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Non-neuronal cell heterogeneity in the nervous system during health and disease

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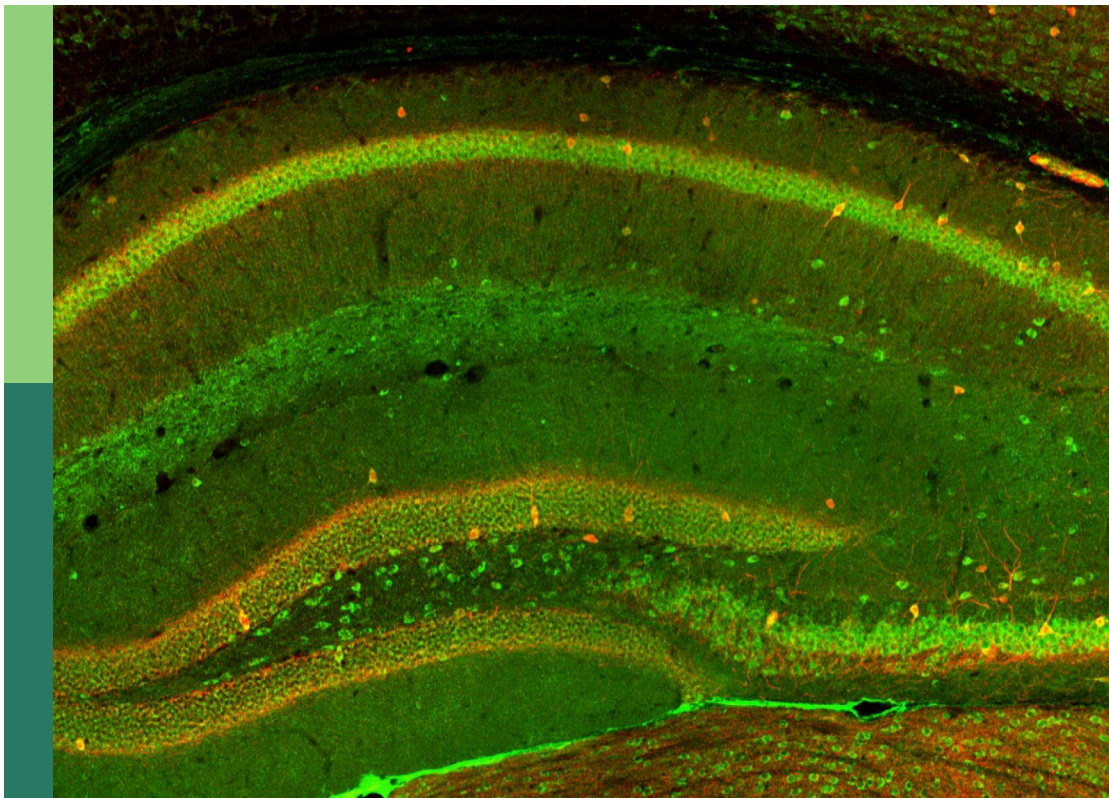

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Non-neuronal cell heterogeneity in the nervous system during health and disease

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Editorial: Non-neuronal cell heterogeneity in the nervous system during health and disease

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Editorial on the Research Topic
Non-neuronal cell heterogeneity in the nervous system during health and disease

Non-neuronal cells, which are just as numerous as neurons within central nervous system (CNS), have been historically understudied (Barres, 2008; Allen and Lyons, 2018). This is largely attributable to the precision by which electrophysiologists could measure neuronal activity. It was these measurements that first revealed the incredible diversity of neurons in their firing patterns, stimuli, connectivity and ultimately function, making neurons the center of neuroscience research. In recent years, non-neuronal cells have received increasing attention and considerable diversity has also been revealed by new single-cell approaches, most notably single-cell (sc) or single-nucleus (sn) RNA sequencing (RNAseq). The work contained within this Research Topic discusses diversity within major non-neuronal cell types in the CNS: astrocytes, oligodendroglia, ependymal cells and microglia. It also touches on the diversity of the meninges, which span both the CNS (leptomeninges) and the periphery (dura). These publications highlight the importance of non-neuronal diversity in the development, function, and disease progression within the nervous system.

How the brain is wired in development and damaged in disease are fundamental questions to treat those suffering from neurological conditions. Neuronal wiring is established during early embryonic development when neuronal progenitors migrate from the neuroepithelium to precise locations, adopt specific cell fates, and form circuits.

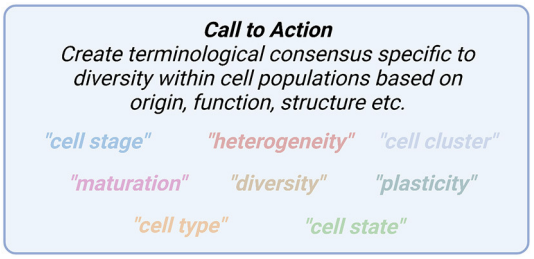

FIGURE 1
A number of terms to describe cell state differ between researchers and fields and require more precise definitions.

The findings that non-neuronal cells like astrocytes exhibit regional and layer-specific heterogeneity (Batiuk et al., 2020; Bayraktar et al., 2020), considerable diversity in response to neuro-inflammation (Hasel et al., 2021) and disease (Itoh et al., 2018; Diaz-Castro et al., 2019) has raised great interest in how these cells may be altering neuronal circuits. In a comprehensive review on inflammatory astrocytes, Reed and Kuipers describe the bidirectional feedback between astrocytes and inflammatory cells which can trigger neurodegeneration and also postulate that these inflammatory states could be targeted to reduce neurodegeneration. Moulson et al. reviewed astrocyte diversity in the context of CNS pathologies such as ischemic stroke, CNS demyelination, and traumatic injury and introduced the idea that the observed diversity might represent plasticity in these pathologies. Lastly, Lo et al. highlight the advancement of single-cell approaches and their challenges for characterizing astrocyte heterogeneity. They conclude that astrocytes display heterogeneity in health but with indistinct cellular hierarchies relative to neurons and are polarized to a number of different subpopulations following demyelination, not merely neuroprotective or neurodegenerative.

Microglia are highly plastic cells in which even subtle changes in homeostasis alter gene expression, morphology and function. Huarte et al. review microglia heterogeneity and highlight how single-cell technologies have allowed for the identification and further stratification of novel subsets of microglia in health and disease. Many of these microglial states are context-derived, and thus may reflect changes to their niche. Oligodendrocyte progenitor cell (OPC) diversity may also arise from changes to their niche and not necessarily from intrinsic differences between cells. In a mini review, Sherafet et al. highlight that diversity in OPCs are typically a process of age, differentiation state and location and argue that OPCs have not been shown to be segregated into functionally distinct subtypes. Sherafat et al. propose a number of factors in the pericellular environment influence OPC diversity and highlight the proximity to the vasculature to be critical for OPC migration, differentiation and vulnerability to demyelinated disease. OPCs and microglia are both shaped by their microenvironment and are poised to respond precisely and rapidly to changes in homeostasis.

Single-cell technologies have revealed an underappreciated diversity in non-neuronal cells, but also provided insights into novel functions of cellular subpopulations. MacDonald et al. use available data in single cell transcriptomic datasets to propose a critical role of ependymal cells in metal ion homeostasis. Additionally, they identify transcriptionally distinct subtypes of ependymal cells dependent on spatial location in the CNS. Like ependymal cells, the meninges are often an overlooked population that were long thought to be primarily for structural support and physical protection. Derk et al. thoroughly review the cellular composition of the meninges, their developmental origins, and functions. During health, the meninges regulates the entry and exit of solutes from the brain, and in disease or injury regulate scarring and inflammation (Dorrier et al., 2021). Bouadi and Tay argue single-cell approaches will be key to understanding how to reduce neuro-inflammation and scarring that accumulates around multi-electrode arrays and neuroprosthetics. Scarring diminishes the effectiveness of these structures over time. They postulate that these devices offer enormous therapeutic promise in pathologies like hearing loss or spinal cord repair.

The articles within this special edition highlight the considerable progress that has been made on identifying non-neuronal diversity, aided in large part by scRNAseq. Additionally, novel technologies will continue to expand the types of data that can be derived to distinguish diverse cell populations. For example, spatial transcriptomics has now been used to identify astrocyte heterogeneity over cortical layers (Bayraktar et al., 2020) and will likely be applied to other non-neuronal cell types. RABID-seq offers an intriguing approach to analyze cell-to-cell interactions (Clark et al., 2021). However, as valuable as these approaches are, mRNA levels often have a low correlation to subsequent protein expression (Tian et al., 2004; Koussounadis et al., 2015). Single-cell proteomics on large numbers of cells may become feasible as advances in mass spectroscopy and barcoding continue (Perkel, 2021). It will also be crucial in coming years to move beyond identifying transcriptionally diverse populations and to truly define their functional roles. To do so, targeted genetic access for specific cell subpopulations will be needed. scRNAseq often identifies unique genes which are expressed in particular subpopulations that could be used to develop Cre-expressing lines (Tran et al., 2019). It is an exciting time to study non-neuronal cells and novel technologies are providing insights into the diversity and critical functions of these cells.

Articles from within this editorial have articulated the importance of carefully choosing appropriate terminology to describe diversity between and within cell populations (Figure 1). Terms such as heterogeneity, cell state, diversity, plasticity, maturation and others may be overused without

clear definitions. Defining these terms through consensus with carefully chosen terminology will lead to precise biological conclusions that are imperative to deepen our understanding of cell heterogeneity and function.

Author contributions

GD, PA, and JS planned the outline for the Editorial. GD composed the initial draft of the Editorial. PA drafted the figure. PA, JS, BA, and JP all edited and improved the Editorial. All authors approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
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Microglia in Health and Disease: The Strength to Be Diverse and Reactive

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Microglia are the resident immune effector cells of the central nervous system (CNS) rapidly reacting to any perturbation in order to maintain CNS homeostasis. Although their outstanding reactive properties have been elucidated over the last decades, their heterogeneity in healthy tissue, such as across brain regions, as well as their diversity in the development and progression of brain diseases, are currently opening new avenues to understand the cellular and functional states of microglia subsets in a context-dependent manner. Here, we review the main breakthrough studies that helped in elucidating microglia heterogeneity in the healthy and diseased brain and might pave the way to critical functional screenings of the inferred cellular diversity. We suggest that unraveling the cellular and molecular mechanisms underlying specific functionalities of microglial subpopulations, which may ultimately support or harm the neuronal network in neurodegenerative diseases, or may acquire pro- or anti-tumorigenic phenotypes in brain tumors, will possibly uncover new therapeutic avenues for to date non-curable neurological disorders.

Keywords: microglia, heterogeneity, brain regions, neuroinflammation, neurodegenerative diseases, brain tumors

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INTRODUCTION

Microglial cells are the innate immune cells of the brain and key players in maintaining the homeostasis of the central nervous system (CNS) (Sierra et al., 2016). Microglia originate from erythro-myeloid precursors in the yolk sac and migrate to the brain around embryonic day 9.5 in mouse (Ginhoux et al., 2010), while they colonize the human cerebrum between the 4th and 24th week of gestation (Menassa and Gomez-Nicola, 2018). Their ontogeny, together with their slow turnover, which differentiate them from most other hematopoietic lineages in adult individuals (Réu et al., 2017), as well as the local environment in the CNS, make microglia a distinct immune cell population (Sousa et al., 2017). Until approximately 20 years ago, microglia have been considered as a resident resting cell type of the healthy CNS able to react to pathogens or toxic elements. However, this paradigm has shifted into the concept of "surveillant" and "supporting" microglia exerting additional multiple functional roles, such as neuromodulation and phagocytosis (Gomez-Nicola and Perry, 2015). For example, during development microglia contribute to building the neuronal circuit through synaptic pruning and stripping, phagocytosis of dying neurons and secretion of neurotrophic factors (Paolicelli et al., 2011; Ekdahl, 2012; Schafer et al., 2012; Paolicelli and Ferretti, 2017; Scott-Hewitt et al., 2020). Further, it has recently been shown that an ATP-dependent microglia-driven negative feedback mechanism operates similarly to inhibitory neurons and is essential for

protecting the brain from an excessive activation (Badimon et al., 2020). Taken together, due to their multiple critical functional roles in the homeostatic brain, various neurological disorders, including neurodegenerative diseases and brain tumors, implicate microglia. Briefly, in Parkinson's disease (PD) and Alzheimer's disease (AD), a mix of beneficial and detrimental roles of microglia have been suggested (Wyss-Coray and Mucke, 2002; Glass et al., 2010; Bodea et al., 2014; Joers et al., 2017; Salter and Stevens, 2017; Wolf et al., 2017; Duffy et al., 2018). For example, in AD microglial cells have been associated with the phagocytosis and degradation of amyloid-ß plaques, but the subsequent excessive release of cytokines is supposed to contribute to neuronal loss (Salter and Stevens, 2017). Similarly, in PD, where microglia are able to recognize and engulf alpha-synuclein, but the concomitant release of reactive oxygen species (ROS) or pro-inflammatory mediators can actively contribute to neurodegeneration (Glass et al., 2010). In brain tumors, microglia, along with tumor-infiltrating macrophages, constitute the predominant immunological cell types (Graeber et al., 2002) and have been shown to affect tumor progression as well as patient survival (Morimura et al., 1990; Gieryng et al., 2017; Sorensen et al., 2018). Indeed, tumor-associated microglia/macrophages (TAMs) are key players along tumor development by contributing to the establishment of a tumor-supporting microenvironment (Hambardzumyan et al., 2016; Grabowski et al., 2020; Maas et al., 2020).

In this context, the hypothesis that several microglial cell subsets exist in the brain has gain momentum in the recent years and microglial heterogeneity has been addressed from different points of views, including morphology, cellular density, proliferation capacity as well as transcriptional and proteomic signatures (De Biase and Bonci, 2018; Silvin and Ginhoux, 2018). Additionally, significant advances have been made by taking advantage of the recently developed single-cell technologies, including RNA-sequencing and mass cytometry (CyTOF). Indeed, several studies using these approaches have now confirmed that microglial cells represent a complex population constituted by different subsets, both in the healthy and diseased brain, displaying specific neuroimmunological adaptations in a context-dependent manner (Stratoulias et al., 2019; Masuda et al., 2020; Provenzano et al., 2020).

Here, we will review the main breakthrough studies that helped to elucidate microglia heterogeneity in the healthy and diseased brain. We suggest that unraveling the cellular and molecular mechanisms underlying specific microglia subpopulations might contribute to uncover new therapeutic targets for brain disorders with an immunological component, including neurodegenerative diseases and tumors.

MICROGLIA HETEROGENEITY IN THE HEALTHY BRAIN

An extensive characterization of microglial heterogeneity encompassing fundamental aspects, including development, gender specificities and spatial distribution has been conducted in the healthy brain. Hence, along this chapter, we chronologically review the main studies that contributed to acquire the current knowledge of microglia diversity under homeostatic conditions (**Figures 1**, **2**).

Lawson et al. (1990) have conducted the first study addressing microglial heterogeneity in the 90's. The authors showed that, in the mouse brain, microglial ramifications and cell shapes were region-dependent. By using the macrophage marker F4/80, they also reported divergent microglial densities across specific brain regions, with higher density in the hippocampus, thalamus and amygdala compared to the cortex and cerebellum (Lawson et al., 1990). Concomitantly to this study in mice, Mittelbronn et al. (2001) studied microglial density across human brain regions using myeloid-specific-immunological markers, including CD68, MHC-II, and IBA1. In this study, the authors described a higher content of microglial cells within the white-matter when compared to gray-matter (Mittelbronn et al., 2001). Seven years later, de Haas et al. (2008) took advantage of *ex vivo* flow cytometry analyses to investigate regional differences in the expression of immunoregulatory proteins across the mouse spinal cord and various brain regions, such as the cerebral cortex, hippocampus, cerebellum or striatum. Interestingly, the authors described that CD40 was overexpressed by microglia located in the cerebellum when compared to cortical microglia. Similarly, CD45 or CXCR3 were described to be less expressed in the hippocampus in comparison to the other regions, while TREM2 was differentially expressed between microglia in the cerebellum and cortex (de Haas et al., 2008). Later on, Doorn et al. (2015) investigated baseline differences in microglial expression of genes in brain regions associated with PD, including substantia nigra, striatum, olfactory bulb, hippocampus, or amygdala of rats. The authors did not detect differences in the expression levels of *Aif1*, *Cd11b*, or *Tlr2* genes in microglia isolated from those regions. However, the expression levels of the phagocytic and pro-inflammatory markers *Cd68* and *Il1b* were higher in the olfactory bulb compared to the other brain regions. Besides, *Cd68* expression was higher in the striatum compared to the amygdala and *Tnf* was overexpressed in the substantia nigra (Doorn et al., 2015).

Along with a deeper understanding of microglial heterogeneity across brain regions, the raise of single-cell transcriptomic technologies has been a breakthrough toward further revealing the cellular diversity of the brain at single-cell resolution. In this context, a pioneer work from Tasic et al. (2016) enabled to build a cell taxonomy atlas of the murine cortex identifying up to 49 transcriptomic cell types, 7 of them being non-neuronal cell types, including microglia. In the same year, two key studies helped to understand microglia diversity across brain regions, although not at single-cell resolution yet. More specifically, Grabert et al. (2016) conducted a large RNA-sequencing analysis of isolated microglial cells from the mouse cortex, hippocampus, striatum, and cerebellum. This study demonstrated that the most variable gene ontology terms discriminating the analyzed brain regions were related to metabolism and immune regulation. Besides, microglial transcriptomic heterogeneity clustered into three different signatures: the "cortex, hippocampus, and striatum," the "cerebellum and hippocampus," and the "cerebellum"

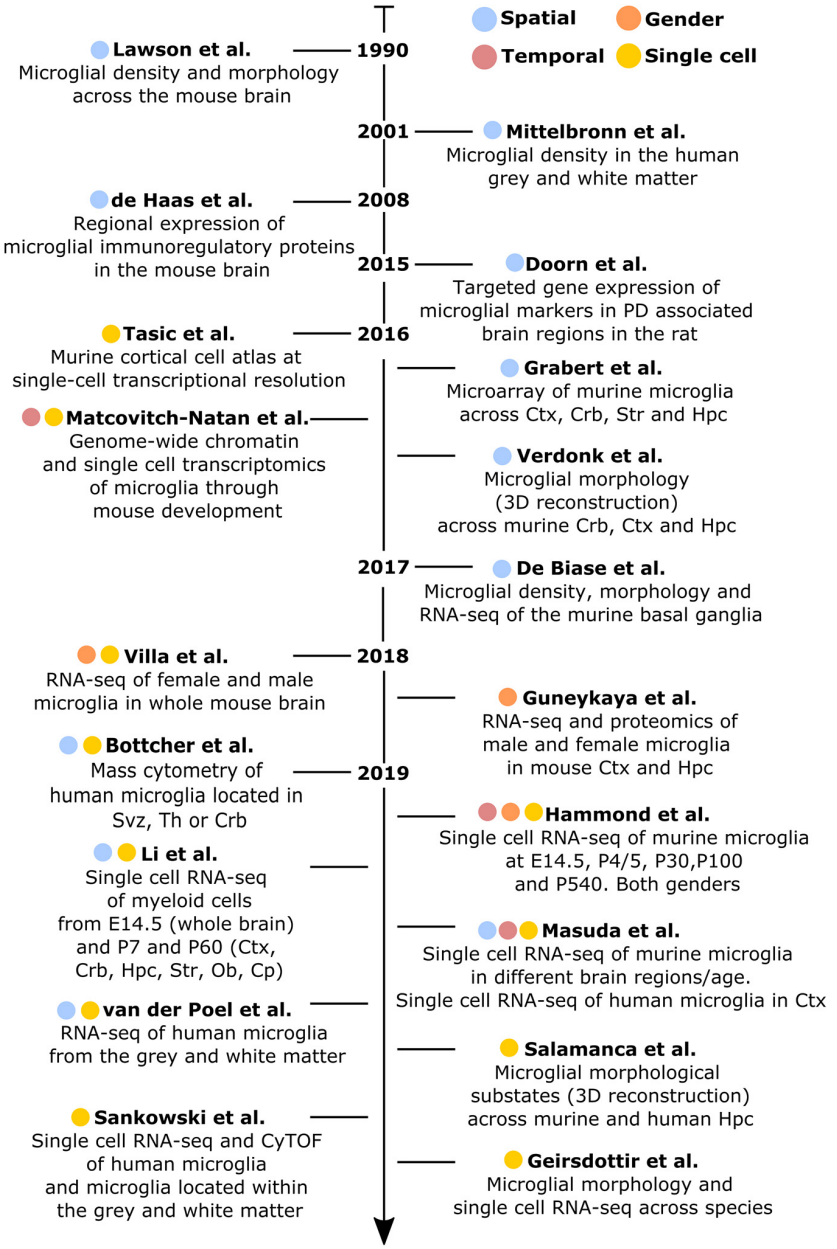

FIGURE 1 | Chronologically ordered list of the main studies that have contributed to elucidate microglial diversity in the healthy brain. Studies are color-coded based on their corresponding addressed topics: spatial (blue), temporal (red), gender (orange), or single-cell resolution (yellow). Ctx, cortex; Crb, cerebellum; Str, striatum; Hpc, hippocampus; Svz, subventricular zone; Th, thalamus; Ob, olfactory bulb; Cp, corpus callosum.

transcriptomes. Importantly, the authors showed that microglia from the cerebellum exhibit a specific "immune-vigilant" transcriptional signature when compared to the others brain regions (Grabert et al., 2016). In addition, Verdonk et al. (2016) expanded the knowledge on regional heterogeneity by shedding light on microglia morphology using an automated method based on 3D reconstruction. With this technique, microglial morphologies have been analyzed according to the complexity of primary ramifications (CI, complexity index) and the total 2D area covered by ramifications (CEA, covered environment area). In line with the transcriptomic findings obtained by Grabert et al. (2016), microglial morphology from the cerebellum was the most diverse. Indeed, cerebellar microglia had smaller CI and CEA in comparison to microglia from the hippocampus, frontal cortex and striatum, which exhibit similar cell body and cellular area (Verdonk et al., 2016). Still in 2016, the first single cell study addressing microglia diversity along development was published by Matcovitch-Natan et al. (2016) who specifically defined early-stage cycling (e.g., *Dab2*, *Mcm5*, *Lyz2)*, synaptic pruning (e.g., *Crybb1*, *Csf1*, *Cxcr2*), and adult immune surveillant (e.g., *MafB*, *Cd14*, *Mef2a*) microglia.

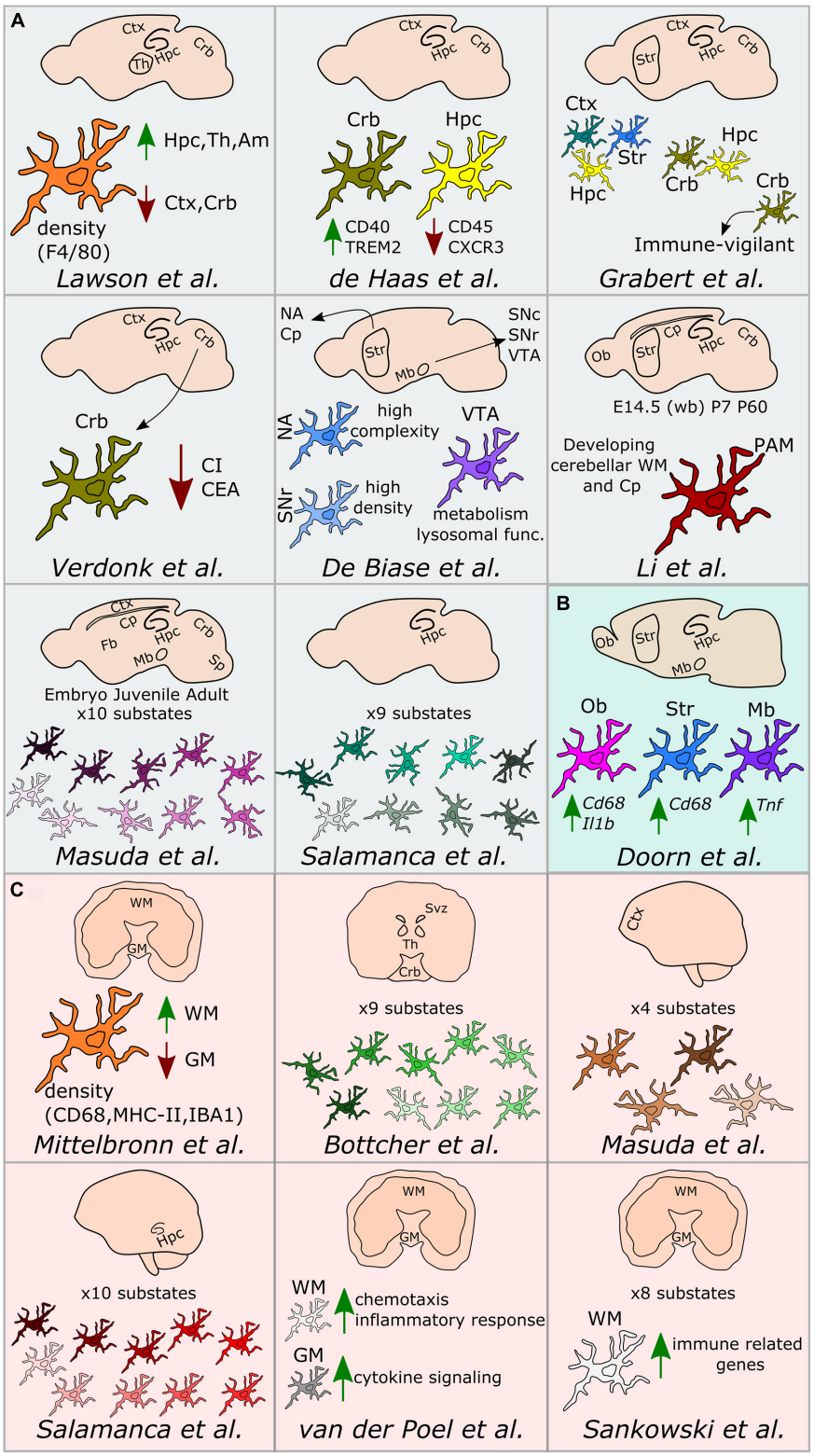

FIGURE 2 | Regional microglial heterogeneity in the healthy brain. Schematic representation of the main findings over the main studies addressing microglial heterogeneity across mouse **(A)**, rat **(B)**, and human **(C)** brain regions. Ctx, cortex; Crb, cerebellum; Str, striatum; Hpc, hippocampus; Svz, subventricular zone; Th, thalamus; Ob, olfactory bulb; Cp, corpus callosum; Mb, midbrain; NA, nucleus accumbens; VTA, ventral tegmental area; SNc, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; Sp, spinal cord; Fb, forebrain; WM, white matter; GM, gray matter; PAM, proliferative-region-associated microglia; WB, whole brain; CI, complexity index; CEA, covered environment area.

It was in 2017 when De Biase et al. (2017) investigated microglia diversity across different areas of the basal ganglia, including the nucleus accumbens, ventral tegmental area, substantia nigra pars compacta, and substantia nigra pars reticulata, using a combination of morphological and transcriptomic analyses. The authors observed that microglia in the striatum and the nucleus accumbens displayed a higher complexity when compared to the others sub-regions. Further, they reported variability in terms of microglial density within the basal ganglia, detecting a higher number of microglial cells within the substantia nigra pars reticulata when compared with the substantia nigra pars compacta and the ventral tegmental area. Interestingly, they also reported a uniform density of all the other cell types within the basal ganglia, interrogating the origin of the region-specific microglial proliferation capacities. Lastly, they described different microglial transcriptional signatures, especially within the ventral tegmental area, where genes related to mitochondrial function, metabolism, oxidative signaling, or lysosomal function were differentially expressed when compared to the other regions (De Biase et al., 2017).

In 2018, two major studies addressing sexual dichotomy at the transcriptional level have been conducted. In both cases, biological processes related to the immune phenotype (e.g., cytokine production, expression of antigen-presenting cell markers and purinergic receptors) were over-represented in male microglia (Guneykaya et al., 2018; Villa et al., 2018). Further, female microglia have been linked to neuronal processes (e.g., perpetuation of neuronal transmission or promotion of neuroprotective mechanisms), as well as have been described to be more susceptible to microbiota modifications (Villa et al., 2019).

Doubtless, 2019 has been the year when microglial heterogeneity has been mostly elucidated at single-cell resolution. For example, Bottcher et al. (2019), by using mass-cytometry, described nine different human microglial substates across the subventricular zone, thalamus, cerebellum, and the temporal and lateral lobes, with the subventricular zone displaying a more distinct signature compared with the other brain regions. In mice, Hammond et al. (2019), by using single cell technology, found high microglial heterogeneity in young mice (E14.5 and P5), with eight different substates of microglia, for which the expression of markers such as *Arg1*, *Rrm2*, *Hmox1*, or *Spp1* differed. In addition, the authors aimed to identify gender-specific differences of microglia across three different ages (E14.5, P4/P5, and P100), but did not detect main differences between males and females, except for the expression of chromosome-specific genes, such as *Eif2s3y* and *Xist* (Hammond et al., 2019). In addition to the study of Hammond et al. (2019), Li et al. (2019) described the so-called proliferative-region-associated microglia (PAM), a specific microglial subtype located in the developing cerebellar white matter and corpus callosum, which is characterized by the expression of *Spp1* and *Gpnmb*. Lastly, Masuda et al. (2019) conducted a more detailed single-cell study, mainly focused on microglia, in which their heterogeneity has been addressed across specific brain regions, such as the corpus callosum, cerebellum, cortex, hippocampus, and facial nucleus at different mouse ages. The authors identified 10 main substates during development with differences in the expression of microglial markers, such as *Tmem119*, *Malat1*, *Lamp1*, or *Apoe* (Masuda et al., 2019). Notably, in the same study, four different substates of microglia have also been described in the cortex of the human brain (Masuda et al., 2019). In parallel, microglial diversity between the gray and white matter has been studied at the transcriptomic level in the human brain (van der Poel et al., 2019). While microglia located in the gray matter were enriched in genes related to cytokine signaling, microglia in the white matter were characterized by genes involved in chemotaxis and inflammatory responses (van der Poel et al., 2019). In the same year, an automated method named MIC-MAC (Microglia and Immune Cells Morphologies Analyzer and Classifier) enabled to evaluate microglial density and morphologies at single-cell level in the hippocampi of human and mouse brains. This method allowed the clustering of microglial subpopulations based on their similarities in a 3D environment and assisted the identification of 10 different microglial subsets within the mouse and the human hippocampus, revealing a unique subset of human microglia (Salamanca et al., 2019). Still regarding microglia diversity in the human brain, Sankowski et al. (2019) recently applied high dimensional techniques and identified up to eight clusters with differential expression of microglia core genes (e.g., *CX3CR1* and *TMEM119*), genes related to major histocompatibility complex II (e.g., *HLA-DRA* and *CD74*) as well as chemokines and cytokines (e.g., *CCL2* and *IL1B*). In the same study, the authors explored regional-associated differences in the temporal lobe between the gray and white matter and detected higher expression levels of immune genes in microglia located in the latter (Sankowski et al., 2019). Later in 2019, Geirsdottir et al. (2019) studied microglia diversity across a various range of species across the evolutionary tree, including mouse and human. The authors used a combination of single cell transcriptomics and 3D reconstruction to study microglia morphology. In this particular study, the authors showed a conserved morphological pattern of parenchymal microglial cells across the species. However, they called attention on variations observed in terms of dendrite length, number of segments, branch points, and terminal points between the cortical and cerebellum microglia in mice and humans. From the transcriptomic point of view, microglia express a set of core genes that are conserved across species, with human microglia displaying a more pronounced heterogeneity than other mammals. Lastly, microglial genes related to the complement pathway, phagocytosis, or genes implicated in neurodegenerative diseases differ between rodents and primates (Geirsdottir et al., 2019).

MICROGLIA ADAPTATION AND DIVERSITY IN BRAIN DISEASES

Microglial cells scan the brain parenchyma and react to specific threats to avoid a disturbance of the critical, fine-tuned activities of the CNS. As mentioned previously, the phenotypic analysis of microglia in the healthy brain parenchyma revealed specific poised subsets, which might eventually support or harm the neuronal network under specific vulnerabilities. For example, this

is all the more important for the understanding of CNS disorders exhibiting regional-specific and cellular pathological hallmarks, as seen in various neurodegenerative diseases, including AD (entorhinal cortex and hippocampus) and PD (nigrostriatal pathway). Therefore, in this chapter we extend the study of microglial heterogeneity in a disease-associated context. More specifically, we tackle microglia diversity associated with neuroinflammatory and neurodegenerative diseases as well as with brain tumors (**Figure 3**).

Neuroinflammatory and Neurodegenerative Diseases

In an attempt to elucidate potential heterogeneous responses of microglia under neuroinflammatory conditions, we analyzed *ex vivo* pre-sorted cells from the mouse brain following a peripheral acute endotoxin challenge, a common model used as a paradigm to study the effect of systemic bacterial infections, ultimately leading or not to neurodegeneration (Bodea et al., 2014). By applying single-cell RNA-sequencing, we demonstrated that the microglial response associated with a peripheral injection of lipopolysaccharide (LPS), most probably induced by a transient serum cytokine storm, rather than stimulated by the response to the TLR ligand that might not reach the brain parenchyma (Banks and Robinson, 2010; Shemer et al., 2020) is heterogeneous. Specifically, we identified two discrete reactive states characterized by various levels of activation and showed that inflammation-induced microglia signatures are distinct from neurodegenerative disease-associated profiles (Sousa et al., 2018). Notably, a similar study conducted a year later showed that microglia isolated from the midbrain of peripherally LPS injected mice adopt an immunosuppressive phenotype in comparison to microglia located within the striatum (Abellanas et al., 2019), thus suggesting that distinct microglia reactions toward neuroinflammatory threats might be region-dependent. In this context, the analysis of microglia phenotypes associated with chronic peripheral inflammation in TNF transgenic mice revealed distinct signatures across different brain regions, including the cortex, striatum, hippocampus, thalamus, and cerebellum. Indeed, microglia located in the cortex, striatum, and thalamus of the transgenic mice clustered together, and their transcriptome significantly differed from the other brain regions. More specifically, microglial cells located within the cortex, striatum and thalamus were characterized by the overexpression of inflammatory genes, such as *Cxcl13*, *Ccl2*, *C3*, and *C4b*, thus suggesting a more pronounced reactive state of microglia under persistent inflammation in these specific regions (Süß et al., 2020).

In the context of neuroinflammatory diseases, multiple sclerosis (MS) is the most common inflammatory, demyelinating and neurodegenerative disorder of the CNS. In this perspective, Hammond et al. (2019) studied microglia diversity in the white matter of mice injected with lysolecithin (LPC), a commonly used mouse model of MS. In this model, microglial cells initially segregated into two clusters, with the so-called injury responsive 1 (IR1) cluster mainly composed by microglial cells of the control group, whereas the IR2 cluster constituted by microglia from LCP injured mice. Further, the authors revealed that IR2 subset was composed by four sub-clusters representing different microglial subtypes or responses in LCP mice differing in the expression levels of proliferative (e.g., *Birc5*) and inflammatory (e.g., *Cxcl10* or *Ccl4*) markers, suggesting that microglia acquire different phenotypes to respond to demyelination (Hammond et al., 2019). Similarly, Masuda et al. (2019) investigated microglial heterogeneity in a MS and neurodegeneration-associated mouse model, respectively the cuprizone and the unilateral facial nerve axotomy models. In this study, the authors describe up to nine different subtypes of microglia displaying differences in the expression levels of inflammatory (e.g., *Spp1*, *Ccl4*, *Cybb*) or MHC-II-related (e.g., *Cd74*, *H2-Aa*, *H2-Ab1*) markers among others (Masuda et al., 2019). Remarkably, a similar microglial heterogeneity has been confirmed in MS patients. More specifically, microglial cells segregated into seven different subtypes, with differential expression levels of chemokines and cytokines (*CCL4* or *ERG2*), MHC-II-related proteins (*CD74* or *HLA-DRA*), and activation markers (*SPP1* or *CTSD*) (Masuda et al., 2019).

In the context of neurodegenerative diseases, Doorn et al. (2014) took advantage of immunohistochemical analyses to examine the expression of TLR2, a known receptor involved in the activation of microglia following its interaction with alpha-synuclein in the substantia nigra and hippocampus of patients with PD and incidental Lewy Body Disease (iLBD), a prodromal state of PD. In iLBD patients, TLR2 microglia expression regionally differed between the substantia nigra and hippocampus. Additionally, Mastroeni et al. (2018) studied the human regional microglial profile associated with PD and AD. More specifically, they conducted RNA-sequencing of isolated microglia from the two most vulnerable brain regions affected in PD and AD, the substantia nigra and the hippocampus CA1, respectively (Mastroeni et al., 2018). They uncovered regional differences highlighting 313 differentially expressed genes between microglia located within the substantia nigra of PD samples and the corresponding cells located in the hippocampus CA1. These differential expressed genes reflected changes in behavior, synaptic transmission or regulation of transport. In the AD samples, 104 differential expressed genes associated with synaptic transmission, cell-cell signaling, or metal ion transport have been detected between microglia located in the hippocampus CA1 and substantia nigra (Mastroeni et al., 2018). In a similar context, Keren-Shaul et al. (2017) used a classical mouse model of AD, the 5XFAD, to study microglial subsets associated with AD at the single-cell level. Notably, the authors depicted a specific population of microglial cells associated with AD, namely disease-associated microglia (DAM). They uncovered that the Trem2 associated pathway, which confers them a higher phagocytic capacity, drives the acquisition of the DAM phenotype. This specific population is supposed to be located around the amyloid-ß plaques and has been confirmed to be also present in the brains of AD patients (Keren-Shaul et al., 2017). Consistently, a transcriptional phenotype of dysfunctional microglia in neurodegenerative diseases, termed "microglial neurodegenerative phenotype" (MGnD), driven by the TREM2-APOE pathway, has been concomitantly described

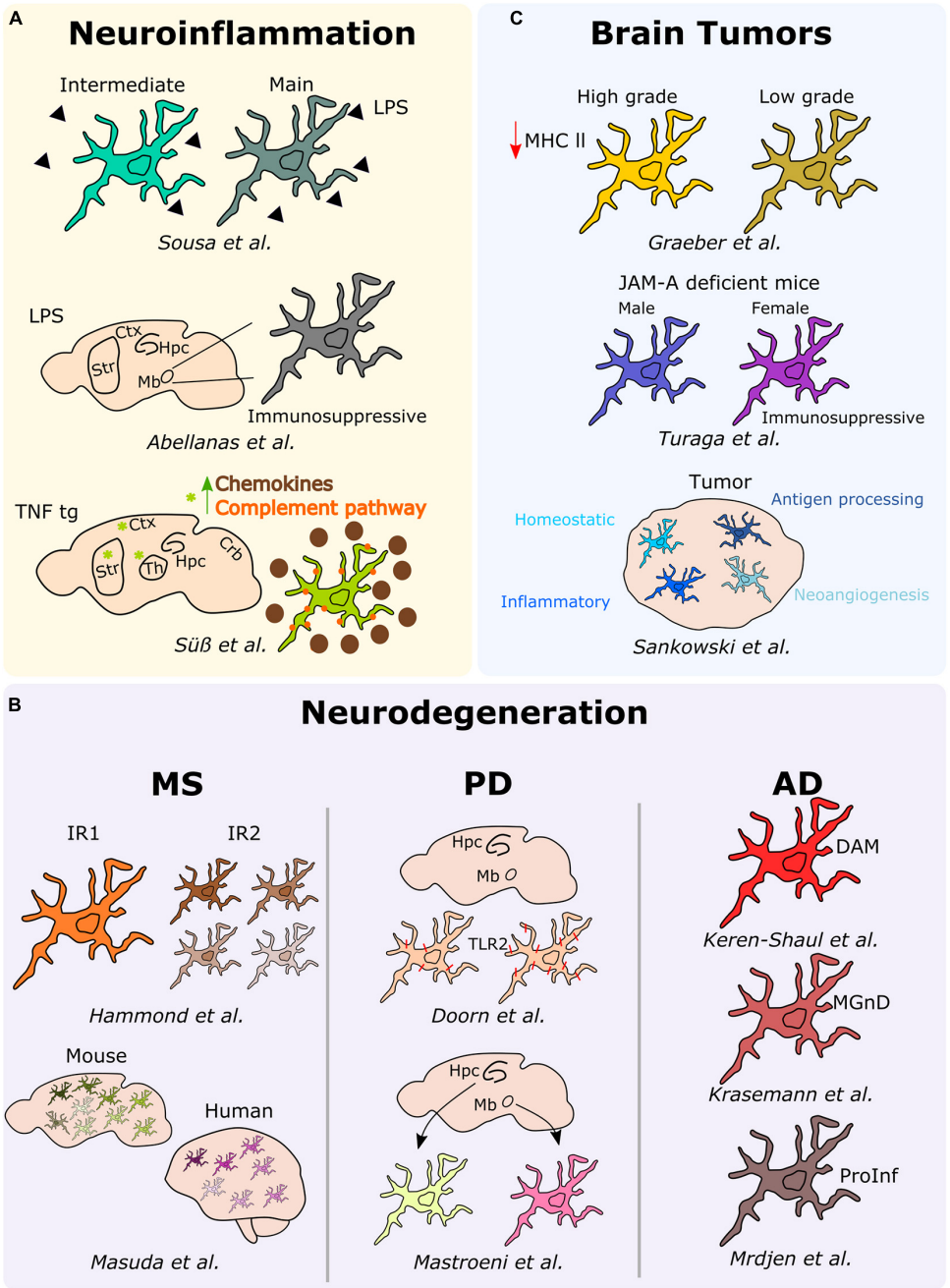

FIGURE 3 | Microglial heterogeneity in different neurological diseases. Schematic description of the main results over the main studies addressing microglia heterogeneity in neuroinflammation, neurodegeneration (MS, PD, and AD), and brain tumors. Hpc, hippocampus; Mb, midbrain; Ctx, cortex; Str, striatum; Th, thalamus; DAM, disease-associated microglia; MGnD, microglial neurodegenerative phenotype; ProInf, pro-inflammatory; IR1, injury responsive 1; IR2, injury responsive 2.

(Krasemann et al., 2017). Further, Mrdjen et al. (2018) took advantage of the CyTOF to identify a microglial subtype in another mouse model of AD, the APP/PS1. Indeed, they identified a subset of microglial cells characterized by the overexpression of phagocytic (e.g., *Cd11c* and *Cd14*), activation (e.g., *Cd86* and *Cd44*) as well as MHC-II-associated markers. In this subset, in line with a pro-inflammatory phenotype, the expression levels of the homeostatic microglia markers (e.g., *Cx3cr1* or *Siglec-H*) were downregulated (Mrdjen et al., 2018).

Brain Tumors

In the past, inter-tumor microglial morphological heterogeneity has been described, for example, across different gliomas, where microglia display a more pronounced amoeboid morphology

in high-grade tumors, while they are ramified in low-grade tumors. These differences were also associated with lower levels of MHC-II expression in high-grade gliomas when compared to their low-grade counterparts (Graeber et al., 2002). Interestingly, microglia diversity associated with brain tumors, and specifically in GBM, has been investigated also taking into account gender specificities. Turaga et al. (2020), while conducting a study regarding the implication of junctional adhesion molecule-A (JAM-A), noticed a poorer prognosis in GBM implanted female JAM-A deficient mice when compared to the corresponding implanted males. Notably, the authors reported an upregulation of the anti-inflammatory genes *Fizz1* and *Ifi202b* in microglia from female JAM-A deficient mice compared to their male counterparts (Turaga et al., 2020). Sankowski et al. (2019), by combining scRNA-seq and CyTOF analyses on human brain samples, including samples obtained from GBM patients, discovered various subpopulations of microglial cells within the tumor. Indeed, they defined a continuum from control-enriched clusters to glioma-associated microglial clusters, the latest being characterized by a decreased expression of core microglial signature genes (*CX3CR1*, *SELPLG*, *P2RY12*, *CSFR1*) and an increased expression of metabolic, inflammatory and interferon-associated genes (*CD163*, *APOE*, *LPL*, *IFI27*, *IFI44*, *SPP1*). Interestingly, the in-between clusters were exhibiting differential expression levels of the previous cited genes, but also hypoxia-related (*VEGFA*, *HIF1A*) and antigen processing MHCI-related genes. Additionally, CyTOF analyses enabled the detection of differential proportions of HLA-DR, TREM2, APOE, and GPR56, confirming major differences between control and glioma-associated microglia also at the protein level (Sankowski et al., 2019). Although only poorly investigated to date, also secondary brain metastases can alter microglial properties, or—vice versa—microglia may even pave the way for an enhanced cerebral dissemination of peripheral tumor cells via increased secretion of IGF-1 and CCL20 together with a reduced expression of SIRP-alpha, the latter leading to impaired phagocytic properties (Simon et al., 2020; Wu et al., 2020). Very recently, the first experimental studies addressing the composition of primary and secondary myeloid cell populations at single-cell level have been performed in mouse models indicating that the genetic programming of brain metastasis-associated myeloid cells is a very early and stable event (Schulz et al., 2020).

CONCLUSION AND PERSPECTIVES

Undoubtedly, the ability to acquire different resting and activated phenotypes confers microglia the advantage to be a plastic and adaptive cell type in the CNS. Along with this review, we have highlighted a large number of studies demonstrating that microglia are far from being a resting or homogenous cell population. For example, in the healthy mouse brain, microglia heterogeneity across various regions has been described in terms of density, morphology, molecular signatures, and metabolism (Tan et al., 2020). For translational purposes, it will be critical to consider that microglia heterogeneity has been suggested to be even higher in the human brain compared to mouse (Prinz et al., 2019). Notably, regarding neurodegenerative diseases little is known about microglial heterogeneity in PD. Hence, future efforts will need to be directed at understanding if specific microglia subsets might differently contribute to PD pathology. In the context of brain tumors, with the difficulty to define reliable markers deciphering macrophages and microglia, the majority of the transcriptomic studies have been focusing on defining a glioma-associated microglial signature in different models, but not assessing their heterogeneity within the tumor mass (Darmanis et al., 2017; Walentynowicz et al., 2018; Maas et al., 2020). The study by Sankowski et al. (2019), being the first one highlighting specific heterogeneous signatures of microglial cells in GBM, calls attention on the lack of knowledge on microglial heterogeneity in that context. Hence, further studies would need to be directed at understanding the implication of different microglial subsets in glioma development and progression (Sankowski et al., 2019). Similarly detrimental, however, much more frequently than primary brain tumors, also brain metastases, at least experimentally, show a relevant contribution of microglia in the establishment and progression of secondary brain tumors, therefore constituting a target for future treatment strategies.

Taken together, microglial variety embraces fundamental aspects, such as spatial-temporal organization, which is present in the healthy and diseased brain. Thanks to the development of high-throughput technologies, including single-cell approaches, different microglial subsets have been unraveled, indicating that microglia are able to adapt to specific environments across particular niches in the healthy brain. In addition, single-cell analyses have been conducted to study microglia associated with inflammation and neurological disorders untangling specific subsets of cells that might differently contribute to each specific pathology (Masuda et al., 2020). Notwithstanding, a crucial aspect that would need to be tackled in future studies would be to understand the functional implication of specific microglial subpopulations across particular neurological diseases, which might enable to explore novel avenues to target neuroinflammation and microglial cells in a specialized context-dependent manner.

AUTHOR CONTRIBUTIONS

OUH and AM conceived the manuscript. OUH, LR, and AM wrote the manuscript. OUH created the figures. OUH, LR, MM, and AM critically revised and approved the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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OPINION
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More Than Cell Markers: Understanding Heterogeneous Glial Responses to Implantable Neural Devices

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INTRODUCTION

Recent publicity surrounding a coin-size computer chip in a pig's brain has placed the spotlight on the field of neurointerfaces (Lewis, 2020). Implantable microelectrode arrays (MEAs), or neural probes, enable the study of brain activity and present promising treatment and therapeutic options for neurological conditions (Boehler et al., 2020). These range from motor and sensory impairments such as spinal cord injuries and hearing loss, to neuropsychiatric disorders including dementia, clinical depression and insomnia. Application-specific MEAs that, for example, record field potentials and neuronal activity have been validated in non-human primates and could help understand mechanisms underlying motor functions and epilepsy (Barz et al., 2017; Gerbella et al., 2021). Key design considerations for biocompatibility, efficacy and longevity of microelectrodes to maintain long-term neuronal recording and stimulation are highly dependent on brain tissue response (Polikov et al., 2005). The functional capacities of a biosensor depend on the number of surrounding neurons in a given radius (50–350 μm) (He et al., 2020). Probe insertions generate inflammatory responses to acute tissue injuries and the introduction of foreign bodies, known as "foreign body response" (FBR). Chronic neuroprosthetic implants in rats at 16 weeks in contrast to 8 weeks have been shown to increase neuronal and dendritic loss, correlate with tau hyperphosphorylation seen in Alzheimer's disease and other tauopathies, and impede regeneration and recording of activity surrounding the device (McConnell et al., 2009). Assessments of acute proinflammatory events and chronic progression have largely centered on histological analyses of non-neuronal central nervous system (CNS) cells such as microglia, astrocytes and oligodendroglia, including their contribution to neuroinflammation and glial scars (Kozai et al., 2015; Prodanov and Delbeke, 2016). However, immunohistochemistry provides qualitative answers and rarely discriminates between heterogeneous cellular states (Wellman et al., 2019). Here we highlight developments that expand our knowledge of context-dependent heterogeneity of glia and blood-brain barrier cells, proposing new approaches to examine the diverse contributions of non-neuronal CNS cells after probe implantation. Having a holistic understanding of multiple glial responses will advance neuroengineering that temper neuroinflammation and tissue scarring, thereby improving functional neuroprosthetic integration.

MICROGLIA AT THE BRAIN-MACHINE INTERFACE

Microglia are myeloid cells of extra-embryonic origin that form the brain-resident macrophages (Ginhoux et al., 2010). Tissue damage triggers microglia-driven repair mechanisms including phagocytosis of cellular debris, chemotaxis, and initiation of cell death pathways through cytokine release (Prinz et al., 2019). As first responders of the CNS that potentially contribute to sustained neuroinflammation, microglial reactivity is widely assessed after microelectrode implantation to examine changes elicited by insertion injury and FBR (Kozai et al., 2012). Intracortical implantation of non-functional microelectrodes in rats has led to the elevation of oxidative stress markers (Ereifej et al., 2018). Microglia have been shown to increase acidosis and inflammation by the release of reactive oxygen species (ROS) in a controlled cortical impact (CCI) mouse model for neurotrauma (Ritzel et al., 2021). ROS can be detrimental to long-term functionality of an implanted sensor (Takmakov et al., 2015). Prolonged microglial reactivity or adherence to the electrode surface could threaten device efficacy and longevity in the recipient brain and diminish recording quality (Huang et al., 2020). Moreover, microglia secretion of proinflammatory cytokines such as tumor necrosis factor (TNF) and interleukin 1 (IL-1) may induce neurotoxic reactive astrocytes (Liddelow et al., 2017) to envelope the implant. Together with cell recruitment, glial scar formation and electrode encapsulation, proinflammatory microglia have earned the reputation of being noxious (Kozai et al., 2016). Yet depletion of microglia was shown to be unfavorable for scar formation, wound healing and survival of neurons and oligodendrocytes (Bellver-Landete et al., 2019), supporting the notion that they promote the stable integration of implanted MEAs. Microglial heterogeneity in the healthy, developing and diseased brain is very well-described (Stratoulias et al., 2019; Masuda et al., 2020), even if mammalian microglia mostly originate from a single erythromyeloid progenitor source in the embryonic yolk sac (Alliot et al., 1999; Ginhoux et al., 2013). However, prevailing studies of MEAs do not reveal the spectrum of neuroprotective or neurotoxic microglial subtypes.

Common markers for microglia and microglial reactivity, such as ionized calcium-binding adaptor molecule 1 (IBA-1), integrin alpha M (ITGAM, or CD11b) and CD68 (also ED-1), are frequently used in immunohistochemical analysis of the brain-electrode interface as readout for tissue damage caused by implantation trauma and FBR (McConnell et al., 2009; Luan et al., 2017; Huang et al., 2020) (**Figure 1**). Transmembrane protein 119 (TMEM119) (Bennett et al., 2016) and purinergic receptor P2Y12 (P2RY12) (Butovsky et al., 2014) are excellent markers for homeostatic microglia, but are thus far rarely used for neurointerfaces. Neuroengineers considered the normalized intensity of microglial cell markers to proportionately represent the degree of inflammation (Lo et al., 2018). For instance, signal intensities of microglial cell markers surrounding insertion sites of explanted probes were examined at acute (1–3 days) or sub-chronic (up to 28 days) phases to evaluate the brain-machine interface (Lind et al., 2013; Wellman et al., 2019). Studies on neurotrauma and FBR showed that microglia could upregulate proinflammatory inducible nitric oxide synthase (iNOS) (Madathil et al., 2018) or anti-inflammatory arginase 1 (Arg1) (Sawyer et al., 2014). Descriptions of microglial cell morphologies in the assessment of FBR after implantation of MEAs include "ramifying" and "amoeboid," which are, respectively, associated with steady and reactive states (Huang et al., 2020). Additional classifications such as "primed," "hypertrophic" and "hypo- or hyper-ramified" are also relevant for the phenotypic characterization of neuroprotective or neurotoxic microglia in pathological conditions (Verdonk et al., 2016). Current immunohistochemical analyses however mostly disregards microglial diversity at the implantation site.

ASTROCYTES AND SCARRING AT NEUROINTERFACES

Astrocytes are star-shaped, heterogeneous glial cells that provide significant neurotrophic support through their interaction with every component of the CNS parenchyma (Verkhratsky and Nedergaard, 2018). They support synapse formation, maturation and pruning, and modulate pre- and post-synaptic transmission in homeostatic CNS (Sofroniew and Vinters, 2010). Tissue damage unleashes reactive astrocytes that adopt neuroprotective or neurodegenerative properties (Liddelow and Barres, 2017). Glial fibrillary acidic protein (GFAP) is the most frequently used immunohistochemical marker for reactive astrocytes in analyses of brain-electrode interface and is positively correlated to astrogliosis and glial scar formation (Polikov et al., 2005; Seymour and Kipke, 2007; Kozai et al., 2015; Prodanov and Delbeke, 2016) (**Figure 1**). $GFAP^+$ astrocytes contribute to scarring through secretion of extracellular matrix chondroitin sulfate proteoglycans (CSPGs) such as neurocan, phosphacan and brevican (Fawcett and Asher, 1999; Matsui et al., 2002). CSPGs are inhibitors of axonal growth and remyelination that are frequently found in multiple sclerosis lesions where they reduce adherence of oligodendrocyte precursor cells (OPCs) for myelin repair (Galtrey and Fawcett, 2007; Lau et al., 2012). Inserting pieces of nitrocellulose filter into adult rat brain cortices induced infiltration of $GFAP^+$ astrocytes into the implants and continued release of CSPGs even at 1 month after tissue injury (McKeon et al., 1991, 1999). Reactive astrocytes formed the principal cell type that increasingly compacted around and encapsulated a silicon microprobe implanted for up to 12 weeks in rats (Turner et al., 1999). This was similarly observed in a marmoset brain carrying an array with 32 Teflon-coated 50-μm-large microelectrodes for 7 months (Budoff et al., 2019). High levels of CSPGs were concomitantly observed with neuronal loss after an uncoated silicon neural probe was implanted in rat brains (Zhong and Bellamkonda, 2007). Recording performance of multichannel, 16-shank, silicon "Utah" MEAs embedded year-long in feline sensorimotor cortex reportedly dropped when neuronal action potentials were recorded (McCreery et al., 2016). This implicates astrocytic glial scar and neuronal death in the loss of biosensor performance. However, reactive astrocytes

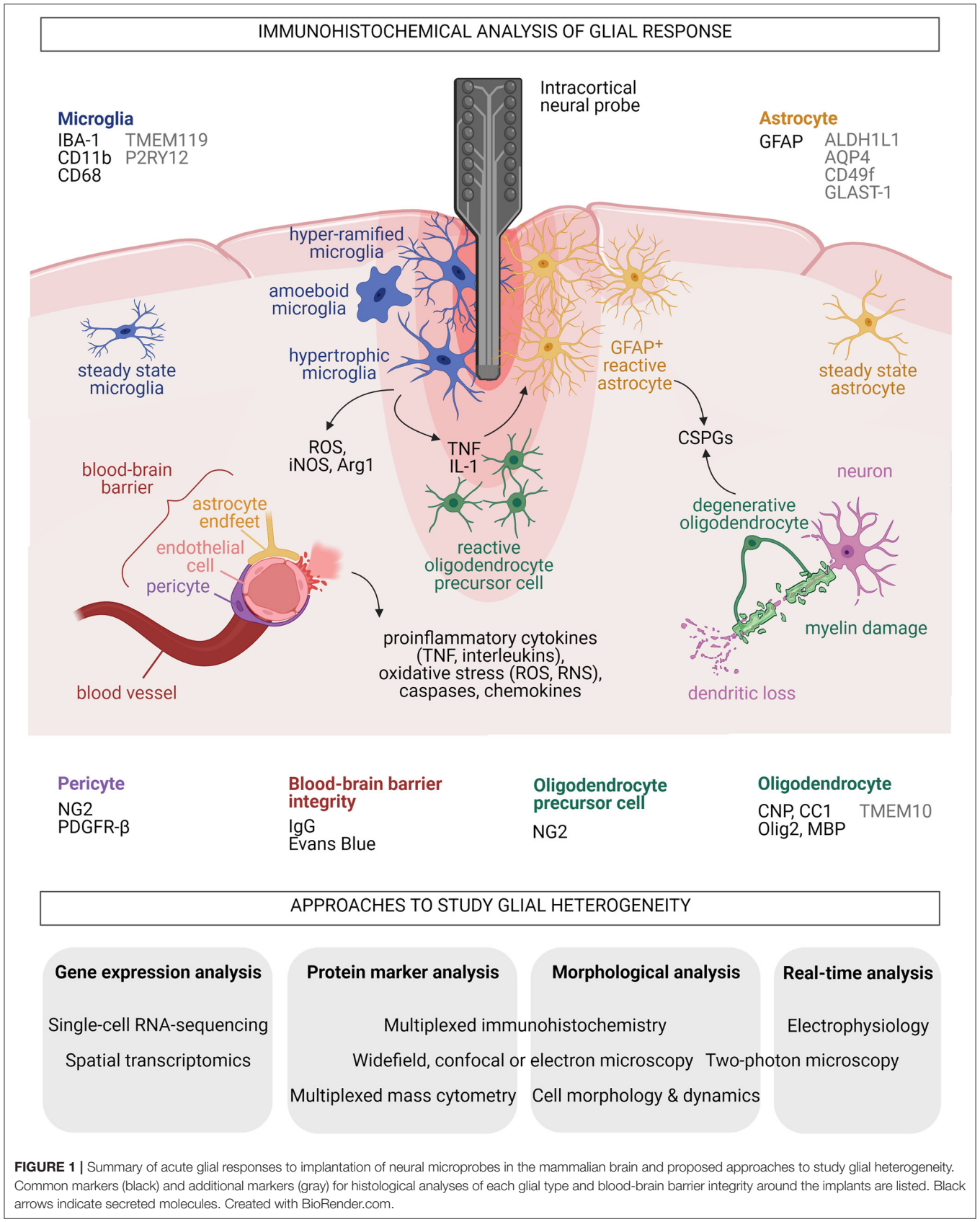

FIGURE 1 | Summary of acute glial responses to implantation of neural microprobes in the mammalian brain and proposed approaches to study glial heterogeneity. Common markers (black) and additional markers (gray) for histological analyses of each glial type and blood-brain barrier integrity around the implants are listed. Black arrows indicate secreted molecules. Created with BioRender.com.

unlikely lead to only destructive outcomes. Conditional ablation of astrocytes after CCI, stab or crush injuries augmented lesion formation, demyelination and death of neurons and oligodendrocytes (Faulkner et al., 2004; Myer et al., 2006). The multifaceted roles of astrocytes suggest they are also vital promoters of repair.

Elucidating long-term changes in astrocytic FBR and scarring at neuroprosthetic implantation sites requires an understanding of astrocyte heterogeneity. Astrocytic diversity is well-described in healthy, developing and diseased CNS (Khakh and Sofroniew, 2015; Chai et al., 2017; Lanjakornsiripan et al., 2018; Clavreul et al., 2019; Pestana et al., 2020) and cannot be represented by the GFAP marker. Markers for homeostatic astrocytes include aldehyde dehydrogenase 1 family member L1 (ALDH1L1) (Cahoy et al., 2008), glutamate aspartate transporter 1 (GLAST-1, also known as excitatory amino acid transporter 1, EAAT-1) (Hurwitz et al., 1993) and aquaporin-4 (AQP4) found in astrocytic end feet (Yoneda et al., 2001) (**Figure 1**). A newly described population of human induced pluripotent stem cells-derived, proinflammatory cytokine-stimulated reactive astrocytes specifically upregulate CD49f (Barbar et al., 2020). From gray matter protoplasmic astrocytes to white matter fibrous astrocytes, diverse astrocytic morphologies in the healthy and diseased brain are well-documented (Zhang and Barres, 2010; Molofsky et al., 2012; Bardehle et al., 2013; Bayraktar et al., 2014). Immunohistochemical analyses to date however exclude the heterogeneity of astrocytes surrounding an implant.

IMPACT OF NEUROPROSTHETICS ON OLIGODENDROCYTES AND THEIR PROGENITORS

Differentiation of OPCs, also known as NG2-glia, gives rise to oligodendrocytes that produce and maintain myelin sheaths, which provide neurotrophic support and optimize brain electrical signaling (Bradl and Lassmann, 2010; Nave and Werner, 2014). OPCs and newly derived oligodendrocytes are essential for remyelination and CNS repair following demyelinating diseases or brain injury (Young et al., 2013; Bechler et al., 2015). Immunohistochemical analyses of oligodendrocytes at neurointerfaces have involved markers including 2′,3′-Cyclic-nucleotide3′-phosphodiesterase (CNP) (Chen et al., 2021) and CC1 (a monoclonal antibody against adenomatous polyposis coli) for mature oligodendrocytes, oligodendrocyte transcription factor 2 (Olig2) for immature oligodendrocytes, and myelin basic protein (MBP) for myelinating oligodendrocytes (Wellman et al., 2018, 2019) (**Figure 1**). TMEM10, a type 1 transmembrane glycoprotein, was recently verified to be specific for mammalian CNS myelin (Golan et al., 2008; de Faria et al., 2019).

With limited antioxidant capacity and high iron content, oligodendrocytes are sensitive to elevated ROS and reactive nitrogen species (RNS) levels arising from glial response to acute implantation injury and FBR (Smith et al., 1999). Similar to $GFAP^+$ astrocytes, OPCs and oligodendrocytes release axonal growth-inhibiting CSPGs including NG2 and myelin-associated glycoprotein (Fawcett and Asher, 1999). Studies on passive multi-channel, four-shank "Michigan" MEAs in murine visual cortex revealed acute oligodendrocyte injury and degeneration, myelin degradation, and reactive swarming of OPCs toward the implant within 12 h (Wellman and Kozai, 2018; Chen et al., 2021). Severe reduction of electrophysiological recording quality from neurons at various tissue depths and observations of decreased neuronal firing in a mouse model of demyelination highlighted the importance of myelin integrity for microelectrode function (Wellman et al., 2020). A clearer picture of the renewal, maturation and function of various oligodendroglia at implantation sites will allow to determine the degree of cohesiveness at the brain-machine interface.

IMPACT OF NEUROPROSTHETICS ON BLOOD-BRAIN BARRIER INTEGRITY

A breach of the blood-brain barrier (BBB) is inevitable during implantation for microelectrodes to reach the neurons. The BBB is composed of endothelial cells, pericytes, and astrocytes, forming the neurovascular unit together with surrounding microglia and neurons (Sweeney et al., 2016; Bennett et al., 2019). Cerebrovascular endothelial cells are seamlessly joined by active protein complexes known as tight and adherens junctions (Tietz and Engelhardt, 2015). Pericytes also regulate BBB permeability and are involved in neuroinflammatory response, clearance of toxic metabolites and promotion of angiogenesis (Hill et al., 2014). Pericytes are typically identified by the colocalization of NG2 and platelet-derived growth factor receptor beta (PDGFR-β) markers (to differentiate them from $NG2^+$ OPCs) (Wellman et al., 2019) (**Figure 1**). Vasculature integrity is commonly assessed by histological detection of immunoglobulin G leakage or Evans blue staining for plasma membrane damage (Nolta et al., 2015; Falcone et al., 2019) (**Figure 1**). Decreased expression of junctional proteins in the compromised BBB promotes neuroinflammation through higher expression of proinflammatory cytokines and chemokines and increased infiltration of peripheral immune cells (Marchetti and Engelhardt, 2020). BBB leakiness, astrogliosis and neuronal death in brain tissue surrounding the implanted device were shown to reduce the number of measurable electrophysiological responses of single neurons and degrade the overall recording performance of the biosensor (Nolta et al., 2015). High-speed pneumatic intracortical insertion of Utah MEA in rat cortex has led to down-regulation of endothelial tight and adherens junction protein markers, and correlated with increased oxidative stress and elevated inflammation levels indicated by upregulation of caspases, chemokines, interleukins and TNF (Bennett et al., 2018, 2019) (**Figure 1**). Notably, BBB release of ROS, RNS, and proinflammatory cytokines and chemokines likely promote microglial and astrocyte reactivity, and loss of neurons and oligodendrocytes.

CURRENT APPROACHES TO STUDY GLIAL HETEROGENEITY

Single-cell transcriptomic technologies that simultaneously quantify hundreds or thousands of expressed genes of individual cells in a given population have unmasked heterogeneous cellular identities and developmental trajectories, and revealed biomarker information (Aldridge and Teichmann, 2020). These powerful methods provide unique insights into health and disease in contrast to bulk transcriptomic and classical histological analyses. Single-cell RNA-sequencing and spatial transcriptomic approaches have shown that glia isolated from healthy and disease-associated brain regions respond across broad cellular states for microglia (Tay et al., 2018; Hammond et al., 2019; Jordão et al., 2019; Li et al., 2019; Masuda et al., 2019), astrocytes (Cahoy et al., 2008; Zamanian et al., 2012; Boisvert et al., 2018; Bradley et al., 2019; Batiuk et al., 2020; Bayraktar et al., 2020; Das et al., 2020), and oligodendrocytes (Jäkel et al., 2019; Spitzer et al., 2019; Floriddia et al., 2020). Advances in single-cell proteomics have also enabled the high-throughput investigation of key biological questions involving protein binding, modifications, and degradation, that cannot be assessed at the transcriptomic level (Slavov, 2020). Multiplexed mass cytometry and multiplexed immunohistochemistry have unveiled regional and pathology-dependent heterogeneity of human peripheral myeloid cells, microglia and astrocytes (Böttcher et al., 2019; Park et al., 2019). Furthermore, multiplexed immunohistochemistry, electron microscopy and *in vivo* two-photon imaging techniques are increasingly applied to study acute and chronic oligodendrocyte and OPC reactivity after microprobe implantation (Bogoslovsky et al., 2018; Michelson et al., 2018; Wellman and Kozai, 2018; Chen et al., 2021) (**Figure 1**). Clearly, advancing MEA technology requires a comprehensive examination of glial responses at neurointerfaces by integrating quantitative single-cell multi-omic analyses with assessments of cell morphology and dynamics, and electrophysiological recordings, as has been recently demonstrated in neurons (Cadwell et al., 2016).

DISCUSSION

Implantation of single-shank or multi-shank MEAs will inevitably trigger changes in glia and the BBB due to acute tissue trauma and FBR. Microglia surrounding the lesion will immediately undergo significant state changes to limit physical damage through microgliosis, phagocytosis of dying cells and debris, and release of proinflammatory cytokines and stress-induced molecules. Microglial reactivity likely elevates the population of reactive astrocytes, which could lead to extensive unwanted glial scarring. In concert with astrocytes, degenerative oligodendrocytes also secrete growth-inhibiting extracellular matrix components, and result in electrode encapsulation and dysfunction. Loss of BBB homeostasis also exacerbates proinflammatory responses of microglia and astrocytes to favor neuronal and myelin loss. CNS repair however necessitates acute inflammatory events contributed by neuroprotective subpopulations of non-neuronal brain cells. To harness the endogenous, neuro-regenerative properties of glia and promote electrode biocompatibility and longevity (Gulino et al., 2019), we propose to investigate context-dependent glial responses at brain-machine interfaces using combinatorial approaches in addition to immunohistochemical assays of protein markers. Probe fabrication breakthroughs in material, size and geometry have limited implantation trauma and reduced probe encapsulation (Patel et al., 2016; Luan et al., 2017; Rivnay et al., 2017). Devices coated with dexamethasone to alleviate neuroinflammation (Kozai et al., 2016; Boehler et al., 2017), or laminin to restrict glial reactivity at implantation sites (He et al., 2006), have shown great promise. A deeper molecular understanding of diverse glial responses at neurointerfaces will identify further candidates for promoting neuroprosthetics development.

AUTHOR CONTRIBUTIONS

OB wrote the first draft of the manuscript and designed the figure. TLT supervised the project and extensively revised the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Living on the Edge of the CNS: Meninges Cell Diversity in Health and Disease

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The meninges are the fibrous covering of the central nervous system (CNS) which contain vastly heterogeneous cell types within its three layers (dura, arachnoid, and pia). The dural compartment of the meninges, closest to the skull, is predominantly composed of fibroblasts, but also includes fenestrated blood vasculature, an elaborate lymphatic system, as well as immune cells which are distinct from the CNS. Segregating the outer and inner meningeal compartments is the epithelial-like arachnoid barrier cells, connected by tight and adherens junctions, which regulate the movement of pathogens, molecules, and cells into and out of the cerebral spinal fluid (CSF) and brain parenchyma. Most proximate to the brain is the collagen and basement membrane-rich pia matter that abuts the glial limitans and has recently be shown to have regional heterogeneity within the developing mouse brain. While the meninges were historically seen as a purely structural support for the CNS and protection from trauma, the emerging view of the meninges is as an essential interface between the CNS and the periphery, critical to brain development, required for brain homeostasis, and involved in a variety of diseases. In this review, we will summarize what is known regarding the development, specification, and maturation of the meninges during homeostatic conditions and discuss the rapidly emerging evidence that specific meningeal cell compartments play differential and important roles in the pathophysiology of a myriad of diseases including: multiple sclerosis, dementia, stroke, viral/bacterial meningitis, traumatic brain injury, and cancer. We will conclude with a list of major questions and mechanisms that remain unknown, the study of which represent new, future directions for the field of meninges biology.

Keywords: meninges, fibroblast, meningeal lymphatic system, arachnoid barrier, blood-CSF barrier, border-associated macrophages

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INTRODUCTION

The meninges are the multifaceted structure surrounding the brain and spinal cord with three structurally and cellularly distinct layers: the pia, arachnoid, and dura. The meninges house a variety of cell types including the largest population of CNS fibroblasts, three different vascular networks, specialized immune populations, neural stem cells and suture stem cells. The meninges were once considered a simple protective structure, but we now understand that the diversity of cells found in the meninges mediate multiple CNS functions. In this review, we will detail the different cell

populations of the meninges and discuss their role in CNS development, homeostasis, injury, and disease.

MENINGEAL CELL TYPES

The meninges contain two compartments: the leptomeninges (collective term for pia and arachnoid layers) and the dura (**Figure 1A**). The vascular make-up, fibroblast, and immune cell populations are different between the two compartments, as are their roles in development, homeostasis, and disease. In this section, we will review the cellular, molecular, and developmental identities of meningeal cells, with a particular focus on the fibroblast populations.

Fibroblasts

Fibroblasts, once thought of as purely structural cells, are now known to execute a variety of functions throughout the body. These functions, which are often organ and tissue specific, include the regulation of neighboring blood vessels, immune cells, and lymphatic vessels, through the production of growth factors, cytokines, and extracellular matrix remolding (Liu et al., 2008; Mueller and Germain, 2009; Barron et al., 2016; Chapman et al., 2016; Furtado, 2016; Roulis and Flavell, 2016; Pikor et al., 2017; Buechler and Turley, 2018; Tallquist, 2020; Wang et al., 2020). Consistent with diverse roles, single cell transcriptome studies of the CNS and non-CNS organs demonstrate transcriptional heterogeneity in fibroblast populations that can correlate with specific locations within a tissue (Saunders et al., 2018; Vanlandewijck et al., 2018; Dobie et al., 2019; Tsukui et al., 2020). Our recent single cell RNA sequencing (scRNAseq) of developing mouse meninges showed pia, arachnoid and dura fibroblasts are molecularly distinct and likely have layer specific functions (DeSisto et al., 2020). However, we are just beginning to understand how the molecular and cellular features of meningeal fibroblasts emerge during development and how these specializations translate to function.

Primitive, non-layer specific meningeal fibroblasts are first observed as a mesenchymal layer called the "primary meninx" which surrounds the nervous system by embryonic day 10.5 (E10.5) in the mouse (McLone and Bondareff, 1975; Dasgupta and Jeong, 2019). Meningeal fibroblasts in different CNS regions have different origins; forebrain meningeal fibroblasts are neural crest derived whereas mid-, hindbrain and spinal cord meninges are derived from the mesoderm (Jiang et al., 2002; Yoshida et al., 2008). By E12, the mouse meninges is organized into a thin outer layer of flat cells and a loose inner layer, which show distinct molecular profiles (Dasgupta et al., 2019; DeSisto et al., 2020). By E13.5 the three layers of meninges (pia, arachnoid, and dura) can be identified in the ventral forebrain, with fibroblasts from each layer having established unique transcriptional signatures and cellular specializations (DeSisto et al., 2020). Maturation and differentiation of the meningeal layers continues in a ventral to dorsal pattern over the forebrain (Vivatbutsiri et al., 2008; Siegenthaler et al., 2009; DeSisto et al., 2020).

The pia is a single layer of fibroblasts that sits adjacent to the glia limitans, a basement membrane (BM) that contacts the brain parenchyma and serves as the attachment point for radial glia cells during development and later for astrocytic end feet (McLone and Bondareff, 1975; Zhang et al., 1990; Sievers et al., 1994; Beggs et al., 2003). The transcriptional profile of pial fibroblasts shows enriched expression of extracellular matrix (ECM) genes, many of which are key components of the pial basement membrane (DeSisto et al., 2020). This supports pial fibroblasts as critical for pial BM maintenance. Embryonic pial cells uniquely express genes not seen in arachnoid or dura fibroblasts, such as *S100a6* and *Ngfr*, and have sub-populations that correlate with specific brain regions (DeSisto et al., 2020). The functional relevance of pial fibroblast heterogeneity in the developing meninges is not known nor is it known if this persists in adult meninges.

Arachnoid fibroblasts are organized into a meshwork of column-like trabecular structures that create space for large blood vessels and open pockets for cerebrospinal fluid (CSF) (**Figure 1**). Studies using transmission and scanning EM in rat and human show arachnoid trabeculae are pillars of collagen fibrils surrounded by fibroblasts (Alcolado et al., 1988; Saboori and Sadegh, 2015; Mortazavi et al., 2018; **Figure 1B**). Trabecular pillars are not seen in mouse arachnoid cells, and EM studies instead show long cellular processes of fibroblasts that span the subarachnoid space (McLone and Bondareff, 1975; **Figure 1C**). Fibroblasts associated with human arachnoid trabeculae have been previously described in EM studies as un-specialized leptomeningeal fibroblasts (Alcolado et al., 1988). However, we found that the molecular profile of human and mouse arachnoid fibroblasts, including in human those associated with trabecula, differs from other leptomeningeal fibroblasts in the pia. Arachnoid fibroblasts express RALDH2 and CRABP2, a retinoic acid (RA) synthesizing enzyme and an RA binding protein, respectively (**Figures 1D,E**; DeSisto et al., 2020). Production of RA by the arachnoid layers critically regulates brain and neurovascular development (Siegenthaler et al., 2009; Choi et al., 2014; Bonney et al., 2016; Mishra et al., 2016; Haushalter et al., 2017). Arachnoid fibroblasts are also uniquely enriched in other secreted factors such as *Wnt6*, *Angptl2*, and *Bmp4* that may act locally in other meninges cells or adjacent structures such as the brain or calvarium (Dasgupta et al., 2019; DeSisto et al., 2020).

Fibroblasts in the mouse dura are organized into an outer layer that adhere to the underside of calvarium bones and an inner layer that contact arachnoid barrier cells (**Figure 1A**). Fibroblasts in the outer dural layer are essentially the periosteal cells of the calvarial bones and consist of abundant collagen fibrils. The inner layer of dural fibroblasts are called dural border cells and are immediately adjacent to arachnoid barrier cells (Nabeshima et al., 1975; Alcolado et al., 1988). Of note, a scRNAseq study of the developing mouse cranial suture indicate that outer periosteal dural fibroblasts and inner dural border fibroblasts are likely molecularly distinct fibroblast populations (Farmer et al., 2021). Additionally, dural fibroblasts have been shown to play a role in suture patency during calvarium expansion to accommodate brain growth (Cooper et al., 2012; Yu et al., 2021).

The last class of fibroblasts seen in the meninges are perivascular fibroblasts (**Figure 1A**). Perivascular fibroblasts are found around large diameter blood vessels throughout

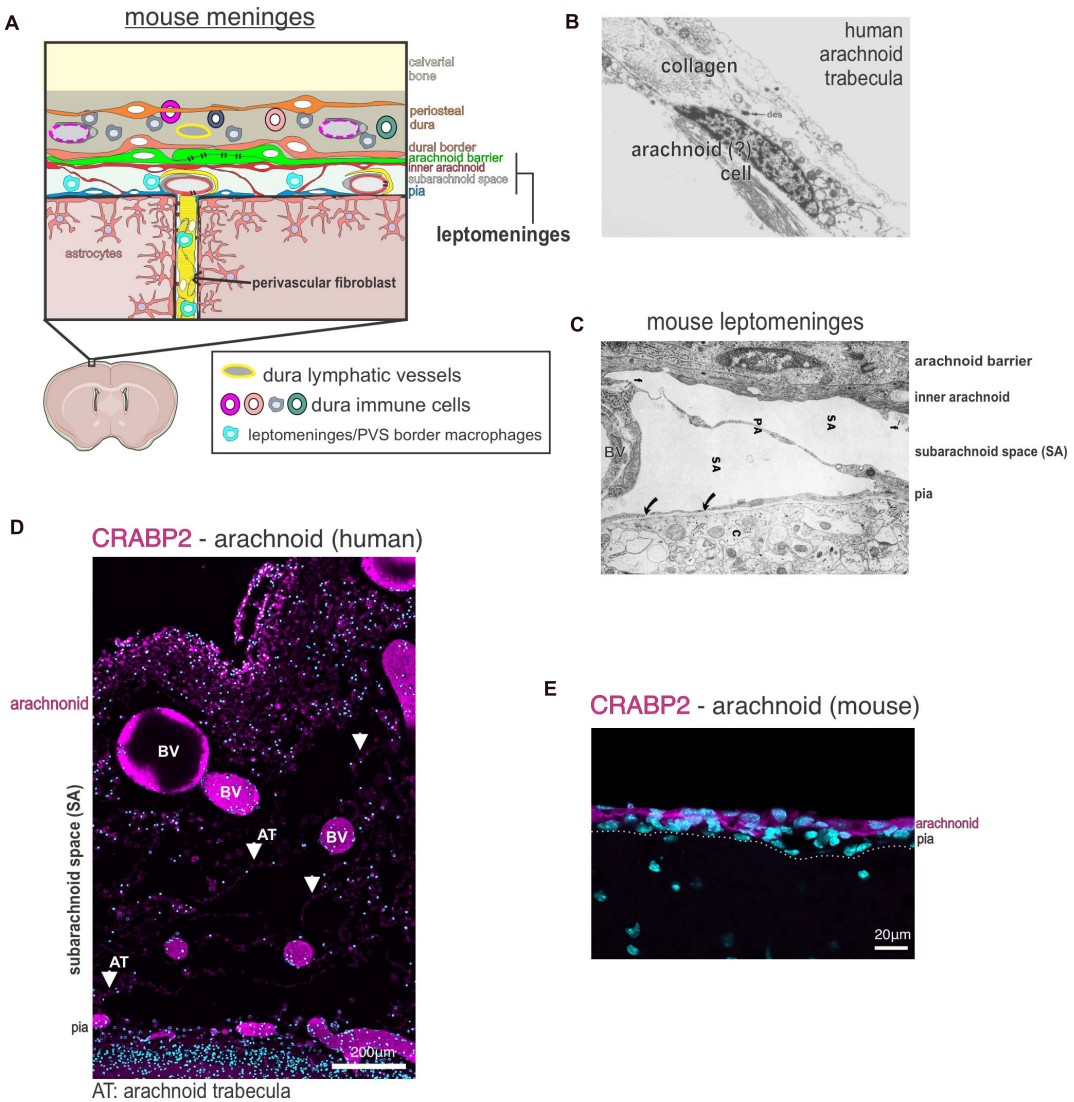

FIGURE 1 | Meninges structure and cellular heterogeneity **(A)** Schematic of cellular make up and structure of the mouse meninges and contiguous perivascular space. **(B)** Electron microscopy image of a human arachnoid trabecula, the collagen fibril structures that span the wide of the sub-arachnoid space. A cell, potentially an arachnoid, is seen associated with the collagen fibril. Image reproduced with permission from Alcolado et al. (1988). **(C)** Electron microscopy image of the mouse leptomeninges, a "pia-arachnoid" cell process (PA) spans the subarachnoid space (SA) containing blood vessels (BV). Cells of the inner arachnoid are immediately adjacent to the arachnoid barrier cell layer, which contains microfibrils (f) of extracellular matrix material. Image reproduced with permission from McLone and Bondareff (1975). **(D)** Immunofluorescence image of human fetal leptomeninges in the Sylvian sulcus labeled with CRABP2 (magenta) and DAPI (cyan). CRABP2 immunoreactivity in the meninges is limited to the arachnoid layer and cells associated with the arachnoid trabecula (AT) in the subarachnoid space (SA). Image reproduced with permission from DeSisto et al. (2020). BV, blood vessels. **(E)** Immunofluorescence image of mouse leptomeninges at postnatal day 14 labeled with CRABP2 (magenta) and DAPI (cyan). CRABP2 immunoreactivity is seen in cells of the arachnoid layer but not in pia-located cells. Image reproduced with permission from DeSisto et al. (2020).

the pia and sit immediately adjacent to the vascular smooth muscle layer (Zhang et al., 1990; Hannocks et al., 2018; Riew et al., 2020; Bonney et al., 2021; Sato et al., 2021). Perivascular fibroblasts are also found around penetrating arterioles and pre-capillary arterioles but not capillaries in the CNS parenchyma of human (Zhang et al., 1990) and rodent (Soderblom et al., 2013; Kelly et al., 2016; Hannocks et al., 2018; Bonney et al., 2021; Dorrier et al., 2021). Recent work using 2-photon live imaging in adult mice detailed the topography of perivascular fibroblasts, showing that perivascular fibroblasts in the brain parenchyma can extend over 200µm on cerebral penetrating arterioles but only extend very short distances on ascending venules (Bonney et al., 2021). Studies of human and rodent meninges described perivascular fibroblasts as "pial" or "leptomeningeal" cells that form an adventitial layer (Zhang et al., 1990; Hannocks et al., 2018; Riew et al., 2020). However, the exact molecular identity of perivascular fibroblasts has not yet been fully elucidated. For example, are perivascular fibroblasts

a homogenous population or are the specialized based on tissue location (meninges vs. parenchyma) or type of blood vessel they surround? It is also unknown how perivascular fibroblasts compare to non-vascular meningeal fibroblasts (such as those in the arachnoid or arachnoid barrier). Do they differ transcriptionally, developmentally, or functionally? Perivascular fibroblasts share some of the same markers as pial fibroblasts such as collagen-1, laminin α1 and Platelet-derived growth factor receptor-α (PDGFRα) (Kelly et al., 2016; Hannocks et al., 2018; Vanlandewijck et al., 2018). Perivascular fibroblasts are of high interest in the context of brain injury and neurological disease because their activation contributes to fibrotic scar formation and inflammation (discussed in detail below). Very little is known about perivascular fibroblasts during CNS development or adult homeostasis, and we expect that future work detailing perivascular fibroblasts will yield important results.

Arachnoid Barrier Cells

The arachnoid barrier, part of the blood CSF-barrier, is an epithelial-like cell layer that separates and controls transport between the dura and CSF filled subarachnoid space (Saunders et al., 2008, 2013; **Figures 1**, **2**). The subarachnoid space, defined by the arachnoid barrier cells on top and pia below, is continuous with perivascular spaces in the brain parenchyma, meaning that molecules in the CSF can access brain tissue (Iliff et al., 2012; Hannocks et al., 2018). Thus, the arachnoid barrier creates a border between the fenestrated vasculature of the dura and CNS (**Figure 2A**). Arachnoid barrier cells express a variety of transporters and can be altered by disease or infection (detailed below), but few functional studies have been done on the arachnoid barrier in comparison to other CNS barriers like the blood brain barrier (BBB). Further, little is known regarding arachnoid barrier development. We recently found that arachnoid barrier cells can be first seen in the mouse forebrain around E13 and they differentiate from mesenchymal cells of the primary meninx (DeSisto et al., 2020).

EM studies first illuminated that the arachnoid barrier cells are laden with tight junctions over 40 years ago (Nabeshima et al., 1975; **Figure 2B**). These findings were preceded by tracer studies demonstrating a functional barrier between the dura and subarachnoid space (Rodriguez, 1955), later confirmed using horseradish peroxidase (HRP) tracer (Balin et al., 1986). Tight junction containing arachnoid barrier cells are observed in the meniges of multiple species from jawless fish to humans, demonstrating evolutionary conservation (Nabeshima et al., 1975; Nakao et al., 1988; Vandenabeele et al., 1996; Rascher and Wolburg, 1997; Brøchner et al., 2015). Arachnoid barrier cells express the tight junction protein Claudin 11, best known for its role in the blood-testes barrier and maintenance of myelin wraps, along with E-Cadherin, consistent with their epithelial-like identity (**Figure 2C**; Rascher and Wolburg, 1997; Bhatt et al., 2013; Brøchner et al., 2015; Uchida et al., 2019b; Ximerakis et al., 2019; DeSisto et al., 2020).

Like other CNS barrier cells, arachnoid barrier cells are enriched in a variety of transporters, including: ABCB1 (Pglycoprotein or Pgp), ABCG2 (BCRP), ABCC4 (MRP4), and SLC transporters OAT1 (Slc22a6) and OAT3 (Slc22a8) (**Figure 2A**; Ek et al., 2010; Møllgård et al., 2017; Yaguchi et al., 2019). Functional studies show that arachnoid barrier OAT1 and OAT3 participate in solute clearance out of the CSF (Yasuda et al., 2013; Uchida et al., 2019a). The exact role for arachnoid barrier cells in controlling CSF composition and CNS transport is still unclear, but it's likely that a detailed understanding of arachnoid barrier cell function could be leveraged to improve CNS drug delivery. Finally, understanding if the arachnoid barrier plays a role in maintaining the vascular and immune specialization seen in the dura and leptomeninges (detailed below) could hold clinical importance.

Blood Vessels: Leptomeninges and Dura

The blood vessels of the leptomeninges, often referred to as the pial vasculature, are connected to the parenchymal CNS vasculature, and located in the subarachnoid space. Several studies show this vasculature has barrier properties and are therefore part of the meninges blood-CSF barrier. Structural studies show that pial blood vessels have tight junctions, including expression of occludin and claudin proteins, as well as adherens junctions, that link to actin filaments (Nabeshima et al., 1975; Nakao, 1979; Cassella et al., 1997; Mazaud-Guittot et al., 2010; Zihni et al., 2016; Liebner et al., 2018; Rua and McGavern, 2018). Further, the pial vasculature is not permeable to peripheral injections of horseradish peroxidase (44 kD) (Balin et al., 1986), and has a high trans-endothelial electrical resistance (Butt et al., 1990; Revest et al., 1994). Pial blood vessels lack expression of PLVAP (Daneman et al., 2010), a component of blood vessels fenestrations, and show high expression of glucose transporter GLUT-1 (Sabbagh et al., 2018). Of note, the pial vascular plexus lacks proximate astrocytic end feet, which the BBB possesses pervasively (Blanchette and Daneman, 2015). Pial vasculature also lacks capillaries; thus, the relevance of its barrier properties may serve more in controlling the movement of immune cells and other blood contents into and out of CSF than fine-tuning solute transport. Overall, the barrier structures seen in the pial vasculature regulates the free movement of molecules and cells into the CSF and CNS.

The pial vasculature forms via vasculogenesis starting at E8 in the mouse, first around the spinal cord and expanding to encapsulate the forebrain starting at E10. This developmental blood vessel system is referred to as the perineural vascular plexus (PNVP) (Nakao et al., 1988). PNVP formation is initiated by VEGFA secretion from the neural tube (Hogan et al., 2004) and is strongly influenced by fibroblasts of the meninges. For example, retinoic acid produced by arachnoid fibroblasts regulates endothelial Wnt-ß-catenin signaling to promote PNVP growth (Mishra et al., 2016). Endothelial Wnt-ß-catenin signaling likely controls the acquisition of barrier properties in the PNVP, as it does in parenchymal vasculature (Zhou et al., 2014; Mishra et al., 2016). Interestingly, VEGF and Wnt ligands are expressed by pia and arachnoid fibroblasts, respectively, during development (DeSisto et al., 2020) and these signals likely regulate PNVP development. The PNVP continues to grow and mature postnatally (Coelho-Santos and Shih, 2020), but the exact role for the meningeal fibroblasts at these later time points is currently unknown.

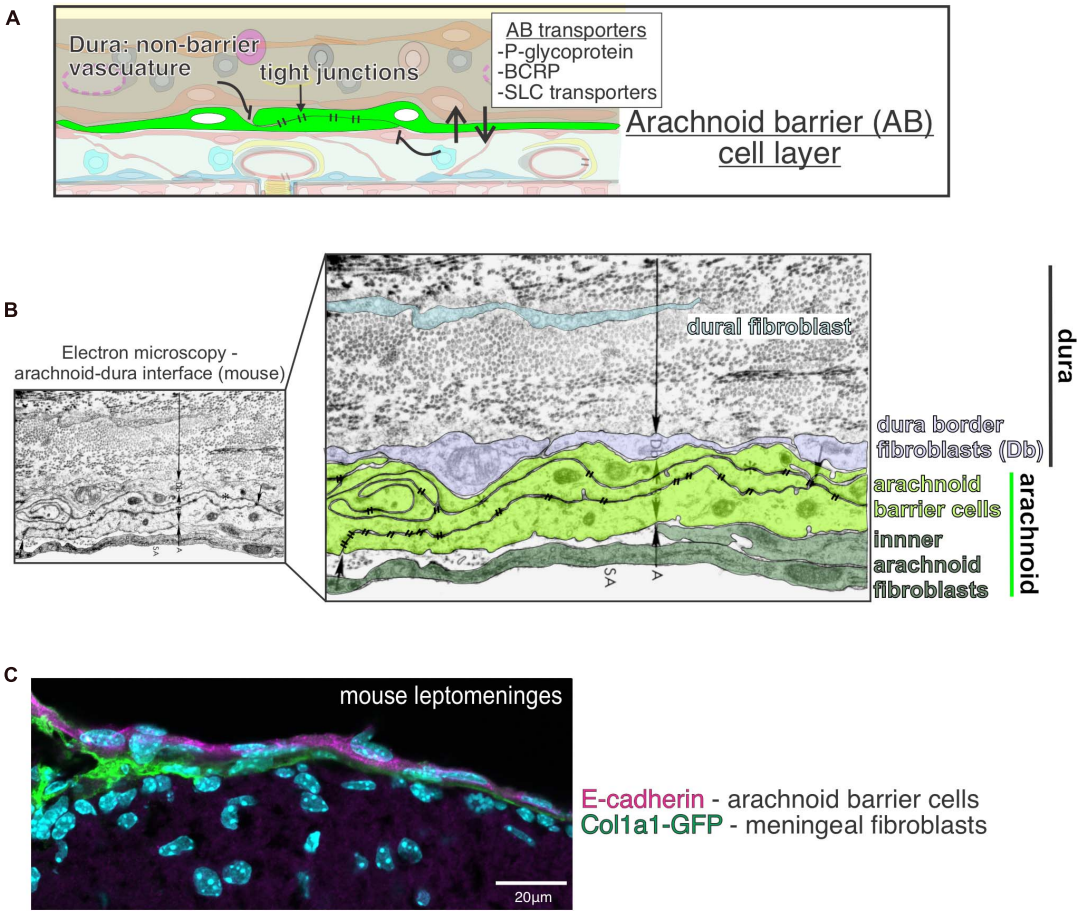

FIGURE 2 | Function and structure of the arachnoid barrier layer in the meninges. **(A)** Graphical depiction of proposed functions of the arachnoid barrier layer of the meninges including (1) as a physical barrier preventing free movement of molecules and cells into the subarachnoid space by virtue of its tight junctions, (2) enriched expression of efflux (P-glycoprotein, BCRP) and solute transporters (SLC) by arachnoid barrier cells support regulated movement of molecules across this barrier layer. **(B)** Electron microscopy image of the mouse arachnoid-dura interface, reproduced with permission from Nabeshima et al. (1975). Pseudo-coloring of the cell bodies highlights close interface between cells of the arachnoid (A) (inner arachnoid fibroblasts and arachnoid barrier cells connected by electron-dense tight junctions) and cells of the dura, [dura border cells (Db) and dural fibroblasts within collagen fibril dense dura layer]. **(C)** Immunofluorescence image of mouse leptomeninges from a postnatal day 14 *Col1a1-GFP* mouse brain with E-cadherin (magenta) and DAPI (cyan). E-cadherin is expressed by arachnoid barrier cells (representing the outer part of the arachnoid layer) but not by inner *Col1a1-GFP*+ fibroblasts, representing pial and inner arachnoid fibroblasts. Image reproduced with permission from DeSisto et al. (2020).

The dura contains an extensive network of blood vessels that include arteries, veins, and fenestrated capillary beds (Shukla et al., 2002, 2003; Coles, 2017; Mecheri et al., 2018). A unique feature of the dura vasculature is the presence of multiple large veins called dural venous sinuses which serve as the main exit for blood from the brain via the cerebral veins (Shukla et al., 2003; Coles, 2017; Mecheri et al., 2018). The non-barrier, fenestrated blood vessels of the dura, are permeable to horseradish peroxidase (MW 44 kD) administered intravenously (Balin et al., 1986) and allow non-selective movement of cells and molecules from the peripheral circulatory system into the dura. The proximity of the leptomeningeal barrier vasculature and non-barrier vasculature of the dura, especially in animals with thin meninges like rodents, raises the question, what are the mechanisms that maintain these specialized properties? Embryonic dural fibroblasts are enriched in Wnt inhibitors *Dkk2* and *Sfrp1* which could prevent development of barrier properties in the dural blood vasculature as occurs in other circumventricular organs (Benz et al., 2019; Wang Y. et al., 2019; DeSisto et al., 2020). Many details of dural blood vessel plexus development have yet to be elucidated. Development of the dura venous sinuses starts around E12 and occurs through the remodeling of three developmental venous plexuses (Tischfield et al., 2017). Proper development of dura venous sinus veins requires paracrine BMP signaling from skull progenitors cells and dural fibroblasts and is disrupted in skull malformations such as craniosynostosis (Tischfield et al., 2017). Away from the dural sinuses, recent work showed that between P0 to P28 there is a gradual reduction in dural blood vessel density and branching (Sato et al., 2021) however, the molecular pathways controlling initial dura blood vascular plexus growth and refinement have so far not been studied in any detail.

Lymphatic Vessels: Dura

Although the structure of lymphatic vessels was first described in 1787 by Mascagni, scientific literature consistently purported that the CNS completely lacked lymphatic vasculature until these vessels were "rediscovered" in 2015 (Bucchieri et al., 2015; Louveau et al., 2015). The dural lymphatics are now recognized as a critical transport system of macromolecules, interstitial fluid, and CSF from the CNS into the cervical lymph nodes (Aspelund et al., 2015; Absinta et al., 2017; Louveau et al., 2018; Ahn et al., 2019). Further, the altered function of dural lymphatics is now implicated in several neurodegenerative diseases (Louveau et al., 2017; Da Mesquita et al., 2018b, 2021).

The lymphatic system in the dura does not form until after birth in the mouse, much later than the peripheral lymphatic system (Izen et al., 2018) and meningeal blood vessels (reviewed above). Dural lymphatic vessels are composed of specialized endothelial cells that express VEGFR-3, LYVE-1, SLC, PDPN, and Prox1 and require VEGF-C signaling and lymphatic flow to properly mature (Antila et al., 2017; Izen et al., 2018; Bálint et al., 2019). By approximately postnatal day 20 in a mouse, the intracranial lymphatic vessels that line the dural sinuses and the extracranial lymphatic vessels that abut cranial nerves, become fully functional, draining content from the CNS into the peripheral lymphatic system at the base of the skull (Aspelund et al., 2015; Antila et al., 2017; Izen et al., 2018; Rustenhoven et al., 2021). Interesting, the dural lymphatic vasculature network is more voluminous in the ventral portions of the skull and display increased complexity, including valves, which the superior lymphatics lack (Ahn et al., 2019).

The three vascular plexuses of the meninges (pial, dural, lymphatics) are strikingly diverse in their developmental timing, barrier properties, and the functions they serve. The variations in barrier integrity have major implications in waste drainage, immune trafficking, antigen presentation, and drug delivery to the brain. Overall, the meningeal vascular systems are critical to CNS function.

Immune Cells

The meninges house an extensive immune cell population that are increasingly recognized to execute important functions in the CNS. The immune cells present in the leptomeninges differ from those present in the dura and several recent reviews detail the transcriptional and functional diversity of meningeal immune cells (Rua and McGavern, 2018; Kierdorf et al., 2019; Alves de Lima et al., 2020). Specifically, Rua and McGavern provide important details for T cells including that CD4+ T cells traffic from the blood to the dura and then the deep cervical lymph nodes, potentially "scanning" the dura meningeal tissue before returning to the lymph node; they also summarize how CD4+ T and B cells mediate homeostatic behavior and that these cells are enriched in the dura with aging. Kierdorf et al. (2019)'s review delves into the barriers that segregate macrophages into various compartments in the CNS at the dura, leptomeninges, choroid plexus, and perivascular compartments and how these populations are differentially regulating homeostasis and disease processes. Finally, Alves de Lima et al. (2020) is a meticulous review of meningeal immune compartments with important speculation on the future directions and importance of meningeal immune cell function. Our goal with this section is to give an overview of meningeal immune cells under homeostatic conditions and direct readers to comprehensive reviews for further details, such as the ones listed above.

The adult leptomeninges primarily harbor macrophages and non-migratory dendritic cells, along with much smaller numbers of lymphoid cells (Mrdjen et al., 2018; Jordão et al., 2019; Van Hove et al., 2019). The adult dura contains macrophages, mast cells, B cells, T cells, neutrophils, innate lymphoid cells, and the largest population of dendritic cells, including migratory dendritic cells, in the CNS (Mrdjen et al., 2018; Jordão et al., 2019; Van Hove et al., 2019). Of note, immune cells of the dura are not evenly distributed through the tissue but rather accumulate around dural venous sinuses (Rustenhoven et al., 2021). Dural venous sinuses are an active site of immune cells trafficking and have emerged as a key neuroimmune interface (Rustenhoven et al., 2021). Stromal cells of the dural sinuses (mural cells and fibroblasts) promote immune cell trafficking and T cell extravasation through their expression of ICAM, VCAM, and Cxcl12 (Rustenhoven et al., 2021). T cells interact with dural antigen-presenting cells laden with CSF derived antigens (Rustenhoven et al., 2021), representing a novel mode of peripheral immune cell surveillance of the CNS. In addition to T cells, gut-educated IgA+ B cells also localize next to the dural venous sinuses to protect against bacterial and fungal brain infection (Fitzpatrick et al., 2020).

Macrophages of the meninges are one of better documented meningeal immune cell and belong to a highly specialized class of macrophages called border associated macrophages (BAMs). BAMs and microglia both originate from yolk sac erythro-myeloid progenitors and can be detected in the brain as early as E10 (Utz et al., 2020). As development continues BAMs and microglia segregate both physically and transcriptionally, with BAMs remaining in the leptomeninges (they are also in the choroid plexus and perivascular spaces) and expressing *CD206* and *Lyve1* which are not expressed by microglia. In the adult, leptomeningeal BAMs are defined by expression of *CD206*, *Lyve1*, *P2rx7*, and *Egfl7* and have significantly different transcriptional profiles from dural BAMs (Mrdjen et al., 2018; Van Hove et al., 2019). Adult dural BAMs don't express *Lyve1* and can also be divided in subgroups. For example, one group of dural BAMs has low expression of major histocompatibility complex II (MHCII^{lo}) and express *Clec4n*, *Clec10a*, *Folr2,* while MHCII^{hi} dural BAMs express greater CCR2, implicating a monocytic origin. Another important difference is that leptomeningeal BAMS are long lived while dural BAMs are continuously renewed by peripheral monocytes (Goldmann et al., 2016; Van Hove et al., 2019). The bone marrow in the calvarium and vertebral column specifically supply monocytes and neutrophils to the dura during homeostasis (Cugurra et al., 2021) and to the meninges and brain parenchyma following brain injury or in neuroinflammation via vascular tunnels connecting the bone marrow and dura (Herisson et al., 2018; Yao et al., 2018; Cai et al., 2019; Cugurra et al., 2021). The unique properties seen among leptomeningeal and dural BAMs is consistent with specialized

functions for these populations in their respective barrier and non-barrier compartments.

While several studies have begun to investigate BAMs in disease (discussed below) very little has been worked out regarding their function in development or homeostasis. Further, the investigations looking into to the details and functions of the other meningeal immune cell populations have only just started.

MENINGEAL RESPONSE TO INJURY AND DISEASE

The next sections will highlight how meningeal cell types and structures respond to CNS injury (traumatic brain injury, stroke, spinal cord injury), infections (meningitis), and disease (multiple sclerosis, cancer, Alzheimer's disease) (**Figure 3**). We also recommend several other comprehensive recent reviews that highlight the meningeal vasculature, immune cells, and lymphatics/glymphatics in disease (Rasmussen et al., 2018; Rua and McGavern, 2018; Mastorakos and McGavern, 2019; Alves de Lima et al., 2020; Bolte and Lukens, 2021).

Meningeal and Perivascular Cells in Acute CNS Injury

Acute injuries to the CNS, such as stroke, traumatic brain injury (TBI) or spinal cord injury (SCI), induce a cascade of immediate events beginning with BBB breakdown, peripheral immune infiltration, acute inflammation, and tissue edema, which ultimately leads to neuronal cell loss (reviewed in Mastorakos and McGavern, 2019). These immediate events are followed by a protracted formation of fibrotic and glial scars, which have both neuroprotective and detrimental effects (Hesp et al., 2018). CNS acute injury responses are driven by a variety of CNS parenchymal cells, perivascular cells and meningeal cell types. For example, formation of the glial scar, which is largely driven by astrocytes, prevents peripheral immune cell invasion and limits inflammation by "sealing" off the infarct area from the healthy CNS tissue. Further, the glial scar works together with the fibrotic scar, which is primarily generated by meningeal and perivascular fibroblasts, to facilitate wound healing (Bundesen et al., 2003; Kelly et al., 2016; Dias and Göritz, 2018; Hesp et al., 2018; Riew et al., 2018). However, both the glial and fibrotic scars also act as major barriers to neural regeneration and axon regrowth long-term (Hesp et al., 2018). The contradictory effects of scar formation underscore the importance of investigating the cell and molecular mechanisms that drive post injury astrocyte and fibroblast behavior. Beyond astrocytes and fibroblasts, other perivascular and meningeal cells and structures like immune cells and lymphatics, also contribute to the CNS injury acute injury response and are discussed in more detail below.

CNS Fibroblasts in Acute CNS Injury

There is ample evidence for fibrotic scar formation following acute CNS injury, but there is some confusion regarding the exact identity of the fibrotic scar forming cells. Beginning ∼3 days post injury, the fibrotic scar forming cells, which are located perivascularly and in all three layers of the meninges, become activated; characterized by proliferation, detachment from the vasculature, increased expression of ECM molecules (vimentin, fibronectin, type I collagens), and upregulation of smooth muscle actin (SMA) (Fernández-Klett et al., 2013; Soderblom et al., 2013; Kelly et al., 2016; Dias and Göritz, 2018; Riew et al., 2018; **Figure 3B**). The fibrotic scar forming cells express PDGFRβ [which is expressed by fibroblasts, pericytes and vascular smooth muscle cells (vSMCs)], along with PDGFRα, and Collagen-1, which are expressed by fibroblasts, but not pericytes or vSMCs (Soderblom et al., 2013; Kelly et al., 2016; Vanlandewijck et al., 2018). Fibrotic scar forming cells are labeled in the *Collagen1a1-GFP* mouse line, which marks fibroblasts but not pericytes, and do not express common markers of pericytes, like desmin and Ng2 (Göritz et al., 2011; Soderblom et al., 2013; Kelly et al., 2016). Thus, the expression pattern of fibrotic scar forming cells is consistent with that of fibroblasts, which are found in both the leptomeninges and CNS perivascular spaces (Soderblom et al., 2013; Kelly et al., 2016). Further support was demonstrated in a mouse model of multiple sclerosis, where the authors used a *Col1a2-CreER*T mouse line, which labels all CNS fibroblasts but not pericytes or vSMCs, with a Cre reporter and found that almost all fibrotic cells within the lesion were labeled by the reporter (Dorrier et al., 2021). In contrast, *Ng2-CreERT* lineage traced cells, which includes pericytes, vSMCs and some neural populations, did not show any expansion in the lesion (Dorrier et al., 2021). While not done in an acute injury model, this evidence further supports that CNS fibroblasts, not pericytes or vSMCs, are the main reactive fibrotic cell type in CNS injuries and disease. It should be noted that some of the confusion surrounding fibrotic scar forming cells comes from a lack of established nomenclature, as scar forming cells are sometimes called stromal cells, mesenchymal cells, Type A Pericytes, Type 2 pericytes and fibroblasts (Göritz et al., 2011; Dias et al., 2018, 2020). In summary, fibroblasts from the meninges and perivascular space are major drivers of fibrotic scar formation following CNS injury however establishing a standard nomenclature would be beneficial.

Another major function of CNS fibroblasts following acute CNS injury is communication with neighboring cells (**Figure 3B**). For example, in a rat spinal cord transection model of SCI, direct signaling between fibrotic cells and astrocytes mediated the glial/fibrotic scar border formation (Bundesen et al., 2003). In an SCI compression injury model, fibrotic scar fibroblasts transiently increased Wnt/β-catenin signaling (Yamagami et al., 2018), and since Wnt signaling is known to drive fibrosis in other organs (Chilosi et al., 2003), and Wnt ligand expression is increased following SCI (Fernández-Martos et al., 2011; González-Fernández et al., 2014), induction of fibroblast WNT signaling likely contributes to fibrosis. In the photothrombotic stroke injury model, activation of TGF-β1 and retinoic acid signaling pathways in meningeal fibroblasts stimulated arachnoid barrier cells and facilitated reconstruction of the blood-CSF barrier (Cha et al., 2014). Fibroblast production of retinoic acid is also implicated as an important regulator of the post CNS injury response by several other studies. For example, perivascular fibroblasts express retinoic acid

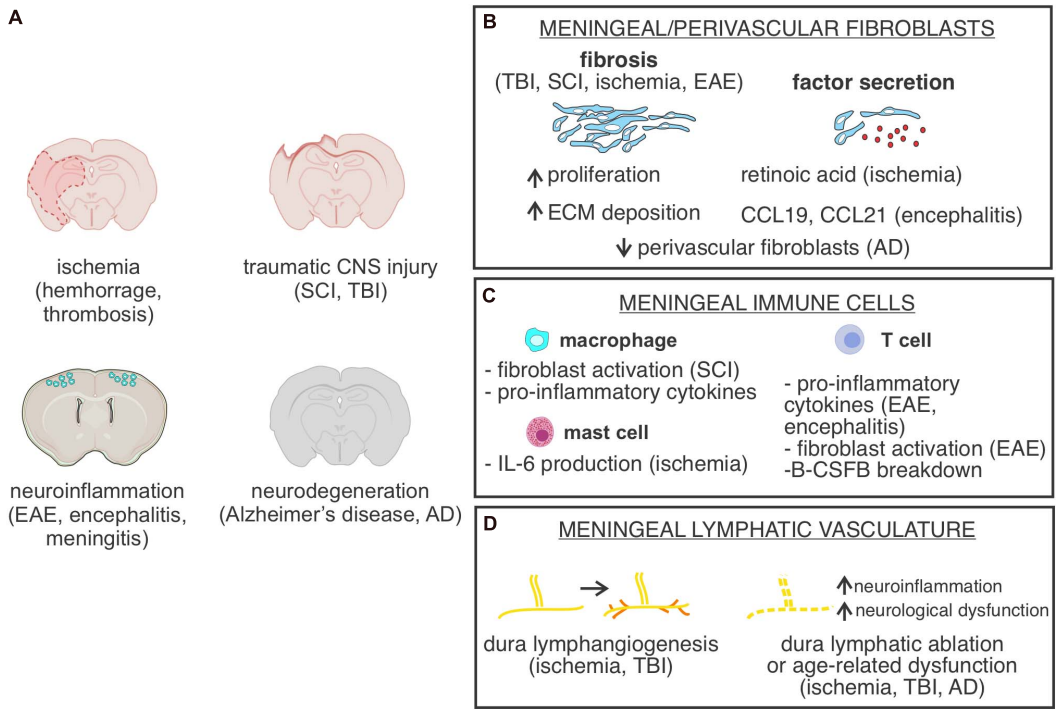

FIGURE 3 | Meningeal cells or structures in CNS injury and disease. **(A)** Depiction of CNS injury or diseases in which meningeal cells, meninges located cell types or parenchyma located perivascular fibroblasts are part of the pathology. SCI, spinal cord injury; TBI, traumatic brain injury; EAE, experimental autoimmune encephalomyelitis. **(B)** Graphical depiction of fibrosis, caused by increased meningeal/perivascular fibroblast proliferation and Extracellular matrix (ECM) deposition and summary of known CNS fibroblast-derived factors identified in specific CNS disease states. AD, Alzheimer's disease. **(C)** Summary of functional roles of meningeal-located immune cells in specific CNS disease states. B-CSFB, blood-CSF barrier. **(D)** Summary of cellular changes in meninges located lymphatic vasculature in response to different CNS injuries and disease states.

synthesizing enzymes RALDH1 and RALDH2 in uninjured brain, and the number of RALDH-expressing fibroblasts increase in the fibrotic scar following stroke injury in mice (Kelly et al., 2016). Retinoic acid signaling was elevated in neurons and astrocytes in the peri-infarct region, suggesting that scar fibroblasts signal to the surrounding CNS cells via retinoic acid release (Kelly et al., 2016). Interestingly, retinoic acid treatment following stroke in rodents reduces neurogenesis while also reducing angiogenesis and gliogenesis in the peri-infarct region (Jung et al., 2007). Thus, fibroblast production of retinoic acid seems to impair CNS recovery while stimulating fibrosis. Overall, continued investigations that detail the cellular and molecular behaviors of post injury CNS fibroblasts will forward our understanding of damage induced pathology and provide therapeutic insight.

Meningeal Immune Cells in Acute CNS Injury

The degree of neuroinflammation following acute CNS injury is a major predictor of clinical outcomes, and numerous studies show that meningeal immune cells play an important role in the brain injury response (reviewed in Arac et al., 2014; Corps et al., 2015; Iadecola et al., 2020). In a study investigating the contribution of meninges-located mast cells in stroke pathology, depletion of mast cells resulted in decreased levels of neuroinflammation, brain swelling, and infarct size following stroke and this was partially mediated by mast-cell derived IL-6 (Arac et al., 2019; **Figure 3C**). Similarly, in a sub-arachnoid hemorrhage model, depletion of macrophages in the leptomeninges and perivascular spaces surrounding large arterioles prior to injury results in improved neurological scoring and reduced levels of inflammation and neuronal cell death (Wan et al., 2021).

Resident meningeal macrophages regulate the acute injury response by influencing the severity of neuroinflammation and fibrosis (**Figure 3C**). For example, in a mild closed-skull compression injury model, Cx3cr1+ meningeal macrophages are observed dying within 30 min of injury induction, leading to formation of a "honeycomb" network of microglia at the glial limitans, likely preventing the spread of reactive oxygen species from the leptomeninges into the brain parenchyma (Roth et al., 2013). In both MCAO and SCI models, macrophages are observed in the fibrotic scar and may associate with fibroblasts (Zhu et al., 2015; Kelly et al., 2016). One study showed that CD11b+ macrophages are partially responsible for recruitment of fibroblasts to the fibrotic scar following SCI (Zhu et al., 2015). The molecular mechanisms by which macrophages in the injury lesion could activate fibroblasts is unclear, though depletion of macrophages prior to SCI leads to depletion of pro-fibrotic cytokines, such as tumor necrosis factor superfamily members Tnfsf8 and Tnfsf13 (Zhu et al., 2015). Inhibition of pro-fibrotic

cytokines could serve as a therapeutic to reduce fibrotic scarring and improve axon regrowth.

Meningeal Lymphatics in Acute CNS Injury

Several recent studies have highlighted the contributions of dual meningeal lymphatics, and more recently, brain perivascular glymphatics, in the neuroinflammatory response following acute CNS injury (Iliff et al., 2014; Plog et al., 2015; Rasmussen et al., 2018). The brain glymphatic and meningeal lymphatic systems work together to clear waste and macromolecules from the brain parenchyma in homeostasis and following injury (Iliff et al., 2014; Plog et al., 2015; Rasmussen et al., 2018). Following TBI, glymphatic fluid flow is disrupted and greater amounts of tau protein is observed accumulating in the parenchyma (Iliff et al., 2014). Additionally, knock-out of *Aqp4*, a water transport channel necessary for interstitial solute clearance and glymphatic function, following TBI, exacerbates the accumulation of tau in the parenchyma (Iliff et al., 2014). Consistent with these results, disruption of glymphatic function results in lower levels of TBI-associated biomarkers in the cervical lymph nodes and bloodstream (Plog et al., 2015). These results highlight the important function of the glymphatic system in clearance of molecular substances from the brain and potential role in facilitating signaling between the brain and periphery.

The dural lymphatic function is also disrupted following acute CNS injury. In a mild closed-skull TBI model, meningeal lymphatic drainage is impaired likely due to increases in intracranial pressure, accompanied by lymphatic morphological changes (Bolte et al., 2020). Interestingly, lymphangiogenesis was observed up to 2 weeks following TBI, though this did not correspond with increased lymphatic function (Bolte et al., 2020). Similarly, lymphangiogenesis was seen following photothrombotic stroke lesion in the mouse cortex, but this effect was not seen following MCAO (Yanev et al., 2020; **Figure 3D**). Thus, the degree of lymphatic impairment and response is dependent on the injury mechanism, and there is likely communication of lymphatic vessels with surrounding tissues following injury.

The importance of meningeal lymphatics post-injury is further highlighted by studies that selective ablation of meningeal lymphatic vessels worsens pathophysiology (**Figure 3D**). In a subarachnoid hemorrhage model, erythrocytes accrue in the cervical lymph nodes via meningeal lymphatics following injury, and ablation of meningeal lymphatic vessels leads to less erythrocyte aggregation and greater neuroinflammation and neurological defects (Chen et al., 2020). Consistent with this observation, pre-existing defects in meningeal lymphatics causes worsened neuroinflammation following TBI (Bolte et al., 2020). Likewise, stimulating meningeal lymphatic vessel outgrowth via VEGF-C administration had decreased levels of gliosis (Bolte et al., 2020). Manipulating VEGF-C/VEGFR3 signaling is a promising avenue for mitigating post-injury complications related to meningeal lymphatic drainage. Blockage of VEGFR3 (expressed by lymphatic endothelial cells) in mice following focal cerebral ischemia resulted in reduced inflammatory response and infarct (Esposito et al., 2019). However, VEGFR3 mutant mice subjected to MCAO develop larger stroke volumes, thus worsened edema (Yanev et al., 2020). Altogether, meningeal lymphatics play a key role in regulating the post-injury response, and further work is needed to tease apart the context-dependent functions and key signaling mechanisms involved in meningeal lymphatic response to acute CNS injury.

Response of the Meninges to Meningitis

A number of infectious agents can induce meningitis, or inflammation of the meninges, including viruses, bacteria, parasites, or fungus (McGill et al., 2017; Wright et al., 2019). Meningitis induces a variety of acute complications (severe headache, fever, photophobia, and neck pain) and regularly induces a range of long term neurological sequelae and sleep defects (Schmidt et al., 2006; Lucas et al., 2016; McGill et al., 2018). Meningitis induced inflammation is driven by the response of resident meningeal immune cells, meningeal fibroblasts and infiltrating leukocytes to the infectious agent (Coles et al., 2017; Manglani and McGavern, 2018; Rua and McGavern, 2018). For example, during murine CNS infections, including mouse hepatitis virus, a murine coronavirus (strains Srr7, cl-2, and A59), the Armstrong strain of lymphocytic choriomeningitis virus (LCMV), all show there is an influx into the brain and subarachnoid space of $CD8^+$ T cells, monocytes and neutrophils into the meninges quickly after infection (Kim et al., 2009; Takatsuki et al., 2009; Cupovic et al., 2016; Watanabe et al., 2016). However, the specific localization of immune cells and neuropathology vary by strain, administration, and dose. Interestingly, in the mouse model of corona virus MHV A59 infection, production of cytokines CCL19 and CCL21 by a meningeal fibroblasts subpopulation, marked by ER-TR7 antigen, and podoplanin, work to recruit anti-viral CD8+ T cells (Cupovic et al., 2016; **Figure 3B**). The role of meningeal fibroblasts in fighting viral infection shares similarities to the function of reticular fibroblasts in the lymph nodes and spleen that help drive immune responses by creating specialized microenvironments (Cupovic et al., 2016; Perez−Shibayama et al., 2019; Morgado et al., 2020). In the LCMV Armstrong mouse model, it was shown that the recruitment of antiviral $CD8^+$ T cells, while necessary to defeat the infection, can also be deleterious. These $CD8^+$ T cells also produce cytokines that can lead to over infiltration of monocytes and neutrophils that drive breakdown of vascular integrity and induce severe edema and brainstem herniation (Kim et al., 2009; Mastorakos and McGavern, 2019), which can ultimately lead to coma and death due to the complex interplay of vascular, hypoxic, and inflammatory changes.

In cases of bacterial meningitis, infections usually start as skin, gastrointestinal or respiratory infections that spread to the blood (Doran et al., 2016; Cain et al., 2019). Once in the blood, the meningitis causing bacteria accumulate in the leptomeningeal vasculature (Mook-Kanamori et al., 2011; Iovino et al., 2013) and move into the meninges by inducing BBB breakdown through multiple mechanisms (van Sorge and Doran, 2012; Kim et al., 2015; Coureuil et al., 2017). Bacterial presence in the meninges induces a strong inflammatory response, including production of multiple cytokines (tumor necrosis factor (TNF)-alpha,

interleukin (IL)-1-beta, and IL-6), and a massive infiltration of neutrophils (Mook-Kanamori et al., 2011; Principi and Esposito, 2020). The inflammatory cytokines and neutrophils work to quell the infection but also drive the breakdown of endothelial cell tight junctions (Banerjee et al., 2011; Barichello et al., 2011; van Sorge and Doran, 2012; **Figure 3C**).

While the reaction of meningeal immune and blood vessels to bacterial meningitis infection has been fairly well characterized, the response of meningeal fibroblasts and arachnoid barrier cells has only just begun to be investigated. All major meningitis causing bacteria can adhere strongly to meningeal fibroblasts and arachnoid barrier-like cells (Hardy et al., 2000; Fowler et al., 2004; Alkuwaity et al., 2012; Auger et al., 2015). The Doran laboratory has shown that Group B Streptococcus bacteria cross the blood brain barrier by binding to vimentin on endothelial cells (Deng et al., 2019). Vimentin is also highly expressed by arachnoid barrier cells (Weller et al., 2018), raising the possibility that this is an additional mechanism of entry for meningitis-causing bacteria, however this has yet to be experimentally assessed. Local cytokine production by meningeal immune cells may also disrupt arachnoid barrier integrity; intracisternal injection of IL1-β, highly upregulated in meningitis, induces rapid meningeal barrier leakage (Ichikawa and Itoh, 2011).

Overall, the pathology of meningitis is driven by a complex interplay between cells of the meninges and the peripheral immune system. Future work to detail the pathological mechanisms will lead to more effective treatments, improved initial diagnostics, and an enhanced understanding of what drives long term sequalae.

TABLE 1 | Future areas of investigation in meninges biology.

Topic	Future areas of investigation
Regionalization	Meningeal fibroblasts show regional gene expression during development, does embryonic regionalization persist in the adult?
	Do meningeal fibroblasts have CNS region-specific functions in the healthy CNS or during disease and injury?
Layer-specific meningeal functions	Layer-specific meningeal stroma/fibroblast populations can impact specific subpopulations of immune cells (Rustenhoven, 2021), do meningeal fibroblasts in other layers serve a similar function(s)?
	Are fibroblasts spatially heterogenous (perivascular vs. layer fibroblasts) in their functions that influence immune and vascular populations?
Arachnoid barrier	What controls the development of the epithelial-like arachnoid barrier vs. arachnoid fibroblasts cells?
	What are the range of functions for the arachnoid barrier and are these functions different between development and adulthood?
	Is a "leaky" arachnoid barrier related to acute or chronic CNS insults?
Dura vasculature	When and how does the dural blood vasculature develop? What mechanisms regulate development and maintenance of diverse vascular properties in the fenestrated blood dura (as opposed to the barrier leptomeninges vasculature)?
CNS fibroblast identity	Many whole brain single cell studies annotate fibroblast containing clusters as "vascular leptomeningeal cells," while other studies refer to these cells as stromal cells, mesenchymal cells, Type A Pericytes, Type 2 pericytes and fibroblasts. There is a lack of consensus on the spatial, transcriptional, and potential functional heterogeneity for these populations. Consistent annotation and analysis are needed to fully advance studies of different CNS fibroblast populations.

This table elucidates the prioritized areas of investigation on key topics that need further study, including: meningeal cellular regionalization, layer-specific functions, arachnoid barrier development and function, dural vasculature development, and CNS fibroblast identity.

Meninges and Perivascular Fibroblasts in Multiple Sclerosis

The leptomeninges are now recognized as a key player in multiple sclerosis (MS), an inflammatory CNS autoimmune disorder characterized by axon demyelination (Russi and Brown, 2015; Pikor et al., 2017; Rua and McGavern, 2018; Wicken et al., 2018). In both MS patients and MS mouse models, immune cells infiltrate the leptomeninges and pathology is commonly seen in cortical areas adjacent to the meninges (Lucchinetti et al., 2011; Mitsdoerffer and Peters, 2016; Pikor et al., 2017). In the experimental autoimmune encephalomyelitis (EAE) mouse model of MS, autoreactive effector T cells first infiltrate the leptomeninges via the pial vasculature, are activated by antigen presenting meningeal/perivascular macrophages, and subsequently enter the CNS parenchyma, triggering lesion formation (Bartholomäus et al., 2009; Schläger et al., 2016). Biopsies taken from early MS patients with cortical demyelination were more likely to have leptomeningeal inflammation, consisting of effector T cells and IgA B cells infiltrates, than those without cortical demyelination. Furthermore, in human cases of MS the severity of cortical lesions is correlated to the extent of meningeal inflammation (Lucchinetti et al., 2011). The presentation of MS induced leptomeningeal inflammation can be variable, ranging from disorganized collections of immune cells to organized ectopic lymphoid follicle-like structures (Choi et al., 2012; Mitsdoerffer and Peters, 2016; Wicken et al., 2018). Emerging work suggests that the ectopic lymph structures seen in MS are potentiated by leptomeningeal fibroblasts which form a reticular cell network consisting of activated fibroblasts in a scaffold-like structure, and that these fibroblasts "scaffolds" might be driven by a subset of meningeal fibroblast that express podoplanin (Pikor et al., 2015, 2017). This is consistent with another report showing that lymphoid follicle-like structures in the cerebral leptomeninges contained $CD20^+$ B-cells, $CD8^+$, $CD4^+$, and $CD3^+$ T-cells, $CD138^+$ plasma cells, and a network of $CD21^+$ and $CD35^+$ follicular dendritic cells (Serafini et al., 2004). It remains unclear if ectopic lymph structures in the leptomeninges potentiate MS pathology or forms because of the pathology, but the meninges are clearly an important player in MS induced neuroinflammation.

Like acute CNS injury, CNS fibroblast activation and expansion contribute to MS induced lesions. Using a *Col1a1-GFP* mouse line in conjunction with an EAE model, two independent groups identified substantial increases in fibroblasts within spinal cord lesion sites (Yahn et al., 2020; Dorrier et al., 2021). Fibroblast expansion was deleterious for oligodendrocyte precursor cell

(OPC) differentiation into mature oligodendrocytes and OPC migration into the lesion, suggesting that fibrosis may be deleterious for remyelination (Yahn et al., 2020). Of interest, a scRNAseq analysis showed that fibroblasts from EAE lesions upregulate interferon γ (IFNγ) signaling, and conditional deletion of IFN receptor γ from fibroblasts partially blocked fibroblast expansion (Dorrier et al., 2021). IFNγ is produced by spinal cord T cells in EAE mice, implicating immune cells in EAE lesions as a source of fibroblast activation signals (Dorrier et al., 2021; **Figure 3C**). Increased number of PDGFRβ-expressing cells are detected in human MS pathology samples (Dias et al., 2020) and increased ECM protein deposition is a common pathological feature of MS lesions (van Horssen et al., 2006), further supporting fibroblast driven fibrosis as a feature of MS. Continued studies on the mechanisms and consequences of MS induced fibrosis will likely provide therapeutic insight.

BBB breakdown is a well-documented feature of MS (Russi and Brown, 2015; Russi et al., 2018; Sweeney et al., 2019) and there is some evidence that MS can also induce disruption of the arachnoid barrier in the meninges, part of the B-CSF barrier (Bartholomäus et al., 2009; Schläger et al., 2016; Uchida et al., 2019b). Claudin 11 is a tight junction protein enriched in arachnoid barrier cells and it is downregulated in late stages of an EAE mouse model (Uchida et al., 2019b). The mechanism of downregulation and if this translates into functional breakdown is currently unknown, however, it may relate to cytokines produced by local immune cell infiltration into the meninges. Previous studies have indicated that cytokine administration into the cisterna magna of mice is sufficient to drive arachnoid barrier and blood-cerebral spinal fluid barrier functional breakdown, however this has not been shown definitively in the EAE models or MS patients (Ichikawa and Itoh, 2011). Activated T cells in the leptomeninges produce cytokines known to perturb tight junction integrity of brain endothelial cells, including IFNγ which is known to drive BBB breakdown in viral encephalitis (Bonney et al., 2019; **Figure 3C**). A disruption to the arachnoid barrier could permit dural and peripheral immune cells to migrate into the leptomeninges, and potentially contribute to leptomeningeal immune infiltrate that is common in MS pathology. However, more detailed investigations into the impact and cellular signals that drive this infiltration still need to be conducted. Overall, the meninges play a critical role in MS and continued work to uncover its exact contributions hold high clinical relevance.

The Meninges and Alzheimer's Disease

Several lines of evidence implicate meningeal-located structures in the genesis and progression of Alzheimer's disease (AD) (for recent reviews see Da Mesquita et al., 2018a; Rasmussen et al., 2018; Nedergaard and Goldman, 2020). AD is a dementia inducing neurodegenerative disorder characterized by the accumulation of amyloid-β containing plaques, which were initially isolated from homogenates of AD patient meningeal tissue (Joachim et al., 1988; Da Mesquita et al., 2018b). The transport of interstitial fluids and macromolecules (including amyloid-β peptides) out of the brain occurs via a complex transport network that utilizes the perivascular glymphatic system of the brain and lymphatic vessels of the meninges (Rasmussen et al., 2018; Nedergaard and Goldman, 2020). Multiple publications show that both the brain glymphatic and meningeal lymphatic systems deteriorate with age (Kress et al., 2014; Ma et al., 2017; Da Mesquita et al., 2018b; Ahn et al., 2019) and their dysfunction can potentiate AD pathology and dementia (Peng et al., 2016; Da Mesquita et al., 2018a,b; Rasmussen et al., 2018; Wang L. et al., 2019; Nedergaard and Goldman, 2020; **Figure 3D**). For example, disruption of meningeal lymphatic vessels promotes amyloid-β deposition, in both the brain and meninges (Da Mesquita et al., 2018b; Wang L. et al., 2019), while the rescue of age-induced meningeal lymphatic defects improved cognitive performance (Da Mesquita et al., 2018b). In the APP/PS1 mouse model of AD, glymphatic dysfunction as measured by CSF clearance rates was impaired (Peng et al., 2016), and injection of amyloid-β into the CSF reduced glymphatic activity (Wang L. et al., 2019). Further, the ablation of meningeal lymphatics impedes anti-amyloid-β therapy by exacerbating microgliosis, neurovascular dysfunction, and behavior defects in the 5XFAD model of Alzheimer's disease (Da Mesquita et al., 2021). Together this supports that impairment of meningeal lymphatics is a feature of AD and a potential therapeutic target.

Meningeal blood vessels, fibroblasts and macrophages are also of interest in AD pathology but their exact roles are unclear. There is overwhelming evidence for cerebrovascular alterations in AD pathology and amyloid-β deposition around leptomeningeal vessels is a hall mark of cerebral amyloid angiopathy, occurring in almost all AD patients (Greenberg et al., 2020). Of interest, recent scRNAseq profiling of human brain vasculature from healthy and AD cerebral cortex showed that perivascular fibroblasts were significantly under-represented in the AD brain single cell data set (Yang et al., 2021; **Figure 3B**). This is in contrast to large increases in CNS fibroblast numbers after acute CNS injuries and in neuroinflammation, demonstrating a different response of perivascular fibroblasts in AD. How accumulation of amyloid-β in the meningeal vasculature may potentiate neuronal impairment or impact meningeal fibroblast and immune cell populations is still being worked out. There are evidence that perivascular and barrier macrophages, found both in the brain parenchyma and leptomeninges, play a role in amyloid-β removal as their depletion causes increased amyloid deposition (Hawkes and McLaurin, 2009). Future studies that further probe AD induced changes to meningeal/perivascular fibroblasts, blood vessels and immune cells will forward our understanding of AD pathophysiology.

Meninges as a Site of Cancer Metastasis

Primary tumors of the meninges are quite rare, however, the leptomeninges is a relatively common site for by contiguous extension of primary tumors of the central nervous system, paranasal sinuses and skull base origin or tumor metastasis which can lead to dissemination into the CNS parenchyma and poor prognosis (Mahendru and Chong, 2009; Waki et al., 2009; Oechsle et al., 2010; Scott and Kesari, 2013). Cancer cells may enter the meninges via the choroid plexus, the brain, by crossing pial blood vessels or by vascular channels that connect the bone marrow and meninges (Redmer, 2018; Yao et al., 2018). To cross

the BBB, tumor cells bind endothelial cells and disrupt their tight junctions (Bos et al., 2009; Kienast et al., 2010; Fazakas et al., 2011; Redmer, 2018). Melanoma cells adhere to and disturb the interaction of brain endothelial cells, which maintain the integrity of the BBB, through a disruption of tight and adherence junction proteins such as Claudin 5 and ZO-1. In addition, proteolytic enzymes such as heparanase and seprase are important for the capacity of metastatic cells to traverse the BBB and occupy the brain (Fazakas et al., 2011). Here, micrometastases give rise to macrometastases through proliferation along brain microvessels (Kienast et al., 2010). Additionally, breast cancer cells express ST6GALNAC5, which is normally exclusively expressed in the brain, allowing for increased adhesion to brain endothelial cells to pass through the BBB (Bos et al., 2009). Further, acute lymphoblastic leukemia cells access the CNS via vascular channels that exist between bone marrow located in the vertebral and calvarium bone and the meninges (Yao et al., 2018).

Once in the leptomeninges, cancer cells can modulate the CSF content in the subarachnoid space content to support cancer growth. CSF is acellular, poor in protein, glucose and cytokine content, which is not conducive to cancer cell proliferation, however, cancer cells are able to grow in the nutrient deficient CSF filled leptomeninges using several notable mechanisms. For example, tumor cells present in the CSF secrete complement component 3 (Boire et al., 2017), which disrupts the barrier functions of choroid plexus epithelial cells, allowing nutrients and macromolecules to enter the CSF (Boire et al., 2017). scRNAseq analysis done on tumor cells isolated from the leptomeninges of patients with metastatic growth showed high expression of the iron-binding protein lipocalin-2 (Lcn2) its receptor *SLC22A17* (Chi et al., 2020). Lcn2 expression, which is induced by macrophage cytokine release, allows tumor cells to grow more effectively in the low nutrient CSF environment. Thus, tumor cells can effectively alter the leptomeninges microenvironment, however, how tumor cells effect leptomeningeal fibroblasts has not been well-studied. Continued studies to the detail the interactions between cancer cells and the leptomeninges could help develop new treatments.

CONCLUSION

Here we have sought to summarize many decades of research on the cellular composition and structures of the meninges, as well as their development and function in the health CNS and contribution to injury and disease. Recent technical advances such as 2-photon *in vivo* imaging in rodents, transgenic mouse lines to better visualize meningeal cell subtypes, scRNAseq to appreciate meningeal cell heterogeneity as well as key conceptual advances in meningeal function (ex: meningeal lymphatics, connection to glymphatics, meningeal immune cell function) has enabled important new discoveries about how the meninges serves as a key interface between the CNS and periphery. There are several novel areas of meningeal biology, particularly as it relates to meningeal fibroblasts and arachnoid barrier, that we would like to highlight as areas of research in the future as well as current challenges that need to be overcome (**Table 1**). Further examination into the precise functions of meningeal subpopulations during homeostasis and disease may provide important insights to develop novel treatments for CNS disorders. Improved capacity to target exact subpopulations of meningeal cells may allow us to slow or halt pathogenesis and restore CNS health by reducing meningeal inflammation and barrier breakdown.

AUTHOR CONTRIBUTIONS

JD, HEJ, CC, BP, and JS contributed to conceptualization, writing, and editing of the review. All authors contributed to the article and approved the submitted version.

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CORRECTION
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Corrigendum: Living on the Edge of the CNS: Meninges Cell Diversity in Health and Disease

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by Derk, J., Jones, H. E., Como, C., Pawlikowski, B., and Siegenthaler, J. A. (2021). Front. Cell. Neurosci. 15:703944. doi: 10.3389/fncel.2021.703944

In the original article **Yao et al., 2018** was not cited in the article. The citation has now been inserted in ***Meningeal cell types***, ***Immune cells***, ***Paragraph 3*** and should read:

Macrophages of the meninges are one of better documented meningeal immune cell and belong to a highly specialized class of macrophages called border associated macrophages (BAMs). BAMs and microglia both originate from yolk sac erythro-myeloid progenitors and can be detected in the brain as early as E10 (Utz et al., 2020). As development continues BAMs and microglia segregate both physically and transcriptionally, with BAMs remaining in the leptomeninges (they are also in the choroid plexus and perivascular spaces) and expressing *CD206* and *Lyve1* which are not expressed by microglia. In the adult, leptomeningeal BAMs are defined by expression of *CD206*, *Lyve1*, *P2rx7*, and *Egfl7* and have significantly different transcriptional profiles from dural BAMs (Mrdjen et al., 2018; Van Hove et al., 2019). Adult dural BAMs don't express *Lyve1* and can also be divided in subgroups. For example, one group of dural BAMs has low expression of major histocompatibility complex II ($MHCII^{lo}$) and express *Clec4n*, *Clec10a*, *Folr2,* while $MHCII^{hi}$ dural BAMs express greater CCR2, implicating a monocytic origin. Another important difference is that leptomeningeal BAMS are long lived while dural BAMs are continuously renewed by peripheral monocytes (Goldmann et al., 2016; Van Hove et al., 2019). The bone marrow in the calvarium and vertebral column specifically supply monocytes and neutrophils to the dura during homeostasis (Cugurra et al., 2021) and to the meninges and brain parenchyma following brain injury or in neuroinflammation via vascular tunnels connecting the bone marrow and dura (Herisson et al., 2018; Yao et al., 2018; Cai et al., 2019; Cugurra et al., 2021). The unique properties seen among leptomeningeal and dural BAMs is consistent with specialized functions for these populations in their respective barrier and non-barrier compartments.

In the original article, there was an error. We omitted reference to an important access mechanism for cancer cells to enter the CNS, vascular channels from the calvarial bone to the meninges described in Yao et al., 2018 *Nature*.

A correction has been made to ***Meningeal response to injury and disease***, ***Meninges as a site of cancer metastasis***, ***Paragraph 1***:

Primary tumors of the meninges are quite rare, however, the leptomeninges is a relatively

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common site for by contiguous extension of primary tumors of the central nervous system, paranasal sinuses and skull base origin or tumor metastasis which can lead to dissemination into the CNS parenchyma and poor prognosis (Mahendru and Chong, 2009; Waki et al., 2009; Oechsle et al., 2010; Scott and Kesari, 2013). Cancer cells may enter the meninges via the choroid plexus, the brain, by crossing pial blood vessels or by vascular channels that connect the bone marrow and meninges (Redmer, 2018; Yao et al., 2018). To cross the BBB, tumor cells bind endothelial cells and disrupt their tight junctions (Bos et al., 2009; Kienast et al., 2010; Fazakas et al., 2011; Redmer, 2018). Melanoma cells adhere to and disturb the interaction of brain endothelial cells, which maintain the integrity of the BBB, through a disruption of tight and adherence junction proteins such as Claudin 5 and ZO-1. In addition, proteolytic enzymes such as heparanase and seprase are important for the capacity of metastatic cells to traverse the BBB and occupy the brain (Fazakas et al., 2011). Here, micrometastases give rise to macrometastases through proliferation along brain microvessels (Kienast et al., 2010). Additionally, breast cancer cells express ST6GALNAC5, which is normally exclusively expressed in the brain, allowing for increased adhesion to brain endothelial cells to pass through the BBB (Bos et al., 2009). Further, acute lymphoblastic leukemia cells access the CNS via vascular channels that exist between bone marrow located in the vertebral and calvarium bone and the meninges (Yao et al., 2018).

The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

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Single Cell Transcriptomics of Ependymal Cells Across Age, Region and Species Reveals Cilia-Related and Metal Ion Regulatory Roles as Major Conserved Ependymal Cell Functions

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Ependymal cells are ciliated-epithelial glial cells that develop from radial glia along the surface of the ventricles of the brain and the spinal canal. They play a critical role in cerebrospinal fluid (CSF) homeostasis, brain metabolism, and the clearance of waste from the brain. These cells have been implicated in disease across the lifespan including developmental disorders, cancer, and neurodegenerative disease. Despite this, ependymal cells remain largely understudied. Using single-cell RNA sequencing data extracted from publicly available datasets, we make key findings regarding the remarkable conservation of ependymal cell gene signatures across age, region, and species. Through this unbiased analysis, we have discovered that one of the most overrepresented ependymal cell functions that we observed relates to a *critically* understudied role in metal ion homeostasis. Our analysis also revealed distinct subtypes and states of ependymal cells across regions and ages of the nervous system. For example, neonatal ependymal cells maintained a gene signature consistent with developmental processes such as determination of left/right symmetry; while adult ventricular ependymal cells, not spinal canal ependymal cells, appeared to express genes involved in regulating cellular transport and inflammation. Together, these findings highlight underappreciated functions of ependymal cells, which will be important to investigate in order to better understand these cells in health and disease.

Keywords: ependymal cell, ependyma, human, mouse, neonatal, adult, brain homeostasis, single-cell RNA-seq (scRNA-seq)

INTRODUCTION

Ependymal cells are ciliated glial cells that form an epithelial barrier, called the ependyma, lining the brain's ventricular system and the spinal cord's central canal. They develop from radial glia along the surface of the ventricles of the brain and spinal canal starting from the first postnatal days, thereby providing an interface between the parenchyma and cerebrospinal fluid (CSF)-filled

cavities throughout life. This interface allows ependymal cells to control the bidirectional passage of immune cells and solutes between the CSF and interstitial fluid (Alvarez and Teale, 2007; Mastorakos and McGavern, 2019); while also providing homeostatic regulation of molecules (Bedussi et al., 2015; Ma et al., 2017); in addition to playing a critical role in sensing and propelling CSF *via* primary and motile cilia, respectively (Bolborea et al., 2015).

Three subtypes of ependymal cells have been described: E1, E2, and E3 ependymal cells. The three distinct ependymal cell subtypes are defined by their cilia number and regional distribution (Mirzadeh et al., 2008, 2017) where E1 ependymal cells possess multiple motile cilia and are the most abundant subtype in the adult brain, occupying the majority of the lateral (forebrain), third (midbrain) and fourth (hindbrain) ventricles. E2 cells possess both primary and motile cilia and are bi-ciliated (Mirzadeh et al., 2008) and line the spinal canal (Alfaro-Cervello et al., 2012) in addition to occupying a portion of the third and fourth ventricles of the brain (Mirzadeh et al., 2017); while E3 ependymal cells have primary cilia, are uniciliated (Mirzadeh et al., 2017), and exist primarily in a small portion of the third ventricle, inhabiting the preoptic and infundibular recesses (Mirzadeh et al., 2017). The multitude of functions and diverse roles that these cells play is understudied (Lorencova et al., 2020).

In this study, publicly available single-cell transcriptomic datasets were screened to identify datasets encompassing ependymal cells across age, nervous system region, and species. Following quality control (QC) scrutiny, we were able to identify four datasets encompassing high-quality ependymal cells, including *mouse* ependymal cells from the adult and neonatal forebrain, and the spinal cord, in addition to ependymal cells from the adult human forebrain. By comparing datasets, we find striking homogeneity across ependymal cells in age, species, and region. This included a highly conserved gene signature related to metal ion regulation functions, which we confirmed using RNAscope—a fluorescence *in situ* hybridization strategy. Yet we also identified gene signatures that distinguished ependymal cells from neonatal and adult contexts, in addition to gene signatures that distinguished between the brain and spinal cord regions, which suggests that ependymal cell states and subtypes also have differing functions and mechanisms for maintaining CNS health.

MATERIALS AND METHODS

Single-Cell RNAseq Data Sourcing

Neonatal mouse forebrain dataset (n = 3) was accessed *via* GEO: GSE123335 (Loo et al., 2019), adult mouse forebrain dataset (n = 4) was accessed *via* GEO: GSE100320 (Shah et al., 2018), while the mouse spinal cord dataset (n = 3) was accessed from the PaglaoDB database under SRA/SRS accession numbers SRA667466/SRS3059989, SRA667466/SRS3059990, and SRA667466/SRS3059991. The human forebrain dataset (n = 5) was provided by Dr. Petrecca and can be made available by the corresponding author upon request.

Single-Cell RNAseq Quality Control

All datasets were imported into the Seurat (v4.0.1) R toolkit for QC and downstream analysis (Macosko et al., 2015). All functions were run with default parameters unless specified otherwise. Cells were excluded during QC selection according to recommended Seurat parameters: if the number of unique genes detected was less than 200, if the number of unique genes detected was greater than 2,500, or if the percentage of mitochondrial genes expressed was greater than 5% (or 10% in the case of the adult brain datasets). Gene expression was log normalized to a scale factor of 10,000. Following QC cut-offs, we identified 18,357 unique features across 41 ependymal cells in the neonatal dataset, 15,259 unique features across 142 ependymal cells in the adult mouse brain dataset, 24,506 unique features across 344 ependymal cells in the spinal cord dataset, and 18,312 unique features across 33 ependymal cells in the adult human brain datasets.

Single Cell Analysis

All codes are available at Github[1]. Pseudotime was performed using the Slingshot (v1.8.0) package (Street et al., 2018) where analysis was conducted on ependymal cell and radial glia clusters identified by the expression of marker genes *Foxj1* and *Gfap*, respectively. Default parameters were used throughout the workflow. An associated heatmap was generated as part of the standard Slingshot workflow. GO-term analysis was performed using the clusterProfiler (v3.18.1) package (Yu et al., 2012) where the top 200 differentially expressed genes were input (significantly regulated; p-value < 0.05 following Bonferonni correction), and the output figure contained the top 30 most significant GO-terms. P-values were adjusted by false discovery rate during clusterProfiler analysis. clusterProfiler (v3.18.1) was also used to generate the cnetplots provided in **Supplemental Material**, using the top 30 GO-terms, top 200 genes, and otherwise default parameters. Unless otherwise stated, heatmaps were generated using default parameters for the DoHeatmap function in Seurat. For heatmap in **Figure 1E**: immature and mature genes from neonatal pseudotime analysis were input and the heatmap was generated using the adult dataset. Technical limitations prevented us from including one gene (1700009P17Rik) as it was not found in the adult dataset. For heatmap in **Figure 5A**: ependymal cells from each mouse dataset were integrated using SCTransform and the top 60 differentially expressed genes were determined (significantly regulated; p-value < 0.05 following Bonferonni correction) by comparing genes up in neonatal ependymal cells, genes up in adult ependymal cells, genes up in spinal cord ependymal cells, genes down in neonatal ependymal cells, genes down in adult ependymal cells, and genes down in spinal cord ependymal cells compared to all other ependymal cells in the merged dataset. Heatmap was created with parameters adjusted to include the top 10 significant genes for each comparison.

[1] https://github.com/StrattonLab/scRNAseq_Frontiers

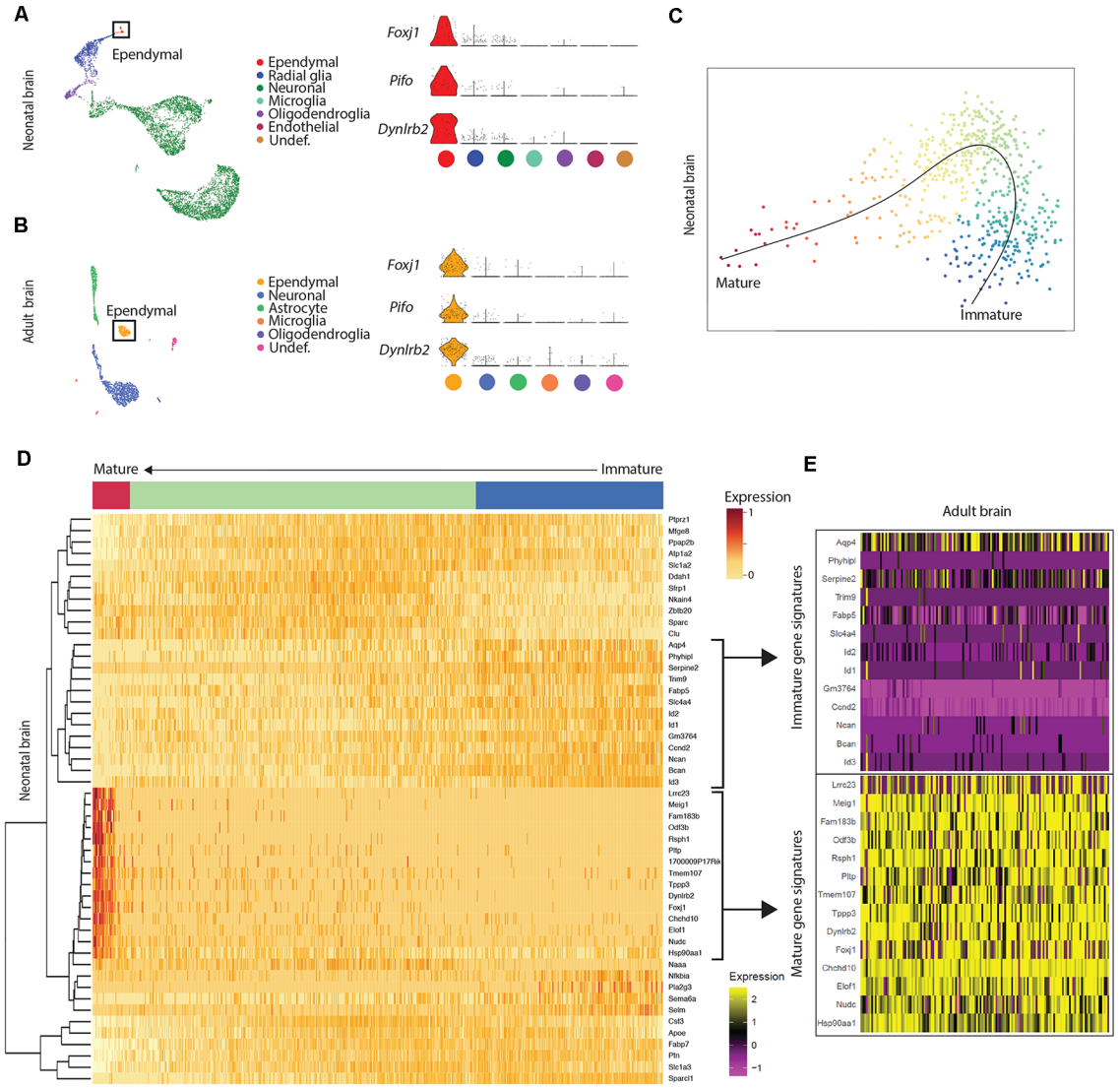

FIGURE 1 | Mature ependymal cells are detectable in the brain by postnatal day 0 and are maintained in adulthood. **(A)** UMAP plot of single-cell transcriptional profiles from neonatal mouse brain. One cluster in the neonatal brain was identified as ependymal cells (box) as per the expression of ependymal cell marker genes such as *Foxj1*, *Pifo*, and *Dynlrb2* (presented in violin plots). **(B)** UMAP plot of single-cell transcriptional profiles from adult mouse brain. One cluster was identified as ependymal cells (box) as per the expression of ependymal cell marker genes such as *Foxj1*, *Pifo*, and *Dynlrb2* (presented in violin plots). **(C)** PCA plot depicting pseudotime trajectory of ependymal cell development in neonatal mouse brain. Ependymal cells (*Foxj1*+ cluster) and radial glia (*Gfap*+ cluster; ependymal cell precursor cells) were extracted from the neonatal dataset and then pseudotime ordering was performed using Slingshot. This revealed a curved trajectory (line) where radial glia (immature ependymal cells, blue) give rise to mature ependymal cells (red) *via* an ordered progression of transcriptional changes. X- and Y-axis represent the first and second raw component scores, respectively. **(D)** Heatmap of differentially expressed genes generated from pseudo-ordered data in **(C)**. This revealed the top 50 most dynamically expressed genes across the pseudo-ordered state (Mature, red; Intermediate, green; Immature, blue). **(E)** Heatmap of adult brain ependymal cells. Selected genes representative of immature ependymal cells (blue in **D**) and mature ependymal states (red in **D**) from the neonatal dataset were plotted demonstrating a striking overlap between mature genes in neonatal and adult ependymal cells. Note the lack of immature gene signatures in adult ependymal cells is consistent with the adult ependymal cell population being fully mature by this stage.

Tissue

C57B6/J mice were used (Charles River Laboratories). Neonatal animals were postnatal day 0–1 (n = 3 per group), while adult animals were 12 weeks old (n = 3 per group). Mice were housed in a vivarium under pathogen-free conditions in cages of up to five animals. Studies were approved by the McGill University Animal Care and Use Committee (UACC) under animal use protocol 2019-8102. UACC guidelines on the ethical use and care of animals were followed. Human brain autopsy tissue was provided by Dr. Prat (U of Montreal) under McGill University Health Centre REB ethics #2020-6185.

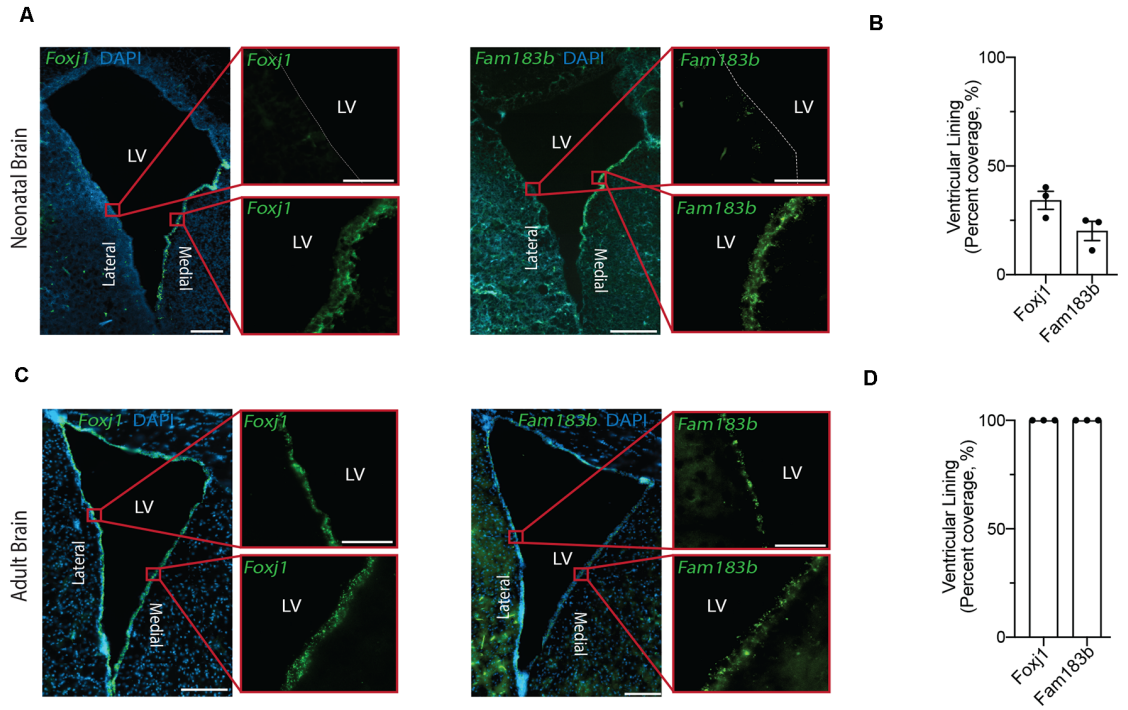

FIGURE 2 | *In situ* detection of mature ependymal cell genes in the ventricular lining of neonatal and adult brain validates the presence of mature ependymal cells from postnatal day 0 onwards. **(A,C)** Representative images of neonatal **(A)** and adult **(C)** mouse brains demonstrate the expression of *Foxj1* (left panels) and *Fam183b* (right panels) along the ventricles at both ages (green). Note the localization of mature ependymal cells along the medial wall of the neonatal ventricle suggesting the temporal progression of ependymal cell development follows a medial to lateral dynamic. LV = Lateral Ventricle. Scale bars: 200 μm (**A,C** overview); 50 μm (**A,C** zoomed). Dotted lines show ventricle-ependyma interface. **(B,D)** Quantification of the percentage of cells (DAPI+) expressing *Foxj1* or *Fam183b* along the ventricular lining of the neonatal **(B)** and adult **(D)** mouse brain (n = 3; mean ± SEM).

In situ Hybridization

Following cardiac 4% PFA perfusion, brains were harvested and post-fixed in PFA for 2 h. Brains were then kept in 30% sucrose at 4°C until they sank (48 h for mature brain, 24 h for neonatal brain), at which point brains were individually snap-frozen in O.C.T. (Tissue-Tek) and stored at −80°C. Tissue was coronally cryosectioned at 15 μm on Superfrost Plus slides (Fisher) at −20°C and stored at −80°C until processing for RNAscope (Advanced Cell Diagnostics, ACD). Human tissue was freshly collected then snap-frozen in OCT. Following cryosectioning, human tissue was fixed on-slide in 4% PFA for 15 min. For all tissue, RNA *in situ* hybridization was performed using RNAscope® (ACD). Tissue was processed according to the protocol set out in the user manual for fixed-frozen tissue unless otherwise stated. Briefly, slides were incubated in a target retrieval solution at 95°C for 15 min. Sections were then washed with 100% EtOH and air-dried for 5 min at room temperature. Sections were ringed using hydrophobic barrier pen (ImmEdge, Vector Labs; Cat. No. 310018) and then incubated with protease III for 20 min at room temperature. Sections were then washed once with PBS before carrying out RNAscope multiplex assays according to the manufacturer's protocols, albeit we used one single wash following each incubation to minimize tissue erosion. All incubations were at 40°C and used a humidity control chamber (HybEZ oven, ACD; Cat. No. 321711). Mouse probes were: Foxj1-C2 (Cat. No. 317091-C2), Fam183b (Cat. No. 515951), Mt3 (Cat. No. 504061), and Rarres2 (Cat. No. 572581), in addition to one human probe MT3-C2 (Cat. No. 525421-C2). Opal dye 520 and 570 fluorophores (Cat. No. FP1487001KT and Cat. No. FP1488001KT, respectively; Akoya Biosciences) were diluted in RNAscope TSA dilution buffer (1:1,000; Cat. No. 322809). Nuclei were labeled with DAPI (1/5,000; Invitrogen). Slides were mounted in PermaFluor Mounting Media (Thermofisher). Sections were imaged using a Zeiss Axio Observer (20× tile scan for the overview, 40× for zoomed images).

FIJI (ImageJ) was used to measure ventricle lining length and to measure the length of probe coverage along lining to calculate a percentage value that represents the coverage over the ventricle or canal. For *Rarres2* quantification, we measured the length of probe coverage along the medial ventricular lining. Statistical analyses were conducted with Graphpad Prism 8 and significance levels were obtained using one-way ANOVAs using Holm–Sidak's test to correct for multiple comparisons.

RESULTS

Publicly available single-cell transcriptomic datasets were screened to identify datasets encompassing ependymal cells

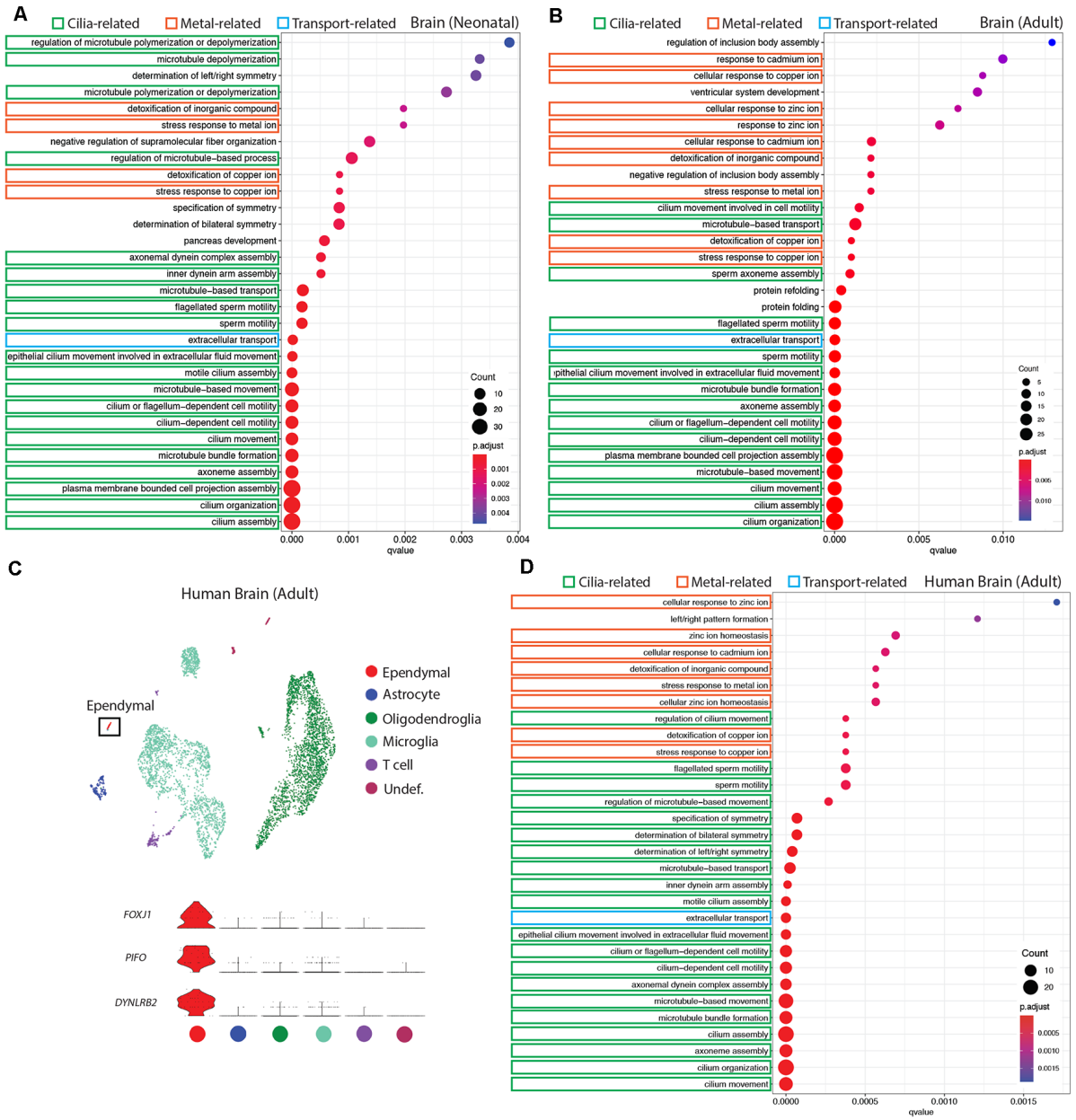

FIGURE 3 | Conservation of ependymal cell GO-terms in the ventricular lining of the brain across age and species. **(A,B,D)** Dot plots representing GO-terms generated from the top 200 differentially expressed genes in ependymal cells (vs. all other cell types in the respective dataset) were generated using clusterProfiler. The top 30 most significant GO-terms were plotted. GO-terms clustered in three major categories that were shared across age and species: cilia-related terms (green), metal ion-related terms (orange), and transport-related terms (blue). Dot size represents gene counts in the respective pathway. Node color intensity shows enrichment degree (*p*-values adjusted by FDR). *Q*-values were assigned to the horizontal axis to control for false positive discovery rate. **(A)** GO-terms in the neonatal mouse brain (20/30 terms are cilia-related, 4/30 are metal ion-related, 1/30 is transport-related, and 5/30 were other functions unique to the neonatal dataset). **(B)** GO-terms in the adult mouse brain (15/30 terms are cilia-related, 9/30 terms are metal ion-related, 1/30 is transport-related, and 5/30 were other functions unique to the adult dataset). **(C)** UMAP plot of single-cell transcriptional profiles from the adult human brain. One cluster was identified as ependymal cells (box) as per the expression of ependymal cell marker genes such as *Foxj1*, *Pifo*, and *Dynlrb2* (presented in violin plots). **(D)** GO-terms in the adult human brain (20/30 functions are cilia-related, 8/30 terms are metal ion-related, 1/30 is transport-related, and 1/30 is related to a miscellaneous function).

across age, region, and species. We extensively screened the literature and single-cell databases (PanglaoDB, Single Cell Portal, Single Cell Expression Atlas, etc.) to identify ependymal cell populations from diverse environments and conditions. Following QC scrutiny, we were able to identify four datasets encompassing high-quality ependymal cells, including ependymal cells from adult and neonatal forebrain, and from spinal cord in the mouse,

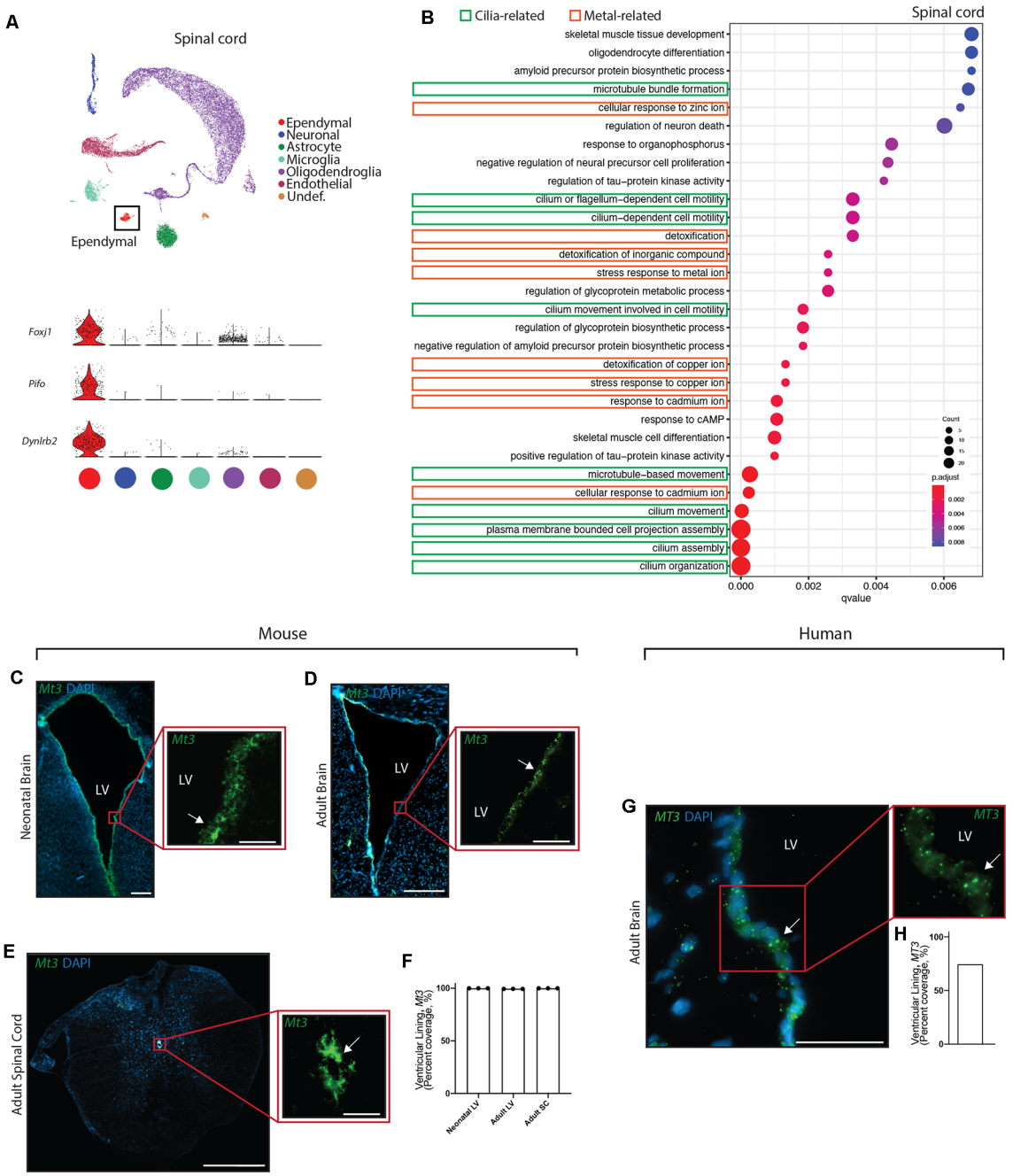

FIGURE 4 | Conservation of ependymal cell GO-terms and genes related to metal ion regulatory functions across age, species, and region. **(A)** UMAP plot of single-cell transcriptional profiles from adult mouse spinal cord. One cluster was identified as ependymal cells (box) as per the expression of ependymal cell marker genes such as *Foxj1*, *Pifo*, and *Dynlrb2* (presented in violin plots). **(B)** Dot plots representing GO-terms generated from the top 200 differentially expressed genes in ependymal cells (vs. all other cell types in the respective dataset) were generated from the adult mouse spinal cord dataset. The top 30 most significant GO-terms were plotted. GO-terms clustered in two major categories: cilia-related terms (green) and metal ion-related terms (orange; 9/30 terms are cilia-related, 8/30 terms are metal ion-related, and 13/30 were other functions unique to the spinal cord dataset). Dot size represents gene counts. Node color intensity shows enrichment degree (*p*-values adjusted by FDR). *Q*-values were assigned to the horizontal axis to control for false positive discovery rate. **(C–E)** Representative images of the neonatal ventricular lining **(C)**, adult ventricular lining **(D)**, and spinal canal **(E)** demonstrates the expression of a gene associated with metal ion buffering (*Mt3*) in mouse tissue (arrow). **(F)** Quantification of the percentage of cells (DAPI+) expressing *Mt3* along the ventricular and canal linings in mouse brain and spinal cord ($n = 3$; mean ± SEM). Note the localization of *Mt3* along the entire lining, including in the neonatal brain along the lateral border where mature ependymal cells do not reside. This suggests that metal ion related functions of the ventricular lining may be initiating even before the full maturation of ependymal cells. **(G)** Representative image of the adult human brain demonstrates the expression of *MT3* in the ventricular lining (arrow). **(H)** Quantification of the percentage of cells (DAPI+) expressing *MT3* along the ventricular lining in human brain. Scale bars: 200 μm (**C–E** overview), 50 μm (**C–E** zoomed; and **G**). LV = Lateral Ventricle.

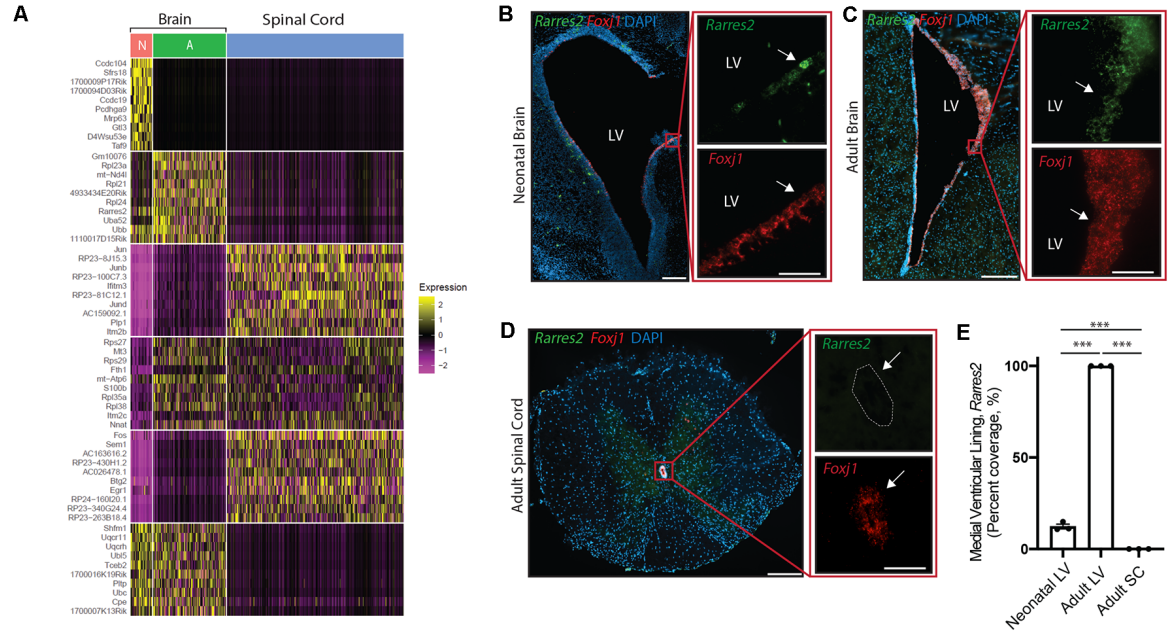

FIGURE 5 | Unique ependymal cell signatures across age and region. **(A)** Heatmap displaying the top 60 differentially expressed ependymal cell genes from the neonatal mouse brain (N, red), the adult mouse brain (A, green), and the mouse spinal cord (blue). **(B–D)** Representative images demonstrating expression of *Rarres2* (green) and *Foxj1* (red) in ependymal cells (arrow) of the neonatal mouse brain **(B)**, adult mouse brain **(C)**, and the mouse spinal cord **(D)**. Note the complete lack of expression of *Rarres2* in ependymal cells in the spinal canal as well as only a subset of cells expressing *Rarres2* in the ventricular lining in early development compared to 100% expression in the adult ventricular lining. Scale bars: 200 μm (**B–D** overview); 50 μm (**B–D** zoomed). **(E)** Quantification of the percentage of cells (DAPI+) expressing *Rarres2* along the ventricular (medial) and spinal canal lining (n = 3; mean ± SEM). ***$p < 0.0001$. In **(D)**, dotted lines show the central canal-ependymal lining interface.

in addition to ependymal cells from the adult human brain.

Mature Ependymal Cells Are Detectable in the Brain by Postnatal Day 0 and Are Maintained in Adulthood

Our goal was to compare ependymal cells at their earliest stage of maturity with ependymal cells that were well established mature cells in the adult forebrain. By comparing ependymal cells at two polar ages where the microenvironments are highly unique, we hypothesized that any similarities would likely reflect the most conserved and relevant gene signatures underlying critical ependymal cell-related functions for the maintenance of the brain. As such, we first sourced a single cell dataset at postnatal day 0, an age that others have shown to be one of the earliest stages of mature ependymal cell generation in the forebrain (Harkins et al., 2021). Indeed, our clustering analysis in postnatal day 0 brain identified one defined cluster expressing genes that were highly specific to mature ependymal cells (**Figure 1A**). This included genes such as: *Foxj1*, a transcription factor which acts as a master regulator of ciliogenesis (Jacquet et al., 2009); *Pifo*, a protein-coding gene involved in ciliary movement (Kinzel et al., 2010); and *Dynlrb1*, a protein-coding gene involved in dynein-mediated transport (Terenzio et al., 2020). We similarly identified this same ependymal cell population in an adult brain dataset (**Figure 1B**). To establish whether mature cells from postnatal day 0 had an extensive gene signature reflective of a mature ependymal cell state, we defined the most mature gene signatures detectable in neonatal ependymal cells and compared this to adult ependymal cells. To accomplish this, we performed pseudotime ordering of ependymal cells as well as their precursor cells (radial glia) which were also detectable in the postnatal day 0 mouse brain (**Figure 1C**). This approach allowed us to order cells based on their developmental progression and identify gene signatures reflective of both the most mature and immature states (**Figure 1D**). Strikingly, 14/15 genes most associated with the mature state in the postnatal day 0 brain (including *Lrcc1, Meig1, Fam183b, Foxj1* etc.) were also highly expressed in adult ependymal cells suggesting that fully mature ependymal cells are detectable at postnatal day 0 (**Figure 1E**). Similarly, the majority of genes that were characteristic of the immature state (*Phyhipl, Ncan, Bcan* etc.) were not expressed by adult ependymal cells, consistent with the adult ependymal cell population being fully mature in adulthood. All further bioinformatic analysis was completed on the mature ependymal cell cluster from the neonatal and adult brain. We also performed validation experiments using RNAscope technology (Wang et al., 2012) on tissue sections collected from neonatal mice and postnatal week 12 adult mice (n = 3 per group). The two genes we assessed, which are associated with the mature state (*Foxj1* and *Fam183b*) were expressed in the ventricular lining at both ages (**Figures 2A–D**).

Albeit the entire ventricular lining of the adult brain expressed *Foxj1* and *Fam183b*, only a portion of the lining of the neonatal brain expressed these markers (*Foxj1*: 34.22% ± 4.18%; *Fam183b*: 20.12% ± 4.45). This expression in the neonatal lining was restricted to the medial wall suggesting that the temporal progression of ependymal cell development follows a medial to lateral dynamic as others have described (Abdelhamed et al., 2018).

Conservation of a Homogenous Ependymal Cell Transcriptome Across Age, Species, and Region

It is well established that E1 ependymal cells monopolize the lateral ventricles of the forebrain and are the major ependymal cell subtype in the brain (Ohata and Alvarez-Buylla, 2016). In order to determine how this ependymal cell subtype is affected by age and species, we used gene ontology (GO)-term analysis to compare *mouse* neonatal and adult ependymal cells (from datasets described above), as well as adult *human* ependymal cells, all derived from the forebrain (**Figures 3A,B,D**, **Supplementary Figure 1**). As with *mouse* neonatal and adult datasets, clustering analysis was performed on adult *human* brain tissue in order to identify any cluster expressing genes that were specific to mature ependymal cells in human tissue. Indeed, we found one cluster expressing *Foxj1*, *Pifo*, and *Dynlrb1* (**Figure 3C**). All ependymal cell clusters across datasets were compared to all other cell types in their respective datasets to generate the top 200 differentially expressed ependymal cell genes (for all genes significantly upregulated, $p < 0.001$ following Bonferroni correction, see **Supplementary Table 1**) then clusterProfiler was used to generate the top 30 most significant GO-terms for each dataset. GO-terms clustered in three major categories that were shared across age and species, including cilia-related terms, metal ion-related terms, and transport-related terms. Strikingly, GO-terms associated with metal ion-related functions made up 4/30, 9/30, and 8/30 of GO-terms in *mouse* neonatal, *mouse* adult, and *human* adult datasets, respectively, demonstrating conservation of this *critically* understudied function for ependymal cells (Zatta et al., 2008). Not surprisingly 20/30, 15/30, and 20/30 of GO-terms in *mouse* neonatal, *mouse* adult, and *human* adult datasets, respectively, were related to cilia consistent with the field focusing largely on this aspect of ependymal cell biology (del Bigio, 2010). Finally, we also noted 1/30 GO-terms in each dataset relating to transport. This is consistent with the knowledge that these cells regulate the bidirectional transport of factors between CSF and interstitial fluid (Trillo-Contreras et al., 2019). There were also unique GO-terms relating to each dataset, but these were minimal and mostly did not have a common theme in comparison to those that were shared (5/30, 5/30, 1/30 unique GO-terms in *mouse* neonatal, *mouse* adult, and *human* adult datasets, respectively).

Given the striking conservation of ependymal cell GO-terms across age and species, we were interested in assessing whether this conservation was maintained across ependymal cell subtypes. To assess this, we sourced a single cell transcriptomic dataset from the *mouse* spinal cord, which is composed of E2 ependymal cells (Alfaro-Cervello et al., 2012). As with the forebrain datasets, clustering analysis was performed on spinal cord tissue in order to identify any cluster expressing genes that were specific to mature ependymal cells in the spinal cord. Indeed, we found one cluster expressing *Foxj1*, *Pifo*, and *Dynlrb1* (**Figure 4A**). This ependymal cell cluster was then compared to all other cell types in this dataset to generate the top 200 differentially expressed ependymal cell genes (for all genes significantly upregulated, $p < 0.001$ following Bonferroni correction, see **Supplementary Table 1**) then clusterProfiler was used to generate the top 30 most significant GO-terms (**Figure 4B**, **Supplementary Figure 1D**). As with forebrain ependymal cells, GO-terms are clustered into several major categories, including cilia-related terms and metal ion-related terms. Strikingly, GO-terms associated with metal ion-related functions made up 8/30 of the GO-terms demonstrating conservation of this *critically* understudied function, even in E2 ependymal cells. Not surprisingly 9/30 GO-terms were related to cilia consistent with the field focusing largely on this aspect of ependymal cell biology (del Bigio, 2010) but no transport-related GO-terms were detected. There were also unique GO-terms which made up far more of the top 30 GO-terms in the spinal cord (13/30) compared to the forebrain datasets which may suggest a more diverse repertoire of functions in E2 ependymal cells.

We performed validation experiments to confirm metal ion-related findings using RNAscope technology (Wang et al., 2012) on tissue sections collected from the brains of neonatal mice ($n = 3$ per group), postnatal week 12 adult mice ($n = 3$ per group), and adult human autopsy ($n = 1$), as well as from the spinal cords of postnatal week 12 adult mice ($n = 3$ per group). A primary gene involved in metal ion buffering is metallothionein 3 (*Mt3*) and we found that this gene was highly expressed in all ventricular and canal linings (74.6–100%, **Figures 4C–H**). Interestingly, *Mt3* was usually localized along the entire lining of the ventricles and the canal, including in the neonatal brain along the lateral border where mature ependymal cells do not reside. This suggests that metal ion-related functions in the ventricular lining may be initiated even before the full maturation of ependymal cells.

Unique Ependymal Cell Signatures Across Age and Region

Albeit our analysis suggests a striking maintenance of ependymal cell function across diverse contexts, we were interested in further exploring potential differences between ependymal cells across age and region. To do this, we integrated *mouse* datasets from the neonatal brain, adult brain, and spinal cord in order to perform direct comparisons. Interestingly, the most striking difference between datasets existed when contrasting the spinal cord with brain datasets (**Figure 5A**) consistent with the spinal cord having more unique GO-terms (**Figure 4B**) and these cells being E2 cells in contrast to the E1 cells found in the neonatal and adult brain (Redmond et al., 2019). A top differentially expressed gene upregulated in spinal

cord ependymal cells was *Plp1* (**Figure 5A**) consistent with a unique spinal cord GO-term: "oligodendrocyte differentiation" (**Figure 4B**). While neonatal and adult brain ependymal cells shared more similarities, there were some upregulated genes unique to the neonatal brain (including *Ccdc104* and *Ccdc19*, involved in the development of cilia) and some unique to the adult brain (including *Rarres2*, involved in initiating inflammation; **Figure 5A**).

We performed validation experiments on *Rarres2* to confirm its unique expression in the adult brain at the exclusion of the neonatal brain or spinal cord. This gene was chosen given its involvement in the inflammatory response and the topographic distribution of inflammatory lesions that appear to be highly prevalent in the parenchyma lining the ventricular system in the adult brain (Hatrock et al., 2020). We used RNAscope technology (Wang et al., 2012) on tissue sections collected from neonatal mice, postnatal week 12 adult mice, and spinal cord tissue (n = 3 per group; **Figures 5B–E**). Consistent with our transcriptomic analysis, we found that *Rarres2* was highly expressed in adult brain (100%), minimally expressed in the neonatal brain (12.28% $\pm$ 1.31%), and undetectable in the spinal cord (0%).

DISCUSSION

Our functional genomics analysis has confirmed a prominent role for cilia-related functions in ependymal cells consistent with the field (del Bigio, 2010; Wallmeier et al., 2019; Lorencova et al., 2020), which was apparent across age, species, and ependymal subtypes. In addition, we have uncovered an underappreciated function for ependymal cells: metal ion regulation, which is remarkably overrepresented, and conserved across species, age, and ependymal subtype. Although largely homogenous, ependymal cells across age and region also have some unique predicted functions as well. Together, our data proposes several underappreciated shared and unique ependymal functions that warrant further investigation.

Our analysis demonstrated that the top 30 GO-terms included four to nine metal ion-related terms across age, species, and ependymal subtypes. Albeit the adult mouse brain dataset revealed the most numerous metal ion-related GO-terms (nine out of the top 30 terms were metal ion-related), these terms were also well represented in the adult human brain and spinal cord (8 out of 30 terms), which suggests a critical role for metal ion regulation in the maintenance of adult brain homeostasis irrelevant of ependymal subtype. Metal ion regulation is critical for the maintenance of homeostasis throughout the body, which, leads to oxidative stress and toxicity, if dysregulated (Valko et al., 2005; Salim, 2017). Indeed, several diseases such as cancer (Valko et al., 2006), autism (Nadeem et al., 2020), Wilson's disease (Kitzberger et al., 2005), and Alzheimer's disease (Lovell et al., 1998; Miller et al., 2006; Everett et al., 2021) are associated with a dysregulation of metal ion homeostasis. Very few studies have analyzed the role of metal ion regulation in the brain, nor how ependymal cells might be involved. In 2008, Zatta et al. (2008) investigated metal ion accumulation in the brain of aging cattle and found that the accumulation of copper, zinc, and manganese was dependent on age. They also found that the major cell types expressing metallothionein, which is the major metal-ion binding protein that buffers heavy metals, were ependymal cells and astrocytes. In our analysis, we also identified metallothionein in both ependymal cells and astrocytes, although it was only ependymal cells, not astrocytes, that were enriched in GO-terms associated with metal ion regulation (astrocyte data not shown), which could suggest a more prominent role for ependymal cells in regulating metal ion homeostasis. There are three major metallothioneins. MT1 and MT2 are found non-specifically across mammalian organ systems, while MT3 is predominantly CNS-specific (Coyle et al., 2002; Miyazaki et al., 2002). Interestingly, MT3 is downregulated in Alzheimer's brains (El Ghazi et al., 2006) but how this contributes to disease pathogenesis and whether a dyshomeostasis in ependymal cell-specific metal ion detoxification function contributes to disease is unknown (Everett et al., 2021).

Interestingly, neonatal ependymal cells had the least number of metal ion GO-terms (4 out of the top 30 terms). Rather these ependymal cells are dominated by GO-terms associated with ciliogenesis and cilia motility, consistent with these cells developing cilia at this early-stage of life (Jacquet et al., 2009). The presence of other GO-terms associated with developmental processes such as determination of left/right symmetry also appeared in neonatal ependymal cells. This is consistent with ependymal cells playing a role in determining the symmetrical development of mammals across the medio/lateral plane (Chen et al., 1998; Wallmeier et al., 2019). Indeed, the absence of functional genes in ciliopathies such as in primary ciliary dyskinesia (PCD) and Kartagener syndrome, can lead to asymmetrical development. For instance, in *FOXJ1* mutant patients, left/right asymmetry occurs and leads to organ laterality defects (Catana and Apostu, 2017). It is thought that during embryogenesis, when ciliary beating patterns are disrupted, the laterality of organ placement is disrupted, and in the most severe cases can result in long-term defects including congenital heart defects. The contribution of ependymal cells in maintaining body symmetry during development has not been studied.

We noted the presence of immune-related genes, such as retinoic acid receptor responder protein 2 (*RARRES2* or Chemerin) which was largely restricted to adult ventricular ependymal cells. *RARRES2* is a 14 kDa protein secreted in an inactive form (prochemerin) which is then activated through the cleavage of the C-terminus by inflammatory serine proteases (Zabel et al., 2005). It is a potent chemoattractant that regulates the recruitment of circulating immune cells *via* binding of the G protein-coupled receptor CMKLR1 (Wittamer et al., 2003; Zabel et al., 2008; Huang et al., 2020). Interestingly, in multiple sclerosis—a common chronic inflammatory disease of the CNS, the most consistent region of inflammatory lesion formation is the periventricular area, directly adjacent to ependymal cells lining the brain's ventricles (Haider et al., 2016). Why immune cells target this defined region is not known. While lesions also form in the spinal cord, these lesions are not as commonly found lining the spinal canal, which raises the possibility that ependymal cells may play a critical role in the recruitment

of immune cells in the brain *via* chemoattractants such as *RARRES2*.

Based on our GO-term analysis, the presence of genes responsible for transport functions in ependymal cells from both the human and mouse ventricles suggests that E1 cells play a critical transport role, which appears to be less prominent in E2 cells from the spinal cord. Aquaporin-4, which is a membrane channel protein, is one transport-associated gene driving the extracellular transport GO-term in our forebrain datasets. Aquaporin-4 responds passively to osmotic gradients and is responsible for the fast transportation of water through its cell membrane. This is critical for the efficient operation of the glymphatic system, important for waste removal, as well as fine-tuning potassium homeostasis. The mechanisms of glymphatic waste clearance in the CNS have been studied extensively in the brain but not the spinal cord (Dupont et al., 2019). These findings suggest glymphatic clearance may have unique regulatory mechanisms in the spinal cord compared to the brain, warranting further investigation.

E2 cells have previously been shown to be capable of contributing to the regenerative response following spinal cord injury (Johansson et al., 1999; Namiki and Tator, 1999; Mothe and Tator, 2005), consistent with spinal cord ependymal cells in our dataset being associated with a GO-term related to the regulation of precursor cell proliferation (“negative regulation of neural precursor cell proliferation”). This GO-term was not detected in brain ependymal cells suggesting that E2 cells may be uniquely involved in actively suppressing proliferative responses in normal health, but under disease or injury conditions, this suppression may be lifted unleashing a dormant regenerative capacity. Interestingly, our previous data assessing the regenerative potential of ependymal cells in the lateral ventricles of the adult mouse did not detect any regenerative potential of E1 cells (Shah et al., 2018). Further studies are needed to clarify the functional similarities and differences between E1 and E2 ependymal cells in relation to their regenerative potential.

Our data implicate ependymal cells in several under-characterized functions related to maintaining brain homeostasis throughout life. Most immediately, deciphering the role of ependymal-mediated metal ion regulation will be critical to understanding the contribution of these cells to regulating oxidative stress and toxicity in health and disease.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by McGill University Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

AM carried out most bioinformatic and RNAscope work. BL, DH, and NC-D supported tissue processing and RNAscope work. MC supported bioinformatics work, while GB supervised MC. AM prepared figures and drafted the manuscript. JS conceived and designed the project, supervised the work, and prepared and approved the final manuscript. KP provided human single cell data. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fncel.2021.703951/full#supplementary-material.

SUPPLEMENTARY FIGURE 1 | GO-analysis networks displaying gene-GO-term connections for each ependymal cell cluster. Top 30 GO-terms (tan nodes) for each ependymal cluster are displayed, demonstrating the genes (gray nodes) that are associated with GO-terms. All included genes are part of the top 200 upregulated genes for that ependymal cluster. Ependymal clusters are displayed for neonatal mouse brain **(A)**, adult mouse brain **(B)**, adult human brain **(C)**, and mouse spinal cord **(D)**.

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REVIEW
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Diversity of Reactive Astrogliosis in CNS Pathology: Heterogeneity or Plasticity?

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Astrocytes are essential for the development and homeostatic maintenance of the central nervous system (CNS). They are also critical players in the CNS injury response during which they undergo a process referred to as "reactive astrogliosis." Diversity in astrocyte morphology and gene expression, as revealed by transcriptional analysis, is well-recognized and has been reported in several CNS pathologies, including ischemic stroke, CNS demyelination, and traumatic injury. This diversity appears unique to the specific pathology, with significant variance across temporal, topographical, age, and sex-specific variables. Despite this, there is limited functional data corroborating this diversity. Furthermore, as reactive astrocytes display significant environmental-dependent plasticity and fate-mapping data on astrocyte subsets in the adult CNS is limited, it remains unclear whether this diversity represents heterogeneity or plasticity. As astrocytes are important for neuronal survival and CNS function post-injury, establishing to what extent this diversity reflects distinct established heterogeneous astrocyte subpopulations vs. environmentally dependent plasticity within established astrocyte subsets will be critical for guiding therapeutic development. To that end, we review the current state of knowledge on astrocyte diversity in the context of three representative CNS pathologies: ischemic stroke, demyelination, and traumatic injury, with the goal of identifying key limitations in our current knowledge and suggesting future areas of research needed to address them. We suggest that the majority of identified astrocyte diversity in CNS pathologies to date represents plasticity in response to dynamically changing post-injury environments as opposed to heterogeneity, an important consideration for the understanding of disease pathogenesis and the development of therapeutic interventions.

Keywords: reactive astrocytes, heterogeneity, plasticity, single-cell RNA sequencing, ischemic stroke, CNS demyelination, traumatic brain injury (TBI), spinal cord injury (SCI)

INTRODUCTION

Astrocytes are critical for the functioning of the adult central nervous system (CNS) in health and disease with a myriad of well-documented roles encompassing the spectrum of physiologic functions from metabolic support to blood-brain-barrier (BBB) integrity to synapse regulation (for review see Volterra and Meldolesi, 2005; Abbott et al., 2006; Koehler et al., 2008; Sofroniew and Vinters, 2010a; Clarke and Barres, 2013; Bayraktar et al., 2015; Khakh and Sofroniew, 2015; Khakh and Deneen, 2019). There is growing consensus that astrocytes are highly plastic in response to environmental fluctuations, particularly in the dynamically changing environment of CNS pathological states. This has shifted our understanding of this ubiquitous glial cell population from that of a binary-population of fibrous and protoplasmic types to one with significant variation across multiple variables, including temporal, topographical, sex, and age, both within and across pathological states. Accordingly, there is significant interest in better understanding and further defining this potential diversity within the astrocyte population.

For the purposes of this review, we define diversity broadly as any distinguishable morphological, physiological, transcriptomic, proteomic, metabolic, or functional difference within the astrocyte population, whether transient or not. The development of technologies enabling detailed descriptions of these responses has led to an accumulation of evidence for diversity within the astrocyte population in the healthy CNS but also across many different models of disease/injury. Here, we briefly address the origin of astrocytes during development and what is currently known about the diversity of astrocytes in the healthy adult CNS prior to an in-depth exploration of diversity in CNS injury/disease, which is the focus of this review. Finally, we initiate a discussion on whether diversity should be sub-divided into more biologically meaningful categories, such as "plasticity" and "heterogeneity." We propose that the use of these definitions provides a framework that will be important as more is discovered about astrocyte diversity in CNS pathologies with specific relevance for future therapeutic development.

ASTROCYTES IN DEVELOPMENT AND ASTROCYTE DIVERSITY IN ADULT HOMEOSTASIS

Astrocytes in the Developing CNS

At the foundation of our discussion on astrocyte diversity in the adult CNS is the multitude of studies on the developmental origin of astrocytes, which we only discuss in brief here (for more details see the following reviews: Bayraktar et al., 2015; Molofsky and Deneen, 2015). The majority of astrocytes originate from subventricular zone (SVZ) resident neuroepithelium-derived radial glial (RG) cells (Noctor et al., 2002; Anthony et al., 2004; Kriegstein and Alvarez-Buylla, 2009), with additional contributions from marginal zone progenitor cells in superficial cortical layers (Costa et al., 2007; Breunig et al., 2012). Importantly, embryonic astrogliogenesis accounts for only a fraction of adult astrocytes, as the majority of murine gliogenesis occurs postnatally (Bandeira et al., 2009) through the symmetric division of differentiated astrocytes (Ge et al., 2012). Direct transformation of RG cells is also a documented source of astrocytes (Merkle et al., 2004; Ghashghaei et al., 2007). Furthermore, NG2 glia (also referred to as oligodendrocyte progenitor cells—OPCs) have been reported to generate a distinct sub-type of ventral forebrain astrocytes (Zhu et al., 2008, 2011; Huang et al., 2014; Nishiyama et al., 2015). Once generated, astrocyte progenitors disperse radially from their site of origin within the confines of a single column leading to the establishment of a diverse population (Magavi et al., 2012; Gao et al., 2014). In the spinal cord, patterning of astrocyte progenitors is initiated by dorsoventral gradients of secreted molecules that facilitate radial organization. For example, three neural tube progenitor domains give rise to three spatially distinct ventral white matter (WM) astrocytes clusters (Hochstim et al., 2008) which are then likely influenced by a combination of intrinsic and extrinsic factors (for review, see Ben Haim and Rowitch, 2017). More fate-mapping analysis is required to establish the extent to which this developmentally established diversity persists into the adult CNS and contributes to the observed adult astrocyte diversity, both in the healthy CNS and in pathological states (e.g., Tsai et al., 2012).

Diversity of Astrocytes in the Healthy Adult CNS

Beginning with Cajal's descriptions of diverse morphologies amongst human and rodent astrocytes over 100 years ago, astrocyte diversity has been a recognized feature of the healthy adult CNS (Zhang and Barres, 2010; Boulay et al., 2017; Lin et al., 2017; Buosi et al., 2018; Matias et al., 2019). Early histologic description designated protoplasmic and fibrous astrocytes as unique subsets (Kölliker, 1889; Raff et al., 1984; Raff, 1989; Andriezen, 1893) based upon differences in location [WM vs. gray matter (GM)], cell body morphology, and interaction with neighboring neuronal structures (Bushong et al., 2002; Oberheim et al., 2012; Lundgaard et al., 2014). Transcriptional-based approaches have expanded upon this initial description (Cahoy et al., 2008; Batiuk et al., 2020; Bayraktar et al., 2020) with unique astrocytic gene profiles demonstrated across various brain regions (Chai et al., 2017; Morel et al., 2017; Duran et al., 2019; Bayraktar et al., 2020). Using fluorescence-assisted cell sorting (FACS) and immunohistochemical approaches, Lin et al. (2017) identified five distinct astrocyte populations in the mouse CNS, which displayed diverse synaptogenesis mechanisms. Similarly, using a transcription factor motif discovery approach, Lozzi et al. (2020) found region-specific astrocytic expression profiles in astrocyte populations from the olfactory bulb, hippocampus, cortex, and brainstem. Furthermore, astrocyte reporter mouse lines exposed molecular differences between different astrocyte populations within the adult cortex (Morel et al., 2019).

Importantly, these unique transcriptomic gene profiles are correlated with neural-circuit-based functional differences (Höft et al., 2014; Chai et al., 2017). Variable expression of key functional components in astrocytes has been noted, including

glutamate receptors, transporter proteins, and ion channels (Matthias et al., 2003; Isokawa and McKhann, 2005; Olsen et al., 2007), as well as in calcium (Ca^{2+}) signaling dynamics (Takata and Hirase, 2008), which is theorized to have a functional role in astrocyte-neuron communication (Bazargani and Attwell, 2016; Chai et al., 2017; Yu et al., 2018). Using multi-photon confocal imaging, Takata and Hirase (2008) demonstrated significant variance in astrocytic Ca^{2+} activity between cortical layer I and layers II/III. Diversity amongst astrocyte populations in different cortical layers has been identified in other studies as well (Lanjakornsiripan et al., 2018; Bayraktar et al., 2020). For example, single-cell RNA-seq (scRNA-seq) analysis identified five transcriptionally distinct clusters distributed amongst cortical layer I and III-V (Bayraktar et al., 2020) and quantification of astrocyte marker expression across cortical layers in the developing mouse brain revealed significant diversity across functionally distinct cortical areas (Batiuk et al., 2020).

Glial fibrillary acidic protein (GFAP)—a major component of intermediate filaments in astrocytes—is a widely used marker of astrocytes (Eng et al., 1971). Importantly, the basal level of GFAP in astrocytes in the healthy CNS is variable (Griemsmann et al., 2015; Ben Haim and Rowitch, 2017). For example, hippocampal astrocytes display higher GFAP expression than striatal astrocyte populations (Chai et al., 2017). Furthermore, GFAP expression is higher amongst spinal cord astrocytes compared to the brain (Yoon et al., 2017). Interestingly, astrocytic GFAP expression is modulated by various extrinsic stimuli, including global physical activity (Rodriguez et al., 2013), exposure to enriched environments (Rodriguez et al., 2013), and glucocorticoid treatment (O'Callaghan et al., 1991), suggesting that plasticity may play a role in shaping the regional diversity of GFAP expression observed in the healthy CNS. Interestingly, GFAP expression also fluctuates with circadian rhythms in the suprachiasmatic nucleus of the thalamus (Gerics et al., 2006). Also, GFAP expression amongst progenitor cells depends upon the developmental stage, highlighting more diversity in the expression of this marker (Cahoy et al., 2008; Kriegstein and Alvarez-Buylla, 2009; Roybon et al., 2013). While an important marker used to identify astrocytes, it is important to note that GFAP is not considered sufficient as an identifier of astrocyte populations, either in the healthy or injured CNS. A combination of multiple astrocyte markers is generally viewed as an improved approach [e.g., GFAP, aldehyde dehydrogenase-1 (Aldh1L1), and glutamine synthetase (GS); Serrano-Pozo et al., 2013]. Importantly, diversity in the expression of these additional markers is also seen (Anlauf and Derouiche, 2013; Waller et al., 2016). For example, diverse expression of Aldh1L1 amongst cortical astrocytes (Waller et al., 2016) and GS amongst entorhinal cortical astrocytes is observed (Anlauf and Derouiche, 2013).

As astrocytes are critical to the normal functioning of local neuronal populations (Chai et al., 2017; Matias et al., 2019), further characterization of the extent of this diversity (for review see Khakh and Sofroniew, 2015; Ben Haim and Rowitch, 2017; Khakh and Deneen, 2019), and the functional implications for neural circuit functioning (for review see, Nagai et al., 2021b) in the healthy CNS is vital. Furthermore, it is imperative to establish a baseline (Tsai et al., 2012; Batiuk et al., 2020; Bayraktar et al., 2020) against which identified diversity in models of CNS insult can be interpreted in order to develop targeted interventions aimed at manipulating aberrant and/or pro-pathogenic responses (Batiuk et al., 2020; Sofroniew, 2020).

DIVERSITY IN THE CONTEXT OF REACTIVE ASTROGLIOSIS

Defining Reactive Astrogliosis

Various terms have been used to describe the range of astrocytic responses to CNS insult and/or environmental perturbation (Eddleston and Mucke, 1993; Anderson et al., 2014; Pekny and Pekna, 2014; Sofroniew, 2015). In line with a recently published consensus statement (Escartin et al., 2021), we define "reactive astrogliosis" as the process by which astrocytes change in response to pathology. This can include changes in transcriptional regulation, or biochemical, morphological, metabolic, and physiological remodeling potentially associated with functional adaptation to the post-injury environment. Reactive astrogliosis was long viewed as homogenous and functionally passive, consisting of a stereotyped set of changes driving the conversion of homeostatic astrocytes to a distinct phenotype—the "reactive astrocyte" (Eddleston and Mucke, 1993; Anderson et al., 2014; Pekny and Pekna, 2014; Sofroniew, 2015). However, current evidence challenges this view, instead pointing to the existence of remarkable diversity in terms of morphology and transcriptional profile in varied CNS disease states (Hamby and Sofroniew, 2010; Zhang and Barres, 2010; Oberheim et al., 2012; Anderson et al., 2014; Schitine et al., 2015; Yoon et al., 2017; Zeisel et al., 2018; Masuda et al., 2019; Matias et al., 2019; Valori et al., 2019; Escartin et al., 2021). This begs the question of how extensive this diversity really is (Cahoy et al., 2008; Ståhlberg et al., 2011; Yoon et al., 2017; Batiuk et al., 2020; Bayraktar et al., 2020).

Role of Astrocytes in CNS Disease

Reactive astrogliosis is observed in virtually all neurological conditions, including epilepsy (Steinhäuser et al., 2015), neoplastic disease (Priego et al., 2018; Heiland et al., 2019), demyelination (Woodruff and Franklin, 1999; Williams et al., 2007; Tassoni et al., 2019; Rawji et al., 2020), traumatic injury (Faulkner et al., 2004; Filous and Silver, 2016; Boghdadi et al., 2020a), neurodegeneration (Vargas et al., 2008; Ben Haim et al., 2015), and ischemic stroke (Zhao and Rempe, 2010; Zamanian et al., 2012; Rakers et al., 2019), as well as microbial CNS infections (Drögemüller et al., 2008; Soung and Klein, 2018; Geyer et al., 2019) and neurotoxin exposure (O'Callaghan et al., 2014; Wheeler et al., 2019). Reactive astrogliosis enables astrocytes to serve key roles in CNS pathological states, including metabolic support of vulnerable neurons, regulation of BBB permeability, remodeling of extracellular matrix (ECM), mobilizing progenitors, as well as immunomodulation, synaptic remodeling, and neurite outgrowth (Woodruff et al., 2004; Sofroniew and Vinters, 2010a; Anderson et al., 2014;

Pekny and Pekna, 2014; Escartin et al., 2019, 2021). This process is regulated by a wide range of factors, both intrinsic and extrinsic to the CNS, and mediated through various cell surface receptors and intracellular signaling pathways (Sofroniew and Vinters, 2010a; Burda and Sofroniew, 2014; Sofroniew, 2015, 2020). Manipulation of these components in models of CNS injury/disease alters functional and histologic outcomes, demonstrating the importance of reactive astrogliosis and of understanding the extent and nature of its diversity (Brambilla et al., 2005, 2009; Okada et al., 2006; Herrmann et al., 2008; Haroon et al., 2011; Spence et al., 2011; Bonneh-Barkay et al., 2012; Wanner et al., 2013).

Reactive Astrogliosis and the Aged CNS

Reactive astrogliosis is also a prominent feature of physiological aging in rodents, non-human primates, and humans (Nichols et al., 1993; Kanaan et al., 2010; Cerbai et al., 2012; Rodríguez et al., 2014; Jyothi et al., 2015; Robillard et al., 2016). Previous studies have noted regional diversity in astrocyte morphology, GFAP expression, and cellular density (Nichols et al., 1993; David et al., 1997; Hayakawa et al., 2007; Lynch et al., 2010; Cerbai et al., 2012; Geoffroy et al., 2016; Rodríguez et al., 2016). Aged astrocytes also demonstrate altered responses to CNS injury. For example, aged astrocytes display increased GFAP upregulation following SCI as compared to younger controls (Geoffroy et al., 2016), however, the functional implications of this remain unclear (Matias et al., 2019; Sutherland and Geoffroy, 2020).

Transcriptional approaches have once again greatly expanded our characterization of astrocyte diversity in the aged CNS (Soreq et al., 2017; Boisvert et al., 2018; Clarke et al., 2018). These studies have collectively revealed that aged astrocytes adopt a more pro-inflammatory phenotype (Orre et al., 2014), consistent with the concept that physiological aging is characterized by chronic low-level inflammation (i.e., "inflamm-aging"; Franceschi et al., 2000). Aged astrocytes from distinct brain regions display unique transcriptional profiles in both murine and human brains (Soreq et al., 2017; Boisvert et al., 2018; Clarke et al., 2018). Boisvert et al. (2018) performed RNA-seq analysis on 4 month and 2-year-old astrocyte-ribotag mice, demonstrating significant upregulation of pro-inflammatory and synapse elimination-related genes and decreased expression of cholesterol synthetic enzymes in the aged mice, with significant regional diversity (Boisvert et al., 2018). Expanding on this, Clarke et al. (2018) demonstrated regional diversity amongst aged astrocytes isolated (using the Bac-Trap method) from the hippocampus, striatum, and cortex, with upregulated genes related to astrocyte reactivity, immune response, and synapse elimination. Soreq et al. (2017) extended these findings to post-mortem human tissue of patients ranging in age from 16 to 102 years, revealing significant regional diversity of astrocyte-specific genes (Clarke et al., 2018). Characterization of the astrocyte secretome during the aging process may help validate many of these observed transcriptomic changes (Rawji et al., 2020). Intriguing questions remain as to the functional relevance of these age-related changes and the role of astrocyte diversity in the spatial propensity of various age-related disease processes (Ben Haim et al., 2015; Rodríguez et al., 2016; Matias et al., 2019). Furthermore, comparison of the transcriptional profiles of astrocytes across pathological states with those seen in the aged CNS may yield novel insights into disease pathogenesis, physiological aging, and the overlap between these states.

Reactive Astrogliosis Diversity: Plasticity or Heterogeneity?

We broadly define astrocyte diversity as any distinguishable morphological, physiological, transcriptomic, proteomic, metabolic, or functional difference within the astrocyte population, whether transient or not (**Figure 1A**). With this definition, and those that follow, we suggest that semantics are important, as a proper classification of diversity will likely lead to greater accuracy in the understanding of function and the directed development of therapeutic strategies. We therefore propose strict definitions to further classify reactive astrocyte diversity.

Heterogeneity has become a catch-all phrase that remains poorly defined, necessitating the need for clearer and unambiguous definitions. The term heterogeneity is derived from the Greek *heteros-* meaning "two, other, or different," and the Latin *-genesis* meaning "origin or development" (Oxford English Dictionary). In distinguishing heterogeneity, we adhere to the definitions offered in a recent discussion of diversity in the oligodendrocyte lineage (Foerster et al., 2019), namely that heterogeneity implies distinct origin, as suggested in the definition, in combination with the demonstration of diverse functions (**Figure 1B**). In support of this, we look to other neural cell lineages. Firstly, heterogeneity amongst neurons is demonstrated by developmentally distinct neuronal subtypes with different transmission modes and firing patterns (i.e., function). A particularly relevant example of heterogeneity in the context of pathology lies within the microglia population. Dogma suggested that in mice, microglia progenitors arise at E7.5 from the yolk sac and then colonize the brain at E9.5 but mutant mice lead to the discovery that *Hoxb8* microglia (which express the *Hoxb8* transcription factor) represent a distinctive subpopulation of cells that are derived from a second wave which do not populate the brain until E12.5 (De et al., 2018). Although the non-*Hoxb8* microglia and *Hoxb8* microglia are very similar with few differentially expressed genes, they occupy distinct distributions in the post-natal mouse brain and demonstrate unique functional characteristics including their ability to participate in synaptic pruning and their response to injury. For example, the two microglia subpopulations are indistinguishable in their response to a stab wound injury in the acute phase (<30 min) but *Hoxb8* microglia demonstrated a greater tendency to accumulate at the injury epicenter at 7 days post-injury (dpi) compared to non-*Hoxb8* microglia (De et al., 2018). A similarly relevant example of heterogeneity in the context of pathology can be seen in the oligodendrocyte lineage. In the context of remyelination, OPCs arising from distinct ventral and dorsal domains during development have differential responses. For example, dorsally derived OPCs in the adult CNS demonstrate enhanced recruitment and differentiation into oligodendrocytes in response to demyelination as compared to their ventrally derived counter-parts (Crawford et al., 2016). Furthermore,

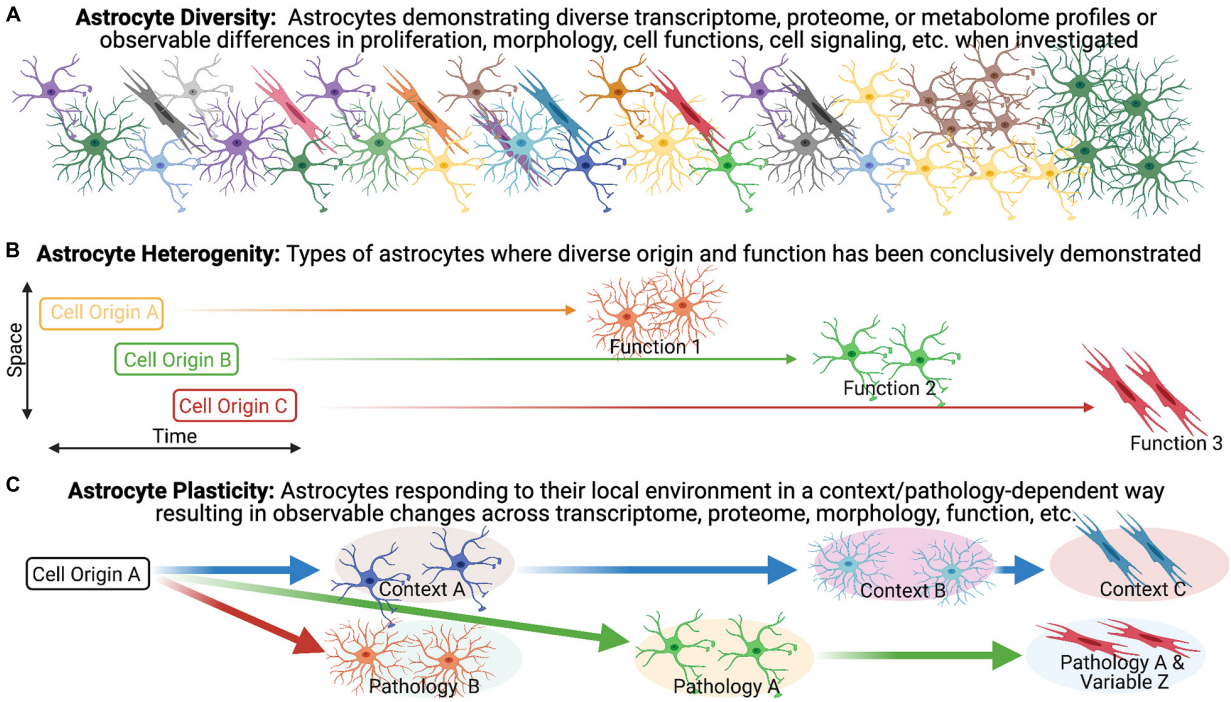

FIGURE 1 | Astrocyte heterogeneity vs. astrocyte plasticity. **(A)** Evidence for diversity within the astrocyte population is becoming increasingly recognized and is particularly robust in the context of pathology/disease. **(B,C)** We highlight the importance of distinguishing astrocyte heterogeneity from astrocyte plasticity, as we define them, to direct our understanding of reactive astrogliosis and inform potential treatments.

dorsally derived OPCs demonstrate increased susceptibility to the age-associated differentiation impairment observed in the context of demyelination (Crawford et al., 2016). These findings illustrate the influence of heterogeneous populations on disease-associated variables (e.g., aging) in pathological settings (Crawford et al., 2016).

In the absence of pathology, one example of astrocyte heterogeneity (as we define it) is the demonstration that postnatal region-restricted spinal cord astrocytes have unique functions. In spinal cord development, spatially distinct astrocytes are specified through a homeodomain transcriptional code from positionally distinct progenitor populations (Hochstim et al., 2008) and spatially distinct domains remain stable throughout life in both mouse brain and spinal cord (Tsai et al., 2012). Specifically within the spinal cord, spatially distinct astrocytes with unique origins were shown to express postnatal region-specific genes and the ventral population plays a distinct role in sensorimotor circuit formation (Molofsky et al., 2014). This region-specific expression pattern of genes was further demonstrated across the cortical and subcortical adult mouse brain. Furthermore, astrocytes from these brain regions exhibited region-matched astrocyte to neuron communication specific to their ability to promote neurite growth and synaptic activity *in vitro* (Morel et al., 2017). Therefore, these spatially distinct astrocytes are a prime example of heterogeneity, as we define it, within the uninjured astrocyte population due to the direct evidence underlying their distinct origin and functions.

In contrast to our heterogeneity definition, plasticity would manifest as malleable morphological and/or phenotypic profiles among cells of a common origin in response to changing environmental conditions (**Figure 1C**). An illustration of this concept can be seen in the macrophage lineage. Monocyte differentiation into effector phenotypes occurs in accordance with local microenvironmental signals, accounting for the diverse macrophage effector functions in various tissues, and in response to different insults (Mosser and Edwards, 2008; Italiani and Boraschi, 2014; Lavin et al., 2014; Xue et al., 2014; Kuznetsova et al., 2020). Applied to the astrocyte lineage, plasticity would imply the CNS is populated with a homogenous population of astrocytes that undergo specialization at their final location as directed by local environmental features. For example, in the healthy postnatal CNS, the functional maturation of cortical astroglia is modified by the loss of neuronal glutaminergic signaling (Morel et al., 2014). In the adult healthy CNS, sonic hedgehog released from local neurons plays an active role in regulating both astrocyte function and the astrocytes' molecular profile (Farmer et al., 2016) demonstrating that astrocytes can respond to cues from neurons that drive their properties/functions. We suggest that for distinct reactive astrocyte populations to be considered heterogeneous, definitive demonstration of distinct origins and functions need to be established to effectively exclude plasticity.

While we segregate plasticity and heterogeneity here for conceptual purposes, it is likely that there is a dynamic interplay between the two with varying contributions across multiple

disease variables. An example of this can be seen in the oligodendrocyte lineage. While the diversity of myelin internode length appears to be a function of the axon characteristic and not oligodendrocyte diversity (i.e., plasticity; Chong et al., 2012; Tomassy et al., 2014), diverse oligodendrocytes isolated from either the spinal cord or cortex form myelin sheaths of different lengths when provided artificial microfiber as a substrate for myelination (Bechler and Byrne, 2015), suggesting at least a degree of intrinsic determination (i.e., heterogeneity; Crawford et al., 2016). Importantly, diversity in internode length is reduced when oligodendrocytes were cultured with dorsal root ganglion neurons or brain slices, implicating both intrinsic (i.e., heterogeneity) and extrinsic factors (i.e., plasticity) in determining the outcome. It is likely that a similar dynamic combination exists for astrocytes as well. Importantly, this is not just a semantic argument, as recognition of the contributions of plasticity to the observed diversity has the potential to reveal novel targets amendable to extrinsic manipulation *via* targeted therapeutic approaches across multiple aspects of disease pathology.

Tools to Better Understand Cell Diversity in Reactive Astrocyte Populations

scRNA-seq or single-nuclei RNA-seq (snRNA-seq) have enabled the direct quantification of single-cell or nuclei RNA complements at an increased resolution (Tang et al., 2009;

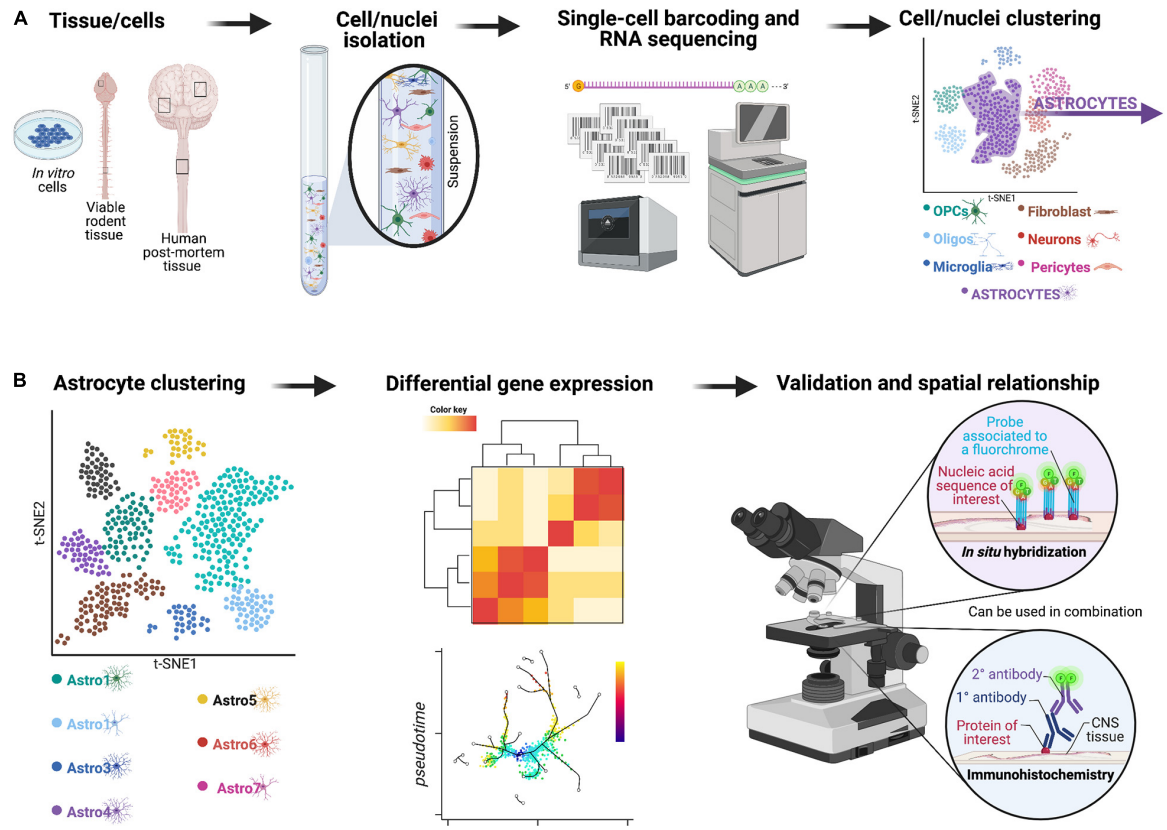

FIGURE 2 | Overview of a potential workflow featuring droplet-based sc/snRNA-seq approaches to investigate astrocyte diversity across different CNS pathologies. **(A)** In the past few years, a technological revolution in RNA-sequencing technology has made it possible to profile the entire transcriptome of individual cells on a massive scale—a technique known as single-cell RNA-sequencing or scRNA-seq (Svensson et al., 2018). Initially, scRNA-seq relied on manual cell picking (Van Gelder et al., 1990; Eberwine et al., 1992) or FACS-based sorting (Ramsköld et al., 2012; Shalek et al., 2013). Innovative analyses revealed a surprising degree of transcriptional heterogeneity in seemingly homogenous cell populations. Subsequent advances in microfluidic instrumentation (Shalek et al., 2014; Treutlein et al., 2014) and droplet-based methods (Klein et al., 2015; Macosko et al., 2015) have since driven experimental costs down significantly to now permit sequencing of tens to hundreds of thousands of cells in a single experiment (Cao et al., 2017; Schaum et al., 2018). The rapid pace of methodological and computational progress has fostered initiatives to profile the mRNA landscape within every single cell of various model organisms (Cao et al., 2017; Schaum et al., 2018) and, ultimately, in humans (Rozenblatt-Rosen et al., 2017). This framework now enables comprehensive interrogation of the molecular etiology of human disease at single-cell resolution (Stubbington et al., 2017; Cheung et al., 2019). For example, single-cell transcriptomics offers an opportunity to elucidate how individual types of cells coordinate their activity to drive pathophysiological processes, and how cell type-specific responses might be targeted to treat disease. Indeed, in only the past few years, scRNA-seq has been applied to asthma (Braga et al., 2019), inflammatory bowel disease (Martin et al., 2019; Parikh et al., 2019; Smillie et al., 2019), obesity (Svensson et al., 2018), Alzheimer's disease (Mathys et al., 2019), and TBI (Arneson et al., 2018), among other disorders. These technologies and analyses enable clustering of all viable cells/nuclei included in the original sample based on gene expression, and for example to identify astrocyte-like subpopulations can be isolated for further analyses. **(B)** The astrocyte-like subpopulations can be further clustered and examined using approaches, such as differential gene expression to yield important information about heterogeneous astrocyte populations.

Zeisel et al., 2018; Habib et al., 2020). Approaches, such as droplet-based sc/snRNA-seq approaches (**Figure 2**) have proven immensely powerful across various disease and injury models, revolutionizing our capacity for cellular characterization (Chen et al., 2018; Ding et al., 2020; Jäkel and Williams, 2020). Importantly, distinct clusters of astrocytes identified through these single-cell technologies need to then be validated to make sure results are not just noise and the spatial relationship needs to be re-established using *in situ* hybridization or immunohistochemistry alone or in combination. Use of these techniques also allow for analysis on human post-mortem tissue and *in vitro* culture systems (Grubman et al., 2019; Mathys et al., 2019) which has significantly enhanced our knowledge of species-specific differences in reactive astrogliosis, a critical hurdle for translation of pre-clinical findings in rodent model systems to human patients (Nichols et al., 1993; Oberheim et al., 2009, 2012; Soreq et al., 2017). When used alone these tools provide a powerful means of assessing astrocyte diversity but do not clearly distinguish between heterogeneity and plasticity unless they are combined with other complementary experiments, such as those looking at lineage-tracing. With that being said, recent papers highlight the potential of these single-cell technologies to yield information about lineage (Weinreb et al., 2020) as well as connectivity analysis (Clark et al., 2021) to be performed in high-throughput, with cell-type resolution. Furthermore, new techniques, such as sc-ATAC-seq will provide information about chromatin accessibility, which is likely to be an important determinant of astrocyte plasticity (Buenrostro et al., 2015). These new approaches will be influential in the task of determining whether reactive astrocyte clusters represent heterogeneity (with district origin and functions) or plasticity.

The Spectrum of Reactive Astrogliosis

Similar to the healthy CNS, transcriptional analysis has revealed multiple clusters of reactive astrocytes in various models of CNS insult (Adams and Gallo, 2018; Tassoni et al., 2019; Yang et al., 2020). Various schemes have been proposed to categorize this diversity, including the classification of reactive astrocytes as either proliferative border-forming or non-proliferative in models of CNS trauma (Sofroniew, 2020). The most well-known is the categorization of astrocytes as neurotoxic "A1" or neuroprotective "A2." In rodent models, intraparenchymal lipopolysaccharide (LPS)-injection induces a neurotoxic astrocyte phenotype stimulated by microglia-derived factors (Liddelow et al., 2017), whereas cerebral ischemia induces astrocytes to adopt what was later coined an "A2" phenotype that appeared to be neuroprotective (Zamanian et al., 2012). Astrocytes resembling what was later referred to as "A1" phenotypes have been identified in various disease states, including models of amyotrophic lateral sclerosis (ALS; Sun et al., 2015), Alzheimer's disease (Sekar et al., 2015; Wu et al., 2019), prion disease (Smith et al., 2020), glioblastoma (Heiland et al., 2019), Parkinson's disease (Chen et al., 2009), and Huntington's disease (Diaz-Castro et al., 2019; Al-Dalahmah et al., 2020). While these "A1" vs. "A2" distinctions are useful, they also likely represent an oversimplification of the much larger continuum of reactive astrocyte states that are present in CNS pathologies. Indeed, the authors themselves stated that these two states were likely only a subset of many potential reactive states (Zamanian et al., 2012; Liddelow and Barres, 2017; Liddelow et al., 2017). In the recently published consensus statement, Escartin et al. (2021) highlight the shortcomings of using these binary divisions of reactive astrocytes, such as "A1" vs. "A2," good vs. bad, or neurotoxic vs. neuroprotective and advocate for the assessment of multiple molecular and functional parameters moving forward (Escartin et al., 2021). Furthermore, a clear neurotoxic role for "A1" astrocytes is not always clearly demonstrated, highlighting the challenge with this dichotomous classification. For example, deletion of a subset of "A1" astrocytes accelerated neurodegenerative progression in a mouse model of prion disease (Hartmann et al., 2019), suggesting that these astrocytes states are considerably more nuanced. As will be discussed in the disease models below, transcriptional analysis has provided further evidence that astrocyte diversity exists along a spectrum of states likely driven by local microenvironments (Zhang and Barres, 2010; Anderson et al., 2014; Escartin et al., 2021). In this review, we still refer to "A1" vs. "A2" terminology for studies conducted in the past but adhere to the consensus put forward by Escartin et al. (2021) to avoid these terms in future research. Indeed, recent studies in models of ischemic stroke (Rakers et al., 2019; Androvic et al., 2020), demyelination (Yoon et al., 2017; Tassoni et al., 2019), and traumatic injury (Burda et al., 2016; Boghdadi et al., 2020b) have revealed substantial disease-specific, regional, and temporal reactive astrocyte diversity.

Potential Variables Shaping Diversity in the Context of Reactive Gliosis

There are many variables potentially influencing the diversity of reactive astrogliosis. Here, we review the current state of knowledge on astrocyte diversity in the context of three representative and clinically relevant CNS pathologies: ischemic stroke, demyelination, and traumatic injury across multiple injury-associated variables (e.g., temporal, topographical, sex, and age). Plasticity can be conceptualized as the diversity in a response to environmental factors. In pathological states this includes features of the post-injury environment (e.g., cytokines, inflammatory cells, etc.). In contrast, heterogeneity manifests as diversity that results from differential origins (either developmental and adult-derived) and functionality. Importantly, plasticity and heterogeneity are not mutually exclusive in our model and elucidating this relationship as it manifests in various conditions of CNS pathology is critical to understanding the contributions of astrocytes in the injured CNS and how these responses can be manipulated. As our understanding of the extent of astrocyte diversity develops, it will become ever more important to integrate and compare astrocyte responses across pathological states to be able to interpret and apply the substantive datasets gleaned from single-cell transcriptomic approaches. To that end, we expand our discussion beyond the confines of a singular pathology and aim to highlight key limitations of our current knowledge, propose areas for future research, and discuss the relevance of this

knowledge for therapeutic development. We specifically focus on the importance of differentiating the contributions of plasticity and heterogeneity to observed astrocyte diversity across multiple variables in CNS pathologies.

ISCHEMIC STROKE

Stroke is the primary cause of severe disability and a leading cause of death worldwide, associated with enormous socioeconomic burden (Cassidy and Cramer, 2017; Campbell et al., 2019; Campbell and Khatri, 2020). Accounting for 75–80% of all strokes (Cassidy and Cramer, 2017; Campbell et al., 2019; Campbell and Khatri, 2020), ischemic stroke results from the occlusion of a cerebral artery by a blood clot that either forms locally (i.e., thrombotic stroke) or more commonly, travels from another location, such as the heart or another proximal vessel (i.e., embolic stroke; Cassidy and Cramer, 2017; Campbell et al., 2019; Campbell and Khatri, 2020). Clinically, the extent of the resultant injury depends on several factors, including the severity and duration of ischemic injury and the quality of collateral blood flow to the affected perfusion territory (Bang et al., 2008; Maud et al., 2021). Despite recent advances in reperfusion techniques, therapeutic options remain limited and largely ineffective in attenuating the progressive neuronal loss and consequent functional impairment (Matei et al., 2021).

Various pre-clinical models of ischemic stroke have been employed (**Figure 3A**; Fluri et al., 2015; Rakers and Petzold, 2017; Sommer, 2017). Among these, the middle cerebral artery occlusion (MCAO) model is largely considered to most closely resemble human ischemic stroke (Longa et al., 1989; Rakers and Petzold, 2017; Sommer, 2017). Other frequently used models include direct mechanical occlusion of a cerebral vessel *via* clipping, ligation, or cauterization (Robinson et al., 1975; Chen et al., 1986; Brint et al., 1988; Hossmann, 2012), stereotactic administration of potent vasoconstrictors (e.g., endothelin-1) to induce vasospasm (Robinson and McCulloch, 1990; Sozmen et al., 2009; Roome et al., 2014; Fluri et al., 2015), and targeted activation of systemically administered photosensitive dye *via* transcranial illumination to induce localized thrombosis (i.e., the photothrombotic model; Watson et al., 1985; Kim et al., 2000; Kleinschnitz et al., 2008). Comparing tissue damage and astroglia responses across the range of pathology encompassed by these models will be informative. Another important consideration is the use of transient occlusive models, which mimic timely recanalization therapy (e.g., thrombolytics or endovascular thrombectomy; Sommer, 2017).

Role of Astrocytes in Ischemic Stroke

The peri-infarct region following ischemic stroke (i.e., the zone immediately surrounding the ischemic lesion; **Figure 3B**), is commonly segregated into a monocyte/macrophage-dense inner region directly bordering the lesion and an astrocyte-rich outer region (Schroeter et al., 1995; Pekny and Nilsson, 2005; Gliem et al., 2015). The peri-infarct reactive astrocytes that populate this outer region secrete a variety of pro-inflammatory cytokines, chemokines, and matrix metalloproteinases that disrupt the BBB and recruit peripheral leukocytes, which are predominant contributors to secondary injury (Gliem et al., 2015; Yang and Rosenberg, 2015; Choudhury and Ding, 2016). Furthermore, astrocytes in this region demonstrate process elongation and polarization, as well as upregulation of several factors involved in ECM reorganization and reactive astrocyte clustering (Hirsch et al., 1994; Peng et al., 2013; Gliem et al., 2015). Using an RNA-seq approach in a MCAO rodent model, Rakers et al. (2019) demonstrated a significant upregulation of various neurotoxic genes associated with the "A2"-specific transcripts at 72 h post-stroke compared to control tissue. In total, >1,000 genes were differentially expressed after focal ischemia, including 38 transcription factors (Rakers et al., 2019), demonstrating the pronounced transcriptional changes that take place in response to ischemia. Importantly, many peri-infarct neurons survive the initial ischemic insult but undergo delayed degeneration due to progressive secondary damage to the surrounding spared tissue (Marchal et al., 1996; Liu et al., 2010; Chamorro et al., 2016). As astrocytes provide critical neuronal support and survive the ischemic insult in large numbers they represent an attractive target for therapeutic manipulation to promote, or at least mitigate, this neuronal loss and provide an increased substrate for repair (Zhao and Rempe, 2010; Liu and Chopp, 2016; Becerra-Calixto and Cardona-Gómez, 2017). As astrocytes are key players in both the pathogenesis of ischemic stroke and recovery process following insult, they are of significant interest. Clarification of the interplay between astrocyte plasticity and heterogeneity across multiple disease-associated variables (e.g., temporal, topographical, age-related, sex-related) promises to open novel therapeutic avenues aimed at mitigating stroke-associated morbidity and mortality.

Astrocyte Diversity in Ischemic Stroke

Transcriptomic studies have revealed astrocyte diversity in models of ischemic stroke (Zamanian et al., 2012; Rakers et al., 2019; Boghdadi et al., 2020a). For example, ischemic insult induces differential expression of a host of genes in diverse subsets of astrocytes, including genes involved in neuroinflammation, apoptosis, and transepithelial migration of leukocytes (Zamanian et al., 2012) as well as cell division and migration (Rakers et al., 2019). Application of snRNA-seq provided refinement of this diversity in an endothelin-1-induced ischemic stroke model in marmosets, revealing 19 discrete reactive astrocyte subsets in the primary visual cortex (Boghdadi et al., 2020a). Interestingly, these subsets were noted to express a mixture of "A1" and "A2" genes (Boghdadi et al., 2020a), further highlighting the limited utility of applying binary categorization to what is most likely a nuanced spectrum of activation states. Interestingly, certain astrocyte subsets in the peri-infarct region expressed Nogo-A (Boghdadi et al., 2020a), well-known as a robust neurite outgrowth inhibitor (Chen et al., 2000; GrandPre et al., 2000; Kim et al., 2004; Schwab, 2004). Astrocyte diversity in models of ischemic stroke most likely represents predominant contribution of plasticity in response to the changing post-injury environment superimposed upon a background of heterogeneity, manifested in the post-natal brain as regionally specific astrocyte subsets with variable responses

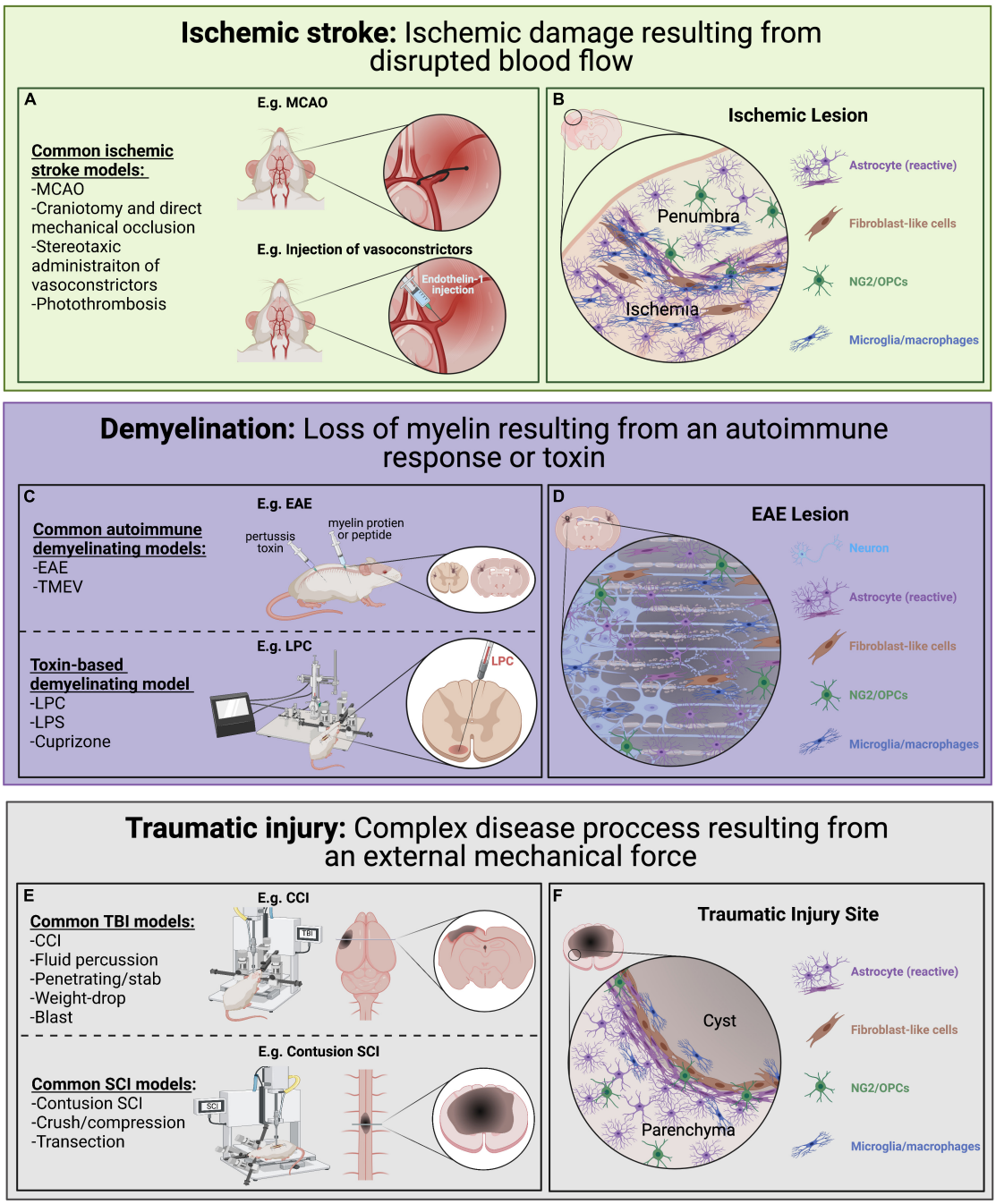

FIGURE 3 | Pre-clinical models of ischemic stroke, CNS demyelination, and traumatic injury used to look at astrocyte diversity. Each of these models have advantages and disadvantages with regards to modeling human disease and induce diverse astrocyte injury responses. **(A)** Common ischemic stroke models. Ischemic stroke results from disrupted blood flow leading to ischemic damage, cell death and associated loss of function. Common animal models of ischemic stroke involve the transient or sustained blockade of normal blood flow to an area of the brain through occlusion of a blood vessel (e.g., MCAO). **(B)** A simplified illustration of an ischemic lesion where the ischemic area is predominately populated by immune cells and reactive astrocytes with a slow gradient toward an inner cluster of microglia/macrophages and surrounded by an outer layer of astrocytes. **(C)** Common demyelination models can be initiated through either an autoimmunity-based or toxin-based route, each highlighting different pathological features and chosen based on the research questions being pursued. **(D)** A simplified illustration of an EAE induced lesion where there is a loss of oligodendrocytes and their myelin sheaths (beige) within the lesion. Demyelination lesions are often filled with immune cells, including microglia/macrophages, and NG2/OPCs which contribute to repair. In the situation where remyelination does not take place, axon degeneration can result. **(E)** Common traumatic injury models focusing on different mechanical injuries applied to the brain and spinal cord, each with its own complex secondary injury cascade. **(F)** A simplified illustration of a typical CNS traumatic injury where a pronounced secondary injury cascade often leads to the loss of tissue (sometimes forming a fluid-filled cyst) at injury epicenter. Generally, there is an inner accumulation of fibroblast-like cells closest to epicenter surrounded by densely packed reactive astrocytes which have an important role in protecting the parenchyma tissue.

to ischemic insult. Elucidating the relative contributions of these processes to observed diversity along several disease-associated variables will require fate-mapping and functional interrogation of astrocyte subsets to reveal the prospective therapeutic potential.

Potential for Astrocyte Plasticity Based on Disease-Associated Variables in Ischemic Stroke

In line with our definition, we propose that identified astrocyte diversity in ischemic stroke occurring along variables of time after injury (i.e., temporal), distance from lesion epicenter (i.e., topographical), age at onset, and sex of the individual, likely represent a greater contribution from plasticity than heterogeneity.

Astrocytes undergo dramatic morphological changes following ischemic insult that evolve over time (Lukaszevicz et al., 2002; Shannon et al., 2007; Benesova et al., 2009; Matyash and Kettenmann, 2009; Li et al., 2014). Using a murine photothrombosis model, Li et al. (2014) revealed increased GFAP expression by day 2 post-insult, with acquisition of stellate morphology and cellular hypertrophy by day 4, and dense astrocyte clustering by day 6. Interestingly, reactive astrocytes became less hypertrophic after day 6 post-insult with gradual lengthening of cellular processes by day 10, reflecting maturation of the reactive astrocyte clustering. Li et al. (2014) then went on to assess astrocyte proliferative dynamics, revealing a peak at day 3 post-insult, with a decline through day 14. Similarly, using an endothelin-1-induced vasospasm rat model, Mestriner et al. (2015) demonstrated significant increases at 30 days post-insult in astrocyte density and increased process ramification and length, as compared to uninjured controls. They also compared these dynamic changes across stroke types, using a time-matched post-hemorrhagic stroke model as comparison. Despite not revealing observable differences in several different GFAP-immunohistochemistry-based measurements, this cross-model comparison approach remains important as it enables isolation of intrinsic vs. extrinsic influences on astrocyte responses in the post-stroke brain (Mestriner et al., 2015). Use of higher resolution transcriptomic studies comparing reactive astrocyte responses over time and across different stroke types (e.g., ischemic, hemorrhagic) and vascular territories (e.g., middle cerebral artery, posterior cerebral artery, etc.) are likely to yield important information on reactive astrocyte diversity.

Astrocytic Ca^{2+} signaling dynamics also vary temporally in rodent models of ischemic stroke, in brain slice culture models of ischemia, and oxygen-glucose deprivation (OGD) models, both acutely and chronically (Duffy and MacVicar, 1996; Ding, 2013, 2014). For example, significant variability in latency to increased Ca^{2+} levels is observed in the initial minutes following ischemic onset in an OGD model (Duffy and MacVicar, 1996; Ding, 2013, 2014), suggesting that differing environmental factors may play a role. Furthermore, application of two-photon imaging in an *in vivo* photothrombosis murine model revealed reactive astrocyte subsets with dynamically changing amplitude and frequency of Ca^{2+} signaling, attributed to fluctuations in extracellular glutamate and GABA levels (Ding et al., 2009), in keeping with our definition of plasticity. Diversity in astrocytic Ca^{2+} signaling can also be seen outside of the acute post-injury period (Winship and Murphy, 2008; Choudhury and Ding, 2016). Using a murine photothrombotic model, Winship and Murphy (2008) demonstrated a progressive increase in magnitude of penumbral astrocytic Ca^{2+} signaling for 2 months post-insult, driven by stimulation of neural circuits *via* limb stimulation. Once again this represents modulation of astrocyte diversity in response to environmental manipulation (e.g., neural circuit stimulation), thus is offered to represent plasticity. Ca^{2+} signaling diversity may have important functional consequences for the functioning of local neural circuits, thus representing a significant parameter to further characterize. As neural circuit remodeling is an important mechanism of functional recovery post-stroke, manifested by the relative success of physical therapy in stroke patients over the sub-acute to chronic period, it will be a priority to optimize environmental variables that promote supportive astrocyte phenotypes. Further elucidation of astrocyte diversity, especially plasticity vs. heterogeneity, in this context is an important aspect of understanding neural circuit remodeling and functional recovery in the post-stroke brain.

Significant astrocyte diversity is also seen in rodent models of ischemic stroke with respect to distance from the lesion site (i.e., proximal topographical variation). For example, in a rat MCAO model, GFAP immunostaining, process volume, diameter, length, and branching points were increased in close proximity to the cortical infarct border zone compared to distances slightly further from the ischemic area (Wagner et al., 2013). Using a photothrombotic murine stroke model, Li et al. (2014) demonstrated an outwards gradation of astrocyte proliferation from the lesion core in parallel with higher densities of GFAP+ reactive astrocytes. After ischemia due to endothelin-1-induced vasospasm in the primary visual cortex of marmosets, astrocyte subsets were shown to differ in their expression of multiple reactive astrocyte markers, immunomodulatory genes, and various cytokine pathways (Boghdadi et al., 2020a), as well as transcriptional regulators and cell surface receptors involved in cell-matrix adhesion and migration (Dzwonek and Wilczynski, 2015; Boghdadi et al., 2020a). Interestingly, a number of neuronal genes were also upregulated, including Growth Associated Protein 43 (*Gap-43*), which is involved in post-infarct plasticity and expressed by neurons after axotomy (Skene and Willard, 1981; Tetzlaff et al., 1991; Frey et al., 2000; Hung et al., 2016). Topographical diversity in astrocyte Ca^{2+} signaling is also seen, with reduced magnitude amongst penumbral astrocytes compared to the lesion core (Winship and Murphy, 2008). Moreover, restricted astrocyte Ca^{2+} signaling has been shown following single vessel occlusion photothrombosis (Zheng et al., 2013). As most work done to date examines proximal changes near the site of ischemia (i.e., within a singular brain region), this is most likely reflective of plasticity in response to gradients of injury-associated factors (e.g., cytokines, inflammatory cells, blood-borne elements) radiating out from the epicenter. One notable caveat is the observation of extensive changes amongst astrocytes occurring over significant distances (i.e., between different brain regions) post-stroke. For example,

in a MCAO rodent model, Rakers et al. (2019) demonstrated significant changes in gene expression in both the ipsilateral and contralateral hemispheres compared to non-injured control tissue. They demonstrated a 2- and 12-fold increase in *Gfap* and a 2- and 20-fold increase in vimentin (*Vim*) in the contralateral and ipsilateral hemispheres, respectively, compared to uninjured control tissue (Rakers et al., 2019). Diversity noted amongst such spatially separated regions in response to injury could represent a relatively greater contribution from heterogeneity as the astrocytes in these regions most likely have different origins. Despite this, plasticity is still likely a key driver of the observed diversity, albeit proportionally less than for changes observed in close proximity to the lesion.

As ischemic stroke is predominately a disease of older individuals, understanding age-related astrocyte diversity is critical for therapeutic development (Badan et al., 2003; Orre et al., 2014; Androvic et al., 2020). Physiologic aging enhances ischemia-induced astrocyte reactivity, resulting in exaggerated glial responses and accelerated formation of densely packed astrogliosis borders as compared to younger controls (Badan et al., 2003; Popa-Wagner and Badan, 2007). As this is likely to be a consequence of extrinsic/environmental factors, we propose that this age-related diversity represents plasticity, at least within the confines of a singular brain region. Using the MCAO model in 18 month old aged mice, Androvic et al. (2020) demonstrated a predominance of aged astrocytes toward a neurotoxic "A1" phenotype as compared to younger animals, which was associated with worse functional outcomes (Badan et al., 2003; Androvic et al., 2020). This neurotoxic predominance is thought to be largely driven by aged microglia, which tend to be more pro-inflammatory and reactive to insult (Orre et al., 2014; Androvic et al., 2020), consistent with plasticity in response to an altered environment. Using a targeted striatal infarction model in rats, Lively et al. (2011) demonstrated an age-related reduction in astrocyte-derived synaptic cleft-1 (SC1) expression, an ECM molecule associated with neural plasticity, a key aspect of recovery following stroke (Badan et al., 2003; Lively et al., 2011; Sohrabji et al., 2013; Cassidy and Cramer, 2017). Furthermore, ischemia-activated primary astrocyte cultures isolated from aged rats display reduced glutamate uptake compared to younger controls (Lewis et al., 2012). Impaired astrocyte-mediated buffering of glutamate levels in the post-stroke brain may contribute to the increased infarct volumes and worse functional outcomes seen in aged rodents following ischemic stroke (Popa-Wagner and Badan, 2007; Selvamani and Sohrabji, 2010), further suggesting an environmentally dependent effect. Further investigation with lineage tracing studies in the context of the aged CNS is an important area to elucidate potential contributions from heterogeneity to this observed diversity.

Although age-specific stroke incidence and mortality are higher in men, stroke-related morbidity is greater amongst women (Reeves et al., 2008; Petrea et al., 2009), making sex an important disease-associated variable to investigate astrocyte diversity. Adult female rodents demonstrate smaller infarct volumes than age-matched males (Hall et al., 1991; Alkayed et al., 1998; Manwani et al., 2011; Selvamani et al., 2014), an effect which reverses with advancing age (Manwani et al., 2013) suggesting that the aged female brain is more susceptible to ischemic insult than its male counterpart (Chisholm and Sohrabji, 2016; McCullough et al., 2016). Importantly, these gross differences are underlain by sex-specific diversity amongst cellular populations, including astrocytes. Using a mouse MCAO model, Morrison and Filosa (2016) demonstrated sex-specific differences in frequency of astrocytic Ca^{2+} elevations as well as a more robust reactive astrocytic response in male mice as compared to age-matched females. Sex hormones appear to be a key player in this observed difference. Indeed, astrocyte-derived estradiol conveys neuroprotective and anti-inflammatory effects in rodent models of global ischemia (Zhang et al., 2014). Furthermore, cultured astrocytes isolated from female rodents are more resistant to *in vitro* ischemic insult and glucose deprivation, mediated in part by increased P450 expression and aromatase activity compared to male astrocytes, thus altering estrogen levels in the cells (Liu et al., 2007; Liu et al., 2008). Importantly, ischemia induces astrocyte-specific aromatase expression and activity *in vivo* as well (Carswell et al., 2005). Consistent with this, live imaging studies in a unilateral MCAO rodent model demonstrated significantly increased estrogen-dependent GFAP expression in adult female mice compared to adult males (Cordeau et al., 2008) and estrogen induces astrocytic expression of glutamate transporters GLT-1 and GLAST (Pawlak et al., 2005; Lee et al., 2009), suggesting that female astrocytes *in vivo* may be more effective at glutamate clearance than their male counterparts. Considering that much of this observed diversity is thought to be driven by sex hormones (e.g., estrogen, progesterone), we suggest that this astrocyte diversity is predominantly representative of plasticity, although we cannot exclude the potential of heterogeneity also being a contributing factor (McCullough et al., 2016). This is particularly interesting given the notable sex-specific difference in clinical outcomes (Reeves et al., 2008; Petrea et al., 2009) and the potential for modulation of hormone-responsive signaling pathways as a means of conveying neuroprotection, which are commonly targeted by therapeutics for malignancies (e.g., tamoxifen, trastuzumab; Jordan, 2003; Cameron et al., 2017; Shagufta and Ahmad, 2018; Xu et al., 2019). Adaptation of these therapeutics for the modulation of astrocytic responses in the post-stroke brain may be a viable approach for neuroprotection and/or post-stroke recovery. This requires detailed characterization of the extent of astrocyte diversity present following ischemic stroke, the functional implications of that diversity with respect to neural survival and function, and the identification of potentially malleable targets for therapeutics.

Potential for Astrocyte Heterogeneity Based on Disease-Associated Variables in Ischemic Stroke

In contrast to plasticity, heterogeneity as a contributor to astrocyte diversity in ischemic stroke represents potential diversity that exists amongst astrocyte populations with established differences in origin and function. According to this definition, we propose that this would be diversity observed between regionally specific astrocyte populations (i.e., diversity across brain regions). In the context of ischemic stroke, this could

be seen as diversity amongst astrocyte responses in diverse brain regions following a similar injury (i.e., infarction in different vascular territories). Mestriner et al. (2015) used endothelin-1-induced vasospasm rat model and compared ischemic lesions in the sensorimotor cortex and dorsolateral striatum and noted variation at 30 days post-insult in terms of GFAP-immunohistochemistry based measurements including cellular optimal density and primary process length (Mestriner et al., 2015). Using a MCAO murine model, Lukaszevicz et al. (2002) demonstrated differential functional responses of protoplasmic and fibrous astrocytes (thus effectively comparing WM and GM lesions), including differences in morphological response and cell death (Lukaszevicz et al., 2002), consistent with demonstration of differential ischemic sensitivities amongst astrocyte subsets in models of ischemia-reperfusion injury (Shannon et al., 2007). Importantly, it remains unclear whether protoplasmic and fibrous astrocytes have distinct origins considering that both can be derived from the postnatal SVZ during corticogenesis (Levison and Goldman, 1993; Parnavelas, 1999; Marshall and Goldman, 2002). Lineage tracing has shown that SVZ-derived multipotent neural stem cells (NSC) have the ability to migrate and contribute to the reactive astrocyte population in the context of multiple stroke models (Faiz et al., 2015) but it is unclear how different their function is from other reactive astrocytes in the region. Further direct lineage tracing experiments are required to establish these populations as truly distinct from an origin and functional perspective, and thus fitting of our offered definition of heterogeneity.

Due to limited high-quality fate-mapping studies, there is a scarcity of data on the extent of astrocyte heterogeneity in ischemic stroke. Furthermore, most data accumulated to date focus on astrocyte diversity as a function of changing environmental parameters, and therefore represent plasticity. Ischemic stroke has the potential to affect multiple vascular territories, and thus diverse populations of astrocytes. Determination of the impact of regionally specific astrocyte diversity to various post-stroke outcome measures is of great interest, both clinically and for understanding stroke pathophysiology. For example, hemorrhagic transformation of infarcted tissue (i.e., hemorrhage developing within infarcted tissue) is a devastating post-stroke complication caused by disruption of the BBB (Warach and Latour, 2004; Khatri et al., 2012; Sussman and Connolly, 2013), of which astrocytes are a key component. Identification of malleable aspects of astrocyte diversity that alter susceptibility of neurovascular degradation may be a viable approach to reduce occurrence in the post-stroke population. To that end, expanded study of reactive astrocyte diversity in ischemic stroke models affecting different vascular territories (e.g., middle cerebral artery, posterior cerebral artery, etc.) using combined fate-mapping and functional assays is warranted.

CNS DEMYELINATION

The most common demyelinating disorder is multiple sclerosis (MS), a complex immune disease characterized by inflammation, primary demyelination (loss of myelin from an intact axon), and neuronal/axonal damage/degeneration (Molina-Gonzalez and Miron, 2019; Rawji et al., 2020). Various pre-clinical models have been employed to replicate the complexity of the MS disease process (**Figure 3C**; Mix et al., 2010; Baker and Amor, 2015; Lassmann and Bradl, 2017; Baecher-Allan et al., 2018). One of the main models is experimental autoimmune encephalitis (EAE; Constantinescu et al., 2011; Ben-Nun et al., 2014), which involves the administration of myelin peptides or CNS homogenate to induce an autoimmune-mediated demyelinating insult to the rodent CNS (Denic et al., 2011; van der Star et al., 2012; Baker and Amor, 2015). While EAE models the immunopathogenesis of acute MS lesions and has been instrumental to discover many disease modifying treatments (Plemel et al., 2017), it is of limited value for assessing the neurobiological aspects of remyelination (Owens, 2006; Haanstra et al., 2015; Procaccini et al., 2015; Plemel et al., 2017; Stimmer et al., 2018). Another immune-mediated model of MS is the use of Theiler's murine encephalomyelitis virus (TMEV) to induce spinal cord demyelinated lesions (Dal Canto and Lipton, 1977; Owens, 2006; Denic et al., 2011; McCarthy et al., 2012). Toxin-based models of demyelination have also been used widely in pre-clinical studies on neurobiological aspects of demyelinating disease. Most commonly employed are LPS (Liddelow et al., 2017), cuprizone (Praet et al., 2014), lysophosphatidylcholine (lysolecithin; LPC; Blakemore and Franklin, 2008; Lassmann and Bradl, 2017), and ethidium bromide (EB) models (Woodruff and Franklin, 1999; McMurran et al., 2019). LPS is a model of neuroinflammation initiated by the injection of a bacterial endotoxin and results in neurotoxic astrocyte phenotype (Liddelow et al., 2017). Cuprizone is a copper chelating agent that is administered orally and induces acute CNS demyelination, particularly in the corpus callosum and cerebellar peduncles (Praet et al., 2014). LPC by contrast, is focally injected into white matter tracts, inducing damage to the myelin sheaths (Blakemore and Franklin, 2008; Lassmann and Bradl, 2017) and acting as a chemoattractant for monocytes, thus triggering a focal inflammatory response (Blakemore and Franklin, 2008; Lassmann and Bradl, 2017). Many toxin-based demyelination models represent a simpler system with predictable and reproducible spatiotemporal patterns to study the remyelination process (McMurran et al., 2019) and have been used as tools to highlight both the permissive and inhibitory roles of astrocytes (reviewed by Rawji et al., 2020).

Role of Astrocytes in CNS Demyelination

Astrocytes are central to the demyelination response and the success of remyelination (Molina-Gonzalez and Miron, 2019; Rawji et al., 2020), as they release anti-inflammatory cytokines, proteins that modulate myelin regeneration (remyelination), actively maintain extracellular ionic and neurotransmitter concentrations, and provide critical neuronal support (Hinks and Franklin, 1999; Jessen et al., 2015; Liddelow et al., 2017). Astrocytes also release pro-inflammatory cytokines, promote BBB permeability, and inhibit OPC maturation (Sisková et al., 2006; van Horssen et al., 2007; Lau et al., 2013; Stoffels et al., 2013). These contrasting functions are thought to represent

changing functional roles at different stages of the remyelination process (reviewed in Rawji et al., 2020). Importantly, astrocyte responses are often dependent on the type of lesion (Rao et al., 2019) where acute active lesions are filled with immune cells and hypertrophic astrocytes with increased GFAP expression and an associated upregulation of proinflammatory chemokines and cytokines (Williams et al., 2007; Franklin and Goldman, 2015). Alternatively, inactive lesions (**Figure 3D**) have less inflammation and the astrocytes typically are more densely packed with long thick processes in close proximity to extensively demyelinated axons (Franklin and Goldman, 2015). In the rodent cuprizone model, astrocytes have been shown to regulate the recruitment of microglia which then facilitates myelin debris removal, an imperative step for subsequent repair (Skripuletz et al., 2013). Astrocytes also play an important role in influencing the balance of OPC-derived oligodendrocyte vs. OPC-derived Schwann cell-mediated CNS remyelination (reviewed by Chen et al., 2021). The ability of astrocytes to take on different roles may reflect distinct sub-populations or dynamic changes in malleable phenotypes driven by spatiotemporal environmental fluctuations (e.g., microglia-derived factors, cytokines; Cerbai et al., 2012; Horstmann et al., 2016). The relative contributions of heterogeneity and plasticity to these dynamically changing roles remains to be elucidated, as well as the potential for manipulation of astrocytes to phenotypes that support a pro-remyelination lesion environment.

Astrocyte Diversity in CNS Demyelination

Similar to other CNS pathologies, transcriptional evidence has expanded our understanding of astrocyte diversity in CNS demyelination, including the use of bulk transcriptional analysis (Rothhammer et al., 2016, 2018; Itoh et al., 2018; Chao et al., 2019; Wheeler et al., 2019). For example, RNA-seq analysis of EAE and MS tissue identified several clusters of astrocytes with differential expression of *S100b*, *Gja1*, *Aldh1l1*, *Gfap*, and *Aqp4*, consistent with a spectrum of astrocyte transcriptional states in EAE (Wheeler et al., 2019). Furthermore, astrocytes in EAE and MS tissue were demonstrated to have variable expression of the transcription factor *Nrf2* and *Mafg* and *Mat2a* signaling, leading to DNA methylation and increased CNS pathology (Wheeler et al., 2020). These data identify epigenetic modifiers as potential therapeutic candidates to modulate pathology in MS. Importantly, as discussed below, there is a need to separate plasticity and heterogeneity in models of CNS demyelination to improve our understanding of factors that promote a pro-remyelination lesion environment and eventually guide therapeutic development. To that end, we propose here a framework for making that distinction using current evidence for astrocyte diversity in models of CNS demyelination as an example.

Potential for Astrocyte Plasticity Based on Disease-Associated Variables in CNS Demyelination

We argue that astrocyte diversity across variables, such as disease stage (early or late disease progression), lesion status (status of demyelination or remyelination), age at disease onset, and sex of the individual are examples where plasticity is the predominant contributor to astrocyte diversity. In comparison to other CNS pathologies, MS and EAE have extensive spatiotemporal lesion variability and extensive immunological activity, complicating the interrogation of astrocyte diversity. For example, lesions present in the same individual at a given time can be at different stages (i.e., active, chronic active, inactive, remyelinated, etc.; Rao et al., 2019) and in different CNS regions (spanning WM and GM). Furthermore, analogous lesions can be present in individuals of different age and/or sex, complicating comparisons between spatiotemporally similar lesions. Given those complexities, we highlight the challenge in distinguishing plasticity and heterogeneity without clear fate-mapping studies to establish a baseline in regionally and temporally disparate CNS regions against which comparison of variability can be assessed.

Temporal evolution of astrocyte diversity in inflammatory demyelinated lesions has been noted. For example, in EAE models astrocyte cholesterol gene expression decreases late in the disease course in both the spinal cord and optic nerve (Itoh et al., 2018; Tassoni et al., 2019). Furthermore, astrocytic major histocompatibility complex-II (MHC-II) expression is markedly increased in early EAE brain and spinal cord lesions followed by a late decrease (Itoh et al., 2018), while optic nerve astrocytes in EAE optic neuritis show an early increase in MHC-II expression that persists through the disease course (Clarkson et al., 2015; Tassoni et al., 2019). Due to the role of MHC-II in immunomodulation, it is tempting to speculate about the functional relevance of this diversity, compounded by the fact that optic neuritis is commonly the initial presentation of MS in patients (Polman et al., 2005; Montalban et al., 2010; Amato et al., 2018). Diversity along the temporal component most likely represents response to a dynamic lesion environment, and thus plasticity. Despite this, it remains important to establish a baseline of heterogeneity, especially between CNS regions, as well as identify newly generated astrocytes in the context of demyelination injury as a potential source of heterogeneity.

Astrocyte diversity is also noted across lesion types in MS models (Rao et al., 2019). For example, acute lesions display hypertrophic astrocytes with increased expression of GFAP and several pro-inflammatory cytokines, chemokines, and remyelination-associated molecules (Woodruff et al., 2004; Williams et al., 2007; Franklin and Goldman, 2015; Rawji et al., 2020). By contrast, inactive lesions contain transcriptionally inactive astrocytes with small cell bodies and long filamentous processes that contribute to an increased density of reactive astrocytes (Franklin and Goldman, 2015; Ludwin et al., 2016). For example, acute lesions demonstrate reduced astrocytic connexin-43 (CX43) expression and disrupted CX43/CX47-mediated astrocyte-to-oligodendrocyte connections (Masaki et al., 2013). Loss of CX43 expression is associated with progressive MS disease, oligodendrocyte pathology, and astrocyte degeneration (Masaki et al., 2013) and patients with reduced CX43 expression have a more rapid clinical disease progression (Masaki et al., 2013). Furthermore, astrocytic chitinase 3-like 1 (CHI3L1) expression, associated with chronic inflammation and neurotoxicity (Cantó et al., 2012; Matute-Blanch et al., 2020),

is observed in chronic MS lesions but absent from other lesion types (Cantó et al., 2012). One potential driver of this diversity is microglial-derived factors (e.g., activin-A), which have been shown to modulate astrocyte activation in demyelinated lesions (Miron et al., 2013; Rawji et al., 2020), further consistent with plasticity as a predominant contributor.

Age is an important factor in the pathogenesis of MS, with clinical disease onset rare after the age of 50 (Tremlett et al., 2010; Amato et al., 2018; Baecher-Allan et al., 2018). Furthermore, age at onset appears to be a key prognostic factor for MS patients (Tremlett et al., 2010; Amato et al., 2018). Progressive MS is also correlated with increasing age (Koch et al., 2009; Tutuncu et al., 2013). Despite the multitude of factors that may be contributing to this observation, including age-related changes in hormones, immune function, neural and non-neural cell populations, it is tempting to speculate regarding the potential contribution of age-related diversity in astrocyte subpopulations to this disease susceptibility. Aged-related astrocyte diversity observed is thought to be at least in part secondary to the increased pro-inflammatory function of aged microglia (Kanaan et al., 2010; Cerbai et al., 2012; Jyothi et al., 2015), thus likely representing plasticity. Aged astrocytes are more reactive and respond to demyelination with a more pronounced cellular hypertrophy (Robillard et al., 2016; Boisvert et al., 2018; Clarke et al., 2018). Aged astrocytes were also demonstrated to have decreased expression of key cholesterol synthetic enzymes (Boisvert et al., 2018; Clarke et al., 2018), which may contribute to the reduced success of remyelination with age (Shields et al., 2000; van Wijngaarden and Franklin, 2013). Further study will help to elucidate the impact of age-related astrocytic changes to remyelination success, either directly through immunomodulatory effects or indirectly through interactions with other neural cell lineages populating the lesions, as well as reveal astrocyte features potentially amendable to therapeutic targeting.

MS affects 2–3 times more women than men (Whitacre et al., 1999), but men are more likely to have disease progression (Savettieri et al., 2004; Koch et al., 2009; Tomassini and Pozzilli, 2009; Shirani et al., 2012). Sex-specific diversity of astrocytes is notable in models of auto-immune demyelination (Chowen et al., 2017), reflecting this clinical observation. In EAE optic neuritis, optic nerve astrocytes in female mice display more robustly increased *C3* expression (a component of the pro-inflammatory complement cascade; Stevens et al., 2007) and dampened upregulation of thombospondin-1 (*Thbs1*) when compared to their male counterparts (Tassoni et al., 2019), consistent with a more pro-inflammatory environment. Importantly, this is correlated with exaggerated retinal ganglion cell (RGC) and axonal loss in female mice (Tassoni et al., 2019), with a significant negative correlation between astrocytic expression of *C3* and RGC density (Liddelow et al., 2017; Tassoni et al., 2019). This is particularly interesting given the female predominance of clinical MS, as well as the frequency of optic neuritis as a presentation in female MS patients (Voskuhl and Gold, 2012; Baecher-Allan et al., 2018; Voskuhl et al., 2018). Importantly, these sex-specific differences were not observed in spinal cord astrocytes in the same model (Tassoni et al., 2019), highlighting notable regional diversity (Itoh et al., 2018). Further investigation to differentiate plasticity (e.g., hormone effects) from heterogeneity and to elucidate the functional relevance of this observed sex-specific diversity are important goals for future research (Chowen et al., 2017; Gilli et al., 2020).

Potential for Astrocyte Heterogeneity Based on Disease-Associated Variables in CNS Demyelination

In contrast to plasticity, we propose that astrocyte heterogeneity in models of CNS demyelination likely manifests as differences in regionally specific astrocyte populations in disparate CNS regions (e.g., brain regions, spinal cord, etc.). For example, scRNA-seq analysis of isolated murine astrocytes after EAE revealed distinct expression profiles for spinal cord, cerebellar, and hippocampal astrocytes (Itoh et al., 2018; Tassoni et al., 2019). Cholesterol synthesis pathways were significantly downregulated in WM-rich regions (e.g., spinal cord, cerebellum, optic nerve) due to altered ApoE-mediated cholesterol transport (Itoh et al., 2018; Tassoni et al., 2019). This is consistent with the downregulation of cholesterol synthesis observed in chronic demyelinated lesions in mouse MS models (Boisvert et al., 2018; Clarke et al., 2018), thought to be detrimental for remyelination given the lipid-rich nature of myelin sheaths (Itoh et al., 2018; Rawji et al., 2020). There are also notable differences between astrocytes that populate subcortical WM and GM lesions (Albert et al., 2007; Prins et al., 2015). Combined snRNA-seq and transcriptomic lesion mapping in MS tissue enabled mapping of dysregulated genes to either GM or WM astrocyte subpopulations (Schirmer et al., 2019) revealing that GM astrocytes in cortical lesions (i.e., GPC5+/SLC1A2+ cells) had decreased expression of genes involving glutamate and K^+ homeostasis, whereas WM astrocytes in subcortical lesions (i.e., CD44+/LINC01088+ cells) displayed upregulated genes including *GFAP*, the transcription factors *BCL6* and *FOS*, and endothelin receptor B (Schirmer et al., 2019). Spinal cord astrocytes in LPC focal demyelination models were shown to have increased GFAP expression as compared to the cerebral cortex (Yoon et al., 2017). Molecular pathway analysis identified the antigen-presentation and interferon signaling pathways as specifically enriched in spinal cord and cerebellar astrocytes (Itoh et al., 2018). Antigen presentation by astrocytes has been previously shown in EAE models (Cornet et al., 2000; Constantinescu et al., 2005) and thought to be functionally important in astrocyte-mediated immunomodulation (Rawji et al., 2016, 2020). Regional diversity can also be seen in EAE optic neuritis models (Horstmann et al., 2016; Nolan et al., 2018; Tassoni et al., 2019). scRNA-seq revealed astrocyte-specific upregulation of the complement cascade and *Thbs1*, a gene involved in RGC synaptic plasticity and visual recovery following demyelination (Tassoni et al., 2019). Astrocytic *Thbs1* expression peaks early in disease (Tassoni et al., 2019), consistent with early astrocyte regulation of neural plasticity in demyelinated visual circuits. Optic nerve astrocytes also displayed reduced expression of cholesterol synthetic genes and increased expression of antigen presentation genes (Tassoni et al., 2019), similar to that seen

in spinal cord EAE lesions (Itoh et al., 2018). Diversity in gene expression was also noted between optic nerve and retinal astrocytes (Tassoni et al., 2019).

Regional diversity in EAE and MS is particularly interesting considering the multifocal nature of the disease (Baecher-Allan et al., 2018; Rawji et al., 2020). One can speculate that regional differences in astrocyte and other glial cell populations create an environment (e.g., ECM, cytokines, chemokines, etc.) more conducive to immune-mediated demyelination and/or for remyelination success. Astrocytes are known to exhibit regional specific responses to T-cell cytokines, leading to regionally specific neuroinflammatory responses in the hindbrain and spinal cord (Williams et al., 2020), suggesting that diverse astrocyte features may contribute to the anatomical propensity for demyelinated lesions to occur in certain locations (e.g., periventricular WM, spinal cord, optic nerve). Expression of pattern recognition receptors (PRRs) and interferon-induced genes, both at a basal level and in response to IFN induction, were higher in cerebellar than cortical astrocytes (Daniels et al., 2017), consistent with regionally specific astrocytic innate immune responses. Moreover, activation of the unfolded protein response alters the astrocytic secretome, generating a unique reactivity state and impairing synaptogenic function *in vitro* (Smith et al., 2020). Understanding the interplay between these environmental features and astrocytes is likely a fruitful area for therapeutic targeting, given the multitude of roles that astrocytes have in CNS demyelination. For example, GM lesions remyelinate more efficiently compared to WM (Albert et al., 2007; Bai et al., 2016; Strijbis et al., 2017), which could reflect, at least in part, contributions of regional specific astrocyte diversity. This will require detailed fate-mapping studies in models of CNS demyelination across lesion types, brain regions, and throughout the natural history of the condition.

TRAUMATIC INJURY

Traumatic CNS injuries remain the major causes of disability, premature death, and long-term neuropsychiatric impairment (Fitzharris et al., 2014; Gbd, 2016; National Spinal Cord Injury Statistical Center, 2021). Traumatic spinal cord injury (SCI) results from the application of an external force on the spine (e.g., motor vehicle accident, fall, sports-related injury, violent injury) damaging the underlying neural tissue in a variable manner (Ahuja et al., 2017; National Spinal Cord Injury Statistical Center, 2021). The primary insult results in massive damage to neural cells and triggers a complex cascade of secondary injury mechanisms that culminate in neuronal and glial cell death, ischemic injury, and inflammation (Alizadeh et al., 2019). This is generally followed by significant reorganization of spinal cord structure, including the formation of a densely packed astrogliosis border surrounding a cystic cavity at the lesion site (Sofroniew, 2009; Adams and Gallo, 2018; Alizadeh et al., 2019; Tran et al., 2021). Traumatic brain injury (TBI) is highly variable mechanistically with a wide range of causative insults and severities. Moreover, TBI has a complex post-injury course with numerous sequelae that form part of a gradually evolving syndrome associated with chronic behavioral disturbances, seizure disorders, and protracted neurodegenerative diseases (Maas et al., 2008; Kovacs et al., 2014; Sharp et al., 2014). Reflecting the variable clinical presentation, TBI tissue pathology is highly inconsistent according to the type and severity of the injury and brain region involved (Sofroniew, 2009, 2015; Burda and Sofroniew, 2014). Due to the myriad and seemingly conflicting roles of astrocytes in the post-trauma environment, characterization of the contributions of heterogeneity and plasticity seems vital to the identification of targets for therapeutic manipulation both acutely for neuroprotective approaches and in the sub-acute/chronic period to enable for more effective axon regenerative approaches, which will continue to be the focus of extensive research efforts (Hilton et al., 2012; Cregg et al., 2014; Anderson et al., 2016; Geoffroy et al., 2016).

Several pre-clinical models of traumatic CNS injury have been utilized (**Figure 3E**; Jakeman et al., 2000; Metz et al., 2000; Dunham et al., 2010). Importantly, given the complexity of human traumatic injury, no one model alone can recreate all aspects of the injury process, therefore models are employed based on the study objectives and are thus limited in generalizability. In terms of injury mechanisms, SCI models can be broadly classified as contusion, crush, and transection (Hilton et al., 2013, 2019; Cheriyan et al., 2014), and recently emerging distraction and dislocation (Chen et al., 2016), with each mechanism having a unique tissue including glial response pattern. Contusion models are the most used due to their perceived clinical relevancy, although this relevancy has yet to be confirmed in a successful human trial of a neuroprotective treatment. The most common TBI models are controlled cortical impact (CCI) models and fluid percussion injury models, with the notable addition of models that mimic blast injuries or repeated mild TBI (i.e., concussion; Xiong et al., 2013). Differences in injury mechanism between the models and resultant secondary injury lead to differential astrocytic responses (Adams and Gallo, 2018; Boghdadi et al., 2020b), a consideration when attempting to generalize astrocyte responses across models.

Role of Astrocytes in Traumatic Injury

Astrocytes (Sofroniew, 2020), OPCs (Hackett et al., 2016), and fibroblasts (Goritz et al., 2011; Soderblom et al., 2013; Dias and Göritz, 2018) have all been shown to contribute to the densely packed accumulation of cells in close proximity to injury epicenter following SCI and TBI, with continual modulation by microglia and infiltrating innate and adaptive immune cells (Silver and Miller, 2004; Silver, 2016; O'Shea et al., 2017; Bradbury and Burnside, 2019). For the purposes of this review, we focus on data pertaining to the astrocytic component (Faulkner et al., 2004; Anderson et al., 2018; Gu et al., 2019; Yang et al., 2020) but recognize that reactive astrogliosis is often accompanied by clusters or intermingling fibroblast-like cells and OPCs (Faulkner et al., 2004; Anderson et al., 2018; Dias et al., 2018; Gu et al., 2019; Yang et al., 2020). In agreement with a recent suggestion to avoid using the term "glial scar" or "scar" (Sofroniew, 2020), which should be reserved for mesenchymal or stromal scar tissue, we will refer to the previously termed "glial scar" as

densely packed astrogliosis borders or densely packed reactive astrocytes. At the sub-acute/chronic traumatic injury epicenter (**Figure 3F**), there is a drastic loss of cells which overtime can result in the formation of a fluid-filled cyst surrounded by an inner layer of fibroblast-like cells and an outer layer of densely packed reactive astrocytes (Tran et al., 2021). Astrocytes are rapidly activated following traumatic injury culminating in the formation of a prominent densely packed astrogliosis border (Sofroniew, 2009; Adams and Gallo, 2018). Densely packed reactive astrocytes after TBI are partially regulated by monocyte invasion where reducing invasion using CCR2−/− mice results in increased astrocyte proliferation but perhaps surprisingly, decreased GFAP+ scar area, ECM deposition, and lesion size compared to controls (Frik et al., 2018). While traditionally viewed as a barrier to axon regeneration and thus functional recovery, the post-traumatic densely packed astrogliosis border is also ascribed a beneficial function in the mitigation of further damage to the surrounding spared tissue (Adams and Gallo, 2018). For example, attenuating densely packed astrogliosis through signal transducer and activator of transcription 3 (*stat3*) deletion results in increased inflammation, lesion volume, and reduced motor recovery compared to controls (Herrmann et al., 2008). Importantly, the role of the densely packed astrogliosis border appears to be dynamic throughout the post-injury period, reflecting the multiplicity of functions ascribed to it (Anderson et al., 2016; Adams and Gallo, 2018).

Astrocyte Diversity in CNS Traumatic Injury

Diversity of reactive astrocytes following CNS trauma is thought to be driven largely by fluctuations in the inflammatory and cellular milieu of the post-injury environment (Hara et al., 2017; Adams and Gallo, 2018; Boghdadi et al., 2020b). RNA-seq performed on astrocytes 2 weeks following a spinal cord crush injury revealed differential expression of over 6,000 genes (Anderson et al., 2016), similar in magnitude with that observed post-ischemia (Zamanian et al., 2012; Anderson et al., 2016). Importantly, these reactive astrocytes demonstrate significant environmental-dependent plasticity (Hara et al., 2017; Boghdadi et al., 2020b). For example, integrin/N-cadherin-dependent collagen-1 signaling shapes astrocyte phenotypes in the post-injury spinal cord (Hara et al., 2017). Moreover, reactive astrocytes grafted into the spinal cord adopt an environmentally congruent phenotype (Hara et al., 2017). For example, reactive astrocytes grafted into the injured spinal cord retain the reactive phenotype, while those transplanted into the uninjured spinal cord revert phenotypically into resting astrocytes, confirmed by transcriptional analysis (Hara et al., 2017). After repetitive diffuse mild TBI, an atypical reactive astrocyte population was observed which featured a lack of GFAP expression, a downregulation of homeostatic proteins, and pronounced astrocyte coupling impairments, highlighting the importance of characterizing diversity across different types of injury (Shandra et al., 2019).

Extensive research has focused on manipulating the astrogliosis-associated deposition of growth-inhibitory ECM molecules (e.g., chondroitin sulfate proteoglycans; CSPGs) in the context of traumatic injury (McKeon et al., 1995; Ponath et al., 2018) in an attempt to improve axonal regeneration (Bradbury et al., 2002; Cregg et al., 2014; Burda et al., 2016; Tran et al., 2018). In a rat model of TBI, CSPG expression is increased in a subset of peri-lesional astrocytes, peaking at 7 days post-injury, which is temporally correlated with a reduction in peri-neuronal net density and increased GAP-43+ neurons (Harris et al., 2009, 2010; Yi et al., 2012). This is consistent with the observed increase in neural plasticity, which is an important intrinsic mechanism of functional recovery following trauma. Additionally, subsets of astrocytes were observed to express the complement component C3 as early as 3 days post-injury with further increase through 28 days post-injury, mainly clustered around the lesion core (Qian et al., 2019). Whether these C3+ astrocytes represent neurotoxic or pro-inflammatory phenotypes akin to "A1" astrocytes (Liddelow et al., 2017) remains to be determined. The potential role of these cells in axonal loss and retraction of transected axons from the lesion edge would be interesting to investigate as they remain key hurdles impeding regenerative and neuroprotective strategies in models of CNS trauma (Anderson et al., 2016; Geoffroy et al., 2016; Liddelow and Barres, 2016; Hilton et al., 2017).

Potential for Astrocyte Plasticity Based on Disease-Associated Variables in Traumatic Injury

In the context of traumatic injury, we propose that astrocyte diversity across the disease-associated variables of time post-injury (i.e., temporal), distance from injury (i.e., topographical), age at which injury is sustained, and sex of the individual are examples where plasticity is likely to predominant over heterogeneity. Extensive work has been performed to characterize reactive astrogliosis in the context of SCI (Filous and Silver, 2016; Silver, 2016; Bradbury and Burnside, 2019), focusing predominately on temporal changes in GFAP and CSPGs expression and morphological changes. Importantly, there are limited peer-reviewed transcriptomics data available to date. scRNA-seq was used to generate transcriptomics data based on temporal changes post-injury after mouse contusion SCI at 1, 3, and 7 dpi (Milich et al., 2021). Gene Ontology (GO) Enrichment Analysis for differentially expressed genes performed on astrocytes at 1 dpi were "translation" and "biosynthetic processes," while by 3 dpi astrocytes were defined by "mitochondrial function" and "oxidative phosphorylation," and at 7 dpi astrocytes were related to "lipid processing" (Milich et al., 2021). Despite numerous remaining questions regarding the extent and functional relevance of temporal astrocyte diversity following CNS traumatic injury, it appears probable that dynamic changes are evident following injury with shifting roles in the evolving injury and recovery processes, hence representing plasticity in response to environmental fluctuations. Further elucidation with fate-mapping studies and functional interrogation is key to revealing effective means of manipulating these processes to promote neuroprotection and recovery following injury.

As with other CNS pathologies, the degree of reactive astrogliosis in traumatic injury is highly dependent on the distance from the lesion, secondary to the gradation of pathology radiating outwards from the lesion epicenter, as reflected by parallel gradients of axonal injury, vascular disruption, ischemia, and inflammation (Wanner et al., 2013; Okada et al., 2018; Boghdadi et al., 2020b). GFAP expression, CSPG expression, astrocyte proliferation, and astrocyte density is highest directly adjacent the lesion, with a decreasing taper with increasing distance (White et al., 2009; Wanner et al., 2013). Importantly, in the injured spinal cord there appears to be diversity amongst locally intermingled astrocytes equidistant to the lesion, including variable expression of GFAP, NESTIN, and brain lipid-binding protein (BLBP; White et al., 2009) and only certain astrocyte subsets proliferate and/or polarize in response to a cortical stab injury (Bardehle et al., 2013), which are predominately juxta-vascular, reflecting the influence of blood-borne elements (Bardehle et al., 2013). These findings highlight the complexity and extent of astrocyte diversity that exists in CNS trauma, largely a reflection of the complex post-injury environment, in keeping with our definition of plasticity.

Despite the increasing prevalence of aged SCI patients, there is limited data on reactive astrocyte diversity in the aged CNS. One study examined astrocytes in young (i.e., 4 month old) and aged mice (i.e., 18-month-old) at 1, 3, and 7 days after a controlled cortical impact TBI (Early et al., 2020). Histological analysis demonstrated a significant increase in GFAP+ area in the aged mice compared to young, but notably only at 7 dpi (Early et al., 2020). Generally, transcriptomics data performed on these astrocyte populations found disproportionate changes in genes associated with reactive astrocytes in the aged mice after TBI compared to their young counterparts, as might be expected based on findings from other pathologies and normal aging process. Importantly, these profiles were not aligned with classic "A1/A2" phenotypes, once again highlighting the existence of a spectrum of reactive astrocyte subsets rather than a binary classification. Given the increasing importance of age in clinical presentation of traumatic CNS injury and the significant astrogliosis observed in animal models in the chronic SCI setting (months to years after injury; Silver and Miller, 2004), further work is needed to better characterize reactive astrogliosis diversity in the aged CNS and determine the functional relevance of these changes to disease outcomes (Burda et al., 2016; Okada et al., 2018).

Males are more commonly affected by CNS trauma than females (Cripps et al., 2011; Devivo, 2012; Gupte et al., 2019), attributed to increased risk-related behaviors. Despite this, there is limited data on sex-related astrocyte diversity in CNS trauma. In fact, because of the bladder problems associated with SCI models, 71% of pre-clinical SCI models are performed in female mice (Stewart et al., 2020), whose shorter urethra eases the stress of bladder expressions. In murine models of severe TBI, females display a larger GFAP+ area compared to males in the first week after injury which resolves by 30 dpi (Villapol et al., 2017). Moreover, chemokine C-C motif Ligand 2 (CCL2) expression was reduced after cortical injury in female astrocytes as compared to their male counterparts, potentially reflecting altered immune cell recruitment (Acaz-Fonseca et al., 2015). In contrast, no sex-specific differences were noted in reactive astrocytes between male and female mice in a model of repetitive diffuse brain injury (Shandra et al., 2019), reflecting the importance of the specific model used. One of the main drivers of sex-specific differences is likely sex hormones, namely estrogen, which is in keeping with our definition of plasticity. The role of estrogens in astrocyte activation has been previously established (Arevalo et al., 2015) and recent data demonstrate that inhibiting estradiol synthesis promotes reactive astrogliosis in female mice after a controlled cortical impact TBI (Vierk et al., 2012), an effect not seen in males (Vierk et al., 2012). Therefore, the female CNS may be more sensitive to CNS trauma as compared to the male counterpart as was suggested previously (Vierk et al., 2012; Tabatadze et al., 2015; Bender et al., 2017). Further investigation of the influence of estrogens in models of CNS trauma is an important means of differentiation between hormonally driven effects (i.e., plasticity) and specific patterns of gene expression that are linked to sex chromosome codification (i.e., heterogeneity). 17β-estradiol has been shown to have neuroprotective effects in pre-clinical models of SCI and TBI (Siriphorn et al., 2012; Day et al., 2017), and therefore targeted manipulation of estrogen signaling pathways may be a viable therapeutic target.

Potential for Astrocyte Heterogeneity Based on Disease-Associated Variables in Traumatic Injury

In contrast to plasticity, astrocyte heterogeneity in the context of traumatic injury may manifest as regionally variable responses to injuries at different locations across the neuraxis (i.e., brain vs. cervical spinal cord vs. thoracic spinal cord, etc.). Further characterization of astrocyte responses across models of CNS trauma utilizing fate-mapping and clonal analysis are important approaches to elucidate the extent of heterogeneity. For example, clonal analysis using the Star Track approach of proliferative astrocytes in a cortical stab injury model revealed a distinct progenitor cell origin as compared to non-proliferative astrocytes (Martín-López et al., 2013), consistent with our definition of heterogeneity. In the injured spinal cord, NSC-derived astrocytes more effectively constrain inflammation and the expansion of secondary damage (Sabelstrom et al., 2013), suggesting that distinct cellular origins may be a contributor to the diverse behavior of astrocytes observed following CNS trauma. Interestingly, a subset of reactive astrocytes in the post-trauma CNS displays certain characteristics of NSCs, including proliferative ability, multipotent lineage potential *in vitro*, and expression of several neurogenesis-associated genes (Buffo et al., 2008; Sirko et al., 2013). The potential for manipulation of this population has been demonstrated (Adams and Gallo, 2018; Magnusson et al., 2020; Zamboni et al., 2020) and represents a potentially attractive therapeutic approach to replace lost cell populations following trauma. Importantly, in models of CNS trauma there is also a potential contribution to observed diversity from astrocytes derived from other cells, including ependymal cells (Barnabé-Heider et al., 2010) and NG2 glia (Komitova et al., 2011; Hackett et al., 2016). For example, 2% of the astrocytes

after SCI were lineage-traced to FOXJ1 ependymal cells, which was dependent upon injury proximity to the central canal (Ren et al., 2017). Fate mapping approaches also revealed that NG2+ cells can give rise to a small percentage of densely packed GFAP+ astrocytes, both after a cortical stab injury (Komitova et al., 2011) and contusive SCI (Hackett et al., 2016). Distinct functionality and transcriptomic differences have not yet been established for these identified cell populations, representing important areas for further research.

As for ischemic stroke and demyelination, additional fate-mapping data in models of CNS trauma to establish the extent of heterogeneity is required. As CNS trauma can occur at various locations across the CNS, regional differences in the astrocyte populations may have a significant impact on injury outcome, as well as the success of functional recovery. For example, diverse astrocyte populations may be a key determinant of the extent of primary and secondary injury, considering the finding that elevated GFAP protein levels in cerebral spinal fluid after SCI are correlated with both baseline injury severity and poorer neurological outcomes (Skinnider et al., 2021a). Furthermore, differences in the local neuronal populations and the interplay between astrocytes and neurons in the post-injury environment may influence the success of neural circuit remodeling and thus functional recovery clinically. Understanding of the contribution of heterogeneity across brain regions, injury types (e.g., contusion, crush, etc.), and across stages of the disease process (e.g., acute, sub-acute, chronic) is therefore critical. This can be accomplished with performance of fate-mapping studies combined with high-resolution transcriptomic approaches and functional assays to interrogate the functional relevance of identified astrocyte diversity in diverse models of CNS trauma (e.g., SCI, TBI, concussion, etc.).

PERSPECTIVES ON ASTROCYTE DIVERSITY IN CNS DISEASE

Effective treatments are lacking for many neurological pathologies, including stroke, MS, and CNS trauma, resulting in significant morbidity and mortality. As astrocytes serve multiple vital roles in the post-insult CNS, they represent key players in disease pathogenesis, as well as promising targets for therapeutic manipulation. Characterization of the extent and functional relevance of astrocyte diversity in conditions of CNS insult is likely to aid in this endeavor: however, given the complexity of astrocyte reactivity (Lukovic et al., 2015; Anderson et al., 2016; Liddelow and Barres, 2016; Silver, 2016; Escartin et al., 2021) this will require a significant research effort. In this review, we summarize the current state of knowledge of astrocyte diversity in three representative and clinically relevant CNS pathologies; ischemic stroke, demyelination, and traumatic injury, highlighting the need for further characterization and functional interrogation of the contributions of heterogeneity vs. plasticity across temporal, topographical, age-specific, and sex-specific variables as a foundation to guide future development of therapeutic treatments to mitigate the impact of these conditions.

Areas of Future Research

Importantly, several knowledge gaps exist in the areas explored in this review. Most notably, there is a relative paucity of scRNA-seq studies characterizing astrocyte diversity in models of CNS trauma, as compared to demyelination and ischemic stroke. Moreover, as the vast majority of pre-clinical research has been performed in rodent models, increased use of non-human primate models or human tissue specimens is an important future avenue, as functional differences between species might pose a hurdle for clinical translation if not thoroughly addressed. Indeed, human astrocytes are known to be structurally more complex and diverse than rodent astrocytes (Oberheim et al., 2009; Zhang et al., 2016), as well as propagate Ca^{2+} waves approximately 4-fold faster than rodents in response to glutamate stimulation (Zhang et al., 2016), suggesting potential functional differences. Another critical area for future study is the influence of age on astrocyte diversity. This is particularly important not just for ischemic stroke, but for traumatic injury as well as older ages represent an increasing proportion of SCI and TBI, which is projected to increase in line with increasing age demographics in developed nations, and for progressive MS and the age-related decrease in remyelination efficiency. Considering that life expectancies of many of these clinical populations is increasing secondary to improved care and treatments, it will be essential to investigate astrocyte diversity after significant time periods after the onset of pathology (i.e., investigations in the 1–2 year post-injury chronic lesion sites within rodent models).

To meet these needs will require a combination of (1) increasing throughput of single-cell biology assays to achieve robust results across experimental conditions and biological replicates (Cao et al., 2020), (2) the application of multi-omic approaches to the context of CNS pathologies to avoid undue reliance on the functional interpretation of single-cell transcriptomes (Cao et al., 2018), and (3) bioinformatic methods development to aid biological investigators in differentiating cell type from cell state (Butler et al., 2018), to prioritize cell subsets most involved in the disease of interest (Skinnider et al., 2021b), and to avoid false positives that have the potential to drive research in unfruitful directions (Squair et al., 2021). Finally, these data will need to be met with stringent reporting standards to enable cross-disease investigations and to understand the conserved and differential responses of astrocytes across neurological disease.

In addition to conducting scRNA-seq-based characterization across multiple variables, it is increasingly important to further validate diverse astrocyte clusters in terms of origin and functionality, as most scRNA-seq analysis only provides a snapshot of gene expression within a specific context. Therefore, parallel assessments need to be performed to (1) validate mRNA and associated protein expression data, (2) establish the origin of identified clusters, and (3) assess functional differences. Furthermore, use of RNA-seq has the potential to identify cluster-specific, context-specific, or lineage-specific astrocyte markers across CNS states that will enable a more detailed interrogation of functionality and origin (e.g., with fate-mapping studies). New methods are also surfacing with the ability to predict ligand-targets links between interacting cells

(Browaeys et al., 2020) and using RABID-seq facilitates the simultaneous investigation of the transcriptome of specific cells interacting with your cell type of interest (Clark et al., 2021) in disease modeling which could further be a powerful tool. In addition, recent tools, such as transposase-accessible chromatin profiling (sc-ATAC-seq; Jia et al., 2018) have the potential to yield information about how chromatin accessibility dictates astrocyte diversity. New technology is also paving the way for sequencing both genomic DNA and mRNA in the same cell which could facilitate a direct comparison of genomic diversity and transcriptomic diversity (Dey et al., 2015). Multi-omics is allowing for increasingly complicated analysis with the potential to, for example, compare genomics, transcriptomics, proteomics, and metabolomics in the same cells. In addition, considering the importance of mitochondrial dynamics in astrocytes (Jackson and Robinson, 2018), future work looking at mitochondrial DNA as a potential source of genomic diversity will likely be important. As the definition of heterogeneity hangs on the demonstration of distinct functionality, detailed assessments across identified astrocyte clusters focusing on a wide range of function-related outcome measures is essential (e.g., Ca^{2+} signaling, neurotransmitter uptake/buffering, inter-cellular connectivity, neurotrophic factor production, etc.; for comprehensive list, see Escartin et al., 2021). Further characterizations of potential functional differences in how astrocytes respond to neuronal activity through G-protein-coupled receptor signaling *in vivo* will likely prove worthwhile (Nagai et al., 2021a). Further areas of research should focus on metabolic profiling to interrogate other potential functional differences between clusters of interest and the continued use of conditional knockout mice for loss-of-functions studies. The other requirement for demonstrating heterogeneity is cells with distinct origins which will require sound fate mapping follow-up studies. Recent sequencing methods are providing more insight specific to tracking clones of cells across time (Weinreb et al., 2020) which likely yields a powerful tool for demonstrating the potential district origin of cell clusters.

Astrocytes as Therapeutic Targets in CNS Disease

Astrocytes are promising therapeutic targets in several CNS diseases (Hamby and Sofroniew, 2010; Bradbury and Burnside, 2019; Valori et al., 2019). One potential approach is the use of viral vectors. Adeno-associated viruses (AAVs) demonstrate astrocyte-specific targeting in adult mice and non-human primates (Foust et al., 2009; Samaranch et al., 2012; Chan et al., 2017). Astrocyte-specific tropism may be increased through use of astrocyte-specific promoters (e.g., *Gfap*, *Aldh1L1*) and other viral capsid modifications (von Jonquieres et al., 2013; Meng et al., 2015; Vagner et al., 2016; Koh et al., 2017; Taschenberger et al., 2017). Lentiviral vectors pseudo-typed with glycoproteins from lymphocytic choriomeningitis virus (LCMV) or Moloney murine leukemia virus (MuLV) demonstrate astrocyte-specific targeting following intraparenchymal injection in rats (Cannon et al., 2011). Nanoparticles have also been used to target astrocytes (Zhou et al., 2018; Sharma et al., 2019), including the targeted regression of astrocyte-derived glioblastoma multiforme in mouse models (Jensen et al., 2013), inhibition of astrocyte-specific human immunodeficiency virus (HIV) replication *via* siRNA delivery (Gu et al., 2017), and astrocyte-specific delivery of mRNA *via* intraventricular administration (Tanaka et al., 2018). Alternatively, coupling of biologically active hydrophilic molecules (e.g., peptides, nucleic acids) to cell-permeable molecules can enable cell-specific delivery, as demonstrated in a mouse model of ALS (Martorana et al., 2012). Use of astrocyte-specific tropic factors, such as the phage AS1 homing peptide (Terashima et al., 2018) or herpes simplex virus type 1 (HSV-1) proteins (Valiante et al., 2015; Falanga et al., 2018) is a promising approach. Alternative approaches also include the use of Crispr-Cas9 gene-editing approach to specifically modify astrocytic genes (Huang and Nair, 2017; Kunze et al., 2018) and small molecules to modulate key signaling pathways (Zhang et al., 2015; Gao et al., 2017). As astrocytes are more resilient to the hostile post-injury environment in the CNS, direct cellular transplantation approaches may also be effective (Lepore et al., 2008; Izrael et al., 2018), although perhaps more limited in conditions, such as MS due to the disseminated nature of the lesions. Interestingly, dampening astrocyte reactivity (i.e., *via* STAT3 pathway inactivation) increases the number of OPC-derived Schwann cells in rodent demyelination lesions (Monteiro de Castro et al., 2015). In the context of the pronounced astrocyte loss seen in SCI, OPCs generate the majority of Schwann cells found in the contused lesion site (Assinck et al., 2017) and manipulating astrocytes to facilitate increased OPCs to differentiate into Schwann cells could be a promising alternative to invasive Schwann cell transplantation treatments which has been previously shown to promote repair and recovery in rat SCI models (Sparling et al., 2015; Assinck et al., 2020). Furthermore, astrocytes may represent an attractive source of cells for direct reprogramming to replace lost neurons or oligodendrocytes (Berninger et al., 2007; Heinrich et al., 2010). Forced expression of the transcription factors *Ngn2*, *Mash1*, or *Pax6* converts astrocytes to glutaminergic neuron-like cells in rodent models (Berninger et al., 2007), while overexpression of *Dlx2* or *Ascl1* converts them to GABAergic neuron-like cells (Heinrich et al., 2010; Liu et al., 2015). Further research is required to characterize the stability and functionality of these trans-differentiated "neurons," but this is an intriguing approach. As transcriptional approaches and functional assessment further reveal the extent and relevance of astrocyte diversity in conditions of CNS disease there will undoubtedly be an expanded interest in astrocytes as therapeutic targets. Expansion of that knowledge is therefore critical to foster this development, specifically the identification of functionally relevant aspects of astrocyte diversity that are amendable to extrinsic manipulation (i.e., plasticity). This necessitates further research including expanded transcriptional studies, functional assays, and fate-mapping analyses to firmly establish and characterize this diversity.

Astrocyte Diversity: An Interplay of Plasticity and Heterogeneity

Using the framework proposed above, it remains unclear as to what proportion of the characterized astrocyte diversity in models of ischemic stroke, CNS demyelination, and traumatic

injury represent heterogeneity vs. plasticity. We propose that without unequivocal demonstration of functional differences and clear fate-mapping data on the origin of the identified astrocyte clusters, most of the identified diversity to date likely proportionally represents plasticity in response to a dynamically changing injury environment. This is a point that has been argued for diversity in the oligodendrocyte lineage (Foerster et al., 2019) and most likely holds true for astrocytes. It is likely that combinations of both heterogeneity and plasticity exist across a non-exhaustive number of variables including spatial, temporal, age, and sex, reflecting the superimposition of plasticity in response to fluctuating and complex environments upon a background of developmentally pre-determined variance. This has significant therapeutic implications, as our definition of heterogeneity defines a population of astrocytes with a unique origin and function that may be more amendable to intrinsic manipulation approaches (**Figure 4A**). In contrast, plasticity implies a degree of malleability and thus would be amendable to extrinsic manipulation (**Figure 4B**). We propose here a simple model of intrinsic (e.g., viral targeting, DREADDs, optogenetics, transgenic manipulation, etc.) and extrinsic (e.g., manipulation of immune response, fibrotic scarring, ECM and stromal-derived matrix, exogenously administered cytokines, etc.) approaches for astrocyte-specific targeting, with the important caveat that extrinsic factors profoundly influence intrinsic mechanisms and no cell exists in complete isolation from their external environment, thus this distinction is not as clear-cut as presented here. Nonetheless, distinguishing heterogeneity from plasticity will yield a better understanding of the mechanisms of reactive astrogliosis and aspects of this response that can be targeted therapeutically. Given the significant interest in astrocytes as potential targets for therapeutic manipulation

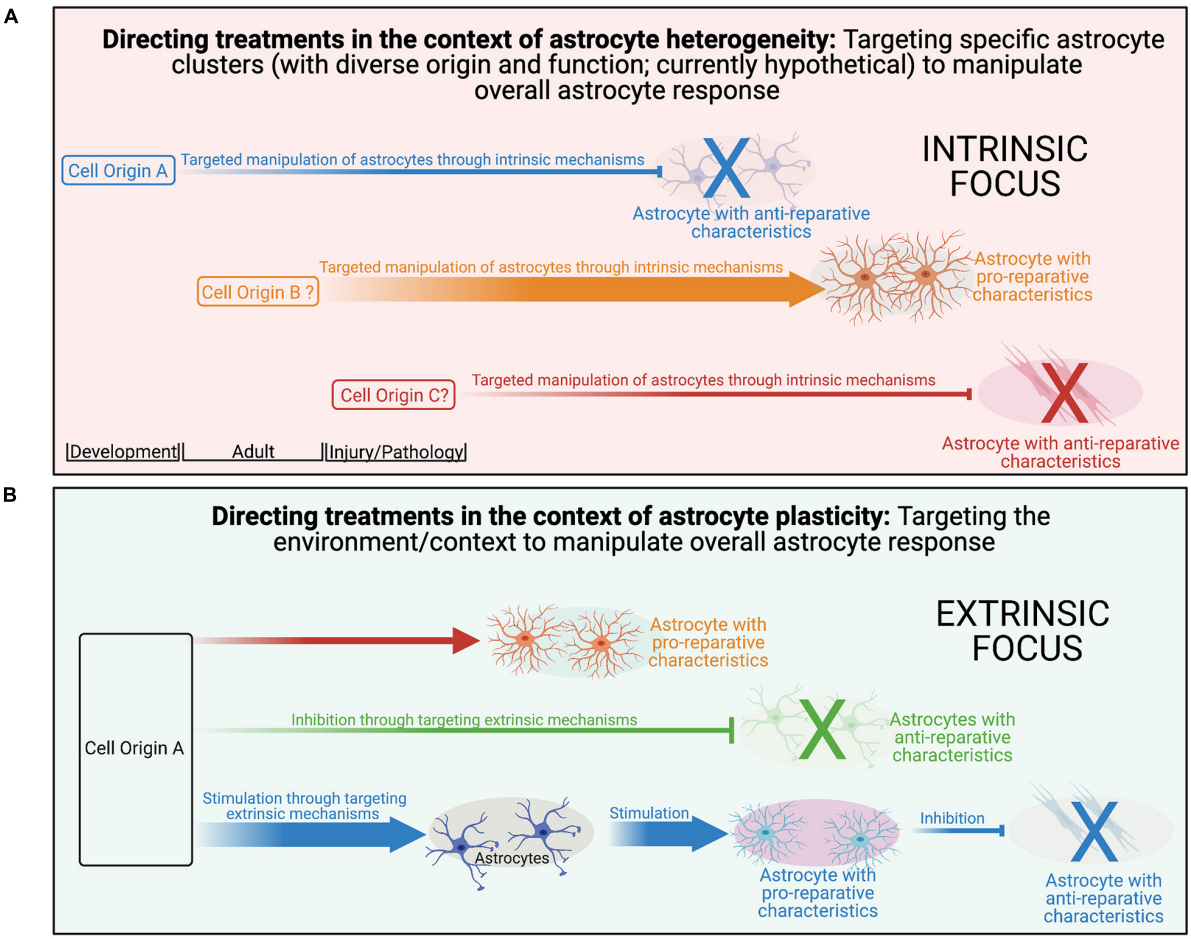

FIGURE 4 | Distinguishing heterogeneity from plasticity is an important first step to ultimately direct treatments geared toward manipulating reactive astrogliosis at the right time and place through intrinsic mechanisms, extrinsic mechanisms, or both. **(A)** To the best of our knowledge, in the context of reactive astrogliosis and pathology, there are no perfect examples of astrocyte heterogeneity as defined in this review. It is likely, however, that further study will reveal evidence, as has been seen in other glial lineages. Identification of populations with distinct origins and unique functions will present opportunity for the therapeutic targeting of intrinsic pathways to manipulate the astrocyte response in the context of pathology. **(B)** Currently, with the multitude of data specific to astrocytes and their plasticity to local environmental changes, most evidence of astrocyte diversity in the context of pathology is suggested to fit into the plasticity category. Further research is needed to understand methods to manipulation the extinctic astrocyte environment to direct astrocytes in ways that will be beneficial in the context of pathology.

(Zhang and Barres, 2010; Schitine et al., 2015; Matias et al., 2019) and the recent increase in tools to assess cell diversity, astrocyte diversity in CNS pathologies remains an exciting and promising field of future research.

CONCLUSION

Astrocytes display substantial diversity in ischemic stroke, CNS demyelination, and CNS traumatic injury, including temporal, topographical, sex-specific, and age-specific differences. Single-cell transcriptional approaches have significantly expanded our knowledge of the extent of this diversity, in the process altering our view of reactive astrocytes from a binary categorization to that of a spectrum of nuanced activation states involving hundreds of differentially expressed genes. Despite this, there are notable gaps in our knowledge, most notably whether this identified diversity represents heterogeneity or plasticity. As discussed throughout the review, the combination of transcriptomics approaches to further characterize the extent of diversity present with parallel functional assessments and expanded fate-mapping studies are critical areas of future study to fully develop our understanding of this diversity and to guide development of therapeutic approaches aimed at mitigating the morbidity and mortality of those afflicted with these conditions.

AUTHOR CONTRIBUTIONS

AJM, RJMF, WT, and PA contributed to the conception of review. AJM and PA contributed to design, original drafting, and figure preparation of the review. AJM, JWS, RJMF, WT, and PA contributed to writing, editing, and approval of final manuscript. All authors contributed to the article and approved the submitted version.

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Astrocyte Heterogeneity in Multiple Sclerosis: Current Understanding and Technical Challenges

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The emergence of single cell technologies provides the opportunity to characterize complex immune/central nervous system cell assemblies in multiple sclerosis (MS) and to study their cell population structures, network activation and dynamics at unprecedented depths. In this review, we summarize the current knowledge of astrocyte subpopulations in MS tissue and discuss the challenges associated with resolving astrocyte heterogeneity with single-nucleus RNA-sequencing (snRNA-seq). We further discuss multiplexed imaging techniques as tools for defining population clusters within a spatial context. Finally, we will provide an outlook on how these technologies may aid in answering unresolved questions in MS, such as the glial phenotypes that drive MS progression and/or neuropathological differences between different clinical MS subtypes.

Keywords: astrocytes, multiple sclerosis, single nucleus sequencing, multiplexed imaging, experimental autoimmune encephalomyelitis

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INTRODUCTION

Due to the resounding success of current MS medications in treating relapsing-remitting MS (RRMS), research interest is increasingly focused on disease progression and neurorepair where current treatments are ineffective. In contrast to RRMS, progressive disease is thought be driven by central nervous system (CNS)-intrinsic processes, where infiltration with peripheral immune cells plays only a minor role. Thus, the attention in MS research is arguably shifting from the peripheral immune system to CNS cells. The CNS-intrinsic processes associated with progression include chronic, diffuse activation of glia cells at the rim of chronic active lesions and in the normal appearing white matter (NAWM) (Chen et al., 2014; Absinta et al., 2019; Sucksdorff et al., 2020), cortical demyelination and neuro-axonal degeneration (Dutta et al., 2006). Identifying the glial subpopulations that contribute to MS progression is likely to yield new targets for therapeutic intervention in progressive MS (Ponath et al., 2018b; Wilbanks et al., 2019; Guerrero and Sicotte, 2020).

In this review, we are focusing on astrocytes. Astrocytes are substantially more numerous than microglia cells, accounting for 20–40% of the total glial population, depending on the brain region (von Bartheld et al., 2016). In a homeostatic state, astrocytes play critical roles in brain function, including synapse formation and elimination (Lee et al., 2021), establishing and maintaining network circuitry, control of neurotransmitters release and uptake (Anderson and Swanson, 2000), modulation of blood-brain barrier (BBB) function (Horng et al., 2017) and blood flow, as well as

maintenance of ion and water homeostasis among others (Sofroniew and Vinters, 2009; Ponath et al., 2018b).

CNS pathologies such as trauma, infection, neurodegeneration, and inflammatory demyelination lead to prominent astrocyte responses with morphological, molecular, and functional remodeling. Reactive astrocytes can promote tissue survival by forming a functional barrier around damaged tissue and contribute to tissue repair (Faulkner et al., 2004; Tyzack et al., 2014; Anderson et al., 2016). However, reactive astrocytes may also become dysfunctional and adopt disease-promoting phenotypes. In MS, this includes recruitment and activation of peripheral immune cells and resident microglia (Ponath et al., 2018a), and potentially direct neurotoxic effects on oligodendrocytes and neurons (Liddelow et al., 2017). In addition, reactive astrocytes have reduced capabilities for glutamate uptake (Pitt et al., 2000; Fang et al., 2012) and other homeostatic functions (Schirmer et al., 2019), which may result in indirect neurotoxicity.

Here, we review the progress made in identifying astrocyte populations in MS and its mouse model, experimental autoimmune encephalomyelitis (EAE). We will discuss the limitations of current snRNA-seq methods in identifying astrocyte subpopulations and the potential of highly multiplexed imaging in further elucidating astrocyte heterogeneity by adding spatial information.

HETEROGENEITY OF RESTING ASTROCYTES

In contrast to oligodendrocytes and microglia, astrocytes are morphologically heterogenous at a resting state. At least nine morphologically distinct astrocyte subpopulations have been identified, of which four subtypes are present within the human neocortex (protoplasmic, radial cells, marginal glia, and perivascular glia) (Matyash and Kettenmann, 2010). Additionally, astrocytes are highly diverse in their functional properties, including calcium dynamics, gap junction coupling, expression of transmitter receptors, membrane currents, and glutamate transporters (Matyash and Kettenmann, 2010).

One of the first efforts to identify astrocyte subpopulations was based on marker expression. Genetically green fluorescent protein (GFP)-labeled astrocytes were isolated and screened for 81 cell surface antigens with fluorescence-activated cell sorting (FACS) (John Lin et al., 2017). This identified five distinct astrocyte subpopulations present across cortex, brainstem, and olfactory bulb, which were subsequently demonstrated to emerge at different developmental stages and to have distinct gene-enrichment and functional properties.

Since their selection as the "Method of the Year 2013" by Nature Methods (2014), single-nucleus and single-cell RNA sequencing (scRNA-seq) have become routine technologies to identify cellular subpopulations based on transcriptome-wide gene expression at single-cell resolution. A study that employed this approach identified astrocyte subtypes in mouse cortex using scRNA-seq and single-cell spatial transcriptomics. By applying multiplexed single-molecule fluorescence *in situ* hybridization (smFISH), the study found that cortical astrocytes segregated into a superficial, mid and deep layer pattern, which did not match the neuronal layering (Bayraktar et al., 2020). However, the spatial clusters did not align with astrocyte clustering based on single-cell transcriptomics, indicating that single cell genomics and smFISH produce different patterns of astrocyte molecular diversity, potentially due to difference in parametric depth.

A separate scRNA-seq study that used Smart-seq2 followed by unsupervised clustering distinguished five distinct astrocyte subtypes in adult mouse cortex and hippocampus (Batiuk et al., 2020). The authors noted that the population structure of astrocytes differed from that of neurons, by lacking distinct cellular hierarchies, suggesting multiple axes of heterogeneity. The transcriptomic differences suggest astrocyte subtype specialization across major astrocyte functions, including synaptogenesis, phagocytosis, synapse function/plasticity, neurotransmission, and others. In addition, the subpopulations were shown to be differentially distributed across the cortex and hippocampus and exhibited distinct morphologies and differential Ca^{2+} signaling (Batiuk et al., 2020).

Together, the single-cell studies provide evidence for transcriptomic heterogeneity of astrocytes, albeit with less distinct cellular hierarchies than neuronal subtypes. The transcriptomic clusters correspond to distinct morphological features and spatial distribution and have implications for different physiological functions. These studies have focused on cortical astrocytes and less is known about astrocyte diversity in white matter (Köhler et al., 2021).

HETEROGENEITY OF REACTIVE ASTROCYTES IN EAE AND MS

A hallmark of virtually all CNS pathologies is prominent astrocyte responses. The contribution of reactive astrocytes to a given CNS disease is complex and likely driven by multiple concurrent astrocyte phenotypes that vary with disease, disease stage, brain region, age and genetic predisposition (Ponath et al., 2018a; Escartin et al., 2021). Therefore, defining the population structures of reactive astrocytes in different CNS conditions will provide critical insight into their pathogenesis.

In EAE, earlier studies determined astrocytic gene expression in different neuroanatomic regions of mice using a Ribo-tagging approach in transgenic animals, which allows isolation of mRNA from astrocytes only (Itoh et al., 2018). This demonstrated regional differences in astrocytes transcriptomes, including differential expression of complement and cholesterol synthesis pathway genes in astrocytes isolated from spinal cord, optic nerve, cerebellum, hippocampus, and cerebral cortex, consistent with heterogeneity of astrocyte reactivity across different CNS regions.

In an early transcriptome study, astrocyte responses were stratified into neurotoxic and neuroprotective phenotypes termed A1 and A2 (Liddelow et al., 2017), in analogy with the now abandoned concept of M1/M2 (macrophages) and Th1/Th2 (lymphocytes) polarization (Ransohoff, 2016). The A1 phenotype exhibited functional deficits affecting phagocytosis

and synapse formation, and was toxic to neurons and oligodendrocytes *in vitro*. A1 phenotypes were found to be present in neurodegenerative diseases and MS, based on the expression of complement component 3, whereas A2 astrocytes, which are characterized by upregulation of several neurotrophic factors, were prevalent in ischemia. This binary classification was initially received with considerable enthusiasm; however, in subsequent studies, only subsets or a mix of A1 and A2 signature genes were found to be upregulated in human diseases and mouse models of acute and chronic CNS injury (Al-Dalahmah et al., 2020; Das et al., 2020; Zhou et al., 2020). A recent consensus statement recommended to move beyond the A1–A2 labels, as they do not capture the functional diversity of reactive astrocytes, and to use multidimensional data to establish distinctive astrocyte phenotypes (Escartin et al., 2021).

A recent study by Quintana et al. that used scRNA-seq and epigenetic analyses in combination with *in vivo* CRISPR-Cas9-based genome editing, identified seven astrocyte subpopulations in EAE (Wheeler et al., 2020). The dominant cluster was characterized by activation of pro-inflammatory and neurotoxic pathways such as the unfolded protein response, activation of NF-κB and inducible nitric oxide synthase pathways. This cluster was driven by downregulation of genes targeted by transcription factor *NRF2*, which limit oxidative stress and inflammation (Wheeler et al., 2020). Moreover, this population was stabilized by epigenetic modifications driven by MAFG and MAT2α signaling, further supporting the notion that epigenetic changes to the CNS contribute to MS pathogenesis (Huynh et al., 2014). In MS lesion tissue, astrocytes were demonstrated to have increased expression of MAFG and decreased expression of NRF2. Recently, the same group identified an anti-inflammatory subset of astrocytes in EAE that was characterized by co-expression of the lysosomal protein, LAMP1, and the death receptor ligand, TRAIL (Sanmarco et al., 2021). This astrocyte population limits inflammation by inducing T cell apoptosis and is driven by IFNγ, produced by natural killer cells within the meninges. A similar population of astrocytes characterized by IFNγ and TRAIL signaling was identified in human brain and this population was reduced by over 80% in MS tissue (Sanmarco et al., 2021).

CHALLENGES OF DETERMINING ASTROCYTE HETEROGENEITY WITH SNRNA-SEQ

Astrocytes can be isolated intact from fresh murine CNS tissue, although enzymatic dissociation has been shown to induce early activation genes (Lacar et al., 2016). In adult human CNS, the method of choice for single-cell transcriptomics is snRNA-seq, because the high cellular interconnectivity makes it difficult to isolate astrocytes and neurons in intact form. snRNA-seq has the additional advantages that it avoids perturbation of gene expression by enzymatic dissociation during whole cell isolation, and that it can be applied to archival frozen material from biobanks (Ding et al., 2020).

Acceptance of snRNA-seq was initially limited, because of differences in RNA quantity and quality between nucleus and cytosol. It was subsequently established that nuclei can be confidently matched to their representing cells, that transcriptomes from single nuclei and whole cells correlate highly, albeit with an abundance of nascent transcripts in nuclei (Lake et al., 2017; Bakken et al., 2018). A recent comparison of snRNA-seq and scRNA-seq data from human microglia demonstrated that a high number of genes implicated in microglia activation in Alzheimer's disease (AD) was substantially reduced in nuclei (Thrupp et al., 2020). This highlights that single-nucleus data are highly sparse and noisy, particularly in glial cells, requiring improved methodologies for recovery of biological signals (see below).

An additional problem of snRNA-seq studies is that the ratios between different CNS cell types differ to those determined by neuro-histological cell quantification (von Bartheld et al., 2016). The neuroanatomic literature suggests that glia cells are approximately 1.5–2 times more numerous than neurons in cortical gray matter, and 3–4 times more numerous in combined cortex and white matter. Oligodendrocytes are the most frequent glial cell type (45–75%), followed by astrocytes (19–40%), and microglia (10%), depending on the brain region (von Bartheld et al., 2016).

In snRNA-seq data, human brain astrocytes and microglia are consistently under-represented. In several landmark studies of human adult cortex, astrocytes constituted between 1.9 and 7.2%, and microglia between <1 and 2.1% of total nuclei (Lake et al., 2018; Zhu et al., 2018; Hodge et al., 2019). In the two snRNA-seq studies of MS white matter lesion tissue, the predominant nuclear populations were neurons, oligodendrocytes and oligodendrocyte precursor cells constituting 80% of total CNS nuclei, whereas astrocyte nuclei accounted for 7 and 11% and microglia nuclei 2.4 and 2.9%, respectively (Jäkel et al., 2019; Schirmer et al., 2019). A possible reason for this underrepresentation is that they are preferentially lost during the preparation steps presumably due to different mechanical properties of astrocyte/microglia nuclei. Moreover, neurons express up to 10 times more RNA than glial cells, which leads to overrepresentation of neuronal RNA in the final library composition (Zeisel et al., 2015). Collectively, this leads to low representation of astrocyte/microglia nuclei, reduced library complexity and consequently low resolution of population structure heterogeneity for glial cells.

In two recent studies, Quintana et al. performed scRNA-seq of astrocytes derived from fresh, rapid autopsy materials of MS patients using 10x Genomics v2.0 chemistry (Wheeler et al., 2020). This data set was integrated with previously published snRNA-seq results of astrocytes in MS and healthy brains. The authors used this approach to confirm the presence of a specific astrocyte population in MS, which was previously identified in EAE and characterized by increased MAFG activation, GM-CSF signaling and pro-inflammatory pathway activity. The authors demonstrated that this population was present in 12 out of 20 MS patients and was strongly expanded in MS compared to controls. The same approach was used to validate that an anti-inflammatory $LAMP1^{+}TRAIL^{+}$ astrocyte subpopulation that was reduced in EAE compared to naïve mice, was also downregulated in MS (Sanmarco et al., 2021).

It is notable that the 48 human samples used in these studies yielded only a total of 9,700 astrocyte nuclei, averaging 200 astrocyte nuclei per sample. This further illustrates that capture of astrocytes/astrocyte nuclei from human brain is sparse and that the analysis is limited by the low number of astrocyte representation.

There is currently a limited understanding of how isolation and purification protocols may change the nuclear yield of various CNS cellular constituents (Box et al., 2020; Denisenko et al., 2020; Ding et al., 2020). New sample preparation protocols have to be designed to address the structural and metabolic vulnerabilities of different nuclei from different brain regions. In addition, standard FACS technology relies on increased pressures (10–70 psi) for hydrodynamic focusing, and application of high voltage charges to the sorted sample path, which inflicts substantial damage on nuclei and may introduce sampling bias by depleting sensitive nuclear populations. Microfluidic sorters have become a mature technology that operates on atmospheric pressure and uses mechanical valves (Utharala et al., 2018; Berlanda et al., 2021). The reduced for electromechanical manipulation greatly aids the preservation of nuclei and the selective enrichment of sensitive and rare nuclei.

Moreover, new genomic protocols and high-throughput strategies are constantly emerging. Since their inception, 3-prime-based scRNA-seq protocols (Ramsköld et al., 2012; Macosko et al., 2015) have improved their sensitivity from initially 5–10 to 20% of RNA molecules captured per cell. Similarly, Smart-seq protocols have doubled their sensitivity from 30–40 to 70% (Smart-seq3), allowing for collection of the majority of transcripts, including isoforms, which ultimately improves the ability to identify biologically meaningful cell clusters (Hagemann-Jensen et al., 2020).

Finally, multi-omics approaches that combine single-cell transcriptomics with epigenetic mapping, proteomics and lineage tracing (Stoeckius et al., 2018; Gaublomme et al., 2019), provide ever-increasing molecular details of cellular states (Ludwig et al., 2019). These details can be further integrated with spatial transcriptomics which provides additional information such as phenotype localization within a given microenvironment and cell-to-cell interactions (Giesen et al., 2014; Liu et al., 2020). Applied to MS, these methodological advances will eventually provide a more complete picture of the cellular constituents that drive MS pathology, including rare and underrepresented cell types.

RESOLUTION OF ASTROCYTE HETEROGENEITY WITH HIGHLY MULTIPLEXED IMAGING

An additional important aspect of defining cell populations is to determine their spatial organization within a tissue environment such as MS lesions, including their spatial interactions and the environmental cues that drive their specific expression profiles. Moreover, the spatial resolution of phenotypes that have been determined by single cell genomics, will confirm and/or further improve the phenotypic separation. As with single cell genomics, substantial progress has been made in recent years with multiplexed spatial profiling of RNA and proteins in tissue.

Spatial RNA Profiling

Technologies to quantify single-cell RNA levels in spatial context have been rapidly evolving. SmFISH and sequential FISH (seqFISH) approaches use fluorescence-labeled small oligonucleotides to probe single mRNA transcripts. SeqFISH uses multiple rounds of hybridization and has been shown to be scalable to the genome level *in vitro* (Eng et al., 2017). SmFISH has been used to define astrocyte subpopulations in different CNS regions (Batiuk et al., 2020; Bayraktar et al., 2020) and to map snRNA-seq-derived phenotypes onto MS lesion tissue (Jäkel et al., 2019; Schirmer et al., 2019). A further development that combines super-resolution microscopy with FISH, termed seqFISH+, achieves, in theory, multiplexing of >20,000 genes in single cells with high accuracy and sub-diffraction-limit resolution (Eng et al., 2019).

Additionally, a method termed deterministic barcoding in tissue for spatial omics sequencing (DBiT-seq) delivers DNA barcodes to tissue at a specific location via a microfluidics platform and sequences the spatially barcoded mRNA and proteins (Liu et al., 2020). Under ideal conditions, DBiT-seq is capable of spatially profiling thousands of mRNAs with next generation sequencing that can be co-mapped with proteins, albeit not at single-cell resolution. These technologies target the whole transcriptome but are currently not as robust as snRNA-seq with lower gene coverage and reading depth.

Spatial Protein Profiling With Highly Multiplexed Imaging

Highly multiplexed imaging has been driven by imaging mass cytometry (IMC), a technology that, like CyTOF, uses antibodies conjugated to lanthanide isotopes (Giesen et al., 2014; Baharlou et al., 2019). Tissue sections are simultaneously labeled with up to one hundred antibodies, laser-ablated at high resolution and followed by time-of-flight mass spectrometry. IMC has several draw-backs that include the need for comprehensive validation and optimization of antibodies, high cost of antibody conjugation and the ablation/mass spectrometry procedure, lack of an amplification step, which reduces sensitivity for low-abundance proteins, and a standard magnification of only 16×, which may provide insufficient resolution. A low tech approach to highly multiplexed imaging is serial immunofluorescence staining of tissue sections with repetitive cycles of immunolabeling, scanning and antibody removal, termed iterative indirect immunofluorescent imaging (4i) (Gut et al., 2018). Similar to classical immunofluorescence labeling, this approach uses off-the-shelf antibodies, and allows for amplifications steps with secondary antibodies, high magnification imaging and acquisition of large tissue areas with a scanning microscope. The number of cycles is limited by decreasing tissue integrity. In our experience, the

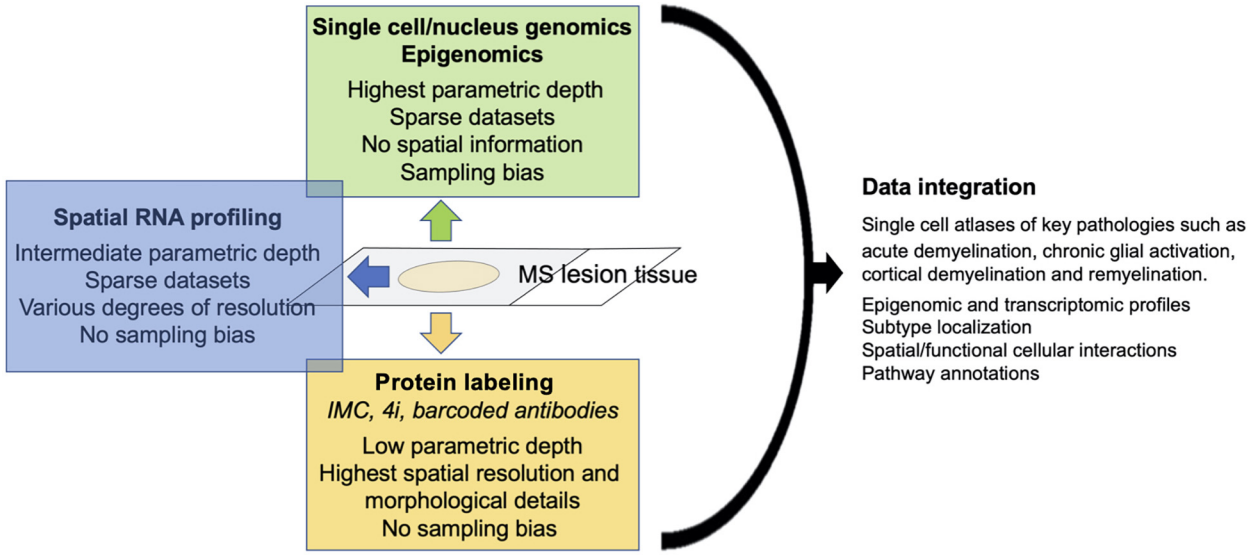

FIGURE 1 | Schematic of strengths/weaknesses of single cell technologies and data output.

multiplexed fluorescent signals are quantitatively reproducible over at least 20 cycles without detectable loss of antigen, increase in background or tissue distortion. Per round, typically 2–4 antibodies can be applied, which allows for staining with a total of 40+ markers. Currently, a microfluidics-assisted version of 4i is being developed that automates most of this method and presumably better preserves tissue integrity. Other multiplexed protein imaging methods, such as nanostring (Decalf et al., 2019; Merritt et al., 2020) and CODEX (Schürch et al., 2020), rely on barcoding of antibodies with unique oligonucleotide sequences. These technologies allow for a one-time antibody application, followed by multiple rounds of binding of unique oligonucleotide reporters, each with a spectrally distinct dye, to assay the corresponding antibody barcodes.

Computational Analysis

The different multiplexed imaging methods all require computational pipelines that include image registration (if multiple scans have been acquired), segmentation of cell types of interest with workflows such as ilastik pixel classification (Berg et al., 2019) and CellProfiler (Carpenter et al., 2006), and extraction of single cell information for cellular phenotype clustering that are visualized with dimensionality reduction methods such as principal component analysis (PCA) and *t*-distributed stochastic neighbor embedding (tSNE) (Amir et al., 2013), as with scRNA-seq or snRNA-seq data. The additional spatial data are analyzed with computational pipelines [e.g., histoCAT (Schapiro et al., 2017)] to localize cellular subpopulations within the tissue environment, to identify spatial interactions and to enrich phenotype transition trajectories for spatial context (Moon et al., 2019). Some of these tools are particularly relevant for tissues with complex cell morphologies such as CNS, where spatial analysis can focus separately on the cell body and peripheral processes. As highly multiplexed imaging methods evolve and are more broadly applied, new computational approaches for studying spatial patterns of cellular and molecular organization are emerging. This includes spatial variance component analysis (SVCA) (Arnol et al., 2019), which uses the marker profiles and spatial coordinates of each cell, to quantify the sources of variation for marker expression, such as cell-to-cell interactions, and intrinsic and environmental effects.

Multiplexed Analysis of Glial Cells in MS Lesions

We have applied IMC to acute MS lesion tissue to investigate the landscape of myeloid and astrocyte phenotypes in acute and chronic active MS lesions (Park et al., 2019). In this study, we identified five astrocyte subtypes and six myeloid cell subpopulations based on expression patterns of 13 glial activation markers. We found that the different glial subpopulations localized to different lesional zones and exhibited subtype-specific spatial interactions. Moreover, we were able to elucidate astrocyte and microglia phenotypic transitions and quantify the effects of cell-intrinsic factors vs cell-to-cell interactions on marker expression in individual cells (Park et al., 2019). In a separate study of acute MS lesions, we were able to demonstrate the activation patterns of lymphocytes and microglia determined in previous MS studies. In particular, we were able to discriminate between the different source of demyelinating macrophages (microglia vs blood-derived macrophages) and to segregate different B and T cell phenotypes (Ramaglia et al., 2019). These studies demonstrate that highly multiplexed imaging, in conjunction with a computational analysis pipeline, provides a wealth of insight into the functional states and spatial organization of glial cells in MS lesions that are not accessible with standard histology.

SUMMARY OF SINGLE CELL APPROACHES TO DETERMINE ASTROCYTE HETEROGENEITY

Single cell genomics, spatial mRNA profiling and highly multiplexed (protein) imaging offer different advantages and disadvantages. While both single cell genomics and spatial mRNA profiling target the whole transcriptome; RNA-seq is superior in isolated cells/nuclei compared to tissue with regard to gene coverage and sequencing depth. In contrast, highly multiplexed protein imaging has a parametric depth of only dozens to perhaps hundreds of markers, but allows for precise outlining of cells with complex, irregular morphologies such as astrocytes, which will improve the spatial analysis of individual cells. Although it is implicitly assumed that mRNA expression correlates with protein expression, this correlation varies substantially with different gene classes (Koussounadis et al., 2015), making it desirable to confirm RNA expression at a protein level, at least for key markers (**Figure 1**). Finally, single-cell gene expression matrix typically contains excessive zero entries (Yuan et al., 2017), resulting in sparse data sets, particularly in underrepresented cell types such as astrocytes. We observed that several key markers of astrocytes and microglia activation that were readily detectable with multiplexed imaging, were absent in single-cell transcriptomic datasets from MS lesions. Of note, spatial RNA transcriptomics and multiplexed protein profiling are not believed to lead to underrepresentation of specific cell types such as astrocytes and microglia. The technology is moving rapidly toward development of spatial multi-omics that incorporate mRNA profiling with protein profiling and epigenomics, as it is now possible with DBiT-seq (Liu et al., 2020).

CONCLUSION

With optimized protocols to capture nuclei from astrocytes and other under-represented CNS cell types such as microglia, improvements in single cell genomics and multi-omics integration will allow generation of complete single-cell atlases of healthy CNS and neurological diseases. These atlases will include epigenomic and transcriptomic profiles, subpopulation localization, spatial and functional (receptor-ligand) cellular interactions and pathway annotations. Applied to carefully characterized MS tissue, this will identify the constituent subpopulations and cellular interactomes that are specific to key pathological processes such as acute demyelination, chronic glial activation in progressive MS, and remyelination. This comprehensive approach will identify therapeutic novel targets to ameliorate disease progression and to promote neurorepair.

AUTHOR CONTRIBUTIONS

CL, MS, MM, SB, ST, and DP wrote the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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She Doesn't Even Go Here: The Role of Inflammatory Astrocytes in CNS Disorders

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Astrocyte heterogeneity is a rapidly evolving field driven by innovative techniques. Inflammatory astrocytes, one of the first described subtypes of reactive astrocytes, are present in a variety of neurodegenerative diseases and may play a role in their pathogenesis. Moreover, genetic and therapeutic targeting of these astrocytes ameliorates disease in several models, providing support for advancing the development of astrocyte-specific disease modifying therapies. This review aims to explore the methods and challenges of identifying inflammatory astrocytes, the role these astrocytes play in neurological disorders, and future directions in the field of astrocyte heterogeneity.

Keywords: astrocytes, reactive astrogliosis, heterogeneity, neuroinflammation, immune mediators, CNS disorders

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INTRODUCTION

Astrocytes are the most abundant glial (non-neuronal) cell type of the central nervous system (CNS). Among many functions, they play a critical role in maintaining blood-brain barrier function (Engelhardt, 2003), supporting neurons and other glia (Jessen, 2004), and reacting to changes in both the local (Henrik Heiland et al., 2019; Shigetomi et al., 2019) and external environment (Rothhammer et al., 2016; Wheeler et al., 2020b). Beyond these homeostatic functions, astrocytes can respond to several stimuli and subsequently display profound genetic, morphological, and functional changes in a process termed reactive astrogliosis (Sofroniew, 2009; Escartin et al., 2021; **Figure 1**). Reactive astrogliosis can be triggered by injury (Cotrina et al., 2015; Okada et al., 2018), inflammation (Hansson et al., 2016), or stress (Chen et al., 2020; Kogel et al., 2021), and can result in a feed-forward process, where an initial stimulus induces a reactive astrocyte response, which triggers the release of intracellular and soluble factors that further drive this response. Reactive astrogliosis, reactive astrocyte response, and (astro)gliosis are terms often used interchangeably in the field of astrocyte biology. While their exact definition might differ between studies, potentially due to the heterogeneity in reactive astrocyte phenotypes and functions (see below), they generally refer to astrocyte responses to stimuli beyond physiological functions, as described above.

While the concept of (reactive) astrogliosis has been known since it was first observed early in the history of neuroscience (Rindfleisch, 1863; Müller, 1904), the heterogeneity of astrocyte phenotypes, function, and reactivity are becoming increasingly appreciated (Escartin et al., 2021). Due to this heterogeneity, the role of reactive astrogliosis in disease processes, such as neuroinflammation, is often multifaceted and remains an active area of research in the field. For example, reactive astrocytes can contribute to inflammation by promoting immune responses, but can also suppress these responses (Cordiglieri and Farina, 2010).

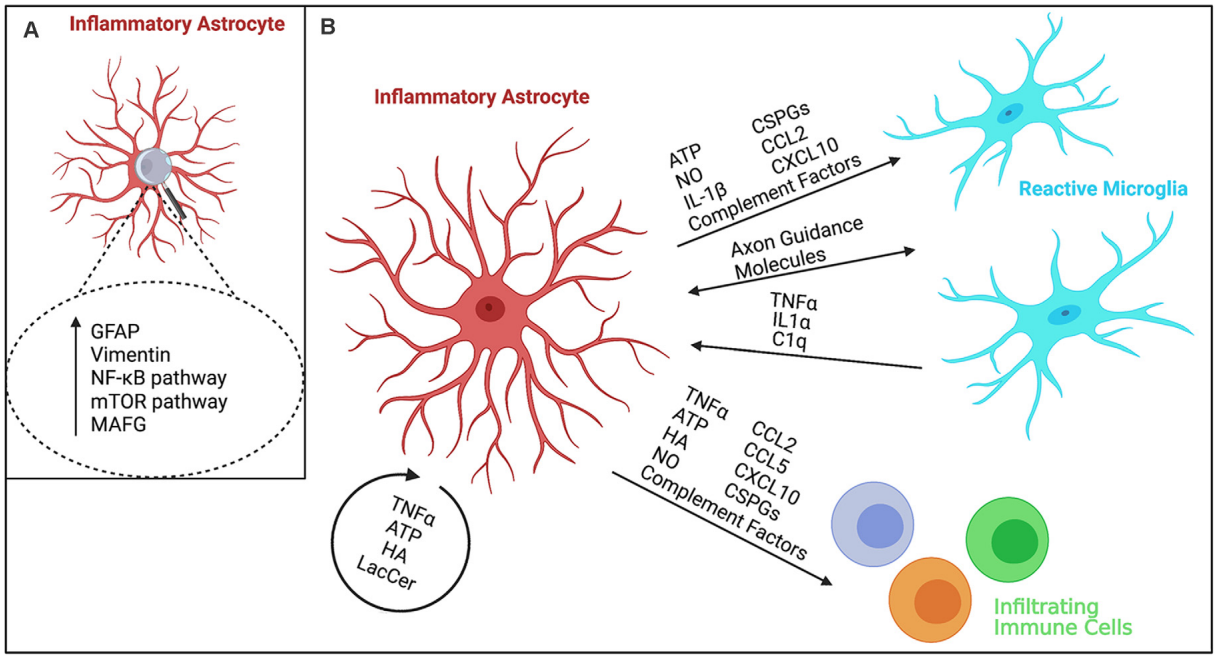

FIGURE 1 | Schematic representation of inflammatory astrocytes and their interactions with other cells during neuroinflammation. **(A)** Markers upregulated in inflammatory astrocytes and pathways regulating their activation. While GFAP and Vimentin are commonly upregulated in reactive astrocytes (Sofroniew, 2009; Escartin et al., 2021), inflammatory astrocytes show increased expression of the NF-κB pathway (Zamanian et al., 2012; Lian et al., 2015) and activation of MAFG and mTOR signaling (Sofroniew, 2009; Zamanian et al., 2012; Li et al., 2015; Wheeler et al., 2020a). **(B)** Schematic overview of various known cytokine, chemokine, ionic, and protein interactions of inflammatory astrocytes with neighboring cells. Inflammatory astrocytes can affect microglia and infiltrating immune cells by secreting immune factors such as cytokines and chemokines (Sofroniew, 2009; Zamanian et al., 2012; Liddelow et al., 2017; Clark et al., 2019), complement proteins (Zamanian et al., 2012; Lian et al., 2016; Liddelow et al., 2017), as well as extracellular matrix molecules such as hyaluronan (HA; Kuipers et al., 2016; Nagy et al., 2019) and chondroitin sulfate proteoglycans (CSPGs; Keough et al., 2016; Stephenson et al., 2018), and cytotoxic factors, such as nitric oxide (NO), adenosine triphosphate (ATP; Orellana et al., 2011), and mitochondrial fragments (Joshi et al., 2019). These cells can, in turn, affect astrocyte reactivity as well (Colombo and Farina, 2016; Rothhammer et al., 2016; Liddelow et al., 2017; Williams et al., 2020; Clark et al., 2021). In particular, microglia have been shown to affect inflammatory astrocyte function (Liddelow et al., 2017; Yun et al., 2018; Joshi et al., 2019), while concurrently inflammatory astrocytes release many microglia-activating factors (Zamanian et al., 2012; Guedes et al., 2018) resulting in a feed-forward loop of activation. In addition, microglia-astrocyte crosstalk (Matejuk and Ransohoff, 2020) has been implicated in driving disease pathology, for example through the release of chemokines/cytokines (Itoh et al., 2017) and direct protein-protein interaction through axon guidance molecules, such as Sema4D/PlexinB2 and EphrinB3/EphB3 (Clark et al., 2021). Astrocytes can also activate themselves in an autocrine manner through the release of cytokines (Sofroniew, 2009; Zamanian et al., 2012; Escartin et al., 2021), ATP (Sofroniew, 2009; Zamanian et al., 2012), inflammatory HA (Kuipers et al., 2016; Nagy et al., 2019), and certain glycolipids such as lactosylceramide (LacCer; Mayo et al., 2014).

In parallel, at least two distinct types of reactive astrocytes were identified in initial studies examining the heterogeneity of astrocyte responses—inflammatory/neurotoxic and neuroprotective astrocytes, originally referred to as "A1" and "A2" astrocytes—analogous to proinflammatory M1 and anti-inflammatory M2 macrophages.

Inflammatory "A1" astrocytes are a classification of reactive astrocytes that are characterized by their neurotoxic, proinflammatory phenotype (Liddelow et al., 2017). They were first defined, alongside their neuroprotective counterparts, "A2" astrocytes, through pioneering experiments conducted in the Barres lab (Zamanian et al., 2012). To assess whether reactive astrocyte responses differ based on the insult given, they analyzed differentially expressed genes in reactive astrocytes that were induced either by experimental ischemic stroke or by neuroinflammation [through systemic administration of lipopolysaccharide (LPS; Zamanian et al., 2012)] and subsequently defined the two distinct activation states described above. However, similar to the evolution of the concept of M1/M2 macrophages, which has been expanded into a more continuous and plastic activation model, recent advances in single cell RNA sequencing (Wheeler et al., 2020a), as well as further analysis of the regional (Itoh et al., 2017; Boisvert et al., 2018; Williams et al., 2020) and phenotypic (Wheeler et al., 2020a) diversity of astrocytes, have made it apparent that the heterogeneity of (reactive) astrocytes extends beyond these two distinct states. In fact, a recent consensus review clarifying various idiosyncrasies in the field of astrocyte biology highlights the need to abandon the limited categorization of A1/A2 astrocytes, as the understanding of distinct astrocyte states has evolved beyond a binary paradigm (Escartin et al., 2021). Instead, a spectrum of reactive astrocyte states, characterized by gene expression signatures, as well as functional features, more accurately reflects astrocyte responses in neuropathology. Nevertheless, it is clear that under certain pathological conditions, astrocytes can adopt distinct

inflammatory features and markers for this inflammatory phenotype are becoming more refined. Therefore, in this review, we will focus on the current state of literature on the role of inflammatory astrocytes in various neurological disorders.

Genes that are differentially upregulated in inflammatory astrocytes (the originally coined "A1" astrocytes) have been identified as critical players in various proinflammatory pathways, including the antigen presentation pathway, the complement pathway, and the interferon response pathway (Zamanian et al., 2012). Activation of the complement pathway can result in detrimental neuroinflammation (Lian et al., 2015; Okrój and Potempa, 2019), and complement component 3 (C3) is markedly enriched in inflammatory astrocytes compared to resting and neuroprotective astrocytes. Therefore, it is now frequently used in histology to identify inflammatory astrocytes, along with the upregulation of general reactive astrocyte markers, such as glial fibrillary acidic protein (GFAP; Escartin et al., 2021; **Table 1**). In addition, a common method to identify inflammatory astrocytes *in vitro* or *ex vivo* is to assess the expression of a set of genes that were found to be uniquely upregulated in the originally defined "A1" astrocytes (Liddelow et al., 2017), by quantitative PCR (**Table 1**). Because there is not one specific marker for this subtype and inflammatory genes can be expressed by other cell types (or even other astrocyte subpopulations) as well, particularly under neuroinflammatory conditions, a combination of markers should be used to properly determine the inflammatory phenotype of reactive astrocytes and rule out contamination of other cell types. In addition, functional features should also be taken into account when defining whether a particular astrocyte subtype observed in a neuropathological condition has inflammatory capacities.

After their first genomic identification, subsequent studies have shown that *in vitro*, inflammatory astrocytes lose many of the homeostatic functions that astrocytes are known for, such as providing neurotrophic support, promoting synaptogenesis, and phagocytosis of synapses (Liddelow et al., 2017). It was shown that inflammatory astrocytes could be induced by soluble factors secreted by LPS-stimulated microglia. Of these factors, IL-1α, TNFα, and C1q, most potently in combination, were shown to be sufficient and necessary to polarize astrocytes to an inflammatory phenotype (Liddelow et al., 2017). Similarly, culturing naïve astrocytes with microglia conditioned media from Amyotrophic Lateral Sclerosis (ALS; Joshi et al., 2019) or Alzheimer's Disease (AD; Xu et al., 2018) models resulted in these astrocytes taking on an inflammatory phenotype.

Inflammatory astrocytes have been the primary focus of neurological disease research, in part because techniques to identify neuroprotective astrocytes have remained elusive, whereas inflammatory astrocytes are more readily identified using the methods described above. As such, the potential role of inflammatory/neurotoxic astrocytes in neurodegenerative and neuroinflammatory diseases has recently been the subject of an increasing number of studies. Here, we discuss the roles that inflammatory astrocytes (may) play in these diseases, the efforts that are being made to pharmacologically target inflammatory astrocytes, and the limitations in studying this specific phenotype.

TABLE 1 | Commonly used techniques to identify inflammatory astrocytes.

Experimental Sample	Technique	Targets*
Tissue/cell culture	Immunohisto- or cytochemistry	Pan-reactive proteins: GFAP, Vimentin, S100β
		Inflammatory proteins: C3, GBP2
Cultured or sorted cells/tissue homogenates	qRT-PCR	Pan-reactive transcripts: Lcn2, Steap4, Serpina3n, S1pr3, Cxcl10, Hsbp1, Timp1, Aspg, Osmr, Cp, Vim, Gfap
		Inflammatory transcripts: C3, H2-D1, Serping1, H2-T32, Ggta1, Iigp1, Gbp2, Fbln5, Fkbp5, Srgn, Amigo2
Cultured/isolated cells/tissue	*In situ* hybridization	Pan-reactive probes: Lcn2, Serpina3n, Slc1a3
		Inflammatory probes: C3, H2-D1, Serping1

**For all detection methods a combination of one or more pan-reactive astrocyte markers, as well as inflammatory-specific markers, is used to confirm the inflammatory phenotype. Sources that contain specific information on antibodies, primer, and probe sequences include: Zamanian et al. (2012), Liddelow et al. (2017), Clarke et al. (2018), and Hartmann et al. (2019).*

ALZHEIMER'S DISEASE

Alzheimer's disease is a progressive, neurodegenerative disease characterized by the accumulation of amyloid-beta plaques and neurofibrillary tangles of the microtubule-associated protein tau (Dickson and Vickers, 2001). The exact role of astrocytes in the propagation (or "seeding") of tau tangles is debated and is a growing, active area of research. Astrocytes have been observed to internalize tau. However, it is yet unclear whether (or when) this internalization leads to degradation or propagation of tau, and whether this contributes to the induction of an inflammatory phenotype in astrocytes (Kovacs, 2020; Reid et al., 2020; Fleeman and Proctor, 2021). In AD, the presence of reactive astrocytes often precedes the formation of the disease's characteristic histopathologies (Heneka et al., 2005; Orre et al., 2014). Moreover, a recent single cell analysis of non-neuronal cell populations in the 5xFAD transgenic mouse model of AD revealed a transient astrocyte response as the disease progresses, from a GFAP-low state to a GFAP-high state, as well as an AD-specific population termed "disease-associated astrocytes" (Habib et al., 2020). As such, there is great interest in determining the role of these reactive astrocytes in the pathogenesis of AD, and advances in RNA sequencing technology drive increasingly refined analyses of their phenotypes and functions.

To quantify inflammatory astrocyte responses, the density of $C3^+$ cells with astrocyte morphology was analyzed in post-mortem AD tissue. $C3^+$ astrocyte-like cells were found to be enriched in the upper cerebral cortex of patients. Interestingly, control tissue also showed significant numbers of $C3^+$ astrocyte-like cells, notably in the lower cerebral cortex and white matter (King et al., 2020). In another study, AD subjects had significantly more $C3^+$ reactive astrocytes compared to matched controls in the entorhinal cortex, one of the first brain regions affected in AD, and the hippocampus (Balu et al.,

2019). The majority of these $C3^+$ astrocytes also co-expressed serine racemase (SR); however, the density of these astrocytes was concentrated primarily in superficial rather than deep layers. SR is an enzyme that is critical for the conversion of L-serine to D-serine, which can bind to NMDA receptors. These human results were confirmed with a murine model of AD using aged TgF344-AD rats and it was additionally found that these rats had increased activation of signaling pathways associated with extrasynaptic NMDAR activation in the hippocampus (Balu et al., 2019). As inflammatory astrocytes have been shown to lose normal astrocyte functions (Liddelow et al., 2017) and extrasynaptic NMDAR activation is linked to the deleterious effects of glutamate on plasticity and neuronal survival (Bading, 2017), these results implicate a potential involvement of inflammatory astrocytes in the progression of AD. Indeed, in a murine tauopathy model, astrocytes were shown to display an inflammatory expression profile in the early stages of neurodegeneration. In addition, C3 immunoreactivity was confined to reactive astrocytes and genetic deletion of C3 resulted in reduced neuronal loss, suggesting that these inflammatory astrocytes might contribute to tau-driven pathology (Wu et al., 2019). In another murine AD model, activation of melanocortin receptors by its agonist D-Tyrosine resulted in a significant decrease in $GFAP^+/C3^+$ astrocytes in the CA1 region of the hippocampus (Lau et al., 2021). This decrease in inflammatory astrocyte numbers correlated with a significant decrease in amyloid plaques deposition and critical levels of toxic amyloid-β isomers in the hippocampus (Lau et al., 2021). These findings suggest that targeting $GFAP^+/C3^+$ astrocytes might be a potential therapeutic avenue in the treatment of AD. In addition, another study showed that *in vitro*, inflammatory astrocyte induction can be blocked by exogenously applied milk fat globule epidermal growth factor 8 (MFG-E8), production of which is reduced in these inflammatory astrocytes (Xu et al., 2018). In a study highlighting the glial effects of amyloid-β exposure in an AD model, activation of the NF-κB pathway, known to be involved in inflammatory astrocyte induction, was detected in astrocytes and subsequent neuronal release of C3 resulted in synaptic dysfunction (Lian et al., 2015). These studies highlight potential pathways to modulate inflammatory astrocyte activation and improve AD pathology.

Cerebral amyloid angiopathy (CAA) is a typical condition of AD pathology and is characterized by cerebrovascular deposition of amyloid protein. While the function of the amyloid protein remains elusive, its accumulation is toxic and known to induce apoptosis and drive neurodegeneration (Chow et al., 2010; Chen et al., 2017). In a murine model of early CAA, immune and glial responses were analyzed, and histology revealed perivascular reactive astrogliosis, identified by GFAP (reactive astrocytes), Thioflavin-S (vascular amyloid), and α-smooth muscle actin (vascular smooth muscle cells) immunoreactivity, in 9-month-old mice. Of note, this phenomenon was absent in 3-month-old mice, suggesting a temporal, progressive astrocyte response. Further characterization of this response revealed a robust inflammatory astrocyte presence, as defined by colocalization of C3 and GFAP, in the hippocampus and cerebellum (Taylor et al., 2020).

As the accumulation of plaques and tangles occurs before the onset of symptoms, and there is a significant benefit of early intervention to patients, there is a strong drive to establish biomarkers for early AD pathology, as well as to develop non-invasive treatments. In this regard, the retina has become a popular area of study given its common embryological origin with the brain (Paquet et al., 2007). In the pursuit of an early biomarker for AD, upregulation of IL-1β in microglia and the additional presence of inflammatory astrocytes, as determined by GFAP and C3 colocalization, was found in retinal tissue from AD patients, indicating that the inflammatory activation of astrocytes is a feature of early AD pathology (Grimaldi et al., 2019). In addition, in a study comparing astrocyte-derived exosomes (ADE) in the plasma of AD patients to those of matched controls, levels of complement proteins and cytokines were analyzed (Goetzl et al., 2019). Complement factors, including C3d and C1q, one of the factors able to induce inflammatory astrocytes *in vitro*, were significantly higher in ADE from AD patients. With respect to cytokine profiles, while there was greater overlap between the two groups, AD ADE contained higher levels of IL-6, TNF-α and IL-1β (Goetzl et al., 2019), cytokines known to be involved in reactive astrogliosis (Choi et al., 2014).

Moreover, exercise is thought to be of benefit as a treatment for AD due to its capacity to stimulate the release of neurotrophic factors (Prado Lima et al., 2018), decreasing deposition of amyloid-β plaques (Prado Lima et al., 2018), and improving tau pathology (Belarbi et al., 2011; Fleeman and Proctor, 2021). In a study using exercise to treat a murine model of AD, rotarod exercise therapy resulted in a decrease of inflammatory astrocytes, along with reduced amyloid-β deposition, neuronal loss, and cognitive decline, showing that astrocyte reactivity correlates with treatment effects as well (Nakanishia et al., 2021).

HUNTINGTON'S DISEASE

Huntington's disease (HD) is a neurodegenerative disease, primarily affecting the basal ganglia, that is caused by a dominantly inherited CAG trinucleotide repeat expansion in the huntingtin gene on chromosome 4 (McColgan and Tabrizi, 2018). In humans, reactive (fibrillary) astrogliosis within the corpus striatum is used to classify progressive stages of HD (Rüb et al., 2016). It has been shown that astrocytes from HD patients become physiologically and morphologically activated when exposed to mutant huntingtin, as determined by increased GFAP staining and morphological changes—specifically, thicker processes and a larger somata (Faideau et al., 2010). Additionally, these reactive astrocytes have significantly decreased expression of the glutamate transporters GLAST and GLT-1, which leads to a subsequent decrease in a critical astrocyte function—glutamate uptake (Rose et al., 2018). This is of interest, as loss of physiological astrocyte functions is characteristic of *in vitro* generated inflammatory astrocytes (Liddelow et al., 2017).

Single-nucleus RNA sequencing of astrocytes derived from the postmortem anterior cingulate cortex of HD and control human tissue went beyond the "A1/A2" classification and

identified several distinct astrocyte "states" as determined by differential gene pattern expression (Al-Dalahmah et al., 2020). Additionally, this study confirmed that astrocytes in the caudate nucleus of HD grades III and IV express markers of an inflammatory state, showing C3 staining alone and double immunostaining for C3 and GFAP (Al-Dalahmah et al., 2020). These results suggest that inflammatory astrocytes in the anterior cingulate cortex are associated with progressive stages of HD.

Genomic and proteomic analysis of striatal astrocytes shows only the inflammatory astrocyte-associated gene *Serping1* to be consistently upregulated across human samples and murine models (Diaz-Castro et al., 2019). However, akin to what has been reported in previous inflammatory astrocyte literature (Liddelow and Barres, 2017), astrocytes from HD striatum undergo significant morphological and transcriptional changes. Moreover, these changes are largely reversed by lowering mutant Huntington protein specifically in astrocytes (Diaz-Castro et al., 2019) showing a direct effect of mutant protein on reactive astrogliosis and highlighting the potential for therapeutics targeting reactive astrocytes in HD.

MULTIPLE SCLEROSIS

Multiple Sclerosis (MS) is a progressive autoimmune demyelinating disease, characterized by infiltration of peripheral immune cells that target myelin within the CNS, and resulting in focal neuroinflammatory lesions, demyelination, and neurodegeneration. Astrocytes are thought to be involved in MS pathogenesis due to their capacity to promote entry of peripheral immune cells to the CNS, as well as to directly affect inflammatory processes in lesion formation (Ponath et al., 2018). One of the most widely used animal models used in MS research is experimental autoimmune encephalomyelitis (EAE), which involves inducing a T cell-driven immune response against myelin that leads to infiltration of these cells into the CNS, activation of resident cells, including astrocytes, and subsequent destruction of myelin, and damage to axons and neurons (Rangachari and Kuchroo, 2013; Lassmann and Bradl, 2017). As the model is driven by an immune response, EAE is often used to study the role of infiltrating and resident inflammatory cells in demyelination, because it recapitulates the inflammatory milieu found in actively demyelinating MS lesions (Lassmann and Bradl, 2017). In addition, MS is often studied in conjunction with optic neuritis, which is also pathologically characterized by peripheral immune cell infiltration (Bettelli et al., 2003; Lassmann and Bradl, 2017). Reactive astrocytes can be found at various stages of MS lesions. In addition to their abundance in chronic lesions, reactive astrocytes are present in the center and the active edge of acutely demyelinating lesions, as well as in bordering white matter (Kuhlmann et al., 2017; Ponath et al., 2017, 2018). In parallel, astrocytes become reactive, as determined by enhanced expression of GFAP, early and throughout EAE pathogenesis (Wang et al., 2005; Luo et al., 2008; Pham et al., 2009). C3 containing astrocytes are abundantly present in the center, as well as the expanding edge of actively demyelinating MS lesions, and can also be found in chronic lesion stages. Interestingly, these $C3^+$ astrocytes are often located in close proximity to activated microglia/macrophages (Ingram et al., 2014; Liddelow et al., 2017).

In EAE, inflammatory astrocytes, as defined by C3 staining and inflammatory-specific transcript analysis, are prevalent in the retina and optic nerve tissue and are associated with retinal ganglion cell loss (Jin et al., 2019). Additionally, the complement cascade was found to be one of the most significantly upregulated pathways in the optic nerve of EAE mice (Tassoni et al., 2019). These results suggest that inflammatory astrocytes could be a potential target against some common visual symptoms of MS resulting from optic nerve degeneration. Additionally, this study observed significantly more C3 expressing astrocytes within the optic nerve of female mice as compared to males (Tassoni et al., 2019). This is of note, as the prevalence of MS is significantly higher in women than in men.

Recently, a pro-inflammatory and neurotoxic signature was also found in an astrocyte subset that is greatly expanded during EAE, identified by single cell RNA sequencing analysis. This subset is characterized by activation of the NF-κB and inducible nitric oxide synthase (iNOS) pathways, reduction of the NRF2 pathway, which limits oxidative stress and inflammation, and increased expression of the master transcriptional regulator MAFG (Wheeler et al., 2020a). Moreover, an astrocyte subset with similar features can be found in a combined scRNAseq dataset containing data from MS and control tissue samples. This inflammatory subset is detected in the majority of patient samples (12 out of 20) and greatly expanded in samples from MS patients compared to control samples (25-fold; Wheeler et al., 2020a).

There are various other models of MS that represent additional facets of its pathogenesis, such as the cuprizone model of demyelination and toxin-induced demyelination and remyelination (Lassmann and Bradl, 2017). However, studies exploring the presence and role of inflammatory astrocytes in these models are very limited.

One of the only known factors to correlate with MS progression is age, the majority of MS patients developing a progressive stage of the disease when they are between 40–50 years old (Tremlett and Zhao, 2017). This is significant, as both immune function and astrocyte functions (Palmer and Ousman, 2018), such as morphological changes (Jyothi et al., 2015), increased GFAP expression (Wu et al., 2005; Clarke et al., 2018), and activation of complement factors (Clarke et al., 2018), are known to change over time. In particular, aging astrocytes take on a more inflammatory phenotype (Clarke et al., 2018). In a study of over 1,000 proteins derived from the cerebrospinal fluid of 431 patients, a cluster of inflammatory astrocyte-derived proteins was found to be significantly upregulated in MS patients and had a significant, reproducible correlation with MS severity (Masvekar et al., 2019). Together, these findings suggest that inflammatory astrocytes may play an active role in various stages of MS pathogenesis and could provide a target for addressing damage to the optic nerve, as well as the CNS parenchyma of MS patients. Moreover, proteins derived from inflammatory astrocytes could prove to be a valuable biomarker to predict the progression of disease in MS.

PARKINSON'S DISEASE

Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized by loss or degeneration of dopaminergic neurons in the substantia nigra in the midbrain and the development of Lewy bodies, protein aggregates that primarily contain the protein α-synuclein (Forno et al., 1986; Braak et al., 2003). It is a disease of unknown etiology commonly associated with aging and family history (Kalia and Lang, 2015). As a result of neurodegeneration, inflammation also plays a key role in PD. When neurons die, they release proinflammatory and cytotoxic molecules (Glass et al., 2010) that promote gliosis and immune responses. These responses lead to a feed-forward cycle wherein activated immune cells further respond by releasing additional proinflammatory factors (Lee et al., 2019), thereby perpetuating inflammation and neuronal damage.

The key contributors to PD pathogenesis with a proinflammatory relationship are astrocytes and microglia. A widely used model for PD is the MPTP model, based on the toxic properties of peripherally administered 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which results in dopaminergic neurodegeneration in the striatum and substantia nigra, a pattern similar to the human disease (Meredith and Rademacher, 2011). In this model, systemic administration of LPS exacerbates microglial activation and induces the conversion of astrocytes to an inflammatory ($C3^+$) phenotype (García-Domínguez et al., 2018). This shows that peripheral inflammation can trigger inflammatory astrocyte conversion under the conditions of dopaminergic neurodegeneration (García-Domínguez et al., 2018). In another murine model of PD, in which LPS is injected into the midbrain, genes associated with inflammatory astrocytes, as well as the potassium channel subunit Kir6.2, were upregulated in the substantia nigra (Song et al., 2021). Kir6.2 is induced by chronic metabolic stress, associated with the degeneration of dopaminergic neurons and can act as an inflammatory mediator (Liss et al., 2005; Du et al., 2014). Kir6.2 was shown to be expressed by astrocytes and its genetic deletion mitigated inflammatory astrocyte expression patterns and prevented dopaminergic neurodegeneration and behavioral deficits (Song et al., 2021). Additionally, it has been shown *in vivo* that NLY01, a glucagon-peptide-1 receptor agonist, is capable of blocking the astrocytic conversion to an inflammatory phenotype by preventing the microglial release of IL-1α, TNFα, and C1q (Yun et al., 2018). NLY01 was shown to be protective in two models of PD: the α-synuclein preformed fibrils (PFF) model of sporadic PD, and the progressive, lethal, constitutive α-synucleinopathy model (Yun et al., 2018). Furthermore, β-sitosterol-β-D-glucoside (BSSG) is a neurotoxin found in cyad seeds and its chronic consumption induces a progressive PD-like disease in humans and rats (Van Kampen and Robertson, 2017). In a study assessing the neuroinflammatory reaction to this neurotoxin, a significant induction of inflammatory astrocytes, verified by co-staining of C3 with GFAP or S100β, was observed with a single BSSG injection to the substantia nigra, correlating with loss of dopaminergic neurons (Luna-Herrera et al., 2020). Together, these studies illustrate the involvement of inflammatory astrocytes in the pathogenesis of PD and provide potential targets to regulate their induction.

PRION DISEASES

Prion diseases are a group of neurodegenerative diseases caused by the conversion of prion protein (PrP) to an abnormal, misfolded form of the protein (PrP^{Sc}). This conversion is characterized by a shift of the normal prion protein α-helical structure to a β-pleated sheet structure, which forms amyloid deposits. The shift of PrP into PrP^{Sc} in prion diseases has a cascading effect, where the misfolded PrP^{Sc} protein acts as a seed and propagates the misfolding of additional proteins. However, the mechanism for this cascade is unknown. Among neurodegenerative disorders, prion diseases are unique because they can occur either spontaneously, genetically, or by transmission. The most common prion diseases are Creutzfeldt-Jakob Disease (CJD), its bovine equivalent Bovine Spongiform Encephalopathy (BSE, "mad cow disease"), and scrapie in sheep and goats (Belay, 1999; Geschwind, 2015).

Reactive astrogliosis is a hallmark of all prion diseases. Astrocytes play roles in prion diseases both in their capacity as proponents of neuroinflammatory response and as promotors of PrP^{Sc} spread and aggregation (Carroll and Chesebro, 2019). In a murine model of prion disease, induced *via* intracerebral injection of scrapie brain homogenate, reactive astrogliosis occurs early and throughout the clinical course and coincides with PrP^{Sc} deposition (Tribouillard-Tanvier et al., 2012). While prion diseases were originally thought to involve a limited neuroimmune response (Belay, 1999; Geschwind, 2015), analysis of cytokines and chemokines in the scrapie inoculation-induced mouse model showed that protein levels of, among many others, IL-1Ra, CXCL10 (IP-10), and CCL5 (RANTES) were significantly increased as the disease progressed (Carroll et al., 2015). These factors are also among many produced by *in vitro* activated human astrocytes (Choi et al., 2014). Additionally, the majority of the inflammatory genes upregulated in scrapie-inoculated mice can be induced by the NF-κB pathway, which is activated in these mice (Tribouillard-Tanvier et al., 2012; Carroll et al., 2015). This is of note as NF-κB pathway activation has been associated with C3 production by astrocytes as well (Lian et al., 2015).

Numerous inflammatory ($C3^+$ or $GBP2^+$) astrocytes can be found in tissues of both murine prion disease and human CJD cases (Hartmann et al., 2019), and expression of C3 and GBP2 is significantly upregulated in CJD brain tissue and is associated with disease duration and risk genotype (Ugalde et al., 2020). Blocking the induction of inflammatory astrocytes in prion-infected triple KO mouse (TKO-mice), lacking TNFα, IL-1α, and C1q expression, however, does not affect PrP^{Sc} protein titers or deposition. Moreover, the disease course is significantly accelerated in these mice, indicating that the inflammatory response of astrocytes might constitute a protective mechanism limiting the damaging effects of PrP^{Sc} accumulation (Hartmann et al., 2019). While their presence and involvement in the pathogenesis of prion diseases is apparent, the overall effect

TABLE 2 | Therapeutic and mechanistic methods of targeting inflammatory astrocytes.

Disease	Method	Primary target	Model system
Alzheimer's Disease	Activation of melanocortin receptor by D-Tyrosine (Lau et al., 2021).	Astrocytes	*in vivo*
Alzheimer's Disease	Exercise (Belarbi et al., 2011; Nakanishia et al., 2021).	Astrocytes	*in vivo*
Amyloid Lateral Sclerosis	Intrathecal transplantation of human-grade astrocytes (Izrael et al., 2020).	Astrocytes	*in vivo* (mouse and human)
Glaucoma Associated Neurodegeneration	Preventing microglial release of IL-1α, TNFα, and C1q by NLY01 (Sterling et al., 2020).	Microglia	*in vivo*
Huntington's Disease	Transcriptional repression of mutant huntingtin protein using zinc finger proteins (Diaz-Castro et al., 2019).	Astrocytes	*in vivo*
Parkinson's Disease	Dopamine D2 receptor agonist inhibition of NLRP3 inflammasome activation in astrocytes (Zhu et al., 2018).	Astrocytes	*in vivo*
Parkinson's Disease	Genetic deletion of Kir6.2 (Song et al., 2021).	Astrocytes	*in vivo*
Parkinson's Disease	Prevent microglial release of IL-1α, TNFα, and C1q by NLY01 (Yun et al., 2018).	Microglia	*in vitro/in vivo*
Prion Disease	Genetic deletion of TNFα, IL-1α, and C1q triple KO (Hartmann et al., 2019).	Microglia	*in vivo*
Multiple Sclerosis	NLRP3 inflammasome inhibition (Hou et al., 2020).	Astrocytes	*in vivo*

of their involvement, whether helpful or harmful, remains to be seen.

DISCUSSION

Inflammatory astrocytes have been shown to play roles in inflammatory and neurodegenerative mechanisms of neurological disease. Of note, in the healthy aging brain, astrocytes have also been shown, *in vivo*, to take on a genetic profile similar to that seen in neuroinflammation-induced astrocytes (Clarke et al., 2018). Aging is a significant risk factor for many CNS pathologies (Palmer and Ousman, 2018; Hou et al., 2019); for example, it correlates with disease progression in MS (Confavreux and Vukusic, 2006; Koch et al., 2009, 2010; Hou et al., 2019). It is therefore critical to explore the role of these aging and potentially inflammatory astrocytes in the exacerbation of disease and injury. It is likely that aging-induced inflammatory astrocytes contribute to neuroinflammation and neurodegenerative processes in general, given the demonstrated neurotoxic functions of inflammatory astrocytes (Liddelow et al., 2017), their prevalence with age (Clarke et al., 2018), and their observed presence in many neurodegenerative diseases (Liddelow and Barres, 2017; Clarke et al., 2018; Goetzl et al., 2019; Hartmann et al., 2019; Tassoni et al., 2019; Song et al., 2021).

A confounding factor in all studies examining the role of astrocytes in disease pathogenesis is the fact that astrocyte and microglial activation commonly happens in concert. Due to obvious experimental challenges, many of the studies discussed in this review do not directly address the potential inflammatory feed forward cycle of inflammatory or generally reactive astrocytes and activated microglia that might contribute to the progression of disease (**Figure 1**). Inflammatory activation of astrocytes can result in the release of proinflammatory cytokines that activate microglia and mediate neurotoxic inflammation. In turn, the proinflammatory factors released by activated microglia can further activate inflammatory astrocytes (Liddelow et al., 2017), thereby creating a detrimental, inflammatory feed forward cycle that exacerbates disease severity. Therefore, disease-modifying treatments targeting inflammatory astrocytes are of great interest, as eliminating a main component of this inflammatory cycle can mitigate its damage.

While still an area to be further explored, there have been some advances in therapeutically targeting inflammatory astrocytes, specifically, during disease (**Table 2**). NLY01, used to block the induction of inflammatory astrocytes by inhibiting the release of IL-1α, TNF-α and C1q from microglia, was successfully used in studies of PD (Yun et al., 2018) and glaucoma-associated neurodegeneration (Sterling et al., 2020) to ameliorate disease severity. With respect to ALS, studies regarding the transplantation of glial precursor cells demonstrated glial transplantation as a method to delay disease onset and ameliorate clinical symptoms (Kondo et al., 2014; Izrael et al., 2020). This has led to a current clinical study (National Library of Medicine, NCT03482050, 2018) of intrathecal transplantation of human-grade astrocytes in the hopes of reducing the large population of inflammatory astrocytes causing damage in patients with ALS (Izrael et al., 2020). These treatments show the potential of this avenue in disease management.

There are significant limitations in studying inflammatory astrocytes, as the mechanism underlying their induction has only been shown *in vitro* (Liddelow et al., 2017) and upregulation of genes associated with inflammatory astrocytes is not ubiquitous in all neurodegenerative diseases and their models. By extension, identifying the inflammatory nature of reactive astrocytes in particular settings is challenging. However, the previously mentioned recent consensus article provides clarity regarding markers and terminology to be used when describing reactive astrocytes (Escartin et al., 2021). Thus far, the methods used to identify inflammatory

astrocytes are generally two-fold: co-expression of GFAP and C3 along with a transcription of a commonly defined subset of inflammatory astrocyte-specific genes. However, C3 upregulation is not unique to inflammatory astrocytes, as the complement cascade is activated in numerous inflammatory conditions (Markiewski and Lambris, 2007). Therefore, co-expression must be clearly shown and verified—preferably through quantitative techniques. Often these methods of identification are used individually or in conjunction to identify the presence of inflammatory astrocytes; however, studies that go beyond this correlation and delve into the mechanisms of inflammatory astrocyte induction and its consequences are still limited.

An additional challenge in determining the contribution of inflammatory astrocytes to neurological disorders is the innate limitations of using animal models. While rodent models provide valuable tools to dissect biological processes, there are various physiological differences between rodents and humans that need to be taken into account when extrapolating findings (Perlman, 2016). With regard to astrocyte responses, while general (reactive) gene expression profiles are similar between human and mouse astrocytes, differences in the molecular pathways induced by some stimuli do exist and it cannot be ruled out that the distinct expression profiles and functions of human astrocytes differ from those in the mouse models used to determine their role in neuropathology (Li et al., 2021). In addition to animal models, the postmortem tissue of patients is a valuable source to determine disease-specific mechanisms. However, many factors that are difficult (or impossible) to control can introduce variation in data and confound findings, such as the cause of death, stage of disease at the time of death, and postmortem interval (time from death to autopsy; Di Lullo and Kriegstein, 2017). For example, available tissue from postmortem sources is generally skewed towards the end or advanced stage of the disease, whereas biopsy material is often from cases that display an abnormal disease pattern. Moreover, obtaining control tissue (either from healthy individuals or non-related neurological conditions) that is properly matched for sex, gender, age, and lifestyle factors is challenging. Therefore, using a combination of techniques, models, and tissue sources is best suited to dissect the intricate interplay of the cellular and molecular mechanisms driving pathology. Moreover, the exact function of specific reactive astrocyte states or subtypes likely depends on the pathological context and stage of disease, due to the suggested transient and/or plastic nature of reactive astrocyte states/subtypes (Mayo et al., 2014; Habib et al., 2020). Only once the exact contribution of inflammatory astrocytes to the various stages of the disease has been mapped, targeting this population specifically at the appropriate stage could provide an effective treatment strategy.

Developments in the field of single cell RNA sequencing have advanced studies of reactive astrocyte responses beyond the initial binary classification of inflammatory/neurotoxic and neuroprotective astrocytes. For example, several unique clusters of reactive astrocytes were identified in EAE and MS tissue (Wheeler et al., 2020a), and differential effects of ablation of reactive astrocytes at different stages of EAE suggest that this astrocyte response might be transient and/or plastic (Mayo et al., 2014). In addition, transient and disease-specific reactive astrocyte populations were observed in the 5xFAD model of AD (Habib et al., 2020). Therefore, as discussed before, a refined view of astrocyte heterogeneity and plasticity allows for a more comprehensive classification of reactive astrocyte populations/states and potentially a greater understanding of their role in disease pathology.

Recent additional advances have also allowed for exploration in the field of cell-cell crosstalk. In a recent study using an mRNA barcoding technique that takes advantage of the pseudorabies virus's capacity to spread between interacting cells (coined RABID-seq), labeling cells interacting with astrocytes showed that pathogenic astrocytes connected to microglia display an inflammatory signature, and that their crosstalk is mediated, amongst others, by axon guidance molecules (Clark et al., 2021). Advances such as these are critical as they allow for an understanding of the complex cellular interactions that perpetuate inflammation. However, it also forebodes that, as the narrative of the involvement of astrocytes in disease continues to develop, it may be that the classification of astrocyte subsets will be defined more by their function in relation to a specific disease state, rather than a specific binary phenotype based on gene expression signatures.

AUTHOR CONTRIBUTIONS

JR performed literature searches, and structured and wrote the manuscript. HK gave structural and contextual input, and edited the manuscript. All authors contributed to the article and approved the submitted version.

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Shaping of Regional Differences in Oligodendrocyte Dynamics by Regional Heterogeneity of the Pericellular Microenvironment

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Oligodendrocyte precursor cells (OPCs) are glial cells that differentiate into mature oligodendrocytes (OLs) to generate new myelin sheaths. While OPCs are distributed uniformly throughout the gray and white matter in the developing and adult brain, those in white matter proliferate and differentiate into oligodendrocytes at a greater rate than those in gray matter. There is currently lack of evidence to suggest that OPCs comprise genetically and transcriptionally distinct subtypes. Rather, the emerging view is that they exist in different cell and functional states, depending on their location and age. Contrary to the normal brain, demyelinated lesions in the gray matter of multiple sclerosis brains contain more OPCs and OLs and are remyelinated more robustly than those in white matter. The differences in the dynamic behavior of OL lineage cells are likely to be influenced by their microenvironment. There are regional differences in astrocytes, microglia, the vasculature, and the composition of the extracellular matrix (ECM). We will discuss how the regional differences in these elements surrounding OPCs might shape their phenotypic variability in normal and demyelinated states.

Keywords: myelin, NG2, PDGF, blood vessels, astrocyte, microglia, neuropilin

INTRODUCTION

Oligodendrocyte precursor cells (OPCs), also known as NG2 glia or polydendrocytes, are uniformly distributed throughout the gray and white matter of the adult central nervous system (CNS). They are lineage committed precursor cells whose primary known function is to differentiate into mature oligodendrocytes (OLs) that myelinate axons and enable fast saltatory conduction of action potentials. OPCs express the cell surface proteins NG2 and platelet-derived growth factor receptor alpha (PDGFRα), which are downregulated during their terminal differentiation into postmitotic oligodendrocytes. The term NG2 cells or NG2 glia is used synonymously with OPCs, regardless of whether the cell differentiates into an OL or continues to exist as an OPC for an extended period of time, as currently evidence is lacking that OPCs comprise cells with dichotomous OL differentiation dynamics.

OPCs arise from discrete germinal zones in the embryo and continue to be generated from the subventricular zone in the mature CNS. Despite the reports that nearly all OPCs are in active cell cycle state in the adult brain (Rivers et al., 2008; Psachoulia et al., 2009; Kang et al., 2010), OPCs are heterogeneous in their proliferative rate. In the normal rodent CNS, OPCs proliferate and differentiate into OLs at a higher rate in the white matter than in the gray matter (Hill and Nishiyama, 2014; Boshans et al., 2020; Nishiyama et al., 2021; **Figure 1**, left). Myelination continues throughout life beyond developmental myelin production and appears to be finely tuned to the local neural network (Monje and Karadottir, 2020; Nishiyama et al., 2021; Pease-Raissi and Chan, 2021).

Damage to myelin occurs in demyelinating diseases such as multiple sclerosis (MS; Lassmann, 2019). Poor remyelination of chronic MS lesions could result from failure of OLs to myelinate axons (Chang et al., 2002) or defects in OPCs (Jäkel et al., 2019; Yeung et al., 2019). Based on the OL dynamics in the normal brain, one would predict that MS lesions in the white matter would be more efficiently remyelinated. Contrary to this prediction, OL dynamics during remyelination in MS is more robust in gray matter lesions (Peterson et al., 2001), despite a higher density of OPCs and OLs in normal appearing white matter than in normal appearing gray matter of MS brains (Strijbis et al., 2017). In this mini review, we will discuss how the regional heterogeneity of the microenvironment surrounding OL lineage cells might contribute to their region-specific behavior in normal and pathological states.

REGIONAL HETEROGENEITY OF OL LINEAGE CELLS

It has been debated as to whether OPCs represent a heterogeneous cell population (Dimou and Simons, 2017; Foerster et al., 2019; Boshans et al., 2020). Unlike neurons which can be classified into functionally and transcriptionally distinct cell types, OPCs exhibit a spectrum of phenotypes that varies with the age and anatomical location. Below are examples of the phenotypic variability among OPCs and an emerging hypothesis that these differences represent different cell and functional states rather than transcriptionally distinct subtypes.

OPC Distribution Does Not Always Parallel OL Distribution

While OPCs are distributed throughout the CNS, their density is higher in white matter than in gray matter (Dawson et al., 2000, 2003; Terai et al., 2003). One determinant of OPC density could be the necessity to generate OLs. Consistently, OPC density is low in CNS regions where unmyelinated axons predominate such as the molecular layer of the cerebellar cortex (Givogri et al., 2002; Sun et al., 2018). However, in other regions the distribution of OPCs does not match that of OLs. For example, in the neocortex OPC are distributed uniformly throughout all cortical layers, whereas myelinating OLs are more abundant in deeper cortical layers (Tomassy et al., 2014). Curiously, OPCs exist in relatively high density in some circumventricular organs (Terai et al., 2003; Zilkha-Falb et al., 2020), where capillaries are fenestrated and their density is high (Gross et al., 1986), suggesting a possible non-progenitor role for OPCs. Indeed, OPCs are involved in feeding and body weight control by modulating leptin-sensing of leptin receptor-expressing neurons in the arcuate nucleus that extend their dendrites into the adjacent median eminence (Djogo et al., 2016).

Higher Rate of OPC Proliferation and OL Differentiation in White Matter

OPCs in white matter proliferate and generate OLs at a greater rate than those in the gray matter (**Figure 1**, left; Dawson et al., 2003; Dimou et al., 2008; Psachoulia et al., 2009; Kang et al., 2010; Zhu et al., 2011; Young et al., 2013). In slice culture, OPCs in the white matter of the developing corpus callosum and cerebellum proliferate more robustly in response to exogenous PDGF AA than those in the neocortex and cerebellar cortex, despite similar levels of PDGFRα (Hill et al., 2013). When OPCs are isolated from adult gray and white matter and transplanted into gray and white matter of recipient adult mice (Vigano et al., 2013), those from the white matter but not gray matter differentiate robustly into OLs in the host gray matter, whereas those from both gray and white matter differentiate into OLs in the host white matter, suggesting both intrinsic and environmental influences. In slice culture, homo- and heterotopic transplantation of 300-μm^3 explants or isolated explant cultures indicated that the proliferative response of OPCs to PDGF AA is determined by signals within the 300-μm^3 microenvironment around the OPCs (Hill et al., 2013). A recent follow-up study demonstrated that neuropilin-1 (Nrp1), a co-receptor for PDGFRα, is expressed by microglia adjacent to OPCs in white but not gray matter and trans-activates PDGFRα on OPCs (Sherafat et al., 2021), providing an example of a regional microglial difference affecting OPC dynamics (see

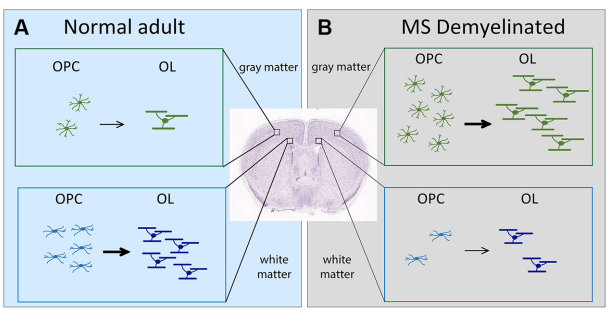

FIGURE 1 | Schematic showing the change in OL dynamics from normal to demyelinated lesion. **(A)** (left, light blue). OPC proliferation and OL differentiation occurs more robustly in the white matter (corpus callosum) than in the gray matter (neocortex) of normal adult brain. **(B)** (right, gray). In MS, OPC recruitment and OL differentiation occurs more robustly in gray matter lesions compared with white matter lesions. The extent of OPC proliferation and OL differentiation is indicated by the number of cells. Arrows indicate differentiation into myelinating OLs. The thickness of the arrows indicates the extent of differentiation. OLs, oligodendrocytes; OPCs, oligodendrocyte precursor cells; MS, multiple sclerosis.

section "Regional Heterogeneity of Microglia Affects OL Lineage Cells" below).

OPCs Are Transcriptionally Homogeneous

Single-cell RNA-sequencing (scRNA-seq) has revealed multiple subtypes of OLs, but OPCs are more transcriptionally homogeneous and fail to cluster into transcriptionally distinct subtypes (Zeisel et al., 2015; Marques et al., 2016; Tasic et al., 2016). In scRNA-seq of OPCs from different ages, the OPC cluster that expresses genes involved in mitosis segregates from the other two clusters found, which express similar transcripts but appear in postnatal day 7 (P7) and juvenile/adult CNS, respectively (Marques et al., 2018). These clusters are not sufficiently distinct to allow classification of OPCs into distinct subpopulations but may represent different cell states described in the next section.

Different Cellular and Functional States of OPCs

OPCs in gray and white matter exhibit different electrophysiological properties shaped by the density of voltage-dependent Na^+ and K^+ channels (Na_v and K_v, respectively; Chittajallu et al., 2004; Clarke et al., 2012; Larson et al., 2016). A recent comprehensive analysis of Na_v, K_v, and glutamate receptor expression and membrane properties of individual OPCs in the cortex and corpus callosum at different ages revealed unique patterns as a function of age and location (Spitzer et al., 2019). When these data are projected against other cellular properties and transcriptional profiles, some correlations emerge. For example, proliferative OPCs have a higher Na_v density, and NMDAR expression coincides temporally with OL differentiation. From these analyses, Karadottir and colleagues propose that OPCs can exist in several different states that include: (1) naive state with no voltage-dependent ion channel expression; (2) proliferative state with high Na_v density and a transcriptomic profile of mitotic cells; (3) state primed for OL differentiation with high NMDAR density and higher transcripts for OL and myelin genes; and (4) quiescent state with reduced Na_v and NMDAR density (Kamen et al., 2021). These states are likely to be affected by their local environment. It is currently not known what specific signals drive them to transition from one state to another, the level of plasticity and convertibility among different states, and how the different OPCs states in turn influence the local neural network.

HETEROGENEITY OF THE PERICELLULAR ENVIRONMENT AROUND OL LINEAGE CELLS

Many excellent reviews have been published on how neuronal activity influences OL lineage cell behavior. Here, we will discuss how non-neuronal elements might affect the behavior of OL lineage cells. Recent studies have revealed regional heterogeneity of astrocytes and microglia. Furthermore, the vascular supply and vascular mural cell function are also likely to contribute to the different behavior of OL lineage cells in the cortex and corpus callosum.

Regional Heterogeneity of Astrocytes Affects OL Lineage Cells

Astrocytes are involved in a variety of functions from homeostasis of extracellular K^+ and glutamate to the regulation of synapses (Khakh and Sofroniew, 2015), and they influence OL development and function in a variety of ways (Barnett and Linington, 2013; Lundgaard et al., 2014; Tognatta et al., 2020). In response to a variety of insults, astrocytes become reactive and upregulate different sets of transcripts depending on the nature of the insults (Escartin et al., 2021). Transcriptional profiling showed that cortical astrocytes are enriched in genes required for cholesterol metabolism, and gray matter astrocytes support *in vitro* myelination more than 2-fold compared with white matter astrocytes (Werkman et al., 2021). Cholesterol is critical for myelination (Saher et al., 2005). Since cholesterol does not cross the blood-brain barrier, cholesterol must be made available to OLs autonomously by biosynthesis in OLs and by horizontal transfer from astrocytes (Camargo et al., 2017). In mice with experimental autoimmune encephalitis (EAE), astrocytes express lower levels of transcripts necessary for cholesterol biosynthesis (Itoh et al., 2018), and astrocytes from MS brains have lower expression of cholesterol biosynthesis genes.

By contrast, astrocytes from the white matter of normal young adult rats express extracellular matrix (ECM) genes more abundantly than gray matter astrocytes (Werkman et al., 2020; **Figure 2**). ECM genes enriched in white matter astrocytes include ECM genes that are upregulated in reactive astrocytes, such as CD44 and collagen genes though they are expressed at a lower level in normal white matter (Zamanian et al., 2012). CD44 is a receptor for the large extracellular proteoglycan hyaluronan which inhibits OL maturation (Buser et al., 2012). Thus, astrocytes from adult white matter appear to be less supportive of myelination compared to those from gray matter. By contrast, those in the gray matter appear to metabolically supportive of OLs and myelin production.

Regional Heterogeneity of Microglia Affects OL Lineage Cells

Microglia and border-associated macrophages make up the tissue macrophages of the CNS (Prinz et al., 2021). Microglia exhibit regional heterogeneity in their morphology and density (Lawson et al., 1990). They are highly plastic cells that switch from a resting homeostatic phenotype to an activated phagocytic state triggered by a variety of insults including MS lesions (Lassmann et al., 2001). In experimentally induced demyelination, activated microglia initially contribute to demyelination (Marzan et al., 2021) but are subsequently replaced by repair-promoting microglia (Lloyd et al., 2019). Microglia not only sense pathology but also play essential roles in normal development and homeostatic processes, such as neurogenesis, synaptic pruning, and regulation of neuronal activity (Butovsky and Weiner, 2018; Thion et al., 2018; Badimon et al., 2020).

Recent studies suggest that there are region-specific interactions between microglia and OL lineage cells. The

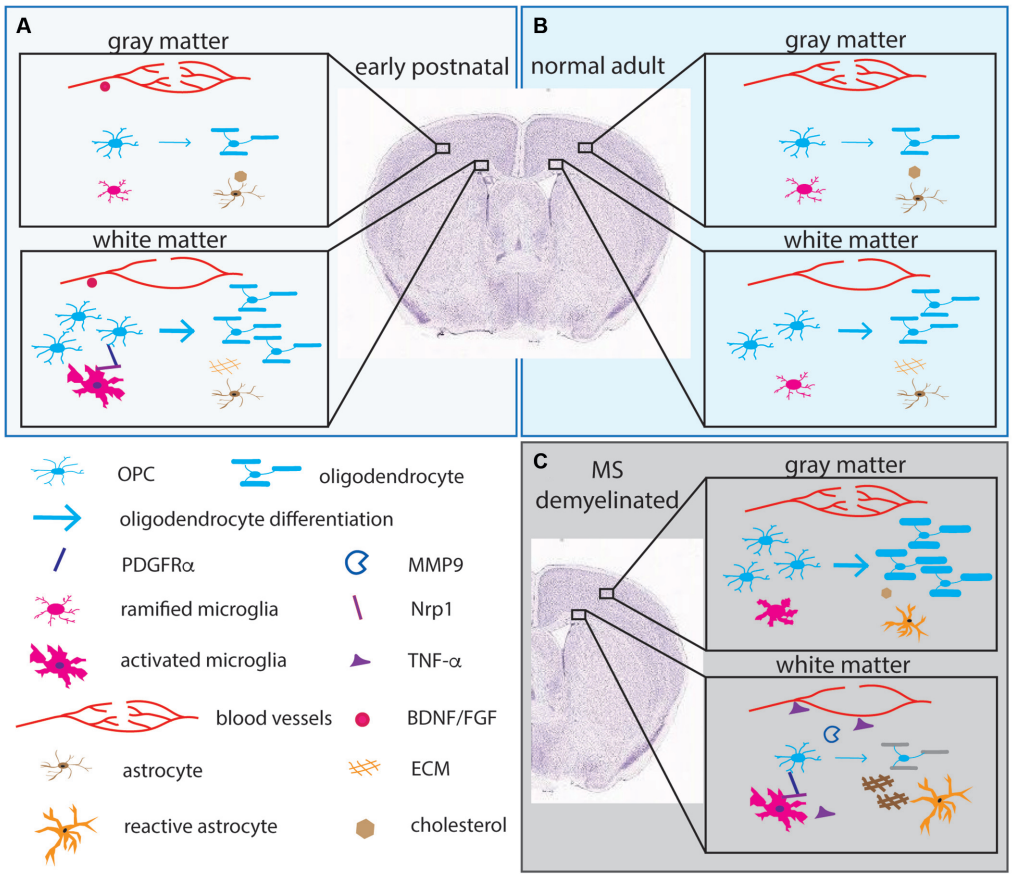

FIGURE 2 | The pericellular microenvironment affecting OPC dynamics under different conditions. **(A)** (upper left box, pale blue). Early postnatal brain **(B)** (upper right box, light blue). Normal adult brain **(C)** (lower right box, gray). Demyelinated lesions in MS brain or experimentally demyelinated lesions. OL lineage cells and their protein products (PDGFRα, MMP9) are depicted in shades of blue. Microglia and their products (Nrp1, TNF-α) are indicated in shades of pink. Astrocytes and their products (cholesterol, ECM) are indicated in orange/brown. Blood vessels and their products (BDNF, FGF) are in red. Arrows indicate differentiation into myelinating OLs. The thickness of the arrows indicates the extent of differentiation. The thick myelin in the gray matter in **(C)** represents successful remyelination. The gray myelin in the white matter in **(C)** represents failure to remyelinate in white matter. The depiction is meant to illustrate examples of the differences and is not meant to be a comprehensive illustration. ECM, extracellular matrix; FGF, fibroblast growth factor; BDNF, brain-derived neurotrophic factor; PDGFRα, platelet-derived growth factor receptor alpha; MMP9, matrix metalloproteinase-9; Nrp1, neuropilin-1; TNF-α, tumor necrosis factor alpha.

early postnatal (P1-P8) corpus callosum contains amoeboid microglia, also referred to as the "fountain of microglia". These are morphologically distinct from the ramified microglia in the cortex and have the transcriptional signature of activated phagocytic cells (Hagemeyer et al., 2017; **Figure 2**). They are likely to correspond to the phagocytic microglia seen by electron microscopy that were seen to have ingested "spongioblasts", which are likely to have been OPCs (Ling, 1976). Depletion of microglia with an antagonist to colony stimulating factor 1 receptor (CSF1R) during the first postnatal week reduces the number of OPCs and OLs (Hagemeyer et al., 2017). Another study showed that a similar population of activated microglia in the early postnatal corpus callosum that express CD11c secrete insulin-like growth factor 1 (IGF1) and play a critical role in developmental myelination (Wlodarczyk et al., 2017). Subsequently published two scRNA-seq studies revealed a microglial subtype that exists in the postnatal white matter that is transcriptionally distinct from developing cortical microglia or adult resting/homeostatic microglia (Hammond et al., 2019; Li et al., 2019). These cells have been termed axon-tract-associated microglia (ATM; Hammond et al., 2019) or proliferative-region-associated microglia (PAM; Li et al., 2019). They express CD68 and other newly identified signature genes such as Spp1 and Clec7A.

Independently, we have shown that activated microglia in the postnatal white matter (ATM/PAM) but not ramified microglia in the cortex express neuropilin-1 (Nrp1), a co-receptor for vascular endothelial cell growth factor (VEGF; Pellet-Many et al., 2008) as well as PDGFRα (Ball et al., 2010; Sherafat et al., 2021). After a demyelinating injury, Nrp1 is strongly upregulated on activated microglia, and microglial Nrp1 deletion severely compromises OPC recruitment and subsequent remyelination (Sherafat et al., 2021). Furthermore, exogenous Nrp1 promotes PDGF AA-mediated OPC proliferation, most potently under

conditions of limited amounts of PDGF AA, and exogenous Nrp1 increases tyrosine phosphorylation of PDGFRα on OPCs (Sherafat et al., 2021), suggesting that Nrp1 on ATM/PAM activates PDGFRα on adjacent OPCs in trans (**Figure 2**). While the mechanism that causes the ATM/PAM to adopt an activated phenotype in the early postnatal corpus callosum remains unknown, apoptosis of early-born ventrally derived OPCs (Orduz et al., 2019) or a sudden local drop in OPC density due to their rapid OL differentiation could trigger their appearance. Future studies could be directed toward elucidating the molecular signaling mechanisms that lead to the emergence of ATM/PAMs in the developing white matter in the context of a homeostatic process for maintaining the correct level of myelin and myelinating cells in the CNS. In demyelinated lesions, the tissue-damaging pro-inflammatory effects of activated microglia, including secretion of inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) are offset by the repair-promoting functions of microglia in a finely coordinated fashion (Lloyd et al., 2019; **Figure 2**).

Regional Heterogeneity of Brain Microvessels and the Oligovascular Niche

Evidence is accumulating for a functional interplay between OL lineage cells and the vasculature (**Figure 2**). In the developing CNS, OPCs migrate along blood vessels (Tsai et al., 2016), and factors from endothelial cells such as fibroblast growth factor 2 (FGF2) and brain-derived neurotrophic factor (BDNF) promote OPC survival and proliferation (Arai and Lo, 2009). Reciprocally, under pathological conditions such as inflammation, OPCs secrete matrix metalloproteinase-9 (MMP9), which increases blood-brain barrier permeability and in turn trigger further tissue damage such as demyelination (Seo et al., 2013). This has led to the concept of the "oligo-vascular niche", which extends the original concept of the "neurovascular niche" to include OL lineage cells. Recent findings suggest that there is reciprocal signaling between OPCs and vascular mural cells (Miyamoto et al., 2014; Kishida et al., 2019), and that neuronal activity affects myelination through altered cerebral blood flow (Swire et al., 2019).

In rat, the capillary density of the cortex is 3–5 times greater than that of the white matter and correlates with glucose utilization (Borowsky and Collins, 1989; **Figure 2**). As pial capillaries perforate the cortex during embryonic development, they bring with them meningeal mesenchymal tissue which remains in the adult as the Virchow-Robin complex (Marin-Padilla, 2012). These regional differences in the capillary network could contribute to the regional differences in the functions of OL lineage cells. We recently reported that 94% of OPC processes have close contact with blood vessels in the neocortex of adult mice, and conversely 92% of the vascular segments are contacted by OPC processes (Pfeiffer et al., 2021), suggesting a significant functional interaction between OPCs and vascular mural cells. It remains to be determined whether there are regional differences in the extent of physical relation between OPCs and the vasculature and the functional significance of the OPC-vascular contacts.

REGIONAL HETEROGENEITY IN MS LESIONS

Since the original description of patients with MS by Jean-Martin Charcot[1] in 1868 and an earlier drawing by Sir Robert Carswell in 1838[2], MS has been known as a disease that affects the white matter, causing demyelination and subsequent scarring and atrophy. More recent studies indicate that MS also affects the gray matter (Brownell and Hughes, 1962). Myelin basic protein immunohistochemistry revealed frequent cortical demyelinated lesions in autopsied brains from active (Peterson et al., 2001) and chronic (Albert et al., 2007) MS patients. Cortical lesions are more commonly seen in a subpial location and contain fewer inflammatory cells. Furthermore, the extent of cortical demyelination is significantly greater in patients with secondary progressive disease of long duration and in primary progressive MS, and pathological hallmarks for neuronal degeneration and apoptosis are present in cortical lesions (Peterson et al., 2001). The subpial cortical demyelination occurs specifically in MS and not in other conditions similar inflammatory mediators and oxidative tissue damage are involved (Junker et al., 2020).

Despite the widespread nature of cortical demyelination, remyelination is significantly greater in cortical lesions than in white matter lesions, particularly in chronic lesions (Albert et al., 2007). This is accompanied by a greater abundance of OPCs and OLs in cortical lesions compared to white matter lesions (Albert et al., 2007; Chang et al., 2012; **Figure 1**). Curiously, the converse is true for normal-appearing tissues in MS brains, where OPCs and OLs are found in greater numbers in normal appearing white matter than in normal appearing gray matter (Strijbis et al., 2017), similar to their distribution in normal rodent brains.

What makes OPCs in the gray matter more competent to respond to demyelinated lesions and promote repair? Some observations suggest inherent differences between OPCs in the cortex and white matter in their ability to respond to inflammatory and recruitment signals. For example, OPCs isolated from rat gray matter are more immature and are less responsive to the inhibitory effects of inflammatory cytokines such as interferon gamma (IFN-γ) and TNF-α on OL differentiation (Lentferink et al., 2018). It is also interesting to note that OPCs that develop later from dorsal germinal zones are recruited more robustly to demyelinated lesions than ventrally derived OPCs (Crawford et al., 2016). A recent scRNA-seq study on OL lineage cells from the white matter of postmortem brain from MS patients revealed a significant reduction in the number of cells with the gene expression profiles of OPCs and intermediate cells that are between OPCs and newly formed premyelinating OLs (Jäkel et al., 2019). Perhaps, gray matter OPCs that have not gone through as many rounds of replication as white matter OPCs suffer less from replicative senescence. A transcriptomic comparison between OL lineage

[1]Charcot JM. Leçons de 1868; Manuscrits des leçns de JM Charcot.: Fonds numérisé Charcot. Bibliothèque de l'Université Pierre & Marie Curie (http://jubilotheque.upmc.fr/subset.html?name=collections&id=charcot).

[2]Pathological Anatomy Illustrations of the Elementary Forms of Disease, London: 1838. https://www.gla.ac.uk/myglasgow/library/files/special/exhibns/month/oct2003.html.

cells in gray and white matter MS lesions may provide some answers to this.

On the other hand, there is quite strong evidence that suggests that environmental factors play a major role in shaping OL lineage cell dynamics that differ between gray and white matter MS lesions. White matter MS lesions contain more extensive reactive astrogliosis and more abundant inhibitors of myelination such CD44 and hyaluronan, many of which are produced by astrocytes (Chang et al., 2012). In addition, inflammatory cells including leukocytes and microglia are more prevalent in white matter lesions and provide an importance source of ECM proteins that hinder myelin repair (Ghorbani and Yong, 2021). After acute demyelination in the white matter, the ability of activated microglia and macrophages to successfully undergo efflux of ingested cholesterol influences the efficiency of subsequent remyelination, and this declines with age (Cantuti-Castelvetri et al., 2018). Furthermore, sterol synthesis in phagocytes is necessary to activate LXR signaling to trigger cholesterol efflux and promote resolution of inflammation and subsequent myelin repair (Berghoff et al., 2021).

Intriguingly, the distribution of cortical lesions appears to coincide with the distribution of the vascular supply (Kidd et al., 1999). The meninges have recently been shown to contain a supply of glial progenitor cells that migrate into the CNS parenchyma and contribute to oligodendrogliogenesis (Dang et al., 2019). These progenitor cells from the meninges could be a more efficient source of remyelinating OLs for subpial cortical MS lesions than the neural progenitor cells in the subventricular zone. Future studies may be directed toward understanding how the meningeal inflammatory infiltrates in cortical MS lesions affect the ability of meningeal progenitor cells to migrate into the cortex and contribute to myelin repair. The greater abundance of blood vessels in the cortex and their associated Virchow-Robin space could play an important pathophysiological role in the dynamics of OL lineage cells in the demyelinated cortex.

CONCLUDING REMARKS

Despite the phenotypic variation of OPCs in different neuroanatomical regions, there has so far been no definitive evidence that OPCs can be segregated into distinct subtypes based on permanent changes in gene expression. Rather, OPCs weave in and out of different cell and functional states depending on their physiological context and microenvironment. The specific signals that cause OPCs to alter their functional states have yet to be elucidated. All the elements surrounding OPCs, including neurons, non-neuronal cells, vascular cells, and the ECM exert specific effects on OPC dynamics, and the nature of these signals can change dramatically under certain pathological conditions.

AUTHOR CONTRIBUTIONS

AS wrote a draft of the manuscript. FP wrote a draft of the manuscript on the vasculature ("Regional Heterogeneity in MS Lesions" section). AN edited the manuscript. All authors contributed to the article and approved the submitted version.

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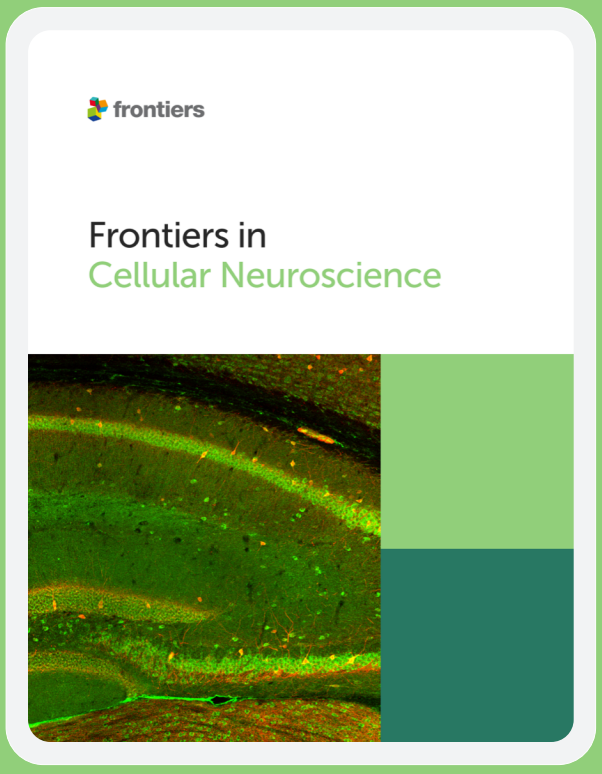

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