The molecular determinants of microglial developmental dynamics

Authors:

Liam Barry-Carroll[2], Diego Gomez-Nicola[1]*

Affiliations:

[1] School of Biological Sciences, University of Southampton, Southampton General Hospital, United Kingdom

[2] Université Bordeaux, INRAE, Bordeaux INP, Nutrineuro, UMR 1286, 33076 Bordeaux, France

*Lead author:

Prof Diego Gomez-Nicola

School of Biological Sciences, University of Southampton. Southampton General Hospital. South Lab&Path Block. Mail Point 840, LD80C. Tremona Road. SO166YD. Southampton, United Kingdom.

E-mail: d.gomez-nicola@soton.ac.uk

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Abstract

Microglia constitute the primary population of parenchymal macrophages in the brain and are considered a unique CNS subset of glial cells due to their extraembryonic origins in the yolk sac. During development, microglia progenitors readily proliferate and eventually colonise the entire brain. Here, we highlight the origins and entry routes for microglia progenitors in the brain and discuss the various molecular and non-molecular determinants of their fate which may inform their specific functions in the brain. Specifically, we explore novel mechanisms involved in microglial colonisation of the brain including the availability of space and how expansion of highly proliferative microglial progenitors facilitates the occupation of the microglial niche. Finally, we shed light on the factors involved in determining the fate of early progenitors towards the microglial identity in the brain opposed to other CNS macrophage subpopulations.

1. Introduction

The central nervous system (CNS) is a diverse environment comprised of various cell types including glial cells. Often thought of as 'support cells' to neurons, glia are highly diversified with multiple origins and functions that are vital for the overall viability of the CNS[1]. In particular, microglia make up a substantial portion of long-lived glial cells and act as the main tissue-resident macrophage population in the brain accounting for between 5 and 12% of all cells in the CNS (Lawson et al., 1990). Original descriptions of microglia noted their unique stellate morphology and apparent capability as phagocytes which led to their original coining as 'mesoglia' owing to their similarity with other cells of mesodermal origin[2,3]. The term 'microglia' was later coined by Rio-Hortega to account for his observation that microglia display a small cell soma and extensive ramifications[3-5]. Indeed, it is these ramifications which allow microglia to constantly survey the local cellular milieu and maintain homeostasis in the CNS[6,7]. Microglial vigilance is facilitated by their expression of a broad battery of pattern recognition receptors allowing them to 'sense' their environment and develop dynamic relationships with other cell types within the CNS[8]. Furthermore, microglia are topographically distributed throughout the brain-parenchyma, akin to a mosaic pattern, which allows them to effectively cover and survey the entire brain in a territory-dependent manner[6,9,10]. Upon sensing disruptions to normal homeostasis, microglia can engage a repertoire of immune functions in a context-dependent fashion[11-14].

The need to understand the developmental trajectory and processes of microglia is increasing as it emerges that they are involved in a number of neurodevelopmental disorders (NDDs)[13,15-18] (**Box 1**). Not only this, but perturbations to normal microglial development may have long term consequences

in the form of immune memory which is linked with pathological outcomes in later life [19-22]. Therefore, it is important to have a benchmark for the 'normal' development of microglia in the healthy brain in order to intervene when this goes awry. Here we discuss the origins and development of microglia with an emphasis on the molecular pathways underpinning these processes.

Text Box 1 Microglial dynamics in Neurodevelopmental Disorders

Neurodevelopmental disorders (NDDs) consist of those with an onset during the developmental period and have longstanding consequences in regard to proper brain function[23] such as autism spectrum disorder (ASD) and schizophrenia[24]. Microglia have been directly implicated in the pathogenesis of NNDs due to their roles in neuronal maturation and wiring during development[25]. NNDs may arise when the normal development and functioning of microglia is impaired due to erroneous environmental input[26,27]. For example, in maternal immune activation (MIA), microglia numbers are increased[15,26] and they display a more advanced profile during early postnatal life[13,28]. Moreover, the microglial release of TNF-α is elevated and associated with altered neurogenesis[5,26,29] and cortical wiring[30]. These mice often display cognitive abnormalities and social deficits in adult life akin to ASD[13]. Other early-life adversities can induce microglial dysfunction that is associated with behavioural alterations[16,31,32]. In humans, post-mortem evidence has also shed light on the involvement of microglia in NDDs[33-35]. For example, a recent study has highlighted the potential involvement of IFN-γ producing microglia in the impairment of myelination in developing white-matter due to interference with oligodendrocyte function[36]. Importantly, several studies have shown that even in the absence of inflammatory stimuli or other environmental influences, impeding the expansion of microglia during development is associated with abnormal brain development in neuronal[25,26,34] and non-neuronal populations including astrocytes[37,38]. In humans, homozygous mutations in the CSF1R gene can result in the partial or complete loss of microglia which associates with an abnormal cytoarchitecture in the corpus callosum and epilepsy during infancy[17,38,3940,41]. Overall, these studies demonstrate the importance of microglia during development in the healthy brain and how disruptions to their normal maturation can be detrimental in later life.

2. Origins and early infiltration

Early yolk sac origins of microglia

Seminal descriptions of microglia highlighted their unique identity among the cellular subpopulations of the CNS due to their suspected mesodermal origins[2-4]. In opposition, another popular hypothesis during the 20th century was that microglial cells were of neuroectodermal origin[40,41]. However, with advancements in immunohistochemical techniques, there was a shift in acceptance towards a myeloid origin of microglia due to reports of overlap in the expression of several antigenic markers between embryonic microglia and macrophages[42]. The exact myeloid origin of microglia was identified by a landmark study which utilised the inducible Cre-recombinase system to fate map various myeloid progenitor sources[43]. Ginhoux et al., demonstrated that microglia are derived from RUNX1-dependent progenitors which give rise to a population of "early" erythromyeloid progenitors (EMPs) (also known as primitive macrophages) in the yolk sac from E7.5 during primitive haematopoiesis[42-44]. Indeed,

further studies have revealed that a portion of these "early" EMPs then differentiate to committed microglial progenitors in the yolk sac at E9 (A2 progenitors[38]) and eventually seed the embryonic brain from E9.5 [38,43]. Microglial progenitors in the yolk sac can be distinguished based on their expression of C-KIT$^-$, PU.1+, CSF1R+, F4/80^{hi}, CD45$^+$, Cx3CR1^{hi} [38,42-44]. Conversely, another portion of "late" EMPs are generated in the yolk sac from E8 and seed the fetal liver where they expand and give rise to cells of the myeloid and erythroid lineage including monocytes [34,35,38,45,46]. The question of microglia ontogeny in humans has been more difficult to address without such high-resolution fate-mapping approaches available in rodents. However, advancements in single-cell RNA sequencing and complimentary analyses have allowed for interrogation of the nature and developmental trajectory of microglia progenitors in humans[47-49]. Indeed, profiling of macrophage progenitors throughout development has led to the identification of MRC1$^+$ CD163$^+$ progenitors in the yolk sac at Carnegie stage (CS) 11 which share the same profile of microglia progenitors in the brain at the respective timepoint[47]. This study compared these aforementioned progenitors akin to "early" EMPs in the mouse[47] suggesting an overlap between species. Moreover, the identification of a second yolk sac macrophage population characterised by *CD34* and *MYB* expression, which are predicted to give rise to monocytes in the liver from CS17 similar to "late" EMPs in the mouse, further strengthens this idea[47]. Differentiation of human induced pluripotent stem cells (iPSCs) towards microglial-like cells provides a promising model for further understand the ontogeny of yolk sac macrophages and microglia in humans[50-55]. Indeed, Hislop et al., recently took advantage of an iPSC-embryoid model to mimic post-implantation embryogenesis[56]. They observed haematopoiesis-like events in extra embryonic yolk sac layers with the emergence of CX3CR1$^+$ myeloid and CD235$^+$ erythroid lineages corresponding to CS7-9 in the *in vivo* scenario[47,56]. Further harnessing the potential of such models will provide unforeseen insight to the origins and developmental kinetics of microglia.

Over the years there have been some studies considering the possible contribution of "late" EMP and/or haematopoietic stem cells (HSCs) sources to the microglia pool, however, it is important to note that any contribution from such sources is zero to minimal[57,58]. One suggested non-yolk sac source of microglia are HSC-derived progenitors arising in the aorta-gonad-mesonephros (AGM) region at E10.5 during definitive haematopoiesis[59-62]. Historical support for this HSC-microglia model is mainly observational and based on early findings that uncommitted HSC progenitors from the periphery can engraft into the microglial niche following lethal irradiation or during blood brain barrier (BBB) breakdown in adult mice[63,64,65,66]. In contrast, it is now broadly accepted that in the absence of irradiation or disease, circulating HSC-derived cells do not contribute to the microglial pool under physiological circumstances[43,45,59,65,67,68]. However, the topic of HSC-derived microglia has resurfaced again in recent years owing to the reports of a HSC-derived Hoxb8$^+$ subset of microglia in the mouse

brain which were estimated to make up to 25% of all microglia at P16[57,69]. Utilising a constitutive Hoxb8^{Cre} reporter, De et al., reported that Hoxb8-derived microglia are HSC derivatives originating in the AGM and fetal liver which enter the brain at E12.5[69], significantly later than bona fide yolk-sac derived microglia[43]. To add to the debate, a recent report suggests that Hoxb8^{+} progenitors give rise to both "late" EMPs and HSCs which seed the fetal liver giving rise to monocytes in an MYB-dependent manner[70]. However, without the use of an inducible Cre line it is difficult to discern the true nature of Hoxb8-derived progeny as even a transient expression of *Hoxb8* in "early" EMPs, as is strongly suggested by the mRNA expression in the yolk sac at E8.5[69], would result in labelling of canonical microglia.

Interestingly, a small number of brain-residing monocytes have been observed during late embryonic to early postnatal periods[71-73] and are estimated to make up around 3-4% of total brain myeloid cells (macrophage/microglia and monocytes) during this time raising the question of a possible monocytic contribution to microglia[72,74]. Similar findings have been reported in human cases where monocyte-like cells have been detected in the postnatal brain[47,75]. Whether fetal monocytes can truly transition to microglia has been addressed by several groups utilising different fate mapping approaches to track embryonic monocytes and has resulted in conflicting reports[73,74]. Our group has previously utilised the lentiviral system to directly induce fluorescent labelling of monocytes arising from the fetal liver at E14[72]. At P0, we detected labelled monocytes across different brain compartments which co-expressed Iba1 and CD206. However, these labelled monocytes were short lived and only persisted in the brain up to P6, therefore not seeming to contribute or transition to microglia. Similarly, utilising an inducible CCR2^{CreER} lines to trace monocytic progenitors between at E14.5-E16.5, Utz et al., reported that the majority of labelled cells at E18.5 were CD11b^{+} Ly6C^{hi} monocytes whereas virtually no microglia were labelled. Other studies utilising Flt3^{Cre}, S100a4^{Cre} and Ms4a3^{Cre} strains of mice commonly used to fate map monocytes fail to show any contribution to the microglia population [46,76]. Moreover, the number of microglia is not affected in CCR2$^{-/-}$ mice[46,59] suggesting that any infiltrating monocytes do not transition to microglia. In contrast, following induction of labelling at E14 in another strain of CCR2^{CreER} mice, Chen et al., observed labelling of P2RY12^{+} cells in the brain parenchyma at P2[73]. Interestingly, the number of labelled CCR2^{+} cells increased following induction of labelling at E17 and persisted up until P24 expressing typical microglia markers such as TMEM119 and P2RY12[73]. The authors suggest that induction of labelling from E17 is likely to capture CCR2^{+} cells arising from BM haematopoiesis as opposed to fetal liver haematopoiesis at E14.5[72,73]. Another possibility for this increased labelling could be due to a transient expression of CCR2 by *in situ* progenitors at this time. Discrepancies between Utz et al., and Chen at al., may also arise due to differences in the CCR2^{CreER} line[73,74] which can influence

selectivity of labelling. Therefore, further work should aim to address these potential issues of selectivity.

In sum, the majority of studies support that microglia in the adult brain are derived from "early" EMPs in the yolk-sac with little to no contribution from "late" EMP or HSC sources.

3. Entry and colonisation of microglia within the developing brain.

Entry to the brain

While there is an extensive expansion of yolk sac macrophages in the extra embryonic yolk sac, the CNS remains devoid of any myeloid progenitors until around E9.5 in the mouse[42-44], while in humans, the first microglia are detected at 4 post-conception weeks (pcw) concomitant with the circulation [77-79]. Indeed, the vasculature appears to be the primary route of entry for these foreign progenitors into the CNS (**Fig. 1a**). Intravital imaging of rodent embryos has revealed that intravascular trafficking of $CSF1R^+$ macrophage/microglia progenitors to the CNS takes place in a timely manner and is restricted to a window between E9.5 until E14.5[80]. In humans, microglia have been reported to localise with capillaries at 5 pcw suggesting blood vessels as a potential entry point [79]. This claim is strengthened by reports where the disruption of circulation clearly impacts and reduces microglial coloniation[43,81]. Another potential source of entry for microglia progenitors is trans-tissue migration through neuroepithelial barriers into the parenchyma[44,82] (**Fig. 1a**). This initial entry of microglia precursors occurs prior to proper vascularisation of the parenchyma[83]. Trans-tissue entry of microglia occurs predominantly at the ventricles where it has been noted that there is a dense population of microglia in the sub-ventricular zone[10,82,84-87]. Indeed, such accumulation of amoeboid microglial cells at the choroid plexus, ventricular and pial borders has also been noted in human cases [77-79]. Reports from post-mortem human cases suggest that microglia initially enter the forebrain by traversing the meningeal, ventricular and choroid plexus borders at around 5 pcw [88]. Convincing real-time evidence of a trans-tissue entry has been recently demonstrated utilising intravital imaging whereby yolk sac macrophages were reported to pass from the ventricles into the cortical plate at E12.5 in mice [87]. It has recently been suggested that the presence of CSF-1 in the embryonic brain is an attractive signal for microglial progenitors and is crucial for their initial entry as neutralising antibodies targeting CSF-1 impede microglial arrival and colonisation into the CNS[89] (**Fig. 1a**). Conversely in the zebrafish, IL-34 signalling is required for the entry of microglia into the CNS during early embryonic development and acts as a pro-migratory signal for microglia from the yolk sac to the brain[90]. Future studies will be needed to elucidate the intricate timings of both mechanisms of microglial entry and further understanding of the role of pro-migratory cues such as CSF-1 and IL-34 is needed.

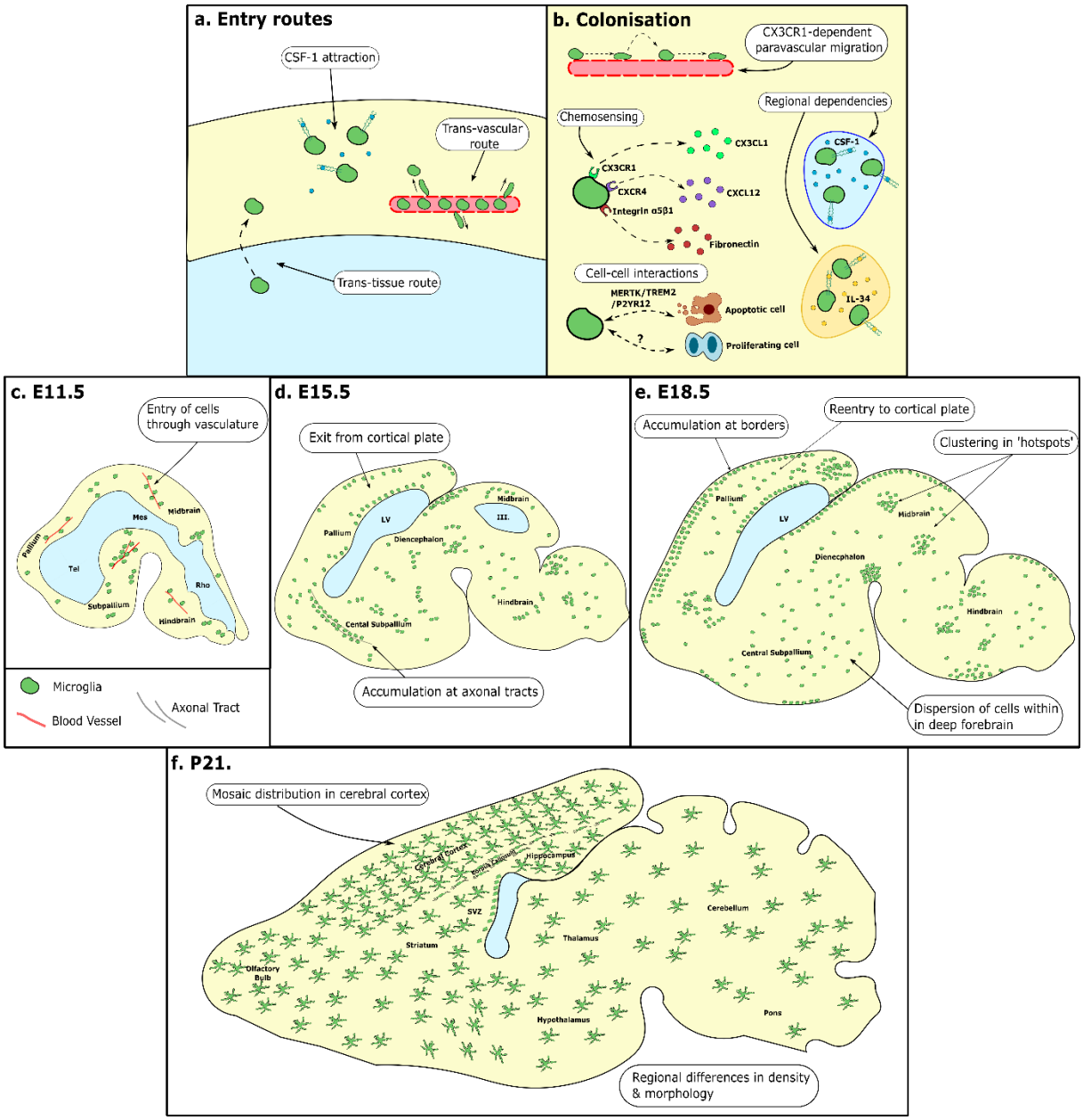
Molecular determination of microglial colonisation

Throughout the course of development, microglia display a heterogenous topographical distribution as they gradually colonise the brain[10]. The developmental changes in the spatiotemporal distribution are influenced by a variety of signalling molecules and cellular interactions [77,85,91] (**Fig. 1b-e**). Of note, the distribution of microglia in the embryonic and postnatal brain is influenced by the regional distribution of IL-34 and CSF-1 within the layers of the developing cortex and also between gray (IL-34) and white (CSF-1) matter regions in the postnatal brain[89,92]. Altogether this implies that the expression of CSF-1 and IL-34 exhibits a level of spatio-temporal control over microglia colonisation patterns resulting in regional dependencies throughout development and adulthood. On a mechanistic basis, the CX3CL1/CX3CR1 axis appears to play an important role in directing microglial-synapse interactions and thereby facilitating development pruning [93-95]. Impaired CX3CR1 signalling results in elevated synapse density and causes hyperexcitability [93,94]. CX3CR1/CX3CL1 signalling may also be important for para-vascular migration of microglia as mice harbouring a genetic deletion of Cx3cr1 displayed delayed migration along blood vessels which resulted in a reduction in the number of microglia in the barrel cortex at P7 and P8 [96]. A similar trend was reported in the hippocampus of Cx3cr1-/- mice[94] suggesting that CX3CR1-CX3CL1 interactions will guide microglial progenitors during development. Another set of pro-migratory cues which influences the migration of microglia is the glycoprotein fibronectin, a component of the extracellular matrix in the CNS. Live imaging of *ex vivo* cortical slices has revealed an age-dependent role for fibronectin in driving microglial migration. Interestingly, blockade of the fibronectin receptor in microglia during early embryonic development slows down migratory microglia [97]. In contrast, blockade during later embryonic development leads to an increase in the velocity of microglia. Altogether this indicates an opposing age-dependent function of the fibronectin receptor on microglial migration capabilities[85,97].

Several incidences of a dynamic spatio-temporal coupling between microglia and various cellular compartments have been described during development suggesting that the interactions between microglia and various cell types will influence their overall colonisation patterns. Historically microglia have been reported to couple with progenitor niches throughout the brain including the deep layers of the cortex and various axonal tracts such as the corpus callosum [85,91,98-100]. This spatial coupling with axonal tracts is also observed in the human which are known as microglial 'nests'[29,88,101]. Within these 'nests', microglia have been suggested to play important roles such as clearance of excess neuronal progenitors cells[91] and synapses[94], as well as aid proper maturation and differentiation of various neuronal and non-neuronal populations [30,91,102]. Therefore, the emergence and development of various neural niches will alter the motility and distribution of microglia [93]. This can be seen in the

neocortex where microglia typically display a random distribution during early embryonic development [25]. However, as development progresses microglia begin disappearing from the cortical plate and accumulate adjacent to the pial surfaces at the meninges and subventricular zone between E15 and E16 [85,86,99]. This change in distribution coincides with the maturation and differentiation of post-migratory neurons within the cortical plate and is facilitated by secretion of CXCL12 from basal progenitors in the meninges and subventricular zone which attracts microglia towards the pial surfaces [86,99]. Interestingly, genetic deletion of the CXCL12 receptor, CXCR4, in microglia impairs the differentiation of post-migratory neurons hinting that this transient exit of microglia from the cortical plate is vital for neuronal maturation [86]. Knockout of CXCR4 is also associated with a regional decrease in microglia in the sub-ventricular zone suggesting that colonisation patterns are driven by local interactions[27]. There is also a decrease in the density of microglia within the cortical plate of humans in late gestational stages[77], however it is unclear whether it is in a similar manner to the mouse[86]. As well as facilitating neuronal maturation, microglia may also influence the maturation and differentiation of oligodendrocyte precursor cells (OPCs)[103,104] through release of soluble factors such as insulin-like growth factor 1 (IGF-1) [102]. Microglia in the developing brain can be seen regionally associated with OPC niches in the hypothalamus[84] and subventricular zone[104]. Together, these reports suggest that the spatiotemporal dependency of emerging neuronal and OPC subsets on microglia will influence their overall colonisation patterns in the developing brain.

As phagocytes, microglia play an active role in the clearance of debris and apoptotic cells, which is particularly important during development when there is a large increase in the number of dying cells in the brain and myelin debris [91,105,106]. Removal and phagocytosis of dead cells is mediated by the expression of several receptors including MERTK, TREM2 and P2RY12 which allow microglia to sense and migrate towards apoptotic bodies [107-109]. Therefore, it has been questioned whether the distribution of apoptotic cells may influence microglia colonisation during development [110]. In support of this notion, recent reports from zebrafish observed that colonisation of microglia was concomitant with an increase in neuronal apoptosis [111,112]. Further, inhibition of apoptosis interfered with microglia colonisation resulting in a drastic decrease in their density and entry into the developing brain [111]. The authors of this study questioned the mechanism behind this response and identified a role for nucleotide signalling in prompting the initial migration of microglia following the release of nucleotides from dying cells [111]. As of yet, these findings are yet to be fully recapitulated in the mouse brain, although it has been recently shown that embryonic and postnatal microglia are spatially associated with apoptotic cells in the parenchyma suggesting there may be an influence of dying cells on the distribution of microglia[9,10].



4. Microglial proliferation during development

Microglia undergo mass expansion during development

Microglia in the rodent brain steadily increase in number during embryonic development and subsequently undergo a rapid expansion during postnatal development when there density peaks 2 and 3-fold during the first few postnatal weeks of life[10,44,72,113,114]. The rate of microglial expansion during development is proportional to the rate of brain growth in an allometric manner, particularly during embryonic stages [10,114]. This expansion phase is followed by a refinement in cell number through apoptosis and egress of extranumerary cells[10,72,114]. Conversely, in humans the expansion of microglia is less linear and follows a wave-like pattern that coincides with various neurodevelopmental milestones that are more complex in nature in comparison to the development of the mouse brain [77].

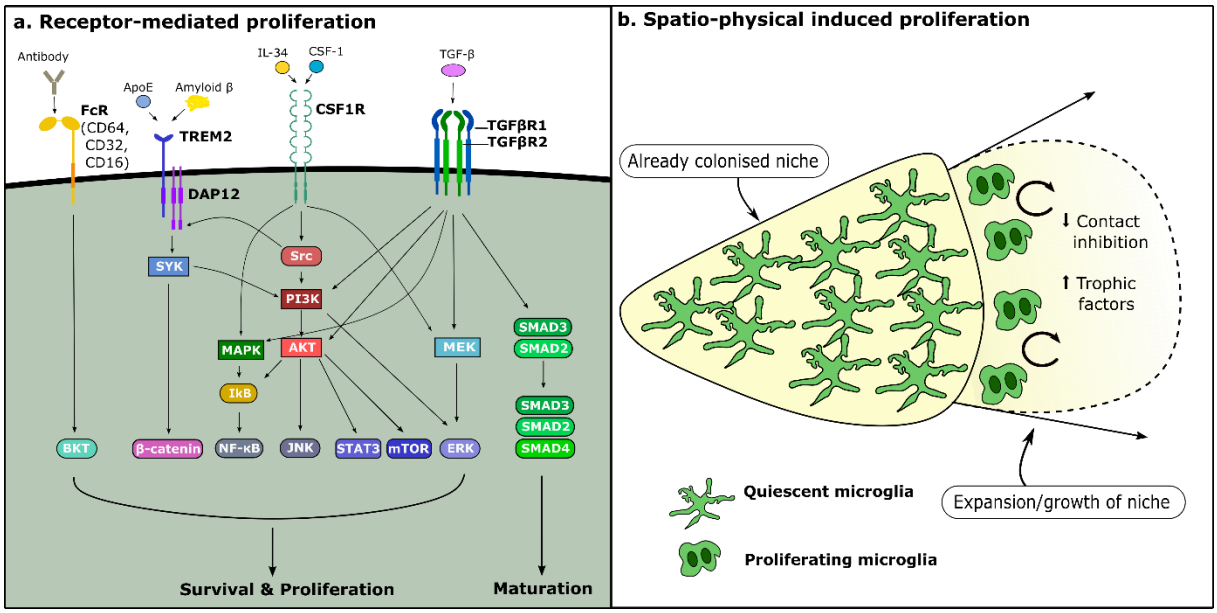
In comparison to microglia in the adult brain[72,115], embryonic and postnatal microglia a display tremendous capacity to proliferate in both humans and mice[13,75,77]. This can really be appreciated in several fate-mapping studies which show that the majority of the adult microglia population are a direct progeny of a small number of the original yolk sac progenitors[10,38,43,46,116,117]. Recent work from our group and others has shown that this profound increase in numbers is facilitated by clonal expansion[10,116]. Interestingly we observed a heterogenous distribution of clone size in the postnatal brain indicating that the proliferation potential is not equal among microglial progenitors [10]. It is unclear whether this heterogeneity in proliferation is an innate feature of certain microglial cells that may represent a stem-like niche or whether this is the product of exposure to certain pro-mitogenic signals in a timely manner. Due to methodological limitations, the clonal dynamics of microglia in the human brain are less understood. Intriguingly, microglia are observed in clusters throughout the developing human brain and a peak in their proliferative potential is observed during embryonic development [77,79] similar to clonally expanding microglia in the mouse brain [10].

Drivers and regulators of microglial proliferation

A number of trophic factors and signalling pathways have been implicated in driving and regulating microglial proliferation (**Fig. 2a**). Of interest, the trophic cytokines CSF-1 and IL-34, which act as agonists for CSF1R, can induce microglial proliferation and regulate microglial survival during development and adulthood [89,118,119]. Genetic and pharmacological approaches to blunt CSF1R signalling in the adult brain result in ablation of the microglial population [120,121]. Within the brain, glial cells are the predominant source of CSF-1 while IL-34 is mainly neuron derived [89,118]. It is important to note that while both ligands share similar tertiary structures, they have unique binding sites on the CSF1R with IL-34 displaying a higher affinity for CSF1R compared with CSF-1 [92,122]. Aside from regulating microglial survival during adulthood, a number of studies have demonstrated that the CSF1R pathway is crucial for microglial expansion during embryonic and postnatal development, as the developmental disruption of CSF1R signalling dramatically reduces the expansion of yolk sac microglial progenitors [25,43,120]. This has been elegantly demonstrated in recent studies whereby deletion of the Fms intronic regulatory element (FIRE), an enhancer within the CSF1R locus, leads to an absence of microglia due to impaired expansion and maturation of yolk sac EMPs[123-125]. CSF1R signalling leads to downstream activation of mTOR and and the transcription factor c/EBPβ which induce proliferation and survival of microglia [126-129]. However, the mechanisms by which CSF-1 and IL-34 induce proliferation are unclear, as the basal levels of CSF-1 and IL-34 expression in the adult brain are associated with microglial survival rather than proliferation[121]. Therefore, it is possible that higher concentrations of CSF-1 and IL-34 are needed to induce a proliferative response, this idea being supported by recent findings

showing that CSF-1 overexpression results in aberrant proliferation of microglial cells that is associated with an elevated density [130].

One of the other major signalling pathways involved in regulating microglial proliferation is the TGF-βR axis which plays a role in regulating the expansion of microglia in the context of development [131]. Genetic deletion of *Tgfb1* in mice was associated with a decline in the number of microglial precursors and microglial specific genes from E14.5, with no change in the number of yolk sac EMPs, suggesting that it is important for the parenchymal expansion of microglia following entry [131]. Similarly, inducible deletion of *Tgfbr2* in Cx3CR1^{+} cells between E10.5 until E16.5 significantly decreases the number of microglia in the embryonic brain [74]. This abolition of TGF-β signalling has a direct impact on microglia proliferation during embryogenesis as demonstrated by a reduction in the number of proliferating Ki67^{+} microglia at E14.5 [74]. In contrast, deletion of *Tgfbr2* during early postnatal development (P1/P3) has no immediate effect on microglia proliferation and numbers demonstrating a time-sensitive role for the TGF-βR axis [74]. Another receptor involved in driving microglial proliferation is triggering receptor expressed on myeloid cells 2 (TREM2) [14,28,132,133]. Similar to CSF1R, TREM2 has been implicated in driving the proliferative response of microglia in response to disease [14,28] and also relies on downstream signalling via DAP12 and the Akt/mTOR and c/EBPβ pathways to induce proliferation [129,134]. Certain lipid species, ApoE and amyloid-β oligomers can activate TREM2 during health and disease [135]. Evidence also suggests a synergistic action between CSF1R and TREM2, as CSF1R activation can induce phosphorylation of the adapter protein DAP12 which forms a transmembrane complex with TREM2 [129,135]. TREM2 can also promote proliferation and survival via β-catenin-dependent signalling. Loss of TREM2 from microglia results in a strong apoptotic response and induces cell-cycle arrest at the G1/S checkpoint and reduced β-catenin expression [132,133]. Interestingly, genetic deletion does not inhibit microglia expansion on the same scale as CSF1R inhibition suggesting that it might be less important for the development of microglia, although further work is needed to investigate the extent of TREM2 dependence during development [133]. An alternative family of receptors associated with microglial proliferation are the Fc receptors FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16) which are found to be important for inducing microglial proliferation in a mouse model of Multiple Sclerosis and relies on downstream activation of Bruton's tyrosine kinase (BTK) to induce a proliferative response [136]. Going forward, it will be interesting to compare the contribution of these canonical pathways during the developmental expansion of microglia. Overall, it appears that there is a significant overlap between the downstream cascades from each of these signalling pathways suggesting that there is a level of convergence in the regulation of microglia in a variety of contexts. This is corroborated by a recent study demonstrating that proliferating microglia share a similar core set of cell-cycle associated genes in a number of different developmental and disease contexts [137].



Space availability as a driver of microglial proliferation

Another potential source of pro-mitogenic cues for microglia is the availability of space or spatial niches (**Fig. 2b**). This type of influence over cellular expansion is referred to as mechanoregulation and describes a process whereby cells use their cytoskeleton to sense mechanical tension posed on them from other cells and surrounding tissue constraints [138]. This process is also known as contact inhibition, whereby proliferation will be inhibited when mechanical stress is imposed, in other words cells will not normally proliferate if they are spatially constrained [138-141]. Furthermore, a spatial checkpoint has been uncovered in epithelial cells at the G1/S phase boundary of cell division which will prevent a cell from dividing if there is not enough space available [142]. In the context of cancer, this checkpoint is overridden which allows for abhorrent tumour growth [142]. It is thought that this feature of cell division is important for reaching stable densities during development and to prevent overcrowding of cells [143].

Indeed, recent evidence suggests that the expansion of the microglial population during development may be associated with space availability as changes in microglial numbers are coupled with changes in brain size. This can be seen by studying the changes in the nearest neighbour distance which inversely correlates with the changes in cell density until a plateau is reached during the third postnatal week [10]. At this point it is thought that contact inhibition is achieved when microglia are reported to have a nearest neighbour distance between 40-50 µm[10] which has been well characterised in the adult brain and is associated with a mosaic-like distribution [144]. Potential regional differences in space availability during development may account for the variation in timings of density peaks observed across different areas of the brain [72,114]. Lack or loss of contact inhibition as a pro-mitogenic signal would also explain the tremendous ability of microglia to repopulate the brain in different paradigms of depletion where the availability of space exponentially increases [144,145]. The repopulation of

microglia continues until the space between cells is full as indicated by a reduction and eventual plateau in the nearest neighbour distance between cells [144] similar to what has been reported during development[10]. Together these findings suggest that microglia within an unoccupied territory will proliferate in order to effectively colonise the spatial niche. Note, it is expected that there would be a higher availability of trophic factors such as CSF-1 and IL-34 in an unoccupied territory compared to a colonised niche which may contribute to the promotion of proliferation[146]. Other evidence of anti-overcrowding mechanisms regulating microglial expansion stems from Vav-Bcl2 mice where apoptosis is dysregulated in myeloid cells [10,72]. In these mice, the density of microglia is significantly elevated throughout development and, unlike wild-type mice, there is no observed refinement of the microglial population. However, instead of continuing to expand in a non-controlled manner, the density of microglia eventually stabilise after the first month of postnatal development which suggests some form of anti-crowding control regulating microglial numbers [72]. Indeed, it is noted that there is a reduction in microglial territory size in order to prevent overlap between cells [10]. The fundamental mechanisms underlying microglial contact inhibition are not well characterised, although membrane-bound cell adhesion molecules (CAMs) may be involved such as Syndecan-4 and other proteoglycans [144,147].

5. Molecular determination of microglial fate in the CNS

Intrinsic and environmental regulation of microglial maturation

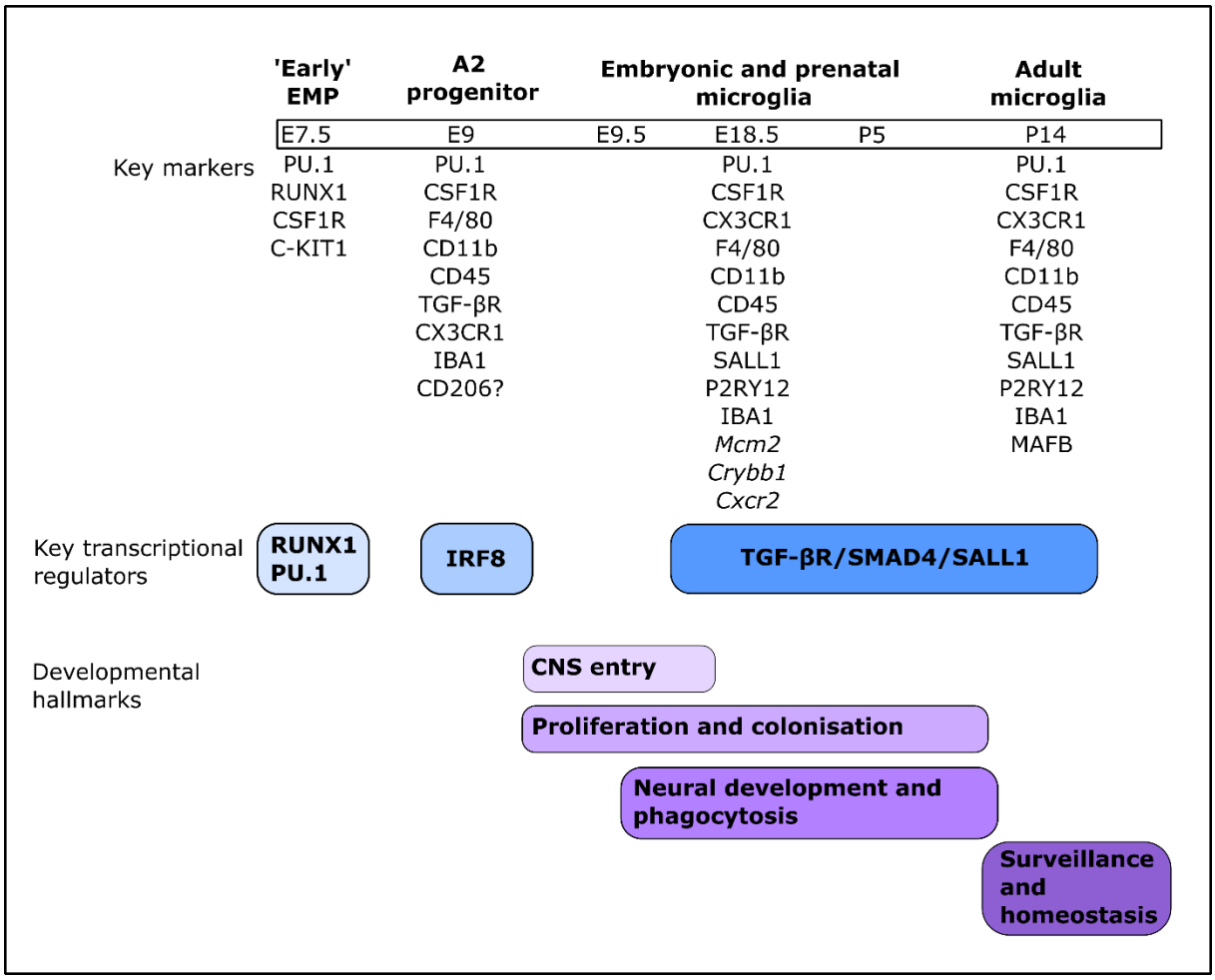
The shift in profile from early yolk sac EMP towards a canonical microglia signature is tightly regulated by a number of molecular and environmental factors which work in tandem (**Fig. 3**). The initial emergence of EMPs in the yolk sac is driven by the transcription factor RUNX1[43,148]. Concomitantly, the transcription factor PU.1 (*Spi1*) acts as a fate-defining regulator of the myeloid lineage and drives the expression of canonical myeloid markers including CSF1R, CSF-1 and CD11b in primitive microglia progenitors of the yolk sac [38,149-153]. Deletion of PU.1 inhibits the maturation and expansion of "early" and "late" yolk sac EMPs [38]. The subsequent transition of yolk sac EMPs towards "A2" microglia progenitors depends on the transcription factor IRF8, which is vital for the emergence of microglia and macrophage-specific genes such as *Cx3cr1*, *P2ry12* and *Aif1* from E9.5 [38,150,154]. The activity of IRF8 is driven by PU.1 expression [38] and deletion of *Irf8* will blunt the induction of such canonical microglial genes and also genes related to microglia 'activation' in response to immunological stimulation [38,155]. Interestingly, while this regulator appears to be important for the emergence and maintenance of the microglial phenotype, it does not regulate microglial proliferation [155-157]. Consequently, deletion of *Irf8* does not prevent microglial colonisation of the developing brain, only the emergence of the homeostatic microglial phenotype. Moreover, the development of yolk sac EMPs occurs independently

of the transcription factor Myb proto-oncogene protein (*Myb*), which is vital for the emergence of HSCs from the fetal liver [43,45-47,150].

As development progresses, there is a shift in the immature profile of yolk sac progenitors towards that of adult homeostatic microglia. This is associated with stereotypical changes in the profile and morphology of microglia which transition from amoeboid to highly ramified cells[13,43,158]. Indeed, interrogation of the transcriptome during embryonic and postnatal stages has revealed that microglia display distinct signatures at various stages of development reflecting their maturity state and temporal functions throughout this window of life [13,156,159]. The majority of embryonic microglia are enriched with genes involved in cell cycle and proliferation such as *Mcm2* indicative of their high proliferative potential, whereas late embryonic-postnatal microglia are enriched with genes involved in neurogenesis and synaptic pruning such as *Crybb1* and *Cxcr2* [13,160]. In contrast, the profile of adult microglia is characterised by the expression of traditional homeostatic genes in a MAFB-dependent manner[13]. This developmental trajectory is also reflected in human microglia which are enriched in genes relating to the cell cycle during gestational development compared with genes related to immune sensing and cytokine signalling in later gestational[47,75,77,161]. Again, the acquisition of a mature microglial phenotype is associated with MAFB in postnatal stages[75]. Another hallmark of developmental microglia is the marked heterogeneity which is in stark contrast to more uniform state observed in adult microglia [98,156,162]. This heterogeneity appears to be driven by the specific needs of local cellular milieu in various brain regions during development and highlight an important role for the microenvironment in driving the diversity and maturation of microglia during development. For example, a subset of microglia are found in close association with axonal tracts (proliferative-associated microglia (PAM)[162] or axon tract associated microglia (ATM) [98]) and are thought to be critical for the clearance of excess myelin during development. Similarly, in humans, broad changes in the microglial transcriptome can be attributed to coincidental neurodevelopmental hallmarks such as neurogenesis and myelination which further drive heterogeneity [75,77,161]. Another interesting finding is that the degree to which microglia in development express typical homeostatic microglia genes is varied, with some subtypes expressing very low or negligible levels of homeostatic genes suggesting immaturity [156,162]. Indeed, Li and colleagues reported that a small number of microglia in the postnatal brain still resemble that of embryonic microglia[162]. This suggests that in the developing brain, microglia exist on a spectrum of maturity with some closer to embryonic or adult states than others.

One of the ways in which the microenvironment can alter the phenotype of microglia is through influence on the enhancer and transcriptomic landscape of microglia. Enhancers are regulatory DNA sequences that bind transcription factors, enhancing gene expression, and are vital for lineage

differentiation and cell signature establishment[163]. Using different transplant techniques, it is possible to study how the enhancers of different cell types can change in response to a new microenvironment. In one setup, microglia and peritoneal macrophages were isolated from mice and cultured. As a result of moving these cells from their typical setting, there was a loss of their core transcriptomic signature[164]. Interestingly, supplementing these cultures with transforming growth factor β 1 (TGF-β1) restored the expression of their core microglial and macrophage signatures. The authors found that changes in the environment were associated with activation of enhancers which lead to the establishment of tissue-specific macrophage signatures[164]. These results are corroborated by a number of studies showing that TGF-β1 is vital for the development and maintenance of the microglial signature [74,131,165]. In particular, genetic deletion of *Tgfb1* in mice was associated with a decline in the number of microglial precursors and microglial specific genes from E14.5, however there was no change in the number of yolk sac EMPs highlighting the tissue-specific role of TGF-β1 signalling in the CNS[131]. Recent work has shown that the transcription factor SMAD4 is vital for TGF-β signalling and the induction of target genes including *Sall1* [166,167]. Indeed, the transcription factor *Sall1* has been shown to play a vital role in driving developmental microglia towards a homeostatic profile and also depends on TGFβ1 signalling[154,168-170]. Similar to the mouse, postnatal microglia in the human engage in TGFβ1 signalling via SMAD4 and SMAD2 dependent pathways which drive the homeostatic phenotype through *Sall1*[47,75]. Taken together these findings implement TGF-β1 as an important environmental cue that mediates the switch between the yolk sac progenitor to parenchymal microglia.



An important aspect of the microenvironment which may be important for microglial maturation and development is sex specification. Subtle regional changes in microglial density, morphology and function have already been described between male and females[171,172]. However, there are conflicting reports with regards to the influence of sex on microglia maturation with only a small number of differentially expressed genes reported between male and female microglia during embryonic and postnatal stages suggesting that the developmental trajectory is unaffected by sex[98,173,174]. Another source of microenvironmental influence on maturation comes from the more distal microbiome [27,175]. Several studies have shown that in germfree (GF) mice that lack a microbiome, the normal maturation of microglia is altered whereby cells display an immature phenotype [13,175]. Further work has demonstrated that this influence of microbiota occurs in a sex-specific and time dependent manner whereby the profile of microglia from male GF mice was significantly dysregulated compared to microglia from females during embryonic stages[27]. Lack of a microbiome is also associated with an increased number of microglia during development[27,176]. The exact nature of this microbiome influence on microglial development remains to be deciphered. Other perturbations in the normal environment such as maternal exposure to infection have a profound impact on the maturation of embryonic microglia and was found to be associated with a more advanced or mature transcriptomic signature[13].

Moreover, these mice display abnormal behaviours associated with ASD[13,15]. Another interesting report demonstrated that the stepwise development of microglia is halted in mice lacking resident CD4 T cells and is associated with memory deficits and anxiety-like behaviour[177]. It is currently unknown which CD4 T cell-derived signals or interactions are required for microglia maturation. Altogether these findings suggest that the normal maturation of microglia is heavily influenced by the microenvironment and that disruption to the normal developmental program of microglia via environmental influences can contribute to the aetiology of neurodevelopmental disorders.

Molecular determination of CNS macrophage fate

Up until recently, it was postulated that other notable CNS macrophage populations such as BAMs derive from blood-borne precursors distinct from parenchymal microglia[178]. However, this notion has been challenged in recent years and it is now considered that microglia and certain BAM subsets such as those at the meninges derive from common "early" yolk sac EMPs[45,74,117,179]. This shared origin between microglia and BAMs has been described in a recent study utilising *in vivo* barcoding of early yolk sac progenitors within the brain at E9.5. Subsequent reconstruction of clones at a single-cell level revealed that microglia and BAMs were derivatives of the same progenitor cells in the yolk sac[116]. Moreover, work from Utz et al., demonstrates that similar canonical genes are expressed by both progenitors, however, there is an emergence of a subset-specific phenotype throughout development[74]. A question of interest is whether or not yolk sac progenitors possess the ability to transition between a BAM or microglia fate considering the aforementioned influence of environmental signals on cell fate of microglia. One possibility is that the fate of specific yolk sac progenitors is already predefined prior to colonisation. Indeed, the observation that CD206 is enriched in certain yolk sac progenitors which go on to form the BAM niche and absent from progenitors giving rise to microglia support this claim [74,125]. Similarly, in the human brain, BAMs are defined by their expression of CD206[75]. In contrast, the discovery of an Ms4a7^{+} subset of microglia at E14.5 which share a significant overlap in signature with BAMs suggests that there is plasticity between BAM and microglia states during development[98]. In support of this, a recent fate mapping study with the inducible Mrc1^{CreER} strain to target CD206 expressing cells arising at E9 revealed that CD206^{+} yolk sac progenitors mature into both BAM and microglia[179]. This transition has been elegantly demonstrated in a series of live imaging experiments which show that intraventricular CD206^{+} BAM progenitors can enter the pallium of the embryonic brain up until at least E12.5[87]. These seeming BAM progenitors transition to *bona fide* microglia following their entry to the parenchyma after 4 hours as defined by a loss of CD206 expression and gain of P2RY12 suggesting an important role of environmental influence on cell fate[87]. One such signal required for the BAM-to-microglia transition may be TGFβ1, as abolition of the TGFβ1/TGFBR2 signalling axis and downstream transcription factor, SMAD4, halts the

emergence of microglia whereas the expansion of BAMs is unaffected[74,166]. Taken together, these findings challenge the idea of hardwired $CD206^+$ BAM or $CD206^-$ microglia subsets within the yolk sac. Further work will be required to unravel the extent of this potential plasticity in cell fate and whether microglia retain the potential to transition to BAMs.

Altogether, it is evident that the establishment of the homeostatic microglia signature relies on a number of external factors in a time-dependent manner during development. Such findings highlight the importance of understanding what 'normal' microglia development looks like and act as a benchmark for healthy development.

6. Conclusion

Our knowledge regarding the development of microglia and their processes during this important window of life has increased massively during the last decade. This has been facilitated in part by the wider availability of fate-mapping models in rodents and an increased access to rare human foetal and postnatal tissues. Here we outlined several key molecular and non-molecular mechanisms governing microglial expansion and colonisation in the healthy brain. Additional characterisation of such mechanisms may provide further clarity about the aetiology of certain NDDs and potential targets for the treatment or prevention of such disorders. Another interesting avenue includes the study of how space availability, as a biophysical mechanism, may regulate microglial expansion as currently most of the evidence supporting this hypothesis is anecdotal or derived from *in vitro* studies. Therefore, further work will be needed to understand the biomolecular properties of this predominantly 'physical' theory of microglial expansion. In general, the influence of biophysical mechanisms influencing brain development is an untapped area with significant potential, considering the intrinsic challenges of growing a highly complex and specialised organ. The advent of more advanced techniques, nested within multidisciplinary teams, will allow uncovering some of these mechanisms. Finally, the field of study of microglial cells in development, ageing and disease, still relies extensively on the use of mouse as a model organism, and the future will need to accelerate the study of human microglia in context. Gaps in our knowledge with regards to microglial origin and development in the human require further attention and perhaps the combined use of iPSCs and 3D cell culture systems will help to address these burning questions. It is now clear that mouse and human microglia are very distinct in terms of identity and dynamics, and it is time for our field to pivot to studying the human brain.

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8. References

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9. Figure Legends

Figure 1 Entry and colonisation of microglia to the embryonic brain.
a, Microglia progenitors enter the brain via trans-tissue and trans-vascular routes and may rely on CSF-1-mediated attraction in the parenchyma. subsequently trafficked to the brain through the vasculature with the onset of circulation. **b,** Colonisation and migration of microglia through the parenchyma is facilitated by a number of processes including para-vascular migration, chemotaxis towards various

cytokines and trophic factors and cell to cell interactions. **c,** During early embryonic development, microglia primarily gain entry to the brain through the vasculature and are sparsely distributed in the brain[43,80]. **d,** Following closure of the BBB, microglia proliferate in situ and colonise the developing brain in accordance with local environmental input such as in the cortical plate where they are transiently absent[25,86] and can be observed accumulating near axonal tracts[25]. **e,** By late embryonic stages, microglia are found in almost all brain regions and can be observed in close proximity to meningeal and ventricular border zones and renter the cortical plate following their transient absence[86]. Microglia also display both dispersed and clustered spatial patterns throughout the parenchyma[10]. **f,** By adult stages (P21) microglia display a mosaic distribution within the cerebral cortex[10]. Regional differences in morphology and density can also be observed[72].

Figure 2 Overview of drivers of microglial proliferation.

a, The proposed receptor-mediated signalling pathways involved in driving proliferation of microglia during development and disease. There is a high level of convergence between CSF1R, TREM2 and TGFβR signalling cascades which act via AKT and MEK/ERK pathways to induce survival and proliferation of microglia. FcR receptors can also induce microglia proliferation via BKT signalling. SMAD-dependent TGFβR signalling is involved in microglia maturation. **b,** Potential spatio-physical niche-like model of microglial proliferation. As a niche expands, microglia progenitors may experience a loss of contact inhibition and higher availability of trophic factors driving proliferation of cells at the frontier of the expanding spatial niche.

Figure 3 The stepwise maturation and diversity of microglia during development.

'Early' EMPs arising in the yolk sac from E7 rely on the transcription factors PU.1 and RUNX1 for expression of a myeloid profile. Maturation of EMPs towards 'A2' microglia progenitors at E9 relies on IRF8 which leads to the expression of several canonical microglia/macrophage genes. Having infiltrated the CNS, microglia progenitors lose any expression of CD206 and further acquire typical microglia markers which relies on TGF-βR signalling and downstream transcription factors SMAD4 and SALL1. Embryonic and prenatal microglia also express a number of genes related to cell cycle and neurogenesis. By P14 the majority of microglia display a transcriptomic profile akin to adult microglia that is dependent on the transcription factor MAFB. The adult microglia phenotype is associated with homoeostatic and surveillance functions.