipids that become glycated after exposure to sugars (81). Advance glycation end-products are associated with aging and have been implicated in neurodegenerative diseases such as AD (82, 83). Additional research has shown that AGEs form early in disease process of AD (84). Engagement of AGEs-RAGE converts a brief pulse of cellular activation to sustained cellular dysfunction and tissue destruction (85). Increasing expression of RAGE on the membranes of neurons and microglia is relevant to the pathogenesis of neuronal dysfunction and death of AD (86). Most pertinent to this discussion is the role of RAGE regarding astrocytes response. Reactive astrocytes surround the β-amyloid plaques and express RAGE (19). It has also been reported that β-amyloid can bind and activate RAGE on astrocytes and induce a pro-inflammatory state via a NF-κB pathway (87). Thus, targeting RAGE has the potential to reduce downstream inflammatory effects.

Transforming growth factor beta (TGF-β) is expressed ubiquitously within the CNS. During development, Transforming growth factor beta helps regulate neuronal survival, neurogenesis, synaptogenesis, and gliogenesis (**Figure 1**) (88, 89). Astroglia expression of TGF-β mediates synaptic refinement as well as glial scar formation (90, 91). Abnormal TGF-β hyperactivation has been detected in neurodegenerative disease and traumatic injury patients, and astrocytes and microglia are the predominate source (92–95). Studies *in vitro* have shown that TGF-β may promote cell survival since supplementing TGF-β protects neurons from β-amyloid toxicity (96). This protective activity was further demonstrated to be antagonized by β-amyloid (97). Furthermore, the expression of the TFG-β type II receptor, mainly expressed by neurons, is reduced in AD brains (72). Therefore, it is clear that TGF-β has both beneficial and detrimental effects. Further work is necessary to determine when TGF-β becomes detrimental in response to neurodegenerative disease.

ASTROCYTE BIOMARKERS IN ALZHEIMER'S DISEASE

Reactive astrocytes have become a focus of study in neurodegenerative disease and are essential players in the pathological process of AD and suggested to be targeted for novel therapeutics (34). Typically, immunohistochemical markers for reactive astrocytes are cytoskeletal components such as GFAP, vimentin, and nestin (98). However, the elevated marker such as GFAP alone is insufficient in categorizing astrocytes as reactive (37). Therefore, multiple markers are necessary to classify astrocytes as reactive.

Alzheimer's disease is classically diagnosed based on clinical criteria while the gold standard of definitive diagnosis is via neuropathology. Diagnosis based on clinical symptoms has a 30% misdiagnosis rate in comparison to neuropathological diagnosis (99). Thus, significant effort has been dedicated to develop clinical tools and tests to establish accurate early diagnosis and monitor the progression of the disease. Initial investigation for CSF markers began with classic astrocyte biomarkers such as GFAP, S100B, and glutamine synthetase, which proved to be not specific candidates due to their associations with homeostatic states of astrocytes and multiple other neurodegenerative diseases (100). Growing research has focused on a new astrocyte CSF biomarker Chitinase-3 protein like-1 (YKL-40), a protein commonly measured as a surrogate marker of neuroinflammation in AD (**Figure 1**) (101, 102). It has been linked to predict progression from normal cognition to mild cognitive impairment (MCI) and MCI to AD (102). Furthermore, elevated CSF YKL-40 has been confirmed to be correlated with phosphorylated tau levels at the early stages of AD (103). However, YKL-40 is not specific to AD alone and can be elevated in other tauopathies. Thus, further evaluation with multiple reactive astrocyte biomarkers is likely required to test for accuracy and progression of the disease.

Imaging modalities such as Positron emission tomography (PET) imaging and Magnetic resonance imaging (MRI) can assess aberrant astrocyte metabolism and detect accelerated brain atrophy. Currently, an inhibitor of the enzyme monoamine oxidase B (MAOB) [11C]-deuterium-*L*-deprenyl has been proposed as a PET imaging biomarker of reactive astrocytes (62, 104–106). MAOB is known to be up-regulated in GFAP-immunoreactive astrocytes. In post-mortem samples of individuals afflicted with AD, both the activity of MAOB and binding of *L*-deprenyl was found to be increased in multiple areas of the cerebral cortex (107). Furthermore, Gulyas et al. demonstrated that the highest binding of *L*-deprenyl occurs in the initial stages of AD (108). This suggests *L*-deprenyl as a promising PET imaging biomarker in the early diagnosis of AD.

The Alzheimer's precision medicine initiative was formed to review current blood-based AD biomarkers (99, 109). At this time no significant blood-based biomarker has proven to

be effective. Therefore, it is crucial to continue investigating and elucidate additional biomarkers for treatment/diagnosis and signaling pathways to target for a better understanding of disease progression. It is unlikely a single biomarker will be found for diagnostic purposes, but rather reactive astrocyte signatures will be required to increase the specificity of diagnostic tests (37).

HETEROGENEITY OF REACTIVE ASTROCYTES IN ALZHEIMER'S DISEASE

Most studies of AD focus on tissue samples that contain many cell types. However, this cannot distinguish the contribution of specific cell types in AD. Although astrocytes could be isolated from Alzheimer's samples based on specific cell markers (110), the functions of each subpopulation are not clear due to the heterogeneity of astrocytes. Most of the current studies for astrocytes emphasize two morphological groups: fibrous and protoplasmic astrocytes, located in the white and gray matter of the brain, respectively (111, 112). However, how many astrocyte subclusters/subpopulations in different regions of the brain and what functions they play are not clear. Recently, the single-cell (scRNA-seq) or single-nucleus RNA-seq (snRNA-seq) methods have been developed and make it possible to analyze cell subtypes or status (113). For human Alzheimer studies, most of the time, only post-mortem frozen samples are available. Single-nucleus RNA-seq is an effective method to analyze individual cells using these samples. Grubman et al. obtained 13,214 high-quality nuclei of entorhinal cortex samples from control and Alzheimer's disease brains (114). Astrocytes (2,171 nuclei) were clustered into eight groups (a1–a8) by bioinformatic analysis. The functional enrichment showed astrocyte subpopulations might have different functions. For example, a1 astrocyte subpopulation was enriched in genes linked to ribosomal and mitochondrial function neuron differentiation and heat shock responses; a2 was enriched in transforming growth factor TGF-β signaling and immune response; a3 and a8 were enriched in cellular responses to lipids and hormones; a4 was enriched in respiratory and mitochondrial genes, whereas a6 was enriched in synapse organization, action potentials, and ion channel activity. Mathys et al. isolated single-nucleus from 48 post-mortem human prefrontal cortex samples (24 individuals with high levels of β-amyloid and other pathological hallmarks of AD, and 24 individuals with no or very low β-amyloid burden or other pathologies) (115). A total of 80,660 droplet-based single-nuclei was sequenced and was used for identifying transcriptionally distinct subpopulations. Three thousand three hundred and ninety-two astrocytes (1,562 cells from no-pathology individuals and 1,830 cells from AD-pathology individuals) were clustered into 4 AD-associated subpopulations (Ast0–Ast3), which were related to the different pathological features of source brains. For example, Ast1 was associated with a high amyloid level, high Braak stage (V), low CERAD (Consortium to Establish a Registry for AD) score, low NIA (National Institute on Aging)-Reagan score, and pronounced cognitive decline, while Ast0 was associated with no pathological traits. They also found astrocyte subpopulations have different responses to AD pathology between female and male individuals: Ast1 was enriched in female cells, whereas Ast0 was enriched in male cells. Zhou et al. analyzed 66,311 individual nuclei from dorsolateral prefrontal cortexes, and found six sub-clusters (Astro0–5) in control (2,955 astrocytes) and AD (2,641 astrocytes) samples (116). Compared with control, Astro3 was depleted in AD. Genes related to the coordination of lipid and oxidative metabolism between neurons and astrocytes, such as *FABP5*, *HILPDA*, and *SOD2*, were down-regulated in AD samples; while the expression of *NCAN* and *COL5A3*, which had functions on the extracellular matrix, were up-regulated in Astro0 and Astro1. These results suggested AD astrocytes might have lost metabolic coordination with neurons in AD. Additionally, Lau et al. sequenced 169,496 nuclei from prefrontal cortical samples of 12 AD patients and nine normal control (NC) subjects (117). From these samples, 17,997 nuclei were of astrocyte origin. The subcluster analysis showed that astrocytes were grouped into nine clusters (a1–a9). The proportion of cells in each subpopulation revealed the relative proportion of a2, a4, a5, a7, a8, and a9 were similar between AD and NC samples. However, the proportion of a1 and a6 were 9.9 and 10.2% larger and a3 were 23.5% smaller in AD, compared to the NC samples. The differential expressed genes across conditions in a1, a3, and a6 demonstrate the DEGs in a1 and a6 were enriched in up-regulated genes and a3 were enriched in down-regulated genes in AD samples. The enriched genes in a1 and a6 were associated with stress response genes, while genes in a3 were associated with neurotransmitter metabolism. All the above results suggest astrocytes from different brain regions might have specific astrocyte subpopulations. These subpopulations can be related to different AD pathology.

Regarding animal models of AD, 5xFAD transgenic mice are commonly used (118). Habib et al. analyzed 54,769 single-nucleus RNA-seq profiles from eight 7-month male mice hippocampus [four WT mice and four transgenic models of AD (5xFAD) mice] to define the role of non-neuronal cells in AD progression (119). Seven thousand three hundred and forty-five WT and AD astrocytes were clustered into six subclusters. A continuous trajectory across astrocyte subclusters showed three end states [*Gfap*-low, *Gfap*-high, and DAA (disease-associated astrocyte, a specific cluster in AD compared with WT)]. In these six clusters, clusters 1 and 2 were *Gfap*-low states and cluster 6 was *Gfap*-high state astrocytes; cluster 5 might be the transitional-like intermediate state between the *Gfap*-low state and *Gfap*-high state; cluster 3 might be a transitional-like intermediate state between *Gfap*-low stage and the DAA (cluster 4). Exemplary studies of astrocyte heterogeneity outlined above is outlined in **Table 1**. The continuous expression spectrum suggested astrocytes have a dynamic activation process in AD.

As astrocyte isolation is challenging, the proportion of astrocytes obtained in total cells is around 10% in the published papers. Also, the current single-cell technologies are limited with low number of transcripts per nuclei/cell compared to bulk RNA-seq. Future improvement of technology and astrocyte isolation will enhance our understanding of astrocyte heterogeneity in AD.

TABLE 1 | Example studies of astrocyte heterogenity in AD.

Species	Reference	Tissue	Total nuclei no.	Astrocyte nuclei no.	Astrocyte clusters
Human	Grubman et al. (114)	Entorhinal cortex	13,214	2,171	a1–a8
Human	Mathys et al. (115)	Prefrontal cortex	80,660	3,392	Ast0-Ast3
Human	Zhou et al. (116)	Prefrontal cortex	66,311	Control (2,955) and AD (2,641)	Astro 0-5
Human	Lau et al. (117)	Prefrontal cortex	169,496	17,997	a1–a9
Mouse (5xFAD)	Habib et al. (119)	Hippocampus	54,769	7,345	Cluster 1–6

DISCUSSION

In summary, there is overwhelming evidence of the vital role astrocytes play in the pathophysiological development and progression of AD. The advent of technologies such as single-cell RNA sequencing and single-molecule imaging provides a greater understanding of the temporal and spatial progression of astrocytes that occurs during AD, which could serve as a framework for researchers to elucidate specific astrocytic biomarkers involved in AD progression (120). Specifically, studies using transcriptomics have allowed us to understand further that reactive astrocytes develop different molecular states during the progression of AD (37). As mentioned earlier, scRNAseq in AD models has demonstrated multiple stage-dependent conditions or subpopulations of reactive astrocytes (114–116). These studies signify the importance of characterizing the complex diversity and function of reactive astrocytes in each individual state to understand further the unique role these changes have in AD progression (114, 115). Therefore, it is not as simple to classify reactive astrocytes in AD as protective or toxic. Understanding the molecular changes at a single-cell level could also provide insight on the time point in which therapeutic intervention against reactive astrocytes can be applied, to harness AD progression and symptoms. The combination of powerful technologies such as viral gene transfer, electrophysiology, and optogenetics with transcriptomics can further elucidate the functions of reactive astrocytes in AD (37, 121).

Additionally, the roles and mechanisms of regulatory RNAs, such as long non-coding RNAs (lncRNAs), are underexplored in AD (122, 123). Currently, studies have demonstrated the regulatory role of lncRNA as it relates to tau hyperphosphorylation and others have suggested the utility of lncRNA as a biomarker for AD (124). These studies have provided the exciting potential of lncRNA as both diagnostic and therapeutic targets for AD.

Another critical consideration in elucidating the pathophysiological mechanisms of AD and determining fruitful therapeutic targets is ensuring we select appropriate *in vitro* and *in vivo* study models. Most cellular spatial information regarding cellular relationships to β-amyloid and neurofibrillary tangles is lost when isolating mRNA samples (125). Similarly, morphological and transcriptomic comparisons on human and mouse reactive astrocytes have revealed significant differences (37). This exemplifies the inherent limitations of *in vitro* studies and animal models in AD, and the difficulty in interpreting results when comparing studies with post-mortem specimens. Human induced pluripotent stem cells are currently increasingly employed in basic science research and can help narrow these differences (37). Furthermore, using multiple genomic techniques in combination, such as spatial transcriptomics and *in situ* sequencing, provides a benefit in preserving cellular spatial information (125). In conclusion, a consensus regarding appropriate research models and the integration of multiple "omic" modalities could provide improved diagnostic and therapeutic targets in reactive astrocytes.

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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