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Review

Microglia function during brain development: New insights from animal models

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ABSTRACT

The role of microglia in healthy brains is just beginning to receive notice. Recent studies have revealed that these phagocytic cells control the patterning and wiring of the developing central nervous system (CNS) by regulating, amongst many other processes, programmed cell death, activity-dependent synaptic pruning and synapse maturation. Microglia also play important roles in the mature brain and have demonstrated effects on behavior. Converging evidence from human and mouse studies together raise questions as to the role of microglia in disorders of brain development such as autism and, schizophrenia. In this review, we summarize a number of major findings regarding the role of microglia in brain development and highlight some key questions and avenues for future study.

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Contents

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1. Introduction

Microglia are the resident immune cells in the brain and much mystery surrounds their function. Despite being described by Rio Hortega over a hundred years ago, these cells did not receive any notice in studies of healthy brain development or function until the past decade. Much of our knowledge about microglia has been in the context of injury and disease. Long viewed as the brain's defenders against biological threats and injury, these chameleon-like cells transform from a resting to 'activated', macrophage-like state when challenged. Following injury or disease, microglia are rapidly recruited to sites of damage where they engulf, or phagocytose, debris as well as unwanted and dying cells. Although critical for the immune response to infection or trauma, microglia also contribute to pathological neuroinflammation by releasing cytokines and neurotoxic proteins (Perry et al., 2010; Ransohoff and Perry, 2009).

The potential role of inflammation and microglial activation in neurodevelopmental and psychiatric disorders has been speculated for some time (Pardo et al., 2005). Postmortem studies suggest that microglial cell density and/or the number of 'activated' microglia may be increased in the brains of individuals with autism, including in regions important for regulating executive functions such as the dorsolateral prefrontal cortex (Morgan et al., 2010; Tetreault et al., 2012; Vargas et al., 2005). Schizophrenia postmortem studies have similar findings, also suggestive of increased microglial cell density and/or activation; further, PET imaging reveals signs of microglial activation in the brains of living individuals with recent-onset schizophrenia (Frick et al., 2013; Monji et al., 2009; van Berckel et al., 2008). Yet these and other intriguing observations in the human brain have remained poorly understood because it is not known whether microglia are mediators of the disease process in these conditions, or simply responders to neuronal dysfunction or both. Put another way, do microglia play an active role in the developmental processes that go awry in autism or schizophrenia, or is their increased activation a byproduct of the pathology? As with any other observation of cells in a pathology sample, the first point of inquiry is determining what these cells do under healthy conditions. Recent studies using animal models have provided key insight.

Significant strides were made in 2005 when pioneering in vivo imaging studies revealed that microglial processes are highly dynamic in the cortices of healthy adult mice—so active, even, that it's estimated they may be able to survey the entire brain parenchyma in 1 h (Davalos et al., 2005; Nimmerjahn et al., 2005). Since these reports, a new line of research examining microglial roles in healthy CNS development has emerged.

It was long thought that microglia derive from peripheral macrophages that enter the brain after birth. In 2010, a landmark fate mapping study challenged this dogma by showing that microglia develop from myeloid progenitors in the yolk sac and make a pilgrimage into the brain very early in embryonic development (Ginhoux et al., 2010). The realization that microglia develop alongside neurons during this critical period of brain development has led to a sea change of thinking about microglia in the healthy brain. New data implicate microglia in many functions required to build and wire the developing CNS, ranging from neurogenesis to synaptic pruning. Here we review some of these key discoveries and highlight the models and emerging tools being used to probe microglia function and signaling in the healthy brain (Tables 1 and 2).

2. Patterning the developing CNS

Microglia have long been recognized as 'professional' phagocytes that rapidly eliminate dead or dying cells and associated debris during CNS disease or injury. They are also known to signal to other CNS cells through a broad range of secreted factors—several that trigger apoptosis, such as tumor necrosis factor alpha (TNFα), reactive oxygen species and glutamate, some that promote survival or proliferation, and others that either promote or fight inflammation (Bessis et al., 2007; Harry, 2013; Pollard, 2009). Recent research has explored how microglia in healthy circumstances may use these phagocytic and signaling functions to pattern the developing CNS.

2.1. *Control of neuronal death*

During early postnatal development large numbers of neural cells undergo programmed cell death, an apoptotic process critical for shaping the landscape of the nervous system. In vertebrates it is estimated that approximately half of all the neurons born originally are eliminated during the course of development (Oppenheim, 1991; Yeo and Gautier, 2004). One of the early clues that microglia may be involved in PCD was their pattern of expression in developing CNS tissues such as the retina or hippocampus—they seem to follow waves of neural expansion and frequently appear in the vicinity of dying neurons (Ashwell et al., 1989; Dailey et al., 2013; Dalmau et al., 1998). A growing body of evidence now supports the idea that microglia are key players in the process of eliminating excess neurons during development. Indeed, they appear to play two distinct roles: (i) responding to PCD by phagocytosis of dead or dying neurons and associated debris, as identified in observational studies, and (ii) actively mediating cell death through soluble or contact-mediated cues, as identified in more mechanistic studies.

Table 1 – Animal models for studying developmental functions of microglia.

Species and CNS region	Developmental processes	Age	Major assays	Key reference (s)
Mouse visual thalamus	Activity-dependent synapse elimination, eye-specific segregation of retinal inputs	First postnatal week	In vivo synapse engulfment assay, anterograde tracing, electron microscopy	Schafer et al. (2012)
Mouse visual cortex	Experience-dependent remodeling of microglia–synapse interactions	Third and fourth postnatal weeks	In vivo two photon imaging, serial 3D electron microscopy	Tremblay et al. (2010)
Mouse hippocampus	Maturation of synapse physiology	Second and third postnatal weeks	Acute slice recordings, response to chemically-induced seizures, biochemical fractionation of postsynaptic density	Paolicelli et al. 2011, Roumier et al. (2004)
	Programmed cell death of neurons	Late embryonic/ neonatal	Caspase staining, detection of superoxide ions	Wakselman et al. (2008)
Mouse motor cortex	Learning-dependent dendritic spine remodeling	First and second months	In vivo two photon imaging, rotarod testing	Parkhurst et al. (2013)
Mouse cerebellum	Programmed cell death of Purkinje neurons	Neonatal	Caspase staining, detection of superoxide Ions	Marin-Teva et al. (2004)
Rat spinal cord	Programmed cell death of motoneurons	Embryonic	TUNEL staining of explants	Sedel et al. (2004)
Chicken retina	Programmed cell death of retinal neurons	Embryonic	Cell death immunoassay in 'optic cup' explants	Frade and Barde (1998)
Zebrafish brain	Phagocytosis of neurons	Embryonic	In vivo imaging	Peri and Nusslein-Volhard (2008)

Parts of this figure were adapted from Table 1 in Schafer, D.P., Lehrman, E.K., Stevens, B., 2013. The quad-Partite synapse: microglia–synapse interactions in the developing and mature CNS. Glia. 61, 24–36.

Table 2 – Genetic tools for targeting microglia in mice.

Protein of interest	Description	Manipulation	Key reference(s)
CX3CR1	Microglial receptor for chemokine CX3CL1, produced by neurons	Knockout, CRE-ER tamoxifen-inducible conditional expression mutant	Paolicelli et al. (2011), Parkhurst et al. (2013)
CR3	Complement receptor 3, part of the classic complement cascade in immune system, thought to be microglia-specific in brain	Knockout	Schafer et al. (2012)
KARAP/ DAP12	Transmembrane polypeptide associated with cell surface receptors in peripheral immune system, appears to be microglia-specific in brain	Knockout	Roumier et al. (2004)

2.1.1. *Evidence for responder role in programmed cell death*

One of the classic studies supporting a phagocytic role for microglia in the brain was a 1990 report using the silver impregnation method of Rio Hortega – fittingly, the neuroanatomist who was the first to use the term 'microglia' – to characterize sections of the rat cerebral cortex during early postnatal development. This method revealed that amoeboid or globular phagocytic cells containing vacuoles appear in the

subplate and cortical layers II/III during the first week after birth, just when these regions are undergoing high levels of PCD (Ferrer et al., 1990). Since then, several studies have shown that microglia migrate to different regions of the CNS at different times, usually right before or during the height of PCD there. Microglia phagocytose neurons in the waves of PCD that occur in the developing retina, spinal cord, cerebellum, hippocampus and cerebral cortex (Bessis et al., 2007; Sierra et al., 2013).

Yet much remains unknown about the molecular pathways mediating microglia's phagocytic actions during PCD. One promising avenue for new research is in vivo imaging in the developing zebrafish, where the process of neurons dying and being eaten by microglia can be visualized in real time (Peri and Nusslein-Volhard, 2008), and a recent genetic screen has identified a receptor protein specifically required for microglial development (Shiau et al., 2013).

2.1.2. *Evidence for mediator role in programmed cell death*

While microglia are responders to PCD in the sense that they can come in and devour the remains of dead or dying neurons, they can also actively mediate the process of cell death and tissue remodeling. Historically, the evidence for this role arose in the peripheral nervous system, where macrophages analogous to CNS microglia are required for elimination of transient structures in the mouse eye. When these macrophages were genetically ablated through expression of diphtheria toxin, entire populations of ocular cells known to disappear on a strict developmental timeline persisted for weeks after their typical time of death (Lang and Bishop, 1993).

Soon matching evidence arose in the CNS. Explants from the embryonic chick eye – "optic cups" grown in a collagen matrix – were used to study the developing retina (the one part of the eye that is CNS tissue). Clear differences in cell death were observed depending on the absence or presence of the retro-ocular mesenchyme, the structure which blood-borne macrophages must traverse to enter the retina. Absence of the mesenchyme correlated with a strong reduction in retinal cell death, unless microglial cells purified from the vitreous bodies of older chick eyes were added to the cultures. Mechanistically, it appears that nerve growth factor released by the microglia may be acting on the p75 neurotrophin receptor of retinal neurons to induce their death (Frade and Barde, 1998).

Similarly, in embryonic explants of the rat spinal cord, microglia are required for PCD of motoneurons. A central mechanism appears to be microglial release of TNFα, which acts on TNF receptor 1 of motoneurons to induce their death (Sedel et al., 2004). Again, in slices of the developing mouse cerebellum, microglia were found to be critical for PCD of Purkinje neurons. A key factor here is the production of superoxide ions by microglia during "respiratory bursts." In this phenomenon, a sharp rise in oxygen usage occurs as the microglia rapidly produce an enzyme necessary for generation of these reactive oxygen species, which then target and degrade the internalized pieces of neurons (Chanock et al., 1994; Marin-Teva et al., 2004).

Beyond these explants and slice studies, in vivo evidence for microglia's role in directing cell death is also emerging. The CD11b integrin and DAP12 immunoreceptor are molecules implicated in cell death in the peripheral innate immune system. In the context of healthy development in the brain, these molecules are selectively expressed by microglia. Transgenic mice deficient for either one display decreased apoptosis in the neonatal hippocampus, suggesting that properly functioning microglia are crucial for PCD in the hippocampus. Similar to the case of PCD in Purkinje neurons, superoxide production by the microglia may be mediating the effect (Wakselman et al., 2008).

Intriguingly, microglia may be sculpting the cellular landscape of the nervous system at a more global level as well, controlling apoptosis of neural precursor cells (NPCs) in addition to postmitotic neurons. Amongst other evidence, when microglia are eliminated in mice in vivo (using in utero injections of liposomes containing the drug clodronate), the number of NPCs in the developing cerebral cortex rises, and conversely, when microglia are pharmacologically activated in vivo (using in utero injection of bacterial lipopolysaccharide) the number of NPCs falls (Cunningham et al., 2013). Of course, these techniques are relatively non-specific and only provide correlative evidence. Further study is required to draw solid conclusions as to whether microglia are directly inducing NPC death during brain development.

2.2. *Control of cell survival and proliferation*

While microglia actively promote neuronal death during brain and spinal cord development, in vitro studies suggest these cells can also influence CNS patterning in opposite ways, by promoting the proliferation of NPCs or survival of neurons (Chamak et al., 1994; Morgan et al., 2004; Nagata et al., 1993). In a recent study of NPCs cultured from the cerebral cortex of mice believed to lack microglia (knockouts for the transcription factor PU.1, controlling the monocyte lineage), there were effects on NPC proliferation and astrogenesis, but not neuronal survival. Microglia appeared to be promoting NPC proliferation, as cultures of NPCs from the cortices lacking microglia had decreased proliferation, but adding microglia from wild type mice to the cultures rescued this effect (Antony et al., 2011).

In vivo evidence for microglia promoting neuronal survival comes from a recent study of the developing mouse cortex. Between postnatal days 3 and 5, inactivating microglia (with the drug minocycline) or ablating them (through injection of a toxin specifically targeting cells under control of the CD11b promoter) resulted in increased neuronal apoptosis in layer V of the cerebral cortex. This reveals that at this stage of development, at least in this brain region microglia are likely providing some form of trophic support. Further examination of the effect suggested that IGF1 signaling downstream of the fractalkine receptor CX3CR1 (expressed almost exclusively by microglia in the postnatal CNS) may be responsible (Ueno et al., 2013). While these results are fascinating, clearly much work remains to be done to understand the positive effects of microglia on cell numbers in the developing CNS.

3. Wiring the developing CNS

Just as the overarching cellular landscape of the nervous system is sculpted by programmed cell death (PCD),

extranumerary synapses and axon branches are sculpted by the process of synapse elimination or pruning (Kano and Hashimoto, 2009; Lichtman and Colman, 2000; Katz and Shatz, 1996; Stretavan and Shatz 1984). Accumulating evidence implicate microglia in regulating synapse numbers, as well as synaptic function and maturation. The field of microglia–synapse interactions has blossomed and novel insights have been gained regarding microglial involvement in synapse development and activity-dependent circuit refinement (Schafer et al., 2013; Tremblay et al., 2011; Paolicelli et al., 2011).

3.1. *Neural activity and microglial dynamics at synapses*

Following the groundbreaking 2005 reports that microglial processes are actively surveying healthy brains, researchers used two photon microscopy to zoom in on microglia–synapse interactions and examine the impact of neuronal activity on these interactions. Visualization of microglia and neuronal processes was carried out using transgenic mice expressing EGFP in microglia and neurons (Iba-1-EGFP/Thy1-EGFP M line) (Feng et al., 2000; Hirasawa et al., 2005; Wake et al., 2009). The findings suggest that microglia directly survey the functional state of synapses. In the cerebral cortex of young adult mice, in layers II/III of somatosensory and visual cortex for instance, microglial processes make brief (5 min) and direct contacts with synapses at a frequency of about once per hour (from the perspective of an individual presynaptic bouton). Reductions in neural activity can lead to a reduced frequency of microglia–synapse contact (Wake et al., 2009).

The hypothesis that microglia play a role in experience-dependent circuit refinement gained further traction in 2010, with a more in-depth imaging study combining immunocytochemical and 3D serial section electron microscopy (EM) reconstruction techniques with in vivo two photon microscopy to characterize microglia–synapse interactions in the mouse visual cortex. These experiments were conducted at the height of the critical period for visual cortex development, when there is significant structural plasticity. Microglia in layer II of the visual cortex were found to contact spines, presynaptic terminals, and synaptic clefts. Spines often changed size upon microglial contact. In general, the microglia preferentially made contact with smaller spines, and many times the spines they contacted turned out to be ones that disappeared in later images. Upon application of a dark adaptation paradigm that promotes synaptic remodeling (dark rearing for 6 days followed by 2 days of re-exposure to light), the ultrastructural properties of microglia–synapse contacts changed in many ways—including altered morphology of the microglial processes, more frequent apposition to synaptic clefts, more extensive enveloping of synaptic elements, and most notably, more phagocytic inclusions (Tremblay et al., 2010). These findings suggested that microglia could be playing a phagocytic role in synapse regulation.

3.2. *Control of synapse number*

In the postnatal brain synaptic connections are formed in excess and must remodel to achieve the precise synaptic connectivity characteristic of the mature organism. Developmental synapse elimination – or synaptic pruning – is a critical part of this remodeling. Synaptic pruning is regulated by both spontaneous and experience-driven neuronal activity, such that subsets of synapses are eliminated while the remaining synapses are preserved and strengthened (reviewed by Shatz 1990; Huberman et al., 2008). A recent study used the mouse retinogeniculate system to test the hypothesis that microglial cells are key to the pruning process. In this classic system, retinal ganglion cell (RGC) inputs to the visual thalamus (dLGN, dorsal lateral geniculate nucleus) are actively refined and remodeled over the first few weeks of postnatal development. Initially, relay neurons in the dLGN are multiply innervated and receive overlapping inputs from the left and right eyes. Between postnatal day 5 (P5) and P10, a remodeling process occurs, called eye-specific segregation, in which overlapping RGC inputs are removed and eye-specific territories are formed (Guido, 2008; Hong and Chen, 2011; Huberman et al., 2008; Jaubert-Miazza et al., 2005). This process is based on activity-dependent competition between the two eyes, as disrupting neural activity or removing one eye impairs the pruning process (Chen and Regehr, 2000; Huberman et al., 2008; Penn et al., 1998; Sretavan and Shatz, 1984, 1986; Stellwagen and Shatz, 2002; Torborg and Feller, 2005). Schafer et al. hypothesized that microglia are one of the major cellular mediators of synaptic refinement and eye-specific segregation. To test this idea, the authors developed a novel in vivo phagocytosis assay in which RGC inputs originating from each eye were labeled by cholera toxin in transgenic mice expressing EGFP in microglia (GFP-CX3CR1). The results revealed that microglia engulf presynaptic RGC inputs in the dLGN during the peak of pruning during eye specific segregation, and this engulfment declines with the emergence of eye-specific territories by postnatal day 10 (Schafer et al., 2012).

The authors were able to distinguish inputs from the left versus right eye because the cholera toxin used as their anterograde tracer was differentially conjugated with red versus blue fluorescent dyes. When an activity-dependent synaptic competition was induced by treating one eye with the drug tetrodotoxin or forskolin (to silence or boost activity, respectively), the less active eye lost territory in the dLGN and its axon terminals were preferentially engulfed by microglia (Schafer et al., 2012). These data suggest the microglia are key mediators of activity-dependent synaptic pruning. But how exactly microglia know which synapses to engulf and eliminate remains an open question.

3.3. *Molecular mechanisms of microglia-mediated synapse pruning*

The classical complement cascade is one molecular pathway implicated in microglia–synapse interactions and activity-dependent synapse pruning (Fig. 1) (Schafer and Stevens, 2010; Stephan et al., 2012; Stevens et al., 2007). Molecules belonging to the classical cascade, C1q and C3, are localized to developing synapses and mediate synaptic pruning in the postnatal mouse visual system (Stevens et al., 2007). Surprisingly, the function of complement proteins in the brain appears analogous to their function in the immune system: clearance of cellular material

that has been 'tagged' for elimination. In the innate immune system, C1q and/or C3 bind cellular material, inducing its removal by several different mechanisms including phagocytic pathways (Gasque, 2004; Lambris and Tsokos, 1986; van Lookeren Campagne et al., 2007). These findings raise the hypothesis that complement, C1q, and C3 target synapses for elimination by microglia, the only resident cell that expresses CR3 in the healthy postnatal brain (Schafer et al., 2012). Indeed, C3 protein is expressed and localized to subsets of synapses in the postnatal dLGN, and C3 receptor is expressed by microglia at high levels at postnatal day 5. Moreover, in C3 or C3 receptor knockout mice, there was reduced engulfment of retinal axons and a sustained deficit in eye-specific segregation as was observed in C1q and C3 KO mice (Schafer et al., 2012; Stevens et al., 2007), implicating microglia as a cellular mediator of complement-dependent synapse elimination. Moreover, disrupting microglia-specific CR3/C3 signaling led to an excess in synapse numbers and connectivity into adulthood (Schafer et al., 2012).

These results provide some hints as to how synapses might be "tagged" for elimination by microglia. It will be important to also determine how the C1q and C3 molecules are localized to specific synapses, and whether changes in neuronal activity impact this 'tagging' process. Another molecular clue regarding how microglia regulate synapse pruning – albeit in a more indirect fashion – comes from experiments in the CX3CR1 (fractalkine receptor) knockout mouse. Fractalkine, or CX3CL1, is a chemokine produced by neurons that binds to the CX3CR1 receptor, expressed selectively on the surface of microglia within a healthy, developing CNS (Ransohoff, 2009). In a 2011 hippocampal study by Paolicelli and colleagues, CX3CR1 knockouts showed signs of a transient decrease in synapse pruning—meaning a temporarily high spine density in the postnatal hippocampus (2-3 weeks) accompanied by increased PSD95 immunoreactivity. Moreover, there were significantly fewer microglia in the postnatal brains of CX3CR1 knockouts, suggesting that the pruning defects may be a consequence of the decreased microglial presence at or near synapses (Paolicelli et al., 2011). In line with the phagocytic inclusions in microglia observed in the visual cortex study, high resolution imaging and EM revealed postsynaptic density protein 95 (PSD95)-positive puncta within the GFP-positive microglial processes in the postnatal hippocampus. EM with double immuno-gold labeling was used to confirm this and demonstrate that the PSD95+ puncta are present both within clathrin and non-clathrin coated vesicles of the microglia (Paolicelli et al., 2011). Most of the effects observed in CX3CR1 KO mice were transient, suggesting a developmental delay in hippocampal synaptic connectivity in KO mice. Whether microglia were targeting specific synapses as part of an activity-dependent developmental pruning process, similar to what was shown in the retinogeniculate system, remains to be explored. Understanding the mechanisms by which fractalkine and complement systems work together to prune specific synapses during development will be an important area of future investigation (Ransohoff and Stevens, 2011).

Interestingly, a new study of learning-dependent structural plasticity in the motor cortex has revealed that depletion of microglia (using a Cre-ER inducible system in which diphtheria toxin is expressed under control of the CX3CR1 gene) results not only in decreased spine elimination, but also decreased spine formation in vivo (Parkhurst et al., 2013). Similarly, a study of hippocampal neurons in culture recently demonstrated that microglia can promote synapse formation in vitro through interleukin-10 signaling (Lim et al., 2013). In future studies of spine and presynaptic terminal dynamics, it will be important to determine if microglia mediate synaptic pruning and remodeling in other brain regions at their peak periods of structural plasticity—to learn if microglia are indeed exerting bidirectional control of synapse number globally, or only in specific circumstances.

3.4. *Control of synapse function and maturation*

Microglia are also key players in the control of synapse maturation and function. The study by Paolicelli et al., for instance, suggests a role for microglia in synapse maturation in the hippocampus. During development, when the CX3CR1 knockouts show a transient decrease in microglia density in the hippocampus, in addition to the increased spine density there are various physiological signs of immature synapses. To begin, the spontaneous EPSC/miniature EPSC (sEPSC/mEPSC) amplitude ratio, which normally increases during development and is thought to be an indication of "synaptic multiplicity" (the number of synapses per axonal input), was decreased in the knockouts. Second, the typical developmental trajectory of LTD was delayed. And third, susceptibility to drug-induced seizures was reduced, consistent with the pattern normally observed in younger wild type mice. All suggest that without an appropriate number of microglia in the hippocampus, synapses do not fully mature (Paolicelli et al., 2011).

Interestingly, although microglia and spine numbers in the hippocampus returning to "normal" levels in young adult mice, the sEPSC/mEPSC ratio remained low. Moreover, EM suggested that this may be due to a failure of CA3 axons in KO mice to form multi-synapse boutons (i.e. axonal boutons forming synapses on more than one dendritic spine) in CA1. There was also a reduction in functional connectivity between the hippocampus and prefrontal cortex, as assayed by the coherence of local field potentials and blood oxygen level-dependent functional MRI (Zhan et al., 2014). Although it remains to be tested directly, abnormal synaptic pruning may underlie this functional connectivity deficit in CX3CR1 KO mice. Separate from the CX3CR1 signaling axis, KARAP/DAP12 is a transmembrane polypeptide associated with cell-surface receptors in hematopoietic cells, and it too appears to be microglia-specific in the brain. Hippocampal slices from mice deficient in KARAP/DAP12 function displayed a subunit composition of NMDA receptors and calcium permeability status of AMPA receptors that are both characteristic of immature synapses, and appear to have enhanced long term potentiation (Roumier et al., 2004).

Another signaling pathway of interest in synapse maturation is the complement cascade. In the mature brain, hypoxia and inflammatory stimuli, when combined, can trigger a long term synaptic depression (LTD) in the hippocampus. When microglial CR3 receptors are activated, the enzyme NADPH oxidase is activated in microglia, and this eventually promotes increased AMPA receptor internalization in nearby

neurons. This can be thought of as a depression in synapse efficacy that serves as a "prelude" to the synapse pruning process (Zhang et al., 2014). Thus, taken together, these studies of different microglial proteins suggest that microglia are important for synapse maturation and plasticity. (Fig 1).

4. Regulating circuits in the mature CNS

Emerging evidence suggests that microglia also play a key role in regulating neural circuits in the mature CNS. While different from the dramatic waves of apoptosis that occur sequentially across brain regions during development, the process of PCD does to some extent continue on in mature animals. A key example is related to the adult neurogenesis that occurs in the hippocampus, in the subgranular zone of the dentate gyrus. While some of the newborn neurons are incorporated into circuits, many are believed to undergo PCD (Song et al., 2012). Microglia seem to be phagocytosing these apoptotic neurons (Sierra et al., 2010).

There is also evidence for deficits in synapse number or function in adult animals whose microglial signaling pathways have been disrupted, such as in adult CX3CR1 deficient mice (Paolicelli et al., 2011; Paolicelli and Gross, 2011), as well as complement (C1q, C3 and CR3) KO mice (Chu et al., 2010; Ma et al., 2013; Schafer et al., 2012; Stephan et al., 2013). In addition, microglia may be regulating long term potentiation, synaptic scaling and basal excitatory and inhibitory neurotransmission in adult animals (Ben Achour and Pascual, 2010; Bessis et al., 2007; Kettenmann et al., 2011).

5. Effects on behavior

The growing awareness of microglial functions at the cellular, molecular and synaptic levels in healthy brains have led to new studies on the impact of these cells at the behavioral level, and also cast new light on observations of microglial abnormalities in a variety of brain disorders.

Neuropathology studies of autopsy samples in autism and schizophrenia have for some time indicated that these disorders have neurodevelopmental origins. There may be defects in cell and synapse number consistent with abnormalities in neurogenesis, programmed cell death, synapse formation and/or pruning in various brain regions, including the prefrontal cortex (Arnold, 1999; Belmonte et al., 2004; Teffer and Semendeferi, 2012; Woo and Crowell, 2005). These observations, paired with findings of increased microglial activation and/or microglial cell density in autopsied brains of individuals with autism or schizophrenia (Monji et al., 2009; Morgan et al., 2010; Vargas et al., 2005), as well as increased microglial markers in cerebrospinal fluid from living patients with autism (Vargas et al., 2005), loosely connect behavioral symptoms with changes in microglia. Given the recent discoveries of microglia playing key roles in CNS patterning and wiring, this correlation raises the question of whether microglial changes in neurodevelopmental disorders are simply a response to the core pathology of these disorders, or something more—such as being one of the developmental abnormalities that actually brings about these disorders.

While this question is very difficult to investigate in humans, mouse models have in recent years begun to provide insights in this area, with some suggesting that microglial abnormalities may indeed be playing central roles in the disease pathology. For instance, in a mouse model of Rett Syndrome – a single gene X-linked disorder with autistic features – a bone marrow transplant that allowed for the infiltration of healthy, wild type microglia into a Rett mouse brain ameliorated a number of disease attributes such as shortened lifespan, breathing difficulties, diminished body weight and locomotor deficiencies (Derecki et al., 2012). Rett syndrome is caused by a mutation in the gene MeCP2, and microglia lacking MeCP2 release abnormal levels of neurotransmitters and inhibit the development of neurons (Maezawa and Jin, 2010). MeCP2-deficient microglia also exhibit impaired phagocytosis, which may be important for clearing away dying cells and debris in the Rett brain (Derecki et al., 2012). Importantly, allowing expression of the deficient gene, MeCP2, in myeloid cells alone (the lineage that microglia are derived from) recapitulates the symptom improvement noted in the bone marrow experiment (Derecki et al., 2012) (see Kipnis chapter, this issue).

Transplantation of wild-type bone marrow also attenuated behavioral phenotypes in a mouse model of obsessive compulsive disorder (OCD). Mice lacking the homeobox protein Hoxb8 show excessive grooming that leads to significant hair loss and skin lesions—reminiscent of humans with the OCD spectrum disorder trichotillomania, which involves the compulsion to pull out one's hair. Unlike MeCP2, which is globally expressed throughout the brain, it appears that the Hoxb8 cell lineage specifically gives rise to brain microglia. But, similar to the MeCP2 study, transplantation of bone marrow from wild type mice into irradiated Hoxb8 mice rescued the excessive grooming and hair loss phenotypes, again suggesting that microglia are mediating core symptoms of the disorder (Chen et al., 2010).

Aside from these genetic models of autism and OCD, microglia may also play a role in environmental stress-based disorder models. In maternal immune activation (MIA) models, for instance, pregnant mice are subjected to an immune challenge (i.e. infection, PolyIC, LPS) in utero and later in life may develop an array of behavioral phenotypes, including deficits in pre-pulse inhibition, decreased social behaviors and exploration learning and memory difficulties. Interestingly, these phenotypes are more likely to occur if the early life infection is followed by later life stress, and are correlated with increases in microglial activation. Early life stress may 'prime' microglia in a way that makes them more vulnerable to later life stresses, and there is some evidence suggesting that a microglial derived factor, interleukin-1β, mediates the learning and memory deficits in such scenarios (Bilbo and Schwarz, 2009; Giovanoli et al., 2013; Harvey and Boksa, 2012; Patterson, 2009; Williamson et al., 2011).

In recent years researchers have also more directly probed the role of microglia in mouse behavior by targeting these cells with genetic manipulations in otherwise typically developing mice. As discussed above, neurons express the chemokine fractalkine, or CX3CL1, and in the CNS the receptor for

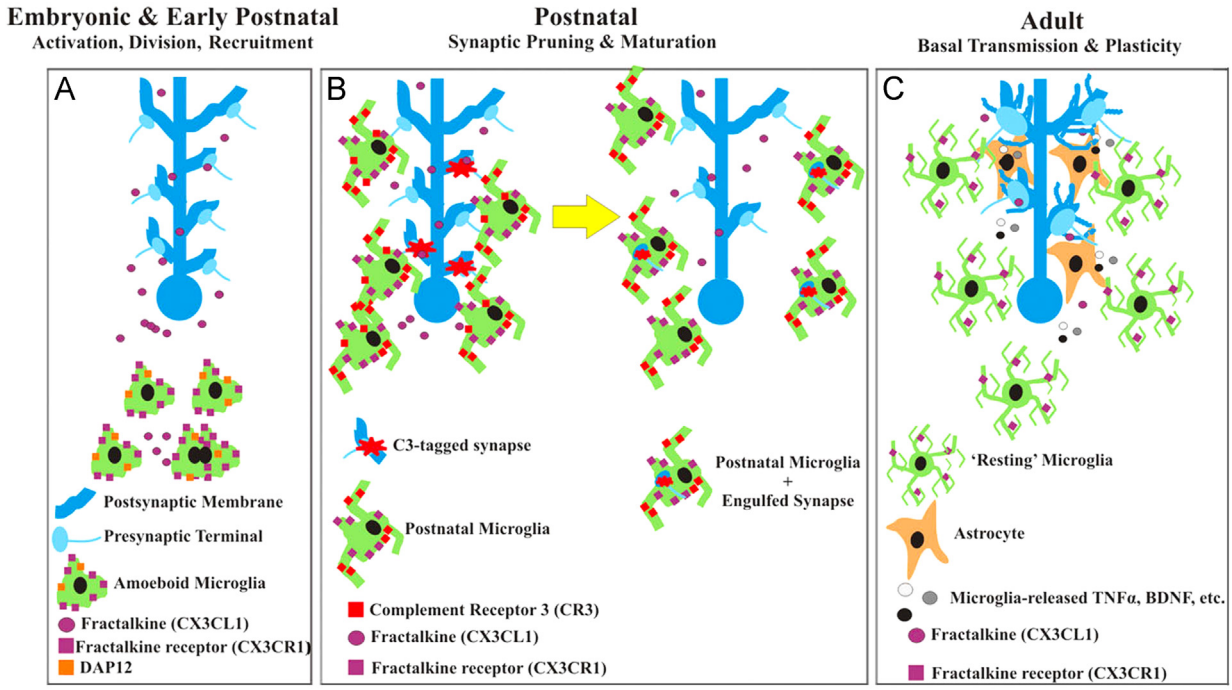

Fig. 1 – Models of microglial function at synaptic sites in the developing mouse brain. **(A) In the embryonic and early postnatal brain, ameboid phagocytic microglia are actively dividing and recruited to regions throughout the CNS. Fractalkine (purple circles), which may be released by neurons, is proposed to act on fractalkine receptors (purple squares) expressed by microglia to regulate their "activation" state, number, and/or recruitment to synapse-enriched regions. DAP12 (orange squares) expressed on the surface of microglia is also thought to affect synapse function during this period, perhaps also by regulating their activation state. (B) In the postnatal brain, process-bearing phagocytic microglia mediate synaptic pruning. Along with the fractalkine receptor (purple squares) and DAP12 (orange squares), these microglia express high levels of complement receptor 3 (CR3, red squares) on their surface. We propose that complement component C3 (red stars) is tagging synapses for removal and have demonstrated that synapses are engulfed by phagocytic microglia in a complement-dependent manner. Disruptions in any of these processes results in deficits in synaptic pruning or maturation. (C) In the adult brain, microglia release soluble factors (gray and black circles) such as BDNF and TNFα, which could affect basal neurotransmission and synaptic plasticity via direct action on neurons or indirectly via astrocytes (orange). In addition, fractalkine signaling via soluble fractalkine (purple circles), most likely released by neurons, and the fractalkine receptor (purple squares), expressed by microglia, modulates microglia–synapse interactions to regulate long term potentiation (LTP) and behavior in the mature CNS. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)** *This figure is a reprint with slight modifications to the legend, reproduced here with permission from Schafer, D.P., Lehrman, E.K., Stevens, B., 2013. The Quad-Partite Synapse: Microglia-synapse interactions in the developing and mature CNS. Glia. 61, 24-36.*

this chemokine, CX3CR1, is expressed exclusively by microglia (Ransohoff, 2009). At least three behavioral studies have been conducted in CX3CR1 mutants. In the first, CX3CR1-deficient adult mice displayed motor learning difficulties, as well as impaired cognitive function as demonstrated by deficiencies in contextual fear conditioning and the Morris water maze. These behavioral phenotypes accompanied the electrophysiological phenotype of impaired long term potentiation discussed earlier. The mice also had increased levels of IL-1β. Treatment with the IL-1 receptor antagonist IL-1ra reversed the LTP, contextual fear conditioning and Morris water maze phenotypes, but not the motor learning deficit. These results suggest that CX3CL1/CX3CR1 signaling between neurons and microglia regulates cognitive function and synapse plasticity, and IL-1β is one key mediator of this signaling pathway (Rogers et al., 2011).

In another study of CX3CR1-deficient mice, synapse maturation defects and impairments in functional connectivity between the prefrontal cortex and hippocampus were accompanied by decreased social interactions and increased repetitive behaviors—phenotypes associated with autism (Zhan et al., 2014). These findings are particularly interesting given the findings of bone marrow transplantation rescuing certain features of Rett syndrome in mice.

More direct evidence of microglia's role in behavior comes from a new study in which investigators specifically manipulated gene expression in microglia using a tamoxifen-inducible Cre-ER protein driven by the CX3CR1 promoter ($CX3CR1^{creER}$) (Parkhurst et al., 2013). While there are peripheral immune cells expressing CX3CR1, these cells have a much higher turnover rate than microglia. Thus, about a week after tamoxifen injection into $CX3CR1^{creER}$ mice,

researchers can achieve mostly microglia-specific recombination of target loci. Using this strategy to either ablate microglia or deplete them of the neurotrophin BDNF, Parkhurst et al. (2013) found significant defects in motor learning and fear conditioning, along with a decrease in dendritic spine formation in motor cortex. These findings are the first to so directly implicate microglia in the control of learning and synapse development.

Intriguing new evidence suggests sex-based differences in microglia in healthy rats. In the preoptic nucleus of the hypothalamus, which regulates expression of sexual behaviors and is known to be different in males and females, researchers recently discovered sex-based differences in the number of activated microglia. Male rats had more activated microglia in this region and females had less. Increasing microglial activation in females led to more "masculine behaviors," and conversely, decreasing microglial activation in males led to more "feminine behaviors" (Lenz et al., 2013). Together, these studies demonstrate that microglial regulation of CNS function impacts a vast range of behaviors, ranging from social and cognitive to sexual behaviors.

6. Summary and conclusions

In the past decade a number of studies have investigated the role of microglial cells in healthy CNS development and function, in the rodent brain. These studies reveal that microglia are crucial for the patterning and wiring of the CNS. They can play both responder and mediator roles in the process of programmed cell death—serving as phagocytes that clear away dead cells, yet also having the capacity to actively promote death via secreted signals. During the process of circuit development, their phagocytic activity is again crucial, this time for pruning away unwanted synapses. They also appear to regulate the maturation and electrophysiological function of synapses, as well as long range functional connectivity between different brain regions. Beyond this, microglia make an impact at the behavioral level, in social, cognitive, learning, grooming and sexual behaviors. In mouse models of neurodevelopmental disorders such as Rett Syndrome and OCD, interventions targeting microglia can produce significant therapeutic benefits. These findings open the floodgates to a flurry of new questions and avenues of investigation.

Clearly, much still remains to be determined regarding the role of these cells in healthy CNS development. Despite the recent attention, they remain one of the most neglected cell types in the brain. It would be worthwhile to examine in more depth the trajectory of microglia development over time, the pathways controlling their migration into the brain, their heterogeneity and presence in different brain regions, their ultrastructure and their dynamics in relation to different neuronal and glial populations. Also, while major strides have been in understanding their control of neuronal apoptosis, and to some degree, survival, much work remains in this sphere, particularly with regard to their control of neural precursor cells. Even more remains to be determined about their control of synapse development and their role in the structural plasticity present during critical periods. What is the role of microglia in critical periods outside the thalamus, for instance? Do they play a role in higher order critical periods? Do they regulate prefrontal or limbic development, as might be expected from their potential involvement in psychiatric disorders?

The few studies of microglia in mouse models of neurodevelopmental disorders conducted to date suggest that the human pathology studies merit further attention—that the increase in microglia activation or density in autopsy brains from individuals with autism or schizophrenia may be more than a late stage response to the disease pathogenesis. It is feasible that microglial abnormalities early in development actually contribute to the pathology in the first place. Thus it will be important in future work to investigate microglial involvement in mouse models of disorders beyond Rett Syndrome and OCD, and to map temporally how any changes in microglial activity line up with other changes, such as those in neuronal connectivity and/or plasticity.

It will also be important to gain a better of understanding of specifically when and where targeting microglia results in the observed amelioration of major disease phenotypes. Additionally, since rescue strategies targeting other CNS cell populations might also produce behavioral improvements, to really understand the developmental basis of these disorders and explore new therapies for humans it will be critical to determine the convergence of any disparate rescue mechanisms—what are their common substrates and downstream pathways? To accomplish all this, it will be important to continue to improve our knowledge of microglial marker proteins and to use new tools such as the CX3CR1^{CreER} mice to exert microglia-specific manipulations. Pursuing this new frontier is likely to shed light not only on microglia specifically, but on nervous system-immune and neuron–glia interactions in a broad sense.

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