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**Migration and differentiation of neuronal precursors in
the postnatal brain: insights from the subventricular
zone and cerebellum**



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***A semente amadurece
com sol e com chuva***

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RESUMO

No cérebro de mamíferos adultos, a neurogénese ocorre constitutivamente na região subventricular (SVZ), na região subgranular (SGZ) do giro dentado (DG) do hipocampo, e em certo nível no cerebelo. Estes nichos neurogénicos são fontes de novos neurónios que integram funcionalmente circuitos existentes. O desenvolvimento adequado do cérebro depende, portanto, do equilíbrio contínuo entre neurogénese e correcto posicionamento final das novas células. Apesar dos enormes avanços da investigação nesta área, há ainda lacunas no conhecimento destes nichos, e dos factores que modulam a sua dinâmica.

Entre os vários factores que afectam o destino das células neurais emergem a histamina e o factor neurotrófico derivado do cérebro (BDNF). A histamina, mediador inflamatório e neurotransmissor, parece promover a diferenciação neuronal. Em células estaminais neurais embrionárias, a histamina induz proliferação e diferenciação neuronal. No entanto, o papel da histamina na neurogénese de SVZ adulta nunca foi devidamente abordado. Em relação ao BDNF, observa-se um anormal desenvolvimento do cerebelo aquando da sua remoção, tendo sido descrita como molécula-chave na promoção da migração celular. No entanto, não há ainda informação sobre os efeitos do BDNF proveniente de diferentes fontes celulares, sobre a migração de células precursoras.

A presente tese dissecar o efeito pro-neurogénico da histamina na SVZ e esclarece o papel do BDNF endotelial durante a migração de precursores neuronais. Para tal, diferentes abordagens biológicas foram usadas, como culturas primárias de células e animais transgénicos. Recorreu-se a metodologias actuais, incluindo imagiologia de cálcio em célula única, cirurgia estereotáxica *in vivo*, imagiologia em tempo real e PCR quantitativo em tempo real.

Esta tese desvenda um novo papel da histamina, como importante modulador da diferenciação neuronal da SVZ; e o BDNF como factor endotelial chave dirigindo a migração de células precursoras neuronais. Em conjunto, os mecanismos celulares revelados contribuem para uma melhor compreensão da dinâmica da SVZ e cerebelo.

PALAVRAS-CHAVE

Célula Estaminais Neurais, Região Subventricular, Cerebelo, Histamina, BDNF

ABSTRACT

In the adult mammalian brain, neurogenesis occurs constitutively in the subventricular zone (SVZ), subgranular zone (SGZ) of the hippocampal dentate gyrus (DG) and at some extent at the cerebellum. These neurogenic niches are sources of new neurons that functionally integrate existing circuits. The proper development of the brain depends thus on continuous balance between new cells' neurogenesis and their proper final placement. In spite of the enormous advances of neural stem cell research, there is still a lack of knowledge about the niches, and factors that modulate their dynamics.

Among the several factors that affect neural cell fate, histamine and brain-derived neurotrophic factor (BDNF) emerge. Histamine, an inflammatory mediator and neurotransmitter, seems to foster neuronal differentiation. In embryonic neural stem cells, histamine induces both proliferation and neuronal differentiation. However, its role in adult SVZ neurogenesis has never been properly addressed. Concerning BDNF, abnormal cerebellum development has been observed upon this neurotrophin removal, and it has been described as a key molecule promoting cell migration. Yet, no data is available on the BDNF dependent cellular source effect controlling cerebellum precursor migration.

The present thesis dissects the proneurogenic effect of histamine in the SVZ, and unravels the role of endothelial derived-BDNF on neuronal cell precursor migration in the postnatal cerebellum. To achieve so, different biological approaches were used, such as primary cell cultures and transgenic animal models. State of the art methodologies were employed including single cell calcium imaging, *in vivo* stereotaxic surgery, time-lapse imaging and quantitative real-time PCR.

This doctoral thesis reveals a novel role for histamine, as an important modulator of neuronal differentiation in the SVZ; and BDNF, as a key player of endothelial-neuronal cell crosstalk that drives cerebellar precursor cell migration. Altogether, the disclosed cellular processes contribute to a better understanding of the SVZ and cerebellum dynamics.

KEY-WORDS

Neural Stem Cells, Subventricular Zone, Cerebellum, Histamine, BDNF

PUBLICAÇÕES • PUBLICATIONS

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LISTA ABREVIATURAS • ABBREVIATIONS

[³H]-thymidine	Tritiated-thymidine
[Ca²⁺]_i	Intracellular calcium concentration
aCSF	Artificial Cerebrospinal Fluid
AD	Alzheimer's Disease
ALS	Amyotrophic Lateral Sclerosis
Ang-1	Angiopoietin-1
AP	Anterio-posterior
BBB	Blood Brain Barrier
BDNF	Brain-Derived Neurotrophic Factor
BLBP	Brain Lipid Binding Protein
BMP	Bone Morphogenetic Protein
BrdU	5-bromo-3'-deoxyuridine
BSA	Bovine Serum Albumin
BV	Blood Vessel
CL	Contra-lateral
CNS	Central Nervous System
CSF	Cerebrospinal Fluid
CXCR4	C-X-C chemokine receptor type 4
DCX	Doublecortin
DEPC	Diethylpyrocarbonate
DG	Dentate Gyrus
DiI	1,1'-dioctadecyl-3,3,3'-tetramethylindocarbocyanine perchlorate
DIG	Digoxigenin
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic Acid
DV	Dorso-ventral
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EGL	External Granule Cell Layer
Ep	Ependymal cell
ERK	Extracellular signal-Regulated Kinase
FACS	Fluorescent Activated Cell Sorting
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FGF	Fibroblast Growth Factor
FGFR	Fibroblast Growth Factor Receptor
FITC	Fluorescein Isothiocyanate
FL-TrkB	Full Length Tropomyosin-related kinase
GABA	Gamma-Aminobutyric Acid
GCL	Granular Cell Layer
GCP	Granule Cell Precursor
GFAP	Glial Fibrillary Acidic Protein
GFP	Green Fluorescent Protein
GL	Glomerular Layer
GLAST	Glutamate-Aspartate Transporter

HAT	Acetyltransferases
HBSS	Hanks Balanced Salt Solution
HD	Huntington's Disease
HDC	Histidine Decarboxylase
HIF	Hypoxia Induced Factor
HVC	High Vocal Center
HxR	Histamine x receptor (x = 1 to 4)
icv	Intracerebroventricular
IGL	Internal Granule Cell Layer
ip	Intraperitoneal
iPSCs	Induced Pluripotent Stem Cells
IPSI	Ipsilateral
JNK	c-Jun N-terminal kinase
KO	Knock-Out
LTD	Long-Term Depression
MAPK	Mitogen Activated Protein Kinase
MCL	Mitral Cell Layer
miRNA	Micro RNA
ML	Molecular Layer
mRNA	Messenger RNA
MS	Multiple Sclerosis
NFkB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NGF	Nerve Growth Factor
Ngn	Neurogenin
NMDA	N-methyl- D-aspartate
NPY	Neuropeptide Y
NSCs	Neural Stem Cells
NT-x	Neurotrophin x (x=3, 4 or 6)
OB	Olfactory Bulb
OD	Optical Density
OSN	Olfactory Sensory Neuron
p75NTR	p75 Neurotrophin Receptor
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
PD	Parkinson's Disease
PDGF	Platelet-Derived Growth Factor
PDL	Poly-D-Lysin
PECAM	Platelet Endothelial Adhesion Molecule
PEDF	Pigment Epithelium-Derived Factor
PFA	Paraformaldehyde
PI3K	Phosphatidylinositol 3-Kinase
piRNA	Piwi-interfering RNA
PKC	Protein kinase C
PLC	Phospholipase C
PLGA	Poly lactic-co-glycolic acid
PNS	Peripheral Nervous System

PRF	Primary Fissure
Prox1	Prospero homeobox protein 1
PSA-NCAM	Polysialic Acid-Neural Cell Adhesion Molecule
PTZ	Pentylene-tetrazole
qChIP	Quantitative Chromatin Immune Precipitation
qRT-PCR	Quantitative Reverse Transcription-PCR
RMS	Rostral Migratory Stream
RNA	Ribonucleic Acid
SAPK	Stress Activated Protein Kinase
SCCI	Single Cell Calcium Imaging
SDF-1	Stroma cell-Derived Factor-1
SEM	Standard Error of the Mean
SF	Secondary Fissure
SFM	Serum Free Medium
SGZ	Subgranular Zone
Shh	Sonic hedgehog
siRNA	Small interfering RNA
Sox2	Sex-determining region Y-box 2
SSC	Saline Sodium Citrate
SVZ	Subventricular Zone
TBS	Tris Buffered Saline
TBS-T	Tris Buffered Saline – Tween 20
TEA	Triethanolamine
TH	Tyrosine Hydroxylase
TNF-α	Tumor Necrosis Factor-alpha
Trk	Tropomyosin-related Kinase
TUNEL	Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling
UTRs	Untranslated Regions
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor
VGAT	Vesicular GABA Transporter
VGLUT	Vesicular Glutamate Transporter
VZ	Ventricular Zone
WT	Wild Type

CHAPTER I

INTRODUCTION

I.1. From the immutable brain to neurogenesis acceptance

Historically, neurogenesis was long believed to be the embryonic and perinatal generation of new functional neurons from their precursors. This immutable scenario was drawn, and minutely described by Ramón y Cajal (Ramón y Cajal, 1899, 1909). Although few studies found mitotic figures in both postnatal and adult rat brain tissue (Hamilton, 1901; Allen, 1912; Bryans, 1959), they were easily doubted and put apart by the long-lasting and strongly accepted Ramón y Cajal's adult cyto-architecture. The lack of appropriate technical methodologies to study, simultaneously, cell division and neuronal phenotype delayed the final contradiction of this dogma.

It was in the 60's that Altman, with tritiated ($[^3\text{H}]$)-thymidine autoradiography, saw radiolabeled newly dividing neurons, with the nucleoside incorporated into their deoxyribonucleic acid (DNA), at the neocortex, dentate gyrus (DG) and olfactory bulb (OB) of the adult rat brain (Altman and Das, 1965, 1966, 1967, 1969). However, their studies were just considered valid with the support of later data provided by Kaplan (Kaplan and Hind, 1977; Kaplan, 1983, 1984). Finally, Nottebohm and colleagues undoubtedly proved that the generation of new neurons in the adult brain was not unconceivable. These authors showed that, not just new cells were generated at the Subventricular Zone (SVZ), but also that they migrated to the high vocal center (HVC) of adult songbirds' brain (Goldman and Nottebohm, 1983; Alvarez-Buylla and Nottebohm, 1988). These new concepts of adult neurogenesis and migration were quickly upgraded with additional data showing that newly born neurons migrate and integrate, forming synapses at the target regions (Burd and Nottebohm, 1985; Alvarez-Buylla *et al.*, 1988) when stimulated (Paton and Nottebohm, 1984).

Afterwards, several groups devoted to the search of these precursor cells in the rodent (Reynolds and Weiss, 1992) and human (Kukekov *et al.*, 1999) brain. With the synthetic thymidine analog 5-bromo-3'-deoxyuridine (BrdU), with identical mode of action as the $[^3\text{H}]$ -thymidine, several *in vivo* studies were conducted together with phenotypic labeling to confirm the neural identity of adult newly generated cells (Kuhn *et al.*, 1996; Kempermann *et al.*, 1997; Eriksson *et al.*, 1998). Being the immutable brain dogma withdrawn, a growing research field fired. Vast studies have been giving insights on the identity of neural stem cells (NSCs) – the recently found new neurons' precursors; as well as on the properties and cellular composition of the neurogenic niches; their cellular dynamics and the migratory behavior of newly generated cells; the survival, integration and functional relevance of these adult born cells within the healthy or damaged brain.

I.2. Neural stem cells

The plasticity of the adult brain is nowadays granted. New cells arise from NSCs, their axons, dendrites and synapses are flexible and their integration into existing circuitries is a truthful event (Lledo *et al.*, 2006). Additionally, although neurogenesis is conserved throughout the mammalian species (Eriksson *et al.*, 1998; Sanai *et al.*, 2004), it is a fundamental biological characteristic of the brain: it is an answer to the demanding environmental changes.

Neural stem cells reside in two specialized niches of the adult mammalian brain: the SVZ, bordering the lateral ventricles (Alvarez-Buylla and Garcia-Verdugo, 2002), and the Subgranular Zone (SGZ) of the hippocampal Dentate Gyrus (DG) (Song *et al.*, 2002). Although at each niche, NSCs have specific characteristics and dynamics, there are three cardinal properties of NSCs: proliferation, self-renewal and multipotency.

Proliferation is the ability to, in the presence of adequate growth factors, generate new cells. Self-renewal is the capacity to go through cell cycles without losing the undifferentiated state. Finally, multipotency is the power to stop cell cycle and differentiate into specialized neural cell types. All these concepts were firstly proved by the *in vitro* experiments of Reynolds and Weiss (1992). They showed that cells isolated from the lateral wall of the ventricle, or from the DG of the hippocampus proliferate generating new floating cell clusters: neurospheres. These ones were capable to form secondary neurospheres when dissociated (self-renewal). Conversely, when removing growth factors from the medium and supplying an adhesion substrate, cells differentiated into new neurons, astrocytes and oligodendrocytes (multipotency). Thus, all the cardinal properties were proved in cells from the two neurogenic niches. Nonetheless, there are some reports showing that isolated cells from other brain regions also have the ability to form neurospheres *in vitro* (Laywell *et al.*, 2000; Itoh *et al.*, 2006).

Still, 20 years after the generalized acceptance of adult neurogenesis, there is not general agreement on the stemness nature of neurogenic niche's cells. Some author's reluctance is clear in their studies: they showed that the SGZ has a limited capacity to self-renew, since its resident cell population had no *in vitro* long-term self-renewal (Seaberg and van der Kooy, 2002; Bull and Bartlett, 2005). Also, in line with this doubting reasoning is the fact that DG neurospheres fail to generate neurons (Seaberg and van der Kooy, 2002). However, and to make the question on whether these cells are true stem cells or not, some of the previously mentioned authors (Bull and Bartlett, 2005) also identified DG endogenous brain-derived neurotrophic factor (BDNF) as responsible for this neuronal multipotency. Thus, they proved the stemness nature of these cells *in vivo* but not *in vitro*. Both SVZ and SGZ are niches populated by cells that continuously proliferate, by symmetric and asymmetric divisions. These divisions yield identical or more committed progenitor cells that will, in due course, differentiate into functional new cells integrating existing circuitries.

As the existence of NSCs was debated, also the identity of these stem cells was under great discussion. Two opposing theories coincided in time. Fiona Doetsch and collaborators

(1999a) pointed a glial origin to NSCs, while Johansson (1999) defended an ependymal source to new neurons. Johansson and colleagues (1999) stood for ependymal cells based on the migration of 1,1'-dioctadecyl-3,3,3'-tetramethylindocarbocyanine perchlorate (DiI)-labeled cells into the OB, together with their neuronal markers expression. They injected DiI intraventricularly and assumed that only ependymal cells, the ones delimiting the lateral ventricle, took up the dye. However, since it is a lipophilic and highly diffusible dye, other cells adjacent cells to the ependymal layer could up take it. Moreover, a population of glial fibrillary acidic protein (GFAP) positive SVZ cells also have a cilium projecting into the lateral ventricle (Mirzadeh *et al.*, 2008). Thus, they had easy access to the injected DiI, giving rise to wrongly interpreted data. Although the ependymal cells' apologists performed the neurosphere assay, presumed ependymal cells isolated on the basis of Notch1 receptor immunosorting, were in fact SVZ astrocytes (Givogri *et al.*, 2006). Notch1 is a key regulator of NSCs (Ables *et al.*, 2011). Furthermore, electron microscopy showed no [³H]-thymidine-labeled cells on the ependymal layer of the lateral ventricles, while at the SVZ there were mitotic astroglial cells (Doetsch *et al.*, 1999b). Few years later, parallel conclusions at the adult hippocampus also pointed to an astrocytic nature of the new neurons at DG (Seri *et al.*, 2001). Despite these data, ependymal cells can in fact be activated, after stroke, and originate new neurons and astrocytes regardless of no self-renewal capacity (Carlén *et al.*, 2009). Moreover, ependymal cells are fundamental for adult neurogenesis. Indeed, they secrete the pigment epithelium derived factor (PEDF), a peptide that promotes self-renewal of SVZ stem cells (Ramirez-Castillejo *et al.*, 2006). Endogenous PEDF modulates Notch transcriptional activity, supporting the balance between symmetrical and asymmetrical divisions which maintains undifferentiated cell stages (Andreu-Agullo *et al.*, 2009).

Finally, the irrefutable *in vivo* evidence that true NSCs have an astrocytic origin is their ability to restore the germinal layer after the ablation of actively dividing cells by anti-mitotic treatment (Doetsch *et al.*, 1999b; Seri *et al.*, 2001). It is still noteworthy that the astrocytic origin of the adult NSCs is similar to developmental neurogenesis. In the last one, radial glia cells are responsible for all neural cell types differentiation. This apparent coincidence is supported by several studies that indicate a common and continuous lineage to both types of neurogenesis (Merkle and Alvarez-Buylla, 2006; Ihrle and Alvarez-Buylla, 2008; Malatesta *et al.*, 2008; Kriegstein and Alvarez-Buylla, 2009). Early in embryonic development, there are neuroepithelial cells around the neural tube, then radial glia cells in young developing brain, followed by age-long adult SVZ/SGZ astrocytes throughout adult neurogenesis.

1.3. Postnatal neurogenesis and the neurogenic niches

Currently, adult neurogenesis is accepted to be an ongoing process happening in two restricted adult mammalian brain regions: SVZ, lining the lateral ventricles (Alvarez-Buylla and Garcia-Verdugo, 2002), and the SGZ of the hippocampus (Song *et al.*, 2002). The SVZ and SGZ are particular regions for neurogenesis not just because they harbor NSCs. Here there are cellular and molecular prosperous conditions for keeping a local population of NSCs, but also for generating newly differentiated cells (Doetsch, 2003). On the other hand, there are regions within the brain parenchyma that easily accept newly differentiated cells, perfectly integrating them within their cellular functional network. These are the OB and the granular cell layer (GCL) of the hippocampus, two plastic areas that have high adaptation capacities in response to environmental stimuli. Newly generated cells from the SVZ migrate towards the OB where they differentiate into mature interneurons. At the hippocampus, newly born neurons migrate locally from the SGZ to the GCL where they differentiate into granule neurons by elongation of axons towards the CA3 region and extension of dendritic trees to the molecular layer (for reviews see Zhao *et al.*, 2008; Ming & Song, 2011).

Other brain regions are not permissive to natural neuronal generation (Agasse *et al.*, 2004). However, some studies showed that neocortex (Gould *et al.*, 1999; Ohira *et al.*, 2010), substantia nigra (Lie *et al.*, 2002; Zhao *et al.*, 2003), striatum (Bédard *et al.*, 2002), amygdala (Bernier *et al.*, 2002), hypothalamus (Yuan and Arias-Carrión, 2011) and piriform cortex (Pekcec *et al.*, 2006) can acquire neurogenic properties, although at a much lower level. Still, caution must be taken since the detection limit and the techniques used to process these tissues differ from the ones used at SVZ/SGZ. Thus, it may bring some bias or artifacts that mislead to a positive neurogenic signal (reviewed in Breunig *et al.*, 2007; Gould, 2007).

Neurogenesis is very well documented in rodents, and has also been demonstrated in primates and humans. In fact, SVZ actively produce neurons in the infant human brain below 18 months of age, destined for the OB and the prefrontal cortex (Sanai *et al.*, 2011). Importantly, SVZ stem cells extracted from the adult human brain retain the capacity to produce neurons *in vitro*, suggesting that neurogenesis in the SVZ may be boosted under proper stimulation. The presence of NSCs in the hippocampus of the adult human brain was firstly described by the seminal study of Eriksson *et al.* (1998). More recently, Doublecortin (DCX) immunoreactive newly born neurons were found across the lifespan from 0 to 100 years of age in the human hippocampus (Knoth *et al.*, 2010). Interestingly, it was also shown that a significant number of cells obtained from human biopsies, from the temporal lobe of epileptic patients, are also capable of expansion and multi-potency *in vitro* (Vaysse *et al.*, 2012). However, one should keep in mind that most of the studies done in humans do not directly measure neurogenesis, but instead evaluate stem cell markers expression in tissue biopsies and cell proliferation, without knowing whether immature neurons derived from stem cells became integrated and functional in the host tissue.

I.3.1. The SVZ – OB axis

The adult SVZ, the major site of adult neurogenesis, is a thin layer of cells situated in the lateral ventricle walls (Altman, 1969; Quinones-Hinojosa *et al.*, 2006; Doetsch *et al.*, 1997). It is delineated on one side by the monolayer of ependymal cells that separate the brain parenchyma from the cerebrospinal fluid (CSF) contained in the ventricles, and on the other side by the striatum. NSCs present in this niche migrate towards the OB, through the rostral migratory stream (RMS).

At this neurogenic niche, B1 cells expressing nestin, sex-determining region Y-box 2 (Sox2), and astrocyte markers such as GFAP and the glutamate-aspartate transporter (GLAST) (Doetsch *et al.*, 1997; Lagace *et al.*, 2007; Platel *et al.*, 2009) have been identified as the resident NSCs. In fact, B1 cells (distinguished from B2 astrocytes which are located close to the striatum) present characteristics of radial glial cells, such as specialized endfeet on blood vessels at their basal domain (Tavazoie *et al.*, 2008) and an apical cilium contacting the ventricle lumen (Mirzadeh *et al.*, 2008; for reviews see Fuentealba *et al.*, 2012). B1 cells divide asymmetrically to originate C cells, which in turn actively proliferate to give rise to immature neuroblasts – A cells. Newly born neurons extensively migrate tangentially along the RMS, a specialized tubular structure. Instead of having the exclusive glial support such as in developmental migration (Marin and Rubenstein, 2003), postnatal neuroblast migration is *homophilic* (Lois *et al.*, 1996). Adjacent migrating neuroblasts align themselves, building a tubular structure until the OB. However, glial contribution is not fully excluded. Specialized astrocytes – also called type B2 (Lois *et al.*, 1996) make up a glial tube that helps *homophilic* neuroblast migration preventing them from migrating away from the RMS (Kaneko *et al.*, 2010). Additionally, blood vessels serve as a delimiting scaffold for migration. Recent data identified blood vessels parallel to the RMS as physical supports for migrating neuroblasts (Snapyan *et al.*, 2009; Whitman *et al.*, 2009). Moreover, vasculature provides molecular cues fundamental for neuroblast migration. Other guidance cues direct RMS migration, either attractive such as prokineticin 2 released by OB cells, or repulsive like Slit that is secreted by the *Choroid plexus* and is distributed as a caudal-to-rostral gradient in the CSF that parallels the RMS flow.

When reaching the OB, A cells change their mode of migration; they detach from chains and start migrating with a scattering radial pattern. This detachment has been demonstrated to be promoted by mitral cell's reelin (Hack *et al.*, 2002) and also tenascin-R (Saghatelyan *et al.*, 2004). Also at this level of radial migration blood vessels are important players, they help OB final cell placement (Bovetti *et al.*, 2007). Neuroblasts then differentiate into granule cells and periglomerular cells in the GCL and glomerular layer (GL) respectively

and replace dead ones. Only half of the newly incorporated cells survive beyond 30 days, survival mostly depends on cellular activity related to sensory experience.

The OB is a small but complex brain structure. The odors are initially detected by olfactory sensory neurons (OSNs) at the olfactory epithelium. These cells, expressing the odor molecule receptors have a long axon entering the OB to synapse with primary dendrites of mitral and tufted cells (reviewed in Lledo *et al.*, 2004). Accordingly, OSNs are translators of chemical signals into electrical stimuli. At the OB, there are two populations of interneurons: granule and periglomerular cells. They connect with mitral cells controlling odor discrimination, memory and learning (Yokoi *et al.*, 1995; Laurent, 2002; Lledo and Lagier, 2006). The functional role of the newly generated interneurons is still elusive. Data are opposing, which might be due to either differences in the way neurogenesis is modulated (odor enrichment, odor deprivation, constitutive or inducible genetic models, pharmacological agents or irradiation), or differences in the behavioral paradigm used to study olfaction (tasks based on spontaneous odor behavior or on associative olfactory learning) (reviewed in Lazarini and Lledo, 2011; Breton-Provencher and Saghatelian, 2012).

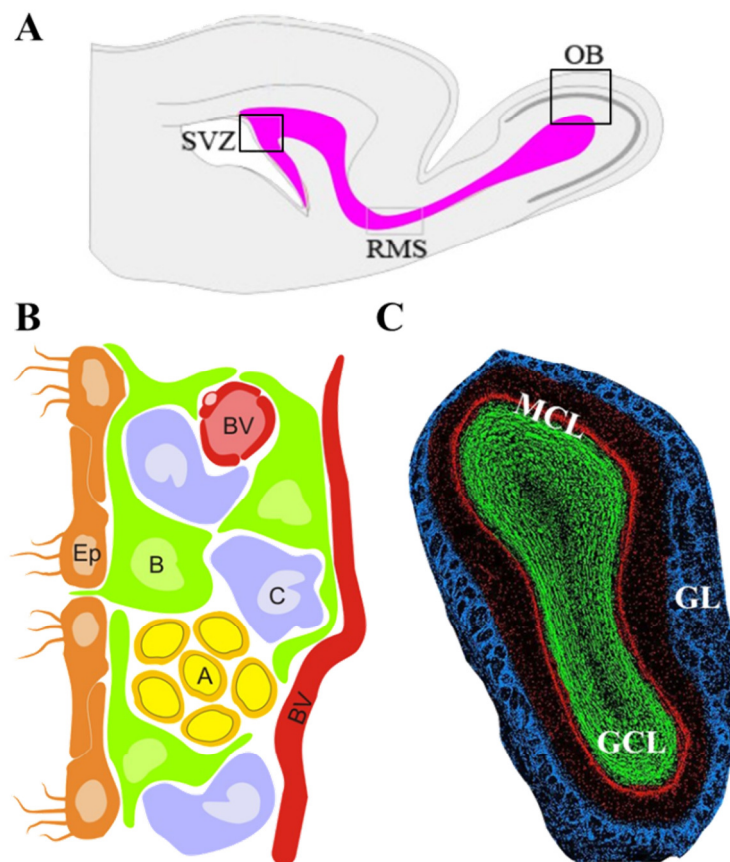


Figure 1. 1. Schematic representation of the SVZ and OB within the adult brain.

A. Localization of the neurogenic niche SVZ, RMS and OB. **B.** SVZ scheme organization. It is separated from the ventricle lumen by a monolayer of ependymal cells (Ep). B cells extend a cilium through the ependymal layer and contact with blood vessels (BV). C cells generate neuroblasts (A cells) that migrate in chains out of the SVZ towards the OB, through the RMS. **C.** OB anatomy in a coronal section. Glomerular layer (GL) is the outmost layer of the OB. Then, a thin layer of mitral cells, at the mitral cell layer (MCL) sets the limit for the core granule neurons from the granule cell layer (GCL).

In the human brain, the cytoarchitecture of the SVZ is different from the one found in rodents. In adult humans, the SVZ is also located at the lateral walls of ventricles, but comprises

four distinct layers: Layer I, a monolayer of ependymal cells surrounding the lateral wall of the ventricles is apposed to Layer II, a layer devoid of cell bodies, also known as the hypocellular gap; Layer III consists of astrocytes surrounding the hypocellular gap; and Layer IV which is enriched in myelin and separates layer III from the brain parenchyma (Quinones-Hinojosa *et al.*, 2006). No clear evidence of a RMS in the human brain has been demonstrated, consistent with the absence of neurogenesis beyond 18 months of age (Sanai *et al.*, 2011).

I.3.2. The SGZ – GCL hippocampal axis

In the adult DG of the hippocampus there is a privileged area housing neurogenesis, the SGZ situated between the hilus and the GCL. It harbours stem cells that are responsible for the production of about 10,000 new neurons per day (Cameron and McKay, 2001).

The cellular organization of the neurogenic DG is similar to what has been described for the SVZ. Type-1 cells (or radial astrocytes, also known as B cells) are considered the NSCs of the DG since they self-renew and are multipotent according to *in vivo* fate mapping studies. Type-1 cells share similarities with B1 astrocytes: they extend a primary cilium and contact blood vessels at their “hilar side” and express GFAP, nestin, and Sox2. However, controversy persists regarding the stem cell properties of these cells, essentially because the sphere forming capacities of these cells *in vitro* are limited: few primary spheres are obtained from adult SGZ cells, they do not passage, indicating no self-renewal capacity, and are mainly unipotent generating astrocytes (Clarke and van der Kooy, 2011). However, the sphere-forming assay as a tool to identify stem cells in a tissue has its limitations. It may not detect quiescent stem cells (such as Type 1 and B1 cells) but rather favours sphere formation from cycling progenitors and “activated” stem cells. In the DG, Type-1 cells asymmetrically divide to generate fast-proliferating nestin-positive cells firstly DCX-negative (Type 2a) and then DCX-positive (Type 2b). These cells, also known as D cells, give rise to immature neurons or Type-3 cells, called G cells as well, which are much less proliferative and nestin-negative. Finally, Type-3 cells migrate locally to the GCL, become post-mitotic and differentiate into calbindin positive mature granule neurons (for reviews see Ehninger and Kempermann, 2008; Zhao *et al.*, 2008).

This neurogenic niche is not only closely associated, but also functionally dependent on the interaction with the basal lamina of blood vessels’ endothelial cells. This crosstalk enables important physical contacts and chemical signaling cues essential to the maintenance of a proliferative and immature pool of neural stem cells.

New granule neurons extend an elaborated dendritic arborization in the molecular layer and an axon on the CA3 region at around day 20 after their birth. However maturation, including spine growth, continues beyond 8 weeks. As in the OB, the survival of these young

neurons is dependent on neuronal activity: a majority of them dies within 3 weeks even after establishing synaptic connections. Once they survive, surpassing apoptosis (Biebl *et al.*, 2000; Zhao *et al.*, 2006), and integrate, they will receive synaptic inputs from the entorhinal cortex. Thus, this constitutive neurogenic process plays an important role in the renewal and refinement of the hippocampal synaptic architecture and probably is involved in processing hippocampal-dependent memory and learning (Gray and Laskowsky, 2007; Kempermann *et al.*, 2008). Interestingly, some studies raised the hypothesis of adaptive hippocampal neurogenesis involved in memory (Feng *et al.*, 2001). According to this theory, the replacement of older granule cells and consequent clearance of their memory traces, is crucial to avoid a massive number of hippocampal connectivities and resulting “catastrophic interference”, as suggested by Wiskott and collaborators (2006).

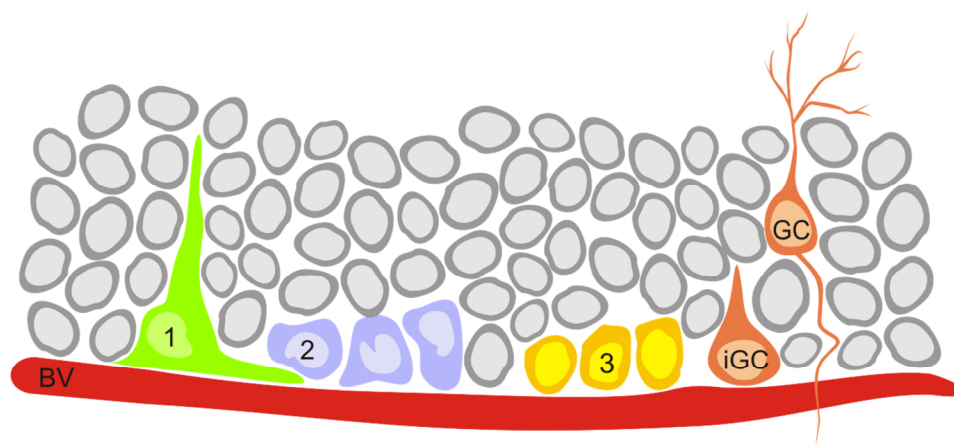


Figure I. 2. Schematic representation of the adult SGZ.

Radial astrocytes or type-1 cells (1) divide to give rise to fast proliferating type-2 cells (2). As maturation proceeds, type-3 cells (3) express markers for immature neurons and continue migration towards the granule cell layer. Immature granule cells (iGC) extend axons and elaborate dendritic trees so as to complete maturation into granule neurons (GC). BV, blood vessels.

On the other hand, Ninkovic and colleagues (Ninkovic *et al.*, 2007) proposed that in the DG, only a small percentage of newly generated neurons contribute to long-term neuron population. Despite neuronal differentiation, they are not affecting too much the plastic neuronal equilibrium. As also previously described (Schmidt-Hieber *et al.*, 2004) neurogenesis in early adult life provides a stable pool of plastic neurons that are kept throughout life. In agreement with this last hypothesis, the proposed continuous insertion of new neurons in the circuits would result in deleterious effects for normal brain function (Meltzer *et al.*, 2005). The proper balance between new neuron generation and integration within existing networks is probably the key to promote plasticity.

I.3.3. Cell based-therapy possibilities

Several recent studies suggest that brain lesion or neurodegenerative processes can stimulate neurogenic niches and the consequent differentiation of new neurons and

oligodendrocytes, depending on the phenotype of the lost cells. This lesion-stimulated neurogenesis/oligodendrogenesis represents a potential important source of brain repairing cells; considering that neuroscientists have been unveiling clues on how to control differentiation, migration, integration and survival of new brain parenchyma cells (Lindvall and Kokaia, 2006; Nait-Oumesmar *et al.*, 2007; Buffo *et al.*, 2008).

Parkinson's disease (PD), Huntington's disease (HD), Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), multiple sclerosis (MS), stroke or spinal cord injury are all examples of possibly treated neurodegenerative diseases based on stem cell-based therapies. Today, medical healthcare is based almost on symptom alleviation. Nonetheless, and since the discovery of adult mammalian NSCs and their modulation possibilities, stem cell-based therapies are becoming increasingly more real.

The acute or chronic loss of neural cells may be challenged by newly generated cells with their origin on NSCs. For that purpose, two strategies might be adopted: the transplantation of NSCs, or their more committed progeny; or alternatively, the mobilization of endogenous NSCs resources within the brain.

The transplantation of NSCs or their committed progeny into damaged brain areas has been explored in the last years. However, this strategy faces several challenges: (1) transplanted cell survival in a damaged, usually inflamed, tissue; (2) transplanted cell proper differentiation into the exact lost cell phenotype; (3) and transplanted cells integration into host tissue perfectly fitting the damaged circuitry. Although several studies reported transplanted NSCs survival together with proper migration at the host tissue, partial differentiation into the needed phenotype and functional improvements in the disease symptoms (Yasuhara *et al.*, 2006; Vazey *et al.*, 2006; Shetty and Hattiangady, 2007; Hicks *et al.*, 2007; Zhu *et al.*, 2009), these observations may all be due to a neuroprotective effect of the transplanted cells in general (Pluchino and Martino, 2005). Thus, the translation of this strategy to human cell therapeutics still needs some proof of principle improvements before being safely applied.

Because of the NSCs multipotency, the mobilization of these resources into a specific cell phenotype relies on the external control of the neurogenic niche. In culture, extracellular soluble factors or genetic manipulations are easily used to trigger a specific phenotype differentiation in expense of others. *In vitro* cell fate determination may increase the success of NSCs transplantation. Thus, one needs to know the specificity of the diseased cell loss to drive, *in vitro*, NSCs before transplantation. Then, also note of worth would be the cell administration way, it also depends on the disease specificity, being a focal (PD, HD, stroke) or multifocal cell loss (MS, AD). The final concern would be the acceptance/rejection of the graft. To avoid so, autologous transplants shall be performed. However, it implies that a first surgery is performed to collect NSCs, followed by a second one to implant the expanded and more committed cells. To circumvent this double surgery procedure, and thus as an alternative to autologous

transplants, could be the use of post-mortem human Central Nervous System (CNS) tissue (Laywell *et al.*, 1999), embryonic NSCs or pluripotent embryonic stem cells obtained from early-stage human blastocyst. All strategies are subjected to the rejection possibility and the former ones raise additional ethical concerns together with the eventual teratoma formation. Thus, a safer and more ethically accepted option would be the reprogramming of somatic autologous cells into induced pluripotent stem cells (iPSCs) by retroviral expression of certain transcription factors (Takahashi and Yamanaka, 2006). Still, tumor formation and genetic instability shall not be forgotten.

To conclude, transplantation cell-based therapies still need optimization to be considered a safe option to treat neurodegenerative diseases. However, a few issues should be kept in mind when approaching this strategy, which include a detailed knowledge on the disease cellular pathology; an optimized animal model for the human disease, previously tested and proved safe; and the certainty on the control of the grafted cells survival, differentiation fate and functional integration.

The opposing strategy to transplantation is focused on the recruitment of the endogenous NSC to the damaged area. Surprisingly, upon injury and in disease models, spontaneous mobilization of these precursors has been observed (reviewed in Curtis *et al.*, 2007a; Kaneko and Sawamoto, 2009). Normal cellular dynamics is perturbed and cells are de-routed from their quiescent undifferentiated state to an active proliferative state so that new NSCs differentiate and migrate to the damaged area.

Both at the SVZ and hippocampus, reports have been published showing recruitment of cells upon injury. Jin *et al.* (2005) showed that, in an HD model, new neurons from the striatum correctly project to the *globus pallidus*. Also ischemic models showed a proliferation trigger with subsequent neuroblast migration into non-neurogenic regions, such as the striatum or cerebral cortex (Arvidsson *et al.*, 2002; Darsalia *et al.*, 2005). Others have shown, also in ischemia, that the new neurons in the striatum contact with existing circuitries (Yamashita *et al.*, 2006). At the other neurogenic niche, and in some lesions, SGZ neurogenesis is increased (Jessberger *et al.*, 2005; Li *et al.*, 2010). However, this increase in cell proliferation and differentiation may cause additional damage, since aberrant projections were observed boosting the pathology symptoms (Parent, 2002; Parent and Murphy, 2008). In temporal lobe epilepsy, the injury-induced neurogenesis worsens symptoms rather than promotes recovery (Parent *et al.*, 1997; Bernardino *et al.*, 2005; Gray *et al.*, 2007; Sperk *et al.*, 2007; Jessberger and Parent, 2008; Yang *et al.*, 2010). Although seizure activity potentiates progenitor proliferation and differentiation of new granular cells, this condition leads to granular cell dispersion into the hilus and aberrant synaptic circuits with mossy fibre sprouting, ultimately leading to network hyperexcitability and consequently worsening the panorama.

Moreover, additional data indicate that these neurogenic niches may represent also resources to brain tumours, especially gliomas, if proliferation is out of control. Brain tumour cells and NSCs share several characteristics including self-renewal property; high proliferative rate; strong association with endothelial cells basal lamina; expression of immaturity cell markers (Sox-2, CD133, nestin); Endothelial Growth Factor (EGF) and Platelet Derived Growth Factor (PDGF) signaling and pluripotency (Reya *et al.*, 2001; Singh *et al.*, 2004; Gilbertson *et al.*, 2007; Pfenninger *et al.*, 2007; Agasse *et al.*, 2007; Coskun *et al.*, 2008). In agreement with these observations, several clinical cases highlight a close association of gliomas with neurogenic niches, especially at the SVZ. Taken together, these data may reveal that under certain circumstances, eventually favoured by genetic susceptibility, changes in the chemical or physical environment of the neurogenic niche may promote a deregulation of immature cell cycle, opening pathways to carcinogenesis while attempting to promote brain recovery (Gil-Perotin *et al.*, 2006; Vescovi *et al.*, 2006; Barami, 2007; Jackson and Alvarez-Buylla, 2008).

On the other hand, there are also diseases in which proliferation is decreased and no physiological compensatory effect is observed at the neurogenic niches. Examples are PD and AD, both with a lower SVZ proliferation rate. In PD, it is observed that during the disease progression dopamine levels drop. This neurotransmitter, which has an important role keeping basal proliferation at the SVZ, is diminished in this diseased condition (Hoglinger *et al.*, 2004). In AD, even prior to the first symptoms arise it is likely that decreased SVZ neurogenesis happens before the accelerated disease progression (Rodriguez *et al.*, 2008; Hamilton *et al.*, 2010; Lazarov *et al.*, 2010). At the SGZ, neurogenesis decrease prior to symptomology is not consensual as some authors claim that there is an increase in hippocampal immature neuronal markers (Jin *et al.*, 2004).

In addition to NSCs, recent data shed new light on alternative resources of repairing cells within brain lesioned areas. It has been shown that in damaged areas, a subset of reactive astrocytes express vimentin and nestin, two cell markers of immaturity. Moreover, these cells can be reprogrammed *in vitro* in order to acquire differentiating capacity (Buffo *et al.*, 2008). These fascinating data open new perspectives for novel brain regenerative therapy strategies based on these atypical astrocytes. Although reactive astrocytes have been accepted for long as present in the brain, their role within this tissue is still elusive. Nevertheless, we witness nowadays a novel fascinating hope in these cells with “stem cell-like potential” as they not only express putative stem cell markers but are also in close association with the angiogenic network present in brain lesion sites. Therefore, we may foresee genetic and pharmacologic manipulations of reactive astrocytes’ resources able to instruct these cells towards differentiation in a diversity of phenotypes useful for particular brain repair approaches.

Finally, and on the contrary to what has been discussed for transplantation-based strategies, there are some positive reports of human neurogenesis in HD and stroke. They all

report an increased neurogenesis upon injury (Curtis *et al.*, 2003; Jin *et al.*, 2006; Macas *et al.*, 2006; Marti-Fabregas *et al.*, 2010), which is very promising data to the future regenerative medicine strategies.

I.3.4. Cerebellum, a special and limited niche

The cerebellum, in Latin “little brain”, is the most posterior part of the rodent brain. Its cortex is a remarkably simple laminar structure and the cerebellar granule neurons are the most prevalent brain neuronal subclass, representing about half of the total brain neurons. Granule cells have an essential role, the coordination of afferent input to the cerebellum through excitatory connections with Purkinje neurons.

Cerebellum is critical for sensory-motor functions, balance control, vestibular ocular reflex but also feed-forward sensory-motor learning, speech and spatial memory (du Lac *et al.*, 1995; Fiez, 1996; Timmann *et al.*, 1999; Boyden *et al.*, 2004; Schmahmann and Caplan, 2006). Mouse mutants with cerebellar abnormalities all have ataxic phenotypes, for instance *staggerer* (Sidman *et al.*, 1962) and *weaver* (Rakic and Sidman, 1973).

This anatomically distinct area of the brain has a prolonged developmental phase. In mice it can reach the 3rd week postnatal, and in humans it takes 2 years to achieve full develop.

I.3.4.1. A layered structure

During embryogenesis, cerebellum arises from the anterior most segment of the hindbrain, the rhombomere 1. Here, two distinct germinal zones will differentially derive from both cellular movements and divisions: the cerebellar cortex and the cerebellar nuclei (Hatten and Heintz, 1995). Each of which will give rise to a limited number of different cell types in a precise sequence. From the cerebellar cortex, Purkinje cells, stellate, basket, Golgi and Lugaro interneurons all arise. On the other hand, from the cerebellar nuclei, at the edge of the neuroepithelium, in a zone called rhombic lip, granule cells and unipolar brush cells originate.

Apart from the granule cells, the most abundant cerebellum cell type, other cells stand out, such as radial glia and Purkinje cells. Radial glia has been reported to be important to this special neurogenic niche (Feng *et al.*, 1994; Anthony *et al.*, 2004; Morales and Hatten, 2006). However, the molecular mechanisms and its interaction with neurons during embryonic development are not yet clear, and some authors even relate it to cancer (Gibson *et al.*, 2010). Purkinje cells are also master neurons from cerebellar cortex. They are born in the embryo and they align in a monolayer that will set the adult limits between molecular layer (ML) and internal granule cell layer (IGL) with their brushy dendritic trees extending into the molecular layer.

During embryogenesis, it is cell migration that puts cerebellum cells in place, forming the layered structure. Cell positioning is well established and sequential (for review Chedotal, 2010; Hatten and Roussel, 2011). However, at birth, the surface of the cerebellum is regular and smooth, cells are correctly placed and ready to make up the foliated adult structure. This inward cell migration will develop the invaginated structure of the adult cerebellum which is only set postnatally (Sillitoe and Joyner, 2007).

I.3.4.2. Postnatal development and migration

Granule cells are the most abundant interneurons of the cerebellum. Their precursors, granule cell precursors (GCP), actively proliferate during the first 2 postnatal weeks at the most superficial layer of the cerebellum cortex: the external granule cell layer (EGL). As they decrease proliferation rate, and exit cell cycle (Espinosa and Luo, 2008), they start radial migration – it is the onset of the postnatal cell migration. EGL disappears and the most internal layer (IGL) increases. At the middle acellular layer (molecular layer) granule neurons can make synaptic contacts with dendritic trees of the Purkinje cells, basket and stellate interneurons. At the IGL, granule neurons synapse with Golgi interneurons, and also receive information from mossy fibers. Purkinje cells extend their dendrites forming connections with in-growing afferent axons from the climbing fibers as well as with cerebellar interneurons. Bergmann glia, specialized astrocytes, provides physical scaffolds for GCP migration (Rakic, 1971) as well as Purkinje cells dendritic outgrowth and synapse formation (Yamada and Watanabe, 2002). Their role is so fundamental that its deletion has several secondary effects on other cerebellum cell types, and an overall cerebellum abnormal development in size and layered structure (Delaney *et al.*, 1996). So far, the postnatal cerebellum development depends on migration only. All cell types orchestrate so that they crosstalk and help each other to reach their final placement and maturation stage. Thus, the full knowledge of cerebellar postnatal migration is crucial to understand its development.

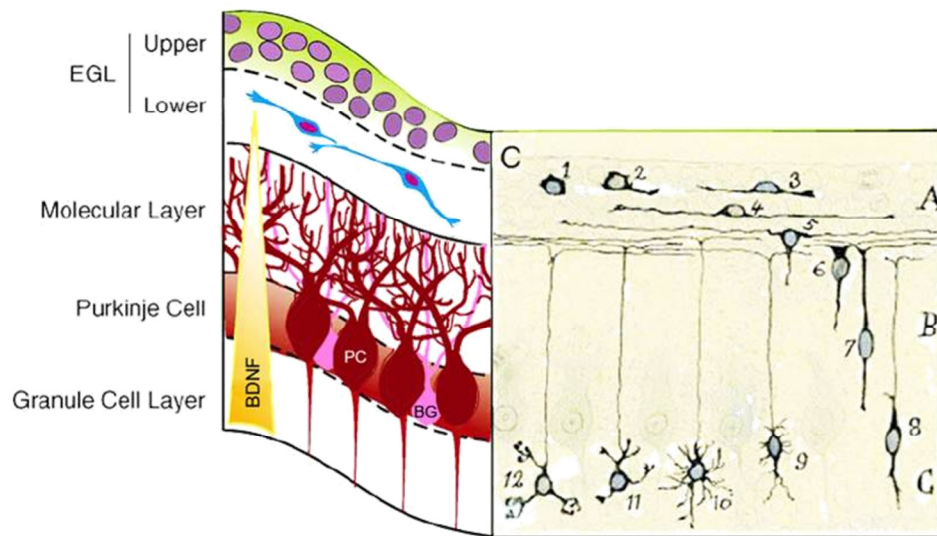


Figure I. 3. Cerebellum postnatal cortex organization.

Right panel: original drawing of Santiago Ramon y Cajal depicting the sequence of differentiation and migration of granule cells from the EGL to the IGL (3–4, tangential migration; 5–8, radial migration). Left panel: Granule cell precursors at the EGL stop proliferating (upper EGL) and start tangential migration at the lower EGL. Molecular and/or genetic cues signal radial migration onset and GCP leave the EGL passing the molecular layer full of Purkinje cell arborization with the help of Bergmann glia processes as physical scaffolds. A BDNF gradient from mature granule cells at the granule cell layer is believed to help in this migratory behavior (adapted from Chedotal, 2010). EGL, external granule cell layer; GCP, granule cell precursor; IGL, internal granule layer.

The out most layer of the postnatal cerebellum, EGL, is transient. It is the result of an intermediate step of proliferation between two migration steps. Embryonically, there is a first (tangential) migration step: cells born at the rhombic lip migrate over the nascent cerebellum to form the EGL; then there is a second (radial) migration step, in which granule cell precursors that were actively proliferating, slow down proliferation, exit cell cycle and move across molecular layer to reach the IGL. The provisional EGL is thus a mitogenic place, for some time. However, which are the triggers that control the shift proliferation-migration? Which are the molecular signals that govern this dual migration control?

Intrinsic and diffusible factors can help in this decision, whether to stay or leave the EGL. Some data points to intrinsic programming of GCP since isolated granule cells can shift their mode of migration without any chemical or physical clue from surrounding cells (Kawaji *et al.*, 2004). Moreover, migration direction seems to be also intrinsically controlled. Tangential migration can spontaneously change while radial migration cannot be reversed (Komuro *et al.*, 2001; Umeshima *et al.*, 2007). However, and in agreement with the latter data, several other reports show the role of diffusible and contact factor helping in migration guidance.

From diffusible factors, neurotrophins play major roles at the cerebellum. Vascular Endothelial Growth Factor (VEGF), for instance, derived from Purkinje cells has recently been pointed to guide granule cell migration (Ruiz de Almodovar *et al.*, 2010). BDNF, another neurotrophin extensively studied, has been pointed as fundamental for cerebellum granule cell migration, both by its gradient across the layers and its wide receptors expression.

Regarding contact factors, they have a foremost role at the shift moment from neuronal supported migration to glial supported migration. When migration with neuronal support (external granule cell layer – tangential migration) changes to migration with glial support (molecular layer – radial migration), it is Semaphorin 6A loss, for instance, that supports this change (Kerjan *et al.*, 2005; Renaud *et al.*, 2008). Plexin-A2 (Kerjan *et al.*, 2005), one of Semaphorin6A receptors, controls the initiation of granule cell migration. Granule cell precursors polarize properly and have neurite extension though they do not move at the EGL.

I.4. Modulation of neurogenesis

Neurogenesis comprises five cardinal steps: proliferation, migration, differentiation, survival and integration, all tightly regulated by intrinsic, extrinsic and environmental factors.

Cell cycle modulators, transcription factors, and epigenetic regulators are intrinsic cues regulating adult neurogenesis. These, in turn, are affected by a huge number of extrinsic factors such as morphogens, adhesion molecules, growth factors, neurotrophins, cytokines, hormones, neurotransmitters and neuropeptides. Moreover, environmental factors as physical exercise, environmental enrichment, diet, social interactions and stress are also regulators of adult neurogenesis. Vasculature, as previously mentioned, also plays a role in neurogenesis and neuroblasts migration.

The global understanding of this wide variety of signalling pathways and cellular interactions will ultimately provide us with precious knowledge for future therapeutic applications.

I.4.1. Intrinsic factors

Intrinsic mechanisms are cell-inherent programs that coordinate the neurogenic niche dynamics. These include epigenetic regulators such as transcription factors, microRibonucleic Acids (microRNAs) and DNA modifying enzymes that do not alter DNA sequence.

Transcription factors, such as Sox2 and NANOG are classic examples of relevant modulators of differentiation. They have been used to reprogram adult somatic cells, such as fibroblasts into NSCs, through an intermediate iPSC state (Takahashi *et al.*, 2007). Forced expression of these transcription factors artificially tune somatic cells for neurogenesis. Sox2 expression is a hallmark of NSCs and is related to stem cell maintenance and proliferation since its down regulation promotes differentiation (Takahashi *et al.*, 2007). On the other hand, certain genes are classified as proneurogenic. This is the case of the basic helix-loop-helix transcription factors Mash1 (Pattyn *et al.*, 2004), Neurogenin-1 (Sun *et al.*, 2001) and the homeobox transcription factor Dlx2 (Suh *et al.*, 2009) that together with Pax6 directs neuronal

differentiation (Doetsch *et al.*, 2002) and promote dopaminergic periglomerular phenotypes in adult mice (Hack *et al.*, 2005; Brill *et al.*, 2008).

MicroRNAs, small interfering RNAs (siRNA) and piwi-interacting RNAs (piRNA) are non-coding RNAs that regulate gene expression (He and Hannon, 2004). miRNA are single strands of 20-25 nucleotides that bind to the 3' or 5' untranslated region of mRNAs and repress translation (Lytle *et al.*, 2007; Rana, 2007). Among them are miR-9 and miR-124. They promote neuronal differentiation of NSCs (Conaco *et al.*, 2006) through inhibition of STAT3, a complex responsible for the expression of GFAP and astrogliogenesis (Krichevsky *et al.*, 2006).

Finally, epigenetics, a field of genetics devoted to gene expression changes that occur regardless of any DNA sequence change, has emerged as a great contributor to the knowledge of neurogenesis modulation. These inheritable changes can occur through covalent DNA methylation or histone modification, among other molecular chromatin alterations (for review Wen *et al.*, 2009). DNA methylation is a crucial mechanism for controlling chromatin remodeling in the adult mammalian nervous system (Levenson *et al.*, 2006; Nelson *et al.*, 2008). During early embryonic development, even if exposed to astrocyte-inducing factor NSCs differentiate exclusively into neurons since astrocytic gene promoters are blocked by hypermethylation (Takizawa *et al.*, 2001). However, once late gestation starts, these promoters become accessible by demethylation and thus NSCs can differentiate into neurons and glial cells (Qian *et al.*, 2000). Histone modifications include a variety of post-translational changes, from which acetylation is the most studied. Acetyltransferases (HATs) are enzymes that acetylate conserved lysine (K) residues on the N-terminus of the nucleosome's histone protein core. Upon this acetyl group addition, the DNA packing into heterochromatin is loosened into euchromatin, which makes it more prone to be actively transcribed (Hsieh and Gage, 2005). Lim and colleagues (2009) showed that differentiating NSCs have the proneurogenic genes *Mash1* and *Dlx2* loci highly methylated.

I.4.2. Extrinsic factors

Panoply of diffusible and contact factors released by the cellular components constituting the neurogenic niche has been also identified to modulate neurogenesis (Moyse *et al.*, 2008).

Ependymal cells, for instance, secrete Noggin, a soluble factor that antagonizes the proglial effect of bone morphogenetic protein (BMP) on SVZ cells, and thereby favours neuronal production (Lim *et al.*, 2000; Peretto *et al.*, 2004). Transgenic Noggin overexpression in the SGZ increases multiple precursor cell populations but especially GFAP-positive cells, suggesting that it acts also on NSCs (Bonaguidi *et al.*, 2008). As Noggin does at the SVZ, it also antagonizes BMP at the DG (Ueki *et al.*, 2003). Astrocytes of both neurogenic areas can be

sources of pro-inflammatory cytokines and morphogens like the soluble sonic hedgehog (shh) that is implicated in self-renewal and proliferation of NSCs (Charytoniuk *et al.*, 2002; Machold *et al.*, 2003; Lai *et al.*, 2003; Ahn and Joyner, 2005).

Among the other soluble factors, cytokines such as Tumor necrosis factor- α (TNF- α), and neuropeptides as Neuropeptide Y (NPY), have been described to stimulate proliferation and differentiation of new neurons. TNF- α strongly influences survival, proliferation and neuronal differentiation of murine SVZ stem cells (Bernardino *et al.*, 2008). NPY induces proliferation of neural progenitors triggering neuronal differentiation *via* Y1 receptor activation, in both neurogenic niches (Howell *et al.*, 2005, Agasse *et al.*, 2008a; Decressac *et al.*, 2009, 2011; Thiriet *et al.*, 2011).

Neurotransmitters, other class of soluble factors, have regulatory effects at the neurogenic niches. Most interestingly, regulatory loops have been identified to control the expansion of proliferating cells in the niche. Indeed, gamma-aminobutyric acid (GABA) is released by SVZ neuroblasts and it acts in an autocrine manner to decrease proliferation and promote migration (Liu *et al.*, 2005). In the adult SGZ, GABA promotes dendritic growth, synapse formation and newborn neuron survival (Jagasia *et al.*, 2009). N-Methyl-D-Aspartate Receptor (NMDAR) signaling regulates survival of neuroblasts in the adult SVZ (Platel *et al.*, 2010) and immature neurons in the adult SGZ (Tashiro *et al.*, 2006). Also, progenitors regulate proliferation and self-renewal of SVZ stem cells through a mechanism involving the interaction of the EGF receptor (EGFR) and Notch pathway. Enhanced EGFR signaling in the SVZ results in the expansion of the progenitor cell pool, reducing NSCs number and self-renewal. This EGFR-mediated mechanism is regulated by Notch signaling (Aguirre *et al.*, 2010).

As just mentioned, growth factors such as EGF or Fibroblast Growth Factor-2 (FGF-2), also may affect the SVZ neurogenic niche. Once administered *in vitro* or *in vivo* they stimulate proliferation (Reynolds and Weiss, 1992; Gritti *et al.*, 1995; Kuhn *et al.*, 1997). Moreover, it was described in rats, that basic Fibroblast Growth Factor (bFGF) also increases neural differentiation (Jin-qiao *et al.*, 2009).

BDNF is an example of a neurotrophin modulating neurogenesis. It is neuroprotective (Li *et al.*, 2010) and also proneurogenic. When administered together with EGF, it promotes neurogenesis within a non-neurogenic tissue – striatum – and helps in stroke functional recovery by the generation of new neurons (Im *et al.*, 2010).

I.4.3. Environmental factors

A vast class of factors can modulate neurogenesis. Environmental factors can acquire special relevance following dramatic events such as brain damage or pathologies, as described in a previous section.

Hippocampal neurogenesis can be boosted by physical exercise (reviewed in Llorens-Martin *et al.*, 2009), social interactions (Gheusi *et al.*, 2009), environmental enrichment (Olson *et al.*, 2006) and dietary caloric restriction (Park *et al.*, 2011). They stimulate neurogenesis at the DG by affecting cell proliferation or survival of new neurons in the DG. Surprisingly, not all mentioned factors have SVZ proneurogenic effect as the one observed at the SGZ. Just the olfactory enrichment seems to affect OB-directed neurogenesis (Alonso *et al.*, 2008; Bovetti *et al.*, 2009; Martončíková *et al.*, 2011).

On the other hand, aging and stress are usually deleterious for neuronal production in both niches. Stress diminishes the production of new neurons in the SGZ, an effect mainly related with glucocorticoid levels (Gould *et al.*, 1998; Tanapat *et al.*, 1998; Schoenfeld and Gould, 2012). In the murine SVZ, the aged reduce neurogenesis was associated with a reduction of both stem cells and EGFR expressing cells (Trapepe *et al.*, 1997; Enwere *et al.*, 2004). It appears that, although NSCs from aged animals retain their potential to proliferate and differentiate into functional new neurons, they are in lower number (Ahlenius *et al.*, 2009). Aging also negatively regulates adult neurogenesis, both SGZ and SVZ, affecting extrinsic and intrinsic factors (review see Lee *et al.*, 2012).

1.5. Neurovascular niche

Vasculature, as briefly mentioned before, is a key cellular component of the stem cell niche. The blood brain barrier (BBB) is an association of cells that protects the brain parenchyma from the free contact with peripheral blood. However, at the SVZ it is modified and stem cells can contact with patches of endothelial cells without the presence of astrocytes endfeet or pericytes, which allows a direct cell-to-cell contact and an easier diffusion of soluble molecules (Shen *et al.*, 2008; Tavazoie *et al.*, 2008). Nestin expressing astrocytes in the DG extend a long apical process through the molecular layer and contact blood vessels (Nikolova *et al.*, 2007). These interactions with blood vessels contribute to control stemness and neurogenesis. Moreover, vasculature is also fundamental in regulating neighboring cells proliferation, survival, and establishment of immune responses, haemostatic balance and control of systemic blood pressure, among other key regulatory mechanisms. Additionally, it has been recently shown that vasculature is denser at the striatal periventricular wall, thus, closer to the neurogenic SVZ (Kazanis *et al.*, 2010).

At the neurogenic niche, the vasculature exerts its role through the secretion of factors that support stem/progenitor cell fate and proliferation (Shen *et al.*, 2004; Plane *et al.*, 2010). Endothelial-derived factors such as BDNF, VEGF or Stromal Cell-Derived Factor-1 (SDF-1) promote neurogenesis. Moreover, extracellular matrix molecules secreted by brain endothelial cells, namely laminin, regulate adherence, stemness and proliferation of SVZ stem cells and

progenitors. Thus, vasculature offers a set of functionalities to the organism's proper development and maintenance.

Blood vessels are hollow tubular structures formed by endothelial cells surrounded by a contractile layer of mural cells (vascular smooth muscle cells and pericytes) and an out most layer of fibroblasts, extracellular matrix (ECM) and perivascular nerves (Girouard and Iadecola 2006). Vasculature is highly present at the meninges and surface of the brain. However, it penetrates deep into the nervous tissue at the same time as blood vessels become smaller and lose their smooth muscle coverage. This way, thin capillaries assure the deep neurovasculature.

I.5.1. Blood vessel structure formation

During embryonic development, angiogenesis is a complex crosstalk between genetic and epigenetic factors that include haemodynamics and tissue oxygenation. Early in development, intrinsic factors, chemical and physical pressures define the hierarchical structure of vasculature: arteries, veins and capillaries.

As soon as the heart begins to beat, blood flow induces remodeling of the vessel network: branching morphology, diameter changes and recruitment of peripheral cell types such as pericytes and vascular smooth muscle cells that help stabilizing the whole network. Both genetic and epigenetic (Chapman *et al.*, 1918; Manner *et al.*, 1995; Wakimoto *et al.*, 2000) aspects control this remodeling. Blood flow influences vasculature remodeling in several ways: it carries oxygen, nutrients and signaling molecules, and it creates physical forces on the endothelial cells of the vessels (Chiu *et al.*, 1998; reviewed in Lehoux and Tedgui, 1998). Postnatal arterial development is thus affected by shear stress (Resnick N *et al.*, 1997), but also by genetic cues: the blood flow within the vessels creates haemodynamic signals sensed by integrins and cytoskeleton (Katsumi *et al.*, 2004). Other proof of genetics importance were the findings by Tzima and colleagues (2005): with the *in vitro* co-transfection of only Vascular Endothelial-Cadherin (VE-Cadherin), Platelet Endothelial Cell Adhesion Molecule (PECAM-1) and VEGF Receptor 2 (VEGFR2) nonendothelial cells aligned perfectly within the flow direction.

Moreover, in other environments it has been shown that, postnatal vasculature undergoes reorganization. VEGF has been implicated in neovascularization (Grunewald *et al.*, 2006) and vasculature remodeling (Bozoyan *et al.*, 2012), together with Hypoxia Inducible Factor (HIF) (Licht *et al.*, 2006) and Ephrins (Colin-Castelan *et al.*, 2011). Thus, the formation and maintenance of this network of blood vessels not just has its role in neurogenesis but also can be influenced back by neurogenesis, in a close crosstalk.

I.5.2. Functional cellular relationship with neurogenic niche

The perception of the tight association of vasculature and the neurogenic niches has been gaining support from several experimental evidences. In 2002, Louissaint and colleagues firstly identified the intricate relationship between neurogenesis and angiogenesis: VEGF as a neuronal/glia secreted factor regulates endothelial cell division at the same time as BDNF from endothelial cell origin modulates survival and migration of Ventricular Zone (VZ) neuroblasts to the HVC (Louissaint *et al.*, 2002).

In mammals, angiogenesis and neurogenesis are tightly regulated by common factors such as FGF-2, VEGF, BDNF or Angiopoietin-1 (Ang-1). Moreover, upon injury, not just neurogenesis but also angiogenesis is increased (Hellsten *et al.*, 2004; Gotts and Chesselet *et al.*, 2005), and both neurogenic and angiogenic factors are overexpressed (Yoshimura *et al.*, 2003; Wang *et al.*, 2004). Within the SGZ of the hippocampus, nestin-expressing radial glia cells are closely associated with blood vessels (Seri *et al.*, 2004) and there is an anatomical relationship between proliferating endothelial cells and proliferating neuronal precursors (Palmer *et al.*, 2000; Jin *et al.*, 2002; Wurmser *et al.*, 2004). In the DG, neurogenesis and angiogenesis are linked processes and they share responsiveness to similar growth factors such as neurotrophins, neuropilins, semaphorins and ephrins (Kraemer and Hempstead, 2003). Moreover, exercise-induced angiogenesis in the hippocampus increases Nerve Growth Factor (NGF) and BDNF protein levels (Ding *et al.*, 2004). Endothelial cells from the neurovascular niches support also the expansion of stem cells in gliomas (Borovski *et al.*, 2009; Galan-Moya *et al.*, 2011; Zhu *et al.*, 2011).

Additionally, upon development, blood vessels give physical support for newly born neurons: radial glia extends from ventricle to pia mater allowing contact with neurons and blood vessels. This relationship is abolished latter in development being re-established in the SVZ with astrocytes contacting the ventricle and capillaries (Alvarez-Buylla and Garcia-Verdugo, 2002). Moreover, and as mentioned, SVZ proliferating cells directly contact blood vessels through the disruption of the BBB at sites lacking astrocyte endfeets (Shen *et al.*, 2008; Tavazoie *et al.*, 2008).

Being that migration is also comprised within neurogenesis, one should not forget the central role of vasculature on neuronal cell migration. At the RMS, evidences indicate vasculature as a key substrate for migration. For instance, endothelial cells synthesize BDNF, which fosters migration *via* p75 neurotrophin receptor (p75NTR) (Snopyan *et al.*, 2009). Additionally, the close interaction between blood vessels and migrating neuroblasts, through their leading processes, suggests that this interaction provides directional guidance to migrating cells (Kojima, 2010). Also at OB, blood vessel and neuroblasts have tight connections. Bovetti and colleagues (2007a) implicated vasculature in OB radial migration. With these so close associations and interdependences, the vasculature present at the neurogenic niche constitutes with the former the neurovascular niche.

I.5.2.1. Diffusible factors

As mentioned before, many secreted endothelial derived factors contribute to the neurovascular niche, such as FGF-2, VEGF, Ang-1 or BDNF, in a paracrine.

FGF-2, a growth factor present at vessel branching sites (Seghezzi *et al.*, 1998), is a potent angiogenic factor at the levels of cell proliferation, migration and differentiation (Biro *et al.*, 1994), at the same time as it is sensed by neural stem cells through FGF Receptor 1 (FGFR-1) (Zheng *et al.*, 2004). Cortical progenitors are proliferative responsive to FGF-2 (Mudo *et al.*, 2009) and even progenitors from non-neurogenic areas are proliferative responsive to FGF-2 (Palmer *et al.*, 1995). Moreover, upon injury (Peng *et al.*, 2008) or in aged tissues (Jin *et al.*, 2003), FGF-2 is crucial to induce neural reparation and promotes migration and differentiation of pre-treated transplanted cells by the enhancement of Neurogenin (Ngn) expression (Vergano-Vera *et al.*, 2009)

VEGF is produced by endothelial cells, but also by ependymal cells and *Choroid plexus*. It stimulates neurogenesis in the SVZ and hippocampus (Jin *et al.*, 2002). Neurospheres express VEGF (Maurer *et al.*, 2003) and its infusion into the lateral ventricle results in an increased neurogenesis by VEGFR2/Flk receptor (Shen *et al.*, 2004; Schanzer *et al.*, 2004; Wada *et al.*, 2006). VEGF can also indirectly act by BDNF stimulation (Louissaint *et al.*, 2002; Shen *et al.*, 2004). More recently, VEGF has been implicated in neuroblast migration during development (Schwarz *et al.*, 2004) and after injury (Wang *et al.*, 2007). Sequestering VEGF during development induces RMS vascular network collapse that then abolishes neuroblast migration (Licht *et al.*, 2010). Its down regulation affected angiogenesis and vasculature growth in the RMS, disturbing thus precursor cell migration (Bozoyan *et al.*, 2012). VEGF increases also neurogenesis and migration after brain injury (Wang *et al.*, 2007; Thau-Zuchman *et al.*, 2010).

BDNF, a neurotrophin simultaneously expressed by neurons, astrocytes and endothelium, has long been associated with vasculature and neurogenesis. BDNF from endothelial cell origin was shown to promote neuronal outgrowth and survival, besides inducing SVZ cells' migration from explants. Leventhal and colleagues (1999) showed that the endothelial cell conditional medium rich in BDNF regulated neurogenic responses. After this report, new studies referred the roles of endothelial BDNF in the songbird HVC (Louissaint *et al.*, 2002), in the rodent RMS (Snapyan *et al.*, 2009) and ischemic striatum (Grade *et al.*, 2013).

Ang-1 is a growth factor produced by endothelial cells that acts on Tie2 tyrosine kinase receptors (Suri *et al.*, 1996). It regulates stem cell differentiation and migration through Tie-2 and CXC Chemokine Receptor Type 4 (CXCR4) receptors (Jones *et al.*, 2001; Imitola *et al.*, 2004), and also promotes cellular adhesion and survival of supporting cells (Papapetropoulos *et al.*, 2000; Patan, 2000; Yancopoulos *et al.*, 2000). Ang-1 can induce neuronal differentiation

from SVZ progenitors (Liu *et al.*, 2009), mouse embryonic cortical and dorsal root ganglion cells (Kosacka *et al.*, 2005; Bai *et al.*, 2009). Ang-1 triggers proliferation *via* activation of Extracellular signal-Regulated Kinases 1/2 (ERK1/2) Mitogen-Activated Protein Kinases (MAPK) pathway without any effect on cell death (Rosa *et al.*, 2010). The same authors showed that Ang-1 is not just proneurogenic but also involved in axonogenesis since it elicited Stress-Activated Protein Kinase (SAPK)/c-Jun N-terminal kinase (JNK) MAPK activation.

I.5.2.2. Contact factors

Several molecules present at the surface of blood vessels contribute to the regulation of the neurovascular niche. ECM has both adhesion and signaling molecules as modulators of the stem cell niche.

Laminin-1, a major protein of the basal lamina maintains SVZ functions. Pollard and colleagues (2006) cultured NSCs from SVZ and gliomas on laminin coatings. It also retains NSCs capacities of DG cells from neonatal mice and embryonic human and mouse cortices (Flanagan *et al.*, 2006; Imbeault *et al.*, 2009). Moreover, human embryonic cortex cells had increased proliferation on laminin rich medium in a $\beta 1$ integrin-dependent manner (Hall *et al.*, 2008).

Both laminin-1 and $\alpha 6\beta 1$ integrin regulate NSCs divisions during neocortical development at the ventricular zone (Lathia *et al.*, 2007b; Loulier *et al.*, 2009). Moreover, during postnatal cerebellum development, GCP division is controlled both by laminin and $\alpha 6\beta 1$ integrin, the former directs centrosome positioning and polarization *via* $\alpha 6\beta 1$ integrins activation (Gupta *et al.*, 2010). This $\alpha 6\beta 1$ integrin is a hallmark of stem cells: during cortical development, $\beta 1$ -dependent anchoring of radial glia NSCs to laminin-rich ventricular surface regulates interkinetic nuclear migration and division orientation of NSC (Belvindrah *et al.*, 2007; Loulier *et al.*, 2009); downregulation of $\alpha 6$ integrin decreases self-renewal and tumour formation (Lathia *et al.*, 2010a); and it is also expressed in non-neuronal tissues (Shinohara *et al.*, 1999; Yovchev *et al.*, 2008; Lathia *et al.*, 2010; Notta *et al.*, 2011).

Furthermore, stroma cell-derived factor-1 (SDF-1) secreted by endothelial cells has a feedback loop and it upregulates the expression of $\alpha 6$ -integrin in C cells, promoting their adhesion to laminin (Kokovay *et al.*, 2010). As the adhesion to the ECM components promotes the exposure to the extracellular factors, the crosstalk with integrin signaling is boosted and this neurovascular relationship is strengthened (Hynes, 2002 and 2007).

I.6. Histamine and neurogenesis

Histamine, firstly described by Dale and Laidlaw (1910), is a molecule that has been always associated with peripheral inflammatory reactions. However, it may act as

neurotransmitter and neuromodulator at both Central and Peripheral Nervous System (CNS and PNS). Its action can be mediated by several receptors within different tissues and the selective activation of each receptor may trigger several biological functions.

This hydrophilic amine is derived from the histidine amino acid by L-histidine decarboxylase (HDC) catalyzed reaction. Once produced, it can either be rapidly inactivated or stored, mainly in mast cells. Its deactivation occurs by the action of either histamine-N-methyltransferases, more frequent at the synaptic cleft, or by diamine oxidases.

I.6.1. Sources of histamine

Histamine triggers inflammatory responses at the immune system as a response to foreign pathogens. Basophils and mast cells, when sensitized by exposure to an antigen/allergen, produce this biological amine. Upon activation, these cells degranulate and release histamine, which increases the permeability of blood vessels to the white cells that will engage the pathogen at the infected tissue. It can thus be categorised as a vasoactive amine.

In the brain, histamine was firstly evident in the studies of Garbarg and Schwartz (1974). Now, we know that there are three cellular stores: neuronal, mast and microglial cells (Orr and Pace, 1984; Katoh *et al.*, 2001). Moreover, histamine can be present in the CSF affecting NSCs either *via* ependymal cells or directly *via* the cilium of SVZ type B cells that contact the ventricle lumen.

In the healthy brain, including postnatal rodent brain, the “bulk” concentration of histamine is very low. Several studies reported that histamine is present at nanomolar concentrations both in the CSF and brain parenchyma of humans and rodents (Khandelwal *et al.*, 1982; Yoshitake *et al.*, 2003; Soya *et al.*, 2008; Kanbayashi *et al.*, 2009; Nishino *et al.*, 2009; Bourgoigne *et al.*, 2011; Croyal *et al.*, 2011). However, low levels of histamine in the CSF or in the brain parenchyma do not reflect the concentrations of histamine reached at the active sites of histaminergic synapses. There is a spatial and temporal peak of histamine concentration in the active zone, sensed by histamine receptors, and it cannot be correlated with the “bulk” histamine concentration (due to histamine metabolism and re-uptake processes). Moreover, it is relevant that brain targets of histaminergic fibers express functional receptors that are activated by micromolar levels of histamine, and that at these concentrations histamine inhibits evoked excitatory post-synaptic potentials (Jiang *et al.*, 2005). On the other hand, it should be highlighted the close physical and chemical contact of the SVZ neural stem cell niche with the CSF, circulating in the lateral ventricles. Histamine, present in the CSF can then affect the neurogenic microenvironment by the contact with ependymal cells (paracrine effect) or by the direct contact with the type B cell’s cilium.

Circulating levels of histamine are robustly increased following brain injury, degeneration or infection (Coelho *et al.*, 1991; Niimi *et al.*, 1994; Alvarez *et al.*, 1996; Anichtchik *et al.*, 2000; Katoh *et al.*, 2001; Yoshitake *et al.*, 2003; Soya *et al.*, 2008; Kanbayashi *et al.*, 2009). Under these conditions, the inflammatory response may elicit *Choroid plexus* mast cells degranulation, which massively increases histamine at the CSF and increases BBB permeability (Zhuang *et al.*, 1996).

I.6.2. Histaminergic system and histamine receptors

Histaminergic neurons, located at the tuberomammillary nucleus of the posterior hypothalamus are pacemakers that fire at slow regular rate. From there, neurons project to widely distributed areas of the CNS, including cortex (through the medial forebrain bundle), hippocampus, basal ganglia and also into the epithelial ventricular zone, thus reaching both neurogenic niches (Panula *et al.*, 1984; Kinnunen *et al.*, 1998; Lintunen *et al.*, 1998). These wide projections make histamine involved in a broad range of physiological and behavioral functions, such as sleep-wake control, emotions, learning and memory (Panula *et al.*, 1984; Brown *et al.*, 2001; Passani *et al.*, 2004; Haas *et al.*, 2003 and 2008). The typical histaminergic synapses are different from normal postsynaptic endings. Instead, histamine is released from varicosities, swelling histamine containing synaptic vesicles located periodically along the axon (Takagi *et al.*, 1986). Thus, histamine release sites and their receptors not always directly appose to each other.

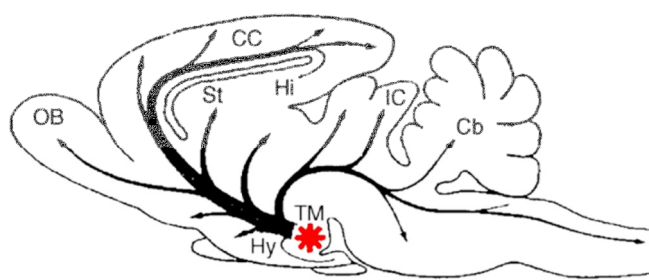


Figure I. 4. Rat histaminergic system.

Cell bodies of the histaminergic system locate at the tuberomammillary nucleus (TM) of the posterior hypothalamus and send nerve fibers to almost all brain regions. Cb, cerebellum; CC, cerebral cortex; Hi, hippocampus; Hy, hypothalamus; IC, inferior colliculus; OB, olfactory bulb; St, striatum. Adapted from Watanabe and Yanai, 2001.

Histamine actions can be mediated through binding to four types of receptors. Two postsynaptic (H1R, H2R), one presynaptic receptor (H3R), and another mainly present in the immune system (H4R) (Chand and Eyre, 1975; Garbag and Schwartz, 1985; Brown *et al.*, 2001; Parsons and Ganellin, 2006). Although all from the family of rhodopsin-like class A receptors coupled to guanine nucleotide-binding proteins (G protein-coupled receptors) (Hill, 1992; Brown *et al.*, 2001), these receptors strongly differ in distribution, ligand binding properties, signalling pathways and functions. Histamine receptors 1 to 3 have broad brain expression, while the fourth receptor, discovered more recently (Nguyen *et al.*, 2001), is mainly expressed at the periphery. Thus, this latter one will be further excluded from brief characterization.

Histamine receptors 1 (H1R) and 2 (H2R) are coded by intronless genes (Yamashita *et al.*, 1991; Gantz *et al.*, 1991) and receptor 3 (H3R) is coded by a single gene by alternative splicing (Lovenberg *et al.*, 1999). H1R mediates excitatory actions on the whole brain activity; it modulates wakefulness, sleep-wake cycle, appetite, learning and memory, stress, seizure and emotion (reviewed in Watanabe and Yanai, 2001; Haas and Panula, 2003). Its antagonists are doxepin, ketotifen, mepyramine, astemizole and antazoline. H2R is thought to have similar functions as H1R within the brain. Famotidine, tiotidine, ranitidine and cimetidine all antagonize H2R. Finally, H3R located at histaminergic neurons and other cell somata, dendrites and axons, is known to regulate the release of neurotransmitters (Brown and Haas, 1999; Schlicker *et al.*, 1992) and thus this receptor is related to psychiatric and sleep disorders as well as cognitive impairments (Cowart *et al.*, 2004; Hancock and Fox, 2004; Passani *et al.*, 2004; Hancock and Brune, 2005; Leurs *et al.*, 2005). Clobenpropit, iodophenpropit and thioperamide are antagonists of this receptor.

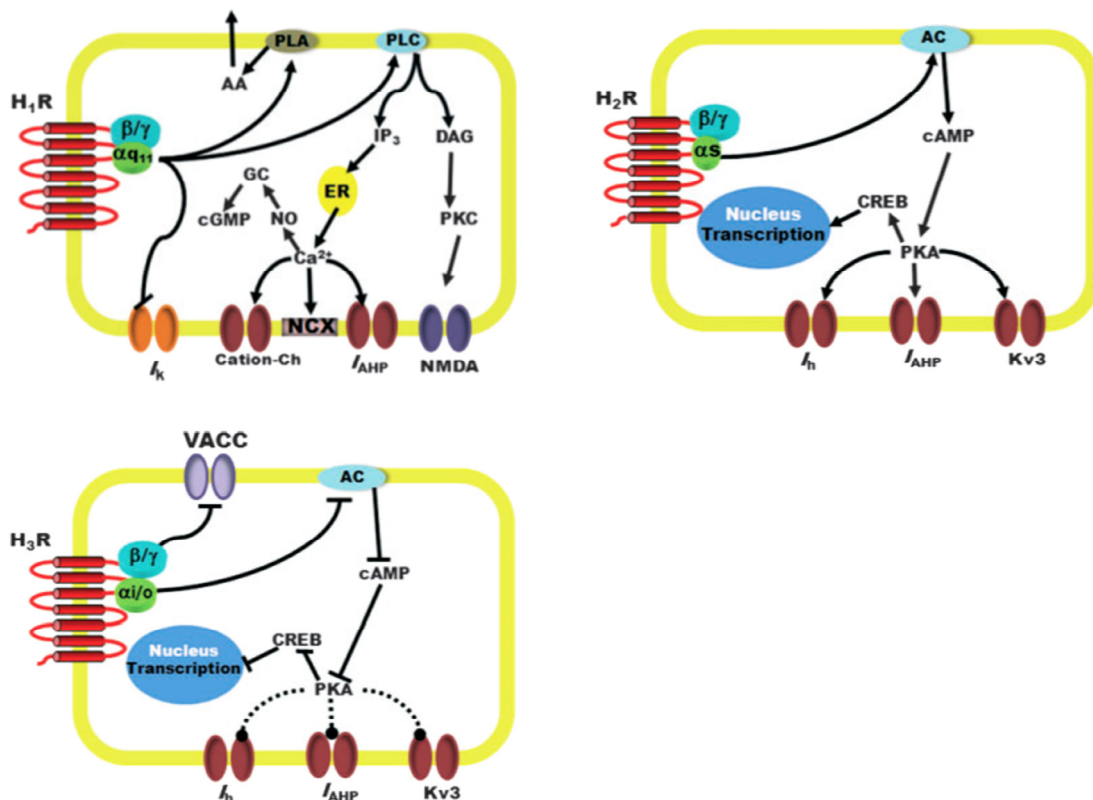


Figure I. 5. Histamine H1R, H2R and H3R main signaling pathways.

A. H1R main signaling pathways. H1Rs interact with $G_{\alpha q/11}$ proteins, which activate several signaling pathways. **B.** H2R main signaling pathways. H2Rs couple to $G_{\alpha s}$ proteins that activate adenylyl cyclases (ACs). ACs produce cyclic adenosine monophosphate (cAMP) that in turn activates protein kinase A (PKA). PKA phosphorylates and activates the hyperpolarization-activated cationic channel (I_h), the small conductance, Ca^{2+} -dependent K^+ current (I_{AHP}), voltage-gated potassium channels (Kv3), and cAMP-response element (CRE)-binding protein (CREB). **C.** H3R main signaling pathways. H3Rs interact with $G_{\alpha i/o}$ proteins whose $\alpha i/o$ subunits inhibit adenylyl cyclases (ACs), consequently reducing the synthesis of cAMP and thus its downstream pathways. The β/γ complexes released upon activation of $G_{\alpha i/o}$ proteins bind to, and inhibit, voltage-activated Ca^{2+} channels. Ch, channel; DAG, diacylglycerol; ER, endoplasmic reticulum; I_{AHP} , small conductance, Ca^{2+} -dependent K^+ current; IP3, inositol-1, 4,5-trisphosphate; NMDA, N-methyl-D-aspartate; NO, nitric oxide; GC, guanylyl cyclase; cGMP, cyclic guanosine monophosphate; NCX, Na^+ - Ca^{2+} exchanger; PKC, protein kinase C; PLA, phospholipase A; PLC, phospholipase C. Adapted from Molina Hernandez *et al.* (2012).

I.6.3. Evidences for a role of histamine in neurogenesis

Histamine has not just been reported to be involved in behavioral functions but also in the modulation of the neurogenic niche. Neuroinflammation (Vizuete *et al.*, 2000), epilepsy (Kukko-Lukjanov *et al.*, 2010) and brain infarction (Motoki *et al.*, 2005) are some dramatic examples of histamine's important role in brain physiology and disease. Under these or other pathological conditions, inflammatory responses may trigger degranulation of mast cells in the *Choroid plexus*, leading to a massive release of histamine in the CSF and consequent BBB permeability. Therefore histamine present in the extracellular space may play a role in neurogenesis.

Despite the fact that the effects of histamine on neurogenesis are poorly understood, it has been shown that H1R are expressed and functionally active in undifferentiated neural progenitors (Agasse *et al.*, 2008b). Indeed, it has been shown that histamine transiently increases intracellular free calcium levels ($[Ca^{2+}]_i$) in SVZ immature/stem cells, embryonic stem and carcinoma cells (Panula *et al.*, 2000; Tran *et al.*, 2004; Agasse *et al.*, 2008b). It was firstly shown by Molina-Hernandez & Velasco (2008) that histamine induces proliferation and neuronal differentiation in NSCs cultures from rat embryonic cerebral cortices. More recently, it was shown that histamine is required during cell proliferation in order to increase neuron differentiation of cortex neuroepithelium progenitors (Rodriguez-Martinez *et al.*, 2012). This effect was mediated by an increase in Ngn1 and Prospero Homeobox Protein 1 (Prox1) expression, both proneurogenic transcription factors. Altogether these reports suggest that histamine can amplify the neurogenic capabilities of NSCs and thus be used for stem cell-based therapies aimed to promote brain repair of the diseased brain.

I.6.4. Role of histamine in the diseased brain

In addition to its function as neurogenesis regulator in several brain conditions, histamine is also found in the host extracellular space of several cancer types, thus suggesting that it may be a crucial factor involved also in tumourigenesis. In fact, levels and activity of HDC are significantly increased in both human, and experimental brain tumours. Moreover, the coexistence of endogenously produced histamine and its specific membrane receptors strongly suggests an autocrine/paracrine loop for histamine in cancer (reviewed in Falus *et al.* 2010).

Many evidences have been showing that histamine may be involved in several cancer cell types' development and progression. Experiments performed in malignant cell lines and experimental tumours *in vivo* suggest that histamine modulates diverse biological responses related to tumour growth, such as proliferation (Medina *et al.*, 2008, 2009; Massari *et al.*, 2011; Francis *et al.*, 2012), survival (Fukuda *et al.*, 2008; Jiang *et al.*, 2010), and modulation of inflammation and angiogenesis (Cianchi *et al.*, 2005). Interestingly, one report showed that

immature myeloid cells, known as myeloid-derived suppressor cells, regulate cancer development *via* their synthesis of histamine (Ruffell and Coussens, 2011). Hdc-deficient mice showed increased susceptibility to chemically induced colon and skin cancers, indicating that histamine restricts tumor growth (Yang *et al.*, 2011). On the other hand, some clinical trials showed increased survival of patients with gastric and colon cancer after treatment with H2R antagonists, such as cimetidine or ranitidine (Matsumoto *et al.*, 2002). These reports exemplify the often-contradictory effects mediated by histamine in cancer cell biology. Besides all these examples, there is yet a long road to clarify the role of histamine in cancer stem cell transformation and in the tumour progression.

Also neurodegenerative diseases have been associated with altered brain histamine and histamine receptors. In living human AD patients, decreased H1R binding has been observed (Higuchi *et al.*, 2000). Moreover, histamine plays an anti-convulsive role in epilepsy through H1R receptor – knocking-out H1R translates into longer electroconvulsion periods and higher susceptibility to Pentylentetrazole (PTZ)-induced kindling (Chen *et al.*, 2003).

1.7. Brain Derived Neurotrophic Factor

Neurotrophins are secreted growth factors that, at both PNS and CNS, regulate survival (Hempstead, 2006), development and function of neurons (Reichardt, 2006; Allen and Dawbarn, 2006). The five neurotrophins - BDNF, NGF and neurotrophin 3, 4 and 6 (NT-3, NT-4 and NT-6) are structurally related and play important roles in the development of the nervous system (reviewed in Bibel and Barde, 2000; Binder and Scharfman, 2004; Chao *et al.*, 2006). They all mediate their actions through binding to two classes of cell surface receptors, p75NTR and trk family of tyrosine kinases (Chao and Hempstead, 1995).

BDNF, originally found in the brain (Barde *et al.*, 1982), is the most widely expressed neurotrophin in this tissue. Despite its name, it is also found in peripheral tissues such as retina (Loeliger *et al.*, 2008), motor neurons (Kishino *et al.*, 1997), kidneys and spleen (Yang *et al.*, 2005). BDNF functional role is well described at the CNS, especially in areas fundamental to learning, memory and higher thinking such as hippocampus, cortex and basal forebrain (Yamada and Nabeshima, 2003). It is fundamental for normal neural development since BDNF knockout (KO) mice suffer from developmental defects in the brain and sensory system, dying soon after birth (Jones *et al.*, 1994). This neurotrophin has been described with relevant involvement in neuronal survival, neuronal differentiation and migration, axonal and dendritic growth and guidance, synaptogenesis, synaptic activity and plasticity, neurotransmission and neuronal apoptosis. A recent metadata study identified several conserved regulatory pathways (Aid-Pavlidis *et al.*, 2009) for BDNF in cellular mechanisms such as synaptic plasticity and transmission, regeneration, skeletal muscle development and angiogenesis among others.

Also several diseases have been associated with altered BDNF levels, such as AD, PD, HD, schizophrenia, depression and bipolar disease (Bibel and Barde, 2000; Weickert *et al.*, 2003; Angelucci *et al.*, 2005; Teixeira *et al.*, 2010; Diniz and Teixeira, 2011; Satomura *et al.*, 2011).

I.7.1. BDNF gene structure

The genomic structure of the BDNF gene is atypical in that it contains multiple promoters that are used to produce messenger RNA (mRNA) containing different noncoding exons, spliced upstream of a common coding exon. These distinct sets of BDNF promoters are used to achieve a precise temporal and spatial control of BDNF production. Since the early 90's that BDNF gene structure has remarkably evolved. Nowadays, its structure and organization are clear and the nomenclature is set.

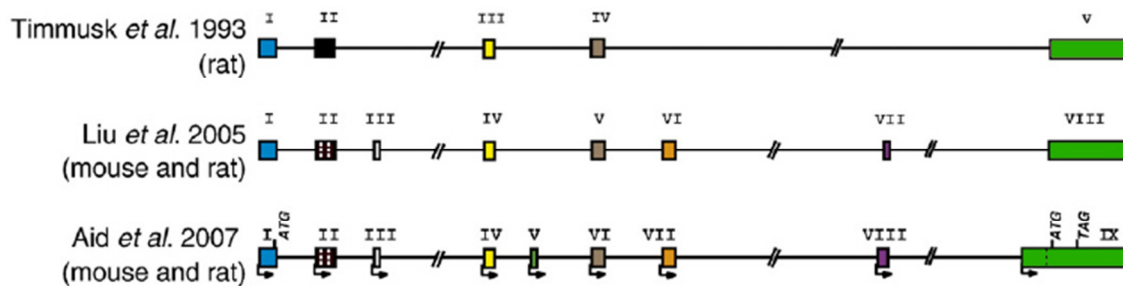


Figure I. 6. Historical perspective from the rodent BDNF gene structure.

First proposed structure by Timmusk *et al.* (1993) of the rat BDNF gene was later changed by Liu *et al.* (2005) – second scheme. The actually accepted mouse and rat BDNF gene structure was proposed by Aid *et al.* (2007). Exons are represented as coloured boxes that identify it, regardless of the evolution of its name. The actual exon IX, in the past called exon V and VIII is the only coding exon, here represented in green. Adapted from Pruunsild *et al.*, 2007.

The first eight (I-VIII) from the nine exons contain 5' untranslated regions (UTRs), linked to individual promoters. Just exon IX contains the entire coding sequence, as well as the 3' UTR (Timmusk *et al.*, 1993; Aid *et al.*, 2007). All exon-intron junctions have conventional splice-donor and -acceptor sites. Translation can be initiated at all the eight untranslated exons, and the resulting different splice variants will generate various mRNAs that still code for the same pre-protein aminoacid sequence (Liu *et al.*, 2006; Aid *et al.*, 2007). If we consider DNA methylation (Chen *et al.*, 2003; Martinowich *et al.*, 2003) and post-transcriptional modifications (Heymach *et al.*, 1996; Mowla *et al.*, 2001), the diversity of mRNAs coding for BDNF increases drastically. Moreover, this variety of transcripts is interestingly differentially expressed during development within tissues, and responds to different stimulations. This differential expression and regulation allows a highly specific and fine-tuned control of BDNF synthesis. For instance neuronal activity regulates BDNF gene expression by calcium mediated pathways (Shieh and Gosh, 1999; West *et al.*, 2001; Mellstrom *et al.*, 2004). Although this diversity of regulatory mechanisms is acknowledged, conflicting data point to specific BDNF

sequences directing trafficking. Some authors state that trafficking is directed by BDNF III-UTR (An *et al.*, 2008); while others (Baj *et al.*, 2011) defend that it is both the coding and its 3' UTR regions that regulate dendritic trafficking.

BDNF mRNA has been detected throughout development in a variety of tissues and brain regions (Ernfors *et al.*, 1990a and b; Hofer *et al.*, 1990; Hohn *et al.*, 1990; Conner *et al.*, 1997). Although it is detectable early in embryonic development, it is postnatally that its expression reaches the highest levels, with the exception of hippocampus (Timmusk *et al.*, 1994; Kawamoto *et al.*, 1996; Conner *et al.*, 1997; Yan *et al.*, 1997; Katoh-Semba *et al.*, 1998). However, not in all BDNF positive structures there is BDNF synthesis. For instance the arcuate nucleus and striatum contain BDNF positive fibers but do not synthesize it (Altar *et al.*, 1997; Conner *et al.*, 1997). This is an indication that BDNF can be anterogradely or retrogradely (Hibbert *et al.*, 2006) transported from distal brain regions.

I.7.2. BDNF synthesis and secretion

BDNF is translated from mRNA as a precursor protein form. This pre-proneurotrophin has a signaling peptide that directs it to the endoplasmic reticulum. Here, this signal is cleaved, and the 32-kDa proBDNF is formed (Halban and Irminger, 1994).

ProBDNF then goes to the Golgi apparatus to be sorted into two different secretory pathways: through small vesicles that easily fuse with plasma membrane to release it constitutively, or by larger vesicles whose fusion is Ca^{2+} -dependent (Lessmann *et al.*, 2003). Although these secretory pathways are common, mature BDNF (matBDNF) can be intracellularly formed and then secreted. Furins at the Golgi, or pro-hormone convertases at secretory vesicles, proteolytically cleave proBDNF into the mature 14-kDa form which is then released. These two alternatives open window for a great debate: proBDNF vs matBDNF and their biological functions.

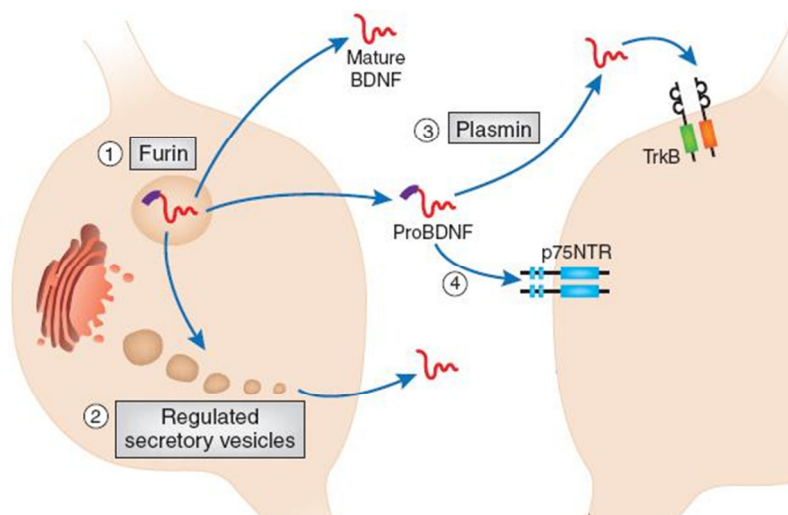


Figure I. 7. BDNF production and processing.

ProBDNF may be processed to mature BDNF by several cellular mechanisms. ProBDNF can be cleaved within the endoplasmic reticulum by furin (1) and in regulated secretory vesicles by proconvertase enzymes (2). If proBDNF reaches the extracellular milieu, it can be processed by plasmin, and the resulting matBDNF activate cell surface TrkB receptors (3). Alternatively, extracellular proBDNF can bind p75NTR (4). Adapted from Barker, 2009.

For some time, proBDNF was believed to be the only BDNF secreted form. Matsumoto *et al.* (2008) argued that it was a transient synthetic intermediate without biological role, as an extracellular secreted ligand. However, a year later, Yang *et al.* (2009) provided data on secreted and functional proBDNF regulating neuronal function. Similarly, others have postulated that proBDNF can bind to receptors, and be either internalized (Bergami *et al.*, 2008) or exert physiological functions (Lee *et al.*, 2001). For example, studies have shown that proBDNF induces neuronal apoptosis (Lee *et al.*, 2001) and facilitates long-term depression (LTD) (Pang *et al.*, 2004; Woo *et al.*, 2005) by activation of p75NTR receptor. This whole discussion is difficult to unveil since the precise time course of all possible BDNF processing is hard to separate. Additionally, in different tissues, different mRNA splice variants can be present, they will be differently targeted and thus its processing can be different. As one can see, these controversies on BDNF processing, secretion and biological functions are still on the table. They are difficult to clarify since different approaches and biological models are used yielding diverse results and interpretations.

This highly debated proneurotrophin topic has been also studied for other neurotrophins, which gained new functions when their pro-forms were under investigation. For instance NT-3, which is extracellularly secreted in a significant part as a precursor form (Farhadi *et al.*, 2000), as well as proNGF (Nykjaer *et al.*, 2004), both have pro-apoptotic roles.

I.7.3. BDNF receptors

There are two distinct classes of transmembranar glycosilated receptors that can bind to neurotrophins: p75NTR – which binds to all neurotrophins, and subtypes of tropomyosin-related kinase (Trk) – which are specific for each neurotrophin.

p75NTR, the BDNF low affinity receptor, recognizes and binds to BDNF and to other neurotrophins with the same low affinity. It has death domain, a cytosolic domain typical of TNF receptors. It signals through activation of nuclear factor- κ B (NF κ B) and JNK regulating RhoA activity. This receptor modulates cell death and survival, neurite outgrowth and arrest, cell migration and myelination. p75NTR may also bind to BDNF Trk receptors, increasing thus its affinity for the neurotrophin.

Trk receptors for BDNF (TrkB) are three isoforms of high affinity that include the full-length TrkB (FL-TrkB), and two truncated isoforms (TrkB-T1 and TrkB-T2) from alternative splicing, lacking the intracellular tyrosine kinase domain (Baxter *et al.*, 1997). Full-length receptors dimerize when binding BDNF and undergo a trans-autophosphorylation reaction. The signaling cascade is mediated by Ras/ERK, phosphatidylinositol 3-kinase (PI3K)/Akt and phospholipase C (PLC)- γ /protein kinase C (PKC) pathways. These cascades usually relate to

neuronal BDNF effects on differentiation and survival. Accordingly, full-length receptors occur in neuronal cells, while truncated receptors are found in glial cells (Reichardt, 2003). Although the function of truncated forms is not yet clear, it has been pointed to be the internalization of extracellular BDNF (Biffo *et al.*, 1995), thus a scavenging function. Moreover, in agreement with its glial location (Reichardt, 2003), TrkB-T1 mediates neurotrophin-evoked calcium signaling in astrocytes (Rose *et al.*, 2003). Regarding inhibition, both truncated TrkB when forming dimers with FL-TrkB become inhibited (Eide *et al.*, 1996).

In addition to the possibilities opened by the two receptors binding to BDNF, the fact that there are two active forms of BDNF (pro and matBDNF) enhances the variations in biological effects. For instance, proBDNF, with its higher affinity for the p75NTR than the mature fully processed matBDNF form (reviewed in Reichardt, 2006), has neuronal apoptotic effects upon p75NTR activation (Lee *et al.*, 2001; Teng *et al.*, 2005). On the other hand, matBDNF has cell survival, neurite outgrowth, synaptic transmission, plasticity and cell migration effects (Dechant and Barde, 2002).

1.7.4. BDNF role in neurogenesis and migration

BDNF role in neurogenesis has long been debated. In 1995, first studies pointed out BDNF as a neuronal survival modulator, in rat SVZ cell cultures (Kirschenbaum and Goldman, 1995). *In vivo* evidences, in agreement with previous reports, showed that mice lacking BDNF had high SVZ cell death (Linnarsson *et al.*, 2000). However, others showed that BDNF might have a deleterious effect at SVZ, with massive increase in BrdU-positive cells at the OB (Zigova *et al.*, 1998; Benraiss *et al.*, 2001); or even in adjacent non-neurogenic regions such as striatum, septum, thalamus and hypothalamus (Benraiss *et al.*, 2001; Pencea *et al.*, 2001). Moreover, there are contradictory reports on BDNF pro-survival or proliferative effect (Ahmed *et al.*, 1995; Cheng *et al.*, 2003). They defend that BDNF has a proneurogenic effect at the differentiation stage. These and other conflicting data, showing decreased SVZ survival upon BDNF intracerebroventricular (icv) injection (Galvão *et al.*, 2008), maintained elusive the effect of BDNF on adult neurogenesis. Still, in non-neurogenic niches, BDNF has been claimed to induce neurogenesis. In the striatum parenchyma, septum, thalamus and hypothalamus, there is an increase in BrdU-positive cells upon BDNF icv administration for 12 days (Pencea *et al.*, 2001). This triggered neurogenesis, correlated with TrkB expression.

However, more consensus has emerged concerning the effect of BDNF in neuroblast migration and also concerning its endothelial cell origin and contribution to the global neurotrophin pool.

BDNF from endothelial cell origin was shown to promote neuronal outgrowth and survival. It was the endothelial cell conditional medium rich in BDNF that regulated the

neurogenic response, inducing SVZ cells' migration from explants (Leventhal *et al.*, 1999). After this breakthrough data, new studies described the roles of endothelial BDNF in songbird HVC (Louissaint *et al.*, 2002), in the rodent RMS (Snapyan *et al.*, 2009) and in ischemic striatum (Grade *et al.*, 2013).

Regarding cerebellum GCP migration, more questions remain to be answered. Although it was in the 90s that Jones *et al.* (1994) firstly identified its role on GCP migration, and that Schwartz *et al.* (1997) postulated that the persistence of EGL was due to an impaired GCP migration to the IGL, still how exactly BDNF exerts its role on GCP is unknown. Later studies confirmed that BDNF has a motility role on GCP migration (Borghesani *et al.*, 2002). It was Zhou *et al.* (2007) who demonstrated the chemotactic BDNF effect requiring TrkB. This was in agreement with previous reports on TrkB role in neuronal migration (Medina *et al.*, 2004). Altogether, these data were consistent with the idea of a gradient of BDNF promoting motogenesis and migration. However, there is still much to disclose than the BDNF gradient as a key player on GCP migration: how is the gradient settled? What is the molecular mechanism that governs BDNF autocrine effect on GCP?

Interestingly, and as previously mentioned, in other brain areas BDNF from endothelial cell origin is fundamental to the proper migratory behavior of neuroblasts. Thus, additional questions to the cerebellum GCP migration in relation to BDNF can be posed. Which is the role of endothelial BDNF to the migratory behavior of GCP? How are the different BDNF cellular contributions regulated through development? Which is the molecular mechanism underlying each BDNF pool mode of action? To answer these and other questions, significant research investment needs to be employed in this robust and exciting scientific area.

1.8. Objectives

Cell-based therapy strategies have evolved in two directions, either grafting NSCs into the host diseased tissue, delivering new possibilities to repopulate the damaged area, or triggering endogenous resources to actively self-repair and substitute the lost cells at the injured places. Still, nowadays, a lot has to be proved concerning clinical procedures for possible transplantation scenarios. Thus, investments in tools to favour endogenous neurogenesis have been multiplied. Histamine, is an example of a molecule long associated with inflammation that has been recently associated with neurogenesis at the SVZ. In this context, part of this doctoral thesis was focused on underlying the cellular and molecular mechanisms that put histamine on the spotlight of proneurogenic factors. In chapter III we concentrate efforts to show that histamine does not affect cell death or proliferation *in vitro*, but rather promotes cell commitment and neuronal differentiation. Histamine role at adult SVZ, both by single pulse

administration and by means of *in vitro* and *in vivo* drug delivery systems, was also the focus of part of this chapter.

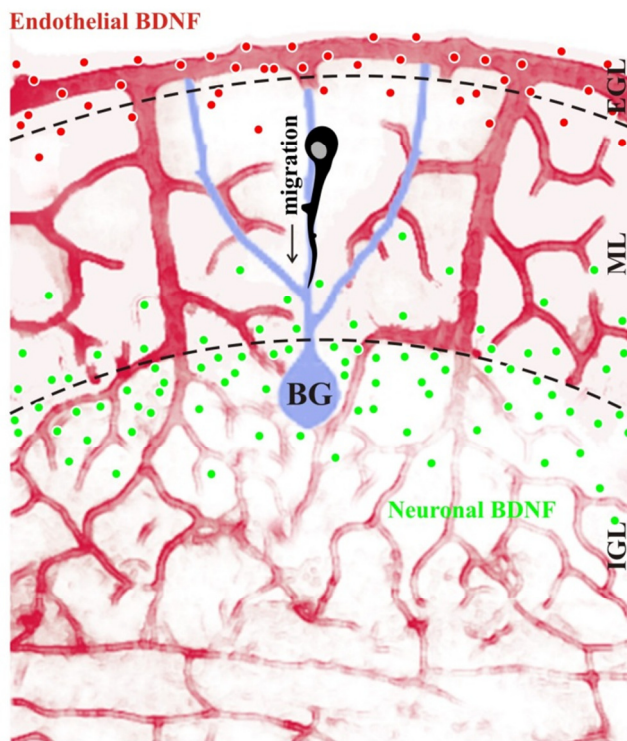


Figure I. 8. Mechanisms of granule cell precursor migration at the cerebellum involving BDNF.

BDNF is one of the modulators of the GCP migratory behavior (arrow indicates the direction of migration towards the IGL). Cerebellum vasculature synthesizes and secretes endothelial BDNF (red dots), which promotes the entrance of GCP in migratory phase. Bergmann Glia (BG) cell processes are used as physical scaffolds for migration. At the IGL, mature granule cells synthesize and secrete neuronal BDNF (green dots). Our hypothesis is that this neuronal neurotrophin fosters migration maintenance. EGL, external granule cell layer; ML, molecular layer; IGL, internal granule cell layer.

On the other hand, regarding the close association of vasculature with neurogenesis, especially migration, in chapter IV we aimed at exploring the role of endothelial derived BDNF to cerebellum postnatal development. Given the pivotal role of vasculature derived BDNF in the RMS neuroblast migration, the high BDNF content of the cerebellum, and its believed gradient from IGL to EGL, we hypothesise that it is the endothelial derived BDNF, the first to be expressed at the cerebellum, the responsible for the precursor cell migration initiation. Thus, in this part of the thesis we focused on unveiling the role of endothelial BDNF to the postnatal development of cerebellum and GCP dynamics.

In summary, the present thesis, with its two pillars on precursor cell differentiation and migration, provides new insights into the fundamental and future clinically scalable knowledge of neuroscience.

CHAPTER II

MATERIAL & METHODS

II.1. Animal handling

All experiments were performed in accordance with institutional approved ethical guidelines for the care and use of laboratory animals: European Union 86/609/EEC, 2010/63/EU and Laval Institute Directives in compliance with Canadian Council on Animal Care.

Animals were housed *ad libitum*, with free access to food (standard diet 4RF21, Mucedola) and water (acidified water pH 3-4), on a 12h light/dark cycle. Cages, 370 or 530 cm² (Tecniplast), with certified autoclaved corn cob as bedding were placed in a climatized room at constant 22 °C and 55% relative humidity.

Table II. 1. Anesthesia and its experimental dosages.

Anesthesia	Dosage (per gram of body weight)
2,2,2-tribromoethanol (Avertin)	0.24 mg
Imalgene 1000 (ketamine)/Rompum 2% (xylazine)	1 mg/0.1 mg
Imalgene 1000 (ketamine)	1 mg

Adult mice were sacrificed by either cervical dislocation (for RNA and protein extraction purposes) or with an overdose of anesthesia (ketamine) for perfusion purposes. For surgery procedures, different anaesthetics were used (Table II.1), according to the duration/purpose of the surgery (mentioned in each section). Anesthesia administration was always intraperitoneal (ip) with 26G (adult) or 31G (pups) sterile needles.

II.2. Animal models

C57BL/6 wild-type (WT) and β -Actin::GFP (β -Actin promoter-driven Green Fluorescent Protein, eGFP animals) transgenic mice (both from Charles River) were routinely used, both for cell cultures and *in vivo* surgeries.

Endothelial conditional BDNF knockout animals were obtained from two mother strains: BDNF^{lox} (*Bdnf* *tm2Jae/J* Stock 004339 from Charles River) and VE-Cadherin::Cre animals (VeCre) (kindly provided by Prof. Aly Karsan from the Pathology and Laboratory Medicine Unit at the University of British Columbia, Canada).

BDNF^{lox} animals harbour *loxP* sites on either side of exon IX of the targeted BDNF gene. This selection cassette was inserted with one *loxP* site downstream of exon IX of the targeted gene, and the other upstream of the same exon. When bred with a strain that expresses Cre recombinase under the control of a specific promoter element in the desired cell types, the resulting mutant mouse strain is useful for studies of cell specific BDNF loss.

Z/EG [Tg(CAG-Bgeo/GFP)21Lbe/J from Jackson Laboratory] reporter animals were

used to check the former transgenic animals cell specificity.

Conditional transgenic mice were generated by mating animals from the mentioned lines with the following design: $\text{VeCre}^{+/-}::\text{BDNF}^{\text{lox/wt}}$ x $\text{VeCre}^{-/-}::\text{BDNF}^{\text{lox/lox}}$ mice. This mating scheme led to 25% of the progeny with expected BDNF KO genotype ($\text{VeCre}^{+/-}::\text{BDNF}^{\text{lox/lox}}$), 50% with BDNF WT genotype ($\text{VeCre}^{-/-}::\text{BDNF}^{\text{wt/fl}}$ or $\text{VeCre}^{-/-}::\text{BDNF}^{\text{fl/fl}}$) and the remaining 25% with BDNF HET genotype ($\text{VeCre}^{+/-}::\text{BDNF}^{\text{wt/lox}}$).

II.3. Cell cultures

II.3.1. SVZ *in vitro* cell cultures

SVZ cell cultures were prepared from 1 to 3-day-old mice as described in Agasse *et al.* (2008b). Brains were removed and placed in Hank's balanced salt solution (HBSS) medium supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin (all from Invitrogen). Brain coronal 450 µm thick slices were prepared with a tissue chopper (McIlwain), from which SVZ fragments were dissected. Digestion was achieved by 0.025% trypsin (Invitrogen) and 0.265 mM EDTA (Invitrogen) incubation at 37°C for 10 min, followed by mechanical dissociation with a P1000 pipette. The single cell suspension was diluted in serum-free medium (SFM) composed of Dulbecco's modified eagle medium [(DMEM)/F12 + GlutaMAXTM-I] supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 1% B27, 10 ng/ml EGF, and 5 ng/ml bFGF-2 (all from Invitrogen). Neurospheres were allowed to develop in an incubator with 5% CO₂ and 95% atmospheric air at 37°C. After 5/6 days, the resulting neurospheres were seeded and allowed to adhere onto 0.1 mg/ml poly-D-lysine (PDL; Sigma-Aldrich) coated glass coverslips in SFM medium devoid of growth factors. Two days after, the medium was renewed with appropriate SFM devoid of growth factors for each experimental treatment.

In the Table II.2, details of the experimental design of each *in vitro* culture protocol are specified according to the purpose pursued.

Table II. 2. Experimental design of the SVZ *in vitro* cell cultures.

Experimental purpose	Culture conditions	Duration
• Single-Cell Calcium Imaging (SCCI) • Immunocytochemistry • Cell death or proliferation	24-well culture plates with glass coverslips; SFM with growth factors (proliferation); SFM devoid of growth factors (differentiation).	2 (proliferation or cell death) or 7 days (differentiation) at 5% CO ₂ and 95% atmospheric air at 37°C
• Cell cycle	Free-floating neurospheres in SFM with growth factors.	48h at 5% CO ₂ and 95% atmospheric air at 37°C
• Cell commitment (Cell pair studies)	Dissociated SVZ cells plated at a density of 6400 cells/cm ² ; Low EGF/FGF-2 medium.	24h at 5% CO ₂ and 95% atmospheric air at 37°C
• Self-renewal (Neurosphere assay)	Single cells from primary neurospheres seeded in low EGF/FGF-2; 2500 cells per well.	6 days at 5% CO ₂ and 95% atmospheric air at 37°C
• Cell phenotype	6-day-old dissociated neurospheres (according to Neurocult dissociation kit instructions - Stem Cell Technology); Single cells, 50000 cells/cm ² allowed to adhere for 1h before experimental treatment; SFM devoid of growth factors.	7 days at 5% CO ₂ and 95% atmospheric air at 37°C
• Axonogenesis	24-well culture plates with glass coverslips; SFM devoid of growth factors.	6h at 5% CO ₂ and 95% atmospheric air at 37°C
• Protein/DNA/RNA extraction	SVZ neurospheres seeded on 6-well cell culture plates; SFM devoid of growth factors.	24 (qChIP), 48 (qChIP and qRT-PCR) or 72 (qRT-PCR) hours at 5% CO ₂ and 95% atmospheric air at 37°C

II.3.2. Histamine *in vitro* treatments

To disclose histamine effect in SVZ cell cultures, different treatments were applied to the previously described cultures. Table II.3 has the details of histamine (Sigma-Aldrich) concentrations used for each experiment in SFM with or without growth factors, depending on the culturing conditions.

Table II. 3. Histamine treatment details.

Experimental purpose	Histamine concentration
• Cell death and proliferation	10, 100 and 500 μ M
• Cell cycle • Cell commitment • Self-renewal • Cell phenotype and SCCI • Axonogenesis • qChIP • qRT-PCR	100 μ M
• Neuronal differentiation	10, 100 and 500 μ M
• Histamine receptor involvement	100 μ M + each receptor antagonist: • 1 μ M mepyramine (Tocris) - H1R; • 5 μ M cimetidine (Tocris) - H2R; • 1 μ M thioperamide (Sigma-Aldrich) - H3R and H4R. At the second day, treatments were renewed.

II.3.3. PLGA histamine-releasing microparticle *in vitro* treatments

Poly lactic-co-glycolic acid (PLGA) microparticles of approximately 2 μ m in diameter were prepared by single emulsion technique. PLGA resomer 752H (Boehringer Mannheim) was dissolved in solvent mixture (1:4 methylene chloride: trifluoroethanol) together with histamine. The solution was added to a stirred chilled polyvinyl alcohol (PVA) solution (5% (w/v)) and the resulting suspension was stirred for 3h. After washing with distilled water, microparticles were freeze-dried. The morphology and diameter of PLGA particles was evaluated by scanning electron microscopy according to Ferreira *et al.* (2007). Blank microparticles, without histamine, were also prepared to test the effect of the microparticle formulation *per se* in SVZ cells.

Release experiments in PBS (0.15M phosphate buffer containing 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4) at 37°C were performed in order to evaluate the release profile of histamine over 30 days, and to assess the loading efficiency of the microparticles. For that, microparticles were dissolved in 1M NaOH overnight and the histamine content assessed by its derivatization with o-phthalaldehyde, which generates a fluorescent compound with emission wavelength at 500 nm (Lerke and Bell, 1976).

A dilution series of microparticles was tested for SVZ cell toxicity as follows: 10 ng/ml, 100 ng/ml and 1 μ g/ml (weight of particles per ml of culture medium). SVZ cells were treated with the mentioned concentrations of blank or histamine-loaded microparticles for 48h. Moreover, cell proliferation was accessed upon 1 μ g/ml microparticles for 48h upon the treatment onset. The experimental controls were non-treated cells. For cell cycle experiments neurospheres were treated for 48h with 1 μ g/ml microparticles (loaded and blank) prior to dissociation for flow cytometry.

To evaluate the influence of PLGA microparticles on SVZ neuronal differentiation, neurospheres were allowed to develop for 7 days with microparticles, either loaded or empty (as control) at the following concentration range: 10 ng/ml, 100 ng/ml and 1 µg/ml (weight of particles per ml of culture medium).

II.3.4. Organotypic hippocampal slice cultures and grafting

Hippocampal slice cultures were prepared from 7 day old C57BL6 WT mice as described in Stoppini *et al.* (1991), and later modified by Kristensen *et al.* (1999) and Noraberg *et al.* (1999). After euthanizing the animals, all procedures were done under sterile conditions. Brains were removed, the two hippocampi isolated, subsequently cut in 350 µm coronal sections (McIlwain Tissue chopper) and slices placed in ice-cold Gey's balanced salt solution (Biological Industries) supplemented with 25 mM D-glucose (Merck), 100 U/ml penicillin and 100 µg/ml streptomycin. Each hippocampus' six individual slices were put onto porous insert membranes (Millipore) that fitted to a 6-well culture tray (Corning Costar). Slices were allowed to settle and grow for 2 weeks in culture medium composed of 50% Opti-minimal essential medium, 25% heat-inactivated horse serum, and 25% HBSS supplemented with 25 mM D-glucose, 50 U/ml penicillin and 50 µg/ml streptomycin (all from Invitrogen), at 5% CO₂ and 95% atmospheric air at 37°C. Medium was changed twice a week.

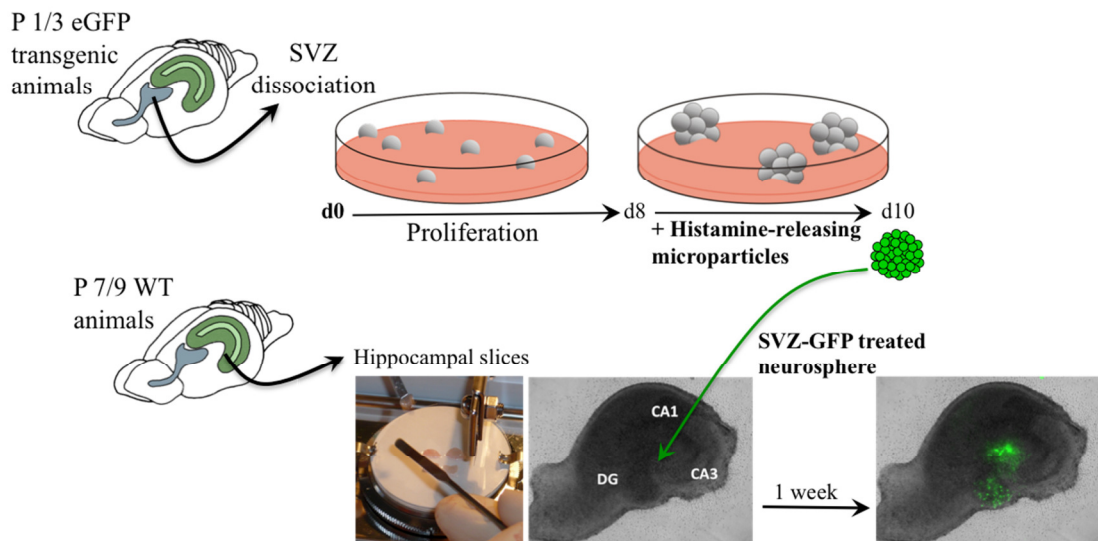


Figure II. 1. Schematic representation of the experimental protocol to evaluate integration and differentiation of grafted SVZ cells on hippocampal slice cultures.

Hippocampal organotypic slices were prepared from P7/9 WT mice. After two weeks of incubation, pretreated SVZ-GFP neurospheres prepared from P1/3 eGFP animals were individually grafted in hippocampal slices. The co-culture was allowed to develop during 1 week.

Before grafting, the medium was replaced by serum-free Neurobasal medium (Invitrogen) containing 1 mM L-glutamine (Sigma Aldrich), 2% B27 (Gibco, Invitrogen), 50 U/ml penicillin and 50 µg/ml streptomycin.

SVZ free-floating neurospheres, obtained from P1 to P3 eGFP mice as described previously were manually delivered to the surface of hippocampal slices with a P10 pipette and a blunt Pasteur pipette within minimal volume. One single SVZ-GFP neurosphere was carefully grafted on the DG a hippocampal slice.

Co-cultures were kept in a 95% air and 5% CO₂ humidified atmosphere, at 37°C, for 1 week. Medium was changed twice a week according to the experimental treatment. After differentiating for 7 days, slices were fixed with 4% paraformaldehyde in PBS (PFA) for 30 min at room temperature prior to immunohistochemistry.

II.4. *In vivo* surgeries

II.4.1. SVZ-GFP adult cell transplantation

SVZ neurospheres prepared as previously described were dissociated and counted. A cell suspension of 50,000 viable cells/µl was prepared in SFM. Weight-matched (approximately 20 g) male WT mice were anesthetized with Avertin prior to surgery.

Unilateral stereotaxic injections of 2 µl cell suspension were performed making use of a 26G Hamilton syringe in the right DG: Anterior-posterior: -1.9 mm, Medial-lateral: -1.2 mm, Dorsal-ventral: -1.8 mm; or striatum: Anterior-posterior: 0.6 mm, Medial-lateral: 1.8 mm, Dorso-ventral: -3.0 mm, both from bregma. After 3 weeks, mice were deeply anesthetized with an overdose of ketamine and perfused transcardially with 4% PFA. Brains were stored overnight at 4°C in PFA and were then transferred to 30% sucrose in PBS at 4°C for cryoprotection. 20 µm thick coronal sections were cut on a microtome (Leica Microsystems) and collected for immunohistochemistry.

II.4.2. Histamine ventricular infusion

Weight matched WT male animals (approximately 20g) were anesthetised with Imalgene/Rompum for surgery according to Table II.1.

Histamine (0.8 mg/Kg) dissolved in artificial cerebrospinal fluid, aCSF (150 mM NaCl, 3 mM KCl, 1.3 mM CaCl₂, 0.8 mM MgCl₂, 0.8 mM Na₂HPO₄, and 0.2 mM NaH₂PO₄) or aCSF alone, as vehicle, were infused for 21 days *via* an osmotic mini pump (Alzet 1004, Charles River, flow rate: 0.10 µl/h). Cannulas were placed on the right hemisphere at the following coordinates (relative to bregma): Anterior-posterior: -0.5 mm, Medial-lateral: 0.7 mm, Dorso-ventral: 3.0 mm. To ensure stable pumping rates, pumps were pre-incubated

before implantation in sterile 0.9% saline at 37°C for 48 h. Pumps were allowed to deliver histamine for 3 weeks after surgery, before sacrifice and tissue processing. Only brains with correct cannula placement were included.

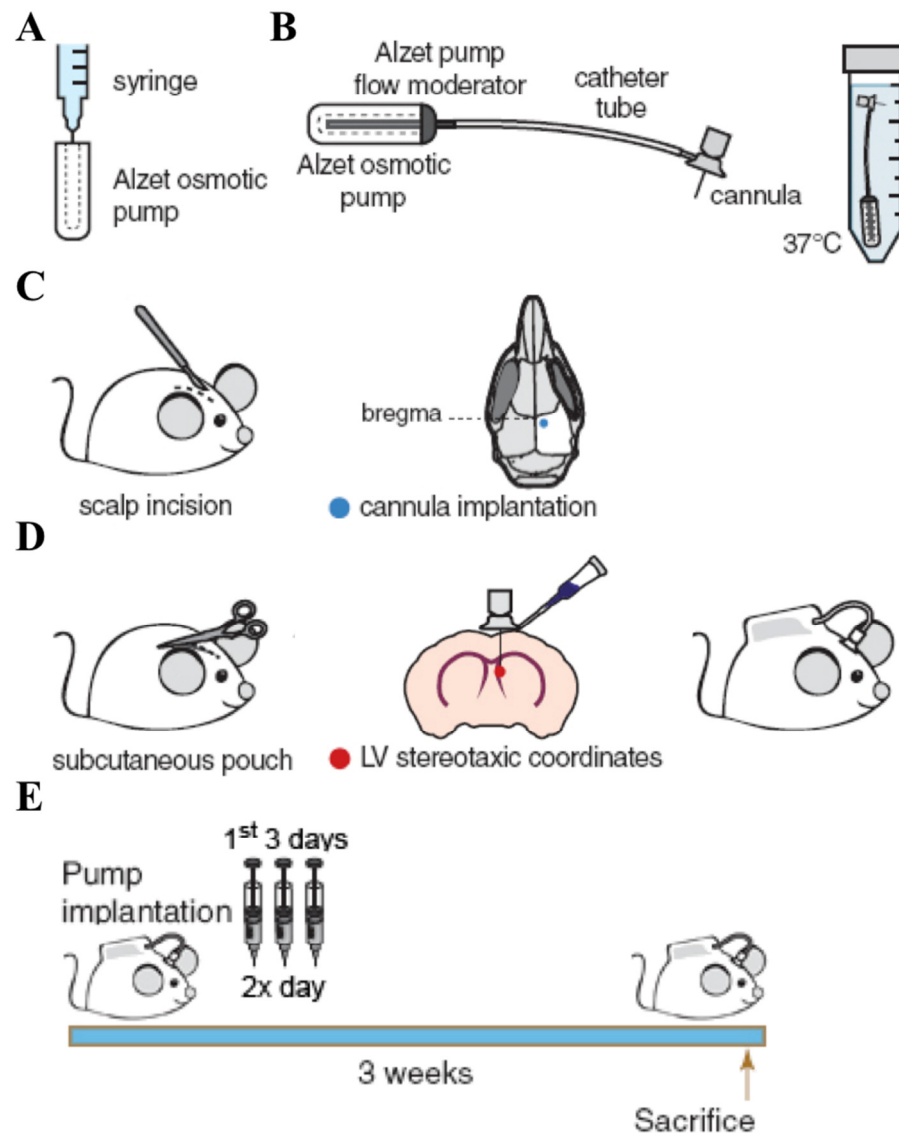


Figure II. 2. Schematic representation of the experimental protocol to prepare and place osmotic mini pumps at the lateral ventricle for long term histamine administration.

A. Pump preparation by filling/loading with histamine in aCSF, or aCSF only for control animals, with a blunt sterile syringe. **B.** Assembling the flow moderator, catheter tube (according to the animal size), and cannula. All pieces are fixed with surgical glue and submerged in sterile saline solution for 48h at 37°C for priming. **C.** Setting the cannula implantation place by making a rostro-caudal incision with a scalpel, which exposes the skull. Elimination of periosteal connective tissue to better locate the bregma suture prior to the setting of the cannula implantation place (blue spot). Drilling a small hole with a needle with precisely the same diameter as cannula diameter is fundamental for perfect placement of the cannula. **D.** Preparation of a subcutaneous midscapular pouch to place the pump before cannula implantation (with a rounded-tip forceps, a pocket is opened in the back of the animal). Pump is placed inside the pocket and carefully descending the cannula through the drilled hole - until the base pedestal is contacting the skull and the tip of the cannula is at the lateral ventricle (red spot) - the whole kit is in place. The pedestal is fixed with surgical glue. After a few seconds gently keeping the pedestal in place, the cannula holder can then be cut and the skin is closed by careful suturing. **E.** Design of the whole 3 weeks experiment. Adapted from Moreno-Estéles *et al.*, 2012.

II.4.3. Retroviral injection in postnatal cerebellum

To label cerebellum GCP, 150 nl of GFP-encoding retroviruses were injected at the primary fissure (PRF) according to the following coordinates from the blood vessel bifurcation anterior to lambda (Shin *et al.*, 2007): Anterior-posterior: -2.9 mm, Medial-lateral: 0.4 mm, Dorso-ventral: 1.0 mm.

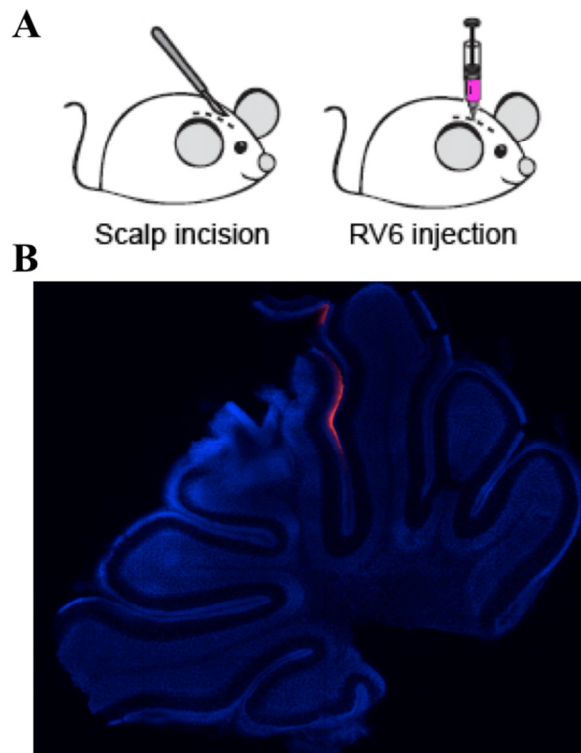


Figure II. 3. Schematic representation of the experimental protocol to label proliferating cells at the EGL of P11 cerebellum, for migration studies.

A. P11 pups were anesthetised and GFP-retrovirus were injected in the proper primary fissure coordinates through a small hole drilled in the skull of the animal, exposed by a small scalp incision. After a small waiting time to avoid reflux, syringe was slowly removed from the brain and skull was sutured. **B.** Representative picture of the target cells by a pink dye test injection. Tissue was stained with nuclear marker DAPI (blue).

For real-time imaging in acute slices, and to evaluate GCP migration parameters, animals were sacrificed 3 to 5 days after injections. During this waiting time, mice pups were together with their mother.

II.4.4. Proliferating cell labeling with BrdU

BrdU (Sigma-Aldrich), a synthetic thymidine analogue that substitutes thymidine in double chained DNA synthesis - occurring in dividing cells – was administered ip at 50 mg per Kg of body weight.

The marker was dissolved in a sterile solution of 0.9% NaCl and 1.75% NaOH (0.4M).

II.5. Experimental procedures

II.5.1. Genotyping knockout animals

II.5.1.1. DNA extraction

Genotyping was performed with DNA extracted from mouse ears. Briefly, ear pieces were placed in lysis buffer (0.5 M Tris HCl pH 8.5, 25 mM EDTA, 1% SDS and 1 M NaCl) containing 0.6 U/ml (20 mg/ml) freshly added Proteinase K (Thermo Scientific) and allowed to digest at 55°C for 3-4h. DNA was precipitated with isopropanol and washed with 100% ethanol before being dissolved in Diethylpyrocarbonate (DEPC)-treated water. DNA was heated for 10 min at 65°C to complete dissolution. If not being used to perform a genotyping PCR, DNA was kept at 4°C.

II.5.1.2. Polymerase Chain Reaction (PCR)

To genotype endothelial BDNF transgenic animals, the presence of VeCre and *LoxP* sites were determined by PCR. Taq Polymerase (Feldan) was activated by a heating step at 94°C for 3 min before the following cycle steps: 39 cycles (VeCre), 35 cycles (BDNF^{lox}) at 94°C for 30 s; 58°C (for VeCre) 1 min followed by a 72°C step for 2 min. Primers (all from Invitrogen) were used at 0.25 pmol/μl final concentration (Table II.4).

Table II. 4. Genotyping primer details.

Gene	Primer 1 (5' - 3')	Primers 2 (5' - 3')
BDNF ^{lox}	TGG ATT GTG TTT CTG GTG AC	GCT TCA TGC AAC CGA AGT ATG
VeCre	GTT TCG AAT TTA CTG ACC	CGC CGC ATA ACC AGT GAA AC

II.5.2. Tissue processing – perfusion

As for histamine neurogenesis studies, mice were deeply anesthetized and perfused transcardially with saline solution (0.9% NaCl), followed by cold 4% PFA. Brains were removed and immersed in 4% PFA overnight at 4°C. Before being cryopreserved, brains were washed in PBS and cryoprotected by an overnight incubation in 30% sucrose solution. Thereafter, brains were sectioned, either coronally or sagittally, on a Leica cryostat, in 40 μm thick slices. Free-floating slices were kept in cryoprotecting medium composed of 30% glycerol pH 7.3, 30% ethylene glycol in PBS at -20 °C before subsequent immunostainings.

Regarding BDNF knockout animals, histological analyses were performed with perfused tissue. Pups were deeply anesthetized and transcardially perfused as described for histamine-treated animals; brains were removed and kept in 4% PFA overnight at 4°C before being sectioned at 40 μm thick sagittal slices with a Leica vibrating microtome. The slices

were kept free-floating sections in PBS.

II.5.3. Immunostainings

II.5.3.1. Immunocytochemistry on SVZ cell cultures

After fixation in 4% PFA or methanol, SVZ cells were simultaneously permeabilized with 0.5% Triton X-100 (Sigma) and nonspecific binding sites blocked with 3% Bovine Serum Albumin (BSA; Merck) in PBS for 30 min at room temperature. Cells were then incubated at 4°C with primary antibodies overnight, according to Table II.5, in 0.1% Triton X-100 and 0.3% BSA, unless otherwise specified.

Table II. 5. Immunocytochemistry antibody concentrations, for SVZ cell culture studies.

Antibody	Supplier	Dilution
Mouse monoclonal anti-NeuN	Chemicon	1:100 ¹
Rabbit polyclonal anti-phospho (Thr 183/Tyr185)-JNK	Cell Signaling Technology	1:100
Mouse monoclonal anti-Tau	Cell Signaling Technology	1:800
Rabbit polyclonal anti-Dlx2	Abcam	1:200
Goat polyclonal anti-Sox2	Santa Cruz Biotechnology	1:500
Rabbit polyclonal anti-DCX	Cell Signaling Technology	1:200
Goat polyclonal anti-DCX	Santa Cruz	
Rabbit monoclonal anti-GFAP	Sigma-Aldrich	1:100
Mouse monoclonal anti-GFAP	Cell Signaling Technology	1:500
Rat monoclonal anti-BrdU	Serotec	1:100
Mouse monoclonal anti-MAP2	Sigma-Aldrich	1:200
Rabbit polyclonal anti-MAP2	Santa Cruz Biotechnology	
Rabbit monoclonal anti-Ki67 (SP6)	Abcam	1:100

After rinsing with PBS, the respective AlexaFluor conjugated secondary antibodies (AlexaFluor either 488 or 594, all from Invitrogen) were incubated at 1:200 concentration in PBS for 1h 30min at room temperature. Following cell nuclei staining with Hoechst 33342 (6 µg/ml in PBS) for 5 min at room temperature, preparations were mounted using Dako fluorescent mounting medium. Cell counting was performed in at least 5 independent fields of duplicates (or triplicates) of each SVZ cell culture (n = one SVZ cell culture), at the border of the neurosphere, where cells form a monolayer.

II.5.3.2. Immunohistochemistry on organotypic cell cultures

Free-floating hippocampal slices were obtained cutting the porous membrane of the insert with a scalpel. Grafted slices were incubated for 1h 30min in 1% Triton X-100 and 3% BSA followed by a 48h incubation at 4 °C with a rabbit polyclonal anti-DCX antibody (1:200; Cell Signaling). Slices were then incubated for 1h 30min at room temperature with a

¹ Incubated in 0.1% Triton X-100 and 6% BSA.

secondary goat anti-rabbit AlexaFluor 594 antibody (1:200; Invitrogen). After rinsing with PBS, cell nuclei were stained with Hoechst 33342 (6 µg/ml in PBS) for 5 min at room temperature. Each slice was mounted on a glass slide with Dako fluorescent mounting medium by leaning the membrane on the slide with the grafted neurosphere facing the coverslip. The percentage of DCX/GFP positive cells *per* total GFP grafted cells was calculated from 5 independent non-overlapping fields around the core of the grafted neurosphere from where SVZ-GFP cells emerged.

II.5.3.3. Immunohistochemistry on brain slices

To evaluate histamine effect on adult brain grafted cells, or histamine long-term effect on SVZ/RMS cells upon pump instalation, 40 µm thick cryostat slices were washed in PBS and permeabilized with 1% Triton-X100, together with 3 % BSA nonspecific binding blockage for 1h. Primary antibody incubation was performed in 0.1 % Triton X-100 and 0.3 % BSA at 4 °C (two overnights). Primary antibody details are listed in Table II.6. Sections were then rinsed in PBS prior to corresponding AlexaFluor conjugated secondary antibody incubation (AlexaFluor either 488 or 594, all from Invitrogen), at 1:1000 in PBS, for 1h 30 min at room temperature. Cell nuclei were stained with Hoechst 33342 (6 µg/ml in PBS) for 5 min at room temperature. Slices were mounted on glass slides with Dako fluorescent mounting medium. Cell counting was performed in five coronal 40 µm thick slices, separated from each other by 5 other adjacent slices.

Table II. 6. Immunohistochemistry antibody concentrations.

Antibody	Supplier	Dilution
Rat monoclonal anti-PECAM	BD Pharmingen	1:100 ²
Rabbit polyclonal anti-BDNF (N20)	Santa Cruz Biotechnology	1:500 ³
Rabbit monoclonal anti-cleaved caspase-3 (Asp175) (5A1E)	Cell Signaling Technology	1:500 ⁴
Rabbit polyclonal anti-Ki67	Abcam	1:1000
Rat monoclonal anti-BrdU	Harlan Sera-Lab	1:500
Rat monoclonal anti-BrdU	Serotec	1:1000
Rabbit polyclonal anti-DCX	BD Pharmingen	1:1000

As for the BDNF KO and WT animals, 40 µm thick sagittal brain sections were obtained in a Leica vybratome. Slices were washed in PBS, incubated in 0.2 % Triton X-100 in PBS for 2h, and then incubated overnight at 4 °C with the primary antibody in 4 % BSA and 0.2 % Triton X-100. Primary antibodies were used in the dilutions present in Table II.6. Slices were then rinsed with PBS followed by AlexaFluor conjugated secondary antibody (AlexaFluor

² Three overnights incubation.

³ Two overnights incubation.

⁴ Incubated in 0.2% Triton X-100 and 5% low fat milk.

either 488 or 594, all from Invitrogen) incubation in PBS for 3h (1:1,000) at room temperature. Cell nuclei were stained with DAPI (1:367; Invitrogen) and slices mounted in Dako fluorescent mounting medium. Cells were counted in whole fissures, from 6 to 10 slices 40 μ m thick, spaced 120 μ m from each other (n = one animal).

II.5.4. Cell death studies

II.5.4.1. TUNEL Labeling

Cell death by apoptosis, in SVZ cells, was evaluated by the terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) method (Gavrieli *et al.*, 1992; Negoescu *et al.*, 1996, 1998). SVZ cells incubated with histamine or with histamine-releasing microparticles were fixed with 4% PFA for 30 min at room temperature, 48h after each treatment setup. Cells were rinsed in PBS, and permeabilized with 0.25 % Triton X-100 (Sigma-Aldrich) for 30 min at room temperature. Thereafter, SVZ cultures were incubated for 20 min in 3% H₂O₂ and reacted for terminal transferase (0.25 U/ μ l) biotinylated dUTP (6 μ M) nick-end labeling of fragmented DNA in TdT buffer (pH 7.5) (all from Roche) for 1h 30 min at 37°C in an humidified chamber. Enzymatic reaction was stopped by 10 min incubation in 300 mM NaCl and 30 mM sodium citrate (both from Sigma-Aldrich) buffer. After an additional rinse in PBS, cultures were incubated for 30 min at room temperature with the avidin-biotin-peroxidase complex (1:100; Vector Laboratories). Peroxidase activity was revealed by the 3,3-diaminobenzidine chromogen (0.025%; Sigma-Aldrich) intensified with 0.08% NiCl₂ in 30 mM Tris-HCl (pH 7.6) buffer containing 0.003% H₂O₂. Before mounting in DEPEX mounting medium (Sigma-Aldrich), cell preparations were dehydrated in ethanol (70% - 2 min; 80% - 2 min; 90% - 2 min; 95% - 2 min; 100% - 2 min) and cleared in xylene (3 min). Cell counting was performed in 5 independent fields from each coverslip, with duplicates or triplicates of each experimental treatment (n = one SVZ cell culture).

II.5.4.2. Caspase-3 Labeling

As for the cleaved caspase-3 (Asp175 5A1E; Cell Signaling) immunostaining, protocol was as described in section 5.3.3 from this Chapter. However, incubation was performed during one overnight at 1:500 dilution in 0.2 % Triton X-100 and 5 % low-fat milk. Cells were counted on whole fissures from 4 to 6 slices, with 40 μ m thick, separated 120 μ m from each other.

II.5.5. Cell proliferation and cell cycle

II.5.5.1. BrdU and Ki67 staining

BrdU (10 μ M; Sigma-Aldrich) was added to control and treated cultures, in the last 4h of each 48h experimental treatment, to label diving cells.

SVZ cells were fixed in 4% PFA for 30 min and rinsed for additional 30 min in PBS at room temperature. BrdU was revealed following 1 % Triton X-100 permeabilization for 30 min at room temperature and 1M HCl incubation for 40 min at 37 °C. SVZ cultures were rinsed in PBS, and nonspecific binding sites were blocked with 3 % BSA (Sigma-Aldrich) and 0.5 % Triton X-100 in PBS for 1h at room temperature. SVZ cultures were then incubated overnight at 4 °C with the conjugated antibody AlexaFluor 594 anti-mouse BrdU antibody (1:100; Invitrogen) in PBS containing 0.1 % Triton X-100 and 0.3 % BSA. After a rinse in PBS, SVZ cell nuclei were stained with Hoechst 33342 (Invitrogen) (6 μ g/ml in PBS) for 5 min at room temperature. Finally, preparations were mounted using Dako fluorescent medium. Cells were counted as for other immunocytochemistries, at the border of neurosphere where migrating cells form a pseudo-monolayer. Five non-overlapping fields were counted for each replica (duplicates or triplicates) of a cell culture.

For *in vivo* proliferation studies, DNA was denatured by an incubation step with 1M HCl at 37°C for 1 h. Slices were rinsed in 0.1 M Borate buffer pH 8.5 before being washed with PBS. Permeabilization was performed as for other immunolabelings with 0.2 % Triton X-100 and 3 % BSA for an hour, before incubation at 4 °C with rat monoclonal anti-BrdU primary antibody (1: 5,000; Serotec) in 0.2 % Triton X-100 and 3 % BSA (2 overnights). After washing with PBS, slices were incubated with secondary antibody and cell nuclei counterstained with 6 μ g/ml Hoechst 33342 (Invitrogen). Slices were mounted using Dako fluorescent medium. Cell counting was done in 6 slices always at the same level, 40 μ m-thick, separated 200 μ m from each other in control and histamine treated animals (one well out of every six, in a total of 24 ordered wells). For SVZ, the most anterior slice counted was the first with a clear triangular lateral ventricle at around 0.8mm anterior-posterior coordinates. For the OB, the most posterior slice was at approximately 3.9 mm anterior-posterior coordinate.

As for BDNF KO animals, protocol for labeling BrdU positive cells was similar. Prior to the 40 min 2M HCl denaturation step at 37 °C, an electron donation step was performed incubating slices in 3% H₂O₂ for 30 min. Permeabilization was performed with 0.2 % Triton X-100. Primary antibody was incubated overnight at 1:500 dilution (rat monoclonal; Harlan Sera-Lab). Biotynilated secondary antibody was incubated for 2h (1:500 anti-rat; Vector Laboratories) after washing. Therefore, avidin-biotin-peroxidase complex (1:100; Vector Laboratories) was added to the slices for 1h. Peroxidase activity was revealed by 3,3'-diaminobenzidine chromogen (0.05%; Vector Laboratories) solution in 0.5 M Tris-HCl pH 7.0 containing 0.003 % H₂O₂. Slices were mounted and dehydrated in ethanol (70% - 2 min; 85% - 2 min; 90% - 2 min; 100% - 2 min) and cleared in xylene (3 min) before being

counterstained with hematoxylin-eosin (Sigma-Aldrich). Mounting was finished with DEPEX mounting medium (Sigma-Aldrich).

Either for *in vitro* or *in vivo* studies, Ki67 labeling was performed together with BrdU labeling to distinguish proliferating cells that were kept or exit cell cycle. Protocol is described in sections 5.3.1 and 5.3.3 from this Chapter.

II.5.5.2. Vybrant DyeCycle Assay

SVZ cell suspension from 48h treated neurospheres was obtained with Neurocult chemical dissociation kit (Stem Cell Technology). After centrifugation for 5 min at 1,000 rpm, cells were fixed with cold sterile 70% ethanol for 20 min at 4 °C. Before labeling, cells were washed with Fluorescent Activated Cell Sorting (FACS) buffer (2% Fetal Bovine Serum (FBS) in PBS). 10^6 cells/ml were labeled with Vybrant DyeCycle Orange (Invitrogen) together with 0.1% Triton X-100 for 45 min at 37 °C.

20,000 washed cells were passed per condition, namely non-labeled control cells, labeled control cells and labeled treated cells, for each cell culture (n = one SVZ cell culture).

II.5.6. Cell commitment studies

After the 24h treatment (or control culture), SVZ cell pairs were fixed in methanol for 15 min at -20 °C before being processed for immunocytochemistry against Sox2 and Dlx2, as described previously. Percentages of Sox2 cell pair populations were obtained from counting about 60 cell pairs in triplicate coverslips obtained from 5 independent cultures.

II.5.7. Self-renewal analysis

For self-renewal assay, after 6 days, primary neurospheres were collected, dissociated as single cells (Neurocult chemical dissociation kit, Stem Cell Technology) and seeded in low EGF/FGF-2 medium as aforementioned. After 6 days, the number of secondary neurospheres was determined. Data was obtained from counting the number of primary and secondary neurospheres in each well, in quadruplicate, per independent cell culture.

II.5.8. Single Cell Calcium Imaging (SCCI)

To analyze the functional differentiation pattern of SVZ cell cultures, variations of $[Ca^{2+}]_i$ in single cells were monitored following 50 mM KCl (Merck) and 100 μ M histamine (Sigma-Aldrich) stimulation at the border of SVZ neurosphere, where migrating cells form a pseudo-monolayer.

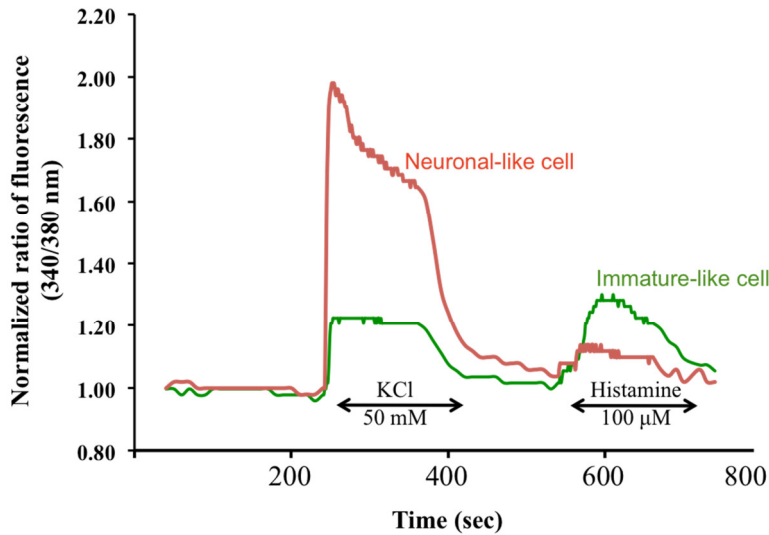


Figure II. 4. Experimental protocol to functionally evaluate neuronal differentiation in SVZ cell cultures by SCCI.

SVZ cells, after being loaded with Fura-2AM, were continuously perfused with Krebs solution. During the 15min protocol, cells were stimulated with 50 mM KCl (5-7min.) and 100 μ M Histamine (10-12min.). Changes in normalized ratio of fluorescence at 340/380 nm were monitored. Red line depicts a representative neuronal-like cell profile and green line an immature-like cell.

SVZ cultures were loaded with 5 μ M Fura-2AM (Invitrogen), 0.1 % fatty acid-free BSA, and 0.02 % pluronic acid F-127 (Invitrogen) in Krebs solution (132 mM NaCl, 4 mM KCl, 1.4 mM $MgCl_2$, 2.5 mM $CaCl_2$, 6 mM glucose, 10 mM HEPES, pH 7.4) for 40 min in an incubator with 5% CO_2 and 95% atmospheric air at 37 $^{\circ}C$. 10 min post-loading, in which cells were kept at room temperature in the same medium without Fura-2AM and pluronic acid, glass coverslips were mounted on an RC-25 chamber in a PH3 platform (Warner Instruments), on the stage of an inverted fluorescence microscope (Axiovert 200; Carl Zeiss). Cells were continuously perfused with Krebs solution in between the two stimuli: high-potassium Krebs solution (50 mM KCl, isosmotic substitution with NaCl – between minute 5 and 7) and 100 μ M histamine (Sigma-Aldrich – between minute 10 and 12). These were added by a fast-pressurized (95% air, 5% CO_2 atmosphere) system (AutoMate Scientific Inc., Berkeley). The variations of $[Ca^{2+}]_i$ were measured by quantifying the ratio of the fluorescence emitted at 510 nm, following alternate excitation (750 millisec.) at 340 and 380 nm, using a Lambda DG4 apparatus (Sutter Instrument, Novato) and a 510 nm longpass filter (Carl Zeiss) before fluorescence acquisition, with a 40X objective and a CoolSNAP digital camera (Roper Scientific Tucson, AZ). Acquired values were processed using MetaFluor Software (Universal Imaging Corp., Marlow).

Analysis was performed in the border of SVZ neurospheres, where migrating cells form a pseudo-monolayer. Each analyzed field contained approximately 100 cells in a total of at least 3 independent cell cultures, where each condition was triplicated.

II.5.9. Vasculature studies

II.5.9.1. Dextran Texas-Red injection

To label functional blood vessels, 100 μ l of high molecular weight dextran-Texas Red (70,000 kDa; Molecular Probes) at room temperature were injected into the heart of deeply anesthetized mice. After waiting 5 min, brains were removed and kept in 4% PFA overnight before being sectioned.

II.5.9.2. Evans Blue injection

In order to study blood vessels leakage, 100 μ l Evans Blue dye (Sigma-Aldrich) were injected into the heart of deeply anesthetized mice. After waiting for 5 min, brains were removed and kept in 4% PFA for 30 min. Right after this short fixation, brains were sectioned in a Leica vibratome and 150 μ m thick sagittal slices were immediately mounted for microscopy.

II.5.10. Time-lapse cell migration

Two to five days after cerebellum retroviral injection, mice were anesthetized according to Table II.1. Brains were quickly dissected in ice-cold sucrose-based aCSF containing: 250 mM sucrose, 3 mM KCl, 1.3 mM MgCl₂, 2 mM CaCl₂, 26 mM NaHCO₃, 1.25 mM NaH₂PO₄, and 20 mM glucose (pH 7.4), bubbled with 95% O₂-5% CO₂. 250 μ m thick sagittal sections prepared in a Leica vibratome were maintained in the dark at 32 °C in 95% O₂-5% CO₂ bubbled aCSF containing: 125 mM NaCl, 26 mM NaHCO₃, 3 mM KCl, 2 mM CaCl₂, 1.3 mM MgCl₂, 1.25 mM NaH₂PO₄, and 10 mM glucose (pH 7.4). Individual slices were carefully placed in an imaging chamber under continuous perfusion with aCSF (1 ml/min). An automatic heating system (TC-344B, Harvard Apparatus) maintained the aCSF at 31-33°C. The chamber was mounted on a wide-field Olympus BX61WI upright microscope equipped with a CCD camera (CoolSnap HQ2) and a Lambda DG4 xenon light source (Sutter Instruments).

For 2h, at every 60s, time-lapse video images were acquired using multiple z-stacks (6-16 sections with 3-7 μ m intervals). Videos were manually processed as Tiff series using ImageJ software (Abramoff *et al.*, 2004) together with Image Stabilizer (Li, 2008) and MTrackJ (Meijering *et al.*, 2012) plug-ins. A threshold discriminating stationary and migratory phases was set at two times the standard deviation between two subsequent acquisitions. Careful visual inspection of cell migration was performed to confirm small displacement values. Cells that remained stationary for the duration of the recordings were not considered for analysis.

II.5.11. Fluorescent-activated cell sorting (FACS)

Cerebella from P11 animals were dissected and tissue was dissociated to single cells by

0.05 % Trypsin-EDTA (Gibco) digestion at 37 °C during two 15 min incubation steps separated by a mechanical dissociation step with a P1000 pippete tip. Cells were then centrifuged and the pellet resuspended in 0.5 % HBSS enriched with 0.9M succrose. Dissociated cells were passed through a 70 µm cell strainer to achieve complete dissociation. Cells were then transferred into sterile PBS to a final cell density of 10⁶ cells/ml.

Before sorting, cells were washed with PBS and passed on a FACSCanto (BD Biosciences) to check labeling and cell integrity. Single-cell suspension was maintained in the dark at 4°C while waiting to be sorted. Cells were sorted on a FACSaria (BD Biosciences) and collected into RNAlater (Qiagen).

II.5.12. Isolation of total RNA

II.5.12.1. RNA from SVZ cell cultures for quantitative RT-PCR

Total RNA was isolated from SVZ cells according to Illustra RNAspin Mini RNA Isolation Kit manufacturer's instructions (GE Healthcare Life Sciences). The full content of a 6-well culture plate was pooled for each experimental condition. Precipitated RNA was resuspended in 40 µl of DEPC-treated water, and samples were stored at -80 °C until further use.

II.5.12.2. RNA from SVZ cell cultures for the evaluation of Histamine Receptors

Isolation of total RNA from SVZ neurospheres (grown in proliferative conditions for 6 days) was performed with Tri Reagent (Sigma-Aldrich) according to the manufacturer's instructions. Cells were gently homogenized in guanidium thiocyanate and phenol. Chloroform was added allowing a clear isolation of RNA, in the resultant aqueous phase. The RNA was precipitated with isopropanol and the pellet washed with 75% cold ethanol. The RNA pellet was dissolved in DEPC-treated water and quantified by optical density (OD) measurements at 260 nm. The RNA quality was evaluated by measuring the OD ratio at 260 and 280 nm (RNA/DNA calculator GeneQuant II) and by gel electrophoresis. RNA extracted from splenocytes was used as a positive control for the detection of histamine receptor 1, 2 and 3 mRNA expressions. To confirm the specificity of the primers, negative controls were also performed replacing RNA samples by water.

II.5.12.3. Total cerebellum extract

RNA from total cerebellum was isolated with TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. Tissue was homogenized in TRIzol and insoluble material

was removed by 12,000 g centrifugation step at 4 °C for 10 min. Chloroform was added to allow a clear isolation of RNA to the resultant aqueous phase. Prior to RNA precipitation with isopropanol, RNase-free glycogen (Invitrogen) was added. RNA pellet was washed with cold 75% ethanol, air-dried and dissolved in DEPC-treated water. Quantification by OD was performed as mentioned in the previous section.

RNA was DNase treated with RQ1 DNase (Promega) according to the manufacturer instructions. Briefly RNA was incubated with 4U of DNase at 37 °C for 30 min. Reaction was stopped by adding RQ1 DNase Stop Solution and inactivating the enzyme at 65 °C for 10 min.

II.5.13. Gene expression studies – quantitative real time RT-PCR

II.5.13.1. cDNA synthesis for histamine receptor study

cDNA was obtained from the transcription of 2 µg RNA using avian microblastosis virus (AMV) reverse transcriptase and Oligo-p(dT) primers (Roche Molecular Biochemicals) according to the manufacturer instructions.

II.5.13.2. cDNA synthesis for SVZ proneurogenic genes and cerebellum BDNF studies

For SVZ proneurogenic studies, cDNA was synthesized starting with 1 µg of total RNA and 10U AMV Reverse Transcriptase according to manufacture instructions (Roche Molecular Biochemicals). The synthesis reaction was performed at 55 °C for 30 min and stopped by a 5 min step at 85 °C.

In the case of cerebellum BDNF studies, cDNA was synthesized directly from FACSorted cells using Roche Molecular Biochemical reverse transcription kit mentioned in the previous section.

II.5.13.3. Quantitative PCR for histamine receptor analysis

With 5 µl template cDNA, a 50 µl reaction was set for cDNA synthesis with Taq DNA polymerase and other reagents according to manufacturer's instructions (Bioron).

Cycle details were as follows: 35 cycles at 95 °C - 30s; at 56 °C - 30s (H1R and H2R) or 58 °C - 30s (H3R) and at 72 °C - 30s. Primers were all used at 10 µM final concentration (all from MWG-Biotech).

Table II. 7. Reverse Transcription-PCR primer details, for SVZ studies.

Gene	Primer	Sequence (5' to 3')
H1R	Forward	GGG CTC AAA GGC CAA TGA C
	Reverse	TCC GCC GGC AAG TAC TCA
H2R	Forward	CTG GCT GTC AGC TTG AAT CG
	Reverse	GCT GCC AGG GAC ACA ATG A
H3R	Forward	CTT CTC TCT CCC AAG ACG ATC TG
	Reverse	GGC TCC GGG ATT AAG GAA GA
β -actin	Forward	GAC TAC CTC ATG AAG ATC CT
	Reverse	ATC TTG ATC ATG GTG CTG

PCR products of each sample were subjected to electrophoresis in a 1.5 % agarose gel stained with ethidium bromide.

II.5.13.4. Quantitative PCR for SVZ proneurogenic genes and cerebellum BDNF studies

For proneurogenic gene expression analysis, as well as for BDNF cerebellum studies, 2 μ l of cDNA sample were added to SYBR Green Master Mix (Bio-Rad), together with primers (all from MWG Operon) according to final concentrations listed in Table II.8.

Table II. 8. qRT-PCR primer details, for cerebellum studies.

Gene	Primer	Concentration	Sequence (5' to 3')
Mash1	Forward	10 μ M	CCC TGG CCA GAA GTG AGA
	Reverse	10 μ M	CTG GGT GTC CCA TTG AAA AG
Dlx2	Forward	10 μ M	AAG CAC TGC AGA AAG TGG GTA
	Reverse	10 μ M	GGA GCC TTA TGT CCT GTT GC
Ngn1	Forward	10 μ M	ATT ACG GGC ACG CTC CAG
	Reverse	10 μ M	CAG CTC CTG TGA GCA CCA AG
BDNF.IX	Forward	0.3 μ M	GGC CCA ACG AAG AAA ACC AT
	Reverse	0.3 μ M	AGC ATC ACC CGG GAA GTG T
GAPDH	Forward	10 μ M	CAA GGC TGT GGG CAA GGT
	Reverse	10 μ M	TCA CCA CCT TCT TGA TGT CAT CA

The thermocycling reaction was initiated with Taq DNA polymease activation by heating mixture at 94 °C during 3 min. The cycles for each gene are detailed in the above Table (Table II.9).

Table II. 9. qRT-PCR cycle details.

Gene	Denaturation Step		Annealing Step		No. cycles
	Duration (sec.)	Temp. (°C)	Duration (sec)	Temp. (°C)	
Mash1	15	94	30	57	40
Dlx2	15	94	30	57	40
Ngn1	15	94	30	64	40
BDNF.IX	10	95	60	57	45
GAPDH ⁵	10	95	60	57	45
GAPDH ⁶	15	94	30	57	40

Fluorescence was measured after the extension step by the iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad). After the thermocycling reaction, the melting curve was performed with slow heating, starting at 55 °C and with a rate of 0.5 °C per 10 sec, up to 95 °C, with continuous measurement of fluorescence to detect any possible nonspecific product. All assays included a non-template control (sample was substituted by sterile water) and a standard curve (in 10-fold steps) of DNA for assessing the efficiency of each set of primers. All reactions were run in duplicates. Cycle threshold values were measured in the exponential phase and were set at the same fluorescence value in each performed run. Data analysis was performed with Bio-Rad iQ5 Software (Bio-Rad). The relative expression of each gene was normalized against GAPDH, using the comparative cycle threshold method as described by Pfaffl's formula (Pfaffl, 2001). The PCR products were subjected to electrophoresis in a 1% agarose gel stained with either ethidium bromide or Green Safe (Nzytech). Photographs were taken in a Versa-Doc Imaging System (Model 3000, Bio-Rad Laboratories).

II.5.14. Quantitative Chromatin Immune Precipitation

After treatment with histamine, as described previously, cells were washed with PBS and chromatin was crosslinked to proteins by incubation with 1% formaldehyde in PBS containing 50 mM MgCl₂ for 15 min at 37 °C. SVZ cells were then incubated with 125 mM glycine for 10 min at room temperature; washed twice with sterile PBS and incubated with lysis buffer [1 % SDS, 10 mM EDTA, 50 mM Tris pH 8.1, containing a cocktail of proteinase inhibitors (Roche)] for 5 min. Chromatin was then fragmented by repeated sonication on ice using Sonics & Materials, Model Vibra Cell (TM) VC 50 (power 20, 10 x 30 sec with 30 sec intervals). After genomic DNA fragment analysis in a 1 % agarose gel, cell suspension was centrifuged at 10,000g, for 5 min, at 4 °C. Supernatant was ten-fold diluted in ice-cold dilution buffer (0.01 % SDS, 1 % Triton X-100, 1.2 mM EDTA, 16.7 mM Tris pH 8.1, 167 mM NaCl and Roche's cocktail of protease inhibitors). Samples were then precleared with 30 µl of 50 % blocked protein G-sepharose beads (Sigma-Aldrich) in PBS, for 30 min at 4 °C,

⁵ Cycle details for BDNF studies.

⁶ Cycle details for Histamine studies.

with rotation. Beads were eliminated by 10,000g centrifugation for 5 min, at 4 °C. 1/10th of the sample was kept as the input and the remaining portion was incubated with 10 µg mouse monoclonal antibody against Histone H3 (trimethyl K4) (H3K4me3; Abcam) at 4 °C overnight; or the unrelated antibody as a negative control (rabbit polyclonal anti-TNFR1, Santa Cruz Biotechnology). Immune complexes were captured by 1h incubation with 40 µl of 50 % blocked protein G sepharose beads, at 4 °C. Then, protein G-sepharose beads were rinsed 7 times for 10 min (TE containing 150 mM NaCl and 0.5 % NP40) and incubated with 50 µg/ml RNaseA/TE, for 30 min, at 37 °C. The input-lysate was prepared in the same way. Proteinase K and SDS were added to 1 mg/ml and 0.5 %, respectively, and samples were incubated overnight at 37 °C, before being incubated at 65 °C for 3h to reverse crosslink. After a centrifugation at 1,000g for 5 min, beads were discarded and the DNA samples were purified by phenol-chloroform extraction and precipitated with 1 µg glycogen, plus sodium acetate and ethanol. DNA pellets were suspended in nuclease-free water and were then subjected to qPCR analysis.

II.5.15. *In situ* hybridization

To detect BDNF mRNA in brain sections from BDNF KO and WT animals, *in situ* hybridization was performed. Antisense RNA probes were generated from plasmids containing mouse BDNF (kindly provided by Dr. E. Castren, University of Helsinki, Finland), which was labeled with digoxigenin (DIG) using DIG RNA labeling kits (Roche Diagnostics) and purified with ProbeQuant G-50 columns (GE Healthcare). Fixed brain 40 µm thick sagittal slices were treated with RIPA buffer (150 mM NaCl, 1 % NP40, 0.5 % sodium deoxycholate, 0.1 % SDS, and 1 mM EDTA in 50 mM Tris-HCl, pH 8.0). Slices were fixed in 4 % PFA followed by PBS washing and incubated in 0.1 M triethanolamine (TEA, pH 8.0), acetylated with 0.25 % acetic anhydride in 0.1 M TEA, and finally washed in PBS. Slices were then pre-incubated in hybridization solution (50 % formamide, 5x saline sodium citrate (SSC), 5x Denhardt's reagent, 500 µg/ml DNA, and 250 µg/ml of tRNA) for 1 h at 60 °C. The riboprobe was added, and slices incubated overnight at 60 °C. Slices were then washed in post-hybridization solution (50 % formamide, 2x SSC, and 0.1 % Tween-20), followed by buffer B1 (100 mM maleic acid, pH 7.5, 150 mM NaCl, and 0.1 % Tween-20), and buffer B2 (10 % FBS in buffer B1). An overnight incubation with anti-DIG antibody (1:2,000 in buffer B2) at 4 °C was then performed. After washing with buffer B1 followed by buffer B3 (50 mM MgCl₂, 100 mM NaCl, and 0.1 % Tween-20 in 100 mM Tris-HCl, pH 9.5), alkaline phosphatase activity was revealed using nitroblue-tetrazolium-chloride/5-bromo-4-chloro-indolylphosphate (Promega). Slices were then washed in buffer B3 and mounted, or were fixed in 4% PFA for 30 min prior to the immunohistochemistry analysis.

II.5.16. Protein isolation and western blot

II.5.16.1. SVZ cell cultures

Protein lysates from SVZ neurospheres were obtained 7 days after treatments. Cells were scrapped and homogenized by sonication in RIPA buffer (150 mM NaCl, 50 mM Tris, 5 mM EGTA, 0.01% Triton X-100, 0.005% DOC, 0.001% SDS pH 7.5 with 10 μ l DTT) containing a cocktail of proteinase inhibitors (Roche). After centrifugation at 14,000 rpm for 10 min at 4 °C, supernatants were removed and frozen at -20 °C. Lysate samples were quantified by the BCA method to be normalized for protein content. Samples were boiled for 5 min in sample buffer (0.4 M Tris-HCl pH 6.8, 30 % glycerol, 10 % SDS, 12 % Phenol Blue) containing 0.5 μ l DTT.

Proteins were separated by SDS-PAGE on 4-12% acrylamide/bisacrylamide gels at constant 100 V (room temperature) using a Mini-PROTEAN Tetra Cell apparatus (BioRad Laboratories). Gels were then transferred at 4°C onto PVDF membranes (Immobilon-P, Millipore) applying constant 300 mA for 1h30min to 2h, with a Mini Trans-Blot Electrophoretic Transfer Cells (BioRad Laboratories). All buffers were prepared in accordance to the manufacturer instructions. Membranes were blocked for 1h in 5% low-fat dry milk in 50 mM Tris Buffered Saline with 0.1% Tween 20 (Sigma-Aldrich) (TBS-T, pH 7.4) at room temperature, followed by incubation in primary antibody diluted in TBS-T containing 0.5 % low-fat dry milk at 4 °C, overnight. After washing in TBS-T with 0.5 % low-fat dry milk, the PVDF membrane was incubated 1h at room temperature with 1:20,000 anti-mouse IgG or 1:5,000 anti-rabbit IgG alkaline phosphatase linked (GE Healthcare, UK). Bands were visualized using ECF substrate (GE Healthcare). Relative protein concentrations were quantified by comparing bands OD, using a VersaDoc digital image analysis system (BioRad Laboratories).

Table II. 10. Western blot antibody details, for SVZ studies.

Antibody	Supplier	Dilution
Mouse monoclonal anti- β -actin	Sigma-Aldrich	1:2,000
Rabbit polyclonal anti-GFAP	Sigma-Aldrich	1:20,000

II.5.16.2. Total cerebellum extract

Total cerebellum extracts were obtained from fresh tissue under sterile procedures. Tissue was dissociated in lysis buffer (50 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 mM Na_3VO_4 , 50 mM NaF, 5 mM $\text{Na}_4\text{O}_7\text{P}_2$, 10 mM sodium β -glycerophosphate, 0.1% 2- β -mercaptoethanol, 1% Triton X-100) enriched with protease inhibitor cocktail III (Calbiochem), with the help of a homogenizer. Samples were sonicated 3 times 10 sec in an ultrasonic water bath before being centrifuged at 13,000 g for 20min at 4 °C. Protein samples

were quantified by the Bradford method before being aliquoted and stored at -20 °C prior to use.

Lysate samples were prepared for gel electrophoresis according to the NuPAGE Novex Bis-Tris Mini Gels (Invitrogen) instructions, as well as all required buffers. Samples were loaded into 4-12 % NuPAGE Novex Bis-Tris Mini Gels for XCell SureLock Mini-Cell system (all from Invitrogen) and run for 1h at constant 200V, at room temperature. Proteins were then transferred into PVDF membranes (GE Healthcare), at constant 17V during one overnight at 4 °C. Membranes were blocked with 7 % low-fat dry milk in washing buffer for 1h at room temperature, before overnight primary antibody incubation at 4 °C in blocking buffer. IgG-HRP conjugated secondary antibody was incubated for 1h at room temperature, after washing the membrane (1:5,000 anti-rabbit and 1:3,000 anti-mouse). Antibody concentrations are listed on the above table (Table II.11). Protein signal was developed with ECL Plus substrate (GE Healthcare) and photographic hyperfilms (GE Healthcare) on a dark film casset. Relative protein concentrations were quantified by comparing OD of protein bands, using a Futura 3000 SV revelation machine and VersaDoc digital image analysis system (Bio-Rad Laboratories).

Table II. 11. Western blot antibody details, for cerebellum studies.

Antibody	Supplier	Dilution
Rabbit polyclonal anti-BDNF (N20)	Santa Cruz Biotechnology	1:1,000
Mouse monoclonal anti-Actin	Sigma-Aldrich	1:3,000

II.6. Statistical analysis

Data are presented as mean $\pm$ standard error of the mean (SEM).

For *in vitro* studies, statistical significance was tested using one-way analysis of variance (ANOVA) followed by Bonferroni post-test for multiple comparisons.

For *ex vivo* and *in vivo* studies, Student's unpaired t test was used for statistical significance (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$).

CHAPTER III
HISTAMINE STIMULATES NEUROGENESIS
IN THE MOUSE SVZ

III.1. Abstract

The SVZ is a niche for neural stem/progenitor cells, and thus, it represents a source of new neuronal cells, potentially useful for brain repair strategies. Therefore, the identification of novel key players that can modulate its neural stem cells differentiation will have a relevant impact in stem cell-based therapies.

In this chapter we describe the unique role of histamine in inducing functional neuronal differentiation in SVZ stem/progenitor cells. This proneurogenic effect is dependent on H1R activation and involves epigenetic modifications, increased expression of *Mash1*, *Dlx2* and *Ngn1* genes, and a commitment of precursor cells without affecting cell proliferation.

For a controlled and prolonged release of histamine, a new drug-delivery system was adopted: biocompatible poly (lactic-co-glycolic acid) (PLGA) microparticles were engineered. They triggered comparably robust neuronal differentiation *in vitro*. In an intermediate step towards *in vivo* studies, preconditioning of SVZ-GFP cells with histamine-loaded microparticles facilitated their neuronal differentiation, once grafted in hippocampal slices. Also, the same preconditioning procedure prompted neuronal differentiation of the SVZ-GFP cells grafted *in vivo* into the hippocampus and striatum of adult mice. Finally, mini osmotic pumps were filled with histamine and implanted in the lateral ventricle of adult mice. The continuous delivery of histamine for 21 days induced a significant increase in neuronal differentiation and an effect on cell proliferation at the SVZ, also promoting an increase in the migration of new neuroblasts towards the OB.

Altogether, this chapter makes evident that histamine is responsible for a neuronal commitment, either *per se* or released from drug delivery systems; thus representing a new tool for brain repair strategies.

III.2. Introduction

NSCs reside in two niches of the adult brain: SVZ lining the lateral ventricles and the SGZ in the DG of the hippocampus (Gage, 2000). SVZ-derived neuroblasts migrate through the RMS towards the OB where they differentiate into functional interneurons (Carleton *et al.*, 2003). Interestingly, upon brain injury, some neuroblasts can leave the RMS to migrate towards damaged areas where they differentiate into the dead cells' specific neuronal phenotype (Lindvall and Kokaia, 2006). Therefore, taking advantage of these unique stem cells' properties, much attention has been taken on the design of experimental strategies to promote brain repair (Pluchino *et al.*, 2003; Hess and Borlgan, 2008). For that purpose it is crucial to identify new proneurogenic factors that can enhance neural stem cells capabilities.

Histamine, an amine acting as a neurotransmitter and neuromodulator, is present in the brain in three main cellular stores: neuronal, mast and microglial cells (Brown *et al.*, 2001). Its actions can be mediated through several receptors: two postsynaptic (H1R, H2R), one presynaptic (H3R), and a forth receptor mainly present in the immune system (H4R). All receptors belong to the family of rhodopsin-like class A receptors coupled to guanine nucleotide-binding proteins (Brown *et al.*, 2001; Hass *et al.*, 2008). Histaminergic neurons, present in the tuberomammillary nucleus, project numerous ramifications throughout the entire adult brain; which makes histamine involved in a broad range of physiological and pathological functions, such as sleep-wake control, emotions, learning and memory (Brown *et al.*, 2001; Hass *et al.*, 2008; Passani *et al.*, 2007). Histamine has also been reported to be involved in several pathologic conditions (Jutel *et al.*, 2002; Kukko-Lukjanov *et al.*, 2006, 2010). Regarding adult neurogenesis, it has been shown that histamine receptors are expressed in undifferentiated neural progenitors. We and others have shown that histamine transiently increase intracellular free calcium levels ($[Ca^{2+}]_i$) in SVZ immature/stem cells, embryonic stem and carcinoma cells (Tran *et al.*, 2004; Agasse *et al.*, 2008b). Moreover, Molina-Hernandez and Velasco (2008) showed that histamine induces embryonic neural stem cell proliferation and neuronal differentiation. In spite of these reports, histamine effects on the SVZ neurogenic niche have not yet been fully addressed. Herein, we unravel the effects of histamine in SVZ cell proliferation, survival and neuronal differentiation, as well as the underlying molecular mechanisms involved. Additionally, we also developed histamine-loaded PLGA microparticles in order to promote a more efficient and controlled *in vitro* delivery of histamine. Furthermore, we used mini osmotic pumps implanted at the lateral ventricle to achieve a similar constant and efficient histamine delivery, directly at the ventricle of adult living animals. Overall, our results identify histamine as a proneurogenic

player at the SVZ, which effects are potentiated through its delivery by microparticles or mini osmotic pumps, highlighting its possible use in future brain repair strategies.

III.3. Results

Since the discovery that classical antihistamines have a sedative action (Uzan, 1979; Tasaka *et al.*, 1989), it has become clear that histamine has relevant actions in the CNS. In fact, the histaminergic system commands various physiologic and pathologic states, but its role in neurogenesis has been elusive. Therefore, in the present work we investigated the role of histamine in SVZ neurogenesis, both in *in vitro*, *ex vivo* and *in vivo*.

III.3.1. Histamine has proneurogenic effects in SVZ cell cultures

III.3.1.1. Histamine receptors are expressed in SVZ cells

It was previously shown that immature cells, nestin and/or GFAP positive cells, can be distinguish on the basis of the selective increase of $[Ca^{2+}]_i$ in response to histamine *via* H1R activation (Agasse *et al.*, 2008b). It was also found by immunocytochemistry that immature cells robustly express H1R. These data suggest that histamine receptors are functional in SVZ immature cells. Moreover, others have shown that embryonic stem and carcinoma cells express functional H1R (Tran *et al.*, 2004; Yanagida *et al.*, 2004). Altogether the data suggests a role for H1R as a marker for undifferentiated neural progenitors.

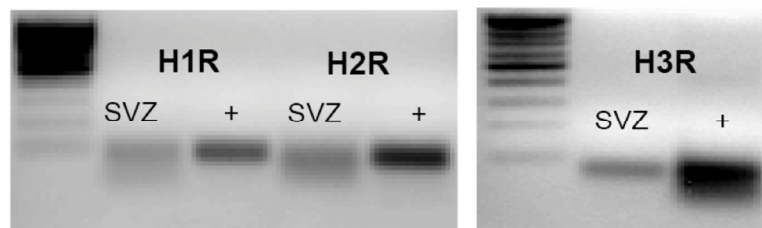


Figure III. 1. Mouse SVZ cells express histamine receptors.

Reverse Transcription-PCR detection of H1R (74 bp), H2R (65 bp) and H3R (69 bp) mRNA expression in SVZ cells grown *in vitro* under proliferative conditions. +: positive controls, total murine spleen mRNA.

To dissect which histamine receptors were expressed by SVZ cells, neurospheres were grown in proliferative conditions, and mRNA expression was analyzed by Reverse Transcription-PCR. As shown in Figure III.1, SVZ neurospheres express all histamine receptors tested: H1R, H2R and H3R. Positive (total murine spleen mRNA) and negative (RNA sample replaced by water, not shown) controls were also performed to confirm the specificity of the primers.

III.3.1.2. Histamine has no toxic effect to SVZ cell cultures

To evaluate the effect of histamine on cell death, the TUNEL assay was performed, since it is a common and adequate method to detect DNA fragmentation resulting from apoptosis (Gavrieli *et al.*, 1992; Negoescu *et al.*, 1996, 1998). SVZ cells were exposed to histamine (10, 100 and 500 μ M) for 48 h and after immunorevelation, the percentages of TUNEL-positive cells were determined and compared to the control.

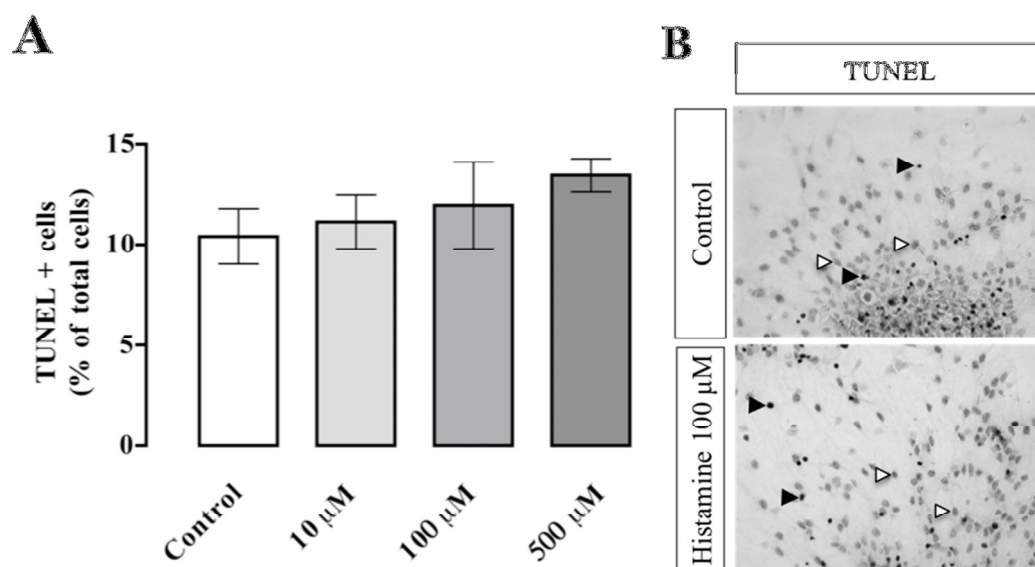


Figure III. 2. Histamine does not exert any effect on cell survival of SVZ cell cultures.

A. Bargram represents the number of TUNEL-positive nuclei expressed as percentages of total number of cells. Cultures were exposed to 10, 100 or 500 μ M histamine for 48h. Data are expressed as mean $\pm$ SEM (n = 2). No statistical significance was observed in the presence of histamine. **B.** Representative microscopy photos of SVZ cell cultures incubated (or not) with histamine (100 μ M). Black arrows point to TUNEL positive cells, white arrows point to TUNEL negative cells. TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling.

At all concentrations tested, histamine induced no significant changes in the percentage of TUNEL-positive nuclei compared with the control (Figure III.2A; control: $10.41 \pm 1.38\%$; histamine 10 μ M: $11.14 \pm 1.34\%$; histamine 100 μ M: $11.95 \pm 2.15\%$; histamine 500 μ M: $13.44 \pm 0.83\%$). These results provide evidence that histamine does not regulate cell survival in SVZ cells, within the concentrations tested.

III.3.1.3. Histamine does not affect cell proliferation

BrdU, a synthetic nucleotide able to substitute thymidine in the DNA of dividing cells, was used to evaluate proliferation. After immunorevelation, percentages of BrdU positive cells were determined and compared to the control, as plotted in Figure III.3A. No statistical significant difference was observed in SVZ cells proliferation upon histamine

treatment (control: $2.44 \pm 0.38\%$; histamine 10 μM : $2.38 \pm 0.85\%$; histamine 100 μM : $2.51 \pm 0.56\%$; histamine 500 μM : $2.66 \pm 0.29\%$).

Being that BrdU labels newly generated cells at the S phase of the cell cycle, other cells in cell cycle, but not yet at the S phase, will not be labeled. Thus, we used Ki67 as a tool to label all cycling cells. Therefore, by counting BrdU-Ki67 double positive cells (as a % total number of Ki67 positive cells), it was possible to determine whether histamine affected the cell cycle by promoting the transition to the S phase (if higher percentage). In agreement with the previous results, histamine did not change the percentage of BrdU-Ki67 double positive cells (Figure III.3B - control: $77.17 \pm 2.00\%$; histamine 100 μM : $82.98 \pm 2.92\%$), suggesting that histamine does not affect cell cycle.

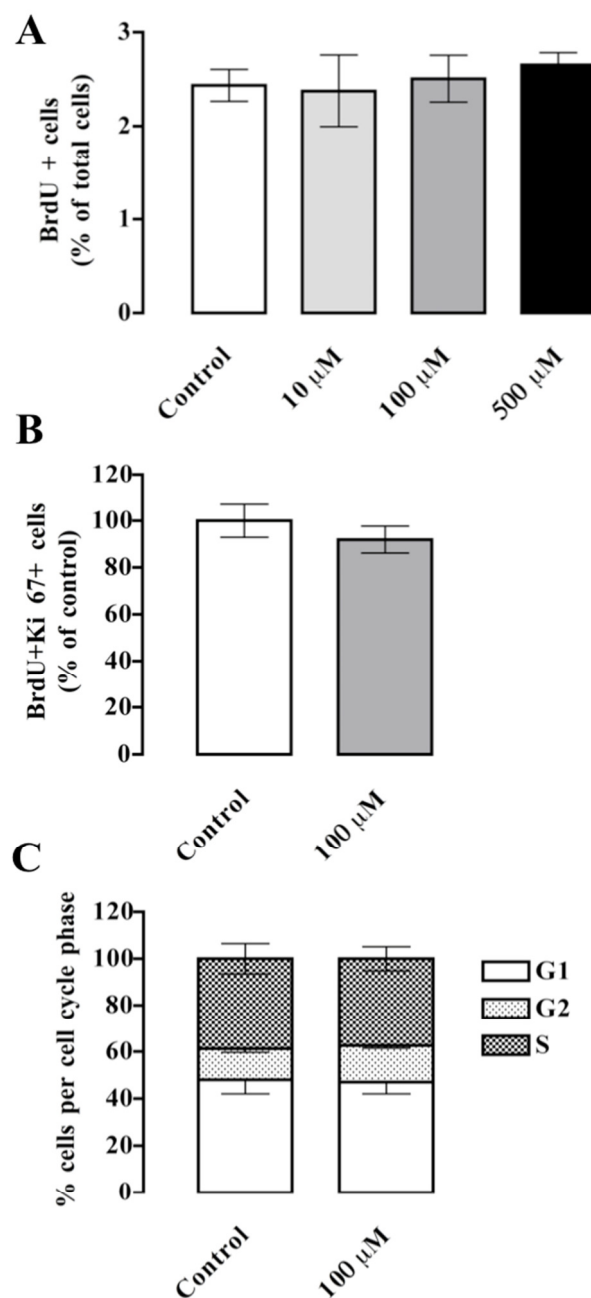


Figure III. 3. Histamine does not exert any effect on cell proliferation or cell cycle progression.

A. Bargram depicts the number of BrdU positive cells expressed as % of total number of cells, in differentiating conditions in the presence (or absence) of 10, 100 or 500 μM histamine for 48h (n=2). **B.** Bargram represents Brdu+Ki67+ cells (as a percentage of total Ki67+ cells), in differentiating cultures exposed (or not) to 100 μM histamine for 48h. (n=3). **C.** Bargram represents the percentage of cells in each cell cycle phase as accessed by Vybrant Dye Cycle Orange in flow cytometry experiments. SVZ cells grown in proliferative conditions (n=4-5). All data are expressed as mean $\pm$ SEM. No statistical significance was observed in the presence of histamine in neither experiment.

Moreover, flow cytometry was used to quantify the amount of DNA present in cells derived from 6 days old dissociated neurospheres (in proliferative conditions), which were previously incubated for 48 h with histamine. Following entry in S phase, cells duplicate their DNA. Therefore, using the DNA-selective stain Vybrant Dye Cycle Orange we were able to determine the relative number of cells present in the different phases of the cell cycle. These additional experiments (Figure III.3C) showed that histamine did not change the percentage of cells in G1 (control: $48.05 \pm 5.96\%$; histamine: $47.10 \pm 5.07\%$), G2 (control: $13.17 \pm 1.48\%$; histamine: $15.51 \pm 1.08\%$) or S phase (control: $38.77 \pm 6.48\%$; histamine: $37.40 \pm 5.12\%$) as compared to untreated cells (control).

Altogether, our results show that histamine does not affect proliferation of rapidly-dividing SVZ cells, neither in differentiation nor in proliferation conditions.

III.3.1.4. Histamine induces progenitor cell commitment

Then, we wanted to further disclose if histamine induces cell commitment of stem/progenitor cells. Uncommitted stem/progenitor cells either divide symmetrically – into 2 stem/progenitor cells; or asymmetrically – into a stem/progenitor and a committed cell. On the other hand, committed cells for differentiation terminally divide into 2 progenitor cells.

The effect of histamine on the type of cell division in single SVZ cells was examined with the cell pair assay. Briefly, Sox2 labels multipotent neural stem cells with the ability to self-renew, whereas committed progenitors are identified by Dlx2 labeling. Cell pairs were categorized in 3 groups, according to Sox2 expression: Sox2 +/+, Sox2 +/- and Sox2 -/-.

Histamine at a concentration of 100 μ M triggers an increase in the percentage of Sox2 -/- cell pairs (control: $30.2 \pm 3.1\%$; histamine: $39.6 \pm 3.3\%$; $p < 0.05$) at the expense of Sox2 +/+ cell pairs (control: $45.2 \pm 5.1\%$; histamine: $31.5 \pm 2.3\%$; $p < 0.01$) as compared with controls (Figure III.4A and C). These results suggest that histamine induces the terminal division of stem/progenitor cells into committed progenitors.

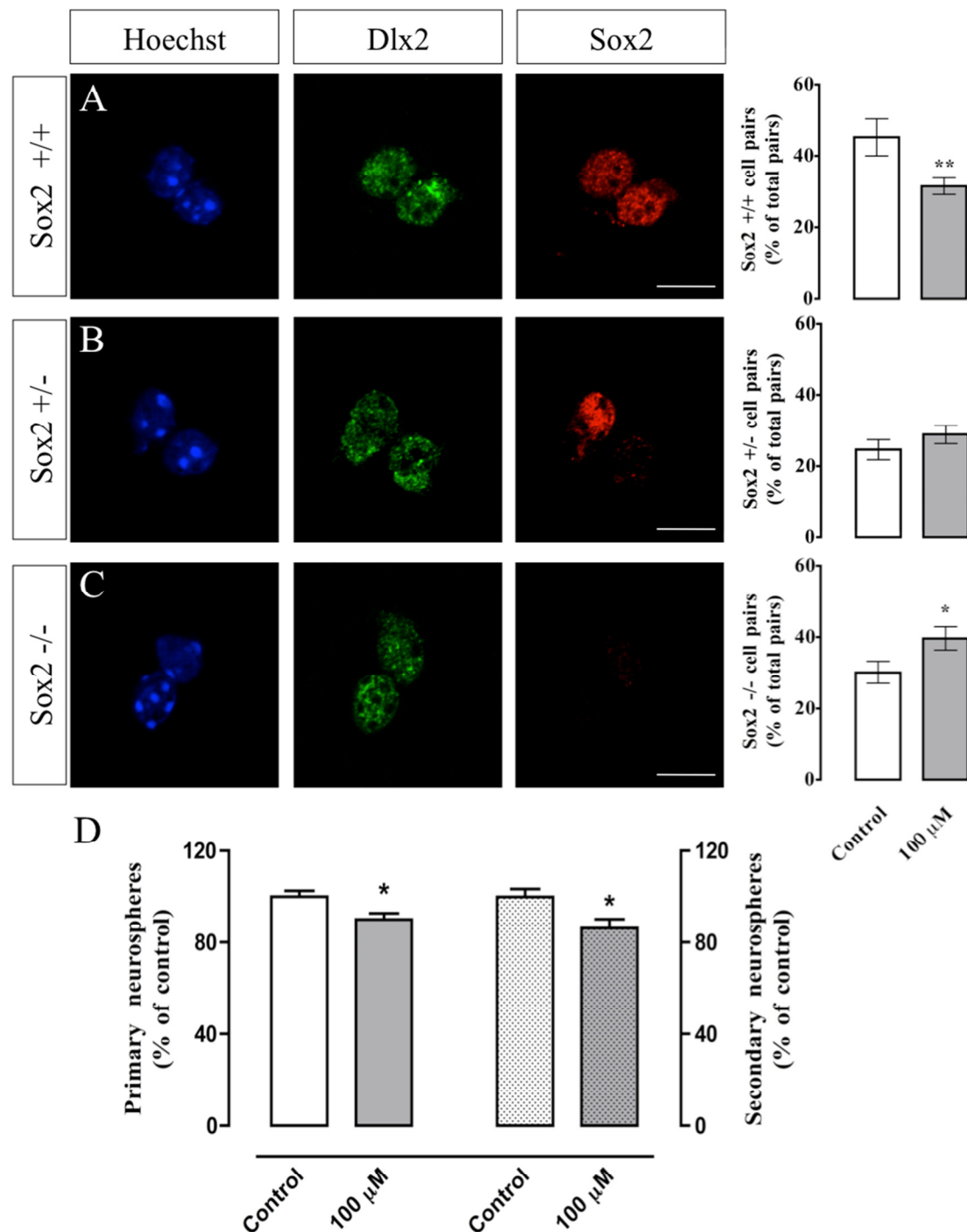


Figure III. 4. Histamine induces a reduction of Sox2-positive stem-like population and an increase of Sox2-negative progenitor cells.

Types of cell pairs characterized by the expression of Sox2 (red) and Dlx2 (green), namely: **A.** two daughter cells that coexpress Sox2 and Dlx2 (Sox2+/+); **B.** two daughter cells that coexpress Dlx2 but just one expresses Sox2 (Sox2+/-); **C.** two daughter cells that only express Dlx2 (Sox2-/-). Scale bar = 10 μm. **A, B, C.** Bargrams illustrate the effects driven by 100 μM histamine on the fate of newly born daughter cells by their expression of Sox2 and Dlx2. Data are expressed as the percentages of specific cell pairs per total cell pairs (n = 5). **D.** Neurosphere forming assay at control and histamine treated cultures. In both experiments, data are expressed as mean ± SEM. *, p < 0.05 and **, p < 0.01.

The reduction of Sox2 +/+ cell pairs upon histamine treatment suggested that a reduction in SVZ cell's self-renewal capacity could be occurring. To further clarify this possibility, the number of secondary neurospheres generated upon histamine treatment was

counted. Indeed, SVZ neurospheres treated with 100 μM histamine generated lower numbers of secondary neurospheres (Figure III.4.D - histamine: $86.3 \pm 4.1\%$; control set to 100%, $p < 0.05$). Overall, these results show that histamine is deeply involved in the reduction of immature cells, favouring neural commitment.

III.3.1.5. Histamine induces neuronal differentiation via H1R activation

Molina-Hernandez and Velasco (2008) showed that histamine induces neuronal differentiation in embryonic stem cells. Nevertheless, there are no reports on the effect of histamine in SVZ stem/progenitor cells differentiation.

To functionally evaluate neuronal differentiation, the variations of $[\text{Ca}^{2+}]_i$ in single cells following 50 mM KCl and 100 μM histamine stimulations (Agasse *et al.*, 2008b) were measured by SCCI. KCl depolarization leads to the massive entry of Ca^{2+} through voltage dependent calcium channels, which can be used as a measure of neural differentiation since differentiated neurons robustly express these channels (Maric *et al.*, 2000; Fiorio Pla *et al.*, 2005). Histamine stimulation increases $[\text{Ca}^{2+}]_i$ in immature cells but not in neurons or in glial cells (Tran *et al.*, 2004; Agasse *et al.*, 2008b). Therefore, the ratio of cellular responses due to histamine or KCl exposure (Hist/KCl) differs significantly within different cell types, being that low Hist/KCl ratio (below 0.8) is characteristic of SVZ-derived neurons. Thus, we quantified the percentage of cells with a low Hist/KCl ratio, as a functional measure of neuronal differentiation (Agasse *et al.*, 2008b).

Stimulating SVZ cells with 100 μM histamine lead to an increase in differentiation of cells with a neuronal-like profile of $[\text{Ca}^{2+}]_i$ responses, when compared with the dominant immature cell profile observed in control cultures (Figure III.5A). When quantifying the percentage of cells displaying a low Hist/KCl ratio, histamine treatment induced a significant increase in the proportion of neuronal-like responding cells as compared with the control (control: $13.8 \pm 3.1\%$, 12 coverslips; histamine 10 μM : $35.2 \pm 3.9\%$, 10 coverslips; histamine 100 μM : $35.2 \pm 3.9\%$, 11 coverslips; histamine 500 μM : $33.4 \pm 3.3\%$, 10 coverslips; $p < 0.01$ control versus histamine 100 μM or 500 μM).

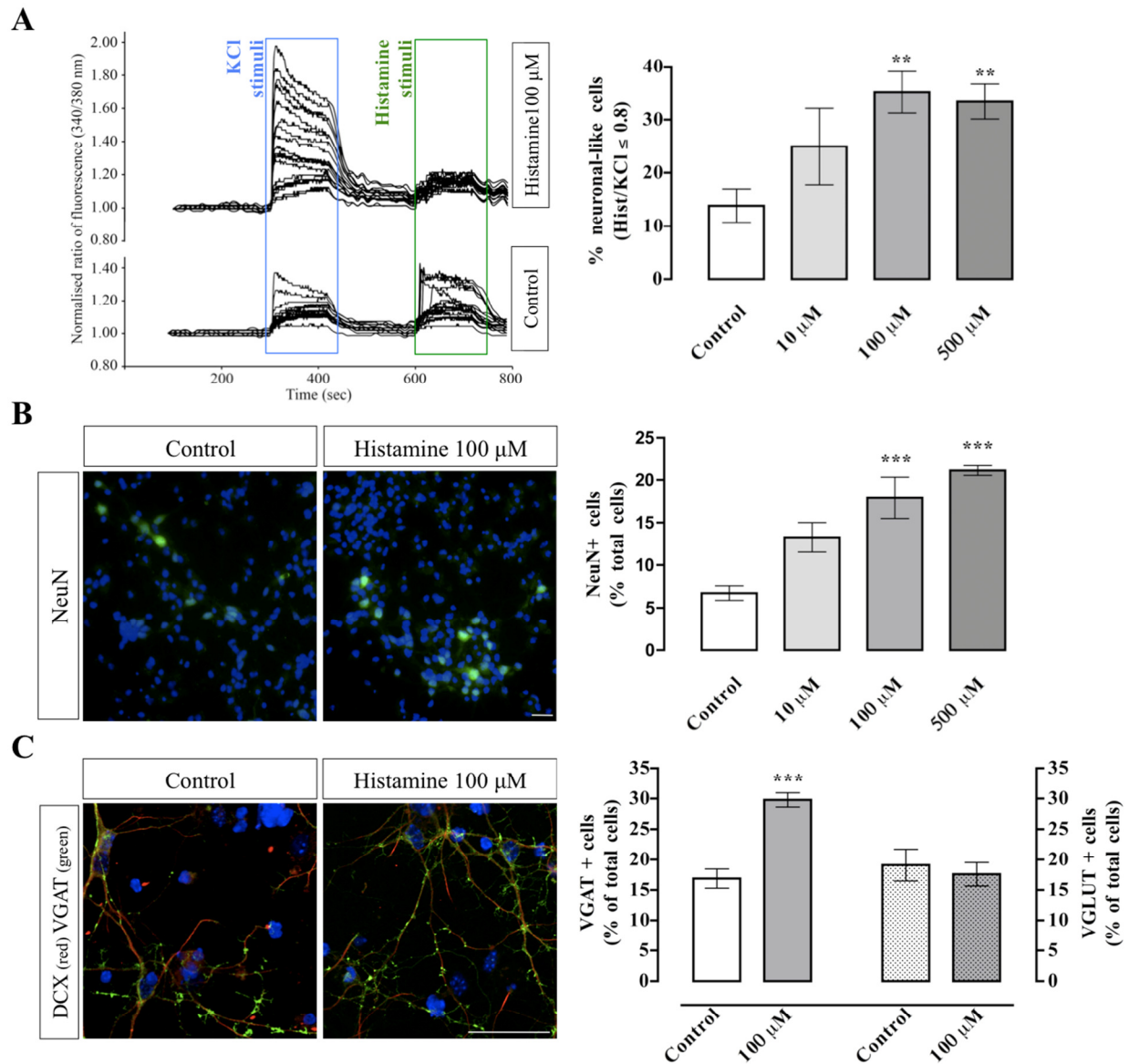


Figure III. 5. Histamine induces neuronal differentiation, favouring the GABAergic phenotype.

A. Left panel: representative SCCI response profiles of 20 cells in a control (bottom panel) and 100 μM histamine treated culture (upper panel). Right panel: bargrams depict the percentages of neuronal-like responding cells as accessed by SCCI analysis ($n = 2-3$; **, $p < 0.01$). **B.** Left panel: representative fluorescent photographs of NeuN-positive neurons (green) and Hoechst 33342 staining (blue) in control and histamine treated (100 μM) culture. Right panel: Bargram depicts the number of NeuN-positive cells expressed as percentage of total number of nuclei per culture. Data are expressed as mean percentage of total cells $\pm$ SEM ($n = 3-5$; ***, $p < 0.001$). **C.** Left panel: representative confocal photographs of VGAT-positive cells (green), doublecortin-positive neurons (red) and Hoechst 33342 staining (blue) in a control and histamine treated (100 μM) cultures. Right panel: Bargram depicts the number of VGAT-positive (left) and VGLUT-positive (right) cells expressed as percentage of total number of nuclei per culture. Data are expressed as mean percentage of total cells $\pm$ SEM ($n = 6$; ***, $p < 0.01$). Scale bars = 20 μm .

To confirm the functional data obtained by SCCI, immunocytochemistry against the neuronal nuclear protein (NeuN), a commonly used marker for mature neurons, was performed. In agreement with the previous functional data, histamine was also responsible for a significant increase in the percentage of NeuN-immunoreactive neurons, as compared to control cultures (Figure III.5B - control: $6.9 \pm 1.3\%$; histamine 10 μM : $13.3 \pm 1.7\%$;

histamine 100 μ M: $19.4 \pm 4.0\%$; histamine 500 μ M: $21.1 \pm 0.6\%$; $p < 0.001$ for control versus histamine 100 and 500 μ M).

To further characterize histamine proneurogenic effect on SVZ cell cultures, the GABAergic and glutamatergic populations were quantified upon histamine treatment relative to non-treated control cultures. Histamine at 100 μ M induced an increase in the percentage of VGAT-immunoreactive (Vesicular GABA Transporter) cells as compared with untreated controls (control: $16.9 \pm 1.6\%$; histamine: $29.8 \pm 1.2\%$; $p < 0.001$), while having no effect on VGLUT (Vesicular Glutamate Transporter)-immunoreactive cells (control: $19.1 \pm 2.6\%$; histamine: $17.6 \pm 1.9\%$). These results suggest that histamine triggers the GABAergic phenotype and has no effect on the subpopulation of glutamatergic neurons, labeled by VGLUT.

Moreover, the histamine receptor that mediates its proneurogenic effect was also disclosed. For that, SVZ cells were co-incubated for 7 days with histamine and the antagonists for each histamine receptor. Mepyramine (1 μ M), an H1R antagonist, completely abolished the increase of the percentage of neuronal-like responding cells induced by histamine (Figure III.6 - histamine 100 μ M: $36.4 \pm 3.8\%$; histamine 100 μ M + mepyramine: $22.1 \pm 3.9\%$, $p < 0.01$). Neither cimetidine (5 μ M), an H2R antagonist, nor thioperamide (1 μ M), a broad antagonist of H3R and H4R were able to block the proneurogenic effect mediated by histamine.

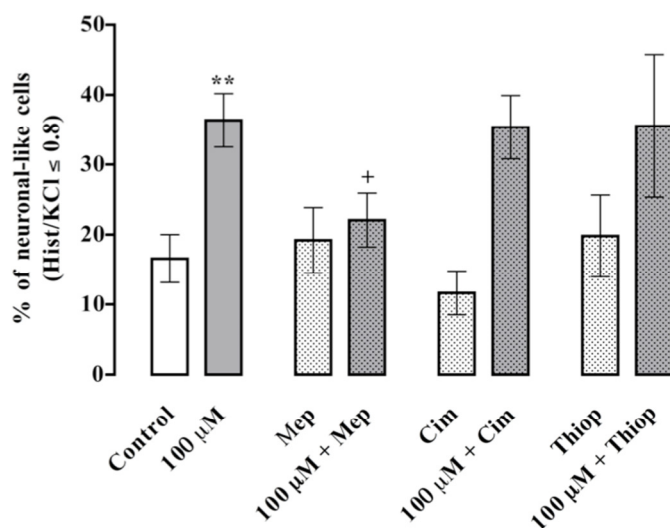


Figure III. 6. Histamine induces neuronal differentiation via H1R activation.

Bar graph depicts the percentages of neuronal-like responding cells. All data are expressed as a mean $\pm$ SEM ($n = 2-3$). Mepyramine (Mep) - 1 μ M; Cimetidine (Cim) - 5 μ M; Thioperamide (Thiop) - 1 μ M. * $p < 0.05$; **, $p < 0.01$.

However, no effect was observed on the immunoreactivity of GFAP, an astrocytic marker (Figure III.7A). This lack of effect on glial differentiation was confirmed by quantifying the relative amount of astrocytes by western blotting (Figure III.7B - control set to 100%; 100 μ M histamine: $99.58 \pm 15.52\%$).

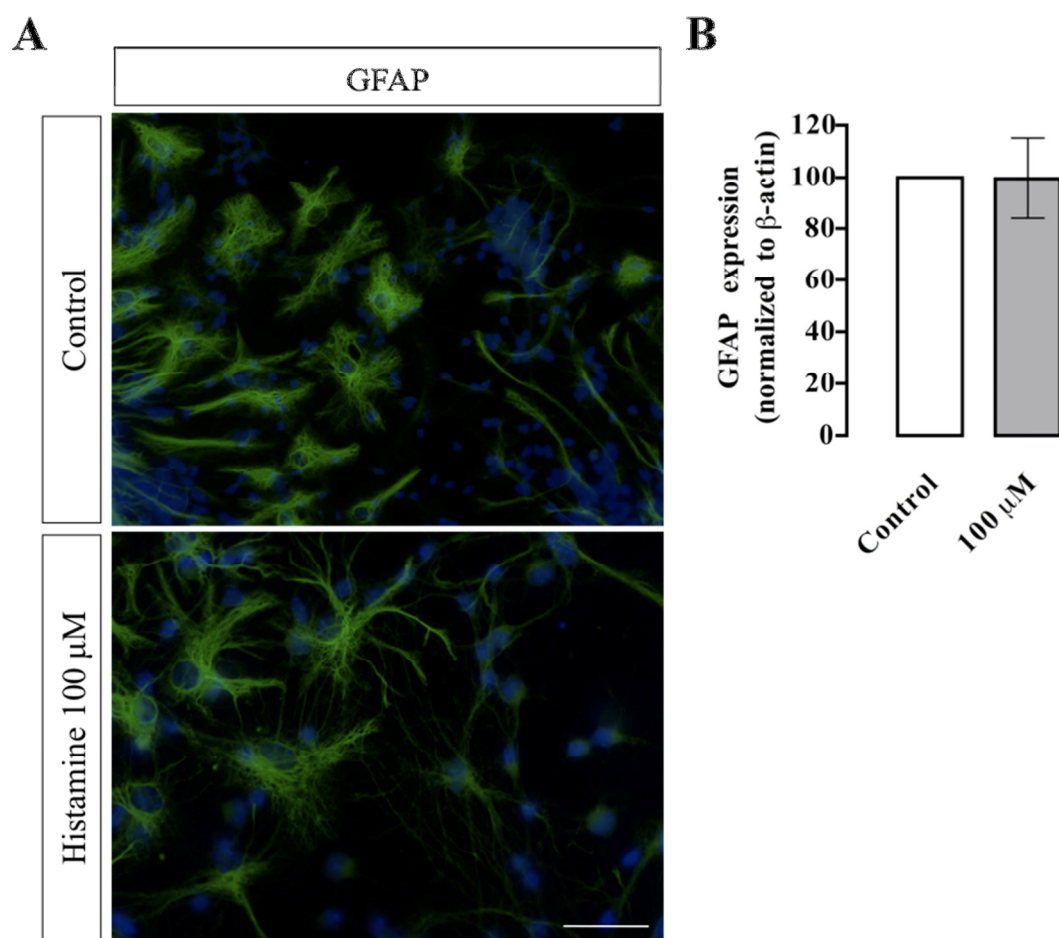


Figure III. 7. Histamine has no effect on GFAP expression.

A. Representative confocal photographs of GFAP (green) and nucleic Hoechst 33342 staining (blue) in control and 100 μM histamine treated cultures for 7 days. Scale bar = 20 μm. **B.** Bargram represents protein expression quantification by western blotting in control and 100 μM histamine treated cultures (n=4). No statistical significance was observed between control and histamine-treated cultures.

Altogether, these results put forward that histamine induces functional GABAergic neuronal differentiation in SVZ cells, *via* activation of H1R receptor, irrespective of cell proliferation, survival, or glial differentiation.

III.3.1.6. Histamine facilitates epigenetic changes leading to expression of the proneurogenic genes Mash1, Dlx2, Ngn1

The proneurogenic effect of histamine might occur through various molecular mechanisms. Epigenetics, namely histone methylation, has been associated with proneurogenic gene's transcription facilitation (Santos-Rosa *et al.*, 2002; Pan *et al.*, 2007). Thus, to disclose if histamine is associated with the transcription of the proneurogenic genes Mash1, Dlx2 and Ngn1, quantitative chromatin immune precipitation (qChIP) targeting the trimethylated lysine 4 form of histone H3 (H3K4m3) followed by a qRT-PCR analysis was performed.

As shown in Figure III.8A, SVZ cells exposed for 24h to 100 μ M histamine showed an increase of approximately 3.5 fold for Mash1 (3.6 ± 1.5 ; $p < 0.05$), and 3 fold for Dlx2 (3.2 ± 0.9 ; $p < 0.05$) promoters trimethylation at H3K4 as compared to control cultures. Interestingly, no statistically significant change was observed regarding the association of the trimethylated form of H3K4 to Ngn1 promoter at 24h (1.1 ± 0.8). However, if incubation with 100 μ M histamine was prolonged for 48h, there was an increase in the recruitment of H3K4Me3 of all promoters, when compared to control (set to 1): Mash1 (2.0 ± 0.6 ; $p < 0.05$), Dlx2 (2.1 ± 0.6 ; $p < 0.05$) and Ngn1 (3.4 ± 1.7 ; $p < 0.05$). These data, although with different kinetics and intensities, point to a robust effect of histamine in the trimethylation of lysine 4 of histone 3, on the promoter regions of the proneurogenic genes Mash1, Dlx2 and Ngn1.

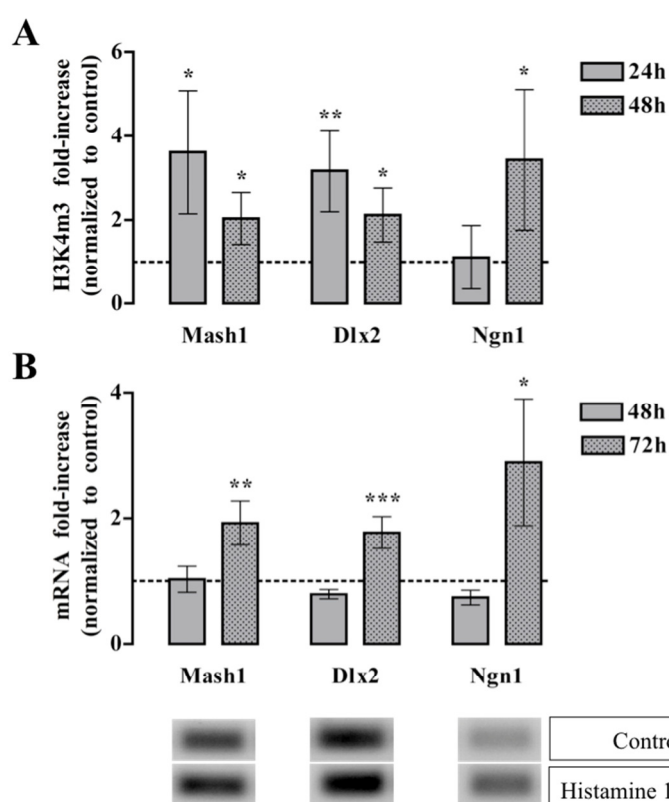


Figure III. 8. Histamine promotes the expression of the proneurogenic genes Mash1, Dlx2, Ngn1.

A. Bargram depicts the fold increase of trimethylated lysine 4 of histone H3 modifications in the promoter regions of Mash1, Dlx2, and Ngn1 genes, measured by qChIP. **B.** Bargram depicts the fold increase of mRNA expression for Mash1, Dlx2, and Ngn1 genes measured by quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis. Below the graph, representative agarose gels illustrate the mRNA expression levels of each gene in cultures under the absence (control) or presence of 100 μ M histamine for 72h. Data are expressed as mean $\pm$ SEM ($n = 4-12$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; control set to 1, dotted line).

To validate that these epigenetic modifications, in fact, make the DNA more prone to be transcribed, qRT-PCR analysis for these three proneurogenic genes was performed. In agreement with qChIP data, 100 μ M histamine treatment for 72h, triggered a significant increase in mRNA levels of Mash1 (1.9 ± 0.3 ; $p < 0.01$), Dlx2 (1.8 ± 0.3 ; $p < 0.001$) and Ngn1 (2.9 ± 1.0 ; $p < 0.05$), as compared with control cultures (set to 1) (Figure III.8B). Although at 48h, no changes were observed in 100 μ M histamine-treated cultures, when compared to control, this result confirms that the H3K4m3 recruitment happens prior to mRNA transcription.

III.3.1.7. Histamine effect on axon growth: the JNK-MAPK pathway

Neuronal differentiation occurs not just by early cell commitment and cell differentiation into neuronal phenotypes, but also by neuronal maturation and axonogenesis.

To evaluate if histamine promotes neuronal maturation and axonogenesis, the phosphorylation and subcellular localization of the JNK-MAPK pathway was studied. SVZ cells exposed for 6h to 100 μ M histamine showed a robust increase of Phospho-JNK (P-JNK) immunoreactivity located on neurites, while control cultures had faint staining with few P-JNK positive neurites and growth cone structures. Histamine significantly increased the number of ramifications per neurosphere (control: 1.3 ± 0.3 ; histamine 100 μ M: 3.5 ± 0.5 ; $p < 0.01$), as well as the total length of ramifications and growth cone-like projections positive for P-JNK per neurosphere (control: 155.5 ± 28.3 μ m; histamine 100 μ M : 315.5 ± 40.8 μ m; $p < 0.05$), compared with control cultures (Figure III.9A Left panel). Interestingly, the P-JNK immunoreactivity induced by histamine was associated with Tau, a microtubule-associated protein that promotes bundling and stabilization of axonal microtubules of both immature and mature neurons (Figure III.9A Right panel). These data suggest that histamine supports axonogenesis and neuronal maturation.

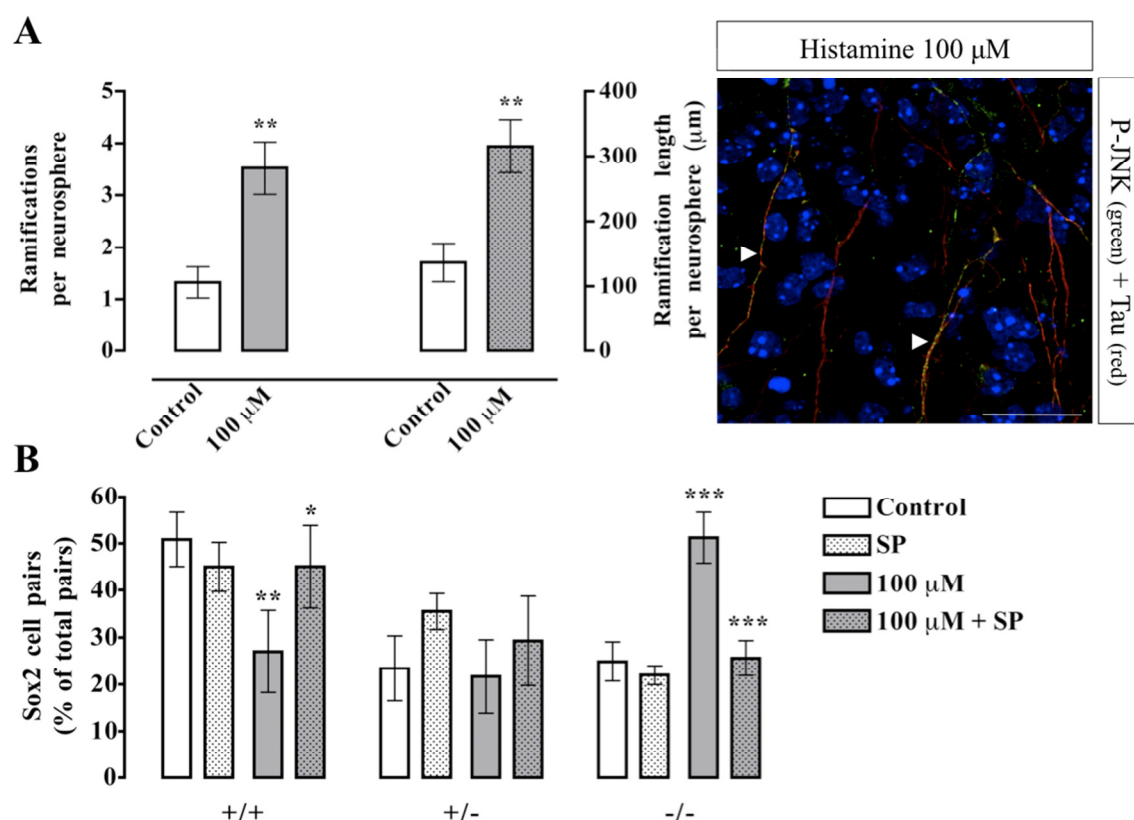


Figure III. 9. Histamine induces activation of JNK-MAPK pathway on growing axons and cell commitment. **A.** Left panel: Bargram depict number of ramifications (left) and total length in μ m (right) of the P-JNK-positive ramifications per neurosphere ($n=6$; **, $p < 0.01$). Right panel: Representative fluorescent confocal digital image of P-JNK (green), Tau (red), and Hoechst 33342 (blue) in cultures exposed for 6 hours to 100 μ M histamine. Arrows depict axons double labeled for P-JNK (green) and Tau (red). Scale bar = 20 μ m. **B.** Cell commitment

studies by cell-pair analysis. Bargram depicts the percentage of Sox2 cell pairs treated (or not) with histamine and 20 μ M JNK inhibitor - SP600125 (SP) (n=5; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). In all experiments, data are expressed as mean $\pm$ SEM.

Since JNK-MAPK may be also involved in previous events of neurogenesis, cell pair assay was performed in the presence of 20 μ M SP600125, a specific inhibitor of JNK activity, to disclose P-JNK involvement in initial phases of cell commitment.

It was observed that SP600125 prevented histamine effect on cell commitment, namely on the decrease of the percentage of Sox2 $+/+$ cell pairs (histamine 100 μ M: $27.0 \pm 3.9\%$; histamine 100 μ M + SP600125: $45.1 \pm 3.9\%$; $p < 0.05$) and the concomitant increase of Sox2 $-/-$ cell pairs (histamine 100 μ M: $51.3 \pm 2.4\%$; histamine 100 μ M + SP600125: $25.6 \pm 1.7\%$; $p < 0.001$), to levels similar to untreated cultures (Figure III.9B). Moreover, SP600125 *per se* did not alter the levels of any of the categorized groups of Sox2 cell pairs. Therefore, these results support the P-JNK involvement in initial phases of cell commitment induced by histamine, with expected and proved impact in cell fate decision and neuronal commitment.

III.3.2. Histamine releasing-PLGA microparticles induce neuronal differentiation in SVZ cell cultures

III.3.2.1. Histamine-loaded microparticle characterization

In order to promote a more controlled delivery of histamine to the SVZ cell cultures, we then developed PLGA microparticles loaded with histamine. This biomaterial-made drug delivery system allows a slower and more efficient delivery of histamine to the cells extracellular *milieu* since they are not internalized, but rather distribute at the cell surface.

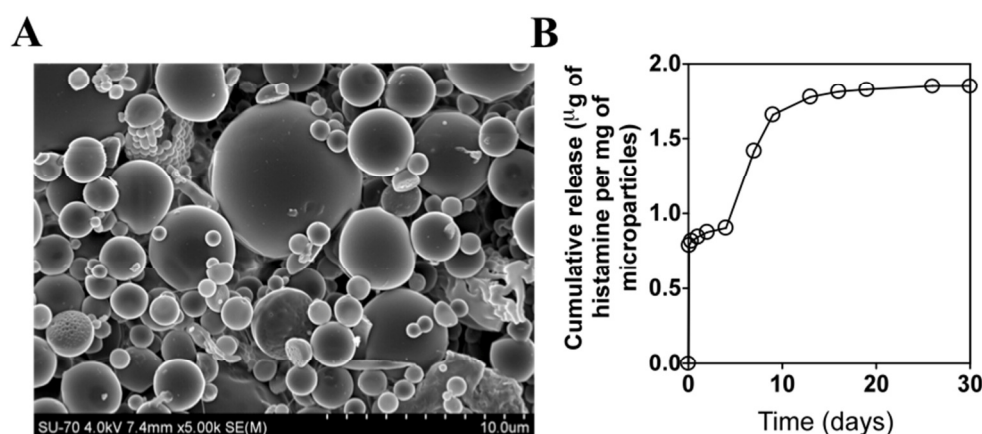


Figure III. 10. PLGA histamine-releasing microparticle characterization.

A. Scanning Electron Microscopy (SEM) micrographs of microparticles obtained by single emulsion. **B.** Histamine-release profile from PLGA-microparticles in PBS aqueous solution at 37°C.

PLGA microparticles (Resomer 502H, Mw = 10,777; Resomer 752H, Mw = 12,470) are composed of 50% lactic acid and 50% glycolic acid, and their morphology, size and cellular internalization were characterized at the Biomaterials Unit of the Center for Neuroscience and Cell Biology (Figure III.10). They have a loading capacity of approximately 5.3 mg of histamine per mg of microparticles. Microparticles' release profile experiments (Figure III.10B) demonstrated that the cumulative histamine released at the end of 7 days is far smaller (1000x less) than the histamine concentration initially tested for the single-pulse experiments (100 µM), as can be seen in Table III.1.

Table III. 1. PLGA microparticle and histamine concentration correspondance.

PLGA microparticle concentration	Approx. histamine concentration released
10 ng/ml	72 pM
100 ng/ml	0,72 nM
1 µg/ml	7,2 nM
10 µg/ml	72 nM
100 µg/ml	0.72 µM

Microparticles with an average 2 μm diameter were selected to prevent their cellular uptake, thus they release histamine at the extracellular *milieu*, as can be observed in Figure III.11.

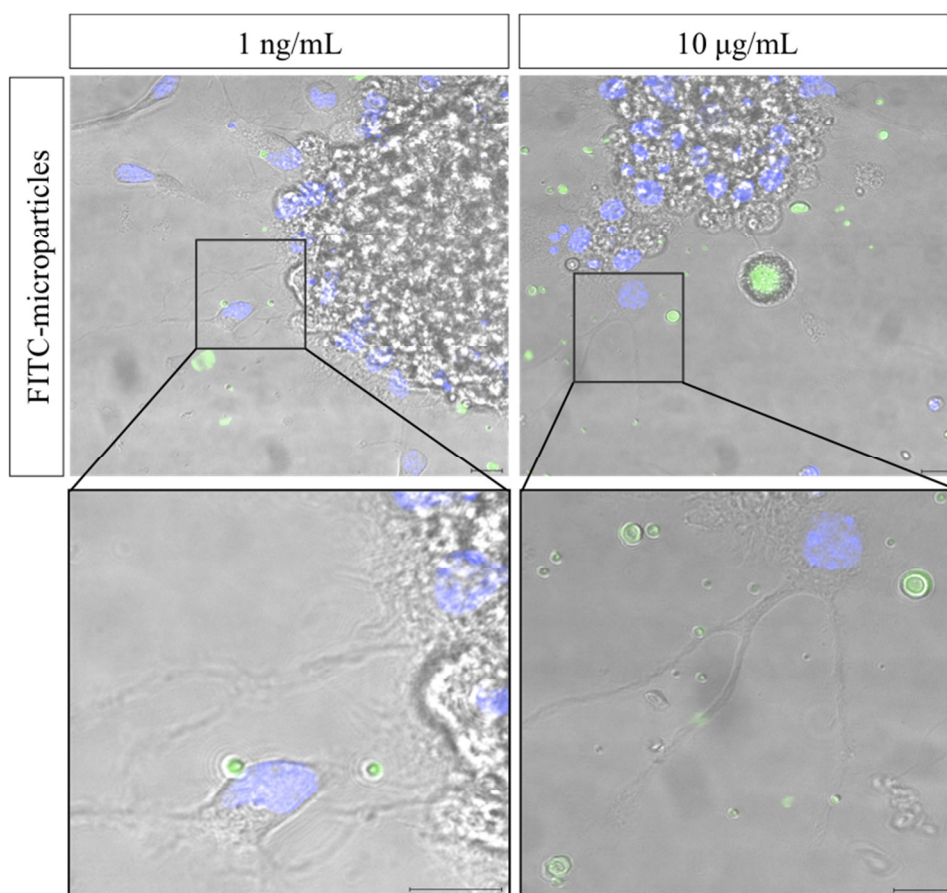


Figure III. 11. Histamine-releasing microparticles distribution in SVZ cell cultures. Representative confocal photos of SVZ cells incubated for 1h in the presence of 1 $\mu\text{g}/\text{ml}$ (right) and 10 $\mu\text{g}/\text{ml}$ (left) histamine-releasing microparticles (fluorescein isothiocyanate (FICT) labeling-green). Cell nuclei were stained with Hoechst 33342 (blue). Scale bars = 20 μm .

III.3.2.2. Cell viability effect of histamine-releasing PLGA microparticles

Blank/unloaded or histamine-loaded microparticles (up to 1 $\mu\text{g}/\text{ml}$ of microparticles) were added to SVZ cell cultures for 48h in order to access their effect on cell death, by apoptosis. Neither histamine-loaded microparticles, nor blank microparticles affected the percentage of TUNEL-positive cells (Figure III.12 - control: $10.85 \pm 1.22\%$; blank microparticles 1 $\mu\text{g}/\text{ml}$: $8.65 \pm 1.31\%$; 10 ng/ml: $9.70 \pm 1.19\%$; 100 ng/ml: $11.35 \pm 2.41\%$; 1 $\mu\text{g}/\text{ml}$: $9.29 \pm 0.99\%$), when compared with control cultures. Thus, none of the two types of microparticles affected cell viability. As expected, the biomaterial formulation had no toxicity on SVZ cell cultures, either *per se* or combined with histamine.

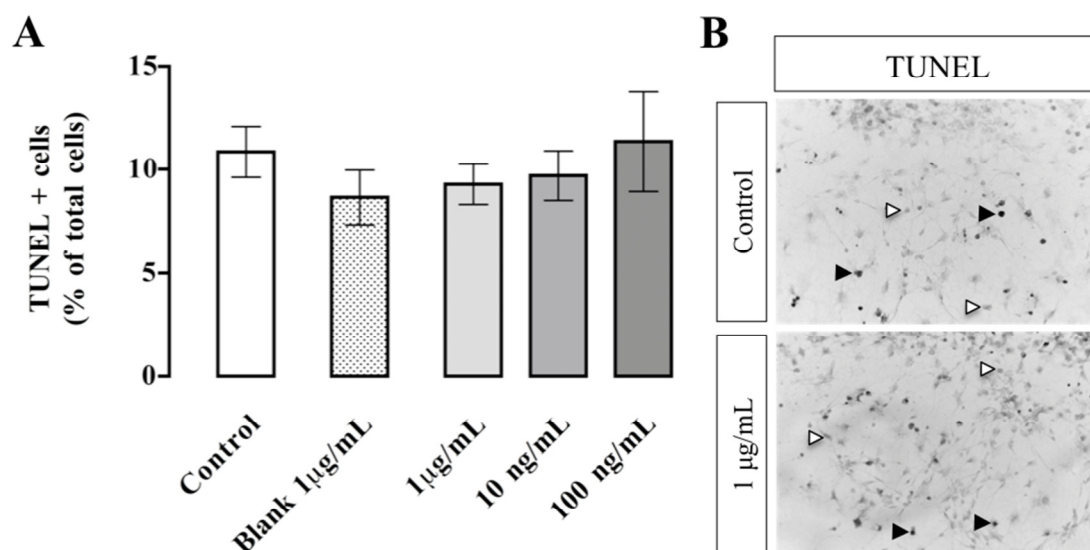


Figure III. 12. Histamine-releasing microparticles do not exert any effect on SVZ cell culture survival.

A. Bargram represents the number of TUNEL-positive nuclei expressed as percentage of total numbers of cells. Cultures were exposed to 1 µg/ml blank microparticles and to 1 µg/ml, 10 ng/ml, 100 ng/ml histamine-releasing microparticles for 48h. Data are expressed as mean $\pm$ SEM (n=4). No statistical significance was observed in the presence of histamine-releasing microparticles. **B.** Representative transmission microscopy photos of SVZ cell cultures incubated (or not) with histamine-releasing microparticles (1 µg/ml). Black arrows point to TUNEL-positive cells, white arrows point to TUNEL negative cells. TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labelling.

III.3.2.3. Evaluation of PLGA microparticle effect on cell proliferation

Similarly to what was performed previously in the case of histamine, proliferation was studied making use of BrdU incorporation in SVZ cell cultures.

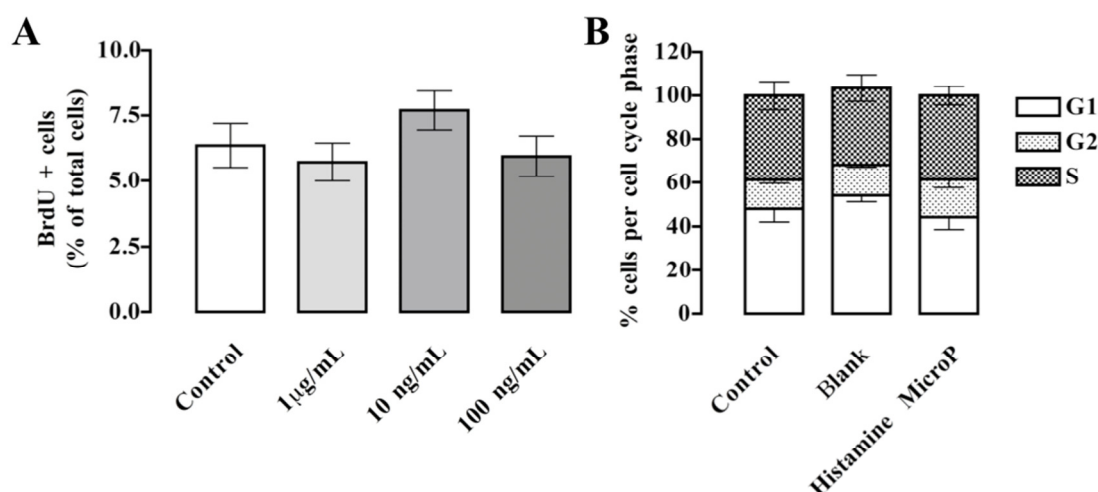


Figure III. 13. PLGA histamine-releasing microparticles do not exert any effect on cell proliferation or cell cycle progression.

A. Bargram depicts the number of BrdU-positive cells expressed as percentage of total numbers of cells, in cultures grown under differentiation conditions, and treated (or not) with 10 ng/ml, 100 ng/ml and 1 µg/ml histamine-releasing microparticles for 48h. (n=2) **B.** Bargram represents the percentage of cells in each cell cycle phase as accessed by using Vybrant Dye Cycle Orange in flow cytometry experiments. SVZ cells were grown in proliferative conditions. Data are expressed as mean $\pm$ SEM (n=4-5). No statistical significance was observed in the presence of histamine-releasing microparticles in neither experiment.

When SVZ cells were incubated with histamine-releasing microparticles, no significant difference in the percentage of BrdU positive cells were observed (control: $6.35 \pm 0.84\%$; 10 ng/ml: $7.70 \pm 0.75\%$; 100 ng/ml: $5.94 \pm 0.77\%$; 1 $\mu\text{g/ml}$: $5.72 \pm 0.72\%$), as compared to control cultures. Also on cell cycle experiments, where the amount of DNA in each cell is measured with Vybrant Dye Cycle, a DNA-selective stain, no differences were observed in the percentage of cells in none of the cell cycle phases (G1 – control: $48.05 \pm 5.96\%$; blank microparticles: $54.14 \pm 2.81\%$; histamine-releasing microparticles: $44.22 \pm 5.66\%$; G2 – control: $13.17 \pm 1.48\%$; blank microparticles: $14.04 \pm 1.09\%$; histamine-releasing microparticles: $17.16 \pm 3.69\%$; S phase – control: $38.77 \pm 6.48\%$; blank microparticles: $35.33 \pm 6.06\%$; histamine-releasing microparticles: $38.61 \pm 4.33\%$) as compared to untreated control cells in proliferative conditions.

Thus, neither histamine, nor blank-PLGA microparticles have effects on cell proliferation or cell cycle.

III.3.2.4. Histamine-loaded PLGA microparticles have proneurogenic effect

Since histamine induced a significant increase in the percentage of new neurons, observed both phenotypically – by NeuN, and functionally – by SCCI, similar approaches as the ones used before were adopted to characterize the effect of histamine-releasing microparticles in SVZ cell cultures.

In accordance with the previous data, histamine-releasing microparticles induced an increase in the percentage of new neurons in SVZ cell cultures, analysed both functionally by SCCI, and phenotypically by immunocytochemistry. As seen in Figure III.14A, histamine-releasing microparticle (1 $\mu\text{g/ml}$) treated cultures showed a significant increase in the percentage of neuronal-like cells (with a $[\text{Ca}^{2+}]_i$ profile of Hist/KCl ratio < 0.8), as compared with blank (unloaded) microparticle treated cultures (histamine-loaded microparticles: $36.6 \pm 3.2\%$; blank microparticles: $15.6 \pm 2.1\%$; $p < 0.01$).

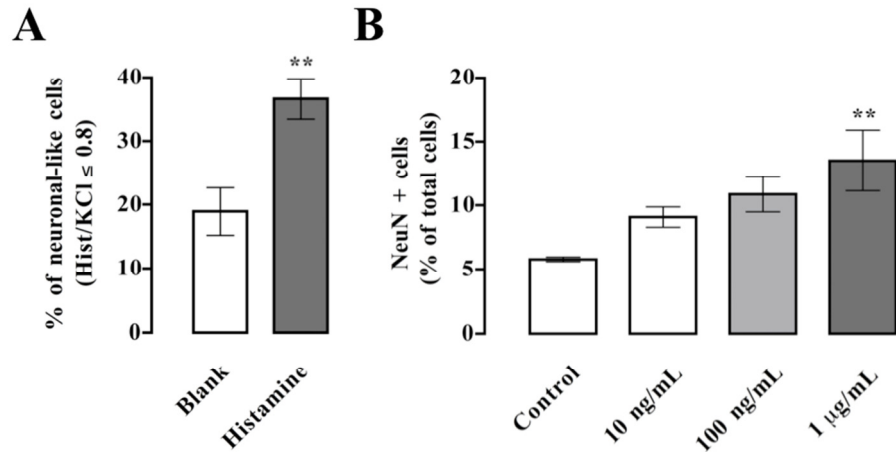


Figure III. 14. Histamine released from PLGA microparticles induced neuronal differentiation *in vitro*.
A. Bargram depicts the SCCI results expressed as percentages of neuronal-like responding cells, with histamine-releasing microparticles (1 µg/ml) compared to control blank microparticle treated cultures (n=2-3; **, p < 0.01).
B. Bargraph represents number of NeuN-positive cells expressed as percentages of total number of cells in SVZ cell cultures, treated with histamine-releasing microparticles with different concentrations (10 ng/ml, 100 ng/ml and 1 µg/ml) as compared to control non-treated cultures (n=2-3; **, p < 0.01). In all experiments, data are expressed as mean $\pm$ SEM.

Also the percentage of NeuN positive cells was increased in SVZ cells incubated with histamine-loaded microparticles (1 µg/ml) for 7 days (Figure III.14B - control: $5.79 \pm 0.19\%$; 10 ng/ml histamine-loaded microparticles: $9.09 \pm 0.79\%$; 100 ng/ml histamine-loaded microparticles: $10.86 \pm 1.37\%$; 1 µg/ml histamine-loaded microparticles: $13.51 \pm 2.38\%$, p < 0.01) as evaluated by immunofluorescence.

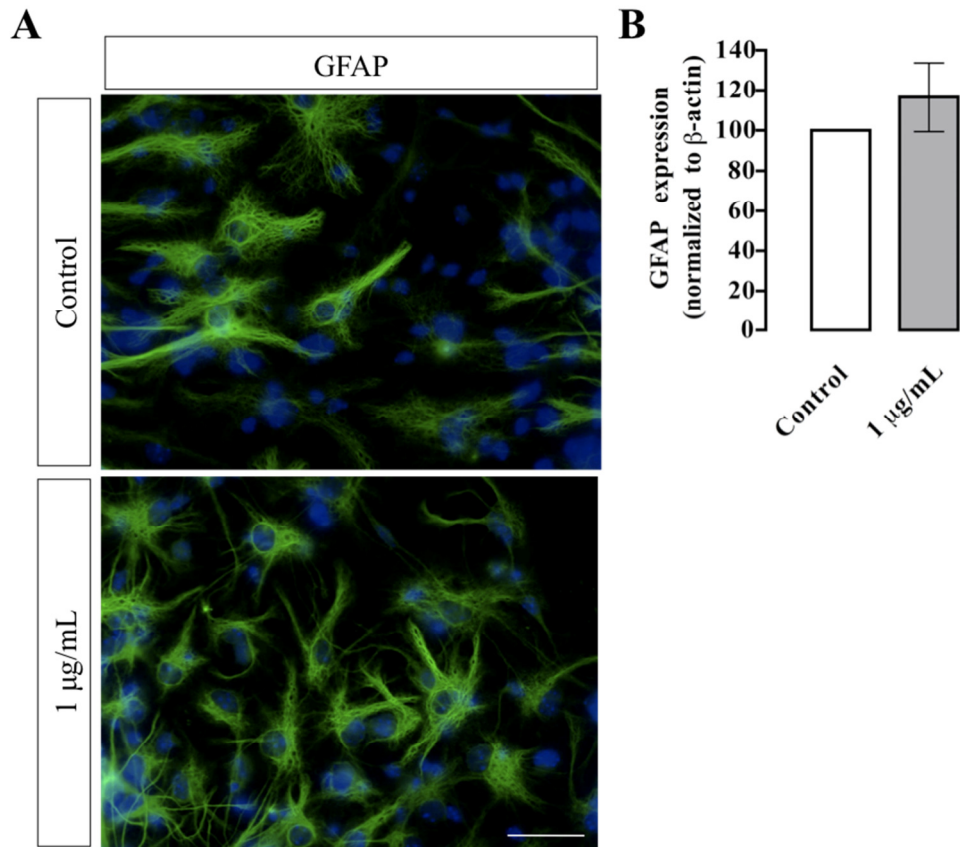


Figure III. 15. Histamine-releasing microparticles have no effect on GFAP expression.

A. Representative confocal photographs of GFAP (green) and nucleic Hoechst 33342 staining (blue) in control and 1 µg/ml microparticle treated cultures. Scale bar = 20 µm. **B.** Bargram represents protein expression quantification by western blotting in control and histamine-releasing microparticle treated cultures (n=4). No statistical significance was observed in the presence of histamine-releasing microparticles.

It should also be noted that histamine-releasing microparticles did not affect astrocytic differentiation, as detected by western blotting against GFAP (control set to 100%, 1 µg/mL: $116.6 \pm 17.18\%$) and by immunofluorescence (Figure III.15).

Overall, our data suggests that histamine released from PLGA microparticles induces robust neuronal differentiation without affecting cell viability, proliferation or in expense of glial differentiation.

III.3.3. Histamine-releasing microparticles have proneurogenic effect in *ex vivo* SVZ grafted cells

To further dissect the role of histamine on neurogenesis, an *ex vivo* model was developed. Histamine-releasing microparticle pretreated eGFP-SVZ neurospheres were grafted on the DG of WT organotypic hippocampal slice cultures. After 1 week of co-culturing, slices were fixed for DCX immunostaining, to label neuroblasts.

As shown in Figure III.16, 1 μ g/ml of histamine-loaded microparticles promoted a robust increase in DCX-GFP double positive cells per total transplanted cells from 4 to 13% (blank microparticles: $4.0 \pm 0.8\%$; histamine-loaded microparticles: $12.9 \pm 1.9\%$, $p < 0.001$).

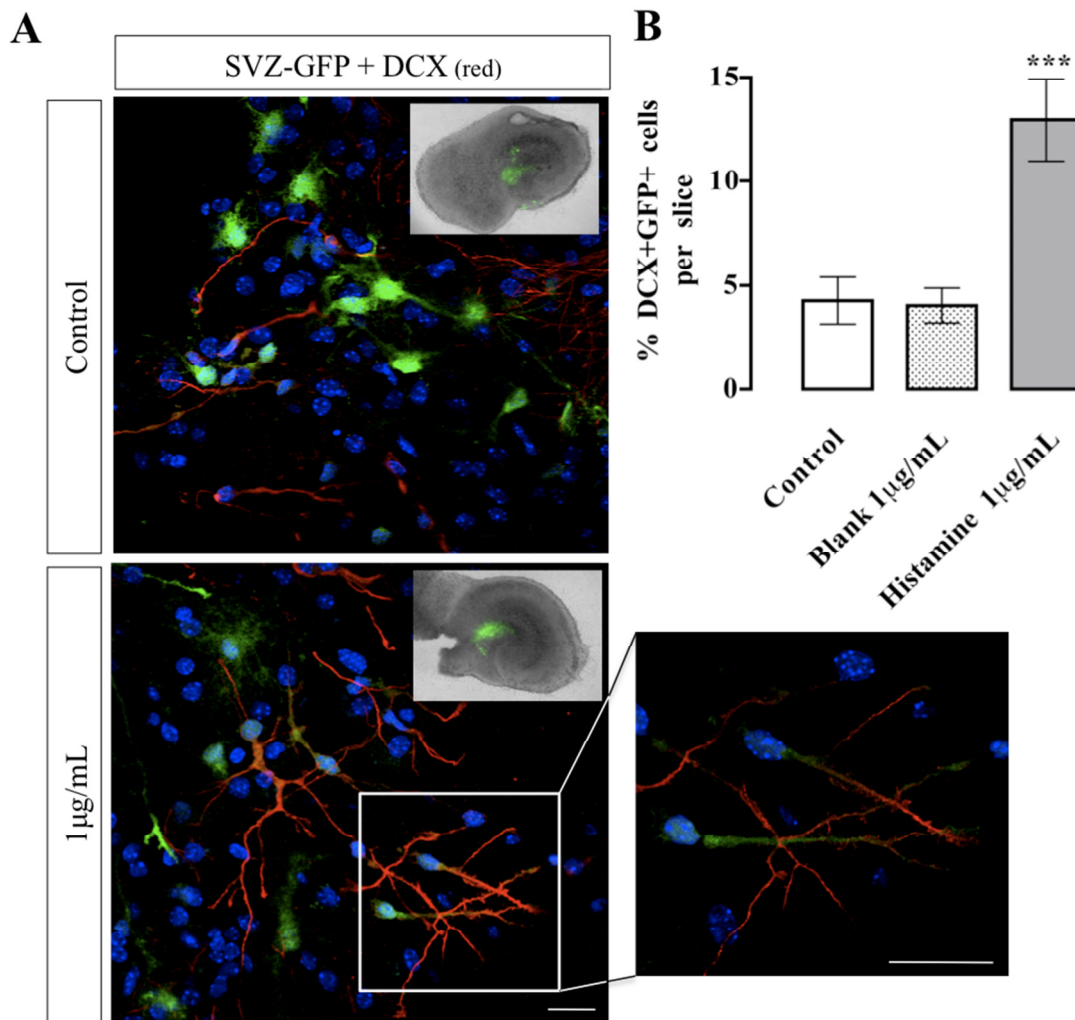


Figure III. 16. Histamine-releasing microparticles induce neuronal differentiation on eGFP-SVZ cells grafted on the DG of hippocampal organotypic slice cultures.

A. Representative confocal digital images of DCX (red), eGFP-SVZ cells (green), and Hoechst 33342 (blue) in organotypic hippocampal slices transplanted with eGFP-SVZ cells previously treated for 48 hours with 1 μ g/ml histamine-releasing microparticles or control. Scale bar = 20 μ m. **B.** Bargraph represents the percentage of DCX+GFP+ cells per eGFP-SVZ total cells counted in hippocampal slices. Data are expressed as mean $\pm$ SEM (n = 6–15; ***, $p < 0.001$).

The new neurons derived from the eGFP-SVZ grafted neurosphere (DCX+GFP+) showed a complex morphology with spines, similar to the structure of endogenous granule neurons present in the host DG (Figure III.16 - detail). Therefore, the eGFP-SVZ transplanted cells not just survive but also are more prone to differentiate into new neurons if pretreated with histamine-loaded microparticles.

III.3.4. The effect of histamine-loaded microparticles in SVZ *in vivo* grafted cells

A similar approach to the one developed for hippocampal organotypic slice cultures was used *in vivo*: eGFP-SVZ dissociated neurospheres were transplanted into hippocampal DG or striatum of WT adult mice. Three weeks after transplantation, some eGFP-SVZ cells pre-exposed to histamine-loaded microparticles integrated the striatum and hippocampal DG (Figure III.17), and differentiated into DCX-positive neuroblasts.

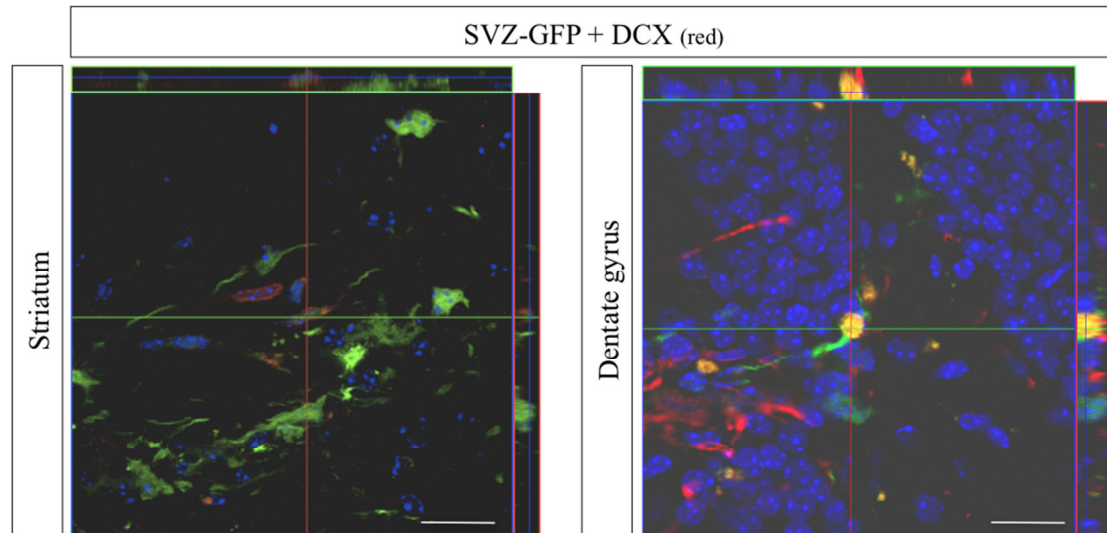


Figure III. 17. Histamine-releasing microparticles induce neuronal differentiation on eGFP-SVZ cells grafted on adult mouse brain.

Representative fluorescent confocal digital images of DCX-positive neurons (red), eGFP (green), and Hoechst 33342 (blue) observed in the grafting site of the striatum (left) and DG (right) of adult WT mouse *in vivo*. eGFP-SVZ cells were pretreated with 1 $\mu\text{g}/\text{ml}$ histamine-releasing microparticles for 48h and then intracerebrally injected in different regions of the mouse brain for 3 weeks. Scale bar = 20 μm .

III.3.5. Intracerebroventricular infusion of histamine induces BrdU and DCX increases at the SVZ and OB of adult animals.

Since many compounds do not reach the brain because of the BBB, they need to be locally delivered which implies an advantage of almost cell specific targeting caused by the local (stereotaxic location) administration (Jin *et al.*, 2002; Kippin *et al.*, 2005; Ramirez-Castillejo *et al.*, 2006). Thus, in this last section, osmotic mini pumps were used to deliver histamine at the lateral ventricles for 21 days. Then, we addressed the effects of histamine on cell proliferation and differentiation of NSC from the SVZ and their progeny.

III.3.5.1. Proliferative and proneurogenic effect of histamine in contralateral SVZ

First, we investigated the potential role of histamine on SVZ cell proliferation in the walls of the lateral ventricles. To address this issue, mini osmotic pumps loaded with histamine (0.8 mg/Kg/h) were implanted at the right hemisphere. In the first 3 days post-surgery BrdU was administered ip twice a day, with 12h interval. 21 days after surgery, animals were perfused and BrdU immunostaining was revealed. Vehicle (aCSF) injected animals were the controls for these experiments and only contralateral (CL, left hemisphere non injected) hemispheres were analysed so that potential injury/inflammation effects derived from the cannula implantation would not mask/boost histamine role *per se* on the fate of early proliferating cells at the SVZ.

Quantification of BrdU positive cells at the SVZ revealed that histamine creates a tendency to increased proliferation (Figure III.18A) during the first days of treatment (control: 25.80 ± 2.75 ; histamine: 34.43 ± 4.77). Since BrdU-positive cells present at the SVZ at this final time point can result from both symmetric and asymmetric divisions of the initial cell that retained BrdU, this tendency may indicate that histamine has a potential role in proliferation of SVZ NSCs *in vivo*. In agreement with this hypothesis is the fact that there was no visible increase in the SVZ thickness. Thus, daughter cells from the initially BrdU-labeled population had two fates: some cells stayed at the SVZ, diving and keeping a stem cell pool; while others stopped mitosis and started to differentiate migrating towards the OB.

In order to identify the proneurogenic potential of histamine at the SVZ, we then double stained for BrdU and DCX. Cells that were initially diving upon pump installation were triggered to differentiate because of histamine stimuli at the ventricle, since there was an increase not only in the total DCX-positive cell population (Figure III.18B) but also in the BrdU-DCX double positive population (Figure III.18C). The total DCX-positive cell population increased from 67.60 ± 3.98 in control aCSF treated animals to 129.1 ± 7.97 upon

histamine treatment ($p < 0.001$). The new population of DCX-positive neuroblasts that come from the initially dividing cells (ones that incorporated BrdU at the first 3 days of treatment) also increased, (10.14 ± 1.94) when compared to similar population from control aCSF treated animals (4.60 ± 0.40 ; $p < 0.05$).

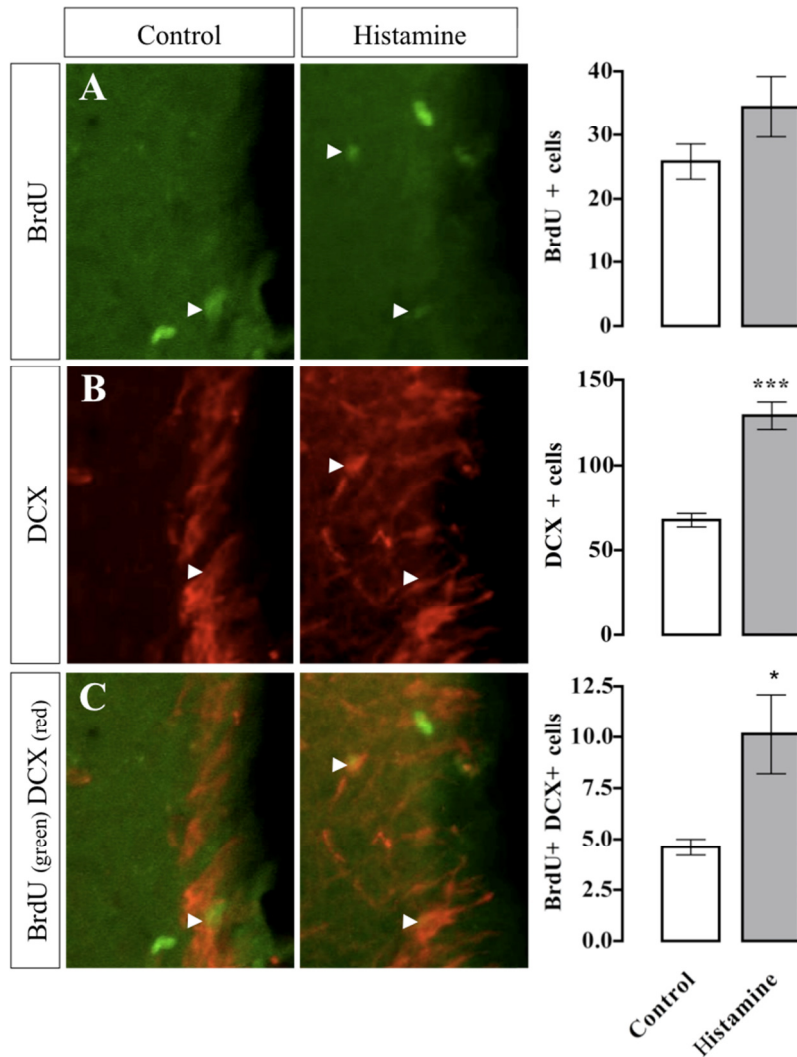


Figure III. 18. Histamine-loaded osmotic mini pumps lead to an increase in proliferation and differentiation of SVZ cells. Representative fluorescent confocal digital images of BrdU (green), DCX (red) and double positive BrdU-DCX cells observed in the SVZ of control and histamine treated animals *in vivo*, 21 days after pump installation. White arrows point to double positive BrdU-DCX cells. Bargraphs represent the total BrdU+ (A), DCX+ (B) and BrdU+DCX+ (C) cells counted in SVZ. Data are expressed as mean ± SEM ($n=5-7$; *, $p < 0.05$ and ***, $p < 0.001$).

Since histamine had a moderate effect in BrdU-positive cells and this cell population represents just part of the potentially dividing cells at the SVZ, we double labeled SVZ for DCX and Ki67 (Figure III.19). Total Ki67-positive population was slightly increased upon histamine treatment (control: 86.20 ± 24.62 ; histamine: 133.0 ± 19.57), which indicates that it has a positive effect on actively dividing cells within the cell cycle.

However, in agreement to what was observed *in vitro*, there was a clear increase in the population of new neuroblasts that are undergoing their final divisions (both DCX-Ki67 positive cells) (control: 29.40 ± 6.85 ; histamine: 49.33 ± 5.05 , $p < 0.05$). Thus, histamine has a role on the terminal division of proliferating cells at the SVZ. This data may also be viewed

as in agreement with the BrdU-positive cell increase trend at the SVZ since this population of cells might be resulting from asymmetric divisions that will thus lead to both NSC and progenitors that will be committed to neuroblasts because of histamine *stimuli*.

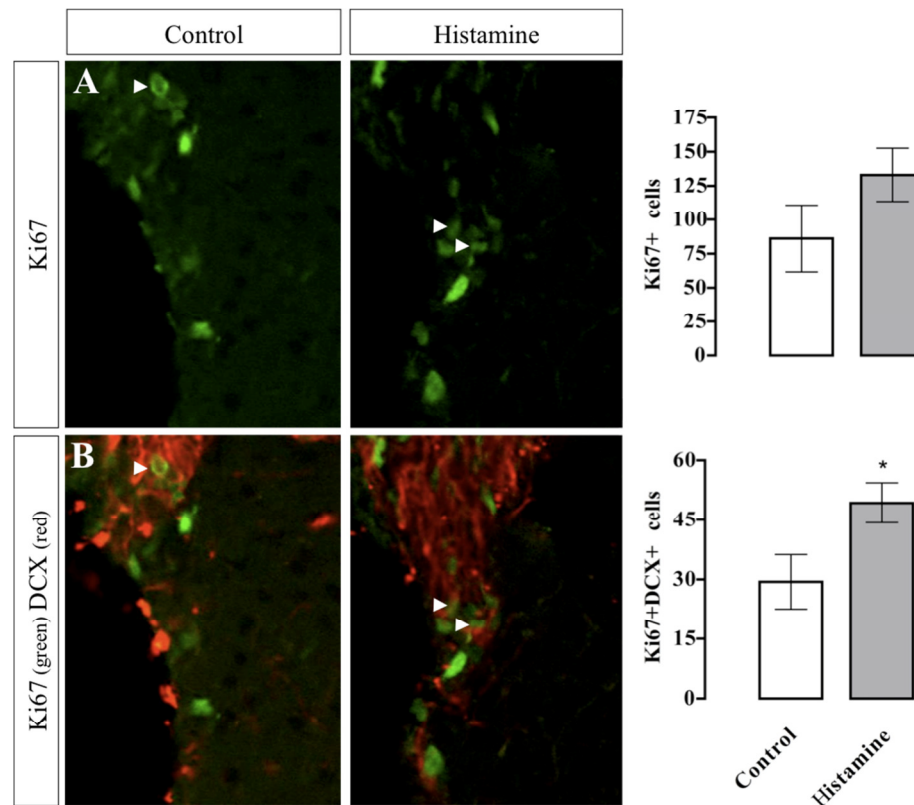


Figure III. 19. Histamine induces an increase in Ki67 and DCX-positive cells in the SVZ of adult animals after 21 days of treatment.

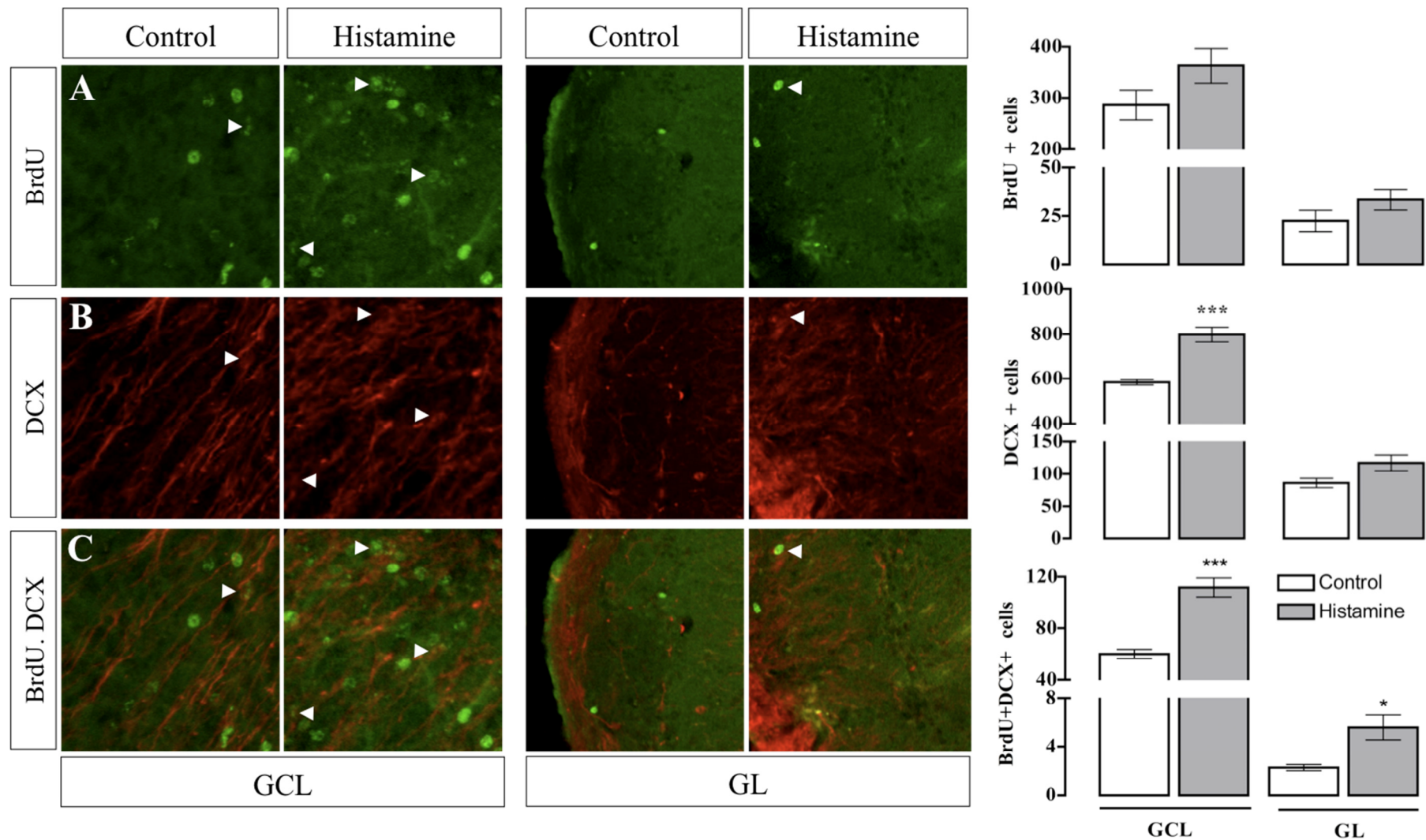
Representative confocal photomicrographs of Ki67-positive (green, **A**) and Ki67/DCX double positive (green/red, **B**) cells in both control and histamine *in vivo* treated animals, 21 days after pump installation. White arrows point to double positive Ki67-DCX cells. Bargraph represents the total Ki67-positive (**A**) and total Ki67/DCX-double positive (**B**) cell numbers counted at the SVZ of control and treated animals. Data are expressed as mean $\pm$ SEM (n=5-6; *, $p < 0.05$). SVZ, Subventricular Zone.

Taken together, our data reveals that histamine is an *in vivo* SVZ key player, mediating cell cycle asymmetric divisions towards neuronal differentiation.

III.3.5.2. Histamine effect on integration and differentiation of new neuroblasts at the contralateral OB

Analysis of the BrdU and DCX expression at the OB revealed a prominent increase of new neurons generated early after histamine-loaded mini osmotic pump installation at both GCL and GL of the contralateral OB (Figure III.20C). This increase in the number of double positive BrdU-DCX cells (Control GCL: 60.00 ± 3.54 ; Histamine GCL: 117.70 ± 7.44 ; Control GL 2.25 ± 0.25 ; Histamine GL: 5.57 ± 1.02) yielded from NSC that were proliferating at the SVZ during the first 3 days of treatment (when BrdU was administered).

Cells were committed to generate new neurons and thus finished their cell cycle divisions and started differentiation and migration through the RMS towards the OB. Since BrdU total cell number is not significantly different upon histamine long-term administration in either layer (Figure III.20A), one can say that although there might be an effect on SVZ cell proliferation, this is not directly reflected in a robust increase of new cells arriving at the OB after 3 weeks, unless they differentiate into DCX-positive neuroblasts. This hypothesis is in line with data stating that if the new cells arriving at the OB do not have a function, they will be “unselected” and die.



III.3.5.3. Comparable effects of histamine in contralateral and ipsilateral hemispheres.

Additionally, to disclose if there was an effect of inherent inflammatory reaction caused by cannulation at the ipsilateral (IPSI, right injected) hemisphere, some animals had both their hemispheres analysed for BrdU and DCX cell counts. The results obtained at the IPSI hemisphere might be biased by inflammatory responses to the injury that cannula implantation produces. Thus, the expected similar results at both CL and IPSI SVZ, since CSF baths both ventricles, may not occur.

The difference observed between CL and IPSI hemispheres in control animals, in which aCSF was delivered, was none (Figure III.21 - IPSI control: 3.50 ± 0.50 ; CL control: 4.00 ± 1.00). Also at the olfactory bulb GCL and GL, there was no statistically significant difference between CL and IPSI. Thus, even with an eventual inflammatory reaction happening at the IPSI hemisphere, in control animals it was not reflected in an increase of BrdU or BrdU-DCX double positive population.

Since control animals do not show any difference in CL and IPSI hemisphere, it was not expected in treated animals either. However, as histamine is an inflammation mediator, its administration could have boosted a reaction that was not observed in control animals. To clarify this possibility, similar analysis was performed in histamine treated animals. The potentiation effect of histamine infusion on the SVZ of the damaged hemisphere was not observed (IPSI histamine: 15.67 ± 1.33 ; CL histamine: 11.33 ± 2.40). Thus, the initial hypothesis that CL hemisphere should be studied instead of IPSI hemisphere because of eventual inflammatory reaction proved to be false. Both hemispheres showed the same relative difference between control and histamine treated animals, excluding any possible bias caused by inflammatory reactions.

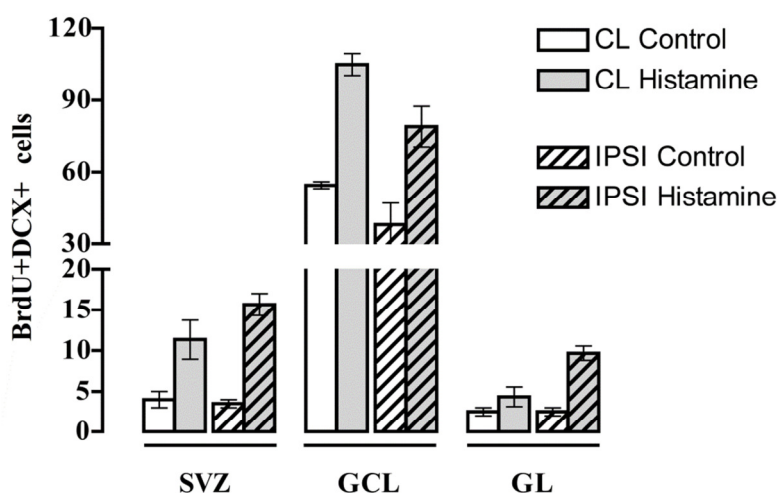


Figure III. 21. Ipsilateral and contralateral effects of mini osmotic pumps installation on BrdU-DCX positive cell population.

Bar graph represents the comparison of ipsilateral (IPSI) and contralateral (CL) total BrdU-DCX double positive cells counted in SVZ, and GCL and GL of the OB of both control and histamine treated animals *in vivo*. Data are expressed as mean $\pm$ SEM (n=3). SVZ, Subventricular zone; GCL, granular cell layer; GL, glomerular layer.

III.4. Discussion

From the time that antihistamines became used in therapy, directly or indirectly because of its sedative action, it became clear that they affect the CNS. Not just as sleep modulators (Uzan, 1979; Tasaka *et al.*, 1989) but also as involved in processes such as seizure convulsion, drug sensitization, stress, memories and learning (Yanai and Tashiro, 2007; Alvarez, 2009). However, the putative effects of histamine on postnatal neurogenesis are still unknown.

Currently, histamine levels in the adult brain are described at the nanomolar range, both at the CSF and brain parenchyma (Khandelwat *et al.*, 1982; Yoshitake *et al.*, 2003; Soya *et al.*, 2008; Kanbayashi *et al.*, 2009; Nishino *et al.*, 2009; Bourgoigne *et al.*, 2011; Croyal *et al.*, 2011). However, under pathological conditions, its levels can be higher since inflammatory reactions trigger *Choroid plexus* mast cells degranulation and consequently, histamine released at the CSF (Soya *et al.*, 2008). Thus, this amine modulates injury-induced neurogenesis since it is on the plethora of soluble factors released at lesioned environments.

Our group has previously showed that immature cells expressing nestin, co-expressing or not GFAP, could be distinguished by the selective increase of $[Ca^{2+}]_i$ as a response to histamine stimuli *via* H1R activation (Agasse *et al.*, 2008b). It was also shown by others that carcinoma and embryonic stem cells express functional H1R, which points to this receptor as being a marker of undifferentiated neural progenitors (Tran *et al.*, 2004). Within this thesis, histamine was found to have no effect on cell death and proliferation but to induce functional neuronal differentiation in SVZ cell cultures through H1R activation.

Besides the contradictory information about histamine effects on proliferation and cell viability, within this thesis no effects were observed at none of these processes. It has been reported that histamine may promote cell proliferation of embryonic stem cells as well as in cancer stem cells (Molina-Hernandez and Velasco, 2008; Pós *et al.*, 2005), and also it can cause impairments in proliferation (Medina *et al.*, 2009) on human malignant melanoma cells, probably due to an increase in hydrogen peroxide levels. These contradictory effects may be explained by both the specificities of each cell type (embryonic stem/progenitor cells versus neonatal SVZ stem/progenitor cells versus cancer stem cells), and the differences in experimental models. Regarding the former, SVZ and tumour-derived neurospheres are heterogeneous mixtures composed of a neural/cancer stem cells and more differentiated progenies, with differential potentials to proliferate, survive and differentiate. It has been reported by others that histamine may also regulate cell proliferation and consequently tumour growth including gliomas, whereas cimetidine can reverse these effects (Pós *et al.*, 2005; LeFranc *et al.*, 2006; Fujikawa *et al.*, 2007). Even if the majority of reports show that histamine promotes tumour growth and proliferation, there are some papers reporting an

opposite effect, probably by increasing hydrogen peroxide levels (Van der Ven *et al.*, 1997; Hernández-Angeles *et al.*, 2001; Medina *et al.*, 2009; Medina and Rivera, 2010). Concerning the last, the differences in experimental models, they can partially explain the opposite effects mediated by histamine since, the same authors (Molina-Hernández and Velasco, 2008) showed in accordance with our data that in differentiation conditions (without bFGF) histamine did not modulate cell proliferation. As mentioned before, and pooling data from different approaches, one can clearly say that histamine does not affect cell cycle in differentiation conditions (without bFGF or EGF) as neither with BrdU, BrdU/Ki67, nor with cell cycle flow cytometry approaches, there were no differences observed.

Histamine promotes a decrease in the number of secondary neurospheres, modifying the self-renewing capacity of SVZ cells. Others showed, in the same line of these results, that NPY promotes SVZ cell culture proliferation but with no effect in increasing the number of secondary neurospheres (Thiriet *et al.*, 2011). Additionally, hepatocyte growth factor has a positive impact on self-renewal, increasing the number of secondary neurospheres, without cell proliferation effects (Nicoleau *et al.*, 2009). Accordingly, using the cell pair assay on freshly dissected and dissociated SVZ immature cells histamine induces terminal division of stem/progenitor cells into more committed/late precursor cells. Indeed, histamine increases the symmetric divisions into two Sox2 daughter cells at the expense of self-renewing divisions giving rise to two Sox2-positive cells. Thus, one can conclude that histamine reduces stemness in SVZ cells, pushing late precursor expansion towards neuronal differentiation.

Regarding neuronal differentiation *in vitro*, no doubts remain about histamine proneurogenic effect: we and others have shown that histamine promotes neuronal differentiation of NSC cultures (Molina-Hernandez and Velasco, 2008; Rodriguez-Martinez *et al.*, 2012; Bernardino *et al.*, 2012). We proved, not just phenotypically but also functionally with the SCCI experiments, that histamine is responsible for an increase in NeuN neurons and also an increase in neuron-like responding cells.

In vivo, SVZ-derived progenitor cells are committed to the OB granule cell and glomerular layers, where they differentiate mainly into GABAergic (Bédard and Parent 2004) but also glutamatergic (Brill *et al.*, 2009) and dopaminergic interneurons (Saino-Saito *et al.*, 2004; Papanikolaou *et al.*, 2008). Herein, it is shown that *in vitro*, postnatal SVZ cells retain the potential to differentiate in GABAergic and glutamatergic neurons, although GABAergic differentiation is favored by histamine treatment. Moreover, tyrosine hydroxylase (TH) immunocytochemistry in differentiated SVZ cultures was also performed. However, with these experimental conditions - 7 days of differentiation upon histamine treatment, no obvious TH immunopositive cells were identified in spite of the later appearance of TH+ neurons (after 15 days under differentiation conditions). Molina-Hernandez and Velasco

(2008) showed that histamine promotes neuronal differentiation in the neuroepithelial stem cells derived from the embryonic rat cerebral cortex *in vitro*, but balanced by a decrease in glial (astrocytic) cell population. On the contrary, we did not observe this shift - GFAP protein levels were not altered.

Neuronal differentiation is a process patterned at the level of epigenetic modifications. Emerging information points to chromatin remodeling and histone modification as mechanisms by which neural stem cells read their fate. Mash1, Dlx2 and Ngn1 are lineage control genes, which are epigenetically activated with a unique combination of histone modifications that make up neuronal cell lineage induction and differentiation (Wen *et al.*, 2009). Consequently, studying the recruitment of H3K4me3 to the promoter regions of these genes disclosed some of the molecular cues of histamine proneurogenic effect. According to the qChIP results, histamine induced the trimethylation of H3K4 on Mash1, Dlx2 and Ngn1 promoters, which ultimately leads to an increase in their mRNA expression levels. In fact, Lim and colleagues (2009) showed that in postnatal mouse differentiating WT SVZ cells, Mash1, Olig2 and Dlx2 loci have high levels of H3K4me3, consistent with their transcription. Mash1 is a key transcription factor essential during embryogenesis, being involved in the production and commitment of neural precursor cells whilst inhibiting their astrocyte potential (Pattyn *et al.*, 2004; Kageyama *et al.*, 2005). Ngn1 was described to be induced in neuronal precursor cells and therefore essential for neurogenesis (Sun *et al.*, 2001). Also Wu and collaborators (2009) showed that the expression of Ngn1 increases drastically in retinoic acid-induced neuronal differentiation of P19 cell lines. Altogether, these reports empower histamine as triggering a signaling cascade that favours histone modifications responsible for the transcription of the mentioned proneurogenic genes in SVZ cells.

Neuronal differentiation and maturation depends also in neurite outgrowth and axon formation. Previously, it was shown by our group that TNF- α and NPY induce axonal formation and maturation in SVZ cells by activating the JNK-MAPK pathway (Agasse *et al.*, 2008a; Bernardino *et al.*, 2008). This pathway is involved in axonal sprouting and neurite outgrowth but not in dendritic growth (Oliva *et al.*, 2006). JNK may mediate axonal outgrowth, microtubule dynamics and neuronal polarization by DCX or MAP-2 phosphorylation (Gdalyahu *et al.*, 2004; Bogoyevitch and Kobe, 2006). Upon histamine treatment, active P-SAPK/JNK immunopositive cells were increased in growth cone-like projections and in axons emerging from the neurospheres, quantified as the number of ramifications per neurosphere. Moreover, the total length of the ramifications was significantly higher in histamine treated cultures. Additionally, JNK immunoreactivity associated with an increased Tau labeling point to histamine as also a player on axonal formation of SVZ cells. Furthermore, histamine-stimulated JNK activation can be also

involved in the initial phases of cell commitment, as observed by cell pair assay in the presence of SP600125, a specific inhibitor of JNK activity. These results are in line with previous reports showing that this pathway is involved in neural differentiation from embryonic stem cells (Amura *et al.*, 2005).

Histamine availability during the 7-day experiments could not be assured. The design of the experiment as single pulse administration should be taken cautiously when extrapolating results to other biological scenarios. *In vivo* drug administration performances take into consideration the long-term drug effect and its kinetics over time. Thus, this approach needed to be updated in order to allow that the observed histamine biological effects within this study can be applied in further therapeutic contexts. For this purpose, a drug delivery system based on a biocompatible biomaterial was developed.

PLGA, an FDA approved biopolymer routinely used for therapeutic devices and extensively investigated for the preparation of drug delivery formulations (Jain *et al.*, 2000; Ferreira *et al.*, 2008; Mungardi *et al.*, 2008) was chosen. It is advantageous over other biomaterials because of its inherent biocompatibility, degradation at physiologic pH and release rates up to days, weeks or months (Mundargi *et al.*, 2008). It degrades in aqueous solutions into lactic and glycolic acid, by-products of its metabolic pathways. In this context, biocompatible histamine-releasing PLGA microparticles represent an advantage in the drug delivery mechanism since this amine is released in a continuous manner and in concentrations that are closer to physiological levels at the SVZ neurogenic niche.

Microparticles with an average size of 2 μm could release histamine in a controlled and sustained fashion at the extracellular *milieu* without affecting cell death, neither in its loaded nor in its blank (unloaded) formulations. PLGA microparticles were not only non-toxic but also had no effect on cell proliferation or cell cycle. On the other hand, histamine-releasing microparticles induced robust neuronal differentiation from SVZ cell cultures, as observed both by functional characterization with SCCI, and by phenotypic characterization with immunohistochemistry. In addition, this effect was comparable to the one observed with single pulse experiments but at a concentration range of approximately 1000 times lower. This is likely due to the spatio-temporal controlled delivery of histamine to the cells. Microparticles tend to locate at the proximity of cell membrane, creating a gradient of histamine. Therefore, low amounts of histamine are required to arouse the same biological effect. Similar results have been obtained previously in the vascular differentiation of human embryoid bodies (Ferreira *et al.*, 2008). The incorporation of polymeric biodegradable particles loaded with specific growth factors into human embryoid bodies had a minimal effect on cell viability and proliferation but a great impact on vascular differentiation. Therefore, microparticles constitute a novel approach for drug delivery systems able to promote brain repair by local and continuous delivery of histamine into neurogenic niches.

Moreover, histamine released from the PLGA microparticles facilitated the differentiation of grafted eGFP-SVZ neurospheres in *ex vivo* and *in vivo* scenarios. Our *ex vivo* data showed that the pre-exposure of eGFP-SVZ cells with histamine-releasing microparticles increased neuronal differentiation of grafted cells in the DG of hippocampal slices. These data support the concept that pre-treatment of neural stem cells with known proneurogenic factors favours neuronal differentiation of transplanted cells. *In vivo*, a comparable approach showed a more discrete but still positive effect, on survival, integration and differentiation of the pre-treated transplanted cells into the host hippocampus or striatum. Previous studies have shown that in the intact adult rodent brain, expanded SVZ cells *in vitro* preferentially produce neurons upon transplantations in homotypic (SVZ) and heterotypic (SGZ) neurogenic niches, whereas upon transplantation in non-neurogenic regions such as the cortex or the striatum, SVZ cells generate mainly glial cells (Richardson *et al.*, 2005; Seidenfaden *et al.*, 2006). These transplantation approaches indicate that the neuronal phenotype determination is tightly regulated by local signals derived from both, the SVZ and SGZ neurogenic niches. As proof of principle, we showed that eGFP-SVZ cells pre-treated with histamine-loaded microparticles differentiate into new neurons at both neurogenic (DG) and non-neurogenic (striatum) niches.

Like PLGA microparticles release histamine at a slow but constant rate, inducing comparable results as single pulse high histamine concentration *in vitro*, osmotic mini pumps perform the same task but *in vivo*. Since ip administration of histamine would probably have biological effects all over the organism, and likely it would not cross the BBB (Theoharides and Konstantinidou, 2007), a drug delivery system able to circumvent the BBB passage without the need for frequent handling of the animal for administration through a canula is mandatory. Osmotic mini pumps were designed for this task. When implanted in the lateral ventricle, they locally deliver the compound with which they were loaded at a constant rate, targeting directly the lateral walls of ventricle; and through diffusion *via* the CSF the compound will bath the SVZ of both ventricles. Hence, the use of these pumps fits perfectly in our experimental plan to disclose histamine proneurogenic effect at the SVZ *in vivo*.

Prolonged histamine icv infusion at the lateral ventricle for 21 days induces a trend increase in the number of BrdU retaining cells at the SVZ. This increase is statistically significant if we consider just the DCX-positive BrdU-retaining cells. Since BrdU was administered long before the perfusion of the animals, right after the pump installation, this increase points to an effect in the asymmetric divisions of NSCs that, with time differentiated into new neuroblasts. This data confirms the previously obtained *in vitro* data (cell pair assay) identifying histamine as a factor of cell commitment in the fate of a NSC to become a differentiated neural cell. To confirm if histamine had an effect in cell proliferation, Ki67 labeling was performed, since this cell marker is associated with G1, G2, S and M phases of

cell cycle. At the SVZ, as with BrdU, Ki67 labeling had a trend for an increase upon histamine treatment that was clearer when looking at DCX-Ki67 double positive cells. Again, as before, histamine is effective in increasing the number of neuroblasts traced to be/have been proliferating at the SVZ.

In order to disclose, if the tendencies observed for both BrdU and Ki67 increase relate with any real increase in proliferation of NSCs or if it was caused, for instance, by an inflammation effect (microglia increase, for instance) we performed a detailed phenotypic characterization. Although there is no unique marker for NSCs, combinations of antibodies can be used to identify the phenotype of BrdU retaining cells at the SVZ with all its advantages and pitfalls (reviewed in Taupin, 2007). Since adult NSCs resemble embryonic radial glia, they both express glial markers such as GFAP and GLAST, and also markers associated with undifferentiated state such as NESTIN, BLBP (brain lipid-binding protein) or the stem cell-related transcription factor SOX2 (Doetsch *et al.*, 1997; Lagace *et al.*, 2007; Platel *et al.*, 2009). Alternatively, others point Prominin-1, Lewis X and an increased ALDH activity as markers of quiescent, slowly dividing and rapidly dividing NSPC populations (Obermair *et al.*, 2010). Thus, as for future prospective plans, a combination of two or more of the above-mentioned markers, as well as CD11b (labels microglia, so that inflammatory responses can be excluded), needs to be performed in order to identify radial glia-like NSC increase.

When looking at the fate of the newly differentiated neuroblasts that leave SVZ towards the OB, it is clear that they reach their destination and that they were not fully eliminated: NSCs labeled with BrdU that go through asymmetric division, differentiate into migrating neuroblasts that reach the OB in about 1 week (Petreanu and Alvarez-Buylla, 2002), and are still visible at both layers of the OB in control and more densely in histamine treated animals. Without doubt, histamine is responsible for the increase in new neurons differentiation at the SVZ, which will ultimately integrate the OB as functional interneurons. Most adult SVZ derived cells differentiate into GABA-containing granule cells (approximately 95%) while the rest differentiate into periglomerular cells in the glomerular layer (Luskin, 1998). Although we do not know if the newly generated DCX-positive neurons will be mature GABAergic neurons, we do know that, as expected, the majority of them differentiated into SVZ-derived cells located at the GCL instead of the GL.

In vivo, there are not yet reports of histamine as a proneurogenic factor. On the other hand, there are promising evidences of histamine involvement in tumours, especially in gliomas. Regarding the similarities between NSCs and cancer stem cells, our data from *in vivo* experiments can have some support, although still being very innovative and breakthrough. Experiments performed in malignant cell lines and experimental tumours *in vivo* suggest that histamine modulates diverse biological responses related to tumour growth,

including proliferation (Medina *et al.*, 2008, 2009; Massari *et al.*, 2011; Francis *et al.*, 2012), survival (Fukuda *et al.*, 2008; Jiang *et al.*, 2010), modulation of inflammation and angiogenesis (Cianchi *et al.*, 2005).

In conclusion, we describe here the comprehensive study of histamine proneurogenic effect at the SVZ, *in vitro*, *ex vivo* and *in vivo*. We demonstrated that histamine is responsible for an increased neuronal differentiation *in vitro* (GABAergic phenotypes favoured), regardless of an expense in glial cells and not affecting cell death, proliferation or cell cycle. Furthermore, we showed that histamine proneurogenic role is effective early in cell fate as it modulates asymmetric cell division. This favoured neuronal differentiation of the NSCs at the SVZ is not an isolated cell-fate effect of histamine. This amine also increases axonogenesis by JNK MAPK activation. The switch from NSCs to neuronal cells is signalled epigenetically at the proneurogenic genes Mash1, Dlx2 and Ngn1. Moreover, biocompatible histamine-loaded microparticles are able to induce comparable *in vitro* effects. As a proof of concept before moving to *in vivo*, we primed SVZ cells with histamine-releasing microparticles and transplanted them into organotypic hippocampal slices and into adult mouse brains. At both paradigms, pre-treated transplanted cells were able to survive, differentiate and integrate at the host tissue. Finally, when histamine was infused at the lateral ventricle of adult animals for a prolonged period of time, there was also an increase in the differentiation of new neuroblasts at the SVZ, as well as at the neuroblasts that reach the OB when triggered by histamine. Taken together, these data clearly indicate that histamine has an important role in modulating adult neurogenesis and thus, shall be further studied for brain repair therapeutic purposes.

CHAPTER IV
ENDOTHELIAL BDNF CONTRIBUTION TO
CEREBELLUM POSTNATAL DEVELOPMENT

The work presented in this chapter was developed at the Cellular Neurobiology Unit from the *Centre de Recherche Université Laval Robert-Giffard* (Québec, Canada), under the direct supervision of Professor Armen Saghatelyan.

IV.1. Abstract

In the adult mammalian brain, neurogenesis occurs constitutively in the SVZ and SGZ of the hippocampus. These neurogenic niches are sources of new neurons that functionally integrate existing circuits. The proper development of the brain depends thus on the balance between the continuous neurogenesis and on the correct final placement of these cells. Thus, their mechanisms of migration are equally crucial to a correct integration and brain function.

Within this chapter, the endothelial BDNF contribution to cerebellum neuronal cell migration and development was unraveled. BDNF with endothelial cell origin is crucial for early cerebellum postnatal cell placement, as well as cellular layer organization and folia formation. A transgenic animal line was used to disclose the effects of endothelial BDNF removal on cell death, proliferation and migration. With a time-lapse imaging technique, the migration parameters of WT and KO animals were investigated. Protein and mRNA levels were quantified to check the contribution of this cellular source of BDNF to the global pool of this neurotrophin. Moreover, being the focus of this chapter the endothelial source of BDNF, a detailed study of the vasculature structure was also performed to check for autocrine changes that indirectly might have affected cerebellum precursor cell placement throughout postnatal development.

In conclusion, in this chapter we show that blood vessels secreting BDNF have an important role in cerebellum postnatal development: endothelial BDNF drives GCP migration, possibly by controlling cell cycle exit and entry in differentiation and migration phases. The changes observed on the vasculature *per se*, by BDNF endothelial removal, may also contribute in an indirect way to the overall cerebellum developmental changes.

IV.2. Introduction

Neurogenesis, as the new generation of neurons in the brain, occurs in the mammalian adult brain, mainly in two restricted regions: the SVZ lining the lateral ventricles and the SGZ in the DG of the hippocampus. However, in the past decades, dedicated research reported that, not only newly born neurons are generated in the adult brain, but also that neuronal precursors migrate towards their final target place within the brain, where they differentiate and functionally integrate. The vast majority of cells migrate at embryonic ages, however, in some few regions of the postnatal brain such as OB and cerebellum, neuronal displacement still occurs. Also, recent evidences of vasculature-guided migration have emerged. The close association of neural precursors with blood vessels (Bovetti *et al.*, 2007; Snapyan *et al.*, 2009; Whitman *et al.*, 2009) has lead to the concept of *vasophilic migration* (Saghatelian, 2009). This close and constant association indicates that important clues from endothelial cells are sensed by neuronal precursors.

BDNF is a member of the neurotrophin family: a class of five related growth factors – NGF, NT-3, NT-4, NT-6 and BDNF (reviewed in Lewin and Barde, 1996). They are structurally related and mediate their actions through binding to two classes of cell surface receptors, p75NTR and Trk family of tyrosine kinases (Chao and Hempstead, 1995). BDNF role in neuronal migration during development has already been addressed (Polleux *et al.*, 2002; Marín and Rubenstein, 2003) and endothelial BDNF has also been shown to foster migration of neuroblasts in RMS (Snapyan *et al.*, 2009). However, its role in the migration of GCP in the cerebellum, where the massive migration in the postnatal brain occurs, has not yet been addressed. Moreover, the early postnatal lethality of BDNF full KO animals suggests a wider function for this neurotrophin.

In early postnatal cerebellum development, GCP present at the EGL are highly proliferative cells that migrate into the IGL, where they will constitute the most abundant cell population of the cerebellum (Hatten, 1993; 1999). GCP migrate along Bergmann glia cells and receive cues from the environment, such as BDNF. The role of this neurotrophic factor to the migration of GCP from EGL to the IGL is well established (Schwartz *et al.*, 1997; Boghesani *et al.*, 2002; Zhou *et al.*, 2007), being that BDNF deficient mice display disrupted cerebellum foliation pattern, EGL persistence, and abnormal coordination and ataxia (Jones *et al.*, 1994; Schwartz *et al.*, 1997). However, the role of BDNF on cerebellum development and granule cell migration has always been related to a neuronal gradient of this neurotrophin. Recently, Rauskolb *et al.* (2010) showed that conditional deletion of neuronal BDNF has only slight alterations in neuronal circuitries, leaving an open window for other BDNF sources. Moreover, endothelial BDNF has been shown to have a role on angiogenesis (Leventhal *et*

al., 1999) and on the crosstalk angiogenesis-neurogenesis (Louissaint *et al.*, 2002; Guo *et al.*, 2008). Thus the contribution of endothelial BDNF for the proper brain development and cerebellum correct foliation pattern needs to be clarified.

The disclosure of endothelial BDNF role to the cerebellum development will lead to important advances on the knowledge of brain development and cerebellum granule cell migration. In this chapter, we unraveled the role of BDNF from endothelial cell origin to cerebellum development, through the generation of a new mouse line with BDNF excision mediated by Cre-recombinase, which was inserted in the VeCadherin locus – a gene expressed in endothelial cells. Detailed analysis of these mutant animals revealed an unexpected complete removal of this neurotrophin responsible for the big phenotypic and morphological changes in cerebellum development. Cell cycle exit and migration onset control rely on this cell source specific BDNF pool. Although there is no change on proliferation and cell survival, GCP remain at the EGL through cerebellum development leading to a deregulation of layer thickness and consequent abnormal cerebellum development. This data, together with the vascular changes observed, highlight the fundamental role of endothelial BDNF to the proper cerebellum development.

IV.3. Results

The major objective of this chapter was to examine the impact of BDNF endothelial deprivation at early postnatal ages. For that, we firstly characterized BDNF mRNA endothelial expression, then we generated and characterized an animal line in which BDNF was selectively removed from endothelial cells (VeCre::BDNF^{loxP}). As KO mice were not viable more than 18 to 21 days, and they showed big phenotypic changes when compared to WT, a detailed cellular characterization of the cerebellum was performed at P11 to understand which was the role of endothelial BDNF in the normal cerebellum postnatal development.

IV.3.1. Endothelial BDNF

Although the vast majority of publications defended neurons as the only source of BDNF, there are already some evidences pointing to the importance of endothelial BDNF not just on angiogenesis but also on the angiogenesis-neurogenesis crosstalk (Leventhal *et al.*, 1999; Louissaint *et al.*, 2002; Kermani and Hempstead, 2007). These data suggest that endothelial cells are a possible source of BDNF, which plays an important role in neurogenesis. To clarify this possibility, we needed to access if endothelial cells express BDNF mRNA at postnatal developmental ages. *In situ* hybridization for BDNF mRNA was performed together with immunohistochemistry for PECAM, a marker of endothelial cells.

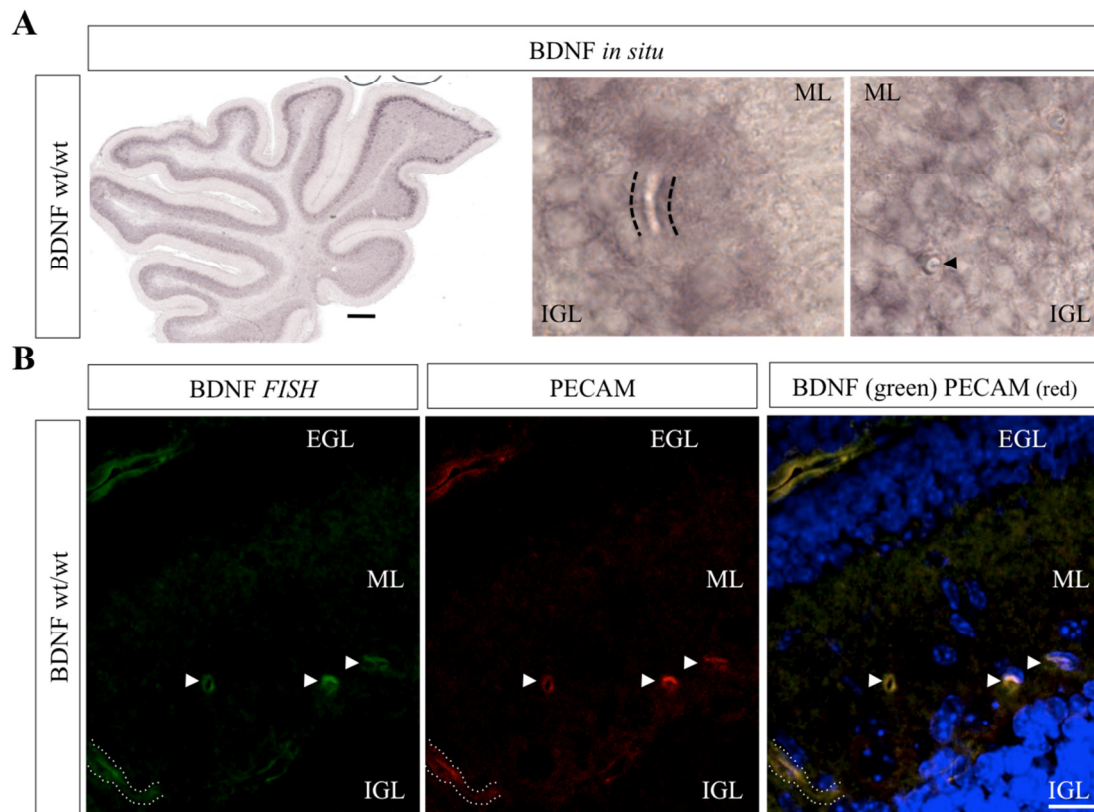


Figure IV. 1. BDNF mRNA expression in P11 WT animals' blood vessels.

A. *In situ* hybridization for BDNF mRNA. Right panels show details of BDNF mRNA-positive blood vessels. Scale bar = 100 μ m. **B.** Fluorescent *in situ* hybridization for BDNF mRNA (green) and immunostaining for PECAM (red). Cell nuclei (blue) stained with DAPI. Dash lines highlight sagittally cut BDNF-positive blood vessels; arrowheads point to coronally cut BDNF-positive blood vessels. Scale bar = 10 μ m. FISH, Fluorescent *in situ* hybridization; EGL, external granule cell layer; ML, molecular layer; IGL, internal granule cell layer.

As it can be seen in Figure IV.1A, there is clear BDNF mRNA expression by granule cells at the IGL. Little or no BDNF mRNA is observed at the ML, and more importantly, high magnification images revealed that BDNF mRNA was expressed in blood vessels (detailed images on the right panel from Figure IV.1A). Trace lines and arrowheads point to sagittally and coronally cut BDNF-positive blood vessels, respectively. Moreover, once *in situ* hybridization was combined with cell specific immunolabeling for blood vessels (Figure IV.1B), all BDNF mRNA positive blood vessels were also PECAM-immunoreactive (arrowhead and trace line). At this point, no doubts remained about the BDNF mRNA expression by endothelial cells in the postnatal cerebellum.

IV.3.2. Generation and characterization of endothelial BDNF knockout animals

Having shown that BDNF mRNA is highly expressed by cerebellum endothelial cells at this developmental age, we devoted to characterize a transgenic animal model in which BDNF was exclusively removed from endothelial cells.

IV.3.2.1. Phenotypic characterization

At birth, KO animals were of equal size as their WT littermates. However, during the first postnatal week their growth was impaired and they stayed behind WT in size (Figure IV.2A). They did not gain weight as WT (Figure IV.2B) and displayed bursts of hyperactivity, in which they moved in circles, intercepted with long periods of inactivity. Additionally, when they moved, they showed abnormal gait and severe motor coordination impairments (Figure IV.2C).

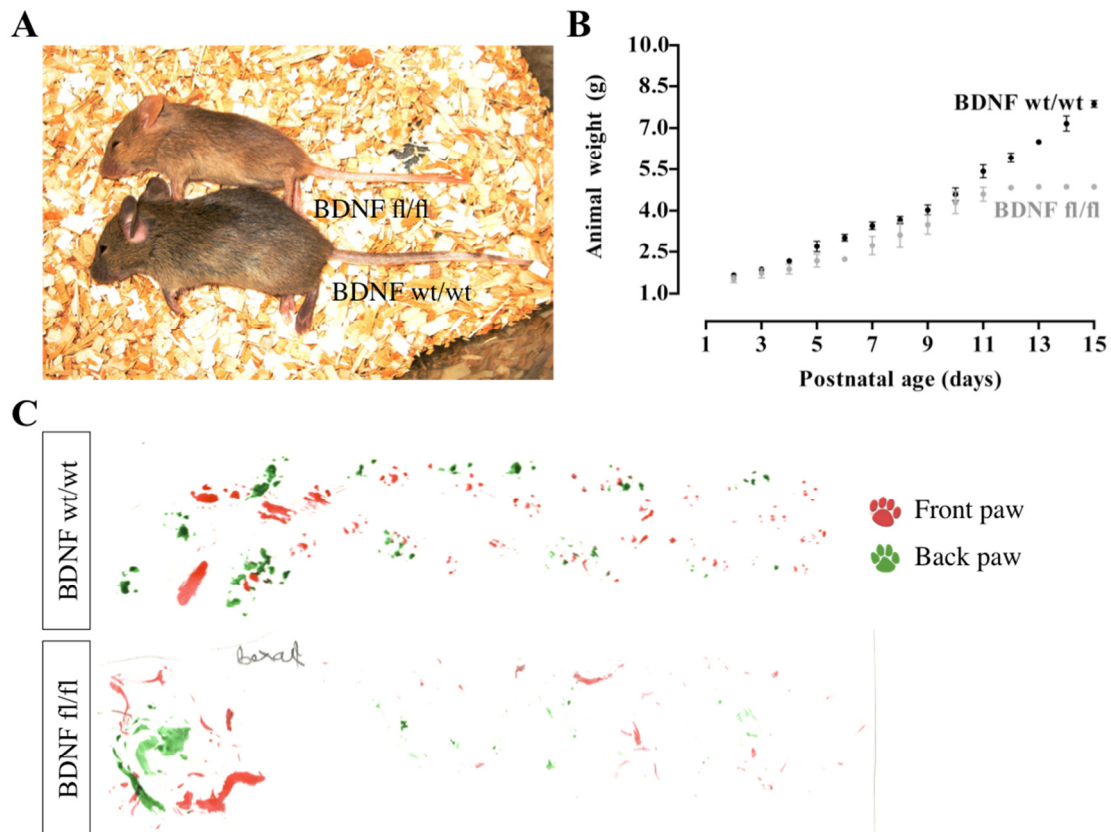


Figure IV. 2. Phenotypic characterization of VeCre::BDNF colony.

A. Size comparison of WT and KO animals at postnatal day 11 (P11). **B.** Weight increase during postnatal development. Data are expressed as mean $\pm$ SEM (n=3-5). **C.** Paw-print test comparing the path and coordination of front (red) and back (green) paws of WT and KO animals.

Consistent with their growth pattern, mutant mice do not survive more than 18 to 20 days. Heterozygote animals had normal phenotype, motor coordination and growth, being undistinguishable from their WT littermates, even at the second postnatal week. With regard to gender, no differences were observed in behavior or weight gain between males and females.

IV.3.2.2. Specificity of Cre recombinase expression

The phenotype observed in mutant animals, similar to the one reported for full BDNF deficient animals (Schwartz *et al.*, 1997; Jones *et al.*, 1994), impelled us to check if the mice

under study had any leakage on Cre-recombinase expression, and if the BDNF excision was truly specific for endothelial cells.

Dextran Texas-Red, a fixable heavy (70,000MW) hydrophilic polysaccharide dye was used to label functional blood vessels. We observe that Dextran Texas-Red filled blood vessels express Cre-recombinase (Figure IV.3A) and that no other cellular structures were immunopositive for Cre-recombinase. A full match was observed between the Dextran Texas-Red and the immunolabeling. Furthermore, by crossing VeCre animals with Z/EG reporter mice, endogenous GFP expression was observed every time recombination occurred (Novak *et al.*, 2000). Thus, as it can be observed in Figure IV.3B, these double reporter animals had basal GFP expression co-localizing with Cre-recombinase expression, in a blood vessel pattern. Additionally, regardless of the basal GFP expression, we performed GFP immunolabeling to enhance any possible low signal away from blood vessels. The right most panel of Figure IV.3B clarified that Cre-recombinase expression was exclusive from endothelial cells, since no enhanced immunoreactivity was observed else where than in blood vessel.

Given that Cre expression was specific from endothelial cells and that LoxP sites were flanking exon IX, the only common coding exon from all BDNF splice variants, we were sure that the animal model being used was a conditional (VE-cadherin - endothelial) BDNF KO, without any leakage of Cre expression. Therefore, the phenotypic changes observed were exclusively due to the endothelial BDNF removal.

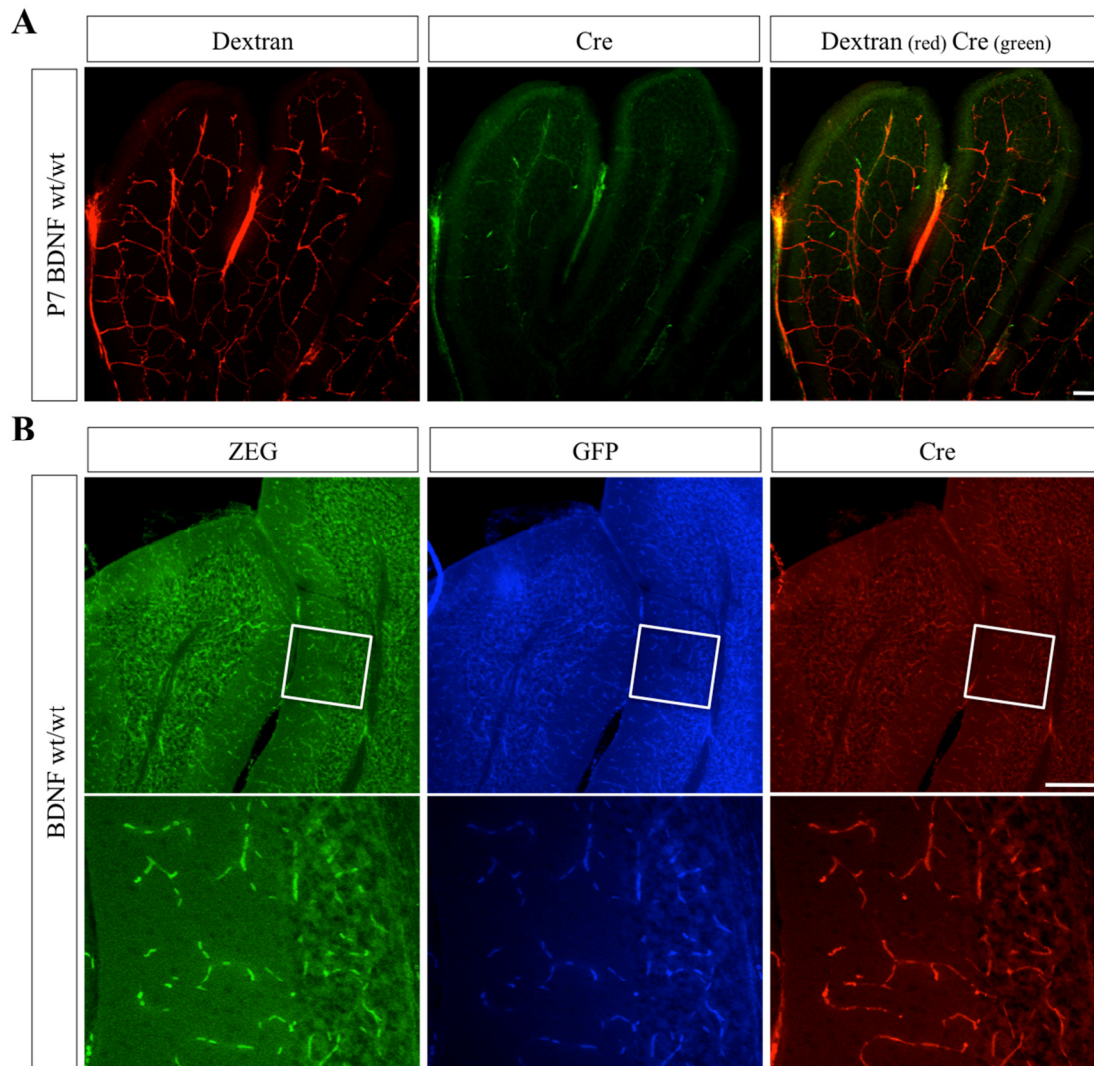


Figure IV. 3. Cre-recombinase expression in blood vessels of transgenic animals.

A. Confocal microscopy representative pictures of Dextran Texas-Red filled blood vessels (red) and immunostaining for Cre recombinase (green) in P7 wild type pups. **B.** VeCre::ZEG double reporter animal confocal representative pictures for basal GFP expression (green), Cre recombinase (red) and amplified GFP signal (blue). Scale bar = 50 μ m. Lowest panel are high magnification pictures showing the blood vessel pattern.

IV.3.2.3. Cerebellum morphological characterization

Gross anatomical changes were expected at these mutant animals' cerebellum. Considering that full KO animals have the same phenotype as the animal model being used, similar morphological changes were anticipated. To analyse so, cresyl violet staining was performed at early postnatal ages (P12, P15, P23) on sagittal sections of both KO animals and their WT littermates. As expected, abnormal foliation pattern and swaller fissures separating different folia were observed in KO animals (Figure IV.4). At the cellular level, the three layers displayed abnormal thickness in KO animals as compared to WT (Figure IV.4, high magnification picture). The thickness of EGL is dramatically increased, whereas ML and IGL are decreased. This cell accumulation, responsible for the abnormal

development of the cerebellum layers, might be due to GCP death, proliferation or migration defects. Other cellular changes – at the level of Bergmann glia, Purkinje cells or even vasculature structure – may also indirectly affect GCP correct differentiation, migration and placement.

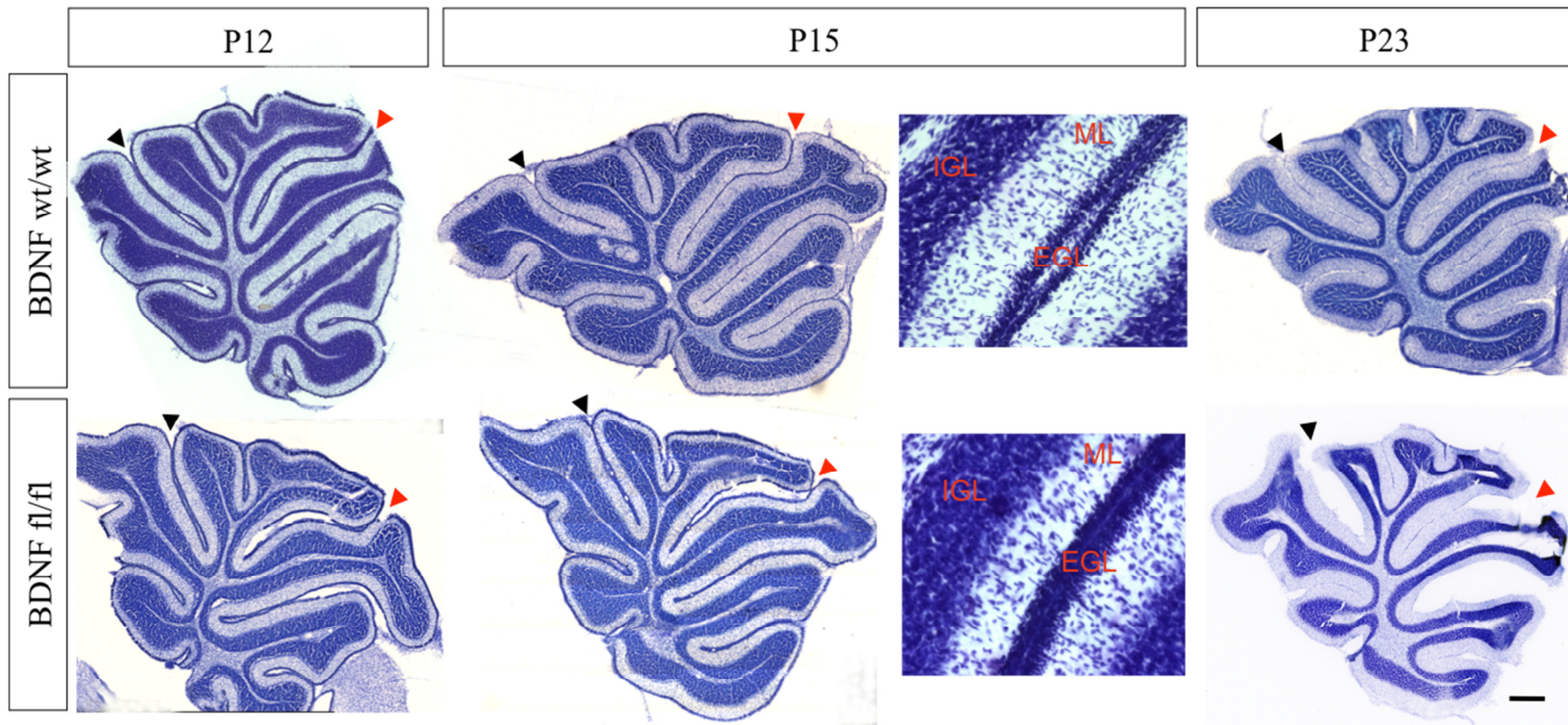


Figure IV. 4. Cerebellum cresyl violet staining on WT and KO BDNF animals from different postnatal ages (P12, 15 and P23).

Zoomed panel is a detailed view from PRF of both WT and KO P15 animals. Red arrows point to primary fissure, black arrows point to secondary fissure. Scale bar = 100 μ m. EGL, external granule cell layer; ML, molecular layer; IGL, internal granule cell layer.

IV.3.3. Role of endothelial BDNF to cerebellum post-natal development

IV.3.3.1. BDNF mRNA is almost absent in P11 knockout animals

Being that the phenotypic and anatomical changes observed at the cerebellum of these conditional KO animals resemble in all the ones reported for the full BDNF KO animal, BDNF mRNA reduction level was quantified by Reverse Transcription-PCR.

BDNF mRNA levels from total cerebellum extracts were drastically dropped off. BDNF mRNA was barely detected by the quantitative Reverse Transcription-PCR in the KO animals (Figure IV.5A - BDNF wt/wt: 1.00 ± 0.29 ; BDNF wt/fl: 0.54 ± 0.14 ; BDNF fl/fl: 0.13 ± 0.13). GAPDH, the housekeeping gene, was detected at approximately the same cycle number in WT and KO animals (data not shown), which eliminated any doubt about the sample quality. Moreover, we performed a sensitivity test: we used dilution series of a WT sample mix to determine the detection limit of the method. The very low 1:500 dilution was still detectable with an exponential phase starting around the acceptable 35th cycle of amplification (Figure IV.5B). This experiment confirmed that the lack of BDNF mRNA detection was not an artefact but a real complete removal of BDNF upon endothelial BDNF.

So far, data pointed to a full dependence on endothelial cells as source of BDNF at early postnatal ages. Therefore, to clarify this complete removal of BDNF mRNA, we performed FACS to isolate endothelial population from all other cell types and analyse their BDNF mRNA content. To shortcut the labeling protocol and optimize the sorting yield, we used a CC-GFP reporter animal crossed with a Tie2::Cre animal. These animals are endowed with all endothelial Tie2-positive cells endogenously labeled with GFP, which will be detected by the sorter as clear FITC-positive signal avoiding labeling procedures that doubtless loose cells (Figure IV.5C). When we compare the BDNF mRNA content of the two populations - depleted and enriched in endothelial cells - relative to a non-sorted population (set to zero, BDNF wt/wt whole tissue) it is obvious that the cellular source of BDNF mRNA at this developmental age is exclusively endothelial cells. Endothelial enriched population have a relative BDNF mRNA content of 7.87 (n=1), while non-endothelial cells have a BDNF mRNA relative level of -5.71 ± 0.46 (n=2). These unexpected data, regarding the majority of published data mentioning BDNF neuronal source, are very promising for our endothelial cell source hypothesis. However, more animals should be added to this experiment, together with KO animal samples to strengthen the data.

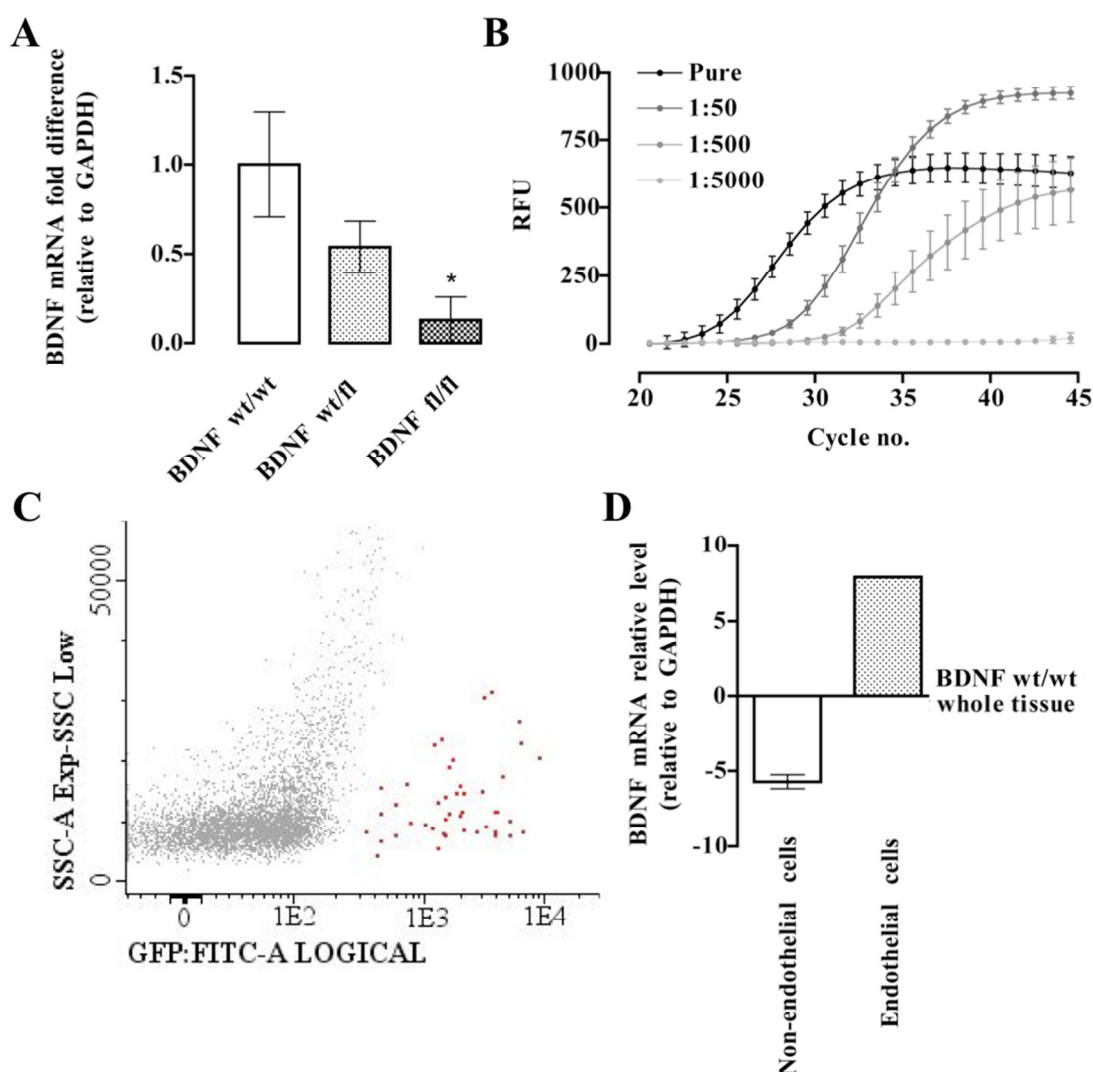


Figure IV. 5. BDNF mRNA decrease in KO animals, measured by quantitative Reverse Transcription-PCR. **A.** Bargram depicts the total cerebellum RNA extracts fold difference in total *BDNF* mRNA levels ($n=4-5$; *, $p < 0.05$). **B.** Amplification plot illustrating the detection limit of quantitative Reverse Transcription-PCR assay accessed with a dilution series of WT RNA sample mix. **C.** Dot plot showing GFP-labeled endothelial cells (represented as red dots) identified by low forward (not shown) and side-scatter (SSC) characteristics, together with high FITC signal. Cell debris and death cells were excluded based on scatter signal. **D.** Bargram depicts the FACSsorted cell specific extracts relative mRNA difference in total BDNF. Enriched populations were normalized to whole tissue sample. Data are expressed as mean $\pm$ SEM ($n=1-2$). RFU, random fluorescent units.

IV.3.3.2. BDNF protein levels are decreased but still present at P11 mutant animals

Having shown that BDNF mRNA at P11 is almost exclusively from endothelial cell origin, we wanted to investigate the level of BDNF protein expression.

BDNF protein expression, at the cellular level, could not be accessed by immunohistochemistry. The labeling pattern was dotted (Figure IV.6A), since it is a secreted factor, and thus we would not be able to state if the BDNF being observed was from endothelial or neuronal origin. For this reason, although western blotting could not clarify us on the cellular source, it was adopted to more accurately quantify BDNF protein levels.

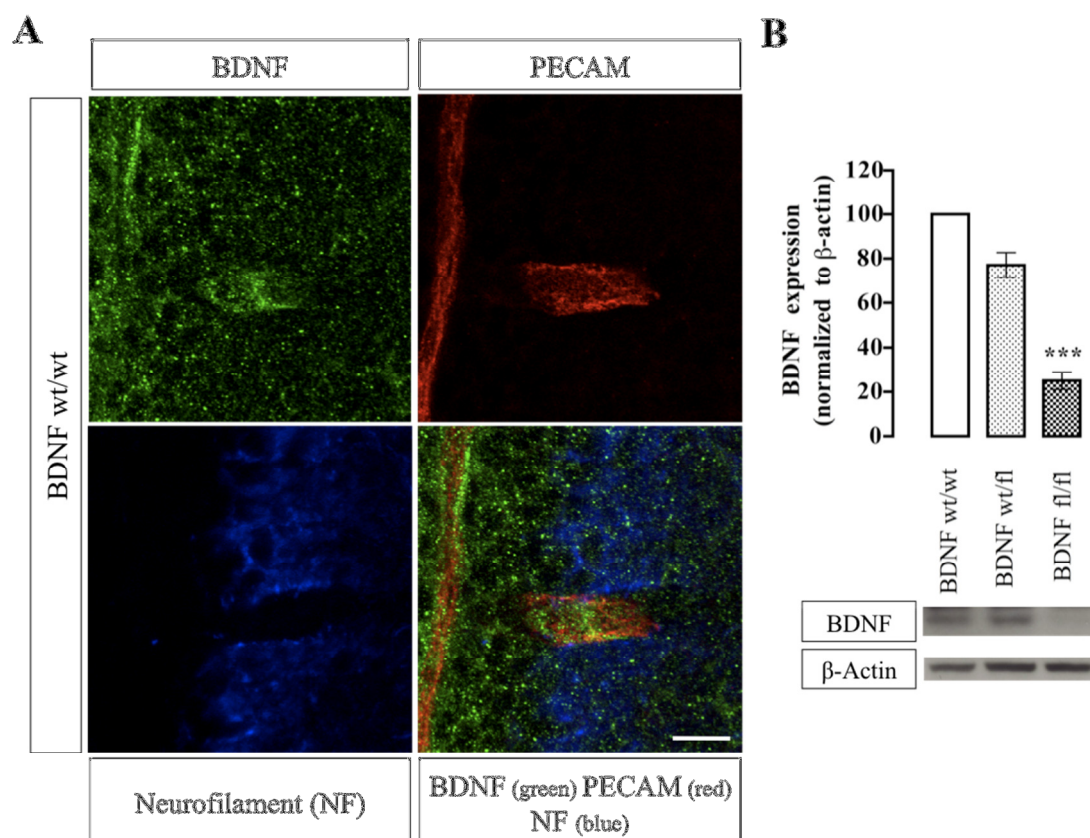


Figure IV. 6. BDNF protein expression, and reduction pattern upon its endothelial removal.

A. Immunostaining for BDNF (green), PECAM (red) and Neurofilament (blue, NF) on P11 WT animals at the level of ML of primary fissure. Scale bar = 10 μ m. **B.** Bargraph depicts the relative reduction of matBDNF normalized to β -Actin in HET and KO animals relative to their WT littermates, quantified by western blotting. Below the graph, representative bands illustrate the protein expression level reduction in animals with the corresponding genotypes. Data are expressed as mean $\pm$ SEM (n=2-3; *** p < 0.0005). ML, molecular layer.

Surprisingly, after the nearly complete removal of BDNF mRNA on KO animals, we still detected a faint band in total cerebellum protein extracts (Figure IV.6). Protein levels showed a similar gene dose-dependent pattern as mRNA. Heterozygotes had a decrease in protein content ($77.06 \pm 5.69\%$, relative to 100% in WT), but it was in KO animals that the effect was maximized ($25.04 \pm 3.99\%$ relative to 100% in WT). It is possible that, with almost no BDNF mRNA present at the cerebellum, the BDNF protein there present has other origin than local. It might be then then transported into the cerebellum, giving rise to positive protein levels whilst no mRNA is present.

IV.3.3.3. Vasculature characterization upon conditional BDNF excision

BDNF, as well as other neurotrophins have been reported as fundamental for angiogenesis. For this reason, it was mandatory to study the eventual effects of BDNF conditional, but drastic, removal on the blood vessel structure/functionality within the cerebellum.

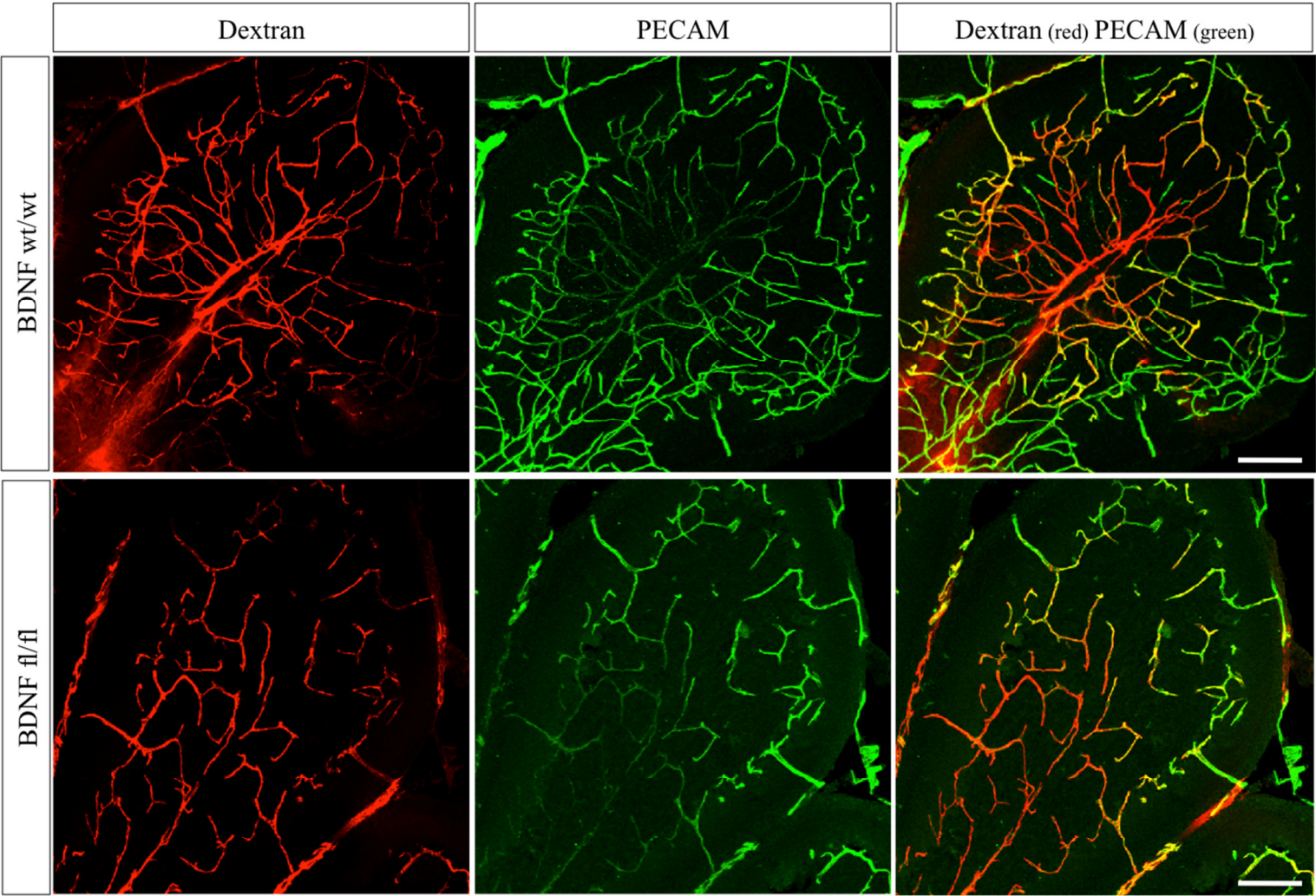


Figure IV. 7. Detail of cerebellum vasculature characterization in P11 WT and KO animals.
Confocal microscopy representative pictures of Dextran Texas-Red perfusion (red) and immunostaining for PECAM (green) at the primary fissure of both WT and KO animals. Scale bar = 50 μ m.

Dextran Texas-Red filled blood vessels fully matched PECAM immunopositive blood vessels (Figure IV.7). The observation of this labeling pattern, however, made clear that some alterations in the blood vessel structure was happening at the KO animals. It could not be the loss of functional blood vessels, as there are no PECAM positive/Dextran Texas-Red negative vessels, but it appeared that there were less blood vessels and/or its overall structure was less complex.

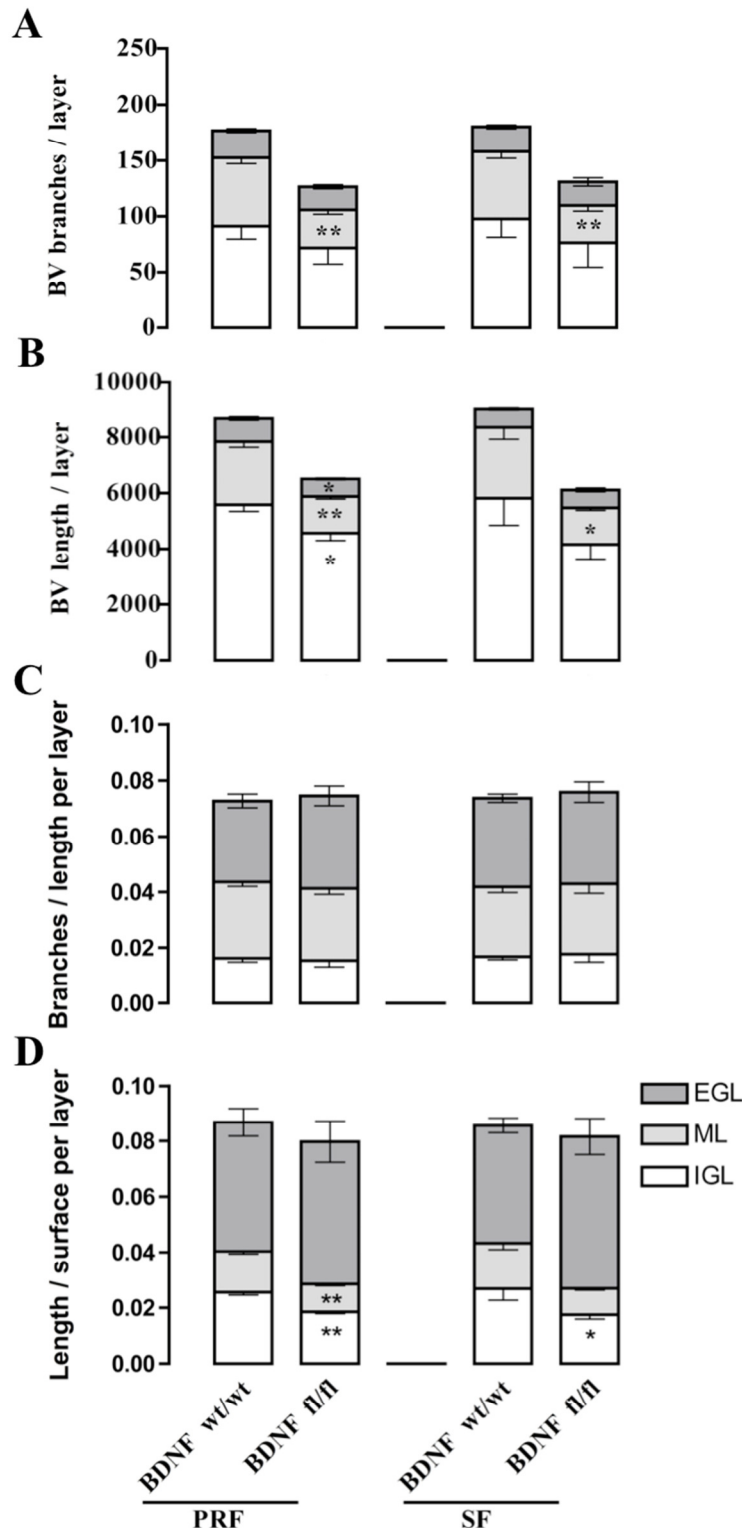


Figure IV. 8. Blood vessel complexity measurements at PRF and SF.

A. Plot depicts the blood vessel branches quantified in each layer. **B.** Plot represents the quantification of the blood vessel length in each cerebellum layer. **C.** Plot represents the normalization of blood vessel branches by their length, per layer. **D.** Plot characterizes blood vessel length normalized by the area of the respective layer of the cerebellum. Data are expressed as mean $\pm$ SEM (n=3-5; *, p < 0.05; ** p < 0.01). PRF, primary fissure; SF, secondary fissure; EGL, external granule cell layer; ML, molecular layer; IGL, internal granule cell layer.

To disclose whether endothelial BDNF had an impact on blood vessels angiogenesis, the number of branches and total blood vessel length was quantified in each layer, as an approximation of complexity and vasculature extent measurement.

When comparing vasculature complexity, by means of the branch number per layer, both fissures had apparently a loss in the ML complexity (Figure IV.8A, Table IV.1). However, this decrease in blood vessel branches was accompanied by a decrease in their length (Figure IV.8B, Table IV.1).

Table IV. 1. PRF and SF blood vessel complexity measurements.

		Branches/layer		Length/layer	
		PRF	SF	PRF	SF
EGL	WT	24.47±1.794	22.08±1.810	854.1±66.16	692.5±44.98
	KO	20.53±1.842	21.12±3.711	625.4±37.16 *	641.3±70.94
ML	WT	61.30±5.169	60.49±6.060	2264±209.8	2537±419.6
	KO	34.10±3.971**	33.22±5.138**	1318±97.96 **	1318±98.20 *
IGL	WT	91.29±11.52	97.77±16.48	5588±237.2	5816±968.7
	KO	71.78±14.53	76.59±21.97	4569±266.9 *	4160±528.9

Blood vessel branches and length measured per layer, in both PRF and SF. Data are expressed as mean ± SEM (n=3-5; *, p < 0.05; **, p < 0.01). PRF, primary fissure; SF, secondary fissure; EGL, external granule cell layer; ML, molecular layer; IGL, internal granule cell layer.

Since both values were decrease when BDNF was conditionally KO in endothelial cells, the ratio branches/length was not changed (Figure IV.8C, Table IV.2). This fact points to a smaller vasculature network instead of a decreased complexity. Nonetheless, it is conceivable that the smaller KO vascular system was caused by an abnormal layer thickness *per se* (as observed before, Figure IV.4). To clarify this possibility, we normalized the length of the blood vessels to the corresponding layer surface area. At the ML of both fissures and at the PRF IGL, removing BDNF from endothelial cells had an autocrine effect on angiogenesis (Figure IV.8D, Table IV.2), since the change was independent of the layer thickness change.

Table IV. 2. PRF and SF normalized blood vessel complexity measurements.

		Branches/Length		Length/Surface	
		PRF	SF	PRF	SF
EGL	WT	0.02915±0.002453	0.03184±0.001455	0.04640±0.004996	0.04228±0.002628
	KO	0.03323±0.003536	0.03294±0.003680	0.05116±0.007418	0.05458±0.006473
ML	WT	0.02739±0.001513	0.02508±0.002011	0.01477±0.0009623	0.01644±0.002316
	KO	0.02589±0.002157	0.02528±0.003357	0.009990±0.0006752 **	0.009447±0.0006787 *
IGL	WT	0.01623±0.001380	0.01683±0.001101	0.02561±0.001056	0.02692±0.004154
	KO	0.01544±0.002346	0.01769±0.002844	0.01861±0.0006964 **	0.01757±0.001636

Blood vessel branches normalized by its length, and blood vessel length normalized by the respective layer surface area. Data are expressed as mean ± SEM (n= 3-5; *, p < 0.05; **, p < 0.01). PRF, primary fissure; SF, secondary fissure; EGL, external granule cell layer; ML, molecular layer; IGL, internal granule cell layer.

Finally, as a way to study blood vessels integrity, an azo-dye that has the ability to pass only through damaged cells was used to perfuse the animals. Evans Blue, with its high affinity for serum albumin, when injected intra-ventricularly shows the leakage of blood vessels, since virtually all albumin-bound Evans Blue should be exclusively within the vessels. A tendency for blood vessels to be leaky was observed at the PRF of KO animals (BDNF wt/wt: 0.078 ± 0.014 ; BDNF fl/fl: 0.099 ± 0.016). At SF, this difference was much clearer, mutant animals had blood vessels with lower integrity (BDNF wt/wt: 0.055 ± 0.009 ; BDNF fl/fl: 0.077 ± 0.002). We can then conclude that conditional depletion of BDNF as an autocrine effect on the maintenance of blood vessels structure that tend to be less stable.

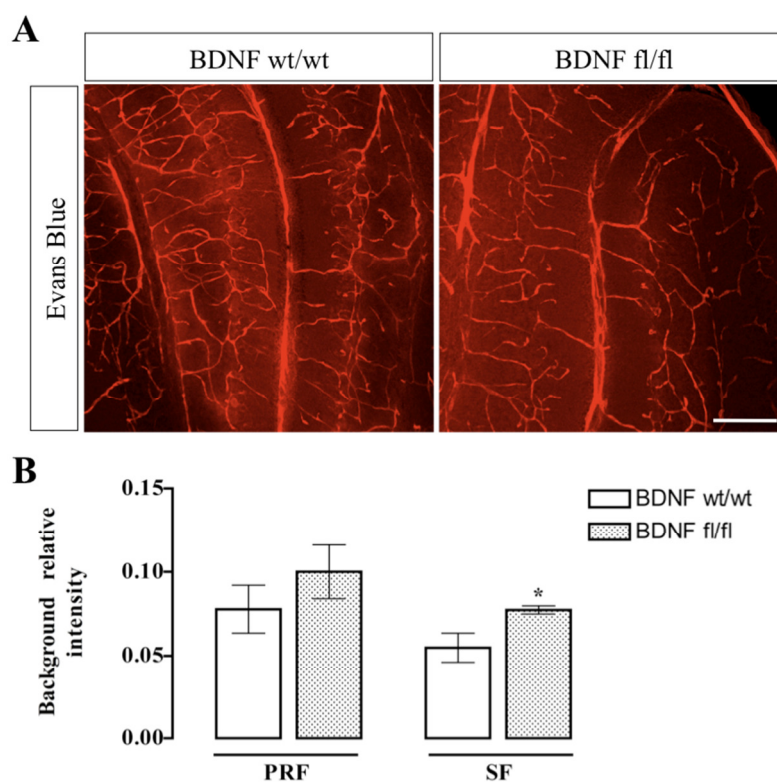


Figure IV. 9. Blood vessel leakage upon endothelial BDNF removal.

A. Representative confocal microscopy pictures of PRF Evans blue labeling. Scale bar = 50 μ m. **B.** Bargram depicts the relative brightness of the background compared to the blood vessel, as a measure of the dye that escaped from damaged vessels. Data expressed as mean $\pm$ SEM (n= 4). PRF, primary fissure; SF, secondary fissure.

Altogether, these data highlight the important autocrine endothelial BDNF effect at cerebellum angiogenesis and blood vessel maintenance. These changes *per se* can influence other cellular changes based on oxygen/nutrient availability, physical support and/or blood vessels molecules extrusion.

IV.3.3.4. Bergmann glia and Purkinje cell change upon BDNF endothelial removal

Like endothelial cells *per se* suffer from selective BDNF depletion, other cells may also be affected. Bergmann glia and Purkinje cells are important cells at the cerebellum development, thus they were also tested for any change upon BDNF conditional removal.

Bergmann glia processes attach to EGL, they serve as guidance for GCP migration, and thus alterations in this cellular pattern may either, affect and be affected by the GCP accumulation at the EGL. Immunolabeling for BLBP, a marker for radial glia fiber in the developing brain showed no alteration in the processes of KO and WT Bergmann glia cells (Figure IV.10, left panel).

Purkinje cells are key players in the cerebellum development. GCP and Purkinje cells have interdependent development and evolution through postnatal growth, being impossible to state which cell type affects the other first. Changes in Purkinje cells arborisation were thus likely to be seen. As expected, once calbindin immunostaining was performed, differences in the arborisation pattern, at the level of ML, were observed (Figure IV.10, right panel).

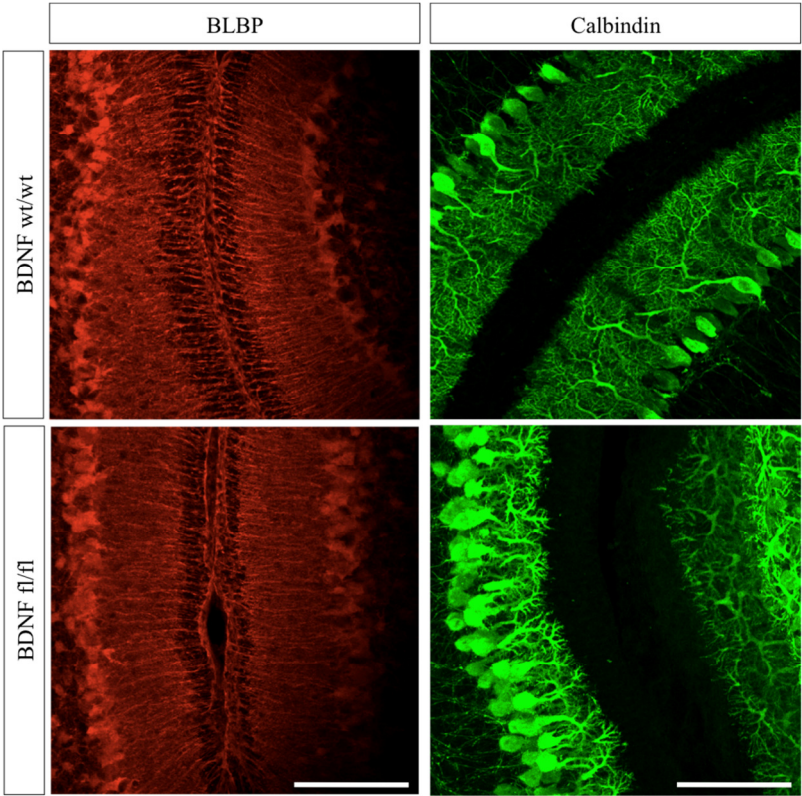


Figure IV. 10. Bergmann glia and Purkinje cell characterization upon endothelial BDNF removal.
Confocal microscopy pictures of primary fissure BLBP (red) and Calbindin (green) immunolabeling to identify, Bergmann glia and Purkinje cells, respectively. Scale bar = 50 μ m.

IV.3.3.5. Endothelial BDNF depletion has no effect on GCP cell death

We observed that GCP accumulate at the EGL of conditional KO animals. This increase in cell density could be due to an alteration in cell survival, signaling cells for cell death by apoptosis.

To disclose any change in cell survival, cleaved caspase-3 immunostaining was performed. There was no change on the density of cleaved caspase-3-positive cells at any of the two layers (EGL – BDNF wt/wt: $17.45 \pm 4.83 \times 10^{-5}$; BDNF fl/fl: $20.02 \pm 1.789 \times 10^{-5}$; ML – BDNF wt/wt: $4.43 \pm 1.353 \times 10^{-5}$; BDNF fl/fl: $2.11 \times 10^{-5} \pm 6.66 \times 10^{-6}$). Since there is a change in layer thickness, looking at cell densities may create a false perspective about any change on the total number of immune-positive cells. However, even if analysing total positive cell number there were no statistically significant changes on cell survival (data not shown).

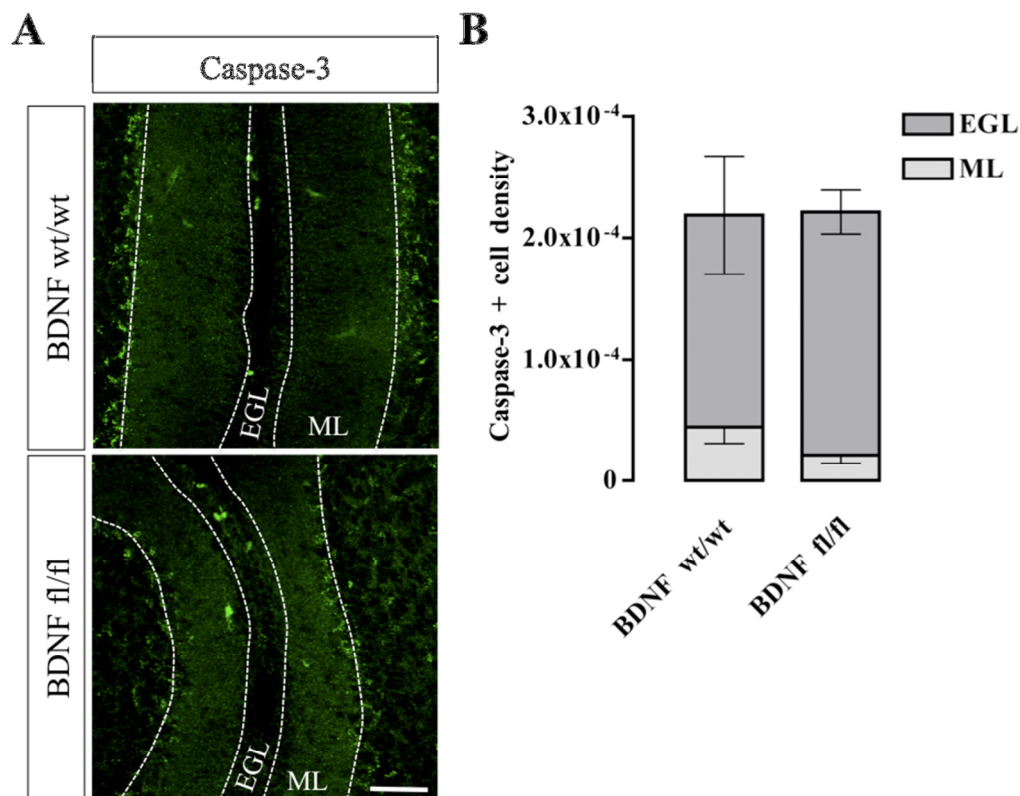


Figure IV. 11. Endothelial BDNF removal does not affect primary fissure cell death.

A. Confocal representative microscopy pictures of cleaved caspase-3 (green) immunostaining. Scale bar = 50 μm. **B.** Bargram represents the density of cleaved caspase-3-positive cells both in EGL and ML of WT and KO animals. Data expressed as mean $\pm$ SEM (n= 4). EGL, external granule cell layer; ML, molecular layer.

Given that no changes were observed, we excluded cell death and keep the hypothesis of cell proliferation and/or migration defects as responsible for maintaining GCP at the EGL.

IV.3.3.6. Depletion of endothelial BDNF affects proliferation and cell cycle

To address any proliferation changes caused by endothelial BDNF removal, a BrdU study was conducted. BrdU was administered ip and animals were perfused within 1h after administration, so that only proliferating cells at the EGL would be labeled. BrdU incorporation showed no statistical significant difference between the proliferation rate in WT and KO animals, as can be seen in Figure IV.12 (BDNF wt/wt: 0.010 ± 0.001 ; BDNF fl/fl: 0.010 ± 0.002).

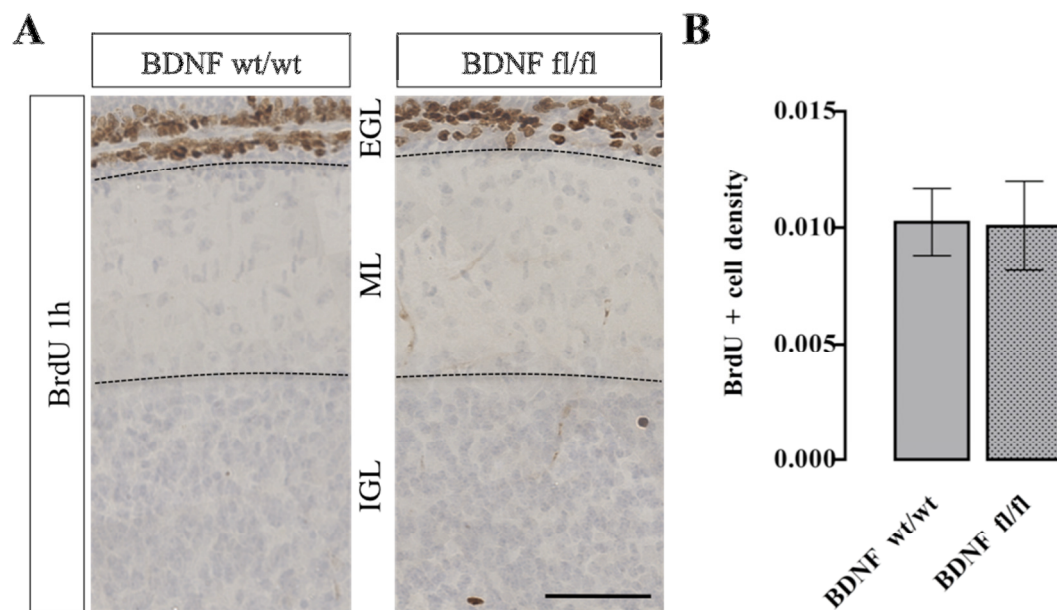


Figure IV. 12. Endothelial BDNF removal has no effect in cell proliferation.

A. Detail of primary fissure immunolabeling of BrdU (brown) incorporating cells at P11. Counterstaining by hematoxylin-eosin. Scale bar = 25 μ m. **B.** Bargram represents the relative density of proliferating cells in the EGL of WT and KO primary fissure. Data expressed as mean $\pm$ SEM (n=4-5).

On the other hand, when there is a 24h interval between BrdU ip injection and the animal sacrifice, there was a tendency to an increase in BrdU cell density at EGL, and a decrease at ML in KO animals (Figure IV.13B, Table IV.3). It is feasible that the difference in cell density was not clear because of the differences in layer thickness. This layer area change may be shading the effect of BDNF conditional removal on BrdU-positive cell number and placement. To elucidate this proliferation/migration issue, data was analysed in a different way, by means of BrdU-positive cell distribution within the three layers: BrdU-positive cell per layer as a percentage of all total BrdU-positive cells. With this strategy, we could clearly detect a statistically significant difference in BrdU-positive cell distribution in KO when compared to WT (Figure IV.13C, Table IV.3).

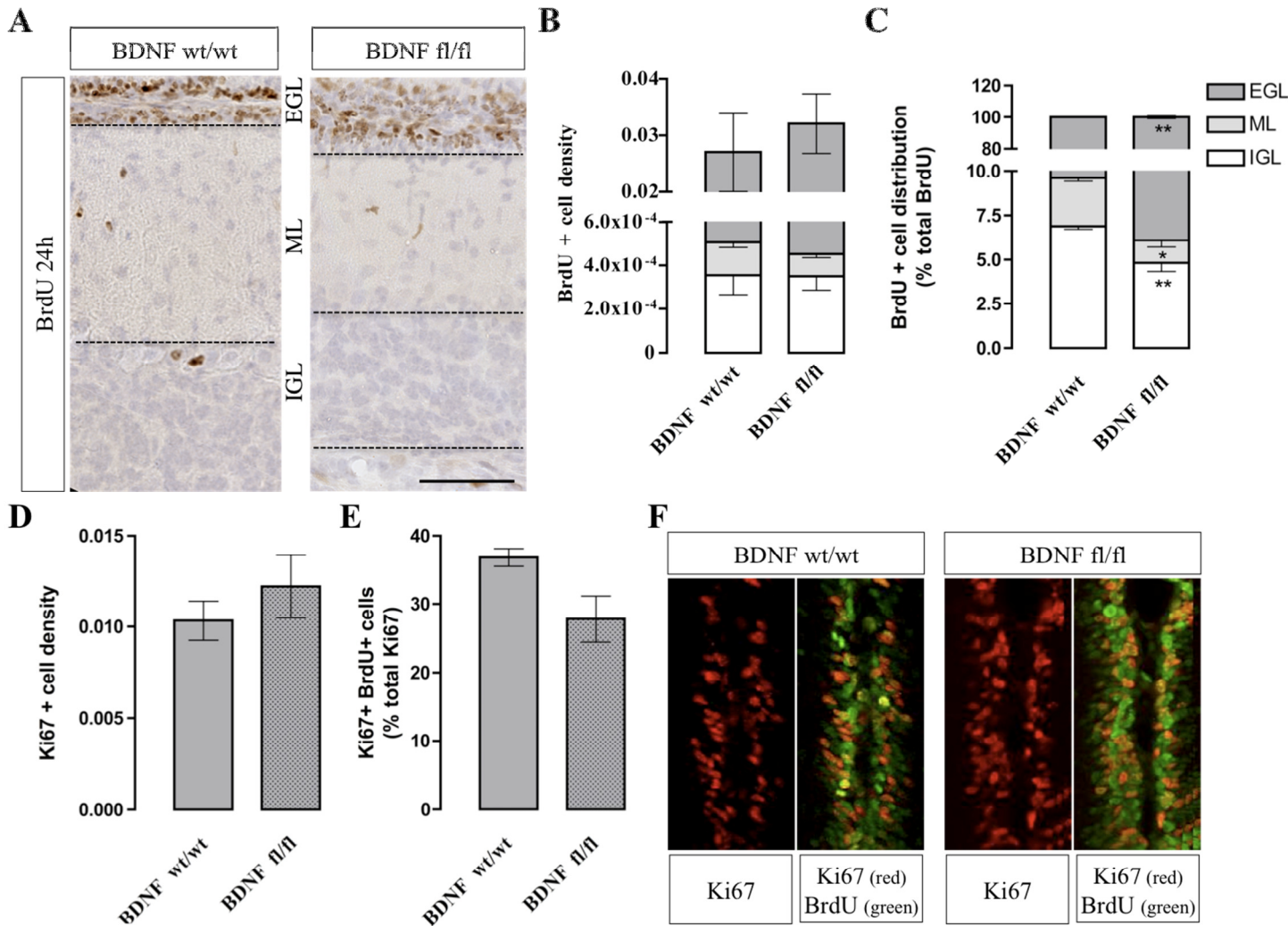


Figure IV. 13. Endothelial BDNF removal has an effect on primary fissure precursor cell distribution and cell cycle.

A. Detail of cerebellum PRF staining with BrdU (24h) in P11 animals. Brown cells are BrdU-positive cells counterstained with hematoxylin-eosin. Scale bar = 25 μ m. **B.** Bargram represents the BrdU-positive cell density across cerebellum layers. **C.** Bargram shows the relative distribution of proliferating cells across cerebellum layers. **D.** Bargram depicts the Ki67-positive cell density at the EGL. **E.** Bargram shows the percentage of Ki67/BrdU double positive cells. **F.** Detail of representative confocal microscopy pictures of Ki67 (red) and BrdU (green) immunostaining at the EGL of PRF. Data expressed as mean $\pm$ SEM (n=3-4; *, p < 0.05; ** p < 0.01). PRF, primary fissure; EGL, external granule cell layer; ML, molecular layer; IGL, internal granule cell layer.

At this point, the migration hypothesis was confirmed. In spite of that, cell cycle defects were not yet excluded. The DNA replication marker BrdU labels only cells at the S-phase, all cells in other phases of cell cycle were not included in this quantification. For that purpose, we counted the number of double BrdU-Ki67 double positive cells at the EGL. KO animals apparently had a shorter cell cycle; they had decreased double labeled population (Figure IV.12E - BDNF wt/wt: $36.82 \pm 1.13\%$; BDNF fl/fl: $27.78 \pm 3.37\%$). Conversely, there were apparently more cells in cycle in KO than in WT (Figure IV.12D - BDNF wt/wt: 0.010 ± 0.001 ; BDNF fl/fl: 0.012 ± 0.002).

Table IV. 3. Primary fissure BrdU-positive cell density and distribution 24h after administration.

		BrdU + cell density	BrdU + cell distribution
EGL	WT	0.02653 ± 0.006977	$90.40 \pm 0.1052\%$
	KO	0.03163 ± 0.005285	$93.83 \pm 0.8489\%$ **
ML	WT	$15.11 \pm 2.400 \times 10^{-5}$	$2.715 \pm 0.1845\%$
	KO	$10.25 \pm 1.652 \times 10^{-5}$	$1.290 \pm 0.3956\%$ *
IGL	WT	$35.54 \pm 9.033 \times 10^{-5}$	$6.893 \pm 0.1759\%$
	KO	$35.00 \pm 6.455 \times 10^{-5}$	$4.815 \pm 0.4991\%$ **

BrdU-positive cell density and distribution across primary fissure cell layers. Data expressed as mean $\pm$ SEM (n=4; *, p < 0.05; **, p < 0.01). EGL, external granule cell layer; ML, molecular layer; IGL, internal granule cell layer.

Altogether, these BrdU and Ki67 data indicate that endothelial BDNF is responsible for cell cycle resume just when GCP are ready to migrate. Thus, in these conditional KO, GCP leave cell cycle faster than in WT, but do not migrate out from EGL.

IV.3.3.7. Endothelial BDNF and GCP migration defects

Finally, to study GCP migration, two methods were used. BrdU incorporation studies were performed as before, but increasing the time interval between incorporation and animal sacrifice. Then, to access migration dynamics independently of any other cellular changes, time-lapse *ex vivo* cell imaging was performed in acute slices.

Since cell death and proliferation were excluded, and cell cycle exit was already accounted, we could study GCP migration by BrdU incorporation with a longer time interval between injection and sacrifice. This time window had to be long enough to allow cells to resume their cell cycle, start to migrate and finally place themselves at the IGL (100h).

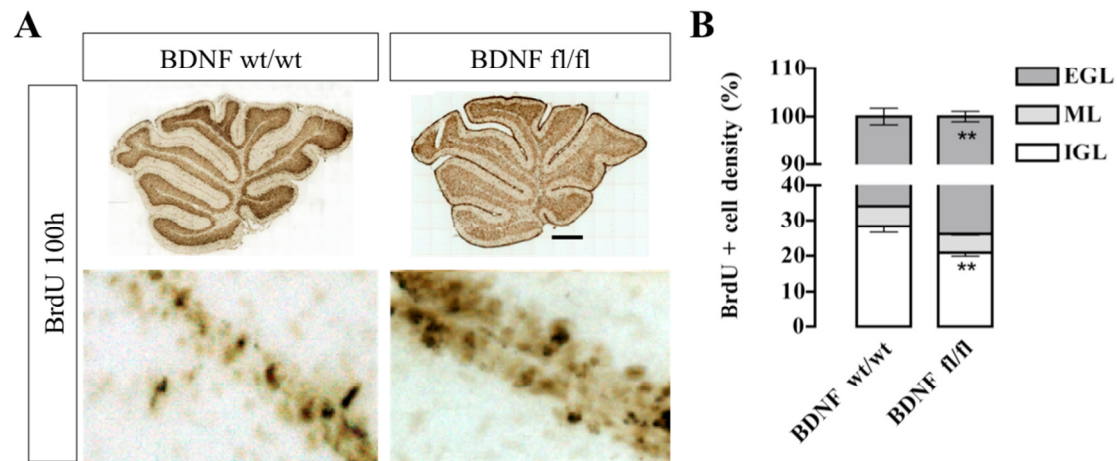


Figure IV. 14. Endothelial BDNF removal has no effect on GCP migration.

A. BrdU staining 100h after its incorporation in P11 pups (both WT and KO). Scale bar = 200 μ m. Lower panel is a detail of primary fissure cell distribution. BrdU-positive cells in brown and counterstaining by hematoxylin-eosin. **B.** Bargram represents the percentage of BrdU-positive cell density of both WT and KO animals. Data expressed as mean $\pm$ SEM (n=3; **, p < 0.01).

We observed that the previously demonstrated difference in cell distribution was enhanced with 100 h waiting time. EGL persisted in KO animals while in WT few cells remained at this layer (Figure IV.14 – BDNF wt/wt: $66.0 \pm 5.92\%$; BDNF fl/fl: $73.9 \pm 3.75\%$). Also IGL cell density was decreased in KO: much fewer BrdU-positive cells reached this internal layer (Figure IV.14 – BDNF wt/wt: $28.4 \pm 5.86\%$; BDNF fl/fl: $20.9 \pm 3.46\%$). A confirmation of endothelial BDNF as a key player for triggering migration onset of GCP was thus obtained.

Although increasing studies focus on the migratory behavior of cerebellum GCP, their dynamics and migratory parameters have not yet been addressed. We thus made use of a technique developed in the laboratory and previously devoted to study RMS migration. *Ex vivo* cell migration was recorded in acute slices 2 to 3 days after injection of GFP-labeled retrovirus at the EGL of primary fissure. Images were acquired every 30 sec for 2 h.

Table IV. 4. Migration parameters of GCP from WT and KO animals.

		Duration of resting period (s)	Rate of migration (μ m/s)	N° cells/slice/animals
EGL (tangential)	WT	590.63 ± 277.8491	0.85 ± 0.5359	8/6/5
	KO	-	-	-
EGL to ML	WT	500.83 ± 291.8083	1.29 ± 0.5341	3/3/3
	KO	1740.0	2.8	1/1/1
ML (radial)	WT	679.24 ± 309.2173	1.01 ± 0.3110	60/18/12
	KO	602.68 ± 236.0773	1.33 ± 0.4446	6/2/1

Duration of resting period (s) and rate of migration (μ m/s) of GCP recorded in time-lapse imaging of P11 cerebellum acute slices. Data expressed as mean $\pm$ SEM. EGL, external granule cell layer; ML, molecular layer.

GCP tangential migration was recorded in WT animals but not in KO animals. This type of migration was scarcely found and cells barely moved, they had a halting behavior with very low speed of migration (Table IV.4, first two data lines - EGL).

Although there were some KO cells recorded with both radial migration and changing from tangential to radial migration, no conclusions can be taken. Very few cells were found at the ML of KO animals (6 cells), and only one cell was recorded changing its mode of migration. KO animals were smaller, their cerebellum development was impaired and different from WT. Thus, even adapted stereotaxic coordinates were not as stable as in WT animals, and for this reason very few labeled cells were found in injected mutant mice. Being so, we cannot draw conclusions about GCP migration parameters other than that they change once endothelial BDNF is knocked out. Still, with the proper caution that such fragile data demand, we could hypothesise on BDNF endothelial role based on the tendencies observed: endothelial BDNF may have a pivotal role promoting the entry of GCP at the EGL into migratory phase, at the change from tangential to radial migration. No change was observed at any parameter of GCP that had already initiated radial migration (at the ML, even with caution, no big differences were observed between WT and KO parameters).

Taken together with the previous data, we can state that endothelial BDNF is a key player of cerebellum early postnatal development being responsible for the coordination of GCP cell cycle exit at the time when these cells would be triggered to start radial migration into the ML, on their way to IGL.

IV.4. Discussion

For a long time, BDNF has been attributed to exclusive neuronal sources (Jones *et al.*, 1994; Kokaia *et al.*, 1995; Rauskolb *et al.*, 2010). However, more recently microglia glia cells have been also claimed as BDNF producers (Madinier *et al.*, 2009; Béjot *et al.*, 2011). Though BDNF was accepted to be secreted from endothelial cells in other contexts (Leventhal *et al.*, 1999), it was just in 2009 that the first report of the endothelial BDNF role to the cell migration was published (Snappyan *et al.*, 2009; Grade *et al.*, 2013). However, within the cerebellum, the neuronal origin of BDNF has never been questioned. Several authors stated that BDNF has a key role on GCP migration (Schwartz *et al.*, 1997; Borghesani *et al.*, 2002; Zhou *et al.*, 2007), but none questioned its cellular origin. Only in 2010, Rauskolb and colleagues used a Cre line to study cell specific BDNF contribution to cerebellum development. However, these authors focused in a later developmental period than ours. Still, they noticed minor alterations upon neuronal BDNF conditional removal. Thus, it was imperative to address the question whether BDNF from other cellular stores was fine-tuning cerebellum postnatal development through cell migration control. The current chapter focused on this endothelial contribution, to the overall BDNF pool, at the developing cerebellum, unraveling its fundamental contribution to the proper cerebellum development.

In this study, we showed that BDNF is present in blood vessels, being expressed by endothelial cells. This mRNA expression pattern at P11 was observed by *in situ* hybridization, together with immunolabeling for endothelial cells. Previous reports based their conclusions of BDNF expression on immunolabelings (Borghesani *et al.*, 2002). Thus, no clear evidences on mRNA expression could be used since this neurotrophic factor can be trapped in cells that do not secrete it. Examples are the studies by Snappyan and collaborators (2009) and also Grade and colleagues (2013). These authors saw BDNF immune reactivity within astrocytes but failed to detect its mRNA within these cells. On the other hand, both NeuN positive neurons and PECAM positive endothelial cells were also positive for its mRNA, thus leading them to conclude that neurons and endothelium are the sources of BDNF within the striatum. In our study, we showed that BDNF mRNA is being expressed, by endothelial cells, at the cerebellum 11th day postnatal. Although *in situ* hybridization data also show positive mRNA signal within neuronal cells, FACS data point to an endothelial cell origin. Having shown this endothelial cellular contribution to the BDNF postnatal pool, we hypothesize that the BDNF of endothelial origin settles the so-called gradient and triggers GCP migration. Afterwards, when there are already enough mature granule neurons at the IGL, there would be a shift of BDNF production to neuronal cells that then maintain the gradient and keep migratory behavior. However, the

initial BDNF, fundamental for the onset of cerebellum postnatal development, is of endothelial origin, establishing the trigger for GCP migration (Figure I.8).

To address this hypothesis we created an endothelial conditional knockout animal model. Commercial BDNF^{loxP} animals were mated with animals that have Cre-recombinase under the VE-Cadherin promoter. Cre is a bacteriophage P1-derived recombinase which efficiently excises DNA flanked by two repeated loxP recognition sites (Sauer and Henderson, 1988; Sternberg and Hamilton, 1981). Animals harboring Cre-recombinase have its expression under the control of a certain gene promoter that temporally and spatially regulates the Cre-mediated recombination. VE-Cadherin, our selected promoter, is an early marker of endothelial cells playing important role in vascular permeability and remodeling (Vittet *et al.*, 1997; Wong *et al.*, 1999; Dejana *et al.*, 2008). This cadherin, together with two other proteins: PECAM-1 and VEGFR2, can drive non-endothelial cells to acquire the ability to align with the direction of the flow, as endothelial cells do (Tzima *et al.*, 2005). Being that BDNF excision was target to VE-Cadherin expressing cells, the resulting animals had BDNF removed exclusively from endothelial cells. The consequences were phenotypically similar to the ones observed at the full KO animal (Jones *et al.*, 1994; Schwartz *et al.*, 1997): weight loss, abnormal gait and uncoordinated movements, and early postnatal death. This observation strengthened our hypothesis. Nevertheless, one could argue that the changes could be due to a leakage in the promoter. To check for this eventual weakness of the animal model being used, we mated VE-Cadherin::Cre animals with Z/EG animals. Z/EG (Novak *et al.*, 2000) animals have endogenous GFP expression at Cre recombination sites, signaling all cells in which recombination is happening regardless of being targeted on purpose or by chance, in the case of promoter leakage. Endogenous and enhanced GFP positive cells were co-labeled by Cre expression, without any additional immune positive cellular structure. Moreover, by perfusing animals with Dextran Texas-Red, an hydrophilic dye with high molecular weight that stays within blood vessels, we could observe that Cre-immunoreactive cellular structures were restricted to the dye-filled blood vessels. Still, BDNF endothelial removal could be debatable on the sight of the tissue/cell BDNF splice variants specificity and diversity. To circumvent this issue, the BDNF^{loxP} animals used target exon IX, the one and only coding exon that is spliced with all other non-coding exons (Timmusk *et al.*, 1993; Aid *et al.*, 2007). Thus, with this strategy, together with the mentioned experimental tests, we can state that the animal model being used completely removes all BDNF transcripts exclusively from endothelial cells. Additionally, one can say that the BDNF endothelial removal alone was dramatic enough to provoke the same phenotype and cerebellum cellular abnormalities as full KO, without any promoter leakage.

First, we briefly characterized the cerebellum morphological changes by vascular BDNF removal. As observed before at the full KO animals, abnormal cerebellum folia structure and fissure depth were found at these conditional KO animals. Moreover, cell distribution was

impaired with persistent EGL and thinner IGL, again, as observed with full KO models (Jones *et al.*, 1994; Schwart *et al.*, 1997; Borghesani *et al.*, 2002). To account for such dramatic behavior and morphological changes, major BDNF depletion should be achieved with these animals. To address this question, we performed quantitative Reverse Transcription-PCR from total cerebellum extracts. In agreement with our hypothesis, at postnatal day 11, the depletion in BDNF mRNA levels was almost total, when targeting only endothelial cell. This decrease has a gene dose-dependent effect. As this result was in total contrast to all published data on BDNF cell source at the cerebellum, we performed additional experiments to check the detection limit of the equipment used, and also quantitative Reverse Transcription-PCR from sorted cell populations. Being that a sample with a low concentration as 1:500 dilution could be measured, if a sample has no detected signal – as it is the case for BDNF KO animals – it means that the mRNA for the gene under study is not present within the sample. There is no possibility that there is an artefact, especially when the levels and quality of the housekeeping gene measurements are comparable to other samples in which both genes have detected signals. Thus, one can exclude any possible poor RNA quality or equipment failure effects in the KO animal samples that lack quantitative Reverse Transcription PCR signal for BDNF mRNA. Furthermore, we FACSorted endothelial cells and looked at each cell type mRNA contribution separately (endothelial versus non-endothelial populations). Although FACS experiments were performed with WT animals from a reporter animal model using Tie2 as promoter – not the initial VE-Cadherin promoter, the results can be securely accepted as representative of endothelial versus non-endothelial cell mRNA content since Tie2 is a marker of endothelial cells used both *in vitro* and *in vivo* (Kisanuki *et al.*, 2001). Even not being statistically strong, these preliminary results point to the same direction as total RNA extracts did: endothelial cells are the only source of BDNF at cerebellum developmental age P11. Interestingly, protein levels followed the mRNA tendency but did not drop in KO as nucleic acids did. This detectable BDNF protein could be explained by its anterograde transport into the cerebellum, by climbing fibers. It has been published that anterograde/reterograde transport occurs at the cerebellum (Terashina *et al.*, 1997) and thus it may bring BDNF from other tissues that in fact produce it, to tissues that do not secrete it. This may account for the immuno signal observed in several reports. Being secreted elsewhere, and then transported into the cerebellum may justify the detection/quantification of BDNF protein within a tissue. Recently, Rauskolb *et al.* (2010) measured an almost absolute decrease in BDNF protein levels in conditional neuronal BDNF KO animals. However, not just these measurements were performed in adult animals (P56), which are not comparable to our young developing animals; but also the promoter used to drive BDNF excision is debatable. They used a tau::cre line (Korets-Smith *et al.*, 2004), thus BDNF revomal will happened only once mature neurons start to express Tau, which happens during axonal outgrowth (Kosik, 1990). This strategy is far different from the one we used, targeting

early VE-Cadherin mediated Cre-expression during postnatal development. Thus, our data cannot even be said to be contradictory with the one mentioned, since experimental designs are very distinct.

Since the endothelial BDNF might have an autocrine effect, as observed with other endothelial factors (reviewed in Otrrock *et al.*, 2007), we analysed the cerebellum blood vessel structure upon its conditional removal. We observed that endothelial BDNF KO animals have an impaired blood vessel structure without any selective effect on functional blood vessels, since no PECAM positive/Dextran negative blood vessels were found. All blood vessels, even if with a different overall structure, were equally functional in WT and KO animals. Thus, the BDNF autocrine effect on vasculature must be on angiogenesis/remodeling and not on functionality. Structure complexity was measured by the quantification of blood vessel bifurcation in each cerebellum layer. It was observed that endothelial BDNF is important for the complexity of the vascularisation, especially at the ML. This decrease in complexity was accompanied by a decrease in the length of the overall blood vessels. Being that at the ML, GCP migrate from EGL to ML, but not in a *vasophilic* way, the vasculature here present may serve not so much as physical support as observed at the RMS (Snappyan *et al.*, 2009), but more as a chemical support structure, secreting factors and maintaining oxygen and nutrient availability. If in fact, there was a weaker/abnormal vasculature at the cerebellum of KO animals, which may interfere with several cell types at the cerebellum. To clarify this issue, we normalized the branches by blood vessel length as an attempt to rule out complexity over size. No significant differences were observed in neither layer, leading us to hypothesize that endothelial derived BDNF has an impact on the overall cerebellum development and not specifically in a particular layer, or in the complexity irrespectively to the extent of the vasculature. BDNF retrieval was responsible for a uniform decrease in both vasculature length and complexity. Never the less, the decreased extension of cerebellum KO vasculature was independent of the whole cerebellum structure shrinkage, measured by the surface area, which was not affected. Additionally, we analysed blood vessel integrity by measuring Evans Blue dye distribution within/out blood vessels. This dye, when injected intraventricularly circulates in the animal's vasculature since it has high affinity for serum albumin, and thus is kept within non-damaged blood vessels - unable to stain other cells than endothelial ones. However, if endothelial cell's integrity is affected, serum albumin can extrude from vessels, together with plasma, and so does Evans Blue dye (Hed *et al.*, 1983; Hawkins and Egleton, 2006). Analysing its fluorescent intensity in/outside blood vessels allowed a rough quantification of the vasculature leakage. KO animals perfused with this dye had higher fluorescent intensities outside blood vessels than their WT littermates, which lead us to think that there might be an autocrine integrity loss effect upon endothelial BDNF removal. The difference observed between PRF and SF might be due to animal variability or to low number of experimental animals per group, since neither Cre

expression was different at PRF and SF, nor was VE-Cadherin mRNA expression (Allen Mouse Brain Atlas - <http://mouse.brain-map.org/>). Altogether, these results open a new window of research on the angiogenesis field: vascular BDNF is an autocrine mediator of cerebellum postnatal angiogenesis and remodeling.

Blood vessels are the transport system used to deliver oxygen, nutrients and other factors to the cells. They support other cells survival and development. Removing BDNF from endothelial cells has an impact on cerebellum development - observed anatomically, as well as on the blood vessel network itself. These two morphological alterations could be directly interconnected, or could be mediated by other cells within the cerebellum. Bergmann glia cells, which are specialized astrocytes, provide physical scaffolds for GCP migration (Rakic, 1971) as well as Purkinje cell dendritic outgrowth and synapse formation (Yamada and Watanabe, 2002). Bergmann glia role is fundamental for cerebellum development, they affect directly and indirectly GCP migration. Their impairment caused by environmental factors affects GCP migration (González-Burgos and Alejandre-Gómez, 2005); and its postnatal ablation has severe consequences on cerebellum development such as cerebellum size reduction and disrupted layered structure (Delaney *et al.*, 1996). Purkinje cells, on the other hand, are the biggest GABAergic neurons from the cerebella cortex and their interdependence with granule cells has been well characterized. Reelin expressed by granule cells, which ApoER2/VLDL receptors are at Purkinje cells, is an example of an intermediate signaling molecule between these two cell types: mutations in reelin or in its receptors impair proper granule cell development (D'Arcangelo *et al.*, 1995; Trommsdorff *et al.*, 1999). Mutations in cell cycle regulators, or loss of potent mitogenic factors such as Shh - produced by Purkinje cells - leads to strong defects in cerebellar lamination (Wechsler-Reya and Scott, 1999; Wallace, 1999; Traiffort *et al.*, 1999; Dahmane *et al.*, 1999; Lewis *et al.*, 2004). Their deep relation through cerebellum postnatal development is so intricate that it is difficult to distinguish where each cell type starts to influence the other and vice-versa. Thus, looking at Bergmann glia and Purkinje cells was fundamental for the overall analysis of endothelial BDNF impact on cerebellum development. Staining for BLBP did not show any significant alteration in Bergmann glia morphology and glial processes attached to the EGL. Thus, no changes in the scaffold used by GCP for migration were observed, excluding any effect of vascular BDNF on Bergmann glia cells biology. The GCP accumulation observed is not due to a lack/abnormal Bergmann glia physical support. Calbindin staining, on the other hand, demonstrated that Purkinje cell arborization was affected by endothelial BDNF removal. However, as discussed above, these changes observed at the Purkinje cell morphology could be due to a direct impact of the lack of endothelial BDNF, or to an indirect effect mediated by other cells at the cerebellum such as GCP that are not correctly placed at this layered structure.

Altogether, our data point to endothelial BDNF as having a key role to cerebellum development. It was showed to cause abnormal GCP distribution across the layers, deficient angiogenesis and leaky vasculature, as well as Purkinje cell abnormal development. However, GCP accumulation could be due to several altered cellular mechanisms: increased cell death, impaired proliferation, abnormal cell cycle control or migratory defects.

In other tissues, BDNF has debatable effects on cell survival (Kirschenbaum and Goldman, 1995; Galvão *et al.*, 2008). At postnatal day 11, within the cerebellum, low cleaved caspase-3 staining was observed. Few positive cells appeared at the EGL or ML, consistent with a developmental stage of high proliferation and migration, and low cell death. The few cells seen in both types of animals were not statistically different. Thus, the increased GCP density at the EGL was not due an apoptotic effect of endothelial BDNF retrieval.

Cell proliferation and cell cycle are key biological processes to cerebellum postnatal development. At the EGL, highly proliferative precursors divide until their cell cycle is stopped and they start migrating across the layers (Espinosa and Luo, 2008). Thus, these two processes are interconnected and probably related to the GCP accumulation. BrdU ip injection, a synthetic thymidine analogue able to incorporate during DNA synthesis, labels dividing cells, which allows the tracing of proliferating cells through a defined time window. Waiting 1h between injection and sacrifice labels only proliferating cells at the EGL - not enough time to start migration. When comparing these proliferation results between WT and KO animals, no differences were observed. On the other hand, when incorporating cells were allowed to divide and start migration, on a 24h time frame between injection and sacrifice, a different cell distribution was observed. This clearer difference in cell placement was not so visible when measured as cell density – because of the changed layer thickness. However, if we analyse the cell distribution, incorporating cells from WT animals clearly migrate much further than the ones from KO. In the absence of endothelial BDNF, proliferating cells do not migrate out of the EGL so promptly as WT cells do. This result, although pointing to a cell cycle defect is not enough to conclude so, since BrdU only labels cells at the S-phase. To better look at cell cycle, we performed an additional staining – Ki67. This protein is associated with proliferation, labeling all cell cycle phases with the exception of the resting G0 cells (Scholzen and Gerdes, 2000). Ki67 positive cell density showed no significant difference with or without endothelial BDNF. Nevertheless, when analysing the double positive population, both Ki67 and BrdU positive cells, there was a trend for a decrease in the double positive cell population in the absence of vascular BDNF. These double positive cells were in cell cycle when BrdU was administered, but could not exit cell cycle within the 24h – they remained in the S-phase. As KO animals have a decrease in this cell population, endothelial BDNF might be controlling the equilibrium between the proper timing for cell cycle exit and the readiness/correct stimuli to start migration. Thus, transgenic cells resume their cell cycle either too fast or too soon,

however they are not able to migrate away from the EGL remaining in the EGL as accumulated cells.

At this point, our initial hypothesis was further supported: endothelial BDNF has a pivotal role on the onset of GCP migration, even if having other autocrine cellular roles. To finally address this question, two approaches were used. Firstly, and going back to BrdU injection, a longer time period (100 h) between injection and sacrifice was waited. After this interval, animals were processed as previous ones, and an even clearer perspective of cell distribution impairment was achieved. While in WT animals almost no cell was still present at the EGL, in KO animals a big percentage of cells were kept in the outer EGL of the cerebellum. These GCP failed to migrate and accumulated at the EGL. Thus, even if more time is given so that cells can recover their positioning within cerebellum layers, they do not succeed. Additionally, we attempted to compare the migratory parameters of GCP from WT and KO animals, both in their tangential and radial migration. For that, we adopted a well-documented method for time-lapse imaging (Snapyan *et al.*, 2009; Bozoyan *et al.*, 2012; Khilghatyan and Saghatelian, 2012; Grade *et al.*, 2013) to cerebellum migration studies. Majority of migration studies were all based on BrdU incorporation (Borghesani *et al.*, 2002) or dye stereotaxic labeling combined with confocal microscopy (Bovetti *et al.*, 2007a – even if in other tissues). Most are performed *in vitro* and some in organotypic slices (Zhou *et al.*, 2007). No reports have been published with *ex vivo* cerebellum migration studies. Thus, the implementation of this method with all its advantages – cell survival, maintenance of cellular networks, specificity of labeled cells and time interval between acquisitions – was a task *per se* in the course of this study. Unfortunately, it proved to be difficult to execute: (1) viral stereotaxic injection at the EGL of PRF was hard to reproduce because of the foliated and angled structure of the cerebellum of WT animals, and much more difficult in KO animals that have abnormal and impaired development; (2) tangential migration in general was very scarce, and when observed cells were merely hesitating from one point to another without a clear route; (3) the most interesting type of migration with respect to previous results, the shift from one type of migration to the other, corresponding to cell cycle exit and start of radial migration to the IGL, was the most rarely observed in the 2 h period in which cells were being recorded; (4) additionally, in the KO animals there was a percentage of animals that did not recover from surgery, regardless of all procedures to avoid it. This way, the number of KO animals that could be used for imaging analysis was much lower. Nevertheless, and though fragile, we can discuss the few results obtained. When BDNF is removed from endothelial cells, GCP at the EGL seem to have longer resting periods when changing their mode of migration. This is in agreement with the BrdU positive cell accumulation observed in both 24 and 100 h experiments. However, when these KO cells start migration, time-lapse imaging data points to a higher speed of migration. This result is in contrast to what was observed before, since waiting more time

between BrdU injection and sacrifice did not compensate the migration onset delay. This contradictory data should be taken cautiously because of its lack of statistical significance. When looking at radial migration, no clear difference was observed neither at the resting period, nor at the rate of migration. This apparent lack of endothelial BDNF effect on radial migration, if confirmed, strengthens our hypothesis: vascular derived BDNF is responsible for the trigger of GCP migration, being as in the RMS the pivot for the onset of migration.

In conclusion, in this study we pinpointed the essential role of the not yet considered vascular BDNF to the overall cerebellum development. We demonstrated that endothelial cells are responsible for the majority of this neurotrophin production in early postnatal ages, regardless of any residual neuronal contribution. With our animal model, we proved that endothelial BDNF has an autocrine effect on cerebellum angiogenesis, remodeling and vessel integrity. Furthermore, we showed that the anomalous cerebellum development and animal phenotype could have been caused by Purkinje cells abnormal arborization, but most prominently by impaired GCP migration. This cellular accumulation was motivated by the unbalanced cell cycle exit and migration onset, based on the lack of pivotal endothelial BDNF. Taken together, these results clearly indicate that endothelial BDNF has an important role in modulating cerebellum postnatal development and thus, shall be further studied.

To fully complete this breakthrough study and accurately change the actual paradigm on the BDNF role in cerebellum development field of knowledge, similar experiments should be performed with a complementary neuronal conditional knockout animal model.

CHAPTER V

GENERAL DISCUSSION & CONCLUSIONS

IV.5. General discussion

Brain diseases represent a major health challenge worldwide, with particular impact on developed societies. These diseases cause critical human suffering, increase the morbidity and also have an impact on economy and governmental decisions. No effective cure exists for the majority of them, and thus, the search for potential new drug candidates and cell replacement strategies, either by transplantation or endogenous stimulation, and the full knowledge on brain development and disease progression are crucial healthcare and scientific demands. The presence of stem cells in the adult brain raises legitimate hopes and redefines the brain as a plastic and potentially self-curative organ, instead of an isolated and mysterious source of debilitating degenerative diseases.

The present thesis is built on the outstanding brain capacity to generate new cells, set the proper environment for them to differentiate and stimulate balanced migration to occupy target areas. Two main pillars were explored in the current work: A) Histamine effects on mouse SVZ cell dynamics, focusing on neuronal differentiation *in vitro*, *ex vivo* and *in vivo*, either administered as single pulse, delivered by biomaterial designed carriers or by biomedical devices; and B) BDNF dependant cellular source effect on cerebellum postnatal development through granule cell precursor activity. Histamine proneurogenic effect was addressed from an *in vitro* to *in vivo* perspective (more classical procedure that first sets the limits and then goes deeper in molecular pathways to escalate into animal models). On the other hand, endothelial BDNF effect at the cerebellum was explored in a different way: from transgenic animal models that have clear phenotypical changes to deeper cell biology studies building up an hypothesis for the observed changes. Both strategies are correct and gave good clues for additional studies in the respective fields of research.

Previous works started to unveil other functions for histamine rather than the classical inflammatory mediation. However, they were scarce and none provided a comprehensive view of histamine as a proneurogenic factor. To pursue this motivation and disclose histamine effects at a neurogenic niche, we used the mouse postnatal SVZ as model. SVZ-derived neural stem/progenitor cell cultures isolated from newborn WT animals are composed by a big diversity of cells including progenitors, astrocytes, neurons and oligodendrocytes. Although this diversity enhances the challenge to develop novel strategies leading to a specific cell phenotype; on the other hand it reinforces the strength of the obtained data, since it is much more related to what is biologically observed than pure cell line cultures.

In vitro administration of histamine promoted a clear neuronal phenotype in SVZ cell cultures, through histamine receptor 1. This was observed regardless of any toxic effect, without affecting cell proliferation or cell cycle and not interfering with other neural phenotypes. Histamine proneurogenic effect occurs early in cell fate determination since it induces early

progenitor commitment and also activates axonogenesis. New neurons endorsed by histamine are preferentially GABAergic, as has been observed *in vivo* at the OB. At the molecular biology level, histamine induces epigenetic modifications that facilitate proneurogenic genes (Mash1, Dlx2 and Ngn1) transcription. These observations were noticed and coincident both at the mRNA levels and histone H3K4 methylation.

Nowadays, drug delivery systems are common tools used by the pharma industry to better target and control active compounds availability and kinetics. PLGA, a biomaterial FDA approved for biomedical devices was used to conceive histamine-releasing microparticles. These new carriers slowly release histamine in a controlled way. Although releasing a cumulative lower dose of histamine, the results obtained are as effective as high single pulse administration. This might be explained by the continuous activation of histamine receptors and consequent signaling cascades triggering, instead of a single firing event at an initial high concentration, followed by a lack of stimuli. The proneurogenic effect without cell toxicity and proliferative effects observed was comparable, with as less as 1000x lower histamine concentration than single pulse administration. Moreover, cells pretreated with these histamine-releasing microparticles succeed in surviving and integrating into host tissue. In adult WT animals, transplanted primed cells integrate; and in organotypical hippocampal slices they not only integrate but also differentiate into new neurons within the host tissue.

In the attempt to further transpose histamine treatments into more closely related environments as the ones observed biologically, we used mini osmotic pumps to continuously deliver this amine at the CSF of the lateral ventricles of adult WT animals. With this strategy we avoid the BBB and assure a local delivery of this molecule to the SVZ target cells. In these experiments, although within a much more complex system, histamine proved to have similar proneurogenic effects *in vivo* as the ones previously observed *in vitro*. During the 3 weeks of treatment, neural progenitor cells from the SVZ are triggered by histamine to start differentiation and migrate towards the OB. Not only new neurons are observed at the OB granule cell layer, but also dividing cells with neuronal markers (Ki67+DCX+) are increased at the SVZ. This additional *in vivo* data confirms the *in vitro* results that point histamine as a molecular trigger for cell commitment – histamine drives cells to their final divisions and to start differentiation. All the above-mentioned results, observed ipsilaterally to the pump installation site, were reproduced on the contralateral hemisphere, which excludes any possible inflammatory interference on the results obtained at this hemisphere.

On the whole, our results doubtless put histamine on the lights to be considered proneurogenic for further *in vivo* studies in disease models. Although this study opened doors for histamine at the regenerative medicine field with strong supporting data, more research shall be done to fully accomplish the goal of having found a new candidate for neuroregenerative therapies.

A lot of efforts have been devoted to the study of the neurovascular niche at the two classical neurogenic niches, SVZ and SGZ. This allowed the discovery of several soluble factors diffused from endothelial cells that contribute to stem cell properties and dynamics. On the other hand, cerebellum granule cells are widely used for migration studies because of its dynamic biphasic behavior, and its abundance within the several tissue cell types. A lot is known about *in vitro* granule cell migration and the plethora of factors that help regulate its direction and onset. However, nothing is known about the possibility of BDNF from endothelial cell origin on its *in vivo* migratory behavior. It is accepted that endothelial BDNF plays a role on SVZ/RMS migration, it is also accepted that BDNF is essential for proper cerebellum granule cell migration. Nonetheless, it has never been questioned the role of vascular BDNF to solve the dilemma of how is the BDNF gradient settled. In this context, making use of the Cre-Lox system, we studied the conditional depletion of BDNF in a tissue-specific manner devoted to endothelial cells.

Importantly, besides all previous reports on neuronal BDNF cellular origin, we proved that endothelial cells from the cerebellum express BDNF mRNA, as well as the endothelium from ischemic striatum (Grade *et al.*, 2013), or the RMS (Snappyan *et al.*, 2009).

Assessing the decrease in mRNA levels of the transgenic animals confirmed that the ataxic phenotype observed in these mutant animals was caused by a massive drop in BDNF. The lack of weight gain, abnormal gait and balance were similar to the ones observed in full KO animals (Jones *et al.*, 1994; Schwartz *et al.*, 1997) in which BDNF was claimed to be from a neuronal source. Our results drastically contrast with the published data regarding the exclusive neuronal BDNF cellular source, but strengthen the hypothesis of a time-dependent dual cell source of BDNF. Endothelial cells would be the initial BDNF producers, triggering granule cell precursors to start migration. Once this migration is set, the already mature granule cells will then assume the BDNF production and will foster migrating cells at the IGL to keep BDNF expression, increasing this neurotrophin concentration at the most internal part of the cerebellum, which will then settle the gradient.

All our results confirm this hypothesis: Bergmann glia cells are not affected by this conditional neurotrophin removal, and thus no change is sensed by the granule cells that use them as migrating scaffolds; Purkinje cells have defective arborization, which might be the cause (or caused by) abnormal granule cell migration/maturation. Both cell types are interdependent on the cerebellum development and thus it is difficult to take any conclusion from these Purkinje cell observations. Regarding granule cell precursors, the major players at postnatal cerebellum development, their positioning within the layers is anomalous. They accumulate at the EGL. This could have been caused by an increased cell death due to the lack of endothelial BDNF. However, no effect on survival was observed. The equilibrium between

proliferation/cell cycle and migration was thus the only possible, and forecasted, cellular key event controlled by endothelial BDNF. This vascular neurotrophin proved to be responsible for the cell cycle exit and precise migration onset. Mutant animal cells resume their cell cycle faster but fail to start migration, therefore accumulating at the EGL. Moreover, we attempted to determine migration parameters; we estimate a longer resting period in granule cells changing their mode of migration (starting radial migration) at the KO animals.

Additionally we observed an autocrine effect of endothelial BDNF on vasculature formation/remodeling as well as on its integrity, since there are fewer blood vessels in mutant animals, and these tend to be leaky.

Altogether, these *in vivo* studies provide the ultimate integrative scenario that reset the paradigms of cerebellar granule cell migration. New windows for more detailed studies, on the molecular pathways activated by endothelial BDNF controlling cell cycle exit and migration were opened. Also, the field of angiogenesis had additional input from our study since BDNF autocrine effect at the highly vascularised cerebellum was not yet documented. This work shall be continued, aiming at disclosing neuronal BDNF precise effect at cerebellar granule cells, early in these postnatal ages.

The present thesis increases the comprehension on SVZ stemness capacities and histamine alternative roles as proneurogenic factor both *in vitro* and *in vivo*. The current work also redefines the scenario to further investigate cerebellum development and neurotrophins' role. This knowledge is particularly relevant for both new cell replacement strategies and targeted stimulation/correction of endogenous resources in the prosecution of brain reparative therapies.

IV.6. Conclusion

Histamine at 100 μ M concentration is of robust proneurogenic relevance.

Histamine does not affect *in vitro* cell proliferation or cell cycle, but it contributes to the shift from early immature cells towards committed progenitors.

In vitro, histamine promotes SVZ neurosphere neuronal differentiation *via* H1R, a proneurogenic effect observed phenotypically and functionally.

Histamine induces JNK-MAPK activation on growing axons.

The effect of histamine in neuronal differentiation is mediated by the trimethylation of H3K4 on the promoter regions of the genes Mash1, Dlx2 and Ngn1. They all have, then, an increased mRNA expression level.

PLGA microparticles slowly release histamine inducing comparable *in vitro* effects at SVZ neurospheres, as single pulse histamine treatments.

Both *ex vivo* and *in vivo* transplanted cells have positive survival, host acceptance and integration, if pretreated with histamine-releasing PLGA microparticles.

Intracerebroventricular infusion of histamine has comparable proneurogenic effects at the SVZ and OB of treated animals.

Cerebellar endothelial cells are responsible for producing of early postnatal BDNF.

Endothelial BDNF is fundamental for cerebellum development, affecting cell distribution within the layers.

GCP without endothelial BDNF accumulate at the EGL of the postnatal cerebellum, causing abnormal cerebellar development and ultimately animal death.

Endothelial BDNF is fundamental for postnatal angiogenesis and vasculature maintenance.

Cell death and proliferation is not affected by vasculature BDNF removal.

CGP abnormally distribute across cerebellum layers, upon endothelial BDNF removal, possibly because of a disturbed equilibrium between cell cycle exit and migration onset.

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