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REVIEW

Microglia in development: linking brain wiring to brain environment

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Microglia are enigmatic non-neuronal cells that infiltrate and take up residence in the brain during development and are thought to perform a surveillance function. An established literature has documented how microglia are activated by pathogenic stimuli and how they contribute to and resolve injuries to the brain. However, much less work has been aimed at understanding their function in the uninjured brain. A series of recent in vivo *imaging studies shows that microglia in their resting state are highly motile and actively survey their neuronal surroundings. Furthermore, new data suggest that microglia in their resting state are able to phagocytose unwanted synapses and in this way contribute to synaptic pruning and maturation during development. Coupled with their exquisite sensitivity to pathogenic stimuli, these data suggest that microglia form a link that couples changes in brain environment to changes in brain wiring. Here we discuss this hypothesis and propose a model for the role of microglia during development in sculpting brain connectivity.*

Keywords: Microglia, development, synaptogenesis, pruning, mice

INTRODUCTION

Microglia are cells of the myeloid lineage that are found dispersed throughout the brain and spinal cord and make up between 5 and 20% of central nervous system cells depending on the brain structure (Lawson *et al.*, 1990; Lyck *et al.*, 2009). They differ from other glia by virtue of their non-neuronal origin and are thought to infiltrate the brain during development following their birth from hematopoieitic precusors in the periphery. Although their precise origin and the timing and avenue of their migration into the brain are still debated, it is now agreed that a significant fraction of microglia derive from the embryonic yolk sac which is the major site of hematopoiesis in the early embryo (Alliot *et al.*, 1999; Herbomel *et al.*, 2001; Chan *et al.*, 2007; Ginhoux *et al.*, 2010). Myeloid cells from the yolk sac rapidly migrate to colonise embryonic organs, including the brain. During later development there is a sharp increment in microglia density in the brain (Dalmau *et al.*, 1998; Paolicelli *et al.*, 2011), but to what extent this is driven by local proliferation or new recruitment of circulating myeloid cells remains unclear. Experiments based on parabiosis (when two animals are joined surgically to create a common vascular compartment) suggest that infiltration of circulating monocytes into the adult brain is minimal under non-traumatic conditions and that bone marrow-derived cells are recruited only upon brain injury (Ajami *et al.*, 2007; Ginhoux *et al.*, 2010). Other studies, however, provide evidence for the recruitment of circulating monocytes into the unperturbed brain during late developmental stages, leaving open the possibility that a window of opportunity exists during which monocytes can be recruited from the periphery, possibly at a point when the blood–brain barrier is immature and permeable to such cells (Hristova *et al.*, 2010). Lineage tracing studies supporting this idea show that up to 40% of resident microglia in the adult brain are positive for the bone marrow hematopoieitic marker Hoxb8 (Chen *et al.*, 2010). However, these data are at odds with other lineage tracing studies examining the contribution of yolk sack hematopoieitic cells to adult microglia that suggest that the vast majority of microglia have an embryonic origin with minimal replenishment from circulating monocytes (Ginhoux *et al.*, 2010). Thus, microglia remain enigmatic brain cells whose infiltration in the brain appears to be carefully orchestrated with its development and whose function, therefore, is likely to be critically linked to brain maturation as well as adult function.

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MICROGLIA – SURVEYORS OF THE HEALTHY BRAIN

Since they were first described almost one century ago (del Rio Hortega, 1932), microglia have been appointed as the scavengers of the brain due to their peculiar and unique phagocytic function. According to the early view, microglia were thought to remain for the most part in a 'resting' state, waiting for a danger stimulus that would transform them into an 'activated' state in which they were able to phagocytose neuronal debris and dead cells. This view prevailed until modern microscopy techniques made it possible to view microglia in their 'resting' state in the living animal, at which point it was discovered that microglia are in fact highly motile in their baseline state (Davalos *et al.*, 2005; Nimmerjahn *et al.*, 2005; Peri and Nüsslein-Volhard, 2008). By using *in vivo* imaging, Wake

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and co-workers showed that microglia processes make brief contacts (~5 min) with neuronal processes, at a frequency of about once per hour, suggesting a direct role for microglia in monitoring the functional state of neurons (Wake *et al.*, 2009). This led Hanisch and Kettenmann to suggest that the baseline state of microglia is better termed, 'surveying' rather than 'resting', to highlight their constant surveillance of the brain parenchyma (Hanisch and Kettenmann, 2007). Nevertheless, the focus of early microglia research on the activated state and stimuli and conditions that underlie its induction, still pervades the field and it is not until recently that researchers have shown renewed interest in the function of 'resting' microglia, and therefore also their function in normal brain development.

Several studies support a role for microglia in neuronal elimination in the uninjured brain. For example, during hippocampal neurogenesis in the adult brain, newborn granule cells are produced in the subgranular zone (SGZ) with a small percentage of them maturating and becoming part of the mossy fiber circuitry, while the majority undergo apoptosis (Ming and Song, 2011). Surveying microglia, in their basal and ramified status, are responsible for the phagocytosis of these apoptotic bodies, engulfing them to produce 'ball-and-chain' structures at their terminal process (Sierra *et al.*, 2010). Importantly these data show that phagocytosis of neuronal material by microglia do not require the induction of the activated state seen during brain injury and show that surveying microglia can have a phagocytic function. Similarly, in the developing zebrafish brain microglia actively contribute to phagocytosis of apoptotic neurons (Peri and Nüsslein-Vollhard, 2008). Their observation that neurons expressing apoptotic markers are always found engulfed within microglia suggests, furthermore, that microglia are actively involved in the induction of neuronal death, rather than being passive 'garbage collectors' that clean up cellular debris after apoptosis has been completed (Peri and Nüsslein-Vollhard, 2008). These observations point to an intimately orchestrated reciprocal signaling between neurons and microglia.

Finally, *in vivo* imaging studies suggest that microglia have a specific affinity for synaptic structures. A recent study by Tremblay *et al.* used transgenic mouse lines expressing fluorescent proteins in neurons and microglia (Thy1::YFP;Cx3cr1::GFP) to monitor their contacts in the living brain using time-lapse imaging (Tremblay *et al.*, 2010). Looking at the visual cortex, they found that neuronal spines, protruberances from neuronal dendrites that harbour postsynaptic sites, are frequently contacted by distal microglial processes and that, occasionally, with a frequency increased by visual deprivation, spines disappeared after being contacted by microglia (Tremblay *et al.*, 2010). Their data suggested for the first time that microglia might have a role in the selective elimination of synapses in the uninjured brain, a function also supported by observations that dysfunction in microglial activity can lead to synaptic impairment (Graeber, 2010).

MICROGLIA MEDIATE NEURON AND SYNAPSE ELIMINATION DURING DEVELOPMENT

The massive invasion and proliferation of microglia during brain development and the highly conserved feature of microglia colonisation of the brain across species (Pont-Lezica *et al.*, 2011) suggest a critical role for these cells in brain maturation. Studies across several species have highlighted the instructive role of microglia in promoting neuronal cell death during development (Marín-Teva *et al.*, 2004; Mallat *et al.*, 2005; Peri and Nüsslein-Vollhard, 2008; Wakselman *et al.*, 2008). In several species microglia have been closely associated with developing axon bundles (Cuadros *et al.*, 1993; Dalmau *et al.*, 1998; Herbomel *et al.*, 2001; Santos *et al.*, 2008) and post-mortem histological studies in humans describe a prominent accumulation of microglia at 10–12 weeks of gestation at the cortical plate–subplate junction, a position where the first synapses are detected, and later at 19–30 weeks of gestation, at axonal crossroads in white matter. This localised accumulation of microglia at points of axonal and synaptic development suggests that microglia are actively involved in the refinement of neuronal connections, including axonal guidance and synaptogenesis (Verney *et al.*, 2010).

We have recently followed up on these observations with the aim to find evidence for a role of microglia in synapse elimination. This work was based on the observation that the expression of the chemokine fractalkine, Cx3cl1, becomes strongly upregulated in the mouse forebrain during the third and fourth postnatal week (Mody *et al.*, 1999), the major period of synapse formation and maturation, and that its receptor is exclusively expressed by microglia (Jung *et al.*, 2000). This chemokine signaling system appeared to be well placed to serve as a neuron-microglia signal related to synaptogenesis. In fact, we found that mice lacking the fractalkine receptor, Cx3cr1, showed a ~30% decrease in the density of microglia in the brain during the second, third and fourth postnatal week, confirming a critical role of this chemokine in either attracting or promoting the proliferation of microglia during synaptogenesis (Paolicelli *et al.*, 2011). The initial stages of synaptogenesis in cortical structures are associated with a maximal level of synapse formation and elimination (Cruz-Martin *et al.*, 2010) and it is notable that at precisely this point microglia arborisation and density increase dramatically in the forebrain (Dalmau *et al.*, 1998; Paolicelli *et al.*, 2011).

Next, we used immunohistological measures to measure synapse maturation in Cx3cr1 knockout mice and found an increase in the number of synapses. Electrophysiological assessment of synaptic maturation in the Cx3cr1 knockout mice confirmed that excitatory synapses were more abundant, but also revealed that they were weaker, with a failure to develop efficient action-potential stimulated synaptic release as measured by the ratio of spontaneous (sEPSC) and mini (mEPSC) excitatory postsynaptic currents (Paolicelli *et al.*, 2011). This second finding has important implications because it argues that microglia-mediated synapse elimination is required for the strengthening of surviving synapses and suggests the presence of reciprocal signaling between neurons and microglia to orchestrate synaptic pruning. We hypothesised that the combination of increased synapses, but decreased maturation of synapses could be caused by a deficit in synaptic pruning as a result of decreased microglia surveillance. More direct evidence for synaptic pruning by microglia was provided by imaging experiments in fixed tissue that captured examples of both pre and post-synaptic proteins engulfed by microglia in the developing forebrain. Higher-resolution imaging using electron microscopy uncovered evidence for engulfment of synaptic material via a clathrin-coated vesicle endocytosis pathway.

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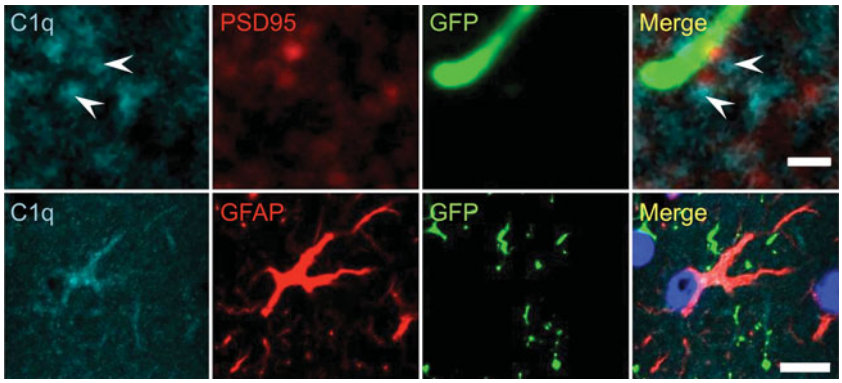


Fig. 1. C1q expression does not overlap with synapses or microglia. (Upper panel) Representative high-resolution confocal single-plane images of CA1 *stratum radiatum* taken from the hippocampus of a *Cx3cr1::GFP*/+ reporter mouse (KO/+) at postnatal day 15 revealed apposed, non-overlapping localisation of C1q (indicated by arrowheads) and PSD95-immunoreactive puncta and GFP-positive microglia processes (scale bar: 1 μm). (Lower panel) Representative low-resolution confocal single-plane images showed C1q staining overlapping with the astrocytic marker GFAP, but not with GFP-positive microglia processes (scale bar: 10 μm).

Similar engulfment and deficient pruning of presynaptic structures were recently reported in mice lacking the complement factors C1q and C3 and the C3 receptor (CR3) in the retinothalamic system (Stevens *et al.*, 2007; Schafer and Stevens, 2011) confirming the hypothesis that the classical complement system is a critical signal for microglia-mediated synapse elimination. Neuronal engulfment via the classical complement pathway is initiated by binding of C1q to desialylated sugar residues on neuronal plasma membrane proteins and the subsequent induction of C2 and C4 cleavage and C3 binding (Linnartz *et al.*, 2012). Engulfment of the complement complex is then mediated by CR3 receptors located on microglia. Intriguingly, both C1q and C3 are expressed transiently in a punctate, synapse-like pattern during the synaptogenetic period in mouse (Stevens *et al.*, 2007) and high-resolution immunofluorescence microscopy revealed these puncta to be directly adjacent to many, but not all pre- and postsynaptic structures. We have recently confirmed these localisation studies, showing that C1q immunoreactive puncta do not overlap with, but are located close to postsynaptic sites during the early postnatal period in mouse (Fig. 1). These C1q immunoreactive puncta do not overlap with microglia processes, and instead appear to colocalise with the astrocytic marker GFAP, suggesting that astrocytes could represent a source for C1q during this development stage. Expression of C1q in astrocytes during this period is supported by mRNA *in situ* hybridisation data that show a distinctive non-neuronal pattern of C1q expression in the hippocampus during the early postnatal stage (Allen Brain Atlas). The precise localisation of C3 at this developmental stage remains unclear, although *in vitro* studies show that it is expressed on neurons and possibly also released by microglia (Walker *et al.*, 1995; Thomas *et al.*, 2000). The CR3 receptor, on the other hand, is exclusively expressed by microglia in the brain (Ling *et al.*, 1990; Stevens *et al.*, 2007).

Although the precise order and timing of events leading from the apposition of C1q to synapses, to its presumed release and binding to neuronal membranes, and the assembly of the remaining complement proteins in preparation for phagocytosis remains to be worked out, these data allow us to propose a four-way synapse model of synaptic pruning. In this model pre- and post-synaptic specialisations are joined by astrocyte and microglia processes, each contributing essential classical complement pathway components to promote microglia-mediated synaptic engulfment and elimination (Fig. 2). Several questions are raised by this model. What are the neuronal signals that initiate the cascade? How important is neuronal activity and/or plasticity in marking synapses for elimination? What is the initiating 'eat me' signal? How does synaptic pruning work in adulthood, when the classical complement factors no longer show punctate expression patterns?

WHEN DANGER CALLS

As mentioned above, a large body of research has documented the role of microglia in responding to pathogenic signals that reach or originate in the brain and in resolving injury in the adult brain. Less is known, however, about the impact of pathogenic signals on microglia function during development. Could the activation of microglia during a time when they are critically involved in synaptic maturation has a detrimental and potentially long-term impact on brain wiring? Under normal conditions microglia activation is suppressed by 'off' signals (e.g. Cx3Cl1, CD22, CD47 and CD200) that are expressed by neurons and maintain microglia in the default, surveying state. 'Off' signals also suppress the expression of pro-inflammatory factors. In contrast, 'on' signals emanating either from neurons or other members of the local brain environment (e.g. glutamate, purines and chemokines) or from exogenous factors that manage to infiltrate the brain (e.g. pathogen-associated molecular patterns or PAMPs) can trigger microglia activation, promoting motility and the release of pro-inflammatory molecules (Fig. 2; Biber *et al.*, 2007; Ransohoff and Cardona, 2010; Saijo and Glass, 2011). Activation of microglia involves dramatic changes in cell shape and motility, including retraction of long processes, transformation into an amoeboid shape, and rapid migration to the site of injury. It is well recognised that microglia can sense and rapidly respond to threats to CNS integrity by expressing a broad pattern of cytokines, either pro- or anti-inflammatory, and by changing their MHC expression profile. Nevertheless, it is important to keep in mind that, despite a common morphological transformation during activation, the final state of microglia upon activation is not an

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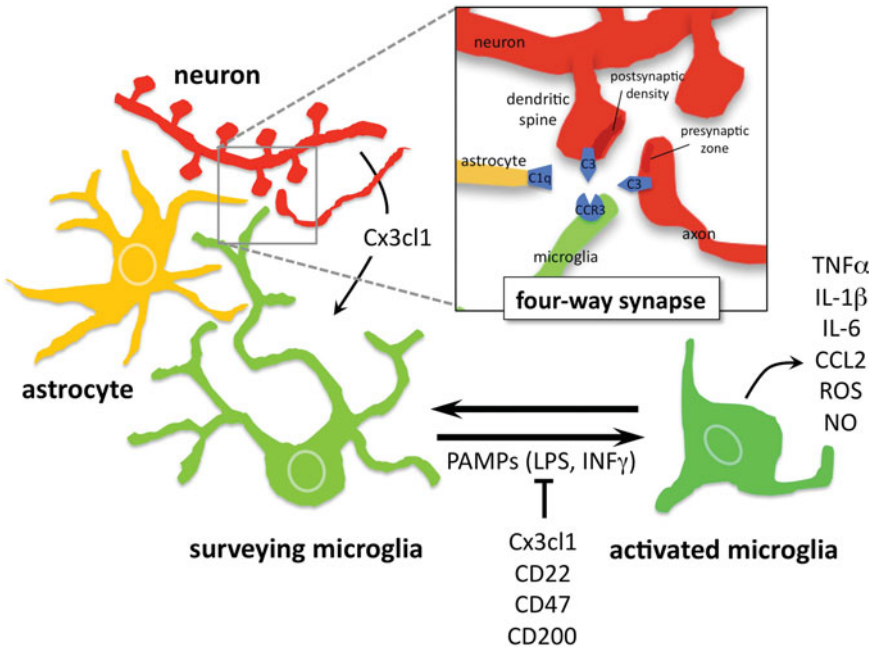


Fig. 2. Role of microglia in synapse elimination during development. Under normal conditions microglia are in a 'surveying' state in which their highly motile processes extend and retract to interrogate the neuronal environment. Together with the more stationary processes of astrocytes, and the pre- and postsynaptic specialisations (see inset) they form a 'four-way' synapse. Neurons produce the chemokine fractalkine (Cx3cl1) to attract and/or promote the proliferation of microglia during synaptogenesis. Critical components of the complement system are produced by each branch of the four-way synapses and are critical for microglia-mediated phagocytosis of unwanted pre- and post-synaptic structures. C1q is expressed by astrocytes, the complement receptor 3 (CR_3) by microglia, and neurons express C_3 (see inset). Under conditions of pathogenic insult or neurodegeneration, pathogen-associated molecular patterns (PAMPs, e.g. LPS, IFN-γ) can drive microglia into an 'activated' state characterised by the secretion of pro-inflammatory factors, including TNF-α, IL-1β, IL-6, ROS, NO. Induction of the activated state is inhibited by fractalkine, Cx3cl1, as well as CD22, CD47, CD200.

all-or-none phenomenon and different activation states can be distinguished by their gene expression signatures.

According to the type and concentration of 'on' signals, activated microglia can express different spectra of pro- or anti-inflammatory cytokines and under certain circumstances, for example, microglia can express an activated pro-inflammatory phenotype without being able to carry out a phagocytic response (Koenigsknecht-Talboo and Landreth, 2005). Microglia can exacerbate inflammatory processes by releasing reactive oxygen species (ROS), nitric oxide (NO) or tumor necrosis factor (TNF)-α, or, on the other hand, can confer neuroprotection and promote neurogenesis (Fig. 2). In some cases fully activated microglia can have neurotoxic effects. In models of neurodegenerative disorders such as Parkinson's disease, microglia activation mediated by Inteferon (INF)-γ has been clearly shown to induce neuronal death (Block *et al.*, 2007; Mount *et al.*, 2007). At the same time, other studies demonstrate that microglia can release neurotrophic factors favoring neuroprotection, neurogenesis, and glia development (Monje *et al.*, 2003; Kitamura *et al.*, 2004). This dual aspect of microglia make understanding their function in a particular neuronal process not straightforward. Cataloguing different activated repertoires and understanding their impact on synaptic pruning and maturation will be critical to understanding the effects of pathological conditions on brain maturation.

However, several external signals have been clearly shown to affect the phagocytic activity of microglia, and thus are likely to impact synaptic pruning. Trophic factors, such as glial-derived neurotrophic factor, M-CSF or the ciliary neurotrophic factor have been shown to promote phagocytic activity (Mitrasinovic *et al.*, 2003; Chang *et al.*, 2006; Lee *et al.*, 2009), while prostaglandin E_2 attenuates phagocytosis through the E-prostanoid receptor 2 (Nagano *et al.*, 2010). Kettenmann and co-workers showed that the neurotransmitter serotonin reduces the phagocytic efficiency of microglia while simultaneously promoting their motility towards the injury site (Krabbe *et al.*, 2012). Evidence suggests that an inflammatory and oxidative milieu, moreover, can negatively regulate microglial phagocytic function. Microglia isolated following IFN-γ/LPS stimulation *in vivo*, for example, exhibited loss of phagocytic and migratory activity (Broderick *et al.*, 2000). Similarly, recent studies showed that stimulation of microglia with IL-1β, LPS or t-BHP (a strong oxidant) significantly reduced phagocytosis in a nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)-mediated manner (Pan *et al.*, 2011). Moreover, in the presence of high levels of the pro-inflammatory factors IL-1β and TNF-α, C_3 receptor-mediated phagocytic activity is reduced, whereas FcR-mediated phagocytosis is not affected, suggesting that more than one distinct signaling pathway controls phagocytic activity (Koenigsknecht-Talboo and Landreth, 2005). As discussed above, C_3 is likely to be a critical 'eat me' signal that instructs microglia for removing synapses (Stevens *et al.*, 2007; Linnartz *et al.*, 2012). Thus, in general, pro-inflammatory factors are likely to impact negatively on synaptic pruning by blunting C_3-mediated phagocytic activity associated with the non-activated, surveying state.

CONCLUSION

Evidence is mounting that microglia have a special role during brain development in sculpting neuronal circuits. Early during

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development they are critical for the elimination of unneeded neurons and later they are involved in removing unwanted synapses. Moreover, multiple data suggest that neurons and microglia have evolved recurrent signaling mechanisms to orchestrate these functions, and that microglia do not just arrive after-the-fact to scavenge neuronal debris, but rather are intimately involved in the selection and elimination process. Finally, data suggest that microglia have a particular crucial role during development in interpreting changes in the brain environment and translating them to modulate neuronal circuit maturation. Any reduction in phagocytic capacity due to increased activation of microglia will have grave consequences for synaptic maturation and potentially irreversible consequences on brain wiring.

Some data suggest that microglia may be more prone to pathogenic stimuli during development, possibly in order to better react to and resolve injury during such a critical period of brain maturation. Such a hypersensitivity to environmental pathogens might explain why bacterial infections at P4, but not at P30, in the rat have been shown to cause significant long-term changes in the cytokine profile of microglia and behavior (Bilbo *et al.*, 2005, 2006, 2008; Meyer *et al.*, 2006, 2008). Early hypersensitivity of the microglia system coupled to its critical role in neuron elimination and synaptic pruning, means that disturbances in microglia homeostasis could be a route to mental illness with a developmental onset. The concept of prenatal inflammation as a risk factor for neuropsychiatric disorders such as autism and schizophrenia has remained current and epidemiological evidence confirms that prenatal exposure to immune challenge can impair later brain maturation (Ciaranello and Ciaranello, 1995; Meyer *et al.*, 2009, 2011; Brown and Derkits, 2010; McAlonan *et al.*, 2010; Buehler, 2011; Patterson, 2011; Vuillermot *et al.*, 2012). For example, cytokine-associated inflammatory events can be critical mediators of the neurodevelopmental effects of prenatal infection (Gilmore and Jarskog, 1997; Meyer *et al.*, 2008) and interleukins such as IL-6 can induce long-term neural and behavioral deficits (Smith *et al.*, 2007). It remains to be determined what role, if any, microglia-mediated neuron elimination and synaptic pruning plays in such developmental programming phenomena.

Statement of interest

None.

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