Microglia and macrophages in brain homeostasis and disease

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Abstract | Microglia and non-parenchymal macrophages in the brain are mononuclear phagocytes that are increasingly recognized to be essential players in the development, homeostasis and diseases of the central nervous system. With the availability of new genetic, molecular and pharmacological tools, considerable advances have been made towards our understanding of the embryonic origins, developmental programmes and functions of these cells. These exciting discoveries, some of which are still controversial, also raise many new questions, which makes brain macrophage biology a fast-growing field at the intersection of neuroscience and immunology. Here, we review the current knowledge of how and where brain macrophages are generated, with a focus on parenchymal microglia. We also discuss their normal functions during development and homeostasis, the disturbance of which may lead to various neurodegenerative and neuropsychiatric diseases.

It has been just over a century since, in 1913, Santiago Ramón y Cajal described oligodendrocytes and microglia together as the 'third element' of the central nervous system (CNS)[1]. A couple of years later, Pío Del Río-Hortega, who once worked with Cajal, phenotypically characterized and officially named the only immune cells in the brain parenchyma, which are now known as microglia. Microglia were initially thought to originate from the neuroectoderm, but this view was later superseded by the idea of a blood monocytic origin, a concept that has dominated for the past three decades[2]. Only recently has it been convincingly shown that microglia arise from embryonic yolk sac (YS) precursors, which also give rise to macrophages in other tissues[3–5]. Microglia maintain their CNS population by self-renewal, with little contribution from blood cells[5–8]. Thus, microglia and most tissue macrophages arise from lineages that are developmentally and functionally distinct from blood-associated myeloid cells, such as monocytes. These advances in our knowledge of microglial ontogeny raise interesting new questions about their developmental mechanisms, which we are only just beginning to answer.

In addition, owing to the misconception that the brain is an immune-privileged site, microglia have long been erroneously considered to be static bystanders in the healthy CNS that have minimal homeostatic functions besides phagocytic scavenging and immune surveillance. With the advent of modern real-time imaging tools, microglia have been found to be more active than once thought, constantly monitoring brain activity by extending and retracting their highly ramified processes[9–11]. It is now becoming increasingly clear that microglia interact with essentially all CNS components and have a marked impact on normal brain functioning and maintenance of tissue integrity[12,13].

During development, the mouse YS myeloid precursors travel to the brain at approximately embryonic day 9.5 (E9.5) before differentiating into immature microglia, which display amoeboid morphology and have distinct molecular and functional properties[5,14]. This occurs concurrently with the birth of neurons and before the generation of astrocytes and oligodendrocytes, which provides microglia with a unique opportunity to 'witness' and participate in numerous developmental events in the CNS, such as neurogenesis[15,16], programmed cell death[17–19], synapse elimination[20,21] and the establishment and remodelling of neural circuits[12,16,22–26]. In addition, microglia quickly adapt to their environment and modify their functions with a broad spectrum of activation states[27,28]. In light of these discoveries, it is perhaps not surprising that microglial activation and dysfunction are increasingly implicated in almost all diseases and injuries of the CNS[29–32].

Adding an extra layer of complexity, there are three other types of CNS macrophage — namely, perivascular, meningeal and choroid plexus macrophages — that are localized at the interface between the parenchyma and the circulation[33]. Perivascular and meningeal macrophages are also generated from YS precursors, whereas choroid plexus macrophages have dual embryonic and adult haematopoietic origins[33,34]. Together with other

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blood-borne myeloid cells that infiltrate the brain under certain conditions, these CNS macrophages constitute an intricate network of heterogeneous cell populations, the study of which has the potential to lead to novel therapies for brain diseases[33,35]. Here, we review recent advances in the research regarding microglial and brain macrophage ontogeny, developmental programmes and functions in health and disease. We discuss some controversial views and remaining questions.

Microglia and brain macrophages

Myeloid cells in the brain. Microglia constitute 5–10% of total brain cells and are the only true CNS parenchymal macrophages[28]. Microglia and perivascular, meningeal and choroid plexus macrophages each occupy a strategic niche, thereby covering the entire CNS[33,34] (FIG. 1). These macrophages adopt different shapes and sizes, and they express a combination of overlapping and unique markers[33]. Their functions are still poorly understood. The CNS also contains an extensive vasculature network, in which circulating myeloid cells, such as monocytes, granulocytes and dendritic cells, reside. There are at least two subtypes of monocytes in mice: Ly6C^{hi} classical inflammatory monocytes (CD14^{hi}CD16^{-} in humans) and Ly6C^{low} non-classical patrolling monocytes (CD14^{+}CD16^{hi} in humans)[35]. Ly6C^{low} monocytes are derived from Ly6C^{hi} monocytes in either the blood or the bone marrow (BM)[36]. During certain diseases or injuries involving breach of the blood–brain barrier (BBB), Ly6C^{hi} monocytes may infiltrate the brain parenchyma and differentiate into microglia-like cells, which are intermingled with the resident microglia, to exacerbate or alleviate disease progression[29,35].

Recent comparative transcriptomic studies have generated useful resources for identifying the molecular signatures of many types of tissue-resident macrophage and blood-borne myeloid cell, including microglia[30,37–41]. These studies led to the discovery of specific bona fide microglial markers, such as transmembrane protein 119 (TMEM119)[41,42]. These new microglia-specific markers should allow for the generation of novel and much-needed tools for observing and manipulating microglial functions without affecting other cell types. However, an important caveat is that the majority of these studies have primarily used bulk RNA sequencing (RNA-seq) of cells isolated acutely based on known markers; it is possible that some subtypes of microglia were missed, in particular those that are present transiently during development, when distinguishable markers are more limited. For example, recent studies show that microglia and macrophages isolated from different regions of the brain have age-dependent and region-dependent differences in transcriptomic profiles[43]. In addition, region-specific cues that establish distinct microglial phenotypes may even be restricted to the level of individual nuclei in an anatomical structure such as the basal ganglia[44]. It is worth mentioning that such transcriptomic heterogeneity has not been documented in single-cell RNA-seq analysis[45,46]. It is unclear whether the current single-cell RNA-seq technology is sensitive enough to detect subtle differences (if they are present) among cells of the same type. Independent confirmation of the reported region-dependent microglial heterogeneity could also help to reconcile some of these discrepancies.

Figure 1 | **The microenvironments and genetic signatures for brain macrophages.** Microglia are the only macrophage population in the central nervous system (CNS) parenchyma, where they can interact with neurons, astrocytes and oligodendrocytes (not shown). They express transmembrane protein 119 (TMEM119), P2Y purinoceptor 12 (P2RY12) and Sal-like protein 1 (SALL1) as specific markers[38,41,77]. Microglia are also distinguished from non-parenchymal macrophages (that is, meningeal, perivascular and choroid plexus macrophages) by low levels of expression of CD45 and MHC class II molecules, although this means of identification is less precise than using specific markers. Non-parenchymal macrophages are less well defined, as many markers have overlapping expression patterns[33,34]. Only choroid plexus macrophage populations receive input from the circulation, and all other brain macrophages, including microglia, maintain their populations by self-renewal under normal conditions. Each brain macrophage population is localized in a specialized niche, which may be instructive for their gene expression and functional properties. Tight junctions between endothelial cells and choroid plexus epithelial cells are primary features of the blood–brain barrier and blood–cerebrospinal-fluid barrier, respectively. These may have a role in confining the territories of different macrophage populations. Inflammatory monocytes (Ly6C^{hi}), which give rise to patrolling monocytes (Ly6C^{low}), may enter the brain parenchyma upon disease or injury. CSF, cerebrospinal fluid; CX_3CR1, CX_3C-chemokine receptor 1.

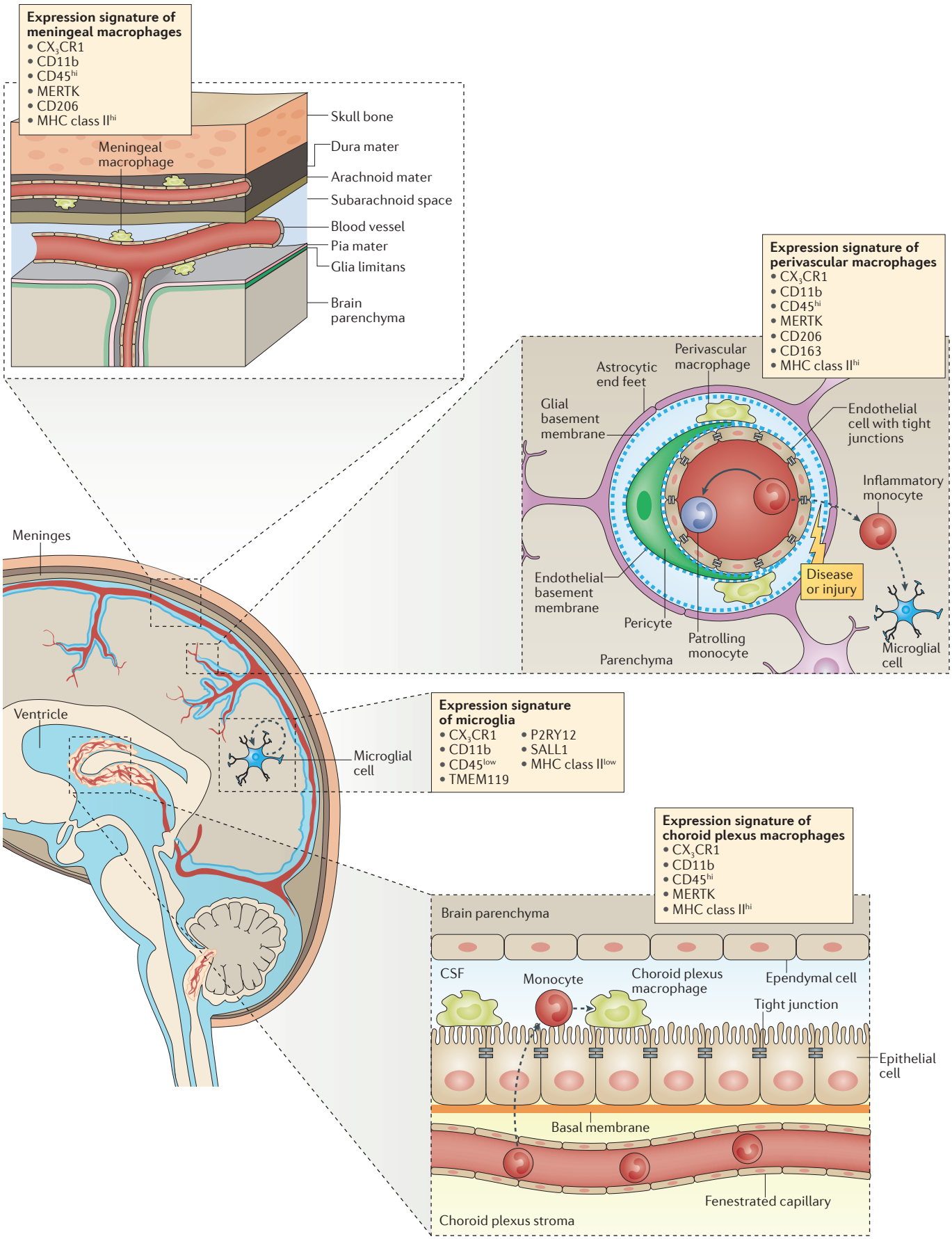
Microglial and brain macrophage ontogeny. In the past few years, a series of lineage-tracing experiments[5,7,8,36,47–49], together with parabiosis experiments[6,7] and neonatal BM transplantations[5,7], have definitively shown the YS ontogeny of microglia[3]. The current consensus view is that microglia are generated from YS erythromyeloid precursors (EMPs), which give rise to YS macrophages — the immediate migratory precursors that colonize the embryonic brain[14,48]. However, the precise nature and time of emergence of these EMPs are still under debate[3,50,51] (FIG. 2).

In rodents, there are at least three waves of haematopoiesis that partially overlap in terms of timing and the tissues involved, which is one reason why microglial and CNS macrophage ontogeny has always been challenging to determine[52]. The first wave starts in the YS blood islands (posterior plate mesoderm) at E7.0, and it generates primitive progenitor cells at approximately E7.5. These progenitors differentiate into nucleated erythrocytes and YS macrophages without passing through monocytic intermediates, hence the name 'primitive' haematopoiesis[3,51]. According to one model (Model 1), it is proposed that these YS primitive progenitors are by nature primary EMPs and that their descendants, the YS macrophages, colonize the entire embryo including the brain to generate all types of tissue-resident macrophage[3,49] (FIG. 2). These populations are commonly referred to as YS-derived tissue macrophages, which are F4/80^{hi}CD11b^{low} (in contrast to F4/80^{low}CD11b^{hi} monocyte-derived cells; see below)[47,49]. The primary

Blood–brain barrier
(BBB). A physiological barrier between blood vessels and brain parenchyma. It is formed by specialized tight junctions between endothelial cells of the blood vessel wall, which is surrounded by a basement membrane and an additional membrane formed from astrocytic end feet, known as the glial basement membrane (glia limitans).

Basal ganglia
A group of interconnected nuclei (clusters of neurons) that lie deep beneath the cerebral cortex. They are responsible for modulating motor control, planning actions and executing habitual behaviours, as well as influencing cognition and emotion.

Parabiosis
An experimental model system in which two animals (most often mice) are surgically joined to establish a common circulation.



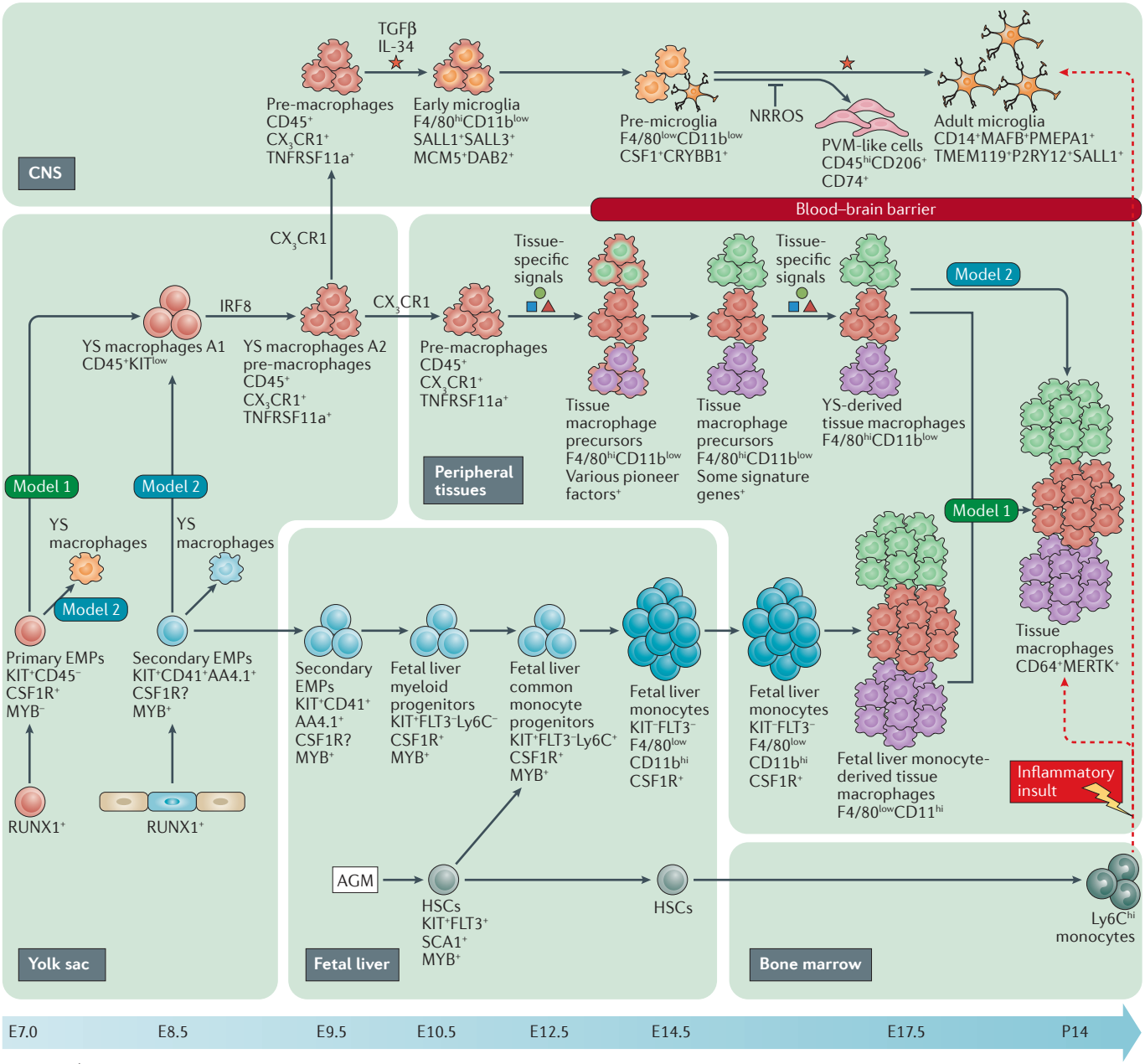


Figure 2 | **Ontogeny and development of microglia and tissue macrophages.** Developmental trajectories of microglia and tissue macrophages are shown in the context of three waves of haematopoiesis. The cellular identities of each intermediate step are defined by surface marker expression and gene signature. A1 and A2 yolk sac (YS) macrophages were defined by Kierdorf *et al.*[14]. Early microglia, pre-microglia and adult microglia were defined by Matcovitch-Natan *et al.*[45]. Highlighted are two models that differ in terms of the precise origins of microglia and other tissue macrophages, as well as the relative contributions of YS macrophages and fetal liver (FL) monocytes to peripheral tissue-resident macrophages. Some key proteins controlling cell fate progression or cell migration are indicated above the arrows. Coloured symbols on some arrows refer to important tissue-specific signals for the differentiation and maturation of a given macrophage population. The brain-derived signals for microglial development are unknown (red star). Between embryonic day 10.5 (E10.5) and E12.5, tissue macrophages start to show differential expression of some genes that persist into the adult stage[82]. Because they have not turned on the full gene signature, these cells are labelled with gradient colours. Expression of colony-stimulating factor 1 receptor (CSF1R) by secondary erythromyeloid precursors (EMPs) is controversial (indicated by a question mark). Only relevant myeloid lineages are shown, and non-parenchymal macrophages are not shown due to the lack of knowledge about their origins. The steps that occur before the aorta, gonads and mesonephros (AGM) region for definitive haematopoiesis are not shown. CNS, central nervous system; CX_3CR1, CX_3C-chemokine receptor 1; HSC, haematopoietic stem cell; IRF8, interferon regulatory factor 8; NRROS, negative regulator of reactive oxygen species; P2RY12, P2Y purinoceptor 12; PVM, perivascular macrophage; RUNX1, runt-related transcription factor 1; SALL1, Sal-like protein 1; TGFβ, transforming growth factor β; TMEM119, transmembrane protein 119; TNFRSF11a, tumour necrosis factor receptor superfamily member 11a.

EMPs express macrophage colony-stimulating factor 1 receptor (CSF1R) and require signalling through this receptor for their survival and differentiation; they are independent of the transcriptional activator MYB[5,49]. At E8.25, the second 'transient definitive' wave of haematopoiesis is initiated in the haemogenic endothelium of

the YS, which generates 'late' or secondary EMPs. The secondary EMPs do not express CSF1R but are MYB dependent[49], which indicates that they have different molecular properties and/or differentiation potential compared with the primary EMPs. According to this model, these secondary EMPs give rise to some macrophages that never exit the YS, and, more importantly, they migrate to and emerge in the fetal liver (FL) at E9.5, where they upregulate CSF1R expression and become myeloid progenitors that have restricted developmental potential[3,49]. Myeloid progenitors further differentiate into F4/80^{low}CD11b^{hi} FL monocytes that have high proliferative capability at E12.5. At approximately E14.5, large numbers of FL monocytes invade all tissues except the brain, where the BBB that has begun to form at approximately E13.5 presumably blocks their entry[49]. These FL monocytes develop into tissue macrophages, diluting the original YS-derived macrophages, and the two populations cannot be distinguished by the adult stage. Overlapping with the second wave of haematopoiesis, at E8.5, the third 'definitive' wave of haematopoiesis starts in the embryo proper, generating immature haematopoietic stem cells (HSCs) from the haemogenic endothelium in the para-aortic splanchnopleura region. At E10.5, this region develops into the aorta, gonads and mesonephros region, from which fetal HSCs start to migrate to the liver, where they join the production of FL monocytes by the secondary EMPs[3,49]. HSCs also differentiate into FL monocytes, thereby at least partially contributing to some tissue macrophage populations[3,8]. Therefore, according to this model, microglia are solely generated from primary EMPs, whereas primary EMPs, secondary EMP-derived FL monocytes and HSC-derived FL monocytes disproportionally contribute to all other tissue macrophages.

In contrast to the proposed sequential generation of heterogeneous EMP subsets[8,49], a different model (Model 2) postulates that a single homogeneous population of EMPs originates in the YS, which corresponds to the secondary EMPs in Model 1 (REFS 48,50) (FIG. 2). These EMPs are thought to be KIT^{+}CD41^{+}CD45^{low}AA4.1^{+} and give rise to essentially all tissue macrophages, including microglia[48,51]. Based on this model, the first wave of haematopoiesis only generates YS-resident macrophages, and FL monocytes derived from either EMPs or HSCs only minimally contribute to tissue-resident macrophages[3]. In addition, these EMPs are thought to express MYB but do not require it for myeloid differentiation[51]. Both Model 1 and Model 2 are consistent with EMPs being the sole origin of microglia and EMP-derived YS macrophages being the immediate precursors that penetrate the CNS during development, but the models differ in the precise nature and timing of EMP emergence in the context of the three waves of haematopoiesis. These discrepancies could be due to our still-poor understanding of the accurate molecular identities of these early haematopoietic precursors. Most fate-mapping studies have relied on the use of a few known surface markers, which may be promiscuous. Interpretation of these studies has been further complicated by the variable labelling efficiency of each reagent and the extraordinary cellular heterogeneity and developmental asynchrony in overlapping tissues, as well as unpredictable delays or residual effects due to tamoxifen-mediated recombination in the face of rapidly changing developmental processes. Future studies will probably benefit from unbiased single-cell transcriptomic analysis of all relevant cell populations to better define their identities and aid in designing more-precise tools.

Recent fate-mapping studies of the origin of non-parenchymal macrophages also challenged the dogma regarding the ontogeny of these cells, which have long been thought to be constantly replenished by blood-derived monocytes. Using the *Cx3cr1*–CreER mouse line (in which expression of Cre recombinase fused to an oestrogen receptor (ER) ligand-binding domain is under the control of endogenous *Cx3cr1* promoter and enhancer elements) for lineage tracing, it was found that perivascular and meningeal macrophages are maintained by self-renewal and are predominantly generated by embryonic haematopoietic precursor cells[34]. By contrast, choroid plexus macrophages were shown to have dual origins from both embryonic myeloid precursors and adult HSCs. This result was confirmed by parabiosis experiments and is also consistent with the observation that only choroid plexus macrophages depend on CC-chemokine receptor 2 for maintenance[33,34]. However, this work did not address the nature of the embryonic precursor cells that generate non-parenchymal macrophages. Considering that the onset of BBB closure at approximately E13.5 might be a mechanism that prevents FL monocytes from entering the microglial pool, it would be interesting to see if non-parenchymal macrophages also arise from a single origin by a similar gating strategy.

Importance of ontogeny. Normally, microglia arise only from EMP-derived YS macrophages. However, other sources, such as FL monocytes or HSC-derived monocytes, can also contribute to the microglial pool under certain conditions, which raises the question of whether ontogeny transmits any intrinsic properties of functional relevance to the differentiated cells. BBB formation coincides with the timing of broad FL monocyte infiltration[53] and it has therefore been speculated to be the main mechanism for blocking FL monocyte accessibility to the embryonic brain[2,49]. Direct evidence for this possibility, however, is lacking. Intriguingly, in the perinatal stage (in particular, at approximately postnatal day 3), myeloid cells of FL origin infiltrate the brain parenchyma and they coexist with the YS-derived cells for approximately 3 days before undergoing complete apoptosis[54]. The relevance of the brief presence of these cells is unknown. Furthermore, when YS macrophages were eliminated by a CSF1R-specific monoclonal antibody at E6.5, a new F4/80^{low}CD11b^{hi} population appeared later in the fetal brain, suggesting that FL monocyte colonization is possible in the absence of YS-derived cells[49] (a caveat is that a toxic effect on the BBB by the CSF1R-specific antibody cannot be ruled out). Similarly, in PU.1-mutant mice that lack microglia and other myeloid-lineage cells, BM-derived cells can infiltrate the CNS and differentiate into ramified cells that are indistinguishable from

microglia, but these cells rarely engraft the brain in wild-type mice even after irradiation[5,6,55,56]. It is unknown how functionally similar these cells are to endogenous microglia. These studies are particularly interesting when we compare microglia with non-parenchymal macrophages, for which the engraftment rates in the brains of wild-type mice were as high as 80%[34]. It is interesting to consider whether this is caused by differences in the sensitivity of brain macrophages to irradiation-induced injuries and what determines the preferences for BM cell colonization.

These findings, together with studies of other tissue-resident macrophages, suggest a niche model that might explain the variations of macrophage ontogeny across tissue types in both steady-state and disease settings[57,58]. For a given situation, the final contribution of possible microglia and macrophage sources to CNS myeloid cells is determined by following a 'niche accessibility–niche availability–precursor competitiveness' decision tree[57]. Each cell source is intrinsically competent to carry out tissue-dependent functions, and phenotypic differences are mainly due to the history of the cells and the existing environmental cues. This model emphasizes the developmental and functional plasticity of macrophages and monocytes, and it trivializes ontogeny as a possible deterministic factor for functions. Future work should address how shifts in microglial ontogeny affect microglial functions at the transcriptomic and epigenetic levels in both the short term and the long term.

Microglial and CNS macrophage development

Microglial precursor migration and CNS colonization. Originating in the YS, microglial progenitors (that is, EMPs) differentiate into YS macrophages before migrating to the embryonic tissues, including to the brain. However, it has not been clear how these precursors disperse throughout the embryo and colonize the brain parenchyma during development. As shown using *Cx3cr1*^{GFP} mice (which express green fluorescent protein (GFP) under the control of the endogenous *Cx3cr1* locus), microglial precursors first emerge in the cephalic mesenchyme at E9.0 and then penetrate the neuroepithelium starting at E9.5 (REFS 5,14). Two indirect observations suggest that the blood circulation is responsible for the long-range transport of YS precursors to distal tissues such as the brain. First, the appearance of YS precursors in the embryo coincides with the establishment of the vasculature. Second, sodium–calcium exchanger 1 (NCX1)-deficient mice, which lack a normal heartbeat and blood circulation, have many fewer microglia in the embryonic brain[5]. As loss of blood circulation could limit nutrient distribution and affect the normal physiology and metabolism of the entire embryo, other possible downstream events could lead to defects in microglial differentiation and migration. Therefore, more-direct evidence is needed to validate this model. Indeed, a circulation-independent mechanism for microglial invasion from brain-surrounding tissues into the parenchyma has been shown by *in vivo* imaging of larval zebrafish, although it is unclear if this migratory behaviour is specific to particular species or developmental stages[59]. Interestingly, CX_3C-chemokine receptor 1 (CX_3CR1) is required for the proper dispersion of macrophage precursors throughout mouse embryos. In mice that lack CX_3CR1 expression, more precursor cells are stuck in the YS and FL instead of colonizing the brain and other tissues. However, this defect is only temporary during embryonic stages, which indicates that there must be other as yet unidentified signals that attract precursor infiltration of the CNS. Studies in zebrafish suggest that lysophosphatidylcholine[60] or nucleotides[61] released by apoptotic neurons might function as chemotactic signals to attract microglial precursors into the brain.

Once inside the brain parenchyma, microglial precursors receive instructive signals from the CNS environment that support their differentiation (see below). They initially have amoeboid morphology and become ramified at approximately E14.5 (REFS 14,62), when they are still highly proliferative, a state that lasts until the first postnatal week[41]. By postnatal day 14, microglia are fully mature and express adult-signature genes[38,41]. This population of cells maintains itself at constant and region-specific densities through tightly coupled local proliferation and apoptosis for nearly the entire lifespan of the animal[54]. It has been shown that microglial turnover rates vary across brain regions, with the olfactory bulb having one of the highest rates (an estimate of 8 months for full turnover in mice[63]), although how fast cortical microglia renew remains controversial[54,63,64]. A recent *in vivo* single-cell imaging study, which presumably offers more accurate assessments, suggests that microglia in the mouse neocortex are long-lived, with a median lifespan of more than 15 months[65]. Microglial self-renewal seems to occur in a stochastic manner without any regional hot spots, but this process shifts towards clonal expansion during pathology[63].

Early myeloid cell differentiation. Microglial differentiation starts as soon as the precursors emerge in the YS. Based on current knowledge of primitive haematopoiesis, primary EMPs (microglial progenitors according to Model 1; discussed above) are generated from the posterior plate mesoderm in the YS, which is independent from the lineages generated in the endothelial haemogenic region that give rise to secondary EMPs[51]. Normally, only the endothelial-to-haematopoietic transition during the generation of secondary EMPs requires runt-related transcription factor 1 (RUNX1), a subunit of the transcription factor core binding factor (CBF)[51,66]. This observation suggests that the emergence of primary EMPs does not depend on RUNX1. However, to the contrary, fate-mapping studies using *Runx1*-CreER mice pulsed with tamoxifen at E7.0–E7.25, during primary EMP generation, demonstrated robust labelling of microglia but not of other tissue macrophages, which suggests that microglia do arise from RUNX1-expressing cells[5]. These conflicting results are one of the reasons why the nature of microglial progenitors is still under debate. It is unknown whether RUNX1 is functionally required for the specification of microglial progenitors.

CSF1R signalling is essential for microglial development and maintenance[67,68]. During embryogenesis, YS macrophages depend on CSF1R signalling for their proliferation, differentiation and survival[5]. In CSF1R-mutant mice, YS macrophages are absent and the subsequent tissue colonization by macrophages fails to occur (see TABLE 1 for microglial phenotypes in other genetic mutants). Similarly, administration of a CSF1R-specific blocking antibody efficiently eliminates fetal macrophages, including developing microglia[49]. Consistent with a role for CSF1R ligands in cell fate specification, they are major components in all protocols for generating induced pluripotent stem cell (iPSC)-derived microglia[69–73] (BOX 1). CSF1R signalling may induce myeloid cell differentiation by activating the myeloid lineage-determining transcription factor PU.1 (REF. 74), which is broadly expressed by haematopoietic cells. PU.1-deficient mice lack microglia and all non-parenchymal macrophages, as well as other tissue macrophages and circulating monocytes[34,47] (TABLE 1). Interestingly, PU.1-mutant mice have reduced expression of CSF1R, CSF2R and CSF3R and consequently do not respond to the corresponding cytokines[75], which suggests that there is a feedback loop between CSF1R signalling and PU.1 regulation.

In addition, constant CSF1R signalling is required to maintain the microglial population in the adult CNS. For example, the CSF1R inhibitor PLX3397 could eliminate more than 90% of microglia when mice were treated for 7 days[76]. This treatment did not cause overt behavioural defects or inflammation, thereby offering a valuable tool for studying microglial functions. The pro-survival function of CSF1R signalling was also demonstrated by the loss of microglia in adult mice when *Csf1r* was specifically knocked out in microglia[77], and recently further demonstrated in a new serum-free *in vitro* culture system for purified microglia[78]. Consistently, mice that lack either CSF1 or IL-34, two partially redundant ligands for CSF1R[79,80], have decreased numbers of adult microglia[5] (TABLE 1).

Table 1 | **Variations in the number of brain macrophages in various mutant mice**

Protein deficiency	Microglia	Perivascular macrophagess	Choroid plexus macrophages	Meningeal macrophages	Refs
PU.1	↓↓		↓↓	↓↓	14,34,47
CSF1	↓				5
IL-34	↓				79,80
CSF1R	↓				5,156
IRF8	↓		↔	↓	14,34
TGFβ	↓↓				38
SALL1	↑				77
NRROS	↑				87
CX_3CR1	↔*↓‡				14,22,82
DAP12	↔§↓↓‖				14,157
TREM2	↔				152
MYB	↔		↔	↔	14,34,47
ID2	↔				14
BATF3	↔	↔	↔	↔	14,34
KLF4	↔				14
CCR1	↔¶				14
CCR2	↔	↔	↓	↔	14,34,56
CXCR3	↔¶				14
NR4A1		↔	↔	↔	34

All quantifications were carried out in the adult stage except for those for which specific stage and tissue information is marked. *Embryonic day 12.5 (E12.5)–E14. ‡E9.5–E10.5, postnatal day 8 (P8)–P28. §E14, P28. ‖10-month old in basal ganglia and spinal cord. ¶E14. Undefined phenotypes are blank. BATF3, basic leucine zipper transcriptional factor ATF-like 3; CCR1, CC-chemokine receptor 1; CSF1R, colony-stimulating factor 1 receptor; CX_3CR1, CX_3C-chemokine receptor 1; CXCR3, CXC-chemokine receptor 3; DAP12, DNAX activation protein 12; IRF8, interferon regulatory factor 8; KLF4, Krüppel-like factor 4; NR4A1, nuclear receptor subfamily 4 group A member 1; NRROS, negative regulator of reactive oxygen species; SALL1, Sal-like protein 1; TGFβ, transforming growth factor-β; TREM2, triggering receptor expressed on myeloid cells 2. The ↑, ↔ and ↓ symbols indicate that the size of a cell population is increased, unchanged or decreased, respectively. The number of symbols indicates the level of changes.

Box 1 | **Stem cell approaches to studying microglia-mediated human diseases**

It is of tremendous interest from a clinical perspective to study human microglia in various disease settings. However, research has been limited by the availability of human cells and lack of reliable ways to maintain them in a state that resembles the expression patterns and functions of endogenous microglia. Using human embryonic stem (ES) cells or induced pluripotent stem cells (iPSCs) to generate the desired population of differentiated cells *in vitro* has been successful in circumventing certain limitations, and cells derived from patients can be used to model human diseases. Previously, protocols to generate human ES cell-derived microglia-like cells relied on the isolation of so-called microglial precursor cells from intermediate precursors belonging to the neuroectodermal lineage[148]. Studies in zebrafish and birds show that the extra-embryonic yolk sac (YS) ontogeny of microglia is likely to be evolutionarily conserved[2]. In humans, YS haematopoiesis occurs at approximately day 19 after conception, which is earlier in relative time than in rodents[149]. These early precursor cells are erythromyeloid precursors (EMPs), which give rise to the YS macrophages that start to infiltrate the human brain at approximately 4.5 gestational weeks[150] (by comparison, mouse YS macrophages enter the brain parenchyma at day 9.5 within a 21-day gestational period). The advances in our understanding of microglial ontogeny and embryonic haematopoiesis have been informative in helping to design strategies for the *in vitro* differentiation of human ES cells or iPSCs into microglia-like cells via steps that mirror the trajectory of endogenous microglial development. At least five protocols have recently been published for this purpose[69–73] (see the table below for comparisons). These *in vitro*-differentiated cells recapitulate many of the functions that are normally carried out by microglia, such as phagocytosis, cytokine release and engraftment into the brain. They express subsets of microglia-specific genes and are closely related to fetal microglia at the transcriptomic level. Future investigations of brain-derived signals that instruct microglial maturation during development should help to improve the protocols in order to generate and culture patient-derived mature microglia for studying human diseases.

Protocol	Starting cells	Important intermediates	Major differentiation factors	Feeder layer or co-culture	Yield (range)*	Time (weeks)	Functional studies	Disease modelled	Advantages
1 (REF. 69)	Human ES cells and iPSCs	YS embryoid bodies (VE-cadherin$^+$KIT$^+$ CD41$^+$CD235$^+$)	IL-34 and CSF1	Murine embryonic fibroblast feeder cells for human ES cell and iPSC propagation	0.5–4	8	• Phagocytosis • Responsiveness to stimuli and endotoxin • 3D culture with neurons and astrocytes from the same iPSCs	Rett syndrome	Compatible with 3D culture
2 (REF. 70)	iPSCs	Haematopoietic progenitor-like cells (CD34$^+$ CD43$^+$)	GM-CSF, CSF1 and IL-3	OP9 murine stromal feeder layer; astrocyte co-culture	2–3	2–4	• Phagocytosis • Production of ROS and cytokines	Murine glioma	Also works for iPSCs
3 (REF. 71)	iPSCs	Haematopoietic progenitors (CD43$^+$)	CSF1, IL-34, TGFβ, CD200 and CX_3CL1	Rat hippocampal neurons during maturation	30–40	5	• Phagocytosis of synaptosomes, tau and β-amyloid • Responsiveness to ADP • Cytokine release	Acute injury in 3D culture with all neural cell types	High purity; high yield; commercially available intermediates; graftable to mouse brains
4 (REF. 72)	Human ES cells and iPSCs	Myeloid progenitors (CD14$^+$ CX_3CR1^+ CD45$^+$)	IL-34 and GM-CSF	No	2–3	8.5	• Phagocytosis • Cytokine release • Responsiveness to ADP with Ca^{2+} transients	NA	Highly pure; can start with fewer iPSCs (10^5)
5 (REF. 73)	iPSCs	Embryoid bodies and MYB-independent macrophages (CD14$^+$CD16^{low} CD163$^+$CD11b$^+$)	IL-34 and GM-CSF (optional)	iPSC-derived neurons	10–43	4	• Increased motility when co-cultured with neurons • Responsiveness to endotoxin • Cytokine release	NA	Genetic evidence for the defined intermediates; efficient and high yield

All protocols with the exception of protocol 2 use serum-free cell culture. CSF1, colony stimulating factor 1; CX_3CL1, CX_3C-chemokine ligand 1; CX_3CR1, CX_3C-chemokine receptor 1; GM-CSF, granulocyte–macrophage colony-stimulating factor; ROS, reactive oxygen species; TGFβ, transforming growth factor β. *Indicates the number of microglia-like cells generated per starting cell.

Of note, CSF1 is broadly expressed by neural cell types, whereas IL-34 is specifically produced by neurons, and the two cytokines are expressed in non-overlapping cortical layers and brain regions[67,81]. Remarkably, cerebellar neurons do not express IL-34 (REF. 67), and the cerebellum and brainstem are the only brain regions in which the number of microglia is not changed in IL-34-mutant animals[79,80].

Once the myeloid lineage is determined by expression of PU.1 and CSF1R, microglial progenitors start to

differentiate into YS macrophages. They first become $CD45^+KIT^{low}CX_3CR1^-$ intermediate precursors and then become the more mature $CD45^+KIT^-CX_3CR1^+$ migratory macrophages[14] (FIG. 2). The transcription factor interferon regulatory factor 8 (IRF8) is required for maturation of the migratory macrophage precursors, which undergo apoptosis in *Irf8*$^{-/-}$ mice, resulting in markedly reduced numbers of embryonic and adult microglia[14] (TABLE 1). Among non-parenchymal macrophages, IRF8 deficiency specifically reduces the number of meningeal macrophages[34]. The exact molecular functions of IRF8 during brain macrophage development remain unknown.

In a recent study, the end products of these early differentiation steps were referred to as 'pre-macrophages' in the YS, which are CX_3CR1^+ and express core genes that are common to all macrophages[82]. They disperse throughout the embryo and only acquire tissue-specific gene signatures after colonization. Remarkably, it was shown that microglia-specific genes such as *Sall1* and *Sall3* start to be expressed by the colonized precursor cells as early as E10.25 (REF. 82) (FIG. 2), which suggests that a rapid, environment-induced fate commitment occurs and that Sal-like protein 1 (SALL1) is a tissue-specific microglia-specifying factor (see below). A similar transcriptomic study further dissected microglial development from E10.5 onwards into three discrete steps, with abrupt shifts occurring at approximately E14 and postnatal day 14 (REFS 41,45). Further work should address the molecular mechanisms that drive these transitions.

Brain environment-triggered signals for differentiation. Microglial and macrophage tissue-specific identities are imprinted by environmental cues[39,40,82]. Transforming growth factor-β (TGFβ) was suggested to be a crucial brain-derived signal for microglial specification[38,40], although anti-inflammatory[77,83] and/or pro-survival functions[78] for this cytokine have also been proposed. Primary microglia cultured with CSF1 and TGFβ have moderate but significant increases in the expression of microglial signature genes compared with those cultured with CSF1 alone[38]. In addition, culture of peritoneal macrophages with TGFβ induces the transcription of some microglia-specific genes[40]. By contrast, microglia in *Tgfbr2*-conditional knockout mice seem to adopt an activated phenotype[77]. However, in these mice, the possibility of non-microglial cell expansion cannot be ruled out, and the long-term survival of microglia was not assessed in this study. A recent study showed that astrocyte-secreted TGFβ, cholesterol and CSF1 function together to strongly support microglial survival *in vitro*[78]. Consistent with this survival function, *Tgfb1*-mutant mice have normal YS precursors but lack endogenous microglia[38]. These functional modes of TGFβ-mediated signalling are not necessarily mutually exclusive. Nonetheless, as these recent studies showed, TGFβ is insufficient to reproduce the ability of the brain environment to induce full microglial differentiation[78,84]. One consequence of chronic exposure to CNS-derived TGFβ, though, is that compared with peripheral cells, microglia are less prone to undergo IRF7-mediated pro- to anti-inflammatory conversion upon injury[85]. Therefore, TGFβ-mediated signalling may intricately modulate microglial activation in pathological conditions[86].

The microglia-specific transcription factor SALL1 and the transmembrane protein negative regulator of reactive oxygen species (NRROS) were recently found to promote quiescent microglial identities in the CNS environment[77,87]. Microglia-specific knockout of *Sall1* leads to rapid loss of microglial signature genes with a concomitant upregulation of genes often associated with other tissue macrophages[77]. In addition, lipopolysaccharide (LPS) treatment downregulates SALL1 expression, which indicates that SALL1 may be an important master regulator for maintaining microglial identity in response to immune challenges. Interestingly, microglia in NRROS-mutant animals also lose expression of many microglial signature genes (such as *Tmem119*, *P2ry12* and *Sall1*) but express higher levels of *Cd45*, *Cd206* (also known as *Mrc1*) and *Cd74*, which are genes that are characteristically expressed by perivascular macrophages[87]. Cluster analysis of RNA-seq results also shows the resemblance of NRROS-deficient microglia to endogenous perivascular macrophages. This microglia-to-perivascular macrophage conversion, together with the downregulation of SALL1 expression, is manifested as early as E14.5, which suggests that NRROS controls the divergence of brain-resident macrophages around the time of BBB closure at least partially through SALL1. Remarkably, NRROS function is not required in the adult to maintain microglial identity, whereas SALL1 is required. This is possibly due to developmental stage-restricted gene regulation or to epigenetic memory at microglia-specific enhancers.

Epigenetic mechanisms regulating CNS macrophage identity. Transcriptional and epigenetic mechanisms form an intricate regulatory network to control microglial and macrophage identities and plasticity[88]. Not only do tissue macrophages express unique genes, but they also have tissue-specific chromatin modifications and enhancer landscapes, which are shaped by ontogeny and environmental cues[39,40]. Lineage-determining transcription factors, such as PU.1, establish the common myeloid enhancers, whilst incorporating incoming brain-derived signals to select subsets of microglia-restricted enhancers for activation[39,40]. Hypothetically, some pioneer factors induced by this activation cooperate with lineage-determining transcription factors to further generate enhancers that are unique to microglia, leading to microglia-specific gene expression. By analysing consensus binding sites around enhancers that are only active in microglia, it was predicted that myocyte-specific enhancer factor 2C (MEF2C) is a brain environment-triggered pioneer factor that may have a role in microglial specification[39]. Similar analyses based on the cooperative binding between PU.1 and candidate factors around

Epigenetic memory
Molecular mechanisms modifying DNA or chromosomal configuration without changing gene sequences that lead to stable changes in gene expression long after the disappearance of initial developmental or environmental signals.

microglia-specific enhancers predict that SMAD family member 3 (SMAD3) is an early master regulator of microglial fate[40].

Interestingly, enhancer landscapes in myeloid cells can be modified by newly introduced environmental cues, which are accompanied by changes in gene expression[40]. For example, microglia quickly lose their gene signatures in culture media that are supplemented with candidate brain-derived factors, but they can regain their typical gene expression profile once transferred back to the brain[78,84]. Identifying the brain-derived signal that induces full microglial differentiation is a major unsolved question in the field.

CNS macrophages in CNS physiology and disease

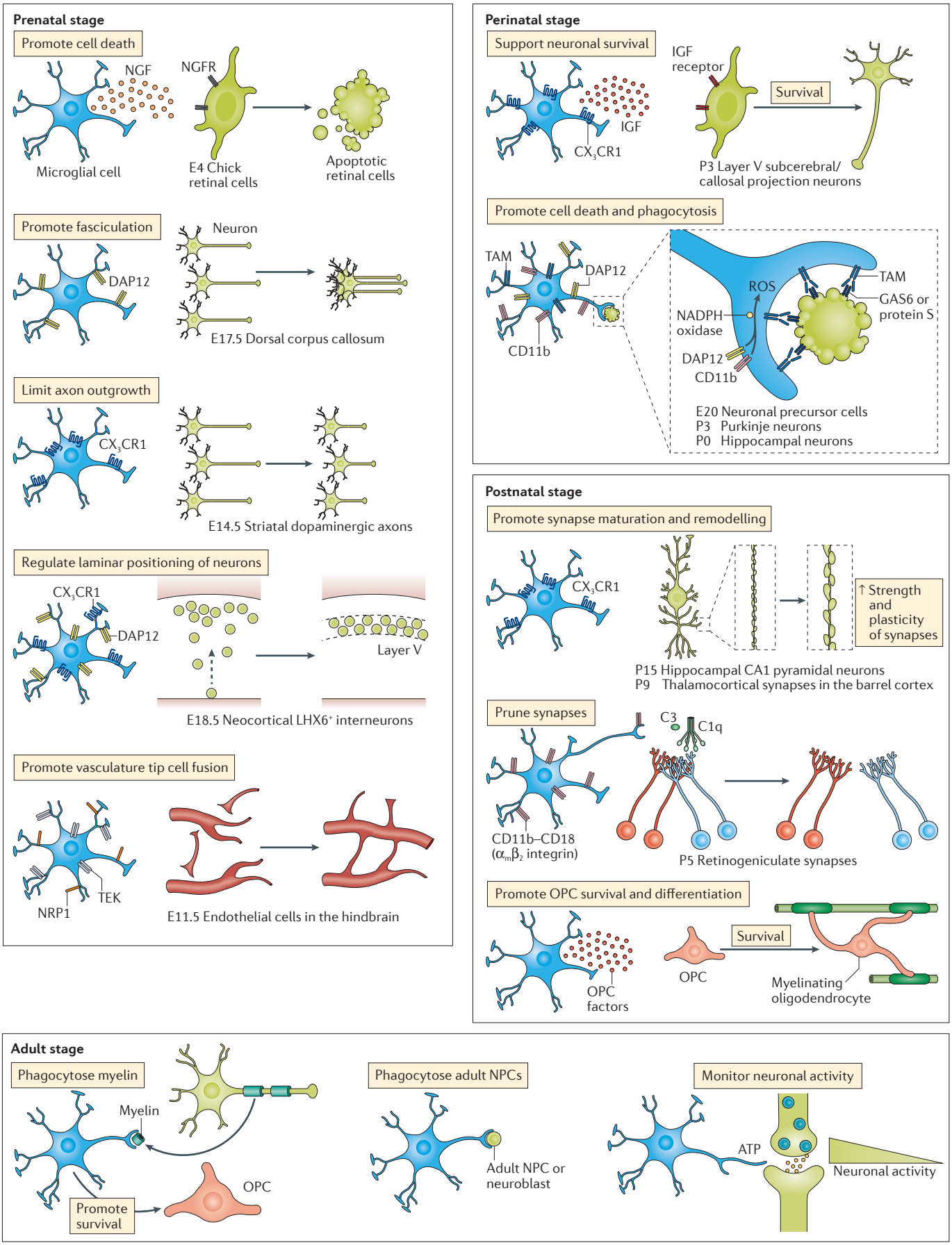
Synapse modulation. Based on time-lapse recording, it is estimated that microglia can scan the entire brain parenchyma every few hours, during which they actively contact other neural components, including synaptic clefts, with their fine processes[9–11]. The functional significance of such microglia–synapse interactions in the adult brain remains largely unknown. In the optic tectum of larval zebrafish, it was shown that microglia preferentially contact neurons with higher levels of activity, and that this activity was attenuated by the interaction[89] (adult stage in FIG. 3). ATP released downstream of NMDA receptor-mediated neuronal activity may function as a 'find-me' signal to direct microglial process outgrowth via purinergic receptor P2RY12 (REF. 90). Mounting evidence has shown that, during development, microglia are actively involved in synapse remodelling and maturation[20,22,25]. In CX_3CR1-mutant animals, the dendritic spine density of hippocampal CA1 neurons transiently increases at approximately postnatal day 15 (postnatal stage in FIG. 3). This phenotype is associated with a temporary reduction in microglial cell numbers (TABLE 1) and with an accumulation of immature synapses, leading to decreased functional connectivity across brain regions and an autism-like behavioural phenotype[22,91]. How CX_3CR1 signalling controls microglial activity to modulate synapse function awaits future investigation. Moreover, it has been shown that microglial P2RY12 signalling is required for synaptic plasticity during the critical period of visual development (postnatal days 21–35 in mice)[92]. The loss of neuronal plasticity in P2RY12-deficient mice is in line with alterations in microglial interactions with synaptic elements and their morphological response to monocular deprivation[92].

Complement and synapse pruning. The classical complement system has a pivotal role in pathogen defence and clearance of cellular debris. Microglia are the main, if not only, cells that express several key complement components in the brain, including complement component C1q (C1q), complement receptor 3 (CR3) and CR5 (REFS 42,93). Under normal, 'sterile' CNS conditions, the function of complement proteins extends far beyond their classical roles in the immune system in targeting bacteria and other microorganisms. They have important roles in developmental synapse pruning, disease-associated synapse loss and cognitive decline in ageing[93]. In the first postnatal week, microglia prune retinogeniculate synapses in the visual system[20,21] (postnatal stage in FIG. 3). C1q and C3 preferentially tag weak synapses, which are then engulfed by microglia via CR3. Beyond the visual system, C1q tagging of synapses is seen throughout the developing CNS, which suggests that it has a widespread role in synapse pruning by microglia. Consistent with this possibility, C1q-deficient mice have spontaneous seizures and have excessive excitatory synapses in the neocortical circuitry[94].

Recently, a series of studies showed that re-activation of complement-mediated developmental synapse pruning by microglia can lead to various neurological and psychiatric disorders (FIG. 4a). In a model of Alzheimer disease, soluble β-amyloid can induce C1q tagging of hippocampal synapses before plaque formation[95]. Remarkably, the synapse loss phenotype, which is a major cause of cognitive decline in Alzheimer disease, can be corrected by knocking out the genes encoding C1q, C3 or CR3. Similarly, in the mouse model for frontotemporal dementia caused by progranulin deficiency[96], in acute

Figure 3 | **Microglial functions in development and homeostasis.** Microglial functions are illustrated based on the stages of development. During the prenatal stage, microglia induce neuronal cell death, promote neuronal fasciculation and limit axon outgrowth. They also regulate the laminar positioning of neurons and increase the complexity of the vasculature network by fusing endothelial cells. During the perinatal stage, microglia support neuronal survival and promote phagocytosis-induced death of neuronal precursors and neurons. During the postnatal stage, microglia promote synapse maturation and remodel neural networks by pruning immature synapses. They also support the survival and differentiation of oligodendrocyte progenitor cells (OPCs). During the adult stage, microglia maintain the OPC pool and phagocytose myelin. In addition, microglia control adult neurogenesis and modulate neuronal activity. Factors that are required for the proper functions of microglia in a given situation are labelled. The precise developmental stages and tissues that were originally used to demonstrate these functions are indicated. Microglial functions under normal homeostatic conditions in adults are less well understood: phagocytosis of myelin can be a mechanism for myelin turnover; phagocytosis of adult neural precursor cells (NPCs) presumably uses the same mechanism as shown for the perinatal stage, when microglia phagocytose embryonic NPCs. Monitoring of neuronal activity by microglia was shown in the optic tectum of larval zebrafish. In this context, microglia preferentially interact with neurons with higher levels of activity and restrict this activity. Microglial chemotaxis is proposed to be mediated by neuronal ATP. C1q, complement component C1q; C3, complement component C3; CX_3CR1, CX_3C chemokine receptor 1; DAP12, DNAX-activation protein 12; E4, embryonic day 4; GAS6, growth-arrest-specific protein 6; IGF, insulin-like growth factor; NGFR, nerve growth factor receptor; NRP1, neuropilin 1; P3, postnatal day 3; ROS, reactive oxygen species; TAM, TYRO3, AXL and MER receptor tyrosine kinases; TEK, angiopoietin 1 receptor.

Complement system
A signalling cascade that can be activated by any of three independent pathways. The classical pathway is activated by antigen–antibody immune complexes. The alternative pathway is triggered by direct hydrolysis of complement component C3. The lectin pathway is activated by the binding of lectin to mannose residues on the surface of pathogens.



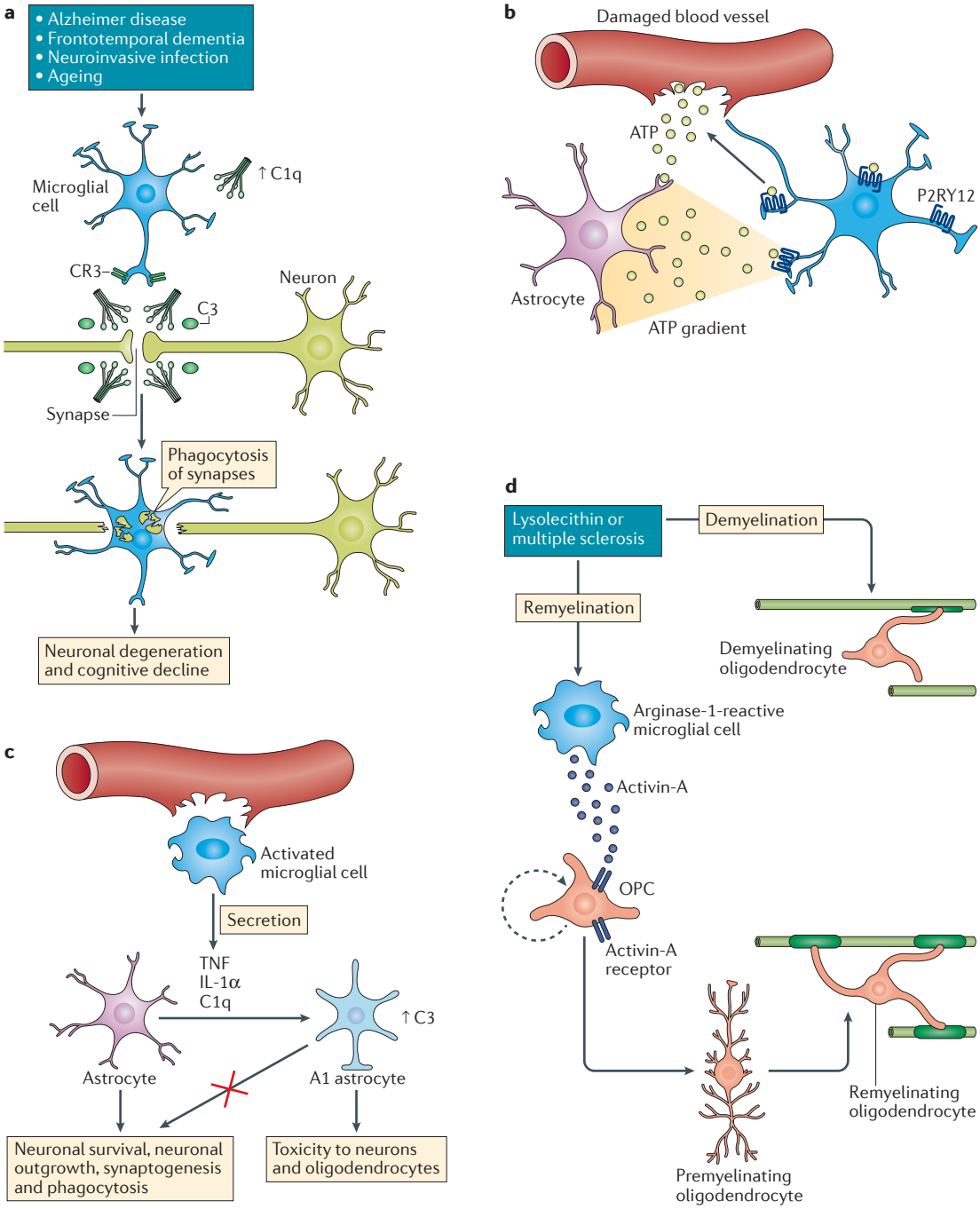


Figure 4 | **Microglial functions during CNS injuries and diseases. a** | Microglia-mediated synapse loss in diseases and ageing. In these processes, microglia secrete higher levels of complement component C1q (C1q), which tags synapses together with complement component C3 (C3). In Alzheimer disease, the upregulation of C1q expression is thought to be induced by β-amyloid oligomers. Microglia then eliminate the tagged synapses via complement receptor 3 (CR3), leading to neurodegeneration and behavioural impairment. **b** | Microglia respond to tissue damage by covering it with rapid process extension followed by movement of their cell bodies towards the injury site. The release of damage-associated ATP triggers an astrocyte-secreted ATP gradient, which is then sensed by microglial P2Y purinoceptor 12 (P2RY12). This process controls microglial chemotaxis to injury. **c** | The induction of A1 reactive astrocytes by injury- or lipopolysaccharide (LPS)-triggered reactive microglia. This classical neuroinflammatory activation of microglia leads to the secretion of IL-1α, tumour necrosis factor (TNF) and C1q, which in turn induce A1 reactive astrocytes. A1 astrocytes, which have higher levels of C3 expression, lose the normal functions of astrocytes and gain new functions such as the ability to kill neurons and oligodendrocytes. This process may underlie neuronal death in many neurodegenerative diseases. **d** | Accelerated proliferation and differentiation of oligodendrocyte progenitor cells (OPCs) induced by arginase 1-expressing microglia in demyelinating diseases. During remyelination, microglia are activated to different states, at least one of which is beneficial. Such activated microglia express arginase 1 and secrete activin A, which binds to activin A receptor on OPCs, promoting their proliferation and differentiation into mature oligodendrocytes.

infection with West Nile virus[97] or in ageing[98], there is a marked increase in microglial expression of C1q, accompanied by C3 synapse tagging and synapse elimination. A theme that is common to all of these settings is aberrant engulfment of synapses by microglia and complement-mediated neurodegeneration, although the brain regions that are affected and the behavioural impairments are disease specific[96,97]. Moreover, human genetic studies have recently identified *C4* gene alleles and copy number variation that cause higher levels of C4A expression as a risk factor for schizophrenia[99]. C4-deficient mice have reduced levels of synaptic C3 and have developmental pruning defects similar to those of mice deficient in C3 or C1q, which suggests that complement-mediated synapse remodelling may also underlie disease pathology in schizophrenia.

It is worth mentioning that an increase in levels of synapse-localized C1q is also implicated in age-dependent cognitive dysfunction unrelated to classical complement signalling or synapse elimination[100]. Thus, major questions remain, including how different pathways and complement components impinge on neural circuitry to affect synapse function and plasticity; how synapses are selectively tagged by complement; and how microglia-mediated synapse remodelling cooperates with other pruning and synaptic plasticity mechanisms induced by other cell types, such as astrocytes[101].

Phagocytosis and neurogenesis. Microglia phagocytose dead, dying and sometimes healthy live cells in the developing and adult brain. During cerebellar development, microglia actively promote engulfment-mediated Purkinje neuron death, by producing reactive oxygen species (ROS)[18] (perinatal stage in FIG. 3). Similar processes occur in the hippocampus, where ROS production depends on microglial expression of CD11b and DNAX-activation protein 12 (DAP12; also known as TYROBP)[17]. Importantly, microglia control the size of the neuronal pool by engulfing neural precursor cells (NPCs) during both embryonic and adult neurogenesis[15,102] (adult stage in FIG. 3). Maternal immune challenge or pharmacological repression of microglial activation *in utero* can respectively enhance or inhibit microglial phagocytosis of NPCs and hence modify the cellular composition of the CNS. This phagocytic process is mediated by microglial expression of the TYRO3, AXL and MER (TAM) receptor tyrosine kinases and their ligands growth-arrest-specific protein 6 (GAS6) and vitamin K-dependent protein S[103] (perinatal stage in FIG. 3). In mice with a microglia-specific deletion of MER and AXL, adult NPCs and their progeny are spared from phagocytosis and contribute to the adult neuronal pool. Interestingly, AXL expression is upregulated in several neurodegenerative disease models[103–105], hinting at a possible link between aberrant microglial phagocytosis and disease pathology.

Microglia and other neural cell interactions. Microglia interact with almost all cell types in the brain to mediate developmental programmes, maintain homeostasis, aid in tissue repair or contribute to disease pathology[12,13]. In developing chick retina, microglia express nerve growth factor (NGF), which can bind neuronal NGF receptor (also known as TNFRSF16 and p75NTR) to induce retinal ganglion cell death[19] (prenatal stage in FIG. 3). By contrast, microglia secrete insulin-like growth factor 1 (IGF1) to support the survival of developing layer V neurons before they reach their distant CNS targets in the postnatal mouse brain[16] (perinatal stage in FIG. 3). In addition, microglia are closely associated with white-matter tracts during development, where they help to control axon fasciculation in the dorsal corpus callosum[24] (prenatal stage in FIG. 3). This function requires DAP12. Similarly, microglia control the outgrowth of dopaminergic neurons and laminar positioning of somatosensory interneurons in the forebrain, thereby contributing to circuitry assembly in a manner dependent on CX_3CR1 and DAP12 (REF. 23). Microglial proliferation and activation have also been implicated in spinal neuropathic pain[106]. Cytokines released by damaged neurons can trigger the upregulation of expression of purinergic receptors, such as P2X purinoceptor 4 (P2RX4), P2RX7 and P2RY12, in microglia, which become hypersensitive to extracellular nucleotides[106]. CSF1 secreted by injured sensory neurons seems to be an essential factor to activate microglia via the CSF1R–DAP12 signalling axis[107]. Upon activation of microglia, induced brain-derived neurotrophic factor interacts with its receptor on neurons, causing a shift in neuronal anion gradients and hyperexcitability of pain-engaging neurons[108]. At the same time, the microglial protease cathepsin S cleaves CX_3CL1 (also known as fractalkine) on the neuronal surface, which in turn acts on microglial CX_3CR1 to maintain pain signalling[109].

Microglia also interact with astrocytes, particularly during brain injury and inflammation. Injury-associated ATP release can induce an astrocyte-derived ATP gradient, which is then sensed by microglia through their purinergic receptor P2RY12, leading to rapid convergence of microglial processes and cell migration[110,111] (FIG. 4b). The same series of activation events occurs in compression and capillary injuries to help seal breached glial limitans or the BBB[112,113]. Such ATP-driven outgrowth of microglial processes can be converted to process retraction during chronic injury, and this conversion for activated microglia is mediated by the downregulation of P2RY12 and concomitant upregulation of the G_s-coupled adenosine receptor A_{2A}, which binds adenosine from the breakdown of extracellular ATP[114]. In addition, injury-activated or LPS-stimulated microglia secrete IL-1α, tumour necrosis factor and C1q, which are necessary and sufficient to induce so-called 'A1' neurotoxic reactive astrocytes[115] (FIG. 4c). A1 astrocytes, which are prevalent after acute CNS injuries and in human neurodegenerative diseases, lose normal synaptogenic and phagocytic functions with a concomitant gain of cytotoxic functions, which enables them to kill both neurons and oligodendrocytes. As A1 astrocytes highly upregulate the expression of C3 and many other complement components, it has been suggested that the normal function of this microglia–astrocyte interaction

Layer V neurons
Neurons in layer V of the six-layered mammalian neocortex. This layer contains excitatory neurons that project either to the contralateral hemisphere or to subcortical brain regions, such as the thalamus and brainstem.

Axon fasciculation
A neurodevelopmental process in which axons that travel in the same direction often adhere together to form a tight bundle.

Corpus callosum
A thick bundle of nerve fibres that connect the left and right hemispheres of the brain and form the most prominent white-matter structure.

may be to protect against bacterial and viral infections but that when activated in other contexts, it may help to drive neurodegeneration in various diseases including Alzheimer disease, Parkinson disease and amyotrophic lateral sclerosis (ALS)[115].

Through pharmacological and genetic depletion, it has been shown that early postnatal microglia are required for the development of oligodendrocyte progenitors and the subsequent myelination process[116] (postnatal stage in FIG. 3). Later in life, microglia continue to maintain the oligodendrocyte progenitor pool under homeostatic conditions[116] (adult stage in FIG. 3). In the context of lysolecithin-induced injury, microglia accelerate remyelination by promoting oligodendrocyte differentiation[117] (FIG. 4d). This effect is proposed to involve arginase 1-expressing microglia that secrete activin-A, which interacts with activin-A receptor on oligodendrocyte progenitor cells in the regenerative process. Moreover, microglia phagocytose myelin, which later in life can form insoluble protein aggregates in microglial lysosomes and compromise their functions[118] (adult stage in FIG. 3). This might be a mechanism for controlling myelin turnover and a cause of ageing. Finally, microglia interact with endothelial cells, as indicated by the observation that microglia mediate blood vessel fusion downstream of vascular endothelial growth factor-dependent tip cell sprouting; the loss of microglia in PU.1- or CSF1-mutant mice results in reduced complexity of the brain vesicular network early in development[119] (prenatal stage in FIG. 3). Interestingly, in zebrafish, microglia can also repair laser-induced microlesions by physically adhering to the broken ends of endothelial cells and pulling them together[120]. This process requires actin polymerization and depends on phosphatidylinositide 3-kinase and RAC1 activity.

Microglial activation in development and degenerative diseases. Mature microglia have highly ramified processes, whereas during development, they adopt an 'amoeboid' morphology with larger, rounder cell bodies and shorter, thicker branches[121]. These morphological features during development are accompanied by higher levels of phagocytic activity as well as by distinct gene expression and secretory profiles. The cells are therefore often viewed as being developmentally activated, which is required for normal brain development as discussed in the case of IGF1 secretion by microglia[16,116]. Similarly, immature microglia were also shown to induce neuronal and oligodendrocyte differentiation by secreting pro-inflammatory cytokines[122]. Upon immune challenge or in brain diseases, microglia tend to show similar morphological changes, sometimes associated with the release of cytokines, chemokines and/or trophic factors. These polarized cells were traditionally categorized as having either 'toxic' (M1 type) or 'protective' (M2 type) states, depending on the expression of a few known markers[27]. However, accumulating evidence suggests that microglial polarization is multidimensional, with extensive overlap in gene expression, as opposed to occurring on a simplified linear spectrum[27].

Microglial activation, irrespective of the particular polarized states, is a salient feature of neuroinflammation that is prominent in almost all neurodegenerative diseases[31,123]. In some settings where the BBB is compromised, blood-borne monocytes having distinct polarization phenotypes could potentially infiltrate the brain or spinal cord, although to what extent such infiltration is implicated in a given disease is a matter of debate. This discrepancy is due to some experimental caveats, such as in BM transplantation, that inevitably create artificial environments that are not normally seen in the course of disease, and a lack of reliable tools for distinguishing microglia from infiltrating myeloid cells. In addition, our knowledge of microglial and macrophage functions in CNS disease is obtained mainly through studying animal models, which may or may not accurately represent human conditions. Nonetheless, differential contributions of infiltrating versus resident myeloid populations have been demonstrated in the pathogenesis of several disease models[124–126]. Therefore, it has been increasingly recognized that investigating myeloid cell heterogeneity with regard to ontogeny and activation status across different stages of disease progression is essential for a mechanistic understanding of neurodegenerative disease pathology. Under this framework, the design of myeloid-cell-based therapeutics should work towards dampening the detrimental effects of microglial and macrophage activation while augmenting the beneficial effects elicited by their 'alternative' activation.

Although microglial activation can clearly alter disease progression, human genetics studies point to a causal role for microglial dysfunction in disease initiation. Prime examples lie in the identification of risk genes for Alzheimer disease, such as *TREM2*, *CD33* and *CR1*, that are exclusively expressed by microglia in the CNS parenchyma[127–133]. These genes are thought to affect the microglial phagocytic activity underlying amyloidosis[134,135]. In particular, it has been suggested that TREM2 and CD33 regulate β-amyloid phagocytosis positively and negatively, respectively. However, a conflicting result showed that depletion of microglia in amyloid precursor protein-transgenic mouse models had little effect on plaque formation or maintenance[136]. Indeed, recent studies of the function of triggering receptor expressed on myeloid cells 2 (TREM2) suggest that activated microglia surrounding β-amyloid plaques mainly function as 'insulators' that confine the neurotoxicity of β-amyloid fibrils[137] (BOX 2). Interestingly, microglia are also implicated in tauopathy, another hallmark of Alzheimer disease, in which microglia may spread microtubule-associated protein tau via exosomes across brain regions[138]. Microglial activation precedes the formation of neurofibrillary tangles in a mouse model of tauopathy[139,140], which suggests that microglia have an active role in driving disease pathology.

Microglial dysfunction also contributes to the onset and/or progression of ALS, a degenerative disease characterized by progressive loss of motor neurons[35]. Genes that are commonly mutated in ALS include superoxide dismutase 1 (*SOD1*), *C9orf72* and TAR DNA-binding

Tauopathy
A member of a class of neurodegenerative disorders that are manifested by intracellular accumulation of hyperphosphorylated microtubule-associated protein tau. These insoluble protein aggregates form neurofibrillary tangles and often underlie the pathological conditions of dementia and Parkinson disease.

Box 2 | **Microglial TREM2 expression and late-onset Alzheimer disease**

Recent genome-wide association studies have shown that many genes that confer risk of neurodegenerative disease are highly, and sometimes exclusively, expressed by microglia[31,84,151]. The expression of triggering receptor expressed on myeloid cells 2 (TREM2) is specific to microglia in the central nervous system, and the rare human polymorphism R47H has one of the highest odds ratios (that is, 2.9–4.5) for susceptibility to late-onset Alzheimer disease[127,129]. Using various β-amyloid-based mouse models of Alzheimer disease, we have gained a substantial understanding of TREM2 functions in microglia-mediated disease pathology, although it is still unclear how these pathways are coordinated at the molecular level. TREM2 is a sensor for a broad range of lipids and lipoproteins[152,153]. More microglia are found in the brains of mice and patients with Alzheimer disease than in those of healthy controls, and these cells tend to surround and phagocytose β-amyloid plaques[137,152]. Parabiosis experiments show that increased numbers of microglia arise from local clonal expansion as opposed to infiltration from the periphery[154]. In TREM2-deficient mouse models or human samples of Alzheimer disease, microglial clustering around β-amyloid plaques is impaired[137,152]. At least five non-mutually exclusive modes of action for TREM2 in Alzheimer disease have been proposed. First, TREM2 senses damage-associated lipids that bind fibrillar β-amyloid, which recruits microglia and facilitates phagocytosis[152]. Second, TREM2 senses various lipids that attach to β-amyloid fibrils, which are enveloped and compacted by microglia so that their neurite toxicity is confined and/or reduced[137,154]. Third, TREM2 senses apolipoprotein E and apolipoprotein J, which bind β-amyloid and function as molecular chaperones for its uptake by microglia[153]. Fourth, TREM2 signalling promotes the survival of microglia so that they can maintain protective activity such as phagocytosis[152]. Fifth, TREM2 signalling induces microglial proliferation and polarizes them to an activated state with protective functions[46,152]. Recently, a possible mechanism that integrates these beneficial effects suggests that TREM2-mediated mechanistic target of rapamycin (mTOR) signalling negatively regulates autophagy, which highlights the importance of maintaining proper microglial metabolic states and fitness during their long-term protective function in Alzheimer disease[155].

protein (*TARDBP*)[141]. Early studies using mice that overexpress the mutant *Sod1* allele showed that genetic removal of this protein in myeloid cells alleviated disease progression and extended survival[142]. Similarly, replacing mutant SOD1-expressing microglia with wild-type BM-derived cells also had beneficial effects, which is consistent with greater release of neurotoxic factor by the mutant microglia[55]. These studies demonstrated non-cell-autonomous pathology of ALS and raised the possibility of targeting microglia for treating neurodegenerative diseases. Recently, the normal function of C9orf72, a hexanucleotide repeat expansion that is associated with ALS and frontotemporal dementia, was studied in a knockout background, in which microglia were found to be strongly activated in the mutant[143]. C9orf72 regulates microglial phagosome and lysosome pathways, and its mutation leads to upregulation of neuroinflammatory factors, reminiscent of C9orf72-mediated ALS pathology[143]. Unexpectedly, TARDBP was also found to regulate microglial phagocytosis[144]. The loss of TARDBP in microglia led to increased β-amyloid clearance and more synapse loss, providing a plausible explanation for the less frequent occurrence of Alzheimer disease among patients with ALS.

Multiple sclerosis is a special case among neurodegenerative diseases, in which neuronal death may occur secondary to myelin loss mediated by autoreactive T cells[123]. Although it remains unclear exactly how the adaptive immune system is activated, myeloid cells have been considered to be essential components driving disease onset and progression[145], as suggested by the strong genetic association between MHC risk loci and multiple sclerosis[146]. It is widely accepted that, compared with microglia, infiltrating monocyte-derived macrophages have a greater role in disease pathology, and therefore, strategies limiting such infiltrating behaviour could alleviate multiple sclerosis symptoms in animal models[145]. Microglia, by contrast, have been suggested to have a beneficial role, particularly during the remission phase. Scanning electron microscopy studies showed that whereas blood-borne macrophages centred around nodes of Ranvier, presumably causing demyelination, microglia seemed to clean up myelin debris, aiding in tissue repair[125]. More importantly, microglia may directly promote remyelination by inducing the differentiation of oligodendrocyte precursor cells[117,147] (FIG. 4d).

Concluding remarks

A scientifically curious inquiry about the origin of microglia has fundamentally shifted our conceptual frameworks in almost every aspect of microglial biology. Now that their origin has been separated from blood-derived lineages, many questions require immediate re-examination, such as their developmental programmes, turnover mechanisms and heterogeneity among other brain myeloid cells. In parallel, the surprising observation that microglia restlessly survey their environment provided perhaps the most obvious rationale for studying their physiological functions during development and homeostasis. These investigations have led to many exciting and often unexpected findings about the non-immune functions of microglia. Such enthusiasm is further fuelled by the newly discovered associations between microglia-enriched genes and a large number of neurodegenerative and neuropsychiatric disorders, which often show signs of neuroinflammation. These observations together raise an important question of whether microglial dysfunction is the primary driving force for the pathophysiology in these diseases. Furthermore, as microglia have extremely diverse functions, it remains unclear whether they have beneficial or detrimental roles during disease progression. In fact, it is probable that they have both such roles, depending on the timing, the particular disease setting and the context of other neural and immune cells. Compared with microglia, our knowledge of the functions of non-parenchymal macrophages in health and disease is currently disproportionally limited, and these cells could be the long-sought missing piece in a puzzle where all components, neural and immune, are highly integrated. Future investigation in this regard may provide insights for our understanding of brain function as a whole. Dissecting the complex roles of microglia and brain macrophages in each scenario is challenging, but equipped with the new tools of unprecedented precision and throughput, we are closer than ever to learning the mystery of the 'third element' in the brain universe and targeting it to fight devastating diseases.

Nodes of Ranvier
Periodic axonal segments, rich in ion channels, that are not covered by myelin sheaths; this allows rapid propagation of an action potential from one node of Ranvier to the next along the fibre.

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