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Serotonin & developmental axonal refinement : microglia contribution ?

Marta Kolodziejczak

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Marta KOŁODZIEJCZAK

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**SEROTONIN & DEVELOPMENTAL AXONAL REFINEMENT:
MICROGLIA CONTRIBUTION?**

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Examining committee members:

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Etienne Audinat	Referee
Alexandre Dayer	Referee
Sonia Garel	Examiner
Anne Roumier	PhD advisor
Luc Maroteaux	PhD director

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List of acronyms

ATP: Adenosine triphosphate	MHC class I: major histocompatibility complex class I
BDNF: Brain derived neurotrophic factor	MHC II: Histocompatibility complex class II
cAMP: Cyclic adenosine monophosphate	NGF: Nerve growth factor
CNS: Central nervous system	NO: Nitric oxide
CR3: Complement receptor 3	P: Postnatal day
CSF1: colony stimulating factor 1	PCPA: (<i>para</i> -chlorophenylalanine), Blocker of 5-HT synthesis through inhibition of Tph2
CSF1R: colony stimulating factor receptor1	PKC: Protein kinase C
CX3CL1, FKN: Fractalkine	Poly I:C: Synthetic analog of viral double-stranded RNA
DAG: Diacylglycerol	RGC: Retinal ganglion cell
dLGN: Dorsal lateral geniculate nuclei	ROS: Reactive oxygen species
DT: Diphtheria toxin	Runx1: Runt-related transcription factor
DTR: Diphtheria toxin receptor	SERT: Serotonin transporter
E: Embryonic day	TGF β : Transforming grow factor beta
FKN, CX3CL1: Fractalkine	TLR: Toll-like receptor
GFP: Green fluorescent protein	TPH: tryptophan hydroxylase
Iba1: Ionized calcium-binding adaptor molecule 1	TTX: Tetrodotoxin, a voltage-gated sodium channel blocker
IGF1: insulin-like factor 1	TXF: 5-OH-Tamoxifen
iNOS inducible nitric oxide synthase	YFP: Yellow fluorescent protein
LPS: Bacterial endotoxin lipopolysaccharides	
MaoA: monoamine oxidase A	

Abstract

Maturation of functional neuronal circuits during development of the central nervous system relies on sophisticated mechanisms. First, axonal and dendritic growth and synapse elaboration should reach appropriate targets. Second, pruning and neuronal death are required to eliminate redundant or inappropriate neuronal connections. Serotonin, besides its functions as a neurotransmitter, actively participates in postnatal establishment and refinement of brain wiring in mammals. Another important role is played by the brain resident macrophages, microglia, in developmentally-regulated neuronal death as well as in synaptic maturation or elimination.

Interestingly, the unpublished laboratory data show a major expression of the serotonin 5-HT_{2B} receptor by postnatal microglia, suggesting that serotonin could participate in temporal and spatial synchronization of microglial functions.

During my thesis, I tested the hypothesis of cross-regulations between microglia and serotonin during postnatal brain development in a mouse model. First, I investigated whether some developmental steps known to be controlled by serotonin, could potentially result from microglia sensitivity to serotonin. Using an *in vivo* model of synaptic refinement during early brain development, the maturation of retinal projections to the thalamus, I observed that *Htr2B*^{-/-} mice present anatomical alterations of the projecting area of retinal axons into the thalamus.

Parallely, I tested the effects of serotonin on microglial cells. Although neither chemotaxis nor phagocytosis assays have revealed any serotonin effect on microglia *in vitro*, a local delivery of serotonin attracted microglial processes on acute brain slices (two-photon microscopy). Indeed, we observed that microglial processes moved rapidly toward the source of serotonin in wild-type mice, but not in *Htr2B*^{-/-} mice lacking the 5-HT_{2B} receptor. Moreover, after comparing mRNA expression level in microglial primary cultures, we have found that some activation markers are upregulated in microglia from *Htr2B*^{-/-} supporting the hypothesis that serotonin modulates developmental microglia via 5-HT_{2B} receptors.

In the second part of my PhD, by using a number of conditional *Htr2B*^{-/-} mice, I investigated which cell type(s) could be responsible for the altered segregation of retinal axons in the thalamus of the total *Htr2B*^{-/-} mice. The analysis of Pet1-Cre mice in which the 5-HT_{2B} receptor is absent specifically in the serotonergic raphe neurons have not revealed any significant abnormalities. The investigation of the involvement of the microglial 5-HT_{2B} in this process is still ongoing.

Overall, my results support the hypothesis that serotonin interacts with microglial cells and that these interactions could participate in brain maturation.

A - INTRODUCTION

A1 Microglia

A1.1 Microglia History

The word *glia* comes from Greek *γλία* which means *glue*. Glia have been described for the first time in 1856 by a German pathologist Rudolf Virchow. He referred to glia as “neuronal glue”, considering them just as a brain filling tissue that keeps the neurons together (Fields 2009). In the early 20th century, Pío del Río Hortega, a student of the famous histologist Ramon Cajal, has reported the existence of three types of glia: astrocytes (Figure 1 A), oligodendrocytes (Figure 1 C), and microglia (Figure 1 B). However, research over the past decades led to the extension of the glial family to include the polydendrocytes (NG2 cells) (Nishiyama et al. 2009).

After a long time during which glial cells have been neglected, their golden time in science has arrived. Although, the glial research is still very young, and we are far from fully understanding glial functions, these cells have already been shown to be in charge of brain development, support and defense.

The proportion of glial to neuronal cells has been a matter of debate for a long time. It varies among species, and also depends upon the quantification method. For example, until recently, it was accepted that glial cells represent even 90% of the human brain, and that this large amount is due to its important size comparing to other species (Allen and Barres 2009). Today however, a novel isotropic fractionator method quantifying proportions of different brain cells simply by using brain cell nuclei suspension, allowed to estimate that the human brain consist 50% of glial cells, whereas mouse brain of 35% (Herculano-Houzel 2014).

Moreover, scientists argue that the glial to neuronal cells ratio is not necessary higher in larger brains but it is rather correlated with lower neuronal density (Herculano-Houzel 2014).

The microglial cells, which were already distinguished by Río Hortega in 1919 as the ‘third element’, are different to neurons and macroglia (astrocytes and

oligodendrocytes). As the name suggests, microglia have much smaller cell bodies than other members of the glial family (Figure 1). Although, these resident brain macrophages are best known for their role in the adult brain (discussed in the chapter: “Microglia in Adult Brain”) recent studies have shown st their involvement in developmental neuronal death, synaptic maturation and establishment of brain wiring.

Before discussing the functions of microglia in adult and developing brains, I will present the current knowledge about their origination from extra-embryonic yolk sac and the transcription factors required for their differentiation.

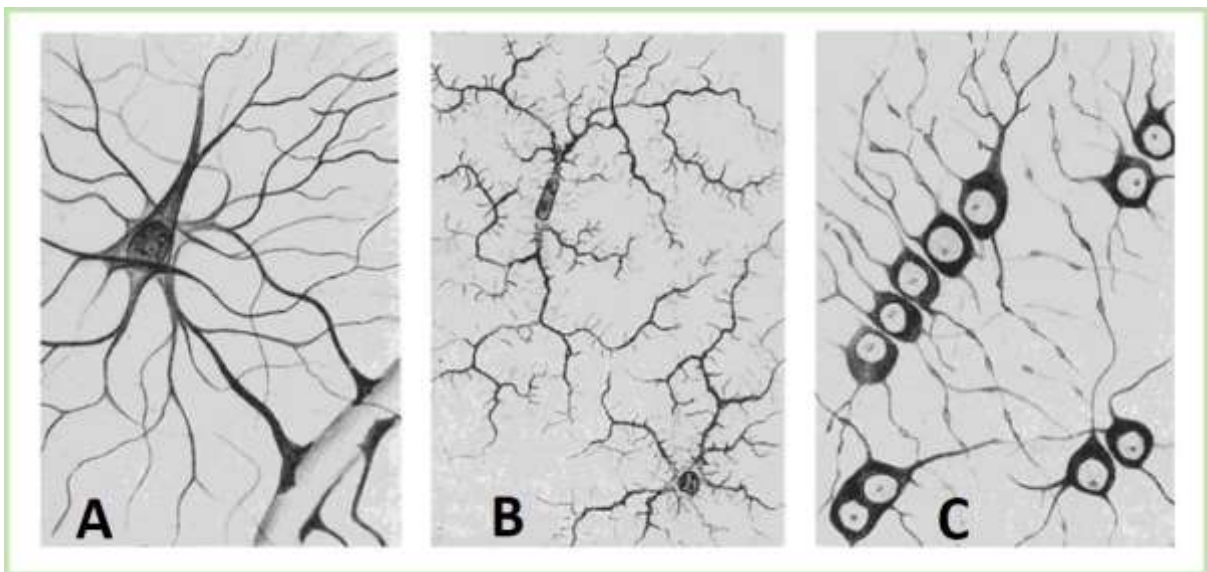


Figure 1: **Different types of glial cells.** A: astrocytes, B: microglia, C: oligodendrocytes. Drawings by Río Hortega, 1920. Source: (Fields 2009)

A1.2 Developmental Origin of Microglia

A1.2.1 Microglia Originate from Extra-Embryonic Yolk Sac

Macroglia (astrocytes, oligodendrocytes and N52 cells), same as neuronal cells, originate from the neuroectoderm. Microglia, however, alike other macrophages, differentiate from hematopoietic precursors originating from the mesodermal tissue. In mouse embryo, there are two distinct waves of hematopoiesis: primitive and definitive. Primitive hematopoiesis takes place in the extra-embryonic yolk sac before E8, whereas

definitive hematopoiesis takes place within the embryo around E10.5 (Ginhoux et al. 2010; Schulz et al. 2012; Kierdorf et al. 2013).

Only recently, generation of a transgenic mouse expressing a green fluorescent protein (GFP) under the control of CX3CR1 promoter (*Cx3cr1^{GFP/+}*), a marker of committed macrophage progenitors, enabled to show that microglia arrive for the first time to the brain through blood vessels around E9.5 (Ginhoux et al. 2010). Before achieving their definitive phenotype in adult animal, microglia are highly proliferative throughout their embryonic life (Ginhoux et al. 2010).

In order to investigate the potential contribution of primitive myeloid precursors to the microglial pool, the Cre recombinase has been expressed under a promoter of runt-related transcription factor (Runx1), which is expressed exclusively in the yolk sac, in mice harboring an YFP reporter construction (Ginhoux et al. 2010). TXF-induced activation of Cre recombinase at E7.5 resulted in YFP expression in 30% of yolk sac macrophages, 30% of microglia and close to 0% of peripheral macrophages. However, when the Cre recombinase has been activated later during embryogenesis there was no YFP positive cells in the brain. Instead, 40% of leukocytes expressed YFP (Ginhoux et al. 2010; reviewed in Prinz et al. 2014). Moreover, this yolk sac cell population of uncommitted stem cells expressing a stem cell growth factor receptor (c-kit), identified in mouse at E8 by flow cytometry experiments, has been reported to represent the earliest potential microglia progenitors (Kierdorf et al. 2013).

Shortly after the formation of blood circulation (around E8.5) the microglial progenitors populate the brain (Kierdorf et al. 2013). These committed developmental microglia express also three other markers: a member of adhesion G protein-coupled receptors family, the F4/80, a subunit of the complement receptor, the integrin CD11b and Runx1 (Ginhoux et al. 2010; Kierdorf et al. 2013; reviewed in Derecki et al. 2014). In the adult brain, the F4/80 and CD11b are upregulated in case of an inflammation.

Another population of the yolk sac-derived progenitors gives rise to a number of tissue macrophages such as liver Kupffer cells, epidermal Langerhans cells or pleural macrophages (Schulz et al. 2012). Definitive hematopoiesis, on the other hand, gives rise to the remaining peripheral macrophages such as circulation monocytes and dendritic cells (Schulz et al. 2012).

A1.2.2 Transition to the Committed Microglia Progenitors

The transition from the uncommitted stem cells to the committed microglial progenitors is regulated by a number of proteins such as Runx1, Pu.1, Irf8 and CSF1R (Figure 2). The absence of one of these proteins results in disturbed microglial differentiation and brain colonization and can have a fatal outcome. For instance, Pu.1 deficient mouse, which lack macrophages, die shortly after birth. The deficiency of interferon regulatory factor (Irf8), which acts downstream of Pu.1, also leads to significant overall decrease in microglia density that persist into adulthood (Kierdorf et al. 2013). Similarly, in mice that lack the colony stimulating factor receptor 1 (CSF1R), microglial cells are missing and the animals die before adulthood. Additionally, the absence of the CSF1R also leads to a severe depletion of most populations of peripheral macrophages. Interestingly, in animals with a null mutation in the main CSF1R ligand, the colony stimulating factor 1 (CSF1 or M-CSF; expressed by the mononuclear phagocytic lineage), the population of the peripheral macrophages is greatly reduced, whereas the microglia are mostly normal. This could be explained by the existence of another CSF1R ligand in the brain. Indeed, an alternative CSF1R ligand, interleukin 34 (IL-34) (produced by neurons), has been proposed to modulate microglia differentiation (Pollard 2009). A more recent study, however, demonstrated that IL-34 is dispensable for developmental microglia and their yolk sac precursors and it is only required for their maintenance in the adult brain (Greter et al. 2012).

Unlike in the case of peripheral macrophages, the expression of transcription factor Myb is dispensable for microglial differentiation.

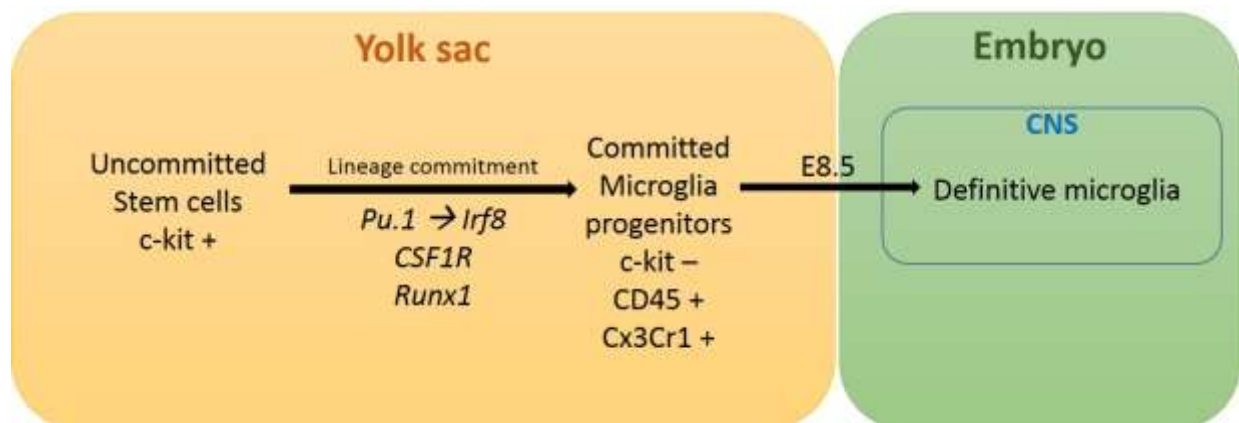


Figure 2: Schematic representation of developmental microglia origin.

The results summarized above show that microglia differentiate early in embryogenesis from yolk-sac derived myeloid progenitors. Their source in the adult brain, however, is still controversial and I will discuss it in the chapter: “Microglia in Adult Brain”.

A1.2.3 Microglia Brain Colonization

As introduced previously, microglial precursors are generated in the yolk sac and start invading the brain shortly after formation of blood circulation. The first microglia precursors arrive to brain at E9.5. These precursor cells have an amoeboid phenotype and accumulate at several sites (e.g. at plexus choroideus and corpus callosum) from where they invade the brain parenchyma. These sites have been first described as “fountains of microglia” by Kershman in 1939 (summarized in Figure 3). Once in brain parenchyma, microglia start to adapt a ramified morphology (Figure 4).

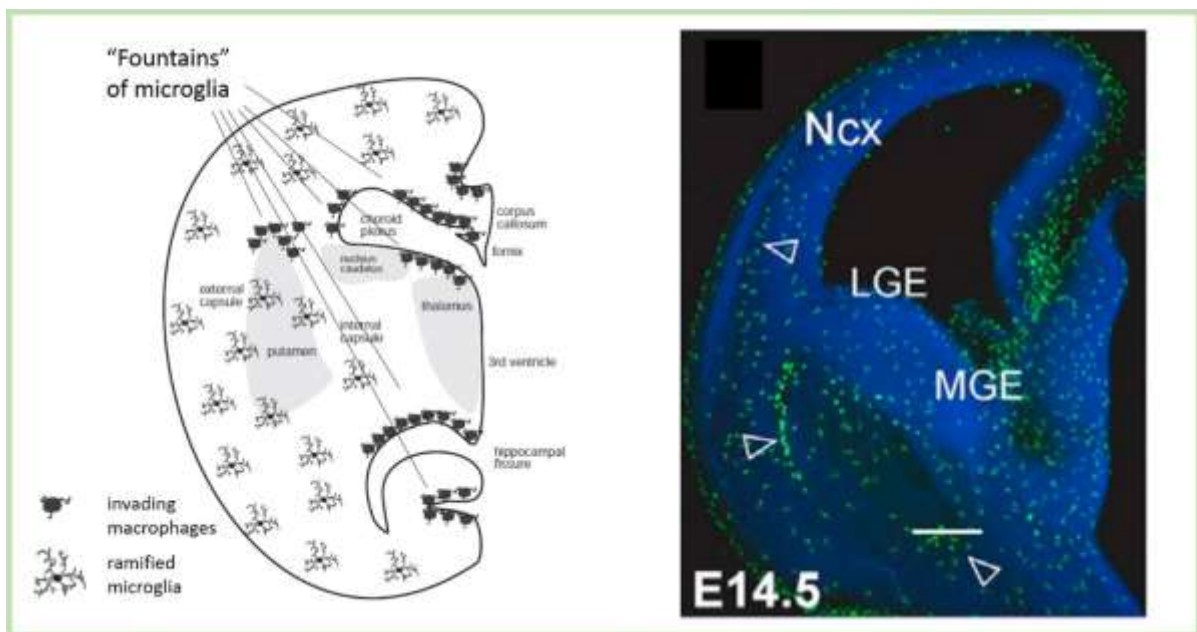


Figure 3: **Microglia colonization of the brain.** On the left: drawing of human embryonic brain and fountains of microglia (from book: “Glial physiology and pathophysiology” 2013, Alexei Verkhratsky, Arthur Butt). On the right: microglial localization in the embryonic mouse forebrain, showing sites of higher concentration of microglial cells (open arrowheads). Scale bar: 500µm. (Squarzoni et al. 2014).

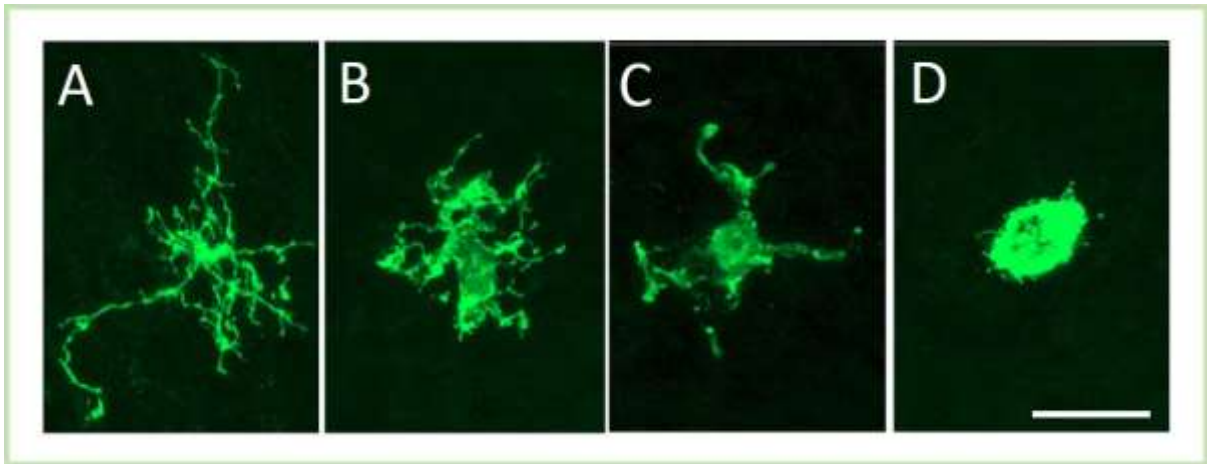


Figure 4 **Classification of developmental microglia morphology** (Iba1 IHC) in the E80 macaque neocortex. Amoeboid microglia (D), ramified microglia (A) and two intermediary phenotypes (B, C). Scale bare: 20 μ m. (Cunningham et al. 2013).

The microglia invasion is a complex process and it remains largely unknown. A very recent study provided some insights into microglia colonization of the mouse cortex. It occurs in three phases. First, from E10,5 to E14,5 a slow increase in microglia number is observed. Next, from E14,5 to E15,5 a rapid phase characterized by a massive increase in microglia takes place. And the last slow phase occurs from E15,5 to E17,5 (Swinnen et al. 2013). At E14.5 amoeboid and more ramified microglia accumulate focally, whereas they are absent in other specific regions such as cortical plate (Arnoux et al. 2013; Squarzoni et al. 2014) (Figure 5). In a timely manner, a progressive invasion of microglia can be observed. However, before achieving their homogenous distribution observed in adult brain, early postnatal microglia are distributed heterogeneously. Until P5, higher densities of microglia have been observed in the vicinity of meninges and ventricular surfaces, whereas there are almost no microglia in cortical layers (Arnoux et al. 2013) (Figure 5).

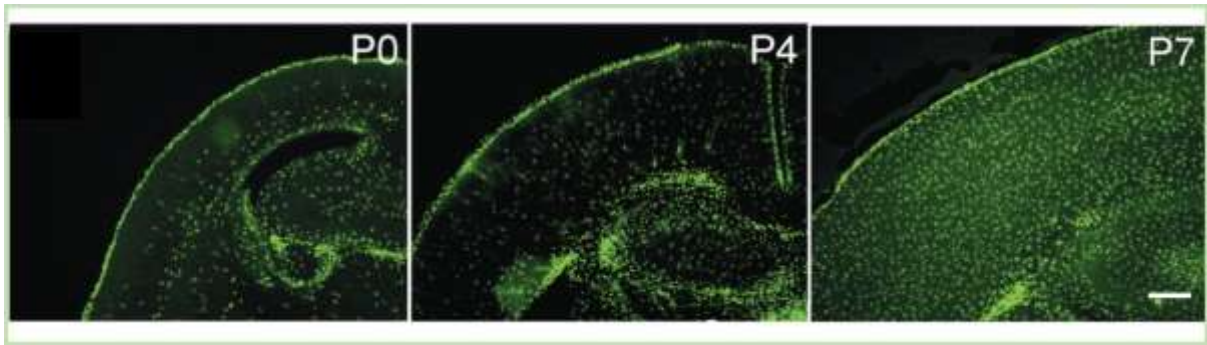


Figure 5: **Postnatal distribution of microglia in the somatosensory cortex** at P0, P4 and P7 of *Cx3cr1^{GFP/+}* mouse. At P0, microglia have an uneven distribution and are notably very rare in the cortical plate. At P4, hotspots of microglia are still visible notably near hippocampal fissure. At P7, the distribution is mostly homogenous. Scale bar: 300 μ m. (Arnoux et al. 2013).

In mouse, the brain colonization by microglia lasts until the closure of the blood brain barrier at around P10 (Verkhratskiĭ and Butt 2013). The gradual increase in microglia density could be explained both by the microglial precursors invasion and by cell proliferation.

A1.3 Microglia in Adult Brain

In this part, I will introduce the role of microglia in an adult brain. First, after explaining their origin, I will present a classification of their phenotypes. Secondly, I will discuss the dynamism of the unchallenged ramified microglia. Next, I am going to review their different activation states and give a brief overview of markers of microglial activation. In the end, I will point out some of the similarities and the differences between microglia and peripheral macrophages.

A1.3.1 Origin of Microglia in Adult Brain

As I have introduced previously, microglia originate from extra-embryonic yolk sac during development. Surgically adjoined parabiotic mice were used in order to investigate the contribution of blood-circulating progenitors to the adult microglia pool. One of these mice had GFP positive hematopoietic cells (except blood cell precursors). The analysis of their brains five months after the surgery have not revealed any GFP

fluorescent microglia (Ajami et al. 2007). This suggests, that in physiological conditions microglial cells are independent from adult hematopoiesis.

Previous approaches to study the origin of microglia in adult brain consisted on a transplantation of GFP positive, mononuclear bone marrow cells into GFP negative mouse (recipient mouse). In order to enable the transplantation, the immune system of the recipient mouse had to be eliminated by irradiation. One of the first experiments of this sort was performed using donor bone marrow cells transduced with a GFP positive vector (murine stem cell virus). Four weeks after transplantation of these cells into an irradiated mouse, a population of ramified GFP fluorescent microglia has been identified in the brain parenchyma. This indicates that, at least after irradiation, adult microglia pool can be replenished from bone-marrow derived progenitors (Priller et al. 2001). A few years later, it has been proposed that the Ly-6^{hi}CCR⁺ monocytic population contains microglial precursors. However, the same study showed that engraftment of circulating microglia progenitors in the healthy brain is dependent on irradiation. The mice with selective-body irradiation (where the head was omitted) were shown to be protected from engraftment (Mildner et al. 2007).

Other experiments have been conducted to investigate whether a drastic increase in microglia number, which is characteristic of many CNS pathologies (injury, disease), is dependent on the peripheral precursors recruitment.

First, five weeks after irradiation/transplantation procedure, an injury (i.e. facial nerve axotomy-induced neurodegeneration) has been inflicted in order to induce a microgliosis in a non-GFP mouse. As a result, around 20% of microglia in the recipient mouse were GFP positive, suggesting that a part of microglial cells originates from blood-circulating microglia precursors (Ajami et al. 2007). Next, a similar injury was inflicted in the recipient mouse after two weeks of parabiosis. Surprisingly, in this model, the injury-induced microgliosis did not result in recruitment of microglia precursors from the circulation (Ajami et al. 2007). The following demonstrates that the GFP positive cells

observed in the brains of transplanted animals originate from immature hematopoietic precursors present in the injected transplantation material, but absent in the blood circulation under physiological conditions (Ajami et al. 2007). These results suggest that the recruitment of GFP positive precursors might be due to the irradiation/transplantation procedure and it does not occur in a physiological situation.

Another approach to study their origin in adult brain consists on inducing microglia repopulation after their pharmacological elimination. Selective CSF1R inhibitor treatment leads to an efficient microglia depletion (most of microglia are eliminated after one week of treatment). Interestingly, microglia repopulation takes place already within one week after stopping drug administration. Further analysis have demonstrated that such a rapid microglia replenishment has been established by a pool of nestin-expressing CNS latent progenitors (Elmore et al. 2014).

In summary, these experiments demonstrate that, in both physiological and pathological conditions, adult microglia pool depends on the self-renewing nestin-expressing CNS latent progenitors, and that it is independent from the bone marrow-derived monocytes and adult hematopoiesis (Ajami 2007, Ginhoux 2010). Throughout life, microglia can use a pool of CNS nestin-expressing progenitors (Elmore 2014, reviewed in Hughes 2014).

A1.3.2 Adult Microglial Phenotype

In adult animals, microglia distribution is homogenous covering the entire brain parenchyma (Figure 6). As discussed before, these brain resident macrophages constitute around 10-30% of total mouse brain cells, depending on the method used for the estimation (Lawson et al. 1992; Herculano-Houzel 2014).

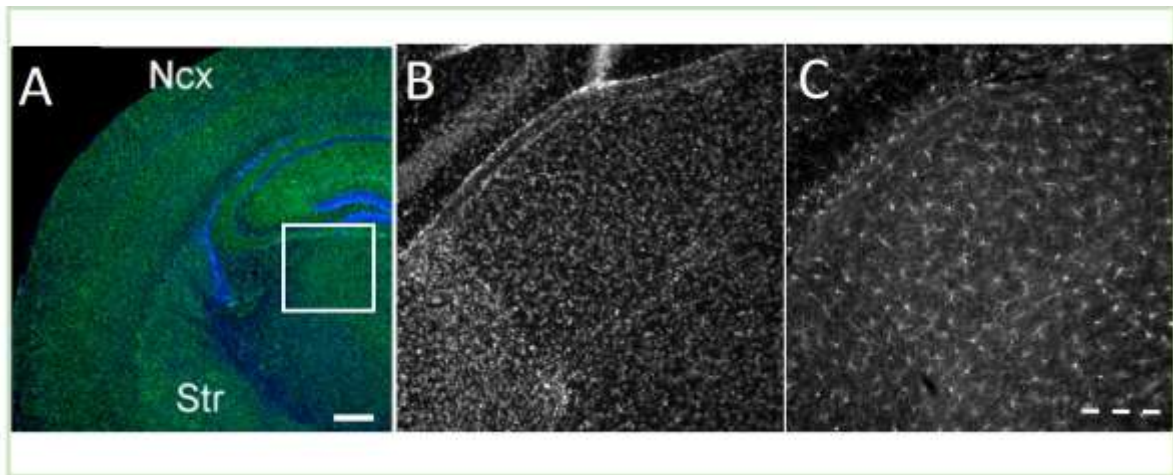


Figure 6: **Homogeneous microglia distribution in adult mouse brain.** A: Coronal section of *Cx3cr1^{GFP/+}* P20 mouse. Green: microglia; Ncx: neocortex; Str: striatum (Squarzoni et al. 2014). B, C: region corresponding to white square on A (thalamic dLGN); B: nuclei stain (Bis-Benzimide); C: microglia stain (Iba1); Scale bare: solid line: 500μm; dashed line (B, C): 200μm.

In physiological conditions, microglia have small soma and long, thin ramified processes (Figure 7). However, in an activated state, their soma might become larger and their processes shorter and thicker. Microglia can also adopt an amoeboid shape resembling peripheral macrophages (Kettenmann et al. 2011)(Figure 7).

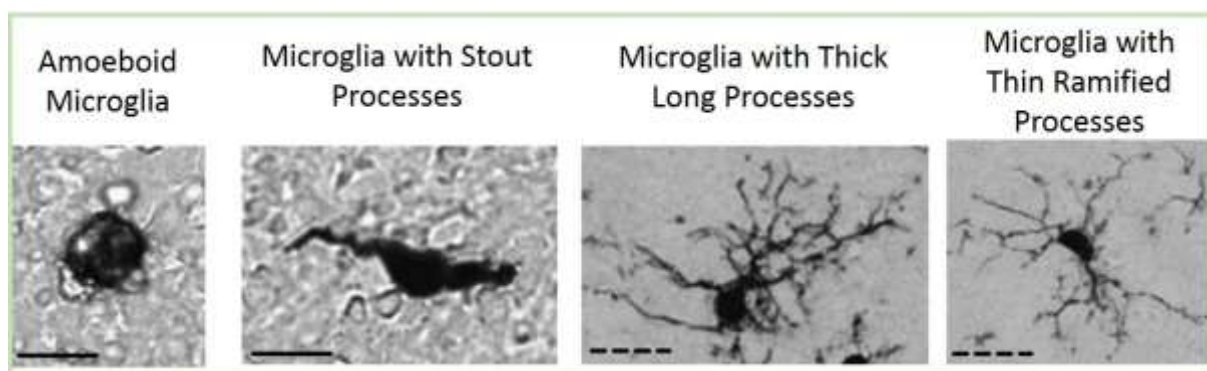


Figure 7: **Classification of microglial morphology** found in adult brain in normal or pathological conditions. Scale bare: solid line: 10 μm, dashed line: 50 μm.

A1.3.3 Microglia in Non-Pathological Conditions

Since there was no direct evidence of movement or activity of ramified microglia in non-pathological conditions, they were long referred to as “resting”. However, their quick transformation in response to even subtle changes of the micro-environment is

contradictory to their quiescent state (Kreutzberg 1996; Nimmerjahn et al. 2005). Only a decade ago, studies using powerful *in vivo* imaging techniques, such as two-photon microscopy, have shown that the ramified microglia are indeed very dynamic. For instance, the time lapse recordings of the brain of *Cx3cx1^{GFP/+}* mouse have demonstrated that, even though the microglial somata are immobile, their processes are very motile. Cortical ramified microglia have been shown to rapidly extend and retract their branches at an average velocity of 1.5 $\mu\text{m}/\text{minute}$ (Nimmerjahn et al. 2005; Davalos et al. 2005). A few years later, another study has shown that the dynamic processes of ramified microglia frequently (once per hour) interact with spines of surrounding neurons, suggesting their ability to carefully monitor brain activity (Wake et al. 2009). Electron microscopy together with three-dimensional reconstructions allowed to visualize appositions of microglial processes with synapses in the visual cortex of a juvenile mouse during normal visual experience (Tremblay et al. 2010). An examination of the optic tectum (brain region responsible for vision reflex) of larval zebrafish has demonstrated that microglia preferentially contact neurons with an increased activity (i.e. in response to a visual stimulation), which in turn leads to a decrease of activity in the contacted neuron (Li et al. 2012). This is summarized in Figure 8.

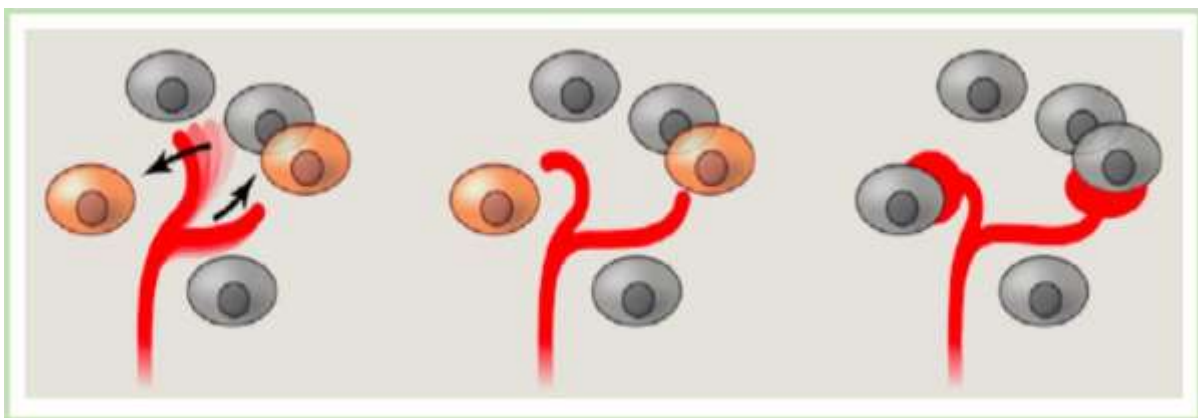


Figure 8: **Microglia control neuronal activity.** Microglial processes (red) preferentially contact more active neurons (orange). This microglia-neuron interaction results in a decrease of activity (grey) of these previously active neurons. (Salter and Beggs 2014).

In summary, these studies clearly show that the ramified microglia are not “resting”. They monitor and control their microenvironment and they stay in a constant dialogue with the surrounding neurons and very probably other neighboring cells.

A1.3.4 Microglia Activation

Alike other macrophages, microglia can quickly respond to changes in their microenvironment. In response to brain injury or infection microglia undergo complex changes including their morphology. Activated microglia can adopt an amoeboid shape resembling peripheral macrophages (Kettenmann et al. 2011) (Figure 7). Their physiologic characteristics and gene expression can also shift to favor e.g. an increased motility and phagocytosis.

Microglial response to injury can be investigated *in vivo* on acute brain slices thanks to two-photon microscopy. Using this technique it has been shown that a laser disruption of blood-brain barrier at the level of a small capillary induces an immediate response of microglia in a radius of 90 μ m. They direct their processes toward the lesion site without translocating their soma. 10 to 15 minutes later, at the site of the lesion, microglial processes form phagocytic spherical engulfments. The number of responding microglia depended on the severity of the injury (Nimmerjahn et al. 2005). Pharmacologically induced hydrolysis of adenosine triphosphate (ATP), a purinergic neurotransmitter, significantly reduced microglial response to the laser injury. Moreover, the same effect has been achieved by administration of an inhibitor of one of the ATP receptors: the P2Y receptor (Davalos et al. 2005). Another study has demonstrated that P2Y₁₂ receptor, which is highly expressed by surveying microglia and downregulated upon microglial activation, is required for early microglial response to injury. Microglia from the P2Y₁₂-deficient mice showed dramatically reduced chemotaxis toward a focal laser ablation *in vivo* and toward ATP *in vitro* (Haynes et al. 2006). Moreover, the P2Y₁₂ receptor has been identified among recently described microglia specific proteins involved in sensing ligands and microbes in microglia environment (so-called microglial “sosome”). These proteins were identified thanks to a novel direct RNA sequencing (DRS) technology which enables robust quantitative analysis of small amounts of RNA without the need for cDNA synthesis or amplification (Hickman et al. 2013).

These examples show that microglia, in response to environmental stimuli, can rapidly switch from their “resting” state and undergo activation. They also show that their response to the laser injury is, at least partially, mediated by ATP.

Different environmental context (i.e. bacterial infection or neuronal death), characterized by different combinations of cytoactive factors, results in a polarized activation of microglia. In parallel to the classification proposed in peripheral macrophages (Mantovani et al. 2002), on the basis of Th1/Th2 lymphocytes classification, two distinct complementary activated states of microglia could be distinguished: classical, M1-like activation, and alternative M2-like activation. This dichotomy has been proposed to describe also neurotoxic and neuroprotective microglia, respectively (Colton 2009). Although this classification is now debated because it is too simple to describe the multiple activation states of microglia. I will describe it briefly.

“Classic” activation generates M1-like microglia, which orchestrate the tissue defense mechanisms. In case of brain injury or infection microglia recognize a pathogen or damage-associated molecular patterns (PAMPs, DAMPs) via their broad range of pattern-recognition receptors (such as TLRs). Upon activation these receptors trigger a microglial signaling cascade resulting in activation of transcription factors and in differential gene expression. The differentiation of M1-like microglia can be triggered experimentally by inflammatory agents such as bacterial endotoxin lipopolysaccharides (LPS), synthetic analog of viral double-stranded RNA (Poly I:C) or IFN γ (Roy et al. 2008; O'Connell et al. 2007). These microglia participate in the “killing phase” of the innate defense of the brain and they are characterized by enhanced antigen presentation and expression of pro-inflammatory and neurotoxic factors such as nitric oxide (NO), TNF α , IL-6, IL-1 β (reviewed in Belarbi and Rosi 2013).

“Alternative” activation generates M2-like microglia, which may be required to terminate the inflammatory response to brain injury or infection. Their differentiation can be induced by anti-inflammatory cytokines IL-4 and IL-13. The neuroprotective function of M2-like microglia is mediated by a panel of anti-inflammatory factors such as IL-4, IL-10, transforming grow factor beta (TGF β), and brain derived neurotrophic factor (BDNF; reviewed in Belarbi and Rosi 2013).

Both, the M1-like and M2-like microglia are equally important and maintaining a balance between these two states is essential for brain homeostasis. Moreover, a disturbance in this balance could be at the origin of numerous brain pathologies. For instance, M1-like

microglia, which normally constitute the first line of defense against pathogens, have been proposed to play a role in neurodegenerative disorders. A recent study has shown that ROS production mediated by microglial NADPH2 enzyme, through a positive feedback loop, activates microglial NFkB and results in persistent neurotoxic microglial phenotype (Qin et al. 2013). Another recent study has demonstrated that decreased microglial proliferation observed in mice lacking galectin-3, a microglia activation marker, results in increased size of an ischemic lesion (Lalancette-Hébert et al. 2012).

These examples clearly show that mechanisms ensuring balance between neurotoxic and neuroprotective microglial states are required. One of such mechanisms involves the alternative arginine pathways. Arginine is a substrate of two distinct enzymes: nitric oxide synthase (iNOS) and Arginase-1. The production of neurotoxic NO is mediated by iNOS, which's expression is upregulated in M1-like microglia. Arginase-1, on the other hand, is an enzyme implicated in cell proliferation and extracellular matrix reconstruction, upregulated in neuroprotective M2-like microglia. Depending on the context one of these two pathways is favored, which creates a balance between neurotoxic and neuroprotective microglial states (Morris et al. 1998; reviewed in Colton 2009).

The M1 and M2-like states constitute the extreme poles along a continuum of activation. There are many more intermediary, parallelly overlapping and complimentary functional microglial states, which share both, inflammatory and anti-inflammatory characteristics.

A recent investigation of microglia at different ages revealed that at any time microglia do not express exclusively M1 or M2 markers and that differential expression of those markers vary with age (Crain et al. 2013).

A1.3.5 Microglia Activation Markers

Switching of microglial functional state does not always require morphological changes. For instance, blood-brain barrier disruption which induces immediate microglia activation and attraction of their processes toward the injured site, does not trigger any major microglia morphological changes (Nimmerjahn et al. 2005). Thus, analysis of morphology alone is often insufficient to identify functional state of microglia. Several

techniques (i.e. immunohistochemistry, FACS, qPCR) allow us to look at the panel of microglial markers, and to better differentiate distinct differentially activated microglial populations. Below, I will present a number of immunohistochemical markers which allow to identify activated microglial cells.

Iba1 (ionized calcium-binding adaptor molecule 1) expression is restricted to monocytic lineage cell line. Indeed, in the brain, microglia are the only Iba1 positive cells (Imai et al. 1996). An upregulation of this marker is induced by activation such as ischemic stroke (Ito et al. 2001) and status epilepticus (Avignone et al. 2008). Another microglial molecule induced upon activation is the beta integrin, CD11b, which together with CD18 forms a heterodimeric complement receptor (named CR3 or Mac1). The markers listed above are present in the unchallenged ramified microglia but their expression level is upregulated in response to an immune challenge. There are also molecules that are absent in basal state and which expression is induced upon activation. For instance, major histocompatibility complex class II (MHC II) is absent in microglia of healthy young rodents. Induction of its expression has been observed in both aged and infected mice. Microglia neither express the galectin-3 (Mac2) in normal physiological conditions. However, its expression has been shown to be required for microglia activation and proliferation in response to ischemic injury (Lalancette-Hébert et al. 2012). Finally, CD68 (or macrosialin) is a cytosolic marker of activated, notably phagocytic, microglia.

A1.3.6 Microglia vs. Macrophages

As I introduced previously, microglial cells are brain resident macrophages. However, despite numerous similarities between microglia and macrophages, there is also an increasing evidence highlighting their unique molecular and functional signature.

On one hand, alike macrophages, microglia can rapidly respond to an immune stimuli: recognize chemotactic and purinergic cues to find the injury site, adapt neuroprotective or neurotoxic phenotype and release inflammatory cytokines, and are able to engulf pathogens via phagocytosis. On the other hand, there is an increasing evidence showing that macrophages and microglia constitute two distinct cell populations. Besides the differences in their developmental differentiation, which I pointed out in one of the previous chapters (“Developmental Origin of Microglia”), they can also be distinguished

by the panel of genes they express. An example illustrating the proportion of similarities and differences between microglia and macrophages is a recent study which compared the top 10% of the most abundant transcripts of microglia with these of peritoneal macrophages. This analysis revealed that 70% of these genes are in both of these cell types, suggesting a substantial number of similarities between microglia and macrophages. The expression levels of the remaining 30% of analyzed genes, however, were dramatically different (fold change ranging from 4.8 to 15.1) contributing to a distinct transcriptomic signature of microglia and macrophages (Hickman et al. 2013).

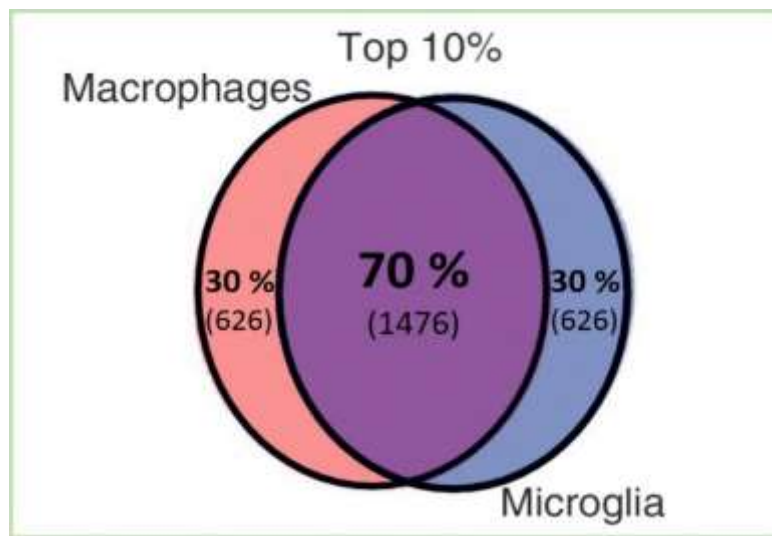


Figure 9: **Proportion of shared genes between microglia and macrophages.** Diagram representing proportion of similarities and differences of the top 10% of transcripts expressed by microglia and macrophages (Hickman et al. 2013).

Another recent study has identified 239 genes and 8 microRNAs which are uniquely or highly expressed by microglia comparing with other myeloid cells (including macrophages). Moreover, this unique microglial signature has been proposed to be dependent on TGF β signaling (Butovsky et al. 2014).

Thus, microglia should be considered “cousins” of macrophages. Despite a shared pool of similarities microglia is a distinct cell type with its own molecular signature.

To summarize, the results presented in this chapter show that adult microglia are able to sense and rapidly respond to changes in the brain microenvironment such as injury, cell death/damage or presence of pathogens. Microglial response to such stimuli is mediated by a wide panel of receptors, including purinergic receptors. Parallely to the

eventual morphological changes, the environmental stimuli might trigger a switch in microglial gene expression profile, increasing their motility or phagocytic capacities. Activated microglia act as double-edged sword: on one hand they can be neurotoxic and actively remove damaged neurons, on the other hand they can secrete neuroprotective and anti-inflammatory factors. A balance between different microglial phenotypes is essential for brain homeostasis.

Additionally to these adult brain functions, microglia might also actively participate in some steps of brain development which I will discuss in the following section.

A1.4 Early Postnatal Microglia

Before discussing microglial involvement in the developmental neuronal death, synaptogenesis and synapse plasticity, I will shortly introduce the phenotype of microglia in an early postnatal brain. Microglial involvement in synaptic pruning and axonal refinement, as well as the behavioral outcome of their alterations will not be discussed here. These subjects will be reviewed in a following chapter (“Developmental Role of Immune System in Psychiatric Disorders”).

A1.4.1 Developmental Microglial Phenotype

In mouse, microglia progenitors populating the brain during embryogenesis have an amoeboid morphology (Hanisch and Kettenmann 2007). Early postnatal microglia share similarities with both ramified and activated microglia. It has been reported that P5 and P9 microglia express CD68, a lysosomes marker specific to phagocytic activated microglia (Figure 10 A, B). CD68 is downregulated in adult P30 ramified microglia (Figure 10 C). On the other hand, P9 developmental microglia have processes characteristic of ramified microglia (Schafer et al. 2012)(Figure 10).

More generally, microglia in developing brain express differentially a number of chemokines-related genes (Kierdorf et al. 2013).

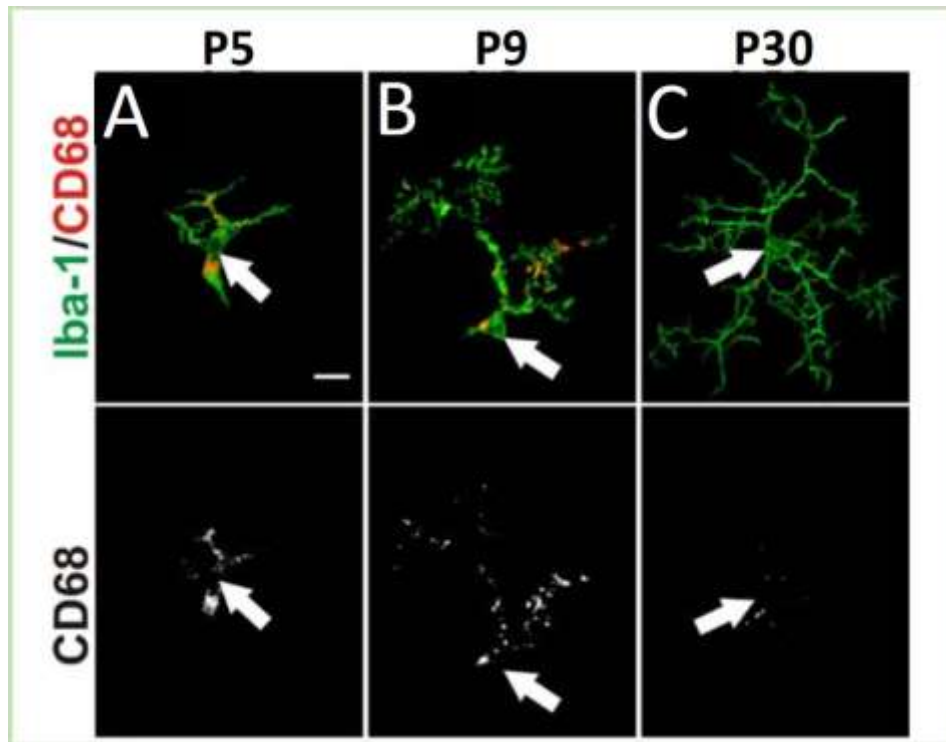


Figure 10: **Developmental microglia express CD68 activation marker.** Green: microglia stain (Iba1); lower panel: CD68 stain; upper panel: merge. Confocal images. Arrows indicate position of cell soma. Scale bar: 10 μ m (Supplementary data Schafer et al. 2012).

Finally, functional electrophysiological studies in mouse cortex have revealed a population of early postnatal microglia which share some characteristics with adult activated microglia. Furthermore, the sharp increase of the number of these cells is observed at the time window of cortex colonization and it is delayed in CX3CR1-deficient mice, in which microglia arrival is retarded. Additionally to their distinguishable electrophysiological properties, these cells also express a particular panel of purinergic receptors. Whereas all of the developing microglia express the P2X purinergic receptors, the heterogeneous expression of P2Y receptors is enriched only in the rectifying microglia population (Arnoux et al. 2013).

These results suggest that developmental microglia are distinct from adult microglia population. Moreover, the existence of differences (such as differential receptor expression) between the developmental microglia supports their putative functions in distinct aspects of brain development.

A1.4.2 Developmental Neuronal Survival

Dynamic changes in cell and synapse numbers during development require rigorous regulation of cell survival. For instance, cell production should ensure but not exceed demand in the developing brain. Furthermore, regulation of cell survival is also essential during maturation of functional neuronal circuits which requires on one hand, axonal and dendritic growth and synapse elaboration with appropriate targets but on the other hand, pruning and neuronal death to eliminate redundant or inappropriate neuronal connections. In the following, I will discuss microglia involvement in this process which has been investigated during the past twenty years.

Microglial production of ROS has been shown to be required for most of the neuronal death in the developing mouse hippocampus. Indeed, CR3, which in the brain is expressed exclusively by microglia, has been demonstrated to act in a convergent pathway with an adaptor molecule, DAP12, to lead to microglial production of superoxide and to induce neuronal death (Wakselman et al. 2008). Moreover, elimination of microglia from organotypic cerebellum slices reduced apoptosis of Purkinje cells, indicating their role in inducing neuronal death in the developing mouse cerebellum (Marín-Teva et al. 2004).

Developmental neuronal death is also observed in the chick retina. Strikingly, a dramatic decrease of neuronal death has been reported in retinas that have been detached from the surrounding tissue before microglia infiltration. Since it was restored by the addition of microglial cells to the retina and since this rescue effect was blocked by nerve growth factor (NGF) antibodies, these results show that the microglia-derived NGF induces developmental neuronal death (Frade and Barde 1998).

Another recent study investigated the role of microglia during perinatal cortical development in macaques and rats. It demonstrated that, early in development, microglia accumulate in the proliferative zones of the cortex where they phagocytose neural precursors and therefore restrain production of cortical neurons (Cunningham et al. 2013) further supporting active microglial contribution to the regulation of developmental neuronal survival.

Moreover, both pharmacologically induced inhibition of microglial activity (with minocycline treatment) and a transient microglia ablation have led to an increased death of cortical neurons in early postnatal mouse brain (Ueno et al. 2013). The microglia ablation was achieved by diphtheria toxin (DT) treatment (at P3) of a transgenic mouse harboring human diphtheria toxin receptor (DTR) under the control of the microglia specific CD11b promoter. The effect of microglia ablation on cortical neurons has been reproduced by inhibition of insulin-like factor 1 (IGF1) signaling. Therefore, since IGF1 is expressed specifically by developmental microglia, these results endorse their survival-promoting effects (Ueno et al. 2013).

The results summarized above support a dual function of developmental microglia which could modulate both, programmed neuronal death and neuronal survival.

A1.4.3 Synaptogenesis, Synaptic Maturation & Plasticity during Development

Developmental microglia function is not limited to the control of neuronal death. Their involvement in synaptogenesis, synaptic maturation and plasticity has also been proposed.

For instance, DAP12 is a transmembrane signaling polypeptide, which was first described in the immune cells, and which expression in the hippocampus is restricted to the perinatal microglia. The absence of DAP12 has been shown to alter the composition of glutamatergic postsynaptic densities and to enhance long term potentiation, suggesting microglial role in synaptic maturation (Roumier et al. 2004).

CX3CR1-deficient animals, which present a delay of microglia arrival to the hippocampus, provide an interesting model to study microglial role in the synaptogenesis, synaptic function and plasticity. In the juvenile (P15) hippocampus of CX3CR1-deficient mice there is an increased number of weak excitatory synapses (Paolicelli et al. 2011). A following study has reported also a decreased functional connectivity between adult brain regions of these mutant mice (Zhan et al. 2014). Moreover, even though in the CX3CR1-deficient mice there was no overall decrease in microglia density in the layer IV of somatosensory cortex, microglia recruitment inside the barrel centers was transiently delayed.

During the maturation of neuronal circuits there is an increase of postsynaptic AMPA receptor proportion and a shift toward GluN2A subunit of NMDA postsynaptic receptors. Both of these changes are impaired in the CX3CR1-deficient animals (Hoshiko et al. 2012) supporting microglia involvement in synaptic maturation.

All the evidence summarized above strongly supports the role of microglia in the synaptogenesis, synaptic function and plasticity.

A1.4.4 Neurogenesis & Plasticity in Adult Brain

Additionally to microglial involvement in the developmental synaptic refinement, there is an increasing evidence supporting their role in the adult neurogenesis and synaptic plasticity, required for neuronal maintenance and learning.

Recently, it has been demonstrated that microglial morphology and the interactions of microglia with synapse-associated elements are modified by visual experience (i.e. light deprivation), supporting the notion of microglial role in modulation of the experience-dependent plasticity in the visual cortex of an adult mouse (Tremblay et al. 2010). Moreover, an *in vivo* time-lapse imaging of ramified microglia in the optic tectum of larval zebrafish have revealed that microglial processes are attracted toward highly active neurons and that neuronal ATP could act as a “find me” signal to guide them. Interestingly, these microglia-neuron interactions result in a reduction of activity of the contacted neurons, supporting the idea that microglia stay in constant dialog with the elements of the surrounding environment (Li et al. 2012).

Another study, showing microglial involvement in adult synaptic plasticity analyzed a model of adult brain microglia depletion. It was achieved by using a transgenic mouse which harbors DTR specifically in microglia. This mouse was obtained by crossing a *Cx3cr1^{CreERT2/+}* mouse, expressing Cre recombinase under the control of *Cx3Cr1* promoter, with a Rosa26-stop-DTR mouse, expressing DTR in presence of Cre recombinase. In this animal, a 5-OH-Tamoxifen (TXF) administration at birth induced activation of Cre recombinase and therefore also the expression of the DTR in all CX3CR1 positive cells (including microglia and peripheral monocytes). 30 days later, the

recombination and thus DTR expression persisted in the long living microglia, whereas it was lost in most of the peripheral macrophages undergoing a rapid turnover. Thus, in these mice, DT injection leads to a specific depletion of microglia. Two-photon imaging has revealed a reduced formation and elimination of dendritic spines on the cortical motoneurons of these microglia-depleted mice. Further analysis of their brains has revealed an altered protein composition of excitatory synapses which has been associated with a decreased spontaneous glutamate release. In the same study, a similar phenotype was observed in mice in which BDNF has been removed specifically from microglia. These results suggest that the microglial involvement in adult synaptic plasticity is BDNF-dependent (Parkhurst et al. 2013). However, the behavioral effects of the microglia depletion in adult brain are still debated. A study reporting an impairment of memory and learning (Parkhurst et al. 2013) is in discord with another study which has not found any behavioral differences in mice where adult microglia were depleted (Elmore et al. 2014). Since in the first study microglia have been depleted during late postnatal period (P19) or young adult (P30) mice (Parkhurst et al. 2013), whereas in the second study only in an adult (P60) mice (Elmore et al. 2014), the observed difference could be possibly due to the age at which microglia were depleted.

An example of microglial role in adult brain plasticity has been also provided by investigation of CX3CR1-deficient mice. It revealed an impaired hippocampal LTP and defects in memory formation. Pharmacological inhibition of the IL-1b receptor corrected this phenotype, suggesting that an increased inflammation is implicated in the synaptic plasticity deficits observed when fractalkine/CX3CR1 signaling is disrupted (Rogers et al. 2011).

Microglia has been also implicated in neurogenesis regulation. The generation of new neurons is not restricted to the developmental period. Indeed, in the hippocampus neurogenesis occurs throughout adulthood. However, only a minority of the newborn neuroblasts in the subgranular zone of the dentate gyrus gives rise to neurons. The vast majority undergo apoptosis and is phagocytized by ramified, unchallenged microglia (Sierra et al. 2010) endorsing their role in adult neurogenesis.

The evidence summarized above demonstrates that microglia are not just a brain “garbage man”, responsible for engulfing neuronal corpses, but that they are also actively

modulating the developmental neuronal death, survival, and synapse formation and function. Moreover, these developmental mechanisms might also underlie the adult plasticity and neurogenesis. In the following chapters we will see that microglia are involved in axonal refinement (see chapter “Immune System & Axonal Refinement”), which could be important for behavior in adulthood (see chapter Developmental Role of Immune System in Psychiatric Disorders”).

A2 Serotonin

Besides microglia, also serotonin has been shown to play a role in the development of functional neuronal circuits. However, before further discussing this subject I will introduce the brain serotonergic system. First I will discuss its anatomy and serotonin biosynthesis. Then, I will introduce brain serotonin receptors and their signaling pathways. Since during my PhD I was investigating the developmental role of 5-HT_{2B} receptor, I will discuss it in more details. In the end, I will give examples of how serotonin could modulate the immune system.

5-hydroxytryptamine (5-HT), commonly known as serotonin, is a monoamine neurotransmitter that has been first identified in 1948 as a vasoconstrictor stored by blood platelets (Rapport et al. 1948). Five years later its presence has been reported also in the brain (Twarog and Page 1953). Serotonin participates in a wide variety of physiological effects including the regulation of intestinal movements, the sleep-wake cycle, pain perception, appetite, and the shaping of emotional and sexual behavior. The pleiotropic effects of serotonin are driven by more than 15 subtypes of its receptors. It has been proposed that serotonin exists since over 750 million years, which could explain such a functional diversity of this monoamine (Peroutka 1995).

A2.1 Anatomy

In the periphery, serotonin is synthesized in enterochromaffin cells of the intestine and it plays multiple physiological roles such as regulation of intestinal movements. Moreover, it is stored in platelets to induce vasoconstriction during clotting. Interestingly, recent studies report presence of serotonin also in immune cells. For instance, human mast cells have been shown to both synthesize and store serotonin, supporting its role in the modulation of the immune system (which I further discuss in the chapter “Serotonin & Immune System Interactions”).

Even though most of serotonin in the body is found in the periphery, as it is stored in platelets, this peripheral serotonin does not enter the nervous system under normal conditions. In the mammalian brain, serotonin is synthesized exclusively in serotonergic

neurons, originating from nuclei of the brainstem. The serotonergic neurons are organized into nine raphe nuclei: B1-B9 (Figure 11 A). Two distinct subdivisions can be distinguished: a rostral division containing nuclei B4-B9 and a caudal division with nuclei B1-B3.

In mouse, serotonergic neurons are generated from E10 to E11. Already one day after, they are able to synthesize serotonin and they start to progressively extend their projections. Serotonergic fibers reach cortex by E14. In mouse brain, a mature pattern of serotonergic axons is achieved by P21 (reviewed in Vitalis et al. 2013; Gaspar et al. 2003).

Early immunohistochemical analysis have revealed that serotonergic fibers originating from the rostral raphe nuclei innervate mostly forebrain targets, whereas the caudal B1-B3 nuclei mainly innervate the spinal cord (Törk 1990). Although, since neurons are often intermingled, the anatomical analysis of serotonin projections were ambiguous. In order to overcome this limitation a novel technique using conditional tracing has been proposed. It consists on injecting viral vectors which express GFP protein under the control of SERT to a small portion of raphe nuclei cells, providing an unambiguous way to determine their targets. Using this technique it has been possible to demonstrate that, for instance, serotonergic neurons originating from B7 terminate, among others, in the thalamic dLGN (Muzerelle et al. 2014)(Figure 11 B).

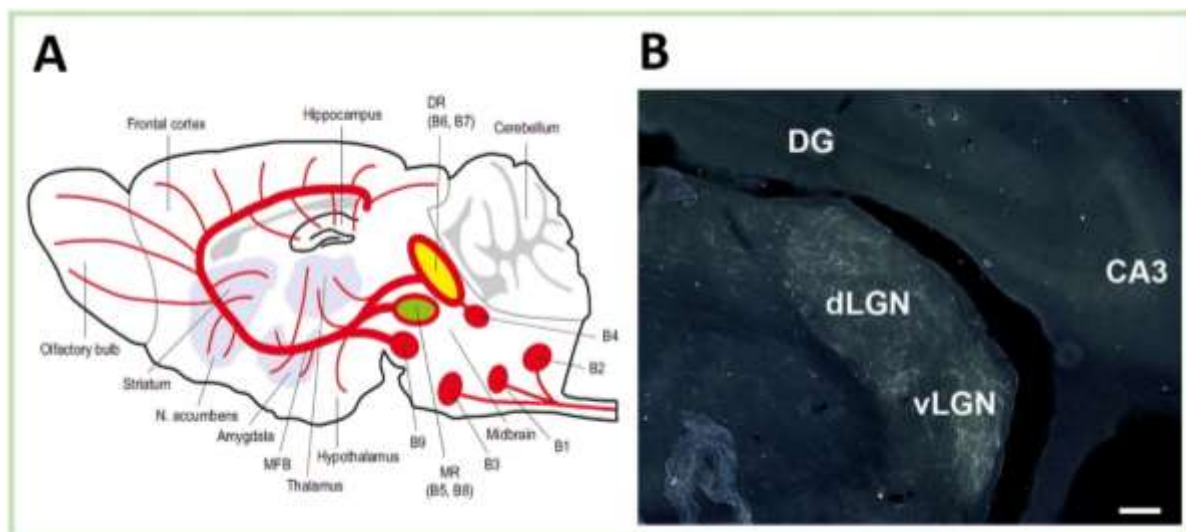


Figure 11: **Serotonergic system in the rodent brain.** A: Schematic representation of raphe nuclei (B1-B9) organization within the brainstem, and of serotonergic fibers distribution (Lesch

2012). B: Projections of serotonergic neurons from B7 raphe nucleus to the thalamic lateral geniculate nuclei. Scale bare: 200 μ m. (Muzerelle et al. 2014).

Additionally to a classical synaptic transmission, where the neurotransmitter released by a presynaptic neuron into the synaptic cleft binds only to the receptors of the postsynaptic neuron, serotonin can also be released in a non-synaptic way from varicosities present along the serotonergic raphe neurons. From there it can diffuse several microns away and activate remote receptor sites by a mechanism called volume transmission (Umbriaco et al. 1995; Bunin and Wightman 1998; Zoli et al. 1999; Daubert and Condron 2010).

A2.2 From Synthesis to Degradation

Serotonin is synthesized from tryptophan, an essential amino acid in animals (Figure 13). There are two enzymes necessary for the 5-HT production: the tryptophan hydroxylase (TPH) and tryptophan decarboxylase (AADC). In mammals, there are two TPH genes, which encode two distinct isoforms of this enzyme. The isoform “1” of TPH is expressed in the periphery (skin and intestine), while isoform “2” is brain specific and expressed in serotonergic neurons of the raphe (Walther et al. 2003; Millan et al. 2008). 5-HT is actively packed into synaptic vesicles by a vesicular monoamine transporter (VMAT2) that transport positively charged serotonin against a proton gradient ($H^+/5-HT$) (Eiden et al. 2004).

The specific serotonin transporter, SERT, uptakes 5-HT from the extracellular environment. The energy to transport the serotonin is generated from the co-transport of sodium ions. Thus, the energy for 5-HT uptake is provided by the Na^+/K^+ ATPase that generates the Na^+ gradient (Figure 12).

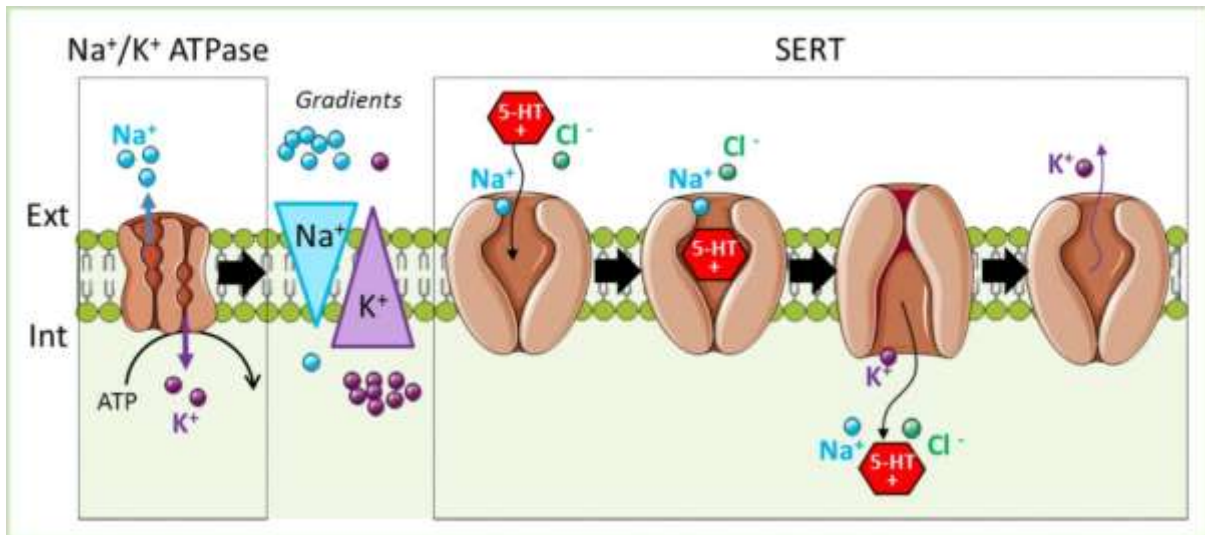


Figure 12: **Serotonin transporter (SERT) functioning.** Serotonin is co-transported with Na⁺, along Na⁺ gradient. Ext: extracellular; Int: intracellular. (From Arnould Belmer).

Additionally to serotonergic neurons, during development, some non-monoaminergic neurons transiently express SERT and VMAT2, and can store and release serotonin (Gaspar et al. 2003). However, being unable to synthesize it by themselves, they use it as a “borrowed neurotransmitter”. Once 5-HT re-entered the cell, it is either recycled and again stored in the synaptic vesicles or degraded. The 5-HT is catabolized by monoamine oxidase A (MaoA), which is localized on the external mitochondrial membrane. The same enzyme is also responsible for degradation of other monoamine neurotransmitters (dopamine, epinephrine and norepinephrine).

As I will show in the following chapters, any disturbance in this fine tuning of serotonin homeostasis could have dramatic consequences, for instance, resulting in inappropriate brain wiring which in turn could increase the risk of neuropsychiatric disorders.

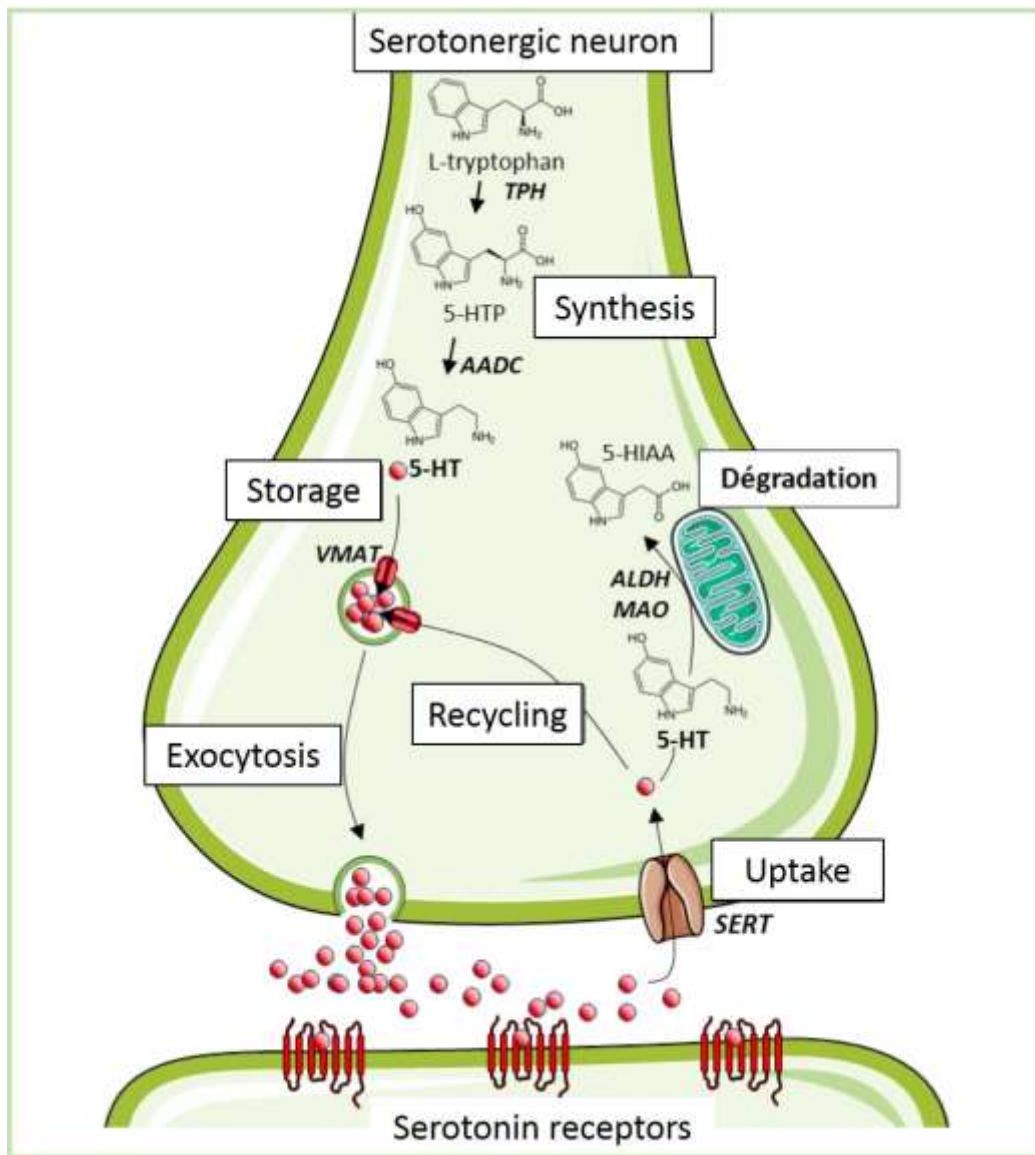


Figure 13: Serotonin metabolism in CNS. (From Arnould Belmer).

A2.3 Serotonin Receptors

Serotonin receptors are classified depending on their structural and pharmacological properties (Peroutka 1995; Choi and Maroteaux 1996). To date, in mammals, there are seven 5-HT

receptor families (5-HT₁ to 5-HT₇). The 5-HT₃ receptors are ligand-gated ion channels which, upon activation, depolarize plasma membrane. All the remaining families are G protein-coupled receptors. These seven-transmembrane domain receptors, which's studying led to the Nobel Prize in Chemistry in 2012 to Robert J. Lefkowitz and Brian K.

Kobilka, constitute an amplifying gate between outside and inside of the cell. Depending on the type of G protein alpha subunit, these receptors activate different signal transduction pathways (Table 1).

The Gi/Go coupled 5-HT₁ and 5-HT₅ receptors inhibit the adenylyl cyclase (AC) and decrease production of cyclic adenosine monophosphate (cAMP) from ATP. In the contrary, the activation of 5-HT₄, 5-HT₆ and 5-HT₇ receptors which are coupled with Gs alpha subunit, increases intracellular cAMP. The family of 5-HT₂ receptors is coupled to Gq/G11 and increases the production of inositol triphosphate (IP3) and diacylglycerol (DAG). These second messengers initiate intracellular calcium increase and activate protein kinase C (PKC) (Table 1) (Millan et al. 2008).

	G protein subunit	Signalling pathway
5-HT ₁ 5-HT ₅	Gi/Go	↓ cAMP
5-HT ₂	Gq/11	↑ IP ₃ , DAG
5-HT ₄ 5-HT ₆ 5-HT ₇	Gs	↑ cAMP

Table 1: Signaling pathways of the various serotonin receptors.

As I was, during my PhD, especially interested in the developmental role of the 5-HT_{2B} receptor, a member of the 5-HT₂ family (next to 5-HT_{2A} and 5-HT_{2C}), I am going to describe it in more details in the following chapters.

A2.3.1 5-HT_{2B} Receptor: Expression

The 5-HT_{2B} receptor has been cloned in rat and mouse in 1992 (Loric et al. 1992; Wainscott et al. 1993; Foguet et al. 1992) and in human in 1994 (Schmuck et al. 1994; Kursar et al. 1994; Wainscott et al. 1996).

The generation of a specific and effective in immunohistochemistry antibody against 5-HT_{2B} receptor has been proven very difficult. This could be explained either by the low 5-HT_{2B} expression in the CNS and/or by lack of high affinity antibodies. A study using the only functional antibody demonstrated 5-HT_{2B} receptor expression in adult mouse stomach, intestine, lung, myocardium and in the cerebellar Purkinje cells (Choi and Maroteaux 1996). Unfortunately, this antibody does not exist anymore.

Nevertheless, mRNA analysis provide some information about gene expression. Indeed, 5-HT_{2B} has been detected in the whole embryo mRNA, demonstrating a peak of its expression at E8-E9, when other 5-HT₂ family receptors are almost absent (Choi et al. 1997). In order to get more precise information about the expression regions a technique of laser cell capturing has been used. It showed 5-HT_{2B} expression in adult rat hippocampus and dorsal raphe (Bonaventure et al. 2002). Even more detailed results were provided by single cell RT-PCR: a recent study has reported 5-HT_{2B} expression in the majority of Pet1 and Tph2-expressing raphe neurons (Diaz et al. 2012).

In situ hybridization (ISH) technique can be used in order to confirm the region of mRNA expression. Whole mount IHC of mouse embryos validated the presence of 5-HT_{2B} transcripts in the neuronal crest cells, heart myocardium and somites (Choi et al. 1997). However, mRNA detection does not guarantee the presence of a functional protein. Thus, binding assays using a radioactive receptor ligand complement the expression data. Indeed, binding experiments on mouse embryo protein extracts confirmed the presence of a functional 5-HT_{2B} receptor at E9 (Choi et al. 1997).

Presence of the 5-HT_{2B} receptor during embryogenesis suggest its developmental role. I will further discuss it in the following chapters.

A2.3.2 5-HT_{2B} Receptor: Pharmacology

Information about the receptor functionality can be obtained by pharmacological analysis.

The 5-HT_{2B} receptor displays high affinity binding to its endogenous ligand, serotonin (K_d ~10 nM). It is also a target of many 5-HT₂ non-selective compounds, metabolites of therapeutic compounds and drugs of abuse. Among its specific agonists we can find BW

723C86, which has been reported to have a 10-fold higher selectivity for the 5-HT_{2B} receptor over the 5-HT_{2C} receptor and a 100-fold higher selectivity over the 5-HT_{2A} receptor. Meanwhile, 2,5-dimethoxy-4-iodoamphetamine (DOI) presents similar affinity to 5-HT_{2A} and 5-HT_{2B} receptors (Knight et al. 2004; Porter et al. 1999). The 5-HT_{2B} receptor can be blocked by high affinity selective antagonists such as RS-127445 (Bonhaus et al. 1999).

These pharmacological tools permit to investigate the specific actions of the 5-HT_{2B} receptor. Comparison of the effects of cell stimulation with a 5-HT_{2B} agonists versus serotonin allows to better distinguish the serotonin effects mediated by this receptor. The use of an antagonists, on the other hand, constitute an alternative to genetic invalidation. However, since the action of pharmacological compounds is often not entirely described and their specificity is limited, the obtained results should be analyzed carefully.

A2.3.3 5-HT_{2B} Receptor: Signaling Pathways

5-HT_{2B} receptor is coupled to Gq alpha subunit and it activates the phospholipase C (PLC) pathway (Choi et al., 1997). As for most of other serotonin receptors, the main signaling pathway is not the only one and it might vary according to the cell type. Indeed, the 5-HT_{2B} receptor has also been shown to mediate serotonin role in cell cycle progression through activation of cyclins E and D1 (Nebigil et al. 2000b). Moreover, its activation leads to the NO production through NOS activation (Manivet et al. 2000), and to arachidonic acid production through activation of phospholipase A2 (PLA2) (Tournois et al. 1998). The signaling pathways possibly initiated by 5-HT_{2B} receptor are summarized in Figure 14.

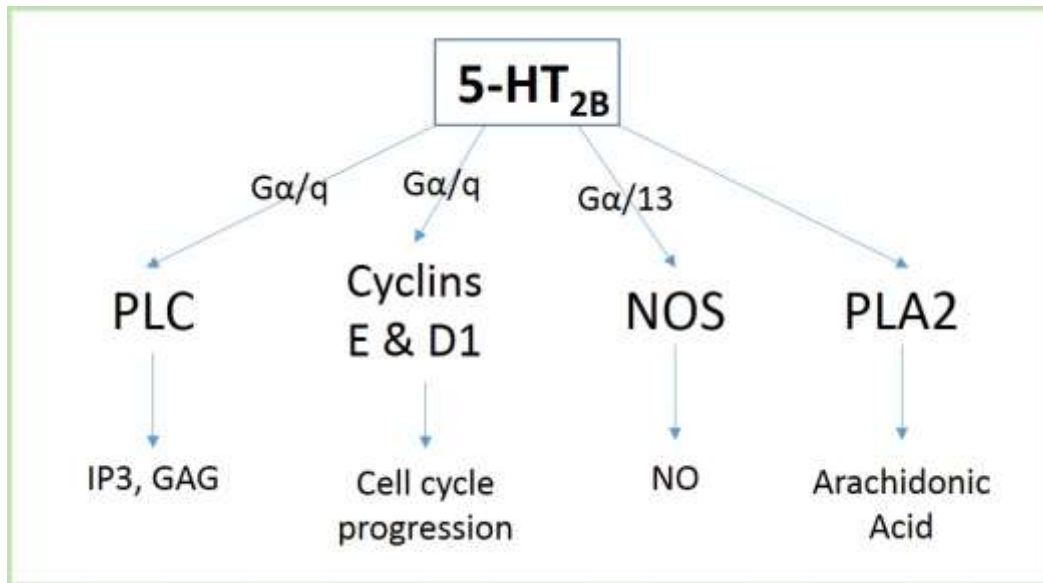


Figure 14: 5-HT_{2B} signaling pathways.

A2.3.4 5-HT_{2B} Receptor: Functions

When embryos were treated with 5-HT_{2B} specific antagonists, it led to morphological defects in cephalic region, neuronal tube and heart, showing that, consistent with its expression time course (see “5-HT_{2B} Receptor: Expression”), the 5-HT_{2B} receptor mediates the serotonin action on embryonic morphogenesis (Choi et al. 1997). 5-HT_{2B} is the only known serotonin receptor which inactivation leads to some embryonic lethality and death in neonates. The 5-HT_{2B}-deficient mouse (*Htr2B*^{-/-}) develops heart malformations (Nebigil et al. 2000a). Recent studies have reported that the physiological and behavioral effects of the psychoactive drug, MDMA (ecstasy), are mediated by the activation of 5-HT_{2B} (Doly et al. 2008; 2009). The anorexic effect of dexfenfluramine, a drug that reduces appetite by an increase in extracellular serotonin level, has also been reported to be mediated by this receptor (Banas et al. 2011). Additionally, 5-HT_{2B}-deficient mice do not respond to serotonin-selective reuptake inhibitors (SSRI) supporting the role of the 5-HT_{2B} receptor in the action of these antidepressants (Diaz Heijtz et al. 2011; 2012). Finally, *Htr2B*^{-/-} mice present an impulsive behavior and are more responsive to novelty compared to wild-type animals (Bevilacqua et al. 2010).

A2.4 Serotonin & Immune System Interactions

A2.4.1 In the Periphery

The first line of defense against a pathogen is assured by the innate immunity. It is constituted by cells such as monocytes, macrophages, dendritic cells, eosinophils, and mast cells. These cells can recognize and quickly respond to pathogens directly at the site of infection. If this defense is not effective to clear the pathogen, an adaptive response takes place. It involves much more pathogen-specific cells, such as T and B lymphocytes, and it takes place in immune organs such as spleen, thymus and lymph nodes.

Serotonin has been shown to modulate inflammation through a set of receptors whose expression pattern varies among cell types. A recent study has demonstrated that human macrophages express different 5-HT receptor levels, depending on their polarization. The 5-HT_{2B} and the 5-HT₇ receptors have been shown to be preferentially expressed by the anti-inflammatory M2-like cells, suggesting the role of serotonin in the polarization of human macrophages. Indeed, serotonin stimulation of these cells triggered an increase in expression of M2 polarization-associated genes and a decrease of the expression of M1 markers. This was shown to be mediated by the 5-HT_{2B} and the 5-HT₇ receptors. Further pharmacological analysis have demonstrated that serotonin, through these receptors, also contributes to cytokine-dependent polarization of human monocytes (las Casas-Engel et al. 2013).

A2.4.2 In the CNS

Much less is known about the effect of serotonin on the CNS immune cells. Very recently, expression of 5-HT receptors by microglia, the brain resident macrophages, has been proposed.

An *in situ* study has reported that serotonin increases microglia motility toward a laser injury. Further analysis showed that serotonin also enhances the chemotactic effect of ATP in a primary cell culture. Moreover, serotonin has been shown to decrease phagocytic capacities of amoeboid microglia on acute brain slices and in a primary cell culture (Krabbe et al. 2012). These results suggest a role of serotonin in modulating the

responsiveness of microglial cells. And indeed, a number of serotonin receptors (5-HT₂, 5-HT₃, 5-HT₅, 5-HT₇) have been detected on these cells (Krabbe et al. 2012).

Another recent study, using murine microglia cell line (BV-2), has also reported expression of serotonin receptors (5-HT₂, 5-HT₄) by microglia. Furthermore, it has been demonstrated that serotonin, via these receptors, mediate microglial exosome secretion and therefore the cytokines release (Glebov et al. 2014).

These results strongly support the role of serotonin in modulation of the immune cells in both the periphery and in the CNS. Moreover, this interaction might not be restricted only to immune functions. The expression of the functional 5-HT receptors on microglial cells could also be required for some steps of brain development.

In summary, there are more than 15 serotonin receptor subtypes. Each of them can act alone or in a synergy with other receptors. The result of their activation is time and place dependent. This great complexity of serotonergic system underlies the pleiotropic effects of serotonin. In addition, since both serotonergic fibers innervation of the CNS and the expression of serotonin receptors occur at an early embryonic age, it came as no surprise that also diverse developmental events depends on a fine tuning of the serotonergic system.

A3 Axonal Refinement

In this chapter I will discuss some of the mechanisms behind the axonal refinement in the early postnatal mouse brain.

First, I will give a definition of axonal refinement and introduce some of the models which are especially useful to study it. As I used, during my PhD, the model of retinal axons segregation in the thalamus, I will explain this model in more details. Then, I will discuss serotonin and microglia contribution to the developmental axonal refinement.

A3.1 Definition of Axonal Refinement

The exuberant perinatal synapse formation results in much more neuronal connections than ultimately will be retained. The subsequent maturation of functional neuronal circuits requires, on one hand, an elimination of early formed inappropriate synaptic inputs and, on the other hand, strengthening of the correct ones (Figure 15). This dynamic process is activity-dependent and requires a bi-directional communication of pre- and postsynaptic elements (reviewed in Cohen-Cory 2002). However, despite scientists' efforts to identify molecules that either modulate the activity or are modulated by the activity (reviewed in Assali et al. 2014), the precise mechanisms behind axonal refinement are still unclear.

During my PhD I investigated the role of microglia, serotonin and their putative interactions in orchestrating some steps of axonal refinement.

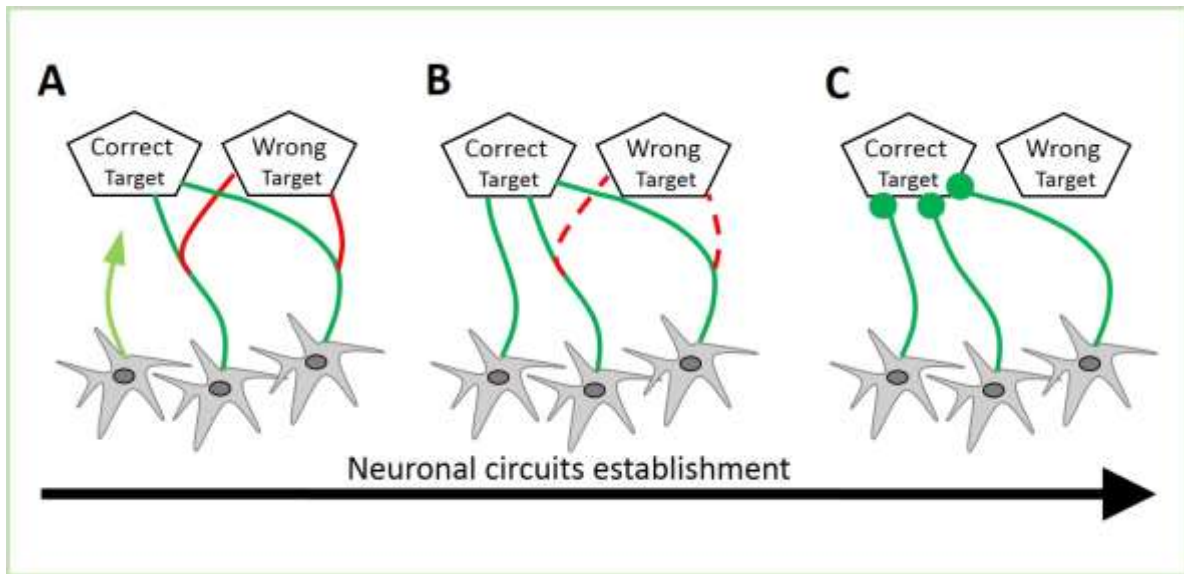


Figure 15: **Main steps of developmental axonal refinement.** A: axonal growth and elaboration of synapses; B: removal of inappropriate synapses; C: strengthening of appropriate synapses

A3.2 Models of Axonal Refinement

The well-described anatomical organization of both visual and somatosensory systems, which are established in the mouse brain during the first postnatal weeks, provides accessible models for studying developmental axonal refinement. On one hand, the retinal axons from both eyes segregate into eye-specific regions in their central targets. On the other hand, thalamocortical fibers carrying information from each given whisker normally terminate in a discrete cluster of layer IV of somatosensory cortex, forming an anatomically distinguishable areas (“barrels”). The analysis of both the segregation of retinal axons in the thalamus and the formation of the cortical representation of the whisker pad are often used to study the involvement of putative mechanisms in the axonal refinement (reviewed in Gaspar et al. 2003). Additionally to the anatomical analysis, the achievement of a mature neuronal circuit can be estimated with electrophysiological techniques which enable to monitor the shift from immature to strong and functional synapses, for instance, in the barrel cortex (Hoshiko et al. 2012) or hippocampus (Zhan et al. 2014). The simple architecture and experimental accessibility of pyramidal synapses are an interesting choice for both electrophysiological (Hsia et al. 1998) and imaging analysis (Cruz-Martín et al. 2010).

All these prominent model circuits are useful to elucidate the universal mechanisms underlying developmental neuronal refinement in the CNS. In the following section of this chapter I will introduce all of them. However, since I used the model of retinal axons segregation in the thalamus during my thesis, I will describe it in more details.

A3.2.1 Barrel Formation in the Somatosensory Cortex

The thalamocortical axons in mice arrive to the layer IV of primary somatosensory cortex early in P0 and they organize into barrel domains between P3 and P5 (Rebsam et al. 2002). In adult rodents, neurons in layer IV densely aggregate as barrel “walls” around the clusters of thalamocortical terminals (Figure 16 A), which can be revealed by simple nuclei stain (Figure 16 C). Since the thalamocortical axons in the barrel cortex are glutamatergic, they can be easily revealed by a stain of vesicular transporter for glutamate (VGlut2; Figure 16 B).

The examination of barrelfield formation and of its cytoarchitecture is extensively used to study developmental axonal segregation.

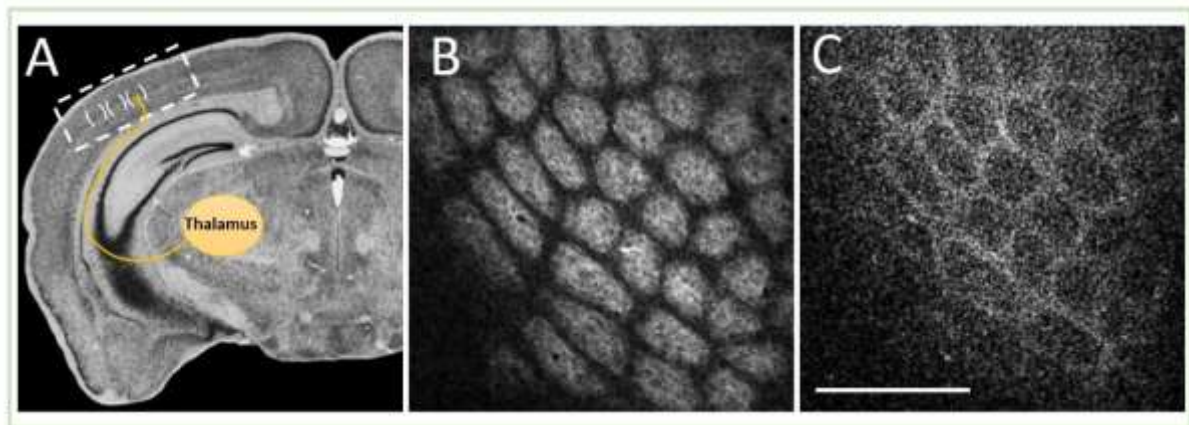


Figure 16: **Barrel cortex.** A: Schematic representation of thalamocortical projections to the barrelfield in the layer IV of somatosensory cortex (inspired by Gaspar 2003); B, C: IHC on flattened cortex B: Vglut2 stain revealing barrelfield; C: nuclei stain (Bis-Benzimide). Image of coronal brain section in A is from Online Mouse Brain Library (www.mbl.org). Scale bare: 450µm.

Another way to assess the maturation level of neuronal circuits using the model of barrel cortex is to examine the complexity of thalamocortical terminals branching. At first, the density of the thalamic afferents arbors is low to achieve a more complex morphology in the adulthood where branches that were not restrained to a single barrel had been

eliminated (Figure 17). A 10-fold increase in number of terminal branches between P1 and P7 has been reported in mice (Rebsam et al. 2002).

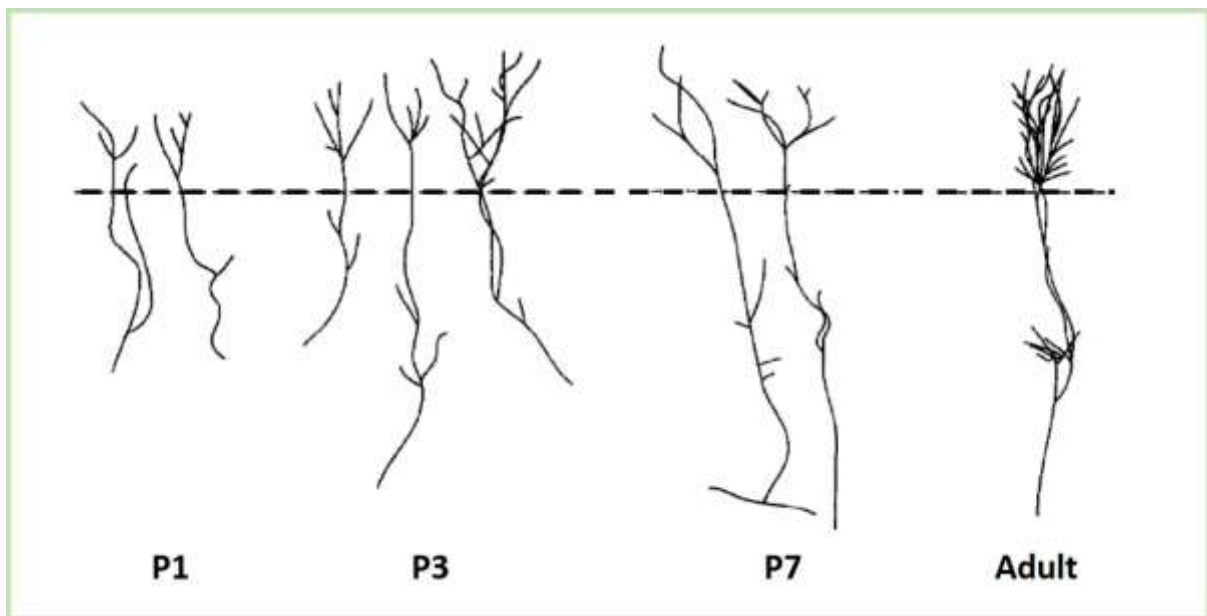


Figure 17: **Terminals of thalamocortical afferents in the rat barrelfield.** The broken line: the lower border of layer IV. (from Killackey et al. 1995)

The thalamocortical neurons are glutamatergic and their synaptic transmission is mediated by NMDA and AMPA receptors. During maturation, NMDA receptor subunits switch from NR2B-dominant to NR2A-dominant. Additionally, the first postnatal week is characterized by the presence of “silent synapses” which contain only NMDA receptors. Later during development the relative amount of AMPA receptor increases and the thalamocortical synapses became stronger (reviewed in Inan and Crair 2007). Using electrophysiological tools, it is possible to examine the functional maturation of synapses between thalamocortical axons and cortical neurons in the barrelfield.

A3.2.2 Maturation of Synapses between Pyramidal Cells

The excitatory synapses made by pyramidal cells, with their simple architecture and experimental accessibility, provide another interesting model to investigate the maturity of neuronal circuits.

Dendritic spines are small protrusions of the dendrite membrane where excitatory synapses are formed. Immature neuronal circuits are characterized by high rate of gain and elimination of these protrusions. In the mouse cortex, the immature filopodia are replaced with mushroom-shaped spines after two first postnatal weeks and their overall dynamics decreases (Cruz-Martín et al. 2010)(Figure 18). Thus, examination of spine morphology provides an information about the maturity of neuronal circuits.

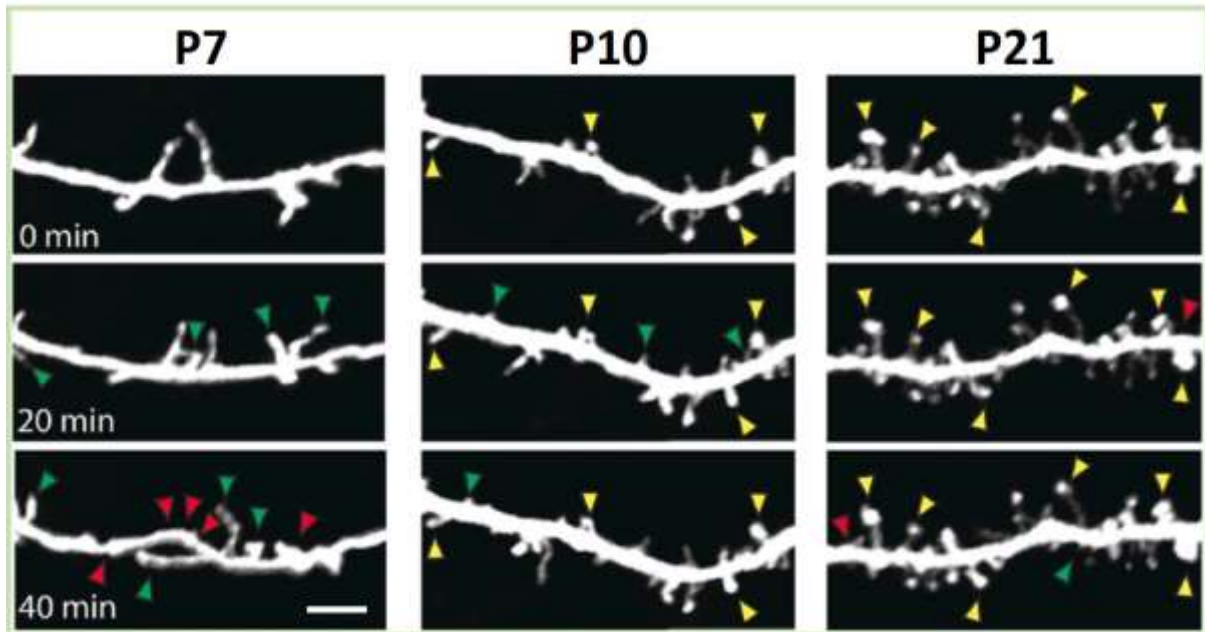


Figure 18: **Dendritic spine dynamics in pyramidal neurons of mouse somatosensory cortex.** In vivo time-lapse two-photon imaging. At P7 the rate of dendritic spine gain (green arrows) and elimination (red arrows) is higher than at P10 and P21 where most of spines are stable (yellow arrows). Scale bare: 5 μ m. (Cruz-Martín et al. 2010).

The electrophysiological examination of hippocampal excitatory synapses made by CA3 pyramidal cells onto pyramidal cells in CA1, complements the morphological analysis of synaptic contacts. For instance, a study comparing the amplitude of the excitatory postsynaptic currents (EPSC) with the amplitude of the spontaneous release, in presence of TTX, of a single vesicles (miniature excitatory postsynaptic currents; mEPSC) into the synapse at different time points of neuronal circuits maturation revealed that the increase in the total number of functional synapses is due to an increase in number of synapses between individual pairs of CA3-CA1 cells pairs rather than an increased number of CA1 cells targeted by a given CA3 neuron. Thus, the developmental consolidation between CA3 and CA1 neuron pairs results from an increased number of

simultaneously releasing synapses per CA3-CA1 cell pair, so-called “multiplicity” (Hsia et al. 1998). This increase in connectivity has been shown to be impaired in mice which lack the microglial CX3CR1 receptor (Paolicelli et al. 2011).

A3.2.3 Segregation of Retinal Axons in Thalamus

In this part I will first describe the segregation of retinal axons in the thalamus and then I will discuss some of the known mechanisms behind this process.

A3.2.3.1 Model Description

Retinal ganglion cell (RGC) axons, originating in the retina, transmit visual information from photoreceptors to their thalamic primary targets, the dorsal geniculate nuclei (dLGN). In mouse, dLGN on each side of the brain contains axonal terminals from both eyes. Just after birth, they are intermingled; the ipsilateral projections are diffused and the contralateral fibers occupy the entire dLGN (Figure 19 Ba). This is due to an excessive number of synaptic connections. During the two first postnatal weeks, a segregation process, which relies on massive, selective synaptic pruning and retraction of ipsilateral and contralateral RGC axon terminals from their inappropriate dLGN regions, results in a “segregated” phenotype. Indeed, in a mature dLGN, the contralateral axon terminals occupy most of its territory except for a small gap containing exclusively fibers projecting from the ipsilateral eye. Both territories are mutually exclusive. These ipsilateral axon terminals are organized into a compact region with a well-defined regular shape (Godement et al. 1984; Upton et al. 1999) (Figure 19 A, Bb). Such a precise eye-specific segregation of the RGC afferents observed within the dLGN of an adult animal is required for binocular vision (Leamey et al. 2007).

This segregation can be visualized in juvenile or adult mouse by ocular injection of a fluorochrome-conjugated B subunit of cholera toxin (CTb), using different color in each eye. 24 to 48h after the injection, CTb has anterogradely traced the RGC axons until their terminals in the dLGN (Figure 19 A).

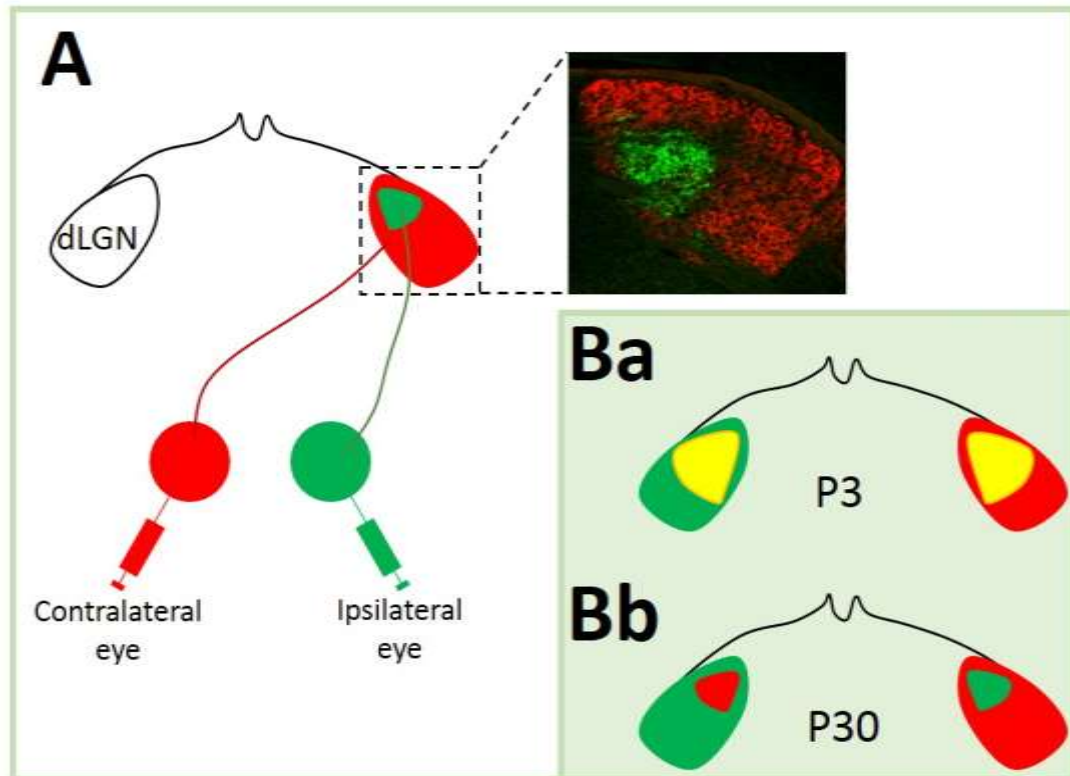


Figure 19: **Visual map formation in the thalamus.** A: RGCs anterograde tracing with CTb to the dLGN; Ba: intermingled RGC axon terminals within dLGN at P3; Bb: segregated RGC axon terminals within dLGN at P30.

A3.2.3.2 General Mechanisms

This surprisingly precise wiring in the visual thalamus is achieved before the opening of the eyes (Upton et al. 1999). It can therefore not be triggered by visual experience. This raises the question of the mechanisms driving the transition from intermingled to segregated projections. RGCs spontaneously fire action potentials prior to the onset of visual experience and this activity has been shown to be involved in the segregation process (reviewed in Assali et al. 2014). Indeed, in rats, when these spontaneous waves are blocked by bilateral injections of a voltage-gated sodium channel blocker, Tetrodotoxin (TTX), the ipsilateral projections are more widespread (Fawcett et al. 1984). Similarly, increasing the amplitude and frequency, with monocular injection of agents elevating cAMP, shows that a stronger eye always occupy more dLGN territory (Stellwagen and Shatz 2002). Thus spontaneous retinal spiking mediate the binocular competition underlying eye-specific segregation. Nevertheless, mechanisms by which the spontaneous activity instructs the axonal refinement remain to be elucidated. The

involvement of serotonin and immune system in developmental axon refinement will be discussed in the following chapter.

A3.3 Serotonin & Axonal Refinement

In this chapter I will discuss some of the evidence showing that serotonin can modulate the developmental axonal refinement. First, I will introduce diverse mouse models that are useful to study developmental serotonin effects. Next, I will discuss the importance of timing for serotonin-dependent circuit maturation.

A3.3.1 Models to Study Serotonin Effects

In order to further investigate the developmental function of serotonin, a number of genetic models targeting genes involved in 5-HT metabolism have been generated.

Two distinct genetic models have been proposed to study the effects of an increased stimulation of brain 5-HT receptors. A mouse in which serotonin catabolic enzyme (MaoA) is removed presents a nine-fold increase in brain serotonin at P1 (Cases et al. 1995; reviewed in van Kleef et al. 2012). On the other hand, the SERT-deficient mouse fails to efficiently remove 5-HT from synaptic cleft, which also leads to an increased extracellular serotonin level. It turns out that an increased 5-HT level causes no striking abnormalities of brain development. However, more subtle effects have been reported.

Both Pet1 and MaoA gene invalidation causes disturbed respiratory rhythms in neonates (reviewed in Gaspar et al. 2003), which suggests a role of serotonin in the maturation of neuronal networks responsible for respiratory control.

A recent study has demonstrated that an excess of serotonin (applied in bath on acute brain slices of E17.5-P0.5 mouse) affects the radial migration speed of the neocortical pyramidal neurons. Since the pyramidal neuron progenitors express 5-HT₆, the authors hypothesized that this receptor could mediate the serotonin effect on radial migration. Indeed, 5-HT₆ agonist treatment had similar effects as serotonin excess, supporting the role of serotonin in this process. Strikingly, the positioning of superficial layer of excitatory pyramidal neurons is altered in the SERT-deficient mice (Riccio et al. 2011).

Furthermore, eye specific segregation of retinal projections in the thalamus also depends on an appropriate serotonin level. Segregation of ipsilateral and contralateral RGCs in dLGN does not occur in neither *MaoA* nor SERT-deficient mice, which present an increased serotonin level (Upton et al. 1999; Gaspar et al. 2003, García-Frigola and Herrera 2010). In these animals the contralateral fibers occupy the entire dLGN and the ipsilateral region is enlarged and more diffused compared with wild-type mice (Figure 20). The alterations associated with an excess of extracellular serotonin could be prevented by early postnatal treatment (P0-P15) with PCPA (Upton et al. 1999). It was also corrected in the double knockout mouse in which both SERT and 5-HT_{1B} genes are invalidated (Upton et al. 2002). Thus, the increased serotonin level has been proposed to inhibit activity dependent segregation mechanisms via the 5-HT_{1B} receptor.

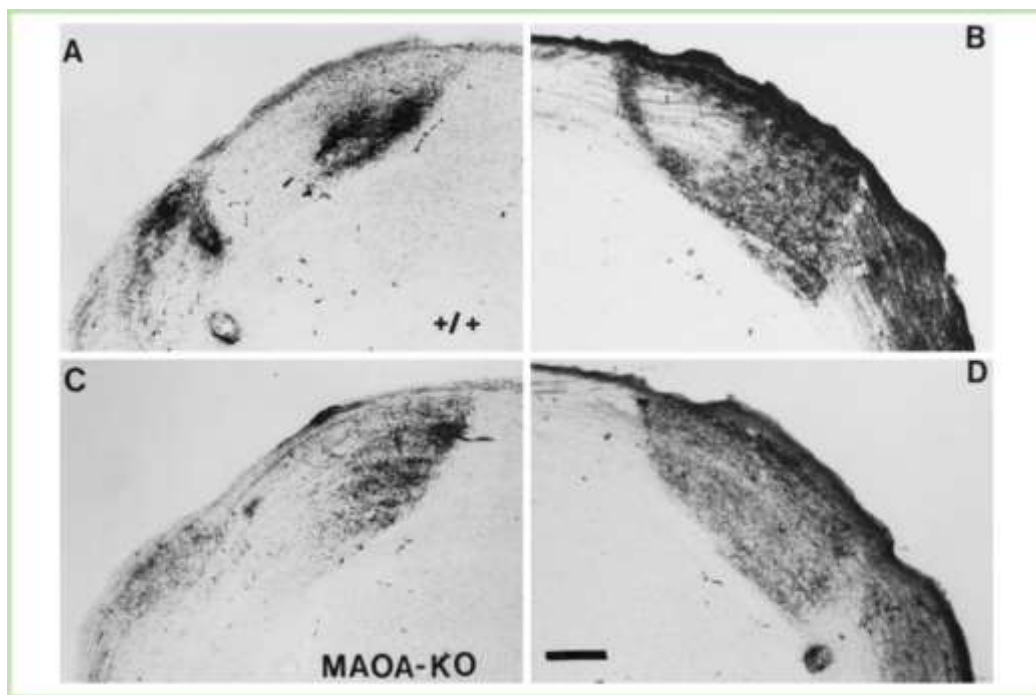


Figure 20: **Deficit of RGC axons segregation in the dLGN of *MaoA*- deficient mouse.** A, B: WT; C, D: KO; left: ipsilateral regions; right: contralateral regions. mouse. Scale bar: 130µm. (Upton et al. 1999).

Developmental maturation of the barrel cortex has also been shown to be modulated by serotonin. Indeed, the barrel cortex does not form neither in *MaoA*-deficient mouse (Cases et al. 1996)(Figure 21) nor in SERT-deficient mouse.

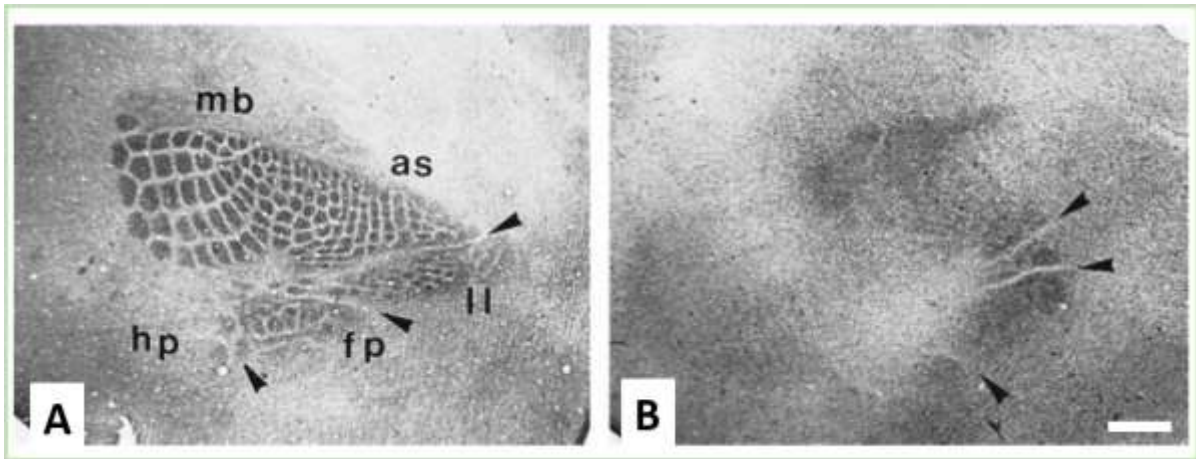


Figure 21: “Barrelless” phenotype in *MaoA*-deficient mouse. A: WT; B: *MaoA* KO. Scale bar: 650 μ m. (Cases et al. 1996).

On the other hand, the effects of serotonin depletion on axonal refinement are not as striking as in the case of serotonin excess (Narboux-Nême et al. 2013; Hendricks et al. 2003). This could be explained by a possible compensation of this deficiency by an exogenous source of serotonin. Indeed, a recent study has demonstrated that both human and mouse placenta can synthesize and supply it to the fetal brain before the generation of serotonergic neurons (Bonnin et al. 2011). Moreover, it is possible that the maternal serotonin also contributes to the fetal serotonin level (reviewed in van Kleef et al. 2012).

A3.3.2 Importance of Timing of Serotonin Action

Previously described defects associated with an increased serotonin level, observed in thalamus and somatosensory cortex of *MaoA* and *SERT*-deficient mice, can be reversed simply by transient administration of PCPA. On one hand, the disturbed segregation of RGC terminals in the dLGN of *SERT* or *MaoA*-deficient mice has been rescued with a P1-P8 and P1-P15 PCPA treatment respectively (Upton et al. 1999; Upton et al. 2002). On the other hand, the PCPA treatment not later than 36h after birth completely restored the normal development of barrelfield in *SERT*-deficient mice (Cases et al. 1996; Persico et al. 2001). These examples demonstrate existence of a very narrow window of time where serotonin action is important for appropriate axonal refinement.

Moreover, even before the establishment of functional serotonergic circuits, serotonin from exogenous sources (mother, placenta) has been detected in the fetal brain. 5-HT neurons, being present already since embryonic day 10 (E10) in mouse brain, are among the first neurons to arise in the developing mouse CNS. As previously introduced, during development, serotonin is also “borrowed” from extracellular environment by a subset of non-monoaminergic neurons, which transiently express a partial serotonergic phenotype. For instance, a subpopulation of ipsilateral retinal ganglion cells (RGCs) has been reported to express SERT between postnatal day 7 and 14 (Upton et al. 1999). Such a transient SERT expression has been also observed in the thalamocortical axons projecting to the barrel cortex (Lebrand et al. 1996). Interestingly, the timing of the transient expression of serotonin transporter in both dLGN and barrelfield coincides with period of developmental axonal segregation in these regions.

Taken together, disturbance of serotonin homeostasis during sensitive developmental periods leads to important deficits of axonal refinement. In one of the following chapters (“Developmental Role of Serotonin in Psychiatric Disorders”) I will discuss the putative consequences of such brain miswiring.

A3.4 Immune System & Axonal Refinement

Researchers have long thought that immune cells played a role in the CNS only in pathological conditions. However, growing evidence during the last couple of decades supports the importance of brain immune system for correct functioning of the CNS in physiological conditions. In this chapter I will discuss immune system elements which have been proposed to be involved in the structural and synaptic brain remodeling through mechanisms analogue to those used in the periphery.

A3.4.1 Mechanisms involving microglia

A3.4.1.1 Complement System

To date, one of the immune elements that is best known for its function during brain development is the complement system. In the periphery, complement proteins are

circulating in the bloodstream “searching” for pathogens to be eliminated. The classical complement pathway is initiated by binding of the Cq1 protein to an antibody (IgG or IgM) that has recognized a pathogen to be eliminated. This triggers a downstream cascade which leads to the opsonization of the unwanted cell with another complement protein: the C3. Macrophages expressing complement receptor recognize C3 protein and phagocyte the pathogen.

During development, complement C1q and C3 proteins are localized to synapses (Stevens et al. 2007). It has been shown that the expression of C1q by RGCs is required for the eye-specific segregation in the dLGN (Stevens et al. 2007). Interestingly, this expression is modulated by a cytokine (a messenger of the immune system). Indeed, blocking the effect of TGF released by astrocytes induced a decreased level of C1q in thalamic RGCs and led to defects of eye-specific segregation in the dLGN (Bialas and Stevens 2013)(Figure 22).

All these results demonstrate the involvement of the complement system in the developmental axonal refinement.

In the brain, CR3 (consists of CD11b and CD18) is expressed exclusively by microglia (Gasque et al. 1998). This suggests that the altered RGCs segregation in the dLGN observed in the complement-deficient mice (CR3 KO) (Stevens et al. 2007) could be caused by defects in the brain resident macrophages. This hypothesis is further supported by the observation that developmental microglia engulf synaptic material in the thalamus and the hippocampus during active synaptic remodeling which endorses their role in the sculpturing of neuronal circuits (Schafer et al. 2012; Paolicelli et al. 2011).

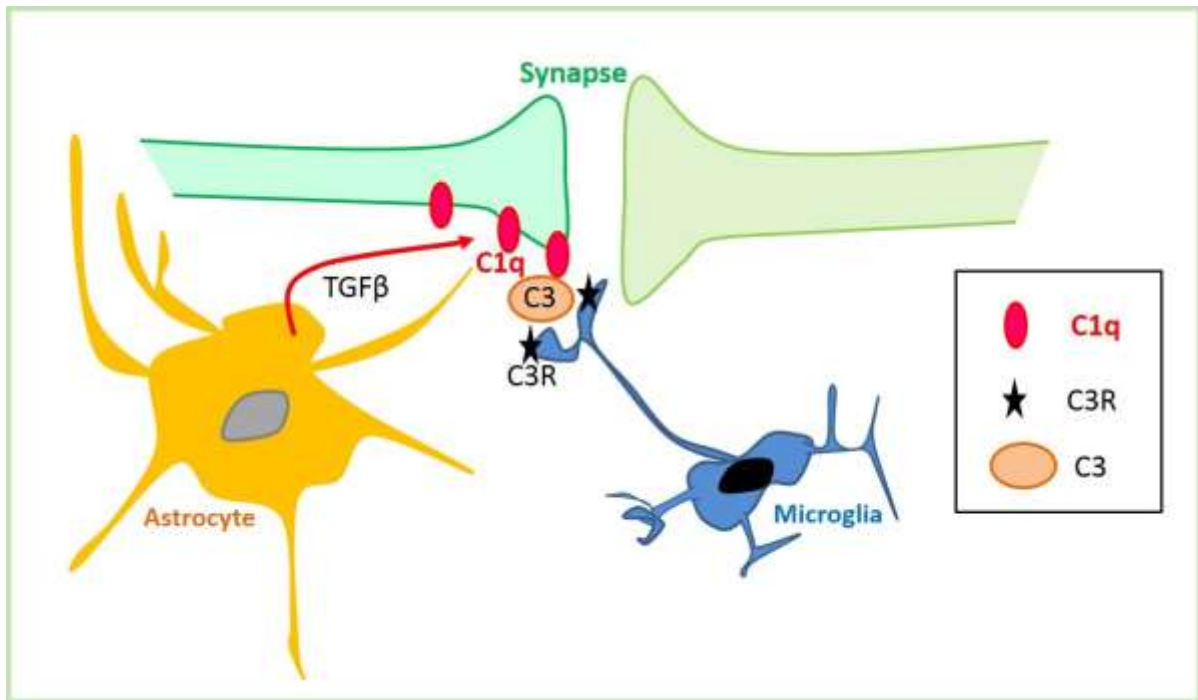


Figure 22: **Complement-mediated synapse elimination during development.** (Inspired by Stephan et al. 2012)

However, molecular actors responsible for the tagging of to be removed neurons are still mostly unknown. A recent cell culture study has proposed that C1q binding to neurons is dependent on their terminal sugar residues (Linnartz et al. 2012). It has been shown that the absence of sialic acid within neuronal glycoalyx predisposes them to bind the complement proteins and be removed via CR3-dependent microglial phagocytosis (Linnartz et al. 2012). Moreover, since microglia have been shown to preferentially engulf synapses of RGCs which activity had been blocked by monocular TTX injection, in the dLGN, we know that at least a part of the synaptic pruning is activity-dependent (Schafer et al. 2012). Thus, complement-dependent and activity-dependent mechanisms have been shown to modulate developmental microglial pruning. However, further investigation is required to find out if those two act through a converging signaling pathway (Figure 23).

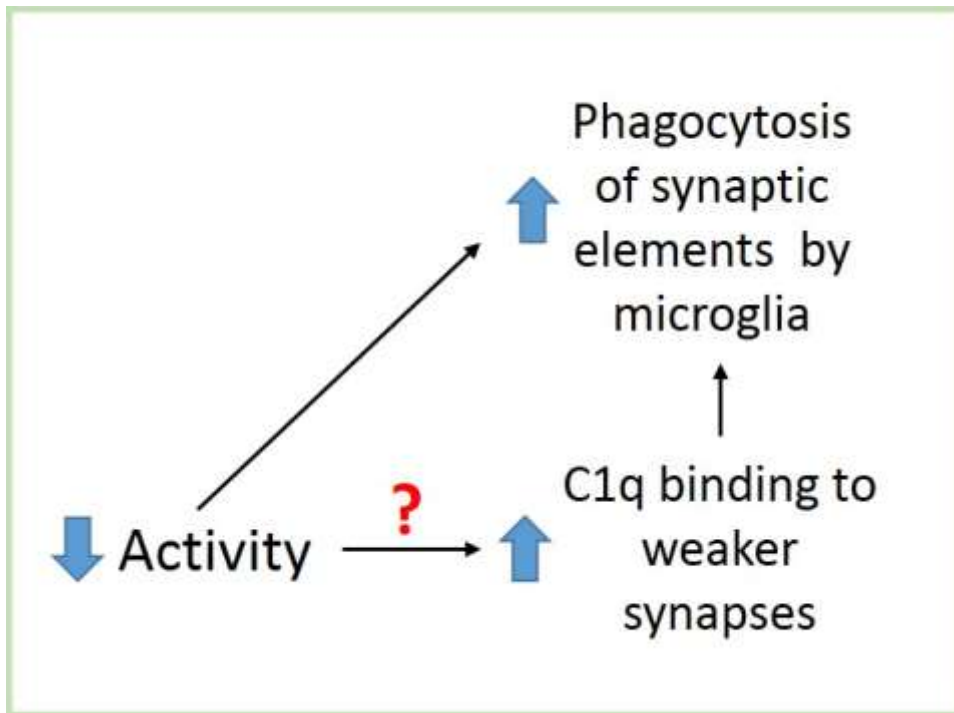


Figure 23: **Both, activity and the complement system regulate phagocytosis of synapses by microglia.**

A3.4.1.2 Fractalkine Receptor (Cx3Cr1)

Fractalkine (CX3CL1; FKN), a chemotactic cytokine, was identified less than 20 years ago. It exists under two forms: soluble and membrane-bound. In the periphery, the membrane anchored fractalkine is expressed by activated endothelium and it enables immune cells expressing the fractalkine receptor (CX3CR1) to reach the inflammation site. In the peripheral immune system, Cx3Cr1 is expressed by circulating monocytes, a subpopulation of NK cells and dendritic cells such as Langerhans cells. Moreover, in the brain, the CX3CR1 receptor is expressed exclusively by microglia (Harrison et al. 1998), whereas fractalkine is expressed by neurons suggesting their interaction (Jung et al. 2000).

An interesting model to study the developmental role of microglia has been provided by fractalkine receptor-deficient mouse (*Cx3cr1^{GFP/GFP}*), in which microglia number was transiently reduced during early postnatal life. Interestingly, in the hippocampus of *Cx3cr1^{GFP/GFP}* animals, the delay of microglia arrival is correlated to a delayed synaptic pruning and an increased number of weak excitatory synapses (Paolicelli et al. 2011). A

following study has demonstrated that in the adult brain the abnormalities observed early in life of the *Cx3cr1^{GFP/GFP}* mice are associated with deficits in synaptic multiplicity and a decreased functional connectivity between brain regions (Zhan et al. 2014).

A3.4.2 Immune System Molecules not (yet) related to Microglia

Most of nucleated cells in the periphery uses the major histocompatibility complex class I (MHC class I) in order to present antigens to effector cells of the adaptive immunity. Since the brain was considered an “immune privileged” organ, it came as a surprise that in a healthy developing and mature brain a subpopulation of neurons had been reported to express the MHC class I. Intriguingly, the expression of this molecule was coincided with time and regions of synaptic plasticity such as adult hippocampus (Corriveau et al. 1998). A following study has reported that MHC class I-deficient mice fail to properly segregate retinal fibers in the thalamic dLGN, demonstrating a role of MHC class I in modulation of activity-dependent brain maturation (Huh et al. 2000). A more recent study has proposed that MHC molecules act downstream of the spontaneous retinal activity and drive the elimination of inappropriate connections (Lee et al. 2014).

Another example of immune molecules which are involved in segregation of RGCs in the dLGN are pentraxins. In the periphery, these innate immunity proteins tag cells that should be phagocytosed and degraded. Pentraxins-deficient mice showed deficits in axonal refinement in the dLGN. Moreover, even though their synaptic number and morphology were normal, the electrophysiological analysis of cultured RGCs from these animals revealed a delay in formation of functional excitatory synapses (Bjartmar et al. 2006).

Although, there is, so far, no clear connection between their mechanisms, the evidence summarized above strongly supports the role of immune system and microglia in modulating early postnatal axonal refinement.

A4 Neurodevelopmental Origin of Psychiatric Disorders

Perturbations during brain development could lead to neuronal miswiring and incorrect axonal segregation, which in turn could have long lasting consequences, potentially increasing vulnerability to a broad range of psychiatric diseases such as autism or schizophrenia. These neurodevelopmental disorders can be modeled and observed in animals in which perturbations in brain homeostasis, even early and transiently, can later impact behavior. In this chapter I will discuss a number of human psychiatric disorders along with their animal models. First, I will focus on disorders associated to serotonin homeostasis perturbation, then, on immune system dysregulation.

A4.1 Developmental Role of Serotonin in Psychiatric Disorders

Alterations in early serotonin homeostasis, such as inappropriate serotonin level or mutation in one of serotonin receptors, are associated with the occurrence of psychiatric disorders including anxiety, addiction, autism and schizophrenia.

A4.1.1 Serotonin level perturbations

Autism spectrum disorders are the psychiatric conditions characterized by deficits in social interactions, impairments in language and communication, and symptoms involving repetitive and restricted behaviors. Since the end of XX century, when an increased blood 5-HT level (hyperserotonemia) has been reported in autistic patients, there has been an increased interest in the possible role of serotonin in the pathophysiology of this disease. Indeed, imaging of radioactive tryptophan using PET scan revealed altered serotonin synthesis capacity with age in autistic children compared to their healthy siblings (Chugani 2002).

Furthermore, a disturbed serotonin metabolism has also been shown to be associated with an enhanced aggressive behavior in humans. A point mutation in the serotonin catabolic enzyme, MaoA, which leads to a complete MaoA deficiency has been associated with an increased aggressive behavior in males of a Dutch family (Brunner et al. 1993). Moreover, an aggressive behavior has been also observed in MaoA-deficient mice,

presenting an excess of extracellular serotonin in the brain of pups (Cases et al. 1995). Another perturbation of serotonin system which has been associated with aggressive and anxiety-like behavior in adult animals is Pet1 deficiency, causing a defective embryonic development of the 5-HT system (Hendricks et al. 2003). Moreover, a shorter polymorphic variation of/in SERT promoter has been reported to reduce the efficiency of serotonin transporter transcription in cultured human cells. This decreased number of SERT mRNA copies should lead to an increased serotonergic transmission. Interestingly, in human the s allele polymorphism has been associated with a higher anxiety-related traits (Lesch et al. 1996), but also with hypervigilance and higher cognition (reviewed in Homberg and Lesch 2011).

Molecules targeting serotonergic system are commonly used in treatment of diverse psychiatric disorders. For instance, inhibitors of serotonin transporter SERT (e.g. serotonin-specific reuptake inhibitors (SSRIs) antidepressants) which increase extracellular serotonin level, are effective in treatment of depression and compulsive obsessive disorder (OCD). A recent prospective study associates fetal neurobehavioral development with SSRIs treatment throughout gestation (Mulder et al. 2011; reviewed in Vitalis et al. 2013).

A recent prospective study associates deficits in fetal neurobehavioral development with SSRI treatment throughout gestation (Mulder et al. 2011; reviewed in Vitalis et al. 2013).

A4.1.2 5-HT_{2B} Perturbations

Impulsive behavior is defined by action without consideration of consequences and decreased inhibitory control. It contributes to multiple psychiatric disorders, including addiction. Recently, a codon stop, exclusive to Finnish population, in the 5-HT_{2B} gene has been identified. This genetic variation in serotonin system has been associated with behavior observed in severely impulsive Finnish criminals (Bevilacqua et al. 2010). Furthermore, this controversial association of a mutation in a human 5-HT_{2B} receptor with severely impulsive behavior, has been also modeled in animals. Mice lacking this receptor present an increased impulsivity alongside with many other neurodevelopmental-like disorders (Bevilacqua et al. 2010).

This small set of examples selected from the rich literature on the subject strongly supports the involvement of 5-HT signaling dysregulation in the pathoetiology of neurodevelopmental psychiatric disorders.

A4.2 Developmental Role of Immune System in Psychiatric Disorders

There is an increasing evidence that an early immune deregulation, which could be caused, for instance, by maternal immune activation during pregnancy, may sometimes explain the occurrence of some psychiatric disorders (reviewed in Patterson 2009).

The link between maternal inflammation during pregnancy and the neurodevelopmental defects of the progeny has been best investigated for schizophrenia. For instance, epidemiological studies have linked both influenza epidemic and rubella pandemic to an increased frequency of schizophrenia diagnosed 20 to 30 years later (reviewed in Brown 2012; Patterson 2009). Studies based on prospective maternal serum biomarkers measurements have shown an association of an increased maternal cytokines level (IL-8, TNF α) with an increased risk of schizophrenia. Additionally, the obstetric records of maternal diseases supports the link between several common maternal infections and schizophrenia (reviewed in Brown 2012). Even though the list of examples associating maternal immune challenge with autism spectrum disorders (ASD) is much shorter, there is some intriguing data. For instance, maternal hospitalization due to viral or bacterial infection during pregnancy was shown to increase the risk of ASD in the offsprings. Additionally, children born to mothers presenting an increased cytokines level (IL4, IL5 INF γ) during pregnancy have also been reported to be at higher risk of autism (reviewed in Brown 2012).

These public health studies are supported by experimental data in rodents, in which maternal immune activation during gestation is well described as a model of neurodevelopmental-like disorders. The potential to induce abnormal behavior has been tested on a wide range of inflammatory agents. Prenatal intranasal influenza infusion and injection of LPS, poly I:C and even of cytokines (IL-6) have all been shown to cause neurodevelopmental-like disorders in mice (reviewed in Patterson 2009, 2011). As the

immunogen injected into pregnant mice generally cannot cross the placenta barrier, other inflammatory components, such as cytokines, are possibly responsible for these developmental anomalies (Nyffeler et al. 2006). Microglia, the resident macrophages of the CNS, which appear early in development (E9.5 in mice), are one of the best candidate for a cytokine releaser in the fetal brain. Indeed, recent work suggests that loss of function of DAP12, a signaling protein transiently expressed by developing microglia, leads to a transient activation of brain macrophages at birth and to synaptic impairment in adult hippocampus (Roumier et al. 2008). Intriguingly, in humans, mutations in DAP12 genes are associated with Nasu-Hakola diseases characterized by early dementia (Paloneva et al. 2000).

Recently, elegant models of embryonic microglia depletion or activation have been used in order to investigate the role of microglia during embryogenesis. Embryonic microglia depletion has been achieved either transiently by injecting anti-CSF1R antibody (E6.5-E7.5) or permanently by genetically removing the Pu.1 transcription factor. These depletion models were compared with mice which underwent prenatal immune challenge, induced by maternal immune activation (LPS at E13,5). Brain analysis of all of these three models revealed perturbations in wiring of the forebrain, which, interestingly, are characteristic of psychiatric disorders (Squarzoni et al. 2014). Another recent study has shown that the disturbed brain connectivity and deficits in synaptic multiplicity observed in adult Cx3Cr1-deficient mice, in which embryonic microglia arrival to the brain is delayed, are associated with impaired behaviors. These mice have been reported to present decreased social interactions and increased repetitive behavior, both of which are characteristic of autism spectrum disorder (Zhan et al. 2014).

The cited studies above suggest that fetal microglia dysregulation might be at the origin of dysfunctions leading to neurodevelopmental disorders in adulthood. Understanding how microglia are regulated and what is their precise role during normal development would help to better explain the consequences of inflammation during early development.

A4.2.1 Role of Adult Microglia in Psychiatric Disorders

All the examples presented in the previous part support the behaviorally relevant developmental function of microglia, implying their participation in permanent developmental brain wiring. However, there is a number of studies demonstrating that in some cases, manipulation of microglia in the adult brain is sufficient to induce behavioral changes.

For instance, in rats, enhanced neonatal maternal care, induced by a neonatal handling paradigm, has been shown to induce a shift in epigenetic programming early in development, resulting in a more anti-inflammatory phenotype of microglia which persists into adulthood and protect against reinstatement of morphine-seeking behavior. This beneficial effect of an increased maternal care has been reproduced by treatment with an anti-inflammatory drug, ibudilast, which targets glia (including microglia) and also reduces reinstatement of morphine-seeking behavior (Schwarz et al. 2011) suggesting that targeting adult microglia could be of interest in treatment of drug addiction.

Another example of the potential therapeutic capacities of adult microglia was presented in a recent study of the mouse model of the Rett syndrome, a devastating disease which is believed to have neurodevelopmental origin and is grouped with the ASD. This disease has been modeled in mouse by invalidation of methyl-CpG binding protein (MECP) 2, a gene which is often mutated in Rett syndrome patients. The experiments of wild-type bone marrow transplantation successfully induced recruitment of wild-type microglia to the brain and halted the progression of the disease. The further investigations revealed that this beneficial effect is mediated by the phagocytic capacities of microglia (Derecki et al. 2013). These results endorses a therapeutic potential of adult microglial. A similar strategy has been used in a study of mice mutated in their *Hoxb8* gene, coding for a DNA binding transcription factor. This mutation has been associated with an excessive grooming. A similar behavior is observed in the human obsessive-compulsive spectrum disorder, trichotillomania. The authors detected the *Hoxb8* brain expression exclusively in a population of microglia (40%). Since the pathologic mice behavior has been rescued by transplantation with wild-type bone marrow, at the time, the authors mistakenly hypothesized that the *Hoxb8* positive microglia could represent a subpopulation of

bone-marrow origin (Chen et al. 2010). As I have introduced in the chapter “Origin of Microglia in Adult Brain”, today it is accepted that the microglia pool depends on self-renewing CNS resident cells and it is independent from the adult hematopoiesis. Nevertheless, the intriguing improvement of the pathologic behaviors in both of the cited studies could be mediated by the recruitment of peripheral microglial precursors, enabled by the irradiation/transplantation procedure, providing new therapeutic implications to treat psychiatric disorders.

Recent studies using models of microglia depletion from an adult brain demonstrate microglial involvement in adult synaptic plasticity (see previous chapters) (Parkhurst et al. 2013; Elmore et al. 2014), which could be behind their therapeutic capacities.

In line with the experimental results summarized above, it is tempting to speculate that both, altered serotonin system and immune dysregulation during gestation, might impend circuit maturation and lead to subtle miswiring problems that can cause behavioral disorders in adulthood. More detailed inspection of brain circuits might help to reveal developmental abnormalities underlying these behavioral changes.

B - RESULTS & DISCUSSION

B1 Article

Since both, serotonin and microglia, appear early in the developing brain and they both have been reported to be required for maturation of functional neuronal circuits during development of the CNS, we addressed in this article the question whether some developmental mechanisms known to be controlled by serotonin, and potentially involving microglia, are impaired when microglia are insensitive to serotonin.

As shown in this article, the hypothesis was supported by our results showing a major expression of the serotonin 5-HT_{2B} receptor by postnatal microglia, suggesting that serotonin could participate in temporal and spatial synchronization of microglial functions.

In order to further investigate the putative interaction between microglia and serotonin I did a collaboration with Nicolas Gervasi, who tested the effect of local serotonin delivery on microglia in acute brain slices of young postnatal mouse (two photon microscopy). We could observe that microglial processes moved rapidly toward the source of serotonin in wild-type mice (Figure 24), but not in *Htr_{2B}^{-/-}* mice, lacking the 5-HT_{2B} receptor.

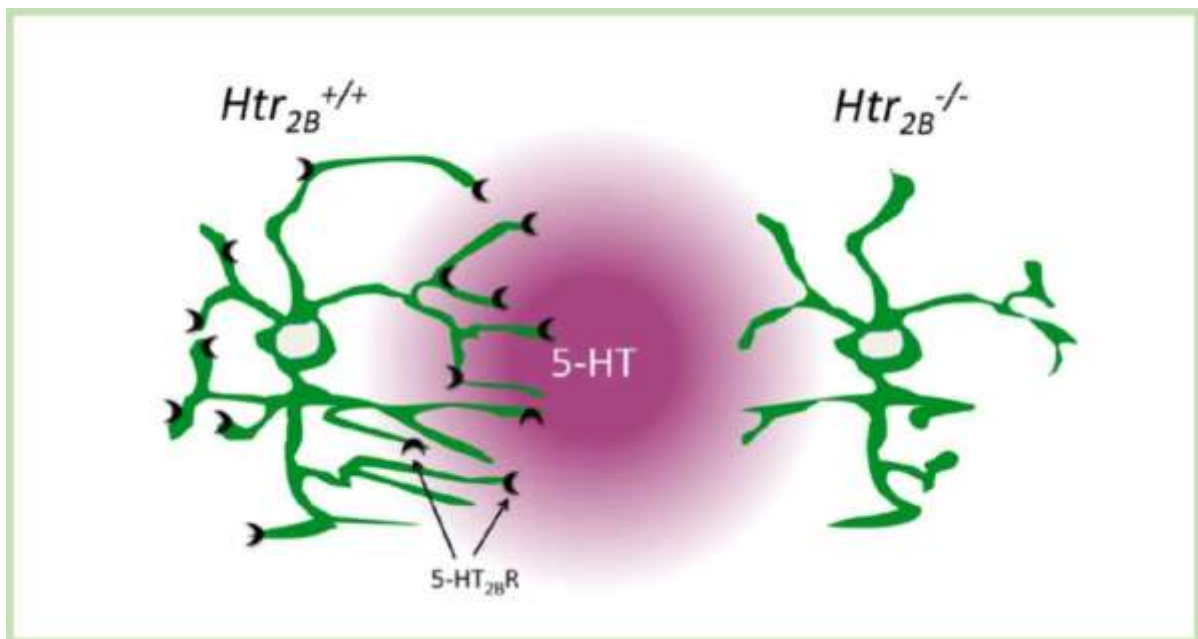


Figure 24: **Microglial processes moved rapidly toward the source of serotonin.** This action is mediated by 5-HT_{2B} receptor.

Next, I used an *in vivo* model of axonal segregation during early brain development: the retinal axons segregation onto the thalamic dLGN. I observed that *Htr2B*^{-/-} mice present the alterations of spatial arrangement of eye-specific territories within the dLGN.

To further test the possibility that the phenotype in the *Htr2B*^{-/-} mice is due to absence of 5-HT_{2B} receptor in microglial cells, we examined microglia from *Htr2B*^{-/-} mice. After comparing mRNA expression levels in microglial primary cultures, we have found that some activation markers are upregulated in microglia from *Htr2B*^{-/-} mice, supporting the hypothesis that serotonin modulates developmental microglia via 5-HT_{2B} receptors.

Overall, these results show that serotonin interacts with and regulates microglial cells. We propose that these interactions could participate in brain maturation.

The following article entitled “**Serotonin modulates developmental microglia via 5-HT_{2B} receptors: potential implication during synaptic refinement of retinogeniculate projections**” has been submitted to the journal *ACS Chemical Neuroscience* on **20th of December 2015**.

***Title.* Serotonin modulates developmental microglia via 5-HT_{2B} receptors: potential implication during synaptic refinement of retinogeniculate projections.**

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Abstract.

Maturation of functional neuronal circuits during development of the central nervous system relies on sophisticated mechanisms. First, axonal and dendritic growth and synapse elaboration should reach appropriate targets. Second, pruning and neuronal death are required to eliminate redundant or inappropriate neuronal connections. Serotonin, besides its roles as a neurotransmitter, actively participates in postnatal establishment and refinement of brain wiring in mammals. Another important role is played by the brain resident macrophages, microglia, in developmentally-regulated neuronal death as well as in synaptic maturation or elimination. Here, we tested the hypothesis of cross-regulations between microglia and serotonin during postnatal brain development in a mouse model. We found a major expression of the serotonin 5-HT_{2B} receptor by postnatal microglia, suggesting that serotonin could participate in temporal and spatial synchronization of microglial functions. Using two-photon microscopy on acute brain slices and local delivery of serotonin, we observed that microglial processes moved rapidly toward the source of serotonin in wild-type mice, but not in *Htr2B*^{-/-} mice lacking the 5-HT_{2B} receptor. We then investigated whether some developmental steps known to be controlled by serotonin, could potentially result from microglia sensitivity to serotonin. Using an *in vivo* model of synaptic refinement during early brain development, the maturation of the retinal projections to the thalamus, we observed that *Htr2B*^{-/-} mice present anatomical alterations of the projecting area of retinal axons into the thalamus. Finally, some activation markers are upregulated in microglia from *Htr2B*^{-/-} neonates. These results support the hypothesis that serotonin interacts with microglial cells and that these interactions could participate in brain maturation.

Keywords.

Microglia; mice; postnatal development; retinal projections; serotonin receptors; thalamus.

Introduction.

Recent evidence indicates that brain resident macrophages, microglial cells, are essential for the proper wiring of neuronal networks at postnatal periods (1, 2). The critical process of developmental elimination of inappropriate synapses involves phagocytic activity of microglia (3, 4), but the influence of microglial cells on synapse development probably extends beyond their phagocytic capabilities. These cells indeed release a large array of signaling molecules, including cytokines, growth factors and transmitters which modulate synaptic functions in pathological conditions (5). In physiological conditions, microglial cells were recently shown to modulate synapse formation (6) and activity (7), and thus to shape neuronal circuits (8). However, mediators controlling the activity of microglia in order to achieve these functions remain largely unknown.

Unlike all other brain cell types, microglial cells have a myeloid origin and derive from precursors produced by primitive hematopoiesis in the yolk sac. Microglial precursors start invading the embryonic brain shortly after blood circulation is established. In mice, the first microglia can be detected in the developing brain from embryonic day (E)9.5 (9). The microglia invasion is a complex process and recent study provided some insight into microglia colonization of the mouse cortex, from E11.5 to E17.5 (10). These precursor cells have an amoeboid phenotype (11) and accumulate at several sites (e.g. at plexus choroid and corpus callosum) from where they start invading the brain parenchyma. Until postnatal day (P)5, higher densities of microglia are observed in the vicinity of meninges and ventricular surfaces, whereas there are fewer microglia in cortical layers (12). The gradual increase in microglia density is explained both by the microglial precursors migration and by cell proliferation to reach adult levels (12). Early postnatal microglia share similarities with both ramified and activated microglia and some P5-P9 microglia express CD68, a lysosomal marker specific to phagocytic activated microglia, which is down regulated in adult P30 microglia (4). Similarly, electrophysiological studies in mouse cortex have revealed that the population of early postnatal microglia, which alike activated microglia, presents a transient outward rectifying current above -30mV mediated by Kv1.3 potassium channels, but do not express other known activation markers like Mac2 or MHCII (12). Additionally, these microglial cells also express a particular panel of specific markers including purinergic receptors (10, 12).

The mouse retinogeniculate system is a classic model for studying developmental synapse elimination. Early in postnatal development, retinal ganglion cells (RGCs) form transient functional synaptic connections with relay neurons in the dorsal lateral geniculate nucleus (dLGN) (13, 14). Before eye opening around P14, many of these transient retinogeniculate synapses are eliminated while the remaining synaptic arbors are elaborated and strengthened in a common pathway to refine synaptic circuits. Notably, microglial engulfment of retinogeniculate inputs occurs during the narrow window of postnatal development (P5–P9) (4). In mice, dLGN on each sides of the brain contains axonal terminals from both eyes. In a mature/adult dLGN, the contralateral fibers occupy most of its territory except for a small gap containing exclusively fibers from the ipsilateral eye. In contrast to the adult situation, at P3, the segregation between ipsilateral and contralateral retinal projections is not yet present and RGC axonal projections from different eyes are intermingled in the dLGN; the ipsilateral projections are diffused and the contralateral fibers occupy the entire dLGN (15). This is due to an excessive number of synaptic connection. During two first postnatal weeks, a maturation period characterized by a massive synaptic pruning, the redundant synapses are eliminated and the correct connections are strengthened resulting in a segregated phenotype (16).

Pruning of pre- and post-synaptic elements by microglia has been observed in the developing hippocampus where the neuron-to-microglia fractalkine signaling pathway control the number of synapses possibly by recruiting microglia in the hippocampus (3). In physiological conditions, microglial cells modulate synaptic activity or stability (7, 8, 17, 18). During the mouse somato-sensory cortex development, fractalkine-dependent recruitment of microglial cells may influence synapse functional maturation (19). In the developing dLGN, the appropriate segregation of eye-specific axonal projections requires microglial cells expressing the complement receptor 3 (CR3), which phagocyte presynaptic elements tagged by complement proteins C1q and C3 (4, 20).

Interestingly, eye specific segregation of retinal projections in the thalamus also depends on an appropriate serotonin (5-Hydroxytryptamine, 5-HT) level. Segregation of ipsilateral and contralateral regions in dLGN does not occur in mice with gene ablation of the monoamine oxidase A (MAOA) or serotonin transporter (SERT) genes, *Maoa*^{-/-} or *Slc6a4*^{-/-}, mice presenting an increased 5-HT level (16, 21, 22). The phenotype associated

with an excess of extracellular 5-HT can be lowered in the double knockout mouse in which both *Slc6a4* and 5-HT_{1B} receptor, *Htr1B* genes are invalidated (23). Thus, the increased 5-HT level has been proposed to inhibit activity dependent segregation mechanisms. For instance, a subpopulation of ipsilateral RGCs has been reported to transiently express SERT between P7 and P14 (16). The timing of the transient expression of SERT coincides with period of developmental axonal segregation in the dLGN. Taken together, the early appearance of brain 5-HT and its heterologous uptake during critical for development periods, suggest the importance of this monoamine during early steps of brain development and wiring. In this work, we assessed putative 5-HT/microglia interactions during brain wiring critical period focusing on refinement of retinal projections in the thalamus in the dLGN area.

Results and Discussion.

Postnatal microglia express 5-HT_{2B} receptors and are in close vicinity of 5-HT fibers.

Although a recent paper identified the presence of 5-HT receptors on primary culture of microglia from neonatal brains (24), there is only few information on 5-HT receptor functional expression in neonatal microglia *in vivo*. We first confirmed using RT-PCR the 5-HT receptor expression of primary cultures of pure microglia, obtained from neonatal brains. The results identified mainly the presence of *Htr_{2B}* mRNA coding for the 5-HT_{2B} receptor in cultured microglia (Fig. 1A). Transcript for *Htr_{2A}* was also detected, but its expression was much weaker (confirmed by qPCR, data not shown). Other 5-HT receptor expression could not be detected, while they are easily detected in whole hippocampus both at P0 and P30, in contrast to *Htr_{2B}* mRNA (Fig. 1A). To confirm this 5-HT_{2B} receptor mRNA expression *in vivo*, we used *Cx3Cr1^{GFP/+}* mice, in which microglial cells express green fluorescent protein (GFP) (25) inserted by knock-in into *Cx3Cr1* gene. We purified microglia (GFP+ cells) by fluorescence-activated cell sorting (FACS) on cell suspensions from cortex or brain stem at early stages of postnatal development: P3 and P9 (Fig. 1B-D). We first confirmed the purification of microglia by the expression of *Mac1*, a microglial marker, specifically in GFP+ fractions. RT-PCR analysis on these samples showed that freshly isolated microglia express *Htr_{2B}* mRNA at both stages P3 and P9 (Fig. 1B-C). *Htr_{2A}* mRNA was not detected in freshly isolated microglia, and *Htr_{2C}* mRNA was visible only in the fraction from P9 cortex and hippocampus. As *Htr_{2C}* is strongly expressed in whole brain, it might be due to a minor contamination of the microglial fraction. Moreover, *Htr_{2B}* mRNA was undetectable in the GFP-negative fractions from cortex and hippocampus indicating a lack or a very low level of expression. In contrast, in brainstem, similar analyses show that *Htr_{2B}* mRNA is expressed in microglia but also in other GFP-negative cells (Fig. 1D), which is consistent with previous results of the laboratory showing 5-HT_{2B} receptor expression in most of the serotonergic raphe neurons (26). This receptor is also expressed in microglia at the adult stage (data not shown and (24)).

Following the hypothesis that microglia and the serotonergic system could interact, it was of interest to investigate if they are spatially close. Using confocal imaging

(Fig. 2A) and three-dimensional reconstruction (Fig. 2B) of brain sections stained for 5-HT and the microglial marker Iba1, we could see points of apposition between 5-HT fibers and microglial processes. Even when not in contact, 5-HT fibers and microglial cells are not more than a few micrometers away, which is consistent with functional interactions, as 5-HT can diffuse and act at slight distances away (about ten μm) from its source of release, a phenomenon known as "volume transmission" (27). These results show that microglia from postnatal mice express the 5-HT_{2B} receptor, which seems to be the main microglial receptor for 5-HT, and might "sense" 5-HT at early stages of postnatal brain development.

Postnatal microglia invasion in the dLGN

We next examined the distribution of microglia in fixed coronal sections of the developing brain, in *Cx3Cr1^{GFP/+}* mice. As previously reported (12), and in contrast to adult brain where microglia are homogeneously distributed in parenchyma, the distribution of microglia in the early postnatal brain (P4) is heterogeneous (Fig. 3A). We focused on the dLGN of the thalamus (Fig. 3A), in which axons from RGCs form synaptic connections with relay neurons, to establish the retinogeniculate pathway of the visual system. Both microglia (4, 28) and 5-HT (16) have been implicated in the refinement of these retinogeniculate projections. At the age of P4, microglial cells are mainly located outside of dLGN, i.e. vLGN, barreloids and the surrounding tissue, with few microglia inside the dLGN (Fig. 3B). With age, the amount of microglial cells inside the dLGN gradually increases: it almost doubles between P4 and P6 ($\times 1.9$; $p < 0.05$), to reach a 10-fold increase in adult animal ($\times 10.3$; $p < 0.01$) (Fig. 3B-C).

Chemoattractant effect of serotonin on microglial processes.

As 5-HT_{2B} receptors are expressed in microglia, we focused on potential chemoattractant effect of 5-HT on microglia. Microglial cells are able to act by extending their processes toward, for example, synapses, and not only by moving their somas (7, 17, 29, 30). As microglia have fewer processes in culture compared to *in vivo*, we investigated the effect of 5-HT on microglia in fresh brain slices using two-photon microscopy. The fluorescent

microglia of *Htr2B*^{+/+}; *Cx3Cr1*^{GFP/+} mice could be observed on 300 μm slices containing the dLGN region. In line with the fact that axonal segregation in thalamic dLGN occurs during two first postnatal weeks, we used P11-P14 mice (Fig. 4). We assessed the effects of a puff of 5-HT on the growth of neighboring microglial processes toward the source, and compared to ATP, a well-known chemoattractant for microglia (24, 31). The effects were quantified by measuring the changes in length of proximal and distal processes in respect to the delivery site. A puff of 5-HT on dLGN slice triggered an immediate increased motility of neighboring microglial processes: extension of proximal processes toward the source was as efficiently as ATP, whereas a release of saline solution did not trigger detectable effects (Fig. 4A-B, Supplementary movies). These results indicate that, at least during the second postnatal week, thalamic microglia express functional 5-HT receptors that regulate the growth direction of their motile extensions. These data complement those from Krabbe et al (24), which showed that bath application of 5-HT increased motility of microglial processes in a lesional context. Next, we repeated this experiment using slices from postnatal brain of *Htr2B*^{-/-}; *Cx3Cr1*^{GFP/+} mice. In the dLGN of these mutant animals, no microglial response to 5-HT could be observed, whereas ATP was as efficient in microglia chemoattraction as in slices from *Htr2B*^{+/+} mice (Fig. 4B). These results show that 5-HT is chemoattractant to microglial processes via 5-HT_{2B} receptors, and also validate the functional expression of these receptors in microglia.

We also investigated in a transwell assay the ability of microglia in culture to migrate through a membrane with 5-μm pores. To investigate the potential effect of 5-HT, purified microglial cells were placed in the upper compartment in plain medium while 5-HT was in lower compartment in order to create a gradient. Such experimental setup could not reveal any significant chemotactic effect of 5-HT on microglia (Fig S1A) while ATP was efficient. No increase in mobility was detected when 5-HT was placed in both compartments, whereas ATP had indeed a chemokinetic effect (Fig S1B).

***Htr2B*^{-/-} mice have defects in dLGN development.**

We then asked whether *Htr2B*^{-/-} mice could be impaired in postnatal synaptic refinement / segregation that is relying both on 5-HT and microglia. For this purpose, we looked at the segregation of ipsilateral and contralateral retinal projections onto the thalamic

dLGN. This segregation could be visualized thanks to an anterograde tracing of the retinal projections using the Cholera Toxin b subunit (CTb) coupled to fluorochromes (each eye was injected with a different color CTb) (Fig. 5A). A common way of analyzing the axonal segregation in the dLGN is to measure the percentage of overlap between the ipsilateral and the contralateral regions. Just after birth the two regions overlap and during the two postnatal weeks they segregate to eye specific areas. At P30, the axonal refinement is likely over and there is very few overlap left between ipsi- and contralateral regions. We looked at the retinal axons segregation in the dLGN at P30 in *Htr2B^{-/-}* and *Htr2B^{+/+}* mice, when segregation of the retinal axons in the dLGN is accomplished. Quantification of the eye-specific segregation did not reveal any significant difference at P30 in *Htr2B^{-/-}* and *Htr2B^{+/+}* mice, although a trend toward a reduced segregation in *Htr2B^{-/-}* mice (Fig. S2). However, we observed a phenotype in the dLGN of *Htr2B^{-/-}* mice, which is especially well visible in the ipsilateral region. Indeed, the ipsilateral region of *Htr2B^{+/+}* mice was rather compact and well defined whereas it was more diffuse and patchy in *Htr2B^{-/-}* mice (Fig. 5A). Such a compact pattern was observed in 70% of *Htr2B^{+/+}* but only in 23% of *Htr2B^{-/-}* mice (Fig. 5B, $p = 0.04$). In addition, the shape and the location of the ipsilateral projections was clearly more variable among *Htr2B^{-/-}* than *Htr2B^{+/+}* animals, as exemplified in Fig. 5C. In order to better visualize and quantify the heterogeneity observed in *Htr2B^{-/-}* animals, all ipsilateral regions were stacked to create a heat map for colocalization within each genotype. Added together those images illustrate that the location of the ipsilateral region in the dLGN is much more consistent within *Htr2B^{+/+}* animals. Their heat map has a large, well organized hot area, which represents the overlap between all the stacked ipsilateral regions, whereas the heat map of *Htr2B^{-/-}* mice is clearly disorganized and hot area is smaller (Fig. 5D). A calculation of the number of overlapping pixels with all other ipsilateral regions for each ipsilateral region revealed that ipsilateral regions of *Htr2B^{+/+}* animals overlap with each other significantly more than those of *Htr2B^{-/-}* animals (Fig. 5D, $p < 0.01$). Altogether, these results show that *Htr2B^{-/-}* mice exhibit anatomical alterations of the retinal projections into the thalamus.

Altered activation status of *Htr2B*^{-/-} microglia.

In *Cx3cr1*^{-/-} mice, the defective elimination of immature synapses in the hippocampus has been correlated to a delayed entry of microglia in the hippocampal structure. We thus compared microglia invasion in the dLGN of *Htr2B*^{-/-} to *Htr2B*^{+/-} animals, following the hypothesis that its alteration could lead to the disorganization of retinal projections observed in this structure. However, the number (Fig 6A) and localization (not shown) of microglia inside the dLGN were similar in *Htr2B*^{-/-} and *Htr2B*^{+/-} mice. Thus, the abnormal shape of the ipsilateral territory is not correlated to a defect in microglia recruitment, while not excluding a role of local recruitment and activation of microglial processes by 5-HT. As 5-HT has been shown to regulate the functional polarization of human peripheral macrophages (32), we checked the activation state of microglia from *Htr2B*^{-/-} mice. The comparison of the expression level of a series of 84 immune markers in microglial primary cultures from *Htr2B*^{-/-} and *Htr2B*^{+/+} neonates revealed a significant overexpression of several markers in the mutant microglia (Fig 6B and S3). Thus, microglia from *Htr2B*^{-/-} mice differs from microglia from *Htr2B*^{+/+} mice in their activation state which could affect their ability to mediate proper synaptic refinement.

Conclusions

It has been acknowledged, since last decade, that microglial cells are involved in promoting neuronal death and clearing apoptotic cells during brain development (33). These immune cells are also essential for the proper wiring of neuronal networks, through the elimination of supernumerary synapses. Pruning of pre- and post-synaptic elements by microglia has been observed in the developing hippocampus where the neuron-to-microglia fractalkine signaling pathway can regulate synapse number and microglia recruitment in the hippocampus (3). This critical process of developmental elimination of inappropriate synapses also involves proteins of the classical complement cascade, a robust immune signaling pathway that tags debris or pathogens for phagocytosis (4). Yet, other signaling pathways might influence microglia recruitment and function during cerebral development. In particular, 5-HT, a major actor of sensory map formation (21), which has been also implicated in various psychiatric diseases (34), has been shown to modulate microglia phagocytosis (24).

In this work, we are reporting evidence that, at early stages of postnatal brain development, microglia and serotonergic system interact. Microglial cells express 5-HT_{2B} receptors and are in close vicinity with serotonergic axons. During brain development microglial 5-HT_{2B} receptors could thus be involved in many functions such as modulation of microglia extensions, chemoattraction and phagocytic capacities. Using focal 5-HT stimulation of microglia on acute postnatal brain slices, we found that 5-HT stimulation induced directional growth of microglial processes. Using slices from *Htr2B*^{-/-} animals, we confirmed that the 5-HT effect was mediated by 5-HT_{2B} receptors. Therefore our results clearly show that 5-HT via 5-HT_{2B} receptors can functionally modulate microglia motility in postnatal dLGN. However, primary cultures of microglia were not responding to 5-HT in chemotactic assays. In such culture conditions, microglia might lose some unique characteristics, which are potentially necessary in response to 5-HT. Another possibility is that, in order to respond to 5-HT, microglia need partners (e.g. astrocytes) or extracellular matrix components that are absent in this assay.

In the healthy developing dLGN, the appropriate segregation of eye-specific axonal projections requires microglial cells expressing CR3, which phagocyte presynaptic elements tagged by complement proteins C1q and C3 (4). Eye specific segregation of retinal projections in the thalamus depends also on an appropriate 5-HT level. The increased 5-HT level has been proposed to inhibit activity dependent segregation mechanisms (23). A subpopulation of ipsilateral RGCs transiently expresses SERT between postnatal day 7 and 14 (16), when developmental axonal segregation and refinement is operating in the dLGN. SERT may create a local 5-HT gradient orienting the microglia processes toward the area where overlapping projections have to be cleared. A defect in sensing this gradient could lead to activation or recruitment of microglia, and thus synapse elimination in improper areas. Analyzing retinal projections in dLGN, we identified here, abnormal organization of retinal projections in *Htr2B*^{-/-} animals, which could be consistent with this hypothesis. On the other hand, 5-HT_{2B} receptors could also be necessary for the microglia to acquire proper developmental phenotype as revealed by differential expression of activation markers in *Htr2B*^{-/-} microglia. Our findings support thus the possibility that 5-HT contributes to axonal refinement during early postnatal development by implicating local recruitment of microglial processes or microglial differentiation. Our data strongly support that in *Htr2B*

/- animals, the phenotype observed is, at least in part, due to the lack of 5-HT activation of 5-HT_{2B} receptors in postnatal microglial cells.

Methods.

Animals.

Wild-type *Htr2B*^{+/+} (wild-type) and *Htr2B*^{-/-} mice were on a 129S2/SvPas background as the embryonic stem cells used for homologous recombination. For two-photon experiments, double transgenic mice were generated by crossing these mice with *Cx3cr1*^{GFP/+} mice (25). Four backcrosses were performed to reduce the contribution of C57/Bl6 genetic background from *Cx3cr1*^{GFP/+} mice. All animals were bred at the animal facilities of the Institut du Fer à Moulin. Young (<P10) animals used for the quantification of microglial cells were littermates. All mice experiments were performed according to the EC directive 86/609/CEE, and have been approved by local ethical committee (N° 1170.01). After weaning, mice were housed in maximum of 5 mice per cage of the same genetic background until the beginning of the experimental protocol. In all the studies, the observer was blind to the experimental conditions being measured.

Reagents.

Solutions of serotonin-hydrochloride (SIGMA) were prepared extemporaneously in water. Aliquots of ATP stock solution in water were stored at -20°C.

Inflammatory cytokines and receptors array.

Quantitative mRNA expression analysis of 84 chemokines and their receptors was performed in primary microglia cultures with the mouse chemokine and receptor RT² profiler PCR array (SABioscience, Qiagen). Total RNA was isolated from primary microglia cultures prepared from *Htr2B*^{+/+} or *Htr2B*^{-/-} pups (n= 4 cultures of each genotype) using the RNeasy mini kit protocol (Qiagen). Equal amounts (500ng) of RNA from each culture were then converted to cDNA using RT² first strand kit (SABioscience, Qiagen). PCRs were performed according to the manufacturer's protocol using RT² profiler PCR array PAMM-011ZA (mouse inflammatory cytokines and receptors). The mRNA expression of each gene was normalized using the expression of the β -glucuronidase (*Gusb*) as a housekeeping gene and compared with the data obtained with the control group (*Htr2B*^{+/+} microglia cultures) according to the $2^{-\Delta\Delta CT}$ method (35).

Anterograde labeling of retinogeniculate projections in dLGN.

P28 mice were anesthetized with an intraperitoneal injections of ketamine-xylazine (ketamine 60 mg/g and xylazine 8 µg/g, in 0.9% saline). 3 µl of 0.2% cholera-toxin subunit B (CTb) conjugated to AlexaFluor 555 or 488 (Invitrogen/Molecular Probes) diluted in 1% DMSO was injected into the eye intravitreally with a glass micropipette. After 2 days, mice were anesthetized with a sublethal dose of pentobarbital and perfused transcardially with 4% paraformaldehyde (PFA) as described in the “Brain slices preparation”.

Brain slices preparation.

Mice were anesthetized with a sublethal dose of pentobarbital, perfused transcardially with 4% PFA in 0.1M phosphate buffer (PB), then their brains were collected and immersed in 4% PFA overnight, cryoprotected overnight in 30% sucrose in PB, and sectioned coronally with a cryotome (55 or 100 µm). Eventually, the sections were processed for immunofluorescence or nuclei staining before being mounted in Mowiol 4-88 (Calbiochem) and imaged using a Leica DM 6000B fluorescent microscope.

Immunofluorescence and epifluorescence or confocal microscopy.

Brain slices were washed one time in PB then incubated in permeabilizing and blocking solution (0.25% gelatin and 0.1% triton in PB) for 30 min. Primary antibodies (Rabbit anti-Iba1, Wako, Japan, 1:600; Rat anti-5HT, Chemicon, 1:100) were diluted in PGT buffer (0.125% gelatin and 0.1% triton in PB). Samples were incubated overnight at 4 °C with gentle agitation. Secondary fluorochrome-conjugated antibodies (Donkey anti-Rabbit-Cy3, Jackson Laboratories; Donkey anti-Rat Alexa488, Molecular Probes) were added in PGT buffer for 3.5 h at room temperature. After rinsing three times with PGT buffer and once with PB, sections were stained for nuclei with bis-benzimide (Sigma) for 15 min, rinsed with PB and mounted in Mowiol. Samples were imaged using a Leica DM 6000B fluorescent microscope, or a Leica SP5 confocal microscope. Three-dimensional reconstruction was performed with Imaris software (Bitplane) based on a stack of confocal sections (0.5 µm apart).

Analysis of distribution of ipsilateral fibers in the dLGN / Image analysis

All image analysis were performed on 10x images obtained with a 10x/0.4 objective of Leica DM 6000B fluorescent microscope. Two images were acquired for each brain section containing dLGN structure: one in a green and one in red channel (for ipsilateral and contralateral region respectively). Animals where injection was incomplete, based on contralateral staining, were excluded from the analysis. For each animal, a median reference section was chosen by a blind-to-the-genotype investigator, based on dLGN and vLGN shape. For heat map analysis, these ipsilateral median images were all aligned in the same orientation using the contralateral region as a reference. For each animal, the ipsilateral image was then cropped into a smallest rectangle containing the entire dLGN visualized on the contralateral image. The intrageniculate leaflet, the vLGN and the optic tract were excluded. Next, these reoriented and cropped images of ipsilateral regions were resized to 400 x 600 pixels (width x height) and converted to binary images. All these were made using ImageJ software. Next the binary images were stack together and heat maps were created using Matlab (Mathworks). The color scale of each pixel corresponds to the number of animals which ipsilateral staining is present in this given location. In order to quantify the heat maps, a percent of overlap between each pair of ipsilateral regions for each genotype was measured using Matlab (Mathworks). The average overlap between every two animals of the same genotype significance was evaluated by two-sided permutation test. The qualification of compact versus diffuse ipsilateral region morphology was determined by a genotype-blind investigator, based on the shape of ipsilateral territories on three consecutive sections (the median and its previous and following sections). Statistical difference between genotypes was determined using Fischer's test.

Primary microglia culture and ex-vivo purification of fresh microglia

For primary cultures of microglia, cortices from P1 pups (*Htr2B^{+/+}* or *Htr2B^{-/-}*) were dissociated by trituration after incubation with 0.25% trypsin. Cells were plated onto poly-ornithine-coated Petri dishes. Cells were grown in DMEM (Invitrogen, France) with pyruvate and low (1g/ml) glucose supplemented with 10% fetal bovine serum (BioWest, France). After 11 days in culture microglia could be detached from the astrocytic layer by gentle shaking and processed for experimentation. For ex-vivo fresh purification, we

used brain tissue from *Cx3cr1^{GFP/+}* animals at P3 or P9 that had been anesthetized and perfused transcardially with PBS. Appropriate anatomical regions (cortex and hippocampus, or brain stem) were rapidly dissected on ice, chopped, digested with Trypsin for 15 minutes at 37°C, rinsed, treated for 5 minutes with DNase, rinsed, mechanically dissociated using a fire-polished Pasteur glass pipet, and filtered on a 70 µm mesh prior to cell sorting on a FACS Aria I (Becton Dickinson). DAPI (Life Technologies) was added to the cell suspensions just before cell sorting in order to gate only living cells. GFP-positive fractions were 97-99% pure.

Two photon microscopy

Brain slices were prepared from mice aged from 11 to 14 days, as previously described (36). Briefly, mice were decapitated, their brain removed, and coronal brain slices (300 µm thickness) were obtained with a HM650 microtome (Microm, France). Slices were prepared in an ice-cold solution of the following composition (in mM): choline Cl 110; glucose 25; NaHCO₃ 25; MgCl₂ 7; ascorbic acid 11.6; Na⁺-pyruvate 3.1; KCl 2.5; NaH₂PO₄ 1.25; and CaCl₂ 0.5 saturated with 5% CO₂/95% O₂. Coronal slices (250 µm) were stored at room temperature in 95% O₂/5% CO₂-equilibrated artificial cerebrospinal fluid (ACSF) containing the following (in mM): NaCl 124; NaHCO₃ 26.2; glucose 11; KCl 2.5; CaCl₂ 2.5; MgCl₂ 1.3; and NaH₂PO₄ 1. During 2-photon imaging, brain slices were continuously perfused with this solution saturated with 5% CO₂/95% O₂, at a rate of 2 ml/min, in a recording chamber of ~1 ml volume maintained at 32°C. A pipette containing ACSF, 5-HT (5 µM) or ATP (500 µM) was placed in the center of the dLGN. After 10 minutes of baseline recording, a gentle positive pressure was applied to the pipette for local application. Movement of microglia processes was followed by recording a fluorescent image every minute with a 2-photon MP5 upright microscope (Leica Microsystems, Germany) with a ×25 0.95 NA water-immersion objective and a Chameleon Ultra2 Ti:sapphire laser (Coherent, Germany) tuned to 920 nm for GFP excitation. Resonant scanners (8 Khz) were used for Z-stack image acquisition. A two-photon emission filter was used to reject residual excitation light (SP 680, Chroma Technology). A fluorescence cube containing 525/50 emission filter and a 560 dichroic filter was used for collecting fluorescence signals.

Statistical analysis

To determine differences between the experimental groups, behavioral parameters and neuroplastic responses were analyzed by either an unpaired Student's t test or a two way analysis of variance (ANOVA) with genotypes and treatments as main factors depending on the experimental design. Bonferroni's test was used for *post hoc* comparisons. In all cases, $p < 0.05$ was considered statistically significant.

Supporting Information Available:

Supplementary data include methods and results for ATP and 5-HT effects on microglia by a transwell assay, for the comparison between ipsilateral projections in dLGN of *Htr2B*^{-/-} vs. *Htr2B*^{+/+} mice, and Volcano plot for mouse chemokines and receptors of *Htr2B*^{-/-} vs. *Htr2B*^{+/+} mice. Supplementary movies display the effects of serotonin application on the growth of neighboring microglia processes. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Abbreviations.

embryonic day (E); postnatal day (P)

retinal ganglion cells (RGCs); dorsal lateral geniculate nucleus (dLGN)

serotonin (5-Hydroxytryptamine, 5-HT); monoamine oxidase A (MAOA)

serotonin transporter (SERT); green fluorescent protein (GFP); fluorescence-activated

cell sorting (FACS); cholera Toxin b subunit (CTb); complement receptor 3 (CR3);

paraformaldehyde (PFA); phosphate buffer (PB); artificial cerebrospinal fluid (ACSF);

analysis of variance (ANOVA).

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Marta Kolodziejczak, performed all the dLGN studies and transwell assays

Catherine Béchade, performed the PCR arrays

Nicolas Gervasi, performed two-photons imaging studies

Theano Irinopoulou, made the Imaris reconstructions

Sophie M. Banas, performed mice breeding and helped with microglia purification

Corinne Cordier, performed the microglia sorting by FACS

Alexandra Rebsam, helped with dLGN experiments and interpretation

Anne Roumier performed the studies on serotonin receptor expression, confocal imaging, experimental design, writing and funding

Luc Maroteaux supervised the analysis, experimental design, writing and funding

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Conflict of Interest

The authors declare no conflict of interest.

FIGURE LEGENDS

Figure 1

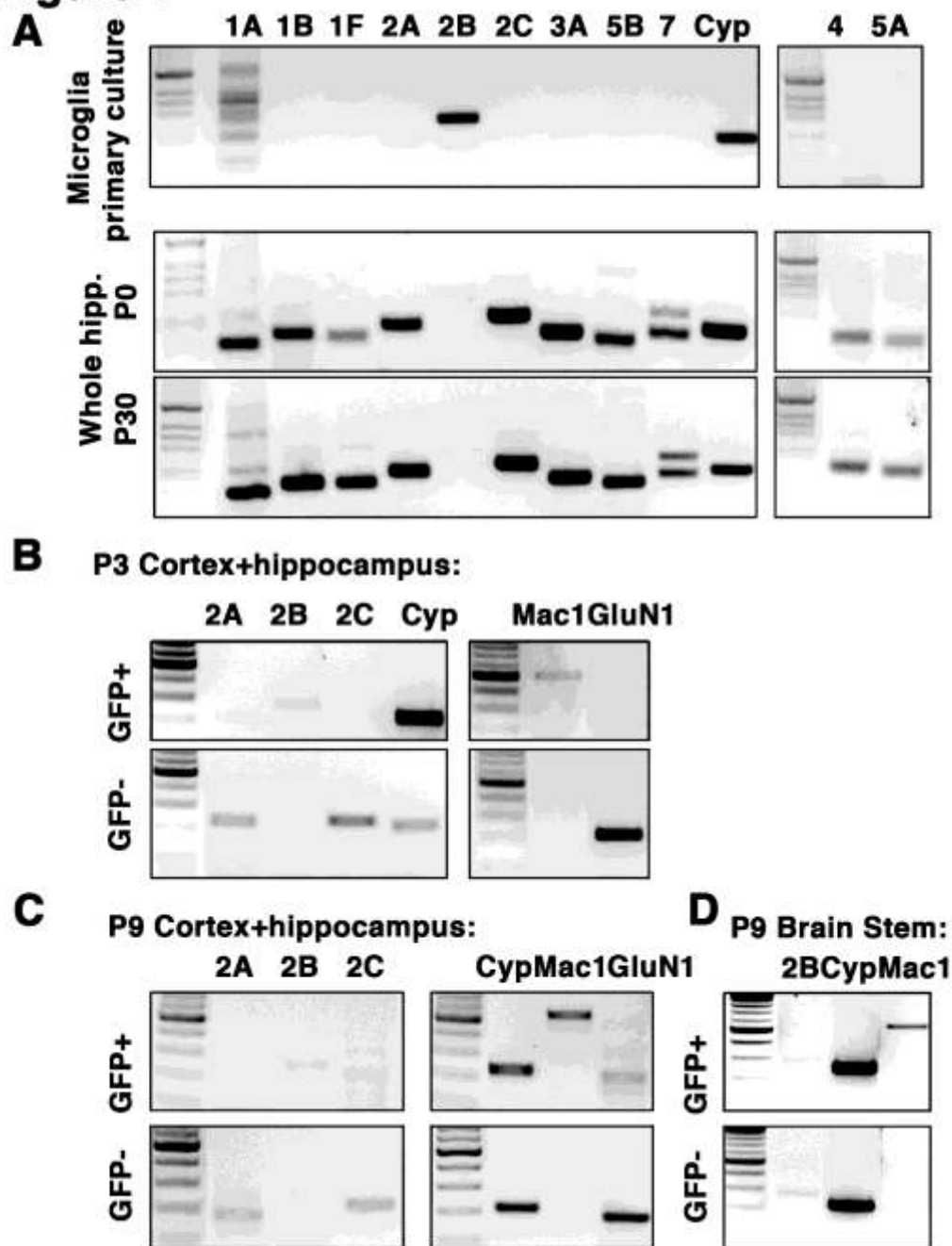


Figure 1: Specific microglial expression of *Htr2B* in primary culture and in the developing brain. (A) RT-PCR experiments on primary culture of microglia (top) and whole hippocampus at P0 (middle) and P30 (bottom) show that *Htr2B* is the main serotonin receptor gene expressed in cultured microglia whereas a large diversity of serotonin receptors subtypes is expressed in whole tissue. Cyp: Cyclophilin-B (reference gene); 1A, 1B, etc: *Htr1A*, *Htr1B*, etc. The bands in lanes 1A and 1F of the top gel, not having the proper size, are non-specific. (B, C) RT-PCR on microglial and non-microglial cells purified by FACS from cortex and hippocampus of *Cx3cr1^{GFP/+}* mice at P3 (B) or P9 (C). At each age, cells were sorted into a GFP positive (GFP+) and a GFP negative (GFP-)

fractions. RT-PCR against the specific microglial gene *Mac1* confirmed that the GFP+ fraction, but not the GFP- fraction, contained microglia. GluN1: NR1 subunit of the NMDAR. (D) RT-PCR on cells sorted from brain stem of *Cx3cr1^{GFP/+}* mice at P9, showing a signal for *Htr_{2B}* in both GFP+ and GFP- fractions.

Figure 2

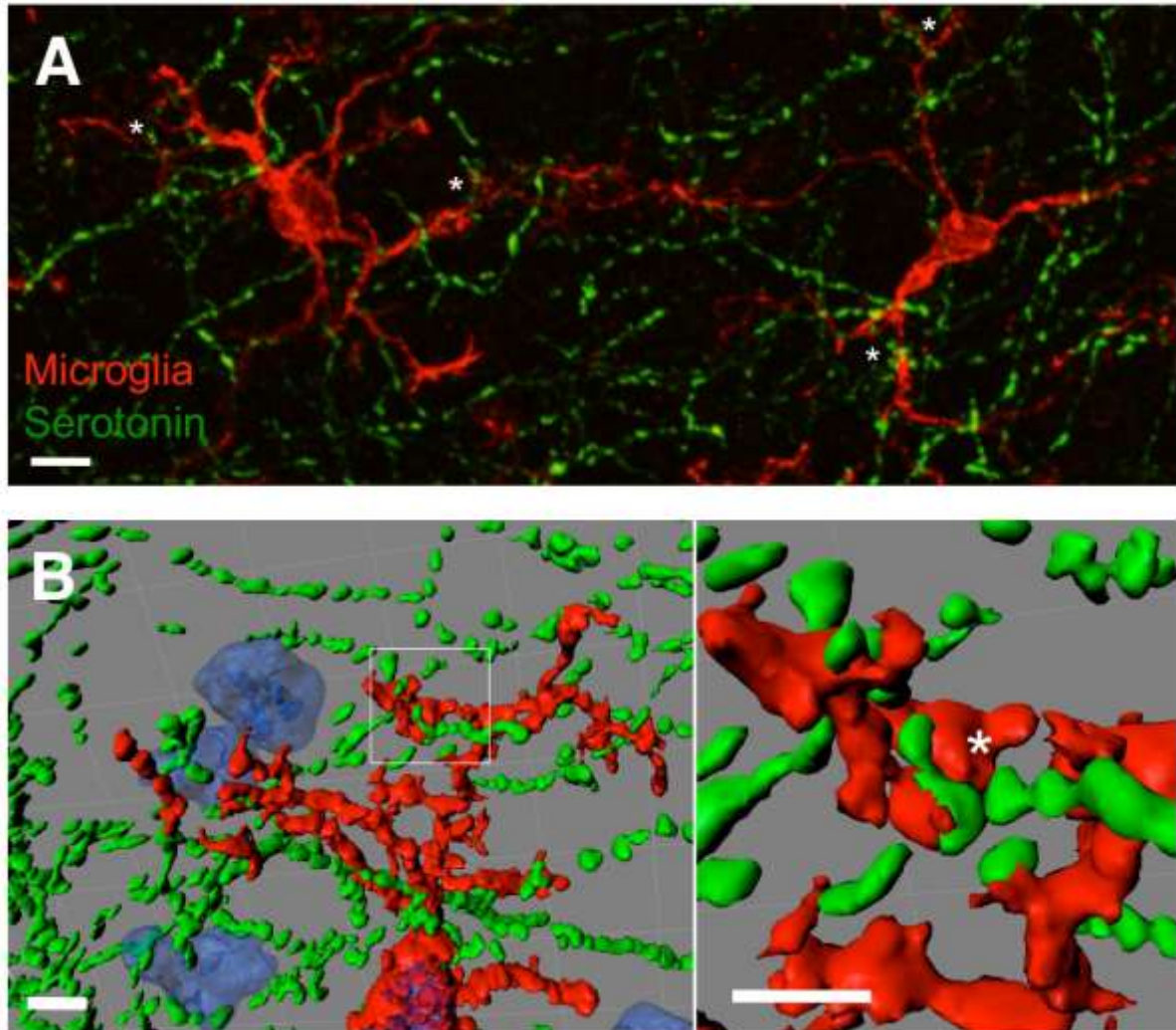


Figure 2: Microglial processes and serotonergic axons are found in close contact *in vivo*. (A) Projection of a 20 μm -thick stack of confocal images from the hippocampus of an adult mouse, showing numerous sites of apposition (asterisks) between microglia stained with anti-Iba1 antibody (red), and serotonergic axons stained with anti-serotonin antibody (green). Note the typical presence of varicosities all along the serotonergic axons. Scale bar: 10 μm . (B) Imaris reconstruction from confocal images. The discontinuous appearance of the serotonergic axons in the reconstruction is due to their particular morphology (varicosities). Nuclei, stained with Bis-Benzimide, are in blue. Left: overview, right: detail showing a microglia process surrounding a serotonin varicosity (asterisk). Scale bars: 5 μm .

Figure 3

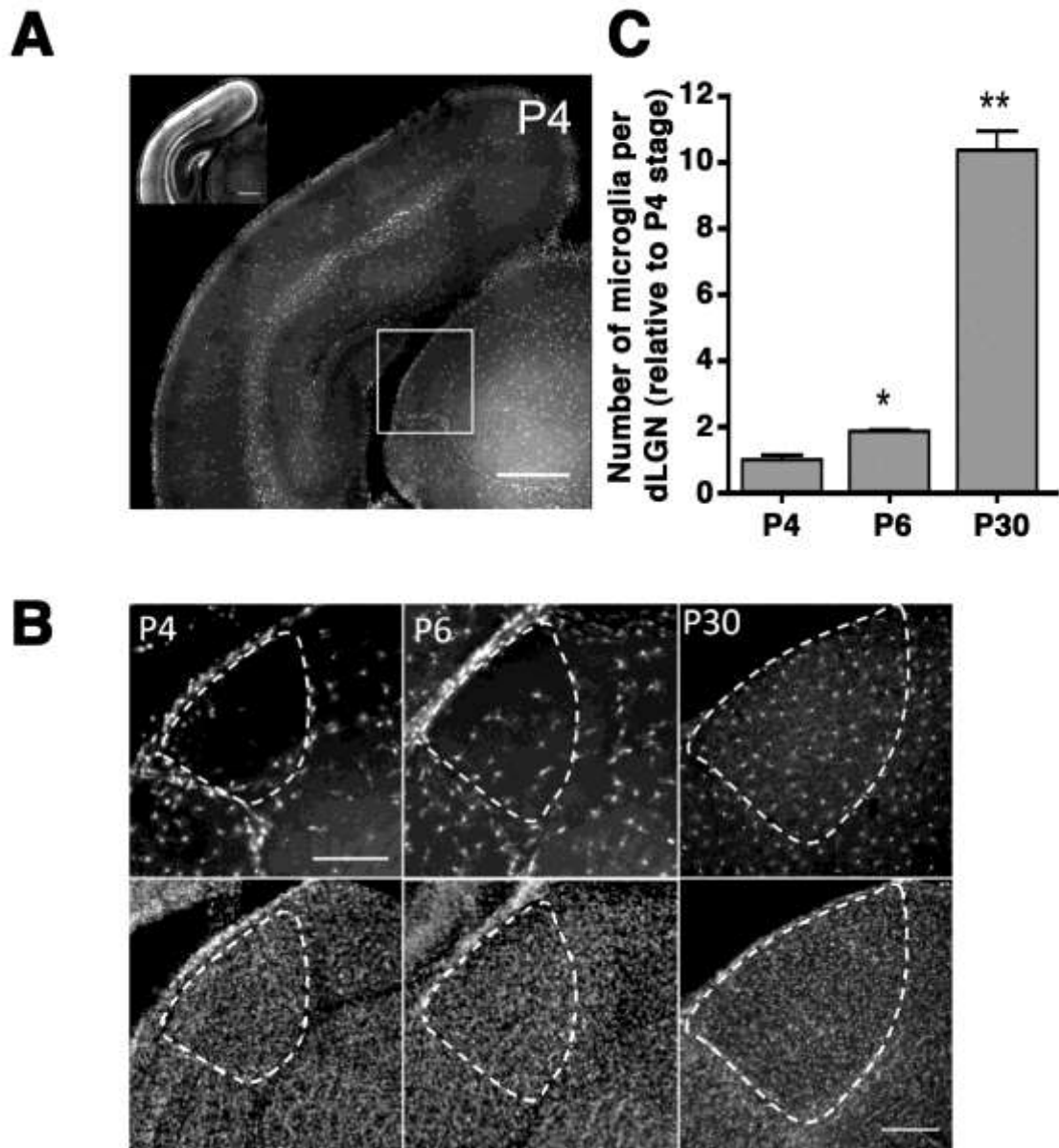


Figure 3: Colonization of the dLGN by microglia during postnatal development.

(A) Distribution of microglia (anti-Iba1 staining) in cortex and thalamus at P4. Scale bar: 500 μm ; upper left inset: nuclei staining with Bis-Benzimide; white square: area magnified in B, including the dLGN. (B) Progressive increase of the density of microglia in the dLGN between P4 and P30. Top : microglia (anti-Iba1), bottom: nuclei (Bis-Benzimide). Scale bars: 250 μm . (C) Relative number of microglia counted in the dLGN per 100 μm -thick brain slice at P4, P6 and P30. P4 (n = 2) and P6 (n = 2) animals used for quantification were littermates, and 2 slices were counted per animal. One-way ANOVA, comparison with P4, (*: $p < 0.05$ and **: $p < 0.01$).

Figure 4

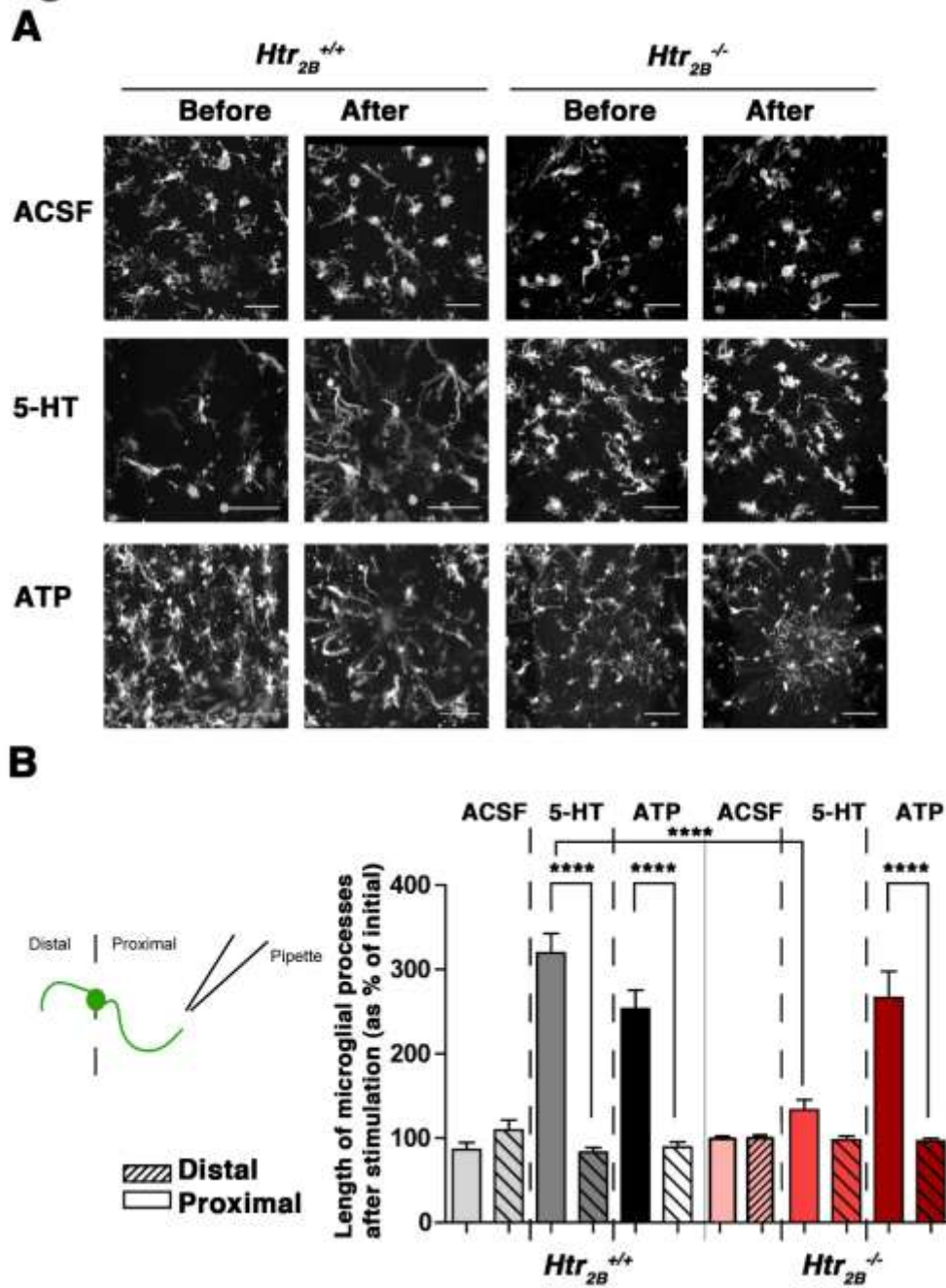


Figure 4: 5-HT_{2B} receptors-mediated chemoattraction of microglial processes toward serotonin in the developing brain. (A) Maximal projections of stacks of 2-photon images taken before and 1 hour after a local application (in the center of the field) of artificial cerebrospinal fluid (ACSF), 5-HT (5 μ M), or ATP (500 μ M) on acute slices of *Htr_{2B}^{+/+}* (left) or *Htr_{2B}^{-/-}* (right); *Cx3cr1^{GFP/+}* mice. All recordings were done in the dLGN area of P11-P14 mice. ATP was used as positive control. Scale bars: 50 μ m. (B) Left: for each slice, processes distal and proximal to the tip of the pipette delivering the ACSF, 5-HT, or ATP solutions, were analyzed separately to show the chemoattractant effect. Right: quantification was done by comparing the length of microglial processes before and after the stimulation. The comparison of proximal vs. distal length was done by one way

ANOVA and Bonferroni post-hoc test (n = 11-25 ****: p<0.0001). See supplementary movies for 5-HT on *Htr2B*^{+/+} or *Htr2B*^{-/-} slices.

Figure 5

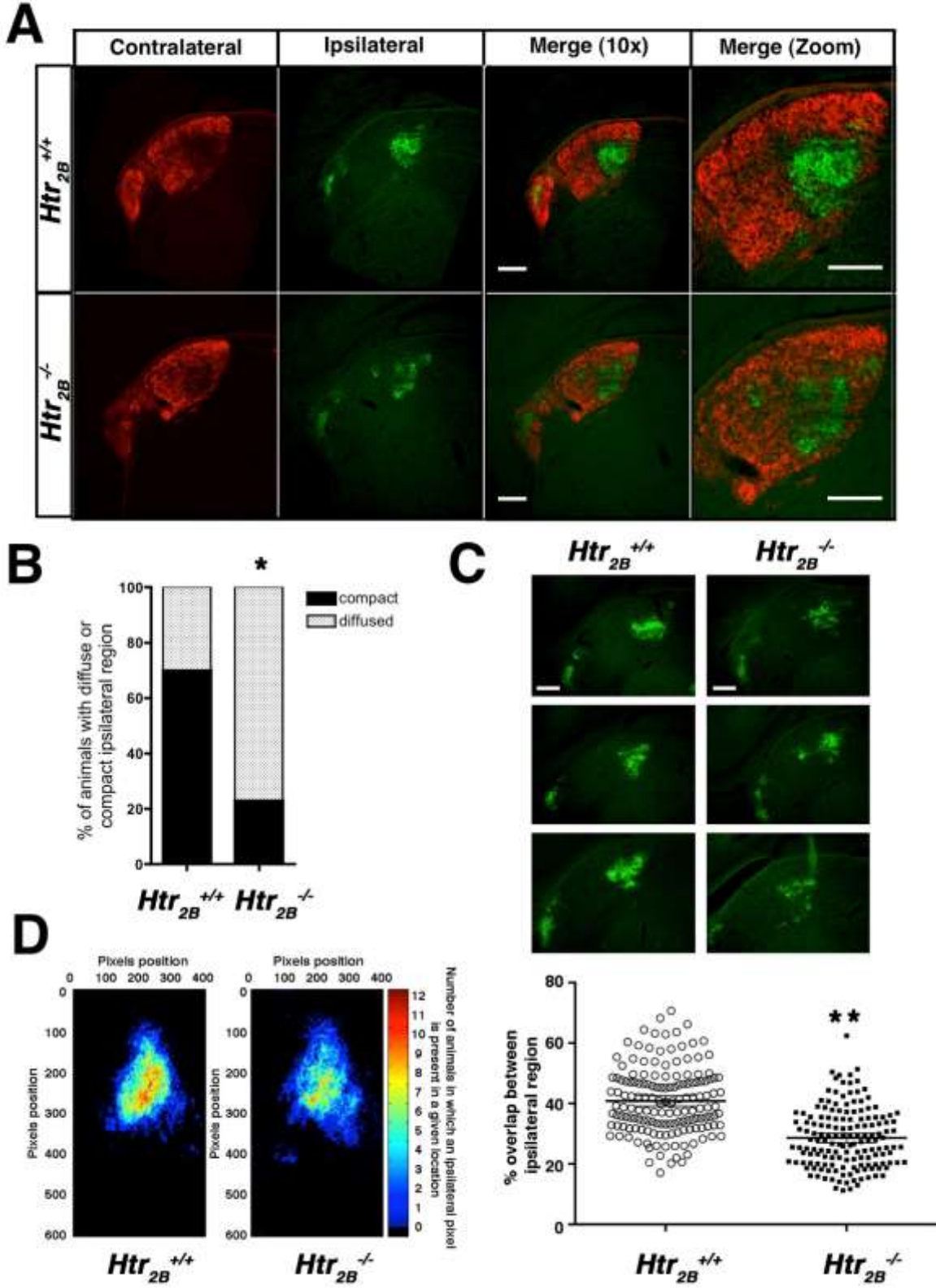


Figure 5: Defects in synaptic refinement in *Htr2B*^{-/-} mice. (A) Dye-labeled retinogeniculate projections in dLGN of *Htr2B*^{+/+} and *Htr2B*^{-/-} mice. Projections from retinal ganglion cells from the ipsilateral retina are in green and those from the contralateral retina are in red. In both *Htr2B*^{+/+} and *Htr2B*^{-/-} animals, projections from ipsilateral and contralateral retinas are segregated, but the region occupied by ipsilateral projections in the *Htr2B*^{+/+} animal is compact and has a regular triangle-like shape, whereas it is diffuse and patchy in the *Htr2B*^{-/-} animal. Scale bars: 100 μm. (B) The majority of *Htr2B*^{+/+} mice have a compact ipsilateral region, whereas majority of *Htr2B*^{-/-} mice have a diffuse ipsilateral region (n = 10-13, p = 0.040 Fischer's test). (C) In each column, pictures from three animals of each genotype illustrate the interindividual stability versus heterogeneity in the shape of the region occupied by ipsilateral projections in *Htr2B*^{+/+} (left) versus *Htr2B*^{-/-} (right) mice, respectively. Scale bars: 100 μm. (D) Heatmaps have been created by superposition of binary images of ipsilateral regions from *Htr2B*^{+/+} or *Htr2B*^{-/-} mice. The color scale corresponds to the number of animals which ipsilateral projections are present in a given location (pixel). The surfaces with "hot" colors, which represent the regions in which most of the mice overlap, are considerably reduced for *Htr2B*^{-/-} comparing to *Htr2B*^{+/+} animals, indicating a defect in the precision of the location of the ipsilateral projections in these mice (n = 12 animals per genotype). (E) The robustness of the location of the ipsilateral projections among individuals was quantified by calculating the average overlap between every two animals of the same genotype. It is significantly reduced in *Htr2B*^{-/-} mice compared to *Htr2B*^{+/+} mice (Two-sided permutation test, p < 0.01).

Figure 6

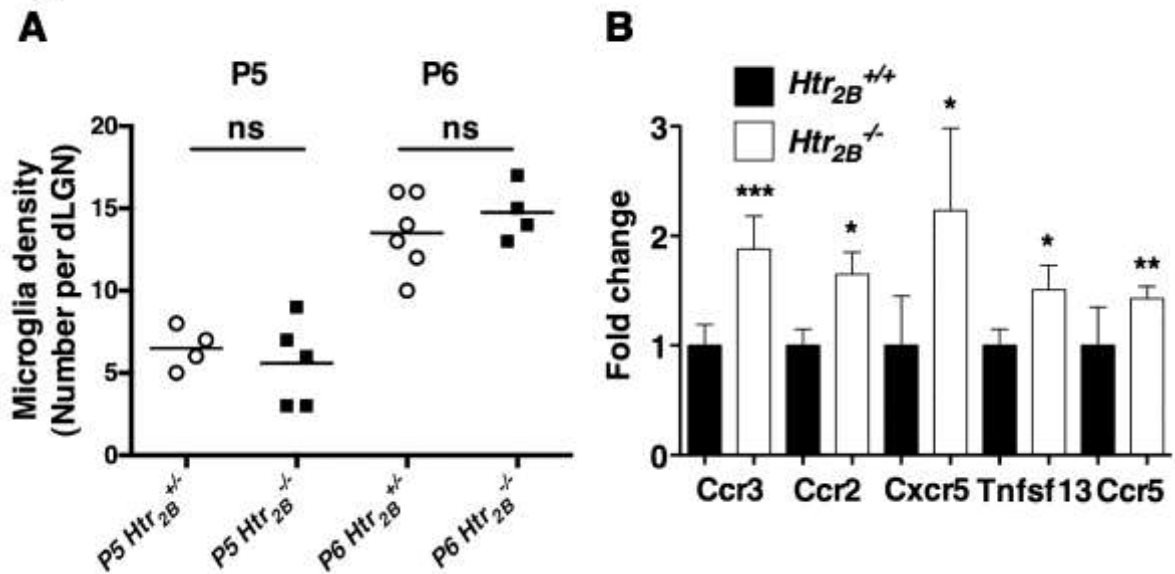


Figure 6: Characterization of *Htr2B*^{-/-} microglia. A) Normal invasion of the dLGN in *Htr2B*^{-/-} mice. Counting of the number of microglia (anti-Iba1 staining) per dLGN in slices from *Htr2B*^{+/+} or *Htr2B*^{-/-} littermates at P5 and P6 revealed no difference due to the genotype. Consistent with Figure 3, a two-fold increase is observed between P5 and P6. One way ANOVA, n = 4-6. B) Overexpression of inflammatory markers in *Htr2B*^{-/-} microglia by comparison to *Htr2B*^{+/+} microglia. cDNAs prepared from primary microglia cultures of *Htr2B*^{+/+} or *Htr2B*^{-/-} pups were subjected to RT² profiler PCR array PAMM-011ZA (mouse inflammatory cytokines and receptors) as described in *Materials and Methods*. The levels of transcripts in the *Htr2B*^{-/-} microglial cultures are expressed relative to the levels in the *Htr2B*^{+/+} microglial cultures. Significant differences between *Htr2B*^{+/+} or *Htr2B*^{-/-} microglia cultures were determined using the Student's t test. (n= 4 cultures of each genotype; *p<0.05; ***p<0.001).

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Supplementary data

Methods

Chemotaxis and chemokinesis in culture (Transwell assays).

Primary cultured microglia, were settled immediately after purification on fibronectin-coated (10ng/ml, Invitrogen) Millicell hanging cell culture inserts in a 24-well plate. These inserts contain a polyethylene terephthalate membrane with 5µm-diameter pores (Millipore). We loaded 50,000 cells per insert. For motility assays, the tested molecules were at the same concentration on both sides of the membrane, and microglia were incubated for 5 h. For chemotaxis assay, the tested molecules were placed exclusively in the lower compartment of the wells, and microglia were incubated for 2 h. In both cases, migration was stopped by fixation with 4% PFA in PB for 15 min. After nuclei staining with Bis-Benzimide (1:1,000 in PB), the inner (upper) part of the insert was cleaned with a cotton swab, the membrane was placed on a glass slide and mounted in Mowiol (Calbiochem; Sigma) and imaged using a Leica DM 6000B fluorescent microscope. The nuclei staining of cells that crossed the membrane was quantified, either manually (cell counting in randomly chosen regions of the membrane) and with a semi-automatized “count nuclei” function in MetaMorph, on series of 6 fields (objective 10x) per membrane (both approaches gave similar results).

Analysis of the segregation of ipsilateral fibers.

Quantification of eye-specific segregation was performed on 10 x images using variable thresholds for the contralateral projection and a fixed threshold for the ipsilateral projection. Using ImageJ software, the boundary of the dLGN was outlined. The intensity threshold for the ipsilateral projection was chosen when the signal-to-background ratio was at least 1.2, especially at the border of the ipsilateral patch, or around the scattered ipsilateral fibers. The overlap between ipsilateral and contralateral projections (pixel overlap) was measured at every 5th threshold value for the contralateral image to obtain the function of overlap between ipsilateral and contralateral projections, as previously described (Rebsam et al., 2009). The portion of dLGN occupied by ipsilateral axons was measured as a ratio of ipsilateral pixels to the total number of pixels in the dLGN region.

Statistical analyses were performed using ANOVA repeated measures. No significant differences were found between genotype.

Reference

Rebsam, A., Petros, T. J., and Mason, C. A. (2009) Switching retinogeniculate axon laterality leads to normal targeting but abnormal eye-specific segregation that is activity dependent, *J Neurosci* 29, 14855-14863.

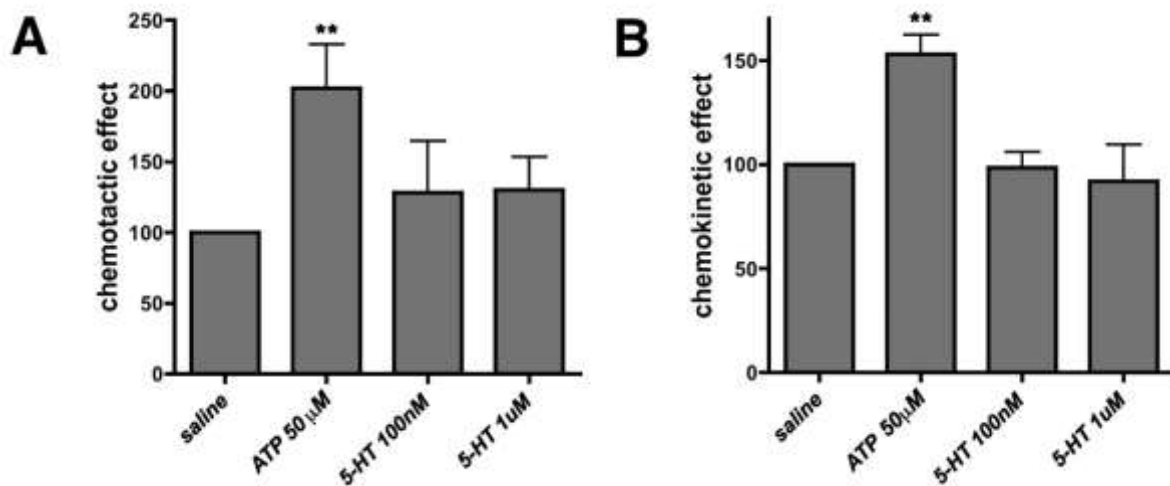


Figure S1: Comparison between ATP and 5-HT effects on microglia by a transwell assay- Primary cultured post natal microglia were settled on top of fibronectin-coated Millicell with 5 μm-diameter pore membrane. A) In chemotaxis assay, 5-HT or ATP were placed exclusively in the lower compartment of the wells at the indicated concentrations. Microglia were incubated for 2 h. B) In chemokinetic (motility) assays, 5-HT or ATP were at the same concentration on both sides of the membrane, and microglia were incubated for 5 h. ATP (50μM) was used as a positive control and shows significant effects. No significant differences were found between 5-HT (100 nM or 1 μM) and saline. Statistical analyses were performed using a Student's t test (n = 3-5, **p < 0.01).

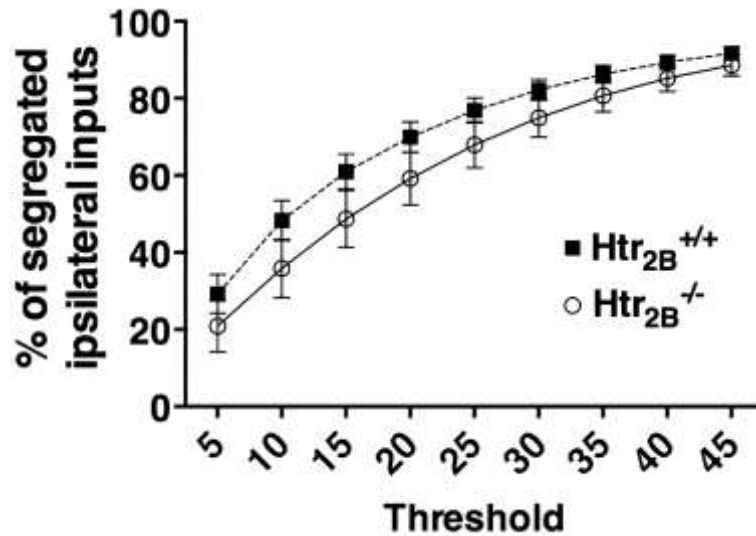


Figure S2: Comparison between ipsilateral projections- The retinal axon segregation on the dLGN at P30 is slightly reduced in *Htr_{2B}^{-/-}* compared to *Htr_{2B}^{+/+}* mice, although not reaching significance ($p > 0.1$). The segregation plot represents the percentage of segregated inputs as a function of the contralateral threshold, the ipsilateral threshold being fixed. Statistical analyses were performed using ANOVA repeated measures.

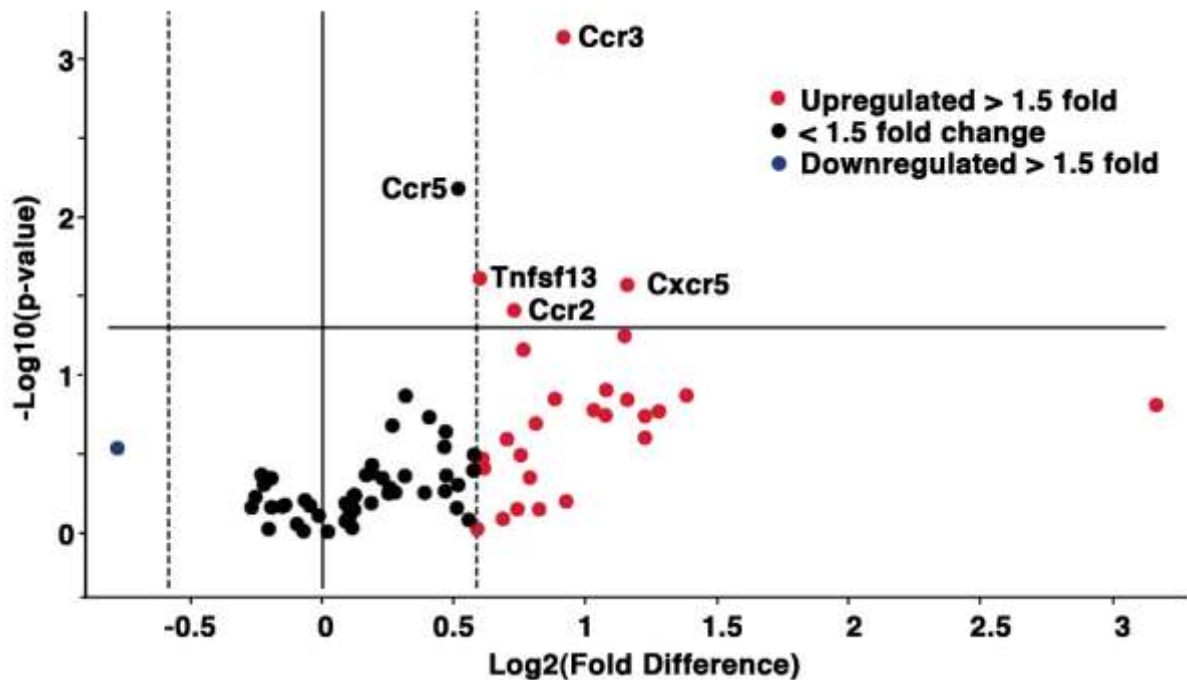


Figure S3. Volcano plot between fold changes for mouse chemokines and receptors of *Htr_{2B}^{-/-}* vs. *Htr_{2B}^{+/+}* vs. p values. cDNA prepared from the microglia cultures were subjected to RT² profiler PCR array for mouse chemokines and receptors, as described

in *Materials and Methods*. Significant differences between *Htr_{2B}^{+/+}* and *Htr_{2B}^{-/-}* microglia cultures were determined using unpaired t-test. Genes positioned above the horizontal line have a p value <0.05. Vertical dotted lines represent a 1.5 fold change in gene expression (threshold for fold difference), (n = 4 cultures of each genotype).

Supplementary movies

Effects of serotonin application on the growth of neighboring microglia processes

The effect of 5-HT on microglia in fresh brain slices was evaluated using two-photon microscopy. The fluorescent microglia of *Htr_{2B}^{+/+}; Cx3Cr1^{GFP/+}* mice have been observed on 300 μ m slices from P11-P14 mice containing the dLGN region. A puff of 5-HT (5 μ M) delivered with a glass micropipet on the center of the field (the time of injection is marked in the movie by "5-HT") on dLGN slice triggered an immediate increased motility of neighboring microglial processes (*Htr_{2B}WT-5-HT*). However, experiment using slices from P11-P14 *Htr_{2B}^{-/-}; Cx3Cr1^{GFP/+}* mice reveals no microglial response to 5-HT (*Htr_{2B}KO-5-HT*) (Scale bars = 50 μ m; movies are 12 frames/s and recordings were 1 frame per min).

B1.1 Axonal Refinement Deficits of *Htr2B*^{-/-} Mice

During my early research, the question arose whether some developmental mechanisms known to be controlled by serotonin, and potentially involving microglia, are impaired when microglia are insensitive to serotonin. In order to investigate this I used an *in vivo* model of axonal segregation during early brain development: the retinal axons segregation onto the thalamic dLGN. Since our results demonstrate that early postnatal microglia express the 5-HT_{2B} receptor, these cells could be involved in RGC afferents segregation. Indeed, as reported in our article “Serotonin modulates developmental microglia via 5-HT_{2B} receptors: potential implication during synaptic refinement of retinogeniculate projections” the full knock-out of this major microglial serotonin receptor leads to alterations of spatial arrangement of eye-specific territories within the thalamic dLGN. Even though the percentage of the overlap between the ipsilateral and the contralateral regions of the *Htr2B*^{-/-} is similar to the wild-type mice, I have identified a difference in their ipsilateral region. At P30, the ipsilateral region of the *Htr2B*^{-/-} mice was more diffuse and patchy whereas it was rather compact and well defined in wild-type animals. In addition, the shape and the location of the ipsilateral region within the dLGN was clearly more variable among the KO than wild-type mice. In the following sections, I will further discuss the altered phenotype observed in the dLGN of *Htr2B*^{-/-} mice.

B1.1.1 Heterogeneity Within wild-type Mice

The anatomical analysis method that I have used revealed a relatively high heterogeneity of the percent of segregation between ipsilateral and contralateral regions within the dLGN of wild-type mice. Curiously, such heterogeneity was not reported in previously published studies (Stevens et al. 2007; Bialas and Stevens 2013). Those studies used C57Bl/6 mice. Since we used 129S2/SvPas mice, the observed increase in basal heterogeneity might be due to their genetic background. Another possibility is that it could result from unstable mice housing conditions. During the study our animal house had been undergoing some changes related to its renovation. This could have in turn resulted in variable sanitary and health state of animals within the study and caused an increased heterogeneity of the basal phenotype.

In order to test this hypothesis, I have recently designed a novel experimental setup where wild-type mice were bred in a “clean” animal house of the Necker Hospital. The comparison of animals bred in our animal house and these bred at the Hospital Necker will show whether the increased basal heterogeneity of the percent of segregation between ipsilateral and contralateral regions within the dLGN is due to housing conditions.

B1.1.2 Axonal Refinement Deficits in $Htr2B^{-/-}$ Mice

The penetrance of the mutant phenotype in $Htr2B^{-/-}$ mice is not 100% and the abnormally shaped and localized ipsilateral region has been observed only in a part of the animals (Article Figure 5). Other knockout mice presented a phenotype similar to wild-types.

Since it was impossible in our case to determine a simple parameter (such as length or roundness of the ipsilateral region), which could be measured for each animal, in order to quantify the difference between genotypes, I measured the level of interindividual variability for each experimental group (as described in the article). I have found that the ipsilateral region of the $Htr2B^{-/-}$ mice is more diffuse, often patchy and have irregular shape comparing to the wild-type animals (Article Figure 5).

The method I have designed has been proved sensitive enough and could be adapted to detect segregation deficits in other brain regions. However, this analysis method requires a number of precautions from the experimenter to avoid several sources of bias. For instance, it is important to pay special attention to the choice of sections for the analysis. DLGN is a three-dimensional structures and its shape changes on the rostrocaudal and nasotemporal axis. Therefore it is important to compare the most corresponding regions. In our case, for each animal, a median reference section was carefully chosen by a blind-to-the-genotype investigator, based on dLGN and vLGN shape. In order to overcome this difficulty one could use three-dimensional image analysis. This would, however, require an experienced investigator with good mathematical and image analysis skills.

Additionally, the quality of the anterograde tracing plays an important role in generating valid results. Both, retina injury and incomplete injection, can deform the perception of ipsilateral and contralateral dLGN regions which would greatly bias the analysis. In order

to confirm the quality of my CTb injections, for each animal, I carefully examined the entire dLGN and eliminated the injured ones.

B1.1.2.1 Retina Injury Consequences

When the retina is damaged, while injecting the CTb to the eye vitreous, this often results in an incomplete stain of the RGC terminals in the dLGN. The damaged RGCs do not transport CTb and such injury is visible on the dLGN section as a “gap” in the fluorescence of axonal terminals. I expected that the information about such an injury at the level of retina would be transmitted to the central targets of the RGCs, for instance, by a release of signaling molecules (ATP, 5-HT etc.).

Since microglia are known to quickly respond to changes in their microenvironment (discussed in the chapter “Microglia Activation”), I expected that the dLGN microglia will detect and respond to the signals potentially released by injured RGCs. I examined microglia morphology in the dLGN regions corresponding to the projection area of injured RGC afferents either one day (P8 mice) or two days (P30 mice) after CTb injection. I revealed microglial cells with an antibody against Iba1 in mice in which only one eye was injected with the CTb. Strikingly, I observed an increased number of microglia within these regions (Figure 25 B, F). Moreover, the cells within the region corresponding to the injured axons presented an activated-like morphology characterized by bigger cell bodies (Figure 25 I, J).

Thus, information about RGCs injury at the level of retina can be transmitted to their central target and very quickly (one day in P8 and two days in P30 mice) trigger microgliosis at the projection sites of their terminals.

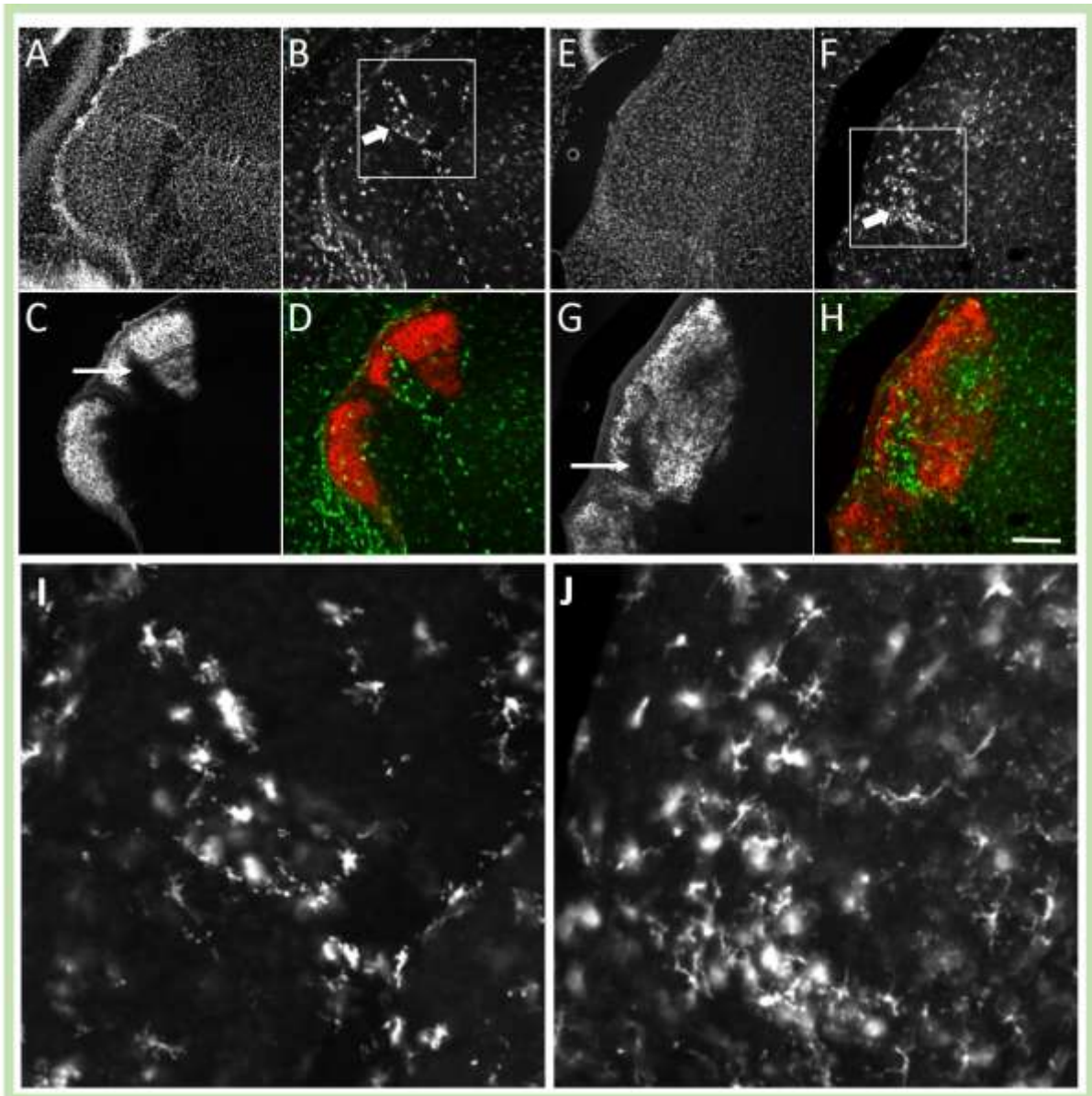


Figure 25: **Retina injury-induced recruitment of microglia to the dLGN.** A-D: P8; E-H: P30; A, E: nuclei stain (Bis-Benzimide); B, F: microglia stain (Iba1); C, G: contralateral region of dLGN (CTb); D, H: merge of microglia and contralateral region stain; I, J: enlargement of the region in the white square of B, F respectively; thick arrow: area corresponding to RGC axons which were injured within the retina; thin arrow: microgliosis. Scale bar: 200 μ m

B1.2 5-HT effect on microglia

B1.2.1 In situ Chemoattractant Effect of Serotonin on Microglial Processes

In order to further investigate the putative interaction between microglia and serotonin we have tested, in a collaboration with Nicolas Gervasi, the effect of local serotonin

delivery on microglia in acute brain slices of young postnatal mouse (by two photon microscopy; Article Figure 4).

A recent study has used a similar, but not identical method to demonstrate another serotonin effect on microglia. They have shown that serotonin, applied in bath on acute brain slices of adult mouse, increases microglia motility toward a laser injury. This result suggested that serotonin enhances microglial responses to environmental cues (such as ATP) signaling an injury (Krabbe et al. 2012).

We, on the other hand, were more interested in the serotonin effect on microglial developmental functionality in physiological conditions. Instead of applying a bath of serotonin we released it at a specific location to create a gradient of this neurotransmitter. We chose a time (P10-P14) and location (dLGN) which match the sensitive developmental period of axonal refinement.

We could observe that microglial processes moved rapidly toward the source of serotonin in wild-type mice, but not in *Htr2B*^{-/-} mice lacking the 5-HT_{2B} receptor. This suggests that serotonin itself could act as a chemotactic signal that attracts microglia to the site of axonal refinement and that this action is mediated by the microglial 5-HT_{2B} receptor.

This is an especially intriguing hypothesis, since it is well established that, during the sensitive developmental periods, a subset of non-monoaminergic neurons transiently express a partial serotonergic phenotype which could result in locally differential serotonin gradients.

B1.2.2 Phagocytosis assay

I have also tested serotonin effects on microglia in the primary cell culture. Besides chemotaxis assay (Article Figure S1), I have also performed a set of phagocytosis assays.

First, I have tested wild-type microglia incubated with or without 5-HT after 24h serum starvation. I have not observe significant 5-HT effect on microglial phagocytic capacities (Figure 26 B). Our results are in disagreement with a recent study which has reported that serotonin decreases phagocytosis both in a primary cell culture and acute brain slices of neonatal brain (Krabbe et al. 2012). This difference might originate from the

type of particles to be phagocytosed (us: *E.coli*; them: latex beads) or from use of different serotonin concentrations. The dissociation constant (K_d) is defined as a concentration of ligand (here the serotonin) at which half of binding sites of a receptor (here the 5-HT_{2B}) are occupied. The K_d of the 5-HT_{2B} receptor to binding serotonin is around 10nM. Therefore, to test the effect of this neurotransmitter on microglia we chose two concentration: 100nM and 1 μ M. The serotonin inhibitory effect on microglial phagocytosis, on the other hand, has been observed after stimulation with 100 μ M of serotonin. Since the K_d of most of G protein-coupled receptors is on the order of nM (10^{-9} M), the use of such a high concentration seems excessive; 100 μ M of serotonin can activate other, non-serotonergic, receptors which have much lower affinity to serotonin. Therefore, it is unjustified to conclude that the effect of microglia stimulation with 100 μ M of serotonin is mediated by serotonergic receptors.

An alternative to the direct microglial phagocytosis stimulation could be a role of serotonin in microglia differentiation. Through the 5-HT_{2B} receptor it might mediate acquisition of microglia developmental properties such as phagocytic capacities. This led to a hypothesis that basal phagocytic capacities of *Htr2B*^{-/-} microglia could be altered. Therefore, I have tested basal phagocytosis (without 5-HT stimulation) of microglia from heterozygotes (*Htr2B*^{+/-}) and full *Htr2B*^{-/-} littermates. This analysis revealed a trend towards increased phagocytic capacities of *Htr2B*^{-/-} microglia (Figure 26 A) although it did not reach significance.

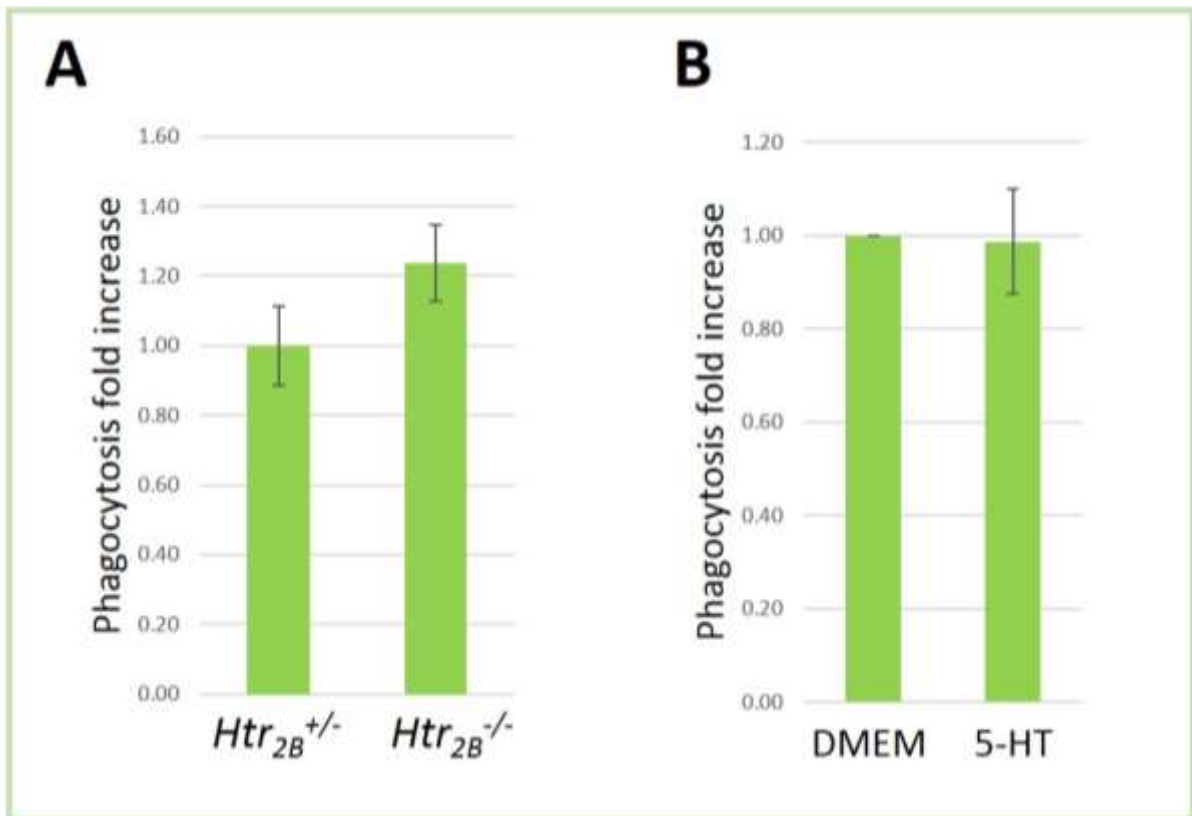


Figure 26: **Microglia phagocytic capacities.** Primary microglia culture. A: basal phagocytosis of *Htr*_{2B}^{-/-} compared with heterozygotes littermates; B: phagocytosis after 45min stimulation with 100nM serotonin versus non treated microglia. P value: 0.16 in A; 0.8 in B (Student T test). Error bar: SEM.

In these experiments, we have used the *Vybrant Phagocytosis Assay Kit* (Thermo Fisher Scientific). Briefly, primary cultured microglia, were settled immediately after purification on a 96-well microplate (100000 cells/well; with or without 100μM5-HT) and incubated with fluorescein-labelled *Escherichia coli*. After 45min incubation time, the amount of engulfed fluorescent bacteria has been quantified using a fluorescence microplate reader. Both, basal and 5-HT-induced microglial phagocytosis have been tested. The presented results have been obtained from four independent experiments.

Since our results showing microglia responsiveness to serotonin on acute brain slices but not in culture, it is possible that the effect of serotonin on microglial phagocytic capacities, motility and chemotaxis are proper to a sensitive developmental periods and locations. Microglia from a primary cell culture are growing for ten days on a Petri dish, which is far from physiological conditions. Microglial response to serotonin might be

dependent on the environmental context and/or require other cell partners (e.g. astrocytes).

B2 Potential Mechanisms & Cells Involved in *Htr2B*^{-/-} Mice Deficits

The phenotype I have identified in the *Htr2B*^{-/-} mice could be mediated by any brain cell which expresses the 5-HT_{2B} receptor. In the following part, I will present different candidate cells and the genetic models (conditional KO mice) I used to investigate their potential involvement in the phenotype observed in the *Htr2B*^{-/-} mice.

B2.1 Cells Expressing 5-HT_{2B} in dLGN

A number of cells present in the dLGN are susceptible to express a functional 5-HT_{2B} receptor (Figure 27).

We have seen that 5-HT_{2B} is a major serotonin receptor expressed on microglial cells. Moreover, serotonergic raphe neurons, which innervate the dLGN (Introduction Figure 11), have also been reported to express this receptor (Diaz et al. 2012). The 5-HT_{2B} could be also expressed by other cell types which project to the dLGN, the RGCs. Indeed, the presence of messenger RNAs for the 5-HT_{2B} receptor has been reported in human retina, where it represents one of the most abundant serotonin receptors (Sharif and Senchyna 2006). Moreover, the unpublished results of a colleague from our institute, Cedric Francius, demonstrate by ISH that the 5-HT_{2B} receptor expression is present in the ganglion cell layer of the eye of a P5 mouse. A co-staining of RGCs is however necessary to confirm the presence of 5-HT_{2B} receptor in these cells.

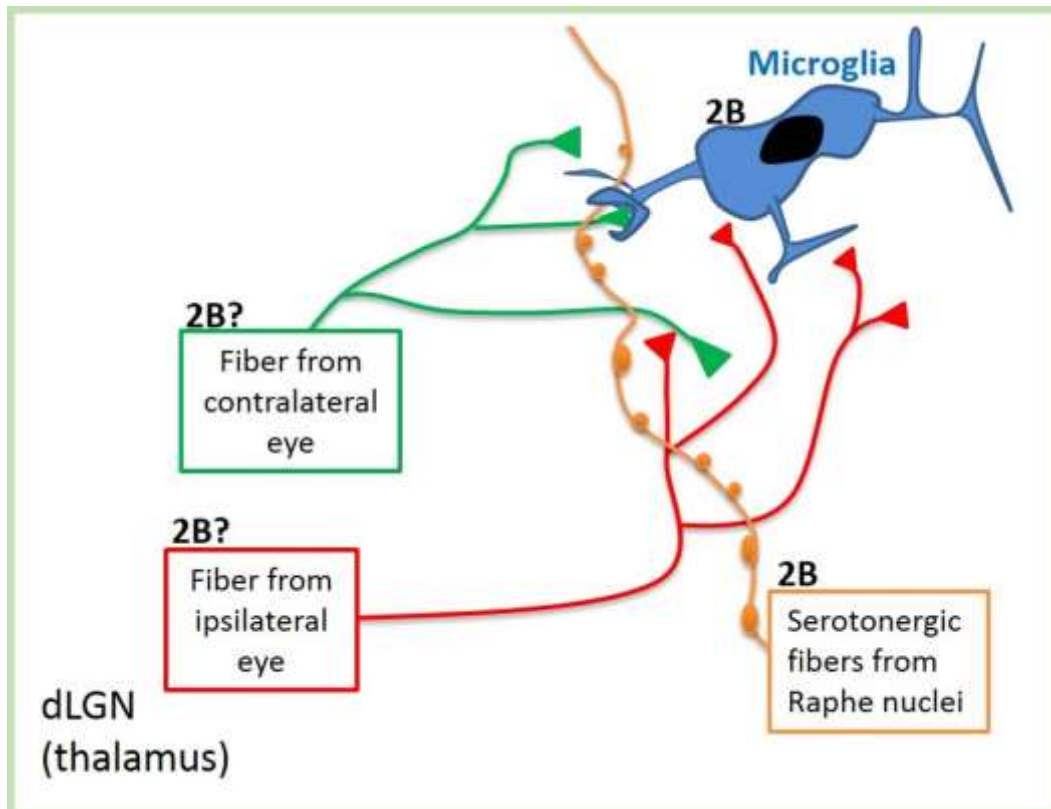


Figure 27: **Cells in the murine dLGN susceptible to express 5-HT_{2B} receptor.** Green and Red: RGCs; Orange: serotonergic raphe neurons (represented with the characteristic varicosities); Blue: microglia.

Low level of 5-HT_{2B} receptor in the brain and the lack of specific antibodies, which would enable its detection in mice, makes it difficult to clearly confirm the presence of this receptor on cells. In order to identify cells responsible for the deficit of segregation of RGCs in the dLGN of *Htr2B*^{-/-} mice, we decided to use a transgenic-based approach and have tested a conditional knockout of 5-HT_{2B} receptor in the candidate cells.

B2.2 Effect of the Invalidation of the 5-HT_{2B} in *Pet1*^{Cre/0}; *Htr2B*^{fl/fl} mice

The study, which demonstrated the 5-HT_{2B} receptor presence in a large majority of serotonergic raphe neurons, has also shown that an increase of extracellular 5-HT in the hippocampus induced by SSRIs can be mimicked by treatment with 5-HT_{2B} agonist and it is absent in the 5-HT_{2B}-deficient mice (Diaz et al. 2012). This demonstrates that 5-HT_{2B} receptor can modulate SERT activity and therefore it could control extracellular

serotonin level. Thus, 5-HT_{2B} receptors expressed by raphe neurons innervating the dLGN could participate in the axonal refinement by modulating the local serotonin concentration. Therefore, we have examined axonal refinement in animals in which 5-HT_{2B} receptor has been removed specifically from these neurons. Mice expressing Cre recombinase enzyme under control of Pet1 promoter (*Pet1^{Cre/0}*) enable to successfully target gene expression in a 5-HT neuron-specific manner (Scott et al. 2005).

Our analysis of RGC axons segregation in the dLGN of *Pet1^{Cre/0}; Htr2B^{fl/fl}* mice revealed a tendency toward a more irregular shape and more heterogeneous localization of the ipsilateral region within the dLGN (Figure 28), reminding the phenotype of total *Htr2B^{-/-}* mice. However, this difference did not reach statistical significance.

This suggests either that these neurons do not mediate this developmental process or that they act in a synergy with, or parallelly to some other 5-HT_{2B}-expressing cells.

Moreover, depending on the targeted gene, the recombination in the *Pet1^{Cre/0}* mice can be more or less efficient. For instance, in *Pet1^{Cre/0}; VMAT2^{fl/fl}*, in which the VMAT2 gene is inactivated specifically in the raphe neurons, approximately 20% of serotonin neurons still contained high 5-HT level. The uniform brain distribution of these 5-HT positive neurons suggests a random incomplete recombination (Narboux-Nême et al. 2013).

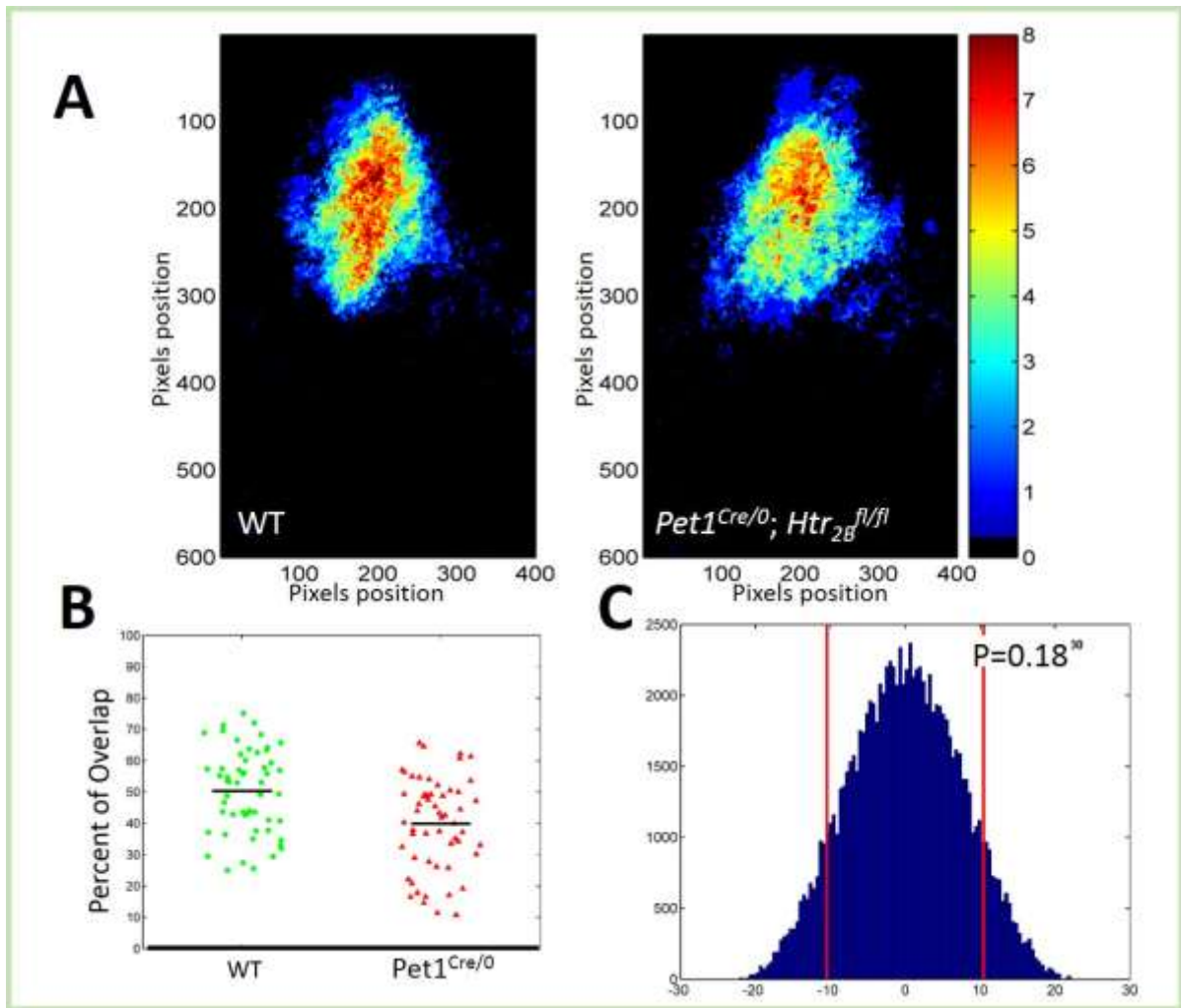


Figure 28: **Synaptic refinement in *Pet1^{Cre/0}; Htr_{2B}^{fl/fl}* mice.** A: Heatmaps have been created by superposition of binary images of ipsilateral regions from *Htr_{2B}^{fl/fl}* (control) or *Pet1^{Cre/0}; Htr_{2B}^{fl/fl}* mice. The color scale corresponds to the number of animals which ipsilateral projections are present in a given location (pixel). The surfaces with “hot” colors, which represent the regions in which most of the mice overlap, are reduced for *Pet1^{Cre/0}; Htr_{2B}^{fl/fl}* comparing to *Htr_{2B}^{fl/fl}* animals (n = 8 animals per genotype). B: The robustness of the location of the ipsilateral projections among individuals was quantified by calculating the average overlap between every two animals of the same genotype. It is not significantly reduced in *Pet1^{Cre/0}; Htr_{2B}^{fl/fl}* mice compared to *Htr_{2B}^{fl/fl}* mice (C: Two-sided permutation test, p=0.18).

B2.3 Putative Role of Retinal Ganglion Cells

A number of previous studies has proposed that the developmental patterning of RGCs could involve serotonin stimulation of RGC afferents themselves. For instance, RGCs have been reported to express 5-HT_{1B} receptor starting at E15 (Upton et al. 1999). Additionally, during early postnatal life, a population of RGCs, mostly restricted to those that project ipsilaterally, transiently expresses SERT (Upton et al. 1999). As I have

previously introduced (see chapter “Models to Study Serotonin Effects”), SERT-deficient mice present alterations in the RGC axons segregation within the dLGN. Interestingly, in double knockouts in which both, SERT and 5-HT_{1B} receptor are absent, the segregation of RGC afferents in the dLGN is wild-type-like (Upton et al. 2002). Yet another study has shown, that in the hamster brain activation of the presynaptic 5-HT_{1B} on the RGCs results in the decreased glutamate release suggesting their role in the inhibition of retinal transmission (Mooney et al. 1994). The amelioration of the segregation deficit in the double KO in which both 5-HT_{1B} and SERT are absent, suggesting that the increased extracellular serotonin level observed in the SERT-deficient mice could result in an overstimulation of the 5-HT_{1B}, which could be responsible for the RGCs segregation deficiently.

Even though the expression of the 5-HT_{2B} receptor by the RGCs has not yet been reported, its role in development of *Xenopus* embryo retina has been demonstrated. 5-HT_{2B} overexpression results in eye morphological defects, including ectopic position of retinal cells (Reisoli et al. 2008). Therefore RGCs constitute a valid candidate that could be responsible for the segregation deficit observed in the dLGN of *Htr2B*^{-/-} animals.

B2.4 Effect of the Invalidation of *Htr2B* in microglia

Microglia with their phagocytic capacities are also good candidates to be implicated in the developmental removal of redundant synapses. As we have demonstrated, just before the onset of the segregation of contralateral and ipsilateral fibers, microglia are distributed outside of the dLGN and their invasion coincides with the critical window of developmental segregation of RGC axons (Article Figure 3B). Moreover, since we have shown that microglia express 5-HT_{2B} receptor, the mutant phenotype observed in the *Htr2B*^{-/-} mice could be due to the absence of this receptor on these brain resident macrophages. Moreover, a recent study, investigating mice with defective microglia (C3R-deficient) has also reported a deficit in dLGN segregation (Stevens et al. 2007), supporting our hypothesis on microglia involvement.

In order to investigate a putative contribution of microglia to the disturbed segregation of retinal axons in the thalamus of *Htr2B*^{-/-} animals, we have generated a mouse with a specific invalidation of 5-HT_{2B} gene in microglia (*Cx3cr1*^{CreERT2/+}; *Htr2B*^{fl/fl}). To do so, we

have crossed males *Cx3cr1^{CreERT2/+}* mouse (Parkhurst et al. 2013) with a female mouse carrying a floxed 5-HT_{2B} gene (*Htr2B^{fl/fl}*). By administrating TXF at birth (P1-P3) the Cre recombinase is activated and it removes the *Htr2B* gene specifically in CX3CR1 positive cells (microglia).

Our analysis has not revealed any significant deficiency of RGCs segregation in dLGN of these *Cx3cr1^{CreERT2/+}; Htr2B^{fl/fl}* mice (Figure 29).

However, since the retinal axon refinement in the thalamic dLGN is an early postnatal event (P3-P9), it is possible that the TXF-induced actual disappearance of the 5-HT_{2B} receptor occurs too late to cause dLGN wiring deficiencies. In this case, TXF gavage of pregnant dams could be required.

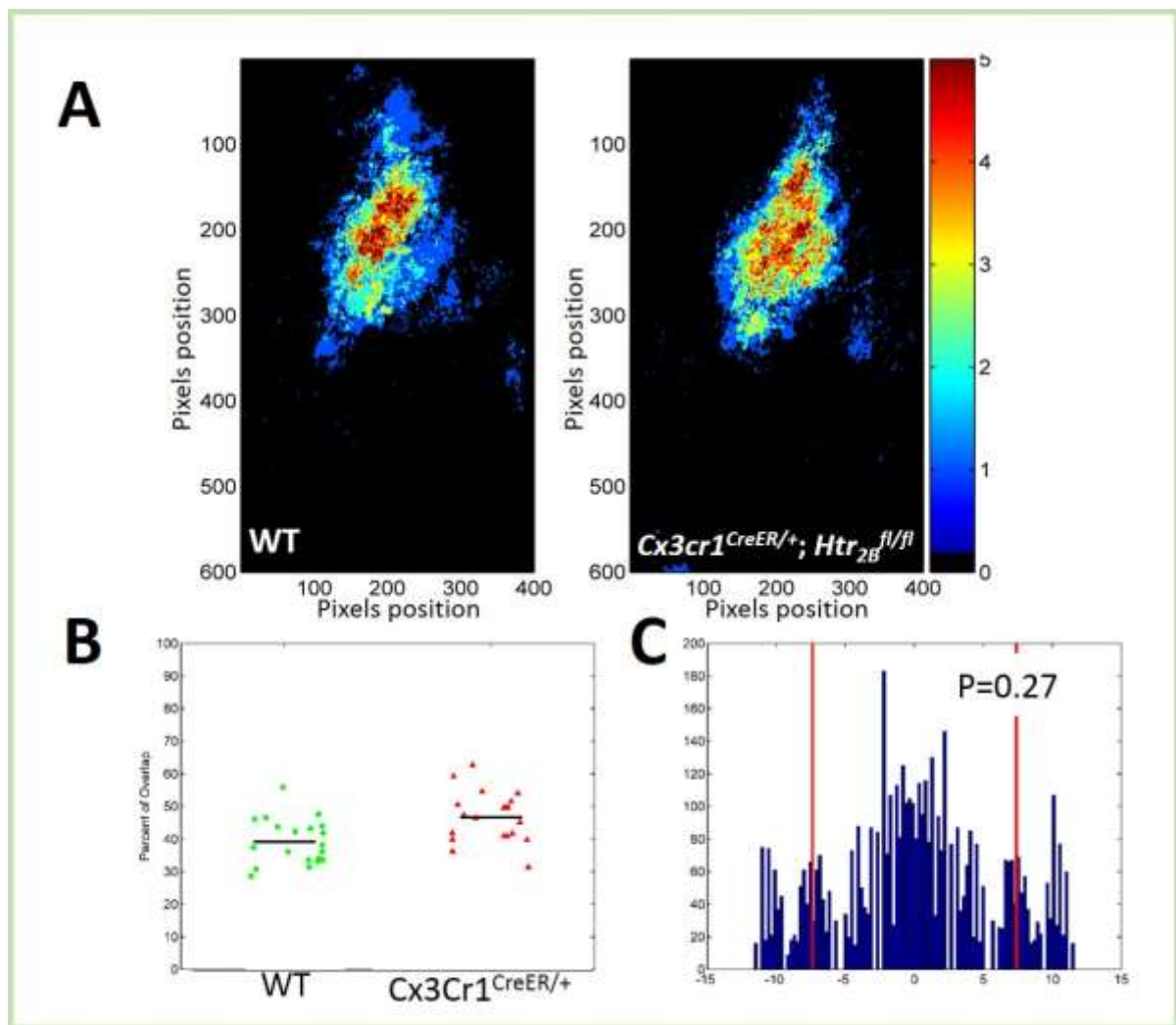


Figure 29: **Synaptic refinement in the *Cx3cr1^{CreERT2/+}; Htr2B^{fl/fl}* mice.** Heatmaps have been created by superposition of binary images of ipsilateral regions from *Htr2B^{fl/fl}* (control) or *Cx3cr1^{CreERT2/+}; Htr2B^{fl/fl}* mice. The color scale corresponds to the number of animals which

ipsilateral projections are present in a given location (pixel). The surfaces with “hot” colors, which represent the regions in which most of the mice overlap, is similar for *Cx3cr1^{CreERT2/+}; Htr2B^{fl/fl}* mice comparing to *Htr2B^{fl/fl}* animals (n = 5 animals per genotype). B: The robustness of the location of the ipsilateral projections among individuals was quantified by calculating the average overlap between every two animals of the same genotype. It is not significantly reduced in *Cx3Cr1^{CreERT2/+}; Htr2B^{fl/fl}* mice compared to *Htr2B^{fl/fl}* mice (C: Two-sided permutation test, p=0.27)

During my PhD, I have tested two distinct conditional mice which allow to target gene expression specifically in microglia: the TXF-inducible *Cx3cr1^{CreERT2/+}* and the *Cx3Cr1^{Cre}* with a constitutively active Cre recombinase (Yona et al. 2013). However, neither of them was optimal. I will discuss the problems I encountered in the following chapter.

B3 Conditional Knockouts of 5-HT_{2B} Receptor in Microglia

B3.1 Cre lox Recombination

In order to further elucidate the precise mechanisms behind the putative role of microglia in the developmental axonal refinement, we have used a novel powerful genetic tool enabling tissue-targeted gene manipulation, the Cre lox recombination. It uses tissue-specific promoters to drive the expression of Cre recombinase, an enzyme able to remove a DNA sequence enclosed between two loxP sites (a “floxed” DNA sequence) through a site-specific recombination (Figure 30 A).

The Cre recombinase can also be fused to a mutated ligand-binding domain of a human estrogen receptor (ER), which can bind a synthetic estrogen antagonist, TXF. Since the ER is mutated, it has low affinity for endogenous estrogen. This system allows to control the subcellular localization of the Cre recombinase, which can be translocated to the nucleus only in presence of the TXF, whereas in the unbound form, it is located in the cytoplasm (Indra et al. 1999). Thus, in the *CreERT2* mice, the recombination takes place only in the presence of TXF (Figure 30 B).

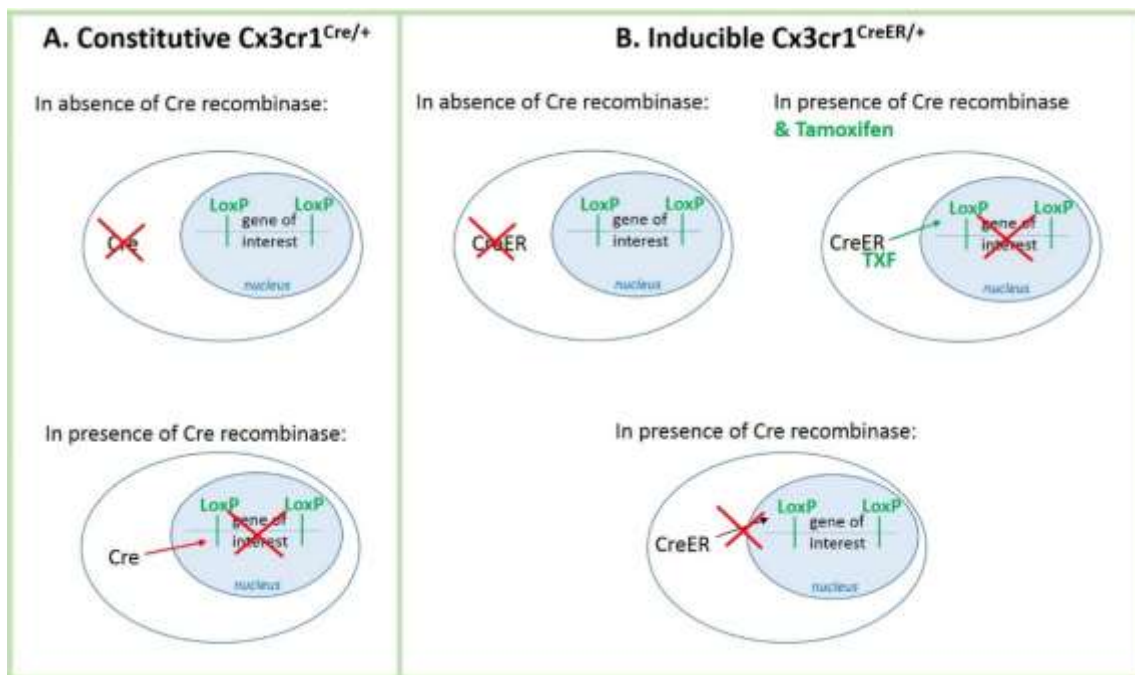


Figure 30: Schematic representation of different invalidations of genes of interest in microglia.

Recently, two transgenic mice harboring a gene encoding a Cre recombinase enzyme inserted in their *Cx3cr1* loci have been generated. One expresses constitutively active Cre recombinase (the *Cx3cr1^{Cre/+}*; Figure 31 A) (Yona et al. 2013), whereas the second one requires TXF administration in order to activate the Cre recombinase (*Cx3cr1^{CreERT2/+}*; Figure 31 B) (Yona et al. 2013; Parkhurst et al. 2013).

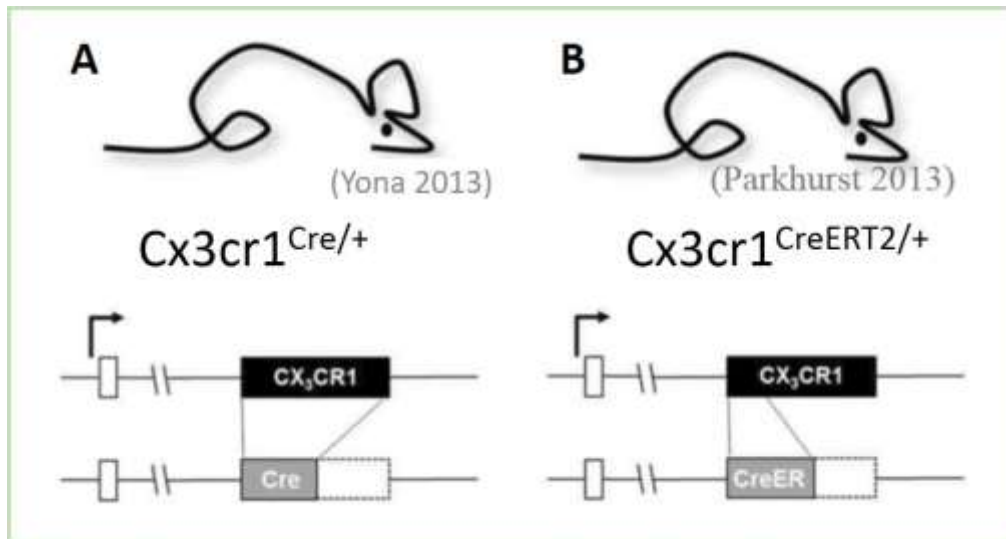


Figure 31: **Schematic of modified *Cx3cr1* loci** of A: *Cx3cr1^{Cre/+}* and B: *Cx3cr1^{CreERT2/+}* mice. (Wolf 2013)

Although, *Cx3cr1* has been reported to be present in multiple myeloid populations in the periphery, in the brain it is expressed exclusively by microglia. Therefore, in order to test the putative role of microglia in the developmental axonal refinement, we have used both of these mice to generate the conditional 5-HT_{2B} receptor deletion specifically in microglia. In the following sections I will discuss both of these models.

B3.1.1 Inducible *Cx3cr1^{CreERT2/+}; Htr2B^{fl/fl}*

In order to investigate a putative contribution of microglia to the disturbed segregation of retinal axons in the thalamus of *Htr2B^{-/-}* animals, we have generated a mouse with a specific mutation of 5-HT_{2B} gene in microglia. To do so, we have crossed a male *Cx3cr1^{CreERT2/+}* mouse with a female mouse carrying floxed 5-HT_{2B} gene, *Htr2B^{fl/fl}* generated in the lab. By administering TXF either at birth or in adulthood, this model can additionally allow to differentiate between the effects of developmental versus adult role of microglial 5-HT_{2B}.

At birth TXF was administrated with three successive injections at P1-P3 (50µl of solution at 1mg ml⁻¹/injection). A number of controls have been performed to confirm the efficacy and specificity of the recombination.

First, presence of recombinant alleles has been confirmed by a PCR reaction (Figure 32) in both brain and tail tissue. In the brain, the recombinant PCR product should come from microglia, whereas in the tail from the peripheral CX3CR1 positive cells such as epidermal Langerhans cells.

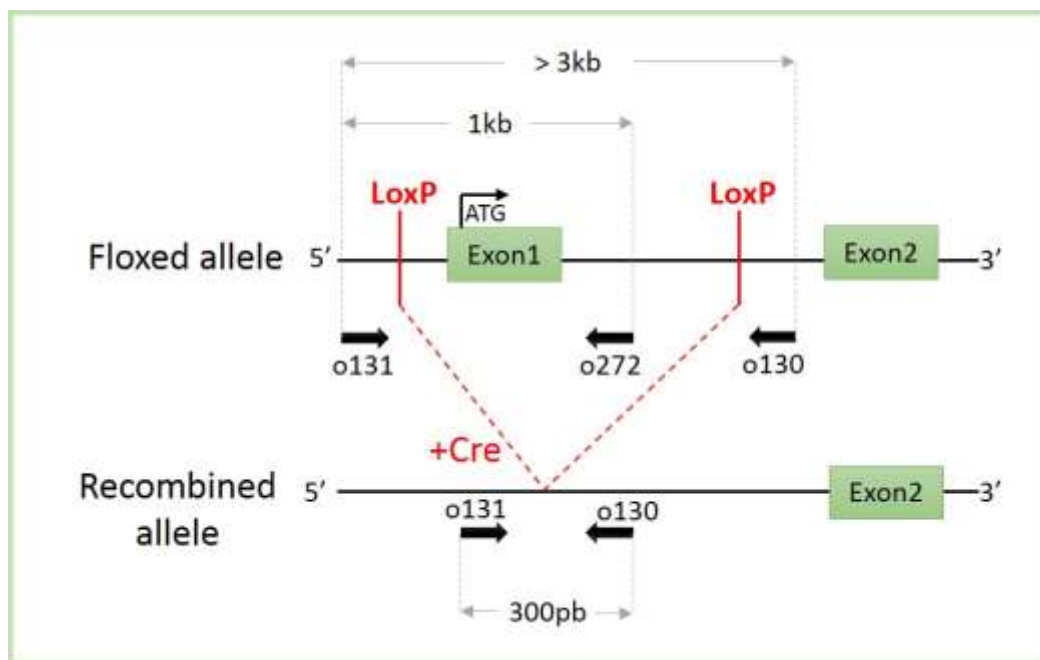


Figure 32: **Recombination of the *Htr2B^{fl/fl}* by the Cre recombinase.** RT PCR with appropriate primers (o130, o131, o272) allows to detect recombinant and non-recombinant alleles.

The PCR results do not give the information about the specificity and efficacy of this recombination. In order to test it, a *Cx3cr1^{CreERT2/+}; Htr2B^{-/-}* mouse has been crossed with a *B6.Cg-Gt(ROSA)26Sor^{tm9(CAG-dtTomato)Heze/J}* (The Jackson Laboratory) reporter mouse, in which the coding sequence for the tomato protein is preceded by a floxed STOP cassette, preventing its transcription. The examination of tomato-positive cells at P11 has confirmed that the recombination is microglia specific. We found that virtually all tomato positive cells were Iba1 positive (Figure 33 B).

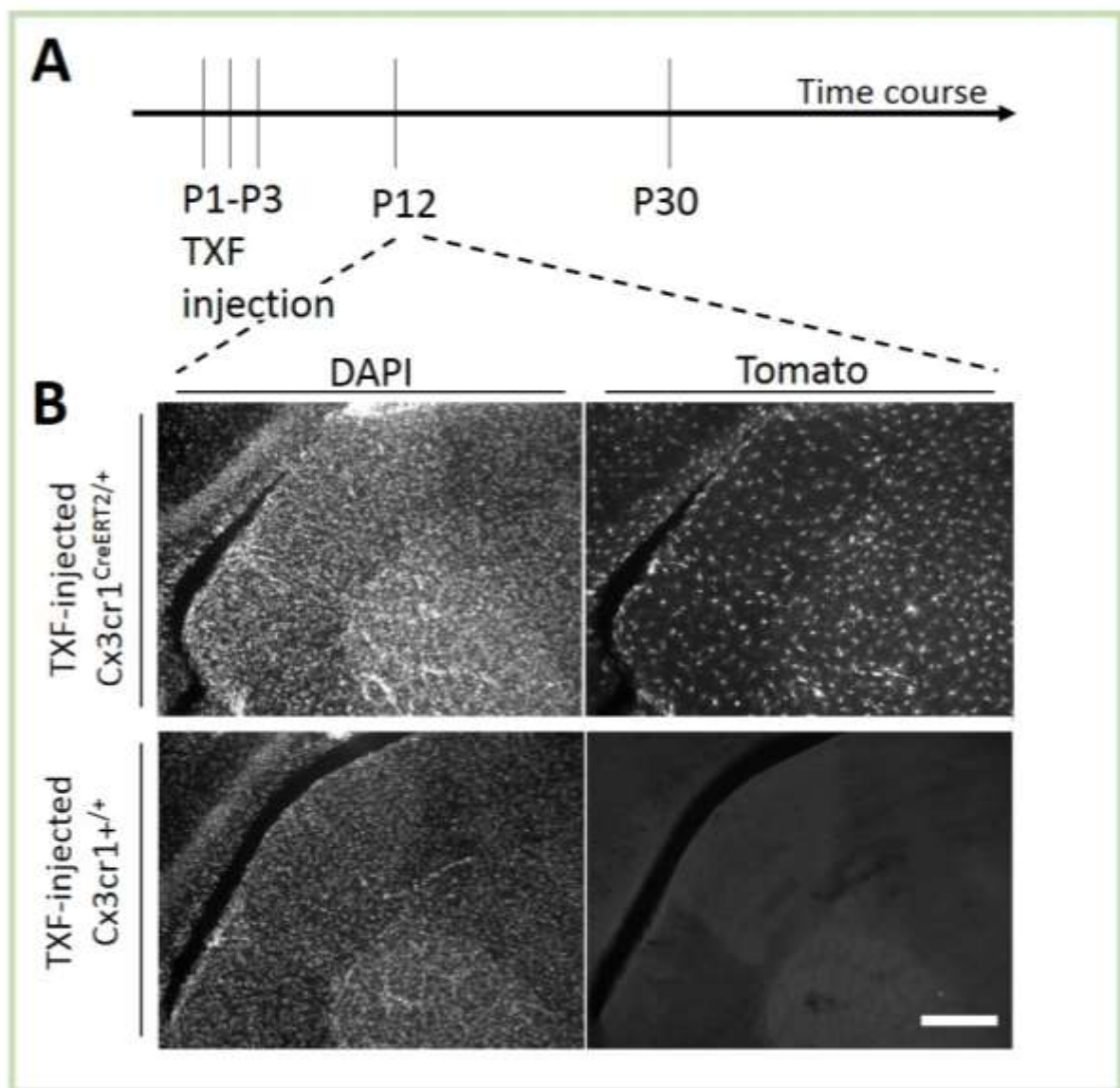


Figure 33: **TXF-induced recombination in *Cx3cr1^{CreERT2/+}; Htr_{2B}^{fl/fl} P11 mouse***. A: Time course of the experiment: TXF was administered at P1-P3 and images were taken at P12. B: tomato staining revealing recombination in the *Cx3cr1^{CreERT2/+}; Htr_{2B}^{fl/fl}* mouse (upper panel) and not in *Cx3cr1^{+/+} Htr_{2B}^{fl/fl}* mouse (lower panel). Scale bar: 200 μ m.

Next, in order to verify if the Cre recombinase expression is TXF-dependent we have examined the tomato expression in the mice that have not been injected with TXF. Surprisingly, at P6, we have found around 30% of tomato-positive microglia. Strikingly, most of these recombinant microglia were aggregated and formed patches, which were homogeneously distributed throughout the brain. Such organization suggests that a single recombinant cell could proliferate and give rise to other recombinant cells.

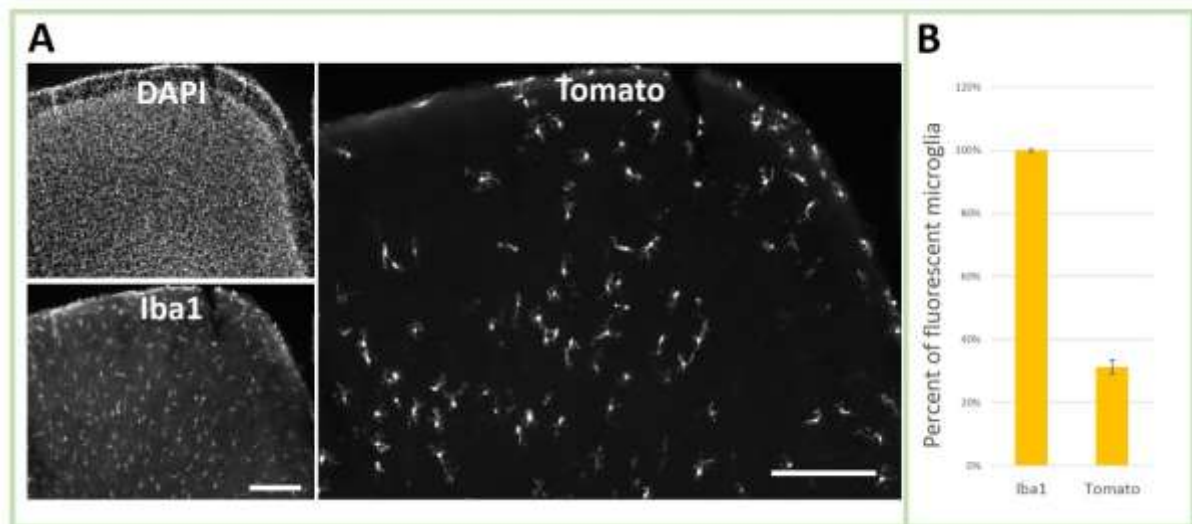


Figure 34: **TXF-independent recombination in *Cx3cr1*^{CreERT2/+}; *Htr2B*^{fl/fl} P6 mouse.** A: Rostral coronal section of the cortex with stain of all microglia (Iba1) and recombinant microglia (Tomato). B: 31% of all microglia were recombinant at P6; P value: <0.0001 (Student T test); Error bar: SEM; Scale bar: 200 μ m.

In theory, without TXF, Cre recombinase cannot be translocated to the nucleus and the recombination is impossible. However, since the *Cx3cr1* promoter is very strong and a lot of the enzyme is produced, translocation of even a very small percentage of it to the nucleus could result in a significant recombination. Moreover, TXF is an analogue of estrogen and it activates a mutated estrogen receptor, which has a very low affinity to the estrogen itself. However, during hormonal peak, for instance at birth, when the concentration of estrogen is very high, a small portion could unspecifically bind to this mutated receptor and induce spontaneous (TXF-independent) recombination. We can also not exclude the presence of estrogen analogues in the water coming from the plastic bottles.

Thus, the TXF injection at birth (P1-P3) efficiently induces a microglia specific recombination in the *Cx3cr1*^{CreERT2/+}; *Htr2B*^{-/-} mice the latest at P11 (earlier ages were not tested). The expression of the tomato protein occurs as soon as the recombination takes place. However, even after *Htr2B* inactivation the protein might stay in the cell still for some time. Therefore, it is important to note that the appearance of the tomato fluorescence might not precisely coincide with the disappearance of the 5-HT_{2B} receptor.

Moreover, I found that the Cre recombinase is active in around 30% of microglia of non-injected P6 mice. Since the retinal axon refinement in the thalamic dLGN is an early

postnatal event (P3-P9), the putative 5-HT_{2B} receptor-mediated role of microglia in this process might be required perinatally. It is possible that the TXF-induced postnatal inactivation of the 5-HT_{2B} receptor occurs too late to cause dLGN wiring deficiencies. On the other hand, the partial spontaneous (TXF-independent) recombination might be not sufficient to reproduce the phenotype observed in *Htr2B*^{-/-} mice. For this reason, in order to induce the 5-HT_{2B} inactivation earlier during development, we could either inject TXF to pregnant females or use animals with constitutive Cre recombinase.

B3.1.2 Constitutive Cx3cr1^{Cre/+}; Htr2B^{fl/fl}

Thus, we have crossed our 5-HT_{2B} floxed mouse with a constitutive *Cx3cr1*^{Cre/+} mouse (Yona et al. 2013) to generate a *Cx3cr1*^{Cre/+}; *Htr2B*^{fl/fl} mouse, in which the recombination should be constitutive in microglia (Figure 30 A). The *B6.Cg-Gt(ROSA)26Sor^{tm9(CAG-dtTomato)Heze/J}* reporter mouse has been used again to control the efficiency and specificity of the Cre recombinase expression. Both P6 and P30 brains have been examined. Strikingly, the tomato positive brains appeared “red” already after dissection (Figure 35 B). Analysis of brain sections has revealed that this intense fluorescence is not exclusively due to microglia recombination. Microglial cells were tomato positive, although the high “background” fluorescence decreased the contrast and the microglia processes were almost not visible (not shown), which was the opposite of the case of *Cx3cr1*^{CreERT2/+}; *Htr2B*^{-/-} where microglia stain was strong and clear (Figure 33 B). In both P6 (Figure 35 A) and P30 (not shown) brains unspecific tomato staining has been identified. Among unspecifically recombinant cells, without additional staining, we were able to identify the granule cells of dentate gyrus (Figure 35 A). This makes these mice functionally close to the total *Htr2B*^{-/-} animals.

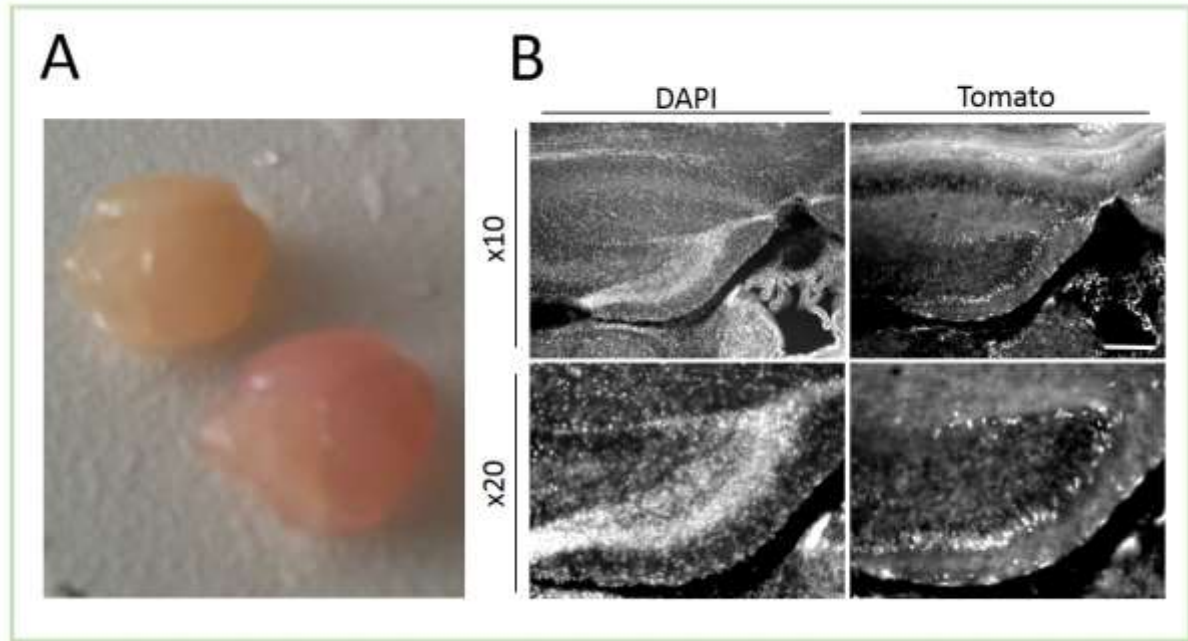


Figure 35: **Recombination in the Constitutive $Cx3cr1^{Cre/+}$; $Htr2B^{fl/fl}$ P6 mouse.** A: dissected brains of wild-type (up) and $Cx3cr1^{Cre/+}$; $Htr2B^{fl/fl}$ mouse (down); B: recombination in granular cells of dentate gyrus. Scale bar: 200 μ m.

The existence of the ectopic Cre recombinase could be explained by a transient developmental expression of $Cx3cr1$ promoter by other non-microglial cells or by a defect in the $Cx3cr1$ promoter that would change its specific activation.

Nevertheless, this observation, which seems to be consistent with some other laboratories, makes this model unsuitable for our study.

In summary, both of the conditional mice we have generated present issues of recombination. We have observed TXF-independent recombination in around 30% of microglia of the $Cx3cr1^{CreERT2/+}$; $Htr2B^{-/-}$ mice (which is not a problem for us) and an unspecific recombination in non-microglial cell in the $Cx3cr1^{Cre/+}$; $Htr2B^{fl/fl}$ mice (Figure 36). In the future, the best model will be prenatal injection of TXF to pregnant dams. This will activate recombination in $Cx3cr1^{CreERT2/+}$ embryos.

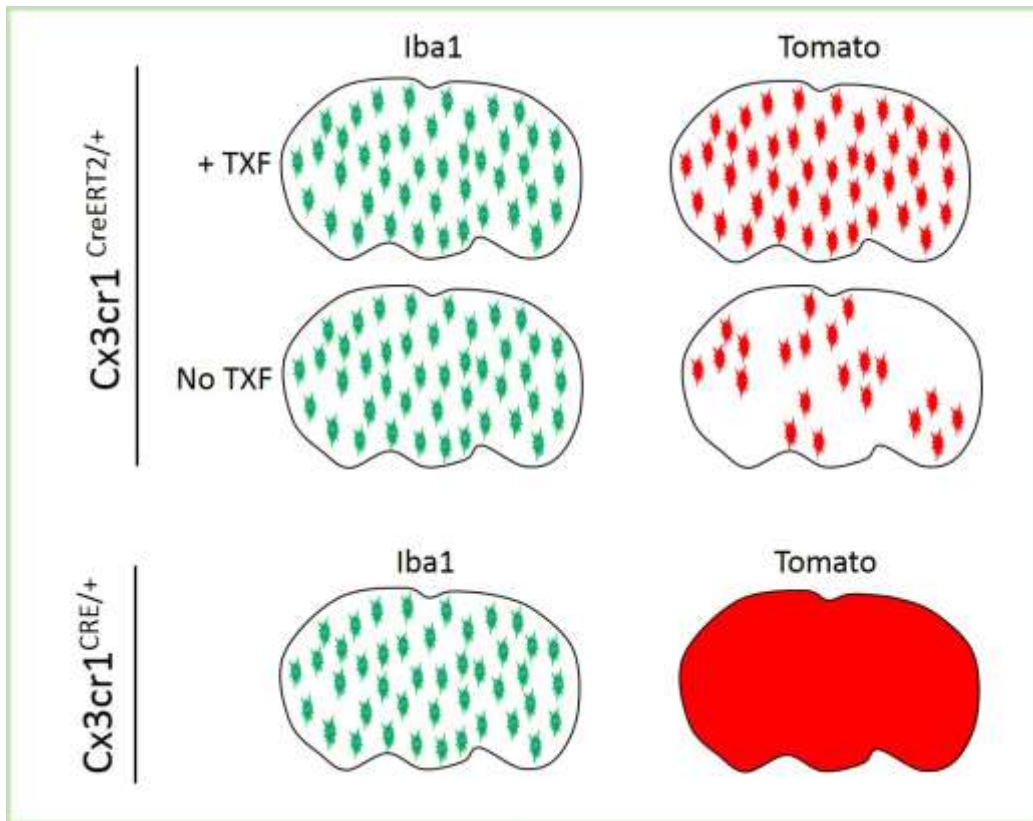


Figure 36: **Recombination efficacy and specificity in the conditional models of 5-HT_{2B} deletion from microglial cells.** Green: all microglial cells; Red: recombinant cells.

B4 Supplementary Data

Other than testing their responses to stimulation, a good way to investigate the effects of serotonin on microglia is to compare cells from an *Htr_{2B}^{-/-}* and from a wild-type mouse. Since 5-HT_{2B} is the major serotonin receptor on microglia, we could hypothesize that in these animals microglia do not sense serotonin or, at least, that their response to it is altered.

B4.1 Invasion of Microglia into dLGN of wild-type Mice

In order to test the hypothesis that 5-HT_{2B} receptor could be required for microglia recruitment to the sites of axonal refinement, we have compared the invasion of microglia in the dLGN between *Htr_{2B}^{-/-}* and wild-type mice. Our investigation however, did not reveal any alterations in the mutant animals. Thus, the segregation deficits observed in the dLGN of *Htr_{2B}^{-/-}* mice is not correlated to a deficit in microglia recruitment, while not excluding a role of local recruitment and activation of microglial processes by serotonin.

At the early postnatal ages I observed that microglial cells are often aligned along tracks crossing along the dLGN. Further immunohistochemistry stain of the blood vessels with antibodies against PECAM revealed that a number of microglia were in close apposition with vessels (Figure 35).

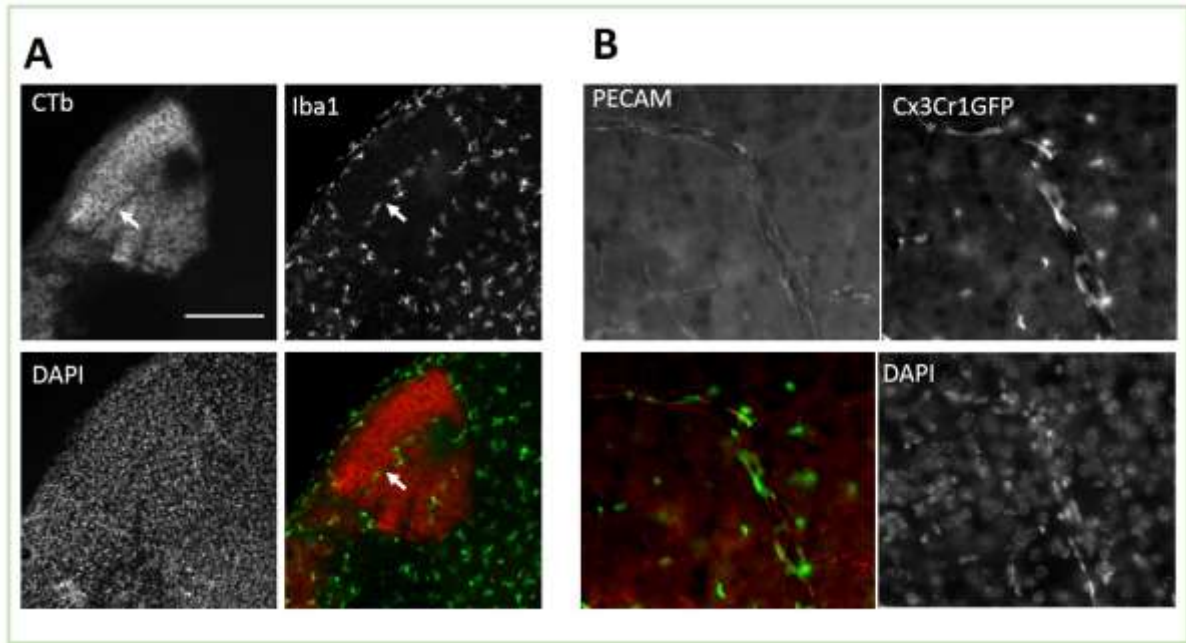


Figure 37: One of the first microglia within the dLGN are aligned along blood vessels. A: track-like structures are visible across the CTb stain of RGC terminals in the dLGN (P7). Microglia are often localized to these tracks. B: endothelial cells stain (PECAM) reveals that the track-like structures are blood vessels. Scale bar: 200 μ m.

The timing of the microglia invasion in the dLGN, which coincides with the beginning of refinement of RGC axons (Article Figure 3), supports the importance of microglia for the developmental axonal segregation in this brain structure. Interestingly, a recent study demonstrates such an association of microglia invasion timing with the axonal refinement in the cortical projection maps of whiskers (Figure 38). Indeed, during development, the recruitment of microglia to the synapses of barrel cortex has been shown to be required for their maturation (Hoshiko et al. 2012). This supports a universal role of microglia during developmental axonal refinement.

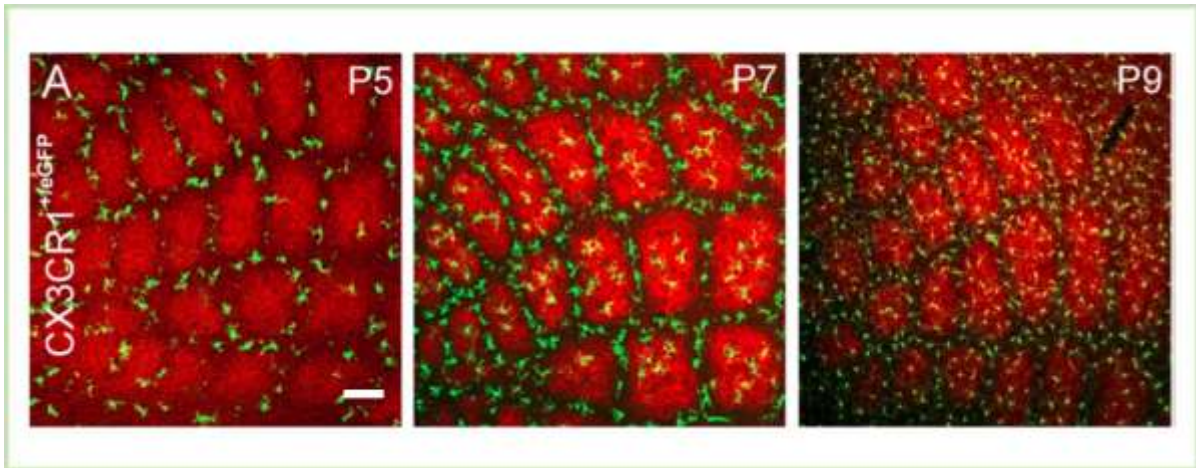


Figure 38: **Microglia invasion in barrel cortex (P5-P7-P9)**. Red: thalamocortical axons (SERT stain); Green: fluorescent microglia (*Cx3cr1^{GFP/+}*). Scale bar: 100 μ m. (Hoshiko et al. 2012).

B4.2 Overexpression of inflammatory markers

In human, it has been demonstrated that, in the periphery, serotonin has an anti-inflammatory effect through the 5-HT_{2B} receptor and mediates polarisation of macrophages toward the M2 state (las Casas-Engel et al. 2013)(discussed in chapter “Microglia Activation”). Since microglia share numerous characteristics with peripheral macrophages (discussed in chapter “Microglia vs. Macrophages”), the microglial 5-HT_{2B} receptor could have a similar effect. Indeed, the analysis of inflammatory markers expression in the *Htr2B*^{-/-} revealed a number of differences to the wild-type mice suggesting that the 5-HT_{2B} receptor is involved in polarization of microglia (Article Figure6).

Strikingly, most of the overexpressed genes in the *Htr2B*^{-/-} mice belong to the family of chemokine receptors (*Ccr1*, *Ccr2*, *Ccr5*, and *Cxcr5*), suggesting that the responsiveness of microglia in the *Htr2B*^{-/-} mice, potentially required for the developmental axonal refinement, might be affected.

B5 Putative Models to Explain *Htr_{2B}^{-/-}* phenotype in dLGN

The analysis of *Htr_{2B}^{-/-}* mice has revealed alterations of spatial arrangement of eye-specific territories within the thalamic dLGN. Even though the percentage of the overlap between the ipsilateral and the contralateral regions in these mice is similar to the wild-type mice, I have identified a difference in their ipsilateral region. At P30, the ipsilateral region of the *Htr_{2B}^{-/-}* mice was more diffuse and patchy whereas it was rather compact and well defined in wild-type animals. In addition, the shape and the location of the ipsilateral region within the dLGN was clearly more variable among the KO than wild-type mice.

The analysis of different conditional animals (see chapter “Potential Mechanisms & Cells Involved in *Htr_{2B}^{-/-}* Mice Deficits”) have not provided conclusive results. At this moment we cannot exclude any cell type expressing 5-HT_{2B} receptor. In the following section, I will propose two models on the mechanisms behind the altered segregation in the *Htr_{2B}^{-/-}* mice: one involving the microglial 5-HT_{2B} receptor and an alternative one.

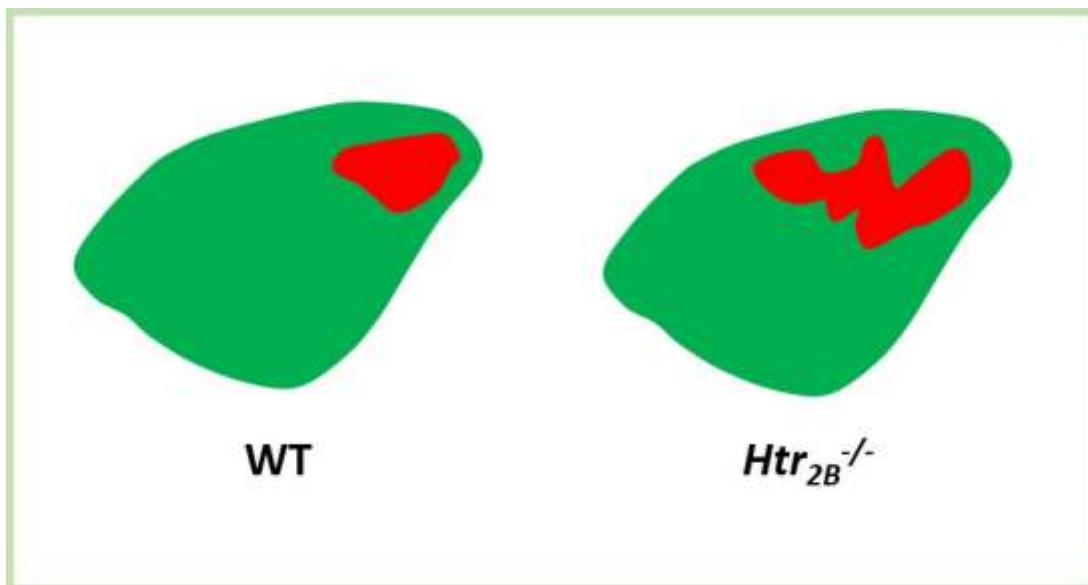


Figure 39: Schematic representation of the RGC axons segregation in the dLGN of the WT and *Htr_{2B}^{-/-}*.

B5.1 Involving Microglia

Results from other groups (Krabbe et al. 2012; Glebov et al. 2014) and ours clearly demonstrate that developmental microglia express 5-HT_{2B} receptor and that they can actively respond to it. This supports the hypothesis that microglial cells could interact with the serotonin system to orchestrate some steps of CNS development.

It is well established that during sensitive developmental periods some non-monoaminergic neurons transiently acquire partial serotonergic phenotype and are able to uptake, store and release serotonin. These neurons can be revealed with the immunolabeling of SERT. As I have introduced before, such a transient SERT expression is present between postnatal day 7 and 14 in the RGC terminals originating in the ipsilateral eye (Figure 40). Interestingly, the timing of the transient SERT expression coincides with the period of developmental axonal segregation in the dLGN, suggesting its importance in the process.

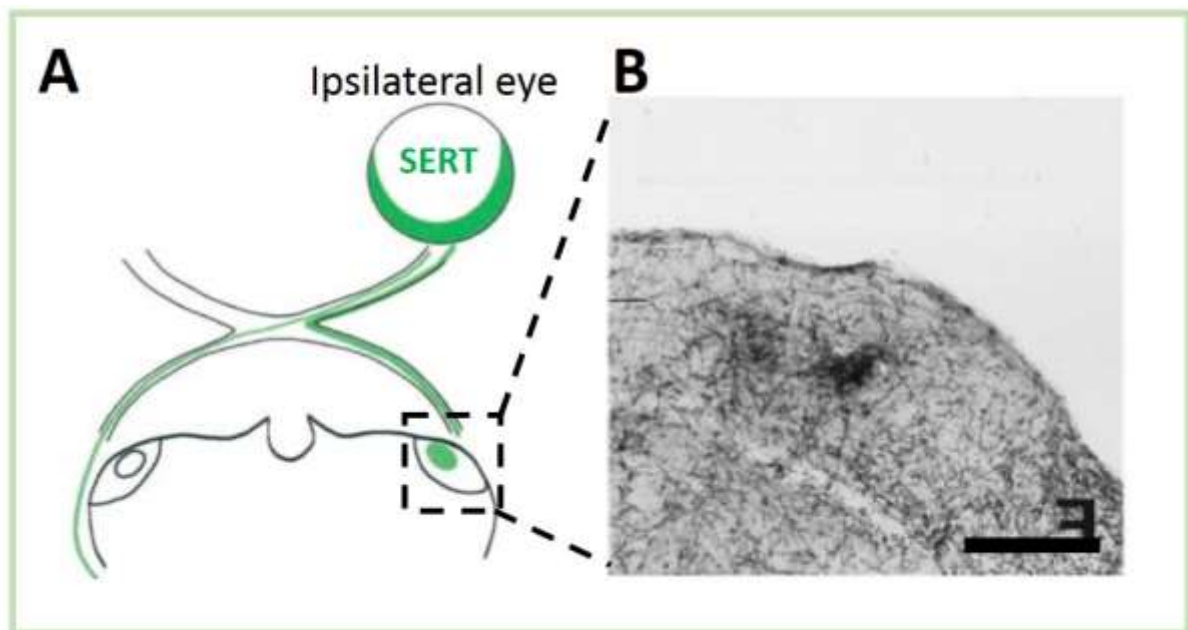


Figure 40: **Transient location of SERT in the dLGN.** A: The area of SERT expression, shown in green, is restricted to the RGC terminals originating in the ipsilateral eye. B: SERT IHC at P8 reveals a stronger stain in the area corresponding to the ipsilateral projections. Scale bar: 150 μ m. (Upton 1999).

The RGCs expressing SERT can uptake the serotonin and therefore locally decrease its concentration. This way a serotonin gradient is formed with a low concentration in the

center of the ipsilateral region and a higher concentration in the contralateral region. Therefore, we speculate that the serotonin-sensitive microglia use this gradient as positioning coordinates (“GPS”) in order to localize themselves within the dLGN. When microglia “sense” low serotonin concentration, they “know” they are in the ipsilateral region, thereby they should remove contralateral fibers.

This hypothesis is supported by the phenotype I have observed in the dLGN of *Htr_{2B}^{-/-}* mice, where the major microglial serotonin receptor is absent. Since the size of the ipsilateral region in these animals is normal and since there is no increased overlap between ipsilateral and contralateral region we could assume that microglia work well (i.e. recognize ipsilateral vs. contralateral fibers). However, since the shape and location of the ipsilateral region are altered it looks like microglia are “disorientated” and removes contralateral fibers at wrong locations.

B5.2 Alternative

We cannot, however, exclude the possibility that the absence of the 5-HT_{2B} receptor on other cell types is responsible for the phenotype observed in the *Htr_{2B}^{-/-}*. As I have introduced previously, the 5-HT_{2B} receptor is present on the majority of the raphe serotonergic neurons, some of which are innervating the dLGN. Moreover, the treatment with 5-HT_{2B} agonist resulted in SSRI-like effect, leading to an increased extracellular serotonin. Therefore, we speculate that in the dLGN of *Htr_{2B}^{-/-}* mice there is a decreased extracellular concentration of serotonin, which in turn perturbs some of the serotonin-dependent mechanisms of axonal refinement such as 5-HT_{1B} signaling (Figure 41).

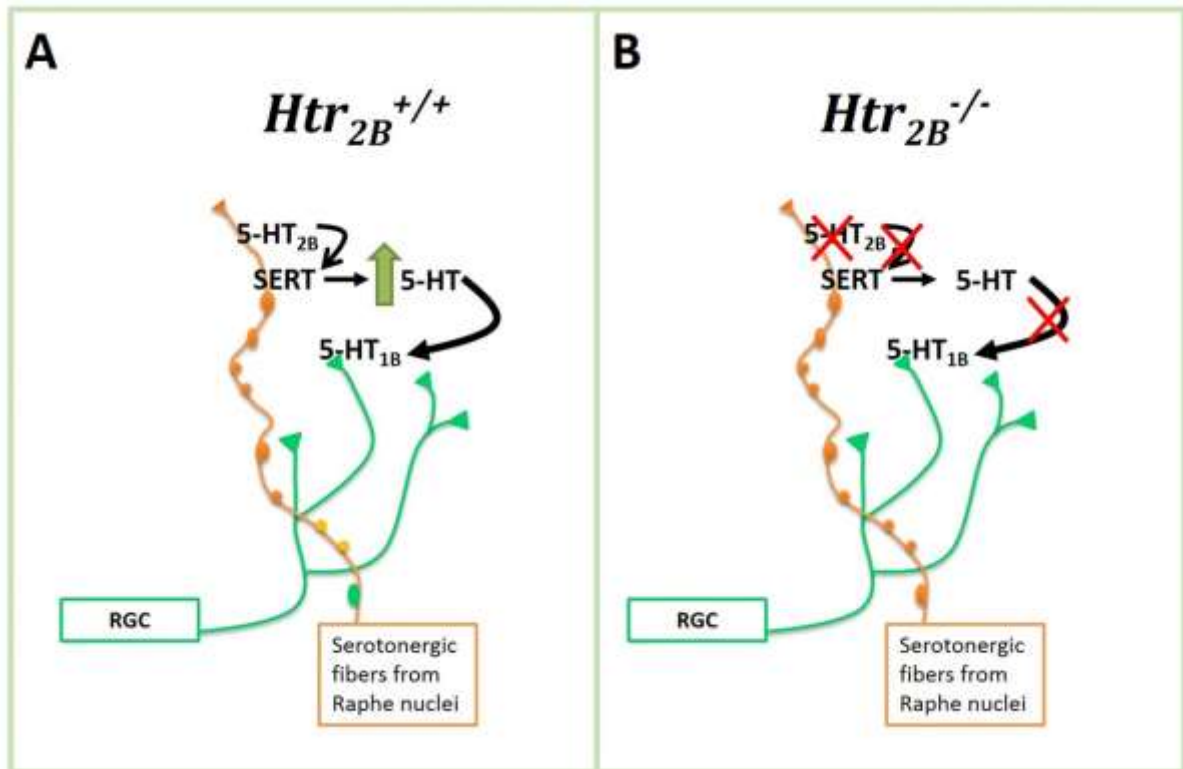


Figure 41: Putative consequences of 5-HT_{2B} receptor absence.

B6 Importance of Results & Perspectives

During my PhD thesis I have demonstrated the role of 5-HT_{2B} receptor in axonal refinement of retinogeniculate projections in the thalamic dLGN. Indeed, I have found that the ipsilateral region of the *Htr_{2B}^{-/-}* mice is more diffuse, often patchy and it has irregular shape comparing to the wild-type animals.

The mechanisms responsible for developmental axonal refinement could be universal for different brain regions. Moreover, such a subtle brain miswiring, could be sufficient to increase vulnerability to a broad range of psychiatric disorders such as schizophrenia or autism.

I have also demonstrated that the developmental microglia can respond to serotonin and that this response is mediated by the 5-HT_{2B} receptor. Since both, serotonin and microglia, appear early in the developing brain and they both have been reported to be required for maturation of functional neuronal circuits during development of the CNS, we speculate that the absence of this receptor in microglial cells is responsible for the segregation deficits in the dLGN of *Htr_{2B}^{-/-}* mice.

As the next step, we should confirm the microglial involvement in the serotonin-mediated synaptic refinement. We tried to do it by using conditional mice in which only microglia do not express the 5-HT_{2B} receptor, but we were faced with limitations of these genetic models. We could also use a model of microglia depletion and analyze the RGC axons segregation in absence of microglial cells.

It would also be interesting to further describe the 5-HT_{2B} expression on microglia. Does it vary with age? Region? A particular microglia subpopulation? Does the microglial response to serotonin is different during the sensitive developmental periods and during the adulthood?

Answering these question might help us understand not only some of the mechanisms involved in the developmental brain wiring but also those involved in the synaptic plasticity in adult brain.

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