STRATEGIES FOR THE USE OF NEURAL STEM CELLS IN BRAIN REPAIR

ESTRATÉGIAS PARA O USO DE CÉLULAS ESTAMINAIS NEURAIS EM REPARAÇÃO CEREBRAL

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FRONT COVER IMAGES

Top panel, from left to right: 1) astrocytes stained for glial fibrillary acidic protein (GFAP; red) and counterstained with Hoechst 33342 (blue nuclei) in subventricular zone (SVZ) cell culture; 2) neurons stained with microtubule-associated protein 2 (MAP-2; red) and counterstained with Hoechst 33342 (blue nuclei) in SVZ cell culture; 3) neuronal precursors migrating in the rostral migratory stream (RMS), labeled by injection of retrovirus with green fluorescent protein (GFP) reporter in the SVZ; 4) GFP-labeled neuronal precursor (green) extending the leading process towards a dextran-Texas Red-filled blood vessel in the ischemic striatum. Bottom panel, from left to right: 1) oligodendrocyte stained for O4 sulfatide in SVZ cell culture; organotypic slice from the hippocampus stained with Hoechst 33342 to visualize the cell nuclei; GFP-labeled SVZ neuroblasts migrating in the RMS along the dextran-Texas Red-filled vasculature. All images were obtained from fixed or live tissue extracted from mouse brain. All images were acquired by Sofia Grade.

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PUBLICATIONS

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ABBREVIATIONS

AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
ANOVA	Analysis of variance
BDNF	Brain-derived neurotrophic factor
BMP	Bone morphogenetic protein
BrdU	5-bromo-3'-deoxyuridine
BSA	Bovine serum albumin
CL	Contralateral
CNP	2'3'-cyclic nucleotide-3'-phosphohydrolase
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
CPZ	Cuprizone
CSF	Cerebrospinal fluid
CTRL	Control
DAG	Diacylglycerol
Dcx	Doublecortin
DG	Dentate gyrus
DIG	Digoxigenin
DiI	1,1',dioctadecyl-3,3,3'3'-tetramethylindocarbocyanine perchlorate
DIV	Days *in vitro*
DMEM/F-12	Dulbecco's Modified Eagle's Medium/Ham's F-12
DNA	Deoxyribonucleic acid
EAE	Experimental autoimmune encephalomyelitis
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
eGFP	Enhanced green fluorescent protein
EGTA	Ethyleneglycoltetraacetic acid
EM	Electron microscopy
eNOS	Endothelial nitric oxide synthase
eNSCs	Embryonic neural stem cells
EPL	External plexiform layer
EPO	Erythropoietin
ERK	Extracellular signal-regulated kinase
ESCs	Embryonic stem cells

FBS	Fetal bovine serum
FGF-2/bFGF	Fibroblast growth factor-2 (or basic fibroblast growth factor)
GABA	Gamma-aminobutyric acid
$GABA_A$	Gamma-aminobutyric acid receptor A
GalC	Galactocerebroside
GAT4	GABA transporter 4
GBSS	Gey's balanced salt solution
GCL	Granule cell layer
GDNF	Glial cell-derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
HBSS	Hank's balanced salt solution
HD	Huntington's disease
HDAC	Histone deacetylases
HIF-1α	Hypoxia-inducible factor-1 alpha
Hist	Histamine
HS	Horse serum
HVC	High vocal center
i.c.v	Intracerebroventricular
IGF-1	Insulin-like growth factor-1
iPSCs	Inducible pluripotent stem cells
IPSI	Ipsilateral
IP_3	Inositol-1,4,5-triphosphate
JNK	c-Jun N-terminal kinase
KO	Knock-out
LGE	Lateral ganglionic eminence
LIF	Leukemia inhibitory factor
LTD	Long-term depression
LV	Lateral ventricle
MAG	Myelin-associated glycoprotein
MAP-2	Microtubule-associated protein-2
MBP	Myelin basic protein
MCA	Middle cerebral artery
MCAo	Middle cerebral artery occlusion
MCL	Molecular cell layer
MCP-1	Monocyte chemoattractant protein-1
MIA	Migration-inducing activity

MMPs	Matrix metalloproteinases
MMS	Medial migratory stream
MS	Multiple sclerosis
NBT-BCIP	Nitroblue-tetrazolium-chloride/5-bromo-4-chloro-indolylphosphate
NFkB	Nuclear factor kB
NGF	Nerve growth factor
NGS	Normal goat serum
NMDAR	N-methyl-D-aspartate receptor
NO	Nitric oxide
NPCs	Neural progenitor cells
NPY	Neuropeptide Y
NRG	Neuregulin
NSCs	Neural stem cells
NT-3	Neurotrophin-3
NT-4	Neurotrophin-4
OB	Olfactory bulb
OECs	Olfactory ensheathing cells
ON	Overnight
OPCs	Oligodendrocyte precursor cells
Opti-MEM	Opti-Minimal Essential Medium
O-2A	Oligodendrocyte-type-2 astrocytes
PAR	Protease-activated receptor
Par-1b/MARK2	Partitioning-defective-1b (Par-1b)/microtubule affinity-regulating kinase 2 (MARK2)
PBS	Phosphate-buffered saline
PD	Parkinson's disease
PDGF	Platelet-derived growth factor
PDL	Poly-D-lysine
PFA	Paraformaldehyde
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PI3K	Phosphatidylinositol 3-kinase
PKC	Protein kinase C
PLC	Phospholipase C
PLP	Proteolipid protein
PNS	Peripheral nervous system
PSA-NCAM	Polysialic acid (PSA)-neural cell adhesion molecule (NCAM)
PTX	Pertussis toxin

p75NTR	p75 neurotrophin receptor
RhoGEFs	Rho-guanine nucleotide exchange factors
RMS	Rostral migratory stream
ROCK	Rho-associated protein kinase
RT	Room temperature
SCCI	Single-cell calcium imaging
SCF	Stem cell factor
SDF-1	Stromal cell-derived factor-1
SEM	Standard error of the mean
SFM	Serum-free medium
SGZ	Subgranular zone
Shh	Sonic hedgehog
Sox2	SRY (sex determining region Y)-box 2
SSC	Saline sodium citrate
SVZ	Subventricular zone
TEA	Triethanolamine
TGF	Transforming growth factor
TH	Tyrosine hydroxylase
Throm	Thrombin
TLE	Temporal lobe epilepsy
TNF-α	Tumor necrosis factor-alpha
tPA	Tissue plasminogen
TrkB	Tropomyosin-related kinase B
T3	Triiodothyronine (or 3,3’,5-triiodo-L-thyronine)
VEGF	Vascular endothelial growth factor
VMPFC	Ventromedial pre-frontal cortex
WT	Wild-type
$[Ca^{2+}]_i$	Intracellular calcium concentration
$[^3H]$-thymidine	Tritiated-thymidine

SUMMARY

Neurogenesis is the process of generation of new functional neurons from precursor cells, and it occurs continuously in the adult mammalian brain. Newborn neurons derive from neural stem cells (NSCs) residing in two restricted niches in the adult brain, the subventricular zone (SVZ) of the lateral ventricles, and the subgranular zone (SGZ) of the hippocampal dentate gyrus. Neurogenic regions often react to brain injury, showing increased proliferation and migration of precursors to the injury site, which suggests that repair mechanisms exist. However, functional integration and cell replacement is scarce or inexistent, and no symptomatic recovery is observed. Two approaches to cell replacement therapies based on the use of NSCs have been considered: the transplantation of NSCs in the injured area, to increase the supply of new cells; and the stimulation of the endogenous self-repair mechanism involving NSCs activation and mobilization to the injured sites. Both approaches face major challenges. Cell transplantation requires the conditioning of NSCs to the specific cell phenotype lost with the injury. On the other hand, intrinsic brain repair mechanisms need to be stimulated *in vivo*, to direct more cells to the injured areas and to improve their local survival.

The present thesis aimed at providing new tools to surpass these issues by 1) developing a method to functionally discriminate oligodendrocytes in the SVZ cultures, which may allow a quick screening of oligodendrogenic factors; 2) evaluating the effect of a demyelinating injury in the differentiation of transplanted SVZ cells; 3) exploring the mechanism of injury-induced migration of SVZ precursors in an animal model of brain ischemia, an important target for manipulation to improve the spontaneous self-repair mechanism.

Considering that each cell type in the mixed SVZ cultures may respond differently to a given stimulus, we used single-cell calcium imaging (SCCI) to assess the pattern of differentiation in neurosphere cultures from

postnatal mouse SVZ, by recording the individual responses of cells under a defined sequence of stimuli. Previously, we showed that neurons and immature cells can be distinguished on the basis of their selective calcium-mediated response to KCl and histamine, respectively. Herein, we demonstrate that thrombin induces an oligodendrocyte-specific increase in $[Ca^{2+}]_i$, an effect mediated by protease-activated receptor-1 (PAR-1)-$G_{q/11}$ and subsequent phospholipase C (PLC)-dependent calcium efflux from intracellular stores. Hence, by monitoring variations of $[Ca^{2+}]_i$ upon application in sequence of KCl, histamine and thrombin we addressed the differentiation pattern within SVZ cultures, since astrocytes displayed no change in $[Ca^{2+}]_i$ with any of the stimuli. The method provides a rapid and reliable way to screen and identify compounds that favor differentiation of SVZ cells in oligodendrocytes, the myelin-forming cells of the central nervous system. This can be of relevance in the context of cell transplantation in demyelinating diseases such as multiple sclerosis.

Importantly, the differentiation of transplanted NSCs may be modulated by the diseased host environment. Indeed, we observed an increased number of oligodendrocytes generated by neurospheres grafted in demyelinated organotypic hippocampal slices, as compared to those grafted in intact slices. These findings indicate that the environment provided by the demyelinated host tissue instructs or favors SVZ cells differentiation towards the phenotype of the lost cells.

Moreover, we explored the mechanism supporting the mobilization of SVZ precursors to ischemic brain sites. In agreement with previous studies, we observed that SVZ precursors migrate to the ischemic striatum using blood vessels as a physical scaffold. Nonetheless, the molecular mechanism sustaining injury-induced vasculature-mediated migration was, hitherto, elusive. Herein, we show that vasculature in the ischemic striatum produces brain-derived neurotrophic factor (BDNF), a neurotrophin known to promote the constitutive vasculature-mediated migration of SVZ precursors in the rostral migratory

stream (RMS). We also demonstrate that neuroblasts recruited to the striatum maintain the expression of p75NTR, a low-affinity receptor for BDNF, whereas reactive astrocytes found in the injured striatum ensheath blood vessels and express TrkB, a high-affinity receptor for BDNF. Albeit devoid of BDNF mRNA, astrocytes were immunopositive for BDNF, indicating that these glial cells bind extracellular BDNF. Curiously, the same pattern of expression was previously observed in the adult RMS, where TrkB-expressing astrocytes bind vasculature-derived BDNF, thus leading to the entrance of migrating cells into the stationary phase. Real-time imaging of cell migration in acute brain slices revealed that cells migrating in ischemic striatum display higher exploratory behavior and longer stationary periods as compared to cells migrating in the RMS, and unveiled a role for BDNF in promoting migration of neuroblasts in the ischemic striatum. This suggests that BDNF may be used to boost migration to the ischemic area, thus increasing the chances of cell replacement by SVZ precursors.

In conclusion, this doctoral work provides a method to screen factors that shape SVZ cells differentiation, a pivotal adjuvant step in the design of cell transplantation therapies; highlights the importance of the injured host environment in the differentiation of the transplanted cells; and explores the mechanism that governs injury-induced migration of SVZ precursors to the ischemic striatum. Altogether, our findings may contribute to ameliorate cell transplantation strategies using NSCs or to amplify the intrinsic self-repair mechanism occurring in the brain upon injury.

RESUMO

A neurogénese é o processo de geração de novos neurónios funcionais a partir de células precursoras, e ocorre continuamente no cérebro adulto de mamíferos. Os novos neurónios derivam de células estaminais neurais (NSCs) que se encontram em dois nichos restritos no cérebro adulto: a zona subventricular (SVZ) e a zona subgranular (SGZ). Os nichos neurogénicos reagem frequentemente à lesão cerebral, observando-se um aumento da proliferação e a migração de precursores para o local de lesão, o que sugere a existência de mecanismos de reparação. Contudo, a integração funcional e restituição celular é escassa ou nula, não havendo recuperação sintomática. Duas estratégias para terapias de reparação celular usando NSCs têm sido consideradas: o transplante de NSCs na área lesionada, e a estimulação do mecanismo endógeno de auto-reparação. Ambas as estratégias apresentam desafios. O transplante celular requer a diferenciação das NSCs para o fenótipo afectado na lesão. Por outro lado, o mecanismo endógeno de reparação tem ser estimulado, *in vivo*, de forma a conduzir mais células para as zonas de dano e melhorar a sua sobrevivência.

A presente tese pretendeu fornecer novas ferramentas para superar estes problemas, ao 1) desenvolver um método que discrimine funcionalmente oligodendrócitos nas culturas SVZ, o que poderá permitir uma triagem rápida de factores oligodendrogénicos; 2) avaliar o efeito de uma lesão desmielinizante na diferenciação de células SVZ transplantadas; 3) explorar o mecanismo de migração dos precursores SVZ, induzida pela lesão, num modelo animal de isquémia cerebral.

Partindo da hipótese de que numa cultura de células SVZ, cada fenótipo poderá responder de forma diferente a um determinado estímulo, usámos imagiologia de cálcio em célula individual (SCCI) para avaliar o padrão de diferenciação em culturas de SVZ de murganho pós-natal, seguindo as

respostas individuais das células a uma sequência de estímulos. Previamente, mostrámos que neurónios e células imaturas podem ser distinguidos com base na sua resposta a KCl e a histamina, respectivamente. Neste trabalho, demonstramos que a trombina induz um aumento de $[Ca^{2+}]_i$ especificamente em oligodendrócitos, um efeito mediado pelo receptor activado por proteases-1 (PAR-1)-$G_{q/11}$ e consequente efluxo de cálcio das reservas intracelulares, dependente da fosfolipase C (PLC). Assim, seguindo as variações de $[Ca^{2+}]_i$ induzidas pela aplicação sequencial de KCl, histamina e trombina, caracterizámos o padrão de diferenciação em culturas SVZ, tendo em conta que a $[Ca^{2+}]_i$ nos astrócitos não é alterada pelos estímulos. Este método proporciona uma maneira rápida e fiável para rastrear e identificar compostos que favoreçam a diferenciação de células SVZ em oligodendrócitos, células que formam a bainha de mielina no sistema nervoso central. Assim, o método poderá fornecer uma importante contribuição no contexto de transplante celular em doenças desmielinizantes, tal como a esclerose múltipla.

No entanto, é importante considerar que a diferenciação de NSCs transplantadas possa ser influenciada pelo ambiente do tecido hospedeiro lesionado. De facto, observámos mais oligodendrócitos provenientes de neuroesferas depositadas em fatias organotípicas de hipocampo desmielinizado, do que de neuroesferas depositadas em fatias intactas. Estes resultados indicam que o ambiente fornecido pelo tecido hospedeiro desmielinizado favorece a diferenciação de células SVZ no fenótipo das células afectadas pela lesão.

Por fim, explorámos o mecanismo que suporta a mobilização dos precursores SVZ para locais de lesão isquémica. De acordo com estudos prévios, observámos que os precursores SVZ migram no estriado isquémico utilizando vasos sanguíneos como um suporte físico. No entanto, o mecanismo molecular que mantém a migração induzida pela lesão era, até à data, elusivo. Neste trabalho, mostramos que a vasculatura no estriado isquémico produz o factor neurotrófico derivado do cérebro (BDNF), uma neurotrofina que foi

identificada anteriormente como promotora da migração constitutiva de precursores SVZ na via migratória rostral (RMS). Verificámos que os precursores recrutados para o estriado expressam p75NTR, um receptor de BDNF de baixa afinidade, enquanto os astrócitos reactivos no estriado isquémico, envolvem vasos sanguíneos e expressam TrkB, um receptor de BDNF de alta afinidade. Apesar de desprovidos de mRNA para BDNF, os astrócitos apresentam marcação imunohistológica para BDNF, o que indica que estas células ligam o BDNF extracelular. Curiosamente, o mesmo padrão de expressão foi observado anteriormente na RMS de murganho adulto, onde astrócitos expressam TrkB e ligam o BDNF proveniente da vasculatura, o que leva à entrada das células migratórias em fase estacionária. Através de ensaios de imagiologia em tempo real, em fatias de cérebro agudamente dissociadas, revelámos que as células que migram no estriado isquémico exibem maior comportamento exploratório e períodos estacionários mais longos, em comparação com as células que migram na RMS, e que o BDNF promove a migração de neuroblastos no estriado isquémico. Isto sugere que o BDNF possa ser utilizado para potenciar a migração para a área isquémica, aumentando as possibilidades de substituição celular por precursores SVZ.

Em conclusão, este trabalho de doutoramento oferece um método para identificar factores que moldem a diferenciação de células SVZ, uma etapa central no desenho de terapias de transplante celular; realça a importância do ambiente apresentado pelo tecido lesionado na diferenciação de células transplantadas; e explora o mecanismo que promove a migração dos precursores SVZ no estriado isquémico. No seu conjunto, os resultados poderão contribuir para melhorar estratégias que visem reparação cerebral através do uso de NSCs.

CHAPTER 1

Introduction

1.1 Neurogenesis in the adult brain: conceptual overview

1.1.1 Historical synopsis

Neurogenesis, defined as the generation of new functional neurons from precursors, was believed to take place during the embrionary and perinatal periods of the mammalian development, exclusively. The elaborated architecture of the adult brain described by works of Ramón y Cajal (Ramón y Cajal, 1899, 1909) was considered immutable, thus turning any view claiming generation of new neurons as inconceivable. Seldom studies on postnatal and adult rat brains detected mitotic figures (Hamilton, 1901; Allen, 1912; Bryans, 1959). However, the long-held tenet was hardly contradicted due to the absence of techniques that allowed detection of cell division together with neuronal and glial labeling. In the late 1950s, tritiated ([^{3}H])-thymidine autoradiography was developed. The radiolabeled nucleoside is incorporated into the deoxyribonucleic acid (DNA) of dividing cells. The method unveiled, for the first time, the presence of new neurons in the neocortex, dentate gyrus (DG) and olfactory bulb (OB) of adult rat brain, in a series of studies conducted by Joseph Altman (Altman, 1962, 1963; Altman and Das, 1965; Altman, 1966; Altman and Das, 1966; Altman, 1967, 1969). These findings were ignored for two decades, as were the supporting reports from Kaplan and colleagues that came out meantime (Kaplan and Hinds, 1977; Kaplan, 1983, 1984). Finally, the concept of adult neurogenesis was revisited in the 1980s by the group of Fernando Nottebohm, with a cohort of evidences that irrefutably proved the constant birth of new neurons in the brain region involved in song learning of the adult songbird. The authors demonstrated that new cells were generated in the border of the lateral ventricles and migrated to the high vocal center (HVC) (Goldman

and Nottebohm, 1983; Alvarez-Buylla and Nottebohm, 1988), where they acquired ultrastructural features of neurons, extended axons into target regions, formed synapses (Burd and Nottebohm, 1985; Alvarez-Buylla et al., 1988) and were stimulated by sound (Paton and Nottebohm, 1984). Later on, the precursors of the newborn neurons, so called neural stem cells (NSCs), were successfully isolated from the brain of adult rodents (Reynolds and Weiss, 1992) and later humans (Kukekov et al., 1999). *In vivo*, the synthetic thymidine analog 5-bromo-3'-deoxyuridine (BrdU) was then used to label the newborn cells in the brain of adult rodents, and combined with cell type markers that confirmed a neuronal phenotype (Kuhn et al., 1996; Kempermann et al., 1997). Like [^{3}H]-thymidine, BrdU is inserted into the DNA of cells in division, but revealed by immunostaining. The field of adult neurogenesis started to gain strength but only witnessed a global acceptance and wide interest when Eriksson et al. (1998) showed the presence of BrdU-labeled neurons in the hippocampal DG of human patients. Since then, a growing number of studies has been providing insights into the identity of the NSCs, the properties of the privileged neurogenic niches, their cellular composition and ultrastructure, the dynamics and regulation of cell proliferation, differentiation and migration, as well as their integration in the recipient tissue and functional relevance for the organism. These subjects will be discussed in the next sections, for a general comprehension on adult neurogenesis, with a special focus on the processes of differentiation and migration documented in brain injury paradigms, which are the pillars of the present thesis.

1.1.2 Neural stem cells in the adult brain

The adult brain is remarkably plastic and dynamic. The active mutability of the adult brain is reflected in the constant alterations of the structure of axons, dendrites and synapses, and the presence of NSCs that continuously produce new neurons capable of integrating in the existing

circuitries (Lledo et al., 2006). Moreover, neurogenesis appears to be highly conserved in mammalian species, including humans (Eriksson et al., 1998; Sanai et al., 2004). The discovery of brain plasticity shed light on the understanding of brain function since it constitutes an important adaptive response of the brain to face internal and external environmental changes.

It is of general acceptance that neurogenesis persists throughout life, in two specialized niches of the adult mammalian brain: the subventricular zone (SVZ), bordering the lateral ventricles (Alvarez-Buylla and Garcia-Verdugo, 2002), and the subgranular zone (SGZ) of the hippocampal DG (Song et al., 2002b). Indeed, the adult brain parenchyma is generally inhibitory to neuronal regeneration (Agasse et al., 2004). Nevertheless, the SVZ and SGZ local niches present peculiar cellular and molecular features that support and maintain the self-renewal and early differentiation of NSCs, holding a permissive specialized milieu for neurogenesis (Doetsch, 2003). Likewise, the parenchyma that receives the new neurons coming from the SVZ and SGZ - the OB and the granule cell layer (GCL) of the DG, respectively - is permissive for their maturation and synaptic integration, consenting the addition and/or replacement of neurons in the pre-established circuitries. The components of such unique niches that embrace neurogenesis will be shortly described in the section 1.1.7.

One may ask why OB and hippocampus are privileged areas, with the capacity to receive new neurons every day, which turns them highly plastic and prompt to adapt to the varying ambience. Interestingly, such areas are both evolutionarily old areas of the brain and, more importantly, they handle large amounts of information, or use particular coding strategies to process that information. Besides, studies have shown evidences that the migratory pathway from the SVZ to the OB, called rostral migratory stream (RMS), and the OB itself, harbor stem cells in the adulthood (Gritti et al., 2002; Liu and Martin, 2003). *In vitro* assays proved that RMS NSCs are multipotent at the clonal level (Gritti et al., 2002), whereas *in vivo* analysis revealed their astrocytic identity

and showed that they supply the granule and periglomerular layers in the OB with new neurons (Alonso et al., 2008; Mendoza-Torreblanca et al., 2008). Outside SVZ-OB and SGZ-GCL axes, neurogenesis appears to be quite limited or inexistent. Some studies claimed the neurogenic status of other regions like neocortex (Gould et al., 1999; Ohira et al., 2010), substantia nigra (Lie et al., 2002; Zhao et al., 2003), striatum (Bédard et al., 2002b; Luzzati et al., 2006), amygdala (Bernier et al., 2002), hypothalamus (Yuan and Arias-Carrión, 2011), brain stem (Bauer et al., 2005), olfactory tubercule (Bédard et al., 2002a) and piriform cortex (Pekcec et al., 2006), although with a much lower production of new neurons, as compared to SVZ or SGZ (Fig. 1.1). Nevertheless, no consistency has been accomplished regarding the existence of neurogenesis in such regions, in the intact mammalian brain. Conflicting data may be due to differences in the sensibility and detection limits of techniques used (since a much lower level of neurogenesis than in SVZ/SGZ was suggested), or to possible histological procedures that may unmask or interfere with the immunodetection of NSCs or progenitors. On the other hand, false-positive labeling may occur, for example by cell fusion, generating misleading conclusions (reviewed in Breunig et al., 2007; Gould, 2007).

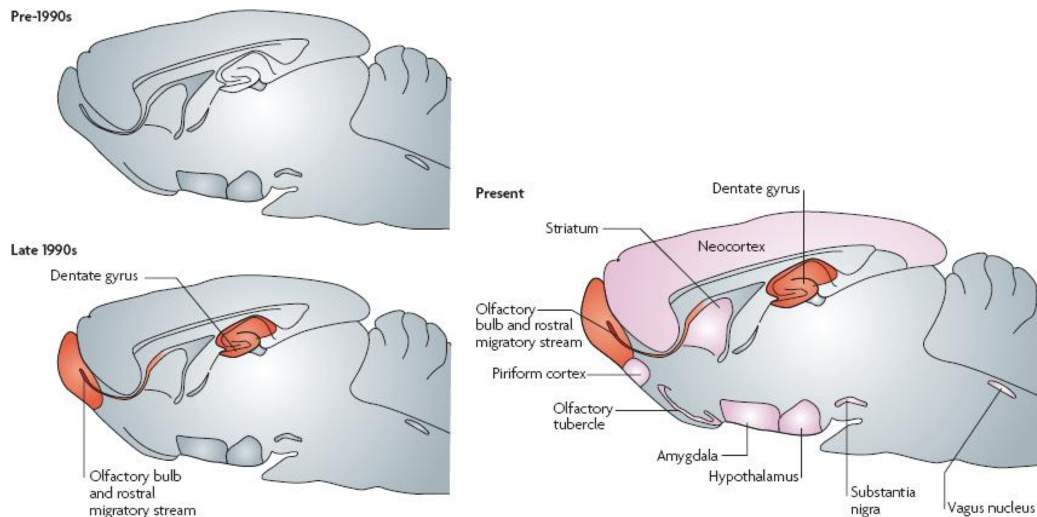


Figure 1.1. Changes in the view of adult neurogenesis in the mammalian brain over the past 20 years shown on a schematic diagram of the adult rat brain. In the pre-1990s, all regions were categorized as "non-neurogenic" (grey). In the late 1990s, only the DG and OB (as well as the SVZ, which gives rise to the RMS) were categorized as "neurogenic" (red). Today, the two known neurogenic regions are shown in red, and areas for which there is controversial evidence for low-level of adult neurogenesis are shown in pink. It should be noted that since not all of these brain regions are present on the same sagittal plane, their location is approximated on the diagram. [Adapted from Gould (2007)].

1.1.3 Neural stem cell properties: proliferation, self-renewal and multipotency

NSCs are defined by three cardinal properties: proliferation, self-renewal and multipotency. These concepts were raised primarily by the characterization of NSCs dynamics *in vitro* (Reynolds and Weiss, 1992). In the presence of the appropriate growth factors, cells isolated from the lateral ventricular wall or from the hippocampal DG of the adult brain, proliferate and generate floating cell clusters, called neurospheres. The ability to form secondary neurospheres from the primary ones unveiled the self-renewal property of these cells. On the other hand, following growth factors withdrawal from the culture medium and provision of a substrate for adhesion, the cells differentiate in neurons, astrocytes and oligodendrocytes demonstrating their multipotency. Noteworthy, neurospheres can be generated from cells isolated

from other brain regions, during the postnatal period, although afterwards this capacity is sustain only in the neurogenic areas (Laywell et al., 2000; Itoh et al., 2006).

Some studies have raised controversy about the true stemness of SGZ cells in the adult brain, i.e., whether the SGZ harbors stem cells or rather a resident population of progenitors that proliferate, but have limited capacity to self-renew, since no long-term self-renewal could be proven *in vitro* (Seaberg and van der Kooy, 2002; Bull and Bartlett, 2005). Also, multipotency was questioned due to the inability of the DG spheres to generate neurons (Seaberg and van der Kooy, 2002). However, Bull and Bartlett (2005) have shown that brain-derived neurotrophic factor (BDNF), a neurotrophin that is present in the endogenous DG microenvironment and lacking in the culture medium, is crucial for the generation of new neurons in the hippocampus. Besides, high levels of bone morphogenetic protein (BMP) signaling occur in SGZ, which hamper NSCs properties *in vitro* (Bonaguidi et al., 2008). Indeed, this study has demonstrated that SGZ cells cultured in the presence of Noggin, a BMP inhibitor which is expressed in the adult DG, are able to self-renew and differentiate in neurons, astrocytes and oligodendrocytes. Furthermore, *in vivo* clonal analysis of SRY (sex determining region Y)-box 2 transcription factor (Sox2)- or nestin-expressing cells in the SGZ demonstrated the self-renewal and multipotency capacities of these cell populations in the adult hippocampus (Suh et al., 2007; Bonaguidi et al., 2011). The authors verified, by tracing $Sox2^+$ or $nestin^+$ cells in SGZ, that they give rise to neurons and few astrocytes.

Regarding adult SVZ cells, it is known for a long time that these cells proliferate forming neurospheres in the presence of epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF or FGF-2) (Reynolds et al., 1992; Gritti et al., 1995). Moreover, they self-renew and differentiate into neurons and macroglia in culture (Gritti et al., 1999). *In vivo*, during the peak of gliogenesis occurring in first postnatal weeks, SVZ progenitors generate not

only neurons but also astrocytes and oligodendrocytes, which colonize the striatum, white matter, and cerebral cortex (Levison and Goldman, 1993). In the adulthood, however, the SVZ almost ceases the production of glial cells, maintaining only a minor contribution on the generation of new oligodendrocyte progenitors that migrate and populate the corpus callosum, striatum, and fimbria-fornix (Menn et al., 2006).

The existence of a tripotent NSC with the capacity to generate neurons, astrocytes and oligodendrocytes in the healthy adult brain thus remains to be demonstrated *in vivo*. In resume, both regions contain cells that proliferate continuously over the lifetime of the animal, either by symmetric divisions, giving rise to two identical daughter stem cells, or by asymmetric divisions, generating progenitors that ultimately give rise to mature and functional cells, mostly neurons that integrate into existing circuitries.

1.1.4 Identity of adult neural stem cells

The identity of the NSCs in the adult brain was a topic under great debate in the late 1990s. Two theories came out in the same year, one pointing for the ependymal origin of the new neurons (Johansson et al., 1999), and other favoring a glial origin (Doetsch et al., 1999b). Johansson and colleagues (1999) inferred that the ependymal cells, which directly contact and delimit the lateral ventricle, were the primary precursors of the new neurons. This assumption was based on the observation of 1,1',dioctadecyl-3,3,3'3'-tetramethylindocarbocyanine perchlorate (DiI)-labeled cells migrating to the OB and expressing neuronal markers, in intraventricularly DiI-infused mice. However, DiI is a lipophilic and highly diffusible dye, being taken by other cells adjacent to the ependymal layer as well. Furthermore, it is worth noting that a population of glial fibrillary acidic protein (GFAP)$^{+}$ SVZ astrocytes, the true stem cells, have a single cilium projecting inside the ventricle and contacting the cerebrospinal fluid (CSF) (Mirzadeh et al., 2008), which facilitates the uptake of

DiI by these cells. The authors claimed to have confirmed their conclusions by the neurosphere formation assay, from putative ependymal cells isolated by Notch1 receptor immunosorting. Nonetheless, it is now known that Notch1 expression also occurs in SVZ astrocytes (Givogri et al., 2006) and constitutes a key regulator of NSCs (Ables et al., 2011). Actually, electron microscopy (EM) analysis of [^{3}H]-thymidine-labeled cells lining the lateral ventricle showed no evidence of ependymal cell division, whereas in the SVZ layer, cells displaying morphological characteristics of astroglia were mitotic (Doetsch et al., 1999b). In agreement, a subsequent study demonstrated that in the adult hippocampus, new granule neurons arise from an astrocytic primary precursor located in the SGZ (Seri et al., 2001). A growing number of studies supported the latest theory, ultimately confirming the glial identity of NSCs residing in the adult SVZ and SGZ. The clearest evidence that some astrocytes in the SVZ and SGZ are the primary precursors *in vivo* is that they are capable of reconstituting the germinal layer after ablation of the actively dividing cells by anti-mitotic treatment (Doetsch et al., 1999a; Seri et al., 2001). It is important to refer, however, that despite the quiescence of ependymal cells under normal conditions, these cells are activated after stroke to produce neurons and astrocytes, but do not self-renew (Carlen et al., 2009). Moreover, a fate mapping study revealed that SVZ astrocytes can give rise to ependymal cells and vice-versa after neuraminidase-induced lesion, revealing a high plasticity of cells in the niche that is recruited under certain conditions (Nomura et al., 2010).

Curiously, the glial origin of adult new neurons resembles neurogenesis in the developing brain, in which radial glia gives rise to all the neural lineages including neurons. The current and well-supported view is that the adult NSCs belong to a continuous lineage starting with neuroepithelial cells that surround the neural tube, through radial glia in the early developing brain, when neurogenesis begins, and resulting in long-lasting adult SVZ and SGZ

astrocytes (Merkle and Alvarez-Buylla, 2006; Ihrie and Alvarez-Buylla, 2008; Malatesta et al., 2008; Kriegstein and Alvarez-Buylla, 2009).

1.1.5 Neurogenesis in the subventricular zone

1.1.5.1 General characteristics

The SVZ is by far the largest pool of NSCs in the adult mammalian brain. A massive progeny of more than 30 000 neuronal progenitors, called neuroblasts, leave the niche every day and migrate away towards the distant OB, in chains enwrapped by specialized astrocytes, along a precisely delineated tangential route, the RMS (Lledo et al., 2006) (Fig. 1.2). Once in the core of the OB, neuroblasts detach individually from the chains and migrate radially towards the bulbar cell layers of interneurons, where they integrate and mature mainly as new gamma-aminobutyric acid (GABA)-producing granule neurons (De Marchis et al., 2004) including some cells that co-express calretinin (Merkle et al., 2007), others as GABAergic periglomerular neurons, including different subtypes co-expressing tyrosine hydroxylase (TH), parvalbumin, calretinin or calbindin (Baker et al., 2001; Saino-Saito et al., 2004; De Marchis et al., 2007; Whitman and Greer, 2007), and another subset as glutamatergic juxtaglomerular neurons (Brill et al., 2009). Herein, the new interneurons establish reciprocal dendrodendritic synapses with the OB principal cells, the mitral and the tufted cells (reviewed in Lledo et al., 2008), while the new juxtaglomerular neurons project their dendritic arbors into several adjacent glomeruli, a typical characteristic of short-axon cells (Brill et al., 2009). Also, a study by Yang (2008) has suggested that SVZ progenitors in the postnatal rodent brain give rise to a subset of interneurons of the external plexiform layer (EPL). Importantly, the GABAergic (De Marchis et al., 2004) and dopaminergic (Baker et al., 2001) phenotypic determination occurs during neuroblast migration in the RMS, constituting lineage-restricted subpopulations of migrating progenitors that target the OB.

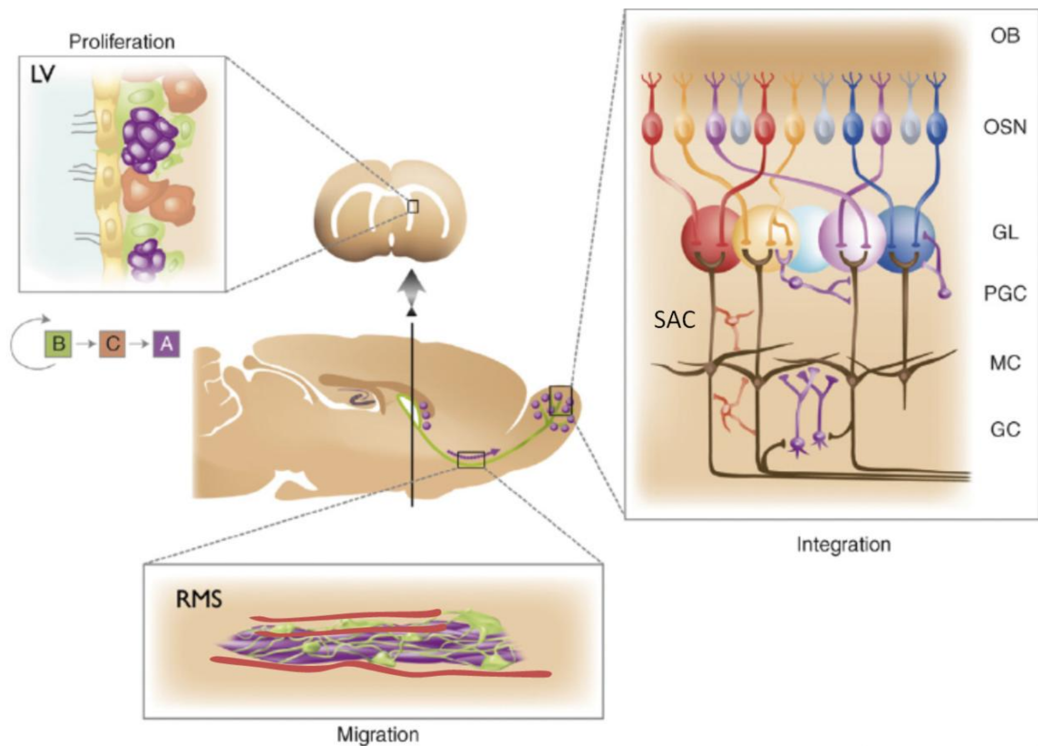


Figure 1.2. The SVZ-OB pathway represents one of the few constitutive neurogenic areas in the adult central nervous system (CNS). Diagram in the center shows a sagittal section of the rodent forebrain. The arrow indicates the tangential migration of neuroblasts (purple dots) toward the OB. New neurons recruited into the OB continually replace local interneurons. Panel on the right illustrates the wiring of the OB. Left panel shows the neurogenic niche. Here, proliferation in the SVZ takes place in the walls of the lateral ventricle (LV), where stem cells (in green, type B cells) divide to generate transit-amplifying cells (in brown, type C cells), which in turn give rise to neuroblasts (in purple, type A cells) that migrate in the RMS, along vasculature and channeled by astrocytes (bottom panel), to their final destination in the OB, where they differentiate into interneurons (PGC and GC, both in purple) or SAC. OSN, olfactory sensory neurons; GL, glomeruli; PGC, periglomerular cells; MC, mitral cells; SAC, short-axon cells (represented in red); GC, granule cells. [Adapted from Lledo et al. (2008)].

Strikingly, the SVZ of the adult human brain harbors NSCs as well, although the niche displays a different cytoarchitecture from the one found in rodents. Indeed, using immunohistochemistry and EM analysis, Alvarez-Buylla's group described a multilayered organization throughout the ventricle wall: a monolayer of ependymal cells lining the ventricle (layer I), a hypocellular gap layer (layer II), a ribbon of proliferative SVZ astrocytes (layer

III) and a transitional layer in the interface with the parenchyma (layer IV) (Sanai et al., 2004; Quinones-Hinojosa et al., 2006). In the *in vitro* neurosphere assay, the SVZ astrocytes from layer III are multipotent, generating neurons, astrocytes and oligodendrocytes (Sanai et al., 2004). In these studies, no evidence of a chain of migrating progenitors leaving the SVZ was found. However, others have found newly generated neurons in the human OB (Bédard and Parent, 2004). Reinforcing the latter, Curtis et al. (2007b) suggested the existence of RMS in the human brain, disposed around a remnant extension of the lateral ventricle towards the OB. Later on, the same group characterized the cellular composition and morphological arrangement of the RMS in the adult human brain (Kam et al., 2009). Recently, however, Sanai et al. (2011) have confirmed the presence of RMS in uninterrupted streams during early postnatal stages of human development, but facing a sharp decline until 18 months old. Indeed, in later childhood and adulthood, occasional neuroblasts were found as individual cells or in pairs. In contrast with Curtis et al. (2007b) and Kam et al. (2009), this study reported no ventricular extension. Moreover, an unexpected additional migratory route targeting the ventromedial pre-frontal cortex (VMPFC) was revealed. In the early postnatal period (4-6 month-old specimens) SVZ-derived neuroblasts branch off the proximal limb of the RMS to diverge into the medial migratory stream (MMS) and supply the VMPFC. The MMS was, however, absent in later stages of development (8-18 month-old specimens) (Sanai et al., 2011). Hence, debate is still ongoing as concern to the existence of RMS in the adult human brain, besides the emergency of new questions regarding the functional relevance of the MMS during the early postnatal period of human development.

1.1.5.2 Cytoarchitecture of the SVZ and lineage relationships

In the late 1990s, researchers explored the lineage relationship between the migrating progenitors and the SVZ stem cells, by EM analysis of the cell composition and organization of the SVZ germinal zone (Doetsch et al., 1997). EM allowed the identification of three main cell types: 1) neuroblasts or type A cells, dark and with an elongated cell body; 2) astrocytes or type B cells (type B1 and B2 cells), light, with irregular shape and abundant intermediate filaments; and 3) type C cells, large, less elongated and with an intermediate electron-density between cells A and C. Thereafter, three-dimensional reconstruction of serial ultrathin EM sections, together with immunohistological analysis, provided a topographic model for the adult SVZ niche and knowledge on the lineage relationships among the cell types (Mirzadeh et al., 2008) (Fig. 1.3). The *bona fide* adult NSCs in the SVZ are the type B1 astrocytes, which are relatively quiescent. They derive from the embryonic radial glia, and resemble their progenitor, with a primary cilium that projects into the ventricle and a long basal process that contacts blood vessels. Interestingly, Mirzadeh and colleagues (2008) revealed a pinwheel organization of the SVZ, given by clusters of ependymal cells surrounding B1 apical surfaces. The slowly-dividing type B1 astrocytes give rise to rapidly-dividing transit-amplifying cells, the type C cells, which in turn, generate migrating neuroblasts or type A cells. Type A cells are also mitotically active along their chain migration in the RMS (Menezes et al., 1995), and are channeled by a tubular glial scaffold made of type B2 astrocytes. In addition to the ultrastructural and dynamic features, the expression of antigens can be used to distinguish the SVZ cell types. Accordingly, for instance, type B cells are GFAP^{+}, type C cells are GFAP-/Dlx2^{+} and type A cells are GFAP-/Dlx2^{+}/polysialic acid-neuronal cell adhesion molecule (PSA-NCAM)$^{+}$ (reviewed in Ming and Song, 2005).

In vitro, SVZ neurospheres were initially believed to be derived exclusively from type B astrocytes (Doetsch et al., 1999b). However,

neurosphere-forming ability is not exclusive of these primary precursors. Indeed, both type B and type C cells can generate neurospheres, and in fact, C cells represent about 70% of the neurosphere-forming activity in the SVZ (Doetsch et al., 2002).

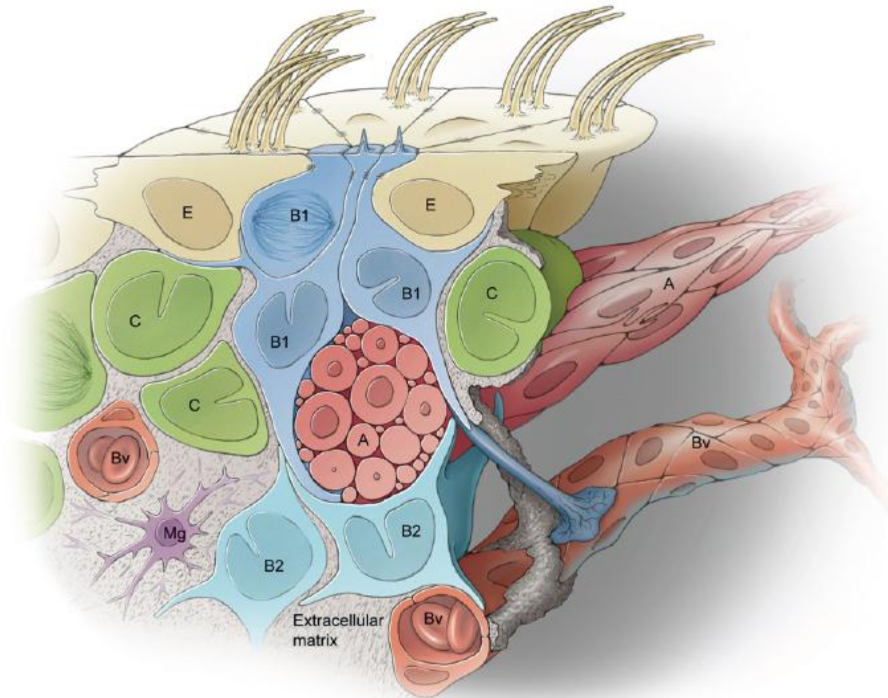


Figure 1.3. Cytoarchitecture of the periventricular adult stem cell niche. The apical ventricular zone and planar surface is shown at top, with the rest of the VZ-SVZ shown in cross section below. Ependymal cells (E in beige) are multiciliated, and the basal bodies of these cilia are oriented in the direction of CSF flow. Ependymal cells form pinwheel-like structures around the apical processes of type B1 cells (shown in blue). Type B1 cells extend a short, non-motile primary cilium into the ventricle. These cells maintain contact with the ventricle but disassemble the primary cilium while dividing. Type B1 cells also frequently extend a basal process with an endfoot that contacts blood vessels (Bv, shown in brown). Type B2 cells, in contrast, have astrocytic characteristics but do not contact the ventricle (shown in lighter blue). Transit-amplifying type C cells (in green) are found close to type B cells. Dividing C cells are also often found in close proximity to blood vessels (shown at left). Type B1 cells also contact their more differentiated progeny, the chains of migrating type A neuroblasts (shown in red). Type A cells migrate tangentially in chains (shown towards right of figure) that ultimately coalesce to form the RMS taking the young neurons to the OB for terminal differentiation. The VZ-SVZ niche also includes extracellular matrix (shaded) that contacts all the cell types in this region, including blood vessels and microglia (in purple). [Adapted from Ihrie and Alvarez-Buylla (2011)].

1.1.5.3 Functional significance of SVZ-OB neurogenesis

The OB is a structure of the vertebrate brain involved in the processing of odor information. The odors are first detected by olfactory sensory neurons (OSNs) in the olfactory epithelium. These sensory cells express receptors that bind odorant molecules, in the nasal cavity, and extend axons that enter the OB and form synapses with the primary dendrites of mitral and tufted cells (reviewed in Lledo et al., 2004). Thus, OSNs convert the chemical signal into an electrical signal that is transferred to the OB. The two populations of interneurons in the OB, granule and periglomerular cells, control the activity of the mitral and tufted cells, and thereby orchestrate the processing of the odor information (reviewed in Lledo et al., 2004).

The synaptic interplay between interneurons and mitral cells is believed to be central in odor discrimination, memory and learning (Yokoi et al., 1995; Laurent, 2002; Lledo and Lagier, 2006). However, the role of the adult-generated new interneurons in the olfactory behavior is unclear. Studies have yielded discrepant results, which may be due to 1) differences in the models used to modulate neurogenesis: odor enrichment, odor deprivation, constitutive or inducible genetic models, pharmacological agents or irradiation; or 2) differences in the behavioral paradigms employed to study olfaction: tasks based on spontaneous odor behavior, or instead, based on associative olfactory learning, which include operant or non-operant learning tasks (reviewed in Lazarini and Lledo, 2011; Breton-Provencher and Saghatelyan, 2012).

Spontaneous odor behavior can be used to evaluate the short-term odor memory upon ablation or stimulation of neurogenesis. Hence, ablation of neurogenesis by anti-mitotic drug treatment results in a marked reduction of short-term memory (Breton-Provencher et al., 2009), whereas odor enrichment increases the number of new bulbar interneurons and improves short-term memory (Rochefort et al., 2002). Nonetheless, neural cell adhesion molecule (NCAM)-deficient mouse, which displays impaired migration of the neuronal

precursors in the RMS, presents decreased numbers of new interneurons in the OB, but unaltered short-term odor memory (Gheusi et al., 2000). It is important to stress that constitutive genetic models can display compensatory mechanisms built during the development of the organism. Besides, the deletion or insertion of a gene may not affect adult neurogenesis solely. Therefore other models offering more specificity and less lateral effects are preferred to ablate adult neurogenesis, as irradiation or anti-mitotic drug treatment.

Associative operant tasks indicated that challenging neurogenesis, either by irradiation (Lazarini et al., 2009) or anti-mitotic treatment (Sultan et al., 2010) causes deficits in long-term olfactory memory, but no effect on learning performance. In contrast, associative non-operant tasks suggested that depletion of neurogenesis by genetic models (Imayoshi et al., 2008) or anti-mitotic treatment (Breton-Provencher et al., 2009) harbors no effect in long-term memory. Mandairon et al. (2011) dissected differences between the two subtypes of associative learning tasks. The authors demonstrated that operant tasks increase the survival of new neurons in the OB, while non-operant procedures have no effect on the survival of the new cells. Hence, the involvement of adult-generated neurons may be influenced by their survival rate, which depends of the learning task. Moreover, analysis of activation levels of different brain structures related to olfaction showed that the contribution of a given brain area to the processing of the sensory information depends on the behavioral paradigm used (Mandairon et al., 2011).

1.1.6 Neurogenesis in the hippocampus

1.1.6.1 General characteristics

Approximately 9000 new cells are daily generated in the SGZ of the hippocampal DG, and travel a short distance, locally in the DG, to the neighboring GCL (Lledo et al., 2006). Along this short path, the migrating cells undergo differentiation and ultimately settle in the GCL of the DG. Herein, the

maturing cells extend axons towards the CA3 pyramidal cell layer, via mossy fiber path, and send dendrites to the molecular cell layer (MCL), becoming new functional granule neurons. The new granule neurons receive inputs from the entorhinal cortex and send outputs to the hilus and CA3, a region that is crucial to memory formation (reviewed in Ming and Song, 2005; Ehninger and Kempermann, 2008).

As briefly aforementioned, Eriksson et al. (1998) detected hippocampal neurogenesis in the adult human brain. The authors examined *post-mortem* human brain tissue from patients with cancer, who had been treated with BrdU for diagnostic purposes. BrdU-labeled cells were found in the DG, in co-expression with neuronal markers, demonstrating that new neurons are generated from dividing progenitor cells in the DG of adult humans. This study was revolutionary, as it proved for the first time the genesis of new neurons in the adult human brain. The next sections offer a simplistic and concise overview on some aspects of DG neurogenesis since the present work is centered, instead, in SVZ neurogenesis.

1.1.6.2 Cytoarchitecture of the SGZ and lineage relationships

Two populations of precursors have been identified in the SGZ of the hippocampus: radial astrocytes, which extend a prominent radial process across the GCL (Seri et al., 2004), are $GFAP^{+}/Sox2^{+}$ (Fukuda et al., 2003; Garcia et al., 2004; Suh et al., 2007), and rarely divide; and non-radial precursors which extend basal processes under the GCL, are GFAP-/$Sox2^{+}$, and actively proliferate (Kronenberg et al., 2003; Seri et al., 2004). Radial astrocytes, referred as type I cells, share ultrastructural similarities with type B cells of the SVZ and have been identified as the primary precursors of the new neurons in the DG (Kempermann et al., 2004; Ehninger and Kempermann, 2008). However, a study suggested that non-radial SGZ $Sox2^{+}$ cells, called type II cells, can self-renew and give rise to neurons and glial cells (Suh et al., 2007). In

addition, the authors proposed the existence of a reciprocal relationship between both $Sox2^+$ precursors, type I and type II. Despite no definitive evidence, the prevalent view is that type I radial astrocytes give rise to type II cells (Seri et al., 2001; Filippov et al., 2003; Fukuda et al., 2003; Kempermann et al., 2004; Steiner et al., 2006). Then, type II cells originate intermediate progenitors, the type III cells, which in turn generate neuroblasts. These precursors migrate to the inner GCL and differentiate in mature and functional granule neurons (van Praag et al., 2002; Seri et al., 2004).

1.1.6.3 Functional significance of the hippocampal neurogenesis

The hippocampus is a brain structure that is responsible for the formation of certain types of memory and involved in emotional behavior. Numerous studies support the current belief that SGZ neurogenesis plays a role in memory and learning. Accordingly, hippocampal neurogenesis in humans is affected by neurological disorders associated with a cognitive decline (reviewed in Zhao et al., 2008). On the other hand, evidences suggest that learning may enhance the survival and incorporation of the new neurons in the GCL circuitry (Leuner et al., 2006). Besides, reducing or ablating SGZ neurogenesis by the use of anti-mitotic agents (Shors et al., 2001) or irradiation (Snyder et al., 2005) produces deficits in hippocampal-dependent tasks. Computational modeling analysis by Deng et al. (2010) has pinpointed an important role for the DG in the pattern separation of inputs coming from the entorhinal cortex, a process that may be helped by the continuous neuronal turnover in the SGZ. Also, SGZ neurogenesis has been implicated in mood regulation. Despite few exceptions, studies have indicated that stress and depression decrease SGZ precursors proliferation, which correlates with deficits in the performance on hippocampal-dependent learning tasks (reviewed in Zhao et al., 2008). However, no direct evidence that adult neurogenesis is required for emotional regulation hitherto exists.

1.1.7 Regulation of adult neurogenesis: the role of niche environment, cell-intrinsic factors and external stimuli

Interestingly, precursor cells isolated from neurogenic areas and transplanted into non-neurogenic regions display very limited neurogenesis (Herrera et al., 1999; Lim et al., 2000; Temple, 2001a). On the contrary, heterotopic precursor cells transplanted into the SVZ, SGZ or RMS can generate neurons that are appropriate to each region. For example, adult rat hippocampal progenitors grafted in the RMS migrate towards the OB and differentiate in TH^+ neurons, a phenotype not normally undertaken by these cells (Suhonen et al., 1996). Moreover, progenitors isolated from the adult rat spinal cord, which normally generate astrocytes and oligodendrocytes, are able to integrate and to differentiate in neurons when grafted in the GCL of the adult hippocampus, but not when grafted in the surrounding areas (Shihabuddin et al., 2000). Altogether, these findings suggested that signals from the brain environment are able to shape the biology of the hosted precursor cells.

Accumulating evidences counteract this stem cell plasticity view, and instead indicate that not all the stem cells are equivalent, but they are actually temporally and regionally restricted (reviewed in Temple, 2001a, b; Lledo et al., 2008). Accordingly, SVZ astrocytes give rise to different interneuron subtypes depending on their location in the SVZ (Merkle et al., 2007). Specific regional labeling and tracking of SVZ precursors suggested that NSCs in the adult SVZ are heterogeneous, organized in a spatial-coded manner, wherein parceled subpopulations have restricted potential to generate different neuronal subtypes in the adult OB. Besides, this study included heterotopic grafting experiments that detected no re-specification of the grafted cells from dorsal or ventral SVZ in one another. Furthermore, heterochronic transplantation of precursors from the embryonic lateral ganglionic eminence (LGE) or from the SVZ, at different stages, to the neonatal or adult SVZ, has shown that grafted cells adopt the phenotypes that are typical of their origin, indicating that they are temporally

restricted and minimally affected by the host age (De Marchis et al., 2007). Importantly, intrinsic cell programmes limit the cell responsiveness to the extrinsic factors. On the other hand, extracellular cues can trigger signaling pathways that interact with intracellular epigenetic mechanisms, resulting in chromatin remodeling and consequent changes in gene expression. Thus, intrinsic and extrinsic mechanisms cooperate to maintain and tightly regulate NSCs in the adult mammalian brain (reviewed in Ihrie and Alvarez-Buylla, 2011).

Environmental cues in the SVZ and SGZ niches have a regulatory and instructive role on maintaining the NSCs and guiding them towards a neuronal fate. Briefly, they comprise cell-cell interactions between the neural cells, but also with non-neural cells, either through direct cell-contact or via soluble molecules. Neighboring cells are important components. In the lateral ventricle, ependymal cells secrete Noggin, which antagonizes BMP-induced glial differentiation, thereby creating a neurogenic milieu (Lim et al., 2000). Also, they have an important role on guiding neuroblasts migration out of the SVZ, by the beating of their cilia on the surface of the lateral wall, which creates concentration gradients of guidance molecules from the CSF (Sawamoto et al., 2006). The CSF components may also be involved in regulating proliferation of SVZ precursor cells (Lehtinen et al., 2011). Others have suggested that the physical force of the CSF flow itself on the primary cilia of type B cells may affect the proliferative state of the cells through a mechanosensory mechanism (Singla and Reiter, 2006). Niche astrocytes themselves work as sensors and regulators of the environment and contribute to support neurogenesis, either through cell-cell contact or via soluble factors (Lim and Alvarez-Buylla, 1999; Song et al., 2002a; Lim et al., 2007). The vasculature has been emerging as a key element of the NSCs niches, as well as the prominent basal lamina and extracellular matrix (ECM) proteins. Together they provide 1) a microenvironment of soluble molecules that keeps proliferation and neuronal

specification, 2) a physical substrate for the cells and 3) the control of cell adhesiveness (Shen et al., 2008; Tavazoie et al., 2008; Goldman and Chen, 2011). For instance, endothelial cells secrete a cohort of factors that are known to influence proliferation, differentiation or survival, namely, bFGF, insulin-like growth factor-1 (IGF-1), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), leukemia inhibitory factor (LIF) and BDNF (Palmer et al., 2000; Mi et al., 2001; Jin et al., 2002b; Louissaint et al., 2002). The role of BDNF in constitutive neurogenesis and ischemia-induced neurogenesis will be discussed in sections 1.1.9 and 1.2.2.2.4, respectively, since it constitutes a central molecule in the work described on Chapter 4. Also, axonal innervations from distant brain regions deliver neurotransmitters that modulate neurogenesis (reviewed in Riquelme et al., 2008). Finally, contact and diffusible signals and their receptors, within the niche, like growth factors, hormones, neurotransmitters, Notch/Jagged signaling, Eph/ephrins and sonic hedgehog (Shh) contribute to a finely tuned regulation of self-renewal and cell fate choice (reviewed in Riquelme et al., 2008; Ihrie and Alvarez-Buylla, 2011). In addition to SVZ niche environment and cell-intrinsic factors, external environmental stimuli like physical exercise, aging and stress greatly affect neurogenesis (reviewed in Ming and Song, 2005). As mentioned, pathological conditions also alter neurogenesis. In this context, ischemia-induced changes in neurogenesis will be discussed in section 1.2.2.2, a subject of special interest for the present thesis.

1.1.8 Migration of newborn neurons in the adult brain

Although it is during brain development that neuronal migration vigorously thrives, in the adulthood it occurs in the neurogenic areas, still impressively, not only in numbers, but also as a highly precise and dynamic process taking place in the very intricate and dense mature brain parenchyma. Daily, cells navigate from their birth place, the SGZ or the SVZ, towards their

final destination, the neighbor GCL or the distant OB, respectively. Along the way, they achieve antigenic characteristics that ultimately allow them to position in the correct place in the target area, get mature, and connect with the local network (Ming and Song, 2005; Ghashghaei et al., 2007).

In the adult rodent brain, SGZ-derived cells show a very limited migratory behavior *en route* to the GCL, located very close to their birth place. Herein, they settle and become functionally incorporated. On the contrary, the SVZ-OB axis witnesses a massive migration of thousands of neuronal precursors *per* day, navigating up to 5 mm through an extremely well-defined tangential path, towards the most anterior parts of the mouse forebrain. This reflects not only a great plasticity in the intact adult brain, but is also suggestive of a finely tuned mechanism of navigation, since no dispersion from the route is observed. The properties and regulation of the migration of SVZ precursors to the OB will be reviewed on the following section.

1.1.8.1 SVZ-RMS-OB migration

1.1.8.1.1 General characteristics

The extensive migration along the RMS has been attracting much attention due to its unique characteristics. In sharp contrast with the developmental neuronal migration, that is characterized by the dependence on glial fibers as a support for neuronal precursors migration (gliophilic migration) (Marin and Rubenstein, 2003), neuroblasts in the RMS lack the scaffold of radial glia processes. Instead, the substrate for tangential navigation is provided by the adjacent migrating cells themselves, building large neuronal chains, where neuroblasts slide along each other *en route* to the OB (homophilic or neurophilic migration) (Lois et al., 1996).

Nevertheless, such a precise and curved route could not rely simply on the homophilic migration. Chains of neuroblasts are encapsulated by specialized astrocytes, previously referred as type B2 astrocytes (Lois et al.,

1996). The existence of this "glial tube" firstly suggested that neuroblasts may use it as a substrate for chain migration. Although, *in vitro*, the astrocytic sheath does not appear to be needed for the proper chain migration (Wichterle et al., 1997), abnormal astrocytic tube formation leads to defects in the chain migration (Anton et al., 2004; Ghashghaei et al., 2006; Belvindrah et al., 2007). A recent study revealed that important molecular interactions between migrating neuroblasts and the ensheathing astrocytes keep the RMS structure (Kaneko et al., 2010). Indeed, the glial tube contributes to restrict the migrating cells in the RMS rather than serving as a substrate for their migration.

In addition, evidences showed that chains of neuroblasts migrate along blood vessels that topographically define the RMS (Fig. 1.4). Such blood vessels display a parallel disposition that physically supports neuroblast navigation in the migratory pathway (Snapyan et al., 2009; Whitman et al., 2009). Interestingly, recent evidences implicated RMS astrocytes in the formation of such well-defined network of parallel vessels, during the postnatal development, via VEGF (Bozoyan et al., 2012). Similarly, within the OB, individual cells use the support of blood vessels to migrate towards their final destination in the bulbar layers (Bovetti et al., 2007). Thus, vasculature acts as a physical scaffold for both tangential and radial migration of SVZ precursors in the adult brain (Fig. 1.4). Also, in neonates, radial migration of SVZ-derived neuroblasts to the cortex uses the radially-oriented vessels of the corpus callosum as a substrate (Le Magueresse et al., 2011). Furthermore, blood vessels provide molecular cues that are critical for the migration of SVZ precursors. This subject will be discussed in the following section. In summary, upon birth in the SVZ, neuroblasts or type A cells assemble together in chains that constitute the emergent RMS and migrate along vasculature, channeled by a glial tube.

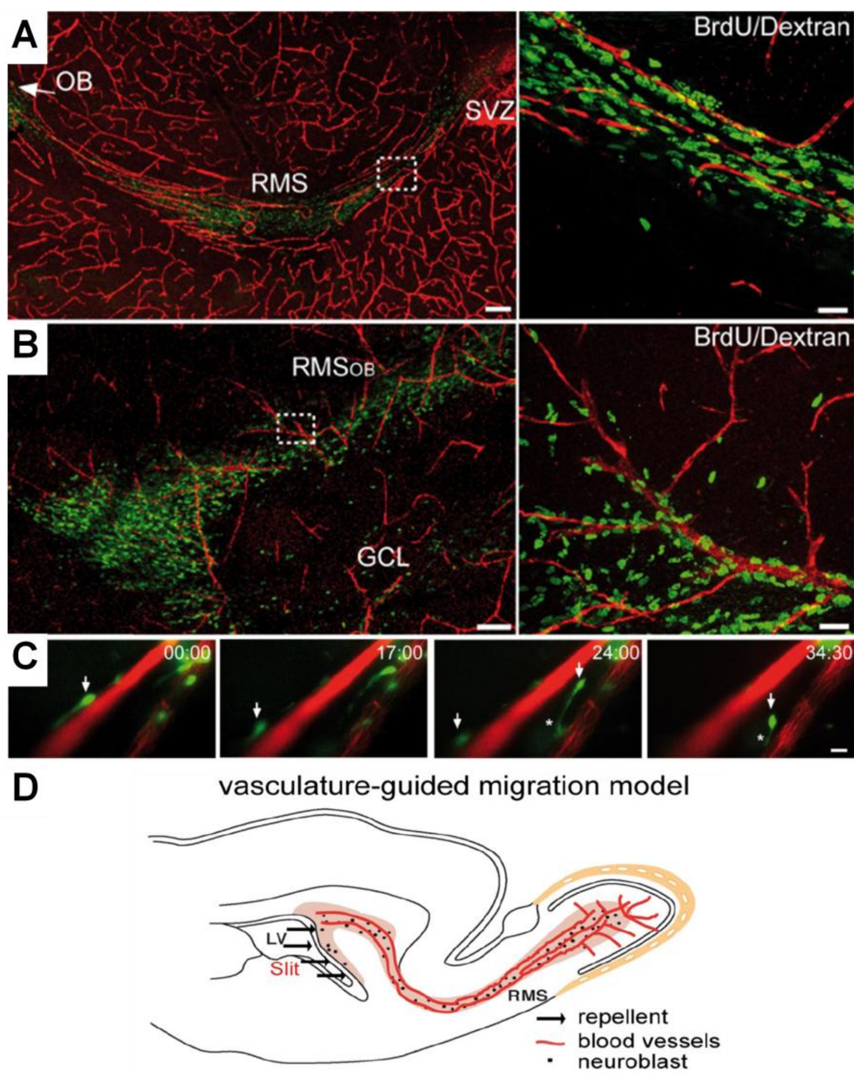


Figure 1.4. Vasophilic migration of neuronal precursors in the adult mammalian forebrain. (**A,B**) Micrographs showing organization of blood vessels in the adult mouse forebrain. Note the close association of tangentially (**A**) and radially (**B**) migrating neuronal precursors (green) with blood vessels (red) that parallel in the migratory stream or scatter in the OB. Newly generated cells in the migratory pathway were labeled by BrdU injection 5 d before analysis. Blood vessels were revealed by injection of dextran-Texas Red to the tail vein. Scale bars: A, B, 100 µm; Aright, Bright, 20 µm. **C**, green fluorescent protein (GFP)-labeled neuronal precursors migrate along blood vessels in acute slices of the adult mouse forebrain. Blood vessels were labeled by injection of dextran-Texas Red to the tail vein 1 h before preparation of acute slices. GFP-expressing retrovirus was injected into the SVZ 3 d before time-lapse imaging in the RMS. Arrows indicate the soma of migratory cells, whereas asterisk shows the leading process. Note that GFP^+ cells are always positioned close to the vasculature either with their soma or leading processes. Time is indicated in minutes in the top right corner of each photograph. Scale bar: 10 µm. **D**, Proposed model for vasculature-guided migration of neuronal precursors in the adult mouse forebrain. According to this model neuroblasts are retained in the RMS and migrate in this pathway as a result of the presence of blood vessels that are oriented parallel to the migratory stream. [Adapted from Snapyan et al. (2009)].

The migrating cells have a very typical elongated morphology, possessing a prominent leading process that extends in the direction of migration, and a short trailing process that contacts the leading process of the nearby cell. Interestingly, time-lapse imaging in acute slices of adult mice revealed that migration of neuroblasts in the RMS is saltatory, i.e., encompassing two phases: a migratory phase, when the leading process is projected, the cell body moves towards it, repositioning nearby, and followed by the trailing process; and a stationary or resting phase that precedes the next displacement (Garcia-Verdugo et al., 1998; Snapyan et al., 2009) (Fig. 1.5). Snapyan et al. (2009) have shown a high speed of migration of the neuronal precursors in the RMS, in acute slices (~120 μm/h). Curiously, while in the SVZ, neuroblasts seem to move randomly in different directions, in the RMS they navigate mostly rostrally (Sawamoto et al., 2006), although they frequently switch direction along the way. Moreover, on their way to the OB, migrating neuroblasts occasionally divide, having a cell cycle time of 14-17 h (Menezes et al., 1995; Smith and Luskin, 1998; Coskun and Luskin, 2002). Interestingly, prior to cell division they consistently withdraw their processes, entering in resting period (Coskun et al., 2007).

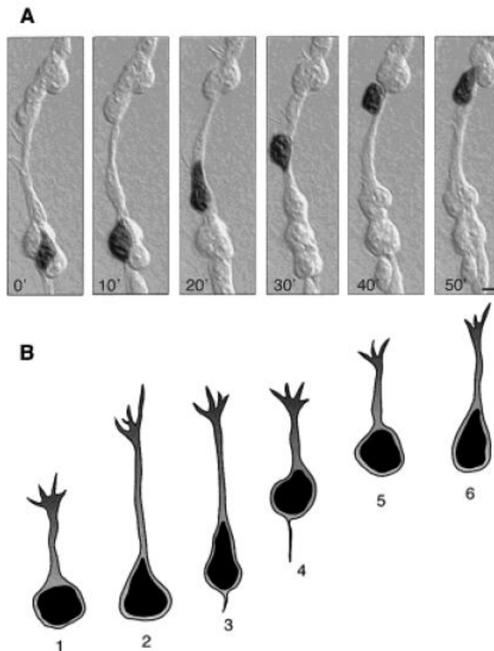


Figure 1.5. Saltatory behavior during neuronal progenitors migration. **A**, Time-lapse series showing neuronal progenitors migrating along chains *in vitro*. Neuronal progenitors formed a network of chains around SVZ explants (1-2 days *in vitro)*. One such chain is shown here with the cell body of one cell highlighted; this cell moves across the field within a 50-min period. Scale bar: 10 μm. **B**, Steps in cell translocation during chain migration. First, cell body may remain stationary for some time while the leading process and growth cone extend or retract actively (1, 2). In a second step, with the leading process in an extended position, the cell body is pulled forward towards the process (3-5). Frequently, a thin, trailing process transiently forms as the nuclei and associated organelles move into the leading process (3, 4). After this translocation step, the cell body becomes temporarily stationary again while the growth cone moves forward elongating the leading process. Repetition of this cycle results in a saltatory movement observed during chain migration. [Adapted from Garcia-Verdugo et al. (1998)].

1.1.8.1.2 Regulatory mechanisms

Understanding the cellular and molecular orchestra that mediates the remarkable migration of SVZ-derived cells throughout the adult forebrain has been an interest shared by many investigators. Contrarily to astrocytes from other brain regions, RMS astrocytes are permissive to accommodate migration, via non-soluble factors (García-Marqués et al., 2010). Additionally, RMS astrocytes can modulate neuroblasts migration by release or uptake of molecules involved in the migratory process. For instance, they secrete a protein with migration-inducing activity (MIA) which promotes neuroblasts migration (Mason et al., 2001). Also, astrocytes express the GABA transporter 4 (GAT4), thus regulating the extracellular concentration of GABA, which is synthesized and released by neuroblasts, and known to decrease neuroblasts migration (Bolteus and Bordey, 2004). Actually, Snapyan et al. (2009) have shown that neuroblasts-derived GABA activates $GABA_A$ receptor in astrocytes membrane,

leading to $[Ca^{2+}]_i$ increase and subsequent Ca^{2+}-dependent insertion of TrkB receptors, the high-affinity receptors of BDNF, on their plasma membrane. As a consequence, RMS astrocytes are able to trap and control the extracellular levels of BDNF, a neurotrophin that promotes migration via p75NTR, the low-affinity receptor, expressed by neuroblasts (Fig. 1.6). Another work explored the glutamatergic signaling in the RMS, and suggested that vesicular release of glutamate from astrocytes controls N-methyl-D-aspartate receptor (NMDAR) activity in neuroblasts, affecting their survival (Platel et al., 2010). A recent study has unveiled another interesting crosstalk between migrating neuroblasts and surrounding astrocytes in the RMS, involving the Slit/Robo receptor system. Briefly, by secreting Slit1, which acts on Robo-expressing astrocytes, the new migrating neurons actively regulate the formation and maintenance of the astrocytic meshwork, channeling it for migration (Kaneko et al., 2010).

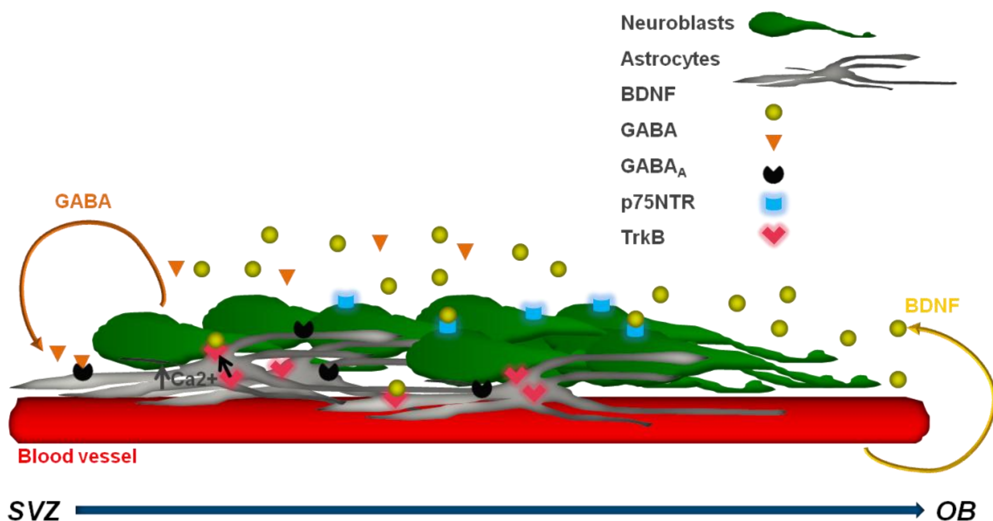


Figure 1.6. Mechanism of neuroblast migration in the RMS involves BDNF. BDNF is one of the modulators of the migration undertaken by new neurons, from their birth place, the SVZ, to the distant OB (arrow indicates the direction of migration towards the OB). RMS vasculature synthesizes and secretes BDNF, which promotes entrance in migratory phase via p75NTR expressed in neuronal precursors. Then, migrating cells release GABA that triggers Ca^{2+} rise in the ensheathing astrocytes. This, in turn, leads to the Ca^{2+}-dependent mobilization of TrkB receptors to the plasma membrane of astrocytes, which scavenges extracellular BDNF. Thus, migrating cells themselves may control their switching behavior from migratory to stationary phase during the saltatory migration, by indirectly regulating the level of vasculature-derived BDNF that is available, via astrocytes.

Chemotaxic molecules have been suggested to act in the initial and sustained polarity of the migrating cells, through the generation of gradients of concentration. Initially, it was proposed that OB could secrete chemoattractant cues for the migrating cells in the RMS (Liu and Rao, 2003), which was supported by the evidence that adult NSCs express chemokine receptors (Tran et al., 2004). Nevertheless, after removal of the OB, precursors still proliferate in the SVZ and migrate rostrally along the RMS, accumulating in the RMS (Kirschenbaum et al., 1999). Thus, long-distance attractant signals coming from the OB are not required for efficient guidance of migrating cells. On the contrary, chemorepulsive signals secreted by tissues nearby the SVZ, namely the septum and choroid plexus, are likely to act as prevalent cues for the initiation of migration away from the SVZ. Slit proteins, Slit1 and Slit2 are highly diffusible chemorepellents secreted in these SVZ surroundings, and accumulated in the CSF (Wu et al., 1999). Notably, the beating of ependymal cells cilia in the walls of the lateral ventricles creates and maintains a flow of Slits-containing CSF, generating a repulsive molecular gradient that pushes away the newborn SVZ neuroblasts (Sawamoto et al., 2006). Accordingly, Slit receptors Robo2 and Robo3 are expressed in the SVZ and RMS. Also, local signals in the SVZ and RMS may also contribute to the directional guidance of cell migration in the RMS. In fact, it was demonstrated that neuroblasts express Slit1 themselves, suggesting that they may have also a cell autonomous role in migration (Nguyen-Ba-Charvet et al., 2004; Kaneko et al., 2010).

Furthermore, cell surface adhesion molecules, together with components of the ECM and integrins have been strongly implicated in maintaining the organization of chain migration and the homophilic tight interaction between the neuroblasts. For instance, removing the PSA moieties from the NCAM in neuroblasts, results in loosely packed chains of cells (Chazal et al., 2000; Hu, 2000). Moreover, in PSA-NCAM knockout (KO) mice, migration to the OB is deeply affected, causing neuroblasts accumulation at the

rostral RMS (Cremer et al., 1994; Hu et al., 1996). Cell-ECM interactions also contribute to the adhesion in between the migrating cells, a *sine qua non* condition for the chain motility. Along the path, local gradients of ECM proteins, such as laminin, tenascin and reelin, influence the migratory process, via integrin receptors. For instance, β1 integrins are critical for chain assembling of migrating neuroblasts (Emsley and Hagg, 2003). β1 integrin-deficient mice have severe defects in RMS chains, besides disruption of the glial tubes, leading to the escape of neuroblasts to the surrounding tissues (Belvindrah et al., 2007).

Once neuroblasts enter the OB, they encounter an extracellular environment that dictates their detachment from the chains, and the radial individual dispersion towards the granule and periglomerular layers. The ECM protein reelin is expressed in the mitral cell layer and olfactory nerve layer of the OB and has a decisive role in the detachment of interneurons precursors from chains (Hack et al., 2002). Similarly, tenascin-R is expressed in the GCL and internal plexiform layer of the OB and not only triggers neuroblasts detachment from chains but also their subsequent radial migration (Saghatelyan et al., 2004). To note that, in parallel, in the OB, the glial tubular structure opens and vessels get scattered, allowing the dispersion of the new neurons towards their final position in the bulbar layers. Furthermore, the migrating neuroblasts express the tyrosine kinase receptor ErbB4. Its ligands, neuregulins 1, 2 and 3 (NRG1, NRG2 and NRG3) are detected in the RMS or nearby tissues. In ErbB4-deficient mice, chain organization is perturbed in the RMS and neuroblasts accumulate in bulbar RMS, suggesting that ErbB4 is likely involved in the switch from tangential to radial migration in the OB or in the placement of the interneuronal precursors within the OB (Anton et al., 2004).

Although extracellular environment has a great impact in modulating neuroblasts migration along the SVZ-RMS-OB pathway, cell-intrinsic mechanisms supporting cell migration, namely cell polarity and cytoskeleton machineries, are critical for directionality and motility in the RMS. Accordingly,

depletion of components of these systems causes disturbances in centrossome orientation, an essential step before cell soma displacement, or in the dynamics of the leading and trailing processes (Higginbotham et al., 2006; Solecki et al., 2006). For instance, deleting the microtubule-associated protein doublecortin (Dcx), affects the speed of migration, the dynamic of the leading process and the soma translocation towards the leading process (Kappeler et al., 2006; Koizumi et al., 2006). Importantly, when affecting migration by Dcx depletion, neuronal maturation in the OB is also altered (Belvindrah et al., 2011). Furthermore, a recent study has shown that reducing the partitioning-defective 1 (Par-1b)/microtubule affinity-regulating kinase 2 (MARK2), a polarity-regulating kinase, leads to impairment of neuroblasts migration in the RMS and consequent lower number of new neurons reaching and integrating in the OB (Mejia-Gervacio et al., 2012).

Concluding, from the assembly of neuronal precursors in chains, in the SVZ, to the dispersion from chains and settling of individual cells in the OB, neuroblasts migration throughout the mature brain requires a synchronized action of cellular and molecular players that together build up the proper scenario for this remarkable mechanism of plasticity in the adulthood.

1.1.9 Role of BDNF in constitutive neurogenesis

BDNF can act in two forms, pro-BDNF and mature BDNF, and through different receptors, which themselves signal via disparate intracellular cascades, thus, a summary of the current knowledge on BDNF signaling is provided in Box 1.1 for a better understanding of the present and later sections.

Box 1.1. BDNF and its receptors

BDNF is the most widely expressed neurotrophin in the brain, and plays paramount roles during development and throughout adulthood, in the CNS and peripheral nervous system (PNS). These include neuronal survival, neuronal differentiation and migration, axonal and dendritic growth and guidance, synaptogenesis, synaptic activity and plasticity, neurotransmission and neuronal apoptosis. The neurotrophin is synthesized from mRNA as a precursor form (pro-BDNF), then proteolitically cleaved by intracellular furins or pro-hormone convertases to form the mature BDNF, which is then released (reviewed in Chao, 2003). Interestingly, studies suggested that the precursor form of BDNF is not just a transient biosynthetic intermediate, but instead can be released and afterwards cleaved by extracellular proteases (Pang et al., 2004; Yang et al., 2009), or can bind to receptors and either be internalized (Bergami et al., 2008), or signal, exerting physiological functions (Lee et al., 2001). For example, studies from the group of Barbara Hempstead have shown that pro-BDNF induces neuronal apoptosis (Lee et al., 2001) and facilitates long-term depression (LTD) (Woo et al., 2005) by activation of p75NTR receptor.

BDNF mediates its actions by binding to two transmembranar receptors with different affinities, tropomyosin-related kinase B (TrkB) and p75 neurotrophin receptor (p75NTR). TrkB receptors include three isoforms with high-affinity for BDNF, the full-length TrkB (TrkB-FL) and the two truncated isoforms (TrkB-T1 and TrkB-T2) that result from alternative splicing and lack the intracellular tyrosine kinase domain (Baxter et al., 1997). It is widely recognized that the truncated forms occur mainly in glial cells, whereas the full-length form is found abundantly in neuronal cells (Reichardt, 2003). The function of TrkB-T1/2 is not entirely understood, however studies have suggested that they can form dimers with TrkB-FL thereby inhibiting their action (Eide et al., 1996), or work as scavenger receptors by sequestering extracellular BDNF and internalizing it (Biffo et al., 1995). Besides, Rose et al. (2003) have shown that TrkB-T1 mediates BDNF-evoked calcium signaling in astrocytes.

When binding BDNF, TrkB-FL receptors dimerize and trans-autophosphorylate, initiating signaling cascades mediated by Ras/extracellular signal-regulated kinase (ERK), phosphatidylinositol 3-kinase (PI3K)/Akt and phospholipase C (PLC)-γ/protein kinase C (PKC) pathways. These signaling cascades are usually associated to the effects of BDNF on neuronal differentiation and survival. p75NTR is the low-affinity receptor for BDNF and also binds the other neurotrophins, nerve growth factor (NGF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4), with the same low affinity. This receptor has a cytosolic death domain - typical of the tumor necrosis factor (TNF) receptors superfamily - that predominantly signals through activation of nuclear factor kB (NFkB) and c-Jun N-terminal kinase (JNK), and modulates RhoA activity. These pathways regulate effects on cell apoptosis, survival, neurite outgrowth and arrest, cell migration and myelination. Besides, p75NTR can interact with TrkB, increasing its affinity to BDNF. It is important to note that pro-BDNF has higher affinity to bind p75NTR than the cleaved mature form (reviewed in Reichardt, 2006).

Modulation of neurogenesis in the adult brain by BDNF has been a topic of intense investigation and some controversies. First studies addressing the role of BDNF in neurogenesis used SVZ cultures from adult rat brain, and detected a very impressive effect of BDNF treatment enhancing neuronal survival (Kirschenbaum and Goldman, 1995). Consistently, Leventhal et al. (1999) identified a humoral regulation of neurogenesis by endothelial cells, through secretion of BDNF. The authors showed that the conditioned medium from endothelial cells contained BDNF and promoted the outgrowth and survival of neuronal cells arising from SVZ cultures, besides inducing their migration from explants. These effects were mimicked my application of BDNF to the SVZ cultures, and blocked by scavenging BDNF in the co-cultures. This study used umbilical and whole brain-derived endothelial cells. Thereafter, the synthesis of BDNF by endothelial cells of the neurogenic regions was demonstrated in songbird HVC (Louissaint et al., 2002) and rodent RMS (Snapyan et al., 2009). In agreement with the pro-survival effect of BDNF in adult SVZ cultures (Leventhal et al., 1999), Linnarsson et al. (2000) reported high levels of cell death in the SVZ of mice lacking BDNF.

Despite of a first wave of evidences from *in vivo* studies suggesting a positive influence of BDNF in neurogenesis, by mediating survival and neuronal differentiation, the current scenario is elusive. Intracerebroventricular (i.c.v) infusion of BDNF or viral-mediated overexpression of the neurotrophin in the SVZ of adult rats has resulted in a massive increase in the number of BrdU-labeled cells in the OB (Zigova et al., 1998; Benraiss et al., 2001). Among these cells, an increased proportion of new neurons was detected by co-labeling of BrdU with neuronal markers. Also, BDNF treatment or overexpression has led to augmented levels of BrdU in the SVZ, and to the aberrant appearance of new neurons in the nearby non-neurogenic parenchyma, including striatum, septum, thalamus and hypothalamus (Benraiss et al., 2001; Pencea et al., 2001). In line with these results, Young et al. (2007) findings suggested that BDNF promotes

neuronal production in the SVZ through p75NTR activity. The authors defined the subpopulation of p75NTR$^+$ cells in the SVZ as highly mitotic BDNF-responsive SVZ precursors with neurogenic potential. Other study has shown that BDNF has actually a dual effect, by decreasing proliferation and increasing differentiation in neurons (Cheng et al., 2003). Accordingly, Ahmed et al. (1995) have described a stimulatory effect of BDNF in neuronal differentiation, and no apparent effect in survival. In contrast, transgenic BDNF-haploinsufficient mice displays drastic deficits in survival but not in proliferation of newborn SVZ cells, and shows impairments in spontaneous olfactory discrimination, effects that were associated to TrkB signaling (Bath et al., 2008). On the other hand, Galvão et al. (2008) have demonstrated that i.c.v BDNF delivery does not enhance adult SVZ neurogenesis, nor does affect survival or proliferation of SVZ cells in mice, but decreases survival of SVZ cells in rats. Also, a study using bioluminescence imaging to visualize SVZ neurogenesis in living animals, together with histology in the fixed brains, has reported diminished neurogenesis with long-term expression of BDNF in the SVZ of the adult mouse (Reumers et al., 2008). Altogether, these studies gather conflicting results in regard to BDNF role in proliferation, differentiation and survival of SVZ precursors, thus further investigation is required. More consensus has been achieved concerning BDNF effect in the migration of SVZ precursors in the RMS, which will be discussed in the next section.

1.1.9.1 Role of BDNF in neuroblast migration in the RMS

As briefly mentioned, Snapyan et al. (2009) demonstrated that BDNF is secreted by the RMS vasculature and fosters neuronal migration via p75NTR expressed by migrating neuroblasts. Moreover, the authors proposed a role for the surrounding TrkB-expressing astrocytes of the glial tube, controlling the availability of extracellular BDNF, by sequestering it on TrkB receptors, and thereby leading to the entrance of neuroblasts in the resting phase. Accordingly,

application of BDNF in acute brain slices triggers the entrance in migratory period, while when exposed to TrkB-Fc, cells shift to stationary phase. This study reported that TrkB expression was mostly astrocytic, and only scarce levels were found in PSA-NCAM neuronal precursors, which is in line with a previous work reporting no expression of TrkB in neuroblasts (Galvão et al., 2008). *In vitro* experiments conducted by Chiaramello et al. (2007) observed, as well, a stimulatory effect of BDNF on migration of SVZ cells from the postnatal mouse brain, but associated the effect to TrkB activation. Also, the authors argued that BDNF acts as an inducer and an attractant on cells migration. However, Snapyan et al. (2009) showed that application of TrkB inhibitor in acute brain slices harbors no effect on neuroblasts migration. Moreover, a recent study excludes the chemoattractant role of BDNF, since similar levels of BDNF were found in the SVZ and OB of postnatal mouse brain, thus no gradient of BDNF concentration exists (Petridis and El Maarouf, 2011). Besides, the authors suggested a concentration-dependent effect of BDNF, being that physiological concentrations (e.g. 1 ng/ml) stimulate migration, whereas high doses induce cell differentiation and reduce migration. Finally, Bagley and Belluscio (2010) claimed the expression of BDNF and TrkB, but not p75NTR, in the neuronal precursors migrating in the postnatal RMS. Using time-lapse imaging in acute slices from postnatal mouse brain, the authors showed that blockade of TrkB signaling or scavenging BDNF by the use of BDNF-IgG increases cell motility and favors anterior migration in the RMS. Intriguingly, application of BDNF to the acute brain slices also induces an increase in cell motility. It is important to note that dynamic imaging in acute slices may not be the best model to conclude about directionality of cells migration, due to the existence of a directional flow in the chamber that may interfere with this parameter.

Although it seems to be clear that BDNF plays an important role in neuroblasts migration, major contradictions are found in regard to the receptor

involved in such function. In the Chapter 4, we explored the role of BDNF in ischemia-induced migration of neuroblasts through non-neurogenic parenchyma. Our study shows that BDNF promotes vasculature-mediated migration of neuroblasts in the ischemic striatum and suggests that p75NTR mediates this effect.

1.2 Neural stem cell-based strategies for brain repair

The discovery that the adult mammalian CNS shelters multipotent and self-renewing NSCs, including in humans, has provoked a strong interest on these cells for cell replacement strategies in the treatment of neurological disorders, and greatly fueled research aiming at this endeavor in the last years. Indeed, NSCs can be propagated in culture, providing a great number of cells for transplantation, and can give rise to neurons, astrocytes or oligodendrocytes.

Neurodegenerative diseases include a wide range of pathologies that claim a major concern, since they tend to be highly debilitating, and for the majority, the available treatments rely only in supportive care to alleviate the symptoms. Among them, Parkinson's disease (PD), Huntington's disease (HD), Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), multiple sclerosis (MS), stroke and spinal cord injury have raised particular interest for stem cell-based therapies. These conditions account for an acute or chronic loss of neuronal or glial cells, local or widespread, that may be challenged by the introduction of new neurons or glial cells derived from stem cells. Two approaches have been witnessing extensive investigation in animal models of brain disease: 1) the transplantation of NSCs or their progeny, and 2) the mobilization of the endogenous NSCs in the adult brain (Fig. 1.7). In the next sections, 1.2.1 and 1.2.2, the scientific bases of these strategies will be introduced, with particular attention to the transplantation of SVZ cells in a

model of MS, and to the endogenous mobilization of SVZ-derived progenitors in a model of stroke.

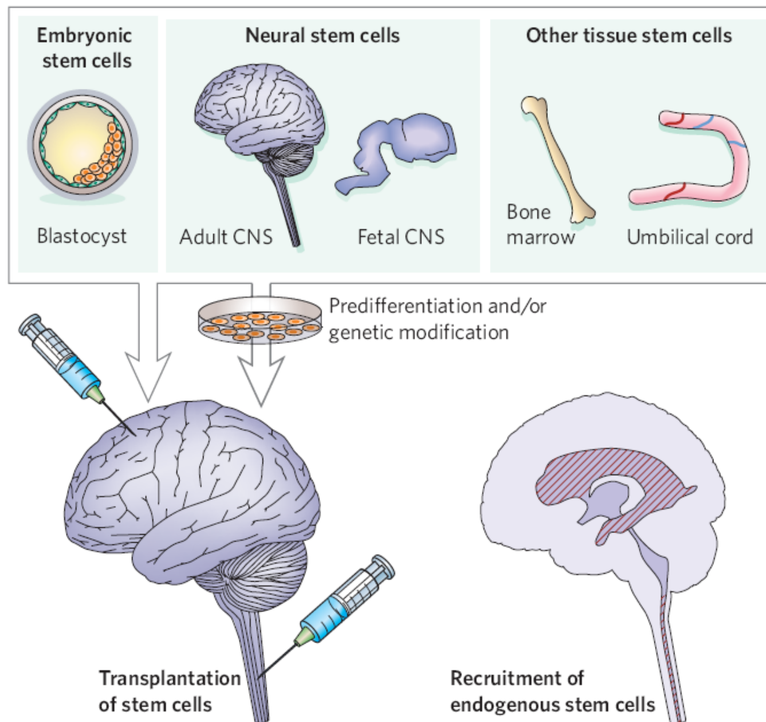


Figure 1.7. Application of stem cells for neurological disorders. Stem cells would be isolated and transplanted to the diseased brain or spinal cord, either directly or after pharmacological/genetic pre-differentiation in culture to form specific types of neurons or glial cells, or cells producing neuroprotective molecules. In strategies relying on the stimulation of the patient's own repair mechanisms, endogenous stem cells would be recruited to areas of the adult brain or spinal cord affected by disease, where they would produce new neurons and/or glia (neurogenic and gliogenic areas along the lateral ventricle and central canal are shown in hatched red). Stem cells could provide clinical benefits by neuronal replacement, remyelination and neuroprotection. [From Lindvall and Kokaia (2006)].

1.2.1 Neural stem cells for transplantation in the diseased brain

Strategies relying on cell transplantation of NSCs in injured areas of the brain have been largely explored in the last decade. To note that, in the damaged areas, grafted NSCs face several challenges, namely 1) to survive in a typically inflamed environment, 2) to differentiate and mature in the phenotype

of the lost cells, and 3) to incorporate in the complex network of the host tissue, successfully reconstructing the affected circuits.

Studies in animal models of PD (Yasuhara et al., 2006; Zhu et al., 2009), HD (Vazey et al., 2006), stroke (Hoehn et al., 2002; Zhang et al., 2003; Li et al., 2006; Hicks et al., 2007), spinal cord injury (Akiyama et al., 2001; Oka et al., 2004), MS (Uccelli and Mancardi, 2010), ALS (Xu et al., 2009) and temporal lobe epilepsy (TLE) (Shetty and Hattiangady, 2007) have generally suggested that transplanted NSCs survive for a long time in the host tissue, have high migratory dynamics homing the injury sites, and a subset of the grafted cells undergo differentiation in the phenotype affected by the course of the injury. Furthermore, a symptomatic functional improvement was observed in some of these reports. Nonetheless, improvements may not be due to a partial restoration of neuronal or glial populations, but to a neuroprotective effect of the grafted cells over the host tissue. Accordingly, Pluchino et al. (2005) demonstrated that systemically administrated mouse NSCs home to demyelinated lesions in the mouse brain and offer neuroprotection by suppressing pro-inflammatory mechanisms. Though some observations in animals are optimistic, a responsible translation into an effective human cell therapy for such pathologies still needs the overcome of important hurdles.

Multipotency of NSCs allows the generation of neurons, astrocytes and oligodendrocytes in culture (Fig. 1.8). Progressive restriction of the cell fate in developing SVZ cultures is specified by extrinsic and intrinsic factors. Accordingly, extracellular soluble factors or genetic manipulation can be used to drive differentiation of SVZ cells towards a certain phenotype, neurons or oligodendrocytes depending if the disease causes neuronal loss or demyelination. In this context, specific types of neurons may also be efficiently generated in culture. Indeed, efforts are being brought together to optimize the *in vitro* fate specification for induction into a certain cell type (pharmacological or genetic induction), a strategy that may substantially increase the outcome

from NSCs transplantation. In acute injuries such as stroke or spinal cord injury, different types of neurons and glial cells die within the affected area. In the case of chronic diseases like PD, HD and ALS, a certain cell type is progressively depleted, or instead, in others like AD, a broad and non-specific degeneration occurs. Thus, it is important to identify the cells that would be ideal for a cellular graft in a given disease, and to understand that some diseases offer higher challenges in the development of a transplantation therapy. Another important disease-dependent issue is the route of transplantation (local *versus* systemic). Indeed, for focal diseases like PD, HD, spinal cord injury, focal stroke and brain trauma, the cell loss occurs in a restricted zone, thereby intralesional transplantation is advantageous. On the other hand, in multifocal diseases like MS and AD, an intravenous or intrathecal transplantation of NSCs may allow the multilocus distribution and settlement of the NSCs, due to the chemoattractant gradient of inflammatory soluble molecules released by the lesioned tissue (reviewed in Martino and Pluchino, 2006).

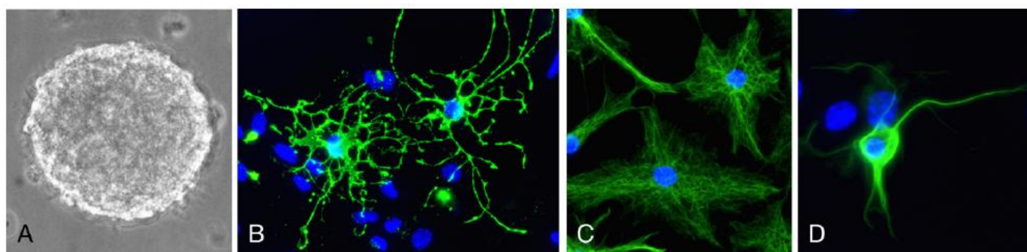


Figure 1.8. *In vitro* characteristics of neuronal precursor cells (NPCs) which may be used for transplantation in CNS disorders. Upon growth factor withdrawal, mouse NPCs (type B and type C cells) grown *in vitro* as free-floating neurospheres (**A**, phase contrast), give rise to progeny containing variable percentages of oligodendrocytes [**B**, Galactocerebroside (GalC)], astrocytes (**C**, GFAP) or neurons (**D**, beta-III tubulin). Nuclei in B–D have been counterstained with DAPI (blue). Magnification 40×. [Adapted from Pluchino and Martino (2008)].

Finally, it is important to note that, in human, cell transplantation therapies using NSCs will rely on an autologous transplant, which requires a first surgery for extraction of NSCs from the patient, and a second surgery for the transplantation of the cells, after an intermediate step of cell type conditioning in culture. As a less invasive alternative, NSCs from *post-mortem* human CNS tissue may be retrieved (Laywell et al., 1999) and are viable for transplantation, although graft rejection may occur. Moreover, NSCs from embryonic origin may be used (eNSCs), or even pluripotent embryonic stem cells (ESCs) obtained from the inner cell mass of the early-stage human blastocyst. However, the use of ESCs/eNSCs comports ethical issues as well as teratoma formation concerns, besides the possibility of graft rejection. Also, skin fibroblasts can be reprogrammed to a pluripotent state by retroviral expression of certain transcription factors, generating inducible pluripotent stem cells (iPSCs) (Takahashi and Yamanaka, 2006; Han et al., 2012). This approach offers no ethical concerns and other important advantages like the unlimited expandability of the cells, broad patterning potential and patient DNA match (autologous transplantation). Nevertheless, tumor formation, genetic and epigenetic instability are a major concern in the use of iPSCs.

In conclusion, to envisage a successful cell transplantation therapy for brain degenerative disorders and injuries, it is mandatory to: 1) harbor a detailed knowledge on the disease pathology; 2) conclude from studies on animal models that faithfully mimic the human disease; 3) optimize the differentiation and functional integration of grafted cells; 4) and probably develop adjuvant therapies to help sustaining survival of the cells and recovery from the disease (for instance, by treatment of the cells with pro-survival factors, or genetic engineering to induce expression of such factors). In the context of the present thesis, the use of NSCs for grafting in the demyelinated brain will be further developed on the next section.

1.2.1.1 MS as a candidate for cell transplantation therapies

Amid neurodegenerative diseases, MS seems to be particularly eligible for cell transplantation therapies. Firstly, albeit axonal loss occurs in MS, the disease is a primary demyelinating disease, i.e., it targets myelin, being axons spared until later stages of the disease (see Box 1.2 for a general overview on the disease). Therefore, engrafted cells have "only" to re-enwrap the nude axons with their membranes, a far more optimistic task than challenging grafted cells to integrate in pre-existing intricate networks and establish functional synapses, by projecting to and receiving signals from the correct counterparts, which is the scenario when transplantation aims at replacing neurons. Moreover, cogent evidences that remyelination is spontaneously taking place in the injured environment supports the view that grafted precursor cells may encounter the proper environmental cues to differentiate and remyelinate. One cannot exclude, however, the contribution of cell-intrinsic mechanisms regulating such capacity. Besides, transplantation strategy in MS aims to be an adjuvant approach to enhance an existing spontaneous repair process, which raises more hopes than repair *per se*. Excitingly, a series of reports has been deciphering an unforeseen "bystander effect" of transplanted NPCs on demyelinating injuries (reviewed in Martino and Pluchino, 2006). In these studies, transplants of NPCs from the SVZ improved the outcome in the experimental autoimmune encephalomyelitis (EAE) model of MS, by releasing a plethora of factors with immunomodulatory or neuroprotective properties. At last, solid evidences have been accumulating from a broad range of sources of remyelinating cells, and a diversity of animal models of demyelinating diseases, and collectively provided proof of principle that cell transplantation in MS can attain effective remyelination in rodent CNS, re-establishment of normal conduction and functional recovery.

Box 1.2. Multiple Sclerosis

MS is a chronic auto-immune disease of the CNS that affects more than 2 million people worldwide, usually beginning in the early adulthood, and more prevalent in females (Flachenecker and Stuke, 2008). Regardless the unclear etiology, likely both genetic and environmental, MS causes disability through demyelination of neuronal axons and consequent impairment of neural conduction.

Membranes of oligodendrocytes, compactly enwrapped around axons, form the myelin sheath. This provides insulation and trophic support to neurons, besides controlling their diameter and distribution of ion channels in the nodes and paranodes. Thus, myelin integrity renders essential for the rapid propagation of nerve conduction and for the maintenance of the axons themselves. Denuded axons become vulnerable and end up degenerating with the progression of the disease, which represents the main cause of debilitation in demyelinating diseases (reviewed in Barres, 2008).

MS may occur in different scenarios: 1) relapsing-remitting MS, which is partially reversible and consists on recurrent episodes of sudden attacks, interspersed by periods of remission with no symptoms; is the most common form, accounting for 80% of the cases, but often develops into 2) secondary progressive form, with gradual and continuous worsening; 3) primary progressive MS, which is irreversible and characterized by a steady neurological decline without attacks, being thus a rapidly debilitating form; and 4) progressive relapsing MS, the rarest, defined by a progressive decline but with periods of remission (reviewed in Joers and Emborg, 2009). Current treatments for MS are based in immunomodulatory agents that simply reduce the frequency and intensity of individual relapses.

Remyelination in MS

Spontaneous and robust remyelination occurs at the early stages of the disease, but is incomplete and eventually fails with disease progression. Compelling evidences indicate that most of the remyelinating oligodendrocytes are derived from resident oligodendrocyte precursor cells (OPCs) widespread throughout the adult brain. Upon demyelinating injury, these cells are induced to proliferate and migrate extensively to the demyelinated area, mature and reinvest the denuded axons, forming new myelin sheaths (reviewed in Franklin and Ffrench-Constant, 2008). In addition, precursors in the rodent SVZ niche become activated and contribute to remyelination (reviewed in Nait-Oumesmar et al., 2008). Indeed, upon demyelination, SVZ-derived cells are able to proliferate and migrate to the nearby corpus callosum, undergo oligodendrogenesis and re-insulate nude callosal axonal tracts (Menn et al., 2006). Analysis of *post-mortem* human tissue from MS patients detected an increased number and overall activation of the cells in and around the SVZ (Nait-Oumesmar et al., 2007). Nonetheless, given the multifocal nature of MS and the neglectable contribution of the endogenous SVZ cells to remyelination, as compared to resident OPCs, the relevance of these cells to promote repair in MS is weak. Therapeutic approaches based on stimulating the recruitment of SVZ cells have been envisaged by several groups. As an alternative, NSCs transplantation has been providing encouraging results in animal models of MS.

Great expectations have been thriving from the considerable body of optimistic evidences obtained with transplantation of stem cells in animal models of MS. Nevertheless, the emergence of a cell therapy for MS based on NSCs entails some concerns. One regards the time for transplantation. Although at later stages the patient has “less to loose”, axonal loss is already ongoing, thus, functional recovery is compromised. In addition, remyelination may be hampered by the astrocytosis and glial scar formation that typically occur in chronically demyelinated tissues. Moreover, it is at earlier stages of the disease that spontaneous remyelination thrives (Franklin and Ffrench-Constant, 2008) suggesting that a proper environment for the reparative process exists. Since MS is a multifocal disease, the site of transplantation could be a major problem. However, studies have demonstrated that systemic administration of NPCs (intravenous or intrathecal) can be therapeutically efficacious in models of multifocal CNS disorders, including EAE, thanks to the capability of blood or CSF-circulating NPCs to pursuit a gradient of chemoattractant molecules released at the sites of lesion (Pluchino et al., 2003; Pluchino et al., 2005). A major hurdle to overcome regards cell differentiation of the transplanted cells. At the moment of grating, the cells need to be highly migratory in order to reach the multiple lesion sites, a characteristic of the round and bipolar early OPCs, but the engrafted reparative cells should be then able to differentiate and mature in the host tissue, ultimately, in myelinating mature oligodendrocytes.

NSCs can be highly expanded in culture and give rise to neurons, astrocytes and oligodendrocytes. SVZ-derived oligodendrocytes undergo all the developmental stages from the early OPCs to the mature myelinating oligodendrocytes. However, under normal conditions, the oligodendrocytic lineage accounts for a small minority of the progeny (Menn et al., 2006). Interestingly, pharmacological or genetic tools can be used to commit NSCs to the oligodendrocytic cell lineage, thus increasing the proportion of oligodendrocytes in the expanded cultures. Thus, induction of the

oligodendrocytic phenotype is a crucial step for effective transplantation strategies. Likewise, treatment of the reparative cells with factors that promote their survival can be an adjuvant approach to achieve better results, since the cells are engrafted in a highly inflamed environment. It is noteworthy that such manipulations, if based on pharmacological drugs rather than genetic tools, should likely be performed before and with, or just with the transplantation, to assure the effect of the pharmacological agents when differentiation and survival are conditioned by the host environment. Also, it s advantageous to graft cells in early stages of differentiation, when they are migratory (critical if the administration is intravenous or intrathecal, as previously discussed) and plastic enough to accommodate in the host tissue. Besides, treatment of the engrafted cells *in locus* even if pre-specified, can be relevant to promote the full differentiation along the oligodendrocytic lineage, namely the expression of myelin proteins and potentiation of remyelination.

On Chapter 3, I describe the development of a functional method that can be used to identify pharmacological compounds that drive differentiation of postnatal SVZ NSCs cells in oligodendrocytes, a crucial step to support grafting approaches for MS. To note, however, that other sources of myelinating cells can be exploited for phenotypic induction and grafting in demyelinated tissue, namely adult NSCs, embryonic or fetal stem cells, iPSCs, bone marrow-derived NSCs, Schwann cells, and olfactory ensheathing cells (OECs).

1.2.1.1.1 Induction of oligodendrocytes by triiodothyronine (T3) hormone

In the field of regenerative medicine for MS, much interest has been gathered on finding molecular targets that drive oligodendrocytes commitment and development. A cohort of evidences supports the fact that the thyroid hormone T3 induces oligodendrocyte differentiation. This was observed not only in cultures, from human embryonic and fetal stem or progenitor cells (Murray and Dubois-Dalcq, 1997; Fritsche et al., 2005; Kang et al., 2007), and

rodent embryonic and adult NSCs (Johe et al., 1996; Whittemore et al., 1999), but also *in vivo,* from the SVZ of young adult rats, following demyelination (Franco et al., 2008). Moreover, the hormone accelerates oligodendrocyte development from the early stage of OPCs into later stages, an effect observed both *in vitro*, in OPC cultures (Barres et al., 1994; Tokumoto et al., 1999; Billon et al., 2002) and *in vivo*, in resident OPCs that are activated upon demyelination (Baas et al., 2002; Calza et al., 2002). Besides, T3 has been shown to promote the synthesis of myelin-specific proteins (Strait et al., 1997; Jeannin et al., 1998) by a direct action on oligodendrocytes, which express T3 receptors (Puymirat, 1992). In agreement, T3 administration enhances remyelination in animal models of demyelinating disease (Fernandez et al., 2004; Franco et al., 2008). It is important to note that remyelination may be a recapitulation of the developmental myelination. In fact, thyroid hormones have a crucial role in the neurodevelopment of vertebrates. Thyroid hormones deficiency during development, leads to structural abnormalities of the brain by affecting cell migration, differentiation, synaptogenesis and myelination. Hypothyroidism delays the deposition of myelin causing hypomyelination in animal and man, whereas hyperthyroidism accelerates it (Walters and Morell, 1981; Legrand, 1986; Dussault and Ruel, 1987). Thus, T3 acts on several stages of the oligodendrocyte development, since cell-cycle exit and terminal differentiation until myelinogenesis. A brief overview on the mechanism of action of T3 hormone is provided in Box 1.3.

Box 1.3. Thyroid hormone and its receptors

Two thyroid hormones are secreted by the thyroid gland, triiodothyronine (T3) and thyroxine (T4). T4 is the main product of the gland and the most abundant in the circulation. Within the cells, T4 is converted in the active form T3, by cytosplasmatic or nuclear deiodinases. T3 acts directly at the transcription level by binding to nuclear thyroid hormone receptors (TRs: TRα1, TRα2, TRβ1 and TRβ2) encoded by TRα and TRβ genes (Rogister et al., 1999). As a result, the hormone activates or represses the transcription of specific target genes, which include genes encoding: the major myelin proteins such as myelin basic protein (MBP), proteolipid protein (PLP), myelin-associated glycoprotein (MAG) and 2'3'-cyclic nucleotide-3'-phosphohydrolase (CNP) (Tosic et al., 1992), neurotrophins and their receptors, components of the extracellular matrix, components of the cytoskeleton, components of the mitochondria, splicing regulators, proteins involved in intracellular signaling, adhesion molecules and co-activators of TR (Konig and Moura Neto, 2002).

T3 controls a number of developmental and physiological processes. The contribution of TRα and TRβ to the diverse effects of T3 remains, however, controversial. It is of general agreement that both OPCs and oligodendrocytes express TRα, and that oligodendrocytes express TRβ, but whether or not OPCs express TRβ remains elusive (Baas et al., 1994a; Baas et al., 1994b; Barres et al., 1994; Carlson et al., 1994; Fierro-Renoy et al., 1995; Carre et al., 1998; Gao et al., 1998; Kondo and Raff, 2000; Billon et al., 2001). Evidences suggest that TRα mediates the effect of T3 on mitosis of neural precursors and timing of OPCs differentiation, whilst TRβ appears to be implicated in the maturation of oligodendrocytes and production of myelin. Importantly, TRα but not TRβ expression was detected in nestin+ precursor cells in the SVZ of adult mouse brain, being apparently required for the maintenance of the cycling activity of these cells (Ben-Hur et al., 1998; Lemkine et al., 2005). In agreement, administration of T3 results in increased Ki67 immunoreactivity in the SVZ (Giardino et al., 2000). On the contrary, Franco et al. (2008) have observed no expression of TRs in the SVZ of intact young adult rat brain, but they detected TRα induction in the SVZ of demyelinated rat brain.

In addition to the oligodendrogenic effect, T3 may exert a protective effect on oligodendrocytes via NGF. In fact, among the target genes of T3, is the gene encoding NGF. Under physiological conditions T3 regulates the endogenous synthesis of NGF in CNS (Calza et al., 1997). Accordingly, a single administration of T4 enhances the level of NGF in the brain (Walker et al., 1979). A study in marmosets has shown that NGF administration protects oligodendrocytes from cell death and ameliorates the pathological scenario in

EAE model of MS, likely through an immunomodulatory effect (Villoslada et al., 2000). Furthermore, neurotrophins can improve remyelination by directly influencing proliferation, differentiation, survival and turnover of oligodendrocytes in the demyelinated lesions (Althaus, 2004). In conclusion, T3 hormone appears to be good candidate to promote oligodendrocyte cell specification, maturation and myelinogenesis of SVZ cells grafted in demyelinated lesions. Nevertheless, an efficient boosting of the oligodendrocytic differentiation from NSCs may have to rely on a cocktail of oligodendrogenic factors. Herein, we used T3 hormone treatment to enrich SVZ cultures in oligodendrocytes. This was useful to validate a method that evaluates the pattern of differentiation of SVZ cultures based on cell-type specific calcium responses (Chapter 3). In addition, the treatment allowed obtaining a sufficient population of oligodendrocytes to study the effect of a demyelinating injury in the differentiation of SVZ cells.

1.2.2 Neural stem cells as an endogenous source for repair

In parallel with research focused on cell transplantation of exogenously pre-committed NSCs in injured area, strategies relying on boosting the intrinsic brain self-repair attempt have been largely explored in the last decade.

Normal homeostasis of adult neurogenic niches and derived migratory routes is perturbed by brain insults. Excitingly, a substantial number of evidences, either in animal models of disease or in human patients, have shown the unforeseen spontaneous mobilization of precursors from the neurogenic niches to the sites of neuronal or glial loss (reviewed in Curtis et al., 2007a; Kaneko and Sawamoto, 2009). Cell production is markedly enhanced in the SVZ in response to stroke, trauma, epilepsy, demyelination and HD models in mice. Upon such injuries, the hitherto quiescent endogenous NSCs are activated and the proliferation of transit-amplifying cells is also increased. Some of these

newborn cells are de-routed from the SVZ/RMS to the damaged area and differentiate in the phenotype of lost cells. New neurons in the striatum of HD animal model were able to correctly project to the globus pallidus (Jin et al., 2005). Moreover, in a model of ischemia, new neurons in the striatum can form synaptic contacts with the existing network (Yamashita et al., 2006). However, no evidence of the establishment of functional synaptic connections exists. Additionally, recruited cells hardly survive, thus, few may contribute to a putative reconstruction of the network. Indeed, no outcome from symptomatology is provided by injury-induced neurogenesis.

In addition to SVZ activation, stroke and epilepsy induce an upregulation of neurogenesis in the hippocampal SGZ (Jessberger et al., 2005; Li et al., 2010a). Nevertheless, whereas injury-induced neurogenesis in the SVZ seems to constitute an attempt to offer beneficial effects in a scenario of pathology, with proper differentiation in the fate of the lost cells being often observed, the overproduction of new neurons in the hippocampus leads to the formation of aberrant projections that end up exacerbating the pathological condition. Indeed, the new abnormal circuitries bring hyperexcitability to the neuronal network and thus may contribute to epileptogenesis (Parent et al., 1997; Parent, 2002; Parent and Murphy, 2008).

On the contrary, in PD and AD, proliferation in the SVZ is decreased. Progressive dopaminergic denervation of SVZ in the course of PD leads to diminished levels of dopamine, a neurotransmitter with an important role maintaining constitutive proliferation in the SVZ (Hoglinger et al., 2004). In the case of AD, an impaired neurogenesis both in the SVZ and SGZ is likely to take place before the hallmarks of the disease and may have a role in the progression of the disease (Rodriguez et al., 2008; Demars et al., 2010; Hamilton et al., 2010; Lazarov et al., 2010). However some controversy still remains, as others reported an increase of immature neuronal markers in the hippocampus of AD patients (Jin et al., 2004).

Excitingly, injury-induced neurogenesis was reported, in human patients of HD (Curtis et al., 2003) and stroke (Jin et al., 2006; Macas et al., 2006; Marti-Fabregas et al., 2010), raising important prospects for the development of cell therapies that may rely on maximizing this pro-neurogenic process. Also, an increased activation of SVZ niche was detected in MS patients suggesting a similar response (Nait-Oumesmar et al., 2007).

1.2.2.1 Envisaging cell replacement by endogenous NSCs: where to act?

Even though an intrinsic self-repair attempt exists in the adult brain, it is extremely limited, with no effective functional recovery. In fact, the newborn precursors de-routed to the site of lesion face inflammation in the damaged parenchyma, rich in debris, cytokines and death signals that compromise their survival and integration. Thus, strategies to be undertaken may include: the promotion of cell survival in the damaged area, the stimulation of neuroblasts migration to drive more cells into the compromised area, and the cell fate commitment to the correct phenotype.

A range of factors is known to foster the production and/or survival or new neurons, including BDNF (Pezet and Malcangio, 2004), erythropoietin (EPO) (Gonzalez et al., 2007), tumor necrosis factor-α (TNF-α) (Bernardino et al., 2008), neuropeptide Y (NPY) (Xapelli et al., 2006; Agasse et al., 2008a) stem cell factor (SCF) (Jin et al., 2002a) EGF and bFGF (Kuhn et al., 1997; Baldauf and Reymann, 2005). A comprehensive knowledge on the effects of administration of the factor, in the scenario of the disease, would be required.

Injury-induced tropism relies on a gradient of chemoattractants, like pro-inflammatory cytokines and chemokines released at the site of lesion (Imitola et al., 2004; Widera et al., 2004; Belmadani et al., 2006). Importantly, other injury-triggered events co-operate with the chemottractant cues to convey precursors towards the injury site. Recently, some studies have shown that SVZ neuroblasts migrating in the ischemic striatum use striatal blood vessels as a

physical scaffold for their migration (Ohab et al., 2006; Thored et al., 2007; Kojima et al., 2010), a process that resembles the vasculature-mediated migration observed in the SVZ-RMS-OB (Snapyan et al., 2009). However the molecular mechanism involved in such vasophilic migration, which can be putatively used to boost the migratory process, is largely unexplored. The work described in Chapter 4, aimed at exploring the molecular mechanism underlying the injury-induced migration occurring after ischemia, taking as a reference the previous knowledge on the constitutive migration taking place in the RMS. Given that the SVZ is the major pool of adult NSCs and located centrally in the brain, it is conceivable to hypothesize that this privileged location brings easy availability to diverse regions in case of injury. Indeed, following a variety of pathological paradigms, SVZ cells were shown to migrate to the cortex (Sundholm-Peters et al., 2005; Faiz et al., 2008), corpus callosum (Nait-Oumesmar et al., 1999; Picard-Riera et al., 2002), striatum (reviewed in Zhang et al., 2008) and even hippocampus (Parent et al., 2006), depending on the area affected.

Besides survival and migration, the de-routed cells face other two challenges to achieve repair: differentiation on the phenotype of the lost cells, and correct functional integration in the complex adult brain. Regarding differentiation, some studies have demonstrated a capacity of the recruited cells to undergo cell specification on the cell type required (Nait-Oumesmar et al., 1999; Fallon et al., 2000; Yamashita et al., 2006), although others observed differentiation in other phenotypes (Liu et al., 2009; Li et al., 2010b; Zhang et al., 2011). As previously mentioned, efforts are being brought together to screen soluble factors or develop genetic tools to instruct the cells into specific phenotypes. In this context, I describe on Chapter 3 the development of a method that rapidly evaluates cell differentiation in SVZ cultures, and may be applied to screen factors that enrich the population of SVZ cells in a given phenotype. Likely, the major roadblock in the therapeutic use of SVZ cells for

cell replacement is the proper reconstruction of the neuronal networks. In such perspective, MS offers a relevant advantage as compared to diseases where the depleted cells are neurons. Among the latest, diseases implying damage in short local circuitries may be better candidates for cell replacement therapies.

Evidences hitherto highly suggest that the brain regenerative capacity may be enhanced in order to help repopulating injured areas. Identification of key targets on injury-induced migration, differentiation and survival is critical for the manipulation of such mechanism, and translation into therapy for the devastating brain injuries.

1.2.2.2 Post-ischemic neurogenesis

A brief comment on the impact of stroke on nowadays societies and the importance of the development of regenerative strategies for stroke is highlighted on Box 1.4.

Box 1.4. Ischemic stroke

With a very high incidence, stroke remains as the leading cause of disability and the third leading cause of mortality in the Western world. Stroke is caused by the interruption of blood flow in a cerebral artery, usually by embolic vascular occlusion. Focal ischemic stroke is the most common form, involving a confined core area of necrotic brain tissue, with irreversible damage, surrounded by the penumbra, a zone with marginal blood supply and partially reversible damage. Currently, the only available treatment for stroke is the acute administration of the recombinant tissue plasminogen activator (tPA) to resume the blood flow (thrombolytic therapy). However, this drug is effective if given within 3-5 hours from the onset of the stroke. Because of such a narrow time window of efficacy, only 5% of stroke victims receive tPA treatment. Although it reduces the infarct volume, most of the patients will still have some neurological deficits. Therefore, it renders imperative to develop neuroprotective and/or neuroregenerative therapies to rescue the ischemic penumbra in a more feasible time window for treatment. Cell replacement strategies have been sought via: 1) stem cells transplantation in the injured area, with encouraging results leading to phase I and II pre-clinical trials (see Burns and Steinberg, 2011); 2) amplification of the naturally occurring injury-induced neurogenesis. In the next sections, I will outline important aspects of the latter, which support the work described in Chapter 4.

Over the past decade, a plethora of studies have demonstrated that cerebral ischemia stimulates neurogenesis in the neurogenic niches, SVZ and DG. Pioneering studies conducted by Liu et al. (1998) indicated a 12-fold increase in cell birth and later neuronal differentiation of the newborn cells, in the DG of adult gerbils, upon global ischemia. Comparatively attracting more attentions, the SVZ of adult brain has been repeatedly shown to expand largely its proliferative pool after focal brain ischemia, and to witness an interesting deviation of the cells from the SVZ-RMS-OB axis towards the ischemic area (Arvidsson et al., 2002; Parent et al., 2002; Ohab et al., 2006; Yamashita et al., 2006; Thored et al., 2007; Zhang et al., 2009) (Fig. 1.9). Moreover, ependymal cells lining the lateral ventricle become activated and give rise to neurons and astrocytes after stroke (Carlen et al., 2009). On the other hand, recent studies suggested the occurrence of neurogenesis in the marginal zone of the adult neocortex, from a small population of resident NPCs that rarely divide under normal conditions (Ohira et al., 2010).

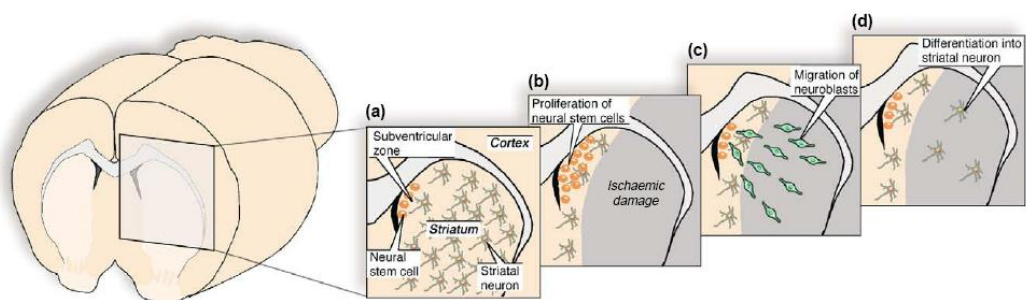


Figure 1.9. Schematic representation of stroke-induced neurogenesis. (**a**) NSCs and NPCs reside in SVZ. (**b**) Focal ischemic insults which lead to pronounced loss of striatal and cortical neurons give rise to increased proliferation of progenitors. (**c**) Neuroblasts formed after and to some extent also before the stroke then migrate to the damaged part of the striatum, (**d**) where they can express markers specific for striatal projection neurons. [Adapted from Kokaia and Lindvall (2003)].

Experimental stroke, typically the classic model of transient ischemia involving middle cerebral artery occlusion (MCAo), stimulates proliferation in the SVZ, neurogenesis, and migration of neuroblasts to the nearby ischemic striatum. Remarkably, analysis of *post-mortem* tissue from stroke patients has provided evidences for active cell proliferation and increased neurogenesis in the ipsilateral SVZ (Macas et al., 2006; Marti-Fabregas et al., 2010), besides the appearance of neuronal progenitors in the ischemic penumbra (Jin et al., 2006). In the compromised area, new cells divide (Zhang et al., 2007) and are found individually, or associated in chains or clusters (Zhang et al., 2004b; Yamashita et al., 2006; Zhang et al., 2009).

Whether or not the deviation of some neuroblasts from the SVZ/RMS to the injured striatum may occur at the expense of the normal migration in RMS, and thereby, alter the number of new neurons integrating in the OB circuitries remains unclear. The first study addressing this question reported that OB from ischemic or from sham-operated mice harbor similar density of BrdU$^+$ cells, and similar percentage of NeuN$^+$ cells among those, in the bulbar GCL, indicating that OB neurogenesis is not affected (Gotts and Chesselet, 2005). Nevertheless, subsequent works raised some controversy, as they indicated less (Ohab et al., 2006; Kernie and Parent, 2010) or more (Koketsu et al., 2006) new cells in the OB. Despite the loss of cells in RMS by mobilization to ectopic injured areas, proliferation in the SVZ is increased, generating more migrating cells in the RMS, and thus it is difficult to predict the outcome in OB neurogenesis. Differences in these studies may result from variations in the model and severity of ischemic injury, in the time point evaluated after injury, and in the quantification methodologies.

Ischemia-induced neurogenesis persists at long-term. Indeed, addition of new neurons to the ischemic striatum occurs even at one year after ischemic insult (Kokaia et al., 2006). Although numerous studies highlight a generous attempt of self-repair from brain endogenous stock of stem cells, the fate of de-

routed cells is, mostly, cell death. Likely, this is due to a failure to integrate, or to cytotoxic effects of the inflammatory environment that cells encounter in the damaged area. In line with this view, treatment with anti-inflammatory drugs seem to allow a stronger neurogenic response to injury (Hoehn et al., 2005), likely by contributing to the survival of the new neurons (Liu et al., 2007c). On the contrary, others have pinpointed a positive role of inflammation in injury-induced neurogenesis, by supporting proliferation and differentiation of NSCs in the SVZ niche (Thored et al., 2009). In addition, inflammatory molecules are important chemoattractants in injury-induced migration (Thored et al., 2006). Inflammation appears thus to have a dual role in neurogenesis, depending on the time window upon injury, and the balance between pro- and anti-inflammatory secreted molecules (Ekdahl et al., 2009).

Few neuronal precursors survive and differentiate into mature neurons in the ischemic striatum (0.2% according to Arvidsson et al. (2002). As regard to the phenotype of these neurons, conflicting results have emerged, and may be due to SVZ regional specificity. Initially, two groups have demonstrated that most of the cells recruited to the ischemic striatum express markers of striatal medium spiny neurons, DARPP-32 and calbindin (Arvidsson et al., 2002; Parent et al., 2002). Subsequently, others have observed mostly parvalbumin-expressing neurons (Teramoto et al., 2003), or almost exclusively calretinin-expressing neurons (Liu et al., 2009). In addition to neurons, oligodendrocytes and astrocytes are generated among the cells mobilized to the ischemic striatum (Li et al., 2010b). Actually, this study emphasizes a more predominant contribution of glial cells than previously appreciated, with 59% astrocytes and 15-20% OPCs within the population of SVZ-derived cells, at 2 weeks after MCAo.

The paucity of the surviving new neurons raises the question about their functional relevance, since such scarce cell number hardly brings any improvement. Besides, despite studies showing functional integration of the

newly differentiated neurons (Yamashita et al., 2006; Hou et al., 2008), no evidence for establishment of the proper intercellular connections exists.

1.2.2.2.1 Modulators of post-ischemic neurogenesis

Gain- and loss-of-function studies revealed important mediators of stroke-induced cell proliferation and neurogenesis. Notch induces SVZ precursors expansion after stroke (Androutsellis-Theotokis et al., 2006), through downstream signaling involving Notch intracellular domain (NICD) and transcriptional targets Hes1 and Shh (Sims et al., 2009; Wang et al., 2009b). Accordingly, one study *in vitro* reported an upregulation of Notch signaling during cell proliferation induced by ischemia, and a downregulation of Notch signaling during differentiation of precursors in new neurons (Wang et al., 2009a). Growth factors, namely EGF, bFGF (Baldauf and Reymann, 2005; Ninomiya et al., 2006; Ma et al., 2008b; Wang et al., 2008), glial cell-derived neurotrophic factor (GDNF) (Kobayashi et al., 2006) and transforming growth factor (TGF) (Ma et al., 2008a; Guerra-Crespo et al., 2009) promote proliferation and early neuronal differentiation during ischemia. The cytokine TNF-α is upregulated at 1 week after stroke and suppresses stroke-induced proliferation (Iosif et al., 2008). In contrast, IGF-1 levels are increased shortly after stroke, and it constitutes a positive regulator of proliferation upon stroke (Yan et al., 2006), Furthermore, EPO is upregulated after ischemia and promotes neurogenesis at the expense of self-renewal (Shingo et al., 2001). Importantly, BDNF, VEGF, GDNF and bFGF may act as neuroprotective agents, and EPO as anti-apoptotic agent, thus exhibiting a pro-survival effect on the pre-existent or on the newly born neurons after ischemia (Marti et al., 2000; Sun et al., 2003; Kobayashi et al., 2006; Ma et al., 2008b; Shi et al., 2009). BDNF expression and neuroprotective role in ischemia is further discussed on the next sections.

Similarly, the aberrant injury-induced migration of SVZ neuroblasts, that provides new cells to the lesioned tissue, relies on a new scenario of injury-

triggered molecular factors. Actually, it entails a concerted action of soluble molecules, cellular contacts and intrinsic autocrine mechanisms, similarly to migration in the RMS. Firstly, the neurogenic niche, which engages a tightly regulated cytoarquitecture with no usual escape of neuroblasts, needs to be disrupted. One may speculate that the physical confinement of the astrocytic meshwork found in the SVZ and RMS becomes loose, likely due to alterations in cell adhesion molecules and components of the ECM. However, this issue is far unexplored and thus deserves further attention.

Then, cells need to sense signals on the surrounding environment that guide them in the direction of the infarct. Focal ischemia leads to a massive inflammatory response in the infarct core and penumbra, including macrophage/leukocyte infiltration, astrocytosis and microglial activation. These activated cells produce several factors, such as cytokines and chemokines that may work as chemoattractants to the SVZ precursor cells, and thus leading to pathotropism through gradients of concentration of such chemoattractants (Aarum et al., 2003). Indeed, the chemokine stromal cell-derived factor 1 (SDF-1) has been consistently identified has a key regulator of pathotropism to ischemic boundary, recapitulating its effect on neuroblasts migration during brain development (Bajetto et al., 2001). Following ischemia, SDF-1 is upregulated in the reactive astrocytes and endothelium of the damaged area, difuses and attracts neuroblasts in the SVZ by interaction with its receptor CXCR4 expressed in these migrating cells (Imitola et al., 2004; Miller et al., 2005; Ohab et al., 2006; Robin et al., 2006; Thored et al., 2006). Blockade of SDF-1/CXCR4 signaling largely inhibits neuroblasts migration to the infarct (Robin et al., 2006). Also, ischemia induces an increase in the expression of the chemokine monocyte chemoattractant protein-1 (MCP-1) in activated microglia and astrocytes. Migrating neuroblasts in the ischemic brain were shown to express the MCP-1 receptor CCR2, and CCR2 KO mice display significantly

lower neuroblasts migration after transient MCAo (Liu et al., 2007b; Yan et al., 2007).

Besides chemoattraction, mechanisms that allow and foster migration through the non-neurogenic parenchyma are required. Matrix metalloproteinases (MMPs) are a family of enzymes that degrade the ECM, allowing cells to advance through the remodeling extracellular matrix. In ischemia, activated endothelial cells and migrating neuroblasts upregulate MMPs (Zhao et al., 2006). MMP2 and MMP9 were shown to mediate cell motility and facilitate neuroblasts migration to the ischemic boundary. Indeed, blocking the expression or activation of MMPs can severely abrogate injury-induced migration (Lee et al., 2006; Wang et al., 2006b; Barkho et al., 2008).

Importantly, the role of vasculature in post-stroke migration of SVZ precursors goes far beyond the production of SDF-1 and MMPs. In fact, histological analysis of fixed ischemic brains unveiled a close proximity of the de-routed cells to striatal blood vessels, suggesting the need for a physical substrate (Ohab et al., 2006; Yamashita et al., 2006; Thored et al., 2007). In agreement, time-lapse imaging confirmed the vasophilic nature of injury-induced migration (Zhang et al., 2009; Kojima et al., 2010), a mechanism that resembles the constitutive migration normally undertaken by these cells (Bovetti et al., 2007; Snapyan et al., 2009). Likewise, Jin et al. (2006) found newly born cells in the vicinity of blood vessels of the penumbral zone of *post-mortem* brain tissue from stroke patients. Interestingly, angiogenesis is induced by ischemia, in a spatiotemporal pattern that coincides with the induced-neurogenesis. It appears that regardless developmental or adult, physiological or pathological, the so called “neurovascular niche” is imperative for neurogenesis.

In stroke paradigm, hypoxia-inducible factor-1 alpha (HIF-1α) is known to mediate downstream gene expression of several factors including the main angiogenic factor VEGF, BDNF, SDF-1 and nitric oxide (NO) (Madri, 2009). Importantly, NO up-regulates SDF-1/CXCR4 and angiopoietin-1(Ang-

1)/Tie-2, thus promoting angiogenesis by the latter, and neuronal migration through both downstream signaling pathways (Cui et al., 2009). Neovascularization upon ischemia is probably mediated through the angiogenic systems VEGF/VEGFR, Ang/Tie, PDGF/PDGF-β, EPO, TGF-β and FGF. As a scaffold structure, both old and new blood vessels serve migration in the ischemic striatum (Kojima et al., 2010). However, suppression of angiogenesis with endostatins or a neutralizing antibody anti-Tie-2 significantly diminishes migration to the ischemic boundary (Ohab et al., 2006), probably by decreasing the extension of the available physical substrate for migration.

On the other hand, vessels also play an essential role in post-stroke neurogenesis and migration as a source of secreted molecules, like VEGF (Teng et al., 2008), Ang-2 (Beck et al., 2000), besides the aforementioned SDF-1 and MMPs. VEGF is an angiogenic and neurogenic growth factor. Indeed, intraventricular administration of VEGF increases neurogenesis in the SVZ and DG of adult mice (Jin et al., 2002a). Hence, activated endothelial cells secrete VEGF which increases neurogenesis. Accordingly, co-cultures of endothelial cells isolated from the ischemic boundary with NPCs from the non-ischemic SVZ, result in a robust increase of the number of neurons generated from SVZ cells, via VEGFR2 (Teng et al., 2008). Reciprocally, NPCs can also enhance angiogenesis. Gene expression analysis of NPCs from the SVZ of ischemic brain revealed that these NPCs express several angiogenic factors including Ang-2 (Liu et al., 2007a). In addition, NPCs from the ischemic area promote angiogenesis *in vitro* as evaluated by capillary-like tube formation assay (Teng et al., 2008).

Regarding neuronal migration towards ischemic area, it is still not clear whether or not VEGF may play a role. Viral-mediated VEGF expression in the SVZ results in the appearance of more $BrdU^+/Dcx^+$ cells in the ischemic area, as compared to the control group (Li et al., 2009). Also, VEGF-overexpressing transgenic mice display increased numbers of $BrdU^+/NeuN^+$

cells in the peri-infarcted cortex (Wang et al., 2007). These studies claimed an effect of VEGF on ischemia-induced migration, however, one cannot conclude about migration from increased numbers of new neurons in the ischemic area. Indeed, the augmentation of the recruited cells may simply result from the effects of VEGF on the proliferation and neurogenesis in the SVZ, increasing the resident stock of precursors but not the migratory process. Concerning endothelial-derived angiopoietins, studies suggested a role in the atypical migration of neuroblasts upon ischemia. Delivery of Ang1 via osmotic minipumps (Ohab et al., 2006) appears to promote neuroblasts migration to the peri-infarcted cortex. Consistently, application of Ang1 on cultures of SVZ explants generated from ischemic brains (Cui et al., 2009) increases migration distance from explants. Altogether these findings provide evidences that vasculature harbors a central role conveying and supporting migration of SVZ precursor cells to the ischemic area.

Finally, following recruitment, migrating bipolar cells need to stop migration and initiate/continue differentiation, integrate and mature, establishing functional synapses with the correct partners. Regulation of these final steps remains poorly understood, likely due to the poor survival of cells once they arrive to the target area.

1.2.2.2.2 Expression of BDNF and its receptors after ischemia

A variety of brain insults, including epileptic, hypoglycemic, ischemic and traumatic, is known to cause changes in gene expression of neurotrophins, such as BDNF. Several *in vivo* studies report an early increase on expression and production of BDNF in the ischemic core and surrounding tissue (Lindvall et al., 1992; Comelli et al., 1993; Takeda et al., 1993; Kokaia et al., 1995; Truettner et al., 2002). In addition, TrkB mRNA levels are induced in the ischemic penumbral cortex (Comelli et al., 1993; Narumiya et al., 1998; Ferrer et al., 2001) or hippocampus (Merlio et al., 1993). Also p75NTR

immunoreactivity is upregulated after ischemia (Lee et al., 1995; Botchkina et al., 1997; Kokaia et al., 1998b; Lambertsen et al., 2007; Angelo et al., 2009). It is widely accepted that neurons are a transient source of BDNF after injury, and such response is generally associated to a role in neuroprotection, to counteract neuronal damage, and in stimulation of sprouting and remodeling. Some studies using experimental models of permanent brain ischemia reported BDNF secretion by activated microglia in injured areas (Madinier et al., 2009; Béjot et al., 2011). In addition to the production of BDNF by brain immune cells, Béjot et al. (2011) claimed the release of the neurotrophin by other cells like ependymal cells, endothelial cells and astrocytes, with different contributions depending on the time point considered after the onset of ischemia.

1.2.2.2.3 Neuroprotective role of BDNF in ischemia

A series of gain- or loss-of-function studies have identified a positive effect of BDNF in stroke tolerance or recovery. Indeed, BDNF infusion or overexpression reduces the infarct volume (Almli et al., 2000; Yanamoto et al., 2000; Berger et al., 2004; Shi et al., 2009) while transgenic mice overexpressing truncated TrkB display larger lesions (Saarelainen et al., 2000). In contrast, other reports observed no differences in infarct size when manipulating BDNF levels, although cell survival or functional outcome was altered. Indeed, a continuous low-dose treatment with BDNF protects the resident striatal medium spiny neurons from stroke-induced cell death (Galvin and Oorschot, 2003). Likewise, intrastriatal delivery of BDNF using viral-vector systems mitigates cell death and leads to improvements in motor deficits (Andsberg et al., 2002; Shi et al., 2009). Also, BDNF treatment diminishes spatial memory deficits (Almli et al., 2000). On the other hand, strategies that reduce BDNF levels result in attenuated stroke-induced angiogenesis, greater locomotor deficits (Qin et al., 2011) and reduced effect of rehabilitation in motor function (Ploughman et al., 2009).

1.2.1.1.2 Role of BDNF in post-ischemic neurogenesis

In addition to the protective effect of BDNF in the ischemic tissue, some studies suggest a role for the neurotrophin in ischemia-induced neurogenesis. Indeed, administration of BDNF in animals subjected to phototrombotic ischemia increases hippocampal neurogenesis ($NeuN^+$/$BrdU^+$ cells per DG) and enhances the number of SVZ-derived neuronal precursors in the ischemic tissue (Schabitz et al., 2007; Keiner et al., 2009). Consistently, Im et al. (2010) reported that BDNF treatment induces a modest increase in the number of new neurons in the ischemic striatum, but a co-infusion of BDNF/EGF amplifies this effect, since EGF expands the proliferative pool of SVZ cells that is responsive to the neuronal instruction given by BDNF. Moreover, post-stroke treatment with a histone deacetylase (HDAC) inhibitor stimulates proliferation and neuronal differentiation in the SVZ and DG, and results in behavioral benefits, through downstream upregulation and secretion of BDNF, and subsequent BDNF-TrkB signaling (Kim et al., 2009). Also, Chen et al. (2005) have shown that endothelial nitric oxide synthase (eNOS) regulates BDNF expression in the ischemic brain. Interestingly, the authors demonstrated post-stroke decreased neurogenesis and angiogenesis, and attenuated functional recovery, in the eNOS KO mice as compared to the wild-type (WT) mice. Since, *in vitro*, BDNF treatment of SVZ cells cultured from eNOS KO mice restores the impaired neurosphere formation, proliferation and neurite outgrowth, the authors proposed that BDNF may mediate the effects of eNOS in functional recovery and plasticity after stroke, although no direct evidence was proven.

In sharp contrast, intrahippocampal viral-based delivery of BDNF gene increases BDNF protein levels in locus and inhibits the ischemia-induced production of new neurons in the DG ($BrdU^+$/$NeuN^+$ or $BrdU^+$/Hu^+) (Larsson et al., 2002). In concert with these findings, i.c.v infusion of the BDNF scavenger TrkB-Fc, instead, promotes ischemia-induced DG neurogenesis (Gustafsson et al., 2003). Furthermore, transgenic $BDNF^{+/-}$ mice with decreased brain levels of

BDNF harbors an increased number of neuroblasts within the ischemic striatum, as compared to the WT littermates (Nygren et al., 2006).

1.3 Main objectives

Efforts envisaging efficient cell replacement therapies for brain injury by the use of NSCs have been gathered, and point to two possible strategies: 1) the grafting of NSCs in the injured area, to supply the depleted zone with new cells potentially able to replace the lost ones; 2) the amplification of the endogenous self-repair mechanism involving NSCs that operates in the injured brain.

Although research on the field has been witnessing great expansion, still translation to the clinical arenas requires many hurdles to be overcome. As regard to cell transplantation strategy, an important step to consider is to instruct the grafted NSCs into the desired phenotype. Thus, identification of soluble factors or genetic tools that favor a certain cell lineage is mandatory. Since MS is a good candidate for a successful transplantation therapy, but oligodendrocytes constitute a small minority on the SVZ cell cultures, the discovery of compounds that spur oligodendrocytic differentiation may be of great impact. In this context, we aimed at developing a method that functionally assessed differentiation patterns in the mixed SVZ cell cultures, useful for a rapid screening of putative oligodendrogenic factors (Chapter 3). Previous studies allowed the discrimination of neuronal cells from immature cells in SVZ cultures by their specific "$[Ca^{2+}]_i$ signatures" upon KCl and histamine stimulations, respectively. Based on the same concept, we proposed to use stimulation with thrombin as a putative tool to identify oligodendrocytes in SVZ neurosphere-derived cultures, considering that thrombin induces $[Ca^{2+}]_i$ rises in an oligodendrocyte cell line (Wang et al., 2004). Accordingly, we designed a

single-cell calcium imaging (SCCI)-based protocol aiming at the full phenotypic characterization of the SVZ culture in terms of differentiation, and used T3 hormone to test our method, since it is a classic tool to boost oligodendrogenesis. Moreover, we investigated the involvement of the protease-activated receptor (PAR)-1 in the stimulation of SVZ-derived oligodendrocytes by thrombin, and explored the thrombin-activated intracellular signaling cascade that leads to the increase of $[Ca^{2+}]_i$ in oligodendrocytes. In addition, because diseased host environment highly differs from the culture conditions, we developed grafting experiments, by depositing eGFP-SVZ neurospheres into intact or demyelinated organotypic hippocampal slices, and kept the co-culture under T3 treatment. By this mean, we aimed at evaluating whether the damaged tissue may modulate the differentiation of new integrating cells (Chapter 3).

On the other hand, strategies to boost endogenous self-repair require better comprehension of the mechanisms underlying the injury-induced proliferation, differentiation and ectopic migration. Herein, we aimed at exploring the migration of neuroblasts to ischemic brain areas (Chapter 4). Given the pivotal role of vasculature-derived BDNF in the daily migration of new neurons along the RMS to the OB, we hypothesized whether BDNF may also contribute to regulate injury-induced migration when these cells are de-routed through the compromised striatum. Several evidences support such hypothesis: 1) BDNF expression is upregulated upon ischemia; 2) after ischemia the meshwork of striatal blood vessels is activated and highly extended via angiogenesis; 3) *in vitro* studies using a mouse endothelial cell line showed that hypoxia stimulates BDNF expression and release by the endothelial cells (Wang et al., 2006a); 4) the close proximity of recruited neuroblasts to striatal blood vessels resembles migration in the RMS; 5) BDNF is important in migration of neuronal precursors in the developing brain and in the constitutive migration in the RMS of the adult (Marin and Rubenstein, 2003; Snapyan et al., 2009); 6) the molecular mechanisms underlying many pathological processes are often a

recapitulation of the constitutive processes occurring in the intact brain. We first analyzed the proximity of migrating cells in the ischemic striatum to blood vessels, by immunohistological analysis or during recording of cell migration in acute brain slices. We then investigated the expression of BDNF, TrkB and p75NTR in the ischemic striatum and identified the cell sources. Finally, time-lapse imaging was used to monitor cell migration in the ischemic area. By following migration in real-time, and applying BDNF or scavenging it from the extracellular solution, we tested the postulated hypothesis that BDNF may modulate injury-induced migration. Furthermore, we explored the dynamics of such atypical migration, and compared with the constitutive migration taking place in the RMS. In brief, the present thesis accents in two pillars of NSCs biology - differentiation and migration - and intends to provide new insights on mechanisms that alter or dictate these processes, with the perspective of favoring cell replacement in brain injuries.

CHAPTER 2

Materials and Methods

2.1 Animals

All experiments were performed in C57BL/6 WT or β-actin promoter-driven enhanced green fluorescent protein (eGFP) trangenic mice (Charles River), in accordance with institutionally approved ethical guidelines for the care and use of laboratory animals [National Institutes of Health (NIH), European Union (86/609/EEC) and Laval Institute directives settled by the Canadian Council on Animal Care]. Animals were housed on a 12 h light/dark cycle at constant temperature (22 °C) and relative humidity (55%), and allowed *ad libitum* access to food and water.

2.2 *In vitro* studies

2.2.1 Cell cultures

2.2.1.1 Subventricular zone neurosphere culture

SVZ cells were obtained from 1- to 3-day-old WT or eGFP mice as described in Agasse et al. (2008a), with minor modifications. Briefly, after decapitation, brains were removed and placed in Ca^{2+}/Mg^{2+}-free-Hank's balanced salt solution (HBSS; Invitrogen) supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen). Meninges were gently removed and the brains were sectioned in 450 µm-thick coronal slices with a McIlwain tissue chopper. Under a dissecting microscope, thin fragments of the SVZ were dissected out and digested in HBSS containing 0.025% trypsin and 0.265 mM ethylenediaminetetraacetic acid (EDTA; Invitrogen) during 10 min, at 37ºC. After mechanical dissociation with a P1000 pipette, the cell suspension

was diluted in serum-free medium (SFM) composed of Dulbecco's Modified Eagle's Medium/Ham's F-12 (DMEM/F-12) with GlutaMAX-I, supplemented with 100 U⁄ml penicillin and 100 μg⁄ml streptomycin, 1% B-27, 10 ng⁄ml EGF and 5 ng⁄ml bFGF (all from Invitrogen). Individualized cells were then plated on uncoated Petri dishes at a density of 3 000 cells⁄cm^2 and incubated during 6 to 8 days in a 95% air and 5% CO_2 humidified atmosphere, at 37ºC. At this stage, free-floating clonal aggregates called neurospheres were formed.

To perform grafting of SVZ neurospheres in demyelinated hippocampal slices, SVZ culture was prepared from eGFP mice, generating $eGFP^+$ primary neurospheres. These experiments were continued as explained in sections 2.3.3 and 2.3.4. On the other hand, to perform the *in vitro* functional evaluation of SVZ cell differentiation, primary SVZ neurospheres were obtained from WT mice. These neurospheres were collected and plated on 0.1 mg/ml poly-D-lysine (PDL; Sigma-Aldrich)-coated glass coverslips. In particular, for the experiments correlating SCCI with subsequent phenotypic immunodetection, the neurospheres were seeded on 0.1 mg/ml PDL-coated microgrid coverslips (CELLocate, Eppendorf) that allow individual cell location. Upon deposition of the spheres, SFM devoid of growth factors was added to the wells. The neurospheres were allowed to adhere during 2 days in a 95% air and 5% CO_2 humidified atmosphere, at 37ºC, before application of the treatments. Under the same conditions, the cultures were then incubated along 7-10 days with 30 nM T3 (Sigma-Aldrich), 10 ng/ml ciliary neurotrophic factor (CNTF; Promokine) or 20 ng/ml SCF (Millipore), in order to direct the differentiation of progenitor cells that emerge from the neurospheres, towards an oligodendroglial, astroglial or neuronal fate, respectively, as supported by previous works (Johe et al., 1996; Murray and Dubois-Dalcq, 1997; Whittemore et al., 1999; Jin et al., 2002a; Ravin et al., 2008). For experiments designed to study mature oligodendrocytes, capable of producing myelin, T3 treatment was mantained for 10 days, thus allowing maturation of cells to later stages of their lineage. Following the

treatment period, SCCI experiments and/or immunocytochemistry were performed.

2.2.1.2 Cortical neurosphere culture

Cortical cells were obtained from early postnatal (P3-5) WT mice. Dissection of the parietal cortex and subsequent neurosphere culture was performed by following the protocol described previously to produce SVZ neurosphere culture (section 2.2.1.1). Likewise, we plated and treated the cortical neurospheres with T3 hormone, during 7-10 days, before performing the SCCI experiments. These experiments were based on the previous knowledge that cortical astrocytes from newborn rodents still retain the clonogenic capacity, therefore forming spheres, as well as the multipotency (Laywell et al., 2000; Itoh et al., 2006).

2.2.1.3 Hippocampal neuronal culture

Primary cultures of hippocampal neurons were prepared from the hippocampi of E18-E19 WT mice embryos. Briefly, the entire hippocampi were dissected out of the brains and treated with 0.06% trypsin (Sigma-Aldrich) in Ca^{2+}/Mg^{2+}-free HBSS (121 mM NaCl, 25.5 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 4.8 mM KCl, 14.3 mM glucose, 10 mM HEPES and 0.001% phenol red, pH 7.4), for 10 min, at 37ºC. The hippocampi were then washed in HBSS supplemented with 1.5 mg/ml of trypsin inhibitor (Sigma-Aldrich) in order to stop trypsin activity. After removing the excess of trypsin and trypsin inhibitor, by washings with HBSS, the tissue was mechanically dissociated. Cells were then seeded on 0.1 mg/ml PDL-coated coverslips (37 500 cells/cm^2) and maintained in chemically defined serum-free Neurobasal medium (Invitrogen), supplemented with 2% B-27, 25 μM glutamate (Sigma-Aldrich), 200 μM glutamine (Invitrogen), 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were kept in a humidified incubator with 5% CO_2 and 95% air, for 7 to 8 days, before

SCCI experiments and immunocytochemistry. Half of the growth medium was changed every 4 days to fresh Neurobasal medium containing 2% B-27, 200 μM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, but without glutamate.

2.2.1.4 Cortical astrocyte culture

Following decapitation, the brains from 6- to 7-day-old WT mice were removed and placed in HBSS solution with 100 U/ml penicillin and 100 μg/ml streptomycin. The entire cerebral cortices were harvested and digested in 0.025% trypsin and 0.265 mM EDTA, then, washed and mechanically dissociated with a P1000 pipette. Cell suspension was centrifuged (65 x *g*, 2 min) and the pellet was resuspended in DMEM/F-12 medium with GlutaMAX-I, supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 0.37 g/l $NaHCO_3$, 10% fetal calf serum (FCS; Invitrogen) and 25 mM HEPES (Invitrogen). Cells were plated onto 0.1 mg/ml PDL-coated coverslips at a density of 25 000 cells/cm^2. On the following day, the medium was changed to remove non-adhering dead cells, and the culture was then allowed to develop in a 95% air and 5% CO_2 humidified atmosphere at 37ºC, during 7 days, before SCCI experiments and immunocytochemistry.

2.2.2 Single-cell calcium imaging

Following the 7- to 10-day differentiation period, we functionally assessed the pattern of differentiation of SVZ cultures, by single-cell analysis of the intracellular calcium variations ($[Ca^{2+}]_i$) upon stimulation with potassium chloride (KCl), histamine and thrombin. We designed this protocol of stimulation as a putative method to functionally determine cell phenotypes in the heterogeneous SVZ cultures, since previous studies have reported that $[Ca^{2+}]_i$ increases 1) in neurons after KCl-induced depolarization (Bading et al., 1993), 2) in stem/progenitors cells following histamine application (Tran et al., 2004),

and 3) in oligodendrocyte cell line OLN-93 after stimulation with thrombin (Wang et al., 2004).

SVZ cultures were loaded for 40 min, at 37ºC, with the calcium probe 5 mM Fura-2-acetoxymethyl ester (Fura-2AM; Molecular Probes) prepared 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) plus 0.1% fatty acid-free bovine serum albumin (BSA; Sigma-Aldrich) and 0.02% pluronic acid F-127 (Molecular Probes). Afterwards, cells were washed in Krebs and the coverslip was mounted on a RC-25 chamber (Warner Instruments). The perfusion chamber was then placed in a PH3 platform (Warner Instruments) on the stage of an inverted fluorescence microscope Axiovert 200 (Carl Zeiss). Cells were continuously perfused with Krebs and stimulated at defined time periods with 50 mM KCl (isosmotic substitution with NaCl), 100 mM histamine (Sigma-Aldrich), and 0.1 U/mL thrombin (Sigma-Aldrich) (Fig. 2.1A). Solutions were applied by a fast-pressurized system (95% air and 5% CO_2 atmosphere) (AutoMate Scientific). $[Ca^{2+}]_i$ was assessed by quantifying the ratio of the fluorescence emitted at 510 nm following alternate excitation (750 msec) at 340 nm and 380 nm, using a Lambda DG4 apparatus (Sutter Instruments Company) and a 510 nm longpass filter (Carl Zeiss). Fluorescence was acquired using a 40x objective and a CoolSNAP digital camera (Roper Scientific). Variations of $[Ca^{2+}]_i$ in approximately 100 cells/field were analyzed and the acquired values were processed with MetaFluor software (Universal Imaging Corporation). KCl-, histamine-, and thrombin-induced peaks, given by the normalized ratios of fluorescence at 340/380 at the specified time periods, were used to calculate the ratios of the responses to histamine/KCl (Hist/KCl) and to thrombin/histamine (Throm/Hist). Indeed, as demonstrated in Agasse et al. (2008a), a Hist/KCl ratio below 0.8 identifies neurons, whereas a Hist/KCl ratio above 1.1 identifies immature cells. On the other hand, glial cells display a Hist/KCl ratio between 0.8 and 1.1, as confirmed by using primary cultures of cortical astrocytes.

According to our hypothesis, Throm/Hist ratio may allow us to discriminate oligodendrocytes among other cells present in SVZ-derived heterogeneous cell populations. A detailed technical description of the method can be found in Grade et al. (2012). Additionally, the experimental protocol was carried out in primary cultures of hippocampal neurons from embryonic mouse brain, cortical astrocytes and cortical neurospheres both obtained from postnatal mouse brain.

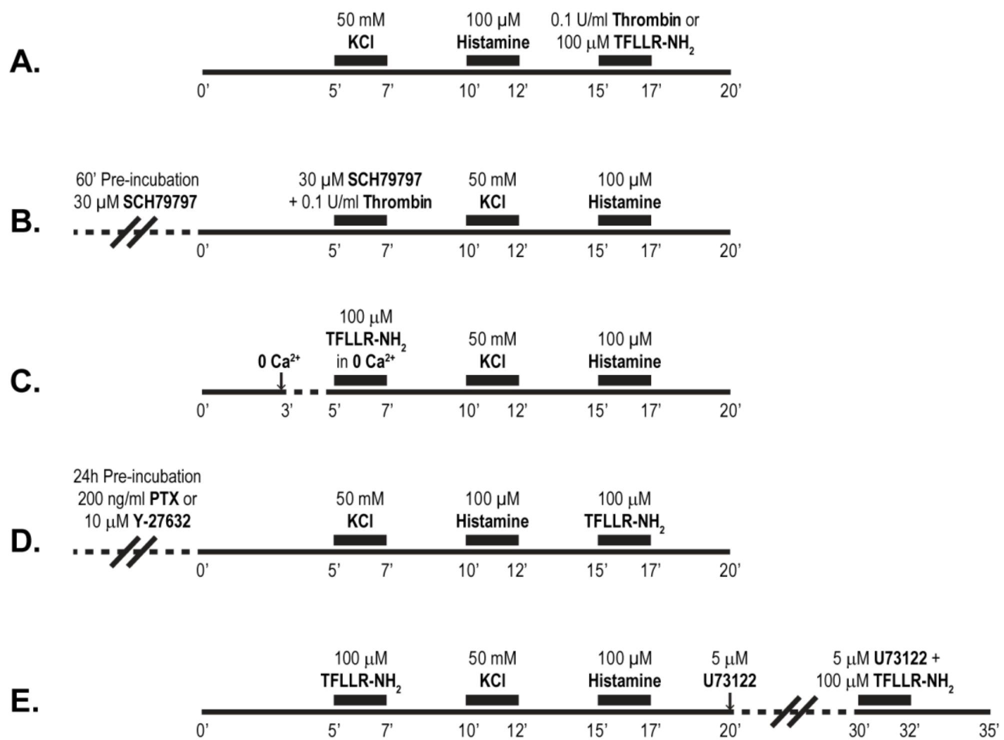


Figure 2.1. Experimental protocols performed in SCCI analysis, aiming at the functional identification of SVZ-derived oligodendrocytes by thrombin response (A), and the clarification of the thrombin-activated intracellular signaling (A-E). SVZ cells loaded with the Ca^{2+} probe Fura-2AM were continuously perfused with Krebs solution and stimulated at defined time intervals with the pharmacological agents, as shown. TFLLR-NH_2, PAR-1 agonist; SCH79797, PAR-1 antagonist; 0 Ca^{2+}, Krebs solution with low calcium concentration; PTX, pertussis toxin, $G_{i/o}$ inhibitor; Y27632, Rho/ROCK inhibitor; U73122, PLC inhibitor.

To identify the receptor and downstream signaling transduction pathway involved in the thrombin-induced $[Ca^{2+}]_i$ rise in SVZ-derived oligodendrocytes, we designed other protocols of stimulation, represented in Fig. 2.1A-E. Among the four members of protease-activated receptors (PARs), PAR-1, PAR-3 and PAR-4 can be activated by thrombin (Coughlin, 1999; Nakanishi-Matsui et al., 2000; Jacques and Kuliopulos, 2003). To investigate whether PAR-1, which mediates most of the actions of thrombin, is involved in the response of SVZ-derived oligodendrocytes to thrombin, a PAR-1 agonist TFLLR-NH_2 (100 μM; Tocris Bioscience) and a selective non-peptide PAR-1 antagonist, SCH79797 dihydrochloride (SCH79797; 30 μM; Tocris Bioscience) (Ahn et al., 2000), were used (Fig. 2.1A,B). To activate PAR-1, bath application of TFLLR-NH_2 was used in the place of thrombin (Fig. 2.1A). Instead, to block the receptor, SVZ cells were pre-treated with SCH79797 during 60 min before the protocol of stimulation with thrombin and SCH79797 co-application, as depicted in Fig 2.1B. Moreover, to disclose whether the elevation of $[Ca^{2+}]_i$ induced by PAR-1 results from Ca^{2+} release from the intracellular stores or from the extracellular Ca^{2+} influx, we replaced the regular Krebs solution by a Krebs solution containing low Ca^{2+} concentration ["0 Ca^{2+} Krebs": regular Krebs with 38 μM instead of 2.5 mM $CaCl_2$, plus 50 μM ethyleneglycoltetraacetic acid (EGTA)] (Malva et al., 2003) 2 min before stimulation with TFLLR-NH_2, prepared in "0 Ca^{2+} Krebs" as well (Fig. 2.1C). To further explore the signaling mechanism triggered by PAR-1 activation, particularly the coupling to heterotrimeric G proteins, a set of SVZ cells was pre-incubated with 200 ng/ml pertussis toxin (PTX; Sigma-Aldrich), which inhibits $G_{i/o}$ proteins, or with 10 μM Y-27632 (Calbiochem, Merck Biosciences), which inhibits the $G_{12/13}$ effectors Rho/ROCK, 24 h before SCCI experiments encompassing TFLLR-NH_2 stimulation (Fig. 2.1D). Finally, we investigated whether the rise in $[Ca^{2+}]_i$ observed after PAR-1 agonist application, results from PLC activation. For this, cells were first stimulated with TFLLR-NH_2, KCl, and histamine, and then

incubated for 10 min with 5 μM U73122 (Sigma-Aldrich), a PLC inhibitor, before a last pulse of TFLLR-NH_2 plus U73122 (Fig. 2.1E). By using this approach, we were able to assess the effect of thrombin in the $[Ca^{2+}]_i$ in thrombin-responsive cells when PLC activity is abolished, giving however, enough time for the cells to recover their intracellular calcium levels before the last pulse of TFLLR-NH_2.

2.2.3 Immunocytochemistry on cell cultures

After the SCCI experiments or directly after the differentiation period, cells were fixed in 4% paraformaldehyde (PFA; Sigma-Aldrich) for 30 min at room temperature (RT), washed in phosphate-buffered saline (PBS; composed of 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4), and incubated in 0.25% Triton X-100 plus 3% BSA, for 30 min, in order to permeabilize membranes and block non-specific binding sites. Subsequently, cells were incubated with the primary antibodies prepared in 0.1% Triton X-100 plus 0.3% BSA, overnight (ON) at 4ºC. The following antibodies were used: rabbit polyclonal anti-NG2 (1:500; Millipore), mouse monoclonal anti-PLP (1:600; Serotec), mouse monoclonal anti-microtubule-associated protein-2 (MAP-2; 1:200; Sigma-Aldrich), rabbit monoclonal anti-glial fibrillary acidic protein (GFAP; 1:100; Sigma-Aldrich), mouse monoclonal anti-nestin (1:200; Millipore), rabbit polyclonal anti-Dcx (1:200; Cell Signaling Technology) and goat polyclonal anti-PAR-1 (1:200; Santa Cruz Biotechnology). Importantly, immunobinding with mouse monoclonal anti-O4 (1:100; Millipore) was performed in living cells, by diluting the antibody in the culture medium and incubating at 37ºC for 30 min, before fixation. Afterwards, cells were washed in PBS and incubated for 1 h at RT, with the appropriate Alexa Fluor-conjugated secondary antibodies, among the following: donkey anti-rabbit Alexa Fluor 488 (1:200), rabbit anti-mouse Alexa Fluor 488 (1:200), rabbit anti-goat Alexa Fluor 594 (1:200), and goat anti-mouse Alexa Fluor 594 (1:200; all from Invitrogen).

Cell nuclei were counterstained with Hoechst 33342 (2 µg/ml in PBS, for 5 min at RT; Invitrogen), and coverslips were mounted on glass slides, with Dako fluorescent mounting medium (Dako). Fluorescent images were acquired with an Axioskop widefield microscope or a LSM 510 Meta confocal microscope (both from Carl Zeiss).

2.3 *Ex vivo* studies

2.3.1 Organotypic hippocampal slice cultures

Organotypic hippocampal slice cultures were prepared from 6- to 8-day-old WT mice in agreement with the interface culture method (Stoppini et al., 1991), as modified by Kristensen et al. (1999) and Noraberg et al. (1999). Following decapitation, the brains were removed, and the two hippocampi were carefully isolated and sectioned into 350 µm-thick-transverse slices using a McIlwain tissue chopper. The slices were placed in ice-cold Gey's balanced salt solution (GBSS; Biological Industries) with 25 mM D-glucose (Merck) plus 100 U/ml penicillin and 100 µg/ml streptomycin, to remove the excess of entorhinal cortex and/or fimbria. Then, slices were transferred to porous (0.4 µm) membrane inserts (Millipore), and these were placed in humidified multiwell culture plates (Corning Incorporated). The wells contained culture medium composed of 50% Opti-Minimal Essential Medium (Opti-MEM; Invitrogen), 25% heat-inactivated horse serum (HS; Invitrogen), 25% HBSS, and supplemented with 25 mM D-glucose, 50 U/ml penicillin and 50 µg/ml streptomycin. The cultures were kept in a 95% air and 5% CO_2 humidified atmosphere, at 33ºC, and the medium was changed twice a week during 2 weeks. At this stage, culture medium was replaced by serum-free Neurobasal medium, supplemented with 1 mM L-glutamine, 2% B-27, 50 U/ml penicillin

and 50 µg/ml streptomycin, and incubator temperature was raised to 36ºC. Treatment with CPZ and grafting experiments were then performed.

2.3.2 Treatment with cuprizone and processing of organotypic hippocampal slices

Organotypic hippocampal slices were incubated with 25 µM of the toxic agent bis-cyclohexanone-oxaldihydrazone, also known as cuprizone (CPZ; Sigma-Aldrich), to induce demyelinating injury. We incubated the hippocampal slices with 25 µM CPZ during 24 h to reach oligodendrocytes death and demyelination. At the end of the toxic treatment, the medium was replaced by fresh serum-free Neurobasal medium, supplemented with 1 mM L-glutamine, 2% B-27, 50 U/ml penicillin and 50 µg/ml streptomycin. Medium change was repeated to ensure total removal of the toxin. Then, grafting experiments were initiated. A subset of slices was instead fixed by immersion in 4% PFA for 30 min at RT, cryoprotected in 30% sucrose, and cut in 20 µm-thick-transverse slices using a cryostat. These thinner slices were placed in adhesive microscope slides (SuperFrost Plus; Thermo Scientific) and then processed for PLP immunohistochemistry to evaluate the extent of demyelination.

2.3.3 Grafting of SVZ-eGFP neurospheres on hippocampal slices

Under sterile conditions SVZ-eGFP free-floating neurospheres were collected individually in minimal volume of medium, using a P10 pipette, and then carefully deposited on the top of the organotypic hippocampal slice (one neurosphere per slice). The neurospheres were placed in a central position within the slices, nearby the DG. Co-cultures were then incubated with 30 nM T3 in the culture medium for 1 week, in a 95% air and 5% CO_2 humidified atmosphere, at 36ºC. Medium was changed twice during the week. Both intact and CPZ-injured slices were grafted with SVZ neurospheres and all co-cultures were treated with T3. This allowed evaluating whether T3-induced

differentiation of SVZ progenitors in oligodendrocytes, is kept or altered when the cells are exposed to a demyelinated tissue. At the end of the 7-day differentiation period, co-cultures were fixed by immersion in 4% PFA for 30 min at RT, and then the whole slice with the implanted neurosphere was processed for immunohistochemistry. The overall protocol regarding the present experiment is depicted on Fig. 2.2.

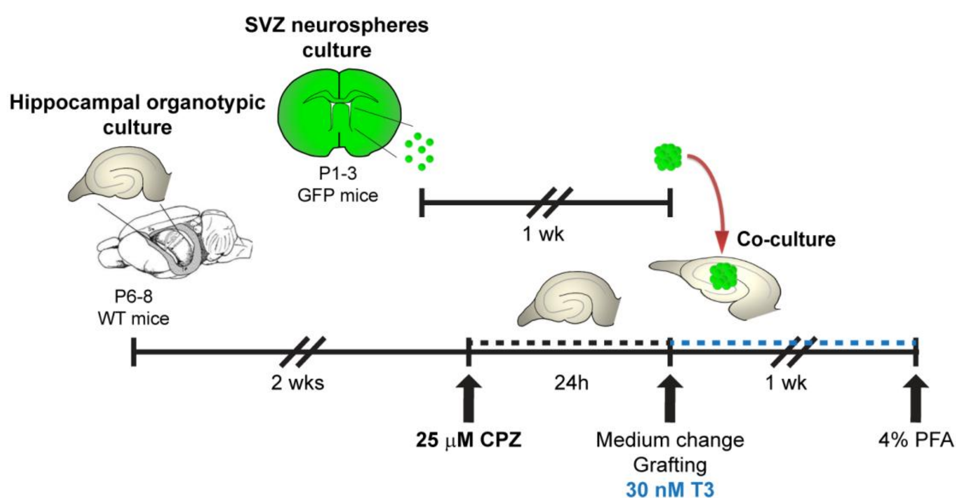


Figure 2.2. Schematic representation of the co-cultures protocol, aiming at exploring whether the *ex vivo* grafting of T3-treated SVZ cells in a demyelinated tissue alters the SVZ cells fate. Hippocampal organotypic slices were prepared from WT mice brains and injured by 25 μM CPZ incubation during 24 h (black dashed line). The toxin was then removed and eGFP-SVZ neurospheres were individually grafted in the intact or demyelinated hippocampal slices (one sphere per slice). The co-culture was allowed to develop during 1 week under 30 nM T3 treatment (blue dashed line).

2.3.4 Immunohistochemisty on organotypic hippocampal slices

20 μm-thick hippocampal slices prepared after the 24 h of CPZ treatment were processed for PLP immunohistochemistry, in order to verify the damage induced by the toxin on the population of oligodendrocytes. Slices were washed in PBS and quenched in 3% hydrogen peroxide (H_2O_2; Sigma-Aldrich) for 10 min. After washing in 0.2% Triton-X100, slices were treated with 10% fetal bovine serum (FBS; Invitrogen) for 30 min, and then incubated with mouse

monoclonal anti-PLP antibody (1:600) prepared in 1% FBS, ON at 4ºC. Sections were then rinsed in 0.2% Triton-X100 and incubated for 1 h in biotinylated secondary anti-mouse antibody (1:200; Vector Laboratories). Following another washing step using 0.2% Triton-X100, to remove the excess of antibody, treatment with avidin-biotin-peroxidase complex (1:100) for 30 min, at RT, was performed. Thereafter, slices were washed in PBS and then in Tris-HCl 0.05 M (pH 7.6). Peroxidase activity was revealed by 0.025% 3,3´-diaminodbenzidine chromogen (DAB; Sigma-Aldrich) in Tris-HCl 0.05 M (pH 7.6) solution containing 0.005% H_2O_2. Reaction was stopped by washings with the buffered solution, and then dehydratation with ethanol (75%, 80%, 95%, 96%; 3 min each, successively) and delipidation with xylene (3 min) were performed before mounting in DEPEX medium (Sigma-Aldrich). Images were acquired using an Axioskop widefield microscope.

Grafted hippocampal slices were processed for immunohistochemical procedure. With the help of a scalpel, the porous membrane of the inserts used for the organotypic slice cultures, was cut around each slice and the immunostaining protocol was carried in these free-floating hippocampal slices. After PBS washings, slices were incubated for 3 h at RT in 10% normal goat serum (NGS; Vector Laboratories), then for 48 h at 4ºC with rabbit polyclonal anti-NG2 (1:100) or mouse monoclonal anti-O4 (1:50), diluted in 1% NGS. Subsequently, slices were washed and incubated for 2 h at RT with goat anti-rabbit Alexa Fluor 594 or goat anti-mouse Alexa Fluor 594 secondary antibodies, respectively (1:200). Nuclei were stained with Hoechst 33342 as described previously. Grafted slices were mounted on glass slides, with Dako, by leaning the membrane on the slide and the neurosphere facing the coverslip. Fluorescent images were acquired with a LSM 510 Meta confocal microscope.

2.4 *In vivo* studies

2.4.1 Stereotaxic injections

To label SVZ-derived cells, GFP-encoding lentiviruses or retroviruses were injected, 3 to 7 days before MCAo, into the dorsal and ventral sub-regions of the SVZ at the following coordinates (in mm): anterior-posterior 0.7; medial-lateral 1.2; dorsal-ventral 1.9 for dorsal SVZ; and anterior-posterior 0.9; medial-lateral 1.0; dorsal-ventral 2.7 for ventral SVZ, respectively. GFP-encoding viruses (150 nl, $1x10^6$-$1x10^7$ TU/ml) were injected into each site in both hemispheres. Immunohistochemistry for Dcx revealed that neuronal progenitor recruitment to the ischemic striatum takes place predominantly along the dorso-ventral axis of the SVZ. By combining two sites of injection in the SVZ, we were able to increase the number of infected cells and, as such, GFP-labeled de-routed neuroblasts. For real-time imaging in acute slices and to evaluate neuroblast/vasculature distances, the animals were sacrificed 3 to 4 weeks after the injections and 2 to 3 weeks post-MCAo.

2.4.2 *In vivo* animal models of disease

2.4.2.1 Cuprizone model of demyelination

To induce demyelination, 2-month-old WT mice were fed with a diet containing 0.2% (w/w) CPZ for 6 weeks. Age-matched control animals were kept on same chow diet but without CPZ. The exposure to this toxin is known to induce demyelination, not only in the corpus callosum (Matsushima and Morell, 2001), but also in the cortex (Skripuletz et al., 2008) and in the hippocampus (Hoffmann et al., 2008; Koutsoudaki et al., 2009; Norkute et al., 2009).

2.4.2.2 Middle cerebral artery occlusion (MCAo) model of brain ischemia

Unilateral transient focal cerebral ischemia was induced as previously described (Lalancette-Hebert et al., 2007; Engel et al., 2011) by intraluminal filament occlusion of the left middle cerebral artery (MCA) for 1 h followed by reperfusion. Briefly, the animals were anesthetized with 2% isofluorane and kept at 37ºC using a heating pad during the surgical procedure and reperfusion period. A midline neck incision was performed, and the internal carotid artery (ICA) was exposed by careful separation from the surrounding tissues under an operating microscope. A 12-mm-long, 6-0 silicon-coated monofilament suture was then introduced via the proximal left external carotid artery (ECA) into the ICA and then into the Circle of Willis to occlude the origin of the MCA. The wound was closed with a suture clip, and symptoms of stroke were monitored. After 1 h, the mouse was re-anesthetized to remove the filament and suture the skin.

2.4.3 Tissue processing and vasculature labeling with dextran-Texas Red

At the end of the 6 weeks of CPZ treatment, mice were deeply anesthetized and perfused transcardially with saline solution (0.9% NaCl), followed by cold 4% PFA. The brains were removed and immersed in 4% PFA ON at 4ºC. Afterwards, brains were washed in PBS and cryoprotected by ON equilibration in 30% sucrose solution, to allow posterior cryopreservation of the tissue. Thereafter, brains were sectioned coronally on a cryostat, in 20-µm-thick slices that were placed in adhesive glass slides for subsequent immunostainings.

As regard to MCA-occluded mice, all the histological analyses were performed 1 and 2 weeks post-MCAo. The mice were deeply anesthetized and perfused as described for CPZ-treated mice. Similarly, brains were removed and kept in 4% PFA ON, at 4ºC. Then, brains were then sectioned into 40-µm-thick

sagittal slices using a vibratome (Leica). The slices were processed as free-floating sections in PBS (sections 2.4.4 and 2.4.5). To evaluate the association of neuroblasts with blood vessels, we either co-immunolabeled the fixed tissues for platelet endothelial cell adhesion molecule (PECAM) and Dcx or assessed retroviral-labeled GFP^+ cells along dextran-Texas Red-filled blood vessels. For the latter case, 200 µl of high molecular weight dextran-Texas Red (70 000 kDa; Molecular Probes) was injected into the heart of deeply anesthetized mice 2 min prior to removing and sectioning the brains. All functional vessels in the brain can be visualized by Texas Red fluorescence using this approach. Dcx^+ or GFP^+ retroviral-labeled neuroblasts that had either the soma or leading process within 3 µm of PECAM or dextran-Texas Red-filled blood vessels were considered as vasculature-associated cells.

2.4.4 *In situ* hybridization

In situ hybridization was performed to detect BDNF and TrkB mRNAs in the brain sections from animals subjected to MCAo. Antisense RNA probes were generated from plasmids containing mouse BDNF (kindly provided by Dr. E. Castren, University of Helsinki, Finland) or the extracellular domain of mouse TrkB (kindly provided by Dr. Lino Tessarollo, National Institutes of Health, Bethesda, MD, USA). The riboprobes were labeled with digoxigenin (DIG) using DIG RNA labeling kits (Roche Diagnostics) and were purified using ProbeQuant G-50 columns (GE Healthcare). Fixed sagittal brain slices (40-µm-thick) were treated with RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and 1 mM EDTA in 50 mM Tris-HCl, pH 8.0). The slices were fixed in 4% PFA, washed in PBS, incubated in 0.1 M triethanolamine (TEA, pH 8.0), acetylated with 0.25% acetic anhydride in 0.1 M TEA, and washed again in PBS. The 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 60ºC.

The riboprobe was added, and the slices were incubated overnight at 60ºC. Afterwards, slices were 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 then buffer B2 (10% fetal bovine serum (FBS) in buffer B1). This was followed by an overnight incubation with anti-DIG antibody (1:2000 dilution in buffer B2) at 4ºC. After washing with buffer B1 and then buffer B3 (50 mM $MgCl_2$, 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 (NBT-BCIP; Promega). The slices were then washed in buffer B3 and mounted, or were fixed in 4% PFA for 30 min prior to the immunohistochemistry analysis.

2.4.5 Immunohistochemistry

To evaluate the degree of demyelination in the hippocampi of CPZ-treated mice, PLP immunostaining was performed on the cryostat-sectioned coronal slices, by using peroxidase-DAB protocol as detailed in section 2.3.4.

As for the brain sections from animals subjected to MCAo, we performed immunohistochemistry using fluorescence. For this, the free floating 40-µm-thick sagittal slices were washed in PBS, incubated in 0.2% Triton X-100 in PBS for 2 h, and then incubated overnight at 4ºC (unless specified otherwise) with the primary antibody diluted in 4% bovine serum albumin (BSA) and 0.2% Triton X-100. The following antibodies were used: goat anti-Dcx (1:1000; Santa Cruz Biotechnology); rat anti-mouse PECAM-1 (1:100, three overnight incubations; BD Pharmingen); rabbit (1:1000; Dako) and mouse (1:1000; Millipore) anti-GFAP; mouse anti-NeuN (1:200; Millipore); rabbit anti-Iba1 (1:400; Wako); rabbit anti-Olig2 (1:1000; Millipore); rabbit anti-BDNF (1:500, two overnight incubations; Santa Cruz Biotechnology, N-20); rabbit anti-TrkB (1:1000, three overnight incubations; Millipore); rabbit anti-p75NTR (1:500, three overnight incubations; Covance), and mouse anti-PSA-

NCAM (1:1000; Millipore). The slices were then washed in PBS and were incubated with Alexa Fluor-conjugated secondary antibodies for 3 h (1:1000 in PBS). Cell nuclei were counterstained with DAPI, and the sections were mounted in Dako fluorescent medium (Dako). An Olympus FV1000 confocal microscope was used to acquire and process images.

2.4.6 Time-lapse imaging

Two to three weeks post-MCAo, the mice were anesthetized, and the brains were quickly dissected in ice-cold sucrose-based artificial cerebral spinal fluid containing in mM: 210.3 sucrose, 3 KCl, 1.3 $MgCl_2$, 2 $CaCl_2$, 26 $NaHCO_3$, 1.25 NaH_2PO_4, and 20 glucose (pH 7.4), bubbled with 95% O_2-5% CO_2. 250 µm-thick sagittal sections were prepared using a vibratome (Leica) and were maintained at 32°C in artificial cerebral spinal fluid (ACSF) containing in mM: 125 NaCl, 26 $NaHCO_3$, 3 KCl, 2 $CaCl_2$, 1.3 $MgCl_2$, 1.25 NaH_2PO_4, and 20 glucose (pH 7.4) bubbled with 95% O_2-5% CO_2. Individual slices were then placed in an imaging chamber mounted on a wide-field Olympus BX61WI upright microscope under continuous perfusion with ACSF (1 ml/min). The microscope was equipped with a CCD camera (CoolSnap HQ2) and a Lambda DG4 xenon light source (Sutter Instruments). The imaging chamber was connected to an automatic heating system (TC-344B, Harvard Apparatus) to maintain the ACSF at 31-33°C.

Time-lapse video images were captured using multiple *z*-stack acquisitions (6–16 *z*-sections, with 3-7 µm intervals) every 30 s for 1 h to track cell migration in the RMS or 2 h to track cell migration in the ischemic striatum. Fewer labeled cells were observed in the injured striatum, and they displayed fewer migratory events per hour. We thus tracked cell migration in the ischemic striatum using a longer acquisition time. The videos were processed and analyzed using Imaris software (Bitplane), which automatically tracks cells in 3D. A 0.03 µm/30 s threshold, which corresponded to the displacement between

two subsequent acquisitions, was set to discriminate between the stationary and migratory phases. A careful visual inspection of cell migration revealed that migratory periods were consistently associated with values above 0.03 while stationary periods were associated with values below 0.03. Often the cell bodies of stationary cells wiggled slightly, when the cell screened the microenvironment with the leading process. The track lengths of migrating neuroblasts were automatically measured using Imaris software and were averaged for each video recording. These values were then averaged across all recordings and were compared. Cells that remained stationary for the duration of the recordings were not considered for analysis. To study the role of BDNF in injury-induced migration, we bath-applied BDNF (10 ng/ml; Peprotech) and TrkB-Fc (1 µg/ml; R&D Systems) and thereby modulate BDNF signaling. We used IgG-Fc (1 µg/ml; R&D Systems) as a control. For these experiments, time-lapse images were first acquired under control conditions for 1 h (ACSF) and then in the presence of the drug for another 1 h. Results are expressed as the percentage of the control value for each group, obtained during the first hour of recording (ACSF). Cells tracked for less than 30 min before or after application of the pharmacological agent were not taken into consideration.

2.5 Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). Statistical significance was tested by using one-way analysis of variance (ANOVA) followed by Bonferroni post-test for multiple comparisons in the *in vitro* studies, or Student's unpaired t test in the *ex vivo* and *in vivo* studies (with $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$).

In SCCI experiments, fluorescence measurements were performed in cells located at the outside border of the seeded neurospheres. Indeed, upon

deposition of the neurospheres on PDL coverslips, cells migrated from the periphery of the spheres forming a pseudomonolayer where individual cells were distinguishable and easily delimited using MetaFluor software, thus allowing single-cell analysis of Fura-2 fluorescence. For the experiments matching the calcium profiles with the phenotypic immunodetection, at least, 8 cells per phenotype were analyzed. Each experimental condition was assessed at least in three different wells (about 100 cells per coverslip). The experiments were replicated at least in three different cultures except where otherwise specified.

As regard to the cell countings on SVZ-eGFP grafting experiments on hippocampal slices, at least 3 fields with easily distinguishable eGFP$^+$ individual cells (at least 10 eGFP$^+$ cells per field) located around the implanted sphere were considered.

CHAPTER 3

Oligodendrogenesis in neural stem cell cultures

- Functional evaluation based on Ca^{2+} transients evoked by thrombin
- Patterns of differentiation upon grafting in demyelinated tissue

3.1. SUMMARY

Current immunosuppressive treatments for CNS demyelinating diseases fail to prevent long-term motor and cognitive decline in patients. Excitingly, glial cell transplantation arises as a promising complementary strategy to challenge oligodendrocytes loss occurring in demyelinating disorders. A potential source of new oligodendrocytes is the SVZ pool of multipotent NSCs. However, this approach has been handicapped by the lack of functional methods for the identification and pharmacological analysis of differentiating oligodendrocytes in the mixed SVZ cultures. Indeed, a method as such could be used to screen factors that prime SVZ cells to oligodendroglial differentiation, useful to commit cells into the desired phenotype before transplantation.

In this study, we questioned whether SVZ-derived oligodendrocytes could be functionally discriminated on the basis of $[Ca^{2+}]_i$ variations following KCl, histamine, and thrombin stimulations. Previously, our group has shown that SVZ-derived neurons and immature cells can be discriminated due to their selective $[Ca^{2+}]_i$ rise upon KCl and histamine stimulations, respectively. Herein, we demonstrate that $O4^+$ and PLP^+ oligodendrocytes do not respond to these stimuli, but display a robust $[Ca^{2+}]_i$ rise following thrombin stimulation, whereas other cell types in the SVZ heterogeneous culture are thrombin-insensitive. We show that thrombin-induced $[Ca^{2+}]_i$ increase in oligodendrocytes is mediated by PAR-1 activation and downstream signaling through $G_{q/11}$ and PLC, resulting in Ca^{2+} recruitment from the intracellular compartments. Moreover, grafting of SVZ neurospheres into demyelinated tissue revealed that the demyelinated host environment *per se* favors oligodendroglia cell lineage among transplanted SVZ

differentiating cells. The calcium-based method described herein allows the functional analysis of cell differentiation in living SVZ cultures, which opens important perspectives on the search of new pro-oligodendrogenic factors to be used prior and/or during grafting, in order to overcome such limitation. As such, it may be a tool of major interest in the development of effective NSCs-based transplantation strategies in the demyelinated brain.

3.2. INTRODUCTION

CNS demyelinating diseases are usually slow-progressing debilitating diseases, resulting in moderate to severe permanent neurological deficits. Triggered either by a viral infection, an autoimmune process, or a genetic abnormality, these pathologies affect oligodendrocytes, the myelin-forming cells of the CNS. Due to the lack of axonal insulation by the myelin sheath, neuronal communication becomes deficient, therefore leading to brain function impairments. In early phases of the disorder, endogenous OPCs spontaneously remyelinate denuded axons in the damaged areas. Nevertheless, as disease progresses, the efficiency of remyelination decreases. Besides, the conventional immunomodulatory therapies are insufficient to prevent the devastating progression of disease (reviewed in Franklin and Ffrench-Constant, 2008).

Recent advances in NSCs biology have raised considerable prospects for the development of remyelinating therapies based on glial cell replenishment. The multipotency of NSCs has been well characterized, unveiling a renewable source of new oligodendrocytes for use in regenerative medicine concerning myelin pathologies. Indeed, SVZ stem cells produce oligodendrocyte progenitors that differentiate into mature and functional myelinating oligodendrocytes (Levison and Goldman, 1993; Menn et al., 2006) able to remyelinate axons in animal models of demyelinating injuries (Keirstead

et al., 1999; Smith and Blakemore, 2000; Akiyama et al., 2001; Pluchino et al., 2003; Cayre et al., 2006). Nonetheless, remaining hurdles still limit the grafting success, including the fact that in normal conditions these cells represent a minority of the SVZ progeny. Therefore, *in vitro* NSCs pre-induction of the oligodendroglial phenotype, either pharmacologically or genetically, has been pinpointed as a key step to substantially favor the outcome from NSCs transplantation on focal demyelination injuries. Moreover, such treatments may be applied endogenously to drive oligodendroglial differentiation, during recruitment of SVZ progenitors to sites of demyelination, ameliorating this spontaneous but inefficient injury-induced self-repair attempt (Nait-Oumesmar et al., 1999; Picard-Riera et al., 2002; Nait-Oumesmar et al., 2007). Thus, much attention has been focused on NSCs differentiation patterns and regulators, specially using *in vitro* NSCs culture model, which is easy to manipulate.

In the present study, we aimed at developing a functional method to analyze oligodendroglial differentiation in mouse SVZ cell cultures, based on single cell calcium imaging (SCCI). Our group has previously developed a calcium imaging protocol that rapidly discriminates neurons and immature cells in SVZ cultures (Agasse et al., 2008a). Indeed, KCl-induced depolarization and a consequent rise in $[Ca^{2+}]_i$ is typical of excitable neuronal cell lineage, whereas histamine triggers a response in immature cells. This method unveiled the pro-neurogenic effects of NPY and TNF-α in mouse SVZ cultures (Agasse et al., 2008b; Bernardino et al., 2008). Interestingly, Wang et al. (2004) have shown that the rat OLN-93 oligodendrocyte cell line displays an increase of $[Ca^{2+}]_i$ following thrombin stimulation, an effect mediated by PAR-1 activation. In line with these findings, we hypothesized that thrombin may be a specific stimulus for SVZ-derived oligodendrocytes in the mixed SVZ culture, thus useful to design a rationale protocol to easily and reliably screen pro-oligodendrogenic factors. In addition, we sought at determining whether differentiation of SVZ cells in oligodendrocytic lineage is altered when cells are in a host environment,

either intact or demyelinated. For this, we produced an *ex vivo* model of demyelinating injury by incubating organotypic hippocampal slice cultures with cuprizone. Indeed, the toxin is known to harbor a detrimental effect on the oligodendrocytic population both *in vivo* (Matsushima and Morell, 2001) and *in vitro* (Cammer, 1999).

Our results show that oligodendrocytes in SVZ cultures can be distinguished from the other cell types, according to the uniqueness of their response to thrombin. The SCCI protocol developed on the basis of the cell type-specific responses to KCl, histamine and thrombin, can thus be important to screen new oligodendrogenic compounds that may improve the efficiency of grafting strategies in demyelinated lesions. Furthermore, we demonstrate that demyelinated host tissue *per se* prompts the cells to undergo oligodendroglial differentiation, although differentiation appears to be generally impaired as compared to the patterns found in SVZ cultures alone. Thus, it is important to retain that transposition (Joers and Emborg, 2009) from the *in vitro* to the *in vivo* paradigm needs to be carefully balanced to assure that pre-commitment of the cells *in vitro* is adjusted to achieve the proper effect *in vivo.*

3.3. RESULTS

3.3.1 Thrombin triggers an increase in $[Ca^{2+}]_i$ in SVZ-derived oligodendrocytes

We have previously demonstrated that neurons and immature cells in SVZ cultures can be rapidly distinguished on the basis of their unique responses to KCl or histamine, respectively (Agasse et al., 2008a). Accordingly, the membrane depolarization evoked by KCl on neurons results in Ca^{2+} entry via voltage sensitive Ca^{2+} channels, whereas histamine activates H1 receptors expressed in immature SVZ cells, inducing the mobilization of Ca^{2+} from the

intracellular stores. Moreover, this study has shown that neither KCl nor histamine trigger a significant $[Ca^{2+}]_i$ rise in astrocytes. Herein, we characterize the intracellular Ca^{2+} profile of oligodendrocytes upon perfusion with KCl and histamine, and we used thrombin as a putative $[Ca^{2+}]_i$ rise inducer in SVZ-derived oligodendrocytes (Wang et al., 2004). For this purpose, primary mouse SVZ neurospheres were seeded on PDL coverslips for 7-10 days. During this differentiation period, cells emerge from the spheres and differentiate in neurons, astrocytes, and oligodendrocytes, albeit the latest to a minor extent, forming a heterogeneous pseudo-monolayer of cells in various stages of maturation. To drive oligodendrocytic differentiation, spheres were treated with 30 nM T3. Indeed, T3 induces the commitment of SVZ progenitors toward the oligodendrocytic lineage; besides, it also increases the differentiation of immature oligodendrocytes into functional remyelinating oligodendrocytes in animal models of demyelinating diseases (Franco et al., 2008; Fernandez et al., 2009). *In vitro*, the T3 hormone is routinely used as a supplement for culture media to increase the number and the differentiation of oligodendrocytes from brain progenitor cells (Johe et al., 1996; Murray and Dubois-Dalcq, 1997). T3 treatment induced a robust increase in the number of $NG2^+$ as well as in $O4^+$ and PLP^+ cells in our cultures (Fig. 3.1A-C). Thus, we used T3-treated SVZ cultures to perform SCCI analysis when we intended to focus on the oligodendrocyte population subset.

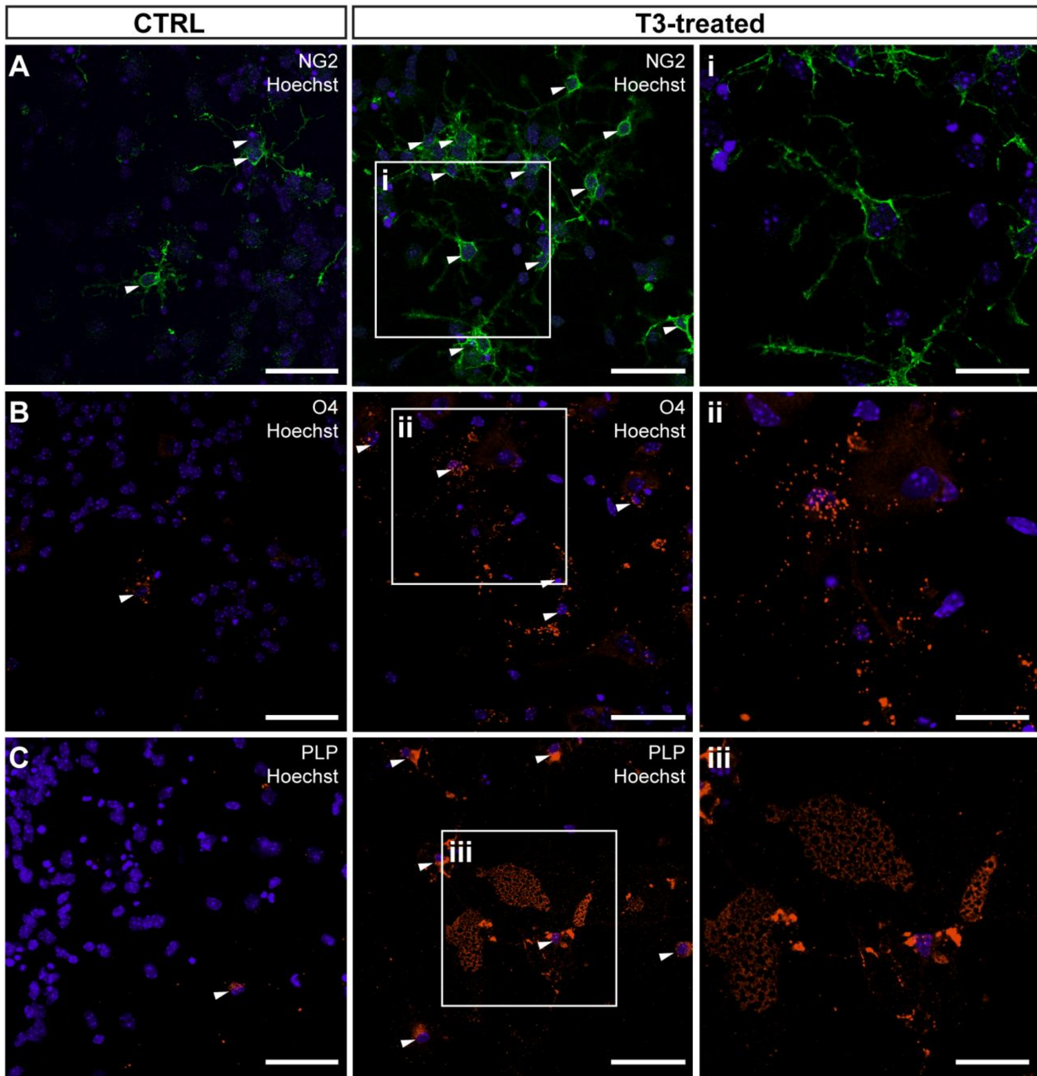


Figure 3.1. T3 increases the production of oligodendrocytes in SVZ cultures. Representative fluorescence images of NG2$^+$ cells (**A**), O4$^+$ (**B**) and PLP$^+$ oligodendrocytes (**C**), counterstained with Hoechst 33342 (blue nuclei) in SVZ neurosphere cultures, treated or not with 30 nM T3, for 7-10 days after spheres deposition and growth factors withdrawal. Arrowheads point to NG2$^+$, O4$^+$ and PLP$^+$ cells in control (non-treated) and T3-treated cultures showing the consistent appearance of more labeled-cells in the treated cultures. Images i-iii consist on detailed representations of the fields indicated with insets in the middle panel. Scale bars: A-C, 50 µm; i-iii, 25 µm.

To correlate profiles of $[Ca^{2+}]_i$ oscillations with the respective cell phenotypes, we performed SCCI analysis in SVZ neurospheres seeded on microgrid coverslips and subsequent immunostaining for the different cell type markers. Cells were pre-loaded with Fura-2AM Ca^{2+} probe and then continuously perfused with Krebs solution. They were stimulated subsequently with 2-min pulses of 50 mM KCl, 100 μM histamine, and 0.1 U/mL thrombin (Fig. 2.1A). Single cell $[Ca^{2+}]_i$ oscillations were given by the normalized ratios of Fura-2AM fluorescence at 340/380. We demonstrated that $O4^+$ oligodendrocytes do not respond to KCl or histamine, whereas thrombin clearly induces an increase in $[Ca^{2+}]_i$ in these cells (Fig. 3.2A). O4 sulfatide expression begins at the stage of pre-oligodendrocytes and remains throughout maturation into mature myelinating oligodendrocytes, when myelin proteins are expressed (reviewed in Baumann and Pham-Dinh, 2001). To further detail the response of oligodendrocytes to thrombin in different stages of maturation, profiles of $[Ca^{2+}]_i$ variations were investigated in $NG2^+$ and PLP^+ cells. NG2 is a chondroitin sulfate proteoglycan expressed in oligodendrocyte progenitors whereas PLP is the major constituent of CNS myelin, being expressed by mature oligodendrocytes. PLP^+ oligodendrocytes respond similarly to the sequence of stimuli as do the $O4^+$ cells, but with higher calcium currents upon thrombin (Fig. 3.2B). The calcium profile of NG2-expressing oligodendrocytes was variable, including cells that responded to histamine and thrombin, others that responded to thrombin only, as well as a subpopulation that was solely responsive to histamine (data not shown). This variability in $NG2^+$ cells response may be correlated with the heterogeneity of progenitors that express NG2. Accordingly, NG2 proteoglycan expression is found in pre-oligodendrocytes and earlier pre-oligodendrocytes progenitors. Besides, expression of NG2 is not restricted to oligodendrocyte progenitors, but also displayed by C-type multipotent cells from the early postnatal SVZ, which is able to give rise to interneurons (Aguirre et al., 2004). Actually, most of the $NG2^+$ cells responded to histamine

confirming an immature stage. It is noteworthy that no NG2$^+$ cell responding to both KCl and thrombin was observed (data not shown).

Increase of $[Ca^{2+}]_i$ upon thrombin stimulation was not seen in MAP-2$^+$ neurons (Fig. 3.2C), or in DCX$^+$ neuroblasts (data not shown), nestin$^+$ immature cells (Fig. 3.2D), or GFAP$^+$ astrocytes (Fig. 3.2E). In agreement with the previous work, cells from the neuronal lineage presented a $[Ca^{2+}]_i$ rise upon KCl stimulation, and none (MAP-2$^+$ neurons) or weak (DCX$^+$ neuroblasts) response to histamine, whereas immature cells expressing nestin responded solely to the histamine pulse.

3.3.2 SVZ-derived oligodendrocytes can be identified on the basis of the selective response to thrombin

Normalized data were used to calculate the ratio of responses to thrombin and histamine (Throm/Hist) (Fig. 3.2E). The high Throm/Hist values for O4$^+$ and PLP$^+$ oligodendrocytes (O4: 1.43 ± 0.04, 14 cells analyzed; PLP: 1.49 ± 0.05, 10 cells analyzed) were due to their marked response to thrombin and no response to histamine, whereas the low values of Throm/Hist for nestin$^+$ cells (0.84 ± 0.01, 8 cells analyzed) reflect their null response to thrombin but high response to histamine. Other cell types had a Throm/Hist ratio of approximately 1 because they did not respond to any of these two stimuli (MAP-2$^+$ neurons: 1.03 ± 0.01, 11 cells; DCX$^+$ neuroblasts: 1.02 ± 0.01, 10 cells; GFAP$^+$ astrocytes: 1.00 ± 0.01, 8 cells). According to these findings, SVZ-derived oligodendrocytes can be functionally discriminated among other cells in SVZ cultures. We concluded that, in our cultures, cells with a Throm/Hist ratio above 1.3 are oligodendrocytes.

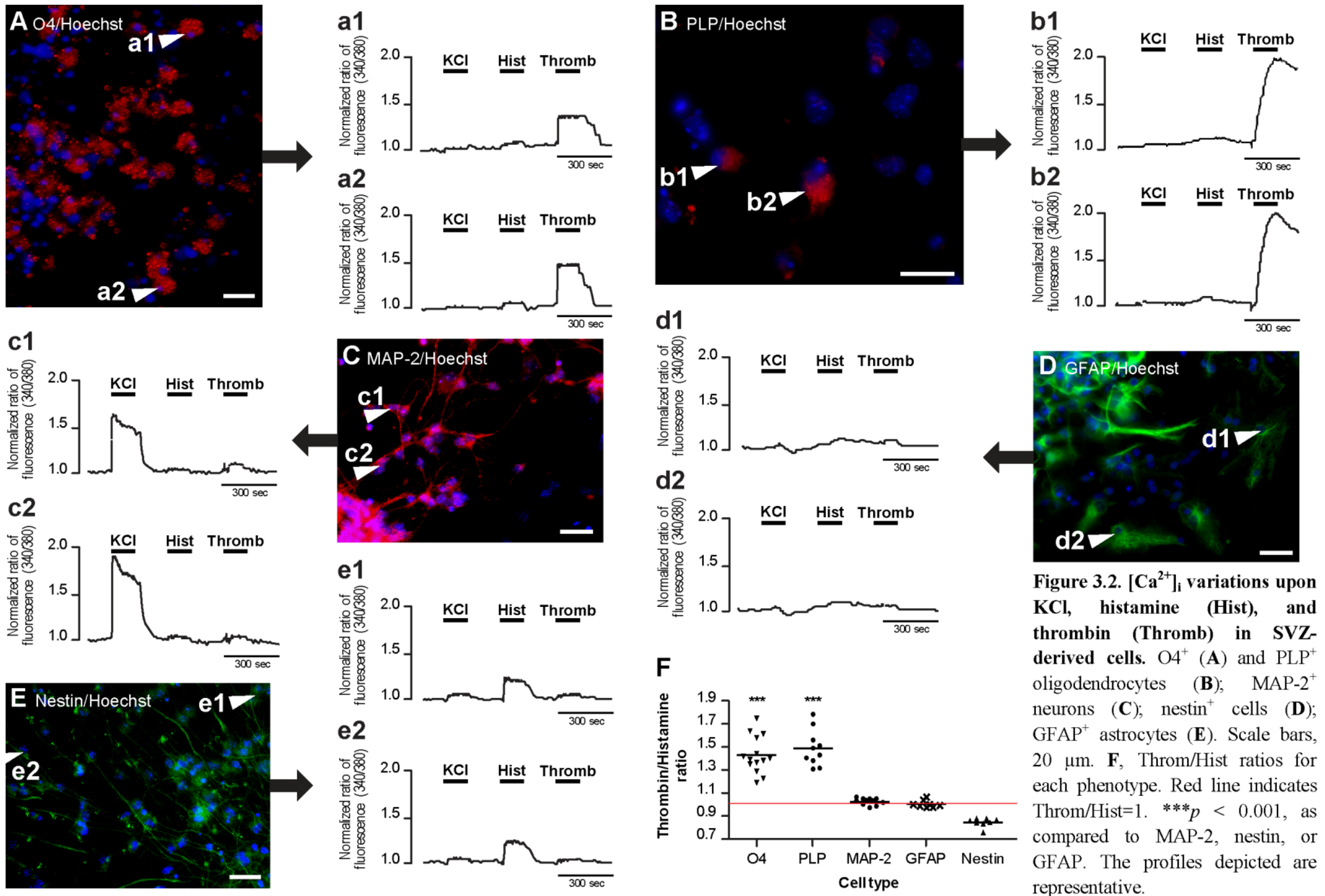


Figure 3.2. $[Ca^{2+}]_i$ variations upon KCl, histamine (Hist), and thrombin (Thromb) in SVZ-derived cells. O4$^+$ (**A**) and PLP$^+$ oligodendrocytes (**B**); MAP-2$^+$ neurons (**C**); nestin$^+$ cells (**D**); GFAP$^+$ astrocytes (**E**). Scale bars, 20 μm. **F**, Throm/Hist ratios for each phenotype. Red line indicates Throm/Hist=1. ***p < 0.001, as compared to MAP-2, nestin, or GFAP. The profiles depicted are representative.

3.3.3 Thrombin-induced $[Ca^{2+}]_i$ increase in SVZ-derived oligodendrocytes is mediated by PAR-1 activation

Many of the effects of thrombin are mediated by PAR-1, being the most extensively studied subtype and the prototype of the PARs family (Coughlin, 1999, 2000). PAR-4 is a low-affinity receptor requiring higher concentrations of thrombin for activation in comparison with the other thrombin receptors (Jacques and Kuliopulos, 2003). PAR-3 does not signal by itself having a possible co-factor role in the cleavage and activation of PAR-4 (Nakanishi-Matsui et al., 2000). On the basis of this knowledge, we focused on PAR-1 expression and function in SVZ cultures. Our hypothesis was also supported by the finding that PAR-1 is the only thrombin receptor functionally expressed in OLN-93, where it was associated with an increase of $[Ca^{2+}]_i$ evoked by thrombin (Wang et al., 2004). In addition, the same authors showed that primary cultured rat oligodendrocytes express high levels of PAR-1 but almost undetectable levels of PAR-3 and no PAR-4.

Therefore, we ran a perfusion protocol consisting on the subsequent application of KCl, histamine, and TFLLR-NH_2, a PAR-1 agonist (Fig. 2.1A), in T3-treated SVZ cultures. We focused on the glial cell population, i.e., responding with a Hist/KCl ratio between 0.9 and 1.1. Cells were highly responsive to TFLLR-NH_2 (Fig. 3.3A, top), showing a similar profile to the one obtained upon thrombin stimulation (see Fig. 3.5A, T3). Additionally we used SCH79797, a selective PAR-1 antagonist. After a 60-min pre-incubation of T3-treated SVZ cultures with SCH79797 (Fig. 2.1B), we verified that thrombin-induced rise of $[Ca^{2+}]_i$ was abolished (Fig. 3.3A, bottom). We measured the peak of response due to thrombin, or TFLLR-NH_2, or thrombin plus SCH79797. A similar magnitude of response to TFLLR-NH_2 (1.42 ± 0.01, 854 cells analyzed) as compared to thrombin (1.41 ± 0.01, 431 cells analyzed) was observed, as well as a complete inhibition of thrombin peak by SCH79797 (1.06 ± 0.01, 381 cells analyzed) suggesting that PAR-1 is the receptor involved in the

thrombin-induced $[Ca^{2+}]_i$ increase in SVZ-derived oligodendrocytes (Fig. 3.3B). In agreement, we observed a dense and clustered PAR-1 punctuated immunoreactivity in every $O4^+$ and PLP^+ cell (Fig. 3.3C). Among other cell types, MAP-2^+ neurons did not express PAR-1, nevertheless some $GFAP^+$ and $nestin^+$ cells expressed PAR-1, albeit not responding to thrombin stimulus.

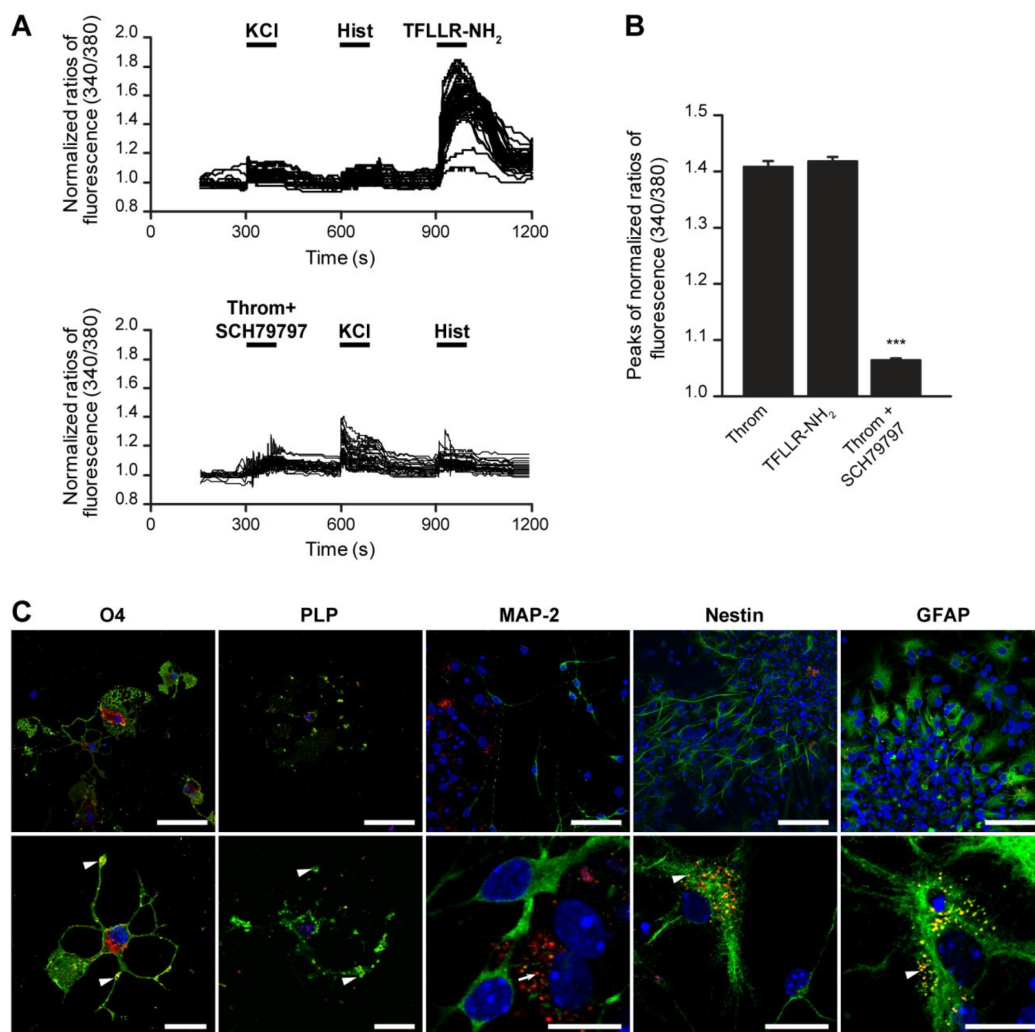


Figure 3.3. Thrombin-induced $[Ca^{2+}]_i$ increase in SVZ-derived oligodendrocytes is mediated by PAR-1 activation. A, Representative profiles upon perfusion protocol as depicted in Fig. 2.1A (TFLLR-NH_2 as third pulse) and 2.1B. **B**, Effect of the application of PAR-1 agonist or co-application of thrombin and PAR-1 antagonist in oligodendrocytes response, as compared to thrombin alone. ***$p < 0.001$. **C**, Expression of PAR-1 (red) in SVZ-derived cell types (green). PAR-1 is expressed in $O4^+$ and PLP^+ oligodendrocytes, and also in some $nestin^+$ and $GFAP^+$ cells. Note PAR-1 (*Figure legend continues.*)

(*Figure legend continued.*) immunoreactivity in red, indicated by arrowheads in $O4^+$, PLP^+, $nestin^+$, $GFAP^+$ cells; or arrows, denoting PAR-1 expression in MAP-2-negative cells). Scale bars: C (top images), 50 µm; C (bottom images), 20 µm. Hist, histamine; Throm, Thrombin; TFLLR-NH_2, PAR-1 agonist; SCH79797, PAR-1 antagonist.

←

Moreover, the signaling mechanism of PAR-1-induced $[Ca^{2+}]_i$ rise in SVZ cultures was investigated. As Fig. 2.1C shows, we replaced the normal Krebs by a "0 Ca^{2+} Krebs" solution 2 min before TFLLR-NH_2 stimulation and during this pulse. Under these conditions, the increase of $[Ca^{2+}]_i$ triggered by application of PAR-1 ligand was as high (TFLLR-NH_2 in "0 Ca^{2+}": 1.38 ± 0.01; 127 cells analyzed) as in normal Krebs, indicating that Ca^{2+} comes from intracellular stores and not from the extracellular solution (Fig. 3.4).

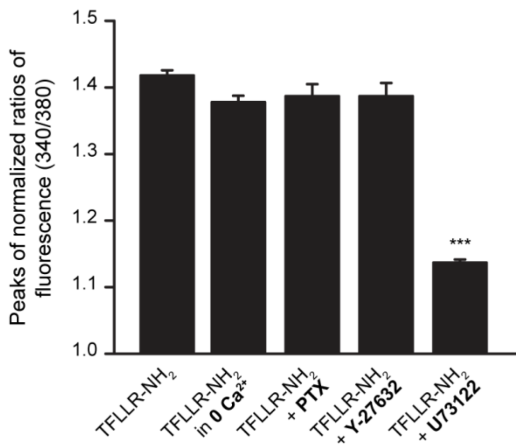


Figure 3.4. PAR-1 signaling in SVZ-derived oligodendrocytes involves $G_{q/11}$ and PLC activity, with calcium recruitment from intracellular stores. Means of peaks of normalized ratios of fluorescence at 340/380 displayed by SVZ-derived oligodendrocytes following perfusion protocols, as in Fig. 2.1*A* (TFLLR-NH_2 as third pulse) and 2.1*C–E*. ****p* < 0.001, as compared to TFLLR-NH_2. TFLLR-NH_2, PAR-1 agonist; 0 Ca^{2+}, Krebs solution with low calcium concentration; PTX, pertussis toxin, $G_{i/o}$ inhibitor; Y27632, Rho/ROCK inhibitor; U73122, PLC inhibitor.

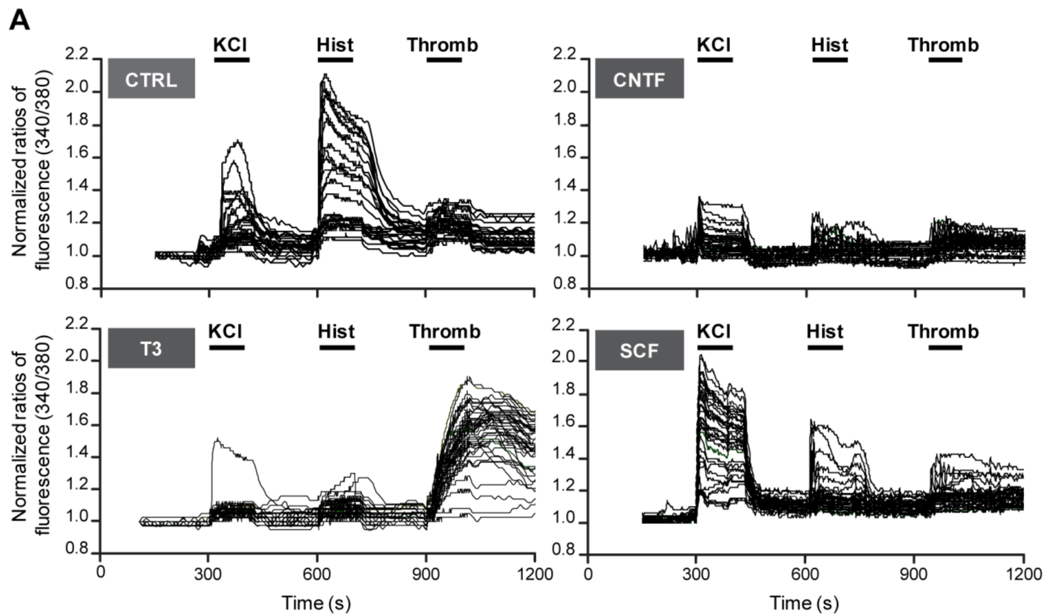
PAR-1 is a transmembrane metabotropic G protein-coupled receptor signaling through $G_{i/o}$, $G_{12/13}$, and $G_{q/11}$ families of large heterotrimeric G proteins (Coughlin, 2000). To disclose the role of G proteins in the thrombin-induced $[Ca^{2+}]_i$ rise, we pre-incubated cells with 200 ng/mL PTX, which inactivates $G_{i/o}$ proteins (Wang et al., 2004) (Fig. 2.1D). PTX incubation did not alter the Ca^{2+} mobilization in SVZ-derived oligodendrocytes, indicating that $G_{i/o}$

are not involved in the PAR-1-induced $[Ca^{2+}]_i$ increase (1.39 ± 0.02, 149 cells analyzed) (Fig. 3.4). Therefore, the signaling cascade triggered by TFLLR-NH_2 includes the PTX-insensitive proteins $G_{q/11}$ and/or $G_{12/13}$. The pathway upon PAR-1-coupled $G_{q/11}$ activation is quite well-described. $G_{q/11}$ activates PLC isoform β (PLCβ), which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol 1,4,5-trisphosphate (IP_3), leading to Ca^{2+} recruitment from the intracellular compartments, and PKC activation. On the other hand, $G_{12/13}$ binds Rho-guanine nucleotide exchange factors (RhoGEFs) initiating a Rho-associated protein kinase-(ROCK) dependent pathway that leads to the activation of PLC isoform ε (PLCε), thus resulting in Ca^{2+} recruitment and PKC activation (Grand et al., 1996; McLaughlin et al., 2005; Hains et al., 2006). To search for the involvement of $G_{q/11}$ and/or $G_{12/13}$ in PAR-1-induced $[Ca^{2+}]_i$ oscillations in SVZ-derived oligodendrocytes, we pre-incubated the cells with 10 μM Y-27632, a ROCK inhibitor, for 24 h, and then ran the SCCI protocol, as depicted in Fig. 2.1D. When ROCK was inhibited, the TFLLR-NH_2-induced $[Ca^{2+}]_i$ increase was still present and at the same magnitude as compared to the peak evoked by TFLLR-NH_2 alone (1.39 ± 0.02, 284 cells analyzed). Furthermore, pre-incubation with 5 μM U73122, a PLC inhibitor (Fig. 2.1E), abolished the response to TFLLR-NH_2, indicating that PLC activation is part of the triggered pathway (1.14 ± 0.01, 398 cells analyzed) (Fig. 3.4).

3.3.4 Oligodendroglial differentiation can be assessed by measuring the variations of $[Ca^{2+}]_i$ upon stimulation with thrombin and histamine

To validate the method described in the present work, we functionally compared non-treated SVZ cultures with T3-treated cultures. Upon SCCI assay consisting of KCl, histamine, and thrombin pulses (Fig. 2.1A), normalized peaks of fluorescence of all the individualized cells were measured and the Throm/Hist ratio was calculated. Indeed, control cultures presented ~3% of cells responding

with a Throm/Hist ratio above 1.3 (3.24 ± 1.06%, 21 coverslips, 1790 cells analyzed) consistent with the normal oligodendrocyte differentiation in SVZ cultures (Gritti et al., 1996; Whittemore et al., 1999). Upon T3 treatment, cultures contained ~35% of these cells (35.07 ± 8.22%, 10 coverslips, 626 cells analyzed) (Fig. 3.5A), which is in accordance with the well-described effect of T3 hormone increasing the OPCs number and promoting the differentiation in oligodendrocytes (Barres et al., 1994; Murray and Dubois-Dalcq, 1997; Zhang et al., 2004a; Kang et al., 2007). Furthermore, SVZ cultures pre-treated with the astrogliogenic factor CNTF (Whittemore et al., 1999; Ravin et al., 2008) or the neurogenic factor SCF (Jin et al., 2002a), during the differentiation period, displayed a similar percentage of cells responding as oligodendrocytes as found in non-treated cultures (CNTF: 2.92 ± 1.19, 8 coverslips, 776 cells analyzed; SCF: 4.74 ± 1.91, 13 coverslips, 1304 cells analyzed). Representative profiles of $[Ca^{2+}]_i$ variations along the SCCI protocol are depicted in Fig. 3.5B. As shown, oligodendrocyte-like responding cells were hardly found in control cultures. On the contrary, when treated with T3, many cells were insensitive to KCl or histamine, and responded markedly to thrombin, thus confirming the shift to an oligodendrocyte phenotype. On the other hand, SCF-treated cultures displayed a neuronal tendency with many KCl-responding cells, and CNTF-treated cultures exhibited a typical astrocytic tendency with many non-responsive cells. In SCF and CNTF-treated cultures the response to thrombin was similar to the one in control cultures, showing that these treatments did not affect the population of oligodendrocytes.



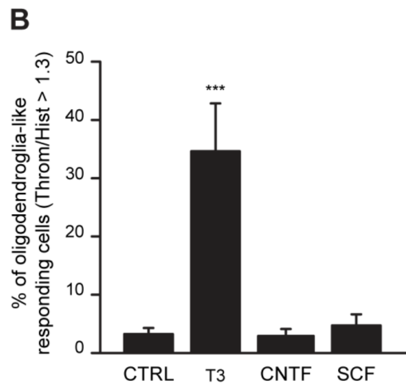


Figure 3.5. Functional evaluation of oligodendrocyte differentiation in SVZ cultures. A, Representative profiles of response elicited upon perfusion with KCl, histamine, and thrombin in non-treated SVZ cultures and in cultures exposed to T3, or CNTF, or SCF for 7–10 days. **B**, Percentage of oligodendrocyte-like responding cells in T3-, CNTF- or SCF-treated cultures. ***p < 0.001, as compared to non-treated cultures. CTRL, control; T3, triiodothyronine; CNTF, ciliary neurotrophic factor; SCF, stem cell factor; Hist, histamine; Thromb, thrombin.

Additionally, SCCI experiments were performed in defined cultures of hippocampal neurons as well as in cultures of cortical astrocytes. Both cultures presented no oligodendrocyte-like responding cells with the established ratio Throm/Hist>1.3 (hippocampal neurons: 0.00 ± 0.00, 8 coverslips, 243 cells analyzed; cortical astrocytes: 0.00 ± 0.00, 7 coverslips, 305 cells analyzed) (Fig. 3.6).

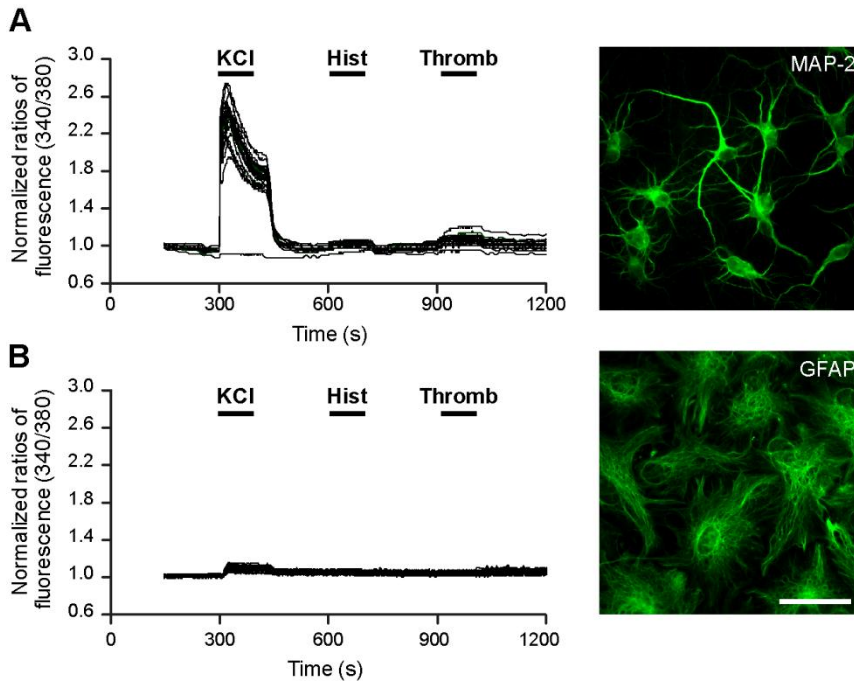


Figure 3.6. Functional evaluation of differentiation in primary cultures of neurons or astrocytes. Representative profiles of response elicited upon perfusion with KCl, histamine, and thrombin in hippocampal neuronal cultures (**A**), which display a KCl-responsive profile, or cortical astrocytes cultures (**B**), which are non-responsive. **a**,**b**, Representative images from the respective cultures, stained for MAP-2 (top) or GFAP (bottom), respectively. Hist, histamine; Thromb, thrombin. Scale bar: 50 μm.

We also investigated if the response to thrombin is exclusive to oligodendrocytes derived from the neurogenic SVZ. For that, we prepared neurospheres from early postnatal cortex, because it has been reported that at this stage the cortex contains astrocytes with stem cell potential (Laywell et al., 2000; Itoh et al., 2006). The neurospheres were seeded on microgrid coverslips, incubated in the absence or the presence of T3, and evaluated under SCCI and immunocytochemistry procedures. Cortical and SVZ-derived PLP^+ oligodendrocytes responded similarly to thrombin (data not shown). However, we also observed some cortex-derived thrombin-sensitive cells that responded to histamine. These data raise interesting questions about the influence of

chemical/cellular environment in cell fate determination and functional responses of differentiating oligodendrocytes.

3.3.5 Demyelinated host environment favors oligodendroglial cell fate in grafted SVZ cells

In vitro pre-commitment of SVZ cells into the phenotype lost in a particular brain disease has been seen as a pivotal step in the development of cell-based brain transplantation therapies. Nevertheless, cell differentiation under oligodendrogenic factors treatment *in vitro* might not be recapitulated the same way when cells are engrafted in the injured tissue. To understand whether demyelinated host environment affects SVZ cell induction on oligodendroglial fate we used an *ex vivo* model of demyelinating injury and evaluated the differentiation of grafted eGFP-SVZ cells. For this, organotypic hippocampal slice cultures were incubated with 25 μM CPZ, for 24 h, as depicted in Fig. 2.2. Administration of CPZ through the diet is widely used as a demyelinating protocol in *in vivo* studies, causing demyelination of the fiber tracts in the corpus callosum (Matsushima and Morell, 2001), cortex (Skripuletz et al., 2008) and hippocampus (Hoffmann et al., 2008; Koutsoudaki et al., 2009; Norkute et al., 2009). Interestingly, *in vitro* studies by Cammer (1999) have shown a detrimental effect of 1 h of CPZ treatment (25 μM) in oligodendrocytes, and reported the presence of swollen or enlarged mitochondria in these cells, recalling the mitochondrial hyperplasia observed in the brains of CPZ-treated mice. Mitochondrial dysfunction is believed to be an early event that leads to apoptotic colapse of oligodendrocytes and consequent myelin sheath degeneration (reviewed in Matsushima and Morell, 2001). Moreover, although *in vivo* studies show that the peak of myelination in rodents occurs between P10 and P60, Haber et al. (2009) have shown that hippocampal slice cultures obtained from P6-7 mice are able to develop myelin. The authors observed a progressive increase of MBP immunoreactivity over the course of 60 days *in*

vitro (DIV), being that, at 10 DIV many MBP fibers were present in CA subregions. In addition, using electron microscopy they detected myelinated axons as early as at 7 DIV. Herein, used hippocampal slices obtained from P6-8 mice and developed for 15 DIV, thus harboring many myelinated axons. We incubated the hippocampal slices with 25 μM CPZ during 24 h to reach oligodendrocytes death and demyelination.

First, to validate the injury model, we performed immunohistochemistry for PLP in the CPZ-treated organotypic slices, as well as in total brain coronal slices obtained from adult mice fed with CPZ-containing diet 6 weeks. In the *in vivo* model of CPZ intoxication, we observed an almost complete demyelination of the white matter tracts of the different hippocampal subregions, in line with previous reports (Hoffmann et al., 2008; Koutsoudaki et al., 2009; Norkute et al., 2009) (Fig. 3.7A,B). Accordingly, CPZ treatment to organotypic hippocampal slice cultures induces a partial demyelination of the hippocampus, given by a decreased PLP immunoreactivity (Fig. 3.7C).

We then grafted eGFP-SVZ neurospheres in the demyelinated hippocampal slices (Fig. 3.8A; one neurosphere per slice) and investigated oligodendrocytic differentiation from SVZ cells under the host environment influence. It is important to refer that upon grafting, T3 was added to the culture medium and kept during the following week, in order to promote oligodendrogenesis, and thus obtain a sufficient number of cells to compare differentiation patterns between intact and demyelinated slices. We demonstrate that grafted SVZ cells maintain the capability to generate oligodendrocytes, including cells displaying NG2 and O4 immunoreactivity, besides the typical highly ramified morphology (Fig. 3.8B). Interestingly, quantification of the percentage of $NG2^+$ and $O4^+$ cells among the eGFP cells, reveals that differentiation of SVZ grafted cells in $O4^+$ or $NG2^+$ cells is promoted by the injured environment (NG2: CTRL, 13.60 ± 2.987%, from n=9 slices, 3158 cells, 2 independent cultures vs. CPZ, 33.092 ± 1.439%, from n=12 slices, 3436 cells,

2 independent cultures; O4: CTRL, 16.40 ± 0.7411%, from n=9 slices, 3180 cells, 2 independent cultures vs. CPZ, 29.72 ± 1.674%, from n=11 slices, 4446 cells, 2 independent cultures; Fig. 3.8C,D). These results highlight the importance of disease environment dictating the fate choice of the transplanted uncommitted SVZ cells.

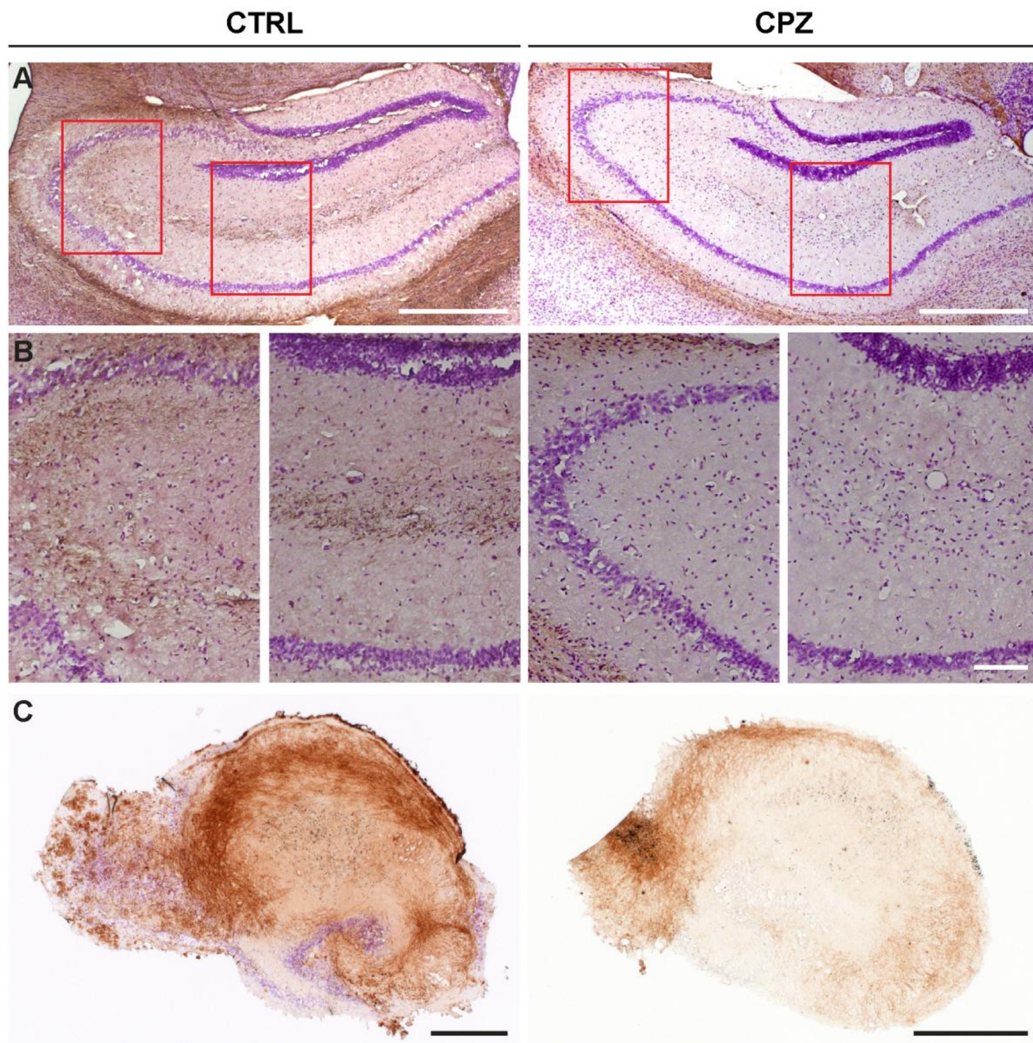


Figure 3.7. Expression of the myelin protein PLP in the adult and postnatal mouse hippocampus after cuprizone treatment. A, Extensive demyelination in the hippocampus of adult mice fed with CPZ (0.2% w/w, 6 weeks; right) as compared to the control group (left). The boxed areas represent the hippocampal regions displayed in high magnification on panel (**B**). **C**, Partial demyelination in organotypic hippocampal slices cultured from postnatal mice brain and treated with CPZ (25 μM, 1 h; right) as compared to non-treated cultures (left). Scale bars: A, 500 μm; B, 100 μm; C, 500 μm.

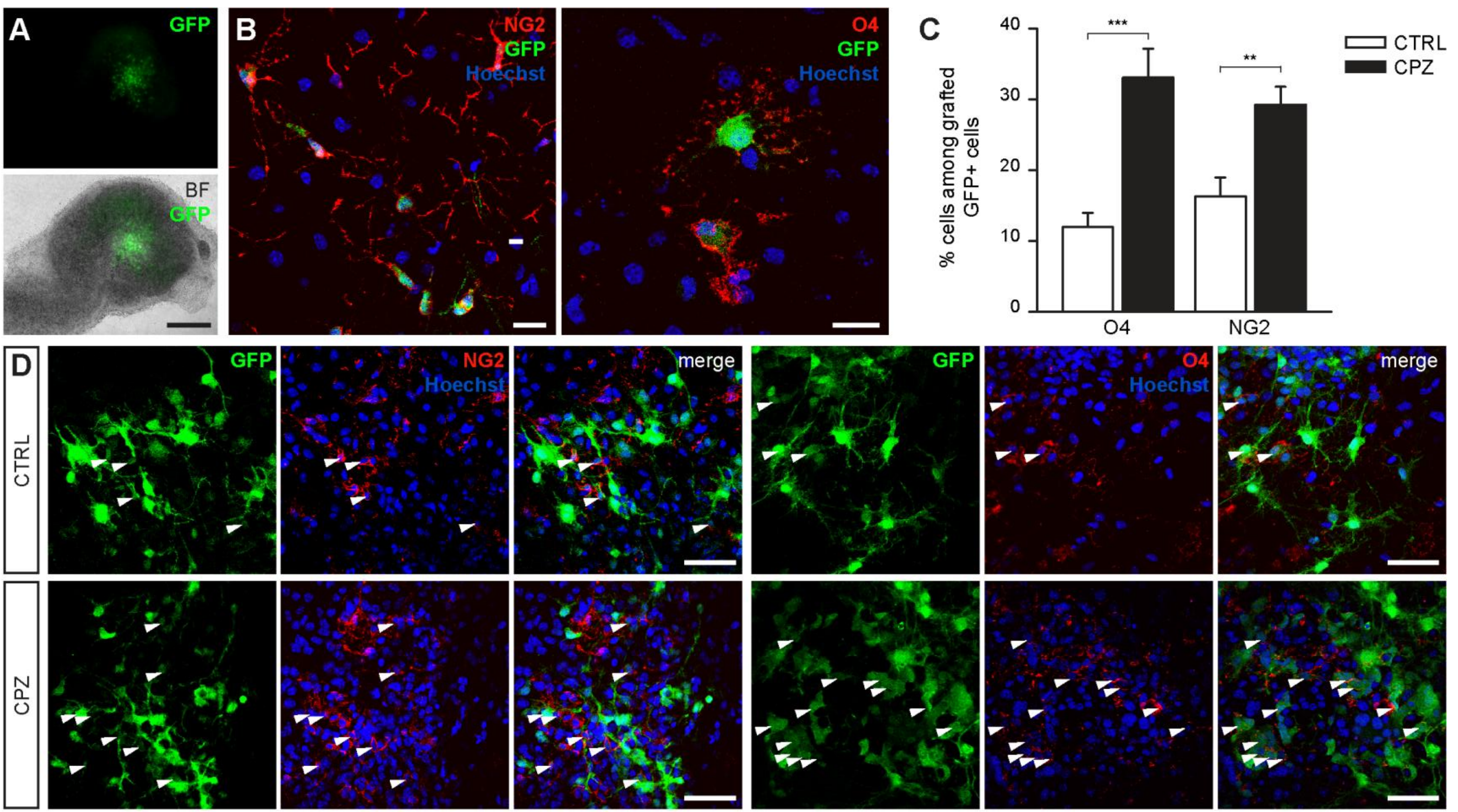


Figure 3.8. Differentiation of eGFP-SVZ grafted cells in demyelinated organotypic hippocampal slices. A, Representative image of an organotypic hippocampal slice sheltering an eGFP-SVZ neurosphere. The co-culture was allowed to develop during 1 week, under T3 treatment. **B**, 1 week upon grafting , $NG2^+$ and $O4^+$ cells were found among the SVZ cells, either implanted in intact or injured slices. **C**, Quantification of the number of $NG2^+$ and $O4^+$ cells among the grafted GFP-SVZ cells in intact or injured paradigm. **D**, Representative images of NG2 (left panel) or O4 (right panel) immunostaining on the co-cultures, counterstained with Hoechst 33342 (blue nuclei). Arrows indicate cells where colocalization of GFP signal with the cell type markers was found. Scale bars: A, 500 μm; B, 20 μm; D, 50 μm.

3.4 DISCUSSION

The main aim of the present work was to develop a novel method to functionally evaluate oligodendroglia differentiation in SVZ cultures. The method consists in measuring the intracellular Ca^{2+} currents evoked by KCl, histamine, and thrombin on the different cell types present in a SVZ culture, and is based on the observation that each cell type displays a distinguishable profile of $[Ca^{2+}]_i$ oscillations during the stimulation protocol (Fig. 3.9).

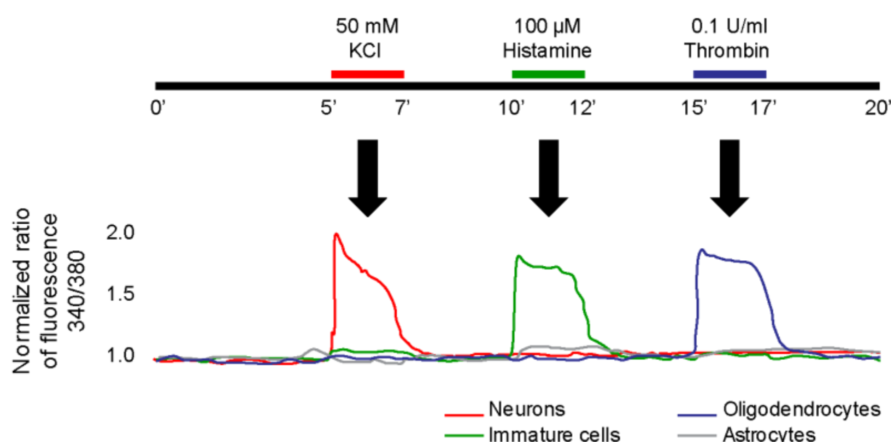


Figure 3.9. Single-cell calcium imaging (SCCI) as a method to assess cell differentiation in SVZ cultures. Experimental protocol performed in SCCI aiming the functional identification of SVZ-derived cells. SVZ cells loaded with the calcium probe Fura-2 AM are continuously perfused in Krebs solution and stimulated at different time intervals as shown by the time sequences. Cell-specific responses are observed when applying the sequence depicted on top: neurons respond to KCl, immature cells respond to histamine, oligodendrocytes respond to thrombin and astrocytes are non-responsive to any of the compounds.

Because oligodendrocytes are rare cells in SVZ cultures, we treated the SVZ spheres with T3 after deposition in PDL and growth factors withdrawal, during 7-10 days, to increase the proportion of oligodendrocytes in culture, and thus obtaining a SVZ cell-derived culture highly enriched in oligodendrocytes. The effect of T3 hormone as a pro-oligodendrogenic factor is

quite well-described. Accordingly, T3 hormone is crucial for normal brain development, being of major importance in the *in utero* development of oligodendrocytes and later postnatal myelination (Rodriguez-Pena, 1999; Bernal, 2005). Barres et al. (1994) concluded that, in the presence of mitogens, T3 stops cell division of bipotential precursors of both astrocytes type 2 and oligodendrocytes, so called oligodendrocytes type 2 astrocytes (O-2A), allowing them to enter into the differentiation stage of the oligodendrocytic lineage. Stem cells may differentiate into oligodendrocytes through an intermediate stage resembling an O-2A cell, although there are still many controversies regarding the lineage paths arising from NSCs. Nevertheless, Johe et al. (1996) have demonstrated that T3 exposure leads rat embryonic and adult multipotent NSCs to a glial lineage and promotes oligodendrocyte differentiation if the treatment is prolonged, revealing an instructive mechanism of T3 action in cell fate decisions. Accordingly, T3 treatment was shown to increase the generation of oligodendrocytes from murine embryonic stem cells (Zhang et al., 2004a), as well as in human embryonic stem cells (Kang et al., 2007). T3 not only accelerates the rate of oligodendrocytes precursor's generation but also the complexity of their morphology toward a mature oligodendrocytes (Murray and Dubois-Dalcq, 1997) and the synthesis of myelin proteins (Baas et al., 2002; Franco et al., 2008). As expected, we observed much more $O4^+$, as well as $NG2^+$ or PLP^+, oligodendrocytes in the T3-treated cultures as compared to the non-treated cultures.

By correlating the Ca^{2+} profiles with subsequent phenotypic immunodetection, we were able to show that (1) similarly to astrocytes, $O4^+$ and PLP^+ oligodendrocytes do not respond to KCl or histamine; (2) $[Ca^{2+}]_i$ is not altered by thrombin in MAP-2^+ mature and DCX^+ immature neurons, as well as in $GFAP^+$ astrocytes; however, (3) $O4^+$ and PLP^+ oligodendrocytes display an increase of $[Ca^{2+}]_i$ following thrombin perfusion. Cells presenting a Hist/KCl ratio between 0.9 and 1.1 are glial cells, as shown in our previous work,

astrocytes or oligodendrocytes, the latest being identifiable by their specific rise of $[Ca^{2+}]_i$ under thrombin perfusion and related Throm/Hist ratio above 1.3. Interestingly, the Throm/Hist ratio characteristic of $O4^+$ oligodendrocytes is variable, and this can be explained by the wide range of oligodendrocyte differentiation stages where O4 sulfatide is present. Moreover, we verified that $NG2^+$ cells have either no response or a variable response to thrombin, which may suggest that PAR-1 expression begins gradually in the stage of progenitors of pre-oligodendrocytes. Thus, these data suggests that thrombin sensitivity in oligodendroglia lineage appears to be gradually increasing with cells maturation. At a latter differentiation stage, a clear response to thrombin was observed in $O4^+$ and especially in PLP^+ oligodendrocytes. According to these findings, thrombin stimulation was identified as a reliable strategy to discriminate oligodendrocytes among other cells in SVZ cultures.

Using PAR-1-specific ligands, we concluded that a thrombin-triggered $[Ca^{2+}]_i$ increase in SVZ-derived oligodendrocytes is mediated by PAR-1 activation. Indeed, PAR-1 immunoreactivity strongly co-localizes with O4 staining, labeling intensively in the oligodendrocyte cell body, and also appearing in clusters on cell processes. A recent study has detected functional PAR-1 receptors in Schwann cell myelin microvilli at the nodes of Ranvier. The authors concluded that PAR-1 activation in Schwann cells leads to a conduction block in the axon, presumably by an influx of calcium and subsequent release of substances that affect the propagation of the action potential (Shavit et al., 2008). Nevertheless, PAR-1 expression is not restricted to oligodendrocytes. Some astrocytes, and occasionally some $nestin^+$ immature cells, also express PAR-1. The scarce $nestin^+/PAR\text{-}1^+$ cells presented a typical astrocytic morphology, suggesting that expression of PAR-1 in astrocytes begins early in their development when they still express the nestin marker of immaturity, but are already committed to become astrocytes. In contrast with the robust PAR-1 labeling in the oligodendrocytes cell body, and their high peak of response to

thrombin stimulus, these occasional nestin$^+$ and GFAP$^+$ cells expressing the receptor presented only a dispersed membranar, rather than clustered, punctuated PAR-1 staining, indicating minor expression. No GFAP$^+$ or nestin$^+$ cells responding to thrombin were found in SCCI analysis, therefore suggesting that low and dispersed expression of PAR-1 makes it insufficient to signal a detectable response by SCCI, or, alternatively, that PAR-1 is not coupled to an increase of $[Ca^{2+}]_i$ in these cell types. On the contrary, mature neurons stained with MAP-2 did not express PAR-1. Previous studies have shown the presence of PAR-1 in motor neurons, olfactory neurons, and postnatal hippocampal neurons (Smirnova et al., 2001; Gorbacheva et al., 2006; Olianas et al., 2007). Moreover, PAR-1-induced low Ca^{2+} transients were observed in rat hippocampal neurons (Yang et al., 1997; Gorbacheva et al., 2006). However, in our experiments, we observed neither PAR-1 expression in SVZ-derived neurons, nor $[Ca^{2+}]_i$ changes in these cells, or in embryonic hippocampal neurons. It is worthwhile to mention that the observed Ca^{2+} transients in hippocampal cultures reported by Gorbacheva et al. (2006) are about 200 nM, a very modest response. Furthermore, Yang et al. (1997) demonstrate the requirement of a 24 h pre-treatment of hippocampal neurons with thrombin to induce calcium response. Also, some authors report the expression of PAR-1 and Ca^{2+}-associated response upon PAR-1 stimulation of newborn rat or mice cortical astrocytes (Wang et al., 2002; Sorensen et al., 2003). We focused on cortical astrocytes from P7 mice and SVZ-derived astrocytes from newborn mice, the last occasionally having PAR-1 expression, but both being insensitive to thrombin stimulation. Differences in culture conditions or age of the animals may be responsible for the different results in PAR-1 expression and thrombin response regarding cortical astrocytes. Concerning the mechanism of PAR-1 downstream signaling toward cytosolic Ca^{2+} rise, our data demonstrate that PAR-1 binding leads to PLCβ activation through $G_{q/11}$ heteromeric protein activity. PLCβ hydrolyzes PIP_2 generating IP_3 and diacylglycerol (DAG), with

consequent IP_3-dependent Ca^{2+} release from the endoplasmic reticulum and PKC activation by DAG. This is consistent with previous studies examining the PAR-1 downstream signaling pathway in oligodendrocytes (Wang et al., 2004), although not so far characterized in SVZ-derived oligodendrocytes.

Finally, the proposed method was validated by comparing the percentage of cells with Throm/Hist ratio above 1.3 (oligodendrocytes) in non-treated SVZ cultures, T3-treated cultures, as well as in CNTF- and SCF-treated cultures. As compared to non-treated cultures, Ca^{2+} profiles in treated cultures completely shifted, differently, reflecting the acquisition of different phenotypes. As expected, T3 induced an increase in the proportion of oligodendrocyte population, whereas treatment with CNTF or SCF conferred no changes in the proportion of oligodendrocytes, further confirming the efficiency of the method.

Besides T3, other factors may be discovered as inducers of the oligodendrocytes cell fate, paving the way to the development of drug cocktails that might be more efficient than the use of one compound only. However, the translation from the *in vitro* cell cultures to the *in vivo* disease paradigm implies other aspects interfering with SVZ cell dynamics, including survival, differentiation and integration in the host inflamed tissue. To broaden our view onto SVZ differentiation in the perspective of ectopic transplantation, we carried out experiments of SVZ grafting in organotypic hippocampal slice cultures pre-injured by CPZ intoxication and evaluated differentiation after 1 week. Importantly, hippocampal demyelination has been observed in the brains of MS patients, in parallel with microglial accumulation and cognitive deficits (Geurts et al., 2007). Our *ex vivo* model of demyelination allowed us to detect an important effect of the demyelinated host tissue favoring the differentiation of grafted SVZ cells into oligodendroglia. In agreement, others studies have described that SVZ cells transplanted in demyelinated areas show a tendency to undergo differentiation in oligodendrocytes, and often myelinate nude axons

(Keirstead et al., 1999; Smith and Blakemore, 2000; Akiyama et al., 2001; Cayre et al., 2006). However these studies were based on transplantations in the large white matter tracts of the corpus callosum and spinal cord, where the intact local environment *per se* is known to trigger oligodendrogenesis of transplanted SVZ cells (Cayre et al., 2006).

In conclusion, we describe here the development of a novel strategy to functionally identify oligodendrocyte differentiation, which resides on the specific stimulation of the cell type with thrombin, acting through PAR-1 receptors. Moreover, we demonstrate that the increase of $[Ca^{2+}]_i$, triggered by PAR-1 activation in oligodendrocytes involves PLC-dependent recruitment of Ca^{2+} from cellular internal stores. As a proof of concept, we treated SVZ cultures with T3, SCF, or CNTF to enrich the cultures in oligodendrocytes, neurons, or astrocytes, respectively. In agreement with our working hypothesis, the resulting profile of responses revealed the efficiency of the procedure. In conclusion, the technological platform described herein is relevant in the context of drug discovery and development, with potential for brain regenerative medicine. Moreover, pre-transplantation studies with new drugs to prime SVZ cells into a certain phenotype should be achieved *in vitro* but complemented by grafting experiments to validate the extent of the effect of the drug. Probably, a cocktail of factors needs to be produced to accomplish a complete and efficient oligodendrocytic differentiation in the host tissue.

CHAPTER 4

Brain-derived neurotrophic factor promotes vasculature-associated migration of neuronal precursors toward the ischemic striatum

CHAPTER 4 – BRAIN-DERIVED NEUROTROPHIC FACTOR PROMOTES VASCULATURE-ASSOCIATED MIGRATION OF NEURONAL PRECURSORS TOWARD THE ISCHEMIC STRIATUM

Note: The work presented on this chapter was performed at the laboratory of Dr. Armen Saghatelyan (Cellular Neurobiology Unit, Centre de Recherche Université Laval Robert-Giffard, Quebec, Canada) under his supervision and in collaboration with Dr. Jasna Kriz (Department of Anatomy and Physiology, Laval University, Centre de Recherche du Centre Hospitalier de l'Université Laval, Quebec, Canada).

4.1. SUMMARY

Stroke induces the recruitment of neuronal precursors from the SVZ into the ischemic striatum. In injured areas, de-routed neuroblasts use blood vessels as a physical scaffold to their migration, in a process that resembles the constitutive migration seen in the rostral migratory stream RMS. The molecular mechanism underlying injury-induced vasculature-mediated migration of neuroblasts in the post-stroke striatum remains, however, elusive. We now demonstrate that endothelial cells in the ischemic striatum produce BDNF, a neurotrophin that promotes the vasculature-mediated migration of neuronal precursors in the RMS, and that recruited neuroblasts maintain p75NTR expression. Reactive astrocytes, which are widespread throughout the damaged area, ensheath blood vessels and express TrkB, a high-affinity receptor for BDNF. Despite the absence of BDNF mRNA, we observed strong BDNF immunolabeling in astrocytes, suggesting that these glial cells trap extracellular BDNF. Importantly, this pattern of expression is reminiscent of the adult RMS,

where TrkB-expressing astrocytes bind and sequester vasculature-derived BDNF, leading to the entry of migrating cells into the stationary phase. Real-time imaging of cell migration in acute brain slices revealed a direct role for BDNF in promoting the migration of neuroblasts to ischemic areas. We also demonstrated that cells migrating in the ischemic striatum display higher exploratory behavior and longer stationary periods than cells migrating in the RMS. Our findings suggest that the mechanisms involved in the injury-induced vasculature-mediated migration of neuroblasts recapitulate, at least partially, those observed during constitutive migration in the RMS.

4.2 INTRODUCTION

Adult stem cells in the SVZ of the lateral ventricle produce neuronal precursors that migrate toward the OB via the RMS. Interestingly, under certain conditions such as cortical or striatal strokes, neuronal precursor cells leave the SVZ and migrate toward ischemic areas (Arvidsson et al., 2002; Parent et al., 2002; Jin et al., 2003a). In recent years, studies on post-stroke neurogenesis have revealed that recruited neuroblasts closely associate with blood vessels (Ohab et al., 2006; Yamashita et al., 2006; Thored et al., 2007) and appear to travel along them (Zhang et al., 2009; Kojima et al., 2010). These data suggest that neuronal precursors require vasculature support for migration in post-stroke areas similar to the constitutive vasculature-mediated migration of neuroblasts in the RMS (Snapyan et al., 2009; Whitman et al., 2009). However, the dynamics and molecular mechanisms driving the vasculature-mediated migration in post-stroke areas remain largely unexplored.

We previously pinpointed an important role for vasculature-derived BDNF in neuroblasts migration along the RMS (Snapyan et al., 2009). We showed that the RMS vasculature serves as a physical substrate and a source of

BDNF that promotes neuroblast migration via the activation of p75NTR expressed by these migrating cells (Snapyan et al., 2009). Curiously, stroke triggers the expression of BDNF in affected areas (Lindvall et al., 1992; Kokaia et al., 1995; Kokaia et al., 1998a), and intravenous (Schabitz et al., 2007) or intraventricular (Keiner et al., 2009) administration of BDNF in animals subjected to phototrombotic ischemia leads to an increased number of SVZ-derived cells in injured tissues. It thus appears that BDNF promotes post-stroke neurogenesis (Schabitz et al., 2007; Keiner et al., 2009; but see Nygren et al., 2006). It is, however, not clear whether BDNF directly affects the migration of neuroblasts in post-stroke areas and, if it does, what the cellular sources of this trophic factor are. It has previously been shown that neurons in compromised areas transiently secrete BDNF (Comelli et al., 1993; Kokaia et al., 1995; Kokaia et al., 1998a). In addition, BDNF immunolabeling has been observed in astrocytes, microglia, ependymal and endothelial cells at distinct times after injury (Béjot et al., 2011). Since BDNF is a secreted protein that can be sequestered by other cell types, a detailed analysis of BDNF mRNA expression in post-stroke areas is required to determine its cellular source.

We studied the expression of BDNF and its receptors in the post-stroke striatum and explored the mechanisms of vasculature-mediated migration of neuronal precursors by focusing on the BDNF pathway. Real-time imaging of cell migration revealed that BDNF promotes neuroblast displacement in the injured striatum along blood vessels that express this trophic factor. We demonstrated that injury-induced migration of neuroblasts shares similarities with the constitutive migration of neuronal precursors in the RMS with regard to (1) vasculature association, (2) expression of BDNF and its receptors, and (3) involvement of BDNF in the initiation of the migratory phase. Our results provide an insight into the mechanisms underlying injury-induced vasculature-mediated migration of neuronal precursors in ischemic areas.

4.3 RESULTS

4.3.1 Neuronal migration in the ischemic striatum is vasculature-dependent and involves astrocytes

In the present study, we used a mouse model of MCAo, which has infarcts mainly in the striatum. To evaluate the extent of neuronal migration from the SVZ to the infarcted striatum, we labeled for Dcx, a marker for newborn migrating neurons in the adult brain. Ischemia-induced neuroblasts recruitment toward the damaged striatum was triggered and was maintained during the two first weeks following ischemia, which is in agreement with previous reports (Thored et al., 2006). Small numbers of Dcx+ neuronal precursors had migrated to the striatum of the ipsilateral hemisphere within 1 week of MCAo (data not shown) while many more labeled cells that had migrated longer distances from the SVZ were observed 2 weeks following the injury (Fig. 4.1A,right). As reported previously (Zhang et al., 2004b; Yamashita et al., 2006; Zhang et al., 2009), Dcx^+ neuroblasts were observed as individual cells (Fig. 4.1B) or assembled in chains (Fig. 4.1C) or spherical clusters (Fig. 4.1D). In contrast, the corresponding contralateral striatum was devoid of Dcx immunoreactivity (Fig. 4.1A, left), resembling the naïve brain. Co-immunolabeling for Dcx and PECAM revealed that the de-routed neuroblasts preferentially localized in the vicinity of striatal blood vessels (Fig. 4.1E,G). Indeed, 86.61 ± 4.27% of Dcx^+ cells (3529 cells analyzed, n=5 mice) after 1 week and 83.95 ± 2.24% of Dcx^+ cells (2860 cells analyzed, n=4 mice) after 2 weeks were located in the vicinity of $PECAM^+$ blood vessels (Fig. 4.1E,G). This finding is consistent with previous reports (Ohab et al., 2006; Yamashita et al., 2006; Thored et al., 2007; Zhang et al., 2009; Kojima et al., 2010) and suggests that neuroblasts use vasculature as a physical substrate to support their migration to the post-stroke striatum.

In the intact adult brain, Dcx expression is restricted to the two neurogenic brain areas, namely the SVZ-OB pathway and the SGZ of the DG. Nevertheless, studies using experimental ischemia or targeted apoptosis have shown an unforeseen induction of neurogenesis in non-neurogenic tissues (Magavi et al., 2000; Chen et al., 2004; Ohira et al., 2010). To ensure that the neuroblasts observed in the ischemic striatum had indeed migrated from the SVZ and had not been produced locally and expressed Dcx following the injury, we labeled the neuronal precursors in the SVZ by stereotaxic injection of GFP-encoding lenti- or retroviruses 3 to 7 days pre-MCAo. GFP^+ neuroblasts were observed in the ipsilateral striatum and were preferentially associated with dextran-Texas Red-filled blood vessels 2 weeks post-MCAo (Fig. 4.1F,G; 80.75 ± 4.44% n=119 cells, n=17 mice).

Astrocytes play an important role in RMS neuroblasts migration by restraining chains within tubular glial structures (Peretto et al., 1997; Kaneko et al., 2010) and by affecting neuronal migration via releasable (Mason et al., 2001) and membrane-bound (García-Marqués et al., 2010) factors. Astrocytes control extracellular levels of GABA to uphold neuroblast migration (Bolteus and Bordey, 2004), modulate vasculature-mediated neuroblast migration (Snapyan et al., 2009), and regulate the formation of migration-promoting vasculature scaffolds (Bozoyan et al., 2012). We thus analyzed the arrangement of the astroglia in the ischemic striatum. Like the RMS, the striatal vessels used for post-stroke migration were ensheathed by astrocytes. We observed a massive astrogliosis in the injured striatum, where some of the reactive astrocytes were in close contact with neuroblasts and blood vessels (Fig. 4.1H,I). This cellular organization, which supports neuroblast migration toward the ischemic striatum, resembles the RMS cytoarchitecture, where constitutive migration of neuronal precursors toward the OB occurs (Snapyan et al., 2009; Whitman et al., 2009). Since vasculature-derived BDNF has been shown to orchestrate neuroblasts

migration in the RMS, we investigated the role of BDNF in injury-induced migration.

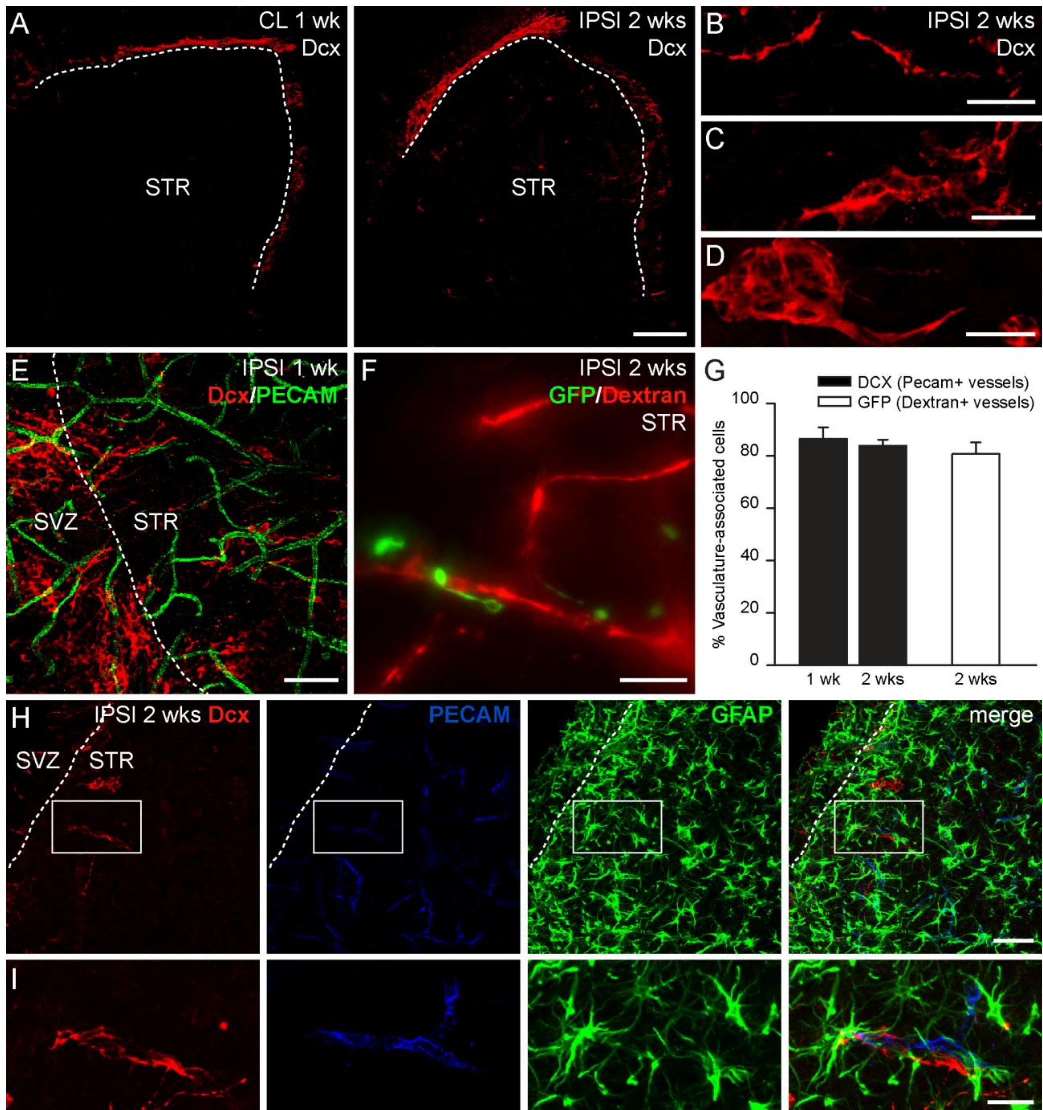


Figure 4.1. Migration of neuronal precursors in the ischemic striatum occurs along the blood vessels and involves astrocytes. A, Dcx immunoreactivity in the ipsilateral (right) and contralateral (left) striatum 2 weeks post-MCAo, showing the presence of neuronal precursors in ischemic areas (right). In ischemic area, Dcx^+ neuroblasts are found as individual cells (**B**) or are assembled in chains (**C**) or clusters (**D**). **E,F**, Dcx^+ neuroblasts (**E**) and GFP^+ neuroblasts (**F**) are found in close proximity to PECAM (**E**) or dextran Texas Red-filled (**F**) blood vessels. **G**, Vasculature-associated cells were quantified by counting the number of neuroblasts within 3 μm of blood vessels. **H**, $GFAP^+$ astrocytes were abundant and enveloped the vasculature that physically supported injury-induced migration. **I**, High magnification image of the inset shown in (**H**). Scale bars: **A**, 200 μm; **B-D**,**F**,**I**, 20 μm; **E**,**H**, 50 μm (SVZ: subventricular zone; STR: striatum; CL: contralateral; IPSI: ipsilateral; wk: week).

4.3.2 Ischemia induces BDNF expression by neurons and endothelial cells in the striatum

In the adult mice striatum, BDNF expression is extremely low and frequently undetectable (Hofer et al., 1990; Zermeno et al., 2009). However, BDNF levels are transiently increased following striatal injuries (Wong et al., 1997; Kokaia et al., 1998a; Batchelor et al., 1999). To study the cellular pattern of BDNF expression, we first performed an *in situ* hybridization to detect BDNF mRNA. BDNF mRNA levels in the contralateral striatum (Fig. 4.2A, left) were similar to the naïve brain at 1 or 2 weeks post-MCAo (data not shown), with no or few positive cells per slice. We did, however, detect an upregulation of BDNF mRNA expression in dispersed round-shaped cells (Fig. 4.2A, middle) in the ischemic striatum 1 week post-MCAo. At 2 weeks post-MCAo, BDNF mRNA expression in these cells had dropped to contralateral levels (Fig. 4.2A, right). *In situ* hybridization combined with immunohistochemistry for cell type-specific markers revealed that the round-shaped cells are $NeuN^{+}$ neurons (Fig. 4.2B). Interestingly, in the ischemic striatum, BDNF mRNA was also detected in dextran-Texas Red-filled blood vessels (Fig. 4.2C). No BDNF mRNA was observed in blood vessels in the contralateral or naïve striatum (data not shown). While BDNF expression in neurons was upregulated 1 week post-MCAo and subsequently dropped to control levels after 2 weeks, BDNF expression in striatal blood vessels persisted 2 weeks after the injury. No BDNF mRNA was detected in other cells types in the ischemic striatum, including Dcx^{+} neuroblasts (Fig. 4.2D), $GFAP^{+}$ astrocytes (Fig. 4.2E), $Iba1^{+}$ microglia (Fig. 4.2F), and $Olig2^{+}$ oligodendrocytes (Fig. 4.2G).

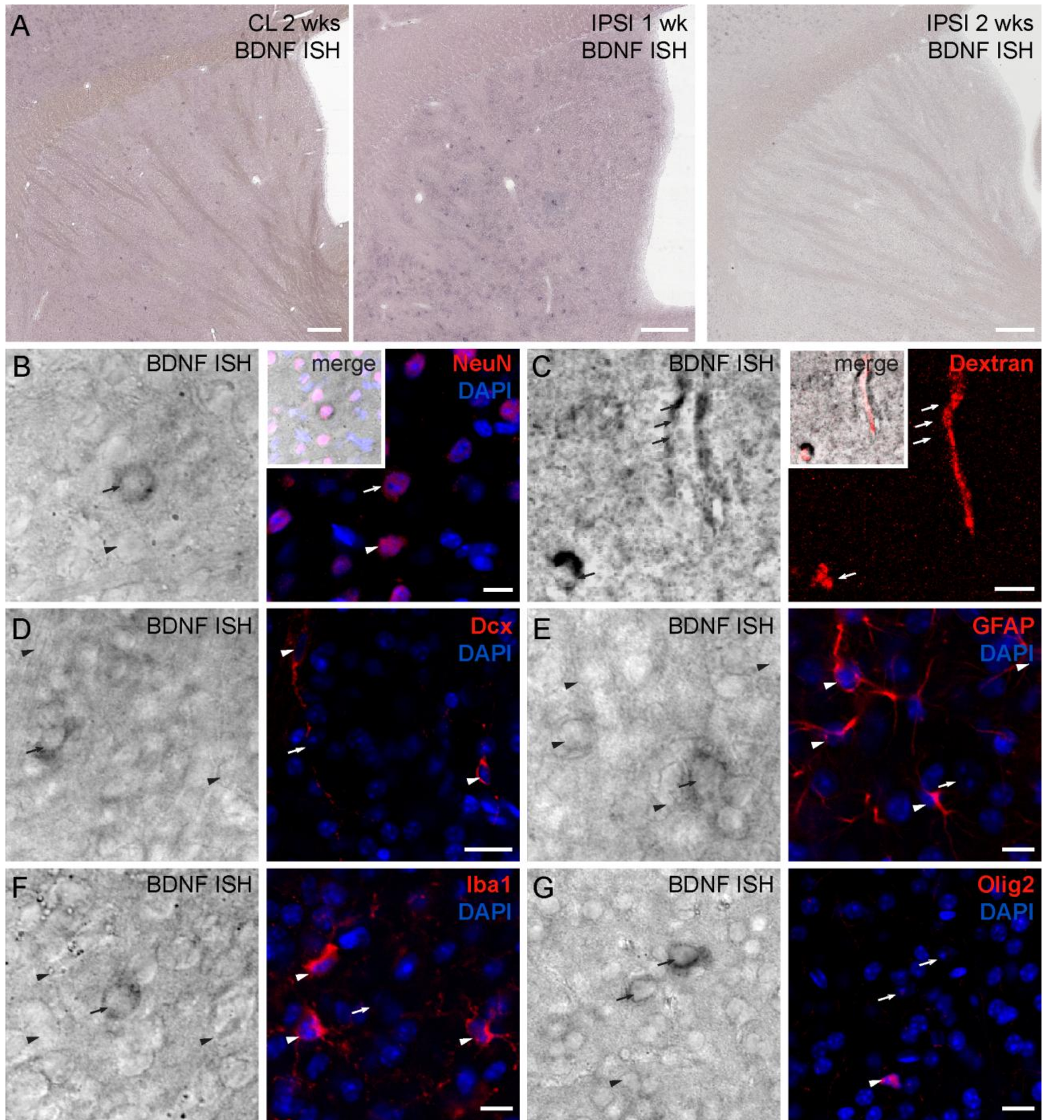


Figure 4.2. Ischemia induces BDNF mRNA expression by neuronal and endothelial cells in the ipsilateral striatum. A, In situ hybridization revealed an upregulation of BDNF mRNA in rounded cells in the ipsilateral striatum 1 week post-ischemia (middle). Little or no BDNF mRNA was found in these cells 2 weeks post-ischemia (right). **B-G**, High magnification images revealed that BDNF mRNA was expressed in $NeuN^+$ striatal neurons (arrow) **(B)** and dextran-Texas Red-filled blood vessels **(C)**. **D-G**, No BDNF mRNA was observed in Dcx^+ neuroblasts (**D**), $GFAP^+$ astrocytes (**E**), $Iba1^+$ microglia (**F**), or $Olig2^+$ oligodendrocytes (**G**) in the injured striatum. Arrowheads indicate cells negative for BDNF mRNA and arrows indicate cells positive for BDNF mRNA. Scale bars: **A**, 200 µm; **D**,**G**, 20 µm; **B**,**C**,**E**,**F**, 10 µm (CL: contralateral; IPSI: ipsilateral; wk: week; ISH: in situ hybridization).

Having shown that BDNF mRNA is expressed, we investigated the expression of BDNF protein. Surprisingly, we detected BDNF immunopositive signals not only in dextran-Texas Red-filled blood vessels and a few neurons (data not shown), but also in astrocytic-like cells, some of which were closely associated with the dextran-Texas Red-filled blood vessels (Fig. 4.3A-D). Co-labeling for GFAP and BDNF confirmed that astrocytes in the ischemic striatum are immunopositive for BDNF (Fig. 4.3D). These results were observed 1 and 2 weeks post-injury. Given that no BDNF mRNA was detected in astrocytes, this finding led us to hypothesize that BDNF secreted by endothelial cells and/or neurons following the injury may be trapped by neighboring astrocytes. Since neuronal BDNF mRNA expression is drastically reduced 2 weeks post-injury, it is conceivable that endothelial cells that continue to express BDNF after 1 and 2 weeks are the main cellular source for this secreted trophic factor. Interestingly, we previously demonstrated that astrocytes in the RMS bind endothelial BDNF via cell-surface TrkB receptors and thus modulate its availability for p75NTR-expressing migrating neuroblasts (Snapyan et al., 2009). This led us to analyze the patterns of TrkB and p75NTR expression in the ischemic striatum.

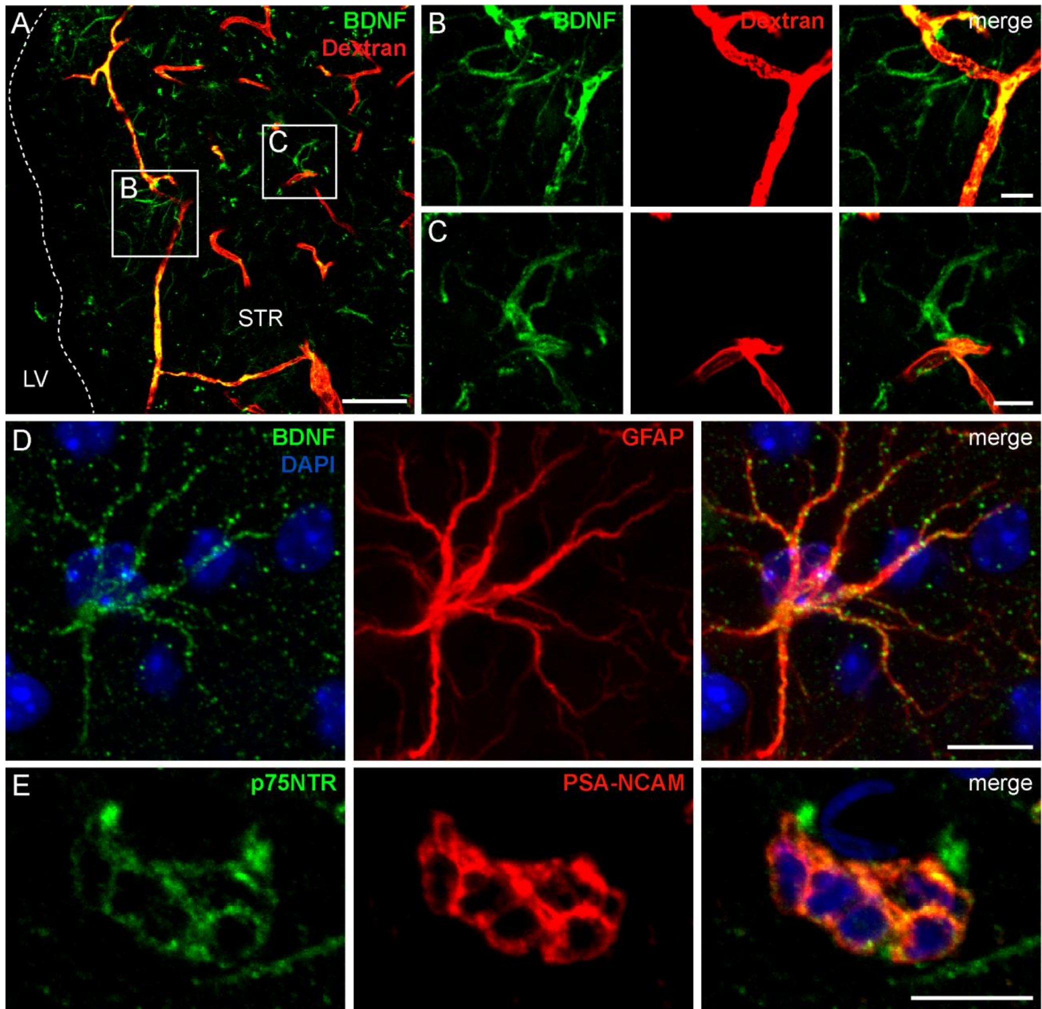


Figure 4.3. Expression of BDNF and p75NTR in the post-stroke striatum. A, BDNF immunolabeling was observed in dextran-Texas Red-filled blood vessels and in astrocytic-like cells. **B**,**C**, High magnification images from insets depicted in (**A**) showing astrocytic-like cells in close proximity to blood vessels. Note that the blood vessels and astrocytes are immunopositive for BDNF. **D**, Co-immunolabeling for BDNF and GFAP revealed the presence of BDNF protein in astrocytes. **E**, PSA-NCAM^{+} migrating neuroblasts maintained p75NTR expression when de-routed to the injured striatal matrix. The pattern of expression shown in these panels was observed 1 and 2 weeks post-MCAo. Scale bars: **A**, 50 µm; **B-E**, 10 µm.

4.3.3 Migrating de-routed neuroblasts express p75NTR while reactive astrocytes in the damaged striatum express TrkB receptor

We observed p75NTR immunolabeling in PSA-NCAM$^+$ neuroblasts that had de-routed to the site of injury (Fig. 4.3E). We also detected p75NTR immunolabeling in astrocytes and some neurons of the ipsilateral striatum (data not shown). These findings were consistent with previous reports showing that p75NTR is upregulated in reactive astrocytes in the ischemic hippocampus (Lee et al., 1995; Oderfeld-Nowak et al., 2003) and in cholinergic neurons in the ischemic striatum (Andsberg et al., 2001).

In situ hybridization for TrkB revealed a robust overexpression of the high-affinity receptor for BDNF throughout the ipsilateral striatum compared to the contralateral striatum (Fig. 4.4A). This pattern of expression was observed both 1 and 2 weeks post-MCAo and coincided with areas of massive astroglial accumulation (Fig. 4.4A,B). TrkB *in situ* hybridization combined with immunolabeling for cell type-specific markers confirmed the expression of TrkB by GFAP$^+$ reactive astrocytes (Fig. 4.4C, arrow). Co-immunolabeling for GFAP and TrkB also showed that these proteins co-localized in the injured striatum (Fig. 4.4D). The expression of the TrkB receptor in astrocytes provided a clue concerning the BDNF immunolabeling results and suggested that the TrkB receptor in astrocytes "traps" extracellular BDNF released by endothelial and/or neuronal cells following ischemia. No TrkB mRNA or protein expression was observed in Dcx$^+$ neuroblasts (Fig. 4.4E), NeuN$^+$ neurons (Fig. 4.4F), Iba1$^+$ microglia (Fig. 4.4G), or Olig2$^+$ oligodendrocytes (Fig. 4.4H). The *in situ* hybridization and immunolabeling results for BDNF and its receptors in the ischemic striatum revealed a pattern that is strikingly similar to the expression pattern observed in the RMS (Snapyan et al., 2009). As in the RMS, p75NTR$^+$ neuroblasts in the ischemic striatum migrate along blood vessels expressing BDNF. Astrocytes that envelope blood vessels and contact neuroblasts likely trap extracellular BDNF through the high-affinity TrkB receptor. We thus

investigated the dynamic properties of injury-induced neuroblast migration to determine whether BDNF acts directly on neuroblasts to guide their migration along the blood vessels in the ischemic striatum.

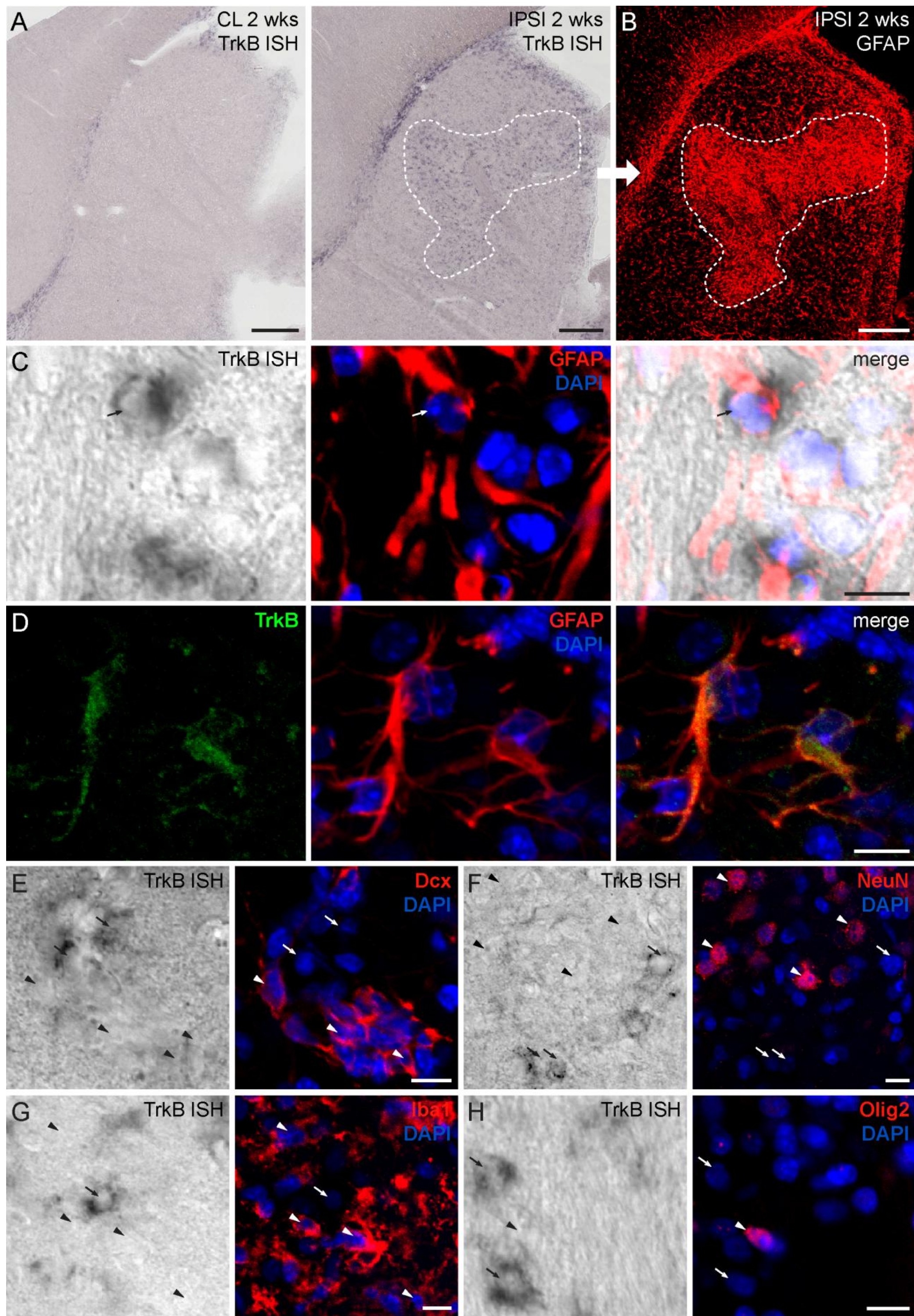


Figure 4.4. TrkB is expressed by reactive astrocytes in the ischemic striatum. A, *In situ* hybridization revealed a massive upregulation of the TrkB receptor in the ipsilateral striatum (*Figure legend continues.*)

(*Figure legend continued.*) (right), as opposed to virtually no TrkB expression in the contralateral side (left). The overexpression coincided with areas of astroglial accumulation (**B**). High magnification images revealed the clear apposition of the TrkB mRNA signal with GFAP immunolabeling (**C**, arrow). **D**, Immunolabeling for TrkB and GFAP revealed that this neurotrophic receptor was expressed by astrocytes in the injured striatum. **E-H,** No TrkB mRNA was detected in Dcx^+ neuroblasts (**E**), $NeuN^+$ neurons (**F**), $Iba1^+$ microglia (**G**)**,** or $Olig2^+$ oligodendrocytes (**H**) (arrowheads; TrkB mRNA is indicated by arrows). The pattern of expression showed in these panels was observed 1 and 2 weeks post-MCAo. Scale bars: **A**,**B**, 200 μm; **C-H**, 10 μm (CL: contralateral; IPSI: ipsilateral; wk: week; ISH: in situ hybridization).

←

4.3.4 Injury-induced neuroblast migration in the striatum is less dynamic than constitutive migration in the RMS

Despite a growing number of studies reporting that SVZ-derived progenitors migrate toward injured areas, the dynamic behavior of neuroblasts during such induced migration has not been fully explored. This issue has been approached by monitoring the migration of DiI-labeled cells, Dcx-GFP-expressing cells, and lentivirally-labeled cells in the striatum of organotypic slices from animals subjected to MCAo (Zhang et al., 2007; Zhang et al., 2009; Kojima et al., 2010). Time-lapse microscopy to monitor the displacement of recruited labeled cells in the striatum was performed, by acquiring multiple *z*-stack images every 15 min (Zhang et al., 2007; Zhang et al., 2009) or 30 min (Kojima et al., 2010). While organotypic slices make it possible to follow cell migration for prolonged periods of times, culturing the slices may alter the migratory properties of the cells. In addition, acquisitions every 15 to 30 min make it difficult to detect and study some migratory properties such as the duration of the stationary and migratory phases, which can be as short as 4 to 10 min (Snapyan et al., 2009). We thus acquired images every 30 s for 1-2 h in acute sagittal brain in order to monitor the patterns and dynamics of injury-induced migration in the striatum and compared them to normal RMS migration. We prepared slices 2 to 3 weeks post-ischemia and 3 to 4 weeks post-GFP-encoding lenti- or retrovirus injection into the SVZ and recorded the migration

of de-routed neuroblasts in the ischemic striatum. We also recorded neuroblasts migration in the contralateral and ipsilateral RMS to determine whether ischemia-induced recruitment of neuronal precursors to the nearby striatum interferes with the normal dynamics of migration in the constitutive pathway. The analysis of neuroblasts migration in the RMS and the ischemic striatum clearly showed that neuroblasts migrated for shorter average distances per hour in the ipsilateral striatum compared to the contralateral (CL) or ipsilateral (IPSI) RMS (Fig. 4.5A-H; CL RMS: 22.98 ± 1.45 μm, 121 cells, n=16 video recordings obtained from 11 mice; IPSI RMS: 19.93 ± 1.09 μm, 117 cells, n=15 video recordings obtained from 11 mice; and STR: 9.80 ± 0.95 μm, 74 cells, n=37 video recordings obtained from 25 mice; $p < 0.001$ with unpaired *t* test, for CL RMS vs. STR and IPSI RMS vs. STR). These results indicated that neuroblasts migrating in the ischemic striatum are less dynamic than those migrating in the normal environment. The difference in the total displacement distance of neuroblasts in the ischemic striatum compared to those in the RMS may be due to the changes in the speed of migration or differences in the duration of the migratory and stationary phases. In our analysis, we assessed the speed of migration solely during the migratory phases, as proposed earlier (Bortone and Polleux, 2009; Snapyan et al., 2009). Interestingly, there was no difference in the speed of migration between neuroblasts migrating in the ischemic striatum and in the contralateral or ipsilateral RMS (Fig. 4.5I; CL RMS: 142.20 ± 2.17 μm/h, 121 cells, n=16 video recordings obtained from 11 mice; IPSI RMS: 136.62 ± 2.58 μm/h, 117 cells, n=15 video recordings obtained from 11 mice; and STR: 128.00 ± 4.88 μm/h, 74 cells, n=37 video recordings obtained from 25 mice). The analysis of the duration of stationary phases revealed that neuroblasts spend significantly more time in the resting period when migrating in the ischemic striatum than in the contralateral or ipsilateral RMS (Fig. 4.5J; CL RMS: 82.18 ± 0.88%, 121 cells, n=16 video recordings obtained from 11 mice; IPSI RMS: 85.46 ± 0.79%, 117 cells, n=15 video

recordings obtained from 11 mice; STR: 92.36 ± 0.64%, 74 cells, n=37 video recordings obtained from 25 mice; $p < 0.001$ with unpaired t test, for CL RMS vs. STR, and for IPSI RMS vs. STR). Thus, fewer migratory periods of neuroblasts may account for the shorter displacement distances (track lengths) in the ischemic striatum (Fig. 4.5F). It also should be mentioned that most cells in the striatum were immotile during the 2-h recording period and were not taken into consideration. Representative traces of individual cells showed that the average distance that cells migrated in injured areas was shorter than in the RMS (Fig. 4.5G). In addition, while cell migration was highly directional in the RMS, once inside the injured striatal matrix, cells seemed to migrate randomly in different directions (in Fig. 4.5B,D,F, the range of colors in the tracks shows the initial (blue) and final (white) positions, and thus the direction of the movement). Interestingly, we also observed that neuroblasts in the RMS ipsilateral to the lesion have longer stationary periods than cells in the contralateral RMS (Fig. 4.5J; CL RMS: 82.18 ± 0.88%, 121 cells, n=16 video recordings obtained from 11 mice; IPSI RMS: 85.46 ± 0.79%, 117 cells, n=15 video recordings obtained from 11 mice; $p < 0.01$ with unpaired t test). It is noteworthy that, unlike our previous studies on neuroblast migration in the naïve RMS (Snapyan et al., 2009; Bozoyan et al., 2012), in the present work we observed lower neuronal precursor motility and longer stationary phases in the contralateral RMS. This difference is likely due to the fact that we monitored cell migration in the RMS 3 to 4 weeks post-viral labeling of neuronal precursors in the SVZ, while in our previous studies we recorded cell migration in the RMS 4 to 7 days post-viral labeling in the SVZ (Snapyan et al., 2009; Bozoyan et al., 2012). Thus, in the present work, we likely tracked a distinct population of neuroblasts that displayed lower motility and that remained in the RMS 3 to 4 weeks post-labeling in the SVZ.

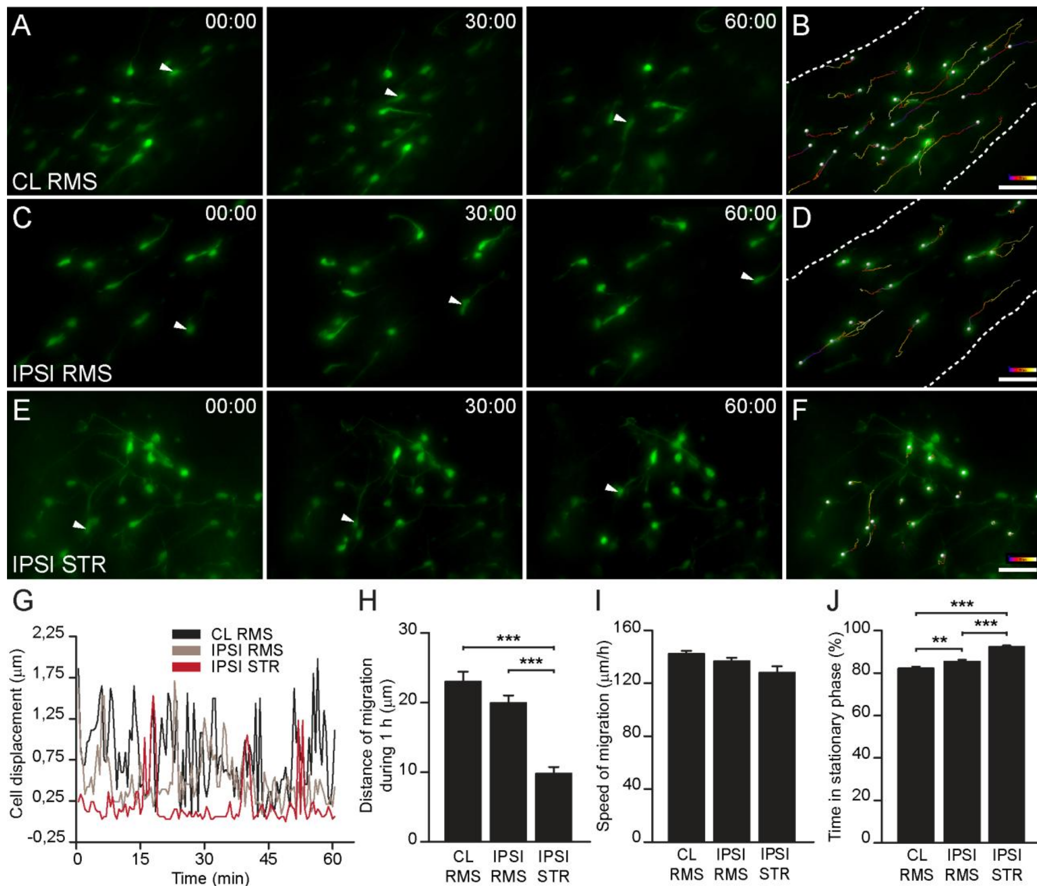


Figure 4.5. Dynamic properties of neuroblast migration in ischemic areas. A-**F**, Timeline snapshots from real-time images of SVZ-derived precursor migration in acute brain slices of MCAo-challenged animals. Cells were labeled by injecting GFP-encoding retroviruses in the SVZ prior to inducing ischemia and were evaluated 2 to 3 weeks post-MCAo in the contralateral RMS (**A**,**B**), ipsilateral RMS (**C**,**D**), and ipsilateral striatum (**E,F**). Arrowheads indicate migrating cells. **B**,**D**,**F,** Migratory tracks of the cells migrating for 1 h in **A,C,E**, respectively (color code: blue in the initial position; white in the final position). **G**, Representative profile of cell displacement over time for individual neuroblasts migrating in the contralateral and ipsilateral RMS, and in the striatum. **H,J,** In the ipsilateral striatum, the recruited neuronal precursors displayed low migratory behavior, spending longer periods in the resting state (**J**), which resulted in shorter displacements per hour compared to the contralateral or ipsilateral RMS (**H**). **I**, No differences in the speed of migration of neuroblasts migrating in the ischemic striatum and ipsilateral and contralateral RMS were observed. Scale bars: 20 µm (CL: contralateral; IPSI: ipsilateral; STR: striatum).

In the ischemic striatum, "resting" cells with a migratory morphology often exhibited a highly exploratory behavior, constantly extending and retracting the leading process with a highly dynamic growth cone in different directions (Fig. 4.6A). The leading process stabilized on a number of occasions,

and the cell soma was translocated in its direction, constituting a migratory event. Interestingly, most of the recruited neuroblasts migrated along blood vessels in the ischemic striatum (Fig. 4.6B). We observed two different types of migration. In the first case, neuroblasts migrated straight along the dextran-Texas Red-filled blood vessels (Fig. 4.6B). We also observed neuroblasts with their soma located close to one blood vessel and a leading process extended toward another blood vessel (Fig. 4.6C, arrowhead; see also Fig. 4.6A). In some cases, the growth cone at the end of the leading process stabilized, leading to the initiation of cell migration. These observations suggested that neuronal precursors may migrate in the post-stroke striatum by "jumping" from one blood vessel to another in addition to the "continuum" migration along individual blood vessels. These two types of migration modes may cooperate to increase the dispersal of recruited neuroblasts in the ischemic striatum.

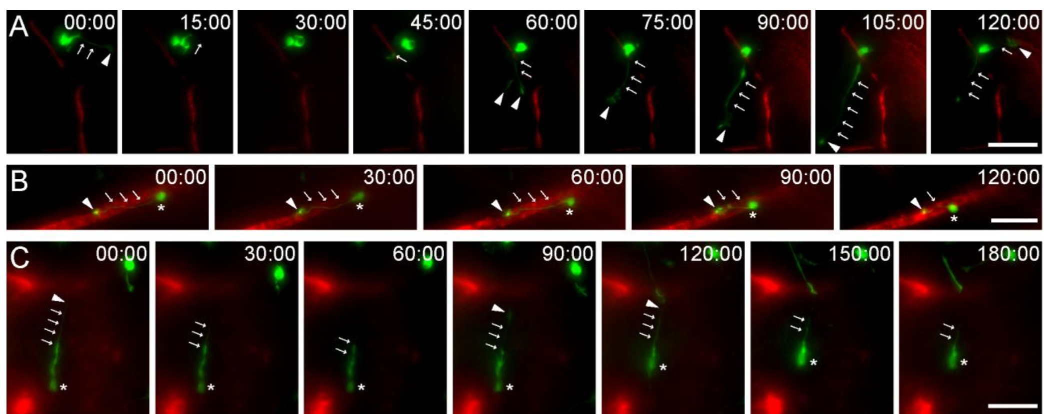


Figure 4.6. Dynamics of vasculature-mediated migration of neuroblasts in the ipsilateral striatum. A-C, Time-lapse videoimages of SVZ-derived neuroblasts migrating in acute brain slices prepared from MCAo-challenged animals. Cells were labeled by injecting GFP-encoding retroviruses in the SVZ prior to inducing ischemia and were evaluated 2 to 3 weeks post-MCAo in the ipsilateral striatum. Blood vessels were visualized by dextran-Texas Red fluorescence. **A**, In damaged areas, "resting" cells actively explored the surrounding environment by extending and retracting leading processes with a highly dynamic growth cone (arrows indicate the processes and arrowheads, the growth cone). **B,** An example of a GFP^+ neuroblast (green) migrating along a dextran-Texas Red-filled blood vessel (red). **C,** Time-lapse images showing that a GFP^+ neuroblast (green) located close to a blood vessel (red) may extend a leading process toward another blood vessel and migrate in that direction by "jumping" from one blood vessel to another. In B and C, arrows indicate the processes, arrowheads the growth cone, and asterisks the cell soma. Scale bars: 20 µm.

4.3.5 BDNF promotes injury-induced migration in the ischemic striatum

To determine whether BDNF modulates the ischemia-induced migration of neuroblasts, we monitored neuroblast migration in the injured striatum of acute slices before and after the bath-application of BDNF or TrkB-Fc in order to scavenge endogenous BDNF. As a control we applied IgG-Fc. The pharmacological agents were applied during the second hour of recording and migration was compared between the first (control: ACSF) and second hour (pharmacological agent) of the recordings. Results are expressed as the percentage of the control (ACSF) for each group. TrkB-Fc, which scavenges exogenous BDNF, making it unavailable to the migrating cells, impaired neuroblast migration in the striatum (Fig. 4.7A). In control experiments, bath-application of IgG-Fc had no effect on migration. In contrast, the bath-application of recombinant BDNF fostered the migratory behavior of progenitor cells in the compromised striatum (Fig. 4.7B). Quantification of neuroblast migration demonstrated that TrkB-Fc decreased the total length of neuroblast displacement per hour (52.77 ± 12.63%, n=15 cells, 6 video recordings obtained from 5 mice; $p < 0.01$ with Student t test; Fig. 4.7C). In contrast, we observed a robust increase in total displacement following the application of BDNF (217.7 ± 47.91%, n=11 cells, 6 video recordings obtained from 4 mice; $p < 0.01$ with Student *t* test; Fig. 4.7C). No effect on migration was observed in control experiments with IgG-Fc application (78.28 ± 26.66%, n=13 cells, 6 video recordings obtained from 4 mice; Fig. 4.7C). We observed no differences in the speed of migration following the application of TrkB-Fc, IgG-Fc, or BDNF (TrkB-Fc: 86.46 ± 6.84%, n=8 cells, 4 video recordings obtained from 4 mice; IgG-Fc: 101.5 ± 10.15%, n=6 cells, 4 video recordings obtained from 3 mice; BDNF: 94.02 ± 4.61%, n=8 cells, 6 video recordings obtained from 4 mice; Fig. 4.7D). Thus, whenever a migratory event was initiated, the cells displaced with the same speed during the migratory phase. However, TrkB-Fc increased the

time spent in the stationary phase (104.2 ± 1.23%, n=15 cells, 6 video recordings obtained from 5 mice; $p < 0.05$ with Student t test), whereas BDNF decreased the time spent by neuroblasts in the resting period (90.2 ± 3%, n=11 cells, 6 video recordings obtained from 4 mice; $p < 0.01$ with Student t test) (Fig. 4.7E). No changes were observed following the bath-application of IgG-Fc (98.66 ± 3.03%, n= 13 cells, 6 video recordings obtained from 4 mice; Fig 4.7E). The changes in migration induced by BDNF and TrkB-Fc were thus due to changes in the time spent in the stationary phase (Fig. 4.7E). BDNF seemed to play a pivotal role in the switch from the stationary to migratory phase by promoting the initiation of migration in cells navigating on the damaged striatum. These effects are similar to that of BDNF on migration in the RMS, where it promotes entry into the migratory phase rather than increasing the speed of migration (Snapyan et al., 2009).

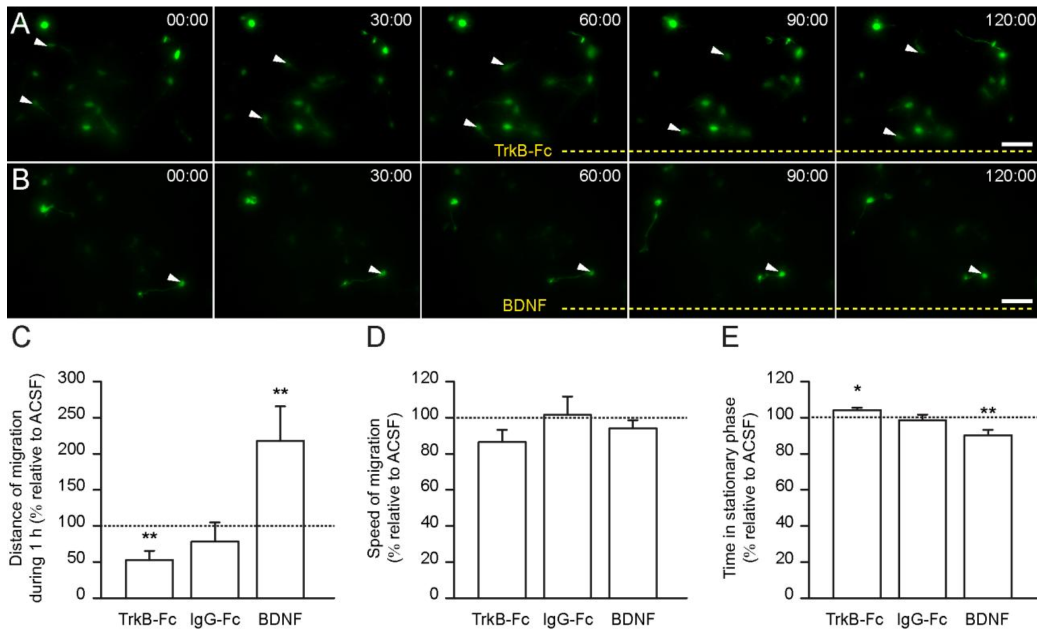


Figure 4.7. BDNF promotes neuroblast migration in ischemic areas. A-B, Time-lapse videoimages of neuroblasts migrating in the ischemic striatum under control conditions (0-60 min) and following the application (60-120 min) of TrkB-Fc (**A**) and BDNF (**B**). Bath-application of TrkB-Fc led to smaller displacement lengths due to an increase in the time spent by neuroblasts in the stationary period (**C,E**). On the other hand, the application of BDNF approximately doubled the total displacement per hour by reducing the time that cells spent in the stationary phase (**C,E**). Frequently, cells that were immotile during the first hour of recording initiated migration following BDNF perfusion (**B,** arrowhead). **D**, No differences in the speed of migration were observed following the application of TrkB-Fc, IgG-Fc, or BDNF. Scale bars: 20 µm.

4.4 DISCUSSION

The goal of the present study was to study the mechanisms of vasculature-mediated migration of neuronal precursors in the ischemic striatum. We demonstrated that, post-MCAo, neuroblasts de-routed to the injured striatum express p75NTR, the low-affinity BDNF receptor, and migrate along striatal blood vessels, which synthesize BDNF in response to the injury. Trapping endogenous BDNF with TrkB-Fc impaired neuroblast migration in the acute slices prepared from the ischemic striatum, whereas recombinant BDNF triggered more migratory events by the de-routed cells. Interestingly, reactive astrocytes were closely associated with blood vessels and expressed TrkB, the high-affinity BDNF receptor. These glial cells may thus modulate the availability of BDNF for p75NTR-expressing neuroblasts by binding free extracellular BDNF. This would in turn modulate the migration of neuronal precursors as previously observed for the constitutive migration of neuroblasts in the RMS (Snapyan et al., 2009). We also showed that neuroblasts migrating in the injured striatum display greater exploratory behavior and thus longer stationary periods than cells migrating in the RMS. Our findings showed that injury-induced migration is less dynamic than constitutive RMS migration, but that it is also controlled by BDNF signaling.

4.4.1 Expression of BDNF and its receptors after ischemia

Unlike the intact striatum, the injured striatum contains considerable amounts of BDNF that is locally expressed by blood vessels and neuronal cells. Until recently, it was generally recognized that neurons are the major transient source of newly synthesized BDNF following strokes (Lindvall et al., 1992; Comelli et al., 1993; Takeda et al., 1993; Kokaia et al., 1995; Kokaia et al., 1998a). The expression of BDNF by surviving neurons in the degenerating ischemic cortex or striatum has been associated with an early autocrine

neuroprotective role that counteracts cell death (Lindvall et al., 1994; Saarelainen et al., 2000; Larsson et al., 2002). In recent years, other groups have shown that microglia transiently produce BDNF within 24 h of permanent focal (Madinier et al., 2009) or multifocal cortical ischemia (Béjot et al., 2011). Béjot et al. (2011) also detected BDNF-immunolabeled ependymal cells on the pial surface and astrocytes ensheathing vessels in injured areas 8 days post-ischemia (Béjot et al., 2011). They also reported that BDNF is expressed in blood vessels 24 h post-injury, but not 8 days post-injury. However, they did not stain blood vessels to validate these results. In addition, since they did not measure BDNF mRNA expression, their conclusions concerning the source of this secreted trophic factor based solely on immunoreactivity results must be treated with caution. In the present study, we used *in situ* hybridization and immunohistochemistry to document the expression of BDNF by neurons and endothelial cells. Interestingly, while BDNF expression in $NeuN^+$ striatal cells decayed from 1 to 2 weeks post-injury, BDNF expression in the striatal vasculature was maintained 2 weeks post-injury. This suggested that neuronal BDNF may be involved in early neuroprotective events, while endothelial BDNF, which persists for a longer time, promotes and maintains the migration of neuronal progenitors in an attempt to replace cells in the injured striatum.

We detected the BDNF receptor p75NTR, but not TrkB, in migrating neuroblasts, suggesting that the low-affinity p75NTR receptor is responsible for the stimulatory effect of BDNF on the neuroblast migration observed in the acute slices of injured striatum. In the SVZ, p75NTR is expressed by neuroblasts, type C cells, and a few astrocytes (Young et al., 2007; Snapyan et al., 2009). Following an injury, some progenitors are mobilized from the SVZ and RMS to the striatum. However, the expression of the low-affinity BDNF receptor is maintained and is likely involved in cell navigation through the striatal matrix. In addition to neuroblasts, a few striatal neurons and astrocytes expressed p75NTR, while virtually no p75NTR was found in the contralateral

striatum. Previous studies have shown that post-stroke neuronal upregulation of p75NTR takes place in a subpopulation of cholinergic striatal interneurons and likely prompts cells to undergo apoptosis (Andsberg et al., 2001; Angelo et al., 2009). The functional role of p75NTR expression by astrocytes following ischemia remains, however, elusive. Studies on the intact cortex (Bergami et al., 2008) and hippocampus (Dougherty and Milner, 1999) have suggested that p75NTR in astrocytes is involved in controlling the extracellular availability of BDNF. TrkB, the high-affinity BDNF receptor, was expressed exclusively in activated astrocytes in the injured area. The expression of the full-length TrkB receptor in neuronal cells undergoes an acute transient increase following brain insults, but this lasts no longer than 24 h (Merlio et al., 1993) or 48 h (Narumiya et al., 1998). This suggests that TrkB plays a role in the early neuroprotective action of BDNF. The *in situ* probe and anti-TrkB antibody used in our study recognized both the full-length and truncated isoforms of TrkB receptor. However, it is widely accepted that the full-length receptor is typically abundant in neurons, whereas the truncated form is mainly found in glial cells (Reichardt, 2003). This suggests that the TrkB receptor overexpressed by astrocytes that we observed in our study 1 and 2 weeks post-ischemia may correspond to the truncated form and that it may be involved in the regulation of neuroblast migration by controlling extracellular BDNF availability. Wong et al. (1997) also described a similar robust overexpression of truncated TrkB that persisted for at least 2 months post-injury.

4.4.2 Molecular mechanisms and dynamics of SVZ neuroblast migration in the ischemic area

We explored the dynamic patterns of post-stroke migration by real-time video-imaging. It has been previously reported that neuroblasts in the ischemic striatum migrate at an average speed of 28.67 ± 1.04 μm/h (Zhang et al., 2009) or 33.1 ± 1.6 μm/h (Kojima et al., 2010). In these studies, images

were acquired every 15 min (Zhang et al., 2009) or 30 min (Kojima et al., 2010), and the calculation of the migratory speed encompassed both the resting and migratory phases (total displacement per time of recording). Since we acquired images every 30 s, we were able to separate the two phases and calculate the speed of cells migrating within the ischemic striatum or along the RMS solely during the migratory period. While our results revealed a marked difference in the duration and periodicity of the migratory and stationary phases in the ischemic striatum and RMS, we observed no changes in the speed of migration. Cells migrating in the striatum were actively screening the environment in different directions, with multiple extensions and retractions of the leading process and a highly dynamic growth cone. However, as soon as the migratory event was initiated, the neuroblasts migrated at the same speed as they do in the adult RMS. The increased exploratory behavior of the neuronal precursors in the injured striatum might have been caused by the "unusual" microenvironment, which does not fully recapitulate the cellular and molecular organization that facilitates neuroblast migration in the adult RMS. Moreover, invading cells face a detrimental environment rich in cell debris and death signals that might affect the migration of new neurons. These factors may account for the higher stationary periods observed during injury-induced migration compared to migration in the RMS.

BDNF plays a pivotal role in neuronal migration during development (Borghesani et al., 2002; Polleux et al., 2002; Marin and Rubenstein, 2003) and in adulthood (Snapyan et al., 2009; Bath et al., 2011). In the present study, we demonstrated that injury-induced migration also relies on a BDNF-dependent mechanism similar to constitutive neuronal migration in the adult SVZ-OB pathway. As in the adult RMS (Snapyan et al., 2009), 1) the migration of p75NTR-expressing neuroblasts occurred along the vasculature scaffold that synthesizes BDNF, 2) migration was fostered by BDNF, and 3) likely regulated by TrkB-expressing astrocytes that envelop the vessels and bind BDNF, thus

controlling the extracellular levels of this trophic factor. The similarities between the mechanisms of constitutive and injury-induced vasculature-dependent migration of neuronal precursors suggests that some of the mechanisms controlling neuroblast migration in the adult RMS might be activated in the compromised striatum to recruit new cells. While weak, the spontaneous recruitment of neuronal precursors in the ischemic striatum suggests that there is potential for developing cell therapies that rely on promoting cell survival and/or injury-induced migration.

CHAPTER 5

General discussion and main conclusions

5.1 General discussion

Tissue regeneration is a biological feature of all multicellular organisms, including fungi, plants and animals. Interestingly, among vertebrates, amphibians and reptiles have their CNS tissue efficiently restored after injury. In stark contrast, however, mammals seem to be specially deprived of a meaningful regeneration of the damaged brain tissue, which may be the price paid for the considerable gain in complexity of the mammalian brain. Accordingly, brain insults in mammals result only in a limited attempt of regeneration, by mobilization of progenitor cells from the endogenous reserves of NSCs to the injured areas, but weak survival and little or none functional integration. Indeed, no reconstruction of the circuitries is achieved.

Along the last decades, regenerative medicine of the brain has been a growing field, witnessing great efforts from researchers to design strategies envisioning brain repair, by either transplantation of stem cells in the depleted injured area, or by potentiation of the endogenous self-repair mechanism. The present thesis explores particular aspects of NSCs biology – differentiation and migration – as main targets to be modulated in the development of cell replacement strategies. As mentioned previously, transplantation strategies may require a directed differentiation of the transplanted cells in the desired cell phenotype, while the spontaneous migration of precursors towards injured areas needs to be bolstered, in order that these strategies may harbor eventual therapeutic potential. In agreement, the present work encompasses three main parts 1) the development of a method that functionally discriminates oligodendrocytes among other cells in postnatal NSCs cultures, thus useful to screen oligodendrogenic factors that are important in the context of

demyelinating diseases; 2) the effect of the altered microenvironment upon demyelinating injury *per se* in the differentiation of postnatal NSCs into the oligodendrocyte cell lineage; 3) the role of BDNF in the vasculature-mediated ischemia-induced migration of NSCs from the adult SVZ to the nearby ischemic striatum.

5.1.1 Functional discrimination of oligodendrocytes among other cells in postnatal NSC cultures by application of thrombin

Oligodendrocytes harbor essential roles in the normal physiology of the CNS, by forming the myelin sheath, which provides insulation to neuronal axons. These cells represent a small minority of the progeny obtained from postnatal or adult SVZ cell cultures (Gritti et al., 2009). Therefore, prospects of repairing demyelinated brain areas through the insertion of SVZ-derived cells with myelinating potential in the depleted area need adjuvant treatments to sculp cells fate favoring the oligodendrocytic lineage. In this context, we developed a protocol that allows a rapid screening of oligodendrogenic factors (see Chapter 3). The method is based on monitoring calcium oscillations at the single cell level, in SVZ cell cultures, under application of a sequence of cell type-specific stimuli. We used bath applications of KCl and histamine to evoke calcium-mediated responses in neurons and immature cells, respectively, in agreement with previous work from our group (Agasse et al., 2008a). We then applied thrombin and registered thrombin-induced calcium rises in oligodendrocytes but not in other cells in the SVZ culture. Interestingly, the increase of $[Ca^{2+}]_i$ upon thrombin appears to be more robust in later stages of the oligodendrocyte development, when cells express PLP, a myelin protein. On the other hand, early stages of the oligodendrocyte development, expressing NG2 proteoglycan, can show sensitivity to histamine only, to both histamine and thrombin, or solely to thrombin. Together, these data may indicate that responsiveness to thrombin is acquired during the maturation of oligodendrocytes. Thrombin-induced

$[Ca^{2+}]_i$ increase in oligodendrocytes is mediated by PAR-1 receptor, and downstream $G_{q/11}$ proteins that, on their turn, lead to the activation of PLC and consequent mobilization of calcium ions from the intracellular compartments. As a proof of principle, the pre-treatment of the SVZ neurospheres with well-described neurogenic (SCF), oligodendrogenic (T3) or astrogliogenic (CNTF) compounds, resulted in evident shifts towards a KCl- or thrombin-responsive, or non-responsive calcium profile, at the expense of a histamine-responsive calcium profile.

Importantly, besides T3, other compounds that spur oligodendrocytes differentiation and myelination need to be identified. Likely, a cocktail of factors may be necessary to provide a meaningful amount of oligodendrocytes precursors, foster their differentiation in the host tissue and facilitate myelination of the host neuronal axons. The technological platform described in Chapter 3 may therefore help revealing drugs that prime NSCs to the oligodendroglial fate, or that induce their maturation in myelin-producing oligodendrocytes. Besides their potential use for cell transplantation strategies to improve remyelination, such drugs can also be applied in the demyelinated brain, in order to favor the oligodendrocytic differentiation of the endogenous SVZ cells that are recruited to the demyelinated areas, and help sustaining their maturation. Noteworthy, as referred in Chapter 1, SVZ cells may be important as a source of myelinating cells but also as immunomodulators of the diseased microenvironment in animal models of MS (Martino and Pluchino, 2006; Ben-Hur, 2008; Giannakopoulou et al., 2011).

5.1.2 Effect of the altered microenvironment provided by a demyelinating injury in oligodendrogenesis of grafted NSCs

Assessing the patterns of differentiation of NSCs *in vitro* constitutes a powerful tool to test a high number of compounds as putative inducers of a specific cell phenotype. However, an approximation to the *in vivo* scenario,

where the disease itself may influence cells differentiation, should be evaluated. With this purpose, we grafted eGFP-SVZ neurospheres in demyelinated or intact hippocampal slices, both cultured from postnatal mouse brain, and investigate the differentiation of the SVZ cells in oligodendrocytes (see Chapter 3). To provide enrichment in oligodendrocytes within the cell population emerging from the neurosphere we treated the co-cultures with T3. We detected an instructive role of the demyelinated tissue environment on the differentiation of SVZ cells toward the oligodendrocytic phenotype. However, the mechanism triggering SVZ cell specification in the oligodendrocytic lineage in a demyelinated environment is not yet understood. This effect may result from secreted or cell-contact cues that are present in the demyelinated but not in the intact tissue. CPZ intoxication leads to the degeneration of oligodendrocytes and consequent disruption of myelin, followed by an accumulation of activated microglia and astrogliosis in the demyelinated tracts (Hiremath et al., 1998; Matsushima and Morell, 2001; Skripuletz et al., 2008; Koutsoudaki et al., 2009). Typically, activated glial cells secrete pro-inflammatory molecules, like cytokines and chemokines, which may guide the differentiation of SVZ cells. One cannot exclude however a possible effect of other elements of the CPZ-induced microenvironment like cell debris and death signals on the differentiation of SVZ cells.

This work is to be continued and aims at dissecting the mechanism of demyelination-induced oligodendrogenesis from transplanted SVZ cells. In detail, we hypothesize that soluble molecules secreted by the demyelinated tissue, possibly inflammatory mediators, may have an instructive role on SVZ cells priming oligodendrogenesis. By running an enzyme-linked immunosorbent assay (ELISA) on the conditioned medium from hippocampal slices subjected to at least 24 h of CPZ treatment, one can identify the molecules whose secretion is increased upon demyelination. A longer exposure of the organotypic slice cultures to the neurotoxicant can be used to better mimic the massive

demyelination of the hippocampus observed in the 6-week CPZ-treated adult mice. Then, the calcium-based method described in Chapter 3 to functionally assess SVZ differentiation patterns *in vitro*, can be used for a quick screen among those molecules to detect which ones may harbor a pro-oligodendrogenic effect on SVZ cells and disclose the receptors involved in such effect. Similar to our experiment of co-culture using T3, the effect of the identified molecules under grafting conditions should be evaluated.

Moreover, we provided here evidence for a sharp demyelination in the hippocampus of adult mice following administration of CPZ during 6 weeks. Having screened a number of endogenous molecules with oligodendrogenic potential in the *ex vivo* model of demyelination, it would be interesting to graft eGFP-SVZ cells in the demyelinated hippocampus of adult mouse and test whether administration of the identified factor or cocktail of factors can increase differentiation of the grafted SVZ cells in oligodendrocytes and remyelination of the axonal tracts, as compared to non-treated mice. Moreover, loss-of-function experiments may be carried, for example, by using SVZ cells from transgenic mice deprived of the receptor involved in the pro-oligodendrogenic effect harbored by the identified soluble factor. Alternatively, one can use the identified molecules to attempt on boosting the differentiation of the endogenous SVZ-derived cells in oligodendrocytes upon CPZ withdrawal, since *per se*, the spontaneous recruitment of SVZ precursors seems to be minor and with a negligenciable role. Altogether these experiments may allow to increase the contribution of SVZ-derived oligodendrocytes in remyelination upon CPZ withdrawal by application of molecules either or not normally secreted in a demyelinated tissue environment.

5.1.3 Role of BDNF in vasculature-mediated migration of neuronal precursors to ischemic brain areas

Previous works reported the recruitment of SVZ cells to ischemic brain areas (Arvidsson et al., 2002; Parent et al., 2002; Jin et al., 2006) and suggested that blood vessels provide a physical support for the injury-induced migration (Ohab et al., 2006; Yamashita et al., 2006; Thored et al., 2007; Zhang et al., 2009; Kojima et al., 2010), revisiting the constitutive migration of neuroblasts along vessels in the RMS and OB. Nevertheless, the molecular mechanism supporting the vasculature-mediated migration in the ischemic striatum was not explored. In Chapter 4 we provide data unveiling a role for BDNF in the vasculature-mediated migration of neuroblasts de-routed from the SVZ to the neighboring ischemic striatum.

We demonstrated that endothelial cells in the ischemic striatum express BDNF, and that the recruited neuroblasts migrate preferentially along blood vessels and maintain expression of p75NTR, the low-affinity receptor for BDNF. Moreover, reactive astrocytes widely distributed in the damaged area were found in close association with the blood vessels and express TrkB, the high-affinity receptor for BDNF. Although no BDNF mRNA was found in astrocytes, these cells were immunopositive for the trophic factor. Importantly, the same expression pattern was observed in the adult RMS where TrkB-expressing astrocytes trap extracellular BDNF allowing thus the entrance of migrating cells into the stationary phase. Using time-lapse imaging in acute brain slices from MCAo-challenged mice, we characterized the dynamics of the injury-induced migration of neuroblasts in the post-stroke striatum, and disclose a role for BDNF in this induced-migration. We showed that neuroblasts migrating in the compromised striatum have higher exploratory behavior and longer stationary periods as compared to neuroblasts migrating in the RMS. Moreover, BDNF promotes neuroblasts migration in the ischemic striatum. Our data thus suggest that the mechanism involved in the injury-induced migration

of neuroblasts in the striatum shares similarities with the mechanism regulating the constitutive migration of neuroblasts in the RMS.

Noteworthy, the majority of the new neurons in the ischemic striatum face cell death likely due to a failure to integrate or to the inflammatory environment. As we show here, although SVZ precursors exhibit tropism to the injury site, one important limitation is the low dynamics of migration in the striatum, which results in the arrival of few cells to the striatum. A possible therapeutic strategy to facilitate cell replacement by the endogenous SVZ after ischemia may include the stimulation of neuroblasts migration, and the control of inflammation and survival. Therefore, the mechanistic insights concerning the injury-induced migration brought up by our work may constitute relevant advances.

Most of the studies on post-stroke neurogenesis, including ours, were performed in young mice, with 2-3 months; however, stroke occurs more frequently in elderly humans. Since neurogenesis abruptly declines with age, a major concern could be that the aged brain may be less prompt to respond to injury by increasing neurogenesis. Nonetheless, it seems that the decrease of neurogenesis with age is due to changes in the environment rather than to an altered potential of the stem cells themselves (Burns et al., 2009). For instance, levels of cytokines that are crucial to sustain neurogenesis, like EGF, bFGF, VEGF and IGF-1, decrease in the course of aging (Enwere et al., 2004; Shetty et al., 2005). However, infusion of such cytokines restores the age-related decrease in neurogenesis (Lichtenwalner et al., 2001; Jin et al., 2003b). Also, isolation of NSCs from young or aged rodents results in similar numbers of neurospheres (Tropepe et al., 1997; Seaberg and van der Kooy, 2002). More importantly, MCAo in young (3 months) and old (15-month-old) rats leads to a comparable level SVZ neurogenesis (Darsalia et al., 2005). Furthermore, post-stroke neurogenesis in humans has been detected in patients up to 84 years old (Jin et al., 2006; Minger et al., 2007). Indeed, immature neurons were observed in the

cortical penumbra surrounding the infarcted area, but never in the naïve human cortex, which is devoid of neurogenesis. Altogether these studies suggest that the neurogenic potential of NSCs in the aged brain may be similar to the one encountered in the young brain, and thus with similar capability of responding to an ischemic brain injury.

Considering the discussed points, the present thesis may add important knowledge on the development of cell replacement therapies based on the transplantation of NSCs in the diseased brain or in the stimulation of the endogenous NSCs.

5.2 Main conclusions

- Thrombin triggers an increase in $[Ca^{2+}]_i$ in oligodendrocytes but not in the other cell types in postnatal SVZ cell cultures.

- KCl, histamine and thrombin can be used to specifically induce rise of $[Ca^{2+}]_i$ in neurons, immature cells and oligodendrocytes, respectively, in a SVZ culture; $[Ca^{2+}]_i$ in SVZ-derived astrocytes remains unaltered during the protocol of stimulation.

- A ratio of the responses to thrombin and to histamine (thrombin/histamine) above 1.3 identifies oligodendrocytes (normalized ratio of fluorescence of the calcium indicator Fura-2AM (340/380) was used to calculate the ratio thrombin/histamine).

- Thrombin-induced $[Ca^{2+}]_i$ increase in oligodendrocytes is mediated by PAR-1 activation, and involves $G_{q/11}$ proteins which, in turn, lead to PLC activation and mobilization of calcium ions from the intracellular stores.

- A protocol based on application of the three pharmacological agents in sequence – KCl, histamine and thrombin – allows the quick evaluation of the differentiation pattern of the SVZ culture.

- SVZ cultures treated with SCF, T3 or CNTF exhibit calcium signatures characterized by an increased number of KCl- or thrombin-responsive or non-responsive cells, as compared to the calcium signature of non-treated cultures, characterized by a high number of histamine-responsive cells.

- Incubation of postnatal hippocampal slice cultures with 25 µM CPZ during 24 h leads to partial demyelination of the hippocampal fiber tracts.

- The demyelinated host tissue environment favors oligodendroglial cell fate in grafted SVZ cells from postnatal mouse brain.

- Neuronal migration in the ischemic striatum is vasculature-dependent and involves astrocytes

- Ischemia induces BDNF expression by endothelial cells, and TrkB receptor expression by reactive astrocytes in the striatum; de-routed neuroblasts migrating in the striatum maintain expression of p75NTR.

- BDNF promotes vasculature-associated migration of neuroblasts in the ischemic striatum

- Injury-induced migration of neuroblasts through the striatal tissue is less dynamic than constitutive migration in the RMS: neuroblasts display higher exploratory behavior and longer resting periods during the saltatory migration in the compromised area.

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