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Microglia and the Brain: Complementary Partners in Development and Disease

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Abstract

An explosion of findings driven by powerful new technologies has expanded our understanding of microglia, the resident immune cells of the central nervous system (CNS). This wave of discoveries has fueled a growing interest in the roles that these cells play in the development of the CNS and in the neuropathology of a diverse array of disorders. In this review, we discuss the crucial roles that microglia play in shaping the brain—from their influence on neurons and glia within the developing CNS to their roles in synaptic maturation and brain wiring—as well as some of the obstacles to overcome when assessing their contributions to normal brain development. Furthermore, we examine how normal developmental functions of microglia are perturbed or remerge in neurodevelopmental and neurodegenerative disease.

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INTRODUCTION

Microglia, the resident immune cells of the brain, are emerging as essential players in the development of the central nervous system (CNS). Once thought to act simply in response to pathogens or other immune-related stimuli, microglia are now known to be important in establishing the circuitry of the brain, with critical roles in brain wiring, synapse development, and synapse function. It is becoming increasingly clear that microglia assume a myriad of states and functions depending on age, brain region, and environmental stimulus (Hanisch & Kettenmann 2007, Ransohoff & Perry 2009). Emerging literature is unveiling a complex developmental program that shapes microglia from progenitors in the yolk sac to mature cells in the developed brain. By postnatal ages, microglia are highly ramified, with multiple fine dynamic processes that form a lattice with which each microglial cell occupies a unique territory. Nascent work is facilitating a greater appreciation for the ability of microglia to detect, transduce, integrate, and respond to a seemingly endless array of environmental cues throughout development. Similarly, new approaches and technologies are enabling a refinement in our understanding of microglia biology, including addressing the limitations of past works.

In this review, we discuss the origin of microglia and the signals that drive their development, as well as key functions that they perform at different stages of CNS development. Additionally, we discuss limitations of the current methods and studies exploring microglial biology and where this field is moving in the future. Uncovering new insights into the roles of microglia in development will offer a deeper understanding of human cognition, learning, and memory and could ultimately lead to new biomarkers and treatments of neurological diseases.

MICROGLIA ONTOLOGY, BRAIN COLONIZATION, AND DEVELOPMENT

Microglia are tissue-resident macrophages, a class of immune cells that provides homeostatic and protective functions to the major organs in the body. There are several distinct types of tissue-resident macrophages, including the alveolar macrophages of the lung, Kupffer cells of the liver,

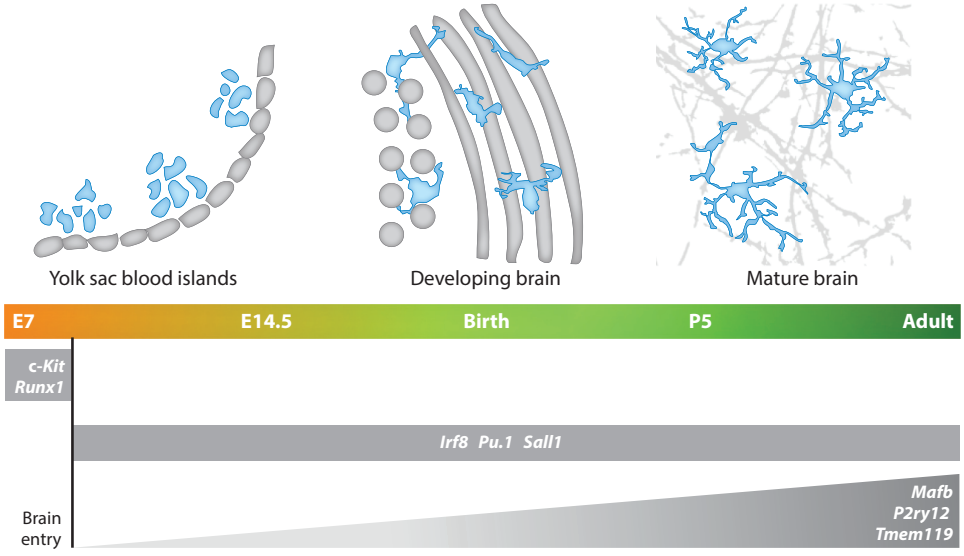

Figure 1

Stages of microglia development. Microglia differentiate from c-*Kit*- and *Runx1*-expressing primitive erythromyeloid progenitors in the extraembryonic yolk sac beginning at embryonic day (E)7.25 in mice. Microglia next migrate to the brain, where they begin to express the transcription factors *Irf8*, *Pu.1*, and *Sall1* to activate the genetic programs that will confer their identity. As microglia continue to develop, they are shaped by the brain environment and transition through several transcriptional phases until they reach their adult state, which is characterized by increased morphological complexity and enriched *Mafb*, *P2ry12*, and *Tmem119* expression.

and Langerhans cells of the skin. These and other macrophage populations in tissues such as the heart, pancreas, and kidneys share many common functions while also maintaining unique features relevant to the tissue they reside in (Davies & Taylor 2015, Okabe & Medzhitov 2016). Until recently, the origin and development of different tissue-resident macrophages were poorly understood; however, the advent of more sophisticated cell-isolation procedures, as well as elegant lineage-tracing studies and next-generation transcriptome sequencing, has enabled the tracking of different populations of immune progenitor and tissue-resident macrophages over time.

Recent fate-mapping studies in mice have shown that microglia are predominantly derived from yolk sac progenitor cells that invade the brain at midgestation, differentiate into microglia, and then self-renew for the life span of the animal (Ginhoux et al. 2010) (**Figure 1**). The process by which microglia migrate from the yolk sac to the brain has remained elusive; however, a functioning circulatory system is required for microglia to reach the brain (Ginhoux et al. 2010). Whether this means microglia actually travel within the vessels or merely use them as structural guideposts is unknown. Given that the brain has very little microvasculature at the time of microglial invasion, microglia likely enter through structures at the brain's borders, including the meninges (Vasudevan et al. 2008), where blood vessels first form and where macrophages accumulate during embryogenesis. A major question remains as to whether macrophages are intrinsically programmed to become specific types of tissue-resident macrophages when they leave the yolk sac or whether they remain multipotent and specialize after reaching a particular organ niche.

It is becoming increasingly clear that specific signals recruit macrophages into the brain to form microglia. In zebrafish, macrophages are attracted by the release of nucleotides from dying

neurons undergoing programmed cell death (Casano et al. 2016, Xu et al. 2016). In mice, they are attracted, in part, by chemokines released from immature neurons occupying the niches next to the brain ventricles, where the choroid plexus resides (Arno et al. 2014). Interestingly, microglia attach to the walls of the lateral ventricles during their migratory window, which would allow them to be exposed to niche-derived factors such as chemokines. No study has identified a signal that completely prevents microglia brain invasion, so a myriad of factors at different stages of microglia migration likely contribute to this process. More detailed fate-mapping and live-imaging experiments, as well as microglia-specific tools, will be needed to determine the exact timing and regulation of microglial brain infiltration and to provide important clues about how microglia reach the brain and achieve their unique identity.

Once microglia enter the brain, signals released by neurons and astrocytes immediately influence microglial identity and differentiate them from other tissue-resident macrophages. In fact, cultured microglia begin to express their unique transcriptional signature only when exposed to neuron-conditioned media or when integrated into complex 3D cultures that contain a diverse array of brain cell types (Abud et al. 2017, Bohlen et al. 2017, Muffat et al. 2016, Takata et al. 2017). Indeed, transplanting tissue-resident macrophages into other tissue environments causes a rapid change in their transcriptomes (Gosselin et al. 2014, 2017; Lavin et al. 2014). Transcriptional and epigenetic analysis has uncovered a handful of critical genes and pathways that are turned on in microglia as they enter the brain and confer microglial identity; among the genes are transforming growth factor beta (*Tgf-b*), Spi-1 Proto-Oncogene (*PU.1*), interferon regulatory factor 8 (*Irf8*), and spalt-like transcription factor 1 (*Sall1*) (Butovsky et al. 2014, Gosselin et al. 2014, Kierdorf et al. 2013) (**Figure 1**). In the absence of TGF-β signaling, microglia fail to assume their unique profile and instead take on a peripheral macrophage-like form (Butovsky et al. 2014, Wong et al. 2017). This signaling pathway, in particular, helps drive a unique microglial identity by utilizing genes downstream of TGF-β that are not expressed by other tissue-resident macrophages and that are potentially necessary for functions particularly relevant in the brain. A direct comparison of gene expression profiles between microglia and other tissue-resident macrophages has been performed only in adult animals, leaving the differences between these two cell types during early development largely unknown.

As the brain develops, microglia undergo significant physical and biochemical changes. Transcriptionally, microglia fall into three general phases associated with different developmental stages: (*a*) early embryogenesis, (*b*) late embryogenesis and early postnatal development, and (*c*) adult microglia [from postnatal day (P)28 onward] (Matcovitch-Natan et al. 2016). Early microglia express genes associated with cell proliferation and the cell cycle, whereas early postnatal microglia predominantly express genes associated with phagocytosis, and adult microglia express genes associated with surveillance and the immune response. However, these terms should not be overinterpreted, as microglia still express phagocytic pathways at high levels throughout their life span and the functions of many surveillance genes are still only partly understood.

In terms of cellular localization, early (embryonic and early postnatal) microglia are unevenly distributed in the brain, are enriched in certain brain regions, and are absent in others. Additionally, they assume many different morphological forms, ranging from small, round amoeboid cells to those bearing multiple long, branched processes (Karperien et al. 2013) (**Figure 1**). As these microglia mature, they extend long, motile processes and begin to tile the brain, forming nonoverlapping domains that delineate an individual cell's surveillance zone. Microglia processes are highly motile; motility is thought to be integral to the ability of microglia to recognize changes in the brain parenchyma, although this capacity has not been directly shown (Davalos et al. 2005, Dissing-Olesen et al. 2014, Nimmerjahn et al. 2005). Recent evidence suggests that this motility is controlled, in part, by the Thik1 potassium channel on the microglia membrane (Madry et al.

2018), and microglia respond rapidly to the release of ATP triggered by neurons under different conditions (Dissing-Olesen et al. 2014, Eyo et al. 2015).

These broad changes in microglia during development are striking, but emerging evidence indicates that subsets of microglia also display unique properties, including discrete morphologies and transcriptional and physiological properties, suggesting that local cues within particular regions further refine microglia identity and function (De Biase et al. 2017, Grabert et al. 2016). In development, these distinct identities might include subpopulations of microglia that perform unique functions, or groups that appear only during restricted developmental windows. In fact, a unique subpopulation of microglia resides in the subventricular zone (SVZ) and supports neurogenesis in adult animals (Ribeiro Xavier et al. 2015). As we begin to develop a deeper understanding of the changing transcriptional and epigenetic landscapes of microglia throughout development, we will be able to codify more specific stages of their development and to perform more detailed functional analysis of microglia subpopulations within each stage. Additionally, we will be able to evaluate whether these stages are synchronized or whether subsets of microglia develop at different rates or in a region-specific manner. Further in-depth analysis of microglia heterogeneity using single-cell sequencing and other approaches will refine our understanding of the myriad of specialized microglia states and could lead to the development of new markers and tools to identify and manipulate these discrete populations.

INTERROGATION OF MICROGLIA FUNCTION: POWERFUL TOOLS WITH LIMITATIONS

Recent development of new tools and approaches to visualize microglia and manipulate their function has led to a number of studies that interrogate microglia function in the developing brain. These tools include genetically modified mice—the *Cx3cr1-EGFP*, *Cx3cr1-Cre*, and *Cx3cr1-CreER^{T2}* mice—along with small-molecule inhibitors and other tools that can be used to deplete microglia, as well as additional knockout animals that target genes identified in recent transcriptomic studies (Cunningham et al. 2013, Elmore et al. 2014, Squarzoni et al. 2014). Each of these systems has been essential for furthering our knowledge of microglia function and biology, but each has its own set of drawbacks that must be taken into account, depending on the question. For example, the *Cx3cr1-EGFP*, *Cx3cr1-Cre*, and *Cx3cr1-CreER^{T2}* knock-in mice yield haploinsufficiency of the *Cx3cr1* gene, which disrupts normal microglia function. Additionally, *Cx3cr1* is expressed in neurons and other immune cells, including other tissue-resident macrophages, which can confound experimental interpretation. This latter issue also extends to the small-molecule inhibitors and knockout mice, which often impact other immune cells throughout the body. These issues have been reviewed in detail elsewhere but must be considered when one is interpreting existing results (Ginhoux & Prinz 2015, Li & Barres 2017).

Studying microglia in a culture setting has also been problematic since microglia assume a biochemical and physical phenotype, including production of inflammatory factors and increased phagocytic activity, that differs considerably from their in vivo state (Bohlen et al. 2017, Gosselin et al. 2017). Therefore, in vitro studies must be interpreted accordingly. Recent advances in culture technique, including identification of factors that confer microglia identity (TGF-β and Il-34) and the removal of serum from microglia protocols, have greatly reduced these artifacts (Abud et al. 2017, Bohlen et al. 2017, Muffat et al. 2016). Further refinement and improvement in these tools will undoubtedly lead to a greater understanding of microglia signaling and function.

MICROGLIA FUNCTION IN PRENATAL DEVELOPMENT

During early embryonic development, neural stem cells in the ventricular zone generate newborn neurons that will eventually form circuits throughout the brain. These intermediate progenitors

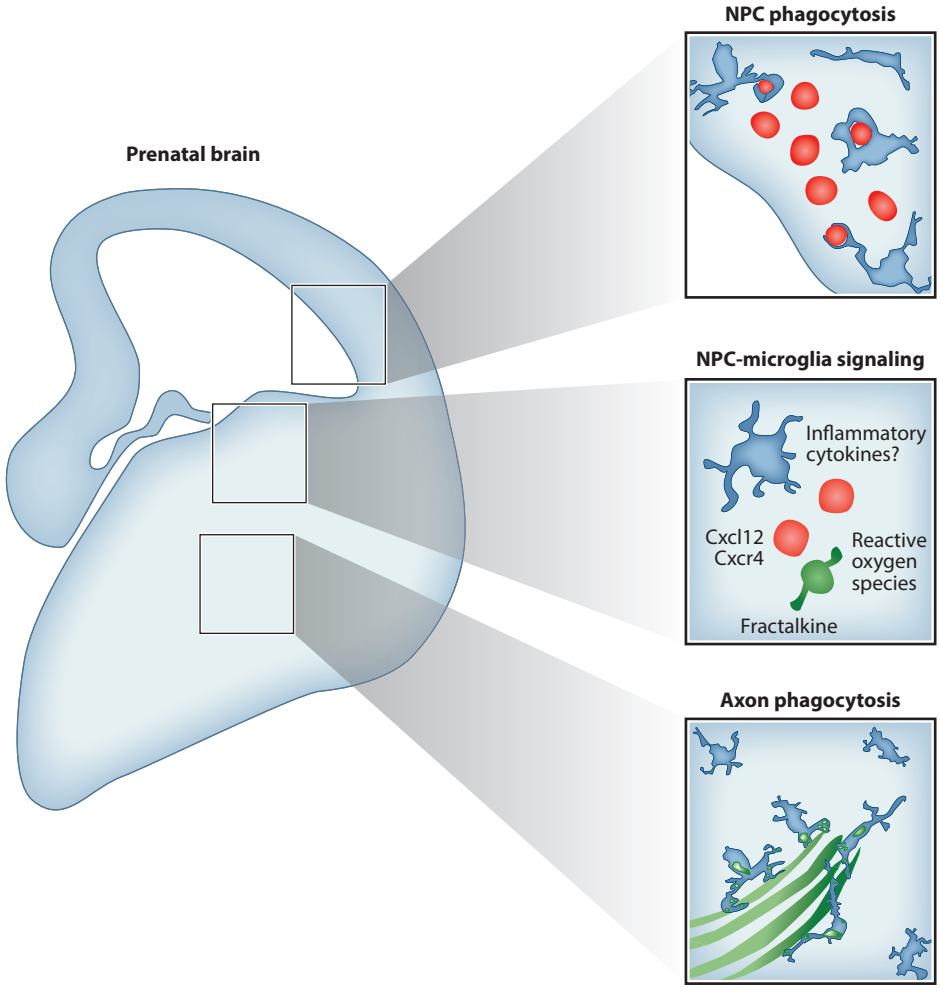

Figure 2

Functions of microglia in the prenatal brain. Prior to birth, microglia perform key functions in the brain that help shape the nascent brain structure. In neurogenic zones near the brain ventricles, microglia phagocytose, or eat, neural progenitor cells (NPCs). Additionally, microglia express and release signaling factors that impact the development and health of neurons. Finally, microglia also phagocytose growing axons during brain wiring to regulate their growth.

are born in the SVZ and differentiate into mature neurons, migrating into the cortex, where they integrate into different cortical layers. Proper neurogenesis requires tight regulation of proliferation and differentiation to maintain the appropriate number of progenitors and mature cells. Microglia have been implicated in this process through cytokine release as well as the phagocytosis of newly born neural progenitors (Cunningham et al. 2013) (**Figure 2**).

Some of the first indications that microglia influence neurogenesis came from studies in adult animals, in which neurogenesis occurs in specialized niches within the SVZ and subgranular zone of the hippocampus. In these areas, neuroinflammation drastically reduced neurogenesis, in part through microglia releasing cytokines like interleukin 6 (IL-6) (Monje et al. 2003). The inhibitory effects of neuroinflammation on neurogenesis have also been observed in many models of brain

injury (Ekdahl et al. 2003, Li et al. 2017, Lucassen et al. 2015, Monje et al. 2003). While many different cell types can initiate neuroinflammation, microglia are thought to be a major source of these inflammatory molecules in an injury context, and experiments in cultured microglia show that proinflammatory M1 microglia directly inhibit neural stem cell differentiation. Interestingly, anti-inflammatory M2 microglia promote neural differentiation, suggesting that microglia can have both positive and negative effects on neurogenesis, depending on their activation state (Butovsky et al. 2006). This notion is supported by evidence that anti-inflammatory drugs, including minocycline, stimulate neurogenesis in vivo, although these drugs can have many off-target effects that are not completely understood. Interestingly, exposure to inflammatory factors caused by injecting the bacterial wall component lipopolysaccharide (LPS) into pregnant dams leads to a loss of neural stem cells and intermediate progenitor cells in the developing embryos, and treatment with the anti-inflammatory drug doxycycline increases neurogenesis and neural precursor number, similar to what is seen in adults (Cunningham et al. 2013, Tronnes et al. 2016). However, there is no evidence that microglia produce inflammatory cytokines under homeostatic conditions, suggesting that microglia might not be the source of these molecules unless they are challenged. Microglia-specific knockouts of pro- and anti-inflammatory cytokines will be important for directly testing whether microglia–neuron cytokine signaling regulates neurogenesis in the healthy brain (Walton et al. 2006, Xavier et al. 2015).

Despite these concerns, two studies show evidence that depletion of microglia leads to altered developmental neurogenesis. The first study used microglia-toxic liposomal clodronate in organotypic slice culture to see what effect the loss of microglia had on neurogenesis (Cunningham et al. 2013). This drug is taken up by phagocytic cells and causes cytotoxicity but can have off-target effects that we do not fully understand. In this study, the authors found a 97% depletion of microglia in the cultures that were kept alive for 3 days. The second study used the colony-stimulating factor receptor 1 (*Csf1r*) knockout mouse; this approach targets a receptor needed for microglia survival, and the knockout mouse lacks more than 99% of microglia. Both liposomal clodronate and the loss of *Csf1r* increased numbers of neural progenitors following depletion, and the *Csf1r*-knockout model had increased numbers of mature neurons in the cortex. Of note, CSF1R signaling can also directly signal to subsets of neural precursors, so the authors generated a neural progenitor–specific *Csf1r* knockout and recapitulated some, but not all, of the effects on neurogenesis, suggesting that the microglia are only partly responsible for the observed phenotypes in this model. Embryonic depletion of microglia using an anti-CSF1R blocking antibody or in the *Pu.1* knockout mouse—which lacks most immune cells—also impacted interneuron number and placement in the cortex; however, peripheral immune cells are also affected in both depletion models, so this could be an indirect effect (Squarzoni et al. 2014). Collectively, these results implicate microglia in developmental neurogenesis. However, whether altered neurogenesis and neuron migration in these models are direct consequences of altered microglia–neuron signaling is unknown and must be followed up using more specific genetic tools that can unequivocally relate these findings to microglia and that can identify the significant signals and pathways.

Microglia not only modulate the differentiation and migration of neural progenitors but also impact how these cells grow once they are in place. In the developing mouse forebrain at embryonic day 14.5 (E14.5), microglia cluster around the leading edges of growing dopaminergic axon tracts and regulate the rate of axonal extension by phagocytosis, the process by which microglia internalize, or "eat," unwanted material in the brain (Squarzoni et al. 2014) (**Figure 2**). Microglia depletion increased the relative extension of these axons, whereas LPS-induced gestational inflammation decreased it, although it is still unclear whether all axons in the tract are phagocytosed or whether specific axons are targeted following interaxon competition or refinement. Intriguingly, other surrounding axon tracts are not engulfed at the same time, raising questions about how this

specificity is achieved and what the functional consequences of disrupted axon growth by microglia are on neural circuit development.

Microglia also regulate neural precursor numbers by phagocytosis. Live imaging of rat slice cultures has recorded microglia engulfing whole neural progenitor cells (Cunningham et al. 2013). In many cases, these neurons were not apoptotic, indicating that microglia may eat otherwise healthy cells. Interestingly, engulfment of neurons by microglia has also been discovered in the brains of developing macaques, suggesting that this mechanism could be conserved between rodents and primates (Cunningham et al. 2013). In the developing mouse cerebellum, microglia directly induce Purkinje cell death through the release of damaging reactive oxygen species (ROS) (Marin-Teva et al. 2004). Blocking ROS-forming pathways or depleting microglia using liposomal chlodronate in slice culture led to increased survival of Purkinje cells, suggesting that targeting microglia in development or disease may lead to the survival of stressed, but otherwise healthy, neurons. However, microglia and neurons in slice culture are artificially activated and stressed, respectively, and could be behaving in a nonphysiological manner. It is important that these results be confirmed in an in vivo context (Marin-Teva et al. 2004).

There are many ways in which microglia could influence how circuits in different brain regions develop and function. The prenatal roles of microglia, including the phagocytosis of neurons and axons, the production of key signaling factors, and the recognition of changes in the environment, are critical for the ability of microglia to continue refining more active networks after birth.

MICROGLIA IN POSTNATAL DEVELOPMENT

Proper brain wiring is accomplished through an intricate interplay between multiple cell types to ensure that appropriate synaptic connections are made and strengthened. Wiring is driven by a number of different processes, including neuronal survival/apoptosis, neuronal activity, cell signaling, and the refinement of axons and synapses.

Under healthy conditions, microglia use their highly motile processes to contact synapses in a dynamic fashion. The frequency of microglia–synapse connections is downregulated by neuronal activity or sensory experience during critical developmental time windows, indicating that these contacts could carry functional implications (Hensch 2004, Paolicelli et al. 2011, Schafer et al. 2012, Tremblay et al. 2010). In fact, microglia are intimately involved in several processes that are important for the proper function and refinement of networks in several brain regions through the expression and release of different neuroactive signals and through the removal of excess synapses (Bessis et al. 2007, Casano & Peri 2015, Ji et al. 2013b, Kettenmann et al. 2013, Paolicelli et al. 2011, Schafer et al. 2012, Sierra et al. 2014, Tremblay et al. 2010, Wu et al. 2015) (**Figure 3**).

Microglia Mediate Synaptic Refinement in the Postnatal Brain

Synaptic plasticity is considered to be a fundamental underpinning of learning and memory (Bliss et al. 2014), leading to experience-dependent changes in connectivity through the strengthening or weakening of synapses from early development into adulthood. Plasticity also involves the remodeling of synaptic terminals (the neuronal buds on either side of the synapse, referred to as the pre- and postsynaptic terminals), including the turnover of postsynaptic terminals on neuronal dendrites. Interestingly, eliminating microglia in adulthood via inhibition with small molecules or blocking production of microglia-produced BDNF (brain-derived neurotrophic factor), a key signaling molecule important for synaptic plasticity, impairs learning and memory and synaptic plasticity (Parkhurst et al. 2013). Depletion of microglia during brain development, by using *Cx3cr1-CreER*^{T2} mice to express diphtheria toxin receptor (DTR) on microglia and macrophages

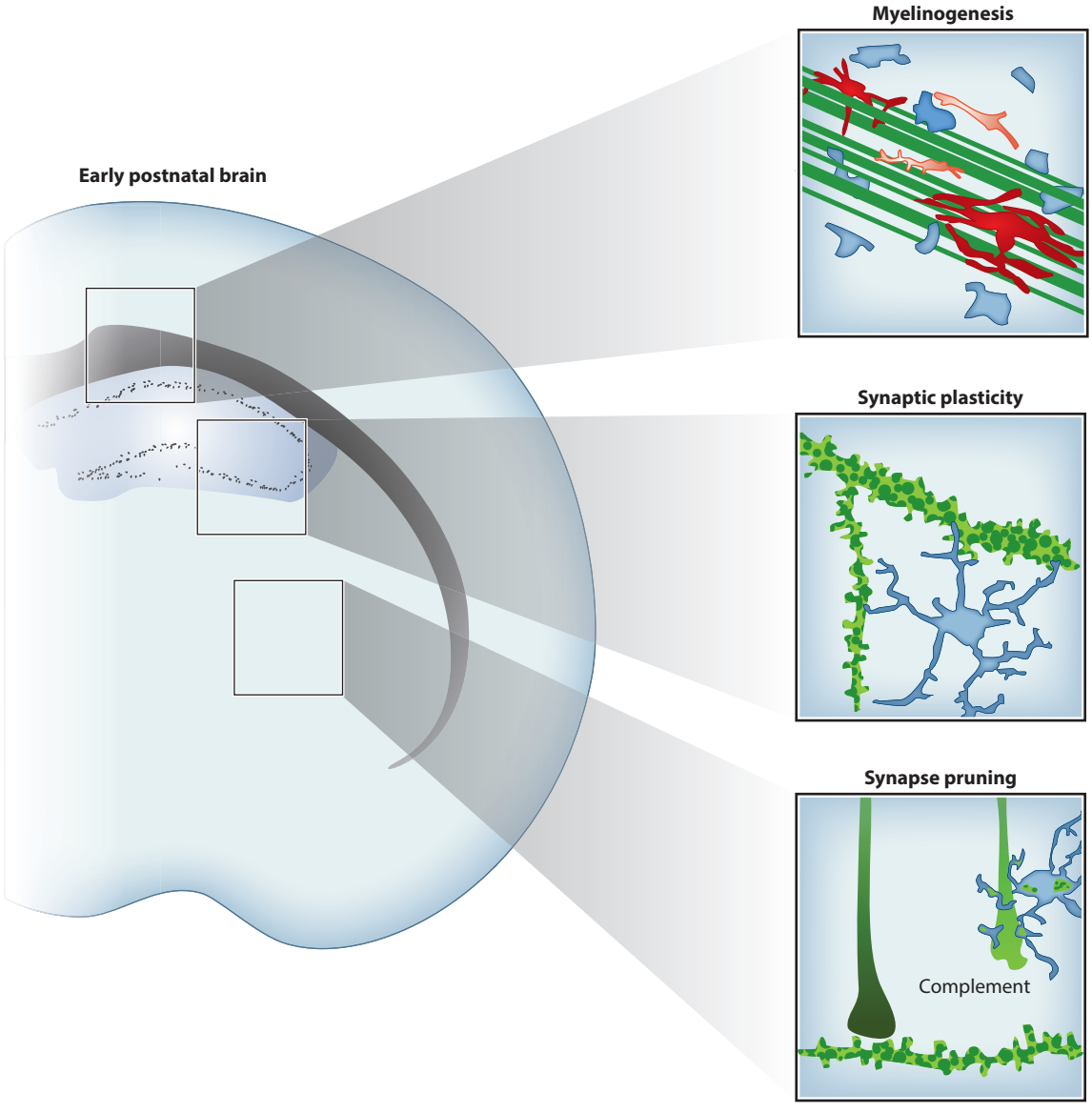

Figure 3

Microglia functions in postnatal brain development. After birth, microglia play essential roles in the continued growth, refinement, and function of neural networks. Microglia are important for the health and development of oligodendrocyte precursor cells (*light red cells*) and the production of myelin by oligodendrocytes (*dark red cells*). Microglia influence learning and behavior by regulating synaptic plasticity. Lastly, microglia refine synaptic networks by pruning unwanted synapses during specific developmental windows through activation of the complement signaling pathway.

followed by timed injection of diphtheria toxin to kill the receptor-expressing cells, also revealed that microglia are involved in the elimination and formation of dendritic spines in the motor cortex (**Figure 3**); such elimination and formation contribute to learning-dependent motor activity (Parkhurst et al. 2013). Depleting microglia at P19 or P30 causes a significant decrease in both spine formation and elimination, including learning-dependent formation and elimination. Depletion of microglia also alters synaptic protein levels and glutamatergic synaptic function (Parkhurst et al. 2013). Another microglia-depletion model also resulted in behavioral changes in rats: Injection of toxic clodronate liposomes into the rat brain at P2 and P4 led to persistent anxiety, along with impaired social and locomotor behaviors (Nelson & Lenz 2017). Intriguingly, such outcomes are reversible, suggesting the dynamism of these cells and their ability to recover and restore function after acute perturbation (Torres et al. 2016). Taken together, these results support an essential role for microglia in the maintenance of synaptic function and underscore the relevance of these cells in postnatal development. While the specific microglia-mediated mechanisms underlying these effects are still largely unknown, one microglial signaling pathway implicated in synaptic plasticity is the fractalkine pathway, in which the microglia receptor CX3CR1 binds to the neuronal ligand CX3CL1. Mice lacking CX3CR1 have markedly fewer microglia in early postnatal development and demonstrate defects in synaptic maturation and refinement in the hippocampus (Paolicelli et al. 2011). Additionally, disrupting the CX3CR1 pathway in microglia reduces connectivity in the brain and negatively impacts social behavior while increasing repetitive behavior in mice (Zhan et al. 2014). These studies demonstrate a microglia-specific pathway that directly impacts postnatal neurodevelopment and function and again support the notion that normal neuron–microglia signaling is essential for circuit development.

Neuroplasticity in adulthood is also affected by microglia. Deleting *Tgf-b* in the CNS leads to aberrant synaptic plasticity in the CA1 area of the hippocampus (Butovsky et al. 2014, Koeglsperger et al. 2013), and knocking out *Cx3cr1* similarly impairs synaptic plasticity and motor and cognitive function (Rogers et al. 2011). Interestingly, depleting microglia in adulthood by using the *Cx3cr1-CreER* ^{T2}-DTR system decreases spine formation without affecting spine elimination (Parkhurst et al. 2013). These effects can also be observed ex vivo in organotypic hippocampal slices: Depleting microglia via clodronate liposomal treatment increases the frequency of postsynaptic currents, which is consistent with a higher density of synapses (Ji et al. 2013a), and a replenishment of the slices with microglia restores normal synaptic currents. These results indicate that a role for microglia in synapse formation persists throughout life (Parkhurst et al. 2013).

While the mechanisms controlling microglial regulation of synaptic plasticity are not completely understood, it is clear that direct manipulation of microglia signaling alters the ability of neurons to wire and function normally. Many other pathways will likely emerge as new tools are developed to specifically manipulate microglia and as new ways are found to measure these highly dynamic and rapid interactions.

Microglia Mediate Synaptic Pruning

In postnatal brain development, an abundance of synaptic connections are initially formed and then refined through a process termed pruning. For a long time, it was assumed that these unwanted connections were lost by shedding excess material or by neuron-intrinsic mechanisms, but recent evidence suggests that microglia can physically remove axons and synaptic terminals as part of the pruning process. Indeed, pre- and postsynaptic structures have been visualized inside lysosomes within microglia in the mouse visual system by using electron microscopy and high-resolution in vivo engulfment assays (Schafer et al. 2012, Tremblay et al. 2010) (**Figure 3**). But how do microglia recognize the synapses that need to be removed versus the synapses that need to stay? Pruning

is mediated in part by neuronal activity and sensory experience, with microglia preferentially engulfing less-active presynaptic inputs (Schafer et al. 2012, Tremblay et al. 2010). Disrupting microglial pruning results in sustained defects in synaptic development, neuroplasticity, and brain wiring (Schafer et al. 2012), with impacts on both development and disease (Butovsky et al. 2014, Hong et al. 2016b, Koeglsperger et al. 2013, Salter & Beggs 2014, Tay et al. 2017).

Several possible neuron–microglia signaling pathways have emerged as possible mediators of pruning, including the classical complement cascade, which is a complex innate immune surveillance system that facilitates the clearance of invading pathogens or cellular debris. The molecule C1q initiates the cascade, while the end product, C3, is an "eat me" signal that coats the offenders and attracts macrophages expressing C3 receptors to phagocytose the target (van Lookeren Campagne et al. 2007). C1q and C3 are expressed in the early postnatal brain (Stevens et al. 2007), and these molecules localize to subsets of immature synapses, tagging them for phagocytosis by microglia, the only CNS cell type that expresses the C3 receptor (Stevens et al. 2007). Depleting critical players in this cascade, including C1q, C3, and CR3, increases synapse number and causes sustained defects in synaptic connectivity in the developing visual system, in which presynaptic inputs from the eye are refined as they innervate relay nuclei in the thalamus. Deficiency can lead to decreased synaptic engulfment [C3 or CR3 knockout (Schafer et al. 2012)] and to enhanced connectivity and epilepsy [C1q knockout (Chu et al. 2010)].

Many important questions remain: What other molecules work in concert with complement to prune specific synapses? Are there "don't eat me" molecules that prevent engulfment of synapses or axons? What ensures that pruning occurs at the right time and place? Do different mechanisms regulate pruning in different contexts? For example, are there distinct mechanisms for disparate regions of the brain, different disease states, or discrete stages of development? And while mechanisms of synaptic pruning are well described in the developing visual system, they remain unclear in other regions of the brain. Whether aberrant pruning during critical developmental periods contributes to neurodevelopmental disorders, such as autism and schizophrenia, remains unknown. These questions represent the next phase of discovery in understanding these critical processes and developing new approaches to improve the prognosis for debilitating neuropathologies.

Microglia Recognize and Phagocytose Dying Cells

Apoptosis plays a critical role in development and homeostasis, especially for determining the size and shape of the vertebrate nervous system (Kuan et al. 2000, Nijhawan et al. 2000). It is important for dying neurons and their degradation products to be quickly cleared from their resident tissue to prevent the diffusion of damaging apoptotic debris and degradation products, and as such dying neurons are quickly identified and cleared via phagocytosis (Lauber et al. 2004, Platt et al. 1998). A key feature of microglia in the postnatal brain is the rapid identification of dying cells, followed by migration and clearance of apoptotic material (Barron 1995, Kettenmann 2007, Peri & Nüsslein-Volhard 2008). It is not entirely clear how microglia detect apoptotic cells under physiological conditions in the healthy brain, but studies have identified the P2Y12 purinergic receptor as a trigger of microglial chemotaxis and phagocytosis in response to neuronal injury (Koizumi et al. 2007, Tsuda et al. 2003). Through the selective targeting of a single neuron in the spinal cord by UV laser ablation, thereby retaining physiological conditions in the surrounding tissue, one group demonstrated that microglia rapidly migrated to the area of injury, recognized the dying cell, and efficiently cleared the apoptotic material through phagocytic engulfment (Morsch et al. 2015). Interestingly, this group observed a significant increase in the speed of microglia upon detection of neuronal injury, highlighting the quick and efficient response of microglia once

activated. Furthering our understanding of these processes is critical to better appreciating how microglia behave not only in physiological conditions but also in neurodegenerative diseases, wherein microglial clearance is symptomatic and microglial activation might contribute to the death of neurons.

Microglia Contribute to Oligodendrocyte Development and Myelinogenesis

In addition to having roles in neuronal wiring, plasticity, and clearance, microglia play critical roles in the development of other brain cell types. Several studies have demonstrated that the protein products of genes expressed by microglia influence oligodendrocyte progenitor cell (OPC) and oligodendrocyte development. For example, microglia-conditioned media enhance OPC survival and maturation, in part through increased expression of PDGF-AA, VEGF, and IGF-1 (Nicholas et al. 2001, Pang et al. 2013). Microglia are also considered to be the primary suppliers of iron to OPCs during developmental myelination; iron is critical for the metabolism required for the proper proliferation and maturation of OPCs (Cheepsunthorn et al. 1998, Connor & Menzies 1996, Todorich et al. 2009, Zhang et al. 2006). Recent work has also demonstrated that a subpopulation of Cd11c-expressing microglia could be important for the generation of OPCs and for subsequent myelination. This subpopulation of microglia is found only in the developing white matter tracts of the early postnatal mouse brain, is transcriptionally distinct from cortical microglia, and expresses genes critical for glial survival and migration and for differentiation of OPCs and oligodendrocytes (Hagemeyer et al. 2017, Wlodarczyk et al. 2017). In further support of these conclusions, a similar population of microglia was found in the early postnatal rat forebrain. In this study, the anti-inflammatory agent minocycline significantly inhibited oligodendrogenesis in the forebrain SVZ of adult rats, whereas LPS-mediated activation of microglia in vitro enhanced oligodendrogenesis (Shigemoto-Mogami et al. 2014). These results support the idea that microglia release cytokines that enhance oligodendrogenesis. However, these studies do not definitively link the small $Cd11c^+$ subpopulation of microglia to these effects, and further studies will be needed to confirm these findings and to elucidate the potential mechanisms.

Recent studies have also illuminated a role for microglia in remyelination, the process of myelin formation after demyelinating injury. For example, microglia and macrophages increase expression of activin-A, a factor that stimulates OPC differentiation, as OPCs begin to differentiate and remyelinate in vivo (Miron et al. 2013). Demyelination models further support the notion that microglia contribute to normal OPC development and myelination. In the cuprizone demyelination model, microglia upregulate factors that influence OPC proliferation and differentiation, including TNF-α, FGF-2, and IGF-1 (Voss et al. 2012). In a mouse model of focal demyelination in adult animals, classical proinflammatory activation of microglia had cytotoxic effects on oligodendrocytes, whereas anti-inflammatory activation of microglia led to phagocytosis of myelin debris. These latter microglia were found to be essential for remyelination due to their ability to promote oligodendrocyte precursor cell differentiation (Kigerl et al. 2009, Miron et al. 2013).

The question remains as to how microglia exert these effects on OPCs and oligodendrocytes. Do microglia express the molecules necessary for oligodendrocyte precursor cell survival or growth? Do subpopulations of microglia have distinct roles from other microglia in ensuring normal myelination and remyelination? Because changes in white matter formation and maintenance have been linked to neurodegenerative and neuropsychiatric diseases, a deeper understanding of the role that microglia play in myelin formation could provide key mechanistic insight into these pathologies and, potentially, novel avenues for therapeutic strategies (Hagemeyer et al. 2012, Poggi et al. 2016).

MICROGLIA IN DISEASE

Many studies have demonstrated that environmental stimuli, such as stress (Merlot et al. 2008), infections (Boksa 2010), dietary intake (Giugliano et al. 2006), diesel exhaust particles (Bolton et al. 2012, 2017), and hyperphysiological levels of glucocorticoids (Caetano et al. 2017), induce acute and chronic inflammatory responses, as well as increased susceptibility to mental diseases characterized by profound synaptic defects (Drozdowicz & Bostwick 2014, Pedersen & Mortensen 2001, Volk et al. 2013). Epidemiological data in humans demonstrate associations between exposure to adverse events early in life and the risk of later-life neuropsychiatric conditions (MacMillan et al. 2001), including autism spectrum disorders (ASDs) (Kinney et al. 2008), psychosis (Varese et al. 2012), and depression (Agid et al. 1999, St. Clair et al. 2015). Additionally, prenatal infections in the first and second trimester of human pregnancy are associated with increased risk of ASD (Atladottir et al. 2010, Di Marco et al. 2016) and schizophrenia (Brown 2012). These studies suggest a link between immune activation and synaptic development; however, the cellular and molecular mechanisms underlying this link remain elusive. Given the importance of microglia in synaptic development and neuronal maintenance, dysregulation of microglia development and function has the potential to contribute, directly or indirectly, to these and other neurodevelopmental disorders.

Microglia in Neurodevelopmental and Neuropsychiatric Disorders

Brain circuit function depends on a careful balance of connections; either too few or too many synapses can be detrimental. Defects in synaptic connectivity and function—including aberrant pruning of cortical synapses—are now believed to be an underlying cause of autism and schizophrenia (Feinberg 1982, Penzes et al. 2011). While direct evidence for a role for microglia in autism and developmental disorders is lacking, such speculation has been ongoing (Pardo et al. 2005). A new perspective on this potential link has emerged via studies into the functions of microglia in the mouse brain. During early embryonic development, as microglia colonize the brain, genetic and/or environmental perturbations could alter microglial development, synaptic pruning, and surveillance, which could directly or indirectly contribute to neurological disorders; however, this hypothesis remains to be tested. A single maternal challenge during embryonic development (E9–E12) with viral or bacterial components, for example, PolyIC, resulted in behavioral changes in offspring, including decreased social behavior, increased repetitive behaviors, increased anxiety, and altered ultrasonic vocalization (Estes & McAllister 2016). A second challenge, such as stress, later in development enhanced the severity of the defects related to anxiety, memory, and cognitive function (Giovanoli et al. 2013). IL-6, a proinflammatory cytokine that is released from macrophages and T cells, mediates these developmental and behavioral deficits, which are typically accompanied by long-term cytokine dysregulation (Garay et al. 2013, Patterson 2011).

Additionally, genetic perturbations affecting different microglia-specific pathways in mice during development result in obsessive-compulsive disorder (OCD)-relevant behaviors (Chen et al. 2010, Zhan et al. 2014) and impaired functional brain connectivity similar to that seen in autism and other neurodevelopmental disorders (Zhan et al. 2014). For example, *Cx3cr1*-knockout animals have impaired synaptic pruning and sustained defects in social behavior and functional long-range connectivity (Zhan et al. 2014). Mutation of the *Hoxb8* transcription factor, which is expressed by precursor cells that give rise to microglia, leads to behavioral abnormalities, including compulsive overgrooming. Wild-type bone marrow transplantation into irradiated *Hoxb8* mutant mice rescued the OCD-related excessive grooming phenotype, as well as hair loss (Chen et al. 2010). In humans, mutation of the *TREM2* (triggering receptor expressed on myeloid cells 2) gene, which encodes an innate immune receptor that is expressed on microglia and myeloid cells, is associated with Nasu-Hakola disease, which is characterized by bone cysts, leukoencephalopathy,

early-mid-life dementia, and neurodegeneration (Paloneva et al. 2001). These phenotypes hint at the importance of microglia in cognition (Bianchin et al. 2004).

Evidence from human studies suggests that microglia are abnormal in ASDs, a complex group of neurodevelopmental disorders that are characterized by impaired social and verbal communication. The heterogeneous clinical and biological phenotypes observed in ASD suggest that a confluence of genetic and environmental risk factors combine or synergize to create a tipping point for dysfunction. Neuroimaging studies using PET/MRI have found putative inflammation in the brains of some patients with ASD (Pardo et al. 2005, Suzuki et al. 2013, Vargas et al. 2005). Postmortem studies have found an increased density of microglia, along with morphological aberration and altered neuronal interaction. Indeed, there is extensive microglial activation in a subset of individuals with ASD (Gupta et al. 2014, Lee et al. 2017, Morgan et al. 2010, Pardo et al. 2005, Takano 2015, Vargas et al. 2005, Voineagu et al. 2011). Most notably, these features are observed in regions that control executive functions such as the dorsolateral prefrontal cortex (Morgan et al. 2010, Tetreault et al. 2012, Vargas et al. 2005). Genome-wide transcriptional analysis of postmortem brain tissue from autism patients reveals altered expression of microglia-specific genes in some individuals, including increased expression of inflammatory markers (Gupta et al. 2014, Suzuki et al. 2013, Voineagu et al. 2011). Consistent with these postmortem studies, an increase in radiolabeled (*R*)-PK11195—a ligand for the translocator protein, which is expressed in both astrocytes and microglia—is observed in the brains of young adults with autism (visualized with PET) (Suzuki et al. 2013). However, [^{11}C](*R*)-PK11195 is not specific for microglia and may be a more general indicator of gliosis and neuroinflammation. Taken together, the patient and functional mouse data suggest that microglia are altered in autism and could contribute to the pathology.

However, it remains to be determined whether microglia are simply responding to aberrant changes in the brain or whether they are contributing to disease. Human genetic studies have not implicated microglia-specific genes in ASD, and insults originating in neurons or other cells may elicit an aberrant response in microglia that then contributes to pathogenesis. An example of this is the mouse model of Rett syndrome (Schafer et al. 2016), a neurodevelopmental disorder typically caused by mutations in methyl-CpG binding protein 2 (*Mecp2*) (Derecki et al. 2012, Maezawa & Jin 2010). Analyzing microglia–synapse interactions in the visual system before, during, and after onset of phenotypic and synaptic regression revealed that the excessive synaptic engulfment in the end stages of disease was independent of microglia-specific loss of MECP2 expression. These data suggest that microglia aberrantly respond to cell-extrinsic loss of MECP2 (in neurons or other cell types). However, one study showed that reconstitution of wild-type microglia into *Mecp2*-knockout mice, or rescue expression of MECP2 in microglia on a knockout background, was sufficient to alleviate many of the disease symptoms (Derecki et al. 2012). In contrast, other studies failed to observe this rescue (Wang et al. 2015).

Another consideration is the differences between sexes. Development of the CNS shows a distinctive sexual dimorphism (McCarthy 2016), including sex differences in microglial function that may play a key role in the overall sexual dimorphism of the brain (Bolton et al. 2017, Hanamsagar et al. 2017, Schwarz et al. 2012). Some of these sex differences may underlie, in part, differences observed in vulnerabilities to, and outcomes of, neurodevelopmental and neuropsychiatric disorders (Hanamsagar & Bilbo 2016). For example, the apolipoprotein E genotype is equally prevalent in men and women but has a stronger effect in women with regard to dementia, implicating sexual dimorphism in Alzheimer's disease (AD). Huntington's disease, autism, and schizophrenia similarly demonstrate sexually dimorphic features of pathology. Sex differences impacting microglial function have been observed in perturbations to the CNS such as traumatic injury, stress, and ischemia (Acaz-Fonseca et al. 2015, Arevalo et al. 2013, Bollinger et al. 2016, Bolton et al. 2017,

McCullough et al. 2016). A better understanding of these differences is critical to understanding disease pathogenesis and therapeutic strategies that will have the greatest benefit to each patient.

Aberrant Reactivation of Developmental Programs in Adult Disease

Could developmental functions become reactivated at an inappropriate time or in an inappropriate context and contribute to disease in the adult brain? Recent studies in animal models suggest that microglia-mediated pruning can become aberrantly activated, stimulating synapse loss in the mature brain during the early stages of neurodegenerative disease. AD is a chronic neurodegenerative disease that is characterized by the loss of synapses and neurons and by the accumulation of amyloid beta (Aβ) plaques. It is well established that complement plays a role in AD pathology in different contexts. Expression and activation of complement have been detected in mouse models of AD and patient tissue (Rogers et al. 1992), along with the upregulation of *C3* and *C4* mRNAs in the temporal cortex of AD patients (Walker & McGeer 1992) and the deposition of C1q, C3, and C4 on Aβ plaques in postmortem tissue from AD patients (Rogers et al. 1992). In the absence of immunoglobulins, Aβ binds to and activates C1q, suggesting that Aβ may activate the classical complement cascade (Rogers et al. 1992) and may facilitate the engulfment of amyloid plaques degenerating cells.

Recent findings suggest that the synapse loss observed in early stages of AD, glaucoma, and other neurodegenerative diseases is caused by an aberrant reactivation of pruning in vulnerable brain regions. In support of this idea, early and region-specific upregulation of complement and deposition of complement onto subsets of synapses were observed in mouse models of AD in the absence of overt inflammation and pathology (including amyloid plaques) (Hong et al. 2016a). Microglia exhibit increased engulfment of synaptic proteins in mice that have been injected with Aβ into the CNS. Importantly, inhibiting the classical complement cascade by deleting the genes for C1q, C3, or the microglia phagocytic receptor CR3 rescues synapse loss, and C3-deficient AD mouse (APP/PS1) models have reduced cognitive impairment, including learning and memory defects (Hong et al. 2016a, Maier et al. 2008, Shi et al. 2017), independent of Aβ plaques (Hong et al. 2016a). Together, these data implicate microglia in early synapse loss via C1q- and complement-mediated synaptic pruning.

Conversely, complement may have beneficial roles in later stages of AD. Expressing the complement inhibitor CRRY or knockout of *C3* increases the deposition of Aβ plaques and neuronal death (Maier et al. 2008, Wyss-Coray et al. 2002). One explanation for this apparent contradiction is that microglia depend on complement to engulf Aβ (Fu et al. 2012). Thus, understanding the signals that regulate specific microglia functions (i.e., engulfment of synapses versus Aβ) at different stages of disease could provide important insights into new therapeutic targets (Salter & Stevens 2017).

New approaches that assess the transcriptomic, proteomic, and epigenomic features of microglia are beginning to uncover a discrete set of microglia cell states across both health and disease. A better understanding of when and where microglial dysfunction impacts CNS diseases will aid in disease diagnosis and highlight possibilities of when and where to intervene therapeutically. The development of microglia-based diagnostics may allow for detection far earlier than ones based on neuronal dysfunction. Development of such markers (for example, soluble complement and TREM2 in the cerebral spinal fluid) or neuroimaging approaches using probes directed toward microglial signaling pathways will aid in diagnosing disease and assessing progression and even recovery upon treatment. Additionally, because of the sexual dimorphism observed in microglial function and dysfunction and that observed in CNS diseases, the development of microglia-focused biomarkers and therapeutics will require attention to the pathways and targets

that may differ between men and women. A final important consideration is the therapeutic relevance of preclinical models. While the cellular composition of the brain seems to be conserved from rodents to primates and humans, the translational relevance between models is still in question and needs to be better understood to ensure success in developing clinical applications. Studies are beginning to illuminate the microglial pathways that are unique and conserved between the human and mouse (Gosselin et al. 2017), which will hopefully lead to a better understanding of the mechanisms underlying microglial function and dysfunction in human disease and to a determination of which aspects are viable for therapeutic targeting. Current studies using single-cell RNA, protein, and epigenetic profiling will provide critical insights into region-specific, species-specific, and disease-specific changes in microglia (Keren-Shaul et al. 2017, Macosko et al. 2015).

CONCLUSIONS AND OUTLOOK

The emerging roles of microglia in brain development are opening up exciting new directions that could greatly expand our understanding of how the brain achieves its incredible complexity and specificity in its connections and structure. Once thought to only be mediators of immune responsivity, microglia are now known to extend their influence into many aspects of brain development, interacting with other cell types to provide support, signaling cues, and monitoring of neuronal circuits. Equally importantly, microglia have emerged as critical mediators of neuropathology in diverse neurological disorders.

Several major aspects of microglial biology remain to be explored. First, we must better understand the states that microglia assume in both development and disease. It is widely agreed that the term activation, or the bimodal M1/M2 scheme, does not sufficiently describe the multitude of ways in which these cells can respond to changes in their environment or the diversity of their functional states. Second, we must devise better ways to manipulate microglia so that we can uncover in more detail the molecular underpinnings of each distinct state and response, as well as test the function of specific signaling pathways. An in-depth understanding of these unanswered questions will be the goal of the next decade of microglial research and discovery and will have important consequences for the prevention, diagnosis, and treatment of neurological disease. This is an exciting time for this area of research, with each discovery suggesting possibilities for novel therapeutic strategies.

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