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SMOLDERS, Sophie; KESSELS, Sofie; VANGANSEWINKEL, Tim; RIGO, Jean-Michel; Legendre, Pascal & BRONE, Bert (2019) Microglia: Brain cells on the move. In: PROGRESS IN NEUROBIOLOGY, 178, p. 60-80 (Art N° 101612).

DOI: 10.1016/j.pneurobio.2019.04.001
Handle: http://hdl.handle.net/1942/29638

Total number of words in this manuscript: 15,999

Microglia: brain cells on the move

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Highlights

- Microglia are immigrant CNS immune cells that show motility and mobility, especially in the developing brain
- Microglial motility and mobility is likely steered by the constantly changing microenvironment of the CNS
- Microglia populate the developing CNS by proliferation and intensive migration, of which the molecular mechanisms have just started to be unravelled
- Microglia actively participate in CNS development and homeostasis, even at early developmental stages
- Defective microglial functions during development can be related to neurodevelopmental disorders

Abstract

In the last decade, tremendous progress has been made in understanding the biology of microglia - i.e. The fascinating immigrated resident immune cell population of the central nervous system (CNS). Recent literature reviews have largely dealt with the plentiful functions of microglia in CNS homeostasis, development and pathology, and the influences of sex and the microbiome. In this review, the intriguing aspect of their physical plasticity during CNS development will get specific attention. Microglia move around (mobility) and reshape their processes (motility). Microglial migration into and inside the CNS is most prominent throughout development and consequently most of the data described in this review concern mobility and motility in the changing environment of the developing brain. Here, we first define microglia based on their highly specialized age- and region-dependent gene expression signature and associated functional heterogeneity. Next, we describe their origin, the migration route of immature microglial cells towards the CNS, the mechanisms underlying their invasion of the CNS, and their spatiotemporal localization and surveying behaviour inside the developing CNS. These processes are dependent on microglial mobility and motility which are determined by the microenvironment of the CNS. Therefore, we further zoom in on the changing environment during CNS development. We elaborate on the extracellular matrix and the respective integrin receptors on microglia and we discuss the purinergic and molecular signalling in microglial mobility. In the last section, we discuss the physiological and pathological functions of microglia in which mobility and motility are involved to stress the importance of microglial 'movement'.

List of abbreviations

ASD, autism spectrum disorders; BBB, blood brain barrier; BDNF, brain-derived neurotrophic factor; CCL, C-C motif chemokine ligand; CCR, cysteine-cysteine chemokine receptor; CD, cluster of differentiation; CSF, colony stimulating factor; CSPG, chondroitin sulphate proteoglycan; CXCL, chemokine (C-X-C motif) ligand; CXCR, C-X-C chemokine receptor; DAP12, DNAX activation protein of 12kda; E, embryonic day; ECM, extracellular matrix; FAK, focal adhesion kinase; FN, fibronectin; GDNF, glial-derived neurotrophic factor; GM-CSF, granulocyte-macrophage colony stimulating factor; Hoxb8, Homeobox protein b8; IFN, interferon; IGF, insulin-like growth factor; IL, interleukin; inos, inducible nitric oxide synthase; $ipla_2$, intracellular Ca^{2+}-independent phospholipase A2; KO, knockout; LM, laminin; LPS, lipopolysaccharide; M1, "classically" activated macrophages; M2, "alternatively" activated macrophages; MCP, monocyte chemotactic protein; MECP2, methyl-cpg-binding protein 2; MIA, maternal immune activation; MMP, matrix metalloproteinases; NADPH, nicotinamide adenine dinucleotide phosphate; NF-κb, nuclear factor κb; NO, nitric oxide; NOS2, nitric oxide synthase 2; NOX2, nicotinamide adenine dinucleotide phosphate oxidase 2; NT, neurotrophin; P, postnatal; PCD, programmed cell death; PI3K, phosphatidylinositide 3-kinase; pnns, perineuronal nets; POA, preoptic area; Poly (I:C), polyinosinic:polycytidylic acid; RGD, arginine-glycine-aspartic acid; SDF, stromal cell-derived factor; SVZ, subventricular zone; TGF, transforming growth factor; TLR, toll-like receptor; TNF, tumour necrosis factor; TTX, Tetrodotoxin; VASP, vasodilator-stimulated phosphoprotein; VEGFR1, vascular endothelial growth factor receptor 1; VZ, ventricular zone

Keywords

Microglia; CNS; Migration; Motility; Phagocytosis; Molecular signalling.

Outline

1. Introduction

Microglia are often referred to as the resident macrophage population in the central nervous system (CNS) where they perform essential tasks during development and homeostasis. In the healthy adult CNS, microglia adopt a ramified morphology characterized by a small cellular body and multiple long thin branched processes that can extend up to 50 µm from the soma (Arnoux et al., 2013; Kozlowski & Weimer, 2012; Nimmerjahn et al., 2005). Ramified microglia were classically defined as 'resting' cells, but this view was challenged by the groundbreaking research of Axel Nimmerjahn and Dimitri Davalos in 2005. Through cranial windows (thinned skull preparation) in the adult mouse, they observed highly motile microglial processes constantly protruding and retracting. Using this active scanning behaviour, microglia were proposed to survey the entire brain in just a couple of hours (Davalos et al., 2005; Nimmerjahn et al., 2005). During embryonic development and upon encountering activating stimuli, microglia may take up the amoeboid-like morphology which is characterized by a largely rounded soma with fewer, thicker and shorter processes (Kozlowski & Weimer, 2012; Mosser et al., 2017b; Rigato et al., 2011). It should be noted that a large continuum of microglial morphologies exists between these two extremes, and that their morphology is not necessarily linked to their function (Arnoux et al., 2013; Hagemeyer et al., 2017; Hanisch, 2013; Karperien et al., 2013; Olah et al., 2011; Scheffold et al., 2016; Streit et al., 2014).

Although almost a century old, Del Rio-Hortega's postulations about the microglial capacity to migrate and to phagocytose still hold true today (Del Rio-Hortega, 1919; Kettenmann et al., 2011). Despite the recent advances in understanding the various roles of microglia in brain development and homeostasis, many aspects of the cellular and molecular physiology underpinning their migration and process motility remain unresolved. In addition, the relation

between migration, branch motility, and their physiological functions remains highly speculative. In this review, we first define microglia as a heterogeneous population in place and time within the CNS based on their transcriptome and functions (chapter 2). Next, the origin of microglia (chapter 3) and their invasion in the CNS is discussed with a clear view on their migration route (section 4.1) and molecules that attract them towards the CNS (section 4.2). Their migration pathway (section 4.3) and behaviour (section 4.4) within the developmental brain is further discussed in detail, focusing on the interaction of microglia with their local environment, which is region-dependent and changes over time (section 4.5). In this regard, we bring forth new hypotheses from which molecules and developmental processes might steer microglial movement. The microglial maturation process and sexual dimorphisms conclude the fourth chapter (section 4.6 and 4.7). The molecular mechanisms underlying microglial movement in the brain are discussed in chapter 5. Finally, the importance of microglial mobility and motility is addressed in the frame of the physiological functions of microglia in development (chapter 6) and neurodevelopmental disorders (chapter 7).

2. The microglial gene signature and functional heterogeneity

Microglia are the brain parenchymal macrophages that possess a highly specialized gene expression signature which may vary depending on the region where they reside in the CNS, age and sex of the organism (Buttgereit et al., 2016; Crotti & Ransohoff, 2016; Galatro et al., 2017; Gautier et al., 2012; Gosselin et al., 2014; Grabert et al., 2016; Guneykaya et al., 2018; Hanamsagar et al., 2017a; Hickman et al., 2013; Holtman et al., 2015; Lavin et al., 2014; Matcovitch-Natan et al., 2016; Nelson et al., 2018; Silvin & Ginhoux, 2018; Thion et al., 2018b; Villa et al., 2018; Wes et al., 2016). The transcriptomic differences underscore the functional

heterogeneity of the microglial population. The different tasks microglia fulfil depending on the developmental needs may be reflected in their transcriptional signature. The distinct functions of microglia in both development and adulthood have been extensively reviewed (Lenz & Nelson, 2018; Schafer & Stevens, 2015; Silvin & Ginhoux, 2018; Tay et al., 2017). Indeed, microglia have unique gene expression profiles depending on the age of the organism (Bennett et al., 2016; Matcovitch-Natan et al., 2016). Matcovitch-Natan et al. (2016) discriminated three groups of microglia in mice dependent on the differential expression of genes across developmental time points: "early microglia" (embryonic day (E)10.5 - E14), "pre-microglia" (E14 – postnatal day (P)9) and "adult microglia" (4 weeks postnatal and onwards) (Figure 1). Early microglia are characterized by their high expression of genes involved in cell cycling and differentiation, such as *Mcm5 and Dab2*, while expression of cytokine *Csf1* and chemokine receptor *Cxcr2* peaked in pre-microglia. Adult microglia are characterized by their high expression of genes for the fractalkine receptor *Cx3cr1,* lysosomal enzyme *Hexb*, transcriptional regulator *Sall1*, transmembrane protein *Tmem119* and transforming growth factor *(TGF)*-β*1* (Bennett et al., 2016; Butovsky et al., 2014; Buttgereit et al., 2016; Goldmann et al., 2016; Matcovitch-Natan et al., 2016). *Sall1* is exclusively expressed by the parenchymal microglia population and by no other CNS-resident (meningeal macrophages, perivascular macrophages and choroid plexus macrophages) or peripheral macrophage populations, and is therefore the preferred gene to perform microglia specific manipulations *in vivo* (Buttgereit et al., 2016; Goldmann et al., 2016). However, it must be noted that neuronal and glial progenitors during mouse embryogenesis highly express *Sall1*, which unfortunately precludes this gene for microglia-specific approaches during development (Harrison et al., 2012). In the steady state, microglia specific depletion of *Sall1* or *TGF*-β*1* resulted in a shift from a resting microglia to an inflammatory macrophage phenotype (Buttgereit et al., 2016; Zoller et al., 2018). Thus, *Sall1*

and *TGF-β1* are key genes that keep mouse microglia in a homeostatic phenotype. Furthermore, microglial homeostatic functions including phagocytosis and immune surveillance fail upon loss of CX3CR1 signalling in mice (Liang et al., 2009; Raoul et al., 2008), thereby adding Cx3cr1 to the microglial homeostatic gene signature. However, it must be noted that the homeostatic microglial signature is lost in pathological processes such as neurodegeneration, neuroinflammation and peripheral immune challenges, which indicates that the microglial signature might not be entirely stable under all circumstances (Hirbec et al., 2018; Holtman et al., 2017; Smolders, S. Et al., 2018; Zrzavy et al., 2017). Changes in the expression profile of mouse microglia upon neurodegeneration correlate well to the human situation (Holtman et al., 2015; Keren-Shaul et al., 2017; Krasemann et al., 2017). However, the overlap in aging profiles between mouse and human microglia is limited (Galatro et al., 2017), implicating that data from studying aging of microglia in mice must be interpreted with care and cannot be directly extrapolated to human microglia.

In addition to age-dependent changes, microglia show regional transcriptional heterogeneity. For example microglia from the cortex, spinal cord, hippocampus, and olfactory bulb show subtle differences in gene expression levels, while microglia from the cerebellum and eyes seem to be less comparable to microglia from previously mentioned regions of the adult mouse brain (Butovsky et al., 2014). Additionally, Grabert et al. (2016) showed that adult mouse microglia have distinct region-dependent transcriptional identities. In contrast, recent deep single-cell RNA sequencing data suggest that adult mouse microglia with homeostatic signatures have a remarkable similar transcriptome, regardless of the examined brain region (Li et al., 2018). The observed regional differences in adult mouse microglia shown in Butovsky et al. (2014) and Grabert et al. (2016) may arise from genes expressed by small percentages of TMEM119$^{low/-}$ microglia which were not included in the study of Li et al. (2018) or might be due

to impurities in the selected microglia populations. Interestingly, Li et al. (2018) showed CNS region-dependent transcriptome differences in P7 mouse microglia. Morphology, lysosome content, membrane properties, and transcriptomes also differ between microglia from distinct mouse basal ganglia nuclei, starting from P12 (De Biase et al., 2017). The transcriptional control of microglial phenotypes in healthy and pathological circumstances was recently reviewed (Holtman et al., 2017) and the sex-dependent differences are discussed in section 4.7 of this review.

3. Origin of microglia

Until the 1990s, researchers heavily debated whether microglia are ectodermally or mesodermally derived cells (Ginhoux et al., 2013). Later in 2006, it turned out Del Rio-Hortega had been right all these years about their mesodermal origin. Mice lacking the transcription factor Pu.1, which is a key regulator of hematopoietic development (Iwasaki et al., 2005), were also devoid of microglia (Beers et al., 2006; mckercher et al., 1996). At that point, the hematopoietic - and thereby mesodermal - nature of microglia was established.

In the mouse embryo, starting at day 8.5 post conception (E8.5), erythromyeloid progenitors develop in the yolk sac depending on the expression of the transcription factors Pu.1 and stem cell leukemia/T-cell acute lymphoblastic leukemia 1 (Scl/Tal-1) (mcgrath et al., 2015; Perdiguero & Geissmann, 2016). These cells express tyrosine-protein phosphatase cluster of differentiation (CD)45 and tyrosine-protein kinase c-Kit, and have the capacity to colonize the fetal liver and differentiate into erythrocytes and various myeloid cells, including tissue-resident macrophages. A subset of erythromyeloid progenitors matures into CX3CR1+ cells in the yolk sac and become microglial progenitors. These progenitors migrate into the brain

between E9.5 and E14.5 in the mouse embryo around the time the blood-brain barrier (BBB) is formed. By E11.5, they invade the spinal cord parenchyma (Rigato et al., 2011). Microglia in the embryonic CNS express CX3CR1, CD45 and adhesion G-protein coupled receptor F4/80 (Ginhoux et al., 2010; Kierdorf et al., 2013). Recently, the presence of a second microglia population was established in mice. In contrast to the canonical, non-*Hoxb8* microglia, *Hoxb8* microglia progenitors appear to be generated during the second wave of yolk sac hematopoiesis. They greatly expand in the aorta-gonad-mesonephros and fetal liver, and invade the brain by E12.5 (De et al., 2018). In humans, microglia start to invade the forebrain around 4.5 to 5.5 gestational weeks (Menassa & Gomez-Nicola, 2018; Monier et al., 2007; Verney et al., 2010) and the major influx and distribution starts around 16 weeks (Menassa & Gomez-Nicola, 2018; Rezaie et al., 2005; Rezaie & Male, 1999; Verney et al., 2010). Although some doubt persisted in the last decade about the origin of microglia renewal and of their local expansion in the adult, it was recently established that the maintenance and local expansion of microglia in the healthy adult brain depends exclusively on the self-renewal capacity of CNS-resident cells, with no requirement for influx of circulating progenitors from the blood or bone marrow (Ginhoux & Guilliams, 2018). Recent literature reviews provide more details on the ontogeny of microglia (Hoeffel & Ginhoux, 2018; Low & Ginhoux, 2018; Prinz et al., 2017; Thion et al., 2018a).

4. Microglia are immigrated workers in the CNS

After microglial precursors are born in the yolk sac, they travel to the CNS via the developing blood vessel network (Ginhoux et al., 2010). Microglia infiltrate the brain by crossing the developing barriers and once arrived, they populate the CNS parenchyma by proliferation and

migration. For the sake of clarity in this review, we distinguish here between migration, which is the active displacement of the cell body by interacting with and applying force to the surrounding environment, and motility, which refers to the movement of the cell's processes or protrusions in order to scan the parenchyma.

4.1 Migration route of microglial precursors to and into the CNS

The time of microglial entry and subsequent invasion of the CNS is region-dependent. The colonization of the embryonic mouse retina occurs in two invasion waves, with the first one occurring between E11.5 and E12.5, and the second one from E12.5 on (Santos et al., 2008). At E12.5, a massive rise in microglial cell number was also observed in the embryonic mouse spinal cord (Rigato et al., 2011). In the embryonic mouse cortex, the microglial colonization process occurs in three phases: the first from E10.5 to E14.5 where microglial cell numbers increase modestly, followed by a steep increase from E14.5 to E15.5, and again a slow expansion phase from E15.5 to E17.5. Although more than 20% of the microglial cells remain actively proliferating in the cortical parenchyma from E11.5 to E13.5, this percentage steeply decreases from E14.5 on (Swinnen et al., 2013). This suggests that proliferation of resident microglia plays an important role in the first invasion phase but lesser in the second one. The steep increase in microglial density in the second phase from E14.5 to E15.5 is probably the result of microglia entering the parenchyma from peripheral sources, since immunoreactivity for Ki-67 shows that microglial precursor cells are highly proliferative before invading the embryonic brain and spinal cord (Rigato et al., 2011; Swinnen et al., 2013). Thus, the overall microglial colonization process is mainly dependent on microglial migration towards the CNS instead of proliferation inside the CNS (Figure 1) (Ashwell, K., 1989; Ashwell, K.W. et al., 1989; Billiards et al., 2006;

Figure 1

Dalmau et al., 2003; Marin-Teva et al., 1998, 1999; Rezaie et al., 1999; Rigato et al., 2011; Sanchez-Lopez et al., 2004; Tay et al., 2017).

The exact infiltration route into the CNS and related molecular mechanisms are not yet fully known, but histological studies mainly from birds and humans suggest that microglia might enter the brain from the meninges, ventricles and through blood vessels (Mosser et al., 2017b; Pont-Lezica et al., 2011; Tay et al., 2017). These proposed entry routes are based on the high microglial density in the meninges (Cuadros & Navascues, 1998; Monier et al., 2007), the presence of microglia inside the ventricles (Monier et al., 2007), and their close association with blood vessels (Pont-Lezica et al., 2011). Also in mice it has been suggested that microglial progenitors may enter the parenchyma by crossing the blood vessel wall before E16.5. Before this embryonic age, the brain is already vascularized, while the BBB is not fully established yet (Cuadros & Navascues, 1998; Hagan & Ben-Zvi, 2015; Rigato et al., 2011). In addition, microglia populate the brain mainly from “hot spots" near the pial membrane and the parenchyma lining the ventricle towards the inner zones of the cortex, suggesting that also the invasion routes via the meninges and ventricles are applicable in mice. From E15.5 on, during the third invasion phase, microglial cells are mainly located in the ventricular and intermediate zones of the cortical wall, and this localization is possibly linked to their role in the proliferation and development of the progenitor cells located in the ventricular zone of the cortex. Indeed, *in vitro* primary cultures indicate that microglial cells can influence progenitor proliferation as well as neurogenesis, astrogenesis and oligodendrogenesis (Antony et al., 2011b; Shigemoto-Mogami et al., 2014; Walton et al., 2006; Yuan et al., 2017).

During the first two postnatal weeks in mice, microglial cell numbers significantly increase in different brain regions with a peak in density at P14 (Figure 1) (Alliot et al., 1999; Arnoux et al.,

2013; Askew et al., 2017; Garay et al., 2012; Kim, I. Et al., 2015; Nikodemova et al., 2015; Paolicelli et al., 2011). This postnatal increase in microglial cell numbers is due to *in situ* proliferation of microglial cells which can be regulated by granulocyte-macrophage colony stimulating factor (GM-CSF), colony stimulating factor (CSF)-1, neurotrophin-3 (NT-3), interleukin (IL)-4 and 5 and migration inhibitory factor 1-α (Arno et al., 2014; Mosser et al., 2017b; Navascues et al., 2000). Despite variations across CNS regions, species and proliferation marker used, microglial cell proliferation seems to decrease steadily from the second postnatal week in rodents (Figure 1) (Dalmau et al., 2003; Eyo et al., 2015b; Mosser et al., 2017b; Xavier et al., 2015). It is noteworthy that Askew et al. (2017) reported an infiltrating monocyte wave into the parenchyma that peaked at P3 in mice. However, this population quickly underwent apoptosis between P3 and P6 and did not contribute to the adult microglial population. Further knowledge about the function of this monocyte-derived wave is lacking, though the authors speculate that these cells have a role in inducing cell death in a subpopulation of yolk sac-derived microglia (Askew et al., 2017). Nevertheless, multiple lines of evidence in rodents indicate that the adult microglial density is acquired after a rapid decrease of 50% in cell number between the third and the sixth postnatal week resulting from decreased proliferation and increased apoptosis, to remain steady during adulthood (Figure 1) (Askew et al., 2017; Nikodemova et al., 2015; Paolicelli et al., 2011; Tay et al., 2017).

4.2 Attracting molecules for microglia entry into and colonization of the CNS

The mechanisms underlying microglial recruitment into the CNS have been partly assessed. Signalling of IL-34 and CSF-1 through their receptor CSF-1R in microglial is essential for microglial survival and precursor entry into the mouse CNS (Elmore et al., 2014; Ginhoux et al., 2010). Whether these attractants are required for microglial progenitor production in the yolk

sac, to travel to the brain or to infiltrate the CNS, remains unclear (Ueno & Yamashita, 2014). *IL-34* mRNA is detectable at E11.5 in the embryonic mouse brain and precedes the expression of *Csf-1* mRNA (Wei et al., 2010). Interestingly, both *IL-34* and *Csf1* transcripts are expressed in the cortex during development, but in complementary regions (Nandi et al., 2012). At E15.5, *IL-34* is restricted to the marginal zone while *Csf-1* is present in the subventricular and the ventricular zone. From P0-P20, *IL-34* is found in cortical layers V to II, while *Csf-1* expression is restricted to layer VI. These complementary expression patterns suggest different functions of these CSF-1R ligands during embryonic CNS invasion by microglia. Furthermore, IL-34 attracts microglial precursors to the proximal brain regions of zebrafish larvae. Embryonic macrophages in IL-34- and CSF1Ra-deficient zebrafish larvae fail to colonize the CNS, but their initial development and colonization of peripheral tissues remain largely unaffected (Wu, S. Et al., 2018).

Additional studies have shed light on the attractive cues for microglia inside the cortex by using multiple mouse gene knockouts (KO) and inhibitor approaches *in vivo* (Arno et al., 2014; Lelli et al., 2013). Microglia in the mouse embryonic cortex accumulate in the ventricular zone/subventricular zone (VZ/SVZ) starting from E14.5, which are regions where C-X-C motif chemokine 12 (CXCL12, alternatively stromal cell-derived factor (SDF) 1) is highly expressed by basal progenitors (Arno et al., 2014). Ablation of basal progenitors as well as impairing their CXCL12 production through genetic depletion or pharmacologically interfering with its signalling pathway resulted in local decreases in microglial density in the mouse embryonic cortex (Arno et al., 2014). Both CXCR4 and CXCR7 are receptors that bind CXCL12, but CXCR4 appears to be the most important receptor to induce signalling in rodents (Arno et al., 2014; Lipfert et al., 2013; Schonemeier et al., 2008; Yoshida, 2003). Indeed CXCR7 expression cannot compensate for the loss of CXCR4 in rats (Lipfert et al., 2013), and importantly it has been

shown that microglial cells do not express CXCR7 in the developing and adult rat brain (Schonemeier et al., 2008). Accordingly, the CXCL12/CXCR4 signalling axis was proposed to be involved in microglial recruitment to the VZ/SVZ. Notably, CXCR4 and CXCL12-induced migration was reported in both microglial cell lines and primary cultures (Lipfert et al., 2013; Lu et al., 2009; Tanabe et al., 1997). Additionally, *ex vivo* brain slice imaging revealed that this chemokine axis is also important for the surveying behaviour of microglia in the mouse embryonic cortex (E14) (Hattori & Miyata, 2018). Other chemokines were proposed to be involved in microglial recruitment to the CNS as well. Yolk sac progenitors express high mRNA levels of various chemokine and chemokine receptor pairs (i.e. Cxcl4 and Cxcr3, Cx3cl1 and Cx3cr1, Ccl2 and Ccr2, Ccl9 and Ccr1), however none of these genes influenced microglial numbers and morphology in the embryo at E14 in mouse single KO strains (Kierdorf et al., 2013).

Microglial cell density in CX3CR1 KO mice was increased in the subcortical white matter at P5 (Ueno et al., 2013), while it was transiently decreased in both the postnatal hippocampus between P8 and P28 (Paolicelli et al., 2011) and in the somatosensory barrel cortex at P6-P7. However, due to the unchanged microglial cell density in CX3CR1 KO mouse embryos (Hoshiko et al., 2012), the CX3CR1 pathway is unlikely to be involved in brain colonization by microglia precursors during the embryonic developmental period. Microglial density was also decreased in the SVZ of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 2 (NOX2) deficient P3 mice. Again, such a defect was not observed in the embryos and thus was not due to a decrease in microglial proliferation rate. NOX2 acts downstream of vascular endothelial growth factor receptor 1 (VEGFR1) and CSF-1R signalling in primary microglia. Accordingly, microglial precursors inside the ventricle of P3 mice that lack the VEGFR1-tyrosine kinase domain necessary for signalling of this receptor did not show NOX2 activation. Consequently, microglial

infiltration into the SVZ was decreased. Presumably, hydrogen peroxide generated by NOX2 at the front edge of microglial extensions controls the remodelling of the actin cytoskeleton and promotes microglial migration in mice. However, such a consequence of the absence of NOX2 activation was not observed in the mouse embryo and no longer observed at P10. At this age, microglial distribution was restored, possibly by a combination of the proliferation and migration of nearby microglial cells. Thus, VEGFR1-mediated NOX2 activation-dependent microglia infiltration into the SVZ from the lateral ventricles is a transient phenomenon that occurs during early mouse postnatal developmental stages (P0-P3) only (Lelli et al., 2013).

Progranulin-a, a soluble growth factor expressed inside the early developing brain (24 hours post fertilization), possibly attracts microglia into neuroepithelial tissues of *in vivo* developing zebrafish. In this case, decreased proliferation of microglial precursors outside the brain cannot be excluded as the cause for decreased numbers of retinal microglia in the absence of progranulin-a since besides cell migration, this molecule stimulates cell proliferation (Walsh & Hitchcock, 2017). *In utero* imaging of conditional microglia-specific knockout animals may be valuable in addressing the chemokine and growth factor pathways involved in steering microglial migration. More feasible approaches for a first-base screening may be the use of several chemoattractant/chemorepellent knockout or functional mutation (loss and gain of expression) models to determine the cues for microglial dispersion within the brain.

In addition to chemokines and growth factors, factors released by apoptotic neurons during naturally occurring programmed cell death (PCD) were proposed to attract microglial cells inside the brain. Microglia have been found to be in close association with apoptotic cells in different CNS regions during several developmental stages of different animal models. Factors released by dying neurons such as CXCL1, lysophosphatidylcholine, sphingosine 1 phosphate,

adenosine triphosphate (ATP) and uridine triphosphate were proposed to act as chemoattractants for microglial cells (Mosser et al., 2017b). Notably, ATP also serves as an extracellular stress signal without the occurrence of cell death, hence inducing rapid microglial responses that include process extension towards locally released ATP (Davalos et al., 2005; Orr et al., 2009). The underlying mechanisms for ATP signalling in microglial dynamics are discussed in sections 5.2 and 5.3 and extensively reviewed in (Rodrigues et al., 2015). Additionally, ATP plays a critical role in pain signalling (Tsuda et al., 2010). Xu et al. Found that apoptotic neurons and lysophosphatidylcholine promoted microglial colonization in the developing zebrafish optic tectum (3-5 days post fertilization) (Xu et al., 2016). In quails, microglial entry and dispersion into retinal explants relies on purinergic signalling by extracellular ATP and uridine diphosphate release and coincides with an increase in retinal cell death, suggesting that microglial cells are attracted towards the dying cells (Martin-Estebane et al., 2017). In the mouse embryo, accumulation of microglia also occurred in the ventral horn of the spinal cord at the onset of PCD of motor neurons during development and coincided with the colonization of the spinal cord by microglia at E11.5 (Rigato et al. 2011). Conversely, live imaging studies on isolated developing mouse hippocampus have shown that microglial mobilization is not dependent on developmental cell death, at least not at P4. Whether apoptotic cues are important for microglia recruitment towards the embryonic mammalian brain remains to be demonstrated (Reemst et al., 2016). Finally, it has been shown that the microglial population was decreased in the E14 brain upon maternal administration of matrix metalloproteinases (mmps) inhibitors at E13, suggesting that mmps play a role in microglial infiltration and/or invasion in the brain. mRNA of MMP-8 and 9, enzymes involved in remodelling of the extracellular matrix (ECM), was highly expressed in microglial progenitors localized outside the mouse CNS parenchyma (Kierdorf et al., 2013). Other molecules such as

semaphorins, netrins, monocyte chemotactic protein 1 (MCP-1, or alternatively CCL2), macrophage inflammatory protein (MIP) 1-α, and the purinergic receptors P2X4R and P2Y12R, may be involved in the recruitment process as well, but will not be discussed here because of lack of *in vivo* evidence (for review see (Mosser et al., 2017b).

Together, these studies indicate that different mechanisms are involved in the attraction of microglia, the invasion of the CNS by these cells and/or the dependency of population size increase on the development stage. After initial recruitment to the CNS, microglia intensively migrate throughout the parenchyma in order to reach their final positions and exert their manifold tasks (Eyo & Dailey, 2013; Michell-Robinson et al., 2015; Swinnen et al., 2013).

4.3 Spatiotemporal localization of microglia within the developing brain parenchyma

After infiltrating the CNS, microglia distribute into the parenchyma according to specific spatiotemporal patterns that are well studied throughout development in different species, such as humans, rodents, avians and zebrafish (Pont-Lezica et al., 2011). As such, microglial colonization of the CNS seems to be a highly conserved process among species, and this pattern is likely related to the functions that these cells exert during development. Interestingly, microglia are not homogenously distributed in the developing brain. Instead, they are found in specific locations such as in areas of cell death, developing vasculature and radial glia, regions containing developing axon fascicles, and acellular spaces (reviewed in (Pont-Lezica et al., 2011)).

Microglial colonization of the rodent brain occurs dorsally to ventrally, and rostrally to caudally (Pont-Lezica et al., 2011). In regions of laminar organization of the developing quail retina, optic tectum and cerebellum, microglia use tangential and radial routes to migrate to their final destinations (Cuadros et al., 1994; Cuadros et al., 1997; Navascues et al., 1995). First, microglia

spread in a full single layer throughout each CNS region, seeming to occur along long tangentially oriented axonal fascicles, which pass near the microglial entry “hot spots”. Tangential migration of microglia in the retina occurs using the end-feet of Müller cells, the local radial glia (Marin-Teva et al., 1998). By doing so, microglia adopt a flat morphology with extensive lamellipodia, likely because of the laminar environment,. Some microglia are clearly polarized in the direction of movement, others are non-polarized with projections radiating in all directions. This possibly indicates that microglia explore their microenvironment in order to orient their movement. In other CNS regions made of axonal fascicles, microglia appear more rounded but show similar morphological characteristics as described above. Additionally, they are in close contact with their substrate (Marin-Teva et al., 1998; Navascues et al., 2000). Then, the cells change direction to migrate perpendicularly to the surface of the CNS and populate the different layers of the nervous parenchyma (Monier et al., 2007; Navascues et al., 2000). In the developing human cortex, histological studies suggest that microglia migrate towards the cortical plate and accumulate at its ventral border, the junction with the subplate (Menassa & Gomez-Nicola, 2018; Monier et al., 2007).

Interestingly, the cortical plate in the developing mouse brain (E15.5) is devoid of microglia, which avoid migrating through this zone (Swinnen et al., 2013). Instead, it seems that microglia accumulate in the layers ventrally to the cortical plate, including the subplate, intermediate zone and ventricular zone, as if they are waiting for a launch signal to populate the cortical layers. This transient microglial absence in the cortical plate has been reported in the human, macaque and rat developing cortex as well (Cunningham, C.L. et al., 2013; Monier et al., 2007; Rezaie & Male, 1999; Verney et al., 2010), but the signals leading to this phenomenon have never been disclosed.

The specific localization of chemoattractive and chemorepellent molecules may underlie the transient absence of microglia in the cortical plate of different species. In the human embryonic brain, microglia could follow the gradient of C-C motif chemokine ligand (CCL) 5 and CCL2 (also known as regulated on activation, normal T cell expressed and secreted (RANTES) and MCP-1, respectively) which together form an increasing gradient from the subventricular zone to a peak in the subplate followed by low levels in the cortical plate (Rezaie & Male, 1999). Additionally, the specific complementary expression of IL-34 and CSF1 during development may play a role in the specific distribution of microglial cells (Nandi et al., 2012). Furthermore, semaphorins could act as chemorepellents for microglia based on their inhibitory properties of axonal outgrowth and specific absence of mRNA from the intermediate zone and subplate between E16 and P0 in rats (Skaliora et al., 1998). In particular, only mRNA of semaphorin type B is present in the cortical plate from E15 until E19 in rats, without concurrent expression in the neuroepithelium, where microglia tend to reside. Adult microglia can also express plexin-B1, the receptor for semaphorin 4A and 4D, in pathologic settings, but it is not known whether embryonic microglia functionally express these receptors (Takamatsu et al., 2010). Slit1, another chemorepellent, is specifically expressed in the cortical plate around E15 in mice (Andrews et al., 2007) and might also repel microglia through Robo signalling, the receptor for Slit proteins, since primary rat microglia were shown to express Robo2 (Liu et al., 2012). In addition, the subplate is the first layer where developmental cell death occurs in mice and where the first thalamocortical synapses are formed starting at E15.5, which might attract microglial cells (Allendoerfer & Shatz, 1994; Ferrer et al., 1992; Mosser et al., 2017b; Stolp et al., 2012). However, cleaved caspase-3 immunoreactivity is not detected in that region at E15.5. Nonetheless, the absence of cleaved caspase-3 immunoreactivity is not conclusive evidence for the absence of apoptosis, since also caspase-independent apoptosis can occur (Sierra et al.,

2013). In addition, microglia in the layers ventral to the cortical plate at E15.5 and E17.5 do not show a phagocytic morphology or Mac-2 immunoreactivity. This is in contrast to microglia that are in the vicinity of dying cells, such as in the choroid plexus primordium, which mostly do exhibit such traits (Peri & Nusslein-Volhard, 2008; Rigato et al., 2011). This microglial morphology is defined as amoeboid or unipolar with endosomal-like compartments in their cell body (Swinnen et al., 2013). Alternatively, synaptogenesis in the subplate layer of mouse brains might be a more plausible event leading to the temporary accumulation of microglia, since microglia can sense neuronal activity (Li et al., 2012; Wake et al., 2009) and their migration speed is likely inversely correlated with neuronal and synaptic activity (Grinberg et al., 2011). Additionally, microglia play a critical role in synapse formation and stabilization (Cristovao et al., 2014) (described in more detail in section 6.3), which implies a bijective relation between synaptogenesis and the presence of microglia.

Contact between microglia and radial glia was also observed in several CNS regions and species, and ECM deposition along radial glia might serve as a mechanical substrate mediating migration (Pont-Lezica et al., 2011). In the E13.5 ventral part of the developing mouse spinal cord, 50% of microglial cells were found to interact with radial glial fibers, which suggest that radial glia may guide radial migration of microglial cells into the spinal cord parenchyma (Rigato et al., 2011). In the retina, microglia adhered to the processes of Müller cells, indicating the use of these cells as a mechanical substrate for radial migration (Navascues et al., 2000). Indeed, a subsequent study indicated that radially migrating microglia in the developing quail retina use the processes of laminin-expressing Müller cells as a substratum and undergo ramification while migrating (Sanchez-Lopez et al., 2004). Noteworthy, the studies mentioned above are all based on immunohistochemical analyses and lack live approaches to follow microglial migration along these structures in real time during embryonic development.

In different CNS regions and species, including mice and human, embryonic microglia were often found touching or aligned along blood vessels (Arnoux et al., 2013; Pont-Lezica et al., 2011; Smolders, S.M. et al., 2017), suggesting that microglial cells use the developing vasculature as migration highways. This possibility was also noted by Del Rio-Hortega who stated: *"In its migration, it follows more or less closely the direction of the vessels"* (Del Rio-Hortega, 1932; Rezaie & Male, 1999). It was recently established that microglia indeed can use blood vessels as migration highways during embryonic development in mice (Smolders, S.M. et al., 2017). In a model of acute brain damage, microglia also use blood vessels to migrate along (Grossmann et al., 2002). The factors that contribute to this chemotaxis remain generally unknown, but microglia show a strong directed migration towards blood vessels in aortic ring explants *in vitro* (Rymo et al., 2011). In the postnatal rat brain, CXCL12 transcripts and proteins were detected in endothelial cells (Tham et al., 2001). Thus, microglia may be attracted towards CXCL12-producing developing blood vessels. Microglia might attach to the blood vessel and subsequently downregulate CXCR4, following a mechanism described in leukemic precursor-B cells (Shen et al., 2001) and in granular cells of the dentate gyrus (Kolodziej et al., 2008). Altogether, it is clear that blood vessels can function as substrates for migration, and so a reciprocal interaction exists between blood vessels and microglia. Some molecular candidates will be discussed later.

4.4 The microglial migration behaviour in the developing brain

Time-lapse imaging demonstrates that mouse embryonic microglia have highly dynamic processes (a phenomenon further referred to as "process motility") and are extremely mobile (further referred to as "migration", "translocation" or "displacement of the cell body") (Smolders, S.M. et al., 2017; Swinnen et al., 2013), properties that have also been described in

the embryonic zebrafish (Herbomel et al., 2001; Svahn et al., 2012). The vast majority of microglia in the developing mouse CNS rapidly change their morphology every 2 minutes (Smolders, S.M. et al., 2017; Swinnen et al., 2013) and more than 96% of the cells translocate within 6 hours (up to E17) (Smolders, S.M. et al., 2017). In the healthy adult brain, only 10-15% of all microglia show cell body translocations within 24 hours. This suggests that the microglial migration speed decreases throughout life (Figure 1) (Eyo et al., 2018). Besides the wandering of embryonic mouse microglia throughout the parenchyma, live imaging indicated that the microglia were constantly sending out and retracting processes during their migration process (Smolders, S.M. et al., 2017; Swinnen et al., 2013), comparable to the surveying behaviour of microglial branches in the healthy adult mouse CNS (Davalos et al., 2005; Nimmerjahn et al., 2005). The scanning property of embryonic mouse microglia processes was observed at E14.5 and E17.5 (and to a lesser extent at E12.5) (Nimmerjahn et al., 2005; Raivich, 2005). Throughout mouse development, the proportion of microglia with extensions and the number of their processes per cell increases; at the age of E14.5, approximately 60% of the microglial cells in the mouse brain had a branched morphology (Figure 1) (Swinnen et al., 2013).

The average migration speed of cortical mouse microglia decreases with development (Smolders, S.M. et al., 2017). This might indicate that microglia gradually acquire their final positions with increasing age. Noteworthy, this reasoning is in contrast with the measurements of Eyo et al. (2016) in mouse hippocampal slices that point to a transient rise in microglial migration speed at postnatal ages. Hippocampal migration speed at P2 (~36 μm/h) is higher than in E17.5 cortex measurements (~25 μm/h). However, the hippocampal microglial migration speed also decreases to ~17μm/h at P6 (Eyo et al., 2016). The studies of Smolders et al. (2017) and Eyo et al. (2016) were executed in different brain regions, thus it cannot be ruled

out that migration speed is determined by region-specific ongoing developmental processes, as previously described for microglial transcriptomics (chapter 2).

Ex vivo time-lapse imaging experiments in acute slice preparations show that mouse embryonic microglia migrate throughout the cortical parenchyma using a saltatory motion pattern with intermittent phases of active migration in the direction of a selected protrusion and pauses, during which the cell seems to scan its environment (figure 2A). Although the overall migration speed decreases, the saltatory migration behaviour of microglia persists during embryonic corticogenesis (Smolders, S.M. et al., 2017). This particular behaviour of microglia during brain development seems to be evolutionarily conserved over species. In late postnatal cultured rat hippocampal slices and developing zebrafish larvae, microglia resemble the same saltatory migration pattern as described in the mouse embryonic brain (Grinberg et al., 2011). Live imaging in the developing zebrafish brain shows that microglia have a high capacity to patrol throughout the parenchyma, which allows them to explore the dense neuroepithelium efficiently and possibly deliver signalling molecules (Herbomel et al., 2001). Adult homeostatic microglia exhibit a similar saltatory migration behaviour but seem to spend more time – i.e. Days – idling in between translocations (Eyo et al., 2018). The capacity to migrate is often attributed to adult microglia in the injured or inflamed adult brain based on increased microglial densities in static histological findings, yet, actual migration should be shown preferentially, for example using time-lapse video imaging in brain slices or in the intact brain (Eyo et al., 2018). In addition, reports of migration of microglia in the injured brain might be muddled with migrating blood born macrophages entering the brain due to experimental procedures (such as injections or stab wounds) (Carbonell et al., 2005), and therefore *in vivo* imaging of microglial migration in brain tissue is necessary.

4.5 Microglial migration in changing environments

Microglial migration speed is likely to be influenced by changes in the local environment. Developmental neuronal apoptosis does not seem to instruct the decrease in microglial migration speed during mouse development (Eyo et al., 2016). Interestingly, in the newborn rabbit brain, microglial migration slowed down as a consequence of bacterial endotoxin-induced *in utero* inflammation (Zhang et al., 2016). In cultured postnatal rat hippocampal slices, microglial movement was significantly decreased by enhanced neuronal activity through lipopolysaccharide (Feranchak et al., 2010) administration or chemical long-term potentiation, but significantly increased by neuronal activity blockade using Tetrodotoxin (TTX). Thus, synaptic activity seems to restrain microglial cells in their current micro-territories and absence of synaptic activity seems to send them away to explore other parts of the parenchyma (Grinberg et al., 2011). However, the observed effect of the administered compounds on microglial migration might occur through a direct effect on these cells and not necessarily through a change in synaptic activity, since LPS was found to suppress migration and process extension in primary microglia cultures (De Simone et al., 2010; Lively & Schlichter, 2013; Orr et al., 2009) and approximately 20% of cultured primary rat microglia express TTX-sensitive voltage-operated Na^+ channels (Kettenmann et al., 2011). However, rapid inward currents typical for voltage-gated Na^+ channels are not observed in microglial cells *in situ* (Kettenmann et al., 2011). Nevertheless, microglial migration speed slows down between E15.5 and E17.5 in mice (Smolders, S.M. et al., 2017) and it is intriguing to speculate that this is due to the emergence of local spontaneous activity starting in the cortex in this time frame. The VZ of mouse embryonic slice preparations shows spontaneous calcium transients, and at E16 some neurons in the mouse neocortex (marginal zone) show repetitive action potential discharges

and spontaneous glutamatergic and γ-Aminobutyric acid (GABA)-ergic synaptic inputs (Luhmann et al., 2016). If microglial migration speed is dependent on local depolarizing activity, this might explain the slowing migration speed in the VZ at E15.5 (Smolders, S.M. et al., 2017). Also, subplate neurons show functional thalamocortical synaptic transmission at E19 in rats (Luhmann et al., 2016), which means that synaptic activity increases towards the end of gestation. These findings suggest a clear correlation between the increasing neuronal activity and the decreasing microglial migration speed during mouse development. However, high extracellular ATP and adenosine levels are associated with increasing neuronal activity (Dunwiddie & Masino, 2001; Lovatt et al., 2012; Zimmermann, 1994), but are also shown to stimulate microglial mobility (Orr et al., 2009) as further explained in sections 5.2 and 5.3. Thus, it is not known whether a causal relationship exists. A way to tackle this question might be to use optogenetic approaches to induce local neuronal firing and study simultaneously the evoked microglial migration behaviour in the *in vivo* animal. A methodological approach for optogenetic manipulation of neonatal neuronal networks is proposed by (Bitzenhofer et al., 2017).

Regional and temporal differences in the expression of chemokines, cytokines and adhesion molecules may play an important role in regulating microglial migration speed as well (Arno et al., 2014; Carbonell et al., 2005; Doyle & Yamada, 2016; Miller, M.J. et al., 2003; Milner & Campbell, 2002a, 2002b; Milner et al., 2007; Petersen & Dailey, 2004). Additionally, changes in ECM composition were found to alter microglial adhesion, morphology and surface marker expression which is likely to impact on migration speed (Milner & Campbell, 2002b; Milner et al., 2007). A change in the expression of ECM proteins and/or their partners, integrins and/or ECM/integrin signalling seem like plausible explanations for the decrease in microglial migration speed, since it is known that such a local environment rapidly changes during mouse

embryonic development (Sheppard et al., 1991; Smolders, S.M. et al., 2017). These mechanisms are further discussed in detail in chapter 5.

4.6 Microglial maturation

Microglia progressively colonize the brain until the end of the second postnatal week in mice. During their colonization process, microglia show highly heterogeneous morphologies ranging from amoeboid-like shape to ramified morphology, which is dependent on factors produced by brain-resident cells. During early development, microglia in the mouse cortical parenchyma show a more amoeboid morphology, with an oval cell body and few short and thick protrusions. The amoeboid morphology might be, but is not necessarily, correlated with activation of the cell in terms of pro- or anti-inflammatory reactivity (Arnoux et al., 2013; Hanisch, 2013; Karperien et al., 2013; Olah et al., 2011; Scheffold et al., 2016). Indeed, microglia in the mouse embryonic cortex show no signs of classical inflammatory activation such as Mac-2, inducible nitric oxide synthase (inos) or interleukin-1β (IL-1β) expression. Nevertheless, all - or almost all in the case of CD68 - cells are immunoreactive for the classical microglia/macrophage markers Iba-1, CD11b and CD68 (Korzhevskii, 2016; Rigato et al., 2011). However, these so-called immature cells actively migrate, move their processes and contribute to brain development. Therefore, it is suggested to refer to the specific developmental stage instead of simply immature versus mature microglia. As previously mentioned, Matcovitch-Natan et al. (2016) discriminated three groups of microglia in mice dependent on the differential expression of genes across developmental time points: “early microglia” (E10.5 - E14), “pre-microglia” (E14 - P9) and “adult microglia” (4 weeks postnatal and onwards) (Figure 1). The study of Butovsky et al. (2014) corroborates these findings and shows that microglia possibly gain their mature transcriptional signature a bit earlier than P21, but definitely after P4 in mice. At that point,

their transcriptional profile is already distinct from macrophages recruited to the CNS in disease settings, from primary microglial cultures (from P1-P2), from embryonic stem cell-derived microglia and from the most commonly used microglia cell lines (BV2 and N9). Interestingly, they found that the transcriptional signature from freshly sorted newborn (P1) and cultured primary microglia closely mimicked the *in vivo* adult mouse microglial signature (Butovsky et al., 2014). Together, these studies found marked differences between the embryonic and the adult mouse microglia transcriptome, which indicates that the series of maturation events are possibly regulated by the rapidly changing local environment (Butovsky et al., 2014; Matcovitch-Natan et al., 2016).

The amount of microglial protrusions increases during embryonic development in mice (Figure 1) (Swinnen et al., 2013), zebrafish larvae (Svahn et al., 2013) and humans (Monier et al., 2007). The actual transformation from "amoeboid" to "ramified" was reported to start around P10 in mice, when transcription factor Runx1 is lost (Nayak et al., 2014; Zusso et al., 2012). By P28, the ramification process is complete (Miyamoto et al., 2016; Nayak et al., 2014). This coincides with a drop in microglial density and the emergence of an adult transcriptional profile (Figure 1) (Matcovitch-Natan et al., 2016; Mosser et al., 2017b; Nayak et al., 2014). Literature often postulates that the ramification process starts when microglia stop migrating and achieve their final location in the CNS parenchyma. In developing zebrafish larvae settlement occurs at 5 days post-fertilization (Svahn et al., 2013). *In vitro* rat primary microglial ramification is induced by astrocyte-derived soluble factors including cytokines (TGF-β, M-CSF, GM-CSF and purines (ATP and adenosine)), even in the absence of physical contact with astrocytes (Mosser et al., 2017b). Furthermore, recent evidence demonstrates a role for the gut microbiota, i.e. The microflora that colonizes the gut, in regulating microglial maturation in mice as well (Erny et al., 2015; Matcovitch-Natan et al., 2016; Thion et al., 2018b). In the absence of the microbiota, microglia

display morphological characteristics and a gene expression profile that correlates to a developmental status, which is maintained throughout adulthood (Erny et al., 2015; Matcovitch-Natan et al., 2016). In addition, developmental maturation of the mouse microglial transcriptional signature appears to be delayed in males compared to females, suggesting an influence of the hormonal system (Hanamsagar et al., 2017b).

In humans, microglia with increased ramifications are widely distributed in the intermediate zone around 22 weeks of gestation, and by 35 weeks the microglial population is highly ramified (Rezaie et al., 2005; Rezaie & Male, 1999; Verney et al., 2010). Several studies in human foetuses describe a general "pattern of microglial differentiation," which is associated with the displacement of microglia from the deeper layers of the cerebral hemispheres towards the cortical plate, with increasing ramification and gradual loss of expression of several markers such as CD68, CD45, CD11b and human leukocyte antigen-antigen D related (HLA-DR, or major histocompatibility complex class II molecule, MHCII in mice) (reviewed in (Rezaie & Male, 1999). It is not clear yet whether the same pattern of differentiation is observed in rodents as well.

Functional (electrophysiological) maturation coincides in general with increased ramification, although high heterogeneity remains in the microglial electrophysiological phenotypes at the same age (P5-P9) in mice (Arnoux et al., 2013; Mosser et al., 2017b). Amoeboid, embryonic mouse microglia show an inward K^+ current, while this current declines during postnatal maturation and transiently switches for an outward K^+ current. Astrocyte-derived diffusible factors such as TGF-β control the upregulation of outward K^+ currents (Mosser et al., 2017b). The function of these voltage-dependent K^+ currents mediated through Kv1.3 channels in embryonic mouse microglia has not yet been elucidated, but may encompass a deactivation process (Schilling et al., 2000) or alternatively, Kv1.3 might be related to integrin-mediated

adhesion and migration of microglia during postnatal development (Arnoux et al., 2013; Artym & Petty, 2002; Levite et al., 2000; Nutile-mcmenemy et al., 2007).

4.7 Sex differences in microglia

The sex differentiation program is a master controller of "brain sex" utilizing many mechanisms, including neural progenitor cell proliferation and survival, developmental apoptosis, synaptogenesis, synaptic pruning and microglial function (Lenz & mccarthy, 2015; Nelson et al., 2018; Nelson et al., 2017a). Males and females exhibit clear differences in the developmental process leading to CNS colonization by microglia, which lead to sex differences in microglial morphology, density and functions in the neonatal (P0), juvenile (P21) and adult mouse (Guneykaya et al., 2018; Nelson et al., 2017a; Nissen, 2017; vanryzin et al., 2018). The morphological differences between rat male and female microglia are present early in its development (Schwarz et al., 2012). Moreover, adult rat microglia (P90) in the prefrontal cortex adapt their morphology gender-specifically upon prenatal administration of the glucocorticoid dexamethasone, suggesting differences in branch motility. This response can be rescued with $A_{2A}R$ blockers, but only in males (Caetano et al., 2017). Therapeutic resistance of adult female mice was also described in a study using an antidiabetic drug to prevent and reverse neuropathic pain and spinal cord microglial activation (Inyang et al., 2018). In addition to morphological differences, microglial function and density are shown to be sex-dependent. *In vitro*, male primary rat microglia (P0-P2) migrated faster while female microglia of the same species and age phagocytosed more fluorescent beads (Yanguas-Casas et al., 2018). In contrast, Guneykaya et al. (2018) showed similar phagocytosis of fluorescent beads between male and female microglia in hippocampal brain slices of 13-weeks old mice. Furthermore, microglial density varies considerably between males and females in the rat parietal cortex, amygdala,

hippocampus and preoptic area (POA) at different stages throughout life (Lenz & mccarthy, 2015; Nissen, 2017). The POA is the brain region essential for male sexual behaviour, and shows clear anatomical differences between both sexes. Estradiol, the aromatized form of testosterone, has emerged as the dominant masculinizing hormone in the developing rat brain. By P4, the male POA shows higher numbers of amoeboid microglia characterized by an enlarged soma, fewer ramifications and shorter process length compared to the female POA. Treatment of female pups with estradiol at P0 and P1 leads to the masculinization of microglial numbers and an increase in the number of amoeboid microglia via the upregulated production of the pro-inflammatory molecule prostaglandin E2 (PGE2). Although the underlying pathway is not known yet, PGE2 also produces a two-fold greater density of dendritic spines in neonatal males compared to females via microglia *in vivo*, suggesting that microglial reactivity in males is necessary to induce the masculine pattern of dendritic spines in the POA, which contributes to the adult sexual behaviour in males (Lenz & mccarthy, 2015; Lenz et al., 2013). Further, the microglial immune reactivity and response to neuropathic pain and chronic stress also vary amongst genders (Hanamsagar et al., 2017b; Tay et al., 2017). It must to be noted that sex differences in microglial function can only occur from late gestation in rats, when the critical period starts (Nelson et al., 2017a). The sex differentiation program is completed by the end of the first postnatal week in rats (Nelson et al., 2017b). The presence of microglial cells during this period is essential for the development of juvenile and adult sexual behaviour in rats (vanryzin et al., 2016). In addition, male and female mouse microglia are differentially affected by the gut microbiome in prenatal and adult periods (Thion et al., 2018b). This central role of microglia in sex differentiation of the brain and behaviour has implications for the incidence of neurodevelopmental disorders in both sexes (Nissen, 2017). Autism spectrum disorder, schizophrenia, Tourette's syndrome, and attention deficit hyperactivity disorder (ADHD) are

more common in males than in females (Nelson & Lenz, 2017). Microglia in post-mortem brains of autistic and schizophrenic patients exhibit an activated phenotype, increased density and abnormal spacing (Nelson et al., 2017b). This suggests that microglia might be involved in these developmental disorders, but whether the increased microglial activity is a cause or a consequence of these disorders remains to be determined.

5. Molecular control of microglial migration, process extension and phagocytosis

Cell migration is a crucial process for the existence of an organism. For example, it is critical for the establishment of the cortical layers in the developing mouse CNS and the colonization of the CNS by microglial cells. As for neurons and many other cell types, the ability of microglia to migrate is indispensable for tissue maintenance and in pathological conditions. However, many aspects of the cellular and molecular control underpinning this microglial migration, especially during development, are not fully understood. In general, cell migration can be described as a series of events that require well-coordinated molecular signalling in space and time (illustrated in detail for microglial migration in Figure 2A). First, a cell senses its environment, which sets its whole molecular and cytoskeletal machinery in motion to eventually polarize into the direction of the cue. This is followed by the coordinated outgrowth of protrusions, formation of adhesions, and translocation of the cell body by contraction towards the adhering zones. Finally, the adhesions are disassembled and the rear is retracted (Vicente-Manzanares & Horwitz, 2011). Thus, in order to move forward the cell needs to transduce its contractile forces to the substratum, mediated through the connections it has established with its surrounding extracellular matrix (ECM) and neighbouring cells. The following section discusses how microglia are amongst the migrating cells using ECM/integrin signalling. Their mobility is likely

Figure 2

to be influenced by the local ECM composition and neuronal activity. In addition, cytokines, chemokines, purines and other signals released by structures such as blood vessels, glial cells, neurons and dying cells influence microglial mobility (Figure 2).

5.1 ECM/integrin signalling in microglia and their mobility

In vivo, and often *in vitro* as well, cells are embedded within or located on top of ECM. The ECM is present throughout the whole body, but its composition and viscoelastic properties can vary greatly (Ahmed & Ffrench-Constant, 2016; Barnes et al., 2017). In the brain, the ECM constitutes 10-20% of the brain volume and it can be localized in three major compartments - namely the basement membrane (around blood vessels or as part of the glia limitans), the perineuronal network (i.e. A special type of pericellular matrix) and the neural interstitial matrix (Kwok et al., 2011; Lau et al., 2013). The ECM's functions during development are to support not only migration but also cell proliferation, differentiation, axonal outgrowth and synaptogenesis, while during adulthood its focus is on cell survival, synaptic plasticity and the response to damage (Soleman et al., 2013). Considering this shift in function, it is not surprising that the ECM landscape is significantly modified between development and adulthood (Figure 3).

Figure 3

As mentioned above, the integrin receptor family plays an important role in establishing cell-matrix interactions and allow cells to quickly respond to changes in the extracellular milieu (Harburger & Calderwood, 2009; Meller et al., 2017). Integrin receptors recognize almost all components of the ECM, and therefore share most of their functions such as cell adhesion, migration and signalling. Integrins are transmembrane receptors composed of non-covalently linked α and β subunits, and 24 different integrin heterodimer combinations (18 α and 8 β subunits) are described in vertebrates with varying ligand binding properties (e.g. Collagen,

fibronectin, laminin) and cell/tissue distributions. These receptors can adopt two extreme conformations: bent, often referred to as “inactive;” or extended, which is an “active” state of the receptor (Figure 4A) (Luo et al., 2007; Shattil et al., 2010; Takagi et al., 2002; Takagi et al., 2003) and activation can be induced via two different yet related signalling cascades. During “outside-in” signalling, a ligand binds the extracellular heads of the integrin and recruits intracellular adhesion signalling proteins, such as talin and kindlin, to the cytoplasmic tail of the β subunit. On the contrary, talin and kindlin are recruited upon a signal coming from inside the cell during “inside-out” signalling. Binding of talin and/or kindlin induces the physical connection of the integrins with the actin cytoskeleton of the cell. Multiple signalling proteins (further referred to as the adhesome), such as paxillin, focal adhesion kinase (FAK) and Src will subsequently associate with the preformed complex to strengthen the connection and induce signalling (Vicente-Manzanares & Horwitz, 2011). For more details regarding the classification, structure, activation pathways and functions of integrins, we further refer to other relevant reviews (del Zoppo & Milner, 2006; Harburger & Calderwood, 2009; Kerrisk et al., 2014; Mui et al., 2016; Park & Goda, 2016; Schmid & Anton, 2003).

Figure 4

Most information available to date on microglial integrin expression and their functions originates from *in vitro* studies using primary rodent microglia or microglial cell lines (Milner, 2002, 2009; Milner & Campbell, 2003; Welser-Alves et al., 2011). However, microglia acutely isolated from embryonic mouse brains show the expression of β1, α5, α6 and αv integrins (Smolders, S.M. et al., 2017; Bennett et al., 2016). Interestingly, the expression level of the α5 integrin is developmentally downregulated from E13.5 to E17.5 (Figure 1) while the α4 integrin is only present on a small subset of microglial cells. Cultured primary microglia derived from early postnatal mice (P0-P2) express various integrins of the three main classes: β1, β2, and αv (Figure 4B) (Milner & Campbell, 2003). These immune cells specifically express the β1 integrins

α4β1, α5β1, and α6β1, and the β2 integrins αlβ2 and αmβ2 (for alternative names see Figure 4B). Alβ2 and αmβ2 are also expressed by microglia in the normal developing and adult rodent CNS (Dalmau et al., 1997; Kloss et al., 1999; Rigato et al., 2011). Amβ2 integrin is mostly known as Mac-1, CD11b or CR3 and plays a major function in microglial phagocytosis and synaptic remodelling in P5 mouse brains (Schafer et al., 2012). From the αv heterodimer class, microglia express αvβ1, αvβ3, αvβ5 and αvβ8 (Milner, 2009; Milner & Campbell, 2003; Milner et al., 2007; Welser-Alves et al., 2011). Avβ5 plays a role in newborn rat microglial phagocytosis of apoptotic rat neurons *in vitro* (Witting et al., 2000). Cultured primary microglia derived from early postnatal mice (P0-P2) do not express α1, α2, αx, β4 and β7 (Milner & Campbell, 2003). Microglial activation and integrin expression are further influenced by exposure to cytokines such as interleukins, tumour necrosis factor (TNF), TGF-β and interferons (ifns), as well as by the ECM *in vitro* (Milner & Campbell, 2003). Cultivation of primary mouse microglia (P0-P2) on fibronectin and vitronectin increases protein expression of the α4β1, α5β1 and αmβ2 integrins, while cultivation on laminin increases αv expression, most likely through outside-in signalling. All three ECM substrates increase expression of αlβ2 integrins. Fibronectin and vitronectin promote microglial pro-MMP9 and MHC class I expression through α5β1 and αvβ5 integrins respectively (Milner et al., 2007). Primary mouse microglia (P0-P2) adhere strongly to fibronectin and vitronectin, but only weakly to laminin, unless they are stimulated (Milner & Campbell, 2002a). It is not known whether vitronectin is expressed in the embryonic brain, but it is deposited in the mouse brain under pathological circumstances such as experimental autoimmune encephalomyelitis (EAE) (Milner et al., 2007). Adhesion of primary mouse microglia (P0-P2) to laminin and astrocyte ECM is regulated via PKC-dependent activation of α6β1 integrin (Milner & Campbell, 2002a). However, these phenomena are however measured in cultured microglia and may be related to the transformation of the cell into a more alerted

or immune-active state due to the culture conditions as shown, for instance, by the upregulation of integrins, MHC class I, the production of mmps and an amoeboid morphology after exposure to fibronectin coating (Milner & Campbell, 2003; Milner et al., 2007).

In addition to influencing microglial adhesion and MMP production, the ECM molecules influence primary mouse microglial (P0-P2) morphology *in vitro* through integrin signalling. Fibronectin and vitronectin promote the lamellipodium-rich amoeboid phenotype while laminin evokes a rounded-up phenotype which is weakly adherent (Milner & Campbell, 2003). These results are corroborated by another *in vitro* study which shows that fibronectin promotes the transformation of isolated embryonic primary rat microglia (E17) from an amoeboid morphology into the process-bearing morphology with a decreased phagocytic capacity of zymosan particles, while laminin reversed this phenotype (Chamak & Mallat, 1991). These results suggest that fibronectin and laminin play a role in the maturation of rat embryonic amoeboid microglia towards the adult ramified phenotype. These *in vitro* data seem to contradict the general idea that interstitial fibronectin and laminin gradually disappear in the brain parenchyma while microglia become highly branched (Figure 1) (Lathia et al., 2007; Liesi, 1985; Sheppard et al., 1991; Smolders, S.M. et al., 2017).

Integrin blockage has led to various outcomes on migration depending on the cell type, the integrin heterodimer and the environmental dimensions. Almost all isolated primary mouse microglia from E13.5 to E17.5 expressed α5β1 integrin. However, its expression level decreased over development and seems to have an age-specific function in microglial migration, as α5β1 blockage decreased migration speed in *ex vivo* acute brain slices at E13.5, but increased speed at E17.5 (Smolders, S.M. et al., 2017). Integrin-mediated functions (migration promoting vs. Inhibiting) seem to vary amongst integrin subtypes and likely switch and/or change importance

during embryonic development. Such a switch might occur for α5β1 integrin, for which it would most likely be at E14, as this is the age reported for mouse microglia to shift gene expression profile from “early microglia (E10.5-E14)” to “pre-microglia (E14-P9)” (Matcovitch-Natan et al., 2016). The long term consequences of defective integrin-dependent microglial migration on brain development and neuronal network functionality remain unknown. Microglia-specific integrin or chemokine receptor knockouts will aid in identifying the effect of this defective microglial migration on radial glia differentiation into neurons, astrocytes and oligodendrocytes, synaptogenesis, myelination and functionality of neuronal networks.

5.2 Purinergic signalling, integrins and microglial mobility

In vitro as well as *in vivo,* purinergic signalling and concurrent intracellular calcium increase seem to play central roles in promoting both microglial mobility, which is used to refer to soma translocation (migration), and process remodelling (motility) (Eyo & Dailey, 2013). In particular, ATP, ADP and adenosine signalling through the metabotropic purinergic receptor P2Y12R, the ionotropic purinergic receptor P2X4R and the metabotropic adenosine receptor A_{2A}R are mostly implicated in regulating microglial process dynamics and chemotaxis in the developing, juvenile and adult rodent brain (Figure 1) (Davalos et al., 2005; Eyo et al., 2015a; Gu et al., 2016; Gyoneva et al., 2009; Kurpius et al., 2007; Nasu-Tada et al., 2005; Noda et al., 2013; Ohsawa & Kohsaka, 2011; Orr et al., 2009; Sunkaria et al., 2016). However, the A_{2A} receptor controls mouse primary microglia (P1-P5) proliferation through BDNF signalling *in vitro* (Gomes et al., 2013), suggesting that this would inhibit microglial migration as microglia are unable to simultaneously proliferate and migrate.

B1 integrin signalling occurs downstream of purinergic receptor activation and plays a pivotal role in regulating microglial mobility. As such, ATP signalling through P2Y12R induces increased

adhesion of neonatal primary rat microglia to collagen, which can be inhibited with RGD peptides, β1 integrin blocking antibodies and P2Y12R antagonists *in vitro*. In particular, RGD and β1 integrin inhibitors inhibit process extension and lead to the accumulation of β1 integrin in the protruding tip (Ohsawa et al., 2010). B1 integrin further regulates rat primary microglial migration towards α-synuclein *in vitro* (Kim, C. Et al., 2014) and mouse microglial migration towards human immunodeficiency virus-1 (HIV-1) Tat protein *in vitro* and *in vivo* (Yao et al., 2013). In newborn primary rat microglia, the latter induces activation of non-muscle myosin light chain kinase, followed by inside-out activation of microglial β1 integrin, outside-in signalling upon ligand binding and finally, actin polymerization *in vitro*. B3 integrin was not involved in primary rat microglial migration towards Tat (Yao et al., 2013). ADP induces β1 integrin translocation to membrane ruffles (motile areas on the cell surface that contain a meshwork of newly polymerized actin filaments) of cultured neonatal rat primary microglia, and in the presence of fibronectin substrate it induces β1-dependent chemotaxis through P2Y12/13R signalling *in vitro*. Details of the signalling pathway(s) generated by β1 integrin translocation and the effect of this outside-in signalling on microglial chemotaxis are not known (Nasu-Tada et al., 2005). Conflicting results are reported regarding the role of β2 integrins: αIβ2 integrin (alternatively CD11a or LFA-1) is proposed to be necessary for normal migration of microglia to sites of excitotoxic injury in organotypic hippocampal slice cultures from P9-P10 mice (Ullrich et al., 2001), while microglial migration to injured neurons is unaffected in cultured slices of 7-days old β2 deficient mice (Kurpius et al., 2006). It should be noted that most of the work described here is from primary microglia in an *in vitro* setting. The molecular machinery that regulates protrusion formation and cell migration however largely depends on the type of environment, e.g. 2D vs. 3D. This stresses the need for *in vivo*- and *in utero*-approaching settings to extrapolate molecular data on migration to the *in vivo* situation (Fraley

et al., 2010). Moreover, as shown by Smolders et al. (2017) for the α5β1 integrin, microglial migration regulators might have age-specific roles that cannot be overlooked.

5.3 Molecular signalling in microglial mobility

In addition to integrin signalling leading to adhesion and cytoskeleton remodelling, ion channels regulating sodium, chloride, potassium and calcium fluxes mediate protrusion formation and migration in primary microglia by inducing actin polymerization and regulation of local swellings and shrinking (Harl et al., 2013; Hines et al., 2009; Lim et al., 2017; Noda et al., 2013; Persson et al., 2014; Schwab, 2001; Swiatkowski et al., 2016; Zierler et al., 2008). Involvement of non-selective cation channels, such as transient receptor potential (TRP) channels, has been extensively described in primary microglial activation and migration as well (Echeverry et al., 2016).

Though the molecular signalling pathways underlying *in vivo* microglial migration remain largely unknown, *in vitro* studies have proposed some mechanistic and signalling events underlying microglial chemotaxis/migration (Fan et al., 2017). Primary rat microglia (P0-P2) do not demonstrate classic types of adhesions during migration, but instead form podosomes. These are 0.4-1µm multimolecular structures with an F-actin core surrounded by a ring of adhesion and structural proteins. Through calcium signalling in these podosomes, microglia are able to adhere to and degrade fibronectin substrates using matrix metalloproteinases (Siddiqui et al., 2012; Vincent et al., 2012). Podosome-based migration seems a plausible mechanism for *in vivo* microglial migration, but this remains to be tested.

The phosphatidylinositide 3-kinase (PI3K) signalling pathway appears to be one of the major signalling pathways in microglia chemotaxis *in vitro*. The activation of PI3K and its localization towards the leading edge membrane is instructive for neonatal primary rat microglial cell

polarity through inducing F-actin polymerization at the cell front *in vitro* (Fan et al., 2017). Activation of this pathway through the purinergic receptors P2X4 and P2Y12 is also involved in microglial migration and process outgrowth in response to ATP and ADP (Irino et al., 2008; Lee, S.H. et al., 2011; Ohsawa et al., 2007; Ohsawa et al., 2010). However, only the study of Ohsawa et al. (2010) examined this microglial behaviour *ex vivo* by making use of hippocampal slices of P4-P7 rat brains. The other findings are found in *in vitro* settings using microglial cell lines and isolated neonatal primary rat microglia. Although ATP-stimulated chemotaxis in microglia requires PI3K activation, membrane ruffling (which can be considered as process motility) of cultured neonatal primary rat microglia does not (Ohsawa et al., 2007). Notably, chemotaxis and chemokinesis occur through two distinct molecular pathways in microglia. Chemotaxis is the directed migration towards a chemical source, while chemokinesis is a non-directional increase of migration in response to a chemical stimulus. ATP stimulates a combination of both chemokinesis and chemotaxis, which are mediated by ROCK signalling, while C5a stimulates only chemotaxis mediated by Rac1 signalling *in vitro*. Further, PI3K is only required for random basal neonatal primary mouse (P1-P3) microglial cell migration and not for directional migration *in vitro* (Miller, A.M. & Stella, 2009). P2Y12R signalling following Toll-like receptor (TLR) 2 activation results in PI3K/Akt and Rac activation, which controls neonatal primary mouse (P0-P3) microglial chemotaxis *in vitro* (Ifuku et al., 2016). In addition to inducing cytoskeletal remodelling, PI3K also induces MMP expression in neonatal primary rat microglia (Ito et al., 2007).

Intracellular Ca^{2+}-independent phospholipase A2 ($ipla_2$) was found to activate PI3K-Akt signalling in the BV-2 microglial cell line through directing Src activation (Lee, S.H. et al., 2011). Active Src phosphorylates paxillin at Tyr^{31}, which is essential for focal adhesion assembly and BV-2 cell migration (Lee, S.H. et al., 2012). $Ipla_2$ also controls the recycling of α6 integrin vesicles

and their delivery to focal adhesion during BV-2 cell chemotaxis (Lee, S.H. et al., 2016). In isolated primary postnatal rat microglia, Akt activation can also be regulated by a phospholipase C (PLC)-mediated increase in intracellular calcium after P2Y12R signalling (Irino et al., 2008). In addition, ERK1/2 (alternatively MAP kinase) signalling in BV-2 cells is directly involved in promoting chemotaxis by regulating phosphorylation states of adhesome proteins such as paxillin, which are required for adhesion dynamics (Lee, S.H. et al., 2012).

While ipla$_2$, PI3K-Akt and ERK1/2 signalling promote migration, PKA signalling is inhibitory and can also occur through ADP stimulation of P2Y12R in BV-2 cells. In this case, P2Y12R signalling induced increased levels of camp and PKA activation, which leads to phosphorylation of vasodilator-stimulated phosphoprotein (VASP). However, prolonged phosphorylation of VASP by PKA disturbs focal adhesion formation/maturation and membrane ruffle formation, resulting in defective chemotaxis. Thus, balanced regulation of phosphorylation and dephospohorylation of VASP is necessary for efficient BV-2 cell chemotaxis (Lee, S. & Chung, 2009). On the contrary, ATP signalling through P2Y12R was found to decrease adenylyl cyclase levels, which normally leads to increased camp levels and PKA activation, and induce increased adhesion of isolated neonatal primary rat microglia to collagen (Ohsawa et al., 2010). Additionally, ADP stimulation through P2Y12R causes β1 to translocate to membrane ruffles in order to promote migration of cultured neonatal rat primary microglia, and this is negatively regulated by PKA (Nasu-Tada et al., 2005).

5.4 Molecular control of process extension and phagocytosis

In addition to their advanced migration capacity in the developing brain, microglia constantly survey the developing CNS by extending and retracting their processes, even in homeostatic conditions (Figure 2B). However, upon CNS injury microglia send out targeted processes to

surround sites of tissue damage. Interestingly, these two modes of motility have recently been shown to differ mechanistically. THIK-1 is a two-pore domain K^+ channel expressed in *in situ* microglia and is required for their appropriate surveillance in acute hippocampal slices from P12 rats or P15-P27 mice. Pharmacological inhibition or gene knockout of THIK-1 depolarizes microglia, hence decreasing their ramification and consequently their surveillance. THIK-1 activity is potentiated by P2Y12 receptors, however blocking these receptors does not influence the microglial membrane potential, nor their ramification or surveillance. In contrast, P2Y12 receptor activation is required to send out targeted processes towards damaged tissue, while THIK-1 is not involved in this mode of motility (Madry et al., 2018).

While surveying the CNS, microglia encounter potentially harmful particles, such as micro-organisms, dying cells, dead cells and protein aggregates. Activation of specific receptors expressed on the microglial cell surface regulates downstream signalling pathways that contribute to the reorganization of the actin cytoskeleton, hence initiating and coordinating microglial engulfment or phagocytosis of these harmful microparticles (> 0.5 µm) (Fu et al., 2014; Vilalta & Brown, 2018). Thus, these receptors are mainly involved in the initial "find-me" and subsequent "eat-me" steps of phagocytosis (Sierra et al., 2013). Phagocytic receptors are generally divided into two distinct types based on their affinity for different harmful substances. The first group consists of receptors such as Toll-like receptors (tlrs) that exhibit a high affinity to bind to foreign microbial pathogens (e.g. LPS and viral nucleotides) and danger-associated molecular patterns (e.g. Deposited amyloid β (Aβ) fibrils and α-synuclein). The second group includes receptors such as the triggering receptor expressed on myeloid cells 2 (TREM-2) that display a high affinity for apoptotic cellular debris (Fu et al., 2014; Rajbhandari et al., 2014; Redlich et al., 2013; Ribes et al., 2010a; Ribes et al., 2010b). In addition to these two types, other receptor families expressed by microglia are shown to participate in phagocytosis of

various harmful elements *in vitro* and *in vivo*. These receptors include Fc receptors (Choi, I. Et al., 2015), complement receptors (Hong et al., 2016; Makranz et al., 2004; Makranz et al., 2006; Reichert & Rotshenker, 2003; Rotshenker et al., 2008; Weinstein et al., 2015), scavenger receptors (Hong et al., 2016; Makranz et al., 2004; Makranz et al., 2006; Reichert & Rotshenker, 2003; Rotshenker et al., 2008; Weinstein et al., 2015), pyrimidinergic receptors (Inoue, 2017; Koizumi et al., 2013; Neher et al., 2014; Sunkaria et al., 2016; Takenouchi et al., 2009), purinergic receptors (Fang et al., 2009), macrophage antigen complex 2 (MAC-2) (Rotshenker, 2009; Rotshenker et al., 2008), mannose receptor (Fu et al., 2014), low-density lipoprotein receptor-related protein (LRP) receptor (Fricker et al., 2012), vitronectin receptor (Neniskyte et al., 2014; Vilalta & Brown, 2018), and MER receptor tyrosine kinase (mertk) receptor (Figure 2B) (Caberoy et al., 2012; Grommes et al., 2008; Nomura et al., 2017; Vilalta & Brown, 2018). Fu et al. (2014) reviewed the intracellular pathways, stimuli, diseases and animal models associated with most receptor types.

Together, these results clearly indicate that microglia can detect changes in their microenvironment through their expression of a wide range of integrins, and changes in ECM reciprocally influence microglial metabolism. Upon purinergic stimulation, microglia effectively use these integrins in regulating their mobility, and β1 integrin - more than β2 and β3 - plays a major role in this process. The underlying molecular signalling pathways are beginning to emerge as well. $Ipla_2$, Scr, PI3K-Akt and ERK1/2 signalling pathways converge on promoting adhesion formation, membrane ruffling, F-actin polymerization and thereby promoting microglial chemotaxis. In contrast, PKA can negatively regulate chemotaxis (Lee, S. & Chung, 2009; Nasu-Tada et al., 2005). Lastly, microglial mobility also influences their ability to phagocytose, which is one of microglia's most studied characteristic.

6. Motility and mobility as a necessity for microglial functions in the developing CNS

Microglia modulate a plethora of events during CNS development such as precursor generation, migration and differentiation, formation of the blood vessel network and synaptic remodelling (reviewed in (Konishi et al., 2018; Lenz & Nelson, 2018; Mosser et al., 2017a; Paolicelli & Ferretti, 2017; Thion & Garel, 2017; Thion et al., 2018a). These processes are tightly orchestrated in time and space (Silvin & Ginhoux, 2018; Thion & Garel, 2017), and as a consequence microglia have to move throughout the brain parenchyma in order to execute their jobs appropriately. Here, we discuss the physiological functions of microglia in the developing CNS in which migration and motility are involved.

6.1 Microglia clear cellular debris

During CNS development, almost 50% of all developing neurons are naturally removed by programmed cell death (Dekkers et al., 2013). It is one of microglia's most evident tasks to quickly clear these enormous amounts of dead cells (Sierra et al., 2013). Extensive cytoskeletal remodelling is indispensable to this process, as microglia have to extend processes or migrate towards the cellular debris to phagocytose it.

Microglia were found to accumulate in regions where developmental cell death occurs such as in the embryonic mouse cerebellum (Marin-Teva et al., 2004), hippocampus (Wakselman et al., 2008) and choroid plexus primordium (Swinnen et al., 2013). Microglia in the mouse embryonic choroid plexus primordium predominantly acquired an amoeboid or unipolar morphology with endosomal-like compartments in their cell body. The phagocytic morphology and immunoreactivity for Mac-2 indicate that these cells have acquired a phagocytic phenotype in

order to clear the apoptotic cells in the embryonic mouse brain (Swinnen et al., 2013). This phagocytic phenotype is also observed in the brain of zebrafish embryos (Peri & Nusslein-Volhard, 2008) and mouse embryonic spinal cord (Rigato et al., 2011).

As microglial aggregation coincides with the occurrence of developmental cell death in different brain regions, a recurrent question is whether microglia merely serve as housekeeping cells to clean up the debris, or whether they actively trigger cell death. *In vitro* as well as *in vivo* evidence shows that microglia can decrease the number of neural precursor cells within proliferative zones in the primate and rodent neocortex (Antony et al., 2011a; Arno et al., 2014; Cunningham, C.L. et al., 2013; Tong & Vidyadaran, 2016). However, this regulation occurs through phagocytosis of viable neurons, a process named phagoptosis, and does not depend on the typical "eat-me" signals (Cunningham, C., 2013). Furthermore, it is shown that microglia promote neuronal cell death in the developing mouse cerebellum (Marin-Teva et al., 2004) and hippocampus (Wakselman et al., 2008). Although microglia accumulate at CNS regions where developmental cell death takes place at that time, it is unclear whether and how microglia regulate this process in different CNS regions (Mosser et al., 2017a).

Microglial phagocytosis exerts a beneficial effect as dying cells need to be cleared rapidly in order to prevent the spillover of pro-inflammatory and neurotoxic molecules (Wolf et al., 2017). However, a limited disturbance can provoke excessive microglial activation and phagocytosis, followed by neuronal damage and potentially the development of neurodevelopmental disorders (Lenz & Nelson, 2018). Therefore, the current view is that microglial phagocytosis is fine-tuned by a plethora of "find-me" and "eat-me" signals and the associated microglial receptors. The finding that the loss of "don't-eat-me-signals" also contributes to the regulation of microglial phagocytosis expands the continuum of microglial activation states even more

(Hanisch & Kettenmann, 2007; Janda et al., 2018; Sierra et al., 2013; Wolf et al., 2017). Therefore, further investigation on the different signals, intracellular pathways and modulation of microglial phagocytosis is needed to reveal a potential therapeutic strategy for neurodevelopmental disorders such as schizophrenia and autism spectrum disorders (Lenz & Nelson, 2018). However, the relative contribution of resident microglia and infiltrating peripheral macrophages and whether they act in a similar way remains controversial, and should be taken into account.

6.2 Microglia support cell survival, proliferation and differentiation

In addition to cleaning up dead neurons, microglia can provide trophic support to promote neuronal survival and proliferation in the developing CNS (Antony et al., 2011a; Arno et al., 2014; Cunningham, C.L. et al., 2013). This support occurs through microglial secretion of factors such as brain-derived neurotrophic factor (BDNF), NT-3, glial-derived neurotrophic factor (GDNF), neurotrophic insulin-like growth factor 1 (IGF-1), CX3CL1, IL-1β, IL-6, TNF-α and IFN-γ (Erblich et al., 2011; Garden & Moller, 2006; Masuda & Prinz, 2016; Mosser et al., 2017b; Shigemoto-Mogami et al., 2014; Ueno et al., 2013). In turn, microglial phagocytosis and migration can be regulated by neural progenitors that release VEGF and CXCL12 (Arno et al., 2014; Mosher et al., 2012). These studies indicate that there is bi-directional communication between developing neurons and microglia with regard to proliferation and survival (Arno et al., 2014; Ueno & Yamashita, 2014).

Next to neuronal support, *in vitro* and *in vivo* studies indicate a role for microglia in regulating glial differentiation through the release of nitric oxide (NO), IL-1β, IL-6 and leukaemia inhibitory factor (LIF) (Antony et al., 2011a; Bechade et al., 2011; Pont-Lezica et al., 2011; Reemst et al., 2016). The processes of radial glia are phagocytosed by microglia postnatally, which might be

a regulatory step in their transformation into astrocytes (Xavier et al., 2015). Besides astrocytic interactions, a subpopulation of amoeboid and highly metabolically active microglia residing in myelinating regions of the mouse brain during the first postnatal weeks was found to be crucial for oligodendrocyte progenitor maintenance, maturation, and the following myelination process (Hagemeyer et al., 2017). In conclusion, microglia support cell genesis, survival, proliferation and differentiation in the developing CNS by phagocytosis and the secretion of a plethora of cytokines, growth factors and other soluble factors. Yet, whether the necessary signalling molecules are locally released by microglia at the target cell – a process which would require targeted migration and/or process extension – or dispersedly released in the extracellular environment remains to be determined.

6.3 Microglia interact with synapses

During CNS development, excess synaptic connections are formed, and in order to establish efficiently functioning neuronal networks, many of these immature synapses are removed by "synaptic elimination"(Presumey et al., 2017; Thion & Garel, 2018; Weinhard et al., 2018). *In vivo* pre- and postnatal microglial depletion and perturbation models demonstrated the essential role for microglia in the formation, maturation and elimination of synapses (Hoshiko et al., 2012; Kim, H.J. et al., 2016; Miyamoto et al., 2016; Nelson et al., 2017b; Paolicelli et al., 2011; Paolicelli & Ferretti, 2017; Parkhurst et al., 2013; Pont-Lezica et al., 2014; Schafer et al., 2012; Schafer et al., 2013; Squarzoni et al., 2014; Torres et al., 2016; Ueno et al., 2013). The developmental stage in which the depletion or perturbation occurred determined to what extent and in which neuronal circuit synapse formation and function were affected. Interestingly, a microglia depletion study suggested that microglial support in synapse formation is maintained across the lifespan, while microglial-mediated elimination of synapses

is restricted to the first post-natal weeks (Parkhurst et al. 2013). Here, we first discuss synaptic support by microglia followed by microglial synaptic elimination.

Recent evidence supports the extensive role of microglia in brain wiring, including synapse formation and function (Cristovao et al., 2014; Schafer et al., 2013). This recognition gave rise to the generally accepted idea of a "quad-partite synapse," which includes pre- and post-synaptic terminals together with perisynaptic microglial and astrocytic processes. In this way, microglia can locally monitor and influence synaptic activity based on the synaptic signals they receive. Imaging studies revealed the dynamic interaction of microglia with synapses in which microglia rapidly respond to changes in neural activity and neurotransmitter release (Schafer et al., 2013; York et al., 2018). In addition to supporting synapses, microglial contact with dendrites of layer 2/3 pyramidal neurons in the developing somatosensory cortex induces Ca^{2+} transients, actin accumulation, and subsequent dendritic filopodia formation. Interestingly, this filopodia formation occurred only in the developmental period of intense synaptogenesis (P8-P10), and not at later postnatal ages (P12-P14 and P26-P30) (Miyamoto et al., 2016).

Microglia are not only involved in synaptic remodelling, but also actively engulf synapses that need to be eliminated, especially during CNS development. The recognition process of "synaptic elimination" most likely occurs through "find-me" (for example neuronal secretion of CX3CL1) and "eat-me" (C1q and C3 expression by neurons) signals (Wu, Y. Et al., 2015), which implies directional migration or process motility of microglia towards the excessive synapses. Indeed, mice lacking the CX3CR1, the receptor for CX3CL1, show transient synaptic pruning defects, leading to immature connectivity and behavioural deficits (Paolicelli et al., 2011; Zhan et al., 2014). Interestingly, CD47-sirpα signalling protects synapses from inappropriate removal

in the developing retinogeniculate system and at least partially regulates the activity-dependent engulfment of synapses. Microglia in CD47 knockout mice indeed fail to display preferential engulfment of less active synaptic inputs, but exhibited increased microglial engulfment of synaptic inputs in general (P5) (Lehrman et al., 2018).

In contrast to previous studies that present indirect evidence for the phagocytic engulfment of dendritic spines in the hippocampus and visual cortex, Weinhard et al. (2018) could not confirm phagocytic elimination of dendritic spines in the developing hippocampus (P15). However, clear evidence indicates microglia contacting dendritic spines and even partial elimination, or trogocytosis, of presynaptic material in the hippocampus. In some cases, microglia eliminated even most of the synaptic bouton. This engulfment is not mediated by the formation of phagocytic cups or pseudopodia at the contact side, but microglia are digesting these structures by invaginations in their membrane, followed by the sinking of the boutons or axonal pinches in their cytoplasm. Hereafter, microglia close their membrane in order to traffic the structures for subsequent degradation (Weinhard et al., 2018). As with phagocytosis, the process of trogocytosis requires well-directed microglial migration or process extension towards the excessive synapses. Dysfunction in this critical process may be linked to neurodevelopmental disorders, including schizophrenia and autism spectrum disorders (reviewed in (Neniskyte et al., 2017)).

6.4 Microglia interact with the CNS vasculature

Microglia invade the brain before blood vessels start to sprout inside the neuroepithelium, and are often found in close contact with and migrating along blood vessels *in vivo* during

development and in the adult mouse CNS (Arnoux et al., 2013; Nimmerjahn et al., 2005; Pont-Lezica et al., 2011; Smolders, S.M. et al., 2017; Zhao et al., 2018). Thus, microglia are ideally positioned to influence the development and remodelling of the CNS vasculature. However, microglia do not seem to be essential for maintaining the CNS vascular system (Kubota et al., 2009), nor for BBB integrity (Elmore et al., 2014). Yet, microglial depletion studies pinpointed that these cells are necessary for augmenting the vascular density/branching in the developing retina and hindbrain by facilitating anastomosis, while not aiding in tip cell extension (Checchin et al., 2006; Fantin et al., 2010; Kubota et al., 2009). Branching inhibiting effects have been demonstrated for microglia in the deep retinal plexus *in vivo*, mediated by Wnt-Flt signalling (Stefater et al., 2011), and in a retina culture model *ex vivo* (Unoki et al., 2010). Concerning the underlying mechanisms of promoting vessel branching, microglia do not contribute significantly to the VEGF pool, which attracts tip cells, and VEGF neither effects microglial survival nor attraction (Fantin et al., 2010). VEGF-A and soluble Flt1 are not amongst the microglial-derived major soluble factors that mediate branching (Rymo et al., 2011). These findings suggest a different mechanism for microglia-mediated branching other than the VEGF-based vascular sprouting (Arnold & Betsholtz, 2013), and conclude that microglia-blood vessel contact enhances but is not essential to promote branching (Rymo et al., 2011). Notch signalling in microglia is involved in mediating microglia-endothelial cell interactions in the retina (Outtz et al., 2011), but the details on how microglial contact with blood vessels is mediated in other parts of the brain remain largely unknown. Although embryonic blood vessels are coated with fibronectin, microglial fibronectin receptors (α5β1 integrins) do not play a role in the contact with or migration along blood vessels (Smolders, S.M. et al., 2017). To conclude, microglia clearly affect vascular branching, though the outcome depends on the local environment and the underlying molecular mechanisms remain to be elucidated.

7. Microglia and neurodevelopmental disorders

Microglia constitute the first line defence in the CNS, and are involved in both the innate and adaptive immune system. They are in the front seat of regulating the inflammatory response by producing cytokines, chemokines and free radicals, such as TNF-α, IL-1β and NO (Chew et al., 2006). Immune activated microglia are capable of proliferation, migration, antigen presentation, inducing cell death and phagocytosis, and upregulating surface markers such as CD11b, MHCII, CD68 and Mac-2 (Chew et al., 2006; Eyo et al., 2018; Rotshenker, 2009). In the case of neuroinflammation and degeneration, microglia adopt a customized phenotype that can be both neuroprotective and neurotoxic, depending on the stimulus and their CNS microenvironment (Colonna & Butovsky, 2017; Hanisch & Kettenmann, 2007). The M1/M2 nomenclature used to categorize macrophages into "classically" activated (M1, driven by the production of pro-inflammatory cytokines), or alternatively activated (M2, related to an anti-inflammatory reaction and tissue repair) was initially used for microglia as well. However, these extreme classifications oversimplify the plethora of *in vivo* phenotypes (Ransohoff, 2016). To accommodate the inconsistencies with regard to ontogeny-, stimulus- and tissue-specific responses of macrophages, Ginhoux et al. (2016) proposed the "multidimensional model of macrophage activation" stressing that future research should encompass high-resolution, single-cell and deep phenotyping techniques in order to develop therapeutic approaches that target specific subsets of macrophages.

Many of the processes in the developing brain which involve proper microglial functioning are disturbed in neurodevelopmental disorders, such as schizophrenia and autism spectrum disorder (ASD), and in corresponding animal models (Bilbo & Stevens, 2017; Fernandez de

Cossio et al., 2017; Kim, H.J. et al., 2016; Knuesel et al., 2014; Paolicelli & Ferretti, 2017; Reisinger et al., 2015; Salter & Stevens, 2017; Squarzoni et al., 2015; Thion et al., 2018a). Schizophrenia and ASD arise from complex interactions among genetic and environmental factors. Concerning the latter, epidemiological and animal studies have established a strong association between an activated immune system in the mother during pregnancy and the risk for the offspring to develop one of both disorders (reviewed in (Knuesel et al., 2014; Nardone & Elliott, 2016). Bacterial or viral infections, such as rubella and influenza virus, during pregnancy cause maternal immune activation (MIA). In animal models, immune stimulation is mostly induced through injection of LPS, an endotoxin from the outer membrane of gram-negative bacteria, or polyinosinic:polycytidylic acid (Poly (I:C)), a synthetic double-stranded RNA mimicking the immune response following a viral infection through activation of the TLR-3 pathway (Alexopoulou et al., 2001; Choi, G.B. et al., 2016; Smith et al., 2007). The offspring from Poly (I:C) injected and LPS injected pregnant mice and primates display core abnormalities associated with ASD, such as deficits in social, communicative and repetitive behaviours, which are also present in the offspring of pregnant mice that suffered from a viral infection (Bauman et al., 2014; Choi, G.B. et al., 2016; Machado et al., 2015; Malkova et al., 2012; Patterson, 2008; Shi et al., 2003; Smith et al., 2007). MIA in rodents creates an inflammatory environment in the fetal brain by elevating levels of pro-inflammatory mediators, such as IL-6 and IL-17a (Choi, G.B. et al., 2016; Knuesel et al., 2014; Smith et al., 2007). Maternal systemic IL-6 and its downstream signalling cytokine IL-17a are key mediators of altered brain development and behavioural abnormalities in the offspring of Poly (I:C) induced MIA mice (Choi, G.B. et al., 2016; Smith et al., 2007).

Because (i) microglia are the immune cells of the CNS and possess the necessary receptors to sense changes in cytokine levels and react upon them (Kettenmann et al., 2011), and (ii) they

guide brain development (Frost & Schafer, 2016), it is tempting to suspect these cells are in the driver's seat of the fetal inflammatory response disturbing brain development and performance. Despite the suggested involvement of microglial cells in neurodevelopmental disorders (reviewed in (Frick et al., 2013; Michell-Robinson et al., 2015), the chicken-or-the-egg problem remains. Microglia could be the first cells to sense the maternally-induced fetal pro-inflammatory cytokines. This may lead to abnormal task exertion during CNS development, which could ultimately result in behavioural disturbances. Alternatively, the cytokine storm could directly alter neuro- and gliogenesis and network formation (Bauer et al., 2007; Deverman & Patterson, 2009; Nakanishi et al., 2007), to which microglia subsequently react. Today, no conclusive answer exists for this question and the role and activation of microglia in MIA has recently been extensively reviewed (Smolders, S. Et al., 2018).

Some animal studies point to a causative role for microglial dysfunction in the development of behavioural deficits reminiscent of neurodevelopmental disorders (Squarzoni et al., 2014). They also show that MIA can have comparable effects on connectivity and brain wiring, as can microglial dysfunction (Squarzoni et al., 2014). Upon genetically- or pharmacologically-disturbed microglial function, cognitive or behavioural abnormalities arise in adolescent and adult mice. For example, CX3CR1 KO leads to impaired connectivity (increased dendritic spines and immature synapses), impaired social interactions and increased repetitive behaviour (Paolicelli et al., 2011; Zhan et al., 2014). However, it is not clear yet whether the behavioural deficits are caused by a lack of CX3CR1 or increased IL-1β signalling in CX3CR1 KO mice (Rogers et al., 2011). In addition, the short-term decreased microglial density observed in the brains of CX3CR1 KO mice during early postnatal development (around P15) may explain these deficits (Paolicelli et al., 2011). Deficits in microglial-mediated synaptic elimination may impair the brain's excitatory versus inhibitory balance, which is frequently suggested as a common

mechanism in a variety of neurodevelopmental disorders (Koyama & Ikegaya, 2015). Mutation of DAP12 in mice, which is only expressed in immune cells, elicits a transient increase in microglial density at P0 along with the exhibition of a pro-inflammatory profile and impaired long-term potentiation (Roumier et al., 2004). Another study showed that DAP12 mutation as well as MIA resulted in microglia exhibiting a downregulation of genes involved in neurite formation and subsequent malformations in the corpus callosum (Pont-Lezica et al., 2014). In parallel, a DAP12 mutation in humans results in the development of an early form of dementia (Nasu-Hakola disease) (Paloneva et al., 2000). Interestingly, LPS-induced MIA in wild type mice generated synaptic alterations reminiscent of the phenotype caused by DAP12 mutation (Pont-Lezica et al., 2014; Roumier et al., 2008). DAP12 KO and CX3CR1 KO partially mimic the effect by LPS injection in pregnant mice, which results in a laminar positioning impairment of a subtype of inhibitory interneurons (Lhx6 subtype) that integrate into the cortical plate (Squarzoni et al., 2014). Genetic and pharmacologic microglial depletion establish the same phenotype (Squarzoni et al., 2014). DAP12 KO results in behavioural alterations, such as reduced startle response and lowered prepulse inhibition, which are associated with schizophrenia (Kaifu et al., 2003). Microglia depletion, as well as microglia-specific KO of BDNF, results in reduced motor learning and decreased fear response (Parkhurst et al., 2013). Furthermore, microglia are responsible for anxiety development upon stress through the recruitment of IL1β-producing monocytes to the brain endothelium (mckim et al., 2017). Knockout of homeobox protein b8 (Hoxb8), a *Hox* gene normally involved in establishing body plans, results in compulsive grooming behaviour, causing hair loss and skin lesions together with deficits in synaptic pruning (Chen et al., 2010). Hoxb8 is specifically expressed by roughly 40% of microglia from P14 onwards and is also involved in maintenance and differentiation of myeloid progenitor cells (Chen et al., 2010; De et al., 2018). Symptoms caused by Hoxb8

deficiency are similar to the obstructive compulsive disorder “trichotillomania” in humans (Chen et al., 2010). Inducible nitric oxide synthase (inos or NOS2) knockout mice also show increased barbering behaviour and could therefore be a putative model of trichotillomania (Casarotto et al., 2018). Progranulin deleted specifically in microglia resulted in excessive grooming as well, which could be prevented by inhibiting nuclear factor κb (NF-κb) in microglia (Krabbe et al., 2017). Knockout of methyl-cpg-binding protein 2 (MECP2) in mice causes a Rett Syndrome-like phenotype, characterized by retarded growth, sleep apnoeas, tremor, impaired gait and locomotor function, and a short life expectancy (Derecki et al., 2012). Two studies reported a rescue of this phenotype by bone-marrow transplantation from wild type mice (Cronk et al., 2015; Derecki et al., 2012), while this was not reproduced by others (Wang et al., 2015). A fourth and most recent study in the visual system determined that excessive synaptic elimination in MECP2 KO mice mediated by microglia is independent of MECP2 expression in microglia themselves (Schafer et al., 2016).

Mutations and allelic polymorphisms in microglia-related genes, such as Csf1r, Triggering receptor expressed on myeloid cells 2 (Trem2), CD33, Irf8, P2x7r and NRROS are associated with an increased likelihood to develop a plethora of neurological diseases, ranging from Alzheimer’s disease to schizophrenia (Frost & Schafer, 2016; Paolicelli et al., 2017; Wong et al., 2017). Most interestingly, the presence of microglia during the first two weeks of postnatal development is crucial for brain development and behaviour. Transient reduction of the microglial population with 50% through depletion during the first postnatal week already resulted in enlarged ventricles and a thinned cortex by P10. Notably, this transient depletion caused sustained alterations in neonatal, juvenile and adult behaviours ranging from deficient prosocial behaviours to working memory deficits to male-specific impairments in sexual behaviour (vanryzin et al., 2016).

Although some findings are still controversial, microglia seem to have a strong contribution at critical periods in the aetiology of neurodevelopmental disorders, and multiple deleterious events, genetic or environmental, are thought to gradually increase the risk of developing such disorders (Hanamsagar & Bilbo, 2017; Thion & Garel, 2017). It is however not clear yet if microglial dysfunction might initiate disease.

8. Conclusion

Microglia are a spatiotemporal heterogeneous population of immigrant immune cells in the CNS parenchyma. At early embryonic ages, they populate the CNS by proliferation and intensive migration from the yolk sac. Within the developing brain, microglial cells intensively displace their cell bodies as well as their long thin processes. This continuous movement has become the signature of microglial cells and has evoked a significant amount of attention amongst scientists. Data obtained during this last decade highlight several fundamental aspects of immature microglial behaviour and related mechanisms in the developing CNS. Even the molecular mechanisms involved in microglial migration have just started to be unravelled, and these findings may explain changes in microglial migration and process motility over development. Microglial dynamics are likely steered by a combination of a constantly changing local microenvironment (e.g. ECM composition), neuronal activity and signal molecules secreted by surrounding cells. For future research, the use of 3D (in contrast to non-physiological 2D models) or even *in vivo* models will be indispensable in decrypting the molecular machinery steering microglial movement and age-specific differences. Thanks to the increasing quality and quantity of research on the non-immune functions of microglia, we now know that CNS population by microglia is a key element for proper CNS development, and that

alterations of microglial functions at critical developmental periods can lead to several neurodevelopmental disorders. However, there are many questions concerning the molecular, cellular and physiological aspects of microglial biology that remain unanswered, some of them being essential to our better understanding of the impact of early microglia dysfunctions on adult CNS homeostasis, paving the way to new research questions on these fascinating moving brain cells.

Acknowledgements

We thank Edna Stewart for reading and correcting this manuscript. Work was supported by grants from Hasselt University (BOF16NI04 and BOF ulg-uhasselt grant), Sint Vincentius Autism research grant, Research Foundation Flanders (FWO 1521619N and 1518419N), and Rotary Belgium (Espoir en Tête - Hoofdzaak er is Hoop).

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Figure legends

Figure 1. Microglial colonization of the developing mouse brain includes strict control of their number and mobility (migration). The decreasing migration during development coincides with decreasing interstitial extracellular matrix components and their respective integrin receptors, here exemplified for laminin (LM) and fibronectin (FN). The complexity of the surveying processes (number, length and amount of branch points) increases gradually while microglia become sedentary.

Figure 2. Microglial mobility (i.e. Migration) and motility (extension and/or retraction of the processes). A) Putative mechanism of saltatory migration. B) The different types of process motility in microglia are regulated by specific signalling molecules and cellular pathways. CR: complement receptors, CX3CR1: CX3C chemokine receptor 1, CXCR4: C-X-C chemokine receptor type 4, fcr: Fragment crystallisable receptor, MAC-2: macrophage 2 antigen, mertk: tyrosine-protein kinase Mer, LRP: low-density lipoprotein receptor-related protein, SR: scavenger receptors, THIK-1: tandem pore domain halothane-inhibited K^+ channel 1, tlrs: Toll-like receptors, TREM-2: Triggering receptor expressed on myeloid cells 2, VEGFR: vascular endothelial growth factor receptor.

Figure 3. Extracellular matrix (ECM) in the central nervous system. ECM components in the CNS are arranged into three major compartments, namely (1) basement membranes that are located around blood vessels and cover the pial membrane, (2) condensed perineuronal nets (pnns) around the cell bodies and dendrites of specific subsets of neurons, or (3) diffusely distributed as the neural interstitial matrix between cells of the CNS parenchyma. The

composition of these three ECM compartments may vary significantly between development and adulthood (Barnes et al., 2017; Lathia et al., 2007; Lau et al., 2013; Richter et al., 2018; Schwartz & Domowicz, 2018; Sheppard et al., 1991; Smolders, S.M. et al., 2017; Soleman et al., 2013; Suttkus et al., 2016). This change in ECM landscape may reflect the differences in function between development and adult, especially in the interstitial matrix. CSPG: chondroitin sulphate proteoglycans, HSPG: heparan sulphate proteoglycan, HAPLN: hyaluronan and proteoglycan link protein. Image was created using Servier Medical Art (https://smart.servier.com/).

Figure 4. Integrin signalling in microglia. A) Integrin changes conformation depending on the activation state. Upon activation, inside-out signalling modulates the binding strength to ECM ligands and binding of ECM enhances clustering and adhesome formation through outside-in signalling. B) Integrin heterodimers shown to be expressed in microglia (acutely isolated embryonic, adult and *in vitro*). In light blue alternative names of the integrins are given and in black the ligands of the specific integrin heterodimer is shown. FN, fibronectin. LM, laminin. VN, vitronectin. TS, thrombospondin. ICAM, intracellular adhesion molecule. VCAM, vascular cell adhesion protein. CD, Cluster of differentiation. LFA1, lymphocyte function-associated antigen 1. MAC-1, macrophage antigen 1. VLA, very late antigen.

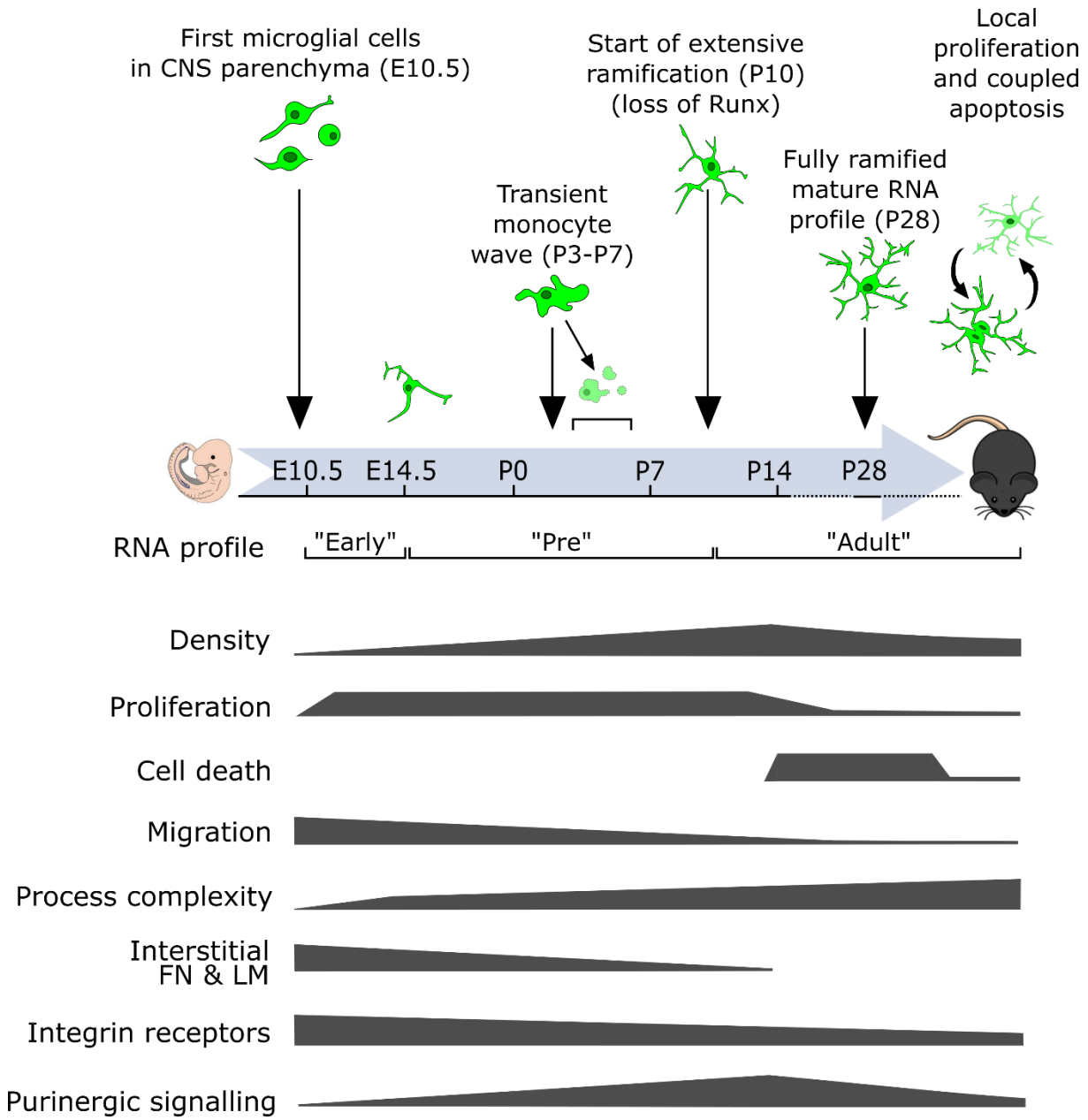

Figure 1

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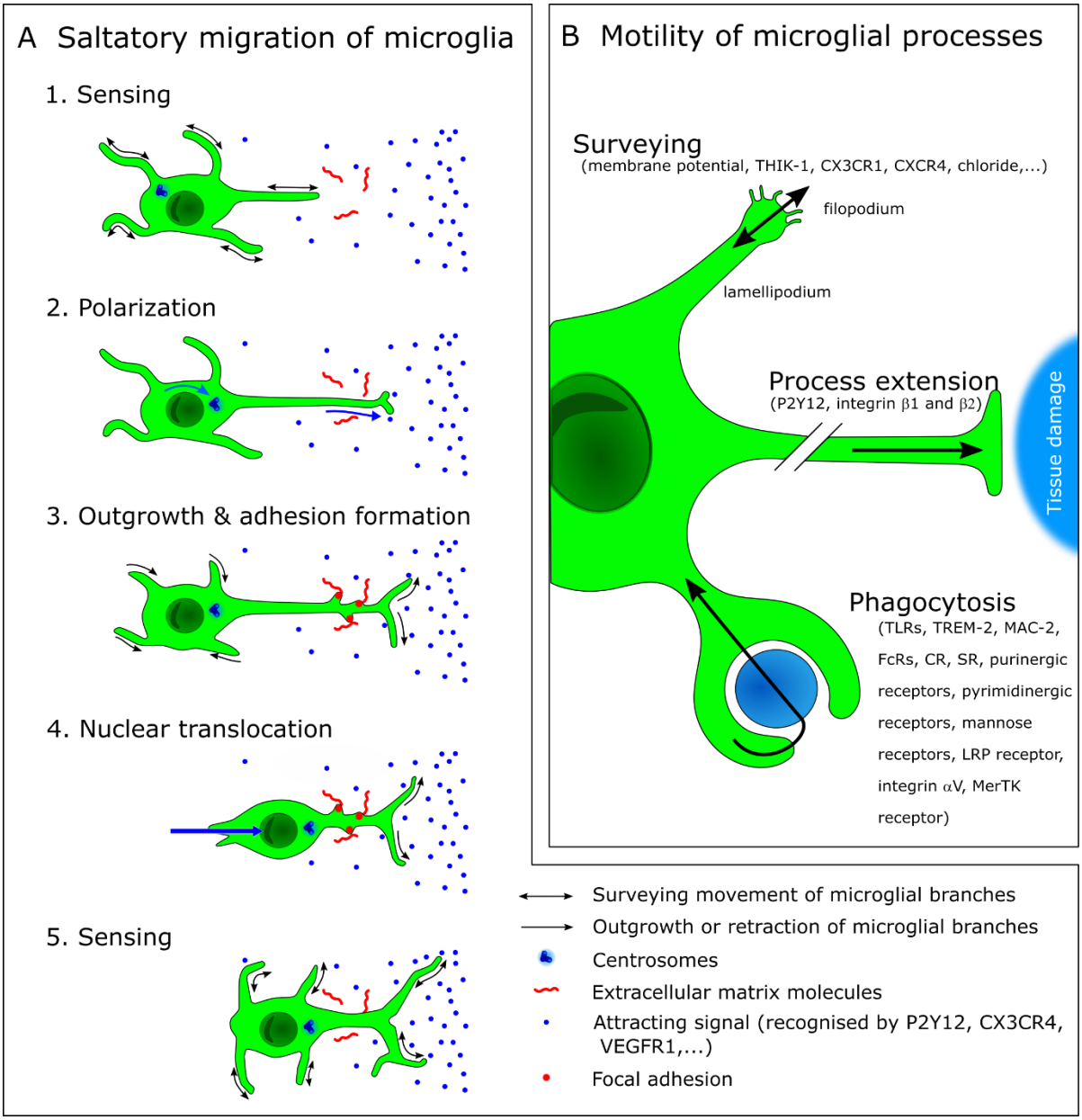

Figure 2

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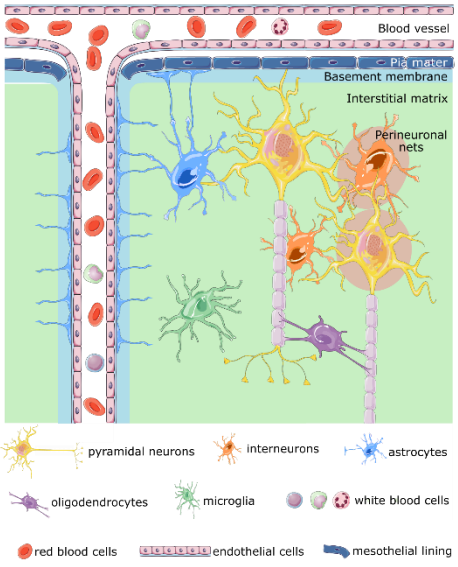

	Expression levels		Function
Basement membrane	Development	Adult	
laminin-nidogen complexes	+	++	neurogenesis cell migration function as a barrier molecular sieve
fibronectin	+	++	
collagen IV	+	++	
HSPGs (e.g. perlecan, agrin)	+	++	
dystroglycan (glycoprotein)	+	++	
Interstitial matrix			*Development* supports cell proliferation cell differentiation cell migration axonal outgrowth synaptogenesis
CSPGs: neurocan and versican	++	++	
aggrecan and brevican	—	++	
hyaluronan	+++	++	
tenascin-R	++	++	
tenascin-C	++	+	
link proteins: e.g HAPLN1	+	+	
collagen IV	low	very low	*Adult* controls cell survival synaptic plasticity response to damage
fibronectin	++	low	
laminin	++	low	
vitronectin	++	+	
Perineuronal nets			*Only in adult* protect from oxidative stress control synaptic stabilization & decrease synaptic plasticity hinder protein aggregation
CSPGs: aggrecan, neurocan,...	—	++	
hyaluronan	—	++	
tenascin-R	—	++	
link proteins: e.g. HAPLN1	—	++	

Figure 3

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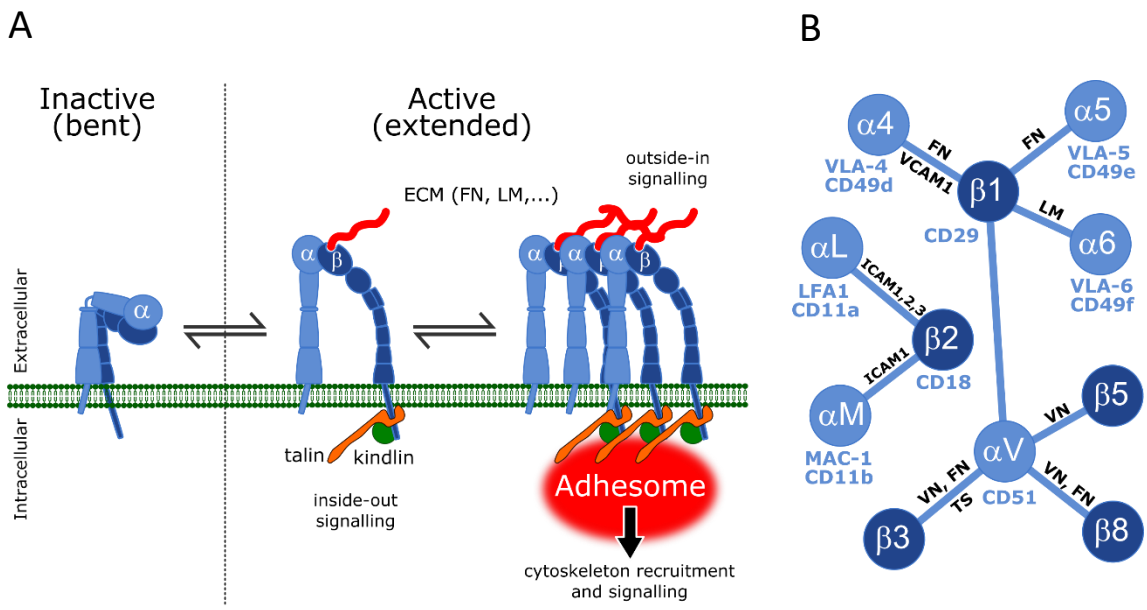

Figure 4

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