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REVIEW ARTICLE

Roles of microglia in brain development, tissue maintenance and repair

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The emerging roles of microglia are currently being investigated in the healthy and diseased brain with a growing interest in their diverse functions. In recent years, it has been demonstrated that microglia are not only immunocentric, but also neurobiological and can impact neural development and the maintenance of neuronal cell function in both healthy and pathological contexts. In the disease context, there is widespread consensus that microglia are dynamic cells with a potential to contribute to both central nervous system damage and repair. Indeed, a number of studies have found that microenvironmental conditions can selectively modify unique microglia phenotypes and functions. One novel mechanism that has garnered interest involves the regulation of microglial function by microRNAs, which has therapeutic implications such as enhancing microglia-mediated suppression of brain injury and promoting repair following inflammatory injury. Furthermore, recently published articles have identified molecular signatures of myeloid cells, suggesting that microglia are a distinct cell population compared to other cells of myeloid lineage that access the central nervous system under pathological conditions. Thus, new opportunities exist to help distinguish microglia in the brain and permit the study of their unique functions in health and disease.

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Abbreviation: EAE = experimental autoimmune encephalomyelitis; LPS = lipopolysaccharide

Introduction

Microglia are brain-resident myeloid cells that belong to the mononuclear phagocyte system, which includes bone-marrow precursors, circulating monocytes and tissue-resident macrophages. In both the healthy and diseased CNS, microglia are perpetually exposed to diverse environmental stimuli that can influence their function and often correspond to a unique molecular and morphological profile. Microglia are highly dynamic innate immune cells that are capable of robust chemotaxis, phagocytosis, antigen presentation, and cytokine production, in addition to a diverse set of newly emerging neurobiological functions. In this review, we explore

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how microglia can impact overall brain function at the molecular and cellular level in both the healthy and pathological context. We discuss the putative roles of microglia throughout mammalian brain development, ageing, and in response to infection and/or injury. Furthermore, discussion is included that provides translational context whereby we can now exploit the phenotypic and functional properties of microglia to help future neuroscience research and provide novel targets of future therapies.

Microglia in the developing CNS

Unique aspects of microglia ontogeny

Microglia are mesodermally-derived mononuclear cells of the CNS that arise during primitive haematopoiesis in yolk sac blood islands (Cuadros *et al.*, 1993; Ginhoux *et al.*, 2010; Golub and Cumano, 2013). In humans, microglia can be identified in the extracerebral mesenchyme as early as 4.5 gestational weeks and invade the parenchyma at ~5 gestational weeks (Monier *et al.*, 2006, 2007; Verney *et al.*, 2010). In rodents, microglia are first detected as primitive tyrosine protein kinase c-kit (now known as KIT) positive erythromyeloid precursors at embryonic Day 8 (Kierdorf *et al.*, 2013), which is consistent with observations that microglia progenitors arise before embryonic Day 8 in rodents (Alliot *et al.*, 1999; Ginhoux *et al.*, 2010). Primitive haematopoiesis may also contribute precursors to the adult haematopoietic stem cell population. Haematopoietic stem cell progeny are responsible for the circulating or 'peripheral' compartment of monocytes, which infiltrate tissues during injury/immune challenge and can replenish populations of certain tissue macrophages throughout life (further discussed in subsequent sections and more detailed in reviews by Sieweke and Allen, 2013; Haldar and Murphy, 2014; Jenkins and Hume, 2014).

In humans, the functional significance of microglia's unique ontogeny remains to be clarified; however, initial insights have revealed that some differences exist in developmental genetic programming when compared to peripheral blood-derived myeloid cell types. Studies using proto-oncogene c-myb$^{-/-}$ mice, a transcription factor that regulates haematopoiesis, demonstrate that unlike haematopoietic stem cells, erythromyeloid progenitors resulting from primitive haematopoiesis do not require c-*myb* for self-renewal and proliferation (Hulshof *et al.*, 2003; Lieu and Reddy, 2009). However, interpreting these data may be complicated given c-myb regulates the colony stimulating factor 1 receptor (CSF1R), which is involved in maintaining and differentiating both monocytes and microglia (Jenkins and Hume, 2014). Microglia differentiation is further subjected to molecular regulation by the transcription factors SPI1 (previously known as PU.1) and interferon regulatory factor 8 (IRF8), which are both implicated in normal myeloid cell development. The fractalkine receptor (CX3CR1) is also commonly used for lineage tracing of microglia in rodent models and is expressed in human microglia (Hulshof *et al.*, 2003; Ginhoux *et al.*, 2010), yet Kierdorf *et al.* (2013) have described genetic and cell-surface markers of rodent microglia before CX3CR1 expression. An early protein tyrosine phosphatase receptor type C positive (CD45+) and CX3CR1 negative (CX3CR1^{-}) myeloid precursor population is dependent on the transcription factor SPI1, while loss of IRF8 causes a maturational defect and apoptosis of microglia precursors before acquiring CX3CR1 expression. IRF8 is linked downstream of SPI1, thus implicating this factor in early microglia differentiation and development (Kierdorf *et al.*, 2013). Interestingly, the C57Bl/6 genetic background requires SPI1 for yolk sac macrophage development, whereas outbred *Spi1*$^{-/-}$ mice do not (Lichanska *et al.*, 1999; Jenkins and Hume, 2014). Additionally, Minten *et al.* (2012) reported an increase in microglia number in the same IRF8 knockouts crossed with CSF-1R-eGFP C57Bl/6 mice (Holtschke *et al.*, 1996; Minten *et al.*, 2012). These discrepancies highlight the complexity and importance of genetic background in animal studies focusing on microglia; it is unclear how these findings translate to humans.

In humans, the spatial distribution of embryo-colonizing microglia in histological sections suggests they enter the brain primordium via the developing meninges, ventricular zone, and choroid plexus (Monier *et al.*, 2006, 2007; Verney *et al.*, 2010). Initially, microglia have an amoeboid morphology and eventually acquire a mature, ramified morphology. This developmental amoeboid shape suggests they may be activated during development (Verney *et al.*, 2012; Supramaniam *et al.*, 2013). Immature rodent microglia express high levels of specific chemokines and their cognate receptors (e.g. CX3CR1, CCR2, CCR1, CXCR3) during maturation in the brain parenchyma (Goings *et al.*, 2006; Verney *et al.*, 2010; Kierdorf *et al.*, 2013; Shigemoto-Mogami *et al.*, 2014). In the respective gene knockout animals, the morphology and numbers of microglia remain constant and suggests these molecules are not necessary for distribution in the developing parenchyma (Kierdorf *et al.*, 2013). Members of the matrix metalloproteinase (MMP) family, including MMP8 and MMP9, also regulate distribution and microglia number in the embryonic mouse brain (Kierdorf *et al.*, 2013). With an increasing interest in anatomically spatial differences amongst microglia in various brain regions, the individual factors regulating the migration and positioning of microglia in regions of the developing brain are currently being investigated (de Haas *et al.*, 2008; Doorn *et al.*, 2014; Filho *et al.*, 2014; Kim *et al.*, 2014*a*; Llorens *et al.*, 2014; Silverman *et al.*, 2014).

Bidirectional interaction of microglia and the developing neural architecture

The human cerebral cortex is composed of 20–25 billion neurons that arise from the ventricular and subventricular zones during embryonic development. It has been estimated that microglia make up 6–18% of neocortical cells in the human brain (Pelvig *et al.*, 2008; Lyck *et al.*, 2009). The early arrival of microglia in the developing brain, together with their activated phenotype and evidence of microgliosis during neurodevelopmental disorders, suggests the possibility of developmental microglia-neuron crosstalk (Monier *et al.*, 2006, 2007; Pelvig *et al.*, 2008; Lyck *et al.*, 2009; Ginhoux *et al.*, 2010; Verney *et al.*, 2010, 2012; Supramaniam *et al.*, 2013; Baburamani *et al.*, 2014; Shigemoto-Mogami *et al.*, 2014). Indeed, a number of studies have implicated microglia in neurodevelopmental contexts including autism spectrum disorders (Gupta *et al.*, 2014), obsessive compulsive disorder (Chen *et al.*, 2010; Nayak *et al.*, 2014), schizophrenia (Kenk *et al.*, 2014; de Baumont *et al.*, 2015), Tourette's syndrome (Lennington *et al.*, 2014), cerebral palsy (Mallar *et al.*, 2014), foetal alcohol spectrum disorders (Guizzetti *et al.*, 2014), fragile X syndrome (Alokam *et al.*, 2014; Gholizadeh *et al.*, 2015), and others.

It has been suggested that soluble factors may be involved during developmental microglia–neuron crosstalk. Pharmacological inhibition of microglia activation using minocycline reduces overall neurogenesis and oligodendrogenesis, whereas neural precursors are sensitive to combinations of the cytokines interleukin 1 beta (IL1β) and interferon gamma (IFNγ) released by microglia (Shigemoto-Mogami *et al.*, 2014). Microglia also enhance oligodendrogenesis via a combination of IL1β and interleukin 6 (IL6); however, the observed effects are targeted towards later stages of oligodendroglial development (Shigemoto-Mogami *et al.*, 2014). *In vitro*, subventricular zone neural stem cell cultures from postnatal Day 8 mice maintain the ability to form neurospheres, but progressively lose the ability to generate neuroblasts; co-culture of subventricular zone neural stem cells with microglia or microglia-conditioned media rescues their ability to generate neuroblasts (Walton *et al.*, 2006). Some studies have suggested that microglia in neurogenic regions behave differently than those in non-neurogenic areas (Goings *et al.*, 2006; Marshall *et al.*, 2014). Indeed, Mosher *et al.* (2012) found that secretory products derived from neural progenitor cells can modulate the cytokine profiles of microglia, while augmenting phagocytosis and migration functions *in vitro*. These effects were apparently due to neural progenitor cell-derived vascular endothelial growth factor (Mosher *et al.*, 2012) and help highlight the bidirectional nature of the microglia-neuron relationship during brain development.

In addition to secretory products, microglia also physically regulate the number of neural progenitor cells in the developing cerebral cortex. In rodents and non-human primates, this phenomenon occurs via preferential phagocytosis of viable neurons (independent of apoptotic markers) within proliferative zones in latter stages of cortical neurogenesis (Cunningham *et al.*, 2013). Large numbers of activated microglia are found within neural proliferative zones, while few microglia are present within the cortical plate and is consistent with studies performed in humans (Monier *et al.*, 2006, 2007; Verney *et al.*, 2010). Interestingly, maternal lipopolysaccharide (LPS) administration negatively impacts the neural progenitor cell population in rodents, presumably by promoting a pro-inflammatory microglia phenotype. Conversely, doxycycline skews the phenotypic ratio towards an anti-inflammatory phenotype and significantly increases the neural progenitor cell population (Cunningham *et al.*, 2013). The mechanisms responsible for the phagocytosis of viable neurons in the developing human CNS are currently unknown; however, relevant phagocytosis-mediators in the adult CNS may provide a starting point (discussed below).

In the developing brain, microglia interact specifically with neuronal synapses. One study addressed the developmental interactions of microglia and synapses by examining the visual cortex during a critical developmental period using the CX3CR1 + EGFP/Thy-1 YFP transgenic mouse (Tremblay *et al.*, 2010). A number of features of visual perception are established during this period in mice, and are associated with well-described changes in dendritic spines (Majewska and Sur, 2003; Bence and Levelt, 2005; Hooks and Chen, 2007; Schafer *et al.*, 2013). This study demonstrated physical contact between microglia and dendritic spines, which were demonstrated to change size and often eliminated. During this developmental period, in response to re-exposure to light following dark adaptation, microglia phagocytosed more synaptic elements compared with dark-adapted controls. These data suggest that in response to visual experience, spine elimination/remodelling is mediated at least partially by microglial phagocytosis (Tremblay *et al.*, 2010; Schafer *et al.*, 2013). Microglia have been shown to directly phagocytose developmental synapses that express C1q via complement receptors on microglia; the loss of C1q expression in the mouse brain results in synapse elimination defects, including a failure to refine synaptic connections (Stevens *et al.*, 2007). In a more recent study, Stevens' group demonstrated that developing retinal ganglion cell presynaptic terminals are pruned in a complement-dependent manner (Schafer *et al.*, 2012; Tyler and Boulanger, 2012). Paolicelli and colleagues used stimulated emission depletion (STED) microscopy to demonstrate that post-synaptic density protein-95 (PSD95, now known as DLG4) immunoreactivity was present within microglial processes in the mouse hippocampus during normal development, suggesting that microglia can uptake pre- or postsynaptic material using clathrin and non-clathrin

coated vesicles (Westphal *et al.*, 2008; Paolicelli *et al.*, 2011).

During development, microglia-synapse interactions are critical. CX3CR1 is expressed by microglia (Jung *et al.*, 2000; Gundra *et al.*, 2014), while its ligand, fracktalkine (CX3CL1), can be either secreted by neurons or found at the cell surface and helps to establish an interaction between microglia and neurons in both the healthy and pathological CNS (Harrison *et al.*, 1998; Hughes *et al.*, 2002). CX3CR1 knockout mice display transient deficits in synaptic pruning, consistent with observations wherein single-cell recordings of CA1 pyramidal neurons revealed a decreased spontaneous excitatory postsynaptic current/miniature excitatory postsynaptic current amplitude ratio, indicating immature connectivity in knockout animals (Paolicelli *et al.*, 2011). In a follow-up study, the authors quantify the effect of this pruning deficit on synaptic transmission, functional connectivity, and behavioural outcomes in mice, suggesting that a repetitive behavioural phenotype is reminiscent of neurodevelopmental disorder in humans (Zhan *et al.*, 2014). CX3CR1 deficit has specifically been shown to impair learning and is consistent with a substantial role for microglia in normal synaptic regulation and network development (Boulanger, 2009; Pernot *et al.*, 2011; Rogers *et al.*, 2011; Voineagu *et al.*, 2011; Fillman *et al.*, 2013). The possibility that defects in microglia function could lead to diverse developmental neuropathologies is an exciting future avenue for a multitude of therapeutic targets.

Pathology and homeostasis in the adult CNS

Maintenance of microglia populations, microgliosis and parenchymal precursors

We are now beginning to understand how microglia are maintained in the adult brain and the factors responsible for regulating their population. It seems that microglia are the dominant tissue-resident macrophages, which maintain their population without contribution from the periphery throughout life (Fig. 1) (Ajami *et al.*, 2007; Mildner *et al.*, 2007; Sieweke and Allen, 2013; Jenkins and Hume, 2014). Colony stimulating factor 1 (CSF1), also known as macrophage colony stimulating factor (MCSF), maintains macrophage populations through its protein tyrosine kinase receptor, CSF1R, which is expressed on committed macrophage precursors, monocytes, and microglia (Pixley and Stanley, 2004; Nandi *et al.*, 2012; Jenkins and Hume, 2014). The osteopetrotic mouse is effectively *Csf1*$^{-/-}$, causing a myriad of skeletal and haematopoietic defects, including a reduction in microglia that ranges from 30% to complete depletion depending on the region (Wegiel *et al.*, 1998; Kondo *et al.*, 2007; Wei *et al.*, 2010; Nandi *et al.*, 2012). Mutations in the CSF1R gene can cause more severe defects, including a >99% microglia reduction brain-wide (Erblich *et al.*, 2011). Elmore *et al.* (2014) found that blocking CSF1R with a multi-targeted tyrosine kinase inhibitor, depleted adult mice of 99% of microglia, reinforcing the importance of CSF1R signalling in microglia maintenance.

In a large functional screen of the human extracellular proteome, Lin *et al.* identified interleukin 34 (IL34), a previously unknown ligand for CSF1R (Lin *et al.*, 2008). IL34 is more abundantly (~10-fold) and broadly expressed compared to CSF1, while their differential regional expression accounts for differences between CSF1 and CSF1R knockout phenotypes (Wei *et al.*, 2010). Using IL34 deficient mice, it was demonstrated that this cytokine is a critical factor for microglia differentiation; neurons represented the primary source of IL34 (Wang *et al.*, 2012). In the adult mouse brain, CSF1 and IL34 are maintained at high levels, whereas CSF1R levels decrease during postnatal development (Nandi *et al.*, 2012). Recently, an alternate receptor for IL34 has been identified, suggesting the possibility of distinct functions for IL34 and CSF1 (Hamilton and Achuthan, 2013; Nandi *et al.*, 2013). Whether IL34 and CSF1 have differential influences on human microglial function is unknown.

Following brain injury, microglia numbers are increased (termed microgliosis) in tissues proximal to injury, exhibit an activated morphology and phenotype, and increase their expression of MHCII/HLA-DR (Monier *et al.*, 2007) and inducible nitric oxide synthase (NOS2) (Verney *et al.*, 2010). In the inflamed brain, the lack of specific markers capable of distinguishing circulating and invading peripheral blood-derived macrophages from microglia in histological sections precludes making accurate distinctions between these cell types. Initially, irradiation and bone marrow reconstitution experiments using congenic mice were used to examine the peripheral and central contributions to the microglia pool under various conditions; however, this model system generated various confounds (Ransohoff, 2007). In parabiosis, syngeneic mice have their circulatory systems joined such that host or donor cells can be tracked using specific markers. Thus, the host microglia population can be distinguished from peripheral cell types under both steady-state conditions and following experimental manipulation. Using this method, it was observed that blood-derived cells did not contribute to microglia turnover under steady-state conditions, nor microgliosis in models using facial nerve axotomy, or overexpression of a transgenic mutant superoxide dismutase 1 ($SOD1^{G93A}$) (Ajami *et al.*, 2007). Using a similar design, yet relying on a more controlled, modified irradiation-reconstitution method, Mildner *et al.* (2007) reported no participation of blood-derived cells in homeostatic microglial turnover, or in experimentally induced microgliosis (facial axotomy, and cuprizone-induced demyelination models).

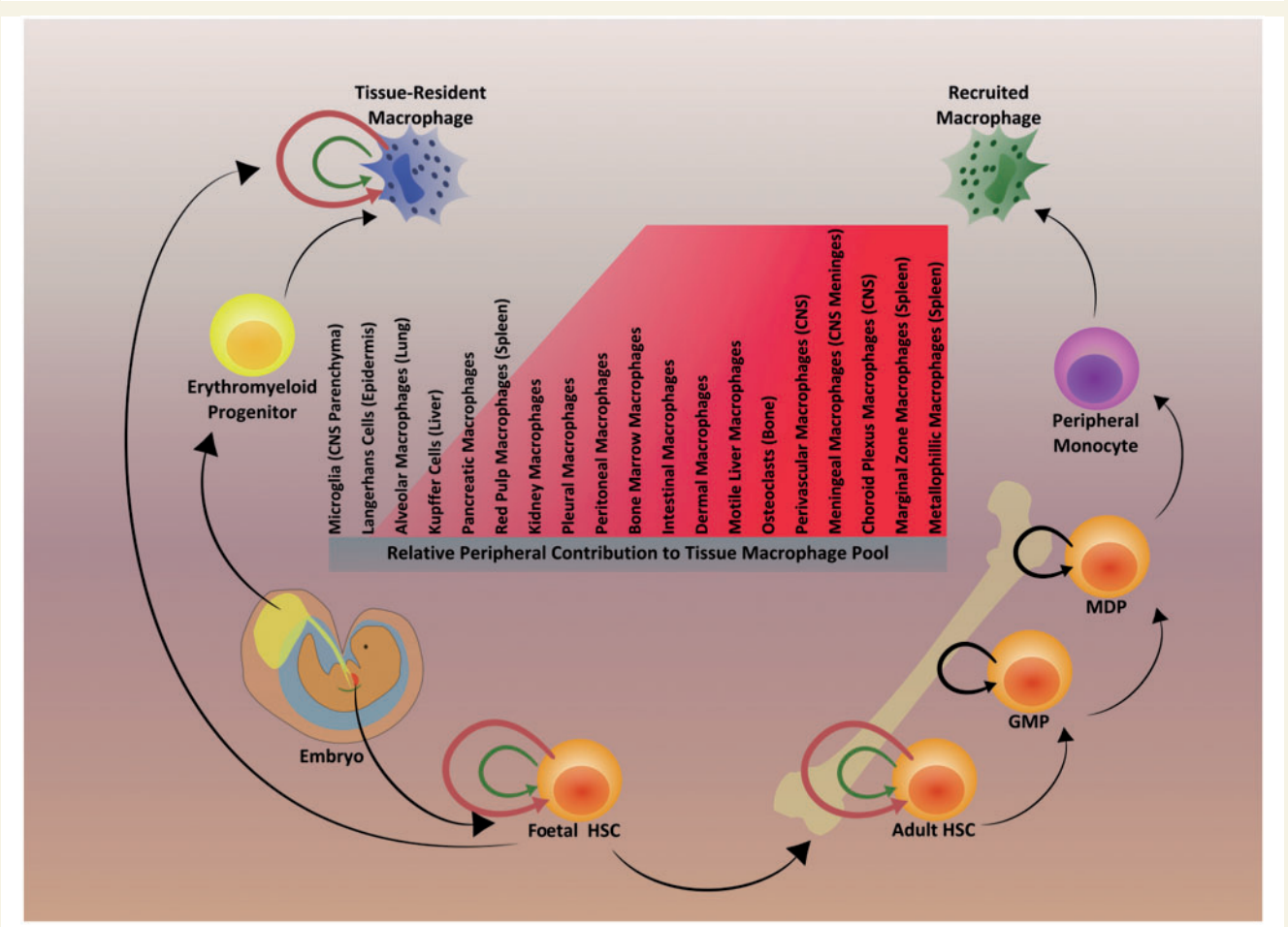

Figure 1 Primitive and definitive haematopoietic contributions to specific tissue macrophage populations. Some tissue macrophages such as microglia are derived from yolk sac erythromyeloid progenitors during primitive haematopoiesis in early embryonic development. In some cases (e.g. Langerhans cells), foetal progenitors within the liver can also contribute to tissue macrophage populations. Tissue macrophage populations are maintained in the adult organism by local self-renewal and/or monocytes recruited from the periphery. In some cases (e.g. microglia) local self-renewal is the only method by which maintenance occurs. *Middle*: The contribution of peripheral myeloid cells to the maintenance of specific tissue macrophage populations is tissue-dependent. Here, it is shown as a list with peripheral contribution to specific tissue macrophage populations increasing from left to right. Near the end of the list, populations of tissue macrophages that are completely maintained by peripheral monocytes are shown (e.g. intestinal macrophages and dermal macrophages). Some types, such as white pulp macrophages (spleen), adipose-associated macrophages, and interstitial macrophages (lung) have an undetermined maintenance mechanism and are not depicted (Sieweke and Allen, 2013; Haldar and Murphy, 2014). Pleural macrophages (lung) and peritoneal macrophages each have two distinct subpopulations: a large majority, which originates from the yolk sac is self-maintained, while a smaller group is maintained by monocytes from the periphery (Haldar and Murphy, 2014). The general mechanisms of primitive and definitive haematopoiesis are shown. Tissue-resident macrophages, such as foetal and adult haematopoietic stem cells (HSC), undergo low rates of homeostatic proliferation to self-maintain their populations (represented by green arrows), and may undergo high rates of proliferation when challenged (injury, infection, irradiation, etc. represented by red arrows) (Sieweke and Allen, 2013). Black arrows represent the transient amplification of granulocyte-macrophage (GMP) and macrophage-dendritic progenitors (MDP) during normal haematopoiesis. Overall the majority of these findings come from experiments performed in rodents.

The literature describing the numerous molecular factors regulating microglia population dynamics at a mechanistic level is vast and are not discussed in detail as they are reviewed sufficiently elsewhere (Kettenmann *et al.*, 2011). Proliferation regulation signals include positive regulation via intracellular and volume-sensitive chloride channels, adenosine receptors, and cannabinoid receptors (Fiebich *et al.*, 1996; Gebicke-Haerter *et al.*, 1996; Schlichter *et al.*, 1996, 2011; Carrier *et al.*, 2004; Hartzell *et al.*, 2005; Ducharme *et al.*, 2007; Milton *et al.*, 2008). Negative regulation may occur via voltage-dependent K_v channels, somatostatin receptors, glucocorticoid receptors, and beta-2 adrenergic receptors (Gehrmann and Banati, 1995; Tanaka *et al.*, 1997; Fujita *et al.*, 1998; Brough *et al.*, 2002). Albumin may also cause microglia proliferation through intracellular Ca^{2+} signalling, providing an interesting candidate signal in the context of injuries that compromise blood–brain barrier integrity

(Hooper *et al.*, 2005; Kettenmann *et al.*, 2011). A recent study found that ganciclovir, an anti-retroviral drug, was a potent inhibitor of rodent microglia/macrophages within the inflamed CNS, without noticeably affecting the peripheral myeloid proliferative response, or subsets of other immune cells. This finding raises the intriguing possibility of therapeutics selectively targeting myeloid proliferation within the CNS (Ding *et al.*, 2014). Furthermore, with the recent molecular profiles for human and rodent microglia, which can be used to help distinguish them from peripheral-derived cells, this may become a therapeutic possibility (Gautier *et al.*, 2012; Chiu *et al.*, 2013; Butovsky *et al.*, 2014).

Despite the suggestion of local maintenance, little is known about microglia precursors in the adult brain. Following experimental injury, nestin expression is upregulated in microglia, but can also be found in many other cell types near stroke infarcts, focal spinal cord injury, and in other injury models (Namiki and Tator, 1999; Sahin *et al.*, 1999; Shibuya *et al.*, 2002; Mothe and Tator, 2005; Takamori *et al.*, 2009; Wohl *et al.*, 2011). It remains unknown whether erythromyeloid precursors express nestin, or if this phenotype is indicative of a more mature precursor cell type found in the brain alone. It is also worth noting that no studies have yet demonstrated a CNS-derived cell with stem characteristics that is capable of generating microglia. Elmore *et al.* (2014) have shown that nestin-positive microglia precursors expressed Iba1, the lectin IB4, and CD45 (PTPRC), with a fraction of cells also expressing Ki67, CD34 and c-Kit (KIT), which decreased in expression upon stabilization of their population (Elmore *et al.*, 2014). In injury models, nestin expression following brain injury often occurs in cells of the perivascular region, suggesting at least one putative niche for microglial precursors (Shin *et al.*, 2013; Hughes and Bergles, 2014).

Immunocentric microglia: activation, polarization and resting states

Microglia possess both immunocentric and neurobiological functions during development and throughout adulthood. In light of the recent expansion of research in the field of macrophage activation, polarization, and plasticity, we support the recent endeavour of Murray *et al.* (2014) to generate standardized myeloid activation/polarization nomenclature and experimental practices (Table 1). This new nomenclature attempts to define polarization by the molecular constituents used to activate the cells. Importantly, in contrast to working with rodent macrophages and microglia in culture, human microglia do not require growth factors for maintenance and/or differentiation *in vitro* (Durafourt *et al.*, 2012). When making direct comparisons with peripheral-derived macrophages, growth factors may or may not be used in attempts to make direct *in vitro* comparisons between peripheral-derived macrophages and brain-resident microglia. Therefore, the null condition M(−) should be clearly defined in each microglia study with these facts in mind (Table 1). 'Polarization' refers to perturbation of microglia with exogenous agents to achieve a particular phenotype, and is evident by the presence of one or more molecular markers and a shift in molecular profile. *In vitro*, the term 'polarization' attempts to simulate environmental conditions that are indicative of microglia function via receptors and mechanisms collectively referred to as the sensome (Hickman *et al.*, 2013). The term 'activation' can be difficult to interpret in the neuroimmunological context because it is derived from a purely immunological context. Although correct in many myeloid cell immunological contexts, activation implies that microglia are 'inactive' under certain conditions. This in fact may not be the case even *in vitro* where human microglia display a high degree of motility and activity, similar to what has been observed *in vivo* (Supplementary Video 1). Use of the term 'basal' instead of 'inactive' may partially resolve this quandary, for now. Alternate considerations for myeloid cell nomenclature may be found in Svahn *et al.* (2014). Given the nature of our review, we use previous widely-cited nomenclature (e.g. M1/M2), which directly relates to the present literature. A table is provided here to aid in relating previous published materials with the novel proposed nomenclature (Table 1).

Polarized myeloid cells, including blood-derived macrophages and CNS-resident microglia, have been historically classified along a spectrum similar to the T helper (T_h1/T_h2) classification of T lymphocytes (Fig. 2). Myeloid cell polarization was initially characterized in peripheral cells whereby classical activation of macrophages was observed following exposure to T_h1-type cytokines such as IFNγ (Mantovani *et al.*, 2004). Building on the observation that in murine macrophages the T_h2-associated cytokine IL4 could induce expression of the mannose receptor CD206 (Stein *et al.*, 1992), a T_h2 driven alternative activation phenotype was described (Gordon, 2003). In the literature, 'classically activated' myeloid cells (LPS ± IFNγ) have been referred to as 'M1', whereas 'alternatively activated' cells (IL4 treatment in the presence or absence of IL13) as 'M2'. At the extremes of this spectrum, the properties of myeloid cells have been often studied by generating monocyte-derived macrophages, followed by exposure to either T_h1 or T_h2 type cytokines to polarize cells into the M1 or M2 lineage, respectively (Sierra-Filardi *et al.*, 2010). Standardized protocols for human myeloid cells, including microglia, have been described previously (Durafourt *et al.*, 2013). The M2 phenotype has been further subclassified according to specific activation stimuli, with M2a generated by exposure to IL4 and IL13, M2b using immune complexes and LPS, and M2c using IL10 and transforming growth factor beta (TGFβ1) (Leidi *et al.*, 2009). The generation of M2a cells with IL4 and IL13 relies on signalling through IL4RA, leading to inhibition of nuclear factor kappa-light-chain-enhancer of

Table 1 Nomenclature of activated macrophages/microglia

Classic nomenclature	Novel nomenclature	Activating molecules	Notes	Citations
M0/Unpolarized	M(-)	None, M-CSF, *or* GM-CSF	M-CSF or GM-CSF may be used to compare with macrophages	Durafourt *et al.*, 2012
M1	M(LPS) M(IFNγ) M(LPS + IFNγ)	LPS, IFNγ or both	a.k.a. Classical activation	Mantovani *et al.*, 2004
M2	M(IL4)	IL4	a.k.a. Alternative activation	Stein *et al.*, 1992; Gordon, 2003; Sierra-Filardi *et al.*, 2010
M2a	M(IL4 + IL13)	IL4 and IL13	Profile associated with anti-inflammatory phenotype in macrophages	Taylor *et al.*, 2005; Leidi *et al.*, 2009
M2b	M(Ig + LPS)	Immune complexes and LPS	Profile associated with pro-inflammatory cell-surface protein expression but IL10 secretion in macrophages	Edwards *et al.*, 2006; Leidi *et al.*, 2009
M2c	M(IL10 + TGFβ1)	IL10 and TGFβ1	Profile associated with tissue remodelling and immune suppression in macrophages	Mantovani *et al.*, 2004; Leidi *et al.*, 2009

GM-CSF = granulocyte-macrophage colony stimulating factor, now known as CSF2; M-CSF = macrophage colony stimulating factor, now known as CSF1.

activated B cells (NF-κB) signalling and an anti-inflammatory phenotype (Taylor *et al.*, 2005). M2b cells are the least well studied; they continue to express M1-associated co-stimulatory molecules and produce nitric oxide (NO), but also secrete anti-inflammatory cytokines, such as IL10 (Edwards *et al.*, 2006). M2c macrophages, originally termed deactivated, are associated with immune suppression and tissue remodelling (Mantovani *et al.*, 2004).

Classically-activated peripheral myeloid cells have been characterized by their rounded amoeboid morphology, which is consistent with a state of hyperactivity. These cells express surface markers such as co-stimulatory molecules CD80 and CD86 and the chemokine receptor CCR7, produced reactive nitrogen species and pro-inflammatory cytokines such as IL12 (Van Ginderachter *et al.*, 2006; Fairweather and Cihakova, 2009). In contrast, M2 cells possess a typical elongated morphology, express cell surface markers including the scavenger receptor CD163, C-type lectins CD206 and CD209, and produce anti-inflammatory cytokines such as TGFβ1 and IL10 (Mantovani *et al.*, 2004; Martinez *et al.*, 2006; Van Ginderachter *et al.*, 2006). In line with this anti-inflammatory profile, the M2 phenotype has been associated with the repair process in animal models of stroke (Desestret *et al.*, 2013), spinal cord injury (Shechter *et al.*, 2013), and demyelination (Miron *et al.*, 2013).

The concept of microglia polarization was first explored in the murine system by Ponomarev *et al.* (2007) and demonstrated that microglia expressed the M2-associated protein Ym1 (now known as CHIL3) in an IL4 dependent manner (Ponomarev *et al.*, 2007). Ym1 is a chitinase-like lectin and has been identified as a marker of M2 macrophages in the murine system, but not in humans (Raes *et al.*, 2002, 2005). Expression of the enzyme arginase 1 has also been reported as a marker of M2 macrophages; however, it remains controversial whether selective expression also translates to humans. Studies characterizing human adult and foetal microglia demonstrated that human microglia can also adopt M1 and M2-like characteristics, while possessing distinct phenotypic and functional properties compared to monocyte-derived macrophages (Durafourt *et al.*, 2012). M1-polarized human adult microglia express similar surface markers as their macrophage counterparts, including CD80 and CCR7. Following IL4 and IL13 activation, human microglia express fewer M2-associated surface markers than macrophages, with CD209 being the most reliable marker. While alternatively-activated macrophages more efficiently phagocytose opsonized targets than their M1 counterparts (Leidi *et al.*, 2009), M2 microglia were also more efficient at phagocytosing myelin compared to either M1 microglia or any polarized macrophage phenotype (Durafourt *et al.*, 2012). In the injured CNS, microglia may therefore be more likely than monocyte-derived macrophages to contribute to the clearance of debris, thus allowing for an efficient repair process to occur (Kotter *et al.*, 2006).

Targeting myeloid cell polarization remains a clinically relevant objective. Murine studies have shown that lack of IL4 in the CNS, but not the periphery, impaired the M2 polarization of microglia and exacerbated symptoms of experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis (Ponomarev *et al.*, 2007). Examination of post-mortem tissue from patients with multiple sclerosis revealed that myeloid cells in active multiple sclerosis lesions abundantly expressed M1 markers, whereas the majority of these cells also co-expressed M2 markers (Vogel *et al.*, 2013). In a demyelination model, following

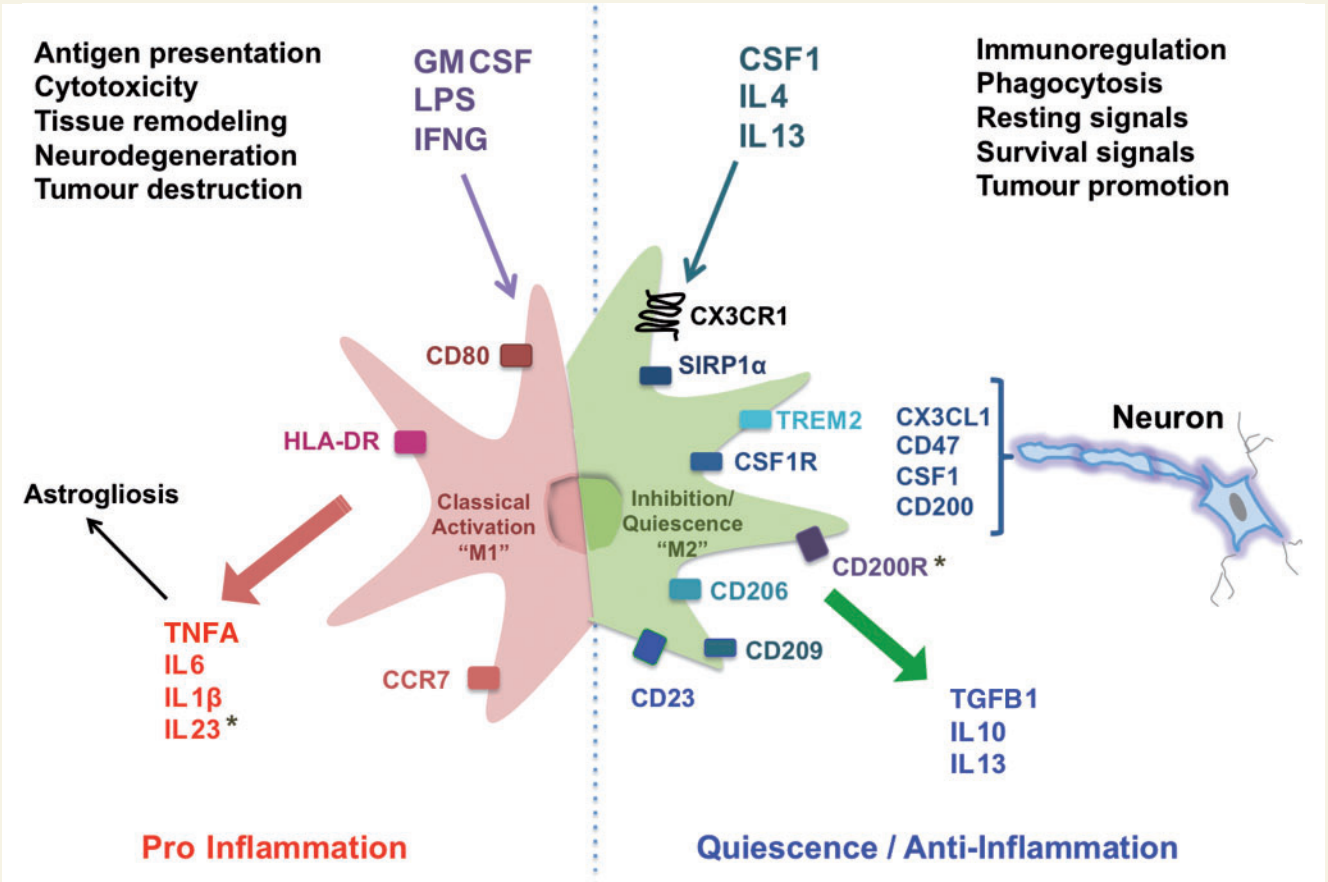

Figure 2 Pro-inflammatory 'M1' and quiescent/anti-inflammatory 'M2' microglia are regulated by the cytokine milieu. Both M1 and M2 microglia phenotypes have several purported roles in the injured CNS, including phagocytosing potential antigens, clearing cell debris, activating cells of the adaptive immune system, and directly contributing to mechanisms related to both neurodegeneration and repair. In response to pro-inflammatory stimuli [e.g. GM-CSF (CSF2), LPS, IFNγ], several cell surface molecules and soluble mediators are upregulated. In the CNS (e.g. CD80, CD86, CCR7), release of TNF and IL6 from microglia can lead to the activation of astrocytes, which in turn can support a permissive microenvironment that harnesses T and B cell recruitment and survival. CSF1 alone or in combination with IL4 and IL13 maintains a quiescent, anti-inflammatory, and/or tissue regenerative phenotype (often termed M2). These cells can express several different Fc receptors (e.g. CD23), C-type lectin molecules (e.g. CD206 and CD209), and release cytokines TGFβ1, IL10 and IL13. In the brain, microglia can also maintain a homeostatic and/or quiescent state by interacting with ligands (e.g. CX3CL1, CD47, CSF1 and CD200) on the surfaces of other neural cells, such as astrocytes and neurons. Asterisk represents quiescence markers and soluble factors not yet validated in human microglia.

stereotactic injection of lysolecithin, murine microglia undergo a switch from the M1 to M2 phenotype. This process was shown to drive oligodendrocyte differentiation and promote remyelination (Miron *et al.*, 2013). In the injured CNS, TNF prevents the phagocytosis-mediated conversion from M1 to M2 type cells, while iron promotes a rapid switch from the M2 to M1 phenotype (Kroner *et al.*, 2014). Dysregulation of myeloid cell polarization has also been implicated in acute neuroinflammatory conditions, including spinal cord injury, traumatic brain injury, and stroke, where M1 cells predominate (Cherry *et al.*, 2014). In a rodent model of spinal cord injury, mesenchymal stem cells were shown to promote M2 macrophage differentiation and improve functional recovery (Nakajima *et al.*, 2012). In another rodent model, M2 macrophages were found to be recruited though the choroid plexus and to contribute to the repair process (Shechter *et al.*, 2013). These studies demonstrate that M2 myeloid cells contribute not only to neuroprotection, but may also promote repair process in the injured CNS and may represent novel therapeutic strategies for treating neurodegenerative conditions.

MicroRNA regulation of microglia status in pathology

Over the past decade, only a small number of molecules have emerged as clinically-used biomarkers of pathogenic processes or treatment responses. Unfortunately, many putative biomarkers do not meet all the criteria for an 'ideal' biomarker (Rajasekharan and Bar-Or, 2012). In several clinical fields of medicine, microRNAs have emerged as important post-transcriptional regulators of gene expression and putative biomarkers that may either indicate or predict disease activity, clinical prognosis, and/or treatment to

individual therapies. MicroRNAs are short non-coding RNAs (18–22 nucleotides) that are stable and abundant molecules present in all biological fluids. Not only have they been suggested as ideal biomarkers, but can also possess disease-relevant biological activity. Most recently, the expression and biological function of several distinct microRNA molecules have been described in myeloid cells, including microglia (Moore *et al.*, 2013; Ponomarev *et al.*, 2013). Compared to other cells of the innate and adaptive immune systems, the relatively high plasticity of microglia, in terms of their contributions to both disease-promoting and tissue-regenerative activities, may be at least in part due to their microRNA expression profiles.

It has been suggested that distinct microRNA expression patterns in microglia may play a significant role in their phenotype and function. The development and differentiation of microglia is controlled by the transcription factors CEBPA and NFIA, which are directly controlled by mir-223 (Fazi *et al.*, 2005). In additional studies by Ponomarev *et al.* (2011) mir-124 was abundantly expressed in the brain and responsible for maintaining microglia quiescence by restricting SPI1 (PU.1) and CSF1 expression. *In vitro* studies measuring microRNA expression in primary rodent microglia have also demonstrated that under standard culture conditions, mir-124 and mir-155 levels decreased over time, whereas mir-146a was increased, suggesting these microRNAs may be responsible for the altered cell phenotype and function associated with ageing (Caldeira *et al.*, 2014). Many additional microRNAs have also been suggested to regulate monocyte differentiation (Forrest *et al.*, 2010), however, the role(s) of these microRNAs in human microglia remains uncertain (Pospisil *et al.*, 2011).

Several microRNAs have been reported to both promote and inhibit innate inflammatory responses (Table 2). In the literature, mir-155 is among one of the most highly referenced pro-inflammatory microRNAs. In primary cells (rodent and human) and several microglia cell lines, mir-155 is upregulated upon activation with several pro-inflammatory factors, including LPS, IFNγ and TNF. In human myeloid cells, including both human fetal and human adult microglia, mir-155 expression is significantly elevated under M1-polarizing conditions (Moore *et al.*, 2013). In human microglia, transfection with a mir-155 mimic alone significantly increases pro-inflammatory cytokine expression, co-stimulatory molecule expression (e.g. CD80, CD86, CCR7), and enhances allogeneic T cell responses in co-culture (Moore *et al.*, 2013); inhibition of mir-155 decreased these phenotypes. This pro-inflammatory phenotype is driven in part by the ability of mir-155 to block *SOCS1* expression, however, additional studies have also demonstrated that mir-155 can also decrease the expression of molecules/receptors associated with promoting the M2 phenotype (Martinez-Nunez *et al.*, 2011). In addition to mir-155, several other microRNAs can also promote M1-like phenotypes in microglia, including mir-9 (Yao *et al.*, 2014) and mir-125b (Parisi *et al.*, 2013). Other microRNAs, including mir-146a and mir-200b, are also thought to contribute to microglia activation, however induction of these particular microRNAs is not thought to promote an inflammatory phenotype, but rather help resolve chronic inflammation (Rom *et al.*, 2010; Jadhav *et al.*, 2014*a*).

Studies performed *in vitro* using human microglia and monocyte-derived macrophages suggest these cell types are phenotypically and functionally distinct upon activation (Durafourt *et al.*, 2012). Differences in cell migration and phagocytosis have also been reported between monocyte-derived macrophages and microglia. The reasons for these differences may be in part due to their unique mRNA and/or microRNA profiles. Recent studies have investigated the molecular signature of microglia compared with other myeloid and immune cell types demonstrating unique microRNAs expression in microglia, including mir-99a, mir-342-3p, and mir-125b-5p (Butovsky *et al.*, 2014). *In vitro*, mir-155 levels are significantly elevated in human blood-derived myeloid cells compared to microglia (Moore *et al.*, 2013). Together with these unique myeloid cell signatures, it may be possible to selectively target biological activities of individual myeloid cell populations, which may have pharmacological and therapeutic value in the context of brain injury and repair.

In several different neurological conditions, including Alzheimer's disease, amyotrophic lateral sclerosis, stroke, multiple sclerosis, and Parkinson's disease, the expression levels of microRNAs are altered in microglia. In multiple sclerosis lesions, activated macrophages/microglia have significantly elevated mir-155 levels compared with microglia in normal autopsied brain tissue. Indeed, *in vivo* silencing of mir-155 ameliorates clinical and pathological outcomes in EAE. In a genetic model of Alzheimer's disease, mir-155 levels are significantly elevated and induced simultaneously with microglia activation (Guedes *et al.*, 2014). Mir-146a, a negative regulator of inflammatory responses, is downregulated in microglia lacking presenilin 2 (*PSEN2*), a gene that is mutated in autosomal dominant Alzheimer's disease, thus increasing the pro-inflammatory phenotype of microglia (Jayadev *et al.*, 2013). In microglia cultured from mice lacking PARK7 (previously known as DJ-1), a Parkinson's disease-associated gene linked to disease onset and progression, mir-155 is increased following activation with IFNγ, but not in wild-type cells (Kim *et al.*, 2014*b*). In an animal model of cerebral ischaemia, decreased mir-424 expression is associated with increased microglial activation and neuronal apoptosis (Zhao *et al.*, 2013), whereas increased mir-181c expression is associated with decreasing TNF and protected neurons from damaging effects of activated microglia (Zhang *et al.*, 2012*a*). A comparison between microRNA expression levels in activated microglia derived from the $Sod1^{G93A}$ mouse versus wild-type microglia identified several dysregulated microRNAs, including mir-155, mir-146b, mir-365, and mir-125b, which were linked to dysregulated IL6 and TNF expression (Parisi *et al.*, 2013). In an unbiased analysis of microRNA expression using microglia and

Table 2 MicroRNAs implicated in microglia function

MicroRNA	Reference	Reported involvement/function
Inflammation-related		
Mir-9	Akerblom *et al.*, 2013; Kong *et al.*, 2014; Yao *et al.*, 2014	Ischaemia, MCPIP1 and NF-κB signalling
Mir-17	Jadhav *et al.*, 2014*b*	NADPH oxidases and reactive oxygen species production
Mir-21	Kong *et al.*, 2014	Ischaemia
Mir-22	Parisi *et al.*, 2013	Purinergic signalling
Mir-29b	Fenn *et al.*, 2013; Thounaojam *et al.*, 2014	Aging, TNFAIP3 and NF-κB signalling
Mir-32	Mishra *et al.*, 2012	TRAF3 signalling
Mir-34a	Alexandrov *et al.*, 2013; Zhao *et al.*, 2013; Su *et al.*, 2014	NF-κB signalling and TREM2-regulated phagocytosis activity, p53 signalling
Mir-124a	Ponomarev *et al.*, 2011; Willemen *et al.*, 2012; Freilich *et al.*, 2013; Kong *et al.*, 2014	CEBPA–SPI1 (PU.1) signalling, cell activation, cell quiescence
Mir-125b	Parisi *et al.*, 2013	Purinergic signalling, STAT3 signalling
Mir-132	Kong *et al.*, 2014	Ischaemia
Mir-134	Kong *et al.*, 2014	Ischaemia
Mir-138	Kong *et al.*, 2014	Ischaemia
Mir-142	Chaudhuri *et al.*, 2013	Viral infections, SIRT1 regulation
Mir-145	Freilich *et al.*, 2013; Su *et al.*, 2014	Contribute to alternative activation, IL4/STAT6 signalling, p53 signalling
Mir-146a	Faraco *et al.*, 2009; Rom *et al.*, 2010; Li *et al.*, 2011; Saba *et al.*, 2012; Pareek *et al.*, 2014	Promoting and resolving inflammation, tumour suppression, NF-κB and JAK-STAT signalling
Mir-146b	Parisi *et al.*, 2013	Purinergic signalling
Mir-155	Tarassishin *et al.*, 2011; Cardoso *et al.*, 2012; Freilich *et al.*, 2013; Lippai *et al.*, 2013; Moore *et al.*, 2013; Parisi *et al.*, 2013; Pareek *et al.*, 2014	Contribute to classical activation state, SOCS1 activity
Mir-181a	Dave and Khalili, 2010; Kong *et al.*, 2014	Ischaemia
Mir-195	Shi *et al.*, 2013	Autophagy
Mir-200b	Jadhav *et al.*, 2014*a*	cJun/MAPK signalling
Mir-221	Kong *et al.*, 2014	Ischaemia
Mir-222	Kong *et al.*, 2014	Ischaemia
Mir-365	Parisi *et al.*, 2013	NFKB signalling, IL6/STAT3 signalling
Mir-424	Zhao *et al.*, 2013	Cell cycle regulation and pro-inflammatory pathways
Mir-689	Freilich *et al.*, 2013	Canonical pro-inflammatory pathway
Mir-711	Freilich *et al.*, 2013	Canonical pro-inflammatory pathway
Growth factor-related		
Let7f	Selvamani *et al.*, 2012	Ischaemia and IGF1 signalling
Mir-29b	Fenn *et al.*, 2013	IGF1 and CX3CL1 signalling
Apoptosis-related		
Mir-21	Zhang *et al.*, 2012*b*	Hypoxia and FasL signalling

monocytes derived from SOD1 mice, differential expression of several microRNAs were also observed between cell types with changes also being observed over disease course (Butovsky *et al.*, 2012).

In neurodegenerative diseases, the participation of microglia and their respective polarized phenotypes in contributing to both tissue injury and repair is becoming increasingly recognized. With the altered microRNA expression profiles in several brain-related conditions, we are now beginning to assign distinct molecular profiles with subtypes of myeloid cells and determine their unique functional roles as they relate to inflammatory and tissue regenerative processes. In the future, not only will microRNAs help elucidate the molecular mechanisms responsible for driving disease pathogenesis, but may also serve as putative pharmacological targets and biomarkers of disease and therapeutic efficacy.

Microglia and the ageing CNS

Microglia are activated in response to numerous signals in the CNS, including viruses, bacteria, cytokines,

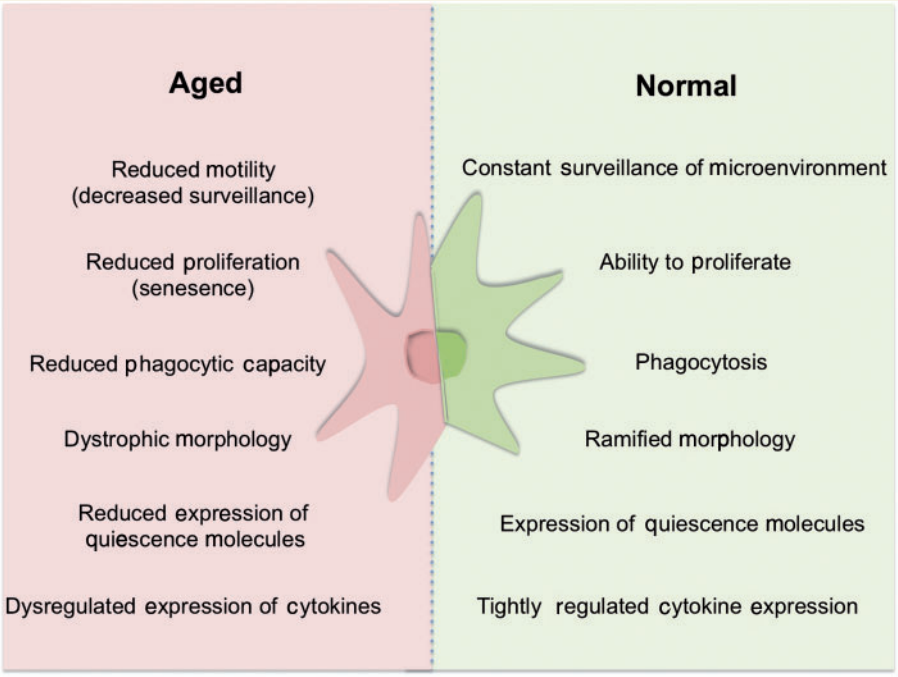

Figure 3 Functional properties of microglia in the normal and aged brain. Ageing has been demonstrated to significantly impact the functionality of microglia. Age-associated changes in motility, proliferation, phagocytosis, and gene expression profiles of microglia are thought to be, in part, as a consequence of cumulative activation in response to systemic infections over time. These changes can lead to a complete loss of function, but also dysfunction and even hyper-reactive responses in aged microglia, thus reducing these cells capacity to effectively survey the CNS environment, maintain homeostasis, and respond both rapidly and efficiently to injury and disease.

chemokines, or aberrant aggregation of endogenous disease-promoting proteins. Alternatively, microglial activation can also occur following the loss of neuronally-derived homeostatic signals. For example, the CD200R present on microglia interacts with CD200 and induces a resting phenotype. CD200 is expressed on the surface of various brain cells, including neurons, oligodendrocytes and astrocytes (Wright *et al.*, 2003; Biber *et al.*, 2007). In the hippocampus of aged rats, a CD200 fusion protein decreased microglial activation (Cox *et al.*, 2012). 'Normal' ageing is often accompanied by a decline in neural function, which can lead to cognitive impairment and thought to be caused by changes in the brain independent of those associated with neurodegenerative disease states. In neurons, such changes include morphological changes (e.g. decreased dendritic length, reduced spine and synapse density), deficits in synaptic plasticity and altered electrophysiological properties (de Brabander *et al.*, 1998; Dickstein *et al.*, 2013). These changes are thought to be due to synaptic remodelling, a decrease in synaptic structures and associated deficits in synaptic plasticity (Jacobs *et al.*, 1997; Duan *et al.*, 2003; Hof and Morrison, 2004). However, neuronal alterations are most likely in response to wider, age-related shifts in glial cell phenotypes, thus resulting in increased release of neurotoxic molecules and decreased glial cell-mediated neuroprotective abilities.

Multiple phenotypic changes have been described for microglia over the lifespan of the cell (reviewed in Harry, 2013) (Fig. 3). Ageing affects microglia in a number of ways by altering the cells' endogenous characteristics and inducing a switch to a less responsive, 'dystrophic' phenotype. Dystrophic changes are morphological changes distinct from the previously described responses to acute challenge, including blebbing of the plasma membrane, reduction/loss of ramified processes and an increase in cytoplasmic inclusions. In aged mice, changes in gene expression reflect the morphologic and functional plasticity of dystrophic cells whereby changes in cytokine production have also been observed; aged microglia produce higher levels of NOS2, TNF, IL1β, and IL6 (Williams *et al.*, 1992). Aged microglia also show elevated expression of markers generally associated with an activated pro-inflammatory phenotype, including, MHC Class II-associated molecules, pathogen-associated molecular pattern (PAMP) receptors and CD11b (Perry *et al.*, 1993). An increase in the number of dystrophic microglia in the aged human brain has been reported, which have a decreased capacity to mount conventional cellular responses to injury (Streit *et al.*, 2004, 2009). A recent *in vivo* study using young, adult and aged mice demonstrated an age-related increase in microglia soma volume, a truncation of cell processes and a more heterogeneous CNS distribution. In addition, the previously described dynamic microglial response to tissue injury was shown to be markedly reduced in the aged animals (Hefendehl *et al.*, 2014). The concept of dystrophic microglia has been challenged with the suggestion that aged microglia

are less altered in terms of morphology and function as previously described. For instance, it has been suggested that aged microglia are merely primed to produce higher levels of both pro- and anti-inflammatory cytokines, relative to an already increased basal expression (Sierra *et al.*, 2007).

In the aged mouse brain, microglia can accumulate phagocytic inclusions, in addition to being less evenly distributed in the cerebral cortex and increasing in overall number (Tremblay *et al.*, 2012). At 12 months of age, murine microglia adopt an M1-like phenotype as shown by increased expression of several pro-inflammatory molecules and cytokines, including NOS2, TNF, IL1β, and IL6 (Crain *et al.*, 2013), while changes in anti-inflammatory cytokines have also been observed (Ye and Johnson, 2001; Sierra *et al.*, 2007).

Research in the Alzheimer's field has yielded much of the work pertaining to the function of aged microglia. In aged mice (14–16 months), microglia produce higher basal levels of pro-inflammatory cytokines compared to microglia derived from young animals (1–2 months) and have reduced glutathione (antioxidant molecule) levels. Aged microglia also display reduced proficiency in the internalization of amyloid-β_{42} (Njie *et al.*, 2012). A clear correlation between microglia and plaque maintenance/clearance in Alzheimer's disease has been established (El Khoury *et al.*, 2007; Bolmont *et al.*, 2008; Floden and Combs, 2011). A study in the APP/PSEN1 mouse model of Alzheimer's disease showed that microglia adopt an M1-like phenotype that progresses with age and correlates with decreased CD11b expression. Over time, the reduced capacity to internalize amyloid-β_{42} suggests that aged microglia have a reduced innate ability to clear disease-relevant protein aggregations (Njie *et al.*, 2012).

Microglial senescence

In response to injury, microglia are thought to undergo a burst of proliferative activity and eventually become apoptotic in attempt to normalize cell numbers to pre-injury levels. Like all somatic cells, it is believed that microglia undergo 'replicative senescence', a phenomenon linked to telomere shortening (Flanary *et al.*, 2007). Cellular senescence is causally linked to the development of age-related pathologies, whereas proof-of-concept experiments suggest that selective removal of senescent cells can delay or prevent age-related tissue deterioration and dysfunction (Naylor *et al.*, 2013). Outside the CNS, senescent cells are rapidly removed from circulation via phagocytosis; however, as microglia are the innate phagocytes of the brain, there lacks a compensatory mechanism for their removal. This observation is supported by studies that show an age-dependent accumulation of microglia within the brain parenchyma. It is suggested that microglial senescence can contribute to the development of dystrophic microglia, which in turn contributes to the pathogenesis of neurodegenerative diseases such as Alzheimer's disease (Flanary, 2005). In a disease context, one might expect that any loss or decrease of phagocytic ability would result in the accumulation of disease related proteins, such as amyloid-β, apolipoprotein E (APOE), and α-synuclein. It has been hypothesized that microglia become compromised because of an onset of senescence, which hinders their homeostatic capability. Consequently, the ability of microglia to fulfil their role in the CNS has a marked effect in the brain parenchyma and may increase the susceptibility to neurodegenerative disorders, such as Alzheimer's disease, Parkinson's disease and multiple sclerosis.

Microglia, neurobiology, endogenous repair in the adult CNS and clinical applications

Microglia-synapse interaction in the adult brain

Since Kreutzberg popularized the facial axotomy model, it has been observed that microglia associate with injured nerve perikarya during synaptic stripping (Kreutzberg, 1966; Blinzinger and Kreutzberg, 1968). Although some have described a potential role for glia-derived ATP or astrocytes in synaptic stripping of motor neurons (Svensson and Aldskogius, 1993; Linda *et al.*, 2000; Yamada *et al.*, 2008), others favour a model involving the direct apposition of microglia with neuronal perikarya and apical dendrites (Blinzinger and Kreutzberg, 1968; Graeber *et al.*, 1993; Cullheim and Thams, 2007; Trapp *et al.*, 2007). Interestingly, the fate of particular synapse terminals seems to depend on the type of input, since Linda *et al.* (2000) found that GABA and glycine terminals were preferentially preserved after motor neuron axotomy, although both excitatory and inhibitory inputs were drastically reduced overall. This may support a transition from information processing towards a mode of survival and repair, protecting injured neurons from over-activity and the secondary effects of excitotoxic molecules, thus helping recovery from injury. Candidate molecular mechanistic targets for the stripping response include the CX3CR1 signalling axis and MHC class 1 molecules as reviewed in Cullheim and Thams (2007). Using two-photon imaging strategies in which microglia and neurons are fluorescently labeled, it has been shown that under basal conditions microglia survey their microenvironment for signals and clearance of debris using highly dynamic processes (Davalos *et al.*, 2005; Nimmerjahn *et al.*, 2005). Wake *et al.* (2009) demonstrated that basal microglia processes make brief and direct contact with synaptic terminals at a rate of approximately once per hour. The frequency of these homeostatic contacts is

activity-dependent and is reduced concomitantly with neuronal activity. In that study, ischaemic insult increased the duration of contact drastically, and was associated with a disappearance of the presynaptic bouton due to engulfment by microglia. Whether this activity is absolutely required for elements of synaptic homeostasis is still unknown, but the mechanisms of this activity are now becoming clearer.

It is conceivable that microglia could engage in crosstalk with neurons and release neurotrophins (Elkabes *et al.*, 1996; Kohsaka *et al.*, 1996; Morgan *et al.*, 2004) or other signalling factors that can regulate synaptic structure and transmission, thereby engaging in modulating synaptic homeostasis. Microglia have a myriad of receptors that can respond to secreted neuronal products and possess the ability to modulate neuronal functions, supporting a bidirectional model of communication (Kettenmann *et al.*, 2011). Brain-derived neurotrophic factor (BDNF) derived from microglia was shown to modulate the neuronal anion gradient underlying neuropathic pain, and directly regulate synaptic function (Coull *et al.*, 2005). Parkhurst *et al.* (2013) recently and definitively showed that microglia depletion in the otherwise healthy rodent brain induced deficiencies in multiple learning tasks and a specific reduction in motor-learning dependent synapse formation. This was largely regulated by microglial BDNF and reproduced in microglia-depleted rodents. In the context of Alzheimer's disease, it was shown that amyloid-β stimulation of NOS2 and reactive oxygen species production in microglia inhibited NMDA-receptor dependent long-term potentiation and provides further evidence for a diverse array of neuronal and synaptic activities that can be modulated by microglia (Wang *et al.*, 2004). Furthermore, the neuroimmune interface with classical neurobiological activities is not reserved for microglia alone, as they have been shown to regulate hippocampal neurogenesis and maintain spatial learning abilities in adulthood through interactions with T cells (Ziv *et al.*, 2006).

Neuronal repair in the adult CNS: the central role of microglia

In the adult CNS, microglia are attractive candidate therapeutic cellular targets for augmenting brain repair. In theory, this involves investigating a means to reduce microglia-induced damage while also boosting pro-neurogenic and/or oligodendrogenic mechanisms that are known to exist in adult microglia. Given the several known mechanisms by which microglia are directly involved in promoting neuroinflammatory damage, we will not detail these mechanisms in this review; for reviews, see (Barres, 2008; Perry *et al.*, 2010; Saijo and Glass, 2011; Rawji and Yong, 2012; Xanthos and Sandkuhler, 2014).

In vitro, LPS-activated microglia blocks neurogenesis in organotypic hippocampal slice cultures; microglia activated by IL4 and IFNγ stimulate neurogenesis. Butovsky *et al.* attributed the blocking activity of LPS-activated microglia to TNF, whereas the augmenting effect of IL4 and IFNγ was attributed to insulin-like growth factor 1 (IGF1) (Butovsky *et al.*, 2005). In a rat model, microglia were found to be pro-neurogenic to striatal neuroblasts derived from the ipsilateral subventricular zone following ischaemia (Thored *et al.*, 2009). Using allogeneic co-cultures of human microglia and neural progenitor cells, Liu *et al.* (2013) showed that co-cultures had higher survival and proliferation rates of both neural progenitor cells and microglia compared to monocultures. Despite evidence that microglia have pro-neurogenic activity (Aarum *et al.*, 2003; Battista *et al.*, 2006), some groups propose that microglia also possess anti-neurogenic activities (Ekdahl *et al.*, 2003; Monje *et al.*, 2003; Goings *et al.*, 2006), highlighting the dual role of microglia during adult neurogenesis, reviewed in Ekdahl *et al.* (2009).

Oligodendrocyte precursor cell differentiation can also be influenced by microglia, making microglia attractive targets for augmenting repair in the context of demyelinating diseases. *In vitro*, Butovsky *et al.* (2005) demonstrated the differential effects of IL4 and IFNγ treated microglia towards augmenting oligodendrogenesis. In a follow-up study, elevated IFNγ conferred a phenotype that blocked oligodendrogenesis with an associated increase in TNF and decrease in IGF1. This phenotype could be reversed by IL4, whereby IL4 activated microglia injected into the CSF of EAE mice improved clinical symptoms and increased oligodendrogenesis in the spinal cord (Butovsky *et al.*, 2006). In a human oligodendrocyte precursor cell model, supernatants derived from LPS and IFNγ-stimulated human microglia (M1) significantly decreased oligodendrocyte precursor cell differentiation; IL4 and IL13 simulated microglia (i.e. M2a) did not exhibit an effect (Moore *et al.*, 2015). Another line of evidence that activated microglia influence neural progenitor cell and oligodendrocyte precursor cell differentiation includes ischaemia studies where activated microglia secretory products influenced neurosphere differentiation with a bias towards oligodendrocytes and neurons (Deierborg *et al.*, 2010). Miron *et al.* (2013) note that M2 cells drive oligodendrocyte differentiation during CNS remyelination in a focal demyelination model. In human gliomas, Sarkar *et al.* (2014) found that brain-tumour initiating cells sphere-forming capacity was markedly reduced by microglia and macrophages derived from non-glioma human subjects and involved the induction of cell cycle arrest and differentiation genes. The authors demonstrated that low concentrations of amphotericin B increased glioma-derived microglial production of IL8 and CCL2 (previously known as MCP-1), which curbed autologous brain-tumour initiating cell growth (Sarkar *et al.*, 2014).

Another interesting prospect is the modulation of phagocytotic properties in microglia, which are often beneficial in models of disease and injury. The surface receptor triggering receptor expressed on myeloid cells-2 (TREM2) is expressed by microglia and often associated with an anti-inflammatory phenotype (Colonna, 2003;

Klesney-Tait *et al.*, 2006). Upon treatment with LPS and IFNγ, TREM2 expression is decreased (Schmid *et al.*, 2002). When overexpressed in microglia, TREM2 increased the capacity to phagocytose apoptotic cell membranes; decreased TREM2 impaired clearance of apoptotic neurons, and increased TNF and NOS2 (Takahashi *et al.*, 2005). In EAE, TREM2 transduced myeloid cells ameliorated disease course by increasing phagocytic activity and inducing an anti-inflammatory cytokine milieu (Takahashi *et al.*, 2007). In humans, mutations in TREM2 and its adaptor DAP-12 (now known as TYROBP) lead to abnormalities in TREM2 function, and are associated with Nasu-Hakola disease, which is characterized by frontal lobe dementia and bone cysts (Paloneva *et al.*, 2002). A rare variant of TREM2 has also been demonstrated to increase susceptibility to late onset Alzheimer's disease (Jones, 2013). By contrast, in Alzheimer's disease brains, the expression pattern of TREM2 is partially associated with amyloid deposition (Jiang *et al.*, 2013); the mRNA for TYROBP is augmented in tissue plaques (Frank *et al.*, 2008). In the CSF of patients with multiple sclerosis, increased soluble forms of TREM2 have been also observed (Piccio *et al.*, 2008).

Similar to TREM2, SIRP-1α (previously known as CD172a) is expressed by microglia and involved in phagocytosis. The interaction between SIRP-1α and its ligand CD47, which is expressed by neurons and astrocytes, inhibits phagocytosis and delivers a 'do not eat me signal' to microglia (van Beek *et al.*, 2005; Biber *et al.*, 2007). This interaction has clinical relevance and has been implicated in multiple sclerosis pathophysiology. Indeed, the upregulation of several microRNAs, including miR-34a, miR-155 and miR-326 within multiple sclerosis lesions, has been suggested to decrease CD47 expression on astrocytes, hence promoting phagocytosis by microglia (Junker *et al.*, 2009). Together, these studies highlight that in the adult brain, microglia can regulate oligodendrocyte precursor cell and neural progenitor cell proliferation and differentiation, a characteristic that can be harnessed for therapeutic application.

Quantifying microglia *in vivo* using PET imaging

PET is a molecular imaging technique able to quantify a molecular target *in vivo*. PET imaging requires the availability of a radiolabelled ligand (radioligand) to bind the target in question with only minimal non-specific binding. The vast majority of PET radioligands used in neuroscience research target neurotransmitter receptors, particularly dopamine and 5-HT receptors. Currently, the 18 kDa translocator protein (TSPO) is the only microglial target for which radioligands are available for clinical use. TSPO is richly expressed in microglia and localised mainly to the outer mitochondrial membrane. Various *in vitro* studies using post mortem human brain tissue have shown increased TSPO density (measured by specific binding of the TSPO ligand; Banati *et al.*, 2000) in diseases characterized by microglial infiltration. These include studies in multiple sclerosis (Banati *et al.*, 2000), Alzheimer's disease (Diorio *et al.*, 1991; Venneti *et al.*, 2008; Gulyas *et al.*, 2009), Huntington's disease (Messmer and Reynolds, 1998), stroke (Venneti *et al.*, 2008), frontotemporal dementia (Venneti *et al.*, 2008), and ALS (Sitte *et al.*, 2001; Venneti *et al.*, 2008).

The limitations of using PET imaging to quantify microglia density

Although the predominant source of TSPO expression in the brain is thought to represent microglia, TSPO is expressed in other myeloid cells, including macrophages. For diseases involving blood–brain barrier disruption and/or monocyte recruitment, TSPO imaging is unable to extract the microglial component of the myeloid signal. Second, astrocytes can also express TSPO under certain conditions in culture (Itzhak *et al.*, 1993) and in animal models (Maeda *et al.*, 2007; Ji *et al.*, 2008). Nevertheless, a substantial autoradiographical literature demonstrates that TSPO expression follows the spatiotemporal pattern of microglial activation (Dubois *et al.*, 1988; Myers *et al.*, 1991; Shah *et al.*, 1994; Stephenson *et al.*, 1995; Conway *et al.*, 1998; Banati *et al.*, 2000; Raghavendra Rao *et al.*, 2000). Hence, it is generally accepted that myeloid cells are responsible for the majority of TSPO expression in CNS diseases.

Currently, the mechanisms responsible for increased TSPO signal are not well understood. It is unclear whether the increase in TSPO signal results from increased cell and/or mitochondrial number, or an actual increase in protein expression within mitochondria. Although *in vitro* experiments in Leydig cells (Rey *et al.*, 2000) and pancreatic islet cells (Trincavelli *et al.*, 2002) show cellular increases in *TSPO* mRNA in response to activation by cytokines, no data is available for microglia. Furthermore, the relationship between microglial phenotype and TSPO expression is poorly understood. It is unknown whether PET signal reflects an increase in cell number or a change in cell phenotype.

Clinical TSPO ligands

^{11}C-PK11195 was first used in humans in 1986 (Charbonneau *et al.*, 1986) and until recently, was the only available TSPO radioligand. Unfortunately, quantification of the ^{11}C-PK11195 signal is complicated by various technical factors, including its high non-specific binding and its adherence to plastic and glass, preventing accurate plasma measurements that are required for signal modelling. In recent years there has been considerable interest in developing new generation TSPO radioligands to overcome these problems.

Hundreds of potential new generation tracers have been synthesized (Chauveau *et al.*, 2008), however, only a few have passed the physiochemical hurdles required to reach clinical evaluation. Early clinical studies with second generation ligands demonstrated the technical improvements over ^{11}C-PK11195, but were compromised by the existence of a polymorphism in the coding region of the gene encoding TSPO, which dramatically alters binding affinity (Owen *et al.*, 2010, 2012). This polymorphism, rs6971, causes an amino acid substitution (Ala147Thr) that reduced the binding affinity of second generation ligands to TSPO in subjects expressing 147Thr (either as a homozygote or a heterozygote) relative to Ala-Ala wild-types. The specific PET signal is determined by both target expression and binding affinity of the radioligand for the target. Therefore, for a given level of TSPO expression, Thr-Thr and Ala-Thr subjects will have a misleadingly low PET signal relative to wild-types. Alanine 147 is highly conserved across species; however, the mutation is only found in humans and was not detected in preclinical development. Current studies now involve genotyping at rs6971 and either enrol Ala-Ala subjects only, or enrol all subjects but correct the data for genotype. Third generation radioligands are now being developed, which have low sensitivity to the polymorphism (Brouwer *et al.*, 2014).

The future directions of PET ligands and CNS research

With the variability caused by the rs6971 polymorphism now understood, PET studies with second generation ligands are in a position to start delivering valuable insights into mechanisms of CNS disease *in vivo*. Ongoing *in vitro* studies into the relationship between microglial phenotype and binding of TSPO ligands will facilitate our understanding of the molecular mechanisms underlying the change in PET signal. In the future, more specific ligands targeting TSPO in the myeloid cell population are likely to become available. New cell surface targets identified in recent studies that distinguish microglia from macrophages (Butovsky *et al.*, 2014) should be amenable to PET and offer the potential to dissect the microglial and macrophage component of the overall myeloid cell signal. Finally, it is likely that ligands will be available to identify specific myeloid cell phenotypes, and will be invaluable in investigating the immunomodulatory effect of novel pharmaceuticals. New generation PET scanners are currently being constructed with much larger gantries. This will markedly improve the sensitivity of the technique, allowing for administering lower doses of radioligand, and more scans in the same patient before reaching radiation safety limits.

Conclusions

This comprehensive review summarizes and emphasizes the importance of microglia in overall brain function. Microglia are dynamic surveyors of their milieu, informing and responding to neurobiological functions intrinsic to CNS development, homeostasis, ageing, and injury. Exciting new pharmacological agents targeting not only the deleterious functions of microglia, but also mechanisms which promote endogenous repair will likely soon be a reality. Furthermore, the development of novel PET ligands for microglia will provide insight into *in vivo* biology of human microglia, providing further correlates to animal data. Overall, future studies will facilitate clinical advancement and interrogation of the microglial role in many aspects of nervous system function.

Supplementary material

Supplementary material is available at *Brain* online.

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