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Role of microglia in the maintenance of forebrain integrity during embryonic morphogenesis

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THÈSE DE DOCTORAT

DE L'UNIVERSITÉ PSL

Préparée à l'Institut de Biologie de l'École Normale Supérieure

**Rôle de la microglie dans le maintien de l'intégrité du cerveau
antérieur au cours de la morphogenèse embryonnaire**

**Role of microglia in the maintenance of forebrain integrity
during embryonic morphogenesis**

Soutenue par

Akindé LAWRENCE

Le 28 septembre 2022

Ecole doctorale n° 515

Complexité du vivant

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Biologie du développement

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LIST OF ABBREVIATIONS

AD	Alzheimer's disease
ATM	Axon-tract associated microglia
ATP	Adenosine triphosphate
BAM	Border-associated macrophage
CAM	CNS-associated macrophages
CC	Corpus callosum
CNS	Central nervous system
CR3	Complement receptor 3
CSA	Corticostriatoamygdalar boundary
CSB	Corticoseptal boundary
CSF1	Colony-stimulating factor-1
CSF1R	Colony-stimulating factor-1 receptor
CX3CR1	CX3C chemokine receptor 1
DAM	Disease associated microglia
DAP12	DNAX-activating protein of 12 kDa
EMP	Erythromyeloid progenitor cell
HOXB8	Homeobox protein Hox-B8
HSC	Hematopoietic stem cells
IGF-1	Insulin growth factor 1
IL-34	Interleukin-34
MIA	Maternal immune activation
NGF	nerve growth factor
PVM	Perivascular macrophage

PVP periventricular vascular plexus

P2Y12 purinergic receptor P2Y, G-protein coupled, 12

TAM Tyro3-Axl-Mertk receptors

TBI Traumatic brain injury

TF Transcription factor

TLR Toll like receptor

TREM2 Triggering receptor expressed on myeloid cells 2

YS Yolk sac

INTRODUCTION

1. Microglia: multifaceted brain macrophages

1.1 General presentation of microglia

1.1.1 Microglia, an atypical glial cell

Microglia are the brain resident macrophages. They were initially described in 1919 alongside oligodendrocytes by Pío del Río-Hortega, who sought to describe the “third element” of the brain, being neither neurons nor astrocytes (Sierra et al. 2016). He was the first one to identify them as phagocytes who display dynamic changes in microglial morphology, from highly ramified to amoeboid (Sierra et al. 2016; Hirbec, Rassendren, and Audinat 2019).

Like all tissue macrophages, microglia play a key role in organ integrity through their contribution to tissue development, homeostasis, repair and in disease (Colonna and Butovsky 2017; Tay et al. 2017; Hirbec, Rassendren, and Audinat 2019; Prinz, Jung, and Priller 2019; Prinz et al. 2021a; Augusto-Oliveira et al. 2022). Microglia constitute the first line of defense of the brain against pathogens or mechanical lesions, and were first described as such. Indeed, like all tissue resident macrophages, they are mononuclear phagocytes and maintain tissue homeostasis through phagocytosis, release of cytokines and chemokines as well as antigen presentation (Colonna and Butovsky, 2017; Hirbec, Rassendren, and Audinat, 2019; Prinz, Jung, and Priller, 2019; Prinz et al., 2021).

Microglia lie within the brain parenchyma, which is partially isolated from the blood circulation by the blood-brain-barrier (BBB), thereby conferring them with unique features (Prinz et al. 2021b; Masuda et al. 2022). On the one hand, microglia are part of the large contingent of glial cells, namely non-neuronal brain cells, which also comprise astrocytes and oligodendrocytes (Jäkel and Dimou 2017; Greenhalgh, David, and Bennett 2020). Astrocytes, initially thought to constitute support cells, acting as regulators of ion concentration, neurotransmitter availability, neuronal excitability and metabolic providers, are emerging as key regulators of neuronal activity (Reemst et al. 2016; Siracusa, Fusco, and Cuzzocrea 2019; Greenhalgh, David, and Bennett 2020). Oligodendrocytes, which are derived from oligodendrocytes progenitor cells (OPC), generate the myelin sheath essential for the regulation of electrical transmission. In addition, both OPC and oligodendrocytes are emerging as highly dynamic actors in

brain functions, for instance by regulating adaptive activity-dependent myelination in the context of learning (Kuhn et al. 2019; Greenhalgh, David, and Bennett 2020; Narine and Colognato 2022). While cross-talk between microglia and other glial cells are only starting to be described, it will be extremely important to understand how the dialogue between glial cells is regulated as well as which cells can act redundantly.

On the other hand, because of the BBB, the brain parenchyma is largely devoid of circulating cells of the adaptive immune system in physiological conditions, making microglia the main contingent of immune brain cells (Prinz et al. 2021a). Nonetheless, microglia are part of a broader community of macrophages, that are present in the brain vicinity, and collectively called border-associated-macrophages (BAMs) or central nervous system (CNS)-associated macrophages (CAMs) (Masuda et al. 2022; Prinz et al. 2021a). BAMs include choroid plexus macrophages, perivascular macrophages (PVM) or meningeal macrophages, located in the dura or leptomeninges (Prinz et al. 2021a; Masuda et al. 2022). Because BAMs lie at a critical interface between brain tissue and adaptive immune cells, they are emerging as important players in brain immunity during physiology and disease (Prinz et al. 2021a; Masuda et al. 2022).

As true glial immune cells, microglia lie at the crossroads of interactions between the immune system and the CNS. Over the past 20 years, landmark studies have revealed that microglia, in addition to their macrophage functions, also exert physiological functions in the neural tissue, regulating many aspects of brain development, physiology and disease (Colonna and Butovsky 2017; Tay et al. 2017; Hirbec, Rassendren, and Audinat 2019; Prinz, Jung, and Priller 2019; Prinz et al. 2021a; Augusto-Oliveira et al. 2022). Indeed, microglia have been shown to regulate neurogenesis, angiogenesis, synaptic transmission via contact with astrocytes as well as synaptic plasticity by pruning inactive synapses or inducing dendritic spine sprouting through modulation of the extracellular matrix (ECM) (Sierra et al. 2010; Nguyen et al. 2020; Diaz-Aparicio et al. 2020; Augusto-Oliveira et al. 2022) (Figure 1).

Importantly, these functions span the entire life, from early steps of development, a feature directly linked to their origin.

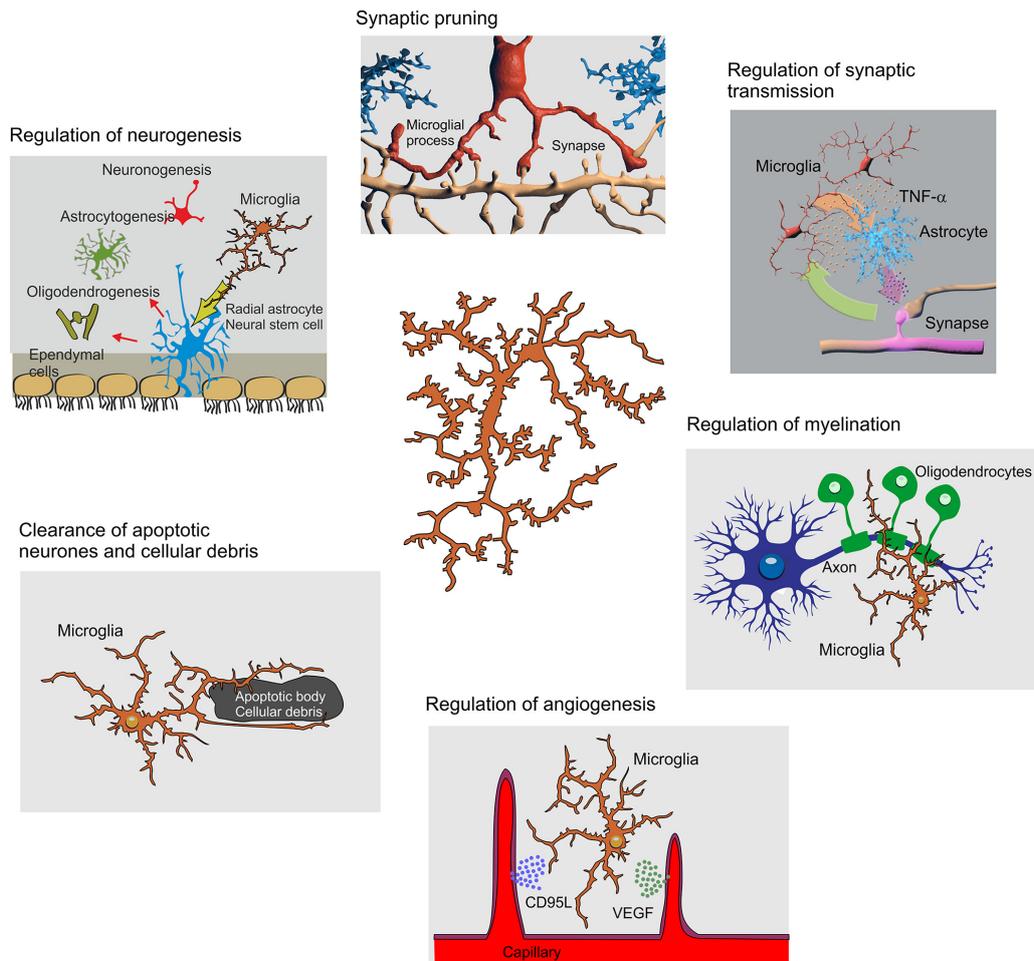


Figure 1. Physiological functions of microglia in the adult brain.

Scheme of physiological roles of microglia in the adult brain. From (Augusto-Oliveira et al. 2022).

1.1.2 Ontogeny of microglia

After the initial observations of Pio Del Rio Hortega who proposed that microglia have a mesodermal origin and are generated outside of the brain (Sierra et al. 2016), it was more recently proposed that microglia derive from monocytes of hematopoietic stem cells (HSCs) origin located in the fetal liver and then bone marrow. Descriptions of microglia at early stages embryonic stages (Perry, Hume, and Gordon 1985; Alliot et al. 1991; Ashwell and Waite 1991; Monier et al. 2007) and more recent lineage tracing studies definitively showed that they are derived from yolk-sac erythromyeloid progenitor cells (EMPs) (Ginhoux et al. 2010; Kierdorf et al. 2013; Hoeffel et al. 2015). Early C-kit⁺ EMPs arise in the extra-embryonic yolk-sac (YS) at E7.5 during the first

steps of hematopoiesis. These EMPs start differentiating into primitive macrophages, a pathway dependent on the transcription factors PU.1 and IFR8 as well as the expression of colony-stimulating factor-1 receptor (CSF1R), which genetic inactivation results in a complete absence of microglia (Schulz and al. 2012; Kierdorf et al. 2013). These primitive macrophages circulate through the blood to exit the YS, then reach the cerebral primordium and start colonizing the neuroepithelium around E9.5 in mice and 4.5-5.5 weeks in humans (Monier et al. 2007; Stremmel et al. 2018). These cells are in close contact with blood vessels as they migrate from the pia and ventricles into the parenchyma, suggesting they could enter through the bloodstream (Stremmel et al. 2018) (Figure 2). Some macrophages enter the parenchyma and become microglia, whereas some remain in the meninges, and other surrounding CNS tissue and become border-associated macrophages (BAMs), including meningeal and choroid plexus microglia, or form tight contact with blood vessels and differentiate into perivascular macrophages (PVM) (Goldmann et al. 2016; Masuda et al. 2022; Utz et al. 2020).

A second wave of microglial invaders has been proposed to come from C-Myb⁺ late EMPs generated at E8.5 in the YS, alongside other EMPs which transition through the fetal liver and will give rise to peripheral tissue resident macrophages. This second wave of microglial precursors has been reported to express HOXB8, and invades the neuroepithelium from E12.5 onwards (De et al. 2018b) (Figure 2). Whether HOXB8⁺ YS-derived microglia really represent an ontogenically different subpopulation is however still highly debated (Sharma, Bisht, and Eyo 2021).

Both waves of early microglia constitute the only glial cells present in the brain until the onset of astrogenesis and oligodendrogenesis in rodents and in humans (Semple et al. 2013; Reemst et al. 2016). Once in the neuroepithelium, microglia start proliferating and maturing to constitute the entire adult population of microglia in the brain parenchyma (Li and Barres 2018; Prinz et al., 2021a). Both waves of early microglia constitute the only glial cells present in the brain until generation of the onset of astrogenesis and oligodendrogenesis in rodents and in humans (Semple et al. 2013; Reemst et al. 2016). Once in the neuroepithelium, microglia start proliferating and maturing to constitute the entire adult population of microglia in the brain parenchyma (Li and Barres 2018; Prinz et al., 2021a).

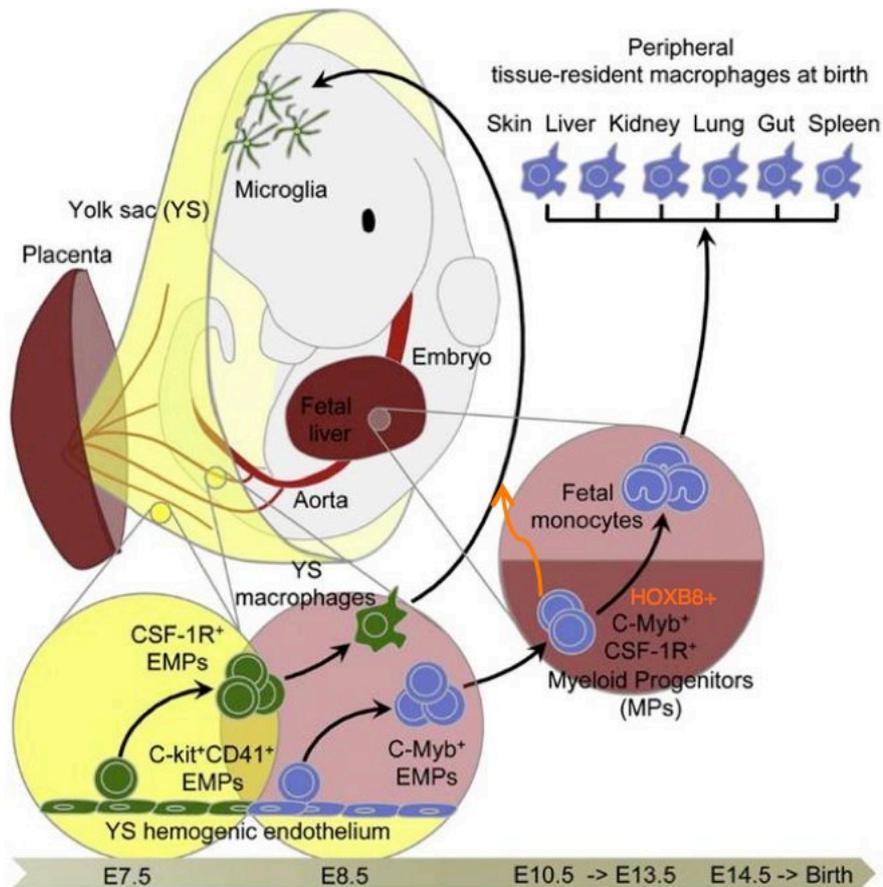


Figure 2. Microglia and peripheral macrophages ontogeny.

In the yolk sac (YS), several erythromyeloid progenitor cells (EMPs) arise. Some early E7.5 EMPs differentiate into YS macrophages through CSF1R signaling, independently of C-Myb and colonize the neuroepithelium where they become the resident microglial population. A later wave of E8.5 EMPs of the YS that express C-Myb, transition through the fetal liver where they become myeloid precursors (MPs) and go on to contribute to populations of peripheral macrophages in other organs. This includes HOXB8⁺ microglia that go on to populate the cortex from E12.5 onwards. Adapted from (Hoeffel et al. 2015).

PVMs and BAMs, but not choroid plexus microglia also self-renew throughout life, showing that, like microglia, they are dedicated to their niche (Li & Barres, 2018; Masuda et al., 2022; Prinz et al., 2021a). Furthermore, since the blood brain barrier closes around E13.5, there is very little contribution of non-microglial immune cells in the brain parenchyma, except in contexts of disease, injury and inflammation (Ginhoux and Prinz 2015) It was recently shown that in humans, microglia have a similar ontogeny and self-renewing abilities (Bian et al. 2020).

1.1.3 How to become and stay microglia: overview and implications

As seen in the previous section, adult microglia thus derive from embryonic YS-macrophage progenitors. While brain colonization by microglia will be described in the next chapter, an important issue is how these macrophages become microglia and how we can define this cell type, in terms of markers and molecular identity. As tissue resident macrophages, microglia respond to the niche that they colonize and acquire a specific cellular and molecular identity, as detailed in section 1.3.2.2 (Hickman et al., 2013; Lavin et al., 2014; Gosselin et al., 2014; Butovsky et al., 2014; Bennett et al., 2016). In particular TGF- β signalling is an essential regulator of microglial identity, notably important for microglial maturation and not for BAM maturation (Buttgereit et al. 2016; Utz et al. 2020; Zöller et al. 2018). Importantly, both acute signals and epigenetic regulators (Datta et al. 2018) are required for the proper maturation of microglia in the brain parenchyma. The interpretation and integration of niche-derived signals is tightly linked to the ability of microglia to respond to their environment via a unique set of endogenous ligand sensing a variety of signals including pathogens called “the microglial sensome”(Abels et al., 2021; Hickman et al., 2013; Hickman & El Khoury, 2019). The integration of niche signals converges onto the induction of the expression of *Sall1*, a transcription factor key for microglia identity (Bennett et al., 2018; Buttgereit et al., 2016; Thion, Low, et al., 2018; Utz et al., 2020). Importantly, circulating monocytes that engraft into the brain in physiological conditions, were reported to lack the capacity to induce *Sall1*, suggesting that, in addition to brain-derived signals, either the cell of origin or the time spent in the neural tissue is essential for a cell to become *bona fide* microglia (F. C. Bennett et al. 2018; Cronk et al. 2018; Ginhoux and Prinz 2015). This first step leads to a progressive maturation that drives the acquisition of important receptors that are also key markers of microglia. For instance, the purinergic P2Y₁₂ receptor (and its counterpart P2Y₁₃) is a central marker of microglia versus BAM identity, as is essential for microglia to respond to injury but also neuronal activity (Eyo et al., 2014; Kyrargyri et al., 2020; Milior et al., 2020). More generally, recent single cell transcriptomic studies in rodents, but also across a variety of species have enabled to define a microglial core signature, also referred to as homeostatic signature as detailed in 1.3.2.2 (Geirsdottir et al. 2019; Masuda et al. 2020). In addition to morphology that has constituted for a long time an important yet ill-defined criteria of

differentiation, this major step has pushed the field into a new era of disentangling ontogeny, identity acquisition, states and function as we will see in the next sections.

Consistently with the existence of a true symbiosis between their niche and microglia, dissociation of brain tissue leads to a rapid downregulation of key factors that constitute hallmark of microglial identity, including P2Y₁₂ (Gosselin et al. 2017). In addition to this, microglia are known to display a genuine and continuous dependence on niche-derived signals. In particular, microglia differentiation and survival is known to rely on the activation of the CSF1R pathway, a receptor that possesses two ligands, CSF1 and interleukin 34 (IL-34) (W. Lin et al. 2019; Kana et al. 2019; Easley-Neal et al. 2019). In terms of CSF1 and IL-34 ligands, distinct cell types (neuron versus glia) seem to produce them in a highly spatiotemporal manner, which will require in depth investigation (Easley-Neal et al., 2019). Nonetheless, CSF1 inactivation seems to overall reduce microglia at early steps of brain development while IL-34 inactivation seems to reduce the density of cells at later stages in the forebrain (Boulakirba et al., 2018; Greter et al., 2012; Muñoz-Garcia et al., 2021). While the ligands appear partially redundant, blocking CSF1-R signaling at any step of microglial life induces death of most of the cells exposed (Easley-Neal et al. 2019). This feature, which is a remarkable feature of the microglial/neural symbiosis, has enabled the generation of a variety of tools to deplete microglia and assess their functions (Eme-Scolan and Dando 2020; Kitic et al. 2019). In particular, global gene inactivation, or blockade of the CSF1R pathway using antibodies (AFS98) or pharmacological compounds (Plexxikon Inc. PLX3397) leads to large scale depletion of microglia (Kim et al., 2021). Importantly, BAMs are also sensitive to CSF1R blockage, implying that this kind of approaches generate models that lack microglia but also BAMs. As a generality, it has been difficult to disentangle the roles of microglia versus BAMs, and most of the studies performed cannot dissociate between the two. More recently, selective cre dependent strategies or additional models are being explored (Kim et al. 2021). In particular, while global genetic inactivation of CSF1R leads to a lack of microglia and BAMs, the depletion of an enhancer of the *Csf1r* gene called FIRE, enables to generate mice that lack microglia (and other resident macrophage populations), but still display, to a certain extent, BAMs (Rojo et al. 2019; Munro et al. 2020). The dependence of microglia to their niche is hence an important feature that enables their manipulation and functional studies.

1.1.4 A cell under influence with life-long consequences

As mentioned, microglia are characterized by a sensome, that enables them to detect a variety of signals (Abels et al., 2021; Hickman and El Khoury, 2019; Hickman et al., 2013). While we will see in 1.2.3 that they respond to local neuronal signals linked to activity, it is also important to stress that microglia can be influenced by a broad variety of signals derived from the body, or the environment (Augusto-Oliveira et al. 2022; Tay et al. 2017; Hirbec, Rassendren, and Audinat 2019). One example is the microbiota, which has been shown to influence microglia in part via short chain fatty acid, and in particular acetate (Erny et al. 2015; Morgane Sonia Thion et al. 2018; Erny et al. 2021). Similarly, nutrition like high-fat diet and exposure to pollutants can modify microglial properties (Delpech et al. 2015; Madore et al. 2020; Martinat et al. 2021). In terms of other exogenous factors, chemotherapy treatments have been reported to affect microglial activity, as well as activity of other glial brain cells, contributing to what is called “chemobrain”, linked to cognitive impairments in patients (Gibson et al. 2019; Gibson and Monje 2021; Gutmann 2019; Whalley 2019).

Another factor that influences microglia in rodents, especially adults, is sexual identity (Thion et al. 2018; Han et al. 2021; Villa et al. 2018; Yanguas-Casás 2020; Lynch 2022). While these differences could be in part mediated by an acute influence of steroid sex hormones, microglia isolated from males and females maintained slightly different properties, with female microglia being more neuroprotective *in vitro* as well as when transplanted *in vivo* (Villa et al. 2018). These observations raise the important possibility that microglia also integrate these influences in the long-term, potentially via epigenetic modifications (Ayata et al. 2018).

Lastly, this capacity of microglia to sense, respond and change for a prolonged timeperiod, is particularly interesting in the context of inflammation. Microglia, as *bona fide* immune cells, respond to inflammation, which in the context of brain studies has been often mimicked by an injection of lipopolysaccharide (LPS), a membrane component of Gram-negative bacteria (Hoogland et al. 2015; Lively and Schlichter 2018; Roumier et al. 2008). In this context, it has been well established that microglia respond differently to an LPS exposure after a previous one, either by presenting an attenuated response (training) or an enhanced one (priming) (Wendeln et al. 2018;

Neher and Cunningham 2019; Roumier et al. 2008). Such modifications, or immune memory, can have long-lasting implications for our understanding of microglial contribution to ageing and normal cognitive decline or responses to neurodegenerative diseases (Koellhoffer, McCullough, and Ritzel 2017; Bulut, Kilic, and Domínguez-Andrés 2021; Costa et al. 2021).

1.2 Adult microglia in health and disease

Microglia deficits have long been directly linked to disease in the context of CSF1R mutations leading to adult-onset leukodystrophy with axonal spheroids (Biundo et al. 2021; Ferrer 2022; Konno et al. 2018). Likewise, homozygous mutations in *TREM2* and *TYROBP(DAP12)* which respectively code for Triggering Receptor Expressed on Myeloid cells 2 (TREM2) and DNAX-Activating Protein of 12 kDa “(DAP12), lead to Nasu-Hakola disease, including an early onset dementia (Konishi and Kiyama 2018; Roumier et al. 2004; 2008; Bianchin et al. 2010; Tay et al. 2017). In addition to these microgliopathies, microglia have been shown over the past decades to regulate several core aspects of brain physiology and pathology. Indeed, a large number of studies have established the main contribution of microglia, and more broadly brain macrophages to the regulation of behaviors and brain function. A first example is “sickness behavior”, a coordinated behavioral response to viral infection or inflammation, leading to reduced social interactions, reduction of appetite and changes in the levels of cytokine and neurotransmitters (Devlin, Smith, and Bilbo 2022; Béchade et al. 2021; Jin et al. 2016). Another striking example is the behavioral deficit induced by the mutation of the *Hoxb8* transcription factor, expressed in microglia and other immune brain cells, leading to obsessive-compulsive behaviors, highlighting the potential roles of microglia in regulating behaviors altered in neurodevelopmental disorders (Van Deren et al. 2022; De et al. 2018b). Finally, microglia are now recognized actors, or at least contributors, to the progression of neurodegenerative diseases. This feature is illustrated with the identification of polymorphisms in the *Trem2* gene as risk factors for the development of Alzheimer’s disease, a gene only expressed in microglia and macrophages in the brain (Song and Colonna 2018)

Based on these studies, microglia are involved in many integrated processes as well as contributors to almost all brain diseases. In this chapter, I have chosen to focus mainly on the cellular properties and functions of microglia.

1.2.1 A central role for microglial phagocytosis

Microglia participate to brain homeostasis through phagocytosis, whereby they remove pathogens as well as dead or dying cells and modulate inflammation, after injury or simply in normal physiological context, such as during neurogenesis in the dentate gyrus of the hippocampus in the mouse brain (Sierra et al. 2010; Diaz-Aparicio et al. 2020; Galloway et al. 2019; Janda, Boi, and Carta 2018). Phagocytic functions of microglia are mediated through several known signaling pathways that underlie attraction of microglia to their target and phagocytosis. ATP/UDP signaling recruits microglia to their target (Koizumi et al. 2007). Furthermore, microglia can recognize pathogens through Toll Like Receptors (TLR) or C-type lectin receptors (CLRs), such as C-type lectin domain family 7 member A (CLEC7A), but also dying cells presenting “eat me” signal phosphatidylserine (PS) via TAM receptors Axl and Mertk (Colonna and Butovsky 2017). Furthermore, microglia recognize apoptotic cells’ complement molecules, in particular via Complement Receptor 3 (CR3) which triggers their phagocytosis (Borucki et al. 2020). Lastly, the receptor complex involving TREM2 and DAP12 also expressed by microglia similarly allows them to recognize and phagocyte pathogens or apoptotic cells (Konishi and Kiyama 2018). In contrast, microglia can also sense “don’t eat me signals” such as integrin-associated protein Cluster of Differentiation 47 (CD47), expressed on the surface of healthy neurons, an effect mediated by receptor Signal regulatory protein α (SIRP α) (Brown and Neher, 2014; Lehrman et al., 2018)(Figure 3).

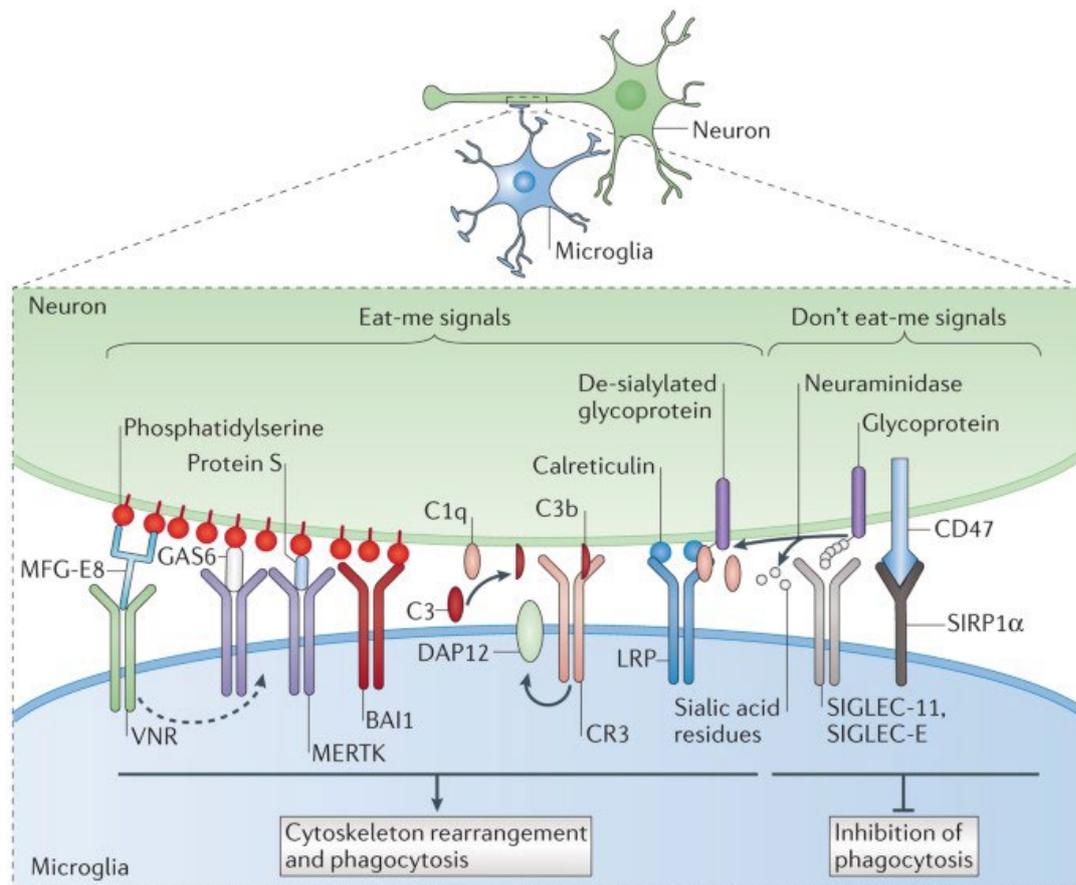


Figure 3. Microglial pathways for sensing and initiating phagocytosis. Microglia sense “eat-me signals” such as phosphatidylserine on the surface of dying neurons or other neural cells. They can also sense “don’t eat me signals” such as CD47 at the cell surface. From (Brown and Neher 2014).

Importantly, all these pathways have been identified as critical regulators not only in the regulation of cell numbers or elimination of debris, but also of synapses (Faust, Gunner, and Schafer 2021; Augusto-Oliveira et al. 2022). As detailed in chapter 2, we will see that similar tagging, recognition and elimination regulates the number of synapses in development but also in the mature brain, thereby contributing to cognitive decline linked to ageing (Cornell et al. 2022)

1.2.2 Microglia: much more than professional phagocytes

If microglia are the professional phagocytes of the brain, this function can also be performed by other glial cell populations such as astrocytes (Jung and Chung 2018;

Faust, Gunner, and Schafer 2021). In addition, microglia contribute to brain functions via many additional cellular properties, notably the production of cytokines including IL1beta, IL6, TNF-alpha and many more (Colonna and Butovsky 2017; Werneburg et al. 2017; Augusto-Oliveira et al. 2022). These cytokines are not only essential for microglial communication to other cells of the immune system, but also are important regulators of synaptic plasticity in a variety of context, acting on synaptic stability but also functional properties (Werneburg et al. 2017). In addition to cytokines, microglia can produce growth factors, such as IGF-1. They can also produce ECM components and their regulators, especially metalloproteases (Mosser et al. 2017; Augusto-Oliveira et al. 2022; Faust, Gunner, and Schafer 2021). This variety of factors produced by microglia that can act onto neighboring cells is large, and I chose to only illustrate how combination of factors can regulate an integrated response, in the context of injury and degeneration (see 1.2.4). Lastly, a major feature of microglia that emerged in the recent years is their capacity to negatively regulate neuronal excitability, notably via the transformation of ATP into adenosine (Badimon et al. 2020; Merlini et al. 2021). Taken together, these reveal ability for microglia to tune brain function in multiple ways. In the next section, their fine sensing of neuronal activity and metabolism is described.

1.2.3 Microglia as sensors of neuronal activity and metabolism

The initial studies of live microglial imaging (Davalos et al. 2005; Nimmerjahn, Kirchhoff, and Helmchen 2005) using the *Cx3cr1^{gfp}* mice that enabled for the first time the *in vivo* visualization of microglia, revealed a complex and rapid surveillance of their surroundings as well as an acute response to lesions. Since these first steps, key studies have deciphered how these cells move, respond to injury and neuronal activity, as well as exert essential roles in the regulation of network activity (Augusto-Oliveira et al. 2022). Beyond a baseline motility of their branches, which was shown to be regulated by the potassium 2P channel THIK-1 (Madry et al. 2018), microglia respond to a variety of signals that are linked to tissue injury, but also neuronal activity. Indeed, from the live studies pioneered in zebrafish (Ying Li et al. 2012), a series of studies have revealed that microglia respond to neuronal activity (Augusto-Oliveira et al. 2022). On the one hand, microglia reorient their processes towards active neurons or synapses, a process that has been shown to rely on P2Y12 and the co-release of ATP

(S.-S. Lin et al. 2021; Augusto-Oliveira et al. 2022; Eyo et al. 2014; Ying Li et al. 2012). On the other hand, a series of studies have highlighted that microglia express a variety of neurotransmitter receptors, rendering them also responsive directly to the release of neurotransmitters (H. Liu, Leak, and Hu 2016; Pannell et al. 2014). As examples recently related to functional responses of microglia, these cells can express the GABA_{B1} receptor and respond to GABAergic activity in the neocortex (Favuzzi et al. 2021), and can also express the Serotonin Receptor 5HT_{2B} and respond to serotonin signaling in the context of sickness behavior (Béchade et al. 2021; Kolodziejczak et al. 2015).

Finally, and importantly, recent studies have revealed that microglia can form somatic junctions with the cell bodies of neurons, notably via P_{2Y12} receptors on the microglial side (Cserép et al. 2020). This interaction also enables the tight link with a network and mitochondria and could constitute a checkpoint of the electrical and metabolic activity (Cserép, Pósfai, and Dénes 2021). Microglia thus seem to exert a complex monitoring of neuronal activity and metabolic activity of their neighbors, not only at the synapse, but also at the cell body. It will be important to dissect out the relative contributions of these monitoring to the adapted response of microglia in the context of normal brain physiology or pathology.

1.2.4 An example of coordinated response to injury and disease

Microglia distribute throughout the surface of the adult CNS parenchyma and are dynamic cells in the adult brain, displaying a highly ramified morphology scanning their environment via motile processes. Through these processes they can rapidly sense damage or perturbation to the tissue. Indeed, several studies have shown through live two-photon imaging that microglia respond to laser ablation of the CNS within a few hours, by extending their cytoplasmic extensions to the site of injury, likely in order to prevent propagation of neurotoxic substances to the surrounding tissue (Davalos et al. 2005; Hines et al. 2009; Dibaj et al. 2010; Nimmerjahn, Kirchhoff, and Helmchen 2005) (Figure 4A). Microglia sense CNS damage signals through expression of specific receptors. These include purinergic receptors such as P_{2Y} purinoceptor 12 (P_{2RY12}) and P_{2X} purinoceptor 4 (P_{2X4}) that can sense ATP released by dying cells (Illes et al. 2020) (Figure 4A).

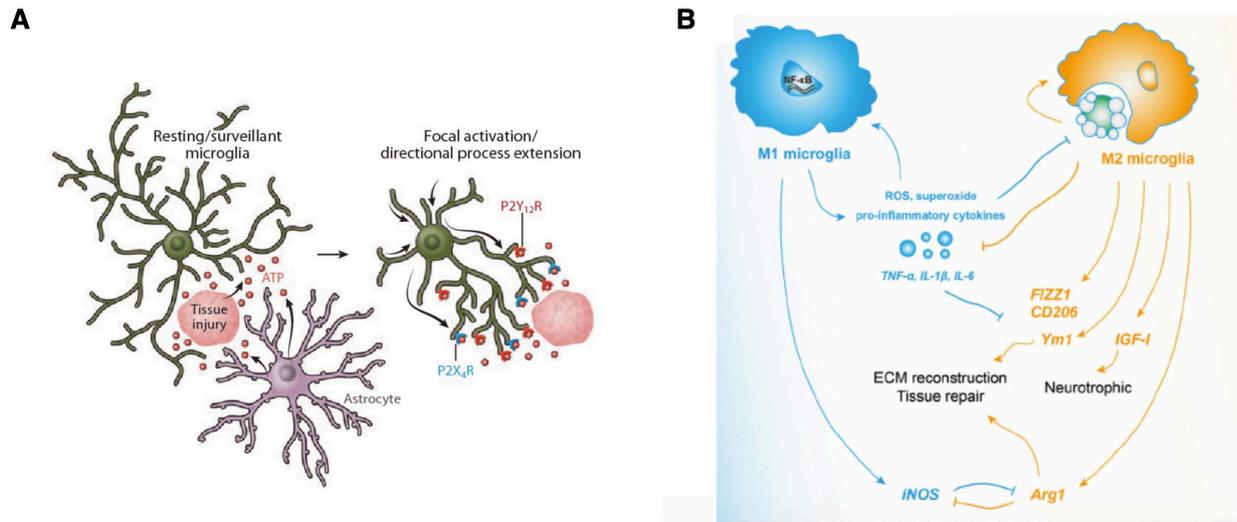


Figure 4. Microglial response in injury and disease. (A) Microglia can sense damage signals such as ATP that are released by neurons or astrocytes during injury. Adapted from (Nayak, Roth, and McGavern 2014). **(B)** Microglia become differentially activated in injury and disease, having both detrimental and protective effects notably in promoting or preventing cell death or encouraging break down or strengthening of the ECM, which has led to the concept of the M1/M2 dichotomy. Adapted from (Tang and Le 2016).

Microglia also respond to more acute injury in the mouse and human brain, such as traumatic brain injury (TBI), axonal breakage, ischemic injuries or in pathological contexts such as Alzheimer's disease (Donat et al. 2017). In these cases, microglia have been observed to migrate to the site of injury. Once there, microglia can have protective but also neurotoxic effect (Tang and Le 2016). They can start producing pro-inflammatory factors, such as IFN- γ , TNF- α or interleukin-6 (IL-6) to fight pathogens and resolve damage, but which can become neurotoxic (Kawabori and Yenari 2015) and lead to exacerbated neuronal damage and death in chronic degenerative diseases (Hickman et al. 2018). On the other hand, microglia may release trophic factors such as pro-neurogenic molecule insulin-like growth factor (IGF-1) to support neuronal survival and/or remyelination (Tang and Le, 2016). It has also been shown that microglia respond to injury through remodeling of the extracellular matrix (ECM)(Kawabori and Yenari 2015; Madsen and Bugge 2013; Kigerl et al. 2009). Indeed, they are involved in glial scar formation through secretion of extracellular matrix chondroitin sulphate proteoglycans (CSPGs), but also its degradation through secretion of matrix metalloproteinases (MMPs) (Kawabori and Yenari 2015). Microglia

can further contribute to ECM repair through expression of enzyme arginase 1 (Arg1) whose products contribute to ECM synthesis and strengthening (Tang and Le 2016). A classical but oversimplified view of the duality of microglial action in disease and injury was represented in the M1/M2 nomenclature (Tang and Le 2016; Ransohoff 2016)(Figure 4B). In this view, M1 microglia are “activated” pro-inflammatory cytokine producing cells that tend to have detrimental effect on the surrounding issue and M2 microglia are pro-repair and anti-inflammatory cytokine producing (Guo, Wang, and Yin 2022). This M1/M2 dichotomy does not do justice to the multiplicity of possible microglial phenotypes in different pathological contexts and the use of this nomenclature tends to disappear (Ransohoff 2016). In the next section, the current view and findings on microglial heterogeneity is described.

1.3 Microglial heterogeneity in health and disease

As seen in the previous section, microglia can perform various functions in the brain in health and disease. Microglia are multifaceted sensors of the CNS and can adapt and react to a broad panel of stimuli. For some time microglia were studied through the prism of M1/M2 activation but it is now assumed that microglia, like other types of CNS cells -neurons, astrocytes, oligodendrocytes, are a heterogeneous population throughout time and space, allowing for this functional diversity (Stratoulis et al. 2019; R. Paolicelli et al. 2022). Indeed, a common pool of microglia can change their “state” in different fashions depending on their local milieu, by changing their distribution pattern, morphology, factor secretion and phagocytic activity (Stratoulis et al. 2019; R. Paolicelli et al. 2022). However, the question remains whether there are “subtypes” of microglia that possess distinct intrinsic baseline properties that can in turn switch states to respond differently to the same stimuli (Figure 5) (Stratoulis et al. 2019; R. Paolicelli et al. 2022). In this section, the current knowledge on microglial heterogeneity is described

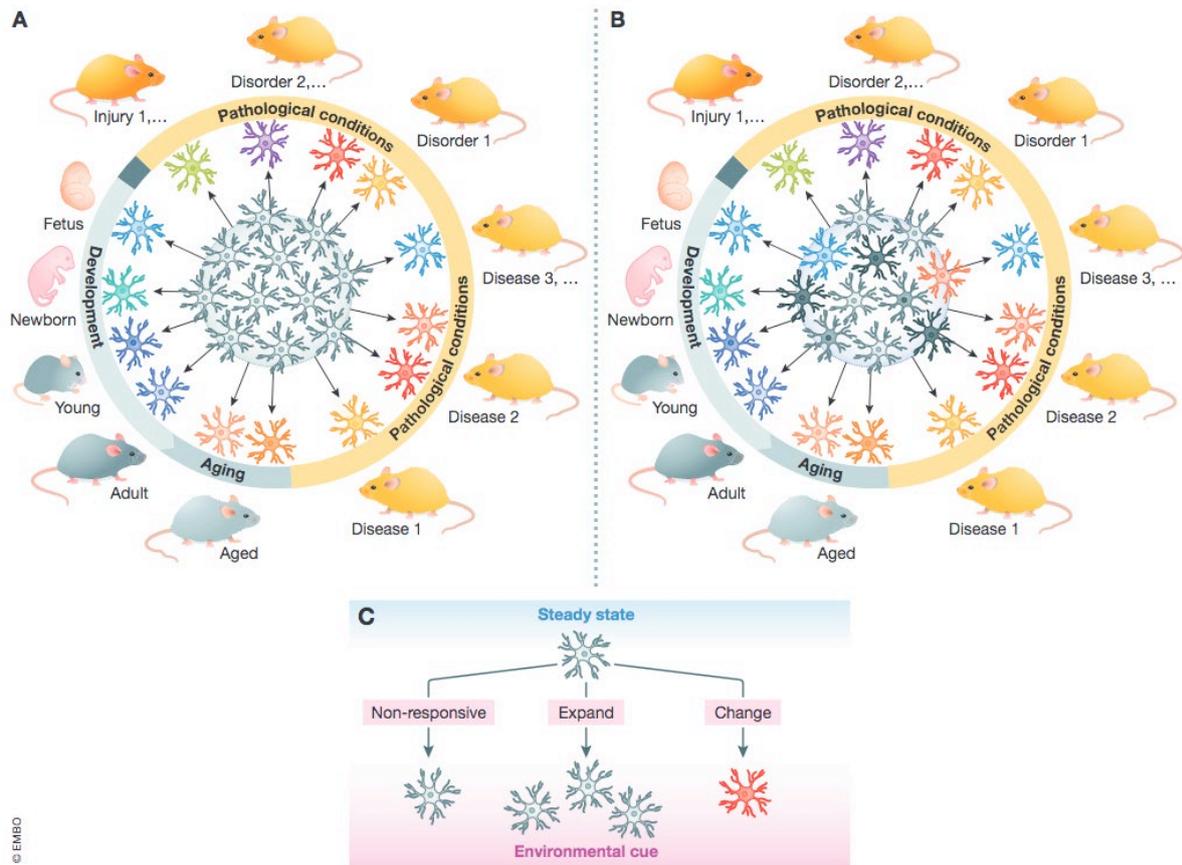


Figure 5. Microglia heterogeneity during the lifespan. (A) Microglial heterogeneity could arise from the same pool of microglia, exposed to different sets of signals in their local milieu that vary spatially, temporally and in health and disease, initiating a change of “state”. **(B)** Alternatively, microglia subtypes with distinct intrinsic characteristics could pre-exist and respond differently to different stimuli, explaining microglial heterogeneity. **(C)** Different subtypes of microglia could respond differently to the same stimulus. From (Stratoulis et al. 2019).

1.3.1 Microglia spatial and morphological heterogeneity in the healthy CNS

In the healthy adult brain microglia are ubiquitously distributed but their density and morphology can vary in different structures (Tan, Yuan, and Tian 2020). Microglial density in the adult mouse brain was assessed by several groups which showed that it was highest in the forebrain compared to midbrain and hindbrain through immunohistochemistry and flow cytometry using different markers such as F4/80, Iba1

or LC1 (Tan, Yuan, and Tian 2020). In particular several studies point to the cortex as a region of high microglia density and the cerebellum as a region of low density (Tan, Yuan, and Tian 2020) (Figure 6A).

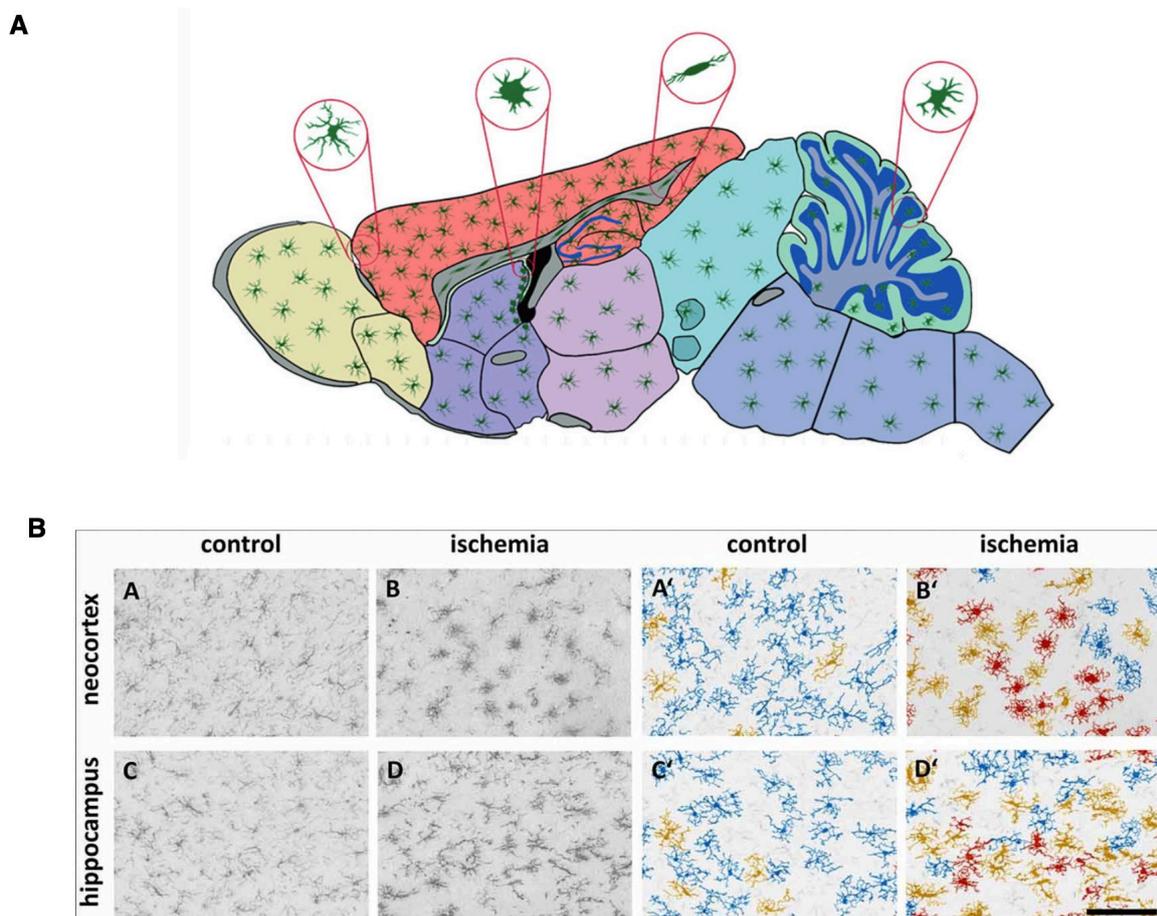


Figure 6. Microglia distribution and morphologies in healthy and diseased brain. (A) In the adult brain microglia populate the brain with slight regional variations in density. In addition, their characteristic ramified morphology varies notably in certain regions like the SVZ. Adapted from (Tan, Yuan, and Tian 2020). **(B)** In conditions of disease or injury, such as ischemia, microglia morphology can vary in differentially according to brain region, as exemplified by distinct response to ischemia of neocortical microglia display more rounded morphology changes compared to hippocampal microglia in this image. From (Leyh et al. 2021).

In addition, microglial density seems to be heterogeneous throughout subregions themselves (Tan, Yuan, and Tian 2020). Furthermore, it has been shown that heterogeneity in distribution and morphology of microglia is greatest during development (described in the next chapter) and microglial morphology is globally ramified in the adult mouse brain, but the degree of ramification varies between different regions (Tan, Yuan, and Tian 2020). Only in certain regions, does this diverge completely, such as in the corpus callosum where microglia are elongated along axons, and regions where the blood brain barrier is leaky such as the subventricular zone (SVZ), where amoeboid microglia can be observed (Tan, Yuan, and Tian 2020)(Figure 6A). These changes reflect differences in microglia interaction with their environment, but the signal and intrinsic characteristics that drive these different morphologies in microglia are not well understood. Importantly, although diversity in microglial morphology suggests heterogeneity and morphology can be associated to certain “states” or functions, this characteristic alone cannot be used to define the “state” or function of a microglia (Stratoulis et al. 2019). Nonetheless, in pathological contexts, microglia can change morphology heterogeneously depending on brain regions or on their proximity to lesions, indicative of the influence of the milieu on microglia, and the relevance of morphology as a proper characteristic to probe microglial functional states (Leyh et al. 2021; Savage, Carrier, and Tremblay 2019)(Figure 6B).

1.3.2 Microglial functional states and transcriptional heterogeneity and in health and disease

1.3.2.1 Identified microglial states in the adult brain

A few subsets with distinguishing features and suspected functional specialization have been uncovered in the adult brain (for review see Stratoulis et al., 2019). These often remain to be further described as “states” or subtypes as they were not always defined based on exhaustive multidimensional characteristics (transcriptomics, spatial distribution, morphology) but rather based on functional specialization or

characteristics such as differential protein marker expression. Interestingly, a putative subtype of microglia present solely in the neocortex was defined as ontogenically different HOXB8 microglia (De et al. 2018a). These HOXB8 microglia differed significantly only by 21 genes from their non-HOXB8 counterparts and although their precise roles are yet undefined, their loss resulted in pathological grooming behavior (De et al. 2018a).

On the other hand, De Biase and colleagues reported microglia from different brain structures can exhibit different “states”, which they propose to be dependent on local cues (De Biase et al. 2017). Indeed, microglia from the cortex or distinct basal ganglia nuclei differ in morphology, lysosomal content, membrane properties and bulk transcriptome and these localization-specific characteristics can be reacquired by repopulating microglia after depletion (De Biase et al. 2017). Further microglial states are also observed in homeostatic conditions. For instance, Keratin sulfate positive (KSPG) microglia are found preferentially in the olfactory bulb, hippocampus and brainstem (Stratoulis et al. 2019). Similarly, certain microglia associate preferentially with the axon initial segment (AIS) of neurons exclusively in the cortex and hippocampus (Stratoulis et al. 2019). Additionally, a group of microglia that have low expression of purinoreceptors regulate neurogenesis in the adult SVZ and rostral migratory stream (RMS) (Xavier et al. 2015). These groups of microglia can react differently to injury or disease, such as AIS microglia that cease AIS contact upon injury or KSPG microglia that associate with motoneurons in amyotrophic lateral sclerosis (ALS) models and could thus represent a disease reactive state (Stratoulis et al. 2019). Furthermore, a phenotype of microglia is almost exclusively present in pathological contexts, and defined as “dark microglia” due to their condensed cytoplasm and nucleoplasm when observed at the ultrastructural level (Bisht et al. 2016). Their functions in pathology remain unidentified (Bisht et al. 2016). There also exist sets of microglia that are resistant to CSF1-R inhibition-induced pharmacological depletion, but the unique properties that allow for their survival are still not well understood (Stratoulis et al. 2019). Some functional microglial states also emerge during development and will be described in the next chapter 3.

1.3.2.2 Transcriptional heterogeneity in adult microglia

It is now possible to track the heterogeneity in microglial transcriptional profiles across time and space, health and disease, using different single cell sequencing methods in mouse and human tissues (Masuda et al. 2020). Several groups have thus reported their findings on the different microglial profiles, as well as comparing them to other border associated macrophages (BAMs), non-parenchymal macrophages associated with the CNS and other myeloid cells. These studies thus also allowed for a refinement of hallmarks of adult microglial identity and homeostatic signature that can be altered in subtypes. The search for a homeostatic signature of microglia was performed in several studies, which showed that TGF- β signaling is important for the specification of commonly identified a hallmark genes signature with *Sall1*, *Tmem119*, *P2ry12*, *Cx3cr1*, *Trem2*, *Siglech*, *Hexb* and *Fcrls* more highly in microglia and little to not in other myeloid cells (Hickman et al., 2013; Lavin et al., 2014; Gosselin et al., 2014; Butovsky et al., 2014; Bennett et al., 2016). Some of these genes, like *P2ry12* and *Cx3cr1* are sense-coding genes that confer specific functions of microglia in response to brain signals. In humans, the homeostatic signature also includes *CX3CR1* and *P2RY12* (Galatro et al. 2017; Gosselin et al. 2017), but microglia show more regional transcriptional heterogeneity (Masuda et al. 2020). These studies commonly show that the homeostatic status of microglia can slightly vary regionally, especially in the mouse cerebellum, albeit to a lower extent in the adult brain than in the developing brain (described in the next chapter), and these differences have yet to be associated to specific functions (Masuda et al. 2020).

Contrastingly, several groups have looked at the transcriptional profile of microglia in disease models and identified a common signature, which was coined disease associated microglia (DAMs) by Keren-Shaul and colleagues (Keren-Shaul et al. 2017). In DAMs, homeostatic microglia gene expression is downregulated and some inflammatory genes are upregulated (Deczkowska et al. 2018b). These genes prominently include ones that code for receptors involved in phagocytosis such as *Axl*, *Tyrobp*, *Clec7A* or *Itgax* (coding for CD11c), lipid metabolism genes such as *ApoE* and *Lpl*, but also tissue repair genes *Gpnmb* and *Spp1* (H. Wang 2021). Consistent with this signature, DAM were initially associated with restriction of A β plaques in Alzheimer's disease (AD) models but similar profiles were broadly found to be protective in other neurodegeneration disease contexts, such as ALS, aging and

demyelination (for review see Benmamar-Badel, Owens, and Wlodarczyk, 2020) (Figure 7A).

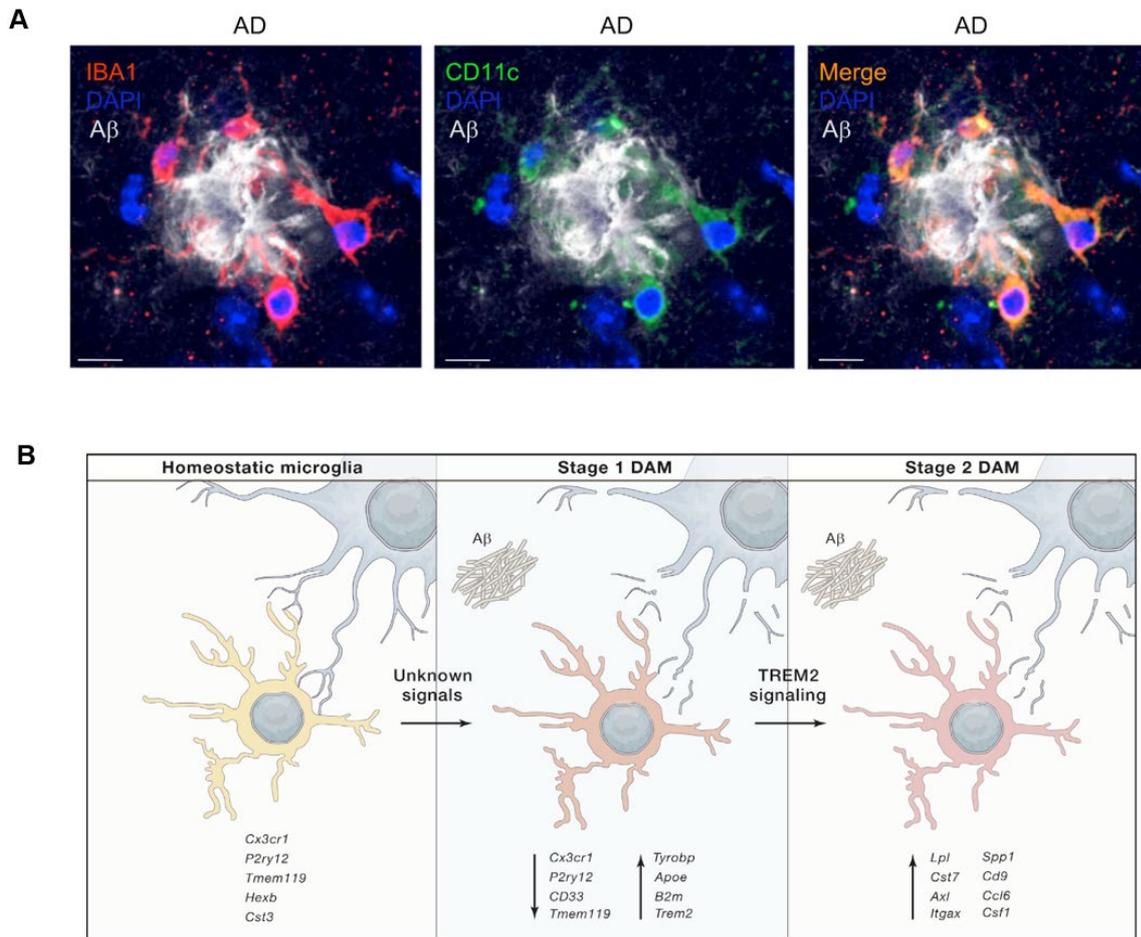


Figure 7. Microglia acquire the DAM profile in Alzheimer’s disease. (A) In some degenerative diseases such as AD the microglial homeostatic transcriptional is lost after exposure to unknown signals, leading to stage 1 DAMs expressing a differential profile. At a more advanced stage microglia acquire a stage 2 DAM profile dependent on TREM2 signalling. (Deczkowska et al. 2018b). (B) CD11c+ DAM microglia accumulate around Aβ plaques in a mouse AD model. (Keren-Shaul et al. 2017).

The emergence of the DAM phenotype was shown to depend on receptor TREM2 signalling, to switch from an early DAM stage 1 to a DAM stage 2 following disease progression (Deczkowska et al. 2018b) (Figure 7B). Indeed, in *Trem2* ^{-/-} mouse

models of AD, A β pathology is worsened (Keren-Shaul et al. 2017) and the DAM profile is never fully acquired as microglia remained in DAM1 stage.

Furthermore, it was proposed that the DAM phenotype is induced by microglia sensing of danger signals such as phosphatidylserine on apoptotic neurons, or signals from myelin debris and A β . Indeed, it has been shown that apoptotic cells can drive the DAM profile *in vitro* and *in vivo*. In humans, similar transcriptomes to DAM have been observed in microglia from AD patients, and mutations in *TREM2* and *DAP12/TYROBP* are involved in an early onset dementia disease, Nasu Hakola (Deczkowska et al. 2018; Konishi and Kiyama 2018). Thus, degenerative diseases microenvironment drives the switch of microglial phenotype to DAM, but the full acquisition of that phenotype is dependent on the TREM2 pathway, showing that there are also different states that microglia can attain due to pathological changes in the tissue but can vary in according to intrinsic properties of microglia (Masuda et al. 2020). Furthermore, there is evidence that more disease context- specific changes in transcriptomic profiles which give rise to clusters distinct from DAM in mouse models and human cases of demyelinating disease, such as autoimmune encephalomyelitis (EAE) and multiple sclerosis (MS) (Masuda et al. 2020).

Taken together, in the adult brain, microglial are multifaceted cells, but their heterogeneity and functional switches are most remarkable in disease contexts. In the next chapter, microglial development and microglial contribution to forebrain development will be described.

2. Microglia and forebrain development

2.1 Forebrain structures and morphogenesis

2.1.1 Structures and tracts of the forebrain

The brain is the anterior part of the central nervous system that derives from a sheet of ectodermal cells, the neural plate, during early development (Figure 8A; for review see (Colas and Schoenwolf 2001)). The neural plate invaginates and folds dorsally to become the neural tube which then gives rise to three compartments: the prosencephalon or forebrain, the mesencephalon or midbrain and the rhombencephalon or hindbrain (Figure 8B) (Colas and Schoenwolf 2001). In this thesis, we will focus on the forebrain, which plays a central role in higher cognition, integration of sensory information, movement and emotions (Medina 2009; Badre 2008; Dumontheil 2014). The forebrain is divided into the telencephalon and more caudally, the diencephalon (Figure 8B).

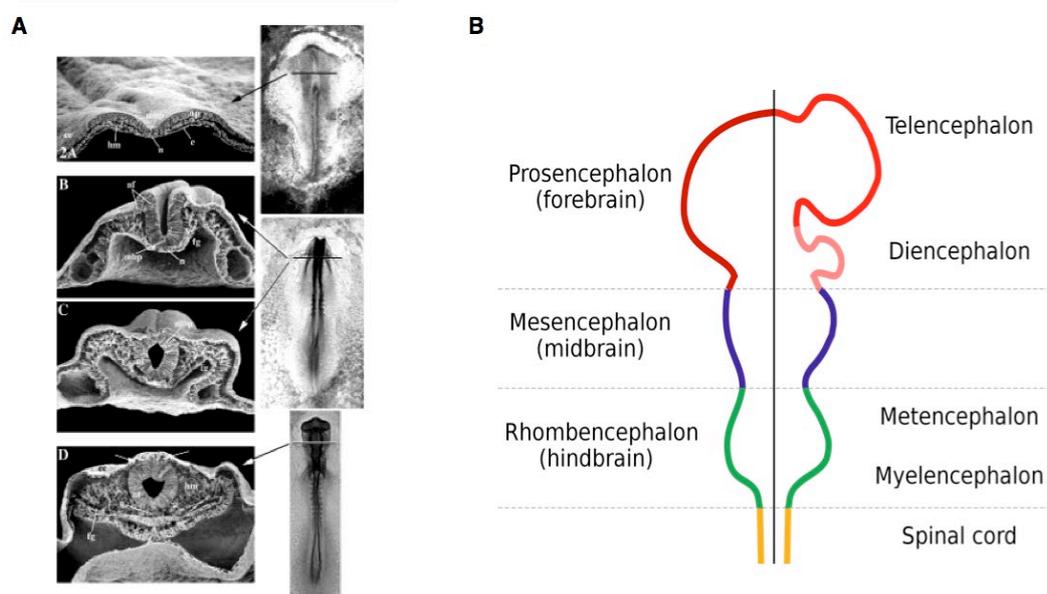


Figure 8. Forebrain. (A) Origin and structures of the forebrain. The forebrain derives from the neuroectodermal cells which give rise to the neural tube. From Colas and Schoenwolf, 2001. **(B)** The anterior neural tube gives rise to the brain, which that can be divided, into 3 main vesicles including the forebrain. Image by (I, Nrets., 2007).

The telencephalon encompasses the pallium and the subpallium. The pallium is made up of two cerebral hemispheres that bear the neocortex and the amygdala, which are

essential for higher cognitive functions and emotional display (Badre 2008; Dumontheil 2014; Medina 2009; Baxter and Croxson 2012). The subpallium contains the basal ganglia, particularly the striatum, which participates in motor control, learning, memory and emotions (Phelps and LeDoux 2005; Groenewegen 2003; Medina 2009) (Figure 9A).

The functional implications of these structures largely depend on the establishment of a correct circuitry, with appropriate specification of their neural components. They also depend on long-range axonal communication between neurons within the forebrain such as commissural tracts. These include the corpus callosum (CC), anterior commissure (AC) and hippocampal commissure which relay information between the cortices of the two different hemispheres, as well as the internal capsule, which contains thalamo-cortical axons, cortico-thalamic axons and cortico-subcerebral axons (Chédotal and Richards 2010) (Figure 9B). In the next section we will describe the development and specification of forebrain structures throughout embryogenesis.

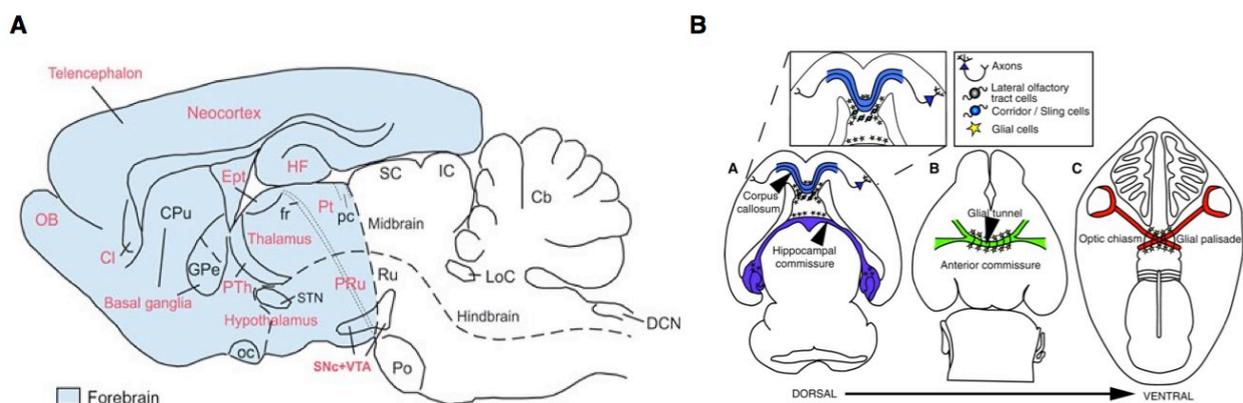


Figure 9. Adult mouse forebrain. (A) Schematic sagittal view of an adult mouse brain, showing the major structures of the forebrain. From (Medina 2009). **(B)** Major commissural axon tracts of the forebrain include the corpus callosum (CC), the hippocampal commissure (HC) and the anterior commissure (AC). Adapted from (Chédotal and Richards 2010).

2.1.2 Forebrain morphogenesis

During morphogenesis, the different structures of the telencephalon and diencephalon are specified and the formation of more or less well-defined borders, such as the pallial-subpallial border (PSB) (Campbell 2003; Rallu, Corbin, and Fishell 2002; Garel, Huffman, and Rubenstein 2003; Hoch, Rubenstein, and Pleasure 2009). In the neural tube, this is orchestrated by morphogen gradients secreted from organizer centers that drive expression of specific combinations of transcription factors to dictate neural cell identity, including neuronal and glial cells (Figure 10A) (Campbell 2003; Rallu, Corbin, and Fishell 2002; Garel, Huffman, and Rubenstein 2003; Hoch, Rubenstein, and Pleasure 2009).

Thus morphogens such as Sonic hedgehog, Wnt, fibroblast growth factors (FGF), retinoic acid (RA) and bone morphogenetic protein (BMP) pattern the forebrain (Campbell 2003; Rallu, Corbin, and Fishell 2002; Garel, Huffman, and Rubenstein 2003; Hoch, Rubenstein, and Pleasure 2009).

Thus, progenitors of the pallium express factors *Emx1*, *Pax2*, *Pax6* or *Ngn2* and give mostly rise to excitatory cells whereas subpallial progenitors express *Gsh2*, *Mash1* and *Dlx* family members and give mostly rise to inhibitory neurons (Figure 10B) (Wonders and Anderson 2006; Medina and Abellán 2009; O. Marín and Rubenstein 2001).

During embryogenesis, the subpallium arises into 4 transitory structures: the medial ganglionic eminence (MGE), the lateral ganglionic eminence (LGE) and the caudal ganglionic eminence (CGE) and the ventral preoptic area (POA). These regions are characterized by specific combinations of gene expression, which define distinct molecular territories from which arise the different nuclei of the basal ganglia (Oscar Marín and Müller 2014; Nóbrega-Pereira et al. 2010; O. Marín and Rubenstein 2001; Oscar Marín et al. 2002). Through radial migration of neurons born there, the MGE thus gives rise to the globus pallidus (GP), LGE thus gives rise to the striatum (Str), the CGE and the POA to the basal magnocellular complex (Oscar Marín and Müller 2014; Nóbrega-Pereira et al. 2010; O. Marín and Rubenstein 2001; Oscar Marín et al. 2002). Some tangential migration of neurons born in those structures participates in subpallial development, but also pallial development. In particular, GABAergic cortical and striatal interneurons are born in the MGE, LGE and CGE (Oscar Marín and Müller 2014).

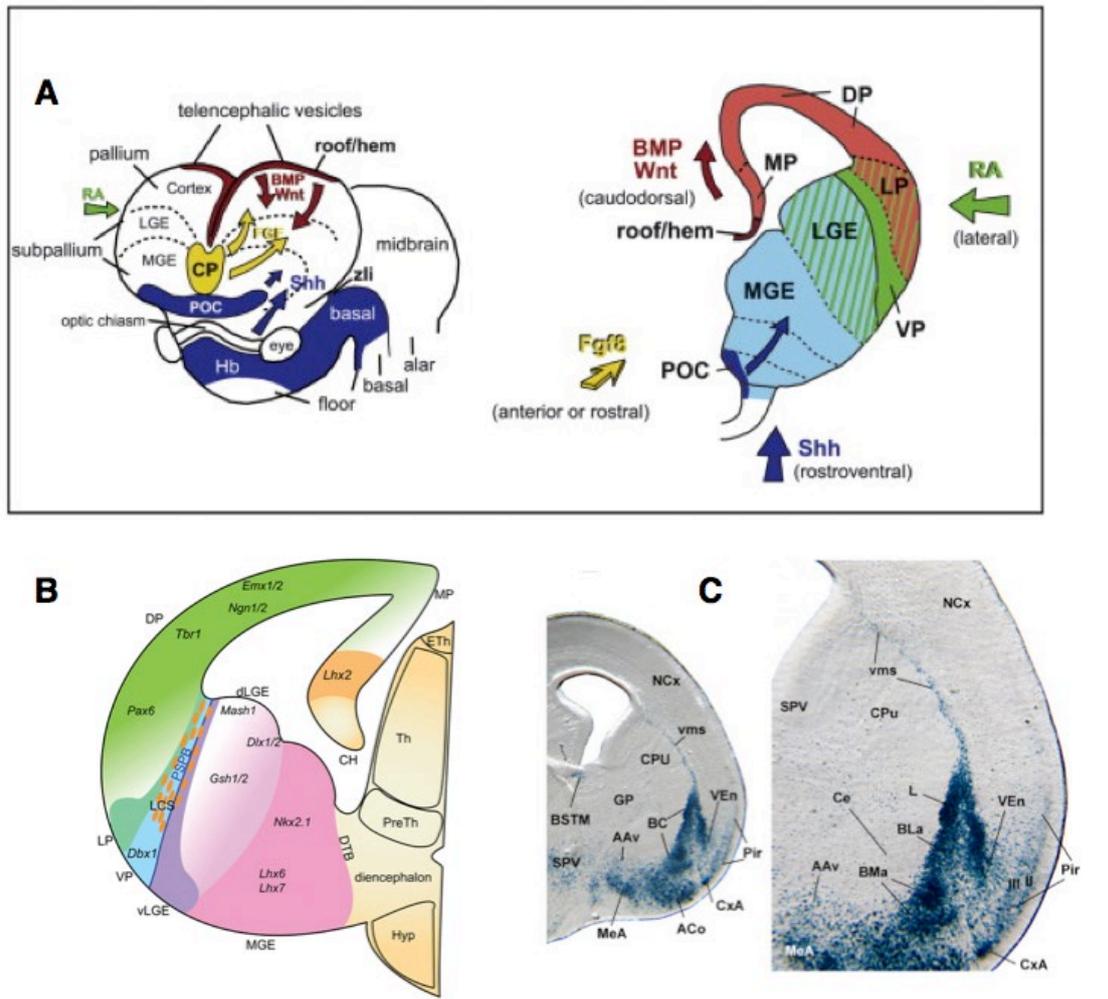


Figure 10. Morphogenetic signals and division of the pallium and subpallium during forebrain morphogenesis. (A) Morphogen gradients dictate pallial and subpallial structures specification. From (Medina and Abellán 2009). **(B)** Distinct combinations of gene expression distinguish pallial and subpallial domains. Axon guidance cues guide thalamic axons to the cortex. From (Grant, Hoerder-Suabedissen, and Molnár 2012). CH, cortical hem; dLGE, dorsolateral ganglionic eminence; DP, dorsal pallium; DTB, diencephalon-telencephalic boundary; Hyp, hypothalamus; LCS, lateral cortical stream; MGE, medial ganglionic eminence; MP, medial pallium; PSPB, pallial-subpallial boundary; LP, lateral pallium; VLGE, ventro-lateral ganglionic eminence; VP, ventral pallium; Th, thalamus. **(C)** Dbx1-LacZ tracing delineates CSA boundary. Adapted from (Puelles et al. 2016). BLA, basolateral amygdala; CPu, caudate putamen (striatum); NCx, neocortex; Pir, piriform cortex; VEn, ventral endopiriform cortex; vms, ventral migratory stream (equivalent of LCS).

The amygdala is part of the ventral pallium and arises from progenitors in the ventral pallium (Medina 2009). The pallial-subpallial boundary (PSB) marks a sharp boundary between the pallium and subpallium and on the pallial side of it comprises migrating

progenitors that have their own transcriptional profile (such as *Pax6*, *Dbx1* and *Sp8* TFs) that migrate along the lateral cortical stream (LCS) giving rise to neuronal diversity in certain amygdalar nuclei, piriform cortex, Cajal Retzius cells and olfactory bulb neurons (Bielle et al. 2005; Carney et al. 2006; Waclaw et al. 2010; Kohwi et al. 2007; Puelles et al. 2016) (Figure 10B).

The PSB runs throughout the rostrocaudal axis, and is mostly rostrally the boundary between the cortex and the striatum (Puelles et al., 2016). However, caudally the PSB becomes the boundary between amygdala and the striatum, which is only dorsally appended to the cortex (Carney et al., 2006; Puelles et al., 2016). This boundary is thus distinguished from what we can define as the cortico-striato-amygdalar boundary (CSA), the boundary that separates the cortex and the amygdala and is prolonged into the cortico-striatal boundary dorsally (Figure 10C).

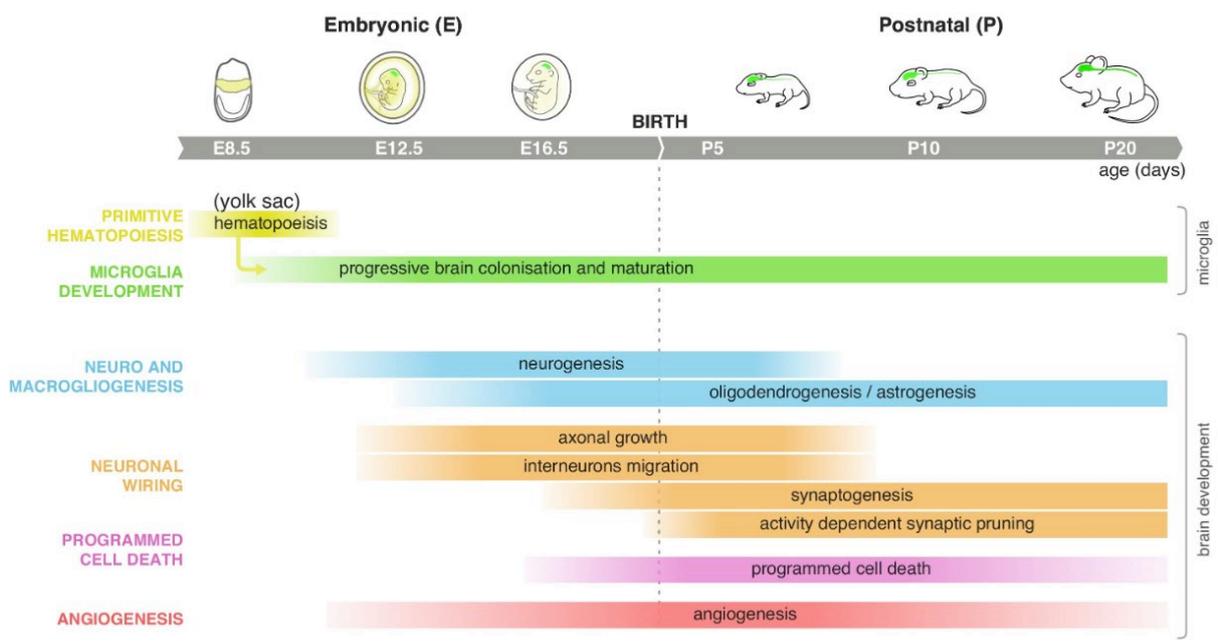


Figure 11. Timeline of developmental events in the forebrain. Microglia are present throughout early and late embryogenesis as well as perinatal developmental processes. From (M. Thion and Garel 2017).

During late morphogenesis, the established structures of the pallium and subpallium continue their growth in a context of morphogenetic tensions with the advent of processes such as neurogenesis and neuronal migration-associated expansion of structures like the cortex as well as mechanical tension associated with axon genesis

(Hankin, Schneider, and Silver 1988; Van Essen 2020; Saito et al. 2019). In this context, microglia are the only glial cells present until late gestation with the onset of genesis of other glial cells (M. Thion and Garel 2017). They are thus present throughout embryogenesis but also during perinatal developmental processes such as programmed cell death and synaptogenesis (M. Thion and Garel 2017) (Figure 11). In the next section, we describe how microglia participate in forebrain development.

2.2. Microglia in development: ontogeny and early functions

2.2.1 Distribution and morphology during forebrain colonization

In spite of the numerous studies performed, we still have a relatively fragmented understanding of the mechanisms by which microglia enter and colonize the brain. Once they start colonizing the brain, microglia show a spatiotemporally precise, stereotypical and uneven distribution pattern in the forebrain, throughout embryogenesis and early postnatal development (Thion and Garel, 2017). This has been observed in many mammalian species such as rodents and primates including humans (Pont-Lezica et al. 2011). In contrast, microglia tile the surface of the forebrain parenchyma more uniformly in the adult brain (Thion and Garel, 2017). For instance, in the embryo, microglia are found to associate with developing blood vessels, prompting the still debated hypothesis of their entry into the brain through the vasculature (Arnold and Betsholtz 2013) and accumulate zones of developmental cell death (Pont-Lezica et al. 2011). This stereotyped pattern of distribution of developmental microglia is exemplified by transient heterogeneous accumulations at different “hotspots” as well as varying morphologies during development, that have been mostly described in mammalian species (Perry, Hume, and Gordon 1985; Swinnen et al. 2013; Squarzoni et al. 2014). Importantly, embryonic microglia in mice also associate with radial glia, and accumulate along growing axon tracts such dopaminergic fibers and corpus callosum (CC) and at the corticostriatoamygdalar boundary (CSA) (Squarzoni et al., 2014; Thion and Garel, 2017) (Figure 12A). Of note, transient amoeboid microglial accumulations were also observed in white matter regions, specifically the CC, in the early rodent postnatal brain (Benmamar-Badel, Owens, and Wlodarczyk 2020). Similar accumulations in the white matter were described already in 1939 as “fountains of microglia” in human embryonic brains at corresponding stages (Kershman 1939)(Figure 12B).

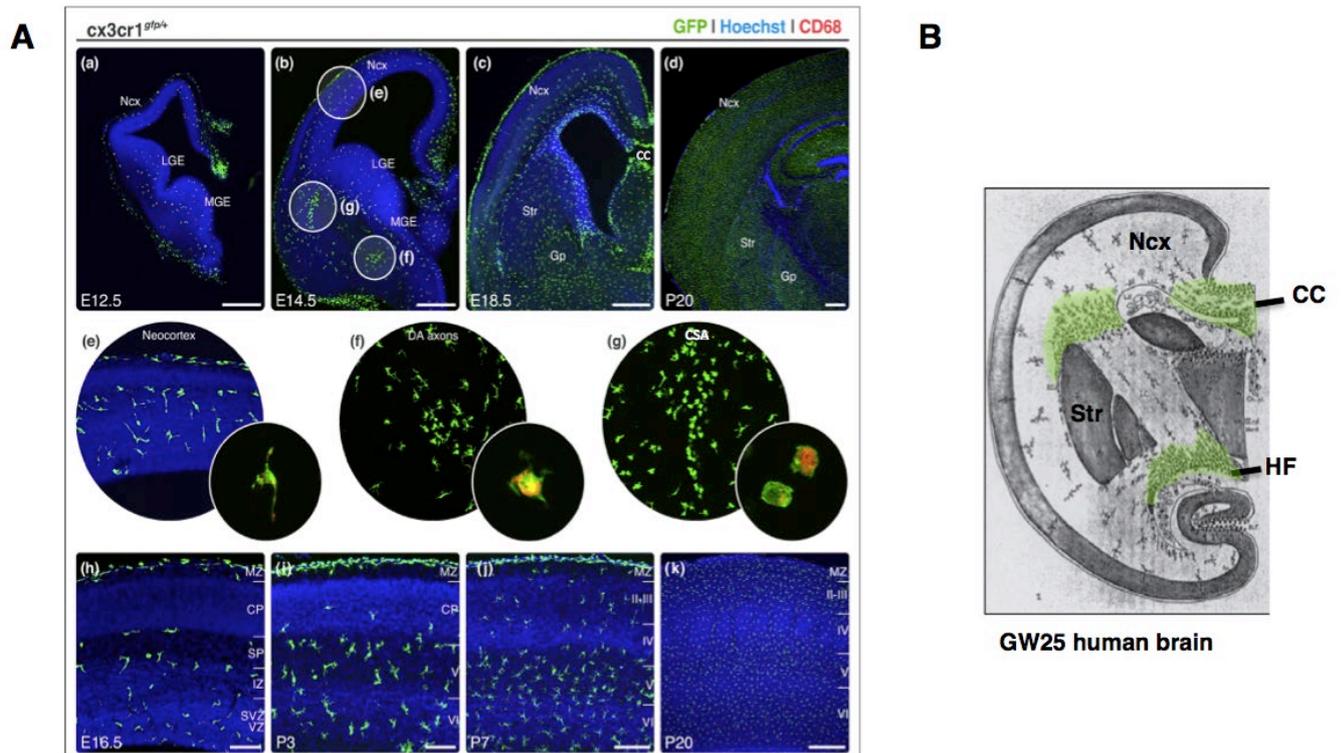


Figure 12. Early distribution of microglia during forebrain development. (A) Microglia visualized by CX3CR1-GFP expression populate the forebrain unevenly in the mouse embryo compared to late postnatal brain and accumulate at hotspots that include the CSA (a-d). Embryonic microglia morphology is less complex than in the adult brain and is more amoeboid at certain hotspots such as the CSA (e-g). Microglia do not invade the cortex homogeneously (h-k). Figure from (M. Thion and Garel 2017). **(B)** Scheme representing “fountains of microglia”, highlighted in green, which can be observed in white matter regions (corpus callosum, CC and hippocampal fissure, HF) and at the border of the striatum in late gestation during human brain development. Adapted from (Kershman 1939).

Another example of the peculiar microglia distribution throughout early development is their colonization of the cortex, which unfolds in a specific fashion: they are first excluded from the cortical plate before E16.5, stay in deeper layers in contact with progenitors and progressively tile the different cortical layers as postnatal development unfolds (M. Thion and Garel 2017)(Figure 12A, h-k). In particular, postnatal development microglia still display some peculiar distributions, as they are excluded from barrel fields of layer IV developing somatosensory cortex before P5 when they start migrating into these structures and interact with young thalamocortical synapses in a CX3CR1 dependent manner (Arnoux and Audinat 2015). From the second

postnatal week, microglia acquire a more homogeneous distribution in the barrels (Arnoux and Audinat 2015).

Finally, although they are generally more rounded and possess less ramified branches, embryonic microglia still vary greatly in morphology, ranging from amoeboid to branched (M. Thion and Garel 2017) (Figure 12A, e-g). Postnatally, microglia start adopting a generally more ramified morphology, explaining why amoeboid “fountains of microglia” stand out (Cengiz et al. 2019)(Figure12B). The diversity in morphology and spatiotemporal specificities of microglial distribution suggests diverse functions related to the structures they associate with during embryonic and perinatal development. These functions are described in the next sections.

2.2.2 The diverse roles of microglia in the developing forebrain

Although their early roles remain largely unexplored, a few precocious embryonic functions of microglia have been identified (Mosser et al., 2017; Pont-Lezica et al., 2011; Thion, Ginhoux, et al., 2018a). An important limitation to all the studies presented herein which conclude on microglial functions, is that the models used usually target both microglia and BAMs, as well as often peripheral macrophages (Hammond, Robinton, and Stevens 2018; Eme-Scolan and Dando 2020; Prinz et al. 2021a). As such, it is important to note that all the functions presented below still need to be precisely attributed to microglia, or to other populations of macrophages. Nonetheless, for clarity and concision purposes, I have kept the terminology “microglia” in the following sections.

2.2.2.1 Prenatal roles of microglia

First, Fantin and colleagues found that appropriate fusion of tip cells to form new branches in the blood vessel network requires the presence of brain macrophages in the embryonic hindbrain (Figure 13A) and the developing retina (Fantin et al. 2010; Arnold and Betsholtz 2013). Close contact between microglia throughout perinatal development in rodents and humans suggests that they may have a more extensive role in early angiogenesis (Mondo et al. 2020). Furthermore, microglia have been shown to regulate axon tract development in the embryonic brain. Indeed, microglia

regulate the growth of dopaminergic axons into the striatum at E14.5, in a CX3CR1-dependent, but DAP12/TYROBP-independent manner (Squarzoni et al. 2014) (Figure 13C). In addition, microglia were shown to regulate CC projections fasciculation at the end of embryogenesis, in a DAP12/TYROBP-dependent manner (Pont-Lezica et al. 2014) (Figure 13C). Interestingly, in both tracts, axonal development was also perturbed by maternal immune activation (MIA), suggesting that MIA could in part act through by altering a normal developmental function of microglia (Squarzoni et al. 2014; Pont-Lezica et al. 2014).

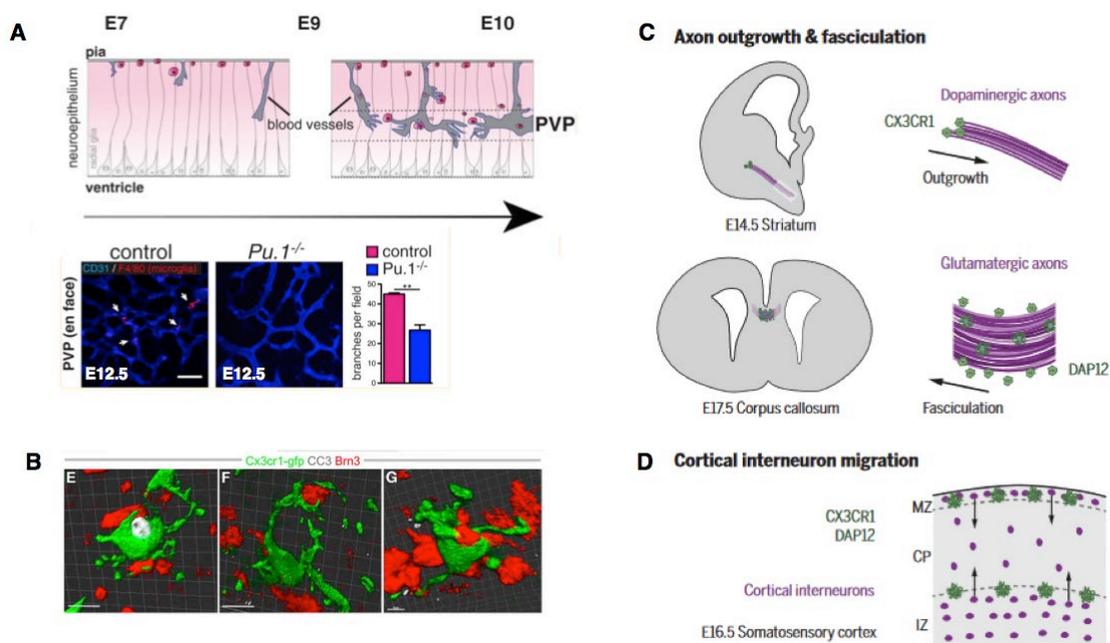


Figure 13. Microglia perform diverse functions in prenatal brains. (A) In the early embryonic hindbrain, microglia associate with blood vessels throughout their sprouting, and their absence in *Pu.1*^{-/-} mice lacking myeloid cells leads to decreased vascular branch points in the periventricular vascular plexus (PVP). Figure adapted from (Arnold and Betsholtz 2013) **(B)** Rendering of 3D reconstructed confocal images of CX3CR1-GFP+ microglia engulfing apoptotic (E) and viable (F-G) Brn3+ RGCs in the embryonic retina. Adapted from (Anderson, Zhang, et al. 2019). **(C-D)** Embryonic microglia are required for appropriate dopaminergic axon outgrowth and CC axon fasciculation as well as positioning of interneurons. Either CX3CR1 or DAP12/TYROBP signaling, or both mediate these functions. From (Thion, Ginhoux, et al., 2018b).

Similarly, Squarzoni and colleague showed that embryonic depletion of microglia, MIA and genetic inactivation of CX3CR1 and DAP12/TYROBP signalling lead to modified interneuron positioning in the embryonic cortex (Squarzoni et al. 2014) (Figure 13D). Consistently, the neocortex of embryonic microglia depleted or MIA offsprings display long term impairments in inhibitory circuits (Thion et al., 2019).

Finally, although microglia functions have mostly been described during postnatal development (see 2.2.2.2), some functions such as the elimination of apoptotic cell bodies and the engulfment of live cells or cell fragments have also been reported prenatally. Embryonic engulfment of apoptotic cells by microglia was observed in different contexts, including E4 chick retinal cells, and E20 rat neuronal progenitors as observed using bi-photon live imaging (Cunningham, Martinez-Cerdeno, and Noctor 2013; Frade and Barde 1998). In a CR3 dependent manner, microglia also engulf apoptotic and viable retinal ganglion cells (RGCs) in the mouse retina (Anderson, Zhang, et al. 2019) (Figure 13B). Importantly, despite the fact that their role in reaction to external insults is not extensively described in the embryo, Rosin and colleagues have recently shown that microglia can induce breakage of AAV infected or *in utero* electroporated damaged radial glia and phagocytose them in the embryonic hypothalamus, through TAM receptors MertK et Axl signaling (Rosin, Marsters, et al. 2021).

Taken together, microglia prove to be multifaceted actors of early embryonic development, where their roles are yet to be fully described. By contrast, we have a

better understanding of their postnatal functions, which will be the focus of the next sections.

2.2.2.2 Neural cell regulation, survival and maturation

As in the adult brain where they were shown to regulate neurogenesis, microglia regulate developmental homeostasis also through regulation of neuronal numbers, survival and maturation (Bessis et al. 2007; Mallat, Marín-Teva, and Chéret 2005; Sierra, Tremblay, and Wake 2014; Su et al. 2014; Hammond, Robinton, and Stevens 2018; Prinz, Jung, and Priller 2019; Augusto-Oliveira et al. 2022). Cell numbers can be controlled through regulation of progenitor cells as well as through regulating survival and apoptosis of differentiated neurons. Microglia are involved in both modes of regulation. Perinatally, microglia engulf P0 hippocampal neurons but also P3 Purkinje cerebellar neurons (Marín-Teva et al. 2004; Wakselman et al. 2008). In these cases, it has been shown that microglia provoke cell death and engulfment by producing reactive oxygen species (ROS) and in the P0 hippocampus, require DAP12/TYROBP expression to perform this function (Wakselman et al. 2008) (Figure 14A). In parallel, microglia regulate neuronal survival through secretion of trophic factors such as IGF1, which promotes the survival of cortical layer V subcerebral and callosal projection neurons during postnatal development (Ueno et al. 2013; Fujita et al. 2020) (Figure 14B-C). Such effect of microglia requires CX3CR1 as well the interaction of microglial NGL-1 and axonal NetrinG1, which enable the accumulation of IGF1-producing microglia along the axons of layer V neurons (Fujita et al. 2020) (Figure 14B-C).

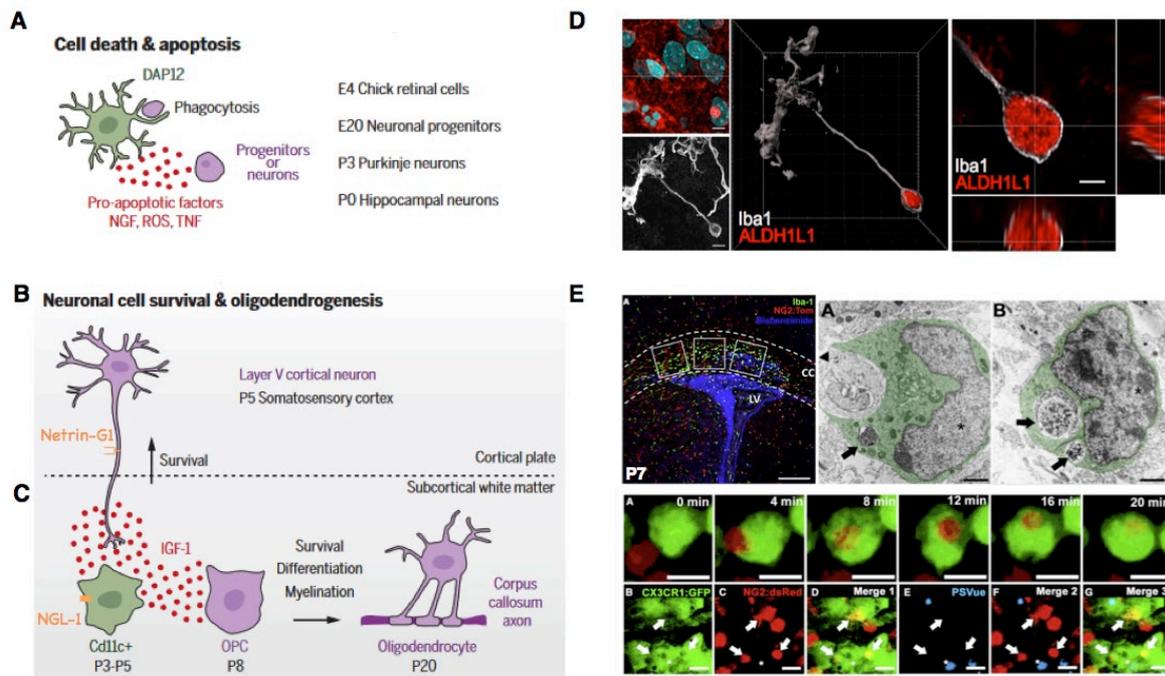


Figure 14. Microglia modulate neural cell populations during early postnatal development. (A) Microglia regulate neuronal cell populations by inducing cell death through factor secretion (such as nerve growth factor (NGF), ROS (reactive oxygen species), tumor necrosis factor (TNF)) and by phagocytosing apoptotic neurons. From (Thion, Ginhoux, et al., 2018b) (B) Microglia promote P5 layer V somatosensory neurons survival through IGF-1 signalling. Adapted from (Morgane S. Thion, Ginhoux, and Garel 2018b) (C) Subcortical white matter microglia interact with layer V neurons by sensing their expression of Netrin-G1 via receptor NGL-1 to promote their survival and regulate oligodendrogenesis and myelination through IGF-1 signalling. Adapted from (Thion, Ginhoux, et al., 2018b) (D) 3D rendering of a confocal imaged Iba1+ microglia engulfing a viable ALDH1L1+ astrocyte at P4 in the amygdala. Adapted from (VanRyzin et al. 2019). (E) In the P7 CC white matter, Iba1+ microglia interact with NG2+ OPCs and newly born oligodendrocytes. Electron microscopy shows that microglia (in green) engulf other cells and live imaging shows CX3CR1-GFP+ microglia engulfing viable NG2+ cells and distinct PSVue+ apoptotic cells. From (Nemes-Baran, White, and DeSilva 2020).

In addition to regulating neuronal numbers, microglia also act onto other glial cells. Several research groups provided evidence that microglia can engulf astrocytes to regulate their numbers during development in the cortex, amygdala and retina (Li et al., 2019; Puñal et al., 2019; VanRyzin et al., 2019). In particular, in the first postnatal week, amygdalar microglia phagocytose viable newborn astrocytes in a sexually dimorphic and CR3-dependent manner, shaping juvenile play behavior in rats

(VanRyzin et al. 2019) (Figure 14D). Furthermore, microglia were shown to regulate oligodendrogenesis in the early postnatal white matter and ultimately myelination (Benmamar-Badel, Owens, and Wlodarczyk 2020). Similarly to astrocytes, several *in vivo* and live-imaging studies have reported that microglia engulf viable oligodendrocyte progenitor cells (OPC) and newly formed oligodendrocytes in the first postnatal weeks (Li et al. 2019; Nemes-Baran and DeSilva 2021; Nemes-Baran, White, and DeSilva 2020; Irfan, Evonuk, and DeSilva 2022) (Figure 14E). Nemes-Baran and colleagues reported that this action is mediated through microglial CX3CR1 and ablation of its coding gene leads to hypomyelination in the white matter in the long-term (Nemes-Baran, White, and DeSilva 2020). Alternatively, but consistent with often proposed dichotomous roles of microglial cells, other groups report that microglial fountains constitute a specialized subtype of CD11c⁺ microglia that can support oligodendrogenesis and promote myelination, in part through trophic IGF-1 signalling (Benmamar-Badel, Owens, and Wlodarczyk 2020; Wlodarczyk et al. 2017). This specialized microglial state is further described in the next section on developmental heterogeneity of microglia.

2.2.2.3 Synaptogenesis

In contrast to the limited knowledge that we have on the functions exerted by microglia and other macrophages during early steps of development, a series of landmark studies have established that these cells play critical roles in postnatal circuit refinement, especially in for synaptogenesis and synaptic plasticity. Microglia were shown to regulate synapses in a dual way, both promoting the formation of synapses and the elimination of weak ones through pruning. In the mouse brain, neocortical microglia can promote synaptic growth by contacting active neurons reminiscent of the same BDNF-driven ability of microglia during motor learning in the mouse adult hippocampus (Parkhurst et al. 2013; Miyamoto et al. 2016) (Figure 15A).

Importantly, microglia also prune synapses during development (Faust, Gunner, and Schafer 2021). It was first found that microglia can prune excitatory hippocampal pre and post-synaptic elements, a process which is impaired in *Cx3cr1*^{-/-} mice (Paolicelli et al., 2011). Concomitantly, it was shown that microglia regulate the activity-dependent pre-synaptic pruning of retinal projections in the dorso-lateral geniculate

nucleus (dLGN) of the thalamus, when axons compete to shape ipsilateral and contralateral territories (Schafer et al. 2012). This process is mediated by tagging of the synapses by the complement and a further elimination via the Complement Receptor 3 (CR3) (Schafer et al., 2012; Werneburg et al., 2017; Faust et al., 2021). Furthermore, synaptic refinement by microglia in the postnatal dLGN can be modulated by microglial serotonin receptor 5-HT2B, which enables them to sense serotonin (5-HT) and be recruited to synapse or switch to a phagocytic state (Kolodziejczak et al. 2015).

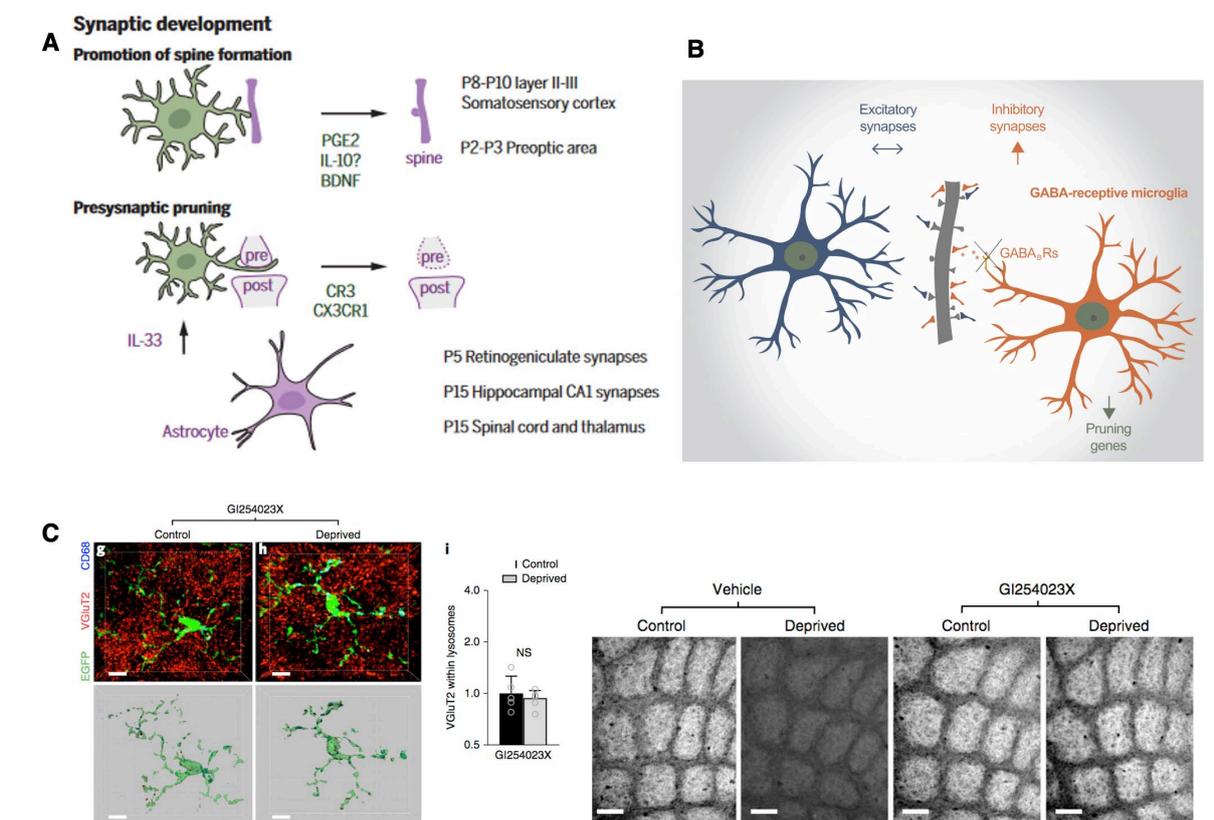


Figure 15. Microglia perform diverse functions in postnatal brains. (A) In the first two postnatal weeks, microglia promote excitatory synapse development dually, via promotion of spine formation and pruning, which can depend on neuronal activity or signaling from astrocytes. From (Morgane S. Thion, Ginhoux, and Garel 2018b). **(B)** Specialized GABA-receptive microglia also shape interneuron GABAergic synapses. From (Favuzzi et al. 2021). **(C)** Synapse elimination by microglia in the sensory deprived barrel cortex is dependent on metalloprotease ADAM10 expression. ADAM10 inhibition through GI254023X inhibitor leads

to poor engulfment of Vglut2 and intact synapses in the sensory deprived barrel cortex. Adapted from (Gunner et al. 2019).

In contrast, in the critical period for somatosensory barrel cortex synaptogenesis, after sensory lesioning, weak synapses were eliminated via a CX3CR1 and CX3CL1-dependent process, requiring metalloprotease ADAM10 upregulation in layer IV neurons and microglia (Gunner et al. 2019) (Figure 15C). Finally, in concert with complement component 1q (C1q), TREM2 drives the elimination of excitatory synapses via the recognition of locally exposed phosphatidyl serine (PS) in specific synapses (Filipello et al. 2018; Scott-Hewitt et al. 2020). This is also the case of adhesion G-protein coupled receptor GPR56 (Taken together, distinct molecular pathways enable the recognition of weaker synapses and the following activity-dependent developmental pruning).

In addition to a microglia/neuronal dialogue, astrocytes can contribute to an additional layer of regulation (Faust, Gunner, and Schafer 2021). For instance, in the developing spinal cord, microglia phagocytosis is promoted by astrocyte secretion of the cytokine interleukin 33 (IL-33) binding to microglial receptor IL1RL1 leading to engulfed synapses in the context of developmental pruning (Vainchtein et al. 2018). Complete IL-33 deficiency led to supernumerary excitatory synapses and overactive circuits (Vainchtein et al. 2018). Of note, this contrasted with IL-33-induced promotion of synapse sprouting during memory consolidation in the adult mouse hippocampus, as a result of microglial ECM remodeling (Nguyen et al. 2020).

Collectively, microglia emerge as essential actors of activity-dependent pruning of excitatory synapses. More recently, Favuzzi and colleagues also reported that specialized GABA-receptive microglia selectively prune inhibitory synapses formed by Parvalbumin (PV) basket interneurons in the somatosensory cortex during the first postnatal weeks (Favuzzi et al. 2021) (Figure 15B). Depleting microglia or impairing GABA sensing via GABA_{B1} receptors in microglia during this critical period led to impaired inhibitory circuitry (Favuzzi et al. 2021). Such GABA_{B1} receptor dependent modulation was also reported in the context of synapse formation of another interneuron subtype called Chandelier cells (Gallo, Van Aelst, PNAS, 2022). Taken

together, microglia emerged as key actors in excitatory and inhibitory synapse development.

2.3 Developmental heterogeneity of microglia in the forebrain

2.3.1 Assessing developmental heterogeneity

As illustrated in the previous sections, the variety of known non-pathological microglial functions is higher during development than in adulthood. Consistently, microglial heterogeneity is most pronounced during prenatal and in early postnatal development, a feature which is only observed in ageing and disease (Figure 16). Microglial heterogeneity during development arises through distinct patterns of distribution, morphology as described in 2.2.1 but also at transcriptional and proteomic levels that are dependent on intrinsic properties of microglia and their environmental milieu. In a yet elusive fashion, microglia respond differently to local signals in the developing brain (Tan, Yuan, and Tian 2020). It appears that heterogeneously distributed developmental microglia could also be focusing on the task at hand in their vicinity and shift their phenotype accordingly (Stratoulis et al. 2021). Importantly, the advent of single cell RNA sequencing has allowed for more in-depth analysis of microglial transcriptional diversity. Several groups have thus reported different clusters or states of microglia identified during development (Hammond et al., 2019; Li et al., 2019; Masuda et al., 2019, 2020; Matcovitch-Natan et al., 2016). Indeed, the term “state” highlights that these are not distinct cell types or subsets, but rather cell states that are triggered in response to local signals, and that microglia can potentially switch states (Stratoulis et al., 2019; Masuda et al., 2020; Paolicelli et al., 2022).

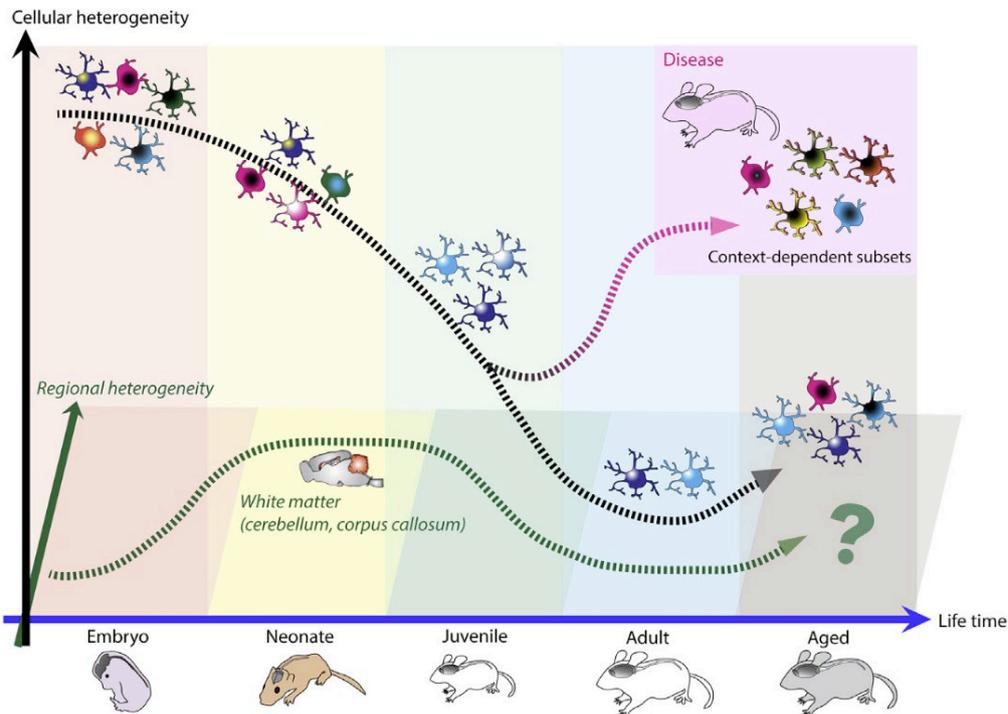


Figure 16. Evolution of microglial heterogeneity throughout mouse lifespan. Microglial cellular heterogeneity is highest in the embryo and its regional heterogeneity is highest during postnatal development and progressively lessens in the adult brain, until aging or disease. From (Masuda et al. 2020).

These landmark studies systematically identified proliferative microglia suggesting that this property is an important hallmark of developing microglia, as expected. In addition, these studies identified a variety of cell states in rodents, which we are only starting to characterize. In some of these single cell studies, top genes expressed were identified and spatial distribution of RNA *in situ* was probed to determine location of the microglial subset or to identify if there were preferential localization of these clusters (Hammond et al., 2019; Li et al., 2019). Moreover, in the human developing brain, studies are still limited but demonstrate similarly high heterogeneity compared to adult microglia including proliferative microglial clusters, ATM-like, but also early immune-sensing homeostatic microglia suggesting that heterogeneity of microglia and potentially functional specialization is conserved but more complex in humans (Bian et al., 2020; Kracht et al., 2020; Li et al., 2022; Popova et al., 2021).

2.3.2 ATM, PAM or CD11c+ microglia

Amongst the diversity of clusters observed in single cell transcriptomic study, one was observed in several studies and emerged as of particular interest. This cluster resembling DAMs, was named axon-tract associated microglia (ATM) or proliferative-associated-microglia (PAM) and localizes in the postnatal white matter of the neocortex and cerebellum (Hammond et al., 2019; Li et al., 2019; Masuda et al., 2020; Matcovitch-Natan et al., 2016). Molecularly, it is characterized by the expression of *Spp1*, which encodes Osteopontin/OPN, *Gpnmb*, *Lgals3*, *Igf1*, *ApoE*, *Lpl*, *Clec7A* and *Itgax*, which encodes CD11c (Hammond et al., 2019; Li et al., 2019; Masuda et al., 2020; Matcovitch-Natan et al., 2016). Li and colleagues went on to show by immunohistochemistry using CLEC7A as a proxy for this subset, that these cells can phagocytose newly formed oligodendrocytes (Li et al., 2019), linking this cluster to a specific functions. Although CD11c (or *Itgax*) was not a top expressed gene in these studies, these specific clusters of microglia likely correspond to “fountains of microglia” and have also been coined CD11c+ microglia (Wlodarczyk et al. 2017; Benmamar-Badel, Owens, and Wlodarczyk 2020). This microglial state regulates oligodendrogenesis dually, through trophic support towards OPCs and through phagocytosis of OPCs and newly born oligodendrocytes, as well as myelination on the long term (Wlodarczyk et al. 2017; Benmamar-Badel, Owens, and Wlodarczyk 2020).

The question of how this phenotype is induced during development, and at the same time why it resembles some adult states such as the DAM is increasingly being studied. In the adult brain CD11c+ microglia have been shown to be upregulated in demyelination conditions, and their induction to be inhibited by SIRP α a receptor that senses “don’t eat me” CD47 expressed on neighboring neurons (Sato-Hashimoto et al. 2019) (Figure 17A). This study thus suggested that CD11c+ state is induced by phagocytosis. Consistently, several groups state that microglial apoptosis in early development could drive this CD11c profile. In the early postnatal retina, where microglial engulfment of apoptotic cells is important (Anderson, Zhang, et al. 2019) CD11c+ microglia expressing high *ApoE*, *Spp1*, *Clec7A* and *Igf1* resembling DAMs and ATMs, require apoptotic cell death to be specified and resistant to CSF1R inhibition (Anderson, Roberts, et al. 2019). Shen and colleagues recently proposed that CD11c microglia emerge as early as the end of embryogenesis upon phagocytosis of apoptotic neurons, mediated through α V β 3-integrin and TAM receptors (Shen et al. 2022). They

further stress that SPP1/OPN expression by postnatal CD11c⁺ microglia is central to preserving their phenotype, and for their functions such as synaptosome engulfment *in vitro* (Shen et al. 2022). Taken together, death or tissue damage has been proposed to regulate the induction of a CD11c⁺ microglia state, with a main difference between developmental ATM/PAM states and disease DAM state: whereas DAM requires TREM2 signaling pathway ATM/PAM induction seems to not require TREM2 presence (Hammond et al., 2019; Li et al., 2019; Masuda et al., 2019, 2020; Matcovitch-Natan et al., 2016).

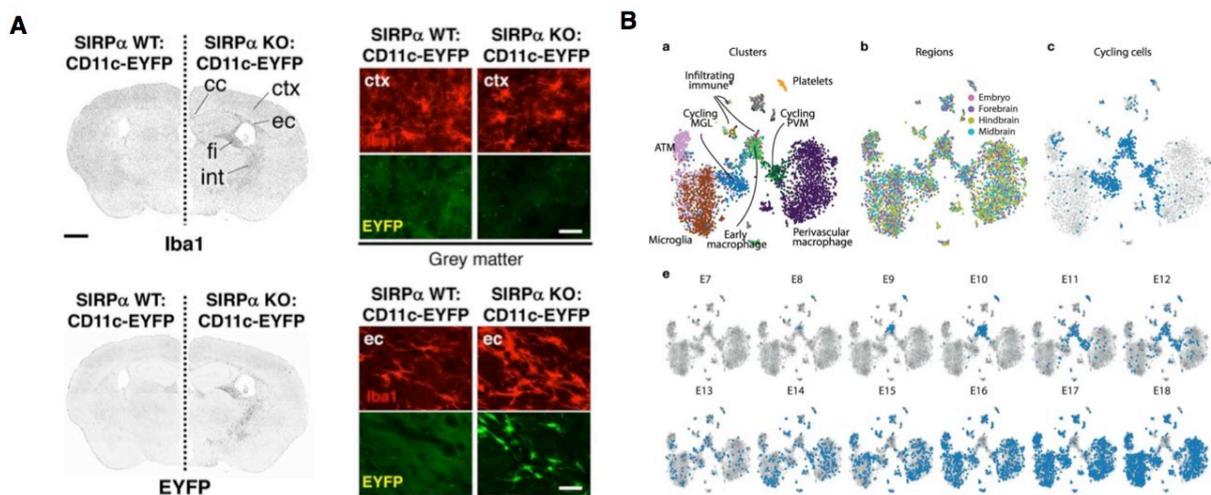


Figure 17. ATM/PAM and CD11c⁺ microglia induction and presence in the embryonic brain. (A) In the adult brain the CD11c⁺ phenotype is suppressed by microglia SIRP α . Invalidation of the latter leads to reinduction of CD11c microglia in forebrain white matter, similar to early postnatal “fountains of microglia”. From (Sato-Hashimoto et al. 2019) (B) ATM-like cells have been transcriptionally identified in a large single cell sequencing study and have been detected as early as E11 and throughout embryogenesis. From (La Manno et al. 2021).

Such ATM-like state was not only detected in normal physiological development but also in the context of injury in the developing spinal cord. Indeed, at early postnatal stages, microglia sharing similarities with ATM were proposed to participate in scar

free wound healing upon crush injury in the mouse spinal cord (Li et al., 2020). At the peak of healing phase, a cluster of microglia in close contact to the injury upregulate top ATM genes including *Spp1*, *Clec7A* and *Igf1*. Of note microglia bordering the injury expressed SPP1/OPN, echoing CD11c+ cell specificities. However, this cluster also uniquely upregulated ECM-related component genes such as *Thbs1*, which codes for thrombospondin and *Fn1*, which codes for fibronectin (Li et al., 2020). The ablation of the latter gene specifically in CX3CR1+ cells led to incomplete wound healing, highlighting possible convergence across developmental and injury-induced states.

Finally, a recent comprehensive transcriptional atlas of developing embryonic mouse brain with 292,495 cells sequenced including all brain cell types revealed the uncovering of a cluster of ATM-like cells in the prenatal brain, as early as E11, suggesting that this microglial profile is also induced and could also play specific roles during embryonic development (La Manno et al. 2021) (Figure 17B).

2.3.3 Other emerging developmental states

Other microglial functional subtypes in development are being investigated but lack thorough characterization, with regards to transcriptional specificity and modes of induction such as GABA receptive microglia that regulate specifically inhibitory synapses in the early postnatal brain (Favuzzi et al. 2021). Of note, Stratoulis and colleagues identified a late postnatal developmental subtype of microglia that express enzyme Arginase 1 (*Arg1*), are enriched locally in the basal forebrain where they are in close contact with cholinergic neurons, and modulate dendritic spine density in the female mouse postnatal hippocampus where these neurons project (Stratoulis et al. 2021) (Figure 18A). These were transcriptionally distinct from ATM/PAM/CD11c+ cells but resembled another identified *Arg1* high expressing cluster that had previously been described but not associated to a function or a localization during postnatal development (Hammond et al. 2019).

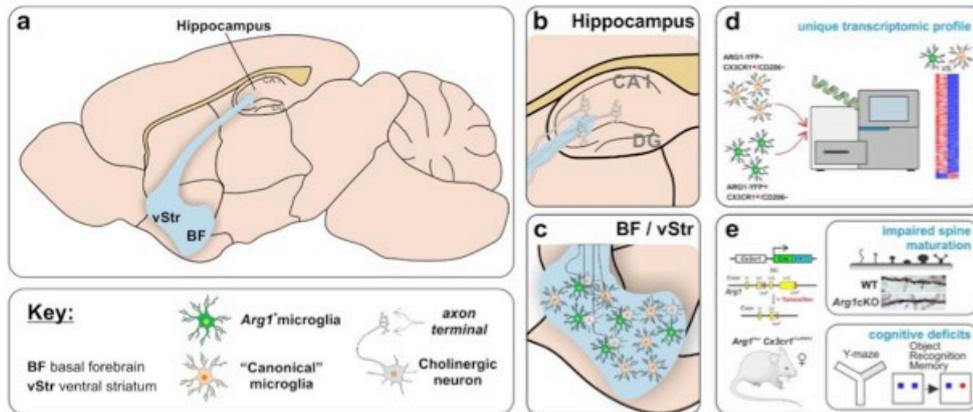
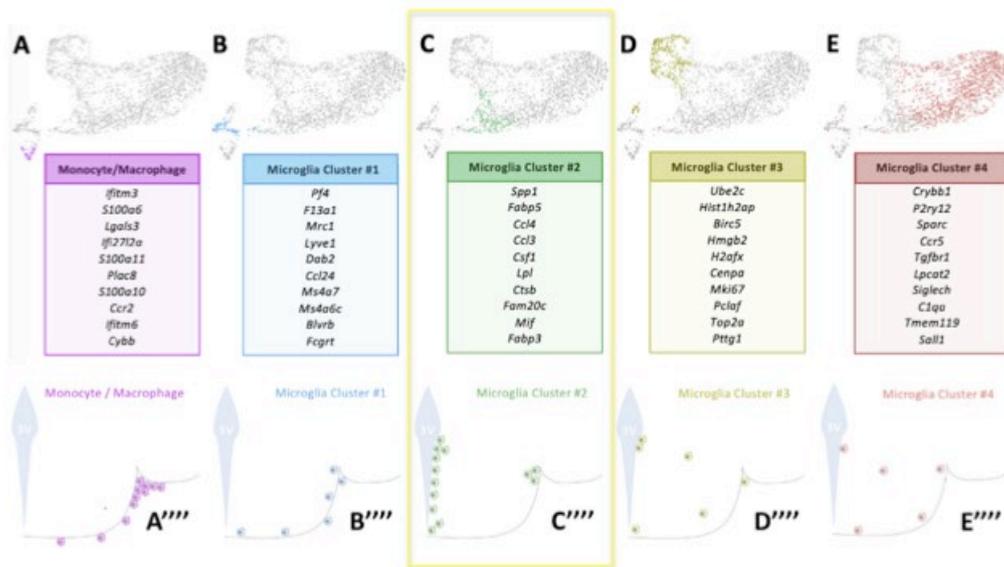
A**B**

Figure 18. Recently emerging developmental microglia states. (A) In the late postnatal brain (P10), a subtype of Arg1⁺ microglia resembling a previously transcriptionally identified cluster was found to be restricted to the basal forebrain and mediate specific functions during hippocampal spine maturation. From (Stratoulas et al. 2021). **(B)** In the embryonic hypothalamus several microglial clusters have been identified and spatially localized. In particular, cluster 2 a high *Spp1* expressing cluster specifically responds to maternal cold stress. Adapted from (Rosin, Sinha, et al. 2021).

To the best of our current knowledge, the only reported embryonic microglial subtype is an E15.5 embryonic cluster that responds to maternal stress by elevation of CCL3 and CCL4 chemokine secretion in the hypothalamus, preferentially regulating male PVN oxytocin neurons and OPCs, possibly through SPP1/OPN (Rosin, Sinha, et al. 2021) (Figure 18B).

Understanding prenatal roles and uncovering other functional will be of great interest to understand how dysfunctional embryonic microglia can affect forebrain development.

OBJECTIVES OF THE THESIS

Brain functioning relies on a number of sequential and stereotyped processes that occurs during embryogenesis and ongoing through early postnatal life. Microglia, resident brain macrophages, have been implicated in multiple postnatal developmental events. However, what microglial cells do during prenatal development remains little explored. Recent studies report that embryonic microglia share transcriptional, morphological and spatial diversity that has been observed in postnatal microglia, suggesting a vast array of roles for these cells during embryogenesis. In particular, specialized functional microglial states have been detected in disease and postnatal development, but whether microglia assume different functional states in the embryo is yet to be explored further. To better understand microglia developmental trajectories and roles during embryonic life, I asked the following questions:

1. Can specific microglial states be detected in the embryonic brain, similar to those in the postnatal brain?
2. What specific pathways upregulated in embryonic microglial states modulate their roles?
3. What is the developmental role of microglial accumulations in the embryonic brain?
4. What are the consequences of impaired roles of these microglia for brain development and pathology?

By addressing these questions, we found in Article 1 (Results section) that a specific embryonic cluster of Axon Tract-associated Microglia-like (ATM) localized as accumulated amoeboid cells at sites of morphogenetic fragility during embryonic forebrain development. We further showed that these embryonic microglia are required for preventing the formation of cavitory lesions at these sites, and that the ATM-like profile is linked to lesion repair. These findings uncover a new role for prenatal microglial heterogeneity in health and disease and shed light on previously unknown mammalian brain morphogenesis processes.

RÉSUMÉ FRANCAIS: INTRODUCTION ET OBJECTIFS

Chapitre 1: La microglie : des macrophages cérébraux aux multiples facettes

1.1 Présentation générale de la microglie

La microglie est présentée comme une cellule gliale atypique, faisant partie du vaste contingent de cellules gliales, à savoir les cellules cérébrales non neuronales, qui comprennent également les astrocytes et les oligodendrocytes. Les microglies sont les macrophages résidents du cerveau, découverts en 1919 par Pío del Río-Hortega, qui les a identifiées comme des phagocytes présentant des changements morphologiques dynamiques. Comme tous les macrophages tissulaires, les microglies jouent un rôle clé dans l'intégrité de leur organe de résidence en contribuant au développement, à l'homéostasie tissulaire, à la réparation et aux maladies. Elles constituent la première ligne de défense du cerveau contre les pathogènes ou les lésions mécaniques. Les microglies se trouvent dans le parenchyme cérébral, partiellement isolé de la circulation sanguine par la barrière hémato-encéphalique, leur conférant des caractéristiques uniques. En tant que cellules immunitaires gliales, les microglies sont au carrefour des interactions entre le système immunitaire et le système nerveux central, régulant de nombreux aspects du développement cérébral, de la physiologie et des maladies.

Les microglies, initialement considérées d'origine mésodermique, dérivant de monocytes issus des cellules souches hématopoïétiques situées dans le foie fœtal puis la moelle osseuse, se développent en fait à partir de cellules progénitrices érythromyéloïdes du sac vitellin, colonisant le neuroépithélium dès les premiers stades embryonnaires pour constituer la population adulte de microglies dans le parenchyme cérébral. Les macrophages associés aux vaisseaux et aux membranes cérébrales, comme les PVM et les BAM, se renouvellent également tout au long de la vie, étant dédiés à leur niche respective. De plus, il y a très peu de contribution des cellules immunitaires non microgliales dans le parenchyme cérébral suivant la fermeture de la barrière hémato-encéphalique vers E13.5, sauf dans des contextes de maladie, de blessure et d'inflammation, et il a récemment été démontré que chez les humains, les microglies ont une ontogenèse similaire et des capacités d'auto-renouvellement.

La maturation des microglies à partir de progéniteurs de macrophages du sac vitellin embryonnaire et leur acquisition d'une identité cellulaire et moléculaire spécifique en réponse à la niche qu'elles colonisent sont essentielles pour comprendre leur fonctionnement. Cette maturation est notamment influencée par la régulation de voies de signalisation telles que le TGF- β et la dépendance au récepteur CSF1R. Cette dépendance des microglies à leur environnement est également illustrée par la nécessité de signaux niche-dérivés, tels que CSF1 et IL-34, pour leur différenciation et leur survie, offrant ainsi des possibilités de manipulation et d'études fonctionnelles.

Les microglies sont caractérisées par un sensome leur permettant de détecter divers signaux, non seulement locaux et liés à l'activité neuronale, mais aussi provenant du corps ou de l'environnement, tels que la microbiote, la nutrition, les traitements chimiothérapeutiques et l'identité sexuelle, influençant ainsi leurs propriétés et leur réponse à long terme, notamment dans des contextes inflammatoires et de vieillissement.

1.2 La microglie adulte en conditions physiologiques et pathologiques

Les déficits des microglies sont directement liés à des maladies telles que la leucodystrophie causée par des mutations du CSF1R, ou la maladie de Nasu-Hakola résultant de mutations homozygotes dans TREM2 et TYROBP(DAP12), montrant ainsi l'importance des microglies dans la physiopathologie cérébrale. De plus, les microglies régulent divers aspects de la physiologie et de la pathologie cérébrales, y compris les comportements, comme en témoigne le comportement de maladie, les troubles obsessionnels compulsifs induits par la mutation du facteur de transcription Hoxb8, ainsi que leur implication dans les maladies neurodégénératives telles que la maladie d'Alzheimer.

Les microglies participent à l'homéostasie cérébrale grâce à leur capacité de phagocytose, éliminant les pathogènes ainsi que les cellules mortes ou mourantes et modulant l'inflammation, que ce soit après une blessure ou dans un contexte physiologique normal, comme lors de la neurogenèse dans le gyrus denté de l'hippocampe chez la souris. Ces fonctions phagocytaires des microglies sont médiées par plusieurs voies de signalisation connues, notamment les récepteurs de type Toll

(TLR) ou les récepteurs de type lectine C (CLR), ainsi que le complexe récepteur impliquant TREM2 et DAP12, qui leur permettent de reconnaître et phagocyter les pathogènes ou les cellules apoptotiques. Ces voies de signalisation jouent un rôle crucial non seulement dans la régulation du nombre de cellules ou l'élimination des débris, mais aussi dans la régulation des synapses, contribuant ainsi au déclin cognitif lié au vieillissement.

Les microglies, en tant que principaux phagocytes du cerveau, partagent cette fonction avec d'autres populations de cellules gliales telles que les astrocytes. De plus, elles contribuent aux fonctions cérébrales par la production de cytokines et de facteurs de croissance, qui régulent la communication intercellulaire et la plasticité synaptique. En outre, les microglies ont la capacité de réguler négativement l'excitabilité neuronale, ce qui leur permet d'ajuster la fonction cérébrale de multiples façons.

Les études initiales sur les microglies grâce à l'imagerie en direct ont montré leur surveillance rapide et complexe de leur environnement ainsi que leur réaction rapide aux lésions. Elles répondent à une variété de signaux, notamment en se réorientant vers les neurones actifs et en exprimant des récepteurs de neurotransmetteurs. De plus, elles peuvent établir des jonctions somatiques avec les neurones, surveillant ainsi l'activité neuronale et métabolique.

Les microglies sont des cellules dynamiques dans le cerveau adulte, réagissant rapidement aux dommages tissulaires grâce à des récepteurs spécifiques comme P2Y12 et P2X4. Elles migrent vers les sites de lésions, où elles peuvent avoir des effets protecteurs ou neurotoxiques en produisant des facteurs inflammatoires. En réponse aux lésions, les microglies remodelent également la matrice extracellulaire pour soutenir la guérison ou la dégradation tissulaire.

1.3 L'hétérogénéité microgliale en conditions physiologiques et pathologiques

La section précédente a montré que les microglies peuvent remplir diverses fonctions dans le cerveau, tant dans la santé que dans la maladie, en agissant comme des capteurs multifacettes du SNC et en adaptant leurs réponses à une large gamme de stimuli, ce qui suggère une hétérogénéité fonctionnelle.

Dans le cerveau adulte sain, les microglies sont réparties de manière ubiquitaire mais leur densité et leur morphologie peuvent varier dans différentes structures, avec une

densité plus élevée dans le cortex et une densité plus faible dans le cervelet. De plus, la densité et la morphologie des microglies semblent être hétérogènes même à l'intérieur des sous-régions elles-mêmes, et bien que leur morphologie puisse être associée à certains "états" ou fonctions, elle seule ne peut pas être utilisée pour définir l'état ou la fonction d'une microglie. En contexte pathologique, les microglies peuvent changer de morphologie de manière hétérogène selon les régions cérébrales ou leur proximité avec des lésions, ce qui témoigne de l'influence du milieu sur les microglies et de la pertinence de la morphologie comme caractéristique pour sonder les états fonctionnels des microglies.

Certains sous-ensembles de microglies avec des caractéristiques distinctives et des spécialisations fonctionnelles présumées ont été découverts dans le cerveau adulte, mais souvent, ils restent à décrire davantage en tant qu'"états" ou sous-types car ils ne sont pas toujours définis sur la base de caractéristiques multidimensionnelles exhaustives. Par exemple, les microglies HOXB8, ontogéniquement différentes, trouvées uniquement dans le néocortex, présentent une perte de fonction associée à des comportements pathologiques de toilettage. D'autre part, les microglies de différentes structures cérébrales peuvent exhiber différents "états" dépendant des signaux locaux, ce qui peut influencer leur réaction à des conditions pathologiques telles que la sclérose latérale amyotrophique (SLA) ou l'atteinte de l'axone initial.

Il est désormais possible de suivre l'hétérogénéité des profils transcriptionnels des microglies dans le temps et l'espace, ainsi qu'en conditions physiologiques et pathologiques, à l'aide de différentes méthodes de séquençage de cellules uniques dans les tissus de souris et humains. Plusieurs groupes ont ainsi rapporté leurs découvertes sur les différents profils microgliaux, ainsi que leur comparaison avec d'autres macrophages non-microgliaux associés au système nerveux, permettant une compréhension plus précise des caractéristiques de l'identité microgliale adulte et de sa signature homéostatique. En outre, plusieurs groupes ont examiné le profil transcriptionnel des microglies dans des modèles de maladies et ont identifié une signature commune, désignée par Disease Associated Microglia (DAM), qui montre une régulation à la baisse de l'expression des gènes microgliaux homéostatiques et une régulation à la hausse de certains gènes inflammatoires.

Chapitre 2 : Microglie et développement du cerveau antérieur

2.1 Structures et morphogenèse du cerveau antérieur

Le cerveau, dérivé de la plaque neurale, se développe en trois parties principales : le prosencéphale, le mésencéphale et le rhombencéphale. Nous nous concentrerons ici sur le prosencéphale, (composé du télencéphale et du diencephale) crucial pour la cognition, les émotions et le mouvement, notamment la division en pallium et subpallium. Ces structures nécessitent un câblage précis et une communication axonale longue distance pour fonctionner correctement.

Pendant la morphogenèse, les structures du télencéphale et du diencephale se spécifient grâce à des gradients de morphogènes qui régissent l'expression de facteurs de transcription spécifiques. Les progéniteurs du pallium et du sous-pallium expriment des facteurs distincts qui déterminent en grande partie la production de cellules excitatrices ou inhibitrices. De plus, la frontière palliale-sous-palliale marque une limite nette entre le pallium et le sous-pallium, définissant ainsi la migration de certains progéniteurs le long du flux cortical latéral, qui contribuent à la diversité neuronale dans certains noyaux amygdaliens, le cortex piriforme, les cellules Cajal-Retzius et les neurones du bulbe olfactif. La frontière cortico-striato-amygdalienne (CSA), qui sépare le cortex et l'amygdale, a pour prolongement dorsal la frontière cortico-striatale. Enfin, pendant la morphogenèse tardive, les structures établies du pallium et du sous-pallium continuent leur croissance dans un contexte de tensions morphogénétiques avec l'avènement de processus tels que la neurogenèse et l'expansion associée à la migration neuronale, ainsi que la tension mécanique associée à la genèse axonale. Les microglies, quant à elles, sont les seules cellules gliales présentes jusqu'à la fin de la gestation, participant aux processus de développement périnatal tels que la mort cellulaire programmée et la synaptogenèse.

2.2. La microglie dans le développement : ontogenèse et fonctions précoces

Malgré les nombreuses études menées, notre compréhension des mécanismes par lesquels les microglies entrent et colonisent le cerveau reste relativement fragmentée. Une fois qu'elles commencent à coloniser le cerveau, les microglies montrent un schéma de distribution spatiotemporellement précis, stéréotypé et inégal dans le cerveau antérieur, tout au long de l'embryogenèse et du développement postnatal

précoce. Par exemple, dans l'embryon, les microglies sont associées aux vaisseaux sanguins en développement, ce qui alimente l'hypothèse toujours débattue de leur entrée dans le cerveau par la vasculature. Enfin, bien qu'elles soient généralement plus arrondies et possèdent moins de branches ramifiées, les microglies embryonnaires varient encore beaucoup morphologiquement, allant de l'amoeboïde au ramifié.

Bien que leurs rôles prénataux restent largement inexplorés, quelques fonctions embryonnaires précoces des microglies ont été identifiées, notamment dans la régulation du développement des tracts axonaux et de l'angiogenèse précoce. De plus, les microglies jouent un rôle phagocytaire important pendant la période prénatale, notamment dans l'élimination des cellules apoptotiques et l'englobement de cellules vivantes ou de fragments cellulaires.

Dans le cerveau postnatal les microglies régulent le développement cérébral en contrôlant les nombres de neurones et en agissant sur d'autres types de cellules gliales, notamment les astrocytes et les oligodendrocytes. Elles participent à la régulation de la survie neuronale par la sécrétion de facteurs trophiques tels que l'IGF1, mais peuvent également provoquer la mort cellulaire en englobant des neurones et des astrocytes par la production de radicaux libres. En outre, les microglies contribuent à l'oligodendrogenèse et à la myélinisation, mais leur rôle dans ces processus peut être à la fois promoteur et inhibiteur, selon les conditions et les signaux environnementaux.

Les microglies jouent également un rôle crucial dans le raffinement des circuits postnataux en régulant la synaptogenèse et la plasticité synaptique. Elles favorisent à la fois la formation de synapses et l'élimination des synapses faibles par élagage. De plus, les microglies sont impliquées dans le marquage et l'élimination des synapses via des mécanismes dépendants de l'activité neuronale et de la reconnaissance de signaux moléculaires spécifiques, ce qui les positionne comme des acteurs clés du développement synaptique.

2.3 Hétérogénéité développementale de la microglie dans le cerveau antérieur

La diversité des fonctions non-pathologiques des microglies est plus élevée pendant le développement que pendant l'âge adulte. Cette hétérogénéité microgliale se

manifeste principalement pendant le développement prénatal et postnatal précoce, mais aussi pendant le vieillissement et les maladies. Les microglies en développement montrent une hétérogénéité au niveau de leur distribution, de leur morphologie et de leur profil transcriptionnel, reflétant ainsi leur adaptation aux signaux locaux du milieu environnant.

Parmi la diversité des clusters observés dans les études transcriptomiques à cellule unique, un cluster a émergé comme particulièrement intéressant, ressemblant aux DAMs et nommé microglies associées aux tractus axonaux (ATM) ou microglies associées à la prolifération (PAM). Ce cluster, localisé dans la substance blanche postnatale du cortex néocortical et du cervelet, se caractérise moléculairement par l'expression de *Spp1*, *Gpnmb*, *Lgals3*, *Igf1*, *ApoE*, *Lpl*, *Clec7A* et *Itgax*. Les microglies CD11c⁺ semblent être induites par la phagocytose, que ce soit pendant le développement ou dans des conditions de démyélinisation chez l'adulte, soulignant ainsi un rôle potentiel de la mort cellulaire ou des lésions tissulaires dans leur induction. Enfin, ces profils de microglies similaires aux ATM ont également été observés dans des contextes de blessure, suggérant une convergence entre les états développementaux et induits par des lésions.

D'autres sous-types fonctionnels de microglies en développement sont en cours d'investigation, mais manquent de caractérisation approfondie tels que les microglies réceptives au GABA. Un sous-type exprimant l'enzyme Arginase 1 (*Arg1*) a été identifié dans le développement postnatal tardif, tandis qu'un cluster embryonnaire répond au stress maternel en régulant les neurones et les cellules gliales dans l'hypothalamus.

OBJECTIFS DE LA THÈSE

Le fonctionnement du cerveau repose sur un certain nombre de processus séquentiels et stéréotypés qui se produisent au cours de l'embryogenèse et se poursuivent jusqu'au début de la vie postnatale. La microglie, macrophage résident du cerveau, a été impliquée dans de multiples événements du développement postnatal. Cependant, le rôle des cellules microgliales au cours du développement prénatal reste peu exploré. Des études récentes indiquent que les microglies embryonnaires démontrent une diversité transcriptionnelle, morphologique et spatiale observée chez les microglies postnatales, ce qui suggère un vaste éventail de rôles pour ces cellules au cours de l'embryogenèse. En particulier, des états fonctionnels spécialisés de la microglie ont été détectés dans des contextes pathologiques et pendant le développement postnatal, mais la question de savoir si la microglie adopte différents états fonctionnels dans

l'embryon reste à approfondir. Pour mieux comprendre les trajectoires développementales et les rôles de la microglie au cours de la vie embryonnaire, les questions suivantes sont à poser:

1. Peut-on détecter des états microgliaux spécifiques dans le cerveau embryonnaire, semblables à ceux du cerveau postnatal?
2. Quelles sont les voies moléculaires spécifiques régulées dans les états microgliaux embryonnaires qui modulent leurs rôles ?
3. Quel est le rôle développemental des accumulations microgliales dans le cerveau embryonnaire ?
4. Quelles sont les conséquences de l'altération du rôle de ces microglies sur le développement et la pathologie du cerveau ?

En répondant à ces questions, nous avons découvert dans l'article 1 (section Résultats) qu'un groupe embryonnaire spécifique de microglies Axon Tract-associated Microglia-like (ATM) se localise sous forme de cellules amiboïdes accumulées dans des sites de fragilité morphogénétique au cours du développement du cerveau antérieur embryonnaire. Nous avons également montré que ces microglies embryonnaires sont nécessaires pour prévenir la formation de lésions cavitaires à ces endroits, et que le profil ATM-like est lié à la réparation des lésions. Ces résultats révèlent un nouveau rôle pour l'hétérogénéité microgliale prénatale dans en condition physiologique et lors de pathologies cérébrales; et mettent en lumière des processus de morphogenèse cérébrale des mammifères jusqu'alors inconnus.

RESULTS

Article 1

(Please note this is a draft of the article. Some experiments and modifications are still to be made before submission)

Microglia maintain brain integrity during embryonic morphogenesis

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ABSTRACT (149 words)

Microglia, the brain resident macrophages, exist in various states in disease and postnatal life, but their prenatal roles remain poorly understood. Here, we identified an embryonic state similar to postnatal Axon-Tract-associated-Microglia (ATM), which transiently accumulates at the fetal cortico-striato-amygdalar and cortico-septal boundaries, two sites of morphogenetic stress. We showed that embryonic microglia and ATM-factor Spp1/Osteopontin maintain tissue integrity at these two boundaries by preventing the formation of cavitory lesions. The ATM boundary niche displayed microcavities and a developmental morphogenetic fragility, suggesting that ATM maintain cohesion by promoting repair. Consistently, ATM-core factors were rapidly induced by mechanical damage, and both microglial repopulation and Spp1/OPN were required for the efficient resorption of large lesions, highlighting remarkable repair capacities. Collectively, our study shows a novel role for embryonic microglia and repair-promoting ATM in maintaining tissue integrity during normal morphogenesis, with major implications for our understanding of brain wiring in health and disease.

INTRODUCTION

Microglia, the brain resident macrophages play key roles in brain circuits and display the remarkable capacity to adopt distinct cellular and transcriptomic states, in development, ageing and disease [1-10]. However, whether distinct states are related to specific functions remains largely to be determined.

From their generation site in the early embryonic yolk-sac, microglia seed the brain primordium at embryonic day (E) 8.5 in mice and gestational week (GW) 4/5 in humans, and progressively colonize the brain in a highly stereotyped and uneven manner [1, 11, 12]. Microglia accumulate at hotspots, avoid selective regions such as the developing cortical plate [13], and show a wide range of morphologies, cellular behaviors and transcriptomic states, before reaching a homogeneous tiling characteristic of adult stages. Microglial uneven distribution starts during embryogenesis and spans into the postnatal period. In particular, specific populations of amoeboid microglia were found in the postnatal cortical white matter, including the corpus callosum [7, 8, 14, 15]. These microglia white-matter “fountains” were recently shown to display a specific transcriptomic state called axon-tract-associated microglia (ATM) [8] or proliferative associated microglia (PAM) [1]. ATM/PAM are characterized by the expression of specific markers including Cd11c (*Itgax*), osteopontin or OPN (*Spp1*), *Lgals3*, *Gpnmb*, *Clec7a* and *Igf1*, which are also shared by disease associated microglia (DAM), identified in several neurodegenerative disorders [2-10]. Intriguingly, postnatal ATM/PAM/Cd11c+ microglia have been reported to exert specific functions in mice including engulfment of nascent glial cells, regulation of myelination and neuronal survival, in part through *Igf1* expression [7, 14-16] [8, 17]. By contrast, embryonic microglial states and their potential roles in brain development remain largely to be characterized.

Here, we explored early microglial heterogeneity and identified in both mice and humans an embryonic ATM-like state. Embryonic ATM formed dense hotspots at two boundaries of the cerebral cortex, the cortico-striato-amygdalar (CSA) and cortico-septal boundaries (CSB), known sites of morphogenetic stress [18, 19]. Using transient depletion and genetic models, we showed that the absence of embryonic microglia induces at both hotspots large cavitory lesions. This novel role in tissue integrity partially relied on the ATM-factor *Spp1*/Osteopontin/OPN, an atypical component of

the extracellular matrix (ECM) [20]. To further understand microglia and ATM functions, we examined the CSA niche and observed physiologically-occurring microcavities as well as a developmental fragility, revealed by increasing morphogenetic stress in a genetic model of cortical heterotopy [21]. These findings raised the intriguing possibility that ATM might maintain tissue integrity by promoting repair. Consistently, we found that ATM-properties are rapidly induced by cortical tissue-lesion as well as during the resorption of cavitory lesions by repopulating microglia that follow embryonic depletion. Such dynamic repair properties relied on Spp1/OPN, supporting a key role for a core-ATM pathway in both tissue integrity and repair. Collectively, this study reveals a novel role of microglia in maintaining tissue integrity during the complex morphogenesis of the mammalian brain, with major implications for our understanding of brain development and the diversity of microglial states and functions.

RESULTS

Embryonic hotspots of ATM localize at boundaries of the cerebral cortex

To explore prenatal microglial heterogeneity, we examined embryonic single-cell (sc) transcriptomic datasets [22] and confirmed the presence of an ATM/PAM/Cd11c-like cluster from embryonic day (E)14.5/15.5 onwards, that we will call embryonic ATM (Fig. 1A, S1A and Table S1). Embryonic ATM share a core genetic signature with previously described ATM (Fig. 1B,C) [8] and PAM (Fig. S1B,C) [7] of the cortical white matter and proliferative zones, that includes *Itgax*, which codes for Cd11c, *Igf1*, *Lgals3*, which codes for Galectin3/Mac2, *Spp1*, which codes for Osteopontin (OPN) and *Gpnmb* (Table S2) [7, 8]. Consistently, the embryonic ATM [22] and the PAM [7] signatures project mainly onto the ATM cluster described by Hammond and colleagues [8] (Fig. S1D-F). Since ATM were involved in postnatal functions, such as myelination [7, 14, 15, 17], we wondered where these embryonic cells locate and what might be their prenatal roles.

To this aim, we performed immunostainings and observed an accumulation of *Cx3cr1^{gfp}*-positive macrophages at the cortico-striato-amygdalar boundary (CSA) between E14.5 and E16.5, that was highly reminiscent of postnatal ATM (Fig. 1D) [7, 8]. First, these cells displayed an amoeboid morphology in spite of being *bona fide* microglia, as assessed by their parenchymal localization (Fig. 1D), the co-expression with the microglial marker P2Y12R, and the lack of expression of the perivascular macrophage marker LYVE1 (Fig. 1D) [1, 23]. CSA microglia further displayed a high expression of the lysosomal markers CD68 and a notable phagocytic capacity, as assessed in *ex vivo* slices (Fig. S1G-H). Finally, *Cx3cr1^{gfp}* or IBA1-positive CSA microglia co-expressed ATM-core markers at E15, a timepoint when the hotspot accumulation is dense (Fig.1E). In particular, using the *Cd11c-eYFP* line and co-immunostaining for Mac2, *Spp1*/OPN and GPNMB (Fig. 1E), we found that more than 60% of CSA microglia co-express proteins encoded by ATM-core genetic signature, in contrast to adjacent regions, where almost no embryonic ATM-like cells could be detected (Fig. 1E). The CSA thus appears as a hotspot of embryonic ATM accumulation, which we also found in human fetal brains from gestational week (GW) 9 to 14 (Fig. 1F and S1I), revealing a conserved pattern between mice and humans.

To determine whether other forebrain regions showed a continuous accumulation of embryonic ATM, we performed a longitudinal study using the aforementioned markers. In addition to scattered cells in the ventral telencephalon and cortical white matter (not shown), we found an ATM hotspot at the septal dorsal midline below the corpus callosum, a region called subcallosal streak or corticoseptal boundary (CSB) (Fig. S2A,B). This accumulation prefigured the distribution of postnatal ATM in the corpus callosum and dorsal cortical white matter [1, 7, 8, 14, 15, 18]. Taken together, our analysis reveals that embryonic ATM accumulate at the CSA and CSB hotspots during normal physiological development.

Microglia prevent the formation of cavitory lesions at sites of ATM hotspots

To investigate the roles of embryonic ATM hotspots, we examined CSA morphogenesis in the absence of all macrophages, including microglia. We first took advantage of a previously set-up transient embryonic depletion [24-26], which relies on the blockade of the CSF1R pathway, an essential pathway for macrophage development and survival, via injections of a CSF1R blocking antibody (AFS) at E6.5 and E7.5 (Fig. S3A). This procedure induces a drastic depletion of microglial progenitors, leading to very few microglia in the embryonic brain until E18.5, when repopulation starts and is largely completed by post-natal day (P)7 [24-26], (Fig.S3A). We could obtain a highly similar microglial-precursor depletion by feeding pregnant dams between E6.5 and E15 with food pellets containing PLX3397[27, 28], a pharmacological inhibitor of CSF1R (Fig. S3B). Finally, we compared both AFS and PLX3397 depletions with *Pu.1* mutant embryos, which lack all myeloid cells [29], to exclude potential confounding effects related to CSF1R pathway. In all conditions in which the brain develops in the absence of macrophages, we observed a cavitory lesion at the CSA boundary, where embryonic ATM normally accumulate (Fig. 2A). Cavitory lesions examined by transmitted electron microscopy (EM) did not present basal lamina and hence resembled pseudocysts reported in several pathologies [30] (Fig. S3D). Such cavities were observed from E14.5 and throughout embryogenesis, as shown by an enhanced tissue disruption at E18.5 (Fig. 2B). To further check if this phenotype was specific to the absence of resident macrophages and not to other populations, we examined models of more transient or more specific depletions. We

first exposed pregnant dams to PLX3397 from E12.5 to E15, or from E12.5 up to E18.5 (Fig. S3C), and consistently observed similar cavitory lesions at the embryonic CSA (Fig. 2A,B). Importantly, we examined *Csf1r*^{ΔfireΔfire} embryos, which lack a specific *Csf1r* gene enhancer leading to an absence of microglia and a relative sparing of other brain macrophages [31, 32], and observed similar CSA cavitory lesions (Fig. 2A,B).

Because lesions were observed after sectioning, a procedure that could enhance tissue fragilities, we wanted to ascertain the existence of such striking CSA cavity by performing whole-brain iDisco tissue clearing and whole-head MRI [33, 34]. iDisco clearing performed and 3D reconstruction performed in two depletion models and *Pu.1* mutants, revealed an embryonic CSA lesion located in the caudal third of the telencephalic vesicles at the border between caudal insular cortex, claustrum, striatum and amygdala, (Fig. 2C and movies S1-7). Similarly, whole-head MRI scanning of PLX3397 E15.5 embryos and newborns pups showed bilateral low intense signals after contrast-enhancement, thereby unambiguously confirming the presence of CSA cavitory lesions (Fig. 2D).

Microglia thus maintain the integrity of the CSA boundary, a site where they normally form an atypical embryonic ATM hotspot. To assess whether this phenotype was selective of the CSA, we examined other forebrain regions. We observed a cavitory lesion at the CSB, another major continuous embryonic ATM hotspot (Fig. S2A). Like the CSA, the CSB is a site of morphogenetic stress and displays a normally occurring cavity called the cavum septum that resorbs over development [18, 35]. Microglial absence or depletion triggered midline cavities in the spot where ATMs normally accumulate, leading to a large cavity close to the cavum septum location (Fig. S2B). Collectively, our findings thus reveal a novel role of microglia in maintaining tissue integrity at dense embryonic ATM hotspots.

ATM-factor *Spp1*/OPN contributes to tissue integrity at cortical boundaries

Microglia involvement in tissue integrity at hotspots of accumulating ATM raises the question of the underlying molecular mechanisms. To address this issue, we first examined whether classical microglial pathways would be required to maintain embryonic brain integrity at the CSA. We examined *Cx3cr1*, *Dap12/TyroBP* and *Complement receptor 3 (CR3)* mutants and embryos exposed to a mild prenatal

inflammation (MIA), which disrupt pathways involved in microglial functions across development, but found no evidence of lesions at the CSA (Fig. S3E). To investigate selective ATM-characteristic properties, we focused on extracellular matrix components Spp1/OPN and secreted proteins GPNMB that have been linked to adhesion and remodeling, in the context of bone or skin [20, 36]. We took advantage of available full knock-outs, as these factors are highly and specifically expressed by microglia and macrophages at these developmental timepoints (not shown). While we observed no phenotype in *Gpnmb* knock-outs (Fig S4), we found that *Spp1* inactivation partially mimicked the absence of microglia, with cavitory lesions present in approximately 50% of mutants at the CSA at E14.5 (Fig. 3A) and 70% of mutants at the CSB at E18.5 (Fig. 3B). Cavities rapidly resorbed overtime, suggesting that while Spp1/OPN contributes to ATM properties, additional factors are likely involved. Importantly, ATM-core factors including Mac2 and GPNMB were still expressed in *Spp1* mutants (Fig. 3C). Our findings thus show that OPN is not required for the induction of ATM-core factors but contributes to microglial function in brain integrity.

The CSA constitutes a site of morphogenetic fragility

The involvement of microglia and ATM-core factor Spp1/OPN in tissue cohesion at cortical boundaries raised the question of what might be specific to these boundaries. The CSA and CSB were both shown to be sites of morphogenetic stress, lying at the border between the cerebral cortex and structures that grow at different paces and into distinct directions [18, 19, 35, 37, 38]. To assess the local niche of embryonic ATM, we focused on the CSA, which has been less characterized than the CSB [18]. Using immunostaining and electron microscopy, we observed that embryonic ATM were systematically abutting apparent microcavities that form during physiological CSA development (Fig. S5A). These microcavities contained fragments of membrane and no basal lamina, suggesting that they could constitute a local tissue fragility, potentially triggered or amplified by morphogenetic stress. To experimentally test this hypothesis, we took advantage of a genetic model of conditional *RhoA* inactivation (*Emx1^{cre/+}; RhoA^{f/f}*) that drastically alters cortical morphogenesis and tissue properties by inducing a large periventricular nodular heterotopia which starts forming around E15.5 [21]. Consistently with the fact that corticogenesis is not drastically altered at early

stages, we observed no changes in CSA morphology of the mutants at E15.5 [21](Fig. S5B). However, from E18.5 onwards, when the heterotopia is large and cortical tissue affected, we started to observe visible lesions at the CSA in 50% of mutant embryos, progressing to a large lesion observed in all cases at P8 (Fig. S5). Importantly, these lesions were not due to apparent changes in microglia colonization or induction of ATM-core factors (not shown). Taken together, these results indicate that the CSA, which normally displays microcavities, constitutes a site of fragility that can lose its integrity in response to morphogenetic modifications.

ATM-core factors are induced by lesions and linked to repair

These findings raised the possibility that embryonic ATM might be induced by microcavities linked to morphogenesis, and required to prevent tissue damage at fragile spots. If so, it would imply that ATM state would be induced by tissue-lesion and have key contributions to repair. To test this hypothesis, we first assessed microglial response to *in utero* lesions (IUL), using a mechanical poking of the neocortex in E14.5 embryos, followed by recovery of the pregnant dams (Fig. 4A-C). We observed after 2.5 hours (h) a rapid accumulation of *Cx3cr1^{gfp}*-positive cells at the lesion site, with approximately 30% of them expressing the core-ATM markers *Spp1/OPN* and *Mac2*, and 10% *GPNMB* (Fig. 4B, C). *In utero* stab-lesions thus extremely rapidly recruit microglia that induce ATM-core markers at the injury site. The short timelag corresponds to a period during which microglia contribute to the repair process, as illustrated by the fact that a majority of PLX3397-treated embryos displayed morphological deficits after 2.5 h (Fig. 4D,E). While these deficits could be corrected overtime, they nonetheless highlight a remarkable involvement in embryonic brain repair, as previously proposed in the neonatal spinal cord [39].

To further explore whether microglia and ATM could repair existing lesions, we took advantage of the fact that microglial embryonic depletion is followed by repopulation during early postnatal life [24-26]. We thus examined the impact of repopulating microglial cells on the cavitory lesions that we had previously characterized (Fig. 2 and S3). In PLX3397-treated pups, we found that CSA midline lesions rapidly resorbed concomitantly to postnatal microglia repopulation (Fig. 4F). Cavities were systematically observed at P0, whereas 60% had closed by P3, with scar-associated

microglial accumulations expressing ATM-markers including Spp1, GPNMB and Mac2 (Fig. 4F,G). At P7 more than 70% of CSA lesions were resorbed, with just a limited number of cases that showed both a cavity and a lack of microglia at the CSA (Fig. S6 and not shown), reinforcing the idea that the resorption correlates with microglia repopulation. Finally, CSA cavities had systematically resorbed by P20 (Fig. 4F), and we observed a very similar timeline of resorption at the CSB (data not shown). CSA and CSB microglia are thus not only important to prevent the formation of large lesions, they are also linked to their prompt closure, thereby highlighting a remarkable repair capacity.

Finally, to assess if repair properties rely on Spp1/OPN, we compared the resorption process in PLX3397-treated controls and PLX3397-treated *Spp1*^{-/-} pups, focusing on P7, a timepoint when a majority of lesions had resorbed (Fig. 4H). We found that in *Spp1*^{-/-} pups, in spite of ATM induction (Fig. 4I), lesions were detected in almost all mutants, highlighting a key role for Spp1/OPN in brain repair.

Taken together, our study reveals a novel role for microglia in maintaining tissue integrity in response to morphogenetic stress linked with corticogenesis. This role involves accumulations of embryonic ATM and Osteopontin/OPN, which not only prevents lesion formation but also promotes their active repair.

DISCUSSION

Our study reveals a novel and genuinely glial/glue role of microglia in preserving brain integrity as the cerebral cortex develops, before the generation of potentially redundant or complementary glial cells, including astrocytes [40, 41]. Indeed, because microglia colonize the brain from early embryonic stages and astrocytes or oligodendrocytes are produced later, microglia constitute a large contingent of embryonic glial cells [1, 12]. Using a variety of experimental approaches, including macrophage embryonic depletions and more specific alterations of resident macrophages using *Csf1*^{Δfire/Δfire} mice [31], we showed that microglia prevent the formation of focal embryonic lesions that are observed throughout embryogenesis.

Lesions are located at specific cortical boundaries that constitute sites of fragility and normally host embryonic ATM, a microglial state previously involved in postnatal myelination [7, 9, 14, 15, 17]. Indeed, we identified embryonic ATM that are remarkably similar to their postnatal counterparts, confirming ATM span the prenatal/postnatal period [8, 22] and further showed that they are linked to a variety of developmental functions possibly through distinct molecular cascades. In particular, while postnatal ATM functions were largely attributed to *Igf1* [14-16], we found that *Spp1/OPN*, an atypical ECM factor previously involved in bone development, wound-healing and inflammation [20, 36, 42], contributes to tissue integrity at cortical boundaries and to the repair of large lesions. While *Spp1/OPN* inactivation is not fully mimicking the impact of microglial depletion and we cannot preclude the involvement of additional cell types, our findings support a contribution of *Spp1/OPN* to microglial functions in both tissue integrity and repair. Importantly, we also reveal that *Spp1/OPN* and other ATM factors are rapidly induced in microglia accumulating at *in utero* tissue-lesion sites, highlighting a remarkable plasticity and capacity to switch states in response to experimentally-induced injury.

Core ATM-factors including *Spp1/OPN*, have been involved in ECM organization, wound-healing or regulation of inflammation, and detected in a wide range of macrophages, during skin lesion, heart-repair after myocardial infarction or liver fibrosis [20, 36, 42-45]. This observation suggests that the “ATM program” involved in brain integrity and repair is likely not specific to microglia, but could constitute a basic feature shared across macrophages important for the general maintenance of tissue

homeostasis, notably in the context of exogenous damage or lesions. In microglia, Spp1/OPN and ATM factors are also found in several damage-induced microglia observed in stroke [46] or during neonatal spinal cord repair [39], as well as DAM and TAM, respectively associated to neurodegeneration or tumors [4, 6, 9, 23, 47]. As such, the “ATM program” could overlap with a basic repair one, triggered by tissue disruption in distinct contexts. Whether Spp1/OPN is systematically involved, which other factors contribute to ATM functions in tissue cohesion and repair, and whether additional cell types could exert related functions in the adult brain constitute key questions to address in future studies.

Such a glial role of developing microglia has not yet been described in other species, for instance zebrafish, in which various genetic models enable to examine brain development in the absence of microglia [48]. Consistently, there are no reported evidence of dense ATM hotspots in zebrafish [48, 49]. These observations raise the intriguing possibility that ATM and their repair properties might have been co-opted during evolution to ensure the proper morphogenesis of a growing cerebral cortex, which is a hallmark of mammals. In agreement, we found that increasing morphogenetic constraints in a model of cortical periventricular nodular heterotopia [21] can lead to a selective “breaking” at the CSA boundary strengthening the idea that CSA is a site of morphogenetic fragility where ATM play key roles in the context of morphogenetic constraints. There is thus a fine balance between tissue fragility, ATM localization and functions, which should enable to further characterize the pathways involved in ATM induction as well as their roles in brain morphogenesis.

In the context of pathological brain wiring, lesions are usually linked to damage induced by an “activated” abnormal microglial activity, in response to a variety of triggers such as hypoxia, inflammation, preterm birth or congenital viral infections. For instance, bilateral cysts in the temporal lobes, where the CSA locates or midline cavities, such as cavum septum pellucidum and midline cystic lesions, have been reported in several neurodevelopmental pathologies [50, 51]. Our study reveals a key role of microglia and the ATM-state in preserving tissue integrity, indicating that lesions might also result from a lack of microglial function during critical steps of morphogenesis. Our findings have thus major implications not only for our understanding of the fundamental mechanisms governing brain morphogenesis and the state-specific functions of

microglia, but also open new avenues for deciphering microglial contributions to brain pathology.

EXPERIMENTAL PROCEDURES

Mouse Lines

Cx3cr1^{+/gfp} [52] (RRID:IMSR_JAX:005582), *Pu.1^{+/-}* [29], *Cd11c-eYFP* [53] (RRID:IMSR_JAX:007567), *Csf1r^{ΔFIRE/+}* [31], *Spp1^{-/-}* [36] (RRID:IMSR_JAX:004936), *CR3^{-/-}* (*Cd11b^{-/-}*) [54] (RRID:IMSR_JAX:003991), *Dap12/TyroBP^{-/-}*, *RhoA^{fl/fl}* [55] and *Emx1^{cre/+}/RhoA^{fl/fl}* [21] mice were maintained on a C57Bl/6 background. C57Bl/6 mice or heterozygotes were used as controls, as they did not exhibit any phenotype, except in repair analyses (Fig. 4), in which we compared *Spp1^{+/+}* and *Spp1^{-/-}* and in periventricular nodular heterotopia analyses (Fig. S5) in which we compared *Emx1^{cre/+};RhoA^{fl/fl}* with cre- littermates. The day of vaginal plug was considered E0.5. Animals were handled in accordance with European regulations and the local ethics committee.

Human fetuses

Fetal tissues were made available in accordance with French bylaws (Good Practice Concerning the Conservation, Transformation, and Transportation of Human Tissue to Be Used Therapeutically, published on December 29, 1998). The studies on human fetal tissue were approved by the French agency for biomedical research (Agence de la Biomédecine, Saint-Denis la Plaine, France, protocol n°: PFS16-002). Three non-pathological human fetuses were obtained at gestational week (GW) 9, GW11 and GW14 from voluntarily terminated pregnancies after parent's written informed consent (Gynaecology Department, Jeanne de Flandre Hospital, Lille, France). Fetuses were fixed by immersion in 4% paraformaldehyde (PFA) at 4°C for 3 days (GW9 fetus) and for 5 days (GW11 and GW14 fetuses). The tissues were cryoprotected in 30% sucrose/PBS at 4°C overnight, embedded in Tissue-Tek OCT compound (Sakura Finetek, USA), frozen in dry ice and stored at -80°C until sectioning. Frozen samples

were cut serially at 16 μm using a Leica CM 3050S cryostat (Leica Biosystems Nussloch GmbH, Germany). Sections were kept at -80°C .

Transcriptomic analysis

Single-cell RNA-seq data reanalysis

10X scRNA-seq microglia data of [8] and [22] were downloaded from the GSE121654 series and loom file <http://mousebrain.org/development> (“dev_all.loom”) respectively. The raw count matrix and metadata for microglia-annotated cells in [22] were extracted from the loom file using R v4.1.2. The data include a total of 1711 cells (510 ATM, 415 cycling microglia, 786 non-cycling microglia cells). Genes were filtered as described by authors (expressed in at least 10 cells for [22] and at least 20 cells for [8]). For the data set of [8], additional metadata such as published t-distributed stochastic neighbor embedding (tSNE) coordinates and reported clusters were provided by the authors to select the 76149 cells, including 2517 ATM cells.

The Seurat v4.1.1 [56] scRNA-seq pipeline was used to produce uniform manifold approximation and projection (UMAP) and tSNE plots of [22] and [8] microglia data. Default parameters were used if not mentioned. For [22], data was first normalized and scaled using SCTransform [57]. PCA was performed and UMAP coordinates were computed using 5 principal components. Plots were created using Seurat v4.1.1, ggplot2 v3.3.6, scCustomize 0.7.0, and the viridis v0.6.2 palette.

Differential expression analysis

First, the differentially expressed genes (DEGs) of ATM-annotated cells from [22] were obtained using the “FindAllMarkers” Seurat function (Wilcoxon Rank Sum test, assay = “RNA”, min.pct=0.1, only.pos=TRUE). DEGs were further filtered (Fold Change > 1.5, Bonferroni adjusted p -value < $1e^{-10}$). To construct venn diagrams, the same threshold was applied to published DEGs of ATM [8] and PAM [7].

Gene set enrichment analysis

To determine gene set enrichment in a scRNA-seq dataset, the “AddModuleScore” Seurat function (ctrl.size = length of gene list, nbin=24) was used. Several gene signatures were tested for enrichment in [22] microglia scRNA-seq data: (i) top

enriched ATM DEGs from [8], corresponding to the nine reported published markers (i.e., *Spp1*, *Gpnmb*, *Igf1*, *Lgals3*, *Fapb5*, *Lpl*, *Lgals1*, *Ctsl*, *Anxa5*), and (ii) top enriched PAM DEGs from [7] (i.e., *Spp1*, *Clec7a*, *Gpnmb*, *Igf1*, *Lpl*, *Pld3*, *Ctsl*, *Ctsb*, *Slc23a2*, *Gpx3*) (Table S1).

Microglial Depletion

Pregnant C57Bl/6 females were administered anti-CSF-1R mAb (α CSF-1R, clone AFS98) or the rat IgG2a isotype control (clone R35-95; BD Biosciences) intraperitoneally at E6.5 and E7.5 as described previously [25]. Alternatively, pregnant mice were given the CSF1R inhibitor PLX3397 (Plexxikon) mixed into standard chow (Ssniff) from E6.5 or E12.5 until E15.5. The dose of PLX3397 was 290 mg/kg and respective controls received standard chow. The efficiency of depletion procedures was verified by immunohistochemistry at embryonic stages and in one newborn P0 per litter for postnatal litters.

Maternal Immune activation

Pregnant females were administered LPS in sterile PBS (0.12 μ g/g mouse; InvivoGen and Sigma) at E13.5 by a single intraperitoneal injection. Control pregnant females were injected at E13.5 with sterile PBS and showed no detectable phenotype.

Immunohistochemistry on sections

For immunohistochemistry, mouse embryonic brains were fixed in 4% PFA at 4°C from 2h to overnight, depending on the developmental stage. Post-natal animals were perfused with 4% PFA, dissected and post-fixed overnight at 4°C and directly cut in PBS. Immunohistochemistry was performed on free-floating 40-100 μ m- thick vibratome mouse brain sections or 25 μ m-thick cryostat human tissue sections. Slices were treated 1h at room temperature (RT) in 0,2% Triton X-100, 0.2% Gelatin in PBS, and then incubated in the same blocking solution with primary antibodies overnight at 4°C: rat anti-CD68 (1/500; Bio-Rad), chicken anti-GFP (1/1000; Aves), rabbit anti-Iba1 (1/500; Wako Pure Chemical Industries), rat anti-Lgals3 (Mac2) (1:1000; Cedarlane

Labs) rat anti-L1 (1/100; Millipore), biotinylated rat anti-Lyve1 (1/200; eBioscience), rat anti-mDectin-1 (Clec7A) (1/30; Invivogen), goat anti-Osteoactivin (Gpnmb) (1/200; R&D Systems), rabbit anti-P2ry12 (1/500; Anaspec), goat anti-Spp1 (1:400; R&D Systems). Sections were rinsed in PBS-0.1% TritonX-100 and incubated from 2h to overnight at 4°C with secondary antibodies (1/400 in PBS, Jackson ImmunoResearch): Alexa 10 Fluor® 488-conjugated donkey anti-chicken, Alexa 10 Fluor® 488-conjugated donkey anti-goat; Alexa 10 Fluor® 488-conjugated donkey anti-rat; Alexa 10 Fluor® 488-conjugated donkey rabbit; Cy3-conjugated donkey anti-goat, Cy3-conjugated donkey anti-mouse, Cy3-conjugated donkey anti-rabbit, Cy3-conjugated donkey anti-rat, Cy5-conjugated donkey anti-mouse, Cy5-conjugated donkey anti-rabbit, Cy5-conjugated donkey anti-rat. Hoechst (1/1000; Sigma) was used for fluorescent nuclear counterstaining.

Tissue Clearing

We used an adapted version of the iDISCO+ clearing protocol previously published [33, 34]. All incubation steps were performed at RT in a fume hood, on a tube rotator (SB3, Stuart) at 14 rpm, using a 15 ml centrifuge tube (TPP, Dutscher) covered with aluminum foil to avoid contact with light. E16.5 brain samples were first dehydrated in a graded series (20%, 40%, 60%, 80%, and 100%) of methanol (MeOH, Sigma-Aldrich) diluted in H₂O, during 1 and a half hour for each step. This was followed by a delipidation step of 30 min in dichloromethane (DCM; Sigma-Aldrich). Finally, samples were cleared overnight in dibenzylether (DBE; Sigma-Aldrich). Samples were then stored in brown glass vial filled with DBE, in the dark and at RT.

Tissue preparation for Electron microscopy (TEM)

E14.5 mouse embryos were perfused with a buffer of 4% PFA, 2.5% glutaraldehyde (EM grade) in PBS. Fixed embryos were kept in aluminum on ice for 30 minutes to allow the glutaraldehyde impregnation. Brains were then dissected and kept overnight at 4°C in a buffer of 4% PFA in PBS. 100 µm vibratome sections were collected, washed 3 times in PBS and postfixated for 2 hours in 2 % osmium tetroxide in PBS. The samples were colored en bloc with 1.5% aqueous uranyl acetate at 4°C for 1.5 hours.

Samples were then dehydrated in graded concentration of ethanol (25%, 50%, 70%, 90% and three times 100%, 10 minutes per step) and rinsed with anhydrous acetone for 10 minutes three times. Samples were infiltrated with graded concentration of Araldite 502 resin (50%, 90%, 1 hour per step) and incubated for 2 hours in pure resin freshly prepared. Samples were then mounted in a minimum amount of resin between two ACLAR 33C films and polymerization of the resin were performed at 60°C for 48 hours. Before cutting, sample blocs were glued paralleled to the flat surface of a cylinder bloc of resin as a support. 70 nm ultrathin sections of the sample were obtained using an ultramicrotome (UC6, Leica). Ultrathin sections were collected on formvar coated slot (2 x 1 mm) grids. Positive staining of grids was performed for 2 minutes incubation in UranylLess aqueous solution (Delta Microscopy) followed with 1-minute lead citrate staining.

MRI scans

After fixation, the embryos were stored in a 1:250 mixture of 0.5mmol/ml gadoteric acid (Dotarem®, Guerbet) and phosphate-buffered saline for at least 72 hours. For imaging, the embryos were embedded in 2% low melting point agar gel (Sigma-Aldrich) and were placed in small Plexiglass containers. All acquisitions were performed on a 17.2 T (1H Larmor frequency = 730.2 MHz) Bruker Biospec preclinical scanner equipped with a 25-mm inner diameter quadrature birdcage volume coil (Rapid Biomedical). 3D images were acquired using a Fast Low Angle Shot (FLASH) sequence with the following acquisition parameters: TE = 7 ms, TR = 150 ms, flip angle = 30°, resolution = 40 × 40 × 40 μm³, signal averaging = 2.

***In utero* cortical lesioning**

Pregnant female mice at E14.5 were anesthetized with isoflurane (3.5% for induction, 2% during the surgery) and subcutaneously injected with 0.1 mg/kg of buprenorphine for analgesia. The uterine horns were exposed after laparotomy. Cortical *in utero* lesions were performed unilaterally using a glass capillary of 100 μm of diameter. Lesioned embryos were left to recover in the mother's womb 2.5 hours after surgery, after which their brains were collected and fixed in 4% PFA overnight at 4°C.

***Ex vivo* phagocytic assay**

Slice preparation was performed as previously described (López-Bendito et al., 2006) with the following modifications. Dissecting medium was prepared from MEM medium (Gibco) with 20mM TRIS powder pH7-9 (Sigma) and 45mM D-glucose (Sigma). After telencephalic brains were cut into 250µm-thick vibratome coronal sections, slices of interests were stored in this medium on ice until use. For *ex vivo* phagocytosis, pH-rodo (Life technologies) was resuspended at 0.5mg/mL in dissection medium, containing 2.5mM CaCl₂, 2.5mM MgSO₄, 1mM NaHCO₃ supplemented with 300uL HEPES (Gibco). for each 50mL of medium. After careful removal of MEM medium, 15uL of this pH-rodo solution was added on top of each slice, which were incubated for 1 hour at 37°C in 5% CO₂. The reaction was stopped by addition of cold pH-rodo suspension medium. After 3 washes slices were fixed in 4% PFA in PBS for 45min and immunohistochemistry was performed as described above.

Image acquisitions and analysis

Slices imaging

Images of immunohistochemistry on sections were acquired with a fluorescence binocular microscope (Leica MZ16 F), a fluorescence microscope (Leica DMI8) and a confocal microscope (Leica TCS SP5 and TSP8). Image analyses were performed with ImageJ, Bitplane Imaris and Adobe Photoshop software.

3D Imaging and Processing

3D imaging was performed with an ultramicroscope II (LaVision BioTec) using InspectorPro software (LaVision BioTec). The light sheet was generated by a laser (wavelength 488 Coherent Sapphire Laser, LaVision BioTec) and two cylindrical lenses. Samples were placed in an imaging reservoir made of 100% quartz (LaVision BioTec) filled with ethyl cinnamate and illuminated from the side by the laser light. Images were acquired with a PCO Edge sCMOS CCD camera (2,560 × 2,160 pixel size, LaVision BioTec). The step size between each image was fixed at 3 µm. For all samples, background fluorescence recorded from exposure to the 488nm wavelength were acquired in order to reconstruct brain morphology and lesions. Images, 3D

volume, and movies were generated using Imaris x64 software (version 9.5, Bitplane). Stack images were first converted to imaris file (.ims) using ImarisFileConverter. File size was next reduced to 8 bits. 3D reconstruction of the sample was performed using “volume rendering” (Imaris). The sample could be optically sliced in any angle using the “orthoslicer” or “obliqueslicer” tools in order to verify phenotypic alterations. Lesions due to microglial depletion could be highlighted using the “surface” tool by creating a mask around the volume. To this end, low intensity pixels resulting from low background fluorescence (and absence of cells in the lesions) in the 488nm exposure condition were selected in order to create the “surface”. 3D pictures and movies were generated using the “snapshot” and “animation” tools. Movies legends were generated using ImageJ software.

Transmitted Electron Microscopy (TEM) acquisition

TEM imaging was performed using a Philips Tecnai 12 Transmission Electron Microscope (Philips/FEI, Eindhoven, The Netherlands) on the electronic imaging department of the Imachem imaging platform (France BioImaging) at Institut de Biologie de l'Ecole Normale Supérieure, Paris. The software Gatan DigitalMicrograph was used to acquire TEM images at various magnifications with a 4K CCD Orius 1000 camera (Gatan).

Quantification and statistical analysis

Co-labelling of IBA1+ or CX3CR1-GFP+ macrophages with P2ry12 microglial marker and ATM markers was quantified specifically in accumulated cells along the CSA, CSB and *in utero* induced lesions in the cortex using Cell Counter on ImageJ software. Lesion severity after embryonic microglia depletion was determined as 0: absence of lesion; 1: mild lesion; 2: severe lesion. The mean scores for lesion severity in different experimental conditions were then compared statistically. All data are presented as mean \pm SEM. Non-parametric two-sided Mann-Whitney U-tests were used to compare two distributions in co-labelling and lesion severity experiments. Fisher's exact test was used to compare contingent presence or absence of CSA/CSB lesions in controls,

Spp1^{-/-} and PLX3397 treated mice. All graphs and statistical analyses were generated using GraphPadPrism 8.0 software. *p < 0.05, **p < 0.01, ***p < 0.001.

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AUTHOR CONTRIBUTION

Conceptualization, A.L., C.B., M.S.T, L.L and S.G.; Formal analysis, A.L., A.C., C.B., N.O., C.L., D.B., L.C, A.C., A.G., D.T., L.C., M.S.T, L.L. ; Investigation: A.L., A.C., C.B., N.O., C.L., G.O., P.S., L.C., L.C., M.S.T, L.L.; Resources, D.M., A.F.,C.A., F.W., J.B.M., C.P, J.P., P.G., F.G. ; Writing – Original Draft, A.L., C.L., M.S.T, L.L. and S.G.; Visualization, A.L., A.C., C.A., N.O., C.L., M.S.T., L.L; Project Administration, M.S.T, L.L. and S.G.; Supervision, M.S.T, L.L. and S.G.; Funding acquisition, S.G.

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FIGURES (MAIN AND SUPPLEMENTAL)

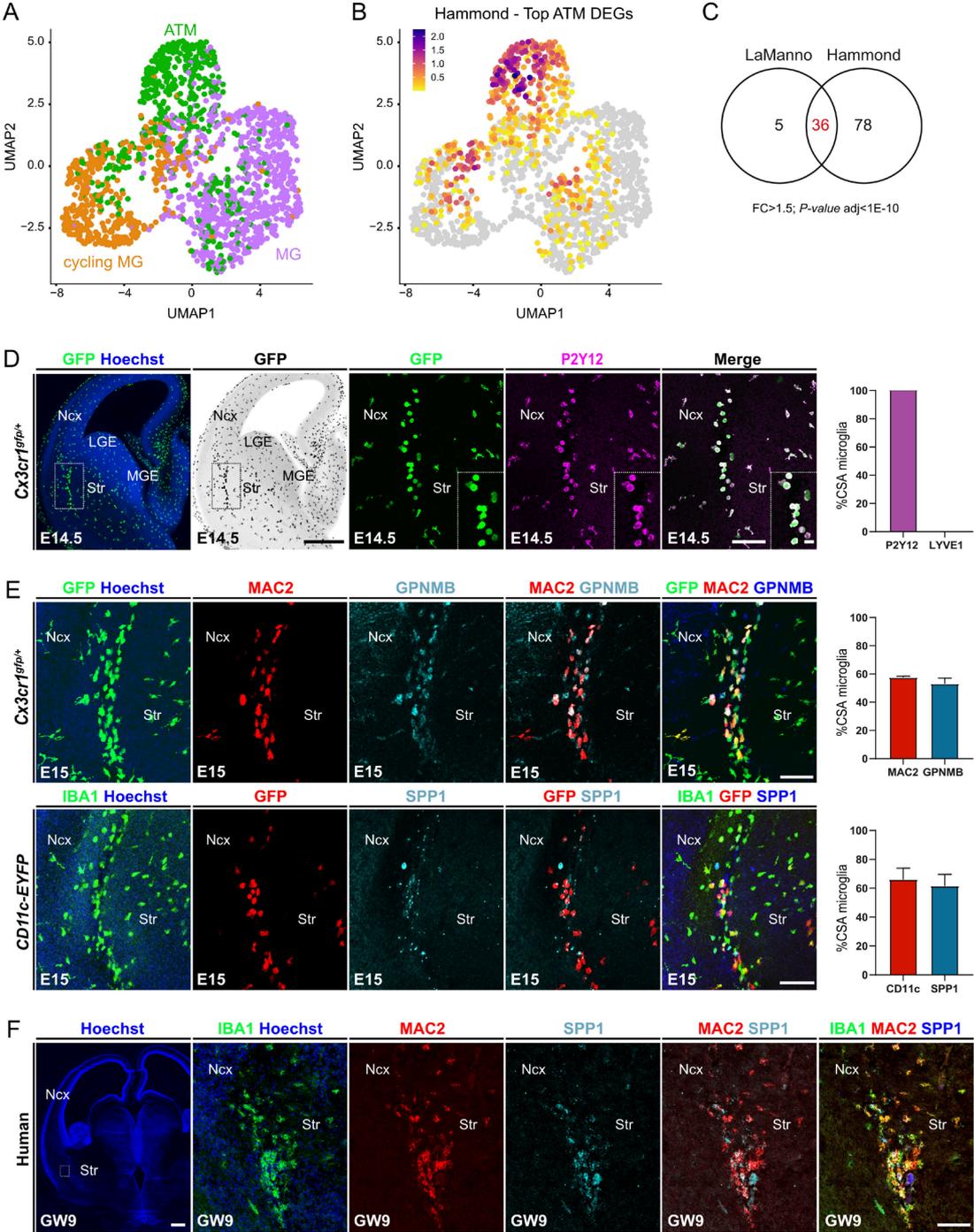


Figure 1. Embryonic ATM are located at the CSA in mouse and human embryos

(A) Single-cell transcriptomic UMAP plot of embryonic microglial cells (n = 1711) extracted from [22] showing homeostatic microglia (MG) (purple), cycling MG (orange)

and ATM (green). **(B)** Projection of the ATM signature (top enriched DEGs) from [8]. **(C)** Venn diagram showing the large overlap between the embryonic ATM signature DEGs identified from the dataset of [22] and the ATM signature characterized in [22] (fold change > 1.5, Bonferroni adjusted p-value < 1e-10). **(D-F)** Immunostainings on embryonic mouse brain sections at E14.5 **(D)** or E15 **(E)** or on human gestational week (GW)9 **(F)**, showing *Cxcr3cr1^{gfp}*-positive or IBA1-positive microglia co-expressing ATM markers at the cortico-striato-amygdalar (CSA) boundary. CSA close-ups are delineated by dotted lines. Immunostaining in mice were performed on at least n=3 from 2 distinct litters and on two human samples. Values are presented as mean ± SEM and scale bars equal 500 μm (D-left panels) 100 μm (D-right panels), 20 μm (D insets), 200 μm (E) and 1mm (F).

ATM, axon-tract-associated microglia; CSA, cortico-striato-amygdalar boundary; DEGs, differentially expressed genes; LGE, lateral ganglionic eminence; MG, microglia; MGE, medial ganglionic eminence; Ncx, neocortex; Str, striatum.

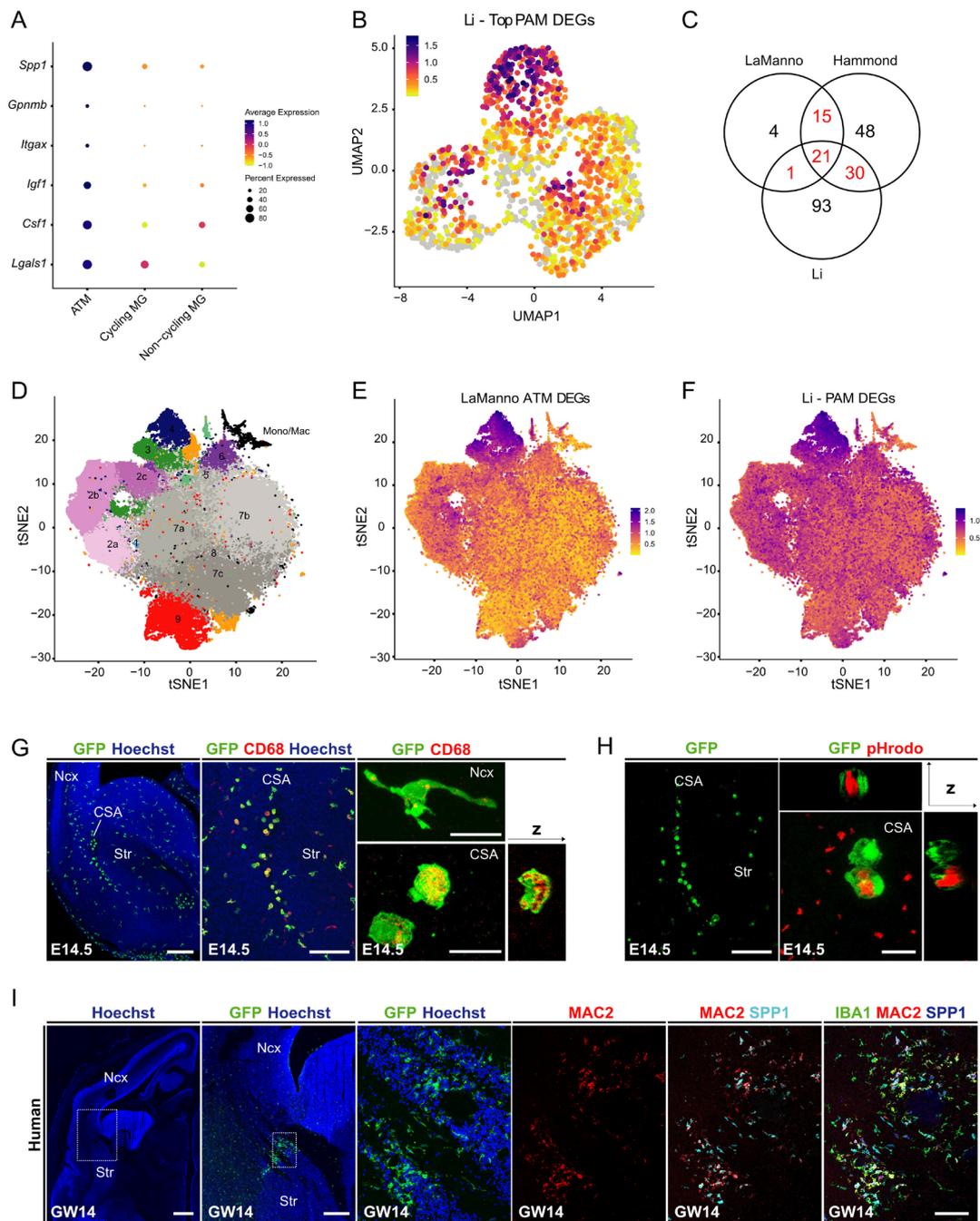


Figure S1. Embryonic ATM share similarities with postnatal ATM

(A) Dot plot reporting the expression levels of ATM-core genes in embryonic ATM, versus in homeostatic or cycling embryonic microglia in the scRNA-seq data set from [22]. The color represents the scaled expression level across all cells within a cluster,

while the size of dots indicates the percentage of cells expressed in that cluster **(B)** Projection of the PAM signature from [7] onto the UMAP plot from Fig. 1A presenting embryonic ATM. **(C)** Venn diagram highlighting the overlap between ATM DEGs identified embryonically in [22] and in ATM from [8] and PAM from [7]. **(D)** Reproduced tSNE plot of the 76149 cells from the scRNA-seq dataset of [8]. **(E,F)** Projections of the embryonic ATM signature defined in [22] **(E)** and the PAM signature defined in [7] **(F)**, highlighting a large overlap with the Hammond ATM cluster 4. **(G-H)** Immunostaining with Cd68 **(G, n=3)** and Phrodo assay in *ex vivo* slices showing **(H, n=4)** an intense staining in CSA microglia at E14.5. **(I)** Immunostaining in human GW14 transverse sections showing co-labeling of ATM markers with IBA1 at the CSA. Scale Bars equal 1500 μm (left panel), 500 μm (second panel) and 100 μm (right panels).

ATM, axon-tract-associated microglia; CSA, cortico-striato-amygdalar boundary; DEGs, differentially expressed genes; Ncx, neocortex; Str, striatum.

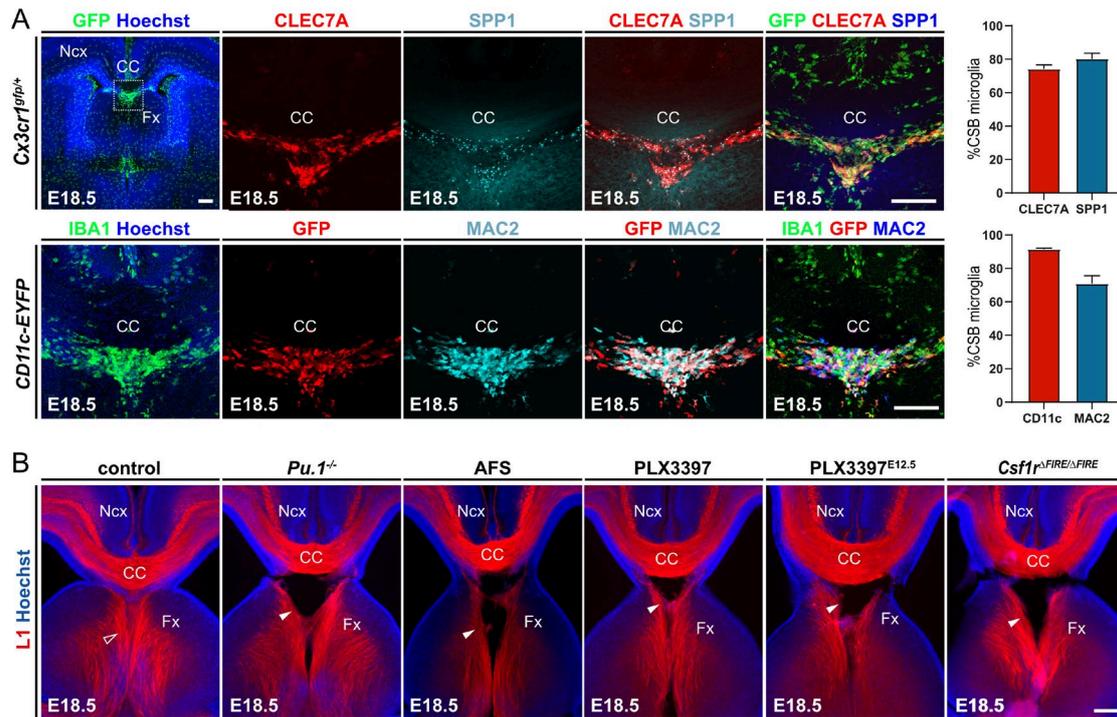


Figure S2. ATM accumulate at the midline CSB and maintain tissue integrity

(A) Immunostaining on E18.5 *Cx3cr1^{gfp}* or *Cd11c-EYFP* coronal brain sections showing co-labeling for ATM markers at the cortico-septal boundary (CSB), below the corpus callosum (CC) (n=4 in at least two litters). **(B)** L1-immunostaining enables the visualization of axons the CC and Fornix (Fx) (open white arrowhead) and highlights a lesion at the CSB (solid white arrowheads) in various pharmacological and genetic models affecting microglial colonization and density (n_{controls}=21, n_{Pu1KO}=4, n_{AFS}=5, n_{PLX3397}=9, n_{PLX3397-E12}=11, n_{Csf1RFire}=5). Scale bars equal 100 μm (A) and 200 μm (B).

CC, corpus callosum; CSB, cortico-septal boundary; Fx, Fornix; Ncx, neocortex; Se, Septum; Str, striatum.

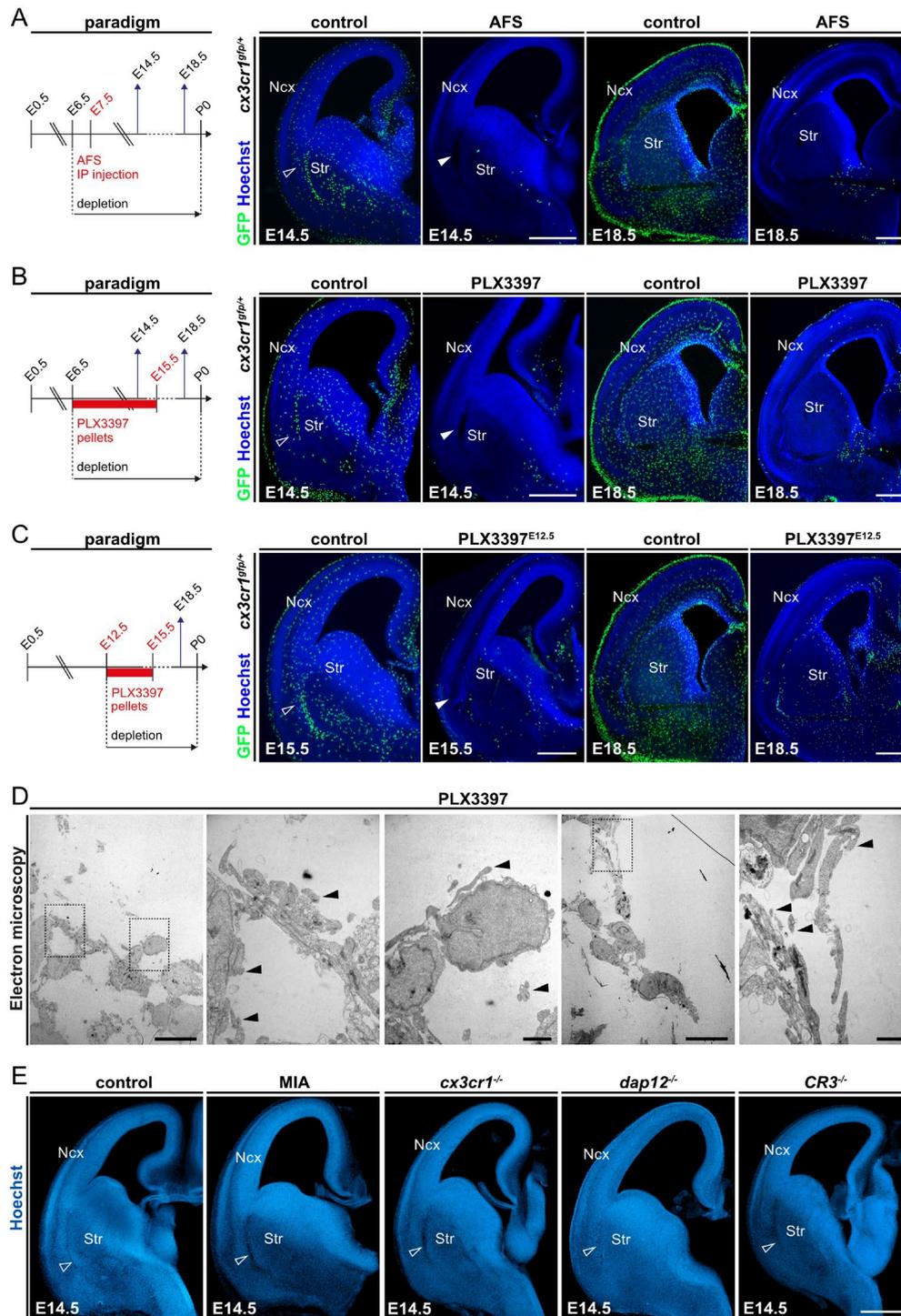


Figure S3. Models of macrophage depletion and functional alteration

(A) Model of embryonic macrophage depletion induced by the injection of a CSF1R blocking antibody at E6.5 and E7.5 which leads to a drastic depletion of brain

macrophages, as highlighted by comparing *Cx3cr1^{gfp/+}* controls and AFS E14.5 ($n_{\text{controls}}=21$, $n_{\text{AFS}}=6$) and E18.5 ($n_{\text{controls}}=15$, $n_{\text{AFS}}=17$) embryos. Open and solid arrowheads respectively indicate the accumulation of GFP-positive cells at the CSA and local CSA tissue lesion in absence of GFP-positive cells. **(B)** Model of embryonic macrophage depletion induced by feeding pregnant dams with PLX3397, a pharmacological inhibitor of CSF1R pathway from E6.5 to E15.5 analyzed at E15.5 ($n_{\text{controls}}=5$, $n_{\text{PLX3397}}=5$) and E18.5 ($n_{\text{controls}}=12$, $n_{\text{PLX3397}}=11$). Open and solid arrowheads respectively indicate the accumulation of GFP-positive cells at the CSA and local CSA tissue lesion in absence of GFP-positive cells. **(C)** Model of embryonic macrophage depletion induced by feeding pregnant dams with PLX3397 from E12.5 to E15.5 analyzed at E15.5 ($n_{\text{controls}}=5$, $n_{\text{PLX3397-E12}}=9$) and E18.5 ($n_{\text{controls}}=12$, $n_{\text{PLX3397-E12}}=10$). Open and solid arrowheads respectively indicate the accumulation of GFP-positive cells at the CSA and local CSA tissue lesion in absence of GFP-positive cells. **(D)** Transmitted Electron Microscopy focused on the CSA tissue lesion in E14.5 PLX3397-treated pups, showing a lack of basal membrane in these cavitory lesions ($n=3$). **(E)** Coronal sections through E14.5 hemibrains of controls, embryos exposed to mild maternal immune activation (MIA), *Cxc3cr1^{-/-}*, *Dap12/TyroBP^{-/-}* and *CR3^{-/-}* mutant embryos showing no lesion at the CSA (open arrowheads) (at least $n=6$ for each condition). Scale bars equal 500 μm (A,B,C,E), 10 μm (D low magnification) and 2 μm (D high magnification).

Ncx, neocortex; Str, striatum.

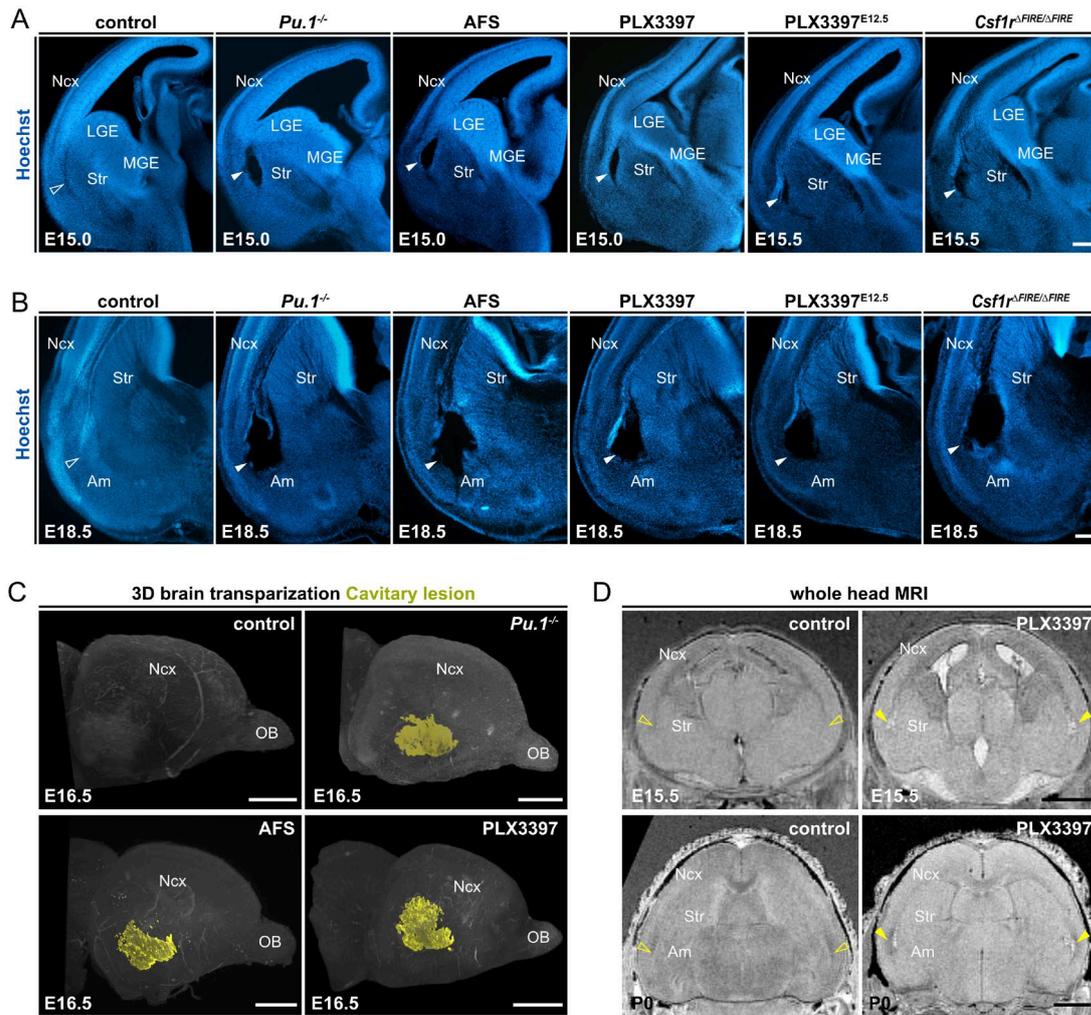


Figure 2. Microglia are required for tissue integrity at the embryonic CSA

(A,B) Hoechst staining of hemibrain coronal sections at E14.5 or E15.5 **(A)** showing CSA integrity in controls (open arrowhead) and cavitory lesions (solid arrowheads) ($n_{\text{controls}}=27$, $n_{\text{Pu1KO}}=6$, $n_{\text{AFS}}=4$, $n_{\text{PLX3397}}=8$, $n_{\text{PLX3397-E12}}=11$, $n_{\text{CSF1RFire}}=7$). Similar staining at E18.5 showing that the cavitory lesions are located at the border between the neocortex (Ncx), striatum (Str) and amygdala (Am) ($n_{\text{controls}}=35$, $n_{\text{Pu1KO}}=5$, $n_{\text{AFS}}=7$, $n_{\text{PLX3397}}=13$, $n_{\text{PLX3397-E12}}=11$, $n_{\text{CSF1RFire}}=6$). **(C)** Cavity reconstruction (yellow) after whole brain clearing using the iDisco method, highlighting no cavities in controls and stereotypically located cavities in 3 models of depletion ($n_{\text{controls}}=9$, $n_{\text{Pu1KO}}=4$, $n_{\text{AFS}}=5$, $n_{\text{PLX3397}}=6$). **(D)** Low intense signals at the CSA of PLX3397-treated embryos were

detected (yellow solid arrowheads) in contrast to controls (yellow open arrowheads) in E15.5 and newborn whole-head MRI scanning. These signals, observed in 3 animals at each stage and condition, confirm the formation of a cavitory lesion in PLX3397-treated embryos. CSA close-ups are delineated by dotted lines. Scale bars equal 200 μm (A, B), 800 μm (C) and 1mm (D).

Am, amygdala; CSA, cortico-striato-amygdalar boundary; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; Ncx, neocortex; OB, olfactory bulb; Str, striatum.

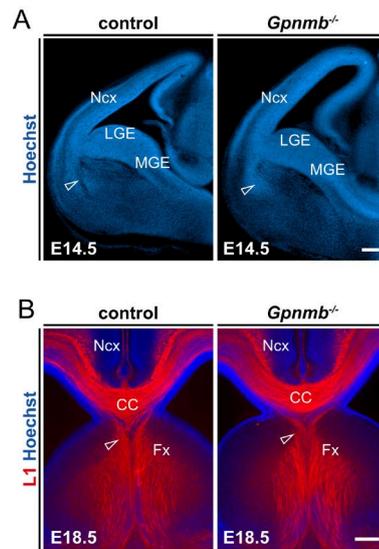


Figure S4. *Gpnmb* inactivation does not alter CSA or CSB integrity

(A) E14.5 coronal hemisection stained with Hoechst showing integrity of the CSA in both controls and *Gpnmb*^{-/-} mutants (open arrowheads) ($n_{\text{controls}}=18$, $n_{\text{GpnmbKO}}=13$). **(B)** L1 immunostaining showing integrity at the CSB in both controls and *Gpnmb*^{-/-} mutants (open arrowheads) ($n_{\text{controls}}=23$, $n_{\text{GpnmbKO}}=8$). Scale bars equal 200 μm .

CC, corpus callosum; Fx, fornix; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; Ncx, neocortex.

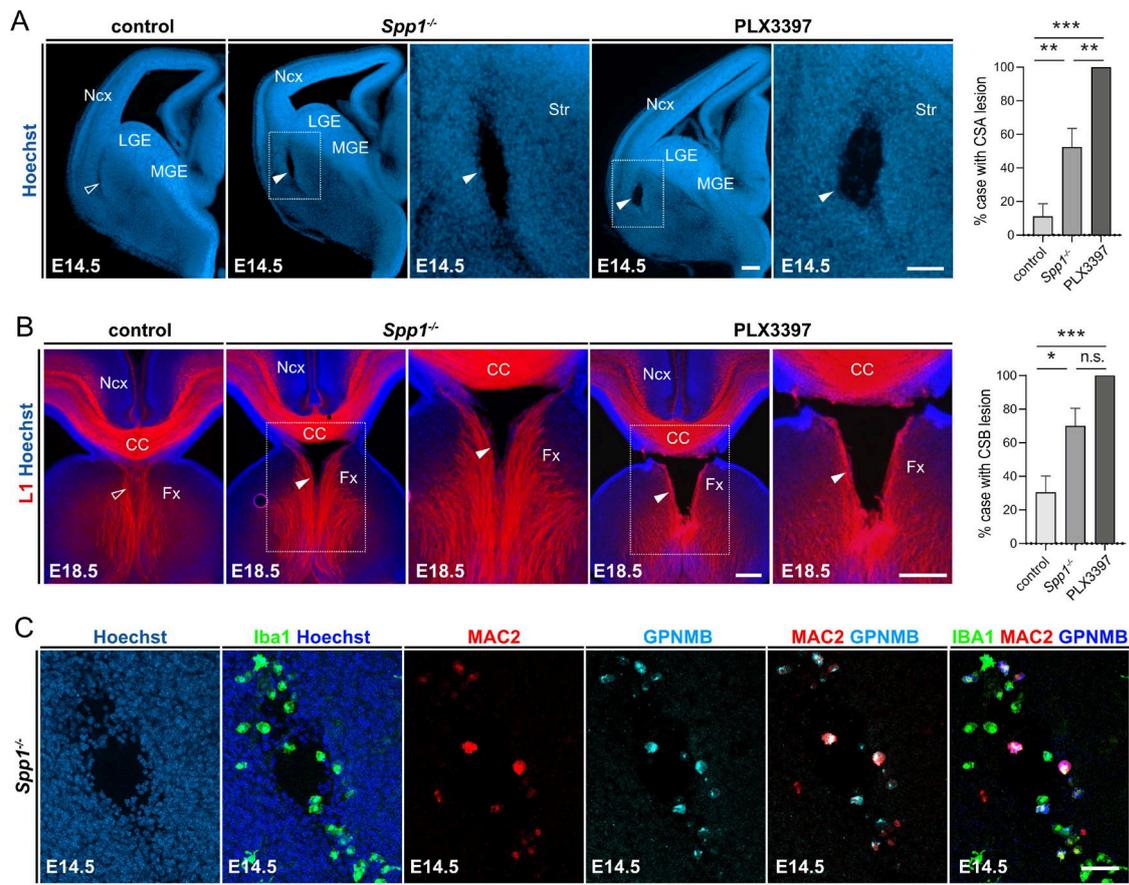


Figure 3. *Spp1*/OPN is required for tissue integrity at the CSA and CSB

(A) Coronal hemisections of E14.5 brains stained with hoechst reveal a CSA disruption in 50% of *Spp1*/*OPN*^{-/-} mutants (solid arrowheads), compared to controls (open arrowheads) and 100% in PLX3397-treated embryos (solid arrowheads) ($n_{\text{controls}}=18$, $n_{\text{Spp1KO}}=21$, $n_{\text{PLX3397}}=13$). **(B)** L1 immunostaining enables the visualization of axons (open arrowheads) and midline lesions (solid arrowheads) in *Spp1*/*OPN*^{-/-} mutants in approximately 70% of the case compared to 100% of the PLX3397 exposed embryos ($n_{\text{controls}}=23$, $n_{\text{Spp1KO}}=20$, $n_{\text{PLX3397}}=8$). **(C)** E14.5 coronal hemisections showing expression immunostaining of Mac2 and GPNMB at the CSA in both controls (open arrowheads) and *Spp1*^{-/-} embryos ($n_{\text{controls}}=3$, $n_{\text{Spp1KO}}=3$). Values are presented as

means \pm SEM and the statistical tests performed are Fisher's exact test, * $p < 0.05$; ** $p < 0.001$; *** $p < 0.0001$. Scale bars equal 200 μm (A,B low magnification), 100 μm (A,B high magnification) and 100 μm (C).

CC, corpus callosum; CSA, cortico-striato-amygdalar boundary; Fx, fornix; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; Ncx, neocortex; Str, striatum.

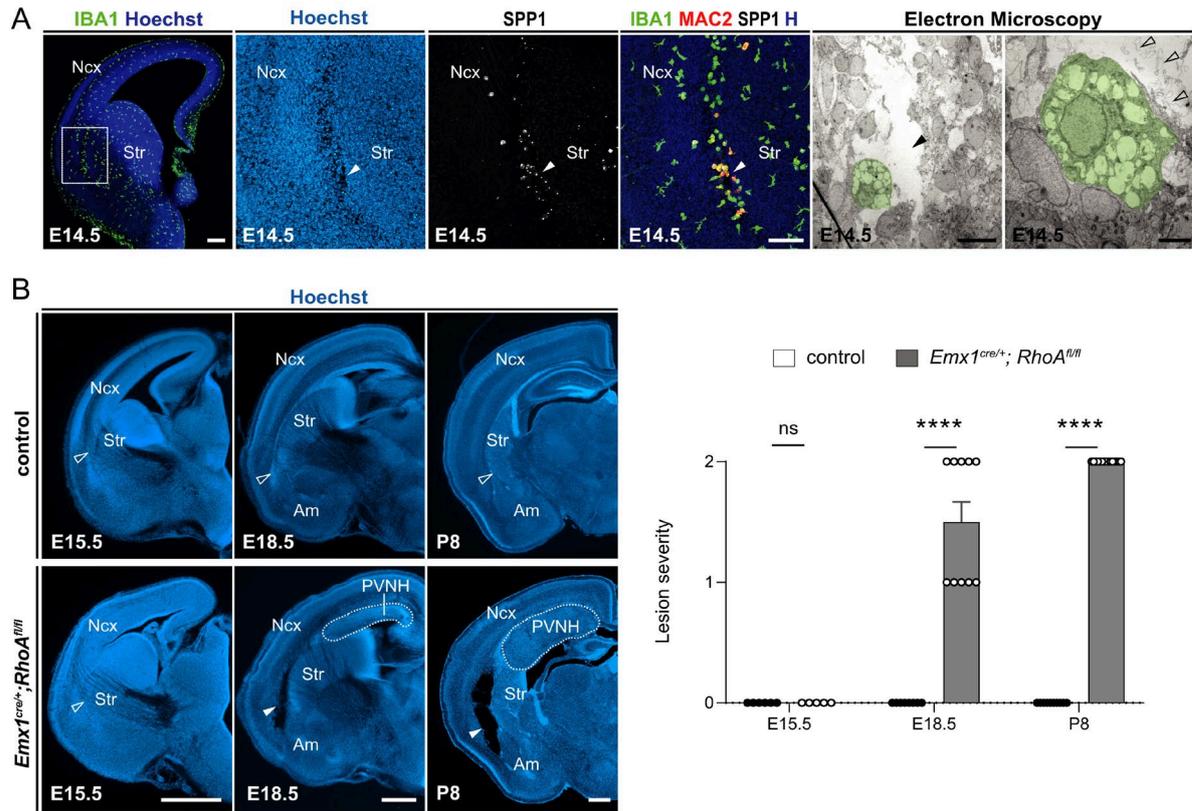


Figure S5. The CSA is a site of developmental morphogenetic fragility

(A) Immunostaining using IBA1, Spp1 and Mac2 antibodies and transmitted EM indicate that at E14.5, ATM line along microcavities visible in hoechst counterstaining and EM (solid arrowheads) (n=6 for immunostaining and n=3 for EM, from at least two distinct litters). **(B)** Coronal hemisections of control and *Emx1^{cre/+}; RhoA^{fl/fl}* brains, a model displaying a periventricular nodular heterotopia (PVNH), visible from E18.5 onwards (dotted lines). Hoechst counterstaining enables to detect no lesion at the CSA of controls and mutants at E15.5 (open arrowheads), a striking CSA lesion in 50% of the mutants (solid arrowheads), followed by a 100% of mutants at P7 (solid arrowheads) (n_{controlsE15}=6, n_{heterotopiaE15}=5, n_{controlsE18}=8, n_{heterotopiaE18}=10, n_{controlsP7}=10, n_{heterotopiaP7}=15). Values are presented as means ± SEM and statistical tests performed are Mann-Whitney U Tests. ***p<0.0001. Scale bars equal 200 μm (A low

magnification), 100 μm (A high magnification), 10 μm in EM low magnification and 2.5 μm in EM high magnification. CSA, cortico-striato-amygdalar boundary; Htp, heterotopia; Ncx, neocortex; Str, striatum.

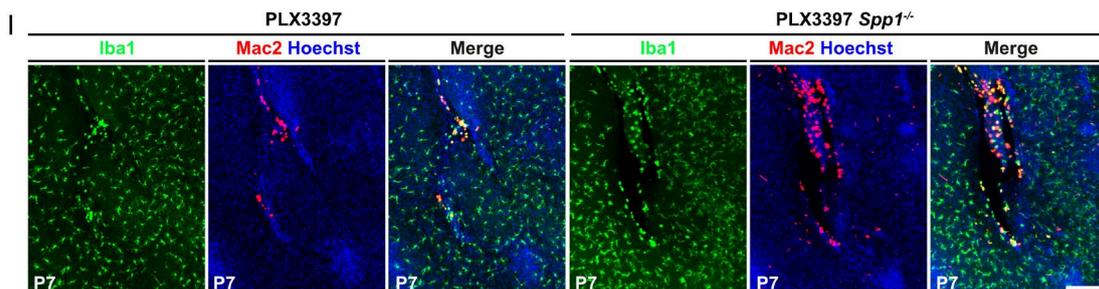
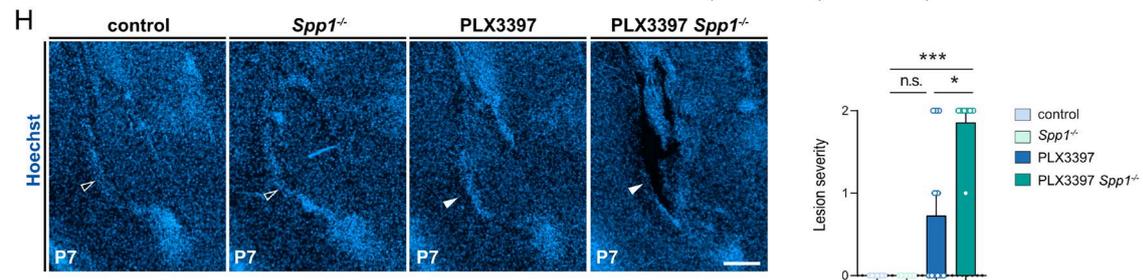
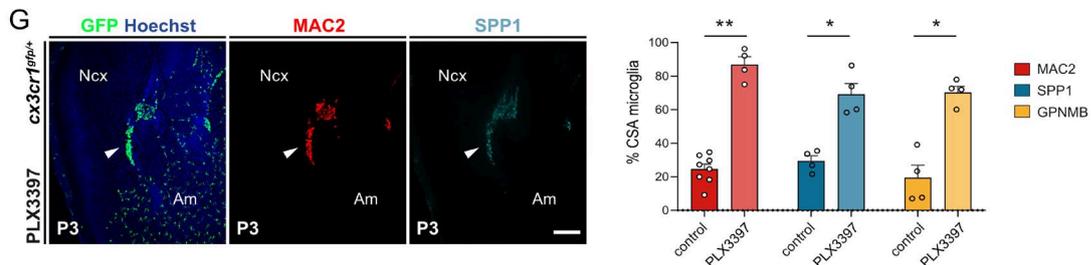
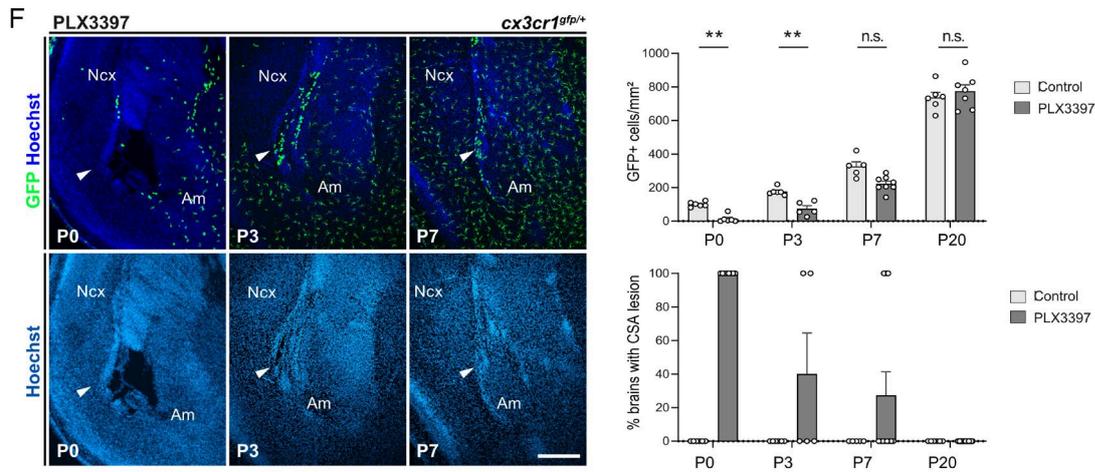
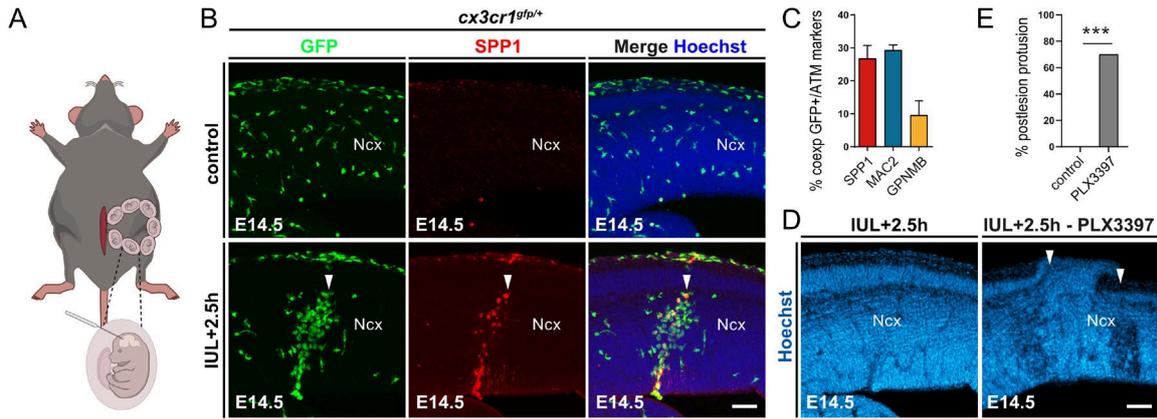


Figure 4. ATM are induced by mechanical damage and involved in lesion repair

(A) Schematic representation of the *in utero* lesion (IUL) procedure mechanically induced by poking the neocortex with a glass capillary. **(B)** Coronal sections through the E14.5 neocortex of control and IUL embryos collected 2.5 hours after the lesion, showing amoeboid *Cx3cr1^{gfp}*-positive cells accumulating at the lesion site and co-expressing *Spp1* and *Mac2* (solid arrowheads), whereas *Cx3cr1^{gfp}*-positive cells are dispersed in controls and do not express ATM markers ($n_{\text{controls}}=3$, $n_{\text{IUL}}=3$). **(C)** Quantification of the percentage of *Cx3cr1^{gfp}*-positive cells co-expressing ATM markers ($n_{\text{controls}}=3$, $n_{\text{IUL}}=3$). **(D)** Coronal sections after IUL in control and PLX3397-treated E14.5 embryos showing an altered morphology (bulge, solid arrowheads) in the absence of microglia. **(E)** Quantification of the morphological defects observed in control and PLX3397-treated embryos ($n_{\text{IUL}}=17$; $n_{\text{IUL-PLX3397}}=13$). **(F)** Coronal sections showing the CSA region (solid arrowheads) in PLX3397-treated embryos during postnatal microglial repopulation at postnatal day (P) 0, P3, P7 and P20. Cavitory lesions progressively resorbed, concomitantly a repopulation by *Cx3cr1^{gfp}*-positive cells which accumulate at the lesion closure site (solid arrowhead) ($n_{\text{controls-P0}}=6$; $n_{\text{PLX3397-P0}}=6$; $n_{\text{controls-P3}}=6$; $n_{\text{PLX3397-P3}}=5$; $n_{\text{controls-P7}}=5$; $n_{\text{PLX3397-P7}}=8$; $n_{\text{controls-P20}}=7$; $n_{\text{PLX3397-P20}}=7$). **(G)** *Cx3cr1^{gfp}*-positive cells accumulating at the lesion closure site co-express ATM markers *Spp1*, *Mac2* and *GPNMB*, as shown and quantified at P3, more specifically in PLX3397-treated embryos compared to controls ($n_{\text{controls}}=4$; $n_{\text{PLX3397}}=4$). **(H)** In contrast to controls, *Spp1^{-/-}* mutants, PLX3397-treated controls which show no CSA lesion by P20, PLX3397-treated *Spp1^{-/-}* mutants reproducibly displayed lesions visible by hoechst staining ($n_{\text{controls}}=7$; $n_{\text{Spp1KO}}=8$; $n_{\text{control-PLX3397}}=11$; $n_{\text{Spp1KO-PLX3397}}=7$). **(I)** While few CSA IBA1-positive cells co-express *Mac2* in resorbed PLX3397-treated controls at P7, they accumulate around the lesions in PLX3397-treated *Spp1* mutants,

indicating that *Spp1* inactivation does not prevent the expression of ATM markers ($n_{\text{control-PLX3397}}=3$; $n_{\text{Spp1KO-PLX3397}}=7$). Values are presented as means \pm SEM, and statistical tests performed are Fisher's exact test (E) and Mann-Whitney U Tests (F-H). * $p<0.05$; ** $p<0.001$; *** $p<0.0001$. Scale bars equal 100 μm (B,D), 200 μm (F-G) and 150 μm (H-I).

Am, Amygdala; CSA, cortico-striato-amygdalar boundary; IUM, *in utero* lesion; Ncx, neocortex;

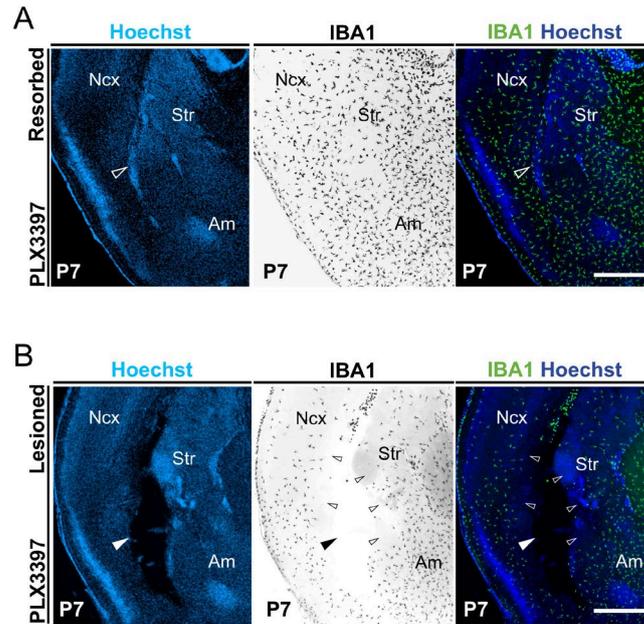


Fig S6. Repair correlates with repopulation at the CSA and CSB

Representative examples showing that **(A)** in most PLX3397-treated pups, repopulation triggers to lesion closure and a “resorbed” phenotype at the CSA by P7 (open arrowheads). **(B)** By contrast, the cases in which the resorption did not occur and were still “lesioned” at P7 (solid arrowheads) correlate with a lack of local CSA repopulation (smaller arrows indicate lack of repopulated microglia at the edges of the lesion) ($n_{\text{Resorbed}}=8$; $n_{\text{Lesioned}}=3$). Scale bars equal 500 μm .

Am, Amygdala; Ncx, neocortex; Str, striatum.

MOVIE LEGENDS

Movie S1. This movie shows a rostrocaudal progression through a E16.5 control hemibrain cleared by iDisco, imaged on a light-sheet microscope and reconstructed using the Imaris software, showing a normal morphology at the CSA and CSB (open arrowheads). The movie progresses through light sheet photographs acquired along the coronal rostrocaudal axis of the sample at a 3 μm Z step size.

Movie S2. This movie shows a rostrocaudal progression through a AFS E16.5 hemibrain cleared by iDisco, imaged on a light-sheet microscope and reconstructed using the Imaris software, showing the cavitory lesion at the CSA (solid arrowheads). The movie progresses through light sheet photographs acquired along the coronal rostrocaudal axis of the sample at a 3 μm Z step size.

Movie S3. This movie shows a rostrocaudal progression through a PLX E16.5 hemibrain cleared by iDisco, imaged on a light-sheet microscope and reconstructed using the Imaris software, showing the cavitory lesion at the CSA (solid arrowheads). The movie progresses through light sheet photographs acquired along the coronal rostrocaudal axis of the sample at a 3 μm Z step size.

Movie S4. This movie shows a rostrocaudal progression through a *Pu.1^{-/-}* E16.5 hemibrain cleared by iDisco, imaged on a light-sheet microscope and reconstructed using the Imaris software, showing the cavitory lesion at the CSA (solid arrowheads).

The movie progresses through light sheet photographs acquired along the coronal rostrocaudal axis of the sample at a 3 μm Z step size.

Movie S5. This movie shows a lateral rotation of a AFS E16.5 hemibrain cleared by iDisco, imaged on a light-sheet microscope and reconstructed using the Imaris software, showing cavitory lesion at the CSA (yellow indicates the reconstructed cavity). Green signal encompasses non-depleted CX3CR1-GFP+ cells and tissue autofluorescence delineating hemibrain morphology. Cavitory lesion was reconstructed and pseudocolored in yellow in Imaris software.

Movie S6. This movie shows a lateral rotation of a PLX E16.5 hemibrain cleared by iDisco, imaged on a light-sheet microscope and reconstructed using the Imaris software, showing cavitory lesion at the CSA (yellow indicates the reconstructed cavity). Green signal encompasses tissue autofluorescence delineating hemibrain morphology. Cavitory lesion was reconstructed and pseudocolored in yellow in Imaris software.

Movie S7. This movie shows a lateral rotation of a *Pu.1*^{-/-} E16.5 hemibrain cleared by iDisco, imaged on a light-sheet microscope and reconstructed using the Imaris software, showing cavitory lesion at the CSA (yellow indicates the reconstructed cavity). Grey signal encompasses tissue autofluorescence delineating hemibrain morphology. Cavitory lesion was reconstructed and pseudocolored in yellow in Imaris software.

RESUMÉ FRANCAIS: ARTICLE 1

Titre:

La microglie maintient l'intégrité du cerveau pendant la morphogenèse embryonnaire

Résumé:

La microglie, macrophage résidant dans le cerveau, existe dans différents états dans des contextes pathologiques et en conditions physiologiques durant la vie postnatale, mais son rôle prénatal reste mal compris. Dans cette étude, nous avons identifié un état embryonnaire similaire à la microglie postnatale Axon-Tract-associated-Microglia (ATM), qui s'accumule transitoirement aux frontières cortico-striato-amygdaliennes et cortico-septales du fœtus, deux sites de tension morphogénétique. Nous avons montré que la microglie embryonnaire et le facteur ATM Spp1/Osteopontin maintiennent l'intégrité des tissus à ces deux frontières en empêchant la formation de lésions cavitaires. La structure où se situent les ATM présente des microcavités et une fragilité morphogénétique développementale, ce qui suggère que les ATM maintiennent la cohésion du tissu en favorisant la réparation. De manière cohérente, nous avons trouvé que les facteurs spécifiques à l'état cellulaires des ATM sont rapidement induits par des dommages mécaniques, et que la repopulation microgliale ainsi que Spp1/OPN sont nécessaires pour la résorption efficace des lésions larges, mettant en évidence des capacités de réparation remarquables. Dans l'ensemble, notre étude montre que la microglie embryonnaire et les facteurs ATM favorisant la réparation jouent un rôle nouveau dans le maintien de l'intégrité des tissus au cours de la morphogenèse normale, ce qui a des implications majeures pour notre compréhension du câblage du cerveau en contextes physiologiques ainsi que pathologiques.

DISCUSSION AND PERSPECTIVES

1. A new role for microglia in maintenance of tissue integrity during embryogenesis

1.1 A new microglia-specific role

We have unveiled a new microglial function, in preserving tissue integrity and cohesion, which had not been previously shown in the embryo (Figure 19). Absence of embryonic microglia in the embryonic brain leads to cavitory lesions at the cortico-striato-amygdalar (CSA) and cortico-septal (CSB) boundaries, two sites of morphogenetic stress and stereotypical developmental accumulation of microglia. A central question in microglial and CNS-associated biology is the identification of unique functions that results from the intrinsic differences between these different macrophages. Indeed, parenchymal microglia are distinct from their BAM counterparts such as LYVE1+ perivascular macrophages or choroid plexus (Utz et al. 2020; Masuda et al. 2019; Prinz et al. 2021b; Masuda et al. 2022). This novel function of developmental microglia coincides with a developmental window where microglia and some other border associated macrophages (BAMs) are the sole glial cells, since generation of astrocytes and oligodendrocytes, which could otherwise have redundant roles, begins at the end of gestation and in early postnatal life (Jäkel and Dimou 2017; Thion and Garel 2017). We thus sought to ensure that the role that we define here is really specific to microglia and not BAMs.

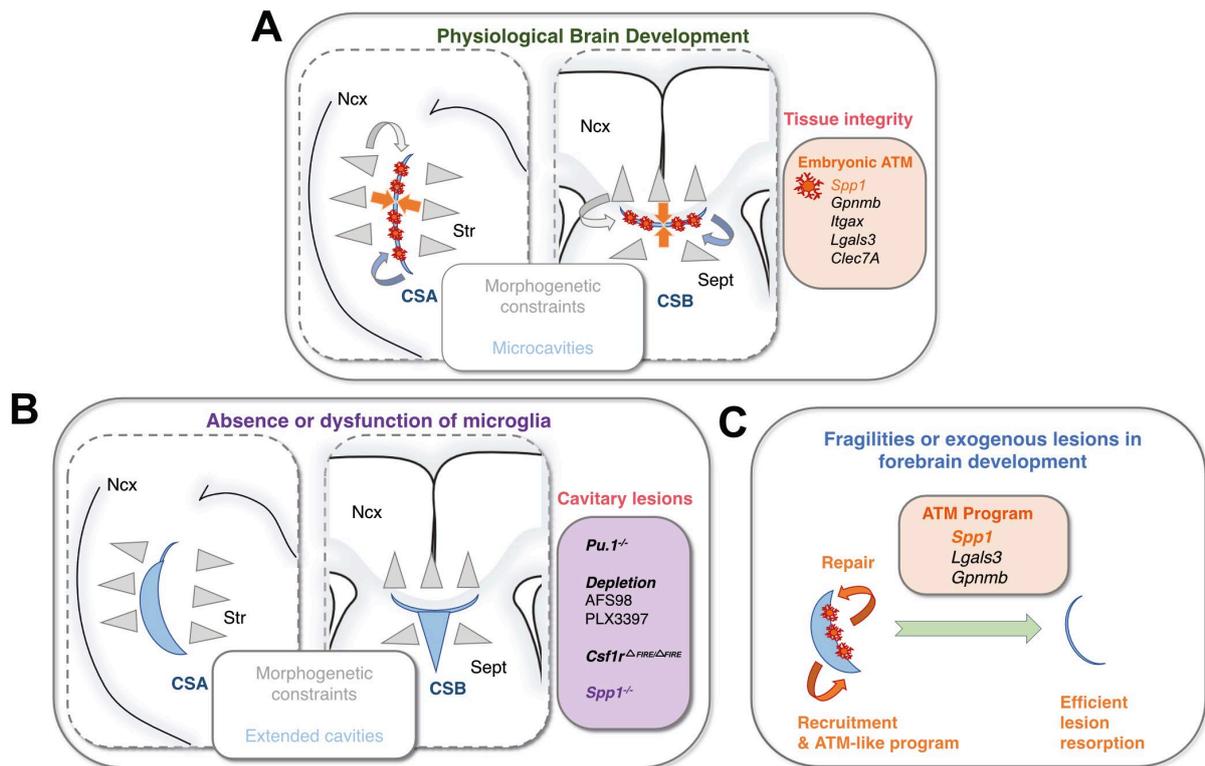


Figure 19. Microglia preserve brain integrity and mediate repair during forebrain development. (A) Microglia displaying an embryonic ATM state accumulate at two sites of morphogenetic constraints bearing microcavities, the CSA and CSB during embryogenesis. Microglia could sense and be recruited by mechanical cues or damage signals at microcavities in order to mitigate exacerbation of local tissue fragility. This function is partially dependent on core-ATM protein SPP1. (B) In the absence of embryonic microglia or upon constitutive ablation of SPP1, microcavities are exacerbated into large lesions. (C) In the context of embryogenic and early postnatal development, microglia can be recruited by fragilities for maintenance of brain integrity, but also by exogenous lesions such as a mechanical poke or CSA lesion. Recruited cells express an ATM program associated with repair. Notably, repair efficiency is dependent on expression of core-ATM protein SPP1 at the CSA boundary.

1.1.1 Specificity of models for investigating roles of embryonic microglia

Most commonly used models to investigate microglia also affect BAMs, including some models used in the present work (Eme-Scolan and Dando 2020). We have identified cavitory lesions at the CSA and CSB in complete absence of microglia or in depletion models where BAMs are also depleted, as exemplified by *Pu.1*^{-/-} mice, where myeloid cells are completely absent (Erblich et al. 2011). We further have investigated models of CSF1-R pharmacological inhibition through injection of AFS98 antibody or supplementation of pregnant dams food pellets with PLX3397 inhibitor (Thion et al., 2018a). Both these models lead to partial depletion of microglia and macrophages, whose survival and differentiation is dependent on this pathway. Indeed, we have observed full depletion of microglia at the CSA in both models, but only partial depletion at the CSB. In addition, we observed some CSF1-R inhibition resistant macrophages in the meninges and an intriguing pool of resistant microglia in the MGE mantle (Article 1 Figure S3). However, we were able to demonstrate the CSA/CSB phenotype in *Csf1r*^{ΔFIRE/ΔFIRE} whereby a mutation in CSF1-R was reported to lead exclusively to absence of parenchymal microglia during development and postnatal life, suggesting the specificity of this model to microglial depletion (Rojo et al. 2019). However, this model must be treated with caution, since more recently, Munro and colleagues demonstrated that subsets of BAMs may also be misgenerated in the context of this mutation (Munro et al. 2020). However, we confirmed that the CSA is a site that normally bears accumulation of exclusively *bona fide* P2RY12+ microglia (Masuda et al. 2022), with no contribution of other BAMs IBA1+ CX3CR1-GFP+ macrophages, such as LYVE1+ perivascular and meningeal macrophages (Masuda et al. 2022; Prinz et al. 2021b; Utz et al. 2020). In the case of the CSB accumulation at E18.5, we observed a vast majority of P2RY12 low IBA1+ or CX3CR1-GFP+ microglia (data not shown), suggesting an alternative state of the microglia accumulated there resembling RMS/SVZ neurogenesis supportive adult microglia (Xavier et al. 2015). In addition, we observed just a handful of LYVE1+ IBA1+ or CX3CR1-GFP+ meningeal macrophages (data not shown) but the paucity of these cells makes it unlikely that they modulate brain tissue integrity at this site. In the light of these findings, we have attempted to deplete microglia specifically during embryogenesis using *P2ry12*^{cre}; DTA mice, in order to selectively deplete microglia through diphtheria toxin induced cell death but reporter expression was not sufficient to induce microglial depletion (Lee et al. 1998)(data not shown). Further experiments using microglia- specific binary cre lines

should be performed in order to clarify this matter (Eme-Scolan and Dando 2020; Kim et al. 2021).

1.1.2 Microglial repair function may be postnatally compensated

Furthermore, we show that postnatal CSA and CSB lesion repair after embryonic microglia depletion is concomitant with microglia repopulation and repair-related properties (an ATM-like profile further discussed in 2.1) but we cannot exclude the possibility that other cells participate in postnatal repair and have the capacity to perform a redundant role in the postnatal brain. To the best of our knowledge, no such lesions as the ones reported in Article 1 were described in condition of acute macrophage depletion in the adult brain, nor did we observe them in the adult *Csf1r^{ΔFIRE/ΔFIRE}* mouse brain (Figure 20), suggesting that other cells, which could be BAMs, recruited immune cells or neural and glial cells can take over the role for microglia in maintaining tissue cohesion, which is indispensable in the early developing brain (Gaudet and Fonken 2018; An et al. 2021; Ghaddar et al. 2021). We have not fully defined the timing of resorption of the lesion in *Csf1r^{ΔFIRE/ΔFIRE}* mouse brain, nor the events occurring during resorption. This possibility should thus be further investigated, as it points to a non-redundant function of microglia in embryonic development, which may become compensated for by other cells in postnatal life.

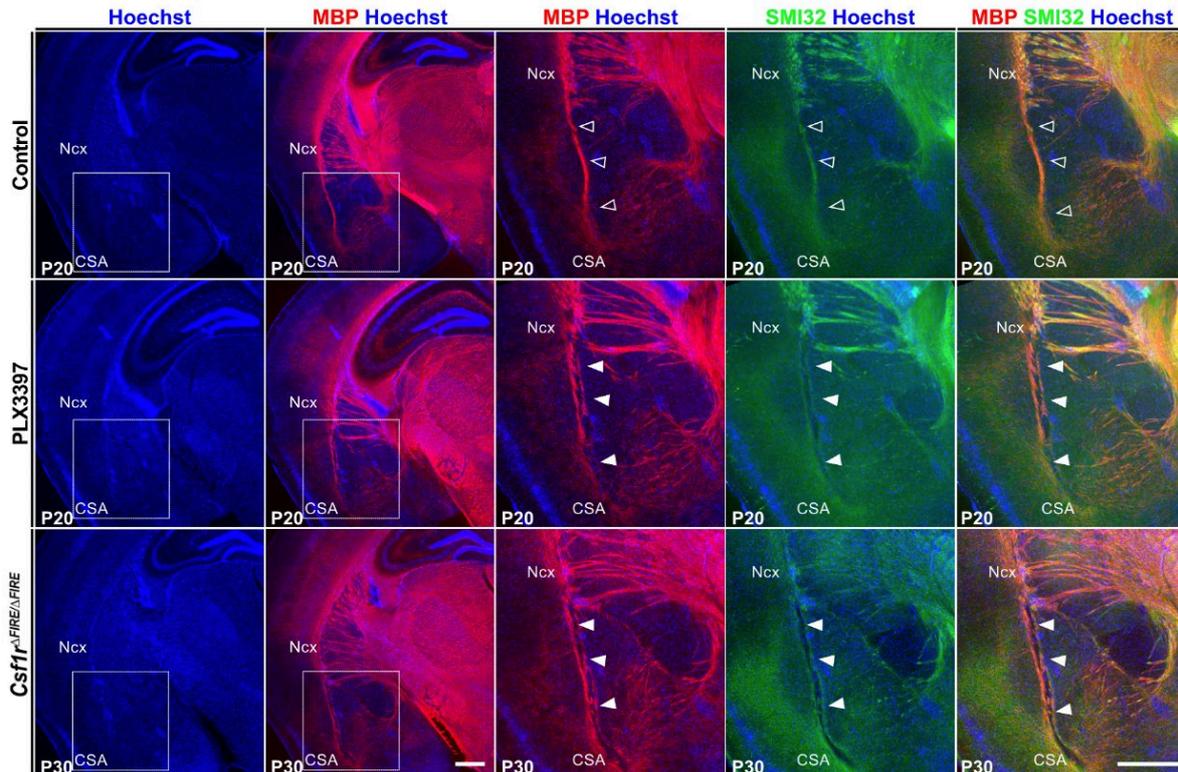


Figure 20. CSA lesions systematically resorb in embryonic depleted PLX3397 and microglia-devoid *Csf1*^{ΔFIRE/ΔFIRE} but lead to abnormalities in abutting fibers. Gross morphology resembles control brains with no signs of lesion in nuclear Hoechst staining in depleted PLX3397 and microglia-devoid *Csf1*^{ΔFIRE/ΔFIRE} respectively at P20 and P30. However, Myelin marker MBP and axonal marker SMI32 indicate that external capsule fibers seem to cluster on the edges of a scar-like space at the CSA abutting the basolateral amygdala.

1.2 Microglial lesion-preventive role is independent of classical pathways but involves ECM protein SPP1/OPN

Moreover, we showed that this microglial role in preserving tissue integrity is independent of classical microglial pathways implicated in phagocytosis, microglial sensing of cell death, tissue damage and injury, or synaptic pruning such as DAP12 (TYROBP)/TREM2, CR3 and CX3CR1 (Augusto-Oliveira et al. 2022; Faust, Gunner, and Schafer 2021; Colonna and Butovsky 2017), or even maternal immune activation driven perturbation of microglial function (Smolders et al. 2018) presented in chapter 1. Indeed, no CSA or CSB lesions were observed in these conditions (Article Figure S3). Of note, these pathways have been implicated numerous developmental functions of microglia from modulation of interneuron positioning, dying cells phagocytosis to synaptic pruning in prenatal and postnatal development (described in 2.2.2). In contrast, we showed that SPP1/OPN, an extracellular matrix protein which was highly expressed by microglia at embryonic hotspots of accumulations, is required for tissue integrity maintenance, since complete invalidation of *Spp1* led to increased lesioning at the CSA at E14.5 and the CSB at E18.5, compared to natural fragilities in control brains (Article Figure 2). We observed no phenotype in full knockouts for *Gpnmb* coding for GPNMB/Osteoactivin, another ECM modulator (Article 1 Figure S4). Of note, the defects observed in *Spp1*^{-/-}, were partial and, when using full knockout mouse models for *Spp1* and *Gpnmb* we cannot exclude compensatory mechanisms to account for the loss of gene expression (Liaw et al. 1998; El-Brolosy and Stainier 2017; El-Brolosy et al. 2019; Rouf et al. 2022). Furthermore, although expression of SPP1/OPN was strongest in microglia, we could not exclude that it was expressed in other forms by other cells, such as radial glia in the vicinity of the CSA/CSB. It will thus be of interest to use macrophage/microglia specific genetic mouse lines such as *Cx3cr1*^{CreERT2}, *P2ry12*^{CreERT2} for targeted conditional invalidation of floxed *Spp1* and *Gpnmb* alleles (M. L. Bennett et al. 2016; Eme-Scolan and Dando 2020), in order to check for specificity of these pathways in microglial maintenance of tissue integrity and repair but also to bypass compensatory genetic robustness. Conditional microglia specific knockouts for *Spp1* may thus display a more drastic phenotype with increased fragilities at the CSA/CSB more closely resembling that in conditions of microglial depletion, as is the case for *Gpnmb* conditional knockout. Thus, to the same extent that the specificity of microglia involvement in maintenance of brain integrity and repair during morphogenesis, it will be required to show specificity of the discovered SPP1/OPN pathway to this function (addressed in 2.2.2).

2. An ATM-like profile associated with tissue repair and cohesion

2.1 Similarities and differences of embryonic ATM-like cells with postnatal ATM and DAMs

We showed that in normal physiology, at the sites where we observe lesions in absence of embryonic microglia, there are microglia that display specific characteristics of postnatal ATM/PAM, consistent with reports of ATM-like clusters in the embryonic brain reported by (La Manno et al. 2021; Masuda et al. 2020). To a certain extent, these microglia were also related to disease-associated microglia (DAMs) (Deczkowska et al. 2018a). DAMs are a state of microglia associated with neurodegeneration patterns, being able to sense damage signals, and are capable of clearing or refraining progression of pathological tissue in neurogenerative diseases (Deczkowska et al. 2018a). On the other hand, postnatal ATM of the developing white matter regulate oligodendrogenesis through trophic secretion of IGF-1 encoded by core ATM gene *Igf1* and via phagocytosis of OPCs (Benmamar-Badel, Owens, and Wlodarczyk 2020). Importantly, it was shown that the full acquisition of the DAM state is dependent on APOE/TREM2/DAP12(TYROBP) while postnatal ATM fate was independent of these signaling pathways (Deczkowska et al. 2018a; Li et al. 2019; Masuda et al. 2020).

We validated through immunohistochemistry that the cells at the CSA and CSB express core ATM molecules SPP1/OPN, GPNMB, CLEC7A, CD11c and MAC2 (*Lgals3*) for which coding genes were showed to be top upregulated genes in DAM and ATM (Deczkowska et al. 2018a; Masuda et al. 2020). These seemingly similar microglial profiles can thus drive different functions and be dependent on distinct pathways. Therefore, the question of how these profiles relate to ATM-like embryonic microglia is important. Our characterization of ATM-like embryonic is not exhaustive as it was based on a given set of core ATM gene encoded molecular marker

expression rather than transcriptional profiling in the case of ATM/DAM (Masuda et al. 2020). In addition, we have not verified protein expression of IGF-1 or possible involvement of this pathway in the function CSA/CSB microglia reported here whereas it was implicated in regulation oligodendrogenesis and myelination by postnatal ATM (Benmamar-Badel, Owens, and Wlodarczyk 2020) and in wound healing in other contexts (Garoufalia et al. 2021). Furthermore, we showed lack of cavitory lesion phenotype in DAP12/TYROBP mutants, which suggests that, like postnatal ATM, this pathway, and TREM2 co-receptor are likely not involved in the function of ATM-like embryonic microglia. However, the implication of APOE remains to be investigated, as it is involved in induction of the DAM state and lipid metabolism, which may be altered in lipid droplet filled embryonic ATM as we have observed them by electron microscopy (Deczkowska et al. 2018b).

Furthermore, the markers that were used to identify ATM-like embryonic microglia did not fully overlap and their onset of expression differed temporally during embryonic development or in *in utero* lesion conditions. SPP1 expression was larger at the CSA at E14.5 (data not shown) and more readily elicited by mechanical wound in the embryonic cortex (Article 1 Figure 4). This strengthens the notion that microglia possess a dynamic profile with likely rapid changes in state and expression of ATM genes and uncovered pathways. It will be of interest to isolate specifically ATM-like cells from the developing mouse forebrain structures where we localized them, use a gating strategy based on SPP1 expression to sort them, and perform highly sensitive RNA sequencing techniques to identify and define their transcriptional profile. It would further be relevant to explore the putative properties that distinguish them from postnatal DAM and ATM (Stark, Grzelak, and Hadfield 2019). A similar experiment has been done in human fetal tissue ATM-like cells have been transcriptionally identified through RNAseq of and primary human microglia cultures (Kracht et al. 2020; Popova et al. 2021) but not localized in human development. We showed for the first time that the ATM-like profile observed at the mouse CSA can be found specifically at an equivalent location during human brain development, raising the intriguing possibility that a microglial state with a conserved function is required during human brain development (Article 1 Figure 1 and S1). It is plausible that sites like the CSA require preserved tissue integrity in humans like in rodents as lesions have been reported at

the site of the CSA in human fetal brains, albeit authors suggested these may be transitory blood vessels (González-Arnay, González-Gómez, and Meyer 2017).

2.2 Mechanisms for repair and maintenance of tissue

integrity of CSA/CSB and injury induced ATM-like cells

2.2.1 Microglial brain glue

Aside from *Igf1* these ATM-like microglia are characterized by the expression of genes that code for proteins involved in cell adhesion like *Itgax*, (coding for CD11c integrin), tissue repair, and ECM components like *Spp1* and *Gpnmb* (Rosmus et al. 2022; Wlodarczyk et al. 2017). These emerging properties suggest that they might act as “gluing” agents capable of maintaining tissue cohesion between detaching structures by a) serving as anchors for cells at either side of CSA/CSB lesions, b) by performing junction by producing ECM bridges between these structures. Conversely, microglia could modulate ECM plasticity by degrading ECM and scar tissue and could contribute in promoting tensile flexibility in an otherwise stiffened tissue exposed to tears during embryogenesis (Hankin, Schneider, and Silver 1988; Van Essen 2020; Saito et al. 2019).

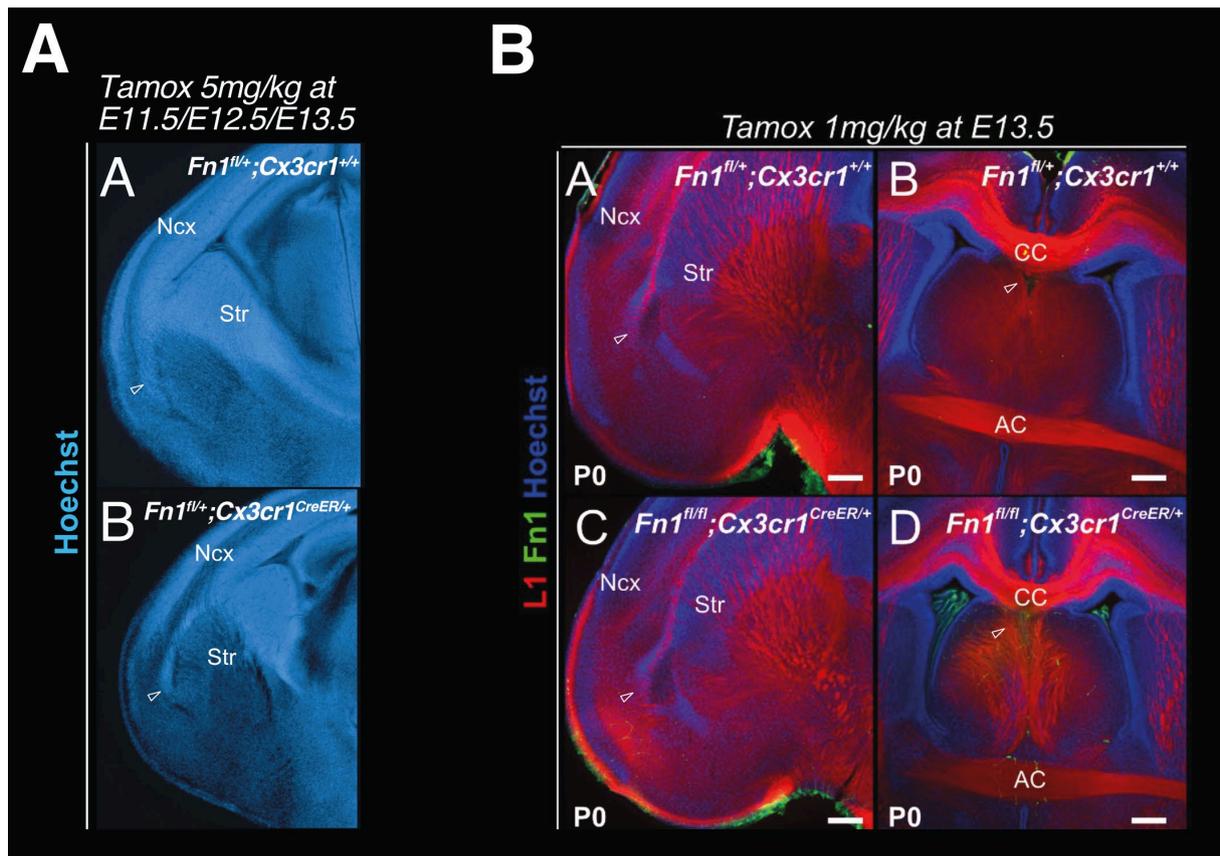


Figure 21. Microglia specific inactivation of Fibronectin1 (Fn1) does not impair in tissue integrity at the CSA and CSB. (A) High dose of tamoxifen to induce inactivation of Fn1 does not lead to impairment at the CSA at E15.0. **(B)** Standard dose of tamoxifen to induce inactivation of Fn1 does not lead to impairment at the CSA and CSB at P0.

The putative repair function of ATM-like embryonic is reminiscent of the recently reported ability of early postnatal microglia to participate in scar free wound healing in spinal cord crush injury (Li et al. 2020). The profile associated with postnatal spinal cord repair also transcriptionally resembled white matter ATM/WAM/PAMs and could be localized in the form of SPP1/OPN expressing microglia around the lesion, similar to what we report in our paradigms. These postnatal spinal cord repair microglia were shown to bridge lesion partly through secretion of fibronectin (Fn1) (Li et al., 2020). We have tried modulating Fn1 expression specifically in microglia during development, but found no evidence of such a role at the CSA/CSB (data not shown). It remains to be determined whether ATM-like microglia also exert their repair function by secreting ECM molecules to bridge lesions at the CSA and the CSB.

2.2.2 Contribution of SPP1 in tissue repair and modulation of microglial state

As previously stated, several lines of evidence suggest that SPP1/OPN plays a role in tissue repair and microglia biology (Rosmus et al. 2022). SPP1/OPN is a multifunctional glycoprotein that can be secreted or expressed on the surface cells (Icer and Gezmen-Karadag 2018). It is an extracellular matrix protein first described in bone tissue where it takes a role in biomineralization (Icer and Gezmen-Karadag 2018). It has been shown to be involved in wound healing, and immune cell migration, adhesion to the matrix, survival and maturation during inflammation (Icer and Gezmen-Karadag 2018). SPP1/OPN is upregulated and has been involved in brain injury and degenerative disease (Rosmus et al. 2022). SPP1/OPN is upregulated at wound site as early as 6 hours after skin incision and sciatic nerve injury in adult mice but also in microglia at 2 days post injury in spinal cord crush injury in neonatal mice, reminiscent of induction of SPP1 we report in the embryonic cortex at 2 hours 30 after injury (Liaw et al. 1998; Yi Li et al. 2020; X. Liu et al. 2017). In addition, *Spp1*^{-/-} mice display ultrastructural defects in skin wounding, particularly ECM disorganization (Liaw et al. 1998) as well as increased damage in injury such as grey and white matter loss in hypoxic-ischemic injury (van Velthoven et al. 2011).

Whereas secreted or membrane surface microglial SPP1 could directly serve as an anchor protein between microglia and ECM and/or neurons and ECM to maintain tissue cohesion, several groups point to the fact that it also constitutes a signal for recruitment of immune and glial cells at injury for repair in the adult brain (An et al. 2021; Riew et al. 2019; Rosmus et al. 2022). In the injured striatum, dying neurons release SPP1/OPN to recruit monocytes and astrocytes for repair and as a trophic signal for surrounding neural cells (An et al. 2021; Riew et al. 2019). Furthermore, it has been proposed that SPP1 acts as an opsonin to recruit microglia/macrophages for phagocytosis of cell debris and pathogens in disease and injury such as Alzheimer's disease (McKee and Nanci, 1996; Shin et al., 2011; Morisaki et al., 2016; Rentsendorj et al., 2018; De Schepper et al., 2022).

At the embryonic CSA/CSB, where microglia are the only immune or glial cells present, unidentified damage signals could elicit microglial expression of SPP1/OPN to further

recruit and prime other microglia for repair of microlesions. This is consistent with Shen and colleagues' demonstration that SPP1 is involved in promoting the CD11c⁺ or ATM state in early postnatal life upon uptake of apoptotic cells (Shen et al. 2022). In our study, however, we still observed expression of other ATM markers (MAC2 and GPNMB) at the CSA/CSB of *Spp1*^{-/-} mice, suggesting that the acquisition of this microglial state is not fully dependent on SPP1/OPN. Nonetheless, it raises the question of whether local apoptosis or other forms of programmed cell death might be involved in the induction of the ATM phenotype. This hypothesis would be consistent with fact that the CSB is a site of massive cell death and microglial phagocytosis in the developing forebrain (Hankin, Schneider, and Silver, 1988 and data not shown). In contrast, we found no sign of apoptotic cell death at the CSA (data not shown), and while ATM-like microglia display an elevated engulfment capacity (Article Figure S1), we never observed the engulfment of whole cells or nuclei, nor the formation of phagocytic cups (data not shown). While other forms of cell death beyond apoptosis could be investigated at this hotspot, it is likely that ATM-microglia located at the CSA rather engulf debris but not whole cells. Interestingly, EM analyses (Article 1) revealed the presence of "lipid" fragments inside cavities normally observed at the CSA, raising the possibility that the ATM-like profile might induced by forms of cell death at this site and this question needs to be addressed in future studies.

3. Lessons from this novel identified role of microglia for general brain morphogenesis and pathology

We showed that sites of developmental fragility at CSA and CSB that are sensitive to the absence microglia during development. The sensitivity of these hotspots during development point to a phenomenon that could be more widespread and stereotypical in different species. Specifically, this observation prompts the interrogation as to what are the microenvironmental events happening which lead to this susceptibility to cavitory lesion and whether they can be explained by morphogenetic events that require regulation by immune cells during (fore)brain development. Indeed, the forebrain is the stage for multiple, sometimes contradictory, morphogenetic forces during development, which can put certain regions under stress and render them vulnerable to lesions (Hankin, Schneider, and Silver, 1988; Van Essen, 2020; Saito et al., 2019).

We found that the CSA is a site that is particularly sensitive and fragile, as demonstrated by the systematic occurrence of lesions correlated with periventricular nodular heterotopia in *Emx1^{cre/+}; RhoA^{fl/fl}* conditional mutants (Cappello et al. 2012) (Article1 Figure S5). The occurrence of lesions in these mutants only correlated with the morphological changes induced by the heterotopia, since no lesions could be observed at the CSA in the absence of heterotopia at E15.5, before systematically appearing as large CSA tears as the heterotopia grew with age (E18.5-P7) (Article 1 Figure S5). This is consistent with reports of hydrocephalic brains where we can observe large tears at the CSA, and sometimes CSB in adult brains (Cao and Wu, 2015; Haward et al., 2020; Ohtoshi, 2008; Xu et al., 2011; Zou et al., 2020). This reinforces the notion that the CSA and CSB as sites that are sensitive to mechanical disruptions, even beyond development. In these cases, we cannot exclude that the disruptions are also elicited by disorganized radial glial organization, and the role of microglia in maintaining radial glial integrity and organization should be investigated. Evidence of lesion resembling CSA lesions in the mouse microglia devoid embryonic brain have been reported in human brains a gestational week 7 in a corresponding

region suggesting that the fragilities occurring in mice are also part of stereotypical human brain development (González-Arnay, González-Gómez, and Meyer, 2017). Furthermore, stereotypical lesion similar to those we report at the CSB in absence of embryonic microglia coined as septum cavum pellucidum are regularly observed in rat and human brains as part of typical development (Hankin, Schneider, and Silver 1988; Nagaraj, Calvo-Garcia, and Kline-Fath 2018). These naturally occurring lesions usually disappear in postnatal life, however in humans, the lack of resorption of such a developmental lesion is loosely associated with neurodevelopmental disorders such as schizophrenia, but its impact on brain functioning and contribution to disorders is still unclear (Das & Dossani, 2022; Trzesniak et al., 2011; Wang et al., 2020). This lesion largely affects the septum, an understudied structure of the brain associated with the limbic system (Sheehan, Chambers, and Russell 2004). We can thus speculate that affective symptoms of disorders such as schizophrenia and depression can find their roots in abnormalities in septal development (Sheehan, Chambers, and Russell 2004).

Furthermore, congenital infectious disorders such as Congenital Cytomegalovirus (CMV) display formation of cystic lesions at hotspots equivalent to CSA and CSB in the human brain, and are accompanied by disabilities (Chaudhari and Ho 2022; Teissier et al. 2014). These observations prompt the interrogation of the links between microglial perturbation in these models and possible consequences on morphology. Lastly, in the light of possible links with human pathology and similar affected regions in congenital disorders it would be of interest to investigate the long-term impact of lesions induced by absence of microglia in the mouse developing brain. Indeed, in our models of embryonic microglia depletion, even after lesion repair we have observed consequences on amygdalar shape (not shown) and amygdala associated axon-tracts (Figure 20). Behavioral testing to explore the consequences of these changes, such as amygdala related fear behavior should be pursued (Phelps and LeDoux 2005; Baxter and Croxson 2012). Further analysis must also be done in the context of the CSB which is a major midline hub of passing commissural axons such as the corpus callosum which could have an impact on cognition and be linked with neurodevelopmental disorders (Chédotal and Richards 2010; Chaudhari and Ho 2022).

Taken together, our findings highlight a new role for repair-associated microglial state during development in maintaining tissue integrity at sites of morphogenetic stress during forebrain development, which could inform the ways in which normal morphogenesis can be affected in human developmental disorders.

RÉSUMÉ FRANCAIS: DISCUSSION ET PERSPECTIVES

1. Un nouveau rôle pour la microglie dans le maintien de l'intégrité des tissus au cours de l'embryogenèse

Nous avons découvert une nouvelle fonction des microglies dans la préservation de l'intégrité tissulaire et de la cohésion, pas encore démontrée dans l'embryon. L'absence de microglies embryonnaires dans le cerveau embryonnaire entraîne des lésions cavitaires aux frontières corticales-striato-amygdaliennes (CSA) et cortico-septales (CSB), deux sites de tension morphogénétique et d'accumulation développementale stéréotypée des microglies. Cette nouvelle fonction des microglies développementales coïncide avec une fenêtre de développement où les microglies et les BAMs sont les seules cellules gliales, car la génération d'astrocytes et d'oligodendrocytes, qui pourraient par ailleurs avoir des rôles redondants, commence à la fin de la gestation et au début de la vie postnatale. Ainsi, nous avons cherché à nous assurer que le rôle que nous définissons ici est vraiment spécifique aux microglies et non aux microglies associées aux frontières.

Les modèles les plus couramment utilisés pour étudier les microglies affectent également les BAMs, y compris certains modèles utilisés dans cette étude. Nous avons identifié des lésions cavitaires au niveau de la CSA et de la CSB en l'absence totale de microglies ou dans des modèles de déplétion où les BAMs sont également déplétées. Cependant, nous avons pu démontrer le phénotype CSA/CSB dans *Csf1r^{ΔFIRE/ΔFIRE}*, où une mutation dans CSF1-R conduit exclusivement à l'absence de microglies parenchymateuses pendant le développement et la vie postnatale, suggérant la spécificité de ce modèle pour la déplétion microgliale. Nous avons également confirmé que l'accumulation à la CSA est un site qui porte normalement l'accumulation exclusivement de microglies P2RY12+ authentiques, avec aucune contribution d'autres macrophages associés aux frontières, comme les macrophages péri-vasculaires LYVE1+. Finalement, nous avons tenté de dépleter spécifiquement les microglies pendant l'embryogenèse en utilisant des souris *P2ry12^{cre}*; DTA, mais l'expression du rapporteur n'était pas suffisante pour induire la déplétion microgliale.

Aussi, nous avons observé que la réparation des lésions postnatales après la déplétion embryonnaire des microglies est associée à leur repopulation et à des propriétés de réparation. Cependant, d'autres cellules pourraient également contribuer à la réparation postnatale, suggérant un rôle non redondant des microglies dans le développement embryonnaire.

2. Le rôle préventif de la microglie à l'égard des lésions est indépendant des voies classiques mais implique la protéine ECM SPP1/OPN

Nous avons observé que des microglies présentant des caractéristiques spécifiques d'ATM/PAM postnatales sont présentes aux sites où nous observons des lésions en l'absence de microglies embryonnaires. Ces profils de microglies semblables peuvent entraîner des fonctions différentes et dépendre de voies distinctes, soulignant l'importance de comprendre leur relation avec les microglies embryonnaires de type ATM. Il est intéressant de noter que le profil ATM-like observé à la CSA chez la souris peut également être trouvé à un emplacement équivalent pendant le développement cérébral humain, suggérant une fonction conservée nécessaire au développement cérébral humain.

Outre *Igf1*, ces microglies de type ATM se caractérisent par l'expression de gènes codant pour des protéines impliquées dans l'adhésion cellulaire telles que *Itgax* (codant pour l'intégrine CD11c), la réparation tissulaire et les composants de la MEC comme *Spp1* et *Gpnmb*. Leur fonction putative de réparation rappelle la capacité récemment rapportée des microglies précoces postnatales à participer à une cicatrisation sans cicatrice lors d'une lésion de compression de la moelle épinière. Il reste à déterminer si les microglies de type ATM exercent également leur fonction de réparation en sécrétant des molécules de la MEC pour combler les lésions à la CSA et à la CSB.

Enfin, comme mentionné précédemment, plusieurs éléments indiquent que SPP1/OPN joue un rôle dans la réparation tissulaire et la biologie des microglies. Il est impliqué dans la cicatrisation des plaies, la migration des cellules immunitaires,

l'adhérence à la matrice, la survie et la maturation lors de l'inflammation. Dans notre étude, nous avons observé l'induction de SPP1 dans le cortex embryonnaire après une lésion, suggérant son rôle potentiel dans la réparation tissulaire embryonnaire. Toutefois, il reste à déterminer si les microglies de type ATM induites par SPP1/OPN sont impliquées dans la phagocytose des débris cellulaires, ce qui nécessitera des études futures pour approfondir cette question.

3. Leçons tirées de ce nouveau rôle identifié de la microglie dans la morphogenèse et la pathologie du cerveau en général

Nous avons observé que certaines régions du cerveau en développement, comme la CSA et la CSB, sont sensibles à l'absence de microglies, ce qui suggère un phénomène potentiellement répandu et stéréotypé chez différentes espèces. Ces sites de fragilité sont sujets à des lésions, comme démontré par la corrélation entre la nodularité hétérotopique périventriculaire et les lésions à la CSA chez les mutants conditionnels *Emx1^{cre/+}; RhoA^{fl/fl}*. Les lésions observées dans ces cas suggèrent que la CSA et la CSB sont sensibles aux perturbations mécaniques, même au-delà du développement, ce qui peut avoir des implications sur la morphogenèse normale et les troubles du développement humain.

ANNEXES

Article 2: Participation in a separate publication

*(Please note this is a draft of the article. Some modifications are still to be made
before submission)*

Contribution of the author of the thesis:

My contribution to this manuscript includes validation of the preterm birth model with the use of the TCA-GFP line that labels thalamocortical fibers that progressively cluster into barrels in the somatosensory in the first postnatal week. It was previously shown that premature birth one day before term accelerates cortical map formation via the accelerated reduction of serotonin (5-hydroxytryptamine, 5-HT) levels (Toda et al., 2013). I showed that preterm birth coherently accelerated clustering of thalamic fibers in at the barrels (Figure 2 and S2).

Secondly, I also took advantage of *Ebf1cKO* mutants that express TCA-GFP transgene by crossing them with TCA-GFP mice (Mizuno et al., 2014). This allowed us to track changes in fibers coverage and distribution in the somatosensory cortex. We used this approach to confirm that there is a reduction in cortical area and inappropriate barrel formation in the *Ebf1cKO* mutants as seen through fiber distribution (Figure 1 and S1). We further showed that preterm birth, PCPA and fluoxetine do not modify these parameters in control and mutant conditions (Figure S4).

A birth-regulated sensitive period enables cross-areal plasticity of thalamic axons

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Summary (148 words)

Primary areas of the neocortex receive sensory input through modality-specific thalamocortical axons (TCA). While TCA are oriented towards their targets during embryogenesis, they have a striking capacity to reorient postnatally to rescue an early mistargeting. How this remarkable adaptive process is regulated remains largely unknown. Here, using a mutant mouse model in which the embryonic TCA trajectory is shifted, we found that TCA rewiring occurs during a short postnatal time-window, preceded by a prenatal thalamic apoptosis, together leading to the formation of reduced primary sensory areas. Furthermore, we showed that TCA plasticity and subsequent sharpening of primary area border are impaired by preterm birth, which acts at the molecular level via regulation of serotonin. Our study defines a novel birth and serotonin-sensitive period that enables concerted adaptations of TCA to cortical areas with major implications for our understanding of brain wiring in physiological and preterm conditions.

INTRODUCTION

The mammalian cerebral cortex is divided into discrete functional areas, each characterized by its own stereotypical pattern of gene expression and cytoarchitecture, as well as reciprocal connectivity with specific thalamic nuclei. In particular, primary visual (V1) and somatosensory (S1) areas receive sensory input directly from the modality-specific dorsal lateral geniculate (dLGN) and the ventral posterior (VP) first order (FO) thalamic nuclei respectively (Cadwell et al., 2019; Caviness and Frost, 1980; Jabaudon, 2017; Jones, 2002; Nakagawa, 2019). Formation of these complex circuits relies on the orchestration of sequential embryonic and postnatal developmental processes. Indeed, during embryogenesis, thalamocortical axons (TCA) grow from specific FO thalamic nuclei and navigate through an intermediate target, the subpallium, in which TCA are organized and directed towards their presumptive primary areas (Backer et al., 2018; Bielle et al., 2011; Cunningham et al., 2022; Dupin et al., 2015; Erzurumlu and Gaspar, 2012; Lokmane and Garel, 2014; Lokmane et al., 2013; Lopez-Bendito, 2018; Molnar et al., 2012; Teissier and Pierani, 2021). Postnatally, TCA invade cortical layers and contribute substantially to the ongoing arealization process by elaborating cortical maps such as the barrel field in S1, corresponding to the whisker map (Erzurumlu and Gaspar, 2012, 2020; Li and Crair, 2011; Martini et al., 2018; Van der Loos and Woolsey, 1973). Thus, the perinatal period appears to be a discrete and critical process required for the emergence of functional cortical maps. In particular, prenatal thalamic spontaneous activity (Anton-Bolanos et al., 2019; Martini et al., 2021; Moreno-Juan et al., 2017) or neuronal activity from thalamic inputs orchestrates the pattern of S1 cortical maps (Erzurumlu and Gaspar, 2020; Genescu et al., 2022; Li et al., 2013; Martini et al., 2018; Matsui et al., 2013; Molnar et al., 2020; Narboux-Neme et al., 2012; Sato et al., 2022). Furthermore, timing

of birth is a fundamental event governing the timing barrels field emergence, as premature birth one day before term accelerates cortical map formation via the accelerated reduction of serotonin (5-hydroxytryptamine, 5-HT) levels (Toda et al., 2013). 5-HT levels have been known to play a central role on TCA growth and barrel field formation for some time (Lotto et al., 1999; Rebsam et al., 2002, 2005; Salichon et al., 2001; Teissier et al., 2017; Vahid-Ansari and Albert, 2021) but the observation that birth triggers the timing of cortical map development via of 5-HT reduction opens up new perspectives regarding TCA plasticity following defective embryonic wiring. A remarkable feature in this system is its high postnatal plastic adaptation. It has been demonstrated that TCA innervation modulates cortical sensory representation as selective reduction or elimination of primary thalamic nuclei (VP or dLGN) triggers adaptive rearrangement of corresponding primary sensory areas at the expense of intercalated HO areas (Chou et al., 2013; Frangeul et al., 2016; Pouchelon et al., 2014; Vue et al., 2013). Conversely, size reduction in primary S1 drives size adaptation of the VP nucleus (Zembrzycki et al., 2013). Overall these experiments revealed the enormous adaptive capacity of thalamic axons and their target cortical areas to match with each other. More impressive, it has been shown that TCA are able to exhibit long-range plasticity during early postnatal life, and match with their correct cortical areas even in case of severe embryonic defects. Indeed, embryonic TCA misnavigation or defective cortical regionalization leads to the innervation of inappropriate cortical targets by TCA but this mismatch is rescued after birth to couple sensory thalamic neurons with their appropriate cortical areas (Little et al., 2009; Lokmane et al., 2013; Shimogori and Grove, 2005). Nevertheless, the time window that supports post-natal cross areal plasticity and the mechanisms controlling it remain largely unexplored.

RESULTS

Rewiring of mistargeted TCA occurs during a short postnatal time-period

To define the critical period allowing the postnatal re-addressing of TCA to their appropriate cortical areas, we took advantage of a unique *Dlx5/6::cre; Ebf1^{fl/-}* model (*Ebf1cKO*) (Lokmane et al., 2013). This model disrupts the development of the subpallium, without altering either cortical or thalamic patterning, thereby non-autonomously triggering a pathfinding defect in embryonic TCA as revealed by L1-CAM and NetG1 (Lokmane et al., 2013; Tinterri et al., 2018) (Figure 1A). Axonal tracing by carbocyanine dye Dil and DiA injections at birth show that this defect is also characterized by a mismatch between TCA subsets and their presumptive cortical areas (Figure 1A). Indeed, while in control animals at postnatal day (P)0, Dil/DiA injections in presumptive visual (V1) and somatosensory (S1) cortex back-labeled visual-dLGN and somatosensory-VP neurons, respectively (dashed delineation in Figure 1A) in *Ebf1cko* mutants the same Dil/DiA injections revealed a more medial retrolabeling exclusively of the VP thalamic nuclei. Furthermore, we observed a complete absence of dLGN retrolabeling in *Ebf1cKO* mutants (dashed delineation in Figure 1A). Altogether these results illustrated an mismatch between TCA and presumptive cortical areas: VP axons target V1 instead of S1, and dLGN axons do not yet reach V1 (Figure 1G). Interestingly, immunolabelling for the apoptotic marker cleaved caspase 3 (cCasp3) at different developmental stages (Figure 1B and S1A) revealed massive apoptosis specifically at E17.5 within the primary visual-dLGN and somatosensory-VP thalamic nuclei in mutants (Figure 1B) with negligible thalamic cell death observed in their respective HO thalamic nuclei (Po and LP) or in any thalamic structures of control animals (Figure S1A). Consistent with apoptosis in these nuclei,

we found a significant reduction in the size of VP and dLGN primary nuclei compared to controls which emerges at P0 and persists after birth (Figure S1BC).

Regarding the role of TCA innervation in cortical sensory representation (Chou et al., 2013; Pouchelon et al., 2014; Vue et al., 2013), we mapped the primary V1 and S1, as well as the interposed HO posterior parietal association area (PTLp) at P7 (Hovde et al., 2019; Lyamzin and Benucci, 2019) using a range of TCA terminals markers including the TCA-GFP line (Mizuno et al., 2014), 5HTT and vGlut2. We observed that TCA targeted clear and discrete cortical domains that correspond to primary sensory S1 and V1 areas, which themselves could be delineated by the endogenous expression of cortical markers *Rorb* and *Lmo4* (Figure 1C and Figure S1D). Nevertheless, while the global cortical surface remained unchanged (Figure 1C and Figure S1D; control=100±1.34%, and mutant=98.03±1.25%), the size of primary cortical areas appeared significantly reduced in mutants, and this appeared at the expense of an increased PTLp (Figure 1C): -36% for the primary sensory V1 area (control=100±2.20%; mutant=63.3±3.64%), -32% for the primary sensory S1 area (control=114.7±3.30%; mutant=82.49±2.71%), and +41% for the PTLp (control=105.7±3.86%; mutant=147.4±8.40%) (Figure 1D). Interestingly, we found that in adults *Ebf1*cko mutants mice the transfer of specific sensory modalities (Ferezou et al., 2006; Petersen et al., 2003) was not affected as visual stimulation or whiskers deflection triggered, albeit reduced, a focal pattern of cortical activity within appropriate V1 and S1 areas (Figure S1F). Thus, embryonic TCA misrouting can be corrected postnatally by a remarkable axonal plasticity, which allows the formation of reduced, but well defined and functional primary cortical areas. Combined these observations led us to question the time window that exists within which this plasticity is possible.

We analyzed the kinetics of plasticity by performing axonal tracing experiments at successive postnatal stages (Figure 1E). We quantified the efficiency of TCA plasticity by establishing a “matching index” (Figure 1F), which represents the percentage of retrolabeled cells in dLGN following Dil injection in V1. At P0 in *Ebf1*cko mutants (Figure 1A,G), in which VP axons targeted V1 (instead of S1) while dLGN axons have not yet reached the cortex, the matching index was very low (Figure 1F; $P0_{\text{mutant}}=7.54\pm 3.18\%$ versus $P0_{\text{control}}=97.7\pm 0.97\%$). Nevertheless, from P1, TCA rewiring is engaged and Dil retrolabeled was observed in the dLGN (Figure 1E). The quantification of these results using our matching index (Figure 1D; $P1_{\text{mutant}}=21.12\pm 8.25\%$ versus $P1_{\text{control}}=96.74\pm 1.91\%$) confirmed simultaneous TCA reorganization, as dLGN TCA start to target V1 and VP TCA rewire from V1 to S1. Later at P2 and P7 (Figure 1E), Dil/DiA double injections in *Ebf1*cko showed the exclusive retrolabeling of dLGN neurons from V1 and a lateral retrolabeling of VP from S1, similar to that observed in control animals (Figure 1F; $P2_{\text{control}}=98.79\pm 0.58\%$; $P2_{\text{mutant}}=95.71\pm 2.65\%$ and $P7_{\text{control}}=99.35\pm 0.32\%$; $P7_{\text{mutant}}=96.04\pm 2.39\%$).

Combined, these experiments using our unique *Dlx5/6::cre;Ebf1^{fl/-}* mice model reveal a short plastic window following birth that allows cross-areal rewiring of TCA and their subsequent matching with the appropriate cortical targets (Figure 1G).

Postnatal plasticity is impaired by preterm birth

We hypothesized that birth could be involved in the temporal regulation of the short postnatal plastic window during which the TCA rewiring occurs (Figure 1G). After birth, neuronal input from the periphery is known to be required for cortical map formation (Van der Loos and Woolsey, 1973). In order to test the involvement of postnatal

peripheral input-mediated neuronal activation on TCA plasticity, we first performed Infra-Orbital Nerve (ION) section from birth to dampen activity from the whisker pad. At P7, we observed comparable patterns of axonal tracing in ION mutants and in unsectioned mutants, demonstrating that TCA plasticity in *Ebf1*cko is independent of sensory peripheral activity (Figure S2A-B).

In order to directly test the role of the birth in TCA plasticity in our mutants, we modified its timing by inducing preterm parturition (PT) in our model. Preterm birth was induced by injection of the progesterone receptor antagonist mifepristone in pregnant mice at E17.5 (Dudley et al., 1996; Toda et al., 2013). Delivery and birth were triggered one day before term without any apparent effects on the survival rate of pups (Figure 2A). As previously shown (Toda et al., 2013), we observed using TCA-GFP line (Figure 2B) or vGlut2 staining (Figure S2C) that preterm birth accelerates the formation and maturation of the cortical somatosensory barrels field. In the untreated full-term condition, we observed a maturation of the barrels field rapidly after birth, in particular between P2 and P3 (Figure 2B), with increased TCA clustering quantified by the “barrel field maturation index” (Figure 2C; Untreated: P2=0.047±0.011, P3=0.21±0.023). In contrast in the PT condition, analysis at equivalent developmental stages P3 and P4, revealed an acceleration of barrels field maturation, with axonal clustering in P3 PT higher compared to P2 untreated (Figure 2B-C; Preterm: P3=0.11±0.013, P4=0.25±0.024). We next performed axonal tracing by cortical Di injections in our *Ebf1*cko model in untreated and PT conditions at P7 (Figure 2D). As expected, in the untreated *Ebf1*cko animals, injections in V1 and S1 revealed efficient axonal rewiring, observed in the exclusive retrolabeling of the dLGN and VP, respectively (Figure 2B,F; Untreated: control=99.53±0.32%; mutant=96.04±2.38%). However, in the PT *Ebf1*cko condition, injection in V1 retrolabeled both VP and dLGN neurons (Figure 2B, F;

Preterm: control=93.45±2.91%; mutant=35.18±14.45%) indicating that a portion of VP somatosensory axons had failed to rewire and were still in V1. PT thus had strong effect on TCA plasticity. More importantly, axonal tracing at P1 revealed the same impairment is present at this stage (Figure 2D), suggesting that in PT *Ebf1cko* pups TCA plasticity begins at birth but then ends prematurely from P1, leading to an innervation of V1 by both VP and dLGN axons (Figure 2D, F; Untreated: control=96.74±1.90%; mutant= 21.12±8.25%; Preterm: control=97.91±2.07%; mutant=19.64±5.80%). We also performed *in vivo* V1 double injections of CTB (Cholera Toxin Subunit-B) at P7 (Figure S2D). In PT *Ebf1cko* we observed a shift in the pattern of dLGN retrolabeling compared to control PT or untreated *Ebf1cKO*, as well as the PT *Ebf1cKO*-specific presence of backlabeled VP neurons (Figure 2E,G; Untreated: control=95.51±2.18%;mutant=87.22±6.12%; Preterm: control=94.85±1.61%;mutant=42.25±7.39%). Overall this indicates that, in case of embryonic TCA misnavigation, preterm birth alters the short plastic window within which TCA are capable of rewiring and results in the persistence of mistargeted VP axons in the V1 premature *Ebf1cko* animals (Figure 2H).

TCA plasticity relies on birth-dependent modulation of 5-HT

As it's known PT induces a premature decrease in 5-HT levels (Toda et al., 2013), we next aimed to explore the role of 5-HT levels in the plastic capacity of TCA (Figure 3A). Our working hypothesis was that the extraordinary axonal plasticity that we observed in our *Ebf1cko* model might require a certain level of 5-HT during the short postnatal window; in that case reduction in 5-HT levels upon the induction of PT would underlie the deficits in TCA rewiring efficiency in those *Ebf1cko* animals. We proceeded to test the effect on PT *Ebf1cko* TCA plasticity of i) pharmacological reduction of 5-HT levels

in *Ebf1*cko mutant by injection of parachlorophenylalanine (PCPA), a 5-HT synthesis inhibitor (Rebsam et al., 2005; Toda et al., 2013), and ii) pharmacological increase of 5-HT levels by fluoxetine injection (Fx), a Selective Serotonin Reuptake Inhibitor (SSRI) (Homberg et al., 2010; Soiza-Reilly et al., 2019), in PT *Ebf1*cko condition to rescue TCA plasticity (Figure 3A). Importantly, the different treatments had no effect on thalamic nuclei size (Figure S3B). Performing axonal tracing at P7 by cortical Di injections we first observed that modulating 5-HT levels after birth in the control condition had no effect on cortical TCA targeting (Figure 3B,C), while forced reduction of 5-HT levels from birth by daily PCPA injections in *Ebf1*cko mutants did partially recapitulate the TCA plasticity defect observed in PT condition (Figure 3B-D; Untreated: control=99.53±0.32%; mutant=96.04±2.38%; Preterm: control=93.45±2.91%; mutant=35.18±14.45%; +PCPA control=99.70±0.21%; mutant=66.78±5.22%). Conversely, increasing 5-HT levels by daily fluoxetine injections in PT *Ebf1*cko fully rescued the defective plasticity, with Fluoxetine-treated PT *Ebf1*cko animals showing the same efficiency of TCA rewiring as in the untreated condition (Figure 3B,C; Preterm+Fx: control=97.65±1.56%; mutant=95.51±1.64%). Double CTB injections in different V1 antero-posterior positions (Figure S3A) confirmed that TCA rewiring is fully rescued in PT *Ebf1*cko condition by Fluoxetine injection from P0 (Figure 3C,E; Untreated: control=95.51±2.18%; mutant=87.22±6.12%; Preterm+Fx: control=92.67±2.52%; mutant=85.92±7.25%). All these results demonstrate that postnatal TCA plasticity is 5-HT levels-dependent and modulated by birth. Thus, while a premature birth or a pharmacological decrease in 5-HT levels affects TCA plasticity in *Ebf1*cko mutants, leading to mistargeted VP axons in V1, forced increase of 5-HT levels after preterm birth rescues the axonal plastic ability and restores TCA the matching with their target cortical areas (Figure 3F).

Efficient plasticity is required for primary area border integrity

It is fully documented that thalamic inputs are required for the establishment of cortical areas identity (Anton-Bolanos et al., 2019; Chou et al., 2013; Monko et al., 2022; Moreno-Juan et al., 2017; Pouchelon et al., 2014; Vue et al., 2013). Given our observation that premature birth or reduction of 5-HT levels from birth (Figure 3 and 4) impairs TCA plasticity in *Ebf1cko*, we also sought to explore their impact on cortical arealization and in particular on V1/S1 border formation. We performed, *in situ* hybridization for cortical markers *Rorb* and *Lmo4* on control and *Ebf1cko* flatten cortex at P7 in untreated, PT, +PCPA and PT+Fx conditions (Figure 4A). Considering the reduction in size of primary areas and concomitant increased in PTLp size in untreated *Ebf1cko* (Figure 1 and S1) we confirmed that each condition did not affect the size of primary areas or PTLp width in control or mutants (Figure S4B-E).

Using *in situ* hybridization for cortical markers (Figure 4A) we observed that in *Ebf1cko* PT condition, in which some VP TCA persist in V1, the molecular identity of V1 border was altered compare to the control (Figure 4A) and blurred *Rorb* and *Lmo4* expression precluded clear delineation of the border. We calculated the average gradient (∇) of cortical markers expression at the V1-PTLp boundary to quantify any deficit in boundary sharpness and observed a significant defect in *Ebf1cko* PT condition compared to the untreated control or untreated mutant conditions (Figure 3B; $\text{control}_{\text{Untreated}}=1.005\pm 0.037$; $\text{control}_{\text{Preterm}}=1.023\pm 0.05$; $\text{mutant}_{\text{Untreated}} = 0.833\pm 0.07$; $\text{mutant}_{\text{Preterm}} = 0.38\pm 0.04$). Furthermore, in the *Ebf1cko* +PCPA condition, which partly recapitulates the PT *Ebf1cko* TCA plasticity defect, a similarly defect in molecular border identity was observed (Figure 4A,B; $\text{control}_{+\text{PCPA}} = 0.909\pm 0.08$; $\text{mutant}_{+\text{PCPA}}$

=0.43±0.03). Finally, in mutant PT+Fx condition in which the thalamic axon plasticity is rescued, we see a full restoration of the sharp V1 border gradient (Figure 4B; control $_{\text{Preterm+Fx}} = 0.87 \pm 0.07$, mutant $_{\text{Preterm+Fx}} = 0.74 \pm 0.08$).

Overall it therefore appears that PT birth affects the early postnatal capacity of TCA to rewire after embryonic misnavigation and prevents successful readdressing of somatosensory VP axons from V1 to S1 in *Ebf1*cko. This persistent mismatch between subsets of TCA and incorrect cortical domains consequently affects the molecular identity and formation of a well-defined V1 border, but this can be restored in PT *Ebf1*cko mutants by forced 5-HT increase from birth, which rescue TCA plasticity and V1 border formation.

DISCUSSION

Environmental perception in mammals relies on the transfer of peripheral sensory modalities to appropriate cortical areas organized in cortical maps via the thalamus (Caviness and Frost, 1980; Jones, 2002). Elucidating the mechanisms underlying the wiring of distinct sensory thalamocortical connections is critical to understand area-sensory circuits and function. Here we revealed that embryonic mistargeting of TCA to inappropriate presumptive cortical areas is rescued within a short postnatal plastic window, during which TCA reroute to the correct cortical areas in birth- and 5-HT-dependent process. Furthermore, we highlight that this remarkable feature is essential for the formation of well-defined cortical areas and relies on sequential developmental events, allowing the plastic adaptation of sensory modalities.

In *Dlx5/6::cre;Ebf1^{fl/-}* conditional mutant mice, embryonic TCA navigation through the subpallium is shifted (Lokmane et al., 2013) causing abnormal cortical area innervation

with VP axons targeting V1 instead of S1, while dLGN axons do not yet innervate the cortex. Surprisingly, we showed at E17.5 a wave of massive apoptosis specifically in FO thalamic nuclei (VP and dLGN) revealing that mistargeted thalamic neurons might trigger apoptosis due to the loss of area-specific trophic factors required for their survival (Lokmane and Garel, 2014; Lopez-Bendito and Molnar, 2003; Ma et al., 2002; Molnar et al., 2012). While other studies reveal perinatal thalamic neuronal apoptosis associated with TCA mistargeting (Little et al., 2009; Zembrzycki et al., 2013) the potential role of neuronal cell death in subsequent TCA plasticity has not been explored. Consistent with previous studies showing a size adaptation between thalamic nuclei and their corresponding primary sensory area (Pouchelon et al., 2014; Vue et al., 2013; Zembrzycki et al., 2013), we observed a postnatal reduction in the size of apoptotic FO thalamic nuclei (VP and dLGN) and their corresponding primary sensory representation (S1 and V1). This primary sensory reduction was associated with the concomitant expansion of the intercalated HO cortical domain. Overall these data demonstrate the remarkable adaptive capacity of the system, which can reshape to match thalamic inputs with proper primary cortical areas.

In addition, we reveal how a discrete postnatal period immediately after birth supports a remarkable plastic window in which misrouted TCA can rewire over long distances to match with their target cortical domain (Armentano et al., 2007; Little et al., 2009; Lokmane et al., 2013; Shimogori and Grove, 2005). In particular, in *Dlx5/6::cre;Ebf1^{fl/-}* mutant mice, dLGN TCA begin to innervate V1 at P1, while VP TCA rewire from V1 to S1, and this results in a complete correction to the canonical dLGN-V1 and VP-S1 thalamocortical pattern of innervation at P2. The precise temporal regulation of TCA readdressing, including the order in which different subsets of TCA start to rewire, remains to be understood.

We demonstrated that timing of birth is crucial for TCA plasticity as triggering premature delivery with mifepristone injection in pregnant dams (Toda et al., 2013) affects the efficiency of TCA plasticity, leading to persistent TCA targeting defects, with some VP TCA failing to rewire and continuing to innervate V1 beyond the plastic window. Furthermore, as preterm birth modulates 5-HT levels (Toda et al., 2013), itself a key modulator of axonal growth and cortical map formation (Erzurumlu and Gaspar, 2020; Lieske et al., 1999; Lotto et al., 1999; Rebsam et al., 2002, 2005; Salichon et al., 2001; Teissier et al., 2017; Vahid-Ansari and Albert, 2021; Vicenzi et al., 2021), we investigated its role in postnatal TCA plasticity. Modulation of 5-HT levels during the postnatal plastic window with postnatal injections of pharmacological tools in *Ebf1*cko revealed its requirement for TCA rewiring. Postnatal reduction of 5-HT levels (by PCPA injections) mimics the effect of preterm birth, inhibiting efficient plastic rerouting of TCA, and impacting both the cortical sensory representation and well-defined border formation of areas. Conversely, increasing 5-HT levels in a preterm condition (by Fluoxetine injections) is sufficient to fully restore TCA plasticity and developmental delineation of cortical sensory areas with clear borders. While the birth-dependent modulation of 5-HT levels appears to be critical for readdressing mistargeted TCA, and it's been shown that thalamic 5HT1 receptors (Bonnin et al., 2006) are required for cortical map formation (Rebsam et al., 2002; Salichon et al., 2001) and timing of its emergence (Toda et al., 2013), we do not know the mechanism through which 5-HT supports this plastic window, whether via a direct effect on the growing axons themselves or other, indirect processes. Furthermore, we know 5-HT can modulate the axonal response to guidance cues (Bonnin et al., 2007; Xing et al., 2015), raising the possibility that a permissive cortical environment might enable TCA homing to specific

cortical areas in response to various guidance cues. Further investigations will be required to decipher the precise molecular role of 5-HT.

In human, preterm birth and dysfunction of the serotonergic system (Gabriele et al., 2014; Muller et al., 2016) constitutes a known risk factor for several neurodevelopmental disorders, including Autism Spectrum Disorders (ASD) and schizophrenia (Brenner et al., 2021; Dimitrova et al., 2021; Johnson and Marlow, 2011; Mwaniki et al., 2012). Moreover, modifications in sensory areas size (Courchesne et al., 2007) or thalamic volume and connectivity (Brenner et al., 2021; Cheon et al., 2011; Jiang et al., 2021; Molnar et al., 2020) have been reported in preterm, ASD or schizophrenia patients. Our findings therefore lead us to speculate that the short postnatal birth- and 5-HT-dependent plastic window identified in our genetic mice model might fulfil a critical function in restoring embryonic axon misguidance; impairment of this plastic window could have pathological consequences which contribute to the complex physiologies associated with specific neurodevelopmental disorders.

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AUTHOR CONTRIBUTIONS

Conceptualization of the study, S.G, L.L and A.S.W; Design and experimental analysis; L.L, S.G, A.S.W, I.F; Experimental investigation and quantification, L.L, A.S.W, A.L, H.C, C.M, I.F; Writing-original draft, A.S.W, S.G and L.L; Supervision, L.L and S.G; Funding acquisition S.G. All authors commented the manuscript. (And M.S, R.P and C.L)

DECLARATION OF INTERESTS

The authors declare no competing interests.

FIGURES AND LEGENDS

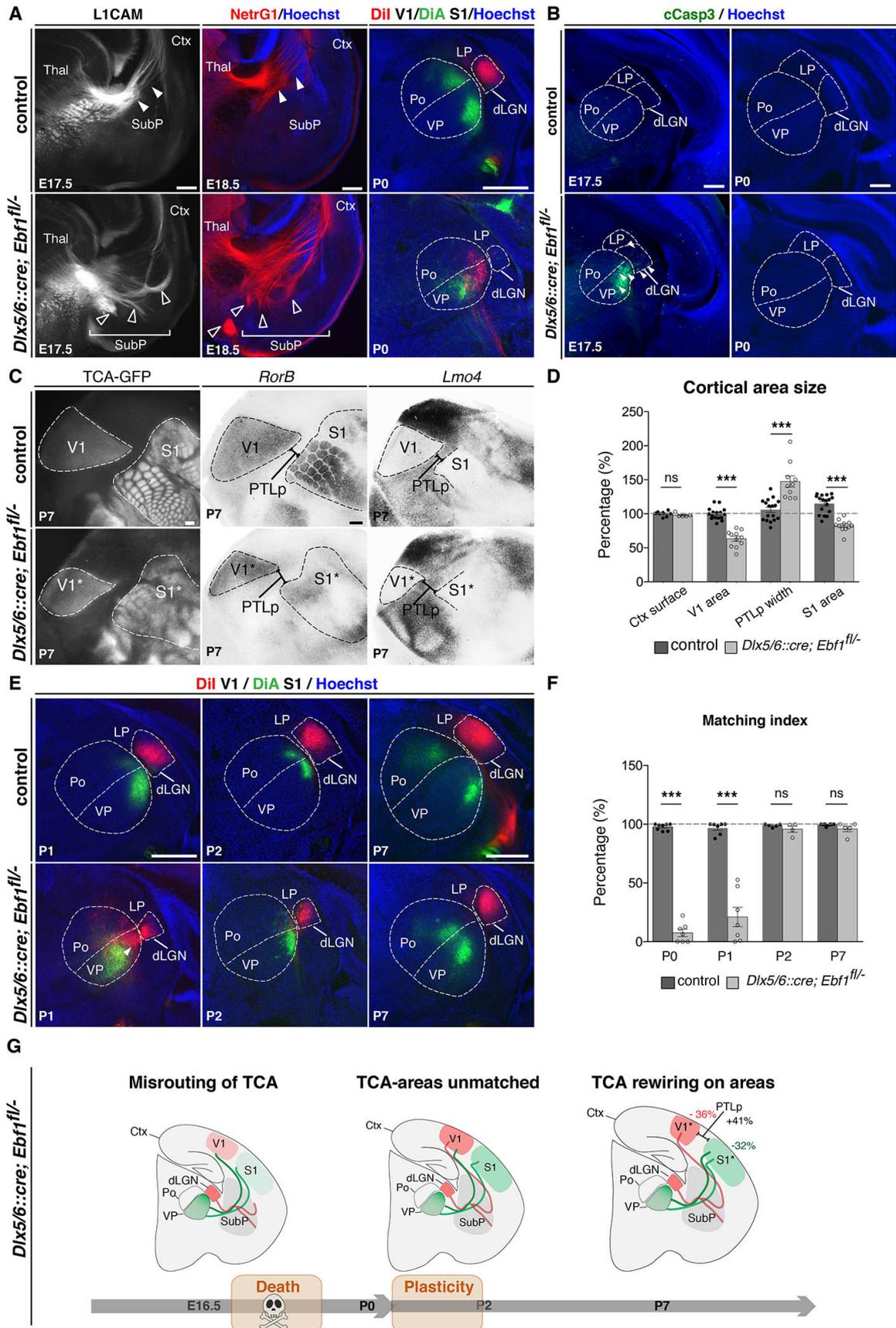


Figure 1: Embryonic TCA mistargeting is rapidly corrected after birth

(A) Staining on coronal sections of L1-CAM at E17.5 ($n_{\text{control}}=3$; $n_{\text{mutant}}=3$) and NetG1 at E18.5 ($n_{\text{control}}=4$; $n_{\text{mutant}}=4$) showing TCA misnavigation in *Dlx5/6::Cre;Ebf1^{fl/-}* subpallium (empty arrowheads) compared to control (arrowheads). Thalamic coronal section after Dil injection in V1 (red) and DiA injection in caudal S1 (green) in control ($n_{\text{control}}=8$) and *Dlx5/6::Cre;Ebf1^{fl/-}* ($n_{\text{mutant}}=7$) showing that TCA target inappropriate cortical domains at P0. **(B)** Coronal section of Cleaved caspase-3 staining at E17.5 ($n_{\text{control}}=3$ and $n_{\text{mutant}}=4$) and P0 ($n_{\text{control}}=4$ and $n_{\text{mutant}}=4$) revealing massive apoptosis in *Dlx5/6::Cre;Ebf1^{fl/-}* primary VP and dLGN thalamic nuclei at E17.5 (arrowheads) compared to control or P0. **(C)** TCA-GFP ($n_{\text{control}}=10$; $n_{\text{mutant}}=5$) staining and *In situ* hybridization of specific cortical markers *Rorb* and *Lmo4* (at least $n=5$ for each condition) on P7 flattened cortex showing strong reduction of V1 and S1 areas (dotted lines delineation) at the expense of intercalated HO PTLp domain in *Dlx5/6::Cre;Ebf1^{fl/-}*. **(D)** Quantification at P7 of the size of the cortical surface ($n_{\text{control}}=8$ and $n_{\text{mutant}}=5$), V1 ($n_{\text{control}}=16$ and $n_{\text{mutant}}=11$), S1 ($n_{\text{control}}=17$ and $n_{\text{mutant}}=11$) and PTLp width ($n_{\text{control}}=17$ and $n_{\text{mutant}}=11$). **(E)** Thalamic coronal section after Dil injection in V1 (red) and DiA injection in caudal S1 (green) in control and *Dlx5/6::Cre;Ebf1^{fl/-}* at P1 ($n_{\text{control}}=7$ and $n_{\text{mutant}}=7$), P2 ($n_{\text{control}}=5$ and $n_{\text{mutant}}=4$) and P7 ($n_{\text{control}}=8$ and $n_{\text{mutant}}=5$) showing rapid TCA rewiring after birth. **(D)** Quantification of the matching index by measuring the proportion of red signals in dLGN nuclei following Dil injection in V1. **(G)** Schematic representation showing sequential checkpoints in *Dlx5/6::Cre;Ebf1^{fl/-}* enable postnatal TCA plasticity but subsequent impact on primary cortical areas (-32% for S1; -36% for V1 and +41% for PTLp domain). Values are presented as mean \pm s.e.m; *** $p<0.001$, 2 ways ANOVA test with Sidak's multiple comparison correction.

Ctx: Cortex; dLGN: dorsal lateral geniculate nucleus; LP, Lateral Posterior nucleus;

Po, posteromedial nucleus; PTLp: Posterior Parietal Cortex; S1, primary somatosensory cortex; SubP, Subpallium; V1, primary visual cortex; VP, ventroposterior nucleus. Scale bars represent 250 μm . See also Figure S1.

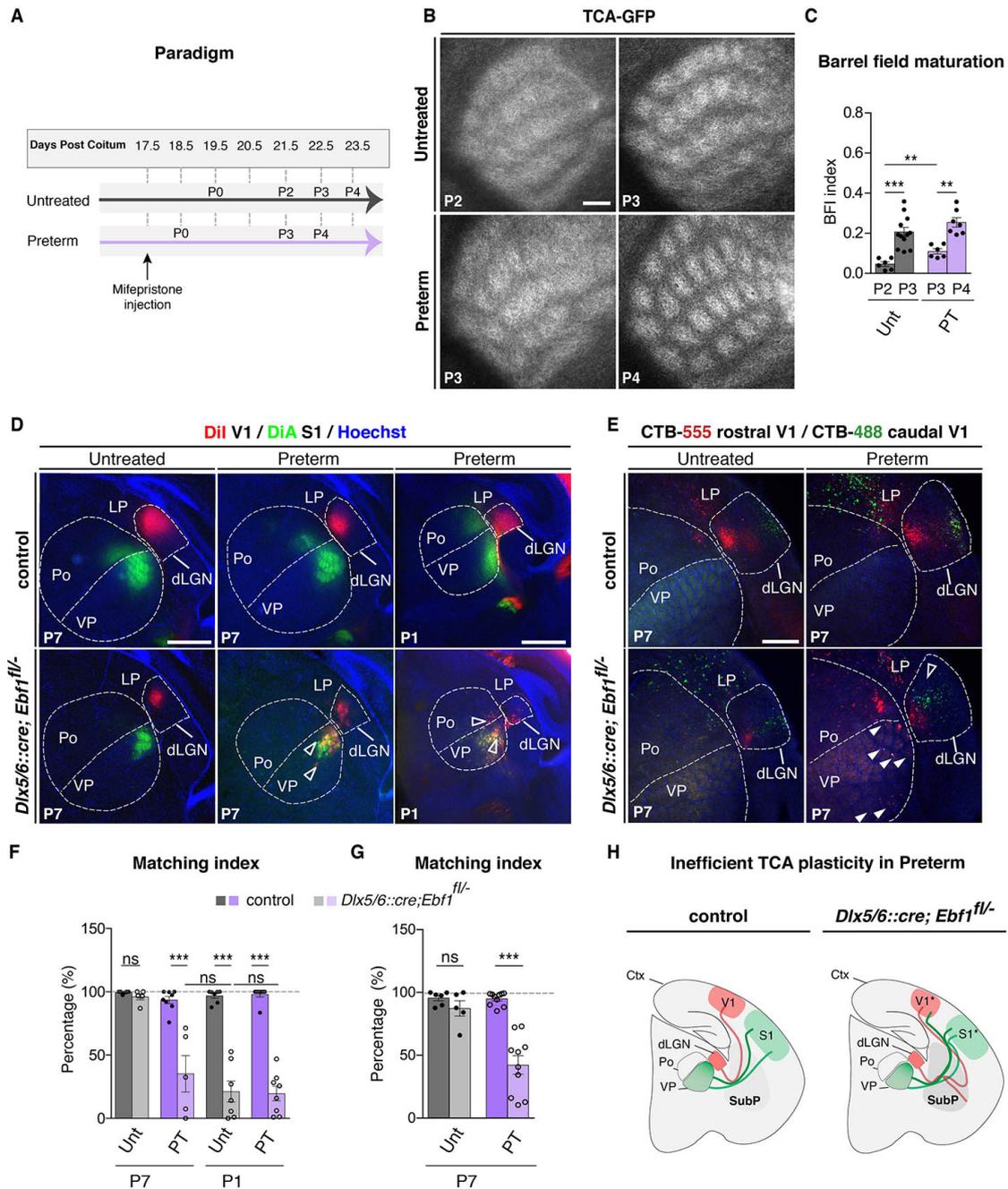


Figure 2: Preterm birth affects TCA plasticity efficiency

(A) Experimental procedure to induce preterm birth using mifepristone injection at E17.5. (B) TCA-GFP immunostaining on flattened cortex of P2-P3 untreated (full term) and P3-P4 preterm pups. (C) Quantification of barrel field maturation with the Barrel Field Intensity (BFI) index using the TCA-GFP line. BFI increases within postnatal stages (Untreated: $n_{P2}=6$; $n_{P3}=12$; Preterm: $n_{P3}=6$; $n_{P4}=7$). (D) Thalamic coronal

section after Dil injection in V1 (red) and DiA injection in caudal S1 (green) in control and *Dlx5/6::Cre;Ebf1^{fl/-}* in untreated (at P7 $n_{\text{control}}=8$; $n_{\text{mutant}}=5$) and preterm (P7: $n_{\text{control}}=8$; $n_{\text{mutant}}=5$ and P1: $n_{\text{control}}=8$; $n_{\text{mutant}}=8$) conditions. In preterm *Dlx5/6::Cre;Ebf1^{fl/-}* at P7 and P1, V1 injection (red) leads simultaneously to a retrolabeling in VP and dLGN thalamic nuclei meaning that VP TCA are targeting V1 instead S1 (empty arrowheads). **(E)** Thalamic coronal section after CTB-555 injection in rostral V1 (red) and CTB-488 injection in caudal (green) V1 in control and *Dlx5/6::Cre;Ebf1^{fl/-}* at P7 in untreated ($n_{\text{control}}=6$; $n_{\text{mutant}}=5$) and preterm ($n_{\text{control}}=10$; $n_{\text{mutant}}=10$) conditions. V1 double injections in preterm *Dlx5/6::Cre;Ebf1^{fl/-}* reveals the defective TCA rewiring as VP TCA are retrolabeled (arrowheads). **(F)** Quantification of the matching index by measuring the proportion of red signals in dLGN nuclei following Dil injection in V1 for (D). **(G)** Quantification of the matching index by measuring the proportion of signals in dLGN nuclei following CTB injections in V1 at P7 for (E). **(H)** Schematic representation showing the defective TCA rewiring at P7 in preterm *Dlx5/6::Cre;Ebf1^{fl/-}* as V1 is targeted by dLGN and VP axons. Values are presented as mean \pm s.e.m; *** $p<0.001$, Mann-Whitney U test for (C) and 2 ways ANOVA test with Sidak's multiple comparison correction for (F-G).

Ctx: Cortex; dLGN: dorsal lateral geniculate nucleus; LP, Lateral Posterior nucleus; Po, posteromedial nucleus; S1, primary somatosensory cortex; SubP, Subpallium; V1, primary visual cortex; VP, ventroposterior nucleus. Scale bars represent 250 μm . See also Figure S2.

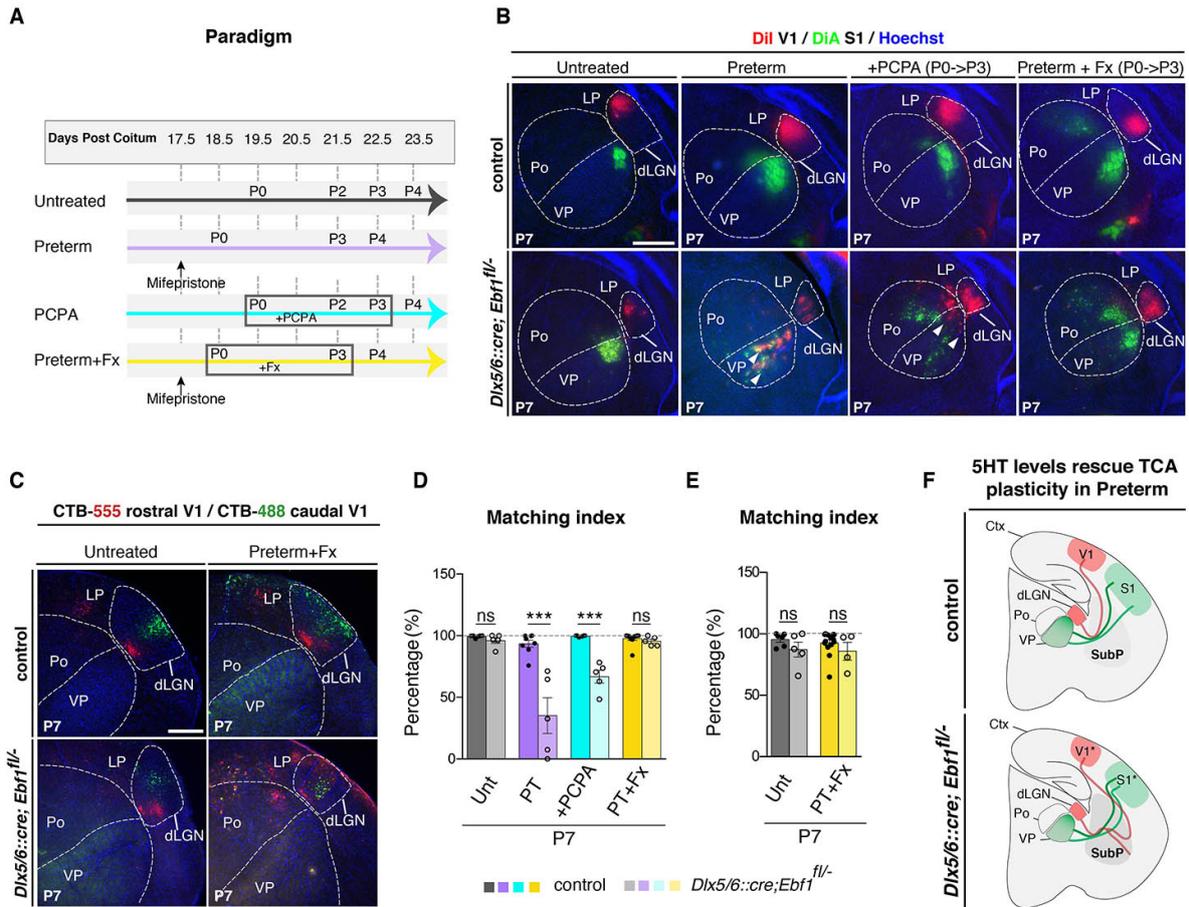


Figure 3: Preterm birth impairs TCA plasticity partly through serotonin levels

(A) Experimental procedure to induce preterm birth by mifepristone injection at E17.5 and reduced or increased 5-HT levels from birth by daily injections of respectively parachlorophenylalanine (PCPA) or Fluoxetine (Fx). (B) Thalamic coronal section after Dil injection in V1 (red) and DiA injection in caudal S1 (green) in control and *Dlx5/6::Cre;Ebf1^{fl/-}* at P7 in untreated ($n_{\text{control}}=8$; $n_{\text{mutant}}=5$), preterm ($n_{\text{control}}=8$; $n_{\text{mutant}}=5$), +PCPA ($n_{\text{control}}=6$; $n_{\text{mutant}}=5$) or Preterm+Fx ($n_{\text{control}}=10$; $n_{\text{mutant}}=5$) conditions. In preterm or + PCPA *Dlx5/6::Cre;Ebf1^{fl/-}* at P7, V1 injection (red) leads simultaneously to a retrolabeling in VP and dLGN thalamic nuclei meaning that VP TCA are targeting V1 instead S1 (arrowheads). Increasing 5-HT levels by Fx injections in preterm *Dlx5/6::Cre;Ebf1^{fl/-}* restore the TCA plasticity. (C) Thalamic coronal section after CTB-

555 injection in rostral V1 (red) and CTB-488 injection in caudal (green) V1 in control and *Dlx5/6::Cre;Ebf1^{fl/-}* at P7 in untreated ($n_{\text{control}}=6$; $n_{\text{mutant}}=5$) and Preterm+Fx ($n_{\text{control}}=14$; $n_{\text{mutant}}=4$) conditions showing that increasing 5-HT levels by Fx injections in preterm *Dlx5/6::Cre;Ebf1^{fl/-}* restores the TCA plasticity. **(D)** Quantification of the matching index by measuring at P7 the proportion of red signals in dLGN nuclei following Dil injection in V1 for (C). **(E)** Quantification of the matching index by measuring at P7 the proportion of signals in dLGN nuclei following CTB injections in V1 for (C). **(F)** Schematic representation showing that increasing 5-HT levels by Fx injections from birth rescue in preterm *Dlx5/6::Cre;Ebf1^{fl/-}* the TCA plasticity defect at P7. Values are presented as mean \pm s.e.m; *** $p<0.001$, 2 ways ANOVA test with Sidak's multiple comparison correction.

Ctx: Cortex; dLGN: dorsal lateral geniculate nucleus; LP, Lateral Posterior nucleus; Po, posteromedial nucleus; S1, primary somatosensory cortex; SubP, Subpallium; V1, primary visual cortex; VP, ventroposterior nucleus. Scale bars represent 250 μm . See also Figure S3.

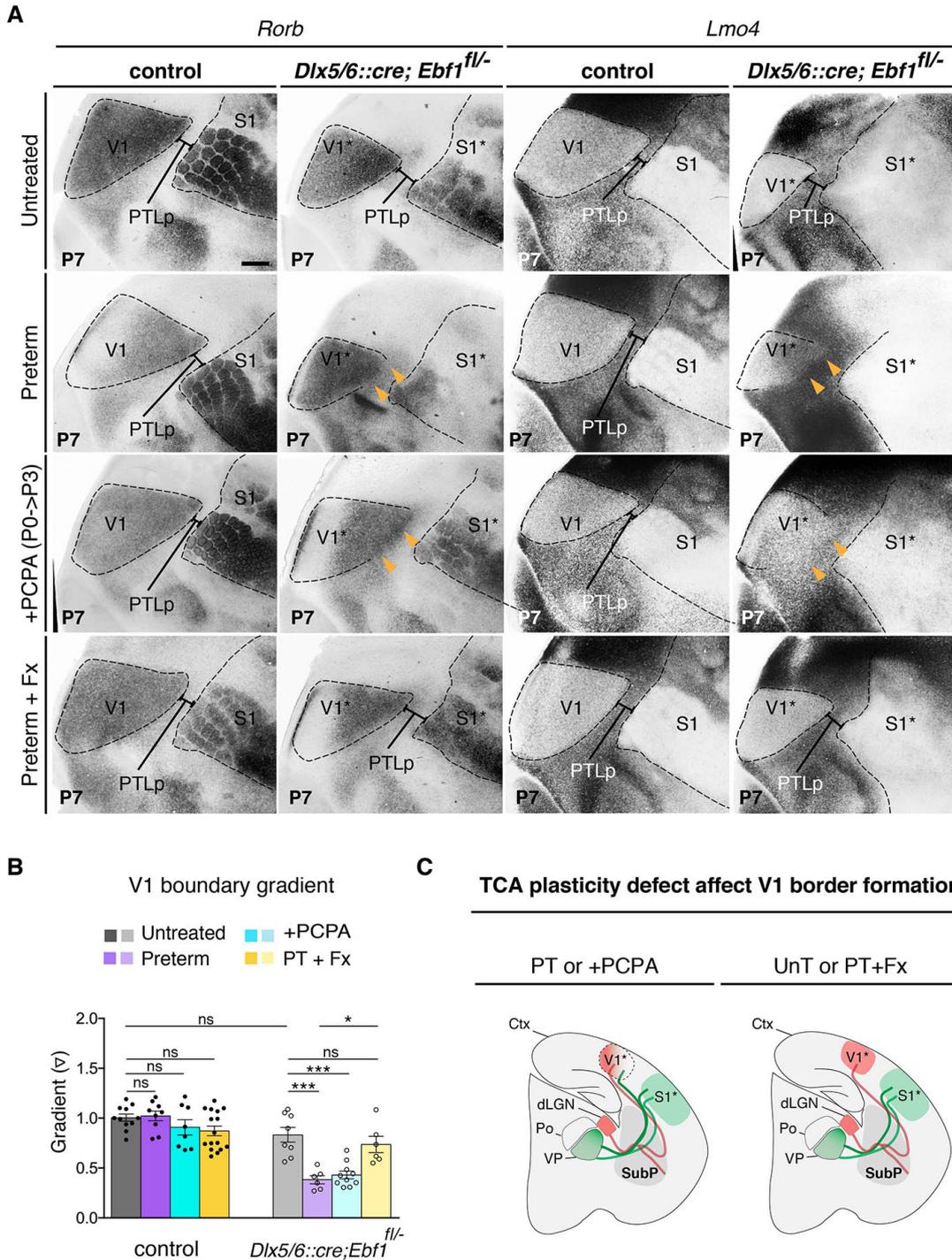


Figure 4: TCA plasticity impairment impact molecular identity of V1 border.

(A) *In situ* hybridization on P7 flattened cortex using specific cortical markers *Rorb* and *Lmo4* in control and *Dlx5/6::Cre;Ebf1^{fl/-}* in untreated ($n_{\text{control}}=11$; $n_{\text{mutant}}=8$), preterm ($n_{\text{control}}=9$; $n_{\text{mutant}}=6$), +PCPA ($n_{\text{control}}=8$; $n_{\text{mutant}}=10$) or Preterm+Fx ($n_{\text{control}}=16$; $n_{\text{mutant}}=6$)

conditions. In preterm and +PCPA *Dlx5/6::Cre;Ebf1^{fl/-}* conditions the molecular identity of V1 border is affected with blurry *Rorb* and *Lmo4* expression (yellow arrowheads) compared to the sharp delimitation in controls or untreated *Dlx5/6::Cre;Ebf1^{fl/-}*. Fx injection in preterm *Dlx5/6::Cre;Ebf1^{fl/-}* conditions restores the well define molecular identity. **(B)** Quantification of the V1 boundary gradient observed in *in situ* hybridization. **(C)** Schematic representation showing the defective V1 molecular identity in preterm and +PCPA *Dlx5/6::Cre;Ebf1^{fl/-}* conditions while Fx injections in preterm *Dlx5/6::Cre;Ebf1^{fl/-}* rescue the boundary V1 sharpness at a comparable level to the untreated control condition. Values are presented as mean \pm s.e.m; * $p < 0.05$, *** $p < 0.001$, 2 ways ANOVA test with Sidak's multiple comparison correction.

Ctx: Cortex; dLGN: dorsal lateral geniculate nucleus; LP, Lateral Posterior nucleus; Po, posteromedial nucleus; PTLp: Posterior Parietal Cortex; S1, primary somatosensory cortex; SubP, Subpallium; V1, primary visual cortex; VP, ventroposterior nucleus. Scale bars represent 250 μ m. See also Figure S4.

METHODS

Mouse lines

Dlx5/6::Cre;Ebf1^{fl/-} mutant animals were obtained by crossing *Dlx5/6::Cre* mice with *Ebf1^{+/-}* mice to generate *Dlx5/6::Cre;Ebf1* offspring. *Dlx5/6::Cre;Ebf1^{+/-}* mice were then crossed with *Ebf1^{fl/fl}* animals to produce *Dlx5/6::Cre;Ebf1^{fl/-}* conditional knockout mice (Lokmane et al., 2013). All these lines were maintained on a C57/Bl6J genetic background. The TCA-GFP Tg line has been kindly provided by Dr Takuji Iwasato (Mizuno et al., 2014) and backcrossed in *Ebf1^{fl/fl}* background. The day of vaginal plug was considered as embryonic day (E) 0.5 and day of birth as postnatal day (P) 0. Animals were bred under French and EU regulations.

Pharmacological and surgical manipulations

Mifepristone treatment: Mifepristone treatment was carried out as described previously with modifications (Toda et al., 2013). Mifepristone (Calbiochem) was dissolved at 10 mg/ml in ethanol and stored at -20 °C. Then we performed subcutaneous injections on pregnant female mice at E17.5 with 150 μ l of 1 mg/ml mifepristone solution diluted in saline between 17:30 and 18:30. Mouse pups born between 12:00 and 18:00 the following day.

PCPA treatment: PCPA treatment was performed as described previously (Toda et al., 2013). PCPA (Sigma) was dissolved at 10 mg/ml in saline. We performed subcutaneous PCPA injections in pups at 300 mg/kg every day from P0 until sacrifice or maximum P3.

Fluoxetine treatment on preterm: Fluoxetine (Tocris) was dissolved at 0.5 mg/ml in 0.9% NaCl solution. We performed subcutaneous fluoxetine injections at 10 mg/kg in pregnant female mice at E17.5 and then in pups at the indicated time points.

Infraorbital nerve (ION) section: ION section was performed on P0 pups. Animals were cryo-anaesthetized on ice. The right ION was sectioned as previously described by (Frangeul et al., 2014). Pups were brought out of cryo-anesthesia on a heating pad and then returned to the mother until sacrifice.

***In situ* hybridization and immunohistochemistry**

For *in situ* hybridization pups were perfused with 4% paraformaldehyde in PBS (PFA), dissected, flattened and post-fixed in 4% PFA overnight at 4°C. For flattening, intact cortices were isolated from subcortical tissue by dissection, sandwiched between two Superfrost microscope slides separated by 1 mm spacers and post-fixed in 4% PFA overnight at 4°C. The following day fixed flattened cortices were cut to 120 µm free-floating sections using a Leica S1000 vibratome and hybridized as described in (Lopez-Bendito et al., 2006), with the following digoxigenin-labeled probes: *Cad8*, *Igfbp5*, *Lmo4* and *Rorβ*.

For immunohistochemistry whole brains or flattened cortices were cut to 70 µm free-floating sections using a Leica S1000 vibratome. Slices were incubated in a blocking solution containing 0.25% Triton X-100 (Sigma), 0.02% Gelatin in PBS for 1h at room temperature (RT) and incubated in the same blocking solution with primary antibodies overnight at 4°C. Primary antibodies were used at the following concentrations: Rat anti-L1CAM 1/100 (MAB5272; Millipore), Rabbit anti-Caspase-3 cleaved 1/400 (AF835; R&D systems), Chicken anti-GFP 1/1000 (GFP-1020; Avès), Goat anti-

NetrinG1a 1/100 (AF1166; R&D systems), Rabbit anti-5HTT 1/1000 (PC177L; Calbiochem), Guinea pig anti-vGLUT2 1/1000 (AB2251; Millipore). The following day, sections were rinsed four times in PBS and incubated with the appropriate secondary antibodies in PBS overnight at 4°C. Secondary antibodies were used at 1/400 and included: A488-conjugated donkey anti-rabbit and chicken, Cy3-conjugated donkey anti-rat and anti-goat, DyLight488-conjugated donkey anti-guinea pig (Jackson ImmunoResearch). Hoechst (Sigma) was used for fluorescent nuclear counterstaining and sections were mounted in Vectashield (Vector).

Axonal tracing: Carbocyanine dye and CTB injections

For axonal tracing using dyes, embryonic and perfused postnatal brains were fixed at least overnight at 4°C in 4% PFA. Crystals of Dil (1,1'-dioctadecyl 3,3,3',3'-tetramethylindocarbo-cyanine perchlorate; Molecular Probes) or DiA (4-4-dihexadecyl aminostyryl N-methyl-pyridinium iodide; Molecular Probes) were inserted into either the thalamus or neocortex of intact hemisected brains. Injected brains were incubated at 37°C and dye diffusion assessed every week by whole-brain examination under a fluorescent binocular set-up (Leica MZ16 F).

For axonal tracing using CTB, P4 pups were cryo-anaesthetized on ice during 1'30'', then 200nl of each Alexa Fluor 488 (C34775) and Alexa Fluor 555 (C34776) Conjugated Cholera Toxin Subunit B (Invitrogen) were injected in specific part of primary visual area (V1) at 1ug/ul. Pups were brought out of cryo-anesthesia on a heating pad and then returned to the mother until P8 when brains were perfused and fixed at least overnight at 4°C in 4% PFA.

Brains were then cut into 100 um-thick free-floating sections using a Leica S1000 vibratome and the sections counterstained using Hoescht (Sigma). Staining were visualized under fluorescent Leica DM5000 B or DMI8 microscope and examined at the level of the internal capsule and/or thalamus.

Image acquisition & quantifications

Images were acquired with a fluorescence binocular microscope (Leica MZ16 F), fluorescence microscope (Leica DM5000 B & Leica DMI8) and a confocal microscope (Leica TCS SP5). Image analyses were performed with ImageJ and Adobe Photoshop software.

Apoptosis quantification

dLGN, LP, Po and VP thalamic structures were identified in immunolabelled coronal sections of mouse brain using anti-vGlut2 (see *Immunohistochemistry*) and Hoescht (Sigma) signal. The level of apoptotic anti-cCasp3 (see *Immunohistochemistry*) signal within each thalamic structure was quantified as follows: anti-cCasp3 signal was thresholded using the automated ImageJ Threshold function and the signal coverage (%) calculated for each demarcated thalamic structure.

Area size quantification

PTLp, S1 and V1 areal structures were identified on tangential sections of flattened cortices using the signal domains of anti-vGlut2 (see *Immunohistochemistry*) from immunolabelling experiments and *Cad8*, *Igfbp5*, *Lmo4* and *Ror β* signal from ISH experiments. The size of an individual areal structure visualized using a specific marker

was calculated as the percentage (%) of the mean size of the same structure visualized using the same marker in littermate controls.

Carbocyanine dye and CTB quantification

dLGN and VP/Po thalamic structures were identified in coronal sections of mouse brains injected in V1 with Dil or CTB using Hoescht (Sigma) signal. The level of fluorescent signal inside the dLGN and VP/Po thalamic structures was quantified as follows: fluorescent signal was thresholded using the automated ImageJ Threshold function and the area of signal (px) calculated separately within the dLGN and VP/Po structures. In order to compensate for the variability in thalamic backlabeling between different injected brains, we calculated and compared the percentage of fluorescent signal in the dLGN versus the dLGN and VP/Po combined; 100% representing fluorescent signal distributed exclusively within the dLGN and absent from the VP/Po.

Barrel Field Intensity index

The barrel field maturation along the time was defined by the Barrel Field Intensity (BFI) index measured according to (Toda et al., 2013). We used GFP-stained tangential sections of the *TCA-GFP* flattened cortex in normal and preterm birth condition at different post-natal stages (P2 to P4). Optical densities were measured along the line of caudal barrels (between C1 and C3) using ImageJ software (NIH). BFI was calculated as the contrast between the intensity of C2 and the average intensity of adjacent spaces separating it from C1 and C3, normalized by the average intensity of the whole barrel region. BFI values thus became increased when TCA axons start to enhance clustering to innervate the barrels.

V1-PTLp boundary quantification

V1-PTLp boundary region was identified on tangential sections of flattened cortices using either *Lmo4* or *Rorb* signal from ISH experiments. We obtained ImageJ Plot Profiles of signal intensity over a length of 100 px set perpendicular to and centered on the V1-PTLP boundary. Plot Profile measurements were taken over three non-overlapping regions of the V1-PTLP boundary for each specimen. We determined the slope of intensity signal across a width of 20 px around the V1-PTLP boundary and the sharpness of the boundary by calculating the average gradient (∇) corresponding to mean of values. A steeper gradient corresponding to a sharper V1-PTLP boundary and *vice versa*.

Voltage-sensitive dye (VSD) imaging

VSD imaging of the cortical activity evoked by visual and tactile whiskers stimulation was performed on adult mice under isoflurane anesthesia (induction: 3-4%, maintenance: 1-1.5%) essentially as previously described (Ferezou et al., 2006; Petersen et al., 2003). Briefly, the left barrel cortex was exposed and stained for 1 h with the VSD RH1691 (1 mg/ml, in Ringer's solution containing [inmM]: 135 NaCl, 5 KCl, 5 HEPES, 1.8 CaCl₂, 1 MgCl₂). After removal of the unbound dye, the cortex was covered with agarose (0.5–1% in Ringer's) and a coverslip. Cortical imaging was performed through a tandem-lens fluorescence microscope (SciMedia), equipped with one Leica PlanApo 1.6x (objective side) and one Leica PlanApo 0.63x (condensing side), a 630 nm excitation filter, a 650 nm dichroic mirror, and a long pass 665 nm emission filter. The resulting field of view was 3.8 x 3.8 mm, with a pixel resolution of 38 x 38 μ m. Visual stimulation was performed using a single brief flash (50 or 100 ms) of a green light emitting diode (LED) placed at about 5 cm in front of the animal, in its

binocular field of view. Tactile whisker stimulation consisted in single brief rostral deflection (2 ms rising time, 2 ms plateau and 2 ms fall) of either the alpha or the C2 whisker on the right side of the snout by means of a multi-whisker stimulator (Jacob et al., 2010). Whiskers were inserted keeping their natural angle in 27G stainless steel tubes attached to the piezoelectric benders (Noliac, Denmark), leaving 2 mm between the tip of the tube and the whisker base. Each whisker deflection consisted of a 95 μm displacement (measured at the tip of the tube). Specific filters were applied to the voltage commands to prevent mechanical ringing of the stimulators. Visual and tactile alpha and C2 whisker stimulations were delivered within pseudo randomized sequences containing extra blank trials (each stimulation being repeated 40 times). The illumination of the cortical surface started 500 ms before each image acquisition to avoid acquiring signal in the steeper phase of the fluorescence bleaching. Recordings were then of 1 second duration, with 200 ms baseline and 800 ms post stimulation. Variations of the fluorescence signals were initially recorded as variations over the resting light intensity (first acquired frame).

Data preprocessing was done using in-house software (Elphy, G. Sadoc, UNIC-CNRS), further analyses were made using custom written routines in IgorPro (Wavemetrics). Subtraction of a pixel by pixel best fit double-exponential from the averaged unstimulated sequence was used to correct for photobleaching. Further subtraction of the averaged signal over three frames just preceding the stimulus was made to focus on sensory evoked activity.

Statistical analysis

All data are presented as mean \pm s.e.m (detailed in each figure legend). A P-value less than 0.05 was considered significant. According to the data structure, we

systematically performed non-parametric tests namely Mann-Whitney U Test, and 2 ways ANOVA test with Sidak's correction, depending on whether we performed single or multiple group comparisons with individual or common controls. Statistics and plotting were performed using GraphPad Prism 7.00 (GraphPad Software Inc., USA). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

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SUPPLEMENTARY FIGURES AND LEGENDS

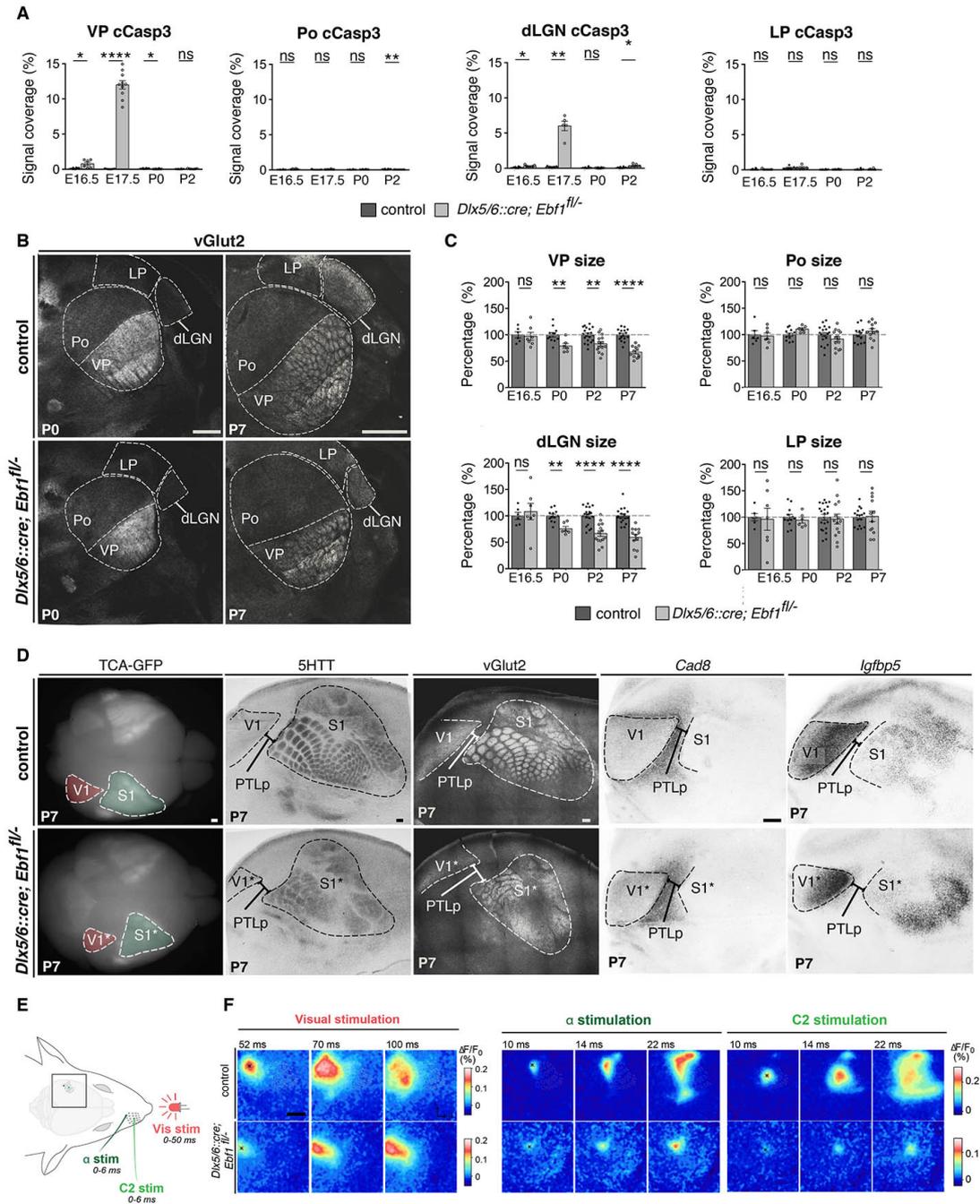


Figure S1: Embryonic TCA mistargeting induces prenatal apoptosis and size reduction of sensory representation

(A) Quantification of percentage of signal coverage by Cleaved capase-3 immunostaining at different stages E16.5 ($n_{\text{control}}=4$; $n_{\text{mutant}}=3$), E17.5 ($n_{\text{control}}=3$; $n_{\text{mutant}}=4$), P0 ($n_{\text{control}}=4$; $n_{\text{mutant}}=4$) and P2 ($n_{\text{control}}=4$; $n_{\text{mutant}}=4$) in each FO (VP, dLGN)

and HO (Po, LP) thalamic nuclei in control and *Dlx5/6::Cre;Ebf1^{fl/-}* embryos. A massive apoptosis is observed in mutant VP and dLGN thalamic nuclei at E17.5. **(B)** Staining on thalamic coronal sections of vGlut2 at P0 ($n_{\text{control}}=5$; $n_{\text{mutant}}=3$) and P7 ($n_{\text{control}}=4$; $n_{\text{mutant}}=5$) showing size reduction of VP and dLGN thalamic nuclei in *Dlx5/6::Cre;Ebf1^{fl/-}*. **(C)** Quantification of control and *Dlx5/6::Cre;Ebf1^{fl/-}* FO (VP, dLGN) and HO (Po, LP) thalamic size in mutants relative to the control at different stages E16.5 ($n_{\text{control}}=6$; $n_{\text{mutant}}=7$), P0 ($n_{\text{control}}=12$; $n_{\text{mutant}}=6$), P2 ($n_{\text{control}}=20$; $n_{\text{mutant}}=14$) and P7 ($n_{\text{control}}=16$; $n_{\text{mutant}}=12$). A significant strong size reduction is observed in mutant FO thalamic nuclei (VP and dLGN) from P0. **(D)** Whole brain of TCA-GFP transgenic line ($n_{\text{control}}=7$; $n_{\text{mutant}}=4$) and immunostaining on P7 flatten cortex of GFP ($n_{\text{control}}=10$; $n_{\text{mutant}}=5$), 5HTT ($n_{\text{control}}=6$; $n_{\text{mutant}}=5$) or vGlut2 ($n_{\text{control}}=7$; $n_{\text{mutant}}=6$) showing reduced cortical domains targeted by TCA (dotted lines delineation) in *Dlx5/6::Cre;Ebf1^{fl/-}*. *In situ* hybridization on P7 flatten cortex using specific cortical markers *Igfbp5*, *cad8* revealing the size reduction of primary areas V1 and S1 (dotted lines delineation) as well as the expansion of PTLp HO domain (at least $n=5$ for each condition). **(E)** Schema of experimental paradigm. The black square represents the window of cortical activity recording following visual stimulation, with brief LED flash (in red), or whiskers stimulation by C2 or α deflection (in green). **(F)** Spatio-temporal dynamics of membrane potential changes imaged using voltage-sensitive dye (VSD) in anesthetized control ($n=3$) and in *Dlx5/6::Cre;Ebf1^{fl/-}* ($n=3$) showing a comparable focal cortical activity after visual stimulation or whiskers deflection. The snapshots display the averaged fluorescence signal for representative cases at three different timings. Scale bar, 1 mm. Values are presented as mean \pm s.e.m; * $p<0.05$, *** $p<0.01$, *** $p<0.001$, Mann-Whitney U test.

dLGN: dorsal lateral geniculate nucleus; LP, Lateral Posterior nucleus; Po, posteromedial nucleus; S1, primary somatosensory cortex; Thal, Thalamus; V1, primary visual cortex; VP, ventroposterior nucleus. Scale bars represent 250 μm

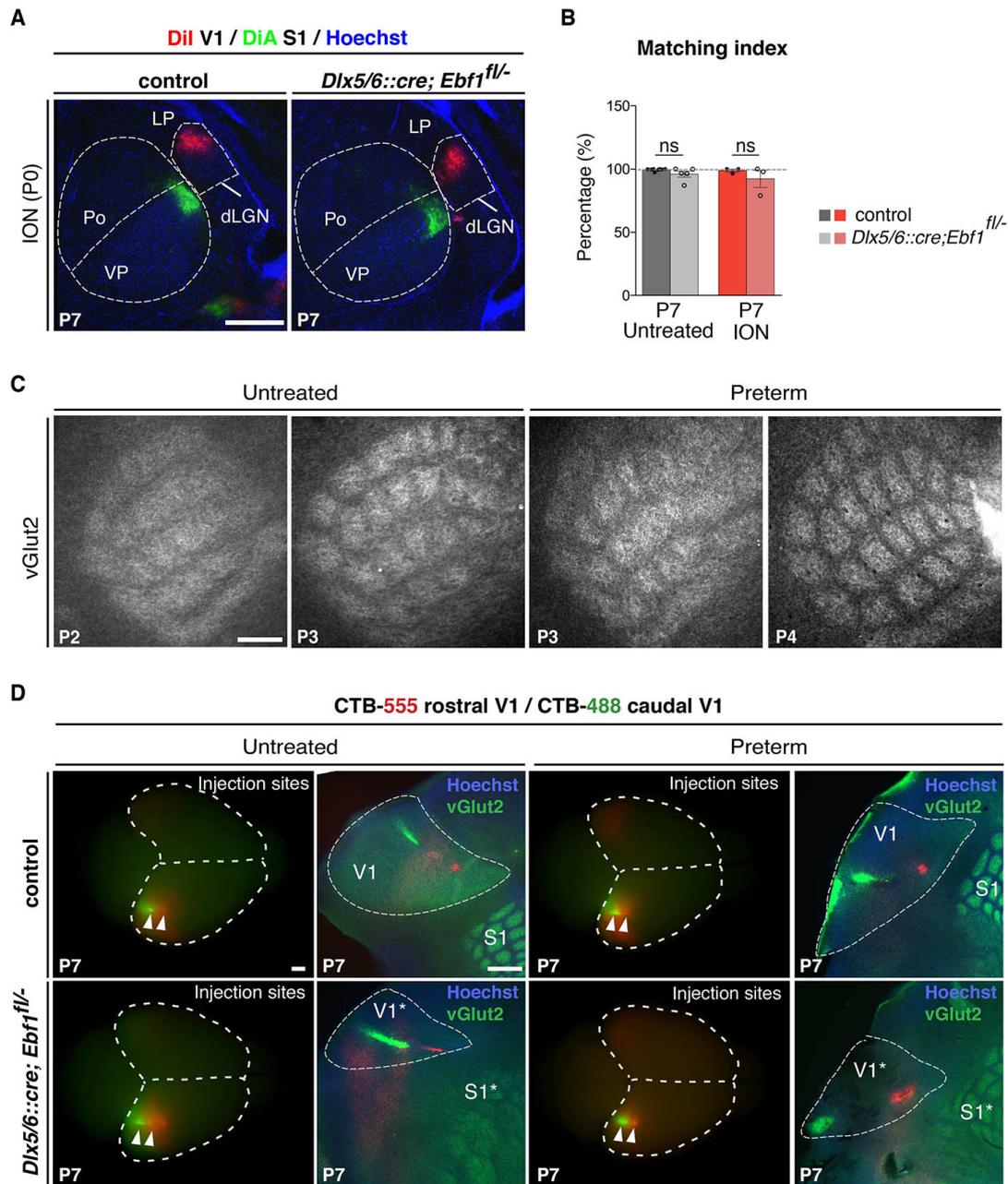


Figure S2: Preterm birth as a model to analyze TCA plasticity

(A) P7 thalamic coronal section after Dil injection in V1 (red) and DiA injection in caudal S1 (green) following sensory peripheral activity deprivation by InfraOrbital Nerve (ION) sectioning at P0 in control ($n_{\text{control}}=8$; $n_{\text{mutant}}=5$) and *Dlx5/6::Cre; Ebf1^{fl/-}* ($n_{\text{control}}=3$; $n_{\text{mutant}}=3$). P0 ION section does not affect postnatal TCA plasticity in mutant. (B) Quantification of the matching index by measuring the proportion of red signals in

dLGN nuclei following Dil injection in V1. **(C)** vGlut2 immunostaining on P2-P3 flattened cortex of untreated (full term) and P3-P4 flattened cortex of preterm pups. In untreated condition, clustering of TCA revealing barrel field maturation is observable between P2 (n=6) and P3 (n=12) or between corresponding postnatal stages P3 (n=6) and P4 (n=7) in preterm condition. **(D)** Localization of CTB injection sites at P7 in untreated (n_{control}=6; n_{mutant}=5) and preterm (n_{control}=10; n_{mutant}=10) conditions on whole brains and flatten cortex showing similar localization in V1 labelled by vGlut2 immunostaining. dLGN: dorsal lateral geniculate nucleus; LP, Lateral Posterior nucleus; Po, posteromedial nucleus; S1, primary somatosensory cortex; SubP, Subpallium; V1, primary visual cortex; VP, ventroposterior nucleus. Scale bars represent 250 μ m.

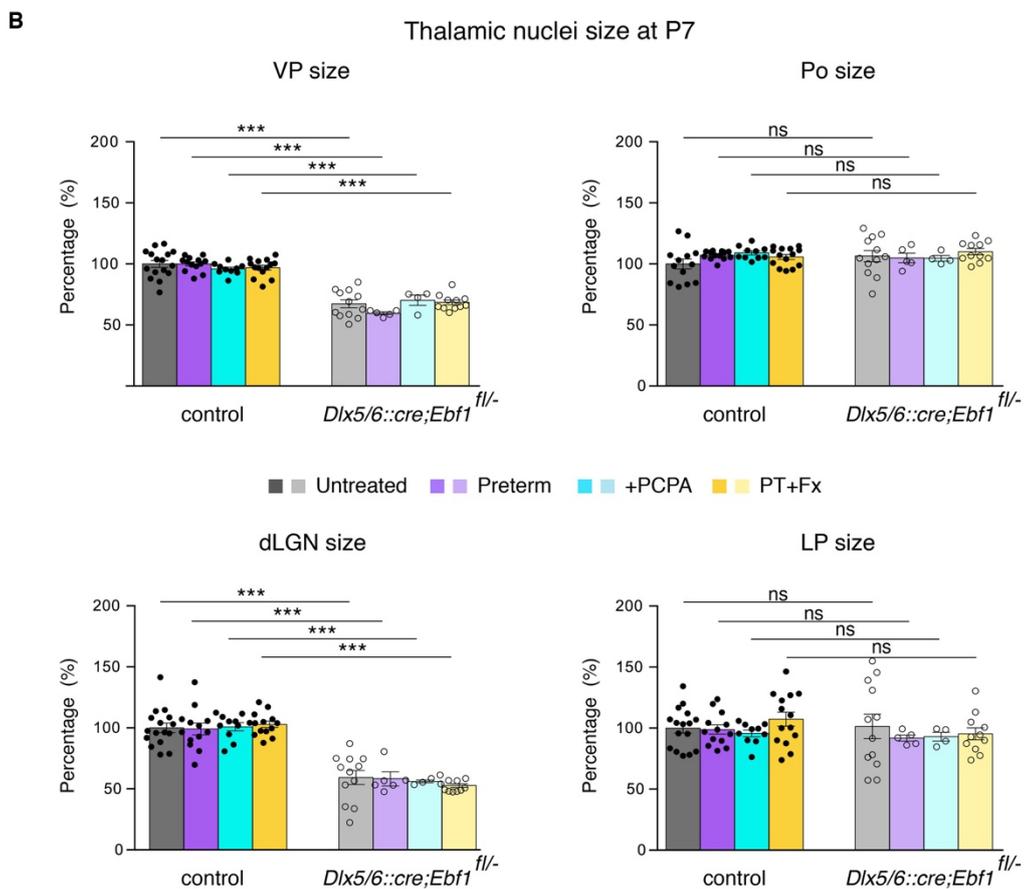
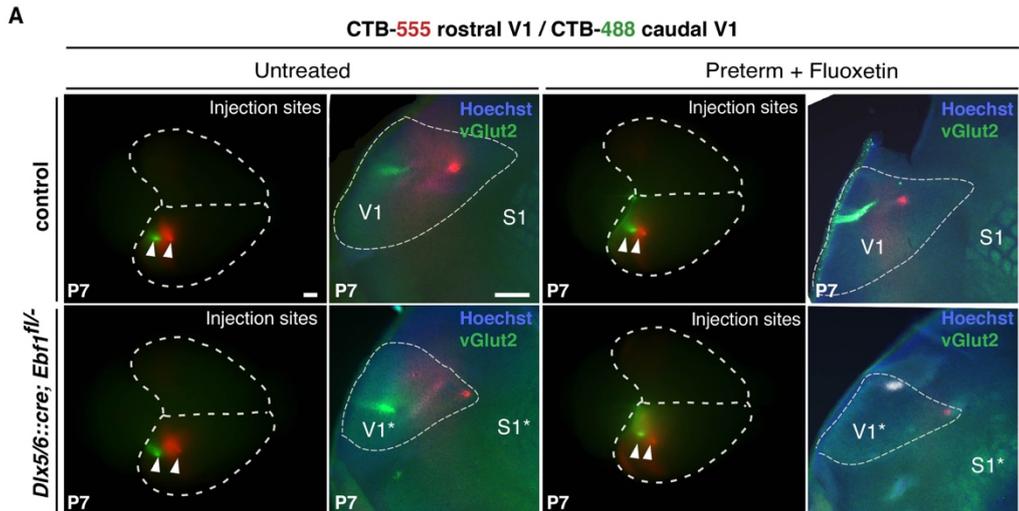


Figure S3: Preterm birth, PCPA or Fx treatments do not affect thalamic nuclei size.

(A) Localization of CTB injection sites at P7 in untreated ($n_{\text{control}}=6$; $n_{\text{mutant}}=5$) and Preterm+Fx ($n_{\text{control}}=12$; $n_{\text{mutant}}=4$) conditions on whole brains and flatten cortex showing similar localization in V1 labelled by vGlut2 immunostaining. **(B)** Quantification

of thalamic size showing that preterm, +PCPA or Preterm+Fx treatments do not impact on FO (VP and dLGN) and HO (Po and LP) representation compared to untreated (Control: $n_{\text{Untreated}}=13$; $n_{\text{Preterm}}=13$; $n_{\text{+PCPA}}=10$; $n_{\text{Preterm+Fx}}=14$ and Mutant: $n_{\text{Untreated}}=12$; $n_{\text{Preterm}}=5$; $n_{\text{+PCPA}}=4$; $n_{\text{Preterm+Fx}}=11$). Values are presented as mean \pm s.e.m; *** $p<0.001$, 2 ways ANOVA test with Sidak's multiple comparison correction.

dLGN: dorsal lateral geniculate nucleus; LP, Lateral Posterior nucleus; Po, posteromedial nucleus; S1, primary somatosensory cortex; V1, primary visual cortex; VP, ventroposterior nucleus. Scale bars represent 250 μm .

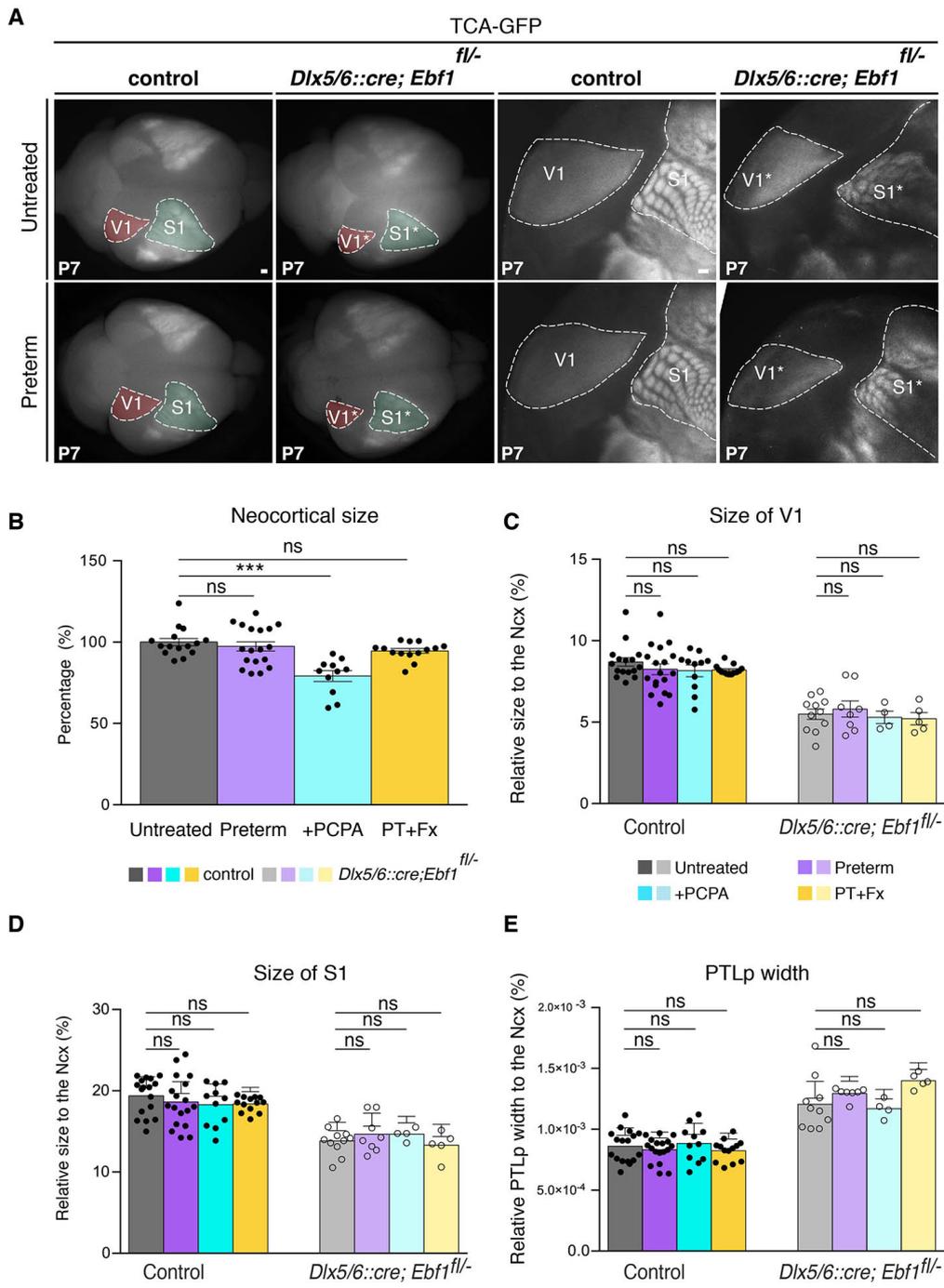


Figure S4: Preterm birth, PCPA or Fx do not affect primary areas size at P7.

(A) Use of TCA-GFP transgenic mice to show at P7 in whole brain and flatten cortex that preterm birth does not affect in control conditions the size areas representation of V1 /S1 and does not enhance the defect in *Dlx5/6::Cre;Ebf1^{fl/-}*. **(B)** Quantification of the neocortical size in control untreated, preterm, +PCPA or PT+Fx conditions. PCPA injection affects the global size of the brain while Preterm or PT+Fx does not impact

($n_{\text{Untreated}}=15$; $n_{\text{Preterm}}=18$; $n_{+\text{PCPA}}=11$; $n_{\text{Preterm}+\text{Fx}}=14$). **(C)** Quantification at P7 of the cortical relative size of V1 representation for each condition (Control: $n_{\text{Untreated}}=16$; $n_{\text{Preterm}}=18$; $n_{+\text{PCPA}}=11$; $n_{\text{Preterm}+\text{Fx}}=13$. Mutant: $n_{\text{Untreated}}=11$; $n_{\text{Preterm}}=8$; $n_{+\text{PCPA}}=4$; $n_{\text{Preterm}+\text{Fx}}=5$). **(D)** Quantification at P7 of cortical relative size of S1 representation for each condition (Control: $n_{\text{Untreated}}=17$; $n_{\text{Preterm}}=18$; $n_{+\text{PCPA}}=11$; $n_{\text{Preterm}+\text{Fx}}=13$. Mutant: $n_{\text{Untreated}}=11$; $n_{\text{Preterm}}=8$; $n_{+\text{PCPA}}=4$; $n_{\text{Preterm}+\text{Fx}}=5$). **(E)** Quantification at P7 of cortical relative size of PTLp for each condition. Control: $n_{\text{Untreated}}=17$; $n_{\text{Preterm}}=18$; $n_{+\text{PCPA}}=11$; $n_{\text{Preterm}+\text{Fx}}=13$. Mutant: $n_{\text{Untreated}}=10$; $n_{\text{Preterm}}=7$; $n_{+\text{PCPA}}=4$; $n_{\text{Preterm}+\text{Fx}}=5$. Values are presented as mean \pm s.e.m; *** $p < 0.001$, 2 ways ANOVA test with Sidak's multiple comparison correction.

PTLp: Posterior Parietal Cortex; S1, primary somatosensory cortex; V1, primary visual cortex. Scale bars represent 250 μm .

RÉSUMÉ FRANÇAIS :

Article 2 : Participation dans une publication distincte.

Titre: Une période sensible régulée par la naissance permet une plasticité inter-zonale des axones thalamiques

Résumé: Les zones primaires du néocortex reçoivent des entrées sensorielles par l'intermédiaire d'axones thalamocorticaux (TCA) spécifiques à chaque modalité. Alors que les TCA sont orientés vers leurs cibles au cours de l'embryogenèse, ils ont la capacité frappante de se réorienter après la naissance pour remédier à une erreur de ciblage précoce. La façon dont ce processus adaptatif remarquable est régulé reste largement inconnue. En utilisant un modèle de souris mutante dans lequel la trajectoire embryonnaire des TCA est décalée, nous avons découvert que le recâblage des TCA se produit pendant une courte période postnatale, précédée d'une apoptose thalamique prénatale, conduisant à la formation d'aires sensorielles primaires réduites. En outre, nous avons montré que la plasticité de la TCA et l'affinement ultérieur des limites des aires primaires sont entravés par la naissance prématurée, qui agit au niveau moléculaire par la régulation de la sérotonine. Notre étude définit une nouvelle période sensible à la naissance et à la sérotonine qui permet des adaptations concertées de la TCA aux aires corticales, avec des implications majeures pour notre compréhension du câblage du cerveau dans des conditions physiologiques et prématurées.

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RÉSUMÉ

Le développement du cerveau antérieur repose sur des étapes séquentielles, notamment la morphogenèse, la croissance axonale et la myélinisation, qui peuvent être altérées dans les troubles neuropsychiatriques. Les microglies, macrophages résidents du cerveau et régulateurs de l'homéostasie cérébrale en conditions physiologiques et pathologiques, émergent en tant qu'acteurs clés du développement du cerveau antérieur. La microglie peut adopter différents états cellulaires et transcriptionnels, une caractéristique remarquable initialement associée au vieillissement et aux maladies, mais que l'on retrouve également au cours du développement cérébral normal. En effet, une signature microgliale connue sous le nom de axon tract associated microglia (ATM), a été trouvée dans la microglie postnatale précoce qui régule la myélinisation du corps calleux. Cependant, les rôles de la microglie et les états microgliaux spécifiques potentiels pendant l'embryogenèse restent largement à explorer. Nous avons identifié des microglies amiboïdes de type ATM dans le cerveau antérieur embryonnaire, qui s'accumulent transitoirement et de manière stéréotypée dans des zones sensibles telles que les frontières corticostriatoamygdaliennes (CSA) et corticoseptales (CSB), deux sites soumis à des tensions morphogénétiques. En utilisant un modèle de déplétion transitoire de la microglie et des modèles d'inactivation génétiques, nous avons étudié les rôles de la microglie dans le développement du cerveau antérieur, en nous concentrant sur ces deux frontières corticales. Nous avons d'abord constaté que l'absence de microglies embryonnaires entraîne des lésions cavitaires au niveau de la CSA et de la CSB. Nous avons également montré que la CSA est un site de fragilité développementale et que le profil embryonnaire ATM-like est associé aux dommages mécaniques et à la réparation du tissu cérébral. Au niveau moléculaire, nous avons découvert que le facteur ATM central Spp1/Osteopontine est partiellement requis pour la cohésion tissulaire au niveau de la CSA et de la CSB, ainsi que pour les propriétés de réparation des microglies. Dans l'ensemble, ce travail révèle un nouveau rôle de la microglie embryonnaire dans le maintien de l'intégrité du cerveau pendant la morphogenèse, mettant en relation un état microglial spécifique avec la cohésion tissulaire et apporte des clés de compréhension des fonctions physiologiques et pathologiques des microglies.

MOTS CLÉS

Microglie, Cerveau antérieur, Intégrité tissulaire, Morphogenèse, Neurodéveloppement

ABSTRACT

Forebrain development relies on sequential developmental steps including morphogenesis, axonal growth and myelination, which can be altered in neuropsychiatric disorders. Microglia, the brain resident macrophages and regulators of brain homeostasis in health and disease, are emerging as key actors of forebrain development. Microglia can adopt various cellular and transcriptional states, a remarkable feature initially associated to ageing and disease, but also found in normal development. Indeed, a microglial signature known as axon tract associated microglia (ATM), was found in early postnatal microglia that regulate myelination of the corpus callosum. However, the roles of microglia and potential specific microglial states during embryogenesis remain largely to be explored. We identified ATM-like amoeboid microglia in the embryonic forebrain, which transiently accumulate at stereotyped hotspots such as the corticostriatoamygdalar (CSA) and the corticoseptal boundaries (CSB), two known sites of morphogenetic stress. Using transient microglia depletion and genetic models, we explored the roles of microglia in forebrain development, focusing on the two ATM-hosting cortical boundaries. We found that the absence of embryonic microglia leads to cavitory lesions at both the CSA and CSB. We further showed that the CSA is a site of developmental fragility and that the embryonic ATM-like profile is linked with mechanical damage and tissue repair. At the molecular level, we found that core ATM factor Spp1/Osteopontin is partially required for tissue cohesion at the CSA and CSB, as well as for the repair properties of microglia. Taken together, this work reveals a novel role for embryonic microglia in the maintenance of brain integrity during morphogenesis, linking a specific microglial state with tissue cohesion with major implications for our understanding of microglial functions in health and disease.

KEYWORDS

Microglia, Forebrain, Tissue integrity, Morphogenesis, Neurodevelopment

