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Review

Microglia and Astrocytes in Disease: Dynamic Duo or Partners in Crime?

Shane A. Liddelow [1,2,3,*] Samuel E. Marsh [4,5,6,*] and Beth Stevens [4,5,6,7,*]

Microglia–astrocyte interactions represent a delicate balance affecting neural cell functions in health and disease. Tightly controlled to maintain homeostasis during physiological conditions, rapid and prolonged departures during disease, infection, and following trauma drive multiple outcomes: both beneficial and detrimental. Recent sequencing studies at the bulk and single-cell level in humans and rodents provide new insight into microglia–astrocyte communication in homeostasis and disease. However, the complex changing ways these two cell types functionally interact has been a barrier to understanding disease initiation, progression, and disease mechanisms. Single cell sequencing is providing new insights; however, many questions remain. Here, we discuss how to bridge transcriptional states to specific functions so we can develop therapies to mediate negative effects of altered microglia–astrocyte interactions.

Changes in Microglia and Astrocyte Function in Health and Disease

Microglia (see Glossary), the resident myeloid cells of the mammalian central nervous system (CNS), colonize the CNS early in embryonic development. They are highly motile cells that interact with all cells in the CNS to mediate normal development, homeostasis, and general brain physiology. Comprising between 5% and 10% of the total number of CNS cells in humans and mice, they are integral to many physiological processes [1]. By contrast, **astrocytes** comprise at least 50% of the brain and spinal cord cells by number in humans and mice. Moreover, astrocytes are fundamental to normal CNS health and functioning [2]. They are required for synapse formation, maturation, and maintenance (reviewed in [3]) and once they begin providing trophic support to neurons, they cannot be removed without disastrous consequences [4]. Although locked in place and unable to move, astrocytes tile the parenchyma with largely non-overlapping domains and are connected by gap junctions between their infinitesimally fine-branched processes, rapidly signaling from cell to cell via calcium-mediated information waves (see [2] for review). Both microglia and astrocytes have robust and profound responses to changes in the normal physiology of the CNS via a response termed '**reactivity**' or 'activation' (Box 1). Changes in glial morphology have been observed by pathologists and neurobiologists in fixed brain tissue for decades. Recent analyses of human brain samples reveal striking changes in microglia and astrocyte transcriptomes in response to a wide range of diseases, infection, and trauma; however, precisely how microglia and astrocytes contribute to specific aspects of disease pathogenesis remains a crucial question. How heterogeneous are microglia and astrocytes in health and disease? How do astrocytes and microglia communicate? How dynamic and reversible are these disease-associated states?

An explosion of new 'omic' approaches and tools to profile and spatially map glial states reveal many different subsets of reactive glia that are present in individual diseases, or across several diseases, and have expanded our understanding of their phenotypes and insights into potential functions. During disease, infection, and following trauma, microglia and astrocytes exhibit altered gene expression profiles that are predicted to affect their function and, in turn, the health of

Highlights

Microglia are resident immune cells of the brain, which derive from a different cell lineage to all other cells in the brain. They are highly motile cells, constantly patrolling the brain parenchyma.

Astrocytes are the largest cell component of the brain and develop from a common progenitor along with neurons and oligodendrocytes. They tile the entire brain and do not migrate during normal physiology. These two cell types are important for normal mammalian brain development and respond rapidly to disease, infection, and trauma.

Microglia and astrocytes interact via contact-dependent and secreted factors to modulate their function during normal health and in disease. Microglia can drive reactivity in astrocytes via the release of specific cytokines, while astrocytes can drive dysfunction in microglia by withholding cholesterol.

Many tools exist to manipulate both microglia and astrocytes, however, complete removal of astrocytes is currently impossible as this results in death.

scRNASeq experiments must be both adequately powered and take into account possible artifacts as a result of subsampling when disseminating results. Ideally, cluster-specific differentially expressed genes should be validated using visualization methods (*in situ* hybridization or spatial transcriptomic approaches) and functional assays.

Caution should be taken in the nomenclature of different 'activation' states of both microglia and astrocytes. While no method is perfect, the field needs to clearly state what constitutes a subset of cells: biologically relevant and functionally characterized descriptions will be the most beneficial.

neurons and other CNS cells; however, there is still a large gap between specific transcriptional states and function. Ultimately, we must move towards a 'functional' understanding of these cells: cataloguing what they can or cannot do under different biological settings (Figure 1, Key Figure). Improved methods to study these functions in culture, in **organoids**, and using stem cells, in combination with new tools to image, isolate, and target both microglia and astrocytes *in vivo*, have provided a wealth of new information about how these two integral CNS glial cells contribute to brain health and development, interact with one another, and mediate injury and disease. Here we highlight several recent exciting discoveries and potential translational paths forward.

Microglia and Astrocyte Trophic Support

The complex interplay between microglia and astrocytes is established during development and changes dramatically in the context of disease, infection, injury, and normal aging. Much of our understanding of both microglia and astrocyte function, as well as their interactions, has largely come from using isolated cell culture systems to study their gene expression, protein amounts, and cellular functions. The earliest of these methods relied on enzymatic digestion and supplementing media with serum components [5,6]. These methods (and variations), while in hindsight not ideal, have provided a wealth of important information about these cells. Methods for the isolation of microglia [7,8] and astrocytes [5] have been improved to enable faster isolation without artifacts induced by enzyme digestion [7] and serum-free culture systems [9,10]. These methods have highlighted non-serum trophic support molecules such as colony stimulating factor 1 (CSF-1), interleukin 34 (IL-34), transforming growth factor beta 2 (TGF-β2), and cholesterol for microglia [10]; and Heparin-Binding EGF-like growth factor (HBEGF) for astrocytes [9]. Microglial trophic support largely comes from fibroblasts (IL-34), endothelial cells (TGF-β2), and astrocytes (cholesterol), and adult *Csf1r*$^{-/-}$ mice that lack the CSF-1 receptor or IL-34 have low numbers of microglia compared with wild type (WT) mice [11,12]. While fibroblasts and endothelial cells likely produce the majority of these microglia trophic factors in the adult brain, developmentally, astrocytes also produce sufficient amounts of all three factors to maintain microglia in mice and in humans [13–16]. In astrocytes, the trophic molecule HBEGF is provided by endothelial cells (with which **astrocyte endfeet** have a close association) [9,17,18]. Although astrocytes can generally maintain gene expression profiles and function at a physiological level without microglia-derived signals, microglia appear to have 'normal' physiological profiles tethered to astrocyte-derived trophic support [10]. This, however, does not mean that this interaction occurs in only one direction. It is also apparent that signaling between microglia and astrocytes is often mediated by secreted signals interacting with cell-specific receptors (see later). But how do interactions between microglia and astrocytes change in response to disease, infection, and injury?

Bi-directional Astrocyte–Microglia Communication

The microglial receptor TREM2 (triggering receptor expressed on myeloid cells 2) has diverse functions, including mediating the uptake of cholesterol bound in apolipoprotein lipoparticles [both APOE and Clusterin (CLU/APOJ)] [19,20]. Lack of TREM2, or disease-associated *TREM2* mutations, can inhibit recognition and uptake of cholesterol-containing lipoparticles [21]. For normal physiological function and trophic support, the interaction of TREM2 with lipoparticles is therefore integral for the transfer of cholesterol from astrocytes to microglia [10]. Multiple mutations in human *TREM2* increase Alzheimer's disease (AD) risk [22–26]. Along with *APOE* [27,28] and *CLU* [29–33], these three genes represent some of the strongest disease-causing genes associated with AD. While *APOE* and *CLU* are largely expressed by astrocytes during homeostasis [17], *Apoe* is dramatically upregulated by microglia during response to injury or disease [8,34,35].

[1]Neuroscience Institute, NYU School of Medicine, New York, NY, USA
[2]Department of Neuroscience and Physiology, NYU School of Medicine, New York, NY, USA
[3]Department of Ophthalmology, NYU School of Medicine, New York, NY, USA
[4]F.M. Kirby Neurobiology Center, Boston Children's Hospital, Boston, MA, USA
[5]Harvard Medical School, Boston, MA, USA
[6]Stanley Center for Psychiatric Research, Broad Institute of MIT and Harvard, Cambridge, MA, USA
[7]Howard Hughes Medical Institute, Boston Children's Hospital, Boston, MA., USA

*Correspondence:
shane.liddelow@nyulangone.org (S.A. Liddelow),
samuel.marsh@childrens.harvard.edu (S.E. Marsh), and
beth.stevens@childrens.harvard.edu (B. Stevens).

Box 1. What Is in a Name?

The field of immunology has long benefited from distinct cell-surface markers to characterize individual cell types. For instance, T helper 17 cells (Th17) are proinflammatory T cells, so-named because they produce IL-17 [139]. The discovery of a marker to distinguish these cells from other immune cells enabled the identification of mediators that drive their production (IL-6 and TGFβ in the case of Th17 cells), contributing to an understanding of their function.

Neuroscience has been less successful in providing similarly comprehensive descriptions of cell subtypes. This has been partly due to a lack of cell-surface antigens, making isolation and sequencing difficult. There has also been considerable trouble in maintaining these cells in culture systems to recapitulate the specific milieu of the brain, resulting in the functional understanding of subsets of microglia and astrocytes lagging behind a growing number of new naming conventions. Early attempts at naming activation states of microglia using the now obsolete M0/M1/M2 terminology was based on *in vitro* studies, but became problematic as complexity and dynamic states of microglia were revealed. Similarly, in astrocytes, an A1/A2 nomenclature gained some traction, but is likewise overly simplistic. More recent naming schemes along disease (disease-associated microglia [34]) or around pathology (plaque-induced genes [140]) have also been met with some resistance based solely, in some instances, on a dislike for any one particular naming convention. Recent investigation shows a lack of correlation between rodent and human studies [141] and suggests that care needs to be taken in associating subtypes based on models of disease rather than on the disease itself.

In astrocytes, while naming conventions are less expansive than in microglia, some additional fidelity is required: reactive astrocytes that form rapidly following acute injury are called 'scar-forming', 'STAT3-dependent', and 'A2', among other names [69,100,142], albeit with what appears to be similar changes in gene expression. Similarly, 'A1', 'proinflammatory', 'aging-associated', 'neurodegenerative', or 'neurotoxic' reactive astrocytes are also used interchangeably [58,97,98,100,143,144]. Problems also arise when original comprehensive transcriptomic and functional characterization of specific subsets in one study or context are less well studied in follow-up studies, leading some to incorrectly attribute new functions, or to generate new nomenclature unnecessarily. Both outcomes are problematic for newcomers and residents of the field alike.

We must combine efforts to consolidate already present (and functionally validated) nomenclature and work collaboratively to generate new comprehensive naming conventions. Though far more difficult, we must also work to connect the dots between transcriptional states and specific functional states. Combined approaches are needed to describe subclasses of microglia and astrocytes on the basis of transcriptomic, proteomic, and functional changes, as well as on the basis of localization to particular CNS regions or to association with pathology (see Figure 1 in main text).

Recent work highlights the fact that astrocytes derived from human induced pluripotent stem cells (iPSCs) taken from AD patients with different isoforms of *APOE* have varying capacities in producing and delivering cholesterol to microglia [36]. These iPSC-derived astrocytes expressing the AD risk variant *APOE4* have increased lysosomal cholesterol amounts and decreased cholesterol efflux relative to astrocytes derived from *APOE2 and APOE3* iPSCs; this, in turn, causes increased secretion of proinflammatory chemokines and cytokines such as CXCL10, RANTES, and IL6. In this study, it remained unclear if these factors were derived from microglia or astrocytes, as these were measured using a mixed culture system [36]. Given that microglia require cholesterol to maintain a physiological state [10], a lack of astrocyte-derived cholesterol is likely responsible for driving this microglial phenotype. Thus, future investigations should be undertaken to test the effects of different isoforms of *APOE* (2/3/4), as well as mutations in the *APOE* gene on each cell type determining the signals and mechanisms driving bi-directional microglia–astrocyte communication in different contexts. Of note, recent work also demonstrated that microglial TREM2 mediates developmental **synaptic pruning** and function in mice [37,38]. This raises questions about the potential involvement and underlying mechanisms of microglia and astrocytes in these same processes beyond synapse pruning.

In addition to its role in lipid metabolism, APOE is present within amyloid plaques in the CNS in patients with AD and in amyloid precursor protein (APP) transgenic mice (a model for AD) [39,40]. Until recently, the cellular source of this APOE was unknown; based on expression studies during homeostasis, astrocytes initially seemed the likely culprit. However, recent studies using combinations of bulk and single cell sequencing (**scRNASeq**) found that microglia

Glossary

5xFAD: mouse model of Alzheimer's disease based on amyloid deposition. The mouse contains five familial Alzheimer's disease mutations [three in amyloid precursor protein (*APP*) and two in presenilin-1 (*PSEN1*) under the control of neuronal-specific Thy1 promoter] driving amyloid production and deposition.
APP/PS1 (Thy1): mouse model of AD based on amyloid deposition, similar to 5xFAD. APP/PS1 (Thy1) mice express single familial *APP* and single *PSEN1* mutations under the control of neuronal-specific Thy1 promoter.
Astrocyte: large, postmitotic macroglia comprising ~50% of cells in the CNS in mammals.
Astrocyte endfeet: terminal processes of astrocytes that interact with blood vessels. They have a high concentration of water channels.
C1q: (complement component 1q) complex glycoprotein complement component protein that associates with C1r and C1s to form the C1 complex. The C1 complex triggers the classical complement pathway. In the CNS, C1q also labels synapses for removal by microglia.
Complement: selection of proteins (the complement system) in the innate immune system; incorporates antibodies and phagocytic cells to clear cell debris, pathogens, and promotes inflammation. In the CNS, complement is also used for promoting synapse pruning by microglia during development and in neurodegenerative disease.
CSF1R: protein encoded by *Csf1r*: type III receptor tyrosine kinase binding CSF1 and IL-34. Essential for the survival and proliferation of many myeloid cells, including microglia.
Droplet transcriptomic approaches: single cell transcriptomic methods using microfluidics to partition single cells or nuclei into nanoliter droplets (e.g., Drop-seq; 10X Genomics).
Frontotemporal dementia: umbrella term referring to a number of neurodegenerative diseases characterized by degeneration of the frontal and temporal lobes of the brain.
GFAP: protein encoded by *Gfap*; intermediate filament protein present in astrocytes in the CNS, but also in ependymal and radial glial cells during development.

Key Figure

Microglia and Astrocyte Interactions in Health and Disease Are Context-Dependent

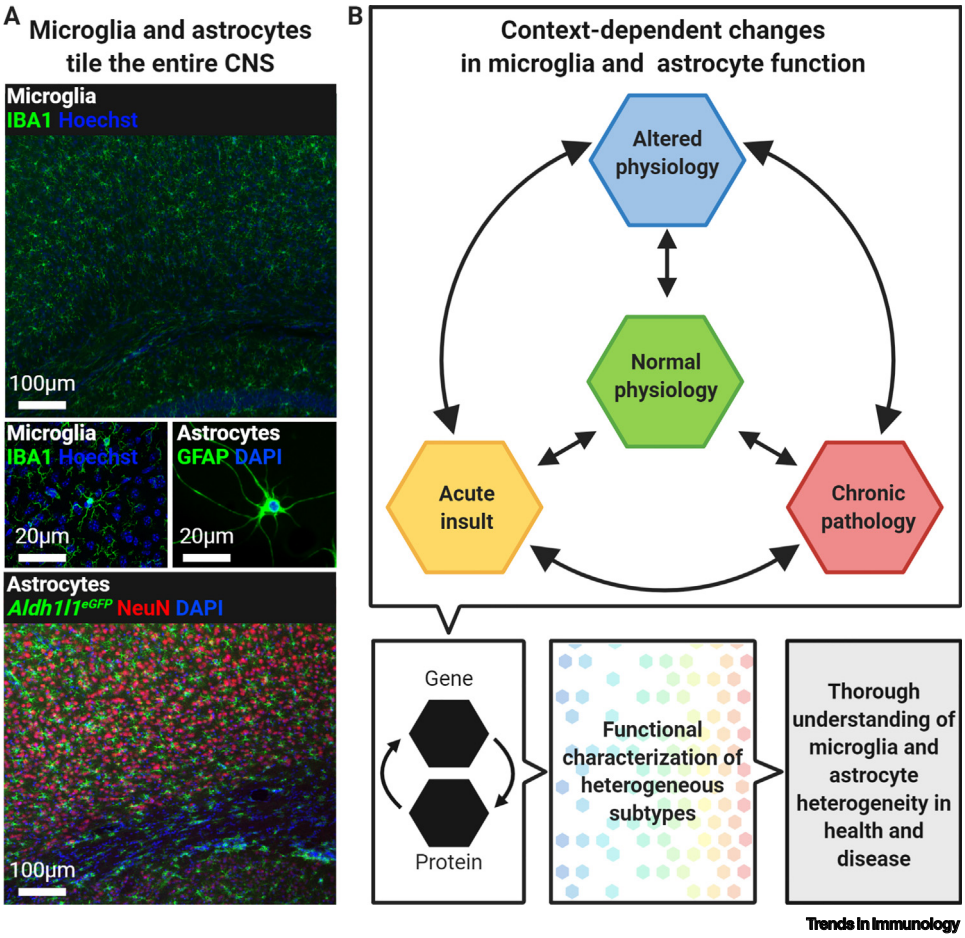

Figure 1. (A) Microglia and astrocytes both have complex morphology that alters in response to changes in normal physiology. They also tile the entire central nervous system (CNS) with non-overlapping domains. Shown here are representative confocal micrographs of mouse cortex microglia; these have been stained with IBA1, and astrocytes; stained with GFAP (in vitro) or visualized by green fluorescent protein (GFP) driven by an *Aldh1l1* promoter. (B) The interaction between microglia and astrocytes may change dramatically depending on context. For instance, physiological contexts may present different interactions between microglia and astrocytes and other CNS cells than during states of altered physiology (e.g., development or aging), acute insult (e.g., trauma or infection), or in chronic pathological conditions (e.g., neurodegenerative diseases). These functional interactions may be sustained or transient. This context is important when inferring changes in gene or protein amounts and how they might affect one another. Ultimately, these changes in the context of development, aging, infection, trauma, or disease should be used to characterize functional changes in microglia and astrocyte subtypes. This figure was created using BioRender (https://biorender.com/). Microglia images courtesy of Dr Daniel Wilton. Astrocyte images courtesy of Rachel Kim.

Glutamate excitotoxicity: pathological process in which excess glutamate is not cleared from the synaptic cleft, causing damage and death of neurons.
Ionized calcium binding adaptor molecule 1 (IBA1): protein encoded by *Aif1*; calcium binding protein present in microglia and macrophages, commonly used for visualization of microglia.
Microglia: small, motile, resident innate immune cell comprising around 5–10% of the CNS in humans and mice.
Multiplex smFISH: (single molecule fluorescence *in situ* hybridization) method enabling visualization of single RNA molecules using targeted probes. Used to provide important validation and spatial context for scRNASeq data.
Oligodendroglioma: tumor of the brain or spinal cord; formed by oligodendrocytes.
Organoids: miniaturized version of an organ, made from differentiated stem cells and maintained in a culture dish. Organoids of nervous tissue can include combinations of neurons and glia.
Prion infection: prions are small misfolded proteins that can propagate their misfolded shape. Prion infection refers to infection of an organ or tissue with a misfolded prion protein.
Reactivity: response of microglia or astrocytes to an external stimulus, normally noxious in nature (e.g., bacterial or viral infection, acute trauma, or pathology associated with disease).
scRNASeq: method that allows measurement of expression of genes in individual cells or nuclei (snRNASeq).
Synaptic pruning: removal of excess synapses by microglia and astrocytes. Most evident during development or disease.

significantly upregulate *Apoe* in a variety of mouse models of injury or disease [8,34,35]. In particular, studies in several AD mouse models [5xFAD, **APP/PS1 (Thy1)**, and variations, including *Trem2*$^{-/-}$ among others] highlight that plaque-associated microglia exhibit the highest upregulation of *Apoe*, raising many questions about the mechanisms of induction and the

functional consequences [34,35,41–43]. Additionally, *Trem2*$^{-/-}$ mice, that carry a loss-of-function variant (T66M) common to **frontotemporal dementia**, exhibit significantly decreased plaque-associated APOE relative to WT mice and similar findings were observed in human AD patient postmortem brain samples bearing *TREM2* risk variants [43]. This plaque-associated APOE may be central to both the microglial response and the accumulation of amyloid, such that targeting APOE may be a viable putative therapeutic mechanism (see Clinician's Corner). Using the APP/PS1 (Thy1) amyloid mouse model of AD crossed to the human *APOE4* mouse, intracerebral infusion with a novel antibody that preferentially targets the nonlipidated aggregates of APOE found in amyloid plaques, was sufficient to reduce amyloid deposition as pathology progressed [44].

Uncertainty remains regarding the role of APOE from both microglia and astrocytes, likely stemming from the fact that these cells have usually been investigated in isolation with minimal understanding of their functional interactions in the context of disease. It is also unclear how well each mouse model of AD recapitulates these functions across species. Nevertheless, there is agreement that in the presence of APOE4 in both rodent models and in human patients with AD, there is higher microglia and astrocyte reactivity, as well as increased CNS pathology [45–49]. In addition, clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9-edited human iPSC-derived *APOE4* astrocytes harbor decreased cholesterol amounts and impaired uptake of amyloid relative to *APOE2* and *APOE3* expressing astrocytes; furthermore, *APOE4* microglia in the same system also exhibit decreased phagocytic capacity compared with *APOE2* and *APOE3* expressing microglia [49]. How the timing and extent of these phagocytic dysfunctions may individually contribute to disease progression and pathology remains unknown. More robust work is needed to understand the complexity of the interactions between microglia and astrocyte-secreted APOE, but the shared nature of the response across multiple disease/injury conditions suggests there may be common pathways that reflect a general program of glial responses to dyshomeostasis.

Similar interactions between microglia and astrocytes in the context of disease have also been reported in mice models of a wide range of pathologies, including inflammation [50], AD [45,50–52], other neurodegenerative diseases such as Parkinson's and Huntington's diseases [53–55], in cancer [56,57], and in **prion infection** [58] or infection with neuropathogenic flaviviruses such as Zika virus and West Nile virus [59–62]. In each instance, microglial cytokine release is paired with some form of astrocyte reactivity, measured by either glial fibrillary acidic protein (**GFAP**) increase, or, in more recent studies, by whole transcriptomic sequencing [55] and functional investigation [45,50,54]. And, while most descriptions of cytokine release and signaling position microglia as a driving force, there is evidence that astrocytes can also act as a major source of immune cell chemoattractants such as CXCL10 and RANTES [63–65].

These responses are often described only in a correlative sense in both animals models and in human patients [i.e., by leading to changes in cell morphology, or in the upregulation of the quintessential markers of reactivity: Ionized calcium binding adaptor molecule 1 (**IBA1**) for microglia and GFAP for astrocytes]. It should be noted, however, that these markers are also present in homeostatic microglia and astrocytes and, as such, their use as markers of 'reactivity' should not be used as a gold standard [66]. This is particularly important given our current understanding of heterogeneity as IBA1 and GFAP are present across several substates of microglia and astrocytes. However, these markers, combined with measurements of changes in cytokine release (from both microglia and astrocytes), as well as measurements of proliferation, changes in protein **complement**, and in some cases, ablation of cells, have been used to determine the functional

role of these cells. Removal of microglia is most often achieved by taking advantage of *Cx3cr1*^{CreER} mice [67] to drive microglia-specific expression of a tamoxifen-inducible diphtheria toxin receptor (DTR), or inhibitors of **CSF1R** to induce large-scale microglial apoptosis [68]. Astrocyte ablation has been less fruitful, likely due to their neuron trophic support role, although this possibility remains to be tested; however, ablation of specific subtypes of reactive astrocytes, using *Gfap*-targeted *Stat3*-depended DTR expression, has proven useful in defining their roles following spinal cord injury in mouse models [69]. These approaches report important roles of microglia and astrocytes in mediating both 'positive' and 'negative' responses to damage, but also suggest that responses to injury or pathology are complex and heterogeneous. For instance, depletion of microglia using a CSF1R inhibitor show no change [70], as well as decreases [71] in plaque pathology in the hippocampi of 5xFAD mice, both with improved neuronal survival depending on the age and length of administration of inhibitor. This improvement in neuronal viability and cognitive ability is also reported in microglia depletion with aging [72], following stroke [73], and in numerous other mouse models of neurodegeneration. In mice that lack *Stat3*-dependent reactive astrocytes (generated by crossing *Gfap*^{Cre} and *Stat3*$^{fl/fl}$ lines), there is extensive axonal dieback, suggesting that these reactive astrocytes are beneficial in promoting recovery from injury in the spinal cord [69]. By contrast, removal of these same astrocytes had no effect on pathology in the 3xTg mouse model of AD [74] (though it remains unclear if this model of AD recapitulates the astrocyte phenotype found in human AD patients, or if the STAT3-mediated reactive astrocyte phenotype is present in patients). Similarly, block of neurotoxic reactive astrocytes appeared to be beneficial in a model of Parkinson's disease (in which preformed fibrils of alpha synuclein are used to seed pathology in mouse brain) [54] and in axotomy to the optic nerve in mice and rats [75]; however, another study also highlighted that these reactive astrocytes were likely to be important in slowing the progression of prion infection in the mouse brain [58].

Accordingly, signaling between microglia and astrocytes in the context of chronic neurodegenerative disease drives many different transcriptomic, proteomic, and functional responses. One form of reactive astrocytes is directly mediated by **C1q** complement, as well as IL1α and TNFα cytokine release by microglia; it appears to drive neuronal death and synapse density loss in mice [50,51,76,77]. These studies, while describing a number of key functional changes *in vitro* with some validation *in* vivo, still require additional investigation to determine the exact pathways driving each functional change. It should also be noted that while an astrocyte-derived neurotoxin mediates this neurodegeneration, the identity of a specific neurotoxin/s has yet to be found. Alternate hypotheses for neuron death include **glutamate excitotoxicity**, or loss of astrocyte-trophic support due to ion dyshomeostasis. It is possible that all three mechanisms exist at different stages of the disease response and this would suggest multiple potential targetable pathway(s) to treat certain neurodegenerative conditions. It is most likely that each of these mechanisms has specific context-dependent drivers and this will be an exciting avenue for ongoing research.

Of particular interest are microglia–astrocyte responses in certain cancers, where interactions with the immunosuppressive chemotherapeutic drug methotrexate can kill childhood **oligodendroglioma** cells. Due to advances in isolation and culture of microglia and astrocytes in serum-free conditions, the mechanism of action of this drug is now known: methotrexate drives expression of many cytokine genes by microglia (e.g., ILs, TNF, among others) in mice both *in vivo* and in culture. These cytokines are capable of driving formation of reactive astrocytes (perhaps in a neurotoxic state), which in turn causes apoptosis of oligodendroglioma cancer cells [57]. Whether this effect also relies on susceptibility by the cancer cells remains unknown (as has been recently shown for neurons in axotomy and bead-occlusion glaucoma models in mice

and rats [75]); however, there is likely some off-target neurotoxicity, given that children treated with methotrexate often have learning and memory deficits that could be attributed to death of neurons or destruction of synapses, other known functions of proinflammatory microglia [51] and astrocytes [50]. As another example, susceptibility may likely drive neuronal death in prion infection in the brain, where reactive astrocytes are associated with prion-infected neurons in mice [58]: astrocytes respond to prion infection by upregulation of many genes associated with inflammatory reactive astrocytes and using *Il1a*$^{-/-}$*Tnf*$^{-/-}$*C1qa*$^{-/-}$ mice that lack the cytokines required to activate this subset of reactive astrocytes led to a significant acceleration of prion disease course relative to WT mice. Moreover, while other reactive transcripts in both microglia and astrocytes remained largely unchanged in these animals, several homeostatic markers in microglia (e.g., *Tmem119, P2ry12*) were lost early during the disease course when astrocyte reactivity was blocked [58]. These homeostatic markers were also lost during isolation and culture of microglia [1,7,8,10,78], but the consequences of this loss of 'microglia' signature remains to be determined.

An important question for future studies is whether subsets of reactive astrocytes drive this loss of microglia homeostasis, or if reactive astrocytes that drive neuron death simultaneously maintain microglia function, suggesting they play an important (beneficial) role in the resolution of disease and injury. Many tools that were previously developed to address these questions may no longer be effective, given the heterogeneity of microglia and astrocyte responses. Our original hypotheses based on bulk tissue or cell sequencing must now be refined in light of complex (and possibly alternate) responses to stimuli by cell subsets. As such, some knockout or transgenic mouse lines, or culture systems, will require retooling as they are unable to maintain this complex heterogeneity.

Microglia–Astrocyte Integration within the Peripheral Immune System

While research into intra-CNS interactions between astrocytes and microglia has received considerable attention, relatively little has focused on the potential impact of peripheral immune cells on astrocytes and microglia. Immune cells residing in the brain borders and brain lymphatics [79,80] as well as circulating immune cells, provide alternate avenues for peripheral signals to affect microglia and astrocytes in either a direct or indirect fashion.

For example, amyloid pathology is enhanced in a mouse model of familial AD (**5xFAD** mouse) by genetically preventing the development of adaptive immune cells (B and T lymphocytes and natural killer cells; *Rag2*$^{-/-}$*::Il2rγ*$^{-/-}$) relative to immune-competent 5xFAD mice [81]. This lack of adaptive immunity dramatically affects microglial number, morphology, gene expression, and function. The study found that microglia in immune-competent 5xFAD mice were prominently bound to endogenous mouse antibodies (IgG), which the immunodeficient mice lacked, lacking antibody production. Moreover, the increased amyloid phenotype of the immunodeficient 5xFAD mice could be rescued by nonspecific antibody (IgG) injection or repletion of peripheral immune cells, which then led to IgG binding to microglia. Taken together, these results suggest that many of these phenotypes appear to be mediated by peripheral signaling (antibody, IgG) across the blood–brain barrier, which did not require direct infiltration of peripheral adaptive antibody-producing cells (B cells or plasma cells) [81]. Moreover, a number of other studies have also found that adaptive immune populations may play key roles in CNS injuries (such as stroke) and neurodegenerative disease [such as Parkinson's disease, AD, and multiple sclerosis (MS)] based on a combination of human genetic and functional studies in mouse models [82–89]; however, a more thorough understanding of how these cells interact with and modulate microglia and astrocyte biology is required.

Emerging research also suggests that the gut microbiome contributes to the function of both microglia and astrocytes in disease. For example, the microbiome of patients with MS varies drastically from nonsymptomatic patients. Specifically, relative to healthy subjects, MS patient bacterial load in the gut is dominated by a decline in *Butyricimonas* spp., but increases in *Methanobrevibacter* spp. and *Akkermansia* spp., ultimately driving IFN and TREM signaling pathways in peripheral $CD4^+$ T cells and $CD14^+$ monocytes, as measured by NanoString and pathway analysis [90]. While it is unknown if these changes in gut microbial populations are drivers of, or a response to MS disease pathology, some evidence in rodent studies suggests the former. For instance, investigation into the role of CNS infiltrating T cells in mouse models of demyelination (experimental autoimmune encephalomyelitis) and dopaminergic cell death [modeled by injection of the toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in brain] suggests increased microglia and astrocyte 'reactivity' relative to controls (as measured by IBA1 and GFAP immunohistochemistry) [88,91,92]. In addition, specific metabolites such as short chain fatty acids, type I IFNs, and tryptophan metabolites produced by gut microbes can alter the activity of both microglia [93] and astrocytes [94], including the astrocyte chemotactic ability to promote migration of $CD11b^+LY6C1^{hi}$ monocytes [94]. Combined, these studies suggest that $CD4^+$, $CD8^+$, $CD25^+$ T cells, and perhaps other peripheral immune cells, might act as integral peripheral mediators between the gut microbiome and CNS-resident microglia and astrocytes. However, the role of the microbiome in driving disease pathology remains controversial, as several studies have been poorly controlled or conclusions drawn prematurely [95]. More work on the putative mechanism(s) of this crosstalk is evidently required. Changes in the gut microbiome and the peripheral immune system can affect microglia and astrocyte responses in the CNS; however, precisely how one compartment interacts with the other is still largely unknown. Moreover, going forward, instead of relying on bulk sequencing or unpurified cell culture experiments to determine such interactions, robustly characterizing disease-associated individual subpopulations in both peripheral and central immune and glial cells is needed.

Does Heterogeneity Matter and How Should We Study It?

Profiling microglia and astrocytes using bulk sequencing methods to describe similarities or differences among different brain regions [96–98], across development, aging [7,8,98,99], and in response to injury and disease [7,8,69,100] suggests ways that these cells may interact with each other and with other CNS cells. We have learned about the development and maturation of synapses by astrocytes (reviewed in [3]). We have also learned how excess production of synapses can be developmentally regulated by microglia- [48,86,87] and astrocyte-mediated pruning [51,101–104]; if such a process remains unchecked, aberrant pruning can have deleterious effects in the context of diseases such as AD, as synapse density loss is associated with decreases in memory function [51]. In addition, reactive astrocytes formed following ischemic stroke (middle cerebral artery occlusion) can become more proficient phagocytes: clearing more synaptic debris and possibly pruning excess synapses above normal levels, as visualized using electron microscopy and immunofluorescence for engulfment of fluoro-jade-labeled neuronal debris [105]. This suggests that like many other functional changes achieved in reactive states of microglia and astrocytes, increased phagocytosis can have both positive and negative outcomes for the CNS as a whole, depending on the context of the initiating pathology.

While originally limited to describing a small number of gene expression changes using *in situ* hybridization, or extrapolating microglia- and astrocyte-specific changes from bulk tissue microarrays and RNA sequencing experiments, the advent of transgenic reporter mice has enabled highly specific transcriptomic analyses. From high throughput approaches sequencing whole cells and single nuclei [106,107], to methods that allow detection of chromatin structure [108–110], copy number variation [111,112], and joint profiling of gene expression and chromatin

in the same cells [113,114], options abound for developing powerful datasets on individual cells. We highlight some of these exciting findings, but also common mistakes and caveats, along with suggestions for moving the field forward.

Single Cell RNA Sequencing of Microglia and Astrocytes

Several recent studies have begun to provide a better understanding of mouse and human microglia using scRNASeq technology [8,115,116]. In mice, the most comprehensive study profiled greater than 75 000 microglia across embryonic, early postnatal, adult, aging, and acute injury conditions [8]. Of note, while microglia exhibit heterogeneity in embryonic and early-postnatal development, that heterogeneity is largely lost by adulthood. **Multiplex smFISH** spatially localized several of these unique populations and/or microglial states during development and in injury models; these populations exhibited specific spatial localization patterns [8]. This study also optimized a previously reported tissue dissociation protocol to extract microglia, using mechanical dissociation with cold temperatures to minimize transcriptional changes that might be associated with common enzymatic dissociation protocols [7,8]. In another report, these results were simultaneously confirmed using an alternative method [115].

Previous work has shown that microglia rapidly alter their transcriptional profile during isolation and *in vitro* cultures [10,117]; thus, care needs to be used when isolating microglia. It is crucial that protocols are similarly optimized and standardized for isolating and profiling microglia nuclei from human brain tissue. Perhaps unsurprisingly, there have been fewer commonalities identified across recent human single cell studies than those performed in mice [116,118–121]. Given their low abundance overall in the brain (~5–10% of total brain cells) one of the greatest challenges in analyzing microglia at single cell resolution is both accruing enough cells from each given patient and obtaining a sufficient number of patients to enable proper sample-level analyses across diagnoses or other clinical stratifications (Table 1). It is the combination of these two factors that will be key in the future for identifying rare microglial subtypes and validating their relevance across large numbers of patients.

The use of scRNASeq in the study of astrocytes is also growing; however, there are additional technical concerns. There is an apparent capture problem: astrocytes are excluded from **droplet transcriptomic approaches** such as Drop-seq and 10X Genomics. This is evidenced when calculating capture rates for astrocytes in whole brain/brain region transcriptomics, as astrocytes appear to account for less than 20% of total cell numbers from a whole brain homogenate [18,114,116,118,119,122–126], when they should account for closer to 50% based on ground-truth evidence and visualization studies [127–129] (Table 1). This raises a key question that should be of concern to all in the field: does this low sampling equate to subsampling of a particular cohort of astrocytes that are overly enriched during sorting or in droplet production? This might skew our interpretation of astrocyte heterogeneity and responses due to the potential artifact of sample preparation. It should be noted that while capture rates for microglia are similarly low, they represent more appropriately the percentage of CNS cells one would expect to be microglia (as detected by other methods; see earlier). While this is not definitive proof that subsampling of microglia does not occur, it appears to be less of a problem. Methods that do not rely on generation of reaction droplets, but instead provide sequencing of cells isolated by fluorescent activated cell sorting into individual wells, are still plagued by low numbers of cells in final sequencing due to the low throughput of such methods (despite being able to provide greater capture rates of astrocytes than droplet methods) [130]. However, whether these low capture rates substantially matter remains unclear (Box 2), but analysis of new large datasets suggests novel populations of microglia and astrocytes are likely lost in smaller underpowered datasets (Figure 2).

Table 1. Microglia and Astrocyte Capture Rates in Recent Human Single Nuclei Transcriptomic Studies[a]

Seq type	Patients (*n*)	Category	Total nuclei	Microglia (*n*)	Microglia capture (%)	Average number of microglia/individual	Astrocytes (*n*)	Astrocytecapture (%)	Average number of astrocytes/individual	Refs
Physiological samples										
10X 3′ V3	5	Control	37 226	1273	3.3	255	4178	11.4	836	[148][b]
10X 3′ V2	24	No pathology	35 166	892	2.5	37	1679	4.8	70	[118][b]
10X 5′ V1	11	Control	32 625	1547	4.7	141	2955	9.1	269	[116]
10X 3′ V2	20	Control	52 556	1822	3.1	91	4644	8.8	290	[149]
10X 3′ V2	9	Control	17 043	136	0.8	15	1571	9.2	175	[150]
Pathological samples										
10X 3′ V2	24	High pathology	34 785	939	2.7	39	1936	5.6	81	[118][a]
10X 5′ V1	11	AD[c]	16 279	919	5.6	84	2641	16.2	240	[116]
10X 5′ V1	10	AD *TREM2* R62H	52 003	1509	2.9	101	7573	14.6	505	[116]
10X 3′ V2	15	ASD[c]	16 594	1520	9.2	152	3423	20.6	342	[149]
10X 3′ V2	8	Epilepsy	6684	42	5.3	5.25	851	12.7	106	[149]
10X 3′ V2	12	MS[c]	31 516	1289	4.1	107	3810	12.1	318	[150]

[a]For illustrative purposes this table shows human microglia and astrocyte capture rates from single nuclei transcriptomic studies using the 10X Chromium controller; these rates are substantially low. Microglia capture averages from 2.9 ± 0.6% capture and 112 ± 43 cells/donor (physiological) to 5.0 ± 1.0% capture and 81 ± 21 cells/donor (pathological); these values are well below the predicted 10% density of microglia in the CNS [127–129,151,152]. Astrocyte capture averages from 8.5 ± 1.1% capture and 328 ± 133 cells/donor (physiological) to 13.6 ± 2.0% capture and 265 ± 65 cells/donor (pathological); these values are well below the predicted 50% density of astrocytes in the CNS [127–129]. These low capture rates (between 10% and 20% of all microglia or astrocytes) lead to an underpowered sample size, bias in substate capture, and artifacts in analysis (Box 2).

[b]Cell-type capture data were obtained from supplementary material in each reference, except for [118,148], where data were reanalyzed from raw data available from the Synapse database[iii] under accession number syn18485175 and syn18915937, respectively. Links to raw code used for reanalysis can be found in the Acknowledgments.

[c]Abbreviations: AD, Alzheimer's disease; ASD, autism spectrum disorder; MS, multiple sclerosis.

Box 2. Artifacts of Low Capture Rates in Single Cell/Nuclear RNASeq

Studying cell heterogeneity, particularly the interaction between individual subsets of cells, has important implications for normal brain development and physiology as well as during disease and infection. The explosion and variety of scRNASeq methods in recent years has enabled expansion of our understanding of the heterogeneous subsets of microglia and astrocytes in both rodents and human patients with a wide range of neurodevelopmental and neurodegenerative diseases. But there are concerns that these sequencing experiments are making mistakes in experimental planning and execution, which further lead to problems in data analysis and interpretation. These two problems fall under two main categories: (i) not enough cells are sequenced (power), and (ii) a select subset of cells are being sequenced (bias).

(i) Power: scRNASeq relies heavily on making up for a lack of depth in sequencing individual cells by pulling from the power of sequencing a wide breadth of samples (cells). Therefore, lowly captured cells, and hence small sample size used in sequencing, may have insufficient power to properly delineate subsamples of cells with differentially expressed genes and important biological functions (see Figure 2 and Table 1 in main text; also see [145] for review). Similarly, an undersampled sequencing dataset may produce clustering that is less biologically accurate than one that is properly powered.

(ii) Bias: are all subsets of cells equally collected using a given isolation protocol? As it is likely impossible to know what genes are expressed by cells not captured, problems that arise when isolation methods select only a few of many substates of a particular cell could greatly bias our understanding of responses to disease, or even differences that are observed in different regions of the CNS. For example, if hippocampal microglia are preferentially excluded over cortical microglia, this could sway the interpretation of sequencing analysis. Similarly, if astrocyte scRNASeq clusters are driven by patient-specific clusters, this suggests a strong subsampling artifact, meaning that researchers could be missing important information about disease responses, or more problematically might be basing future research on biased datasets.

Two systematic experimental overview and comparison studies of several scRNASeq methods have recently been published [146,147] that further describe pros and cons of scRNASeq methods.
For these reasons, well-designed statistically powered experiments with samples collected from several biological replicates (rather than simply a large number of cells from a single sample) to mediate some of these problems is needed. Quality control metrics should confirm that clustering is not due to artifacts (postmortem interval, enzymatic digestion, individual patient variability, sex, technical variation, among other metrics).

We have a unique opportunity to take advantage of powerful sequencing technologies to determine transcriptomic changes in microglia, astrocytes, and a wide array of other interconnected cells. Future studies will need to determine how cell types change across time/disease progression with respect to each other (as they do not exist in isolation). It also remains to be seen how well our current animal models, while often suitable for recapitulating specific aspects of disease pathology, actually do recapitulate the cellular heterogeneity of CNS subpopulations, including disease-responsive cells such as

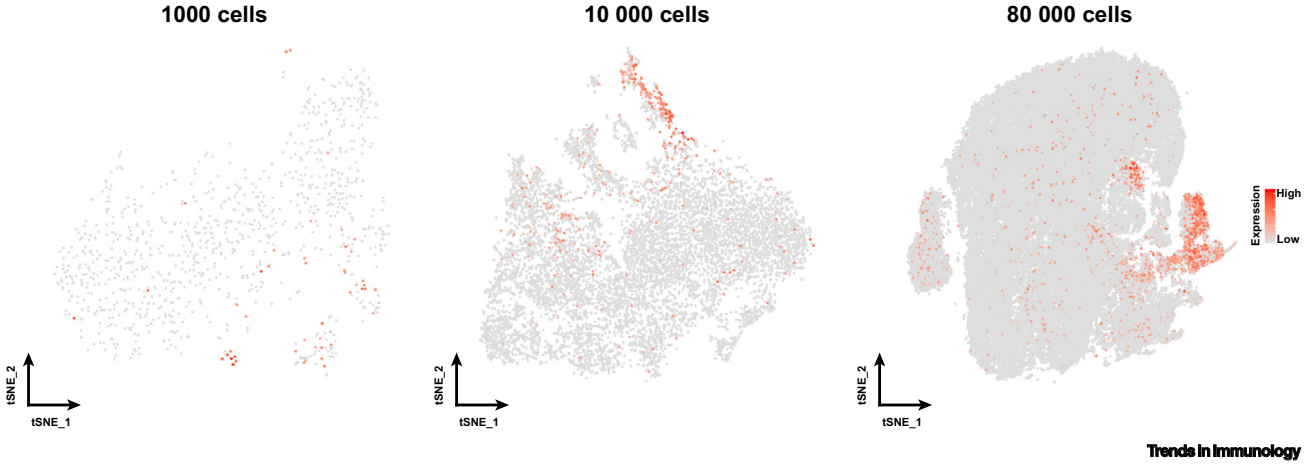

Figure 2. Subsampling Artifacts in Single Cell Sequencing. For illustrative purposes, a feature plot is shown depicting the expression of a single gene in small (1000 cells, left), medium (10 000 cells, center), and large (80 000 cells, right) subsampled datasets taken from a larger single cell RNA sequencing 10× experiment. The red color depicts the expression level of the gene (red is high). While there is strong clustering of the gene in the large dataset, this clustering may be lost with smaller cell numbers. See also [145]. Abbreviation: tSNE, *t*-distributed stochastic neighbor embedding (a method of visualizing complex multidimensional analyses in a reduced two-dimensional space). Data for tSNE feature plots analyzed by Dr Philip Hasel.

microglia and astrocytes. Only through incorporation and comparison of large volumes of scRNASeq data from interacting cells, across species, will we be able to properly understand disease initiation and progression, a requirement for the development of future candidate therapies.

Integration Is the Key

While these single cell and nuclei sequencing efforts have generated large amounts of data, a large discrepancy remains in what constitutes appropriate and 'normal' data quality control and analysis. Several analysis pipelines are widely used, including Seurat [131], SCANPY [132], and a suite of packages on BioConductor [133]. These pipelines are further enhanced by additional specific analysis tools for dataset integration (e.g., LIGER [125]), trajectory analyses such as pseudotime (e.g., Monocle [113]), and RNA velocity (e.g., Velocyto [134]). Finally, big data such as these provide ample opportunity to study multiple cell types in the same model with substantially high resolution. Many studies of astrocyte or microglia function focus on each specific cell type in isolation, when in reality, the actions of these cells are part of complicated intracellular signaling that involves many cell types. New analysis tools that model intercellular communication by linking ligands to target genes across cell types and tissues have recently been created that allow to dissect cell-to-cell communication using single cell data to uncover novel cell interactions in health and disease [135,136].

Unfortunately, there is often ambiguity in describing analysis methods within publications. Often authors state the pipeline used, without describing the specific parameters used or providing details about data correction, integration, regression out of biological and technical effects (e.g., removal of doublets or contaminating cells not of interest, or reporting of mitochondrial DNA contamination), as well as dealing with batch effects, and sharing analysis code. By far, however, the most common error appears to be data integration, where researchers fail to complete batch correction, highlighting specific clusters that are not based on biological differences, but instead, are artifacts of data collection. It will be essential for the field to come together to adhere to common standards when reporting these data, such as those produced for qPCR [minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines] [137], microarray [minimum information about a microarray experiment (MIAME) guidelines] [138], and next-generation RNA sequencing [minimum information about a high-throughput sequencing experiment (MINSEQE)[i]], when those technologies were still young. A similar minimum requirement for study design, quality control, analysis, and validation for modern scRNASeq and single nuclei sequencing experiments should ensure biological reproducibility, ample sample sizes to account for undersampling errors, and ensure that the appropriate analytical pipelines are used to prepare data accurately describing real biological variation.

One simple solution that might solve many of these issues is to require the sharing of raw analytical code similar to the requirement that is in place to share all raw sequencing data. This open science approach has already been embraced by many in the single cell field, but such an additional requirement would be highly beneficial to the field at large. Sharing raw code would allow for others to both easily replicate the figures and analyses performed, but also easily determine what additional analyses or filtering might be relevant. Services such as GitHub[ii] make this process simple and free. Methods sections should still contain many of these details but could do so in a more descriptive, readable language, while raw code could be examined and followed by those wishing for more detail. With agreement and adoption of such guidelines in place, reanalysis of published data and integration with newly prepared samples may be more seamless and help accelerate new discoveries.

Clinician's Corner

Microglia and astrocytes in humans and mice integrate function and trophic support for each other and for neurons during normal physiology; however, alterations in this support during early stages of disease involves combinatorial activation of both cells, driving the death of neurons.

Microglia and astrocyte responses to disease are highly heterogeneous and likely change throughout disease progression. Known dysfunctional pathways provide key targets for future intervention and therapy.

There is evidence that removal of neurotoxic reactive astrocytes (driven by microglia-derived cytokine release) in chronic neurodegenerative disease models and acute trauma is beneficial in maintaining neuron numbers and function [50,54,75]. Meanwhile, other evidence suggests that with prion infection, a block in astrocyte neurotoxic function leads to faster infection rates and quicker death relative to brains containing these reactive astrocytes [58]. It is clear that the complex interactions between astrocytes and microglia with other CNS cells represents a difficult 'moving target' in therapeutic discovery.

It remains unclear at what stage targeting microglia and astrocytes will be beneficial for chronic neurodegenerative disease. However, healthcare providers might be able to treat their patients more effectively if we understood what effect pro- and anti-inflammatory drugs have on microglia and astrocytes.

In the future, it might be possible to manipulate individual subsets of reactive microglia and astrocytes to stop disease initiation, slow progression, or reverse the effects of neurodegeneration. This might be potentially accomplished in the clinical setting by silencing/blocking cytokine release (microglia target), enhancing trophic support (astrocyte target), blocking neurotoxic capacity (microglia/astrocyte target), or by intervening with specialized diets and changing bacterial load in the gut (microbiome target). Robust studies are evidently warranted to test these possibilities.

Concluding Remarks

The study of microglia and astrocytes has seen a renaissance in recent years. This is partly due to an increase in the availability of new tools to enable rapid and cost effective sequencing of individual cells, but also due to new methods to isolate, culture, and target these cells *in vivo*. As discussed earlier, these new tools have provided valuable insights into the functions of microglia and astrocytes during normal development as well as during the early initiation and progressive stages of many chronic neurodegenerative diseases. Most interesting has been the increase in resources and models that integrate both cell types, highlighting how they communicate and integrate functions throughout the brain. Part of this effort will require a deeper understanding of microglia and astrocyte surface proteomes and secretomes and integrating these data with other multi-omics datasets (transcriptomics, epigenetic, proteomic); this will facilitate identifying mechanisms of astrocyte–microglia crosstalk in different genetic and environmental contexts.

It is clear that one of the roles of microglia and astrocytes in many aspects of brain health is to coordinate responses to disease, infection, and injury. We now need to focus our research efforts on functional studies, as these will enable us to move beyond our current shallow understandings and to exploit novel dysfunctional pathways that may enable us to ameliorate a wide range of diseases (see Outstanding Questions). The future looks bright and now we have the tools to address our exciting research questions.

Outstanding Questions

How do microglia and astrocyte responses change in the context of disease and genetic background? For instance, do mutations in disease-causing genes change the way that cells would respond to secondary insults such as inflammation and can these altered response mechanisms shed light on the initiation and progression of chronic neurodegenerative diseases?

How do transcriptomic signatures relate to protein profiles? The journey from gene to protein is complex and consists of two major steps: transcription and translation. While we have been able to measure translation using TRAP mouse lines for some years, we still have precious little information regarding actual protein amounts and post-translational modifications in these cells during health and in disease. Do protein modifications differ across different regions of the CNS? Do they change throughout development and in aging? And how are they altered in the acute and chronic setting of disease and infection? All of these questions are ready to be addressed in the near future.

Is functional heterogeneity as common as transcriptomic diversity? Recent advances in scRNASeq suggest the existence of distinct subtypes of microglia and astrocytes in different contexts, as well as new markers that may be used to label and manipulate glia for functional investigation. The future will no doubt provide numerous studies that expand on these studies.

Are functionally distinct microglia and astrocytes present across a given disease, or is each subtype distinct to the multiple contextual cues driving their formation (e.g., sex, age, brain region, disease state, etc.)?

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Disclaimer Statement

B.S. is a member of the Scientific Advisory Board and minor shareholder in Annexon Biosciences. B.S. is an inventor on multiple patents 'Modulation of Synaptic Maintenance' (US8148330B2, US9149444B2) and is inventor on pending patent 'Biomarkers for Dementia and Dementia Related Neurological Disorders' (WO2015103594A1/US20160327572A1). S.A.L. is an academic founder of AstronauTx Ltd. S.A.L. is an inventor on a pending patent 'Neuronal and Oligodendrocyte Survival Modulation' (US20190248885A1). S.E.M. has no interests to declare.

Resources

[i] http://fged.org/projects/minseqe/

[ii] https://github.com/

[iii] https://adknowledgeportal.synapse.org/

[iv] www.radc.rush.edu

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