Bidirectional tuning of microglia in the developing brain: From neurogenesis to neural circuit formation

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Abstract

The developing brain employs multi-step processes to construct neural circuitry. Recent studies have highlighted that microglia, traditionally known to be the resident immune cells in the brain, have essential roles in these processes, which range from neurogenesis to establishing synaptic connections. Microglia play bidirectional roles for maintaining proper circuitry: eliminating unnecessary cells, axons, and synapses, while supporting the neighboring ones. Although these processes are performed in different parts of the neuron, similar molecular mechanisms are possibly involved. This paper reviews recent progress on the knowledge of the roles of microglia in brain development, and further discusses the application of this knowledge in therapies for brain disorders and injuries.

Introduction

Microglia account for 3–12% of the cells in the central nervous system (CNS) and are commonly referred to as the brain's resident immune cells or tissue macrophages [1,2]. Microglia play diverse roles in pathological conditions, owing to their ability to dramatically change morphology and engage in inflammatory/repair responses [3,4]. Recent technological advances have revealed additional physiological functions of microglia in the healthy brain, particularly, during development where dramatic changes in neuronal circuitry occur daily. Here, we review the newly discovered role of microglia on brain development, and further discuss whether understanding these functions will aid in therapeutic treatment of diseases that target the CNS.

Origin of microglia and their distribution

Although the majority of cells in the brain are generated from neural progenitor cells, microglia are considered to be of monocyte/macrophage lineage because of their shared features. However, whether their origin is distinctly defined during development or recruited from monocytes in circulating blood has been a subject of debate [5,6]. This topic has recently been addressed by a study that used parabiosis to demonstrate that blood-derived monocytes are not provided to the adult brain and microglia can “self-renew” [7]. This implies that there is a specific, predetermined origin of microglia during development. Indeed, expression of microglial markers suggest that they are derived from the embryonic yolk sac [8], and more recent studies using genetic mice have shown direct evidence of this (Fig. 1a) [9-11]. In these studies, taking advantage of early markers such as RUNX1, a transcription factor, and CSF1R, a receptor for CSF1 (M-CSF), the lineage of microglia was traced using *Runx1-CreER* and *Csf1r-CreER;YFP* reporter mice with tamoxifen injection to label

yolk sac progenitor cells in a restricted developmental time window. It was demonstrated that microglia were derived from myeloid progenitors at around E7.5 and the cells were Myb-negative and PU.1-dependent Csf1-receptor-expressing progenitors, which are different from Myb-dependent hematopoietic stem cells that differentiate into other macrophages and monocytes [9,10]. The molecular profiles of these progenitors have been further identified [11].

In mice, cells migrate out of the yolk sac and infiltrate the brain at E9.5 (Fig. 1a and b) [5,8-10,12,13]. Although the route to infiltration is not yet completely understood, histological studies imply that these cells enter from the meninges, ventricles, and intracerebral blood vessels. Once in the brain, microglia gradually increase in number and colonize areas such as subcortical and cerebellar white matter in early postnatal days (Fig. 1d). These colonized regions are referred to as “fountains of microglia” in rodents [14-16] and humans [17,18] and are ultimately distributed to most brain regions presumably through migration and proliferation [12,14,16].

The timely and locally invasive nature of microglia implicates that these cells play a critical role in brain development. In fact, recent reports have revealed that microglia are involved in the construction of neural circuits, which are assembled in multiple stages: neural progenitors proliferating around the ventricular zone (VZ), differentiated neurons migrating to specific areas, and neurons projecting axons to target areas and forming synapses (Fig.1).

Bidirectional roles of microglia in neurogenesis

During the course of brain development, significant numbers of neurons and glial cells (astrocytes and oligodendrocytes) must be precisely generated from neural progenitor cells. Interestingly, microglia have been observed in the neuroepithelium

where progenitor cells proliferate [5,6,8,9,19], implying a role for microglia in neurogenesis (Fig. 1b and c). Microglia have been shown to exert trophic effects *in vitro*. For example, cultured embryonic cortical cells of PU.1^{-/-} mice lacking microglia demonstrate no change in cell survival and neurogenesis of progenitor cells, but exhibit reduced proliferation and astrogenesis [19]. A number of factors known to be expressed in microglia, such as BDNF, FGF2, and IGF1 [14,20], may be involved in this process. *In vivo*, a decline in microglia is correlated with reduced histogenesis of the brain in *Csf1r*^{-/-} mice [21]. This study implies a role for microglia in neurogenesis; however, whether this is a direct effect of microglia remains unclear.

Conversely, it was found an opposite role of microglia in that they can restrict the number of neural progenitor cells *in vivo* [22]. This group observed prominent numbers of microglia in the VZ and subventricular zone (SVZ). In these zones, non-dying progenitors phagocytosed by microglia gradually increased, and this was correlated with reduction in the number of progenitor cells. Deactivating or eliminating microglia by tetracycline or liposomal clodronate, respectively, significantly increased the number of progenitor cells, whereas activating microglia by lipopolysaccharide (LPS) decreased the number of these cells. Therefore, the authors concluded that microglia regulate the number of progenitor cells mainly through phagocytosis. This can be related to the phagocytosis of new born cells in the hippocampal proliferative zone [23].

From these results, it has been postulated that microglia play bidirectional roles in neurogenesis by supporting cell generation and removing unnecessary cells.

Microglia control the survival and death of developing neurons

The regulation of neuronal number is essential to the construction of neural circuitry.

In the developing CNS, cell death occurs extensively to eliminate unhealthy and misconnected neurons [24]. Survival of neurons is presumably maintained by factors derived from target or connected neurons and glial cells. While cell death was thought to be determined autonomously within the neuronal circuit, recent findings indicate that microglia might also be involved (Fig. 1d). In the developing retina, proNGF secreted from microglia have been shown to induce cell death via P75 neurotrophin receptor [25]. In another case, TNF α derived from macrophages adjacent to the spinal cord was shown to commit motor neurons to die [26]. These studies suggest that microglia promote cell death through secreted molecules. One other mechanism that induces cell death is phagocytosis [29,30], which is classically considered to be just the process of eliminating dying cells in both the normal and damaged developing brain [31]. In the postnatal cerebellum, many dying Purkinje cells are phagocytosed by microglia. Eliminating microglia by liposomal clodronate or inhibition of NADPH oxidase can significantly reduce Purkinje cell death [32]. This indicates that microglia aid the process of cell death by engulfment and release of superoxide ions. Although the studies above should be carefully interpreted since they were all conducted *in vitro* in which microglia may easily change their activity (to toxic, in this case) [27,28], the following *in vivo* study demonstrated that DAP12-CD11b-ROS signaling contributed to developmental death of postnatal hippocampal neurons [33], as similarly observed in progenitor cells [22]. Future work is required in order to elucidate whether microglia facilitate cell death, just engulf dying cells, or do both.

While the mechanism by which microglia recognize target cells (“find me” signal), engulf them (“eat me” signal), and induce cell death still remains unclear, some clues have surfaced. Microglia phagocytize healthy neurons in inflammatory conditions and use a similar mechanism to engulf apoptotic cells [30,34,35]. Phosphatidylserine (PS)

exposed on neurons is bound by MFG-E8, which are then phagocytized via the vitronectin receptor on microglia. In this context, phagocytizing glial cells (not categorized as microglia/macrophages) likely use Jedi-1 and MEGF10, previously reported as *CED-1* in *C. elegans* [36] and *Draper* in *Drosophila* [37] respectively, for phagocytosis in developing dorsal root ganglia [38,39]. Again, most of these experiments were conducted *in vitro* and not tested in microglia in the developing brain.

We recently found an opposite function in which microglia can promote the survival of specific neurons in the postnatal cortex (Fig. 1d) [14]. The first aim was to determine the role of postnatal amoeboid microglia which typically accumulated in the white matter [15,16,40,41]. To address this, we inactivated or eliminated microglia by using minocycline treatment, CD11b-DTR transgenic mice, and *Cx3cr1*^{-/-} mice. Interestingly, all of these modifications led to increased apoptosis in layer V neurons at P3-5. This time window correlated with the period when microglia accumulate near the axons of layer V neurons. We also identified microglia-derived IGF1 as a survival factor. Additional factors might be involved in the effects of microglia since IGF1 only partially supported neuronal survival.

Collectively, microglia locally patrolling eliminate neurons showing abnormal signs (“eat me”/“kill me” signals), and support the survival of surrounding healthy neurons not expressing abnormal signs (or expressing “don’t eat me” signals). These processes would result in preventing secondary necrosis and inflammation, and help maintain homeostasis.

Microglia engage in maintenance of axons and synapses during neural circuit formation

Microglia likely perform similar functions with respect to axons and synapses during neuronal wiring. In the initial phase, axons robustly project to a target area and improper connections are pruned in a degenerative or retractive manner [42]. In *Drosophila*, glial cells surround axons from mushroom body, engulfing degenerating ones [43]. Some neuronal signal is responsible for attracting glial cells to these dying axons and glial cells seem to facilitate the degenerative process [43]. The engulfment occurs through Draper and Ced-6, which are also known to be involved in enveloping apoptotic cells [44]. This implicates that apoptotic cells and degenerating axons are removed by a similar molecular mechanism. In the mammalian system, microglia has a capacity for engulfing axonal debris [45] and microglia-engulfing axonal materials are observed where pruning occurs [14,16,42]. Despite their close correlation, the role in axon pruning is still unknown; microglia presumptively engulfs axonal material, but it is not clear whether they facilitate pruning (Fig. 1d).

Along with elimination, microglia may have a supportive effect on axon growth and guidance [41,46]. During the postnatal period, amoeboid-shaped microglia in the white matter where axons are growing express axonal growth factors, such as IGF1 [14], thrombospondin [47,48], and NT3 [49]. They also express guidance molecules, such as Slit, Netrin [50], Sema7a [51], and RGMa [52] in the injured CNS. However, there is no direct evidence *in vivo*, that microglia support neighboring axons during axonal pruning (Fig. 1d).

The role of microglia on synapse formation is a topic of much interest (Fig. 1e) [53]. This comes with the finding that microglia are highly active with motile processes, which was discovered by using the combination of microglia-specific EGFP-expressing mice (*Cx3cr1^{GFP/+}*) and two-photon microscopy [54,55]. Subsequently, *in vivo* imaging and electron microscopic analyses revealed that

microglia frequently contact synapses in a neuronal activity-dependent manner, suggesting a role for microglia in synaptic remodeling [56]. During development, synapses are initially generated superabundantly and then pruned to refine a circuit. In the juvenile visual cortex, dendritic spines are contacted by microglia, and a quarter of them disappear after the contact [57]. Moreover, deprivation of visual input results in increased contact and synapse-like inclusions in microglia. This suggests that microglia eliminate excess synapses to remodel connections. Consistent with this, pre- and postsynaptic molecules have been detected in microglia in the postnatal hippocampus, and decreases in microglial number in *Cx3cr1*-knockout mice increase the number of dendritic spines [58]. Similar correlations have been observed in the postnatal somatosensory cortex [59]. While the molecular mechanism still remains unclear, CX3CL1 (fractalkine)-CX3CR1 signaling may be required for migration into the microcircuit, or for locally targeting the synapses.

Components of the classical complement cascade, C3 abundant in synapses, and its receptor CR3 expressed in microglia, have been shown to be involved in the phagocytic process of synapses in the postnatal retinogeniculate circuit [60]. Microglia engulf presynaptic materials of weak input side when binocular input to the dorsal lateral geniculate nucleus (dLGN) is being remodeled. Engulfed synapses are decreased and refinement of binocular input to the dLGN is impaired in *C3*- and *CR3*-knockout mice [60,61]. It remains unknown whether microglia selectively detect weak synapses via the C3/CR3 system, and this system is generally used in other brain regions.

While the studies above suggest that microglia play an essential role in engulfing unnecessary synapses, these cells may also maintain required synapses given their bidirectional role in other developmental processes. Indeed, contacted pre- and post-

synaptic sites alter their size by both growing and shrinking, and not all the spines are eliminated [57]. Other properties of microglia have been uncovered in the optic tectum of larval zebrafish. Microglial processes were directed to contact active neurons, requiring membrane depolarization-activated pannexin-1 hemichannels in neurons that can release ATP etc., and the contact downregulated neuronal activity [62]. If this is the case in all neurons, microglia would use molecular mechanisms to select specific synapses to contact, eliminate, or preserve (Fig. 1e).

How do microglia migrate into and within the brain: unresolved questions

The mechanism underlying microglial migration is an unresolved question. Although microglia distribute ubiquitously in the adult brain, a variety of developmental tasks are often specialized in each brain region. Therefore, oriented migration and recruitment in a timely and local manner are important processes. Nevertheless, it remains unknown how microglia recognize the brain and their final location (Fig. 1). Although the blood-brain barrier (BBB) is immature enough for cells to enter during development, it is clear that microglia do not just randomly enter the brain from the vasculature. For example, amoeboid microglia are precisely controlled to accumulate in the postnatal white matter [15,16,40]. We have observed that microglia likely invade the postnatal forebrain from the midline, ventricle, or choroid plexus, but it is unclear how these cells are recruited in such a precise time window and location. Interestingly, microglia exogenously introduced into the blood stream specifically target to the brain [63]. This implies that some specific cues are involved in the relocation process. In the experimental autoimmune encephalomyelitis (EAE) model, autoreactive Th17 cells enter the CNS from a specific lumbar region through CCL20-CCR6 signaling, suggesting that some gateways are located in the

CNS that render the invasion of immune cells [64]. Interestingly, CCL20 expression is higher in this region even in the healthy CNS. From this view, it will be possible to imagine that microglia might also use a similar gating system.

The candidates involved in microglia migration could include chemokines, ATP–P2X4/P2Y12 signaling, and glutamate [65-67]. For example, the *panther* mutant in zebrafish, which lacks the M-CSF receptor gene, shows delayed invasion of microglia into the brain [13]. Likewise, microglia are reduced in *Csf1r*^{-/-} mice [9,21]; however, it is still unresolved whether this signal is required to migrate out from the yolk sac, to invade the brain, or for just microglia production in the yolk sac. Some other chemokines can be excluded as the candidates. CCR2 has been shown to be expressed in monocytes but not in microglia and *Ccr2*^{-/-} mutants do not show reduced microglia numbers [11,68,69]. *Cx3cr1*^{GFP/GFP}, *Ccr1*^{-/-}, and *Cxcr3*^{-/-} do not alter entry of microglia into the brain [11,14]. Similarly, *P2y12*^{-/-} mice do not exhibit a reduction of microglia in the adult brain [66], although it have not yet been thoroughly examined in development. One recent study found that VEGFR1-NOX2 signaling is required, in part, for microglial migration from the ventricle to the SVZ [65], but the entire mechanism still remains unknown.

One other possible mechanism is that microglia are attracted by degenerating axons or cells; however, the “find me” molecule has not yet been tested in this context. Importantly, these attracting molecules may also be involved in local migration and motility that would be essential for the other tasks in targeting specific neurons and synapses. Thus, it will be intriguing to investigate the mechanism underlying directional migration. Lastly, if we know the mechanism underlying migration, we may be able to control the number of infiltration microglia/macrophages in CNS diseases.

How are the variable functions of microglia regulated? – application to CNS disease

One key advantage to understanding the variable roles of microglia would be to clarify the multiple functional states and heterogeneous populations of microglia, that apparently contribute to normal brain development and function. For example, only 40% of microglia are derived from Hoxb8-expressing hematopoietic cells, the mutation of which leads to abnormal grooming behavior [12]. However, it is not clear how these heterogeneous populations and functions emerge. Microglia have been traditionally classified as a “resting” state, which have a ramified morphology (or recently “surveying” microglia [70]), and an “activated” state, showing profound changes in shape and gene expression, particularly in pathological conditions. Even if we use this simple classification, it cannot explain the diverse set of functions. For example, “activated” microglia have bidirectional functions, exerting both toxic and trophic effects onto neurons [14,32]. Other classifications, M1 (classic) and M2 (alternative) states, which applied from macrophage literature [71], is not easily applied to the healthy brain and its development. Indeed, microglia in the P3 mouse brain highly express both M1 (iNOS and TNF α) and M2 genes (Arginase-1), compared to adult microglia [28]. Even in diseased conditions, microglia behave differently from macrophage/monocytes [72,73]. Tissue macrophages, including microglia, are considered to be M2 but this rigid view may lead to misunderstandings about heterogeneous functions of microglia.

Therefore, it should be clear that the biggest problem posed in this field is the difficulty in dissecting out the multiple faces of microglia: what activation and deactivation are, in which state phagocytosis, toxic, and trophic functions occur, as

well as how these states are regulated. These multiple factors hinder our understanding of the real roles of microglia. It would be important to understand the mechanism that transits the properties of microglia (i.e., resting (surveying) vs. activated and M1 vs. M2). However, because this simple classification cannot demonstrate whole properties of microglia, we also need to explore the function of each gene expressed in specific microglial populations. This may lead to a more direct understanding of the variable functions.

The tools used to specifically modify microglia *in vivo* are critical factors in advancing in this field. Some drugs frequently used, such as minocycline and LPS, are not specific to microglia. Some genetic and chemical approaches to ablate microglia (liposome clodronate, CD11b HSVTK mice, and CD11b-DTR mice [27]) will ablate heterogeneous microglia, thereby eliminating each individual function. Thus, tools to selectively delete specific genes such as *Cx3cr1-cre*, *Cx3cr1-creER* [74,75], RVG-9dR-siRNA [76], and β 1,3-d-glucan-encapsulated siRNA particles [77], will be more promising approaches. It will be intriguing if these specific deletions can regulate each microglial function such as phagocytosis, or states of activation. These technologies will pave a new way to expand our knowledge of microglia.

As reviewed here, microglia have diverse roles even in the normal brain and its development. This suggests that disruption of microglia function can lead to CNS disorders. For example, obsessive grooming behavior, a model of trichotillomania, can be observed following *Hoxb8* deletion in microglia [12]. Mutation of the *Sod1* gene in microglia promotes late progression of diseases related to amyotrophic lateral sclerosis (ALS) [78]. Phagocytic deficiency in *Mecp2*^{-/-} microglia attributes to the pathology in a mouse model of Rett syndrome, an X-linked autism spectrum disorder,

suggesting that clearance of debris is essential for normal brain function [79]. Many reports have further demonstrated that microglia engage in tissue defense and repair by their diverse functions such as phagocytosis, cell death induction, neuroprotection, axonal modulation, and synapse monitoring. Thus, understanding the molecular mechanisms of each function in brain development will help lead to the establishment of a therapeutic method to regulate microglial functions and attenuate symptoms of CNS diseases.

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Annotation

10* This study demonstrates that some of tissue macrophages including microglia are derived from specific lineage in yolk sac; Myb-independent, PU.1-dependent, and Csf1l-receptor-expressing lineage, which can be distinguished with Myb-dependent hematopoietic stem cell-derived macrophages/monocytes. This concludes that microglia has a specific origin different from definitive hematopoietic stem cells.

11* Following the study in [10], the authors found several differentiation steps of microglia in the yolk sac and specifically c-kit-positive cells as a precursor. They further demonstrated that Irf8 and MMPs are required for microglial development and expansion in the brain.

14** Using different methods to modulate microglial function *in vivo*, the authors demonstrate that postnatal microglia have a role to maintain the survival of specific neurons. This is the first report showing a trophic effect of microglia on neurons during brain development.

22* This study show that microglia engulf neural precursor cells and may regulate the proper size of precursor pool during development. This suggests that microglia do not just clean cellular debris up, but also actively control the number of cells via phagocytosis.

60** This study demonstrate that microglia actively engage in synapse elimination during neural circuit formation, and reveal that CR3/C3 signaling is involved in this process. This study is extended from [61], and is the first report showing the key molecules involved in synaptic engulfment by microglia.

62** This study reveals that neuronal activity steers patrolling microglial processes to contact neurons using pannexin-1 hemichannels, and eventually the contact reduces neuronal activity. This highlights a novel physiological role of microglia to regulate homeostasis of neural circuit.

64** This study demonstrate that, in an EAE model, autoreactive immune cells infiltrate the CNS from a specific site through upregulation of chemokine CCL20 induced by sympathetic nerve activation. This proposes a system that creates an entry site of immune-related cells in the brain.

65* The authors show that NOX2 and VEGF signaling is required for infiltration of postnatal microglia around the ventricle. This study proposes a novel field to investigate the mechanism of microglial migration and infiltration in a developing brain.

75** This report demonstrates that target deletion of interest gene in microglia by using *Cx3cr1*-Cre or *Cx3cr1*-CreER mice is very useful for the analyses of microglial function.

79* The authors demonstrate that replacement with wild type microglia in *Mecp2*^{-/-} mice attenuates the pathology of a mouse model of Rett syndrome, an autism spectrum disorder induced by *MECP2* mutation. Decreased phagocytic activity in *Mecp2*^{-/-} microglia seems to relate to the symptoms. This provides an important clue that phagocytic function of microglia would be indispensable for normal brain development and function.

Figure legend

Figure 1. Bidirectional functions of microglia in the developing brain. Microglia are generated in the yolk sac from E7.5 in mice (a) and migrate out of the sac, infiltrating the neuroepithelium through ventricles, meningeal tissue, or vasculature (b, red dotted arrows). Microglia eliminate unnecessary neural progenitor cells and may also support neurogenesis (b and c, red and blue arrows respectively). Progenitor cells start differentiating into neurons and migrate to their final position (c and d, green dotted arrows), where microglia support the survival of neurons and eliminate unnecessary ones (blue and red arrows, respectively). Neurons then project axons, and microglia transiently accumulate along the axonal tract in the postnatal period (d). How microglia accumulate along the axon largely unknown (red dotted arrow). Microglia may affect axonal growth/survival of neurons and engulf degenerating or pruned axon materials (blue and red arrows, respectively). Microglia distribute in the brain during neural circuit formation (d to e, red dotted arrows) and survey their environment, frequently contacting synapses (e), and engulfing those that are unnecessary. This act may control homeostasis of neural activity. VZ, ventricular zone; SVZ, subventricular zone; ?, unknown or indicating that this has not been completely proved *in vivo*.

Table 1

Molecules involved in developmental functions of microglia

Process	Paradigm
Initial differentiation / proliferation of microglia	<i>in vivo</i>
Migration into the CNS	<i>in vivo</i> <i>in vivo</i> <i>in vivo</i> <i>in vivo</i> <i>in vivo</i>
Cell death induction	<i>in vitro</i> <i>in vitro</i> <i>in vitro</i> <i>in vivo</i>
Cell survival	<i>in vivo</i>
Phagocytosis of neurons	<i>in vivo</i> <i>in vitro</i>
Axonal growth	<i>in vitro</i>
Phagocytosis of axons	<i>in vivo</i>
Synapse elimination	<i>in vivo</i> <i>in vivo</i>