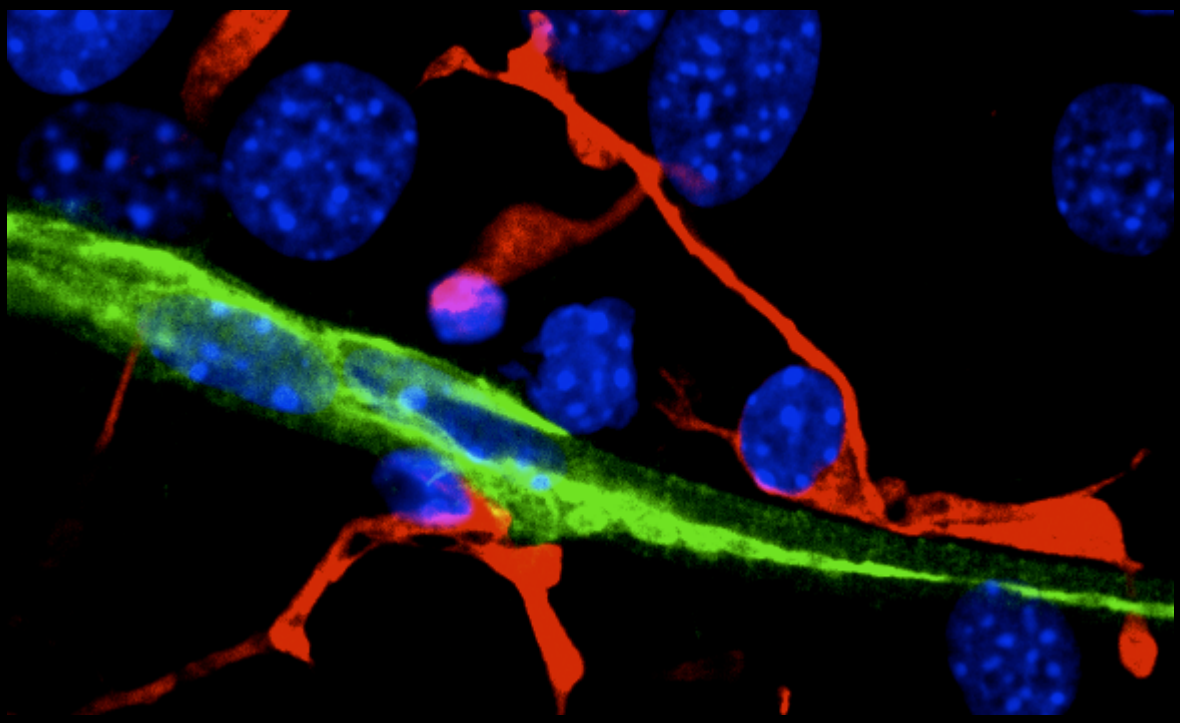
PhD Thesis presented to the Faculty of Medicine, University of Coimbra

Role of intercellular communication between endothelial and stem cells in stemness and neurogenesis



Alexandra Isabel Freitas Rosa, 2011

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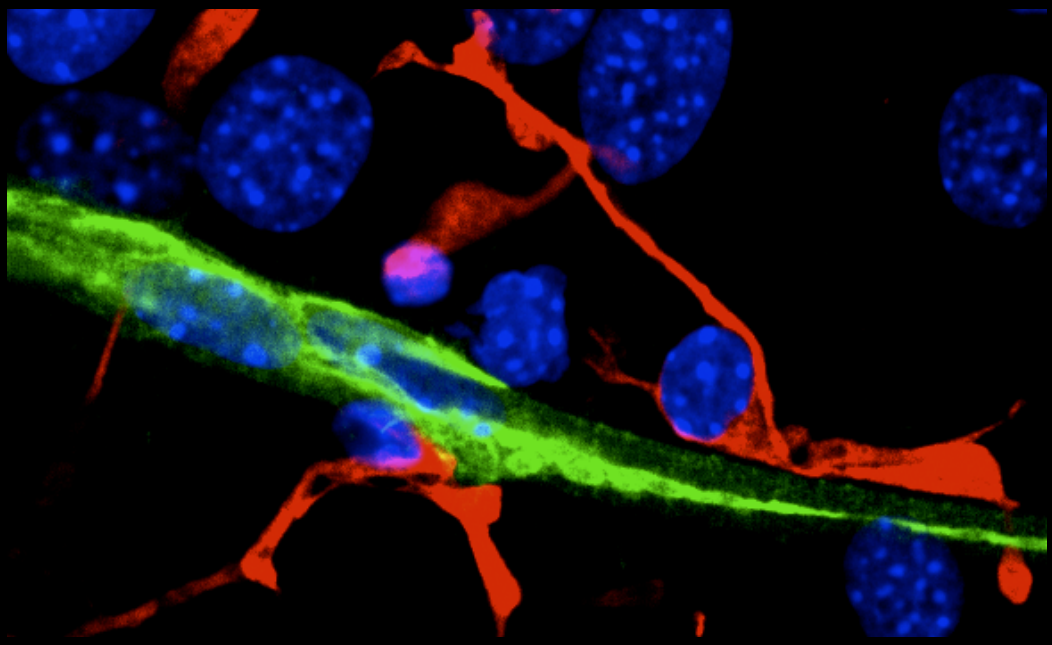
Role of intercellular communication between endothelial and stem cells in stemness and neurogenesis: focus on the soluble factor angiopoietin-1 and direct contact *via* laminin-1/ α6β1 integrin and connexin43 as new targets for brain repair



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PhD Programme
Biologia Experimental e Biomedicina

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Role of intercellular communication between endothelial and stem cells in stemness and neurogenesis: focus on the soluble factor angiopoietin-1 and direct contact *via* laminin-1/α6β1 integrin and connexin43 as new targets for brain repair

Papel da comunicação intercelular entre células endoteliais e células estaminais na *stemness* e neurogénese: papel do factor solúvel angiopoietina-1 e contacto directo *via* laminina-1/integrina α6β1 e conexina43 como novos alvos em reparação cerebral

Dissertação apresentada à Faculdade de Medicina da Universidade de Coimbra, para prestação de provas de Doutoramento em Ciências Biomédicas.

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Index

Abbreviations

α6β1 integrin: Alpha6beta1 Integrin

Ang-1: Angiopoietin-1

Ara-C: Arabinosylcytosine

ATP: Adenosine 5'-triphosphate

BCIP: 5-bromo-4-chloro-3-indolyl phosphate

BrdU: 5-bromo-2-deoxyuridine

BDNF: Brain-derived neurotrophic factor

BBB: Blood-brain barrier

BMP: Bone morphogenetic protein

BSA: Bovine serum albumin

$[Ca^{2+}]_i$: Intracellular calcium

CBX: Carbenoxolone

CD31: Cluster of differentiation 31 (PECAM)

CHX: Cycloheximide

CM: Conditioned medium

CNS: Central nervous system

Cx: Connexin

CXCR4: CXC chemokine receptor 4

DAB: 3,3'-diaminobenzidine

DCX: Doublecortin

DG: Dentate gyrus of the hippocampus

DMEM: Dulbecco's modified eagle medium

ECM: Extracellular matrix

EDTA: Ethylenediaminetetraacetic acid

EGF: Epidermal growth factor

EGFR: EGF receptor

EPO: Erythropoietin

ERK: Extracellular signal-regulated kinase

FAK: Focal adhesion kinase

FBS: Fetal bovine serum

FGF-2: Fibroblast growth factor-2

Fura-2 AM: Acetoxymethyl ester of Fura

GABA: Gamma-aminobutyric acid

GFAP: Glial fibrillary acidic protein

GJ: Gap junction

GJIC: Gap junction intercellular communication

GRO-α: Growth-related oncogene-α

GCL: Granule cell layer

HBSS: Hank's balanced saline solution

HEPES: N-(2-Hydroxyethyl)piperazine-N'-(2-Etanesulfonic acid)

HGF/SF: Hepatocyte growth factor/scatter factor

HVC: High vocal centre

ICV: Intracerebroventricular

IGF-I: Insulin-like growth factor-I

IL-6: Interleukin-6

IL-8: Interleukin-8

JNK/SAPK: c-Jun N-terminal kinase/stress-activated protein kinase

KA: Kainic Acid

LIF: Leukemia inhibitory factor

MAP-2: Microtubule-associated protein 2

MAPK: Mitogen-activated protein kinase

mBEC: Mouse brain endothelial cell

mCD24: Mouse cluster of differentiation 24

mRNA: Messenger RNA

mTOR: Mammalian target of rapamycin kinase

Lam-1: Laminin-1

LT-HSC: Long-term hematopoietic stem cell

MEK: Mitogen-activated protein extracellular signal-regulated kinase

NBT: Nitro blue tetrazolium chloride

NeuN: Neuronal nuclei

NG2: Chondroitin sulphate proteoglycan

NO: Nitric oxide

NPC: Neural progenitor cell

NPY: Neuropeptide Y

NSC: Neural stem cell

OB: Olfactory bulb

PBS: Phosphate buffered saline

PDGF: Platelet-derived growth factor

PDGFRα: Platelet-derived growth factor receptor-α

PECAM: Platelet/endothelium cell adhesion molecule (CD31)

PFA: Paraformaldehyde

Pi3K: Phosphoinositide kinase

PSA-NCAM: Polysiliated neural cell adhesion molecule

RMS: Rostral migratory stream

RT: Room temperature

RT-PCR: Reverse transcription polymerase chain reaction

SCCI: Single cell calcium imaging

SDF-1: Stromal cell-derived factor-1 (CXCL12)

SCF: Stem cell factor

SDNSF: Stem cell-derived neural stem/progenitor cell supporting factor

SE: *Status Epilepticus*

SFM: Serum-free medium

SGZ: Subgranular zone

Shh: Sonic hedgehog protein

SMA: Smooth muscle actin

SVZ: Subventricular zone

TGF: Transforming growth factor

TGFR: Transforming growth factor receptor

TLX: Transcription factor tailless

TUNEL: Terminal deoxynucleotidyl transferase dUTP nick-end labelling

VEGF: Vascular endothelial growth factor

VEGFR2: Vascular endothelial growth factor receptor 2

VWf: Von Willebrand factor

VZ: Ventricular zone

Publications

Rosa AI, Gonçalves J, Cortes L, Bernardino L, Malva JO, Agasse F. The angiogenic factor angiopoietin-1 is a proneurogenic peptide on subventricular zone stem/progenitor cells, J Neurosci, 2010.

Maria Francisca Eiriz, Sofia Grade, Alexandra Rosa, Sara Xapelli, Liliana Bernardino, Fabienne Agasse and João O. Malva, Functional evaluation of neural stem cell differentiation by single cell calcium imaging, Current Stem Cell Research and Therapy, *in press*.

Abstract

The subventricular zone (SVZ) of the adult mammalian brain is an endogenous reservoir of stem cells that produce neurons during the lifespan of the organism by a process designated neurogenesis. Understanding the regulation of neurogenesis in the SVZ is of particular interest when developing new therapeutic strategies for cell replacement. It has been recently shown that the vasculature in neurogenic niches modulates neurogenesis. Indeed, endothelial cell-derived soluble and contact factors are crucial for the maintenance of SVZ cells' characteristics and neurogenesis. However, the cellular and molecular mechanisms underlying this regulation are hardly unveiled. The present thesis was undertaken in order to (i) clarify whether heterocellular contacts between endothelial and SVZ cells regulate stemness and neurogenesis, (ii) disclose the involvement of the endothelial-derived diffusible factor angiopoietin-1 (Ang-1), and direct heterocellular contact molecules such as laminin-1/$\alpha 6\beta 1$ integrin and connexin (Cx) 43, in the observed effects, (iii) assess whether changes in the microvasculature upon injury correlate with enhancement of neurogenesis *in vivo*.

We first showed that Ang-1 and its receptor Tie-2 are expressed by SVZ cells. Ang-1 positively regulates SVZ cell proliferation and neuronal differentiation in cultures. *In vivo*, Ang-1 is found in vessels and ependymal cells, while Tie-2 is expressed by endothelial cells, by stem/progenitor cells and neuroblasts in the SVZ, by neuroblasts in the rostral migratory stream (RMS) and by mature neurons in the olfactory bulb (OB). This study shows that Ang-1, a major player of angiogenesis, promotes SVZ neurogenesis.

We then disclosed that direct heterocellular contacts promote proliferation and stemness as observed in co-cultures of SVZ cells and mouse brain endothelial cells (mBEC). It has been recently shown that interaction between α6ß1 integrin expressed by SVZ cells with vessel-derived laminin-1 may account for the

maintenance of the stem cell state of SVZ cells. Moreover, in the stem cell niche of the bone, junctional communication between endothelial cells and progenitor cells modulates osteoblast differentiation. In our model, we verified that SVZ neurospheres express α6ß1 integrin while mBEC express laminin-1. Both cell types express the gap junction (GJ) channel protein Cx43. Disruption of laminin-1/α6ß1 integrin and gap junction intercellular communication (GJIC) using incubations with a neutralizing antibody against α6 integrin or the GJIC blocker carbenoxolone, respectively, inhibited the effects observed in control co-cultures. Therefore, heterocellular contacts *via* integrins and GJs play a crucial role in maintaining SVZ homeostasis.

Furthermore, because interaction with endothelial cells controls SVZ dynamics, we hypothesized that injury-induced neurogenesis may be triggered by changes affecting the microvascular network. In a model of temporal lobe epilepsy, we observed that the increase in SVZ cells' proliferation correlates with an increase in microvascular network density, 7 days after seizure induction. Although exploratory, these results lead us to foresee microvascular changes upon injury as a pre-requisite for injury-induced neurogenesis. Further experiments have to be completed to test this hypothesis.

This thesis expands the existing knowledge regarding the modulatory role exerted by endothelial cells and vessels on SVZ stem cells, a research area that may become relevant in the cell therapy context.

Resumo

A zona subventricular (ZSV) do cérebro adulto de mamíferos constitui um reservatório endógeno de células estaminais. Recentemente foi demonstrado que a vasculatura dos nichos neurogénicos, e especialmente a interacção entre células endoteliais e células estaminais/progenitoras da ZSV, é crucial para a manutenção das características das células da ZSV e permite a manutenção contínua da neurogénese. No entanto, os mecanismos celulares e moleculares subjacentes a esta regulação ainda são pouco conhecidos. A presente tese procura (i) clarificar se o contacto heterocelular entre células endoteliais e células da ZSV regula a neurogénese e as capacidades estaminais; (ii) avaliar o papel do factor solúvel de origem endotelial angiopoietina-1 (Ang-1) e dos contactos heterocelulares directos mediados por laminina-1/integrina $\alpha 6\beta 1$ e conexina (Cx) 43 nos efeitos observados; (iii) verificar se as mudanças na microvasculatura induzidas após lesão cerebral se correlacionam com um aumento de neurogénese *in vivo*.

Verificámos que a Ang-1 faz parte do nicho neurogénico e é expressa em células da ZSV. Além disso, estas células expressam o receptor da Ang-1, o Tie-2. A Ang-1 regula positivamente a proliferação celular e a diferenciação neuronal em culturas de células da ZSV. *In vivo*, a Ang-1 é encontrada em vasos sanguíneos e células ependimais, enquanto o Tie-2 é expresso em células endoteliais, células estaminais/progenitoras e neuroblastos na ZSV, neuroblastos na via migratória rostral e neurónios maduros no bolbo olfactivo. Este estudo revelou que a Ang-1, crucial na angiogénese, promove a neurogénese na ZSV.

Foi verificado em co-culturas de células estaminais da ZSV e de células endoteliais de cérebro de murganho que os contactos heterocelulares directos promovem a proliferação celular e as características estaminais da ZSV. Estudos recentes demonstraram que a interacção entre a integrina α6ß1, expressa pelas células ZSV, e a laminina-1 endotelial poderá ser responsável pela manutenção do

estado estaminal das células ZSV. Além disto, no nicho estaminal do osso, a comunicação junccional entre células endoteliais e progenitoras modula a diferenciação dos osteoblastos. No nosso modelo, foi verificado que as esferas de ZSV expressam integrina α6ß1 enquanto as células endoteliais de cérebro de murganho expressam laminina-1. Os dois tipos celulares expressam Cx43. A disrupção das interacções laminina-1/integrina α6ß1 e das comunicações intercelulares juncionais através da utilização de um anticorpo neutralizante anti-integrina α6 e de um inibidor de comunicações juncionais, a carbenoxolona (CBX), respectivamente, resulta na inibição da proliferação e *stemness* observados em culturas controlo. Assim, os contactos intercelulares directos *via* laminina-1/integrina α6ß1 ou comunicações juncionais mantêm a homeostase da ZSV.

Finalmente, porque a interacção com células endoteliais controla a manutenção da dinâmica da ZSV, colocámos a hipótese de que após lesão cerebral a estimulação da neurogénese poderia ser despoletada por alterações morfofuncionais da rede microvascular. Num modelo de epilepsia do lobo temporal, foi verificado um aumento de proliferação das células da ZSV, que se correlaciona com um aumento de densidade da microvasculatura nesta região cerebral. Embora exploratórios, estes resultados indicam que as alterações na microvasculatura após lesão cerebral poderão ser um pré-requisito para a neurogénese induzida pela lesão. No entanto, estudos adicionais serão necessários para clarificar este ponto.

A presente tese adiciona mais elementos ao conhecimento relativo ao nicho neurovascular da ZSV, em particular no que respeita às características do nicho neurogénico. Estes dados são de extrema relevância no contexto da terapia celular, na medida em que poderão contribuir para novas abordagens conducentes à substituição celular.

Chapter 1 – Introduction

1. Introduction

The mammalian brain has been, for a long time, considered a rigid structure devoid of any regeneration capacity. According to this concept, at birth the brain is endowed with a stock of neurons that would inevitably decrease with aging and disease. This old dogma has nonetheless been challenged with the discovery of two brain areas capable of originating neurons in the adult animal: the subventricular zone (SVZ) of the lateral ventricle and the dentate gyrus (DG) of the hippocampus. In these areas, neurogenesis occurs from stem/progenitor cells with capacity to proliferate, migrate and differentiate into different phenotypes.

Local environment regulates the dynamics of stem cells. With the introduction of the stem cell niche concept in 1978, Shofield described the existence of an environment capable of keeping stem cells undifferentiated and in a proliferative state (Shofield *et al*., 1978). This concept has barely changed with years and, in a given tissue, the niche can be defined as a microenvironment capable of instructing stem cells to proliferate and differentiate, but also capable of maintaining them in a quiescent state. The stem cell niche is constituted by diffusible factors, cell-extracellular matrix (ECM) interactions and cell-cell interactions (Doetsch, 2003; Li and Xie, 2005; Moyse *et al.,* 2008), with the vasculature also playing a relevant role (Yoshida *et al*., 2007; Shen *et al.*, 2008; Tavazoie *et al.*, 2008). At the long term, a detailed knowledge of the molecular and cellular components of the stem cell niche is extremely relevant to design strategies for cell replacement and cancer therapies.

In this thesis, we have studied the role of intercellular communication between endothelial cells and stem cells in stemness and neurogenesis, with a particular focus on the soluble factor angiopoietin-1 (Ang-1) and on direct heterocellular contacts *via* endothelial laminin-1/ SVZ $\alpha 6\beta 1$ integrin and endothelial/SVZ connexin (Cx) 43 gap junction intercellular communications (GJICs), as new targets for brain repair.

1.1. Stem cells: basic biology

A stem cell is a multipotent cell endowed with the capacity to proliferate and self-renew. Stem cells give rise to progenitors that differentiate into a variety of other cell types. Stem cells with the largest differentiation capacities are present in the embryo at the zygote stage. These cells are totipotent i.e. they retain the ability to divide and produce all the differentiated cells in an organism, including extra-embryonic tissues. About 5 days after fertilization in humans, the blastula is formed (Figure 1.1.A). At this stage the embryo is a spherical structure possessing an inner cell mass (ICM), or embryoblast, which subsequently forms the embryo, and an outer layer of cells, or trophoblast, which later forms the placenta. The trophoblast surrounds the ICM and a fluid-filled blastocyst cavity known as the blastocoele. It is in the ICM that the so-called embryonic stem (ES) cells reside. These cells are capable of originating all the tissues in the organism and are therefore called pluripotent (Evans and Kaufman, 1981; Henderson, 2008). Recently, a lot of attention has been given to induced pluripotent stem (iPS) cells, artificially derived from non-pluripotent cells, typically adult somatic cells, by inducing a "forced" expression of certain genes (Figure 1.1 B). The generated iPS cells are remarkably similar to naturally-isolated pluripotent stem cells and solve the ethical problem of using an embryo to obtain stem cells (Takahashi and Yamanaka, 2006; Yamanaka, 2009).

In the adult, stem cells with the capacity of originating various cell types within one tissue are also present. These cells are called multipotent and possess three main characteristics (Hall and Watt, 1989):

a) Capacity to maintain mitotic activity during the lifespan of the organism;
b) Capacity to self-renewal, i.e. the endogenous stock of cells is maintained by symmetric and asymmetric divisions in which one stem cell originates two similar daughter stem cells or one daughter stem cell and one progenitor cell, respectively;

c) Capacity to generate progenitor cells: the asymmetric division of stem cells produces progenitors or precursors that proliferate temporarily and undergo differentiation in various cellular phenotypes.

1.2. The brain: from immutable to neurogenic

In the beginning of the XXth century, Santiago Ramón y Cajal revealed the complex structure of the brain where several ramified cells are deeply entwined (Figure 1.2.). One of the conclusions derived from such observation was that the brain would be deprived of regeneration capacities, as it seemed virtually impossible that upon neuronal death, the exact same functional contacts could be re-established between a newborn neuron and neighbouring cells. A few decades later, however, Joseph Altman reported the existence of newborn neurons in the adult rat brain in the hippocampus (Altman and Das, 1965; Altman, 1969). The scientific community resisted to this new idea and in 1972, Privat and Leblond identified the SVZ as a reservoir of glial cells. Additionally, a few years later, a study using tritiated thymidine suggested that no new neurons could be generated in the adult brain (Rakic *et al*., 1985). It seemed like, indeed, the brain was immutable and the old dogma persisted.

Neurogenesis studies concerning the adult brain restarted only two decades ago. These studies focused on the generation of new neurons in a region of the adult canary's telencephalon, known as the high vocal centre (HVC), which contributed to the singing process. Newly-generated neurons had their origin in stem cells in the ventricular zone (VZ) of the HVC (Alvarez-Buylla *et al*., 1990). Neurogenesis from stem cells was also reported in the central nervous system of reptiles, amphibians and fish (for a review, see García-Verdugo *et al*., 2002).

It was in 1992 that the presence of stem cells in the SVZ of the adult mouse was finally demonstrated, by Reynolds and Weiss. Single cells derived from the adult SVZ were induced to proliferate in the presence of epidermal growth factor (EGF) and the authors obtained, in this way, clonal aggregates, designated neurospheres,

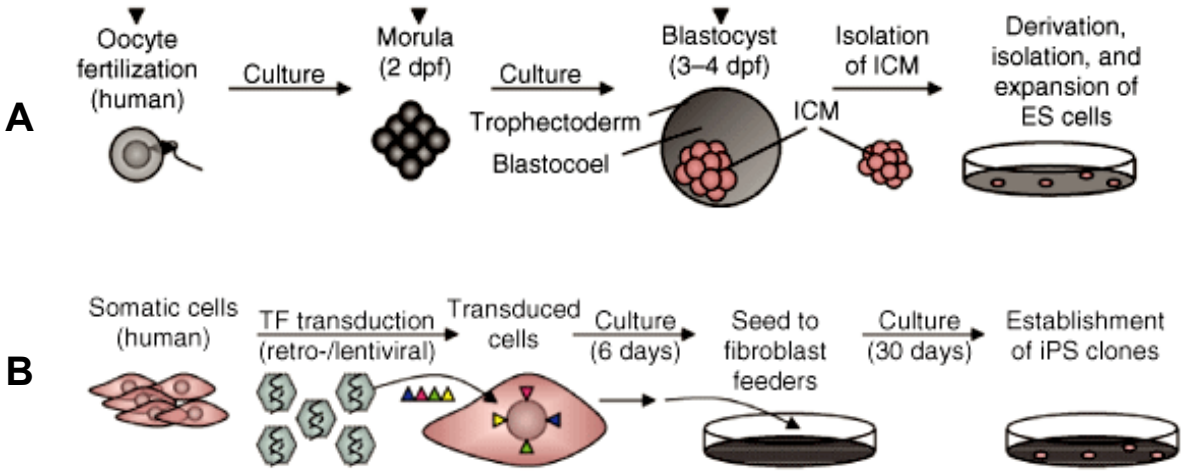


Figure 1.1. ***A***, Derivation of human embryonic stem (ES) cells. Prepared oocytes can be fertilized and cultured *in vitro* resulting in a morula (4-16 totipotent cells), followed by a blastocyst (16-40-cell stage). During this period, the inner cell mass (ICM) becomes apparent. The isolation of pluripotent cells of the ICM from the trophectoderm allows their culture to permanent ES cell lines. Vertical arrowheads indicate points where human material can be donated to generate ES cells. dpf, days post fertilization. ***B***, Generation of induced pluripotent stem (iPS) cell lineages. Somatic cells are transduced with a minimal set of transcription factors required for cellular reprogramming (current protocols use Oct4, SOX-2, and Klf4). Current methods use retroviruses to maximize cellular transfection and reprogramming, but other transduction methods appear feasible. As with ES cells, after nuclear reprogramming, the colonies are typically transferred onto fibroblast feeder layers (however, feeder-free methods also exist). The cultures are subsequently incubated in standard ES media plus basic fibroblast growth factor for 30 days to establish iPS cell colonies. Adapted from Henderson, 2008.

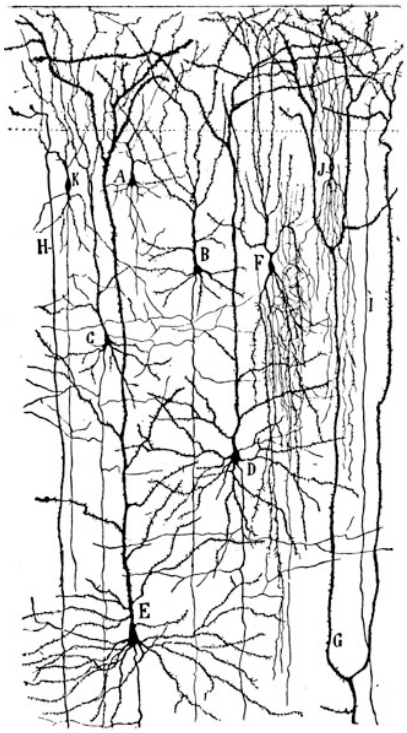


Figure 1.2. Drawing of human frontal cortex neurons by Santiago Ramón y Cajal, revealing the complexity of the neuronal network. ***A-E***, Pyramidal cells; ***F, J*** and ***K***, Interneurons; ***G, H*** and ***I***, Nervous fibers. Adapted from http:// nobelprize.org/nobel_prizes/medicine/articles/cajal/.

constituted by both stem cells and progenitors with limited proliferation capacities. When 6-8 days-old neurospheres were dissociated, 67% of dissociated cells generated new neurospheres, demonstrating the capacity of self-renewal of these cells. After 21 days in culture, differentiated cells were observed in the periphery of neurospheres, such as astrocytes and chains of migrating neurons (Reynolds and Weiss, 1992). It was therefore clear that the SVZ hosts cells that obey the criteria of stem cells (Figure 1.3.A and B). Besides astrocytes and neurons, SVZ progenitors can also generate oligodendrocytes (Levison and Goldman, 1997). *In vivo*, the three cellular types are also generated (Lois and Alvarez-Buylla, 1993; Levison and Goldman, 1993). Glial progenitors migrate through the white matter or *via corpus callosum* and differentiate into astrocytes or oligodendrocytes (Levison *et al*., 1993; Nait-Oumesmar *et al*., 1999; Kakita *et al*., 2003; Menn *et al*., 2006). Newly-generated neuroblasts migrate rostrally into the OB where they differentiate into inhibitory interneurons (Lois and Alvarez-Buylla, 1993; Luskin, 1993; Betarbet *et al*., 1996; Lois *et al*., 1996; Doetsch *et al*. 1997; Luskin *et al*., 1997; García-Verdugo *et al*., 1998; Kornack and Rakic, 2001; Bédard and Parent, 2004; lledo *et al.*, 2006) (Figure 1.4.).

Stem cells from the SVZ are also present in humans and although their functions *in vivo* are poorly understood it is possible they play a role in brain repair (Macas *et al*., 2006; Martí-Fàbregas *et al.,* 2010). SVZ stem/progenitor cells in both animals and humans are maintained during the lifespan of the organism, and decrease with aging (Palmer *et al*., 2001; Maslov *et al*., 2004).

Neurogenesis in the rodent's hippocampus, was firstly observed by Altman and Das in 1965. However, it was not until the late 90's that neurogenesis was associated to the presence of progenitor cells (Gage *et al*., 1998). According to some authors, cells originating neurons in the hippocampus are not stem but progenitor cells since colonies obtained in culture are limited as far as self-renewal and multipotency capacities are concerned (Seaberg and van der Kooy, 2002; Bull and Bartlett, 2005). Nonetheless, other evidences point to the existence of stem cells in

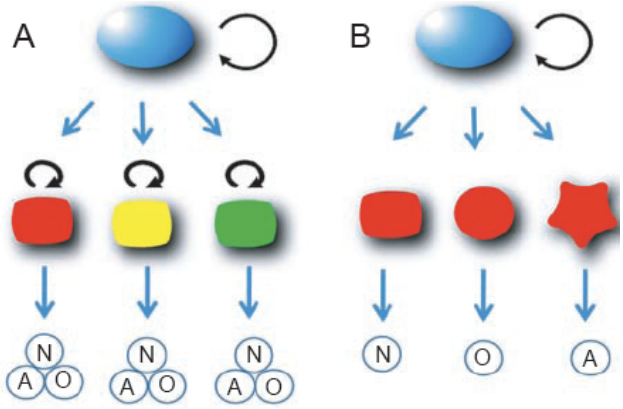


Figure 1.3. The lineage models of adult NSCs in the mammalian brain. **(A)** Adult NSCs (red, green and yellow) generated from primitive NSCs (blue) are intrinsically diverse, exhibiting vastly different developmental potential depending on their regions of distribution and developmental origins. **(B)** Adult NSCs are relatively homogenous (blue) and give rise to a heterogeneous population of lineage-restricted progenitors. Adapted from Ma *et al*., 2009.

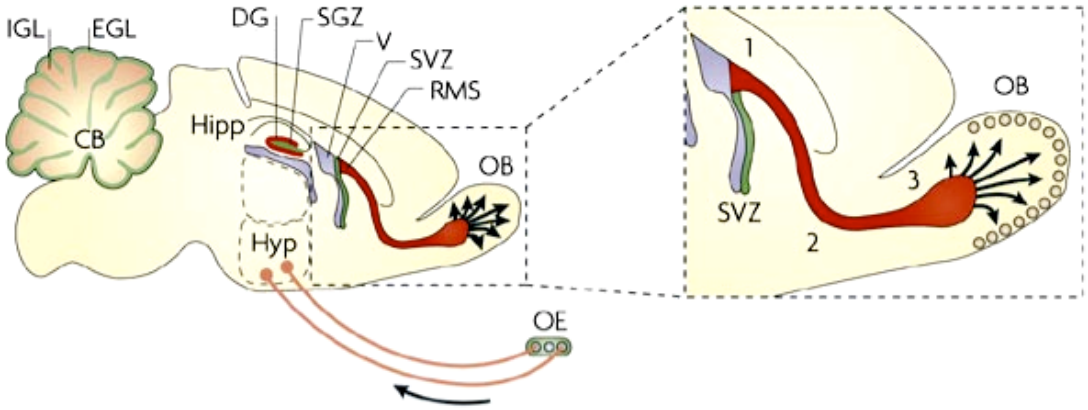


Figure 1.4. In rodent brains postnatal neuronal migration is evident in three main areas: the cerebellum (CB), the hippocampus (Hipp) and the rostral migratory stream (RMS). A small number of neurons also complete their migration into the hypothalamus (Hyp) at around the time of birth. Distinct germinal zones (green) give rise to neurons that migrate to adjacent target zones (red). Lighter shade indicates that migration in these regions occurs primarily during the very early postnatal period and does not persist into adulthood. Cells born in the anterior subventricular zone (SVZ, inset) initiate their migration from the SVZ (**1**) as chains (**2**) streaming towards the olfactory bulb (OB, **3**), where they end their migration. Adapted from Ghashghaei *et al.,* 2007. DG, dentate gyrus; EGL, external granule cell layer; IGL, internal granule cell layer; OE, olfactory epithelium; SGZ, subgranular zone; V, ventricle.

the adult hippocampus (Ables *et al*., 2010; Lugert *et al*., 2010).

Neurogenesis in the hippocampus has been evidenced in non-human primates and in humans (Eriksson *et al*., 1998; Gould *et al*., 1999). In the human, adult neurogenesis seems to contribute to the temporal information of memories of facts and events (Aimone *et al*., 2006; Zhao *et al*., 2008). Impairment of hippocampal neurogenesis is associated with pathologies such as schizophrenia (Eriksson *et al*., 2006) and depression (Sahay and Hen, 2007; Ho and Wang, 2010).

1.3. The adult brain hosts neural stem cell niches

Two main regions in the adult brain harbour neural stem cell niches and sustain the capacity of generating new neurons from stem/progenitor cells. These areas are the SVZ, lining the lateral ventricle, and the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG).

1.3.1. The SVZ, RMS and OB system

1.3.1.1. Cellular organization of the SVZ niche, RMS and OB

The SVZ is located in the lateral walls of the lateral ventricles and it is composed of several distinct cellular types which express different phenotypic markers (Table 1.1.): putative neural stem cells that label for glial fibrillary acidic protein (GFAP) (type B cells), immature precursors (type C cells), immature neurons or neuroblasts (type A cells), tanycytes and ependymal cells. The SVZ is isolated from the ventricular cavity by a layer of non-ciliated cells with microvilli, the tanycytes (type D cells) and ciliated cells, the ependymal cells (type E cells) (Doetsch *et al*., 1997). A closer look at the ventricular surface reveals a striking pinwheel organization. The pinwheel's core contains the apical endings of B cells and in its periphery two types of ependymal cells: multiciliated (E1) and a type (E2) characterized by only two cilia and very complex basal bodies (Figure 1.5.) (Mirzadeh *et al*., 2008). The SVZ also comprises a specialized vasculature, lacking astrocyte endfeet coverage (Tavazoie

Antigenic marker	A cell (Neuroblast)	B cell (Astrocyte)	C cell (Progenitor)	D cell (Tanycyte)	E cell (Ependymal cell)
PSA-NCAM	+	-	-	-	-
β-tubulin III	+	-	-	-	-
GFAP	-	+	-	+	+
Nestin	+	+	+	n.d.	+
Dlx-2	+	-	+	-	-
EGF-R	-	+/-	+	n.d.	n.d.
mCD24	+	-	-	+	+
LeX	-	+	+	-	-
CD133	-	-	-	-	-

Table 1.1. β-tubulin III: neuronal cytoskeleton protein; CD133: transmembranar glycoprotein, also known as prominin: Dlx-2: transcription factor; EGF-R transmembranar receptor for the EGF growth factor; GFAP: Glial Fibrillary Acidic Protein, astrocyte cytoskeleton protein; LeX or LeX/ssEA-1 or CD15: sugar expressed on the cellular surface; mCD24: mouse cluster of differentiation 24, transmembranar protein; Nestin: cytoskeleton protein of neuroepithelial cells; PSA-NCAM: polyasylated form of the Neural Cell Adhesion Molecule, expressed by migrating neuroblasts. +: the antigenic marker is expressed; - : the antigenic marker is not expressed; +/-: a minority of the cells of this cell type express the antigenic marker; n.d.: non determined (Doetsch *et al.*, 1997 and 2002; Belvindrah *et al.*, 2002; Capela and Temple 2002; Coskun *et al.*, 2008).

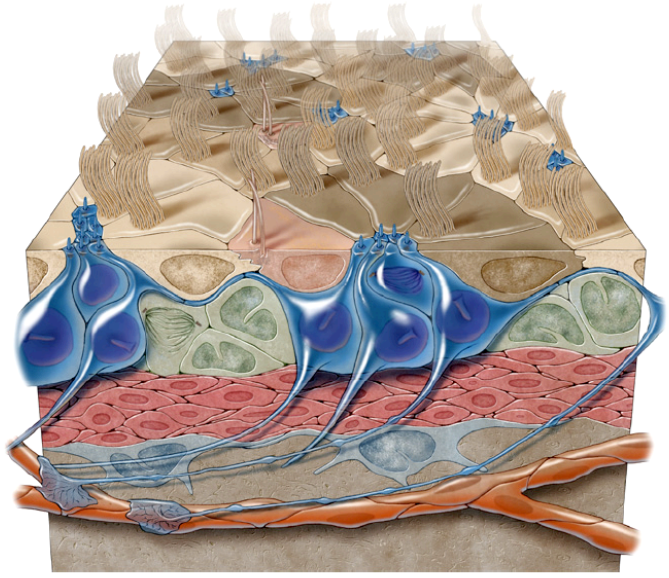


Figure 1.5. Three-dimensional model of the adult subventricular zone (SVZ) neurogenic niche illustrating B cells (blue), C cells (green), and A cells (red). B cells have a long basal process that terminates on blood vessels (orange) and an apical ending at the ventricle surface. Ependymal cells encircling B apical surfaces exhibit a pinwheel organization (light and dark brown). E2 cells are peach. Adapted from Mirzadeh *et al.*, 2008.

et al., 2008). Neural stem/progenitor cells lie close to blood vessels (Tavazoie *et al*., 2008; Shen *et al*., 2008) and extend a basal process terminating on blood vessels in the form of specialized endfeet that may facilitate responses to perivascular ECM (Mirzadeh *et al*., 2008).

B and C cells differ not only from their antigenic profile but also from the duration of their cellular cycle. Type C cells proliferate rapidly, cycling in 12 h, whereas B cells are quiescent: their cell cycle lasts more than 15 days (Morshead *et al*., 1994 and 1998). Moreover, B cells have a cilium that contacts the cerebrospinal fluid of the ventricle (Doetsch *et al*., 1997; Doetsch, 2003), probably sensing molecular cues existent here.

Type A cells or neuroblasts are frequently organized in chains of migrating cells and groups of undifferentiated C cells are associated with these chains. Neuroblasts have the particularity of being able to divide during their migration through the RMS (Menezes *et al*., 1995; Coskun and Luskin, 2001). Evidence *in vivo* has shown that neuroblasts generated in SVZ migrate to the OB through the RMS, inside a tubular structure formed by type B astrocytes (Lois and Alvarez-Buylla, 1994; Doetsch and Alvarez-Buylla, 1996; Lois *et al*., 1996; Kornack and Rakic, 2001; Pencea *et al*., 2001). Recent data show that migrating neurons dynamically remodel the morphology and organization of astroglial tubes to promote long distance, directional migration and they accomplish this by secreting the diffusible chemorepulsive protein Slit1, whose receptor, Robo, is expressed on astrocytes (Kaneko *et al*., 2010). Even though present *in vivo*, the contact with astrocytes is not necessary to migration, since neuroblasts from the SVZ are added to the OB in the absence of GFAP positive cells in the RMS of the newborn rodent (Law *et al*., 1999). In addition, *in vitro*, neuroblasts organize spontaneously as chains from SVZ explants (Wichterle *et al*., 1997). Migration is therefore the result of the concerted action of chemoattractive molecules present for instance in the OB, (Liu and Rao, 2003) and chemorepulsive molecules. These, again, include Slit 1, expressed by A and C cells, in the SVZ and

in the RMS. Curiously, Slit1 is not expressed in the OB and Robo receptors are present in the SVZ and RMS (Chen *et al*., 2001; Nguyen-Ba-Charvet *et al*., 2004). Other surface molecules play a role in neuroblast migration. These include the polysiliated neural cell adhesion molecule (PSA-NCAM), required to chain migration in the adult (Chazal *et al.*, 2000) and α6β1 integrin, as well as its ligand laminin-1, an extracellular matrix (ECM) protein. Interaction between neuroblasts' membranar α6β1 integrins and laminin-1 are important to maintain the RMS structure and neuroblast migration (Emsley and Hagg, 2003). In addition, brain-derived neurotrophic factor (BDNF) and its high affinity receptor tyrosine kinase receptor type 2 (TrkB) regulate both the motility and direction of neuroblasts in the RMS flow (Bagley and Belluscio, 2010). Ependymal cells contribute to neuroblast migration along the RMS, through oriented cilia beating and formation of gradient guidance cues (Sawamoto *et al*., 2006).

When reaching the OB, neuroblasts depart the RMS and migrate radially to different layers of the OB. Molecules required to this process include reelin (Hack *et al*., 2002), prokineticin-2 (Ng *et al*., 2005) and tenascin-R (Saghatelyan *et al*., 2004).

In the OB, neuroblasts differentiate into granular and periglomerular interneurons, expressing the neurotransmitters dopamine and gamma-aminobutyric acid - GABA (Betarbet *et al*., 1996; Baker *et al*., 2001). OB interneurons can be classified according to their expression of calcium binding proteins: calbindin positive cells are primarily observed in the glomerular layer, calretinin expressing cells are seen in the glomerular layer and mostly in the superficial granule cell layer (GCL; Bagley *et al*., 2007).

1.3.1.2. Relevance of SVZ neurogenesis

One can appreciate the relevance of SVZ neurogenesis by looking at actual numbers: thousands of neuroblasts are generated in the rodent SVZ everyday (Morshead and van der Kooy, 2001) and, from these, 30000-60000 new neurons

survive and are added daily to the OB (Lois and Alvarez-Buylla, 1994; Biebl *et al*., 2000).

In the OB, newly-generated neurons integrate and establish new functional connections with pre-existing neuronal networks (Carlén *et al*., 2002; Petreanu and Alvarez-Buylla, 2002; Belluzzi *et al*., 2003). Behavioural tests have demonstrated that bulbar neurogenesis is necessary to odour discrimination and memory (Gheusi *et al*., 2000; Rochefort *et al*., 2002). Furthermore, it was shown that recently generated adult-born olfactory granule neurons and older, preexisting granule neurons, undergo contrasting experience-dependent modifications *in vivo* (Magavi *et al*., 2005). A final piece of evidence that SVZ neurogenesis contributes to odour memory comes from studies in pregnant mice, in which SVZ neurogenesis is increased, probably contributing to recognize the pups' identity (Shingo *et al*., 2003).

1.3.1.3. The SVZ stem cell identity

The identification of the true stem cell in the SVZ has proven to be a difficult task since neural stem cells (NSCs) are very rare in the adult brain (no more than 0.2-0.4% according to Morshead *et al*., 1998) and, in addition, no single molecular marker can unambiguously identify them. As a consequence, NSCs have to be identified functionally, through the demonstration of their stem cell capacities: self-renewal and multipotency. However, unlike the hematopoietic system, no reliable *in vivo* stemness assay of NSCs, at the single-cell level, is currently available. Therefore, identification of NSCs has heavily relied on *in vitro* culture (Doetsch *et al*., 1999a; Johansson *et al*., 1999; Coskun *et al*., 2008). An inherent problem of culture experiments is that slight differences in conditions may result in successful growth of one type of stem cells, but not others.

In 1999, Doetsch and co-workers identified GFAP-positive astrocyte-like cells as the SVZ stem cells. In one study, Doetsch and collaborators showed that SVZ astrocytes, specifically labelled by retroviral infection, produce bulbar neurons

(Doetsch *et al*., 1999a). In another study, they showed that, after treatment with arabinosylcytosine (Ara-C), an anti-mitotic drug which destroys rapidly proliferating A and C cells, B cells are capable of regenerating in 10 days the SVZ cells. C cells are regenerated from B cells and these originate A cells (Doetsch *et al*., 1999b). Also in 1999, Johansson and collaborators reported apparently contradictory results: they provided evidence that ependymal cells retain the characteristics of NSCs. A morphologic property characterizes SVZ stem-like astrocytes: these cells possess a cilium, which extends through the ependymal layer and contacts the ventricular cavity (Doetsch *et al*., 1997, Mirzadeh et al., 2008). The presence of such contact may have been the reason for a supposed erroneous identification of the ependymal cell as the stem cell. By injecting in the lateral ventricle DiI, a coloured lipophylic marker, the authors may have stained not only ependymal cells but also SVZ stem-like astrocytes *via* their cilium, which then originated DiI-labelled neurons in the OB.

In 1999, Chiasson and collaborators demonstrated that ependymal cells *in vitro* are not stem cells since they cannot form spheres, self-renew nor generate neurons. In addition, other evidences seem to corroborate Doetsch's group work: in culture, type B astrocytes display proliferation, self-renewal and multipotency capacities (Chiasson *et al*., 1999; Lim and Alvarez-Buylla, 1999; Morshead and van der Kooy, 2001). Moreover, in the mouse, after specific elimination of SVZ astrocytes, SVZ cells put in culture are no longer capable of generating spheres (Morshead *et al*., 2003; Garcia *et al*., 2004)

On the other hand, recent studies have relaunched the discussion. One such study states that ependymal stem cells are capable of dividing asymmetrically and transfer progeny into the subventricular zone when activated by injury promoted by local delivery of 6-hydroxydopamine. According to these data, stem cells in the ependymal layer may have been missed in many previous studies because they are usually quiescent and divide only in response to strong stimuli (Gleason *et al.*, 2008). Another approach used the immunoreactivity for a stem cell marker, prominin-

1/CD133, present in different types of stem cells, including myogenic and hematopoietic stem cells (Miraglia *et al*., 1997) as well cancer stem cells (CSCs) of glioblastomas (Lottaz *et al*., 2010). Prominin-1/CD133, is exclusively localized to the ependyma, although not all ependymal cells are CD133+. This study also shows that CD133+ ependymal cells are mostly quiescent, but can divide *in situ* under the condition in which actively-dividing cells in the adjacent SVZ are killed off. Finally, *in vitro* culture demonstrates that CD133+ cells exhibit the canonical features of NSCs, i.e., self-renewal and multipotency (Coskun *et al*., 2008). In light of these evidences, it seems that ependymal cells represent an additional, perhaps more quiescent, stem cell population in the mammalian forebrain. Nonetheless, the GFAP monociliated astrocyte remains the SVZ stem cell of excellence. Astrocytes in the SVZ represent 20% of the total cell population (Doetsch *et al*., 1997). However, because stem cells in the SVZ are so few, this implies that only a subpopulation of the GFAP cells are, in fact, stem cells.

Even though there is no unequivocal stem cell marker, numerous markers have been identified that, in combination, allow the identification of SVZ stem cells (Table 1.1.). Nestin is an intermediary filament protein, expressed in immature cells, but its expression by different cellular types of the SVZ limits its value (Doetsch *et al*., 1997). Dlx2, a transcription factor involved in neuronal development in the embryo, is present in A and C cells (Doetsch *et al*., 2002). B cells express the trisaccharide LeX/ssEA-1, however, this antigenic marker is also present in dividing cells (C cells) and cannot be used as a specific marker (Capela and Temple, 2002). No SVZ cell co-expresses LeX and mCD24, a marker of ependymal cells also expressed in neuroblasts (Capela et Temple, 2002). In the SVZ, C cells and a subpopulation of B cells express the EGF receptor (EGFR) (Doetsch *et al*., 2002). Finally, so-called “ependymal stem cells” express CD133 (in contrast to any other cell in the SVZ) but not mCD24. Cells expressing CD133 and mCD24 are ependymal cells with no stem cell capacities (Coskun et al., 2008).

Some functional tests can be used to identify neural stem cells. These include the Hoechst 33342 extrusion test and the aldehyde dehydrogenase (ALDH) test. Stem cells hold the ability to exclude the DNA probe Hoechst 33342 *via* the multidrug transporter ABCG2 of the Adenosine 5'-triphosphate (ATP)-binding cassette family. The Hoechst 33342-negative population of cells is referred to as the side population and contains the stem cells (Mouthon *et al.*, 2006). Stem cells also exhibit high activity levels of the enzyme ALDH and can be selected by FACS sorting using a fluorescent ALDH substrate (Corti *et al.,* 2006).

1.3.1.4. SVZ stem cells: a major hope for cell therapy?

Brain damage, occurring in ischemia, head trauma or epilepsy, is known to induce cellular proliferation in the SVZ as well as the expression of immature neuronal markers PSA-NCAM and doublecortin (DCX) (Szele and Chesselet, 1996; Tzeng and Wu, 1999; Jin *et al*., 2001; Arvidsson *et al*., 2002; Parent *et al*., 2002a and b; Macas *et al*., 2006; Yang and Levison, 2007; Martí-Fàbregas *et al.,* 2010). It therefore appears that the brain holds some intrinsic repairing capacity, which we should be able to manipulate to promote brain repair.

One approach to promote brain repair using SVZ cells could rely on the engraftment of SVZ-derived cells into damaged areas. Therapeutic potential of SVZ cells is limited due to poor long-term survival. For instance, in a recent study of SVZ cell transplantation in rats after focal ischemia, the majority (~99%) of cells died within 2 months of grafting. Cell survival was significantly, and negatively, correlated with microglial activation. The tendency of SVZ cells to differentiate into glia also constitutes an obstacle to their usage in cell replacement therapies, since very frequently the aim is obtaining neurons, of a particular phenotype (Hicks *et al*., 2008). A recent study faced this issue: a better cell survival was obtained upon transplantation in the sclerotic hippocampus, but transplanted cells did not or hardly contributed to neuronal replacement and mainly adopted an astroglial fate (Raedt *et*

al., 2009). Another report states that cultured adult rat neural progenitor cells (NPCs) survived but differentiated into astrocytes 6 weeks after transplantation into the striatum of the normal adult rat brain (Chen *et al.,* 2007). As SVZ tissue contains both neuronal and glial progenitors, one strategy to solve astroglial differentiation consists in separating cellular phenotypes in order to graft only desired ones. As an example of this, Seidenfaden and co-workers selected cells expressing PSA-NCAM and depleted glial progenitors from the dissociated SVZ using magnetic cell sorting. However, ectopic transplantation of obtained cells led again to the generation of glial cells, showing that committed neuron precursors undergo glial differentiation outside their normal environment (Seidenfaden *et al.*, 2006). Nevertheless, some authors have achieved relative success in grafting SVZ cells. In fact, neuronal migration and maturation are obtained if adult rat SVZ cells grafted in the striatum are pre-treated with FGF-2 (Zhang *et al*., 2003). FGF-2-pre-treated neural stem cells from the OB can also give rise to neuroblasts, which migrate and mature efficiently upon grafting (Vergaño-Vera *et al*., 2009). In another study, SVZ progenitors in the cerebellum differentiated into cerebellar-like interneurons and cerebellar-specific astrocytes upon transplantation (Milosevic *et al*., 2008). Furthermore, OB-RMS progenitors can also generate neuroblasts that, upon transplantation, integrate, migrate and differentiate into granule and glomerular neurons (Giachino and Taylor, 2009). A recent study states that, in a mouse model, grafted NPCs after stroke improved cell survival and partial recovery of functional deficits, not by cell replacement itself, but by releasing neuroprotective soluble factors (Doeppner *et al.,* 2010).

The neuronal phenotype is not always the desired one in cell replacement therapies: in the case of multiple sclerosis, an inflammatory disorder of the central nervous system (CNS), there is destruction of myelinating cells, the oligodendrocytes. Accordingly, a group identified recently a novel population of cells in the human SVZ that expresses βIV tubulin and holds myelinating potential. Once transplanted into postnatal myelin-deficient rat brains, not only do these cells survive

but they also myelinate axons quite efficiently revealing the potential of this strategy (Wu *et al*., 2009a).

In addition to the increase in proliferation and neuronal differentiation in the SVZ after brain damage, neuronal progenitors are known to migrate to the injured cortex or striatum (Szele and Chesselet, 1996; Tzeng and Wu, 1999; Jin *et al*., 2001; Arvidsson *et al*., 2002; Parent *et al*., 2002a and b; Yang and Levinson, 2007). It is therefore reasonable to consider the instructive directing of neuronal or glial progenitors towards lesion, even without grafting surgery. For this, however, knowledge of the appropriate guiding molecules is necessary. For instance, it has been shown that upregulation of the chemokines monocyte chemotactic protein (MCP)-1, macrophage inflammatory protein (MIP)-1α and growth-related oncogene (GRO)-α following striatal cell death leads to chemoattraction of SVZ-derived progenitor cells into the damaged striatum (Gordon *et al*., 2009).

All these evidences point to the high potential of SVZ stem cells as tools in brain repair. It is, however, extremely important to identify strategies to obtain appropriate phenotypes from SVZ cells (Milosevic *et al*., 2008; Giachino and Taylor, 2009; Wu *et al*., 2009a) and novel ways to promote neuroprotection (Doeppner *et al.,* 2010). Equally relevant is understanding mechanisms that control cell death and proliferation to guaranty that the grafted cells survive and integrate in the correct circuits but do not generate epilepsy (Bernardino *et al*., 2005) or cancer (Siebzehnrubl *et al*., 2009). Stimulation of the endogenous SVZ stem/progenitor cell pool may also be a reliable therapeutical approach if cells can be directed towards injured sites (Gordon *et al*., 2009).

1.3.2. The subgranular zone

1.3.2.1. Cellular organization

In the hippocampus, adult neurogenesis occurs according to four distinct stages: a precursor cell stage, an early survival stage, a postmitotic maturation

phase and a late survival phase. As seen in Figure 1.6. in the precursor cell stage, radial glial-like cells or type-1 cells, Nestin positive, originate type-2 cells with high proliferative activity, also Nestin-positive. Type-2 cells generate type-3 cells, with little proliferative activity and no longer positive for Nestin but positive for DCX and PSA-NCAM (Steiner *et al.*, 2006). After cell cycle exit, the early survival stage is initiated, where the majority of generated neurons are eliminated by apoptosis (Biebl *et al*., 2000; Kuhn *et al*., 2005). The post-mitotic maturation phase is associated with dendrite and axon extension as well as increased synaptic plasticity (van Praag *et al.*, 2002; Ambrogini *et al*., 2004; Schmidt-Hieber *et al*., 2004; Zhao *et al*., 2006), while the late survival phase represents a period of fine tuning.

1.3.2.2. Relevance of hippocampal neurogenesis

In the adult rat hippocampus, about 10000 new neurons are generated daily from progenitor cells in the DG (Cameron and McKay, 2001). Here, newly-formed neurons establish synaptic connections with pre-existing ones (Carlén *et al*., 2002). These new neurons are probably involved in learning and memory, since suppression of hippocampal neurogenesis with anti-mitotic drugs or irradiation results in a decrease of learning (Shors *et al*., 2001; Snyder *et al*., 2005). More recently, an elegant study demonstrated that removal of a crucial regulator of adult NSC proliferation, the transcription factor tailless (TLX), specifically from adult NSCs, results in marked deficits in spatial learning (Zhang *et al*., 2008). In the human, adult neurogenesis was demonstrated to contribute to the temporal information of declarative memories of facts and events (Aimone *et al*., 2006). Decrease of hippocampal neurogenesis is associated with schizophrenia (Eriksson *et al*., 2006) and depression (Sahay and Hen, 2007; Ho and Wang, 2010).

1.3.3. Adult neural stem cells and brain tumours

CSCs have been isolated from human brain tumours such as gliomas and

glioblastomas (Galli *et al*., 2004; Yuan *et al*., 2004). There is increasing evidence that this population of CSCs is responsible for chemo- and radio-resistance to classical anti-cancer therapies as well as tumour relapse (Bao *et al*., 2006; Wang *et al*., 2010).

CSCs have been proposed to derive from normal brain stem cells following genetic alteration of genes encoding self-renewal pathway components. CSCs are similar to normal stem cells but have an extra capacity: they can initiate tumours (Vescovi *et al*., 2006; Al-Hajj and Clarke, 2004). It has been argued that neural stem cells or neural progenitors can be the source of CSCs in the brain (Figure 1.7.) by undergoing transformation events when they are in a transit-amplifying phase (Hadjipanayis and Van Meir 2009 a and b). However, other authors point to the mutation-induced dedifferentiation of mature brain cells such as astrocytes and oligodendrocytes (Bachoo *et al*., 2002). NPCs were never clearly shown to originate tumours. Curiously, NPCs from embryonic stem cell lines possess the ability to decrease glioma growth as well as to respond to cues from the tumour, demonstrating a crosstalk between NPCs and tumour cells (Staflin *et al*., 2007). NPCs from the SVZ act similarly on glioblastomas and this anti-tumourigenic action decreases with aging and SVZ cell proliferation decline. The proliferative rate of NPCs relies on the expression of D-type cyclins: young mice NPCs express cyclins D1 and D2, but the expression of cyclin D1 is lost upon aging, and in adult NPCs only cyclin D2 remains. Adult mice supply fewer NPCs to glioblastomas and have larger tumours than young mice. In young and adult cyclin D2-deficient mice a reduced supply of NPCs to glioblastomas is observed as well as the generation of larger tumours compared with wild-type mice (Walzlein *et al*., 2008).

1.4. Modulation of neurogenesis

Neurogenesis depends highly on cell signalling molecules and on cell-to-cell interactions, which constitute the stem cell niche. Neurogenesis' regulation cues can be grouped in intrinsic, extrinsic and environmental.

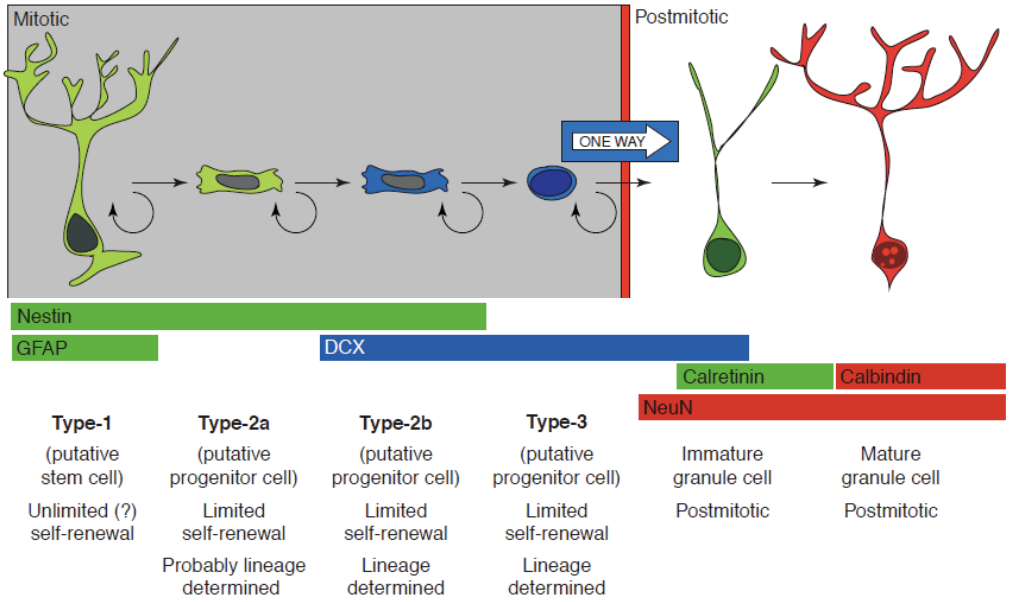


Figure 1.6. Schematic view of neurogenesis in the hippocampus. Radial glial-like cells or type-1 cells, Nestin and glial fibrillary acidic protein (GFAP) positive, originate type-2 cells, also Nestin-positive. Type-2 cells generate type-3 cells, no longer positive for Nestin but positive for doublecortin (DCX). After cell cycle exit (indicated by the "one way" sign) newborn neurons undergo post-mitotic maturation, associated with dendrite and axon extension and initially calretinin expression. Mature granule cells switch from calretinin to calbindin expression. NeuN: Neuronal nuclei. Adapted from Kempermann *et al.*, 2004.

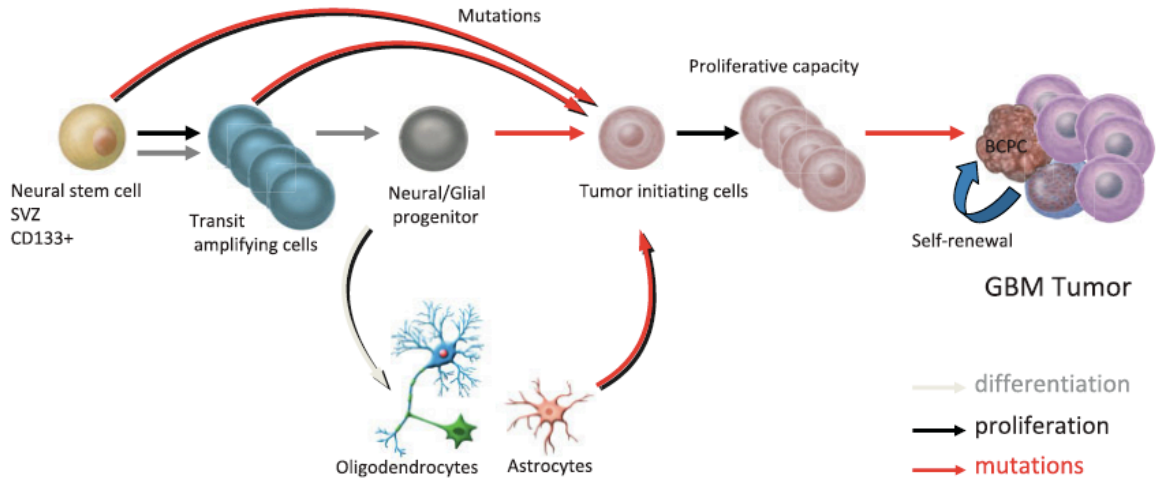


Figure 1.7. Possible lineage relationships for the ontogeny and generation of brain cancer-propagating cells and glioblastoma multiforme (GBM) tumours. During normal central nervous system differentiation, neural stem cells undergo an amplification step to produce transit-amplifying progenitor cells (type C cells), which then differentiate into neural/glial progenitor cells. Mutations generating GBM tumours can occur at all levels within this lineage and produce tumour-initiating cells. These are believed to be stem-like in behaviour based on their ability to self-renew, proliferate, and generate brain cancer-propagating cells (BCPC), differentiated tumour or cancer-like progenitor cells within the tumour mass. Dedifferentiation events may also generate self-propagating cancer cells from astrocytes and oligodendrocytes. SVZ indicates subventricular zone. Adapted from Van Meir *et al.*, 2010.

1.4.1. Intrinsic factors

Subventricular zone cells have intrinsic properties considered fundamental for neurogenesis to occur. These include a combination of transcription factors and chromatin remodelling enzymes, which identities are yet to be fully unravelled.

Relevant transcription factors include Vax1, SOX-2, Mash1, Dlx2, Pax6 and Olig2. Vax1 is relevant to neurogenesis as Vax1 (-/-) mutants show increased proliferation, abnormal differentiation of B cells and failure of neuroblasts to join the OB (Soria *et al.*, 2004). SOX-2 and Mash1 increase the numbers of dividing precursors (Ferri *et al.*, 2004; Parras *et al.*, 2004). Dlx2 is essential to the production of GABAergic interneurons in the OB (Bulfone *et al.*, 1998), while Pax6 is required to the generation of superficial granular and dopaminergic periglomerular interneurons (Hack *et al.*, 2005; Kohwi *et al.*, 2005). Finally, Olig2 may be involved in the production of oligodendrocytes (Rowitch *et al.*, 2002 and 2004).

Transcription factors expression is regulated by a chromatin-remodelling enzymatic machinery consisting in histone acetylases, deacetylases, methylases and demethylases, and by members of the polycomb and trithorax protein complexes, acting in concert to activate or repress target genes (Buszczak and Spradling 2006).

1.4.2. Extrinsic factors

Cell-extrinsic components of the stem cell niche also contribute to neurogenesis. Throughout the past decade, numerous signalling pathways and soluble factors have been implicated in neural stem cell regulation and neurogenesis (Coronas, 2009; Moyse *et al.*, 2008). These include the Wnt/β-catenin, sonic hedgehog (Shh), bone morphogenic protein (BMP) and Notch signalling pathways, as well as signalling cascades associated to growth factors such as epidermal growth factor (EGF), fibroblast growth factor-2 (FGF-2), BDNF, stem cell-derived neural stem/progenitor cell supporting factor (SDNSF), insulin-like growth factor (IGF)-1 and tumour growth factor-α (TGF-α).

The Wnt/β-catenin pathway is a key regulator of neural stem cell behaviour in embryonic development. In adult hippocampal stem/progenitor cells the Wnt/β-catenin pathway is active and Wnt3 is expressed. Overexpression of Wnt3 is sufficient to increase neurogenesis *in vitro* and *in vivo*. By contrast, blockade of Wnt signalling reduces neurogenesis *in vitro* and abolishes neurogenesis almost completely *in vivo* (Lie *et al*., 2005).

The soluble protein Shh, expressed in the SVZ and the hippocampus, is implicated in self-renewal of stem and progenitor cells (Charytoniuk *et al*., 2002; Machold *et al*., 2003; Ahn and Joyner *et al*., 2005). In the hippocampus, Shh also facilitates proliferation (Lai *et al*., 2003).

Neuronal differentiation of progenitors from the SVZ is facilitated by secretion of noggin, a BMP antagonist, by ependymal cells. This soluble factor binds to the neurogenesis inhibitor BMP-4, secreted by SVZ cells themselves, allowing the generation of neuroblasts (Lim *et al*., 2000; Peretto *et al*., 2004). High levels of BMP signalling occur in hippocampal but not SVZ precursors *in vitro*, and blocking BMP signalling with Noggin is sufficient to foster hippocampal cell self-renewal, proliferation and multipotency. Moreover, NSC maintenance requires continual Noggin exposure, which implicates BMPs as crucial regulators of NSC. *In vivo*, Noggin is expressed in the adult dentate gyrus and limits BMP signalling in proliferative cells of the SGZ. Transgenic Noggin overexpression *in vivo* in the SGZ increases multiple precursor cell populations but proportionally increases the glial fibrillary acidic protein-positive cell population at the expense of other precursors, suggesting that Noggin acts on NSCs (Bonaguidi *et al*., 2008). In the hippocampus, the soluble factor neurogenesin-1 (Ng-1), produced by astrocytes, also antagonizes BMP (Ueki *et al*., 2003).

Expression of the Notch receptor in the SVZ and hippocampus contributes to the maintenance of stem cells in a state of quiescence (Chambers *et al*., 2001; Gaiano *et al*., 2000, Ables *et al*., 2010) and self-renewal (Hitoshi *et al*., 2002,

Chojnacki *et al*., 2003). Notch receptor activation induces the expression of the specific target genes hairy and enhancer of split 3 (Hes3) and Sonic hedgehog (Shh) through rapid activation of cytoplasmic signals, including the serine/threonine kinase Akt, the transcription factor STAT3 and mammalian target of rapamycin, and thereby promotes the survival of neural stem cells (Androutsellis-Theotokis *et al*., 2006). A recent study has shown that enhanced EGFR signalling in the SVZ *in vivo* results in the expansion of the NPC pool, and reduces NSC number and self-renewal. This occurs through a non-cell autonomous mechanism involving EGFR-mediated regulation of Notch signalling (Aguirre *et al*., 2010).

Proliferation in the SVZ is affected by growth factors. EGF and FGF-2, once administered to cultures *in vitro* or by ICV *in vivo*, stimulate the proliferation of SVZ cells (Reynolds and Weiss, 1992; Gritti *et al*., 1995; Weiss *et al*., 1996; Kuhn *et al*., 1997). In rats, bFGF treatment has been described to promote not only proliferation but also neural stem cell differentiation into neurons, astrocytes, and oligodendrocytes (Jin-qiao *et al*., 2009). A co-factor of the growth factor FGF-2, cystatine C, has also been identified in the hippocampus, favouring the mitogenic effect of FGF-2 (Taupin *et al*., 2000).

SVZ cells express the FGF-2 receptor FGFR-1 (Gritti *et al*., 1999). Activation of FGFR-1 in GFAP+ cells is required for neuronal recovery after neonatal hypoxic injury, which is attributable in part to enhanced cortical and OB neurogenesis (Fagel *et al*., 2009).

B and C cells express the EGFR and do not respond in the same way after acute stimulation (7h) with EGF (Seroogy *et al*., 1995; Doetsch *et al*., 2002). EGFR has been described to be a key regulator of the expansion of SVZ precursors in response to brain injury. Modulating EGFR signalling represents a potential target for therapies to enhance brain repair from endogenous neural precursors following hypoxic/ischemic and other brain injuries (Alagappan *et al*., 2009).

BDNF has been reported to be neuroprotective (Li *et al*., 2010) and ICV administration of BDNF and EGF promotes striatal neurogenesis and functional recovery in an adult animal model of neonatal stroke. The fact that Ara-C completely blocks functional recovery indicates this effect may be the result of newly-generated neurons (Im *et al*., 2010).

Hippocampal cells secrete another factor, SDNSF, which favours survival of stem/progenitor cells (Toda *et al*., 2003), while in the SVZ, after stroke, elevated numbers of IGF-1-expressing microglia are found. IGF-1 mitigates apoptosis and promotes proliferation and differentiation of NSCs (Thored *et al*., 2009).

Furthermore, in the mouse, when the gene coding for the choroid plexus-secreted growth factor TGF-α is silenced, proliferation is decreased in the SVZ, indicating that in basal conditions, proliferation is facilitated by the presence of this factor in the cerebrospinal fluid (Diaz-Ruiz *et al*., 1993; Seroogy *et al*., 1993; Tropepe *et al*., 1997). Injury response of endogenous SVZ neural stem cells as well as behavioural recovery can be significantly enhanced by application of TGF-α, which represents a potential therapeutic molecule for chronic stroke and other neurological damages in humans (Guerra-Crespo *et al*., 2009).

1.4.3. Environmental factors

Strikingly, neurogenesis is affected by environmental factors. These include, for instance, hormonal regulation, neuronal activity regulation and aging.

Neurogenesis is modulated by hormones, which may be transported to the brain by blood. In non-mammal vertebrates such as reptiles and birds, neurogenesis is more relevant in spring than in summer. In the bird this correlates with a higher production of testosterone, which favours the survival of neurons (reviewed in García-Verdugo *et al*., 2002; Nottebohm, 2002). In adult female zebra finches, following a unilateral penetrating injury to the hippocampus, cell proliferation is enhanced in the ipsilateral hippocampus, as well as in the SVZ proximal to the injury.

Ovariectomy substantially suppresses proliferation, suggesting estrogens are directly involved in the brain's response to injury (Lee *et al*., 2007). In basal conditions, estrogen has been reported to favour the proliferation and the survival of precursors in the DG of female rats, but not SVZ neurogenesis (Tanapat *et al*., 1999). However, following stroke, in mice, estradiol enhances neurogenesis in the SVZ through the estrogen receptors α and β (Suzuki *et al*., 2007). On the other hand, prolactin, acting in concert with TGF-α promotes proliferation and increases the production of neurons in the SVZ of the pregnant mouse (Shingo *et al*., 2003).

Erythropoietin has been shown to stimulate the production of neural stem/progenitor cells and prevent apoptosis in development, but it also serves as a neuroprotective mediator of brain ischemia (Ruscher *et al*., 2002; Kim *et al*., 2008). Moreover, erythropoietin-activated endothelial cells promote the migration of neuroblasts through the secretion of metalloproteinases -2 and -9 (Wang *et al*., 2006).

Thyroid hormones stimulate SVZ neurogenesis in adult rats (Giardino *et al*., 2000). Accordingly, triiodothyronine, a thyroid hormone, enhances fluoxetine-induced neurogenesis in rats with a possible role in antidepressant-augmenting properties (Eitan *et al*., 2010). Curiously, thyroid hormones also promote differentiation of oligodendrocyte progenitor cells and improve remyelination after cuprizone-induced demyelination (Franco *et al*., 2008).

Finally, stress hormones also affect neurogenesis in the adult brain. In the DG, proliferation of neuronal precursors is reduced after stress both in the rat and in the primate. Glucocorticoids secreted in excess, after stress, may be responsible for this phenomenon (Gould *et al*., 1998; Tanapat *et al*., 1998). In the SVZ, neurogenesis is, apparently, not affected by stress (Kippin *et al*., 2004).

Neuronal activity contributes to modulate neurogenesis in the adult brain: mice in enriched environments and with free access to locomotor activity show increased neurogenesis in the DG (Kempermann *et al*., 1997; Kempermann *et al*.,

1998; van Praag *et al*., 1999). These effects are specific to the DG, since in either case neurogenesis is not affected in the SVZ (Brown *et al*., 2003). Nevertheless, exercise after intracerebral haemorrhage does seem to contribute to the enhancement of proliferation and survival of NPCs in the SVZ and their migration towards injured areas (Jin *et al*, 2010). Additionally, if mice are placed in an odour-enriched environment, neurogenesis in the SVZ increases (Alonso *et al*., 2008) and when one nostril is obstructed neurogenesis is diminished uniquely in the ipsilateral SVZ (Corotto *et al*., 1994). Sensory deprivation diminishes the number of newborn cells in the OB but it also reduces the density of granule and periglomerular cells generated before nostril occlusion. Sensory activity also influences positively the development and expression of dopaminergic, but not GABAergic, calretinin or calbindin phenotypes (Bastien-Dionne *et al*., 2010).

Aging also affects neurogenesis: in the hippocampus, the numbers of granule cells generated in the DG are decreased in the aged rat comparing to young animals (Kuhn *et al*., 1996). Reduction of neurogenesis with aging is possibly due to the reduction of the levels of IGF-1 and its receptor, as well as the increase of glucocorticoid levels (Cameron and McKay, 1999; Anderson *et al*., 2002). In the OB of old gerbils, there is a decrease in DCX-immunoreactive neuronal progenitors and its protein levels and this is associated with a reduction of cell proliferation in the SVZ, as well as an increase in α-synuclein in the OB (Choi *et al*., 2010). Neural stem and progenitor cells from aged mice retain their potential for proliferation and differentiation into functional neurons, despite their lower efficacy (Ahlenius *et al*., 2009). In the mouse SVZ, reduction of neurogenesis with aging is associated with a reduction in the number of stem cells and in the expression of EGFR (Tropepe *et al*., 1997; Enwere *et al*., 2004; Maslov *et al*., 2004).

1.5. Vasculature and neurogenesis

1.5.1. Blood vessels and the nervous system: a very precocious

functional relationship

During embryo development, the vasculature is one of the first systems to differentiate (Hirshi *et al*., 1998) and it is the coordinated formation of blood vessels and nerves that originates an intricate network of neurovascular circuits (reviewed in Park *et al*., 2003). The vasculature offers the organism a resource for distribution of nutrients and removal of metabolic products (Hirshi *et al*., 1998; Risau, 1997), but also an essential network for the regulation of neighbouring cells' proliferation and survival, establishment of innate and adaptative immunity, maintenance of haemostatic balance, trafficking of blood cells and blood-borne molecules, as well as control of systemic blood pressure (reviewed in Aird, 2007). Within the brain, balance between blood supply and energy consumption is crucial. Accordingly, the interaction between astrocytic endfeet, mural cells (vascular smooth muscle cells and pericytes) and endothelial cells restricts the flux of harmful agents allowing, nevertheless, the passage of metabolic substances. This functional association of cells that protects the brain parenchyma environment from the free contact with peripheral blood is also known as the blood-brain barrier (BBB) (reviewed in Rubin and Staddon, 1999; Abbott, 2002). The efficiency of the BBB is highly dependent on the presence of tight junctions between endothelial cells and astrocyte endfeet (Abbott *et al*., 2006).

Blood vessels are tube-like structures with a luminal surface composed of endothelial cells, which are surrounded by a contractile layer of mural cells (Hirshi *et al*., 1999). Vascular smooth muscle cells are present in larger vessels (reviewed in Red-Horse *et al*., 2007) and an outer layer is usually formed by fibroblasts, extracellular matrix (ECM) and perivascular nerves (Hirschi *et al*., 1999; Girouard and Iadecola, 2006). Blood vessels are highly abundant at the meninges and cover the surface of the brain, but also penetrate deep into the nervous tissue. The first penetrating arteries and arterioles are separated from the brain by the Virchow-Robin space, but progression into deeper layers result into the disappearance of such a space, allowing the direct contact of endothelial cells with astrocytic endfeet.

Furthermore, as arterioles go deeper and deeper in the brain they become smaller and lose their smooth muscle coverage, originating capillaries, structures essentially consisting of endothelial cells variably covered by pericytes and ECM (Figure 1.8.). This minimal composition of capillaries allows a unique interaction with brain tissue (reviewed in Girouard and Iadecola, 2006; Aird, 2007).

1.5.2. The neurovascular niche

The concept that the vasculature contributes to the stem cell niche and that neural stem cells lay, in fact, in a neurovascular environment has recently gained strong support from experimental evidences. For instance, in the bird, angiogenesis, the generation of new blood vessels from pre-existing ones, and neurogenesis occur simultaneously: VEGF, secreted by astrocytes and neurons, stimulates division of endothelial cells, and the production of BDNF by these cells allows the survival and migration of neuroblasts from the VZ to the HVC (Louissaint *et al*., 2002).

In mammals, angiogenesis and neurogenesis are regulated by common factors such as FGF-2 (Gritti *et al*., 1995; Kuhn *et al*., 1997), VEGF (Jin *et al*., 2002b; Sun *et al* 2010) EPO (Shingo *et al*., 2001), Ang-1 (Bai *et al*., 2009a; Rosa *et al*., 2010), Ang-2 (Liu *et al*., 2009) and PEDF (Ramírez-Castillejo *et al*., 2006; Pumiglia and Temple, 2006). Upon cerebral injury, such as ischemia and epilepsy, angiogenesis is concomitantly increased with neurogenesis (Hellsten *et al*., 2004; Gotts and Chesselet, 2005), and both neurogenic and angiogenic factors are overexpressed (Yoshimura *et al*., 2003; Wang *et al*., 2004; Gotts and Chesselet, 2005). In fact, several recent approaches have used angiogenic factors to promote brain repair. For instance, transforming growth factor (TGF)-α induces angiogenesis and neurogenesis following stroke (Leker *et al*., 2009). VEGF, on the other hand, increases neurogenesis after traumatic brain injury (Thau-Zuchman *et al*., 2010) and enhances cortical newborn neurons' generation, and their neurite development, in the adult rat brain after cerebral ischemia (Wang *et al*., 2009). EPO is able to reduce

hippocampal cell loss and improves the functional outcome following traumatic brain injury, by enhancing angiogenesis and neurogenesis, in rats (Xiong *et al*., 2009). *In vivo* and *in vitro* data indicate that EPO also amplifies stroke-induced oligodendrogenesis, which could facilitate axonal re-myelination and lead to functional recovery after stroke (Zhang *et al*., 2010). One interesting approach uses bone marrow mononuclear cells, which can contribute to the proliferation of endogenous ischemia-induced NSPCs through vascular niche regulation, including endothelial proliferation (Nakano-Doi *et al*., 2010).

Within the SGZ, Nestin-positive radial astrocytes are closely associated with blood vessels (Seri *et al*., 2004) and there is an anatomical relationship between proliferating endothelial cells and proliferating neuronal precursors (Palmer *et al*., 2000; Jin *et al*., 2002b; Wurmser *et al*., 2004). In fact, in the hippocampus, endothelial cells and neuronal precursors proliferate in clusters (Palmer *et al*., 2000). Angiogenesis and neurogenesis are linked processes in the DG of the hippocampus as highlighted by the high levels of VEGF and VEGFR (Palmer *et al*., 2000), and shared responsiveness to similar growth factors such as neurotrophins, neuropilins, semaphorins and ephrins (Shima and Mailhos, 2000; Carmeliet, 2003; Kraemer and Hempstead, 2003). In addition, exercise-induced angiogenesis in the hippocampus increases levels of NGF and BDNF proteins (Ding *et al*., 2004), which also increase neurogenesis (Barnea and Nottebohm 1994; Kempermann *et al*., 1997; Kempermann *et al*., 1998).

Alvarez-Buylla and collaborators proposed in 2002 that, in neurogenic regions, blood vessels supply trophic support for newly-generated neurons *via* cellular contacts. The proximity between stem cells and capillaries seems indeed a condition for neurogenesis. During development, radial glia, identified as neuronal precursors, extend from the ventricle to the pia mater, allowing contact with neurons and blood vessels. This functional syncytium of cells allows trophic support (from capillaries) of newly-generated neurons. At terminal stages of development, radial

glia differentiate into astrocytes and lose their contact with the brain surface by retracting their projections. Interestingly, inhibition of neurogenesis is associated with the disorganization of the syncytium. In the SVZ, however, this functional syncytium is re-established: astrocytes contact the ventricle and capillaries (reviewed in Alvarez-Buylla *et al*., 2002). The SVZ hosts a more stable vasculature comparing to the SGZ and in basal conditions no angiogenesis is observed. However, a modified BBB has been described, lacking both astrocyte endfeet coverage and endothelial cell tight junctions at special sites (Tavazoie *et al*., 2008). Proliferating cells in the SVZ are located close to blood vessels and frequently contact them directly, particularly at sites lacking astrocyte endfeet (Shen *et al*., 2008; Tavazoie *et al*., 2008). Integrin $\alpha 6\beta 1$ has been implicated in proliferation rate and it has been suggested that stem cells contact with the niche's endothelial cells due to the interaction of integrin $\alpha 6\beta 1$ with endothelial laminin-1 (Shen *et al*., 2008)

Recent evidence indicates that the vasculature also serves as a substrate for the migration of newly-generated neuroblasts. Endothelial cells of blood vessels synthesize BDNF that fosters neuronal migration *via* neurotrophin receptor p75 (p75NTR) expressed on neuroblasts (Snapyan *et al.,* 2009). After reaching the OB, a large number of neuronal precursors associate with the blood vessels and migrate radially into the OB (Bovetti *et al.*, 2007). The vasculature-guided (vasophilic) migration of neuronal precursors has been observed not only under normal conditions but also following stroke. In the healthy brain, within the RMS, blood vessel density is higher than in other brain regions, including areas with equal cell density, and the orientation of blood vessels parallels the RMS throughout the caudal to rostral path. Migratory neuroblast chains are longitudinally aligned along blood vessels within the RMS, with over 80% of vessel length in rostral areas of the RMS apposed by neuroblasts (Whitman *et al*., 2009). In addition, half of the radially migrating cells associate with the vasculature in the GCL of the OB. On the other hand, after stroke, NPCs migrate along blood vessels from the SVZ towards the

ischemic region of the striatum. The leading process of migrating NPCs is closely associated with blood vessels, suggesting that this interaction provides directional guidance to migrating cells (Kojima 2010).

Interestingly, stem cells of glioblastoma seem to be dependent on aberrant vascular niches that mimic the normal neural stem cell niche (Gilbertson and Rich, 2007). In fact, angiogenesis is necessary for tumour growth (Jain *et al.,* 2007; Bergers, 2009) and tumours secrete VEGF but also a large number of alternative pro-angiogenic factors, including FGF-2, angiopoietins, PDGF, interleukin-8 (IL-8), and hepatocyte growth factor/scatter factor (HGF/SF). Endothelial cell growth and proliferation is stimulated since endothelial cells in the vicinity of the tumour express VEGFR2. The level of VEGF production in a tumour increases with the degree of malignancy (Schmidt *et al*., 1999; Van Meir *et al*., 2010). Strikingly, anti-angiogenic treatments may selectively target glioma stem-like cells (Hadjipanayis and Van Meir, 2009a and b; Atkinson *et al*., 2009).

1.5.3. Endothelial cells regulate the dynamics of neural stem cells

Within the neurovascular niche, either through diffusible factors, ECM-cell contacts or direct heterocellular contacts, endothelial cells are capable of regulating neural stem cells (Figure. 1.9.).

1.5.3.1. The role of diffusible factors

Many endothelial-derived diffusible factors contribute to the neurovascular niche. Examples include molecules such as the angiogenic factor VEGF, Ang-1, BDNF and FGF-2.

VEGF is produced by endothelial cells, ependymal cells and the choroid plexus and it stimulates neurogenesis in the SVZ and in the hippocampus of mammals (Jin *et al*., 2002b). Neurospheres, as well as reactive astrocytes, express VEGF (Chow *et al*., 2001; Maurer *et al*., 2003) and infusion of VEGF in the lateral

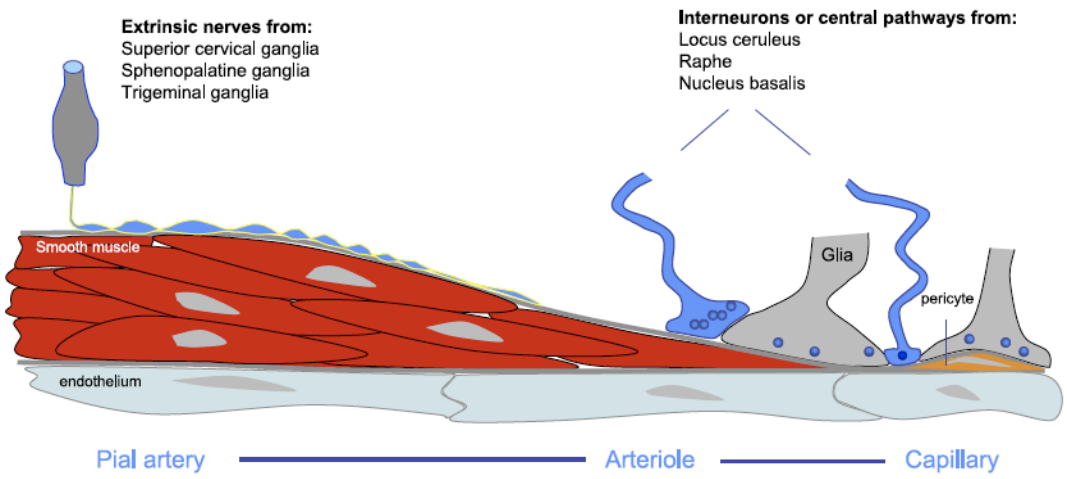


Figure 1.8. Relationship of cerebrovascular cells with neurons, glia, and perivascular nerves. Pial arteries and arterioles are innervated by nerve fibers arising from cranial autonomic ganglia. Smaller cerebral arterioles come in contact with nerve terminals arising from local interneurons and from central pathways originating from distant sites in the brain stem or basal forebrain. These neurovascular associations often terminate on astrocytic endfeet lining the abluminal vascular surface. Pericytes, contractile cells embedded in the capillary wall, are closely associated with astrocytic endfeet and endothelial cells. Adapted from Girouard and Iadecola, 2006.

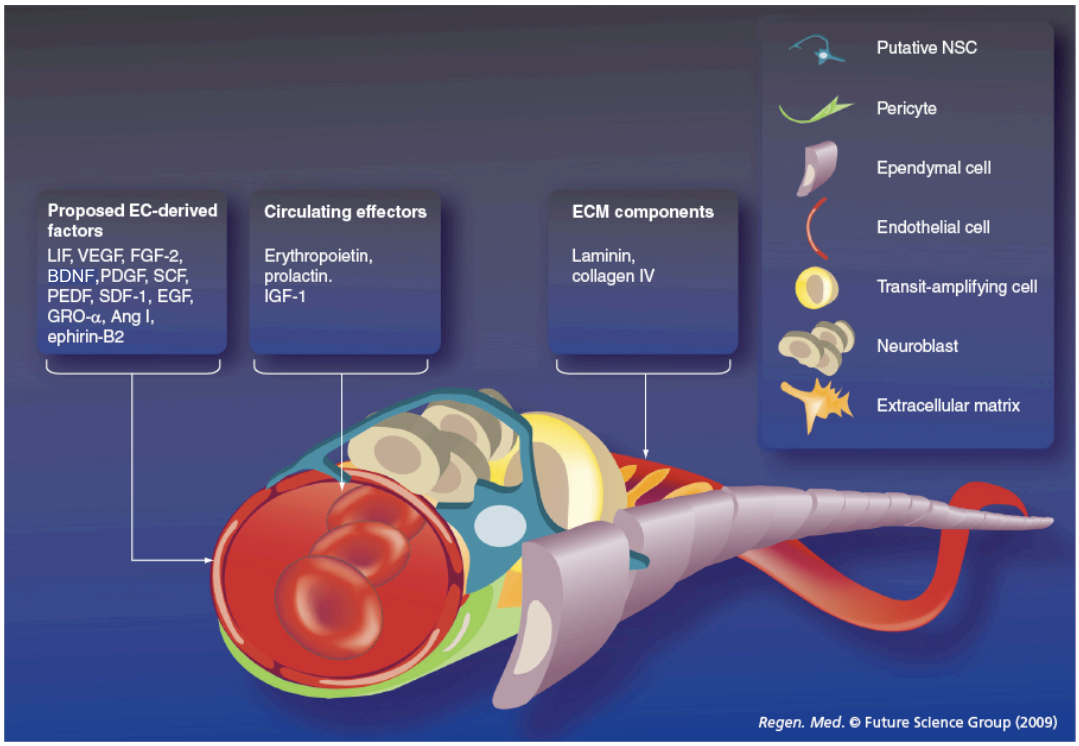


Figure 1.9. Proposed endothelium-derived regulators of the SVZ neurovascular niche. Endothelium-derived regulators can originate from the endothelial cell itself, the blood or the extracellular matrix surrounding the blood vessel. AngI: Angiopoietin-1; BDNF: Brain-derived nerve factor; EC: Endothelial cell; ECM: Extracellular matrix; EGF: Epidermal growth factor; FGF-2: Fibroblast growth factor-2; GRO-α: Growth-related oncogene; IGF: Insulin-like growth factor; LIF: Leukemia inhibitory factor; NSC: Neural stem cell; PDGF: Platelet-derived growth factor; PEDF: Pigment epithelial-derived factor; SCF: Stem cell factor; SDF:Stromal cell-derived factor; VEGF: Vascular endothelial growth factor. Adapted from Goldberg and Hirschi, 2009.

ventricle results into an increase of neurogenesis through the activation of the VEGFR2/Flk receptor (Shen *et al*., 2004; Schänzer *et al*., 2004; Wada *et al*., 2006), which results into promotion of cell survival (Wada *et al*., 2006). VEGF may also act indirectly through the stimulation of BDNF (Louissant *et al*., 2002; Shen *et al*., 2004).

Ang-1 is produced by endothelial cells and acts on the Tie-2 receptor (Suri *et al*., 1996). Its effects on neurogenesis are hardly unveiled, however, previous studies suggest a positive action of Ang-1 on SVZ neurogenesis (Ohab *et al*., 2006; Yamashita *et al*., 2006; Shin *et al*., 2008).

BDNF, on the other hand, is produced by neurons and astrocytes, although it can also be secreted by the endothelium. In the songbird, BDNF induces the recruitment of newly-born neurons (Louissant *et al*., 2002). *In vitro* BDNF's release from endothelial cells supports SVZ-derived neuron outgrowth, survival and migration (Leventhal *et al*., 1999). It has been described to act by a positive feedback loop to reduce proliferation and increase neuroblast differentiation through the release of nitric oxide (NO) by neural stem/progenitor cells (Cheng *et al*., 2003; Packer *et al*., 2003). BDNF mediates exercise-induced synaptic plasticity within the hippocampus (Vaynman *et al*., 2003) and its role has been associated to insulin-like growth factor (IGF)-1: exercise stimulates IGF-1 uptake from the blood into the hippocampus, where it is necessary for the increase of proliferation of neuroblasts and the increased levels of BDNF (Trejo *et al*., 2001; Ding *et al*., 2006). However, it is relevant to point out that intra-ventricular infusion of BDNF yielded different results depending on the animal species, having no effect on neuron production in the mouse SVZ, while decreasing it in rats (Galvão *et al.,* 2008).

Finally, FGF-2 is expressed *in vivo*, in sites of vessel branching and also in ECM in cell culture (Seghezzi *et al*., 1998). While endothelial cells secrete this potent angiogenic factor (Biro *et al*., 1994) to control endothelial cell proliferation, migration and differentiation, neural stem cells also respond to it (Gage *et al*., 1995; Kilpatrick and Bartlett, 1995; Gritti *et al*., 1996; Palmer *et al*., 1997; Ciccolini and Svendson,

1998) as they express the receptor FGFR-1 (Doetsch *et al*., 2002; Zheng *et al*., 2004; Jackson *et al*., 2006). In the CNS, FGF-2 affects neurogenesis and proliferation of cortical progenitors (Vaccarino *et al*., 1999; Raballo *et al*., 2000; Alzheimer *et al*., 2002; Mudò *et al*., 2009). FGF-2 acts in concert with other factors: aside from inducing VEGF expression in endothelial cells, it can prime neural progenitor responsiveness to EGF (Ciccolini and Svendson, 1998). NPY increases the proliferative effect of FGF-2 and increases the expression of FGFR1 on Nestin positive postnatal hippocampal precursor cells, *via* Y1 receptor (Rodrigo *et al.,* 2010).

Other soluble factors contributing to the neurovascular niche, as well as known mechanisms of action, are listed in Table 1.2.

1.5.3.2. The role of contact factors

ECM molecules are expressed on the outer surface of blood vessels, and may contribute to the stem cell niche by sequestrating relevant factors such as FGF-2 (Kerever *et al*, 2007). Most importantly, ECM possesses both adhesion and signalling roles.

Direct interaction of human mesenchymal stem cells with laminin-1 triggers sprouting of neurite-like processes. Function-blocking antibodies directed against α6 or β1 integrin subunits inhibit neurite formation from human mesenchymal stem cells on laminin-1-coated culture plates, confirming the involvement of integrin $\alpha6\beta1$ in neurite outgrowth. Mechanistic studies reveal that cell adhesion to laminin-1 activates focal adhesion kinase (FAK), and mitogen-activated protein kinase kinase/extracellular signal-regulated kinase (MEK/ERK) signalling pathways (Mruthyunjaya *et al*., 2010). Collagen IV inhibits neural progenitor cell proliferation and glial cell differentiation, while promoting neuronal differentiation, in rats (Ali *et al*., 1998). To elucidate the function of ECM proteins on cell differentiation, a recent study describes the usage of biomimetic surfaces that display specific ECM peptide

Endothelial-derived factor	Actions/mechanisms
VEGF	• Stimulates neurogenesis in the SVZ and in the hippocampus of mammals (Jin *et al*., 2002b). • Promotion of neurogenesis occurs through the activation of the VEGFR2/Flk receptor (Shen *et al*., 2004; Schänzer *et al*., 2004; Wada *et al*., 2006), which results into promotion of cell survival (Wada *et al*., 2006). • May also act indirectly through the stimulation of BDNF (Louissant *et al*., 2002; Shen *et al.,* 2004).
BDNF	• Induces the recruitment of newly-born neurons in the songbird (Louissant *et al*., 2002). • *In vitro* BDNF supports SVZ-derived neuron outgrowth, survival and migration (Leventhal *et al*., 1999). • Acts by a positive feedback loop to reduce proliferation and increase neuroblast differentiation through the release of nitric oxide (NO) by neural stem/progenitor cells (Cheng *et al*., 2003; Packer *et al*., 2003). • Intra-ventricular infusion of BDNF yielded different results depending on the animal species, having no effect on neuron production from mouse SVZ, while decreasing it in rats (Galvão *et al.,* 2008). • Mediates exercise-induced synaptic plasticity within the hippocampus (Vaynman *et al*., 2003) by stimulating IGF-1 uptake from the blood into the hippocampus, where it is necessary for the increase of proliferation of neuroblasts and the increased levels of BDNF (Trejo *et al*., 2001; Ding *et al*., 2006). • Endothelial cells of blood vessels synthesize BDNF that fosters neuronal migration *via* neurotrophin receptor p75 (p75NTR) expressed on neuroblasts (*Snapyan et al.,* 2009).
FGF-2	• Affects neurogenesis and proliferation of cortical progenitors (Vaccarino *et al*., 1999; Raballo *et al*., 2000; Alzheimer *et al*., 2002; Mudò *et al*., 2009). • Acts in concert with other factors: it induces VEGF expression in endothelial cells, and primes neural progenitor responsiveness to EGF (Ciccolini and Svendson, 1998). NPY increases the proliferative effect of FGF-2 and increases the expression of FGFR1 on Nestin positive postnatal hippocampal precursor cells, *via* the Y1 receptor (Rodrigo *et al.,* 2010).
PDGF	• Has been described as mitogenic, reported to have differentiation actions on neural progenitor cells (Richards *et al*., 1992; Johe *et al*., 1996) and after ICV infusion periventricular hyperplasias are observed, as well as an increase in oligodendrogenesis at the expense of OB neurogenesis (Jackson *et al*., 2006). • In the SVZ, type B cells express PDGFRα and become activated in the presence of PDGF (Jackson *et al*., 2006).
SCF or Kit ligand	• Stimulates neurogenesis *in vitro* and *in vivo* (Jin *et al*., 2002a). • Induces neural stem cell migration to areas of brain injury (Sun *et al*., 2004).
PEDF	• Promotes self-renewal in SVZ B cells without affecting proliferation, contributing to stem cell maintenance (Ramirez-Castillejo *et al*., 2006).
EGF	• In the SVZ, EGF receptors are expressed by C cells and by a subpopulation of B cells, the stem-like astrocytes. After injection of EGF in the lateral ventricles, the number of transit-amplifying cells increases, while neurogenesis arrests. *In vitro*, transit-amplifying cells form spheres and revert to a more "stem-like" state (Doetsch *et al*., 2002). • EGFR pathway interacts with Notch and regulates neural stem cell number and self-renewal (Aguirre *et al*., 2010).
BMP-2 and BMP-4	• BMP signalling counteracts neurogenesis *in vivo* and *in vitro* (Gross *et al*., 1996; Lim *et al*., 2000; Chen *et al*., 2007) inducing astrocyte formation (Mathieu *et al*., 2008).
LIF and IL-6	• Promote self-renewal of adult neural stem cells (Bauer and Patterson, 2006). • Promote self-renewal of embryonic stem cells to induce astrogenesis, in the presence of BMPs (Yin *et al*., 2003; Bauer and Patterson, 2006).
SDF-1 or CXCL12	• During DG development, activation of SDF-1 receptor, CXC chemokine receptor (CXCR)4, provides migratory cues to neural stem/progenitor cells that migrate from the lateral ventricle to the nascent DG. Neuroblasts expressing CXCR4 migrate towards endothelial cells, which secrete SDF-1 (Hill *et al*., 2004; Imitola *et al*., 2004; Tran *et al*., 2004; Robin *et al*., 2006; Thored *et al*., 2006). • Shared signalling between NSCs and the vasculature through growth-related oncogene (GRO)-α/CXCR4 and Ang-1/Tie-2 has been described (Ward *et al*., 2004; Carmeliet *et al*., 2005). • Transplanted NPCs integrate into the SVZ and associate with the vasculature, a process directed by endothelial-derived SDF-1. SDF-1 may act through the upregulation of $\alpha 6\beta 1$ integrin (Kokovay *et al*., 2010).
Ang-1 and Ang -2	• Ang-1 regulates stem cell differentiation and migration through Tie-2 and CXCR4 receptors (Jones *et al*., 2001; Stumm *et al*., 2002; Arai *et al*., 2004; Imitola *et al*., 2004; Robin *et al*., 2006). • Ang-1, as well as Ang-2, can induce neuronal differentiation from SVZ progenitors through Tie-2 activation (Rosa *et al*, 2010; Liu *et al*, 2009).
TGF-β	• *In vitro* neural stem/progenitor cells express TGFRI, II and III, and TGF-β1 decreases the expansion of these cells by arresting the G0-G1 phase of the cell cycle (Wachs *et al*., 2006).
Eph/ephrin	• Blocks proliferation since infusion of antibody against ephrin-B2 or EphB2 promotes SVZ cell proliferation (Conover *et al*., 2000).

Table 1.2. Soluble factors contributing to the neurovascular niche, as well as known mechanisms of action.

motifs in a controlled manner. Presentation of ECM domains for collagen, fibronectin and laminin influence the formation of neurites by differentiating PC12 cells. Fibronectin and laminin act together in inducing expression of βIII tubulin positive cells, whereas the negative effect of the collagen IV domain in neural differentiation is cancelled out by coexpression of collagen I (Cooke *et al.*, 2010).

Laminin/integrin interaction has been described to play a role in anchoring embryonic NSCs to the ventricular surface and maintaining the physical integrity of the neocortical niche (Loulier *et al.*, 2009). Integrins containing the β1 subunit regulate epidermal stem cell maintenance (Jensen *et al.*, 1999; Zhu *et al.*, 1999; Raghavan *et al.*, 2000) and the higher expression of laminin receptor α6β1 has been implicated in the maintenance of mouse spermatogonial stem cells (Shinohara *et al.*, 1999) and human embryonic stem cells (Xu *et al.*, 2001). Furthermore, a recent study implicated α6β1 integrin in the binding of endothelial-derived laminin-1 to stem cells and promotion of stemness in the SVZ (Shen *et al.*, 2008). *In vitro* SVZ cells express α6β1 integrin and blockade of any of these integrins results in partial incapacity of neurospheres to adhere to endothelial cell monolayers. Disruption of α6 integrin function *in vivo* using a neutralizing antibody causes SVZ progenitor cells to move away from blood vessels and unexpectedly stimulates proliferation, probably due to the presence of endothelial-derived soluble factors. In glioblastoma stem cells, the laminin receptor integrin α6β1 is also expressed, contributing to self-renewal, proliferation and tumour formation capacity (Lathia *et al.*, 2010). In addition, expression of α6β1 integrin increases tumourigenesis in U87, a glioma cell line (Delamarre *et al.*, 2009).

Proximity of SVZ stem/progenitor cells to blood vessels may facilitate the establishment of heterocellular gap junctions (GJs). Functions of GJs are beyond cell coupling since they allow the passage of electrical currents and small molecules but also promote cell-cell adhesion (Lin *et al.*, 2002; Elias *et al.*, 2007) and cell-signalling

(Lampe *et al*., 1998 and 2000; Le *et al*., 2001). In the developing murine cortex, GJ coupling plays a role in cell proliferation (LoTurco and Kriegstein, 1991; Bittman *et al*., 1997; Bittman and LoTurco, 1999; Weissman *et al*., 2004) and neuronal differentiation and migration (Nadarajah *et al*., 1997; Elias *et al*., 2007 and 2010), also contributing to the generation of cortical circuits by mediating patterns of electrical activity (Sun and Luhmann, 2007). In developing and mature astrocytes, Cx43 is the predominant GJ protein (Dermietzel *et al*., 1991; Giaume *et al*., 1991). During postnatal development Cx43 expression is primarily restricted to radial glial cells (Yamamoto *et al*., 1992), which give rise to both astrocytes and neurons (Anthony *et al*., 2004; Casper and McCarthy, 2006; Malatesta *et al*., 2003). Cx43-GJs couple progenitor radial glia only during certain phases of the cell cycle and prior to mitosis, after which other connexins are upregulated (Nadarajah *et al*., 1997; Rozental *et al*., 2000). Cx43 is also expressed in GFAP-expressing cells in the postnatal subventricular zone (Liu *et al*., 2006). GJIC has been shown to regulate homeostasis and differentiation of stem/progenitor cells of the bone (Guillotin *et al*., 2008) as well as attachment of cancer cells to endothelial cells (Elzarrad *et al*., 2008).

1.6. Objectives

Interaction between endothelial cells and SVZ stem/progenitor cells is crucial for the maintenance of SVZ cells' characteristics. In the present thesis work, the role of intercellular communication between endothelial and stem/progenitor cells in stemness and neurogenesis was investigated, with a focus on the soluble factor Ang-1 and on direct contact *via* laminin-1/$\alpha 6\beta 1$ integrin and Cx43, as new targets for brain repair. In an *in vivo* model of temporal lobe epilepsy, we intended to correlate microvasculature changes, namely microvasculature density, to lesion-induced SVZ cell proliferation.

In a first task, we aimed at evaluating the role of the angiogenic factor Ang-1

in SVZ neurogenesis. For this, SVZ cells were tested for the expression of Ang-1 and its receptor Tie-2 and, because SVZ cells are a heterogeneous population of undifferentiated cells, neurons, astrocytes and oligodendrocytes in different stages of maturation, we analysed which cells expressed the Ang-1/Tie-2 system. Having shown that Tie-2 was present in SVZ cells, we verified whether Ang-1 modulated cell proliferation, survival and neuronal differentiation. Finally, we dissected whether stem/progenitors cells in the SVZ *in vivo* expressed the Tie-2 receptor.

In a second task, we established a co-culture of SVZ cells and mouse brain endothelial cells (mBEC) in order to study whether direct heterocellular contacts exerted any effects in SVZ dynamics. For this, parameters such as cell death, cell proliferation, stemness and neural differentiation were evaluated. Furthermore, we wanted to investigate which molecules were involved in these contacts. Having shown that mBEC express laminin-1 and SVZ cells express α6ß1 integrin and that both cell types express Cx43, we investigated if endothelial laminin-1-SVZ α6ß1 integrin-mediated contacts as well as GJIC were involved in the observed effects.

Interaction with endothelial cells control SVZ dynamics, therefore, we hypothesized that injury-induced neurogenesis may be triggered by changes affecting the microvascular network. Thus, in final task, in an *in vivo* model of temporal lobe epilepsy, we tried to correlate SVZ cell proliferation with microvascular network density. For this, microvasculature density was characterized in normal and in injured brains in the neurogenic SVZ and in non-neurogenic areas. SVZ proliferation was assessed and cells were classified according to their distance to blood vessels.

This thesis contributes to further understand the SVZ neurovascular niche and to unravel how SVZ cell characteristics are maintained. This knowledge is potentially relevant in the cell therapy context, contributing to the development of new tools for cell replacement.

Chapter 2 - Materials and methods

2. Materials and Methods

2.1. Experimental models

2.1.1. Animals

Experimental work involving the use of mice and was performed in a way to minimize animal discomfort, according to the ethically approved institutional guidelines and in compliance with national and international laws and policies (EEC Council Directive 86/609, American Association for Laboratory Animal Science Guidelines). C57BL/6 mice were housed at constant temperature (22ºC) and relative humidity (55%) with a fixed 12 h light-dark cycle and free access to water and food. Adult mice were euthanized by cervical dislocation and mice pups by decapitation.

2.1.2. *In vitro* cell cultures

2.1.2.1. Subventricular zone cell cultures

SVZ cells were prepared from 1- to 3-d-old C57BL/6 donor mice as described previously (Agasse *et al*., 2008a). Briefly, mice were killed by decapitation, and the brains were removed and placed in HBSS (Invitrogen, Carlsbad, CA, USA) supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen). Fragments of SVZ were dissected out of 450-µm-thick coronal brain sections, obtained by using a McIlwain tissue chopper, and then digested in 0.025% trypsin (Invitrogen) and 0.265 mM Ethylenediaminetetraacetic acid (EDTA) (Invitrogen) (10 min; 37°C), followed by mechanical dissociation with a P1000 pipette. The resulting cell suspension was diluted in serum-free medium (SFM) composed of DMEM (DMEM/Ham's F-12 medium GlutaMAX-I) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 1% B27 supplement, 10 ng/ml epidermal growth factor, and 5 ng/ml basic fibroblast growth factor-2 (all from Invitrogen). Single cells were then plated on uncoated Petri dishes at a density of 3000 cells/cm^2 and allowed to develop in an incubator with 5% CO_2 and 95% atmospheric air, at 37°C. Six to 8 days (d) after plating, the SVZ neurospheres were collected and seeded onto poly-D-

lysine (0.1 mg/ml)-coated glass coverslips, placed into 12-well cell culture plates for single-cell calcium imaging (SCCI) experiments or 24-well cell culture plates for immunocytochemistry and covered with 1 ml or 500 µl, respectively, of SFM devoid of growth factors. Then, SVZ neurospheres were allowed to develop for 2 d with 5% CO_2 and 95% atmospheric air, at 37°C, before experimental treatments.

2.1.2.2. Mouse brain endothelial cell cultures

2.1.2.2.1. Mouse brain endothelial cell isolation

The protocol established by Wu and collaborators (2003) was used, with modifications. Six to 8 weeks-old C57/BL6 mice were used. For each isolation, 5-6 mouse brains were aseptically collected in sterile phosphate buffer saline (PBS, 0.1 M; containing 137mM NaCl, 2.7mM KCl, 10mM Na_2HPO4, 1.8mM KH_2PO_4, pH 7.4) and the meninges were removed. Brains were then rinsed 3 times in sterile PBS, transferred to RPMI 1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 2% fetal bovine serum (FBS) and cut into small pieces using a scalpel. The tissue was further homogenized with 6-7 strokes in a 7 ml Dounce tissue grinder. The homogenate was suspended in 30% dextran (Sigma-Aldrich, Saint Louis, MO, USA) and centrifuged at 10000 rpm for 10 min. The red pellet, containing the endothelial cells, was washed twice in 2% FBS RPMI and digested with 1 mg/ml of collagenase/dispase (Roche, Basel, Switzerland) in RPMI for 45 min at 37ºC. The enzymatic reaction was stopped by adding 20 ml of 2% FBS RPMI and the digested microvessels were collected by centrifugation at 1200 rpm, for 7 min. The pellet was resuspended in RPMI containing 100 ng/ml endothelial cell (EC) growth supplement (Upstate Biotechnology¸ Billerica, MA, USA), 2 mM L-glutamine, 10 mM HEPES, 24 mM $NaHCO_3$ (all from Invitrogen), 300 units heparin (Sigma-Aldrich), 100 U/ml penicillin, 100 µg/ml streptomycin (Invitrogen), and 10% FCS. This endothelial cell medium was supplemented with 4 µg/ml puromycin (Sigma Aldrich) and mouse brain endothelial cells (mBEC) were plated on 1% gelatin A (from porcine skin, Sigma-

Aldrich)-coated petri dishes. Brain endothelial cells express high levels of glycoprotein-P (P-gp) (Jette *et al*., 1993; Beaulieu *et al*., 1997) and thereby survive puromycin treatment (a P-gp substrate) unlike other brain cells (Perrière *et al*., 2005). After 2 days in culture with 4 μg/ml puromycin, the medium was removed and replaced with fresh endothelial cell medium free of puromycin, and cells were allowed to grow to confluency, time at which they were collected either for immunocytochemistry, western blotting, co-culture studies or cell culture expansion (passaging). Cells were never used above passage 2.

2.1.2.2.2. Mouse brain endothelial cell characterization

Mouse BEC express CD31 or Platelet Endothelial Cell Adhesion Molecule (PECAM) and Von Willebrand factor (VWf). PECAM is a membrane protein, member of the immunoglobulin superfamily, and it is found in endothelial cells, platelets, macrophages, granulocytes, lymphocytes, megakaryocytes, osteoclasts and neutrophils, playing a role in cell adhesion (DiMaio and Sheibani, 2008). VWf is a large multimeric glycoprotein present in plasma and relevant in blood haemostasis. It is produced constitutively in the endothelium, megakaryocytes and subendothelial connective tissue (Sadler *et al.*, 1998). CD31 and VWf expression were verified in mBEC cultures and numbers of positive CD31 cells were determined in four separate cultures. Because mBEC cultures include other contaminant cell types despite puromycin purification, we intended to identify these cells by immuno-labelling for Cd11b (a marker of microglia), GFAP (a marker of glial cells) and SMA (smooth muscle actin, a marker of smooth muscle cells and pericytes). Briefly, confluent mBEC were collected and adhered to SuperFrost Plus glass slides (Thermo Scientific®, Menzel GmbH & Co KG, Braunscheweig, Germany) by centrifugation in a Cellspin I (360 g, 5 min; Tharmac GmbH, Waldsoms, Germany). Referred immunostainings were performed as described in the “Immunostainings” section (2.2.2.).

2.1.2.3. Subventricular zone cell and mouse brain endothelial cell co-cultures

2.1.2.3.1 Subventricular zone cell and mouse brain endothelial cell co-cultures end-point setting and determination of the incubation period of mBEC with cycloheximide

SVZ cell cultures must be performed in serum free media (SFM), as serum decreases proliferation and increases glial differentiation. We assumed that co-cultures could not be maintained for a long period of time since endothelial cells undergo apoptosis in serum-free conditions (Kwak *et al*., 1999). Therefore, to determine a co-culture end-point, cell viability of mBEC cultured in SFM was evaluated at 24 and 48 h using the MTT assay (section 2.2.4.1.).

To reduce cell-cell contact proteins in mBEC we inhibited protein synthesis by incubating cells with cycloheximide (CHX; 1 µg/ml; Sigma) for 1 h, prior to co-cultures assembling. To verify if this treatment had an effect in mBEC viability, we performed MTT assays on 1 h-CHX-treated mBEC at 24 and 48 h, as described in section 2.2.4.1.

2.1.2.3.2. Subventricular zone cell and mouse brain endothelial cell co-cultures

Mouse BEC (20000 cells) were plated on 1% gelatin A-coated glass coverslips in 24-well plates and allowed to develop in endothelial cell medium for 24 h in the incubator. Cells were then treated with or without (control condition) the protein synthesis inhibitor CHX (1 µg/ml) for 1 h and carefully washed 3 times in sterile PBS to completely remove traces of FBS and/or CHX. SVZ spheres were placed on top of the mBEC and the co-culture was covered with 500 µl of SFM devoid of growth factors. Then, the co-cultures were allowed to develop for 24 h in the incubator.

2.1.3. *In vivo* animal model of epilepsy

In a model of epilepsy, C57BL/6 mice received an intraperitoneal (ip) injection of 50 mg/kg 5-bromo-2'-deoxyuridine (BrdU, Sigma-Aldrich) 30 min prior to an ip injection with either 30 mg/kg Kainic Acic (KA, in a NaCl 0.9% saline solution, Sigma) or saline solution (control animals). Epileptic seizures were followed and only animals reaching *status epilepticus* (SE) were considered for further studies. Six hours following the injection of KA or saline solution, all mice were injected again with 50 mg/Kg BrdU. Animals were injected daily with BrdU for 7 days and then euthanized with an ip injection of 100-200 mg/kg ketamine (Merial, London, UK). Prior to cardiac arrest, mice were intracardiacally perfused with 0.9% NaCl (50 ml, 37ºC, pH 7.4, 37ºC) and subsequently with 4% paraformaldehyde (PFA) in NaCl 0.9% (4ºC, 50 ml, pH 7.4). Brains were collected in 4% PFA and left at 4ºC overnight, and then transferred to a 30% sucrose in PBS solution, pH 7.4. Twenty-µm coronal brain slices were obtained by using a Leica CM3050 S Cryostat (Leica Microsystems GmbH, Wetzlar, Germany).

2.2. Experimental procedures

2.2.1. Cell proliferation studies

Cell proliferation was evaluated by BrdU incorporation, a technique that has been used extensively to detect DNA replication during the proliferation of mammalian cells. BrdU is an analogue of thymidine able to incorporate into DNA during the S-phase of the cell cycle (Dolbeare *et al*., 1995).

In *in vitro* assays, 4 h before the end of the culture session, cells were incubated with 10 µM BrdU (Sigma-Aldrich), fixed in 4% PFA for 30 min and rinsed in PBS at room temperature (RT). Brain slices of animals injected with BrdU were rinsed in PBS. BrdU was then unmasked after successive passages in 1% Triton X-100 for 30 min at RT, ice-cold 0.1 M HCl, for 20 min, and finally 2 M HCl for 60 min,

at 37°C. After acidity neutralization in sodium borate buffer (0.1 M $Na_2B_4O_7.10H_2O$, pH 8.5; Sigma-Aldrich) for 15 min at RT, cells were rinsed in PBS, and nonspecific binding sites were blocked with 3% BSA (Sigma-Aldrich) and 1% Triton X-100 in PBS, for 30 min at RT. SVZ cultures and brain slices were then incubated for 90 min with the primary mouse Alexa Fluor 594-conjugated monoclonal anti-BrdU antibody (1:100; A21304; Invitrogen) in PBS containing 0.1% Triton X-100 and 0.3% BSA. After a rinse in PBS, SVZ cell nuclei were stained with Hoechst 33342 (Invitrogen) at 2 µg/ml in PBS for 5 min at RT. Brain slices were processed for double or triple immunocytochemistry as explained in section 2.2.2. Finally, the preparations were mounted using Dako fluorescent medium (Dako). Fluorescent images were recorded using a LSM 510 Meta confocal microscope or an Axioskop 2 Plus fluorescent microscope (both from Carl Zeiss, Göttingen, Germany).

2.2.2. Immunostainings

Mouse BEC and SVZ spheres collected and adhered to SuperFrost Plus glass slides by centrifugation in a Cellspin I (360 g, 5 min), SVZ cells grown on poly-D-lysine-coated glass coverslips, co-cultures of mBEC and SVZ cells on glass coverslips and brain slices were processed for immunocytochemistry. Briefly, cells were fixed in 4% PFA, for 30 min, and rinsed in PBS at RT. Brain slices previously stained for BrdU were rinsed in PBS. Nonspecific binding sites were blocked with 3% BSA (Sigma-Aldrich) and 1% Triton X-100 in PBS for 30 min at RT and then cells/brain slices were incubated for 90 min with a primary antibody in PBS containing 0.1% Triton X-100 and 0.3% BSA, at RT and then overnight at 4ºC. A new rinse in PBS and an incubation with an adequate secondary antibody followed. Table 2.1. shows a list of primary and secondary antibodies used for immunocytochemistry during the course of this work and respective information on their usage. In double and triple immunostainings, the protocol was repeated in order to obtain the desired labelling. After rinsing in PBS, cell nuclei were stained with Hoechst 33342

Primary Antibodies used in Immunostainings

Mouse Brain Endothelial Cell Characterization

Antigen	Company	Catalog number	Source	Dilution
CD31	BD Biosciences	550274	rat	1/200+
vWF	Abcam	ab6994	rabbit	1/200+
CD11b	Serotec	MCA711	rat	1/500+
Smooth Muscle Actin	Abcam	ab5694	rabbit	1/200+
GFAP	Cell Signaling Technology	3670	mouse	1/500+

Modulation of neurogenesis by Ang-1

Antigen	Company	Catalog number	Source	Dilution
Nestin	Abcam	ab5968	rabbit	1/250+
Nestin	Millipore Bioscience Research Reagents	MAB353	mouse	1/200+
Ang-1	Abcam	ab8451	rabbit	1/100+*
Tie-2	R&D Systems	AF313	goat	1/10+*
Tie-2	Santa Cruz Biotechnology	sc-31266	goat	1/50+
DCX	Cell Signaling Technology	4604	rabbit	1/200+*
DCX	Santa Cruz Biotechnology	sc-8066	goat	1/200+
Tau	Cell Signaling Technology	4019	mouse	1/800+
GFAP	Cell Signaling Technology	3670	mouse	1/500+
NG2	Millipore Bioscience Research Reagents	AB5320	rabbit	1/100+
Class III ß-tubulin	Covance	MRB-435P	rabbit	1/750+
NeuN	Millipore Bioscience	MAB377	mouse	1/100+
Phospho-(Thr183/Tyr185)-SAPK/JNK	Cell Signaling Technology	9251S	rabbit	1/100+
BrdU	Serotec	OBT0030	rat	1/50*
BrdU	Invitrogen	A21304	mouse	1/100+*
Epidermal growth factor (EGF) receptor (EGFR)	Millipore Bioscience Research Reagents	04-290	mouse	1/200*
CD31	BD Biosciences	550274	rat	1/100*
Tyrosyne hydroxylase (TH)	Abcam	ab112	rabbit	1/500*

Modulation of neurogenesis by cell-to-cell contacts

Antigen	Company	Catalog number	Source	Dilution
CD31	Santa Cruz	sc-1506	goat	1/200+
CD31	BD Biosciences	550274	rat	1/200+
CD31	BD Biosciences	550274	rat	1/100*
BrdU	Invitrogen	A21304	mouse	1/100+
SOX-2	Santa Cruz Biotechnology	SC-17320	goat	1/100+
DCX	Santa Cruz Biotechnology	sc-8066	goat	1/200+
Laminin-1	Abcam	ab7463	rabbit	1/100+
Cx43	Invitrogen	71-0700	rabbit	1/100+*
α6 Integrin	R&D Systems	MAB13501	rat	1/100+
ß1 Integrin	Invitrogen	44-870G	rabbit	1/100+

Correlation between microvasculature and SVZ cell proliferation in the non-injured and epileptic brain

Antigen	Company	Catalog number	Source	Dilution
CD31	BD Biosciences	550274	rat	1/100*
BrdU	Invitrogen	A21304	mouse	1/100+*
NeuN	Millipore Bioscience	MAB377	mouse	1/100+

+used in vitro; *used in vivo

Secondary Antibodies used in Immunostainings

Secondary Antibody	Company	Catalog number	Dilution
Alexa Fluor 488 Donkey anti-mouse	Invitrogen	A21202	1/200
Alexa Fluor 594 Donkey anti-mouse	Invitrogen	A21203	1/200
Alexa Fluor 488 Donkey anti-rat	Invitrogen	A21208	1/200
Alexa Fluor 594 Donkey anti-rat	Invitrogen	A21209	1/200
Alexa Fluor 488 Donkey anti-rabbit	Invitrogen	A21206	1/200
Alexa Fluor 594 Donkey anti-rabbit	Invitrogen	A21207	1/200
Alexa Fluor 488 Donkey anti-goat	Invitrogen	A11055	1/200
Alexa Fluor 594 Donkey anti-goat	Invitrogen	A11058	1/200
Alexa Fluor 633 Donkey anti-goat	Invitrogen	A21082	1/200

Table 2.1. Information relative to antibodies used in immunostainings during the course of the work in the present thesis.

(Invitrogen) at 2 μg/ml in PBS, for 5 min at RT. Finally, the preparations were mounted using Dako fluorescent medium (Dako). Fluorescent images were recorded using a LSM 510 Meta confocal microscope or an Axioskop 2 Plus fluorescent microscope (both from Carl Zeiss).

2.2.3. Characterization of the brain vasculature using alkaline phosphatase activity

Alkaline phosphatase (AP) is an enzyme expressed in the luminal surface of arteries, arterioles and capillaries, and distinguishes afferent from efferent blood vessels in the brain (Bell and Scarrow 1984), where it plays a role in the blood-brain barrier and transendothelial transport (Vorbrodt *et al*., 1986). AP activity was revealed using 5-bromo-4-chloro-3-indolyl phosphate (BCIP), which is hydrolyzed by the enzyme to form an intermediate that undergoes dimerization once nitro blue tetrazolium chloride (NBT) is added. Briefly, 20 μm coronal brain slices were incubated with a 100 mM Tris-HCl pH 9.5 solution containing 100 mM NaCl, 50 mM $MgCl_2$, 0.53 mM NBT (Sigma-Aldrich) and 0.38 mM BCIP (Sigma-Aldrich), for 10 min at RT. The reaction was stopped by washing with 100 mM Tris-HCl, pH 9.5. Slices were then dehydrated using 75%, 80%, 85% and 96% ethanol solutions and delipidated using xylene, followed by mounting in DEPEX (Sigma-Aldrich).

2.2.4. Determination of cell viability and apoptotic cell death

2.2.4.1. Evaluation of mBEC viability in the presence of SFM and in the presence of SFM plus cycloheximide: MTT assay

This colorimetric assay measures the enzymatic activity of mitochondrial reductase that reduces the yellow tetrazolium salt 3,[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich) into purple formazan. The reduction takes place only when reductase enzymes are active and, therefore, conversion is often used as a measure of viable cells.

To evaluate if SFM or SFM plus 1 µg/ml CHX exerted toxic effects in mBEC, the MTT assay was performed at the end of 24 or 48 h culture period. Briefly, mBEC were plated in a 96-well plate at a density of 10000 cells per well, 3 wells per condition, in mBEC media and left in culture for 24 h. After this time, mBEC were incubated with SFM for 24 and 48 h or with SFM and CHX for 1 h and then with SFM for 24 and 48 h. A set of controls in normal mBEC media was performed. After this time, MTT (0.5 mg/ml, Sigma) was added to the medium, followed by a 4h incubation in the incubator. Then, cells were lysed overnight in MTT solubilisation solution (10% SDS, 0,01% HCl in sterile water) in the incubator. The OD was read at 490 nm using a plate reader apparatus and the SoftMax® Pro Data Analysis Software (Molecular Devices, Sunnyvale, USA).

2.2.4.2. Determination of cell death by apoptosis: terminal deoxynucleotidyl transferase dUTP nick end labelling

Cell apoptosis in SVZ cells was evaluated by the terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL). This method is based on the specific activity of terminal deoxynucleotidyl transferase (TdT), which attaches labelled nucleotides (dUTP) to the 3'-OH ends of the DNA generated during apoptotic-induced DNA fragmentation.

At the end of each 24-h (co-cultures) or 48-h (SVZ cultures) incubation protocol, cells were fixed for 30 min in 4% PFA at RT, rinsed in PBS, and permeabilized in 0.25% Triton X-100 (Sigma-Aldrich), for 30 min at RT. SVZ cultures were incubated for 20 min in 3% H_2O_2 but not co-cultures. Both culture types were reacted for terminal transferase (0.25 U/µl) biotinylated dUTP (6 µM) nick end labelling of fragmented DNA in TdT buffer (30 mM Tris/HCl, 140 mM sodium cacodylate, and 1 mM $CoCl_2$, pH 7.5), all from Roche, for 1 h at 37°C in a humidified chamber. The enzymatic reaction was stopped by 15 min of incubation in 300 mM NaCl and 30 mM sodium citrate buffer (both from Sigma-Aldrich).

In the case of SVZ cultures, after an additional rinse in PBS, cultures were incubated for 30 min at RT with the avidin–biotin–peroxidase complex (1:100; Vector Laboratories). Peroxidase activity was revealed by the DAB (diaminobenzidine) chromogen (0.025%; Sigma-Aldrich) intensified with 0.08% $NiCl_2$ in 30mM Tris-HCl, pH 7.6, buffer containing 0.003% H_2O_2. The cell preparations were then dehydrated in ethanol (75°, 2 min; 80°, 2 min; 85°, 2 min; 96°, 2 min), cleared in xylene (3 min), and mounted using DEPEX mounting medium (Sigma-Aldrich). Photomicrographs of TUNEL-positive cells were recorded using a digital camera (Axiocam HRC; Carl Zeiss) adapted to an Axioskop 2 Plus fluorescent microscope (Carl Zeiss).

In the case of co-cultures, enzymatic reaction stopping was followed by an additional rinse in PBS and cultures were incubated for 1 h 30 min at RT with a Rhodamin-Avidin Complex (1:100; Vector Laboratories). Co-cultures were then washed with PBS and processed for CD31 immunostaining as described in the "Immunostainings" section (2.2.2.).

2.2.5. Cell differentiation studies

2.2.5.1. Single-cell calcium imaging

To determine the functional differentiation pattern of SVZ cells, the variations of free intracellular calcium levels ($[Ca^{2+}]_i$) were analysed in single cells after stimulation with 50 mM KCl or 100 µM histamine (Sigma-Aldrich) (Agasse *et al*., 2008b). KCl depolarization causes an increase in $[Ca^{2+}]_i$ in neurons (Ambrósio *et al*., 2000), whereas stimulation with histamine leads to an increase in $[Ca^{2+}]_i$ in stem/progenitor cells (Tran *et al*., 2004). SVZ cultures were loaded for 45 min with 5 µM acetoxymethyl ester of Fura-2 (Fura-2 AM) (Invitrogen), 0.1% fatty acid-free BSA, and 0.02% pluronic acid F-127 (Invitrogen) in Krebs' solution (132 mM NaCl, 4 mM KCl, 1.4 mM $MgCl_2$, 1 mM $CaCl_2$, 6 mM glucose, 10 mM HEPES, pH 7.4) in an incubator with 5% CO_2 and 95% atmospheric air, at 37°C. After a 10 min postloading period, at RT, in the same medium without Fura-2 AM and pluronic acid, to obtain a

complete hydrolysis of the probe, the glass coverslip was mounted on an RC-20 chamber, in a PH3 platform (Warner Instruments, Hamden, CT, USA), on the stage of an inverted fluorescence microscope (Axiovert 200; Carl Zeiss). Cells were continuously perfused with Krebs' solution and stimulated by applying high-potassium Krebs' solution (containing 50 mM KCl, isosmotic substitution with NaCl) or 100 μM histamine. Solutions were added to the cells by a fast-pressurized (95% air, 5% CO_2 atmosphere) system (AutoMate Scientific, Berkeley, CA, USA). The variations of $[Ca^{2+}]_i$ were evaluated by quantifying the ratio of the fluorescence emitted at 510 nm after alternate excitation (750 ms) at 340 and 380 nm, using a Lambda DG4 apparatus (Sutter Instrument, Novato, CA, USA) and a 510 nm bandpass filter (Carl Zeiss) before fluorescence acquisition with a 40x objective and a CoolSNAP HQ digital camera (Roper Scientific, Tucson, AZ, USA). Acquired values were processed using the MetaFluor software (Universal Imaging, Downingtown, PA, USA). Histamine/KCl values for Fura-2 fluorescence ratio were calculated to determine the extent of neuronal maturation in cultures.

2.2.5.2. Neuronal differentiation/maturation studies

To investigate the influence of potential neurogenic peptides on neuronal differentiation, SVZ neurospheres were allowed to develop for 7 d, in the presence of the putative pro-neurogenic molecules and immunocytochemistry for the neuronal nuclear protein NeuN was performed.

To investigate the involvement of the stress-activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK) signalling pathway in response to Ang-1, SVZ neurospheres were treated with the pro-neurogenic molecule and/ or 20 μM anthra[1,9-cd]pyrazol-6(2*H*)-one (SP600125) (Sigma-Aldrich), an inhibitor of SAPK/JNK, for 6 h at 37°C. At the end of each incubation protocol, immunocytochemistry against phosphorylated (activated) forms of the SAPK/JNK kinase, namely phospho-stress-activated protein kinase (P-SAPK)/JNK, was

performed. To ascertain that P-SAPK/JNK localization was associated with axons, double immunocytochemistry was performed to visualize both P-SAPK/JNK and τ. Each experiment included a series of control cultures not subjected to any drugs. Immunostainings were performed in accordance with 2.2.2.

2.2.6. LAMP assay to image gap-junction-mediated cell-cell communication

Intercellular communication through gap junctions was evaluated by using the LAMP assay, described by Dakin *et al*., 2005. LAMP (local activation of fluorescent probe) is a technique that allows the imaging of cell-cell junctional coupling by gap junctions. It involves loading cells with a non-fluorescent caged coumarin dye (7-hydroxy-6-chloro-coumarin 3-carboxamide: HCCC2). The caged dye, NPE-HCCC2/AM is a lipophilic molecule that diffuses across membranes. While in the cytoplasm, the AM group is hydrolyzed by endogenous esterases stopping the diffusion of the molecule out of the cell. UV-light-mediated uncaging is a photolysis reaction that releases the HCCC2 fluorophore from the NPE (2-nitrosoacetophenone) caging group. When released from the caging group, HCCC2 fluoresces brightly. The molecular weight of HCCC2 is of 450 Da, so far below the molecular passage limit of connexin (Cx) channels, around 1000-1500 Da. Consequently, HCCC2 can diffuse between the coupled cell pair through gap junctions. Local uncaging is performed after choosing regions of interest (ROI) in one of the cells of coupled cell pairs. Uncaging areas in “donor cells” are chosen so as to be at least a 2 µm apart from the cell-cell contacts. Fluorescence variations in “recipient” cells are monitored following the drawing of ROIs encompassing the cytoplasm. The photoactivable probe used in our study has been developed and kindly provided to us by Weng-Hong Li and collaborators (Univ. Texas, Southern Medical Center at Dallas, Dallas, TX, USA). The method has been previously described in papers published by this group (Dakin *et al*., 2005; Yang and Li, 2009).

2.2.6.1. Preparation of the co-cultures for the LAMP assay

Co-cultures of mBEC and SVZ cells were prepared as previously described with the only difference consisting in the use of GFP (instead of WT) SVZ spheres in order to facilitate the identification of mBEC/SVZ cell pairs. Twenty-four-h-old co-cultures were rinsed with completed HBSS (20mM HEPES in HBSS, pH=7.35) and incubated with the loading solution containing the probe (2.24 μg/ml NPE-HCCC2/AM, 0.015 % pluronic acid in completed HBSS) during 20-45 min at 37ºC. Cells were then washed with completed HBSS and kept in Krebs' solution (composition detailed in section 2.2.5.1.) during the experiment.

2.2.6.2. Uncaging and fluorescence imaging

Glass coverslips were mounted on an RC-20 chamber in a PH3 platform (Warner Instruments) on the stage of a confocal microscope (LSM 510 Meta; Carl Zeiss). Fields of interest, containing putative coupled cell pairs of mBEC (non-fluorescent) and SVZ cells (GFP positive) were selected using transmission light (DIC) and green fluorescence (excitation at 488 nm, emission at 505-550 nm) with a 63X oil-immersion objective. A ROI in the cytoplasm of an endothelial cell was selected for UV light-mediated uncaging. Prior to photolysis, blue fluorescence was observed to verify that there was no residual activation of the coumarin probe.

UV light-mediated uncaging was performed through the use of the 405 nm laser in a ROI in the endothelial cell for 2 sec (2 scans, 10 interactions, 100% power of laser 405 nm). Photos in blue (405 nm laser, 1% laser power) and green (488 nm laser, 1% laser power) were taken every 5 s during 4 min (bleach is performed at second 5). At the end of the experiment, a photo encompassing the green, blue and brightfield channels was taken to visualize residual activation of the probe in non-coupled cells.

2.2.7. Isolation of total RNA from SVZ cells

Total RNA was isolated from SVZ spheres and total murine placenta, using TRI reagent (Sigma-Aldrich) according to the instructions of the manufacturer. Cells were gently homogenized in guanidium thiocyanate and phenol and allowed to stand at RT to secure the complete dissociation of nucleoprotein complexes. Chloroform was added, allowing a clear isolation of RNA in the resultant aqueous phase. Then, the RNA was precipitated with isopropanol, and the pellet was washed with 75% (v/v) ethanol, dried at RT, redissolved in diethylpyrocarbonate-treated water, and stored at -80°C until use. The total amount of RNA was quantified by optical density (OD) measurements at 260 nm, and the purity was evaluated by measuring the ratio of OD at 260 and 280 nm (RNA/DNA calculator GeneQuant II; GE Healthcare, Waukesha, WI, USA). In addition, RNA quality was assessed by gel electrophoresis.

2.2.8. Reverse transcription-PCR analysis

Ang-1 and Tie-2 mRNA expression was determined by reverse transcription (RT)-PCR. First, cDNA was obtained from the transcription of 2 μg of RNA using M-MuLV Reverse Transcriptase RNase H^- and oligo-dT_{15} primers (Bioron, Ludwigshafen, Germany). PCR was performed in a 50 μl reaction system (Bioron) containing 5 μl of template cDNA, 1 μl (0.2 μM) of deoxynucleotide mix, 5 μl of 10 x PCR buffer [160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl, pH 8.8, 0.1% Tween 20, 25 mM $MgCl_2$], 0.2 μl (0.2 μM) of upstream primer, 0.2 μl (0.2 μM) of downstream primer, a variable volume of water, and 0.25μl (5000 U/ml) of DNA-free sensitive TaqDNA polymerase (Bioron). Primers used in PCRs were as follows:

Ang-1, forward primer, 5'-TGCATTCTTCGCTGCCATTCT-3', and reverse primer, 5'-ATTGCCCATGTTGAATCCGGT-3';

Tie-2, forward primer, 5'-ATGTGGAAGTCGAGAGGCGAT-3', and reverse primer, 5'-CCCTGAACCTTATACCGGATGA-3' (Sigma- Aldrich);

β-actin, forward primer, 5'-GACTACCTCATGAAGATCCT-3', and reverse

primer, 5'-ATCTTGATCATGGTGCTG-3' (MWG Biotech).

PCR products of each sample were subjected to electrophoresis in a 1.5% agarose gel and stained with ethidium bromide. Negative controls were performed without RNA sample, which was replaced by water. Positive controls were total murine placenta mRNA samples. Photographs were taken in a Versa-Doc Imaging System (model 3000; Bio-Rad).

2.2.9. Western blot analysis

Free floating SVZ spheres or spheres adhered to poly-D-lysine-coated six-well plates, mBEC, placental or whole brain tissue were homogenized in 50mM Tris-HCl, 0.5% Triton X-100, supplemented with 1 mM PMSF, 1 mM dithiothreitol (DTT), 1 µg/ml chymostatin, 1 µg/ml leupeptin, 1 µg/ml antipain, 5 µg/ml pepstatin A (all from Sigma-Aldrich), pH 7.4, at 4°C. The supernatant was collected after centrifugation at 14,000 rpm for 10 min, at 4°C. Protein concentration was measured by the BCA method and samples were treated with SDS-PAGE sample buffer [6x concentrated: 350 mM Tris, 10% (w/v) SDS, 30% (v/v) glycerol, 0.6 M DTT, 0.06% (w/v) bromophenol blue], boiled 5 min at 95°C, and stored at -80°C until use for Western blotting analysis. Proteins were separated by SDS-PAGE on acrylamide/bisacrylamide gels and transferred onto PVDF (polyvinylidine difluoride) membranes using a gel transfer apparatus. After blockade of the nonspecific binding sites with 5% low-fat milk in PBS containing 0.5% Tween (Sigma-Aldrich) (PBST), for 1 h at RT, the membranes were probed with primary antibodies, presented in Table 2.2., at 4°C overnight. Membranes were washed with PBST, incubated for 1 h at RT with appropriate secondary antibodies (Table 2.2.), and visualized using ECF reagent (GE Healthcare, Buckinghamshire, UK) on the Storm 860 Gel and Blot Imaging System (GE Healthcare) or using conjugated secondary antibody fluorescence on an Odyssey® Infrared Imaging System (Licor Biosciences, Lincoln, USA). Quantification was processed using ImageJ (NIHImage, Bethesda USA).

Primary Antibodies used in Western Blot Analysis

Modulation of neurogenesis by Ang-1

Antigen	Company	Catalog number	Source	Loaded protein (µg)	Dilution
Ang-1	Abcam	ab8451	rabbit	60	1/3000
Tie-2	R&D Systems	AF313	goat	80	1/250
GFAP	Sigma-Aldrich	G9269	rabbit	5	1/20000
Class III ß-tubulin	Covance	MRB-435P	rabbit	5	1/1000
ß-actin	Sigma-Aldrich	A1978	mouse	Loading control	1/2000

Modulation of neurogenesis by cell-to-cell contacts

Antigen	Company	Catalog number	Source	Loaded protein (µg)	Dilution
Laminin	Abcam	ab7463	rabbit	25	1/1000
Cx43	Invitrogen	71-0700	rabbit	25	1/100
α6 Integrin	R&D Systems	MAB13501	rat	50	1/100
ß1 Integrin	Invitrogen	44-870G	rabbit	70	1/1000
GAPDH	Santa Cruz	sc-25778	rabbit	Loading control	1/10000

Secondary Antibodies used in Western Blot Analysis

Secondary Antibody	Company	Catalog number	Dilution
Alkaline Phosphatase-conjugated Goat anti-rabbit	Amersham	RPN 5783	1/20000
Alkaline Phosphatase-conjugated Goat anti-mouse	Amersham	RPN 5781	1/20000
Alkaline Phosphatase-conjugated Rabbit anti-goat	Sigma	A4187	1/5000
IRDye® 680-conjugated Goat anti-rabbit	Licor Biosciences	926-32221	1/5000
IRDye® 680-conjugated Goat anti-rat	Licor Biosciences	926-32229	1/5000

Table 2.2. Information relative to antibodies used in Western blot analysis during the course of the work in the present thesis.

2.3. Experimental setting

2.3.1. Study of the modulation of neurogenesis by diffusible and contact factors

2.3.1.1. Modulation of neurogenesis by Ang-1

2.3.1.1.1. Ang-1/Tie-2 system in SVZ cells in culture and *in vivo*

To verify if the Ang-1/Tie-2 system was present in SVZ cells, SVZ spheres, obtained by centrifugation in a Cellspin I (Tharmac), were tested for the co-expression of Ang-1 and Nestin or Tie-2 and Nestin by immunocytochemistry. Presence of Ang-1 and Tie-2 proteins in SVZ spheres was confirmed by western blotting. Non-treated SVZ cells (48 h or 7 d after depositing on poly-D-lysine) were tested for the co-expression of Tie-2 and DCX, τ, GFAP or NG2. In *in vivo* experiments, 6- to 8-week-old mice were daily injected with 50 mg/kg BrdU for 5 d and after this time anesthetized and perfused with 0.9% NaCl and subsequently with 4% PFA. Brains were collected in 4% PFA and left at 4°C overnight, until transference to a 30% sucrose solution. Twenty-μm coronal and sagittal brain slices were obtained by using a Leica CM3050 S Cryostat (Leica Microsystems). Brain slices were stained for BrdU, Tie-2 and DCX or epidermal growth factor (EGF) receptor (EGFR); CD31 and Ang-1; Tie-2 and DCX; Tie-2 and tyrosine hydroxylase (TH). All referred immunocytochemistries were performed as described in the "Immunostainings" section (2.2.2.), except for BrdU immunocytochemistry, described in 2.2.1. Western blotting is described in section 2.2.9.

2.3.1.1.2. Evaluation of the effect of Ang-1 on SVZ cell proliferation

To investigate the effect of Ang-1 on cell proliferation, SVZ cells were treated with 10, 100, and 500 ng/ml Ang-1 for 48 h. To determine whether the Ang-1 receptor Tie-2 was involved in the putative pro-proliferative effect of Ang-1, SVZ cells were treated with 500 ng/ml Ang-1 together with 5 μg/ml anti-Tie-2 neutralizing antibody (Sigma-Aldrich). BrdU exposure and unmasking followed as described in

the “cell proliferation studies” section (2.2.1). We also investigated whether the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase pathway was involved in the proliferative effect of Ang-1. For this, in a set of experiments SVZ cells were coincubated with 500 ng/ml Ang-1 and 20 μM 1,4-diamino-2,3-dicyano-1,4-bis (*o*-aminophenylmercapto) butadiene monoethanolate (U0126) (Sigma), a highly selective inhibitor of both mitogen-activated protein extracellular signal-regulated kinase (MEK)1 and MEK2, a type of MAPK/ERK kinase (Learish *et al*., 2000). BrdU incubation and immunorevelation followed, again, as described in the “Cell proliferation studies” section (2.2.1.).

2.3.1.1.3. Evaluation of the effect of Ang-1 on SVZ cell apoptosis

To investigate the effect of Ang-1 on SVZ cell apoptosis, SVZ cells were treated with 500 ng/ml Ang-1 and/or 5 μg/ml anti-Tie-2 neutralizing antibody for 48 h and cell apoptosis was determined by Tunel staining as described in 2.2.4.2.

2.3.1.1.4. Evaluation of the effect of Ang-1 on neuronal differentiation

2.3.1.1.4.1. Functional evaluation of the effect of Ang-1 on neuronal differentiation by SCCI

To investigate the influence of Ang-1 on neuronal differentiation, SVZ neurospheres were allowed to develop for 7 d with recombinant human Ang-1 (500 ng/ml), at 37°C. To determine whether the Ang-1 receptor Tie-2 was involved in the putative pro-neurogenic effect, SVZ cells were treated with 500 ng/ml Ang-1 and/or 5 μg/ml anti-Tie-2 neutralizing antibody. At the end of these treatments, SCCI experiments were performed according to section 2.2.5.1. To investigate the involvement of the phosphoinositide-3-kinase (PI3K)/AKT signalling pathway in response to Ang-1, SVZ neurospheres were treated with 500 ng/ml human recombinant Ang-1 and/or 20 nM rapamycin (Tocris Bioscience, Bristol, UK) for 7 d. On the second day, the treatments were renewed. Rapamycin inhibits mammalian

target of rapamycin kinase (mTOR), a serine-threonine kinase of the downstream signal molecules of PI3K/AKT kinases. In neurons, mTOR is involved in the control of the size of the soma as well as the directional axonal growth and dendritic tree development (Swiech *et al*., 2008).

2.3.1.1.4.2. Evaluation of the effect of Ang-1 on neuronal maturation

To investigate the influence of Ang-1 on neuronal differentiation, SVZ neurospheres were allowed to develop for 7 d in the presence of recombinant human Ang-1 (500 ng/ml) at 37°C. In order to determine if the Ang-1 receptor Tie-2 was involved in the putative pro-neurogenic effect, SVZ cells were treated with 500 ng/ml Ang-1 together with 5 µg/ml anti-Tie-2 neutralizing antibody (R&D Systems, Minneapolis, MN, USA). NeuN staining was then performed according to 2.2.5.2. To investigate the involvement of the stress-activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK) signalling pathway in response to Ang-1 stimulation, SVZ neurospheres were treated with 500 ng/ml human recombinant Ang-1 and or 20 µM anthra[1,9-cd]pyrazol-6(2*H*)-one (SP600125) (Sigma-Aldrich), an inhibitor of SAPK/JNK, for 6 h at 37°C. At the end of each incubation protocol, immunocytochemistry against phosphorylated (activated) forms of the SAPK/JNK kinase, namely phospho-stress-activated protein kinase (P-SAPK)/JNK, was performed as described in 2.2.5.2.

To disclose whether neuronal differentiation occurred at the expense of glial differentiation, we evaluated the levels of GFAP and βIII tubulin proteins in control *versus* Ang-1-treated SVZ cells. Briefly, 6- to 8-d-old neurospheres were plated onto six-well plates previously coated with poly-D-lysine, allowed to adhere for 24 h in the presence of SFM and treated in the absence (control) or in the presence of 500 ng/ml Ang-1. The medium was renewed after 48 h. Seven days after the first treatment, the cells were harvested by scraping in 0.1% Triton X-100 in PBS and processed for western blot as described in 2.2.9.

2.3.1.2. Modulation of neurogenesis by heterocellular cell-to-cell contacts between SVZ cells and mBEC

To determine whether cell-to-cell interactions between SVZ cells and mBEC contributed to neurogenesis, 4 parameters were evaluated in co-cultures (performed as described in 2.1.2.3.2.): proliferation, stemness, neuronal differentiation and apoptosis. These parameters were evaluated using CD31/BrdU, CD31/SOX-2, CD31/DCX and CD31/Tunel co-labellings, described in sections 2.2.1., 2.2.2. and 2.2.4.2.

To inhibit contact proteins in mBEC, a set of experiments was performed where mBEC were exposed to CHX, a protein synthesis inhibitor, for 1 h, prior to SVZ spheres depositing. This treatment was performed in order to decrease the exposure of ECM proteins in mBEC. However, CHX reduces not only ECM proteins but also the synthesis of soluble factors. Thereby we also investigated whether the observed effects in proliferation and stemness were due to impairment of physical cell-to-cell contacts and not due to endothelial-derived soluble factors. Accordingly, in a set of experiments, mBEC were incubated with CHX for 1 h and, when SVZ neurospheres were deposited on mBEC, conditioned media (CM) from endothelial cells was added during the subsequent culture section. Furthermore, as shown in the present thesis and previously published (Rosa *et al*., 2010), Ang-1, an endothelial-derived factor, promotes neurogenesis in SVZ cultures. These data lead us to further investigate the involvement of soluble factors, and particularly Ang-1, in the observed effect of CHX. Accordingly, we incubated a set of co-cultures with 5 µg/ml anti-Tie-2 neutralizing antibody (R&D Systems).

2.3.1.2.1. Evaluation of contact proteins in SVZ and mBEC cultures

SVZ spheres or mBEC preparations for microscopy analysis were obtained by centrifugating cell suspensions for 5 minutes in a Cellspin I (Tharmac, Waldsolms,

Germany). SVZ spheres were tested by immunocytochemistry for the co-expression of Nestin and α6 integrin, Nestin and β1 integrin or Nestin and Cx43. Mouse BEC were tested also by immunocytochemistry for the co-expression of CD31 and laminin-1 or CD31 and Cx43. The presence of laminin-1 in mBEC and α6 and β1 integrins in SVZ spheres and Cx43 in both cell types were confirmed by western blotting. To verify if Cx43 could mediate gap junctional contacts between endothelial cells and SVZ cells, co-cultures and brain slices (obtained as described in 2.3.1.1.1.) were immunolabelled for Cx43 and CD31.

In order to verify if CHX could reduce the amount of laminin-1 and Cx43 in mBEC, 1 h CHX-treated mBEC were processed for immunocytochemistry and western blotting and compared to untreated (control) cells.

Immunocytochemistry stainings are described in the "Immunostainings" section (2.2.2.). Western blotting protocol is described in the "Western blot analysis" section (2.2.9.).

2.3.1.2.2. Modulation of neurogenesis by endothelial-derived laminin-1 and SVZ-expressed α6β1 integrin and gap junctions

SVZ-mBEC co-cultures were conducted for 24 h in the presence of 5 µg/ml anti-α6 integrin neutralizing antibody (R&D Systems) or 35 µM carbenoxolone (CBX, a gap junction blocker, Sigma-Aldrich) in order to block the interaction of integrin α6ß1 with laminin-1 and the gap junction-mediated intercellular communication between SVZ and mBEC, respectively. CD31/BrdU, CD31/SOX-2, CD31/DCX and CD31/Tunel labellings were then performed, as described in sections 2.2.1., 2.2.2 and 2.2.4.2., to study proliferation, stemness, neuronal differentiation and cell death by apoptosis of SVZ cells in co-cultures.

2.3.1.3. Correlation between microvasculature and cell proliferation in neurogenic *versus* non-neurogenic areas of the adult mouse brain, either

intact or after induction of epilepsy

2.3.1.3.1. Characterization of blood vessel density in the neurogenic SVZ and in non-neurogenic areas, in control and KA-treated mice

In 20-μm brain slices of control and KA-treated mice, obtained as described in 2.1.3., vascular network density was determined using AP activity, as described in 2.2.3.

2.3.1.3.2. Evaluation of cell proliferation in the normal and epileptic brain and association with proximity to blood vessels

In 20-μm brain slices of control and KA-treated mice, obtained as described in 2.1.3., BrdU incorporated in the DNA was stained using the same protocol as detailed previously in 2.2.1. CD31 expression was determined as described in the "Immunostainings" section (2.2.2.).

2.4. Data and statistical analysis

In all experiments, except for immunocytochemistry of cytospin preparations and co-cultures, measurements were performed in the border of SVZ neurospheres where migrating cells form a cell pseudo-monolayer (Gage, 2000). For SCCI experiments, the percentage of neuronal-like responding cells (with a histamine/KCl ratio<0.8) was calculated on the basis of one microscopic field per coverslip, containing~100 cells (magnification, 40x). Percentages of NeuN-, BrdU-, or TUNEL-immunoreactive cells in SVZ cell cultures were calculated from cell counts in five independent microscopic fields in each coverslip with a 40x objective (~200 cells per field). Quantification of the numbers of neuritic ramifications positive for P-SAPK/JNK per neurosphere, as well as the total neuritic length per neurosphere (at 6 h), was performed in two independent cultures (two coverslips for each condition) in~20 non-overlapping fields per coverslip using digital images (magnification, 20x). Software used was Axiovision, release 4.6 (Carl Zeiss).

For the mBEC culture characterization, percentages of total CD31 positive cells were determined and the contaminants (non-CD31 cells) were identified.

In co-culture experiments where cell proliferation, apoptosis and stemness were assessed, SVZ cells contacting CD31+ cells were counted and the percentages of BrdU+/Tunel+/SOX-2+ cells within this population were determined (Figure 2.1.). In untreated (control) co-cultures, percentages of BrdU+/Tunel+/SOX-2+ cells were also determined in the border of SVZ neurospheres, where SVZ cells form a pseudomonolayer or a carpet of differentiating cells. In this particular case, a carpet corresponds to a region where no mBEC are present and, thereby, counted cells are not in contact with mBEC. In co-culture experiments concerning cell differentiation, measurements were performed only in SVZ cells contacting mBEC. Numbers of DCX+ ramifications in contact with mBEC as well as numbers of DCX+ positive cell bodies were expressed as a percentage of total cells contacting CD31+ cells.

Except where otherwise specified, the experiments were at least replicated in three independent culture preparations. Within each experiment, three coverslips for each condition were analysed.

In *in vivo* experiments where the brain vascular network was characterized, the SVZ surface was determined. According to Doetsch *et al*. 1997, the cellular composition of the SVZ comprehends ependymal cells (~30 μm wide), B cells (~10 μm wide), transit-amplifying cells or C cells (~20 μm wide) and migrating neuroblasts or A cells (~10 μm wide). Therefore, we considered the SVZ to be around 70 μm-wide starting from the lateral ventricle and gave it an excess of 30 μm, due to the existence of chains of migrating neuroblasts. Because the anterior SVZ is known to be larger, the excess given was of 80 μm. The total length was considered to be the lateral ventricle length (Figure 2.2.). This procedure allowed us to determine the total surface of the SVZ in each slice, which was essential to determine the total blood vessel surface as a percentage of the total SVZ surface. Six animals were used for

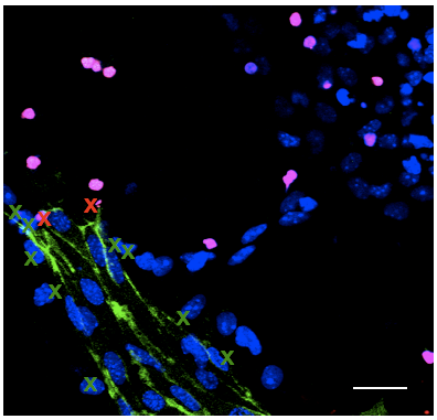


Figure 2.1. Representative fluorescent confocal digital image depicting a control mBEC/SVZ co-culture, stained for CD31, a maker of endothelial cells, in green, and for Tunel (apoptotic nuclei), in red. Hoechst 33342 staining (blue) was used to visualize cell nuclei. Crosses show how cell countings are performed in the population of cells contacting mBEC: green crosses indicate negative nuclei, red crosses indicate positive nuclei. Scale bar, 20 μm.

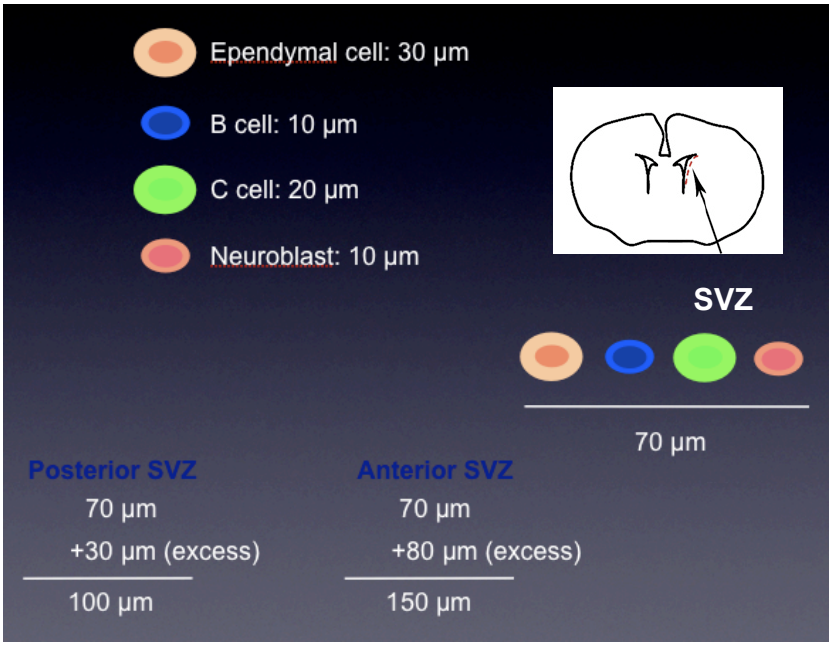


Figure 2.2. In order to characterize brain vascular network, the SVZ surface was determined. According to Doetsch *et al.,* 1997, the cellular composition of the SVZ comprehends ependymal cells (~30 μm wide), B cells (~10 μm wide), transit amplifying cells or C cells (~20 μm wide) and migrating neuroblasts or A cells (~10 μm wide). Therefore, we considered the SVZ to be around 70 μm-wide starting from the lateral ventricle and gave it an excess of 30 μm, due to the existence of chains of migrating neuroblasts. Because the anterior SVZ is known to be larger, the excess given was of 80 μm. The total length was considered to be the lateral ventricle length.

determinations in each condition (a total of 30 and 31 brain slices analysed for control and KA-treated animals, respectively). In *in vivo* experiments where correlation between proliferation and the proximity to blood vessels was investigated, total BrdU nuclei were counted in z-stacks of 20-μm brain slices and classified according to the distance from blood vessels: 0 μm, <10 μm, 10-50 μm and >50 μm. Five animals were used for determinations in control animals, four animals were used for determinations in KA-treated animals (a total of 15 and 12 brain slices analysed for control and KA-treated animals, respectively).

Data are expressed as mean ± SEM. Statistical significance was determined by using the unpaired two-tailed Student *t* test, with $p \leq 0.05$ considered to represent statistical significance. In co-culture experiments, the ANOVA test was used with Bonferroni post-test to compare selected columns of data, with $p \leq 0.05$ considered to represent statistical significance.

Chapter 3 - The angiogenic factor angiopoietin-1 is a pro-neurogenic peptide on subventricular zone stem/progenitor cells

3.1. Abstract

In the adult mammalian brain, the subventricular zone (SVZ) hosts stem cells constantly generating new neurons. Angiopoietin-1 (Ang-1) is an endothelial growth factor with a critical role in division, survival and adhesion of endothelial cells *via* Tie-2 receptor activity. Expression of Tie-2 in nonendothelial cells, especially neurons and stem cells, suggests that Ang-1 may be involved in neurogenesis.

In the present work, we investigated the putative role of Ang-1 on SVZ neurogenesis. Immature cells from SVZ-derived neurospheres express Ang-1 and Tie-2 mRNA and protein, suggesting a role for the Ang-1/Tie-2 system in the neurogenic niche. Moreover, we also found that Tie-2 protein expression is retained upon differentiation in neurons and glial cells. Ang-1 triggered proliferation *via* activation of the ERK1/2 (extracellular signal-regulated kinase 1/2) mitogen-activated protein kinase (MAPK) kinase pathway but did not induce cell death. Accordingly, coincubation with an anti-Tie-2 neutralizing antibody prevented the pro-proliferative effect of Ang-1. Furthermore, Ang-1 increased the numbers of NeuN (neuronal nuclear protein)-positive neurons in cultures treated for 7 d, as well as the numbers of functional neurons, as assessed by monitoring $[Ca^{2+}]_i$ rises after application of specific stimuli for neurons and immature cells. The pro-neurogenic effect of Ang-1 is mediated by Tie-2 activation and subsequent mTOR (mammalian target of rapamycin kinase) mobilization. In agreement, neuronal differentiation significantly decreased after exposure to an anti-Tie-2 neutralizing antibody and to rapamycin. Moreover, Ang-1 elicited the activation of the SAPK (stress-activated protein kinase)/JNK (c-Jun N-terminal kinase) MAPK, involved in axonogenesis.

Our work shows a pro-neurogenic effect of Ang-1, highlighting the relevance of blood vessel/stem cell crosstalk in health and disease.

3.2. Introduction

In the adult mammalian brain, neurogenesis occurs constitutively in the

subventricular zone (SVZ) from glial fibrillary acidic protein (GFAP)-expressing neural stem cells (Lledo *et al*., 2006; Zhao *et al*., 2008; Chojnacki *et al*., 2009).

Neurogenesis is tightly regulated by diffusible factors and some of them are regulators of angiogenesis. In the postnatal and adult brain, angiogenesis occurs by sprouting of new vessels from preexisting ones. Molecular players act in concert to regulate the multistep process of angiogenesis, such as vascular endothelial growth factor (VEGF), which induces mitosis of endothelial cells. Moreover, stabilization and maturation of the sprouts is ensured by angiopoietin-1 (Ang-1), an endothelial growth factor that promotes cellular adhesion of supporting cells and survival (Thurston *et al*., 1999; Papapetropoulos *et al*., 2000; Patan, 2000; Yancopoulos *et al*., 2000). Ang-1 effects are mediated through binding to the Tie-2 tyrosine kinase receptor (Suri *et al*., 1996). Apart from its role in angiogenesis, VEGF is a potent inducer of neurogenesis, increasing proliferation and neuronal differentiation in progenitor cell cultures as well as in the SVZ and subgranular zone *in vivo* (Jin *et al*., 2002b; Schanzer *et al*., 2004; Sun *et al*., 2006; Segi-Nishida *et al*., 2008). Despite the well-described involvement of Ang-1 in angiogenesis, little is still known about its effect on neurogenesis. The Tie-2 receptor is found in neurons (Valable *et al*., 2003; Kosacka *et al*., 2005) and glial cells such as Schwann cells and glioblastoma cells (Poncet *et al*., 2003; Lee *et al*., 2006; Makinde and Agrawal, 2008). In addition, Tie-2 expression is found in stem cells, including embryonic stem cells from human (Parati *et al*., 2002) and mouse brains (Bai *et al*., 2009a), rat liver stem cells (Kuroda *et al*., 2002), and hematopoietic and mesenchymal stem cells (Huang *et al*., 1999; Yuasa *et al*., 2002). Expression outside the vascular compartment suggests a wider biological role of the Ang-1/Tie-2 system than that previously thought and Ang-1 may regulate stem cell dynamics. Indeed, exogenous addition of Ang-1 promotes survival of neurons, mesenchymal stem cells, and neural progenitor cells after exposure to serum deprivation or hypoxia (Valable *et al*., 2003; Bai *et al*., 2009b). In hematopoietic stem cells, Ang-1/Tie-2 is crucial for the maintenance of the stem cell state (Hirao *et al*.,

2004). Moreover, Ang-1 elicits neuronal differentiation and neurite outgrowth in mouse embryonic cortical and dorsal root ganglion cells (Kosacka *et al*., 2005; Bai *et al*., 2009a). After cortical and striatal stroke, angiogenic remodelling is accompanied by neurogenesis in the peri-infarct area and in the SVZ. Stroke elicits an upregulation of endothelial-derived Ang-1 and the migration of newly-born doublecortin (DCX)-positive neuroblasts from the SVZ to the damaged area. These data suggest a positive action of Ang-1 on SVZ neurogenesis (Ohab *et al*., 2006; Yamashita *et al*., 2006; Shin *et al*., 2008).

In the present work, we propose to unravel the role of Ang-1 on SVZ neurogenesis, with a focus on proliferation, differentiation and axonogenesis.

3.3. Results

3.3.1. SVZ cells express both Ang-1 and its receptor Tie-2

The expression of both Ang-1 and the Tie-2 receptor was first investigated in SVZ neurospheres in proliferative conditions. Briefly, primary neurospheres were grown from single SVZ dissociated cells in SFM containing 10 ng/ml EGF and 5 ng/ml FGF-2. After 4–5 d, neurospheres were collected and processed for RT-PCR, western blot, and immunocytochemistry. Transcripts of mRNA for Ang-1 and Tie-2 were detected by RT-PCR in SVZ neurospheres (Figure 3.1*A*). Additionally, by western blotting, expression of Ang-1 and Tie-2 protein was demonstrated in SVZ cells (Figure 3.1*B*). Moreover, Tie-2 and Ang-1 were detected by immunocytochemistry in Nestin-positive SVZ neurospheres (Figure 3.1*C*, *Cc1*, *Cc2* or *D*, *Dd1*, *Dd2*, respectively). These results suggest that SVZ neurospheres secrete Ang-1 that may signal in an autocrine/ paracrine manner to modulate the SVZ cell dynamics. To disclose whether Tie-2 expression is maintained upon differentiation, SVZ neurospheres were seeded onto poly-D-lysine-coated coverslips and allowed to differentiate in SFM devoid of growth factors for 48 h to 7 d. During this period of time, cells migrate out of the neurospheres and form a pseudo-monolayer so-called

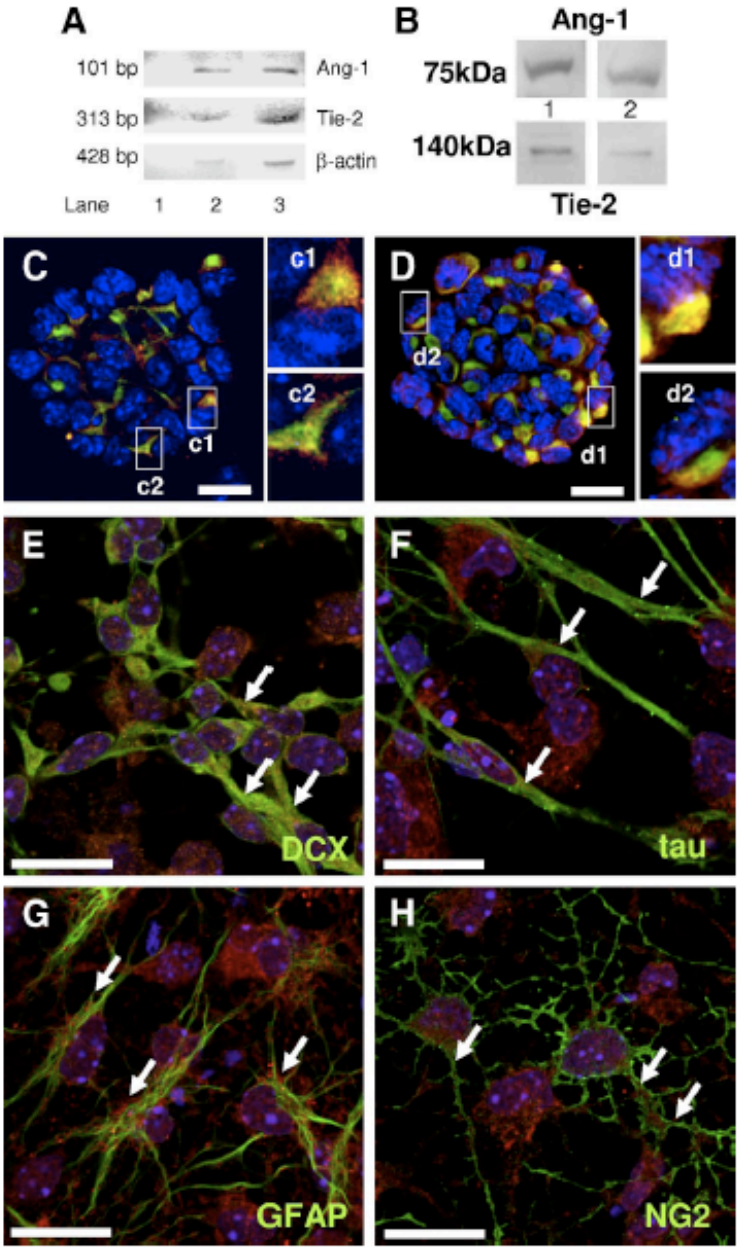


Figure 3.1. SVZ cells express both Ang-1 and Tie-2. ***A***, RT-PCR detection of Ang-1 and Tie-2 mRNAs in SVZ neurospheres. Lanes 1 and 2 correspond to the negative control (nontemplate negative control) and positive control (total mRNAs from mouse placental tissue), respectively. Lane 3 corresponds to SVZ neurospheres. Ang-1, 101 bp; Tie-2, 313 bp. ***B***, Detection of Tie-2 and Ang-1 proteins by western blotting in SVZ neurospheres. Lane 1 corresponds to the positive control (total proteins from mouse placenta), and lane 2 corresponds to SVZ proliferating cells. ***C***, ***D***, Representative fluorescent confocal digital images depicting Tie-2 and Ang-1 immunoreactivity in SVZ neurospheres (red staining for Tie-2 and Ang-1; green staining for Nestin, as a marker of immature cells) ***c1*** and ***c2***, and ***d1*** and ***d2*** are magnifications of squares in ***C*** and ***D***, respectively. ***E***, The Tie-2 receptor is maintained in SVZ cells migrating out of a neurosphere 2 d after plating, and it is expressed in neuroblasts (red staining for Tie-2; green staining for DCX, a marker of immature neurons). ***F***, The Tie-2 receptor is also maintained in SVZ cell-derived neurons after 7 d of differentiation (red staining for Tie-2; green staining for the τ (tau) protein, an axonal marker). ***G***, ***H***, Tie-2 expression is retained in astrocytes (red staining for Tie-2; green staining for GFAP) and oligodendrocyte progenitor cells (red staining for Tie-2; green staining for NG2 chondroitin sulfate proteoglycan, a marker of oligodendrocyte progenitors), respectively. The arrows indicate regions of Tie-2 labelling. Hoechst 33342 staining (blue) was used to visualize cell nuclei. Scale bars, 20 μm.

"carpet of differentiation", constituted of neurons, oligodendrocytes, and astrocytes in different stages of maturation. Expression of the Tie-2 receptor is maintained along the neuronal lineage as immature neurons expressing DCX (Figure 3.1*E*), as well as τ-positive mature neurons (Figure 3.1*F*), are immunoreactive for Tie-2. Nevertheless, expression of Tie-2 is not restricted to the neuronal lineage as both GFAP-positive astrocytes and NG2-positive oligodendrocyte precursors are found immunopositive for Tie-2 (Figure 3.1*G* and *H*, respectively). Negative controls were performed to confirm the specificity of the antibodies used for the detection of the Tie-2 receptor and Ang-1 (data not shown). These results were confirmed with another anti-Tie-2 primary antibody (Figure 3.2*A*-*F*). Notably, we showed that in coincubation with a Tie-2 blocking peptide, the anti-Tie-2 antibody failed to label SVZ cells demonstrating the specificity of the Tie-2 labelling (Figure 3.2*A* and *B*). A brain slice double-labelled for CD31 and Tie-2 was used as a positive control as vessels expressed Tie-2 (Figure 3.3). The wide expression of the Tie-2 receptor suggests that Ang-1 may modulate proliferation and differentiation in the SVZ neurogenic niche.

3.3.2. Ang-1 stimulates cell proliferation and exerts no effect on cell death

We first determined whether Ang-1 modulates cell proliferation. For that purpose, we applied increasing concentrations of Ang-1 (10, 100, and 500 ng/ml) for 48 h on SVZ cells in differentiation conditions. After fixation, BrdU was immunorevealed and positive nuclei were counted. Representative immunostainings for BrdU in control and 500 ng/ml Ang-1-treated conditions are shown in Figure 3.4, *A* and *B*, respectively. A significant increase in the numbers of BrdU-immunopositive nuclei was obtained in cultures incubated with 100 and 500 ng/ml but not with 10 ng/ml Ang-1 compared with control (control: 8.13 ± 0.52%, n = 15 coverslips, 12912 cells counted; 10 ng/ml Ang-1: 7.99 ± 0.67%, n=10 coverslips, 7816 cells counted; 100 ng/ml Ang-1: 10.07 ± 0.53%, n = 7 coverslips, 7567 cells counted, $p < 0.05$; 500

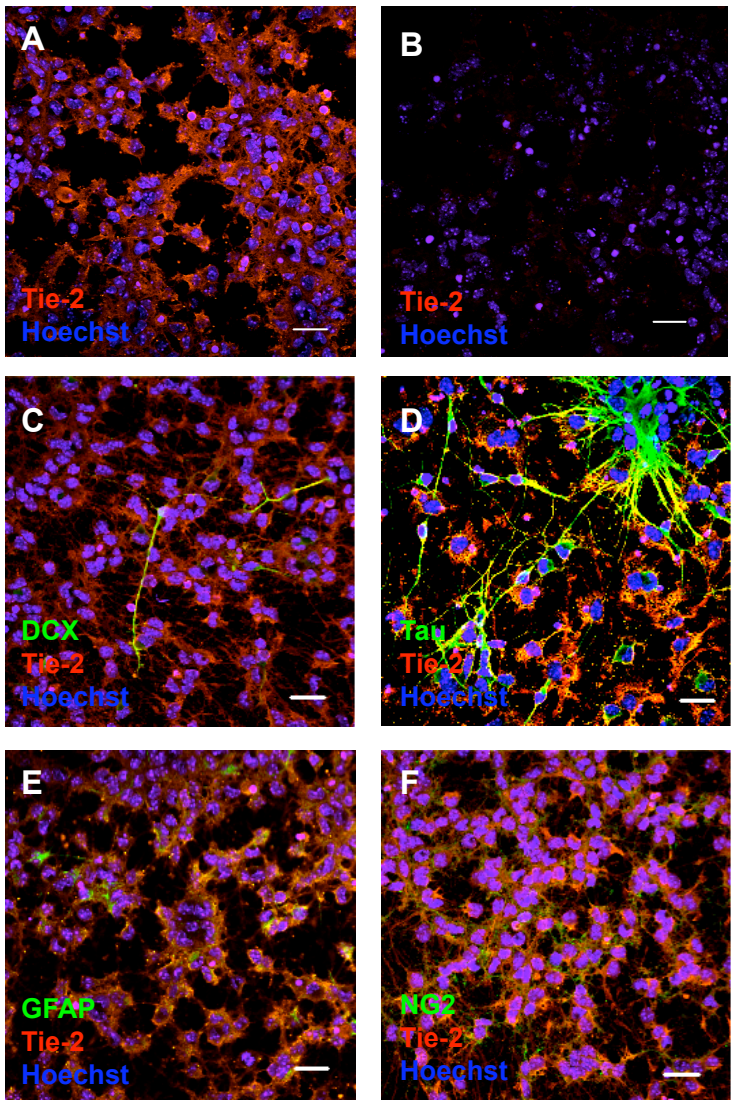


Figure 3.2. Phenotype discrimination of Tie-2 positive cells in SVZ differentiating cells, using a different anti-Tie-2 antibody. To confirm the results shown in Figure 3.1., we used another anti-Tie-2 primary antibody (1:50 from Santa Cruz Biotechnology). SVZ differentiating cells are positive for Tie-2 (***A***) and this labelling is specific, once it is abolished by co-incubating the cells with the antibody and the respective blocking peptide in a 1:10 proportion (***B***). Expression of Tie-2 in DCX (***C***) and τ (Tau) (***D***) positive cells, indicating that it is present in immature and mature neurons. Tie-2 is also expressed in glial cells, namely in GFAP positive astrocytes (***E***) and NG2 positive oligodendrocyte progenitors (***F***). Hoechst 33342 staining (blue) was used to visualize cell nuclei. Scale bars, 20 μm.

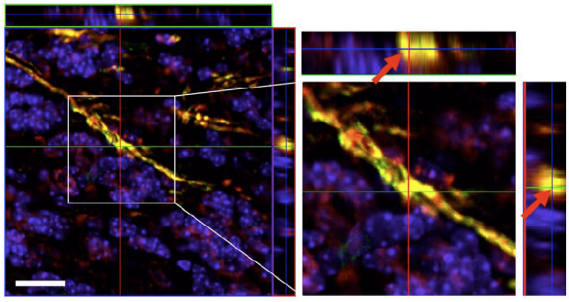


Figure 3.3. Representative z-stack digital image of an immunohistochemistry positive control for the anti-Tie-2 antibody. *In vivo* staining shows blood vessel endothelial cells expressing both Tie-2 (red) and CD31 (green). Red arrows indicate areas of co-expression in the same vessel. Hoechst 33342 staining (blue) was used to visualize cell nuclei. Scale bar, 20 μm.

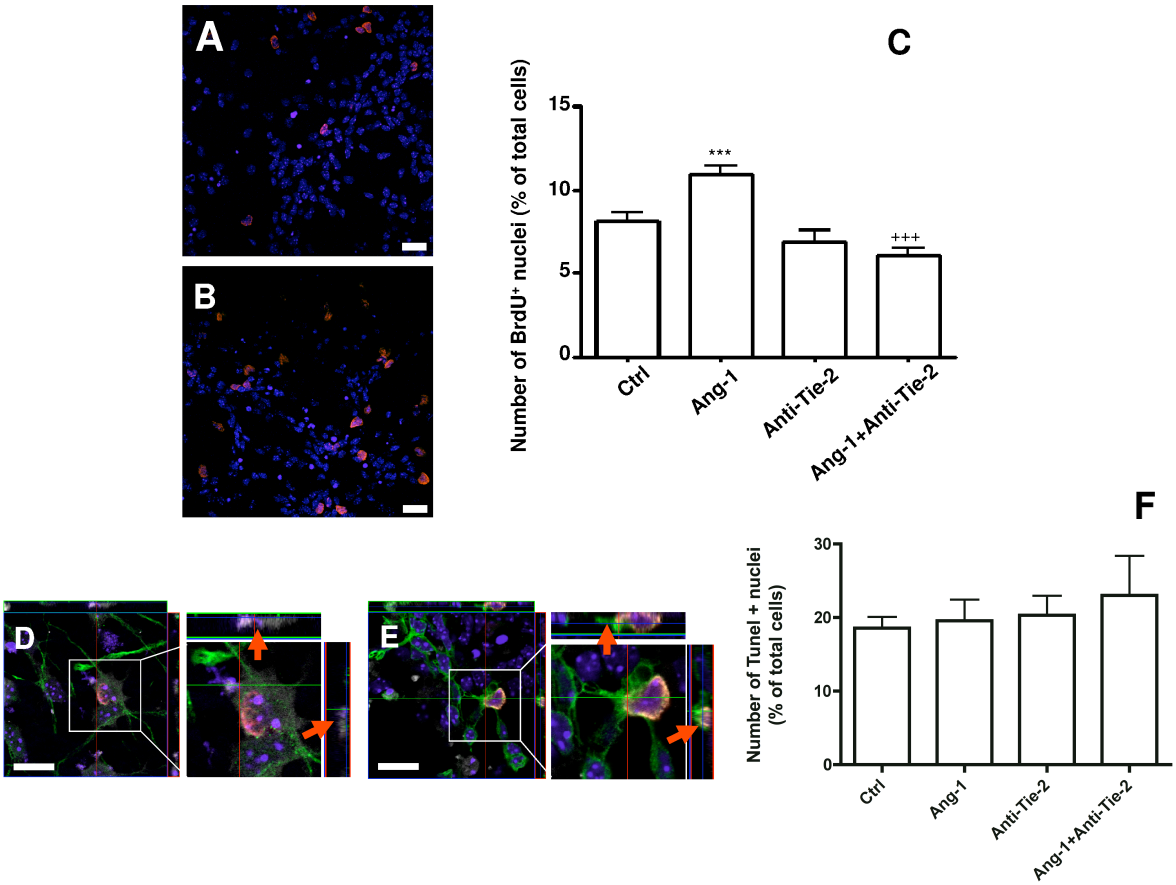


Figure 3.4. Ang-1 modulates cell proliferation in mouse SVZ cell cultures, an effect mediated by the Tie-2 receptor. ***A***, ***B***, Representative fluorescent confocal digital images of BrdU (red nuclei) and Hoechst 33342 staining (blue nuclei) in a control culture and in a culture treated with 500 ng/ml Ang-1, respectively. ***C***, Bar graph depicts the number of BrdU-positive cells, expressed as percentages (%) of the total number of counted nuclei, in control cultures and in cultures exposed to 500 ng/ml Ang-1 and/or 5 μg/ml anti-Tie-2 neutralizing antibody for 48 h. Data are expressed as mean ± SEM. ***$p < 0.001$, using the unpaired Student *t* test for comparison with SVZ control cultures. +++ $p < 0.001$, using the unpaired Student *t* test for comparison with Ang-1-treated SVZ cultures. ***D***, ***E***, Representative *z*-stack confocal digital images of control SVZ cultures showing BrdU-positive cells (in red) positive for Tie-2 (white staining) and DCX (green staining) (***D***) or Nestin (green staining) (***E***). The arrows indicate areas of triple labelling. Hoechst 33342 staining (blue) was used to visualize cell nuclei. Scale bars, 20 μm. ***F,*** Bar graph depicts the number of Tunel-positive cells, expressed as % of the total number of counted nuclei, in control cultures and in cultures exposed to 500 ng/ml Ang-1 and/or 5 μg/ml anti-Tie-2 neutralizing antibody for 48 h. Data are expressed as mean ± SEM.

ng/ml Ang-1: 11.00 ± 0.43%, *n* = 15 coverslips, 14935 cells counted, $p < 0.001$). As the most marked effect was obtained with 500 ng/ml Ang-1, we used this concentration for the following studies, as depicted in Figure 3.4*C*. To investigate whether the Tie-2 receptor mediates the pro-proliferative effects of Ang-1, SVZ cell cultures were coexposed to 500 ng/ml Ang-1 and 5 µg/ml anti-Tie-2 neutralizing antibody, for 48 h, followed by BrdU immunoassays. As depicted on Figure 3.4*C*, the proliferative effect of 500 ng/ml Ang-1 was prevented in the presence of the anti-Tie-2 antibody (5 µg/ml anti-Tie-2 plus 500 ng/ml Ang-1: 6.08 ± 0.44%, *n* = 6 coverslips; 5885 cells counted; $p < 0.001$ *versus* Ang-1 alone). Hence, Ang-1 promotes proliferation of SVZ cells *via* Tie-2 binding. To further disclose the progenitor phenotypes of the cells induced to proliferate on Tie-2 binding, triple immunocytodetections were performed. Some of the BrdU-positive cells were simultaneously positive for Tie-2 and DCX, expressed by neuroblasts (Figure 3.4*D*), and for Tie-2 and Nestin, a marker of immature cells (Figure 3.4*E*). These data demonstrate that Ang-1 *via* Tie-2 binding induces proliferation at least in neuroblasts and immature cells. In addition, we performed TUNEL staining to examine the effects of Ang-1/Tie-2 on apoptosis (Figure 3.4*F*). Exposure of SVZ cells to 500 ng/ml Ang-1 and/or 5 µg/ml anti-Tie-2 for 48 h did not affect the numbers of TUNEL-positive nuclei (control: 18.57 ± 1.53%, *n* = 10 coverslips, 4542 cells counted; 500 ng/ml Ang-1: 19.56 ± 2.88%, *n* = 4 coverslips, 3876 cells counted; 5 µg/ml anti-Tie-2 plus 500 ng/ml Ang-1: 23.02 ± 5.37%, *n* = 6 coverslips, 4468 cells counted).

3.3.3. Ang-1 modulates cell proliferation through the ERK/MAPK kinase pathway

Proliferation in SVZ cultures has been reported mainly to depend on the activation of the ERK/MAPK kinase pathway (Learish *et al*., 2000; Agasse *et al*., 2008a; Bernardino *et al*., 2008; Nicoleau *et al*., 2009). To test whether Ang-1 binding to Tie-2 results in the downstream activation of this pathway, BrdU incorporation

assays were performed in the presence of both 500 ng/ml Ang-1 and 20 μM U0126, a highly selective inhibitor of both MEK1 and MEK2, activators of ERK1/2. As expected, incubation with the MAPK kinase inhibitor alone decreased significantly the normal proliferative activity inherent to SVZ cultures (control: 6.32 ± 0.49%, *n* = 6 coverslips, 5468 cells counted; control plus 20 μM U0126: 3.36 ± 0.31%, *n* = 6 coverslips, 4898 cells counted; $p < 0.001$) (Figure 3.5), demonstrating the specificity of U0126 to inhibit proliferation associated to ERK activation. We verified that DMSO, the solvent used to resuspend U0126, was not toxic, by performing TUNEL. In fact, this solvent, diluted 10,000 times from our 20 mM stock solution, did not increase cell death (control: 18.57 ± 1.53%, *n* = 10 coverslips, 7966 cells counted; DMSO, 1/10,000: 17.04 ± 0.34%, *n* = 2 coverslips, 2185 cells counted). Increase of proliferation obtained with 500 ng/ml Ang-1 is abolished in the presence of U1026 (500 ng/ml Ang-1 plus 20 μM U0126: 4.29 ± 0.50% positive nuclei, *n* = 6 coverslips, 4577 cells counted, $p < 0.05$ compared with control, $p < 0.001$ compared with Ang-1-treated condition) (Figure 3.5). Therefore, the pro-proliferative effect of Ang-1 is associated with ERK1/2 MAPK kinase pathway activation.

3.3.4. Ang-1 induces neuronal differentiation *via* Tie-2 and mTOR activation

To unravel whether Ang-1 promotes neuronal differentiation, cells were treated with 500 ng/ml Ang-1 for 7 d. After that, neuronal differentiation was evaluated after the immunorevelation of the neuronal-specific nuclear protein NeuN (Figure 3.6 *A and B*). Ang-1 induced a significant increase in the numbers of NeuN immunoreactive cells compared with the control condition (control: 11.64 ± 1.25%, *n* = 12 coverslips, 9566 cells counted; 500 ng/ml Ang-1: 18.62 ± 1.22%, *n* = 9 coverslips, 8619 cells counted; $p < 0.001$) (Figure 3.6*C*). Interestingly, addition of 5 μg/ml anti-Tie-2 neutralizing antibody to the culture together with 500 ng/ml Ang-1 prevented the pro-neurogenic effect of Ang-1 (5 μg/ml anti-Tie-2 plus 500 ng/ml Ang-

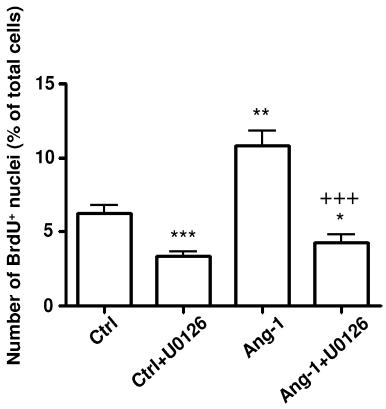


Figure 3.5. Ang-1 modulates cell proliferation in mouse SVZ cell cultures, an effect mediated by the MAPK/ERK kinase pathway. Bar graph depicts the number of BrdU-positive cells, expressed as percentages (%) of the total number of nuclei per culture, in control cultures and in cultures exposed to 500 ng/ml Ang-1 and/or 20 μM U0126, a highly selective inhibitor of MEK1 and 2, for 48 h. Data are expressed as a mean ± SEM. ***p < 0.001, **p < 0.01, *p < 0.05, using the unpaired Student t test for comparison with SVZ control cultures. +++p < 0.001, using the unpaired Student t test for comparison with Ang-1-treated SVZ cultures.

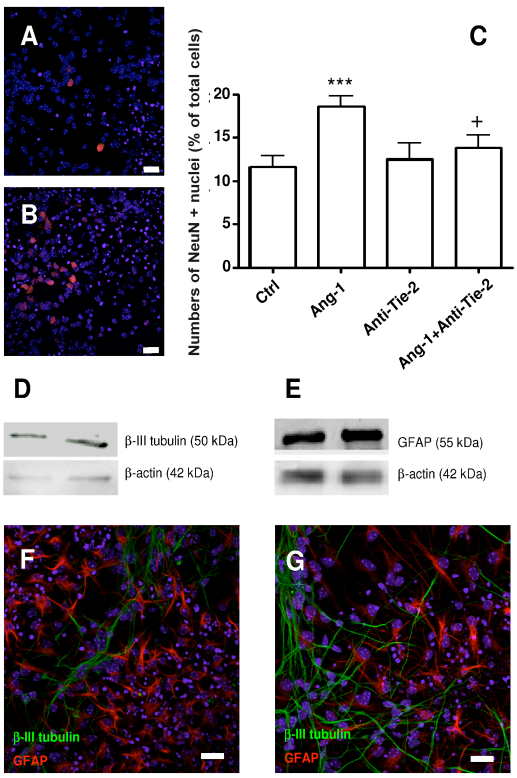


Figure 3.6. Ang-1 induces neuronal differentiation in mouse SVZ cell cultures *via* Tie-2 activation. ***A***, ***B***, Representative fluorescent confocal digital images of NeuN-positive neurons (red nuclei) and Hoechst 33342 staining (blue nuclei) in control SVZ cultures (***A***) and in cultures treated with Ang-1 (***B***)., Scale bars, 20 μm. ***C***, Bar graph depicts the number of NeuN-positive cells, expressed as percentages (%) of the total number of cells per culture, in control cultures and in cultures treated with 500 ng/ml Ang-1 and/or 5 μg/ml anti-Tie-2 neutralizing antibody, for 7 d. Data are expressed as mean ± SEM. ***p < 0.001, using the unpaired Student t test for comparison with SVZ control cultures. +p < 0.05, using the unpaired Student t test for comparison with SVZ cultures treated with Ang-1. ***D***, ***E***, Representative Western blots for βIII tubulin (***D***) and GFAP (***E***) the β-actin blots are provided as loading controls of total protein extract from SVZ cells treated for 7 d in the absence (control) or the presence of 500 ng/ml Ang-1. ***F***, ***G***, Representative confocal digital images of SVZ cell cultures treated for 7 d in the absence (control) (***F***) or the presence of 500 ng/ml Ang-1 (***G***) and stained for βIII tubulin (green staining) and GFAP (red staining). Hoechst 33342 staining (blue) was used to visualize cell nuclei. Scale bars, 50 μm.

1: 13.86 ± 1.42%, *n* = 9 coverslips, 7221 cells counted; $p < 0.05$ *versus* Ang-1 alone) (Figure 3.6*C*). Incubation of the cells with the anti-Tie-2 antibody had no effect on the numbers of NeuN-positive cells (5 µg/ml anti-Tie-2: 12.49 ± 1.93%, *n* = 4 coverslips, 2883 cells counted) (Figure 3.6*C*). These results suggest that Ang-1 increases morphological neuronal differentiation *via* Tie-2 binding. Neurogenesis may occur detrimentally to glial differentiation. To disclose this point, SVZ neurospheres were seeded onto poly-D-lysine-coated culture dishes and incubated during 7 d in the absence or the presence of 500 ng/ml Ang-1. Relative amount of astrocytes and neurons protein markers were quantified by western blotting. Figure 3.6 *D* and *E* depict the immunoblots for βIII tubulin and GFAP. Quantification revealed that the amount of GFAP was similar in control and Ang-1-treated condition (control: 100.00 ± 16.80%; 500 ng/ml Ang-1: 81.94 ± 4.86%; *n* = 3 independent experiments). However, Ang-1 increased the βIII tubulin levels, compared with control (control: 100.00 ± 11.39%; 500 ng/ml Ang-1: 137.20 ± 14.90%; *n* = 3 independent experiments). Consistently, immunoreactivity to βIII tubulin was increased in SVZ cultures treated in the presence of Ang-1 (Figure 3.6 *F* and *G*). This suggests that Ang-1 induces neuronal differentiation but does not decrease the numbers of glial cells generated. We then evaluated the functional neuronal differentiation in SVZ cultures using a method settled at our laboratory, based on the monitoring of the variations of $[Ca^{2+}]_i$ in single cells in response to 50 mM KCl and 100 µM histamine stimulations (Agasse *et al*., 2008b). Membrane depolarization of neuronal cells after exposure to high KCl concentrations leads to the opening of voltage-sensitive calcium channels and massive influx of calcium into the cytoplasm (Ambrósio *et al*., 2000), whereas stimulation with histamine specifically triggers an increase in $[Ca^{2+}]_i$ in immature SVZ cells (Tran *et al*., 2004). Taking this into consideration, we demonstrated that a low histamine/KCl ratio of response (< 0.8) is characteristic of SVZ-derived neurons (Agasse *et al*., 2008b). SVZ neurospheres were treated during 7 d as

aforementioned and then loaded with the Fura-2 AM calcium probe, perfused continuously for 15 min with Krebs' solution, and briefly (2 min) stimulated with 50 mM KCl and 100 µM histamine (Figure 3.7*A*). Figure 3.7*B-D*, depict representative profiles of response displayed by control cultures (Figure 3.7*B)*, Ang-1-treated alone (Figure 3.7*C*), or together with the anti-Tie-2 antibody (Figure 3.7*D*). Quantification of the numbers of cells presenting a neuronal-like profile of response is represented in Figure 3.7*E*. Control cultures showed a predominant immature-like profile, characterized by cells mainly increasing $[Ca^{2+}]_i$ in response to histamine (Figure 3.7 *B* and *E*). In contrast, Ang-1-treated SVZ cells displayed an increase in the numbers of cells increasing $[Ca^{2+}]_i$ in response to KCl but not to histamine stimulation comparing with control cells, consistent with a neuronal-like profile (Figure 3.7*C* and *E*) (control: 7.50 ± 2.20%, 1927 cells analysed, *n* = 14 coverslips; 500 ng/ml Ang-1: 23.50 ± 5.06%, 1419 cells analysed, *n* = 11 coverslips; $p < 0.01$). In the presence of both Ang-1 and anti-Tie-2 antibody, cells responded mainly as observed in the control condition. Indeed, the pro-neurogenic effect of Ang-1 was prevented by incubation with the anti-Tie-2 antibody (5 µg/ml anti-Tie-2 plus 500 ng/ml Ang-1: 6.95 ± 2.52%, 1080 cells analysed, *n* = 9 coverslips; $p < 0.05$ *versus* 500 ng/ml Ang-1 alone) (Figure 3.7 *D* and *E*). In addition, we verified that incubation of cells with the anti-Tie-2 antibody alone did not affect neuronal differentiation (5 µg/ml anti-Tie-2: 10.09 ± 5.02%, 836 cells analysed, *n* = 5 coverslips) (Figure 3.7*E*). Together, these results show that exogenous addition of 500 ng/ml Ang-1 induces neuronal differentiation in SVZ cells, an effect mediated through Tie-2 activation. In another set of experiments, SVZ cells were coincubated for 7 d with 20 nM rapamycin and 500 ng/ml Ang-1. Rapamycin inhibits mTOR, a serine-threonine kinase of the downstream pathway of the PI3K/AKT kinases. In cultures treated with both rapamycin and Ang-1, fewer functional neurons were obtained compared with cultures treated with Ang-1 alone (control: 8.05 ± 2.93%, 2189 cells analysed, *n* = 19 coverslips; 500 ng/ml Ang-1: 34.43 ± 4.99%, 2003 cells analysed, *n* = 14 coverslips,

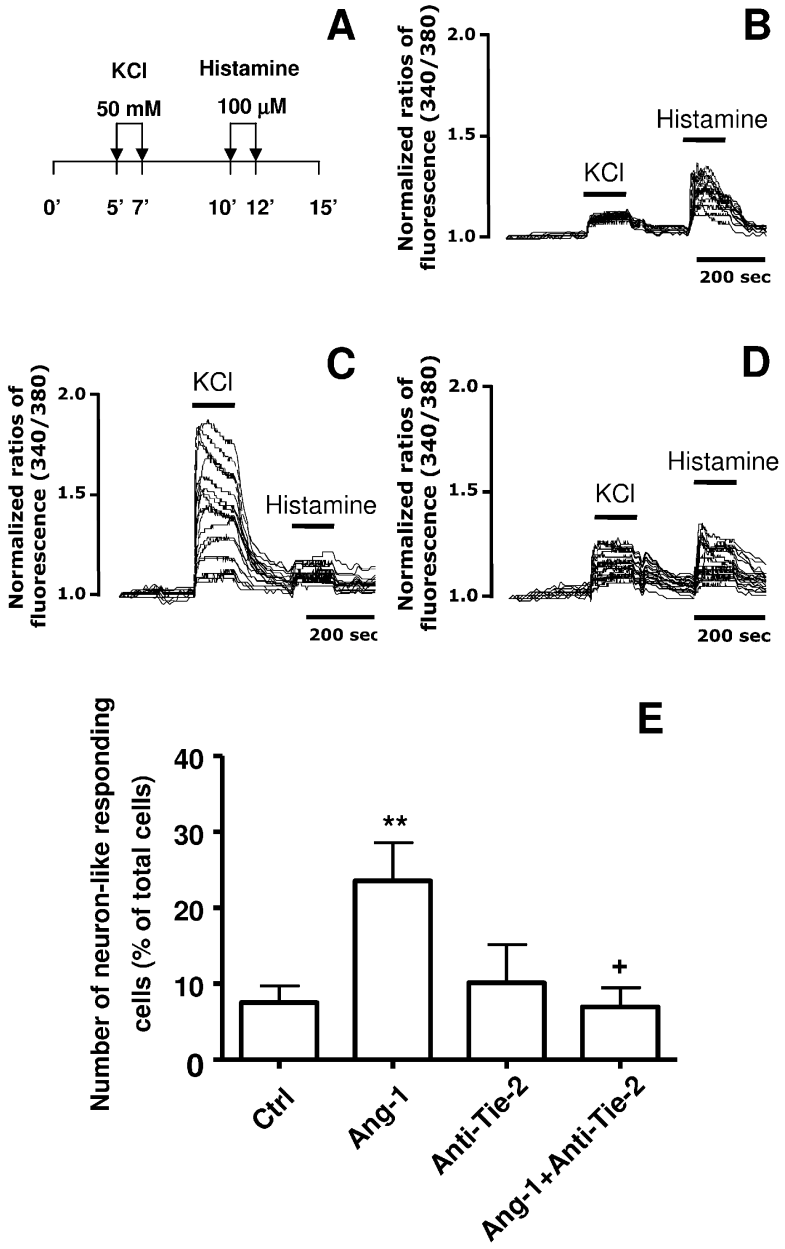


Figure 3.7. Ang-1 increases the generation of neuronal-like responding cells in mouse SVZ cell cultures *via* Tie-2 activation. ***A***, SVZ cultures were perfused continuously with Krebs' solution for 15 min and stimulated for 2 min (from minute 5 to minute 7) with 50 mM KCl and for 2 min (from minute 10 to minute 12) with 100 µM histamine. ***B–D***, Shown are representative single-cell calcium imaging profiles of response of 20 cells in a control culture (***B***), in a 500 ng/ml Ang-1-exposed culture (***C***), and in a culture treated with both 500 ng/ml Ang-1 and 5 µg/ml anti-Tie-2 neutralizing antibody (***D***). ***E***, Bar graph depicts the percentages (%) of neuronal-like responding cells in SVZ control cultures and in cultures exposed to Ang-1 and/or anti-Tie-2 for 7 d. Data are expressed as mean ± SEM.**$p < 0.01$, using the unpaired Student *t* test for comparison with SVZ control cultures. +$p < 0.05$, using the unpaired Student *t* test for comparison with SVZ cultures treated with Ang-1.

p < 0.001; 20 ng/ml rapamycin plus 500 ng/ml Ang-1: 10.57 ± 3.42%, 1435 cells analysed, *n* = 12 coverslips, *p* < 0.001 *versus* 500 ng/ml Ang-1 alone; 20 ng/ml rapamycin: 10.83 ± 2.46%, 1163 cells analysed, *n* = 10 coverslips; DMSO, 1/100,000: 6.65 ± 1.67%, 967 cells analysed, *n* = 8 coverslips) (Figure 3.8), demonstrating that mTOR mediates neuronal differentiation induced by Ang-1 stimulation. Previously, we verified that DMSO, diluted 10,000 times from our 20 mM stock solution, was not toxic by performing TUNEL (as indicated above). Because DMSO used to resuspend rapamycin was even more diluted (1/100,000) we did not repeat the TUNEL assays.

3.3.5. Ang-1 promotes neuronal maturation *via* the activation of the SAPK/JNK pathway

We showed that Ang-1 increased the numbers of morphologically (NeuN expression) and functionally (increase of intracellular calcium concentration after KCl depolarization) differentiated neurons in SVZ cultures. In fact, most neurons extend a single axon to be fully developed and functional. Recently, activation of the SAPK/JNK MAPK pathway has been shown to be related to axonogenesis (Oliva *et al*., 2006). Moreover, we verified that two pro-neurogenic factors, neuropeptide Y (NPY) and tumour necrosis factor-α (TNFα), increased the numbers and total length of ramifications immunoreactive for the phosphorylated form of JNK, P-JNK, in SVZ cultures. Additionally, P-JNK-positive ramifications colocalized with the immunoreactivity to the axon-specific protein τ (Agasse *et al*., 2008a; Bernardino *et al*., 2008) but not with MAP-2 (microtubule-associated protein 2), expressed in dendrites (Figure 3.9). Therefore, we verified whether Ang-1 activates the SAPK/JNK signalling pathway. Exposure of SVZ cells to 500 ng/ml Ang-1 for 6 h increased P-JNK immunoreactivity in neurites and growth cone-like structures emerging from the neurospheres compared with control cultures. Double immunocytochemistry was

performed to visualize whether P-JNK is associated with the axonspecific protein τ (Figure 3.10*A*). P-JNK is indeed localized predominantly in τ-positive axons. Quantification of the total length of ramifications per neurosphere (Figure 3.10*B*) as well as the numbers of ramifications per neurosphere (Figure 3.10*C*) showed that 500 ng/ml Ang-1 increases significantly both parameters compared with control cultures (total length of ramification per neurosphere: control, 128.70 ± 24.21 µm; Ang-1, 479.50 ± 70.51 µm, $p < 0.001$; numbers of ramifications per neurosphere: control, 1.81 ± 0.19; Ang-1, 4.20 ± 0.61, $p < 0.01$). The same experiments were performed in the presence of the 20 µM SP600125, an inhibitor of JNK activity. It has been previously demonstrated that SP600125 reduces the JNK phosphorylation (Oliva *et al*., 2006). Conversely, P-JNK immunoreactivity decreased in SP00125-treated cultures. In fact, both numbers (Figure 3.10*C*) and length of P-JNK ramifications (Figure 3.10*B*) per neurospheres decreased in cultures treated with SP600125 alone or together with 500 ng/ml Ang-1 (total length of ramification per neurosphere: control, 128.70 ± 24.21 µm; Ang-1 plus SP00125, 66.70 ± 5.60 µm; $p < 0.05$; numbers of ramifications per neurosphere: control, 1.81 ± 0.19; Ang-1 plus SP00125, 0.92 ± 0.02; $p < 0.001$) demonstrating the specificity of the P-JNK labelling. Decrease in P-JNK immunoreactivity is associated with a decrease in τ expression, pointing to a crucial role of P-JNK activation in Ang-1-mediated axonogenesis. Together, these data show that Ang-1 promotes axonogenesis *via* activation of the SAPK/JNK pathway in SVZ cultures.

3.3.6. Tie-2 is expressed in neuronal progenitors and neurons along the SVZ-rostral migratory stream-olfactory bulb system *in vivo*

In vivo, neuroblasts and stem/progenitor cells proliferate in the SVZ (Coskun *et al*., 2001; Chojnacki *et al*., 2009). *In vitro*, some proliferating DCX-positive neuroblasts and immature Nestin positive progenitor cells expressed Tie-2,

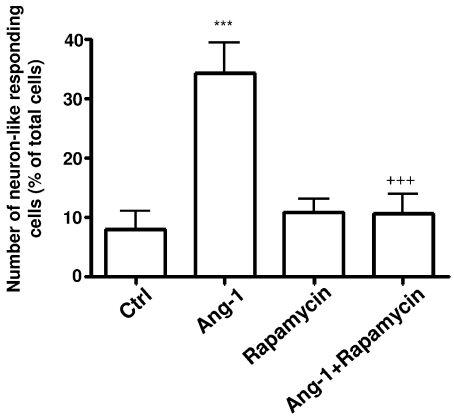


Figure 3.8. Ang-1 increases the generation of neuronal-like responding cells in mouse SVZ cell cultures *via* mTOR. SVZ cultures were perfused continuously with Krebs' solution for 15 min and stimulated for 2 min (from minute 5 to minute 7) with 50 mM KCl and for 2 min (from minute 10 to minute 12) with 100 µM histamine. The bar graph depicts the percentages (%) of neuronal-like responding cells in SVZ control cultures and in cultures exposed to 500 ng/ml Ang-1 and/or 20 nM rapamycin, for 7 d. Data are expressed as mean ± SEM. ***p < 0.001, using the unpaired Student t test for comparison with SVZ control cultures. +++p < 0.001, using the unpaired Student t test for comparison with SVZ cultures treated with Ang-1.

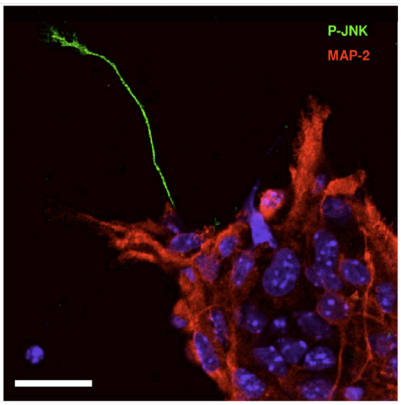


Figure 3.9. Representative confocal digital image of P-JNK (green) and MAP-2 (red) immunostainings in a SVZ cell culture. Hoechst 33342 staining (blue) was used to visualize cell nuclei. Scale bar, 20 µm.

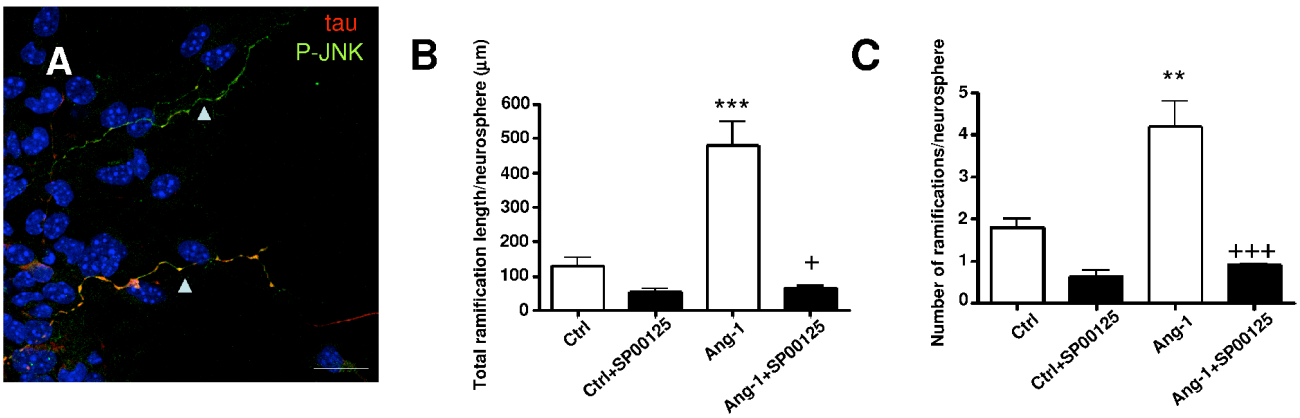


Figure 3.10. Ang-1 induces activation of SAPK/JNK pathway on growing axons. ***A***, Representative fluorescent confocal digital images depicts the P-SAPK/JNK (green), τ (tau) protein (red), and Hoechst 33342 staining (blue nuclei) in cultures treated with 500 ng/ml Ang-1, for 6 h. Growing axons (double labelled for P-SAPK/JNK and τ) are indicated by arrowheads. Scale bar, 20 µm. ***B***, ***C***, Bar graphs depict the total length (in micrometers) of P-JNK-positive ramifications and the number of ramifications per neurosphere. Data are expressed as mean ± SEM. Measurements were done in 20 nonoverlapping fields in each coverslip from two independent culture preparations using digital images (magnification, 20x). **p < 0.01; ***p < 0.001, using the unpaired Student t test for comparison with SVZ control cultures. +p < 0.05, +++p < 0.001 using the unpaired Student t test for comparison with SVZ cultures treated with Ang-1.

suggesting that signalling *via* Tie-2 may induce these cells to proliferate. To determine whether Tie-2 receptors are present in proliferating neuroblasts and progenitors *in vivo*, brain slices from BrdU-injected mice were immunostained for Tie-2, EGFR as a marker of progenitor cells, and DCX. As depicted in Figure 3.11*A* and *B*, some EGFR/BrdU-positive progenitors and DCX/BrdU-positive neuroblasts express the Tie-2 receptor, suggesting that Tie-2 may be involved in proliferation *in vivo*. Moreover, using immunohistochemistry, Ang-1 was detected in microvessels crossing the SVZ and close to the ependymal layer (Figure 3.12). Therefore, it is possible that locally available Ang-1 may favour proliferation *via* Tie-2 binding. Ang-1 triggers neuronal differentiation in SVZ cultures and the expression of Tie-2 by DCX-positive neuroblasts as well as in τ-positive neurons suggests that Tie-2 may be involved in neuronal maturation. Accordingly, Ang-1 promotes axonal development *in vitro*. To further support that Tie-2 signalling may be involved in neuronal maturation *in vivo*, the expression of Tie-2 by migrating neuroblasts and tyrosine hydroxylase (TH) positive periglomerular cells was investigated in the RMS and OB, respectively. Tie-2 expression was found in some DCX positive migrating neuroblasts in the RMS and TH-positive periglomerular interneurons in the OB, suggesting a role of Tie-2 signalling in SVZ-derived neuronal differentiation in the SVZ–OB system (Figure 3.11*C* and *D*, respectively).

3.4. Discussion

The present work intended to disclose the effects of the angiogenic factor Ang-1 on SVZ neurogenesis. We showed that Nestin-positive SVZ cells express Ang-1 and its receptor Tie-2, suggesting an autocrine/paracrine regulation of the SVZ cell dynamics *via* Ang-1. Treatment of SVZ cells with 500 ng/ml Ang-1 did not affect SVZ cell death or survival. However, Ang-1 is known to promote cell survival in endothelial cells (Fujikawa *et al*., 1999; Papapetropoulos *et al*., 2000). In nonendothelial cells, anti-apoptotic properties of Ang-1 have been described, but

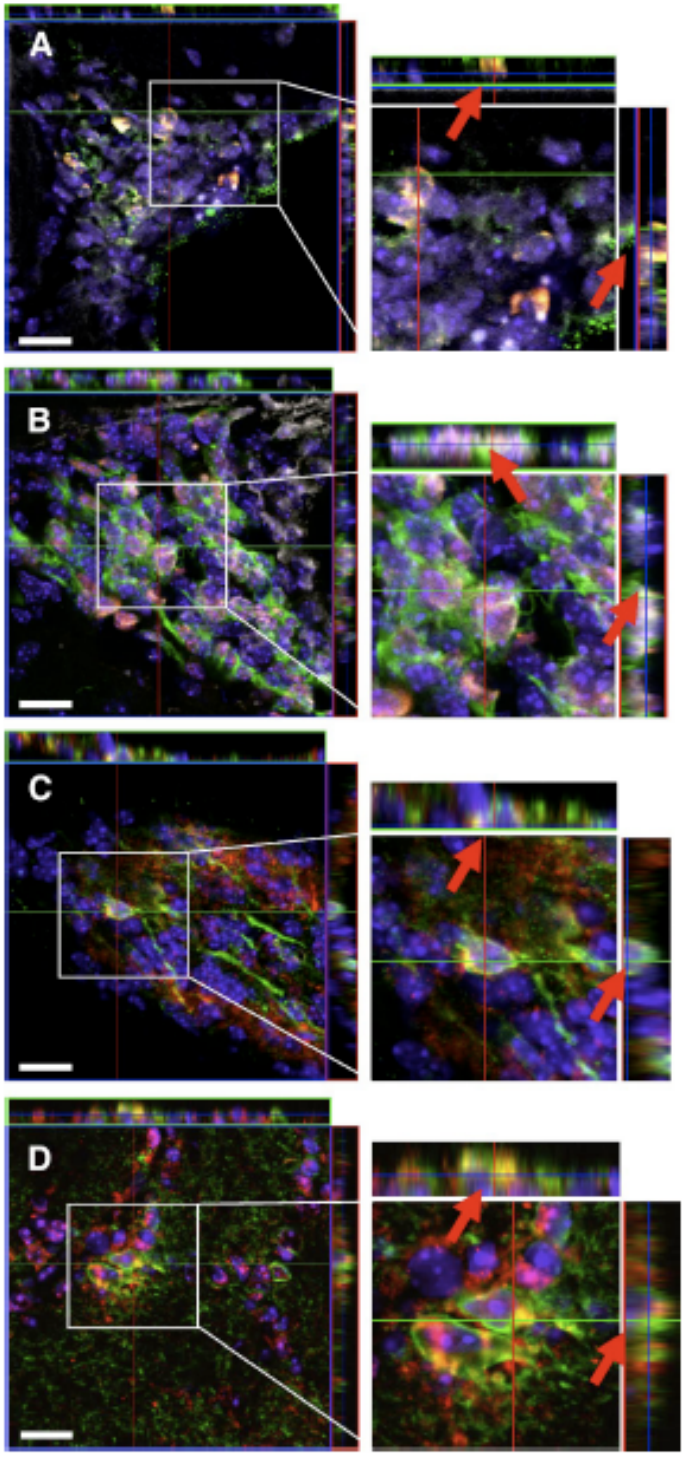


Figure 3.11. Tie-2 is expressed in neurons along the SVZ, the RMS, and the OB. ***A***, ***B***, Representative *z*-stack confocal digital images of the SVZ showing BrdU-positive stem/progenitor cells (red nuclear staining for BrdU and green staining for EGFR) (***A***) and BrdU-positive neuroblasts (red nuclear staining for BrdU and green staining for DCX) (***B***), both cell types expressing Tie-2 (white staining). ***C***, Representative *z*-stack confocal digital image of the RMS showing a DCX neuroblast (green staining) expressing Tie-2 (red staining). ***D***, Representative *z*-stack confocal digital image of TH-expressing periglomerular cells (green staining) expressing Tie-2 (red staining). The arrows indicate regions of double or triple labelling. Hoechst 33342 staining (blue) was used to visualize cell nuclei. Scale bars, 20 μm.

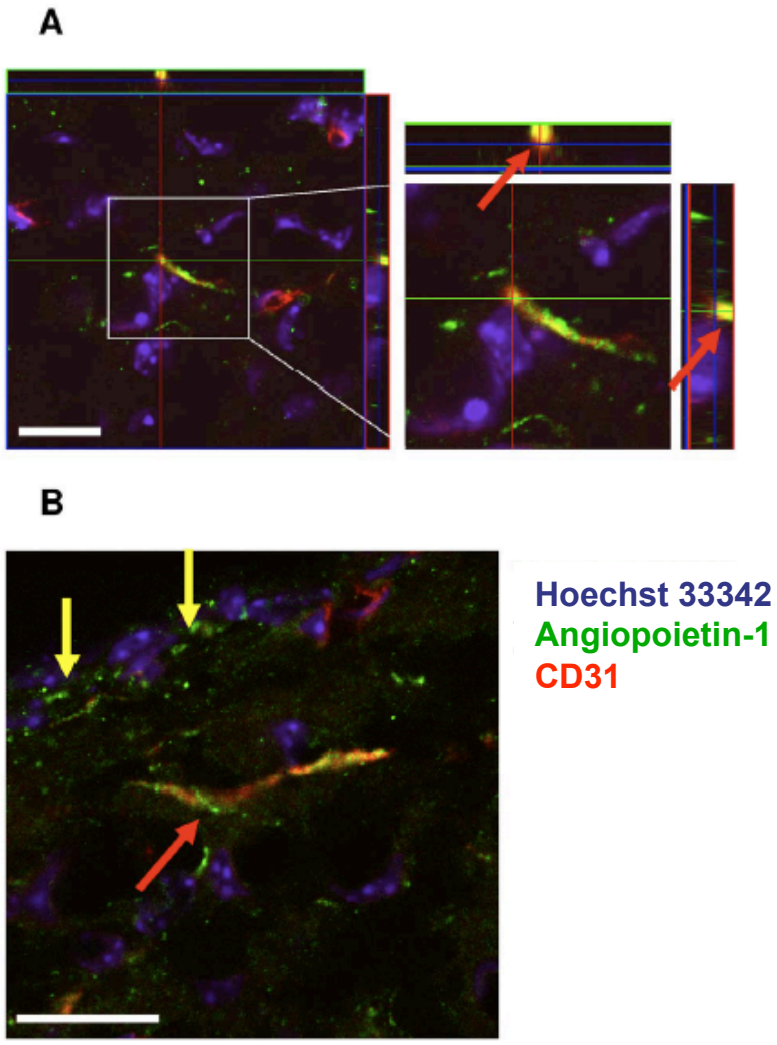


Figure 3.12. ***A***, Representative z-stack digital image of SVZ microvessels expressing CD31 (red) and Ang-1 (green). Red arrows indicate areas of co-expression in the same vessel. ***B***, Representative confocal digital image of Ang-1 (green) and CD31 (red) stainings in the SVZ. Red arrow indicates co-expression of Ang-1 and CD31. Yellow arrows point to expression of Ang-1 in ependymal cell layer. Hoechst 33342 staining (blue) was used to visualize cell nuclei. Scale bars, 20 μm.

only on injury paradigm: Ang-1 protects embryonic cortical neurons and progenitor cells from apoptosis induced by serum deprivation and hypoxia (Valable *et al*., 2003; Bai *et al*., 2009b). Therefore, Ang-1 does not promote survival of SVZ cells in basal conditions but could protect them on injury. Incubation of SVZ cells with 500 ng/ml Ang-1 induced proliferation, an effect mediated *via* Tie-2 and ERK1/2 kinase activation, the canonical pathway mediating proliferation in the SVZ (Learish *et al*., 2000). Although some studies failed to show a pro-proliferative effect of Ang-1 in endothelial cells (Huang *et al*., 1999; Takakura *et al*., 2000; Velazquez *et al*., 2002), others showed that Ang-1 triggers proliferation *via* ERK1/2 in murine brain endothelial cells and human umbilical vein endothelial cells (HUVECs) (Koblizek *et al*., 1998; Kanda *et al*., 2005; Abdel-Malak *et al*., 2008). Activation of the PI3K/AKT kinases also mediates the Ang-1-induced proliferation in endothelial cells (Kanda *et al*., 2005; Abdel-Malak *et al*., 2008). However, in SVZ cultures, ERK1/2 activation seems to prevail on PI3K/AKT pathway as total inhibition of Ang-1-induced proliferation was obtained in the presence of a MEK inhibitor. Exogenous administration of Ang-1 increases the numbers of neurons through the activation of Tie-2. Ang-1 similarly elicits pro-neurogenic effects in embryonic mouse neural progenitors (Bai *et al*., 2009a), increasing the proportion of βIII tubulin neurons through Tie-2 binding and PI3 kinase activation. A growing body of evidence suggests the involvement of mTOR in neuronal differentiation. The serine-threonine kinase mTOR is one of the downstream signalling molecules of the PI3K/AKT pathway. Rapamycin-mediated inhibition of mTOR prevents neuronal differentiation induced by insulin in neuronal precursors from the rat embryonic telencephalon and decreases the numbers of neurons in neuroblastoma cell cultures (Han *et al*., 2008; Zeng and Zhou, 2008). Consistently, fewer neurons were obtained in SVZ cultures coincubated with rapamycin and Ang-1 compared with Ang-1 alone, demonstrating that mTOR mediates Ang-1-induced neuronal differentiation. mTOR may be activated by SAPK/JNK kinases, which, in our study, promote neuronal maturation.

Consistently, SP600125 (SAPK inhibitor II) was shown to decrease mTOR activation in H1299 lung cancer cells (Jin *et al*., 2009). Moreover, mTOR may be one convergence point between Notch and Tie-2 signalling cascades leading to proliferation and neuronal differentiation. Indeed, intracerebroventricular injection of Ang-2 and Tie-2 activation promote the expansion of the hairy and enhancer of split 3 (Hes3)-positive precursors pool in the rat SVZ (Androutsellis-Theotokis *et al*., 2009). The transcription factor Hes3 belongs to the Hes/Hey gene family that mediates transcriptional responses to Notch activation. Intracerebroventricular injection of the Notch ligand Delta-like 4 (Dll4) elicits similar responses to Ang-2, suggesting that Tie-2 and Notch receptors activate similar pathways promoting self-renewal and proliferation (Androutsellis-Theotokis *et al*., 2009 and 2010). In stem-derived cell cultures from the embryonic mouse brain, transcription of the Hes3 gene after Dll4 binding to Notch is mediated by the consequent phosphorylation of AKT and mTOR (Androutsellis-Theotokis *et al*., 2006). Although we did not investigate the involvement of mTOR in Ang-1-induced proliferation, we showed that mTOR mediates the pro-neurogenic effects elicited by Tie-2 activation. Consistently, Androutsellis-Theotokis *et al*., (2006) showed that newly-generated cells after Dll4 intracerebroventricular injection expressed DCX. In addition, Notch activation induces proliferation of neural progenitors after ischemia in the rat brain (Wang *et al*., 2009). Additional studies are needed to unravel the role of mTOR in mediating proliferation and neuronal differentiation after Tie-2 activation. In the present study, the involvement of ERK/MAP kinases in neuronal differentiation was not assessed. However, the ERK pathway is involved in neuronal differentiation induced by bone morphogenetic protein 4, FGF-2, and nerve growth factor in neuronal precursor and mouse bone marrow stromal cells (Yang *et al*., 2008; Moon *et al*., 2009; Washio *et al*., 2009). Here, mTOR inhibition completely blocked Ang-1/Tie-2-mediated neuronal differentiation, suggesting that ERK/MAP kinases may not be critical. Consistently, ERK inhibition is required to induce differentiation in neural stem-derived cell cultures

(Wang *et al*., 2009). We further investigated the capacity of Ang-1 to promote neuronal maturation and neurite outgrowth in SVZ cultures. Ang-1 increases neurite length in neural progenitors derived from the embryonic mouse brain (Bai *et al*., 2009a) and in dorsal root ganglion cell cultures (Kosacka *et al*., 2005, 2006). *In vivo*, Ward *et al*. (2005) showed that Ang-1 increases the dendritic arborization of motor cortex and hippocampal neurons. To assess whether Ang-1 triggers neurite outgrowth, we measured the numbers and length of neurites immunoreactive for P-SAPK/JNK in 6 h-treated cultures. Activation of the JNK pathway is involved in axonal sprouting and neurite outgrowth but not in dendritic growth (Waetzig *et al*., 2005; Oliva *et al*., 2006). Ang-1 treatment increased the numbers and length of P-JNK-positive axons. The SAPK/JNK pathway is generally activated on cellular stress such as stimulation by proinflammatory cytokines, leading mainly to apoptosis (Karin and Gallagher, 2005). In endothelial cells, serum deprivation evokes apoptosis and is accompanied by an increase of the P-JNK levels. Ang-1 attenuates serum deprivation-induced apoptosis *via* inhibition of the SAPK/JNK pathway (Harfouche *et al*., 2003). Regarding neurite outgrowth, Ang-1 triggers neurite outgrowth in PC12 cells without affecting levels of JNK phosphorylation (Chen *et al*., 2009). Despite the fact that the cellular model is different, discrepancies may arise from the method used to evaluate P-JNK levels. Indeed, western blotting requires a considerable amount of protein. We detected P-JNK by immunocytochemistry, which is associated with thin neurites and therefore may not represent a sufficient amount of protein to be detected in western blotting. In the present paper, Ang-1 promotes both proliferation and neuronal differentiation. As differentiation generally requires exit of the cell cycle, there may be an apparent contradiction. However, SVZ cell cultures consist of a heterogeneous population of postmitotic and cycling cells, as well as DCX-positive neuroblasts that retain the capacity to proliferate *in vitro* and *in vivo* (Coskun *et al*., 2001; Li *et al*., 2009). Additionally, other factors able to promote proliferation and neuronal differentiation have been described. NPY, for instance, promotes

proliferation and neuronal differentiation in the rodent dentate gyrus and SVZ (Howell *et al*., 2005; Agasse *et al*., 2008a; Decressac *et al*., 2009). TNFα and VEGF display pro-proliferative and pro-neurogenic capacities in SVZ cultures (Jin *et al*., 2002b; Bernardino *et al*., 2008; Wittko *et al*., 2009). Considering the heterogeneity of SVZ cell types, pro-proliferative factors such as Ang-1 are susceptible of modulating proliferation of cycling cells. Factors promoting neuronal differentiation, Ang-1 here, may commit early postmitotic cells to the neuronal lineage. It has been recently demonstrated that Ang-2 increases the commitment of mouse SVZ cells to neurons, likely involving the binding of the transcription factor C/EBPβ (CCAAT-enhancer-binding protein β) to the promoter of βIII tubulin gene (Liu *et al*., 2009). Retinoic acid promotes proliferation of SVZ cells and commitment to a neuronal fate of P19 cells through epigenetic regulation of the ngn-1 gene expression (T. W. Wang *et al*., 2005; Wu *et al*., 2009b). Hence, Ang-1 pro-proliferative and pro-differentiation effects may account for the diversity of targeted cells. Recently, Ang-2 was shown to increase neuronal differentiation in SVZ cultures *via* Tie-2 binding (Liu *et al*., 2009). Moreover, intracerebroventricular administration of Ang-2 increased proliferation in the rat SVZ (Androutsellis-Theotokis *et al*., 2009). The similarities of Ang-2 and Ang-1 effects on SVZ are quite puzzling as Ang-2 is a competitive antagonist of Tie-2 in endothelial cells and cancels the anti-apoptotic and pro-migratory effects of Ang-1. However, Ang-2 may also stimulate Tie-2. Indeed, in the absence of Ang-1, Ang-2 binds to Tie-2 in HUVECs, promotes Tie-2 and PI3K-AKT activation, and acts similarly to Ang-1 as a pro-survival factor in a serum deprivation paradigm (Yacyshyn *et al*., 2009; Yuan *et al*., 2009). Nevertheless, in the presence of Ang-1, Ang-2 antagonizes the activity of Ang-1 (Yuan *et al*., 2009). Ang-2 is a less potent activator of Tie-2 compared with Ang-1 (Yuan *et al*., 2009). Hence, it is conceivable that Ang-1 and Ang-2 activate the SVZ-expressed Tie-2 receptor. The system Ang-1/Tie-2 may play a role in the SVZ *in vivo*, as Ang-1 labelling is found in ependymal cells and in

microvessels crossing the SVZ. This is in accordance with previous reports showing that sources of Ang-1 include perivascular astrocytes, endothelial cells, ependymal cells and the choroid plexus (Acker *et al*., 2001; Nourhaghighi *et al*., 2003; Ward *et al*., 2005; Ohab *et al*., 2006; Tonchev *et al*., 2007; Fukuhara *et al*., 2008; Horton *et al*., 2010). Moreover, Ang-1 mRNA was detected in the SVZ of adult mice (Liu *et al*., 2009), and we showed that proliferating DCX positive neuroblasts and EGFR-positive progenitors express Tie-2. Together, these observations suggest that the basal neurogenic activity in the SVZ *in vivo* may partly account for the local secretion of Ang-1 and identify Ang-1 as a component of the neurogenic niche.

In conclusion, the pro-neurogenic effect of Ang-1 opens new perspectives for brain repair. A better understanding of the neurovascular niche and of endothelial cell-derived soluble factors may be of extreme relevance to allow the development of new strategies to enhance neuronal replacement using SVZ stem cells.

Chapter 4 - The effect of heterocellular contacts between mouse brain endothelial cells and subventricular cells on stem/progenitor cell dynamics: involvement of direct contact *via* laminin-1/α6β1 integrin and gap junctions

4.1. Abstract

Neurogenesis in the subventricular zone (SVZ) of the adult mammalian brain is tightly regulated by environmental cues derived from either diffusible factors or cell-to-cell contacts. Heterocellular contacts with the microvasculature maintain the neurogenic capacities of stem cells and *in vivo* SVZ stem cells are associated with the abluminal endothelial surface of the vessels. Here, we used co-cultures of mouse brain endothelial cell (mBEC) monolayers obtained from C57BL/6 adult mice, and SVZ cells from early postnatal mice to study the impact of heterocellular contacts on SVZ dynamics and to identify some of the contact molecules involved. The co-cultures were grown for 24 h and then immunostained for CD31 (a marker of endothelial cells) and either Tunel (to detect apoptotic nuclei), BrdU (a marker of proliferation), SOX-2 (a marker of multipotent stem/progenitor cells) or doublecortin (DCX, a marker of SVZ-derived neuroblasts). SVZ cells contacting CD31+ cells were counted and the percentages of Tunel+, BrdU+, SOX-2+ and DCX+ cells were determined within this population of cells. To test whether extracellular matrix (ECM) and membrane proteins were involved in this interaction, mBEC were incubated for 1 h in the presence of 1 μg/ml of the protein synthesis inhibitor cycloheximide (CHX) prior to co-culture. This treatment is expected to decrease the turnover of membrane proteins and thereby to impair normal cell-to-cell contacts between mBEC and SVZ cells. We observed that the relative numbers of BrdU+ and SOX-2+ cells present in the population of SVZ cells in contact with mBEC were increased as compared with the corresponding relative numbers in the SVZ cells that were not in contact with mBEC. Accordingly, the relative numbers of BrdU+ and SOX-2+ SVZ cells not in contact with mBEC were similar to the obtained in the population of SVZ cells in contact with mBEC previously treated with CHX. Our experiments show that viable contacts with endothelial cells support proliferation and stemness in SVZ cells. However, contact with mBEC does not exert any effect on cell death or on the numbers of DCX+ neurons.

A recent study implicated $\alpha 6\beta 1$ integrin in the binding of endothelial-derived laminin-1 to stem cells and promotion of stemness (Shen *et al*., 2008). Moreover, gap junction intercellular communication has been shown to regulate homeostasis and differentiation of stem/progenitor cells of the bone (Guillotin *et al*., 2008) as well as attachment of cancer cells to endothelial cells (Elzarrad *et al*., 2008). To investigate whether these molecules account for the observed effects, co-cultures were treated with 5 µg/ml anti-α6 integrin neutralizing antibody to disrupt the binding of SVZ cells to laminin-1, and with 35 µM of the GJIC inhibitor carbenoxolone, respectively. As the numbers of BrdU+ and SOX-2+, but not Tunel + and DCX+, cells decreased in the population of cells contacting the CD31+ endothelial cells, we concluded that heterocellular contacts *via* laminin-1/$\alpha 6\beta 1$ integrin and gap junctions are involved in proliferation and maintenance of the stem state, but not in cell death nor neuronal differentiation.

4.2. Introduction

In the adult mammalian brain, neurogenesis occurs constitutively in the SVZ from GFAP-expressing, monociliated, neural stem cells, also known as B cells. These cells originate transit amplifying C cells, which give rise to neuroblasts or A cells (Lledo *et al*., 2006; Zhao *et al*., 2008; Chojnacki *et al*., 2009). Neuronal progenitors migrate through the RMS into the OB where they are known to differentiate into interneurons (Luskin *et al.*, 1993) and, to a much lesser extent, oligodendrocytes (Menn *et al*., 2006). Following brain damage, neurogenesis is increased in the SVZ, and neuronal precursors migrate outside the RMS towards sites of injury (Arvidsson *et al*., 2002; Parent, 2002; Parent *et al*., 2002a and b; Park *et al*., 2002; Imitola *et al*., 2004; Park *et al*., 2006; Li *et al.*, 2010).

Neurogenesis and stem cell dynamics are tightly regulated processes. SVZ stem/progenitor cells are closely associated with blood capillaries in the SVZ which, in some sites, lack pericyte and astrocyte endfeet coverage, possibly allowing the

diffusion of soluble molecules (Palmer *et al*., 2000; Shen *et al*., 2004; Rosa *et al*., 2010) and the establishment of heterocellular contacts and cellular-extracellular matrix (ECM) contacts (Tavazoie *et al*., 2008). In addition, neural stem cells extend a specialized process that terminates on blood vessels and may facilitate responses to perivascular ECM (Mirzadeh *et al*., 2008).

Integrins and laminins are among the contact factors that have been proposed to mediate the control of stem cells dynamics by endothelial cells. Integrins are heterodimers of two transmembrane chains, α and β, and receptors of laminins, with different ligand specificities (reviewed in Hynes, 1992). Laminins are heterotrimeric glycoproteins of three chains, α, β and γ. Integrin $\alpha6\beta1$ is the receptor for the ECM protein laminin-1 ($\alpha1\beta1\gamma1$) (reviewed in Colognato and Yurchenco, 2000).

Laminin-1 has been shown to augment the proliferation/survival of human NSCs (Hall *et al.*, 2008) and laminin/integrin interaction has been described to play a role in anchoring embryonic NSCs to the ventricular surface maintaining the physical architecture of the neocortical niche as well as cell proliferation (Loulier *et al*., 2009). Integrins containing the $\beta1$ subunit regulate epidermal stem cell maintenance (Jensen *et al*., 1999; Zhu *et al*., 1999; Raghavan *et al*., 2000) and neural stem cell maintenance (Campos *et al*., 2004) while the higher expression of laminin-1 receptor $\alpha6\beta1$ integrin has been implicated in the maintenance of mouse spermatogonial stem cells (Shinohara *et al*., 1999) and human embryonic stem cells (Xu *et al*., 2001). In fact, $\alpha6$ and $\beta1$ integrins have been proposed as markers of human neural stem cells (Hall *et al.*, 2006). In glioblastoma stem cells, the laminin-1 receptor $\alpha6\beta1$ integrin is expressed and it is responsible for self-renewal, proliferation and tumour formation capacity (Lathia *et al*., 2010). Expression of $\alpha6\beta1$ integrin also enhances tumourigenesis in U87, a glioma cell line (Delamare *et al*., 2009).

A recent study implicated $\alpha6\beta1$ integrin in the binding of endothelial-derived

laminin-1 to SVZ neural stem cells and promotion of stemness (Shen *et al*., 2008). In the present work we hypothesized that physical interaction between mBEC and SVZ cells could be established through endothelial laminin-1 and α6β1 integrin in SVZ cells, and that this specific interaction could modulate SVZ neurogenesis.

Moreover, heterocellular gap junctions (GJs) could modulate mBEC/SVZ contacts and by this way control SVZ stem cell dynamics. GJs are large-diameter channels that form an aqueous pore between adjacent cells, allowing the exchange of small molecules (1<kDa) (Goldberg *et al*., 1999; Goldberg *et al*., 2002) and electrical currents (Kirchhoff *et al*. 1998; Simon *et al*., 1998). Each GJ is composed of two hemichannels, formed by six connexin (Cx) subunits, the connexon (reviewed in Bruzzone *et al*., 1996; Goodenough *et al*., 1996; Harris, 2001 and Alexander and Goldberg 2003). Functions of GJs are beyond cell coupling since they allow the passage of electrical currents and small molecules, such as ATP and glucose, as well as ions, such as calcium. GJs also promote cell-cell adhesion (Lin *et al*., 2002; Elias *et al*., 2007) and cell-signalling (Lampe *et al*., 1998 and 2000; Le *et al*., 2001).

GJs are highly expressed during development and have been implicated in the control of embryonic patterning and morphogenesis (Coelho and Kosher, 1991; Levin, 2002). At least 5 Cxs are highly expressed in the rodent embryonic cerebral cortex including Cx26, Cx36, Cx37, Cx43 and Cx45 (Nadarajah *et al*., 1997; Cina *et al*., 2007). Cx26, Cx37 and Cx45 are largely evenly distributed from the VZ to the cortical plate, whereas Cx36 and Cx43 are more significantly expressed in the VZ (Cina *et al*., 2007; Elias *et al*. 2007). In the developing murine cortex, GJ coupling plays a role in cell proliferation (LoTurco and Kriegstein, 1991; Bittman *et al*., 1997; Bittman and LoTurco, 1999; Weissman *et al*., 2004) and neuronal differentiation and migration (Nadarajah *et al*., 1997; Elias *et al*., 2007 and 2010), also contributing to the generation of cortical circuits by mediating patterns of electrical activity (Sun and Luhmann, 2007).

In developing and mature astrocytes, Cx43 is the predominant GJ protein

(Dermietzel *et al*., 1991; Giaume *et al*., 1991). During postnatal development Cx43 expression is primarily restricted to radial glial cells (Yamamoto *et al*., 1992), which give rise to both astrocytes and neurons (Anthony *et al*., 2004; Casper and McCarthy, 2006; Malatesta *et al*., 2003). Cx43-GJs couple progenitor radial glia only during certain phases of the cell cycle and prior to mitosis, after which other connexins are upregulated (Nadarajah *et al*., 1997; Rozental *et al*., 2000). Cx43 is also expressed in GFAP-expressing cells in the postnatal subventricular zone (Liu *et al*., 2006). Cx43 has been associated with the ability of bFGF to maintain stem/precursor cells in an undifferentiated state *in vitro*, possibly by controlling the diffusion of growth factors between cells (Cheng et al., 2004). Cx43 GJs have also been implicated in progenitor migration as mice deficient in Cx43 show an accumulation of precursors in the intermediate zone due to their inability to migrate into the cortical plate (Fushiki *et al.*, 2003). A follow-up study showed a role for Cx43 in maintaining adhesion of migrating precursor cells on radial glia fibers (Elias *et al.,* 2007).

Cx43-mediated GJs have been shown in co-cultures of human umbilical vein endothelial cells (HUVEC) and human bone marrow stromal cells (HBMSC), promoting HBMSC differentiation possibly due to the diffusion of signalling molecules through the junctional channel (Villars *et al.*, 2002). A later study by the same group reveals that interaction between endothelial cells and primary human osteoprogenitors promotes initially osteoblastic proliferation (Guillotin *et al*., 2008).

Cx43 GJs also seem to play a role in the attachment of cancer cells to endothelial cells: according to Elzarrad and collaborators (2008), adhesion of breast cancer cells to the pulmonary endothelium increases with the overexpression of Cx43 by cancer cells. Upregulation of Cx43 is shown in tumour cell-endothelial cell contact areas *in vitro* and *in vivo*, and in areas of intratumoural blood vessels. (Elzarrad *et al*., 2008).

Based on these evidences we hypothesized that physical interaction between

mBEC and SVZ cells could be established through heterocellular Cx43 GJs, and that this specific interaction could affect SVZ neurogenesis.

4.3. Results

4.3.1. Characterization of mBEC cultures and design of novel co-culture conditions

Mouse BEC cultures were obtained as detailed in the "Materials and Methods", section 2.1.2.2.1. Following puromycin removal, isolated mBEC clusters develop at the bottom of petri dishes as depicted in Figure 4.1.A. Characterization of the cultures was performed on cells from confluent monolayers, i.e. obtained 5-7 days following puromycin removal, enzymatically detached from petri dishes and transferred onto glass slides by cell centrifugation. Endothelial cell cultures are enriched in CD31 positive cells (Figure 4.1.B). Results show that in all tested cultures, CD31 positive cell numbers were above 89% (culture I: 89,50 ± 0,72%, 1390 cells counted; culture II: 89,97 ± 0,42%, 1016 cells counted; culture III: 90,82 ± 2,41%, 1568 cells counted; culture IV: 90,72 ± 3,24%, 1362 cells counted, Figure 4.1.E. Most of the cells also label for von Willebrand Factor (vWF), another endothelial cell marker (Figure 4.1.C). The few contaminating cells in these mBEC cultures consist of smooth muscle actin (SMA) positive cells (Figure 4.1.D), which can either be smooth muscle cells or pericytes. GFAP positive astrocytes and CD11b positive microglial cells were not detected in our co-cultures, even though the respective immunocytochemistries were performed, with the appropriate positive controls (mouse brain slices).

To perform the co-culture, SVZ cells must be cultured in SFM as serum decreases proliferation and increases glial differentiation. However, endothelial cells grow in medium containing serum and undergo apoptosis after serum removal (Kwak *et al*., 1999). In order to optimize the co-culture conditions, in terms of duration of the co-culture session, endothelial cell viability was evaluated by MTT assay. Mouse

BEC were cultured in endothelial cell medium and SFM, for 24 and 48 h (Figure 4.2.A and B, respectively), prior to incubation with MTT. Optical density was measured at 490 nm. As a reference, the values obtained in cultures incubated with endothelial cell medium were set to 100% and all the other measurements were compared with this condition. In SFM, at 24 h, mBEC viability tends to decrease but without statistical significant difference when compared to controls (91.66 ± 4.31% of controls, n=3). However, at 48 h, cell viability decreases significantly to 62.08 ± 2.75 % of controls (n=4, $p<0.0001$). This clearly demonstrates that SFM is detrimental to cell viability at 48 h, but not at 24 h and, therefore, co-cultures should be used at this time point. Figures 4.2.C and D show a 24-h mBEC/SVZ cell co-culture.

In this chapter, we aimed at studying cell-to-cell interactions between mBEC and SVZ cells as well as the proteins involved in these physical interactions. To decrease the turnover of ECM proteins in mBEC and, thereby, impair normal cell-to-cell contacts between mBEC and SVZ cells, we used 1 μg/ml of the protein synthesis inhibitor cycloheximide (CHX), 1 h prior to the depositing of SVZ spheres onto mBEC. Because we intended to affect protein synthesis without inducing relevant cell death, we had to verify whether this treatment had any effect in the viability of mBEC. To do so, we performed a 1 h-pulse of CHX in SFM in mBEC cells, and then replaced the media by fresh SFM. After 24 h, since it was the chosen end point for co-cultures, again, we performed the MTT tests. As depicted in Figure 4.2.A, CHX induces only a slight decrease in cell viability comparing to controls: 82.58 ± 4.62 ($n=3$, $p<0.01$) and, thus, we considered a 1 h-CHX-pulse acceptable for usage in our co-cultures.

4.3.2. Contact between SVZ cells and mBEC promotes SVZ cell proliferation without affecting cell death

To determine if heterocellular contacts between SVZ cells and mBEC regulate SVZ cell proliferation, co-cultures of SVZ cells and mBEC were performed

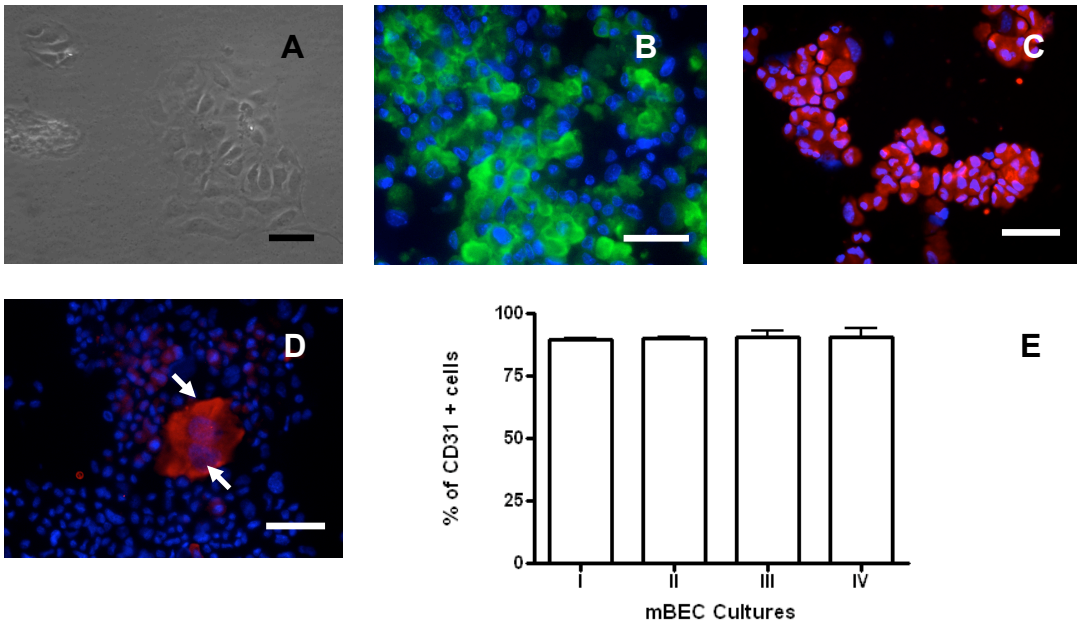


Figure 4.1. Characterization of mouse brain endothelial cell (mBEC) cultures. ***A***, Representative transmission digital image of mBEC cultures after removal of puromycin. Scale bar, 100 μm. ***B-D***, Representative fluorescence digital images showing the immunoreactivity of mBEC cultures: ***B***, Detection of CD31 expression, an endothelial cell marker (CD31 staining in green); ***C***, Detection of Von Willebrand factor (VWf) expression, another endothelial cell marker (VWf staining in red); ***D***, Detection of smooth muscle actin (SMA) expression, a pericyte and smooth muscle cell marker, showing the identity of contaminant cells in mBEC cultures (SMA staining in red). Nuclei are stained with Hoechst 33342 in blue. Scale bars, 50 μm. ***E***, Bar graph depicts the percentages (%) of CD31 positive cells in 4 different mBEC cultures.

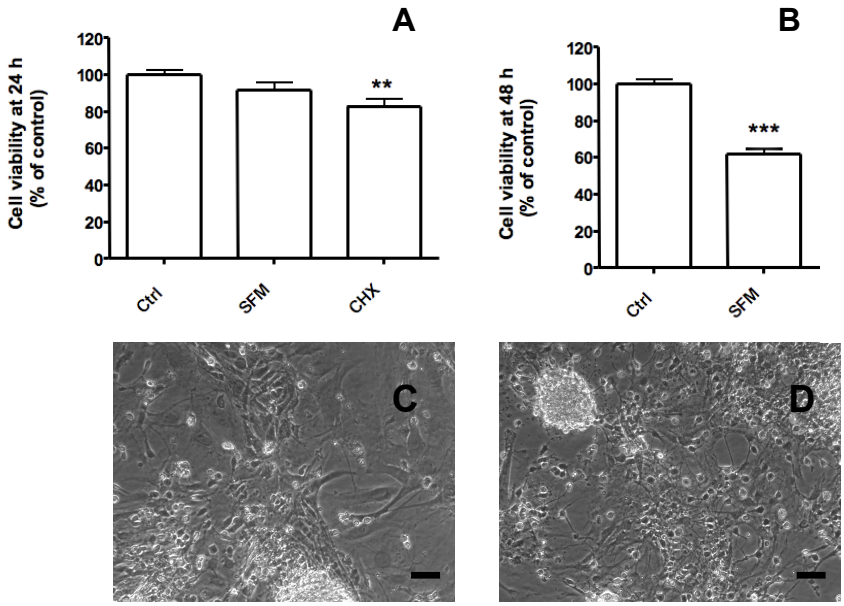


Figure 4.2. Optimization of mouse brain endothelial cells (mBEC)/subventricular zone (SVZ) cells co-cultures. ***A***, Bar graph depicts the cell viability of mBEC, determined by MTT assay, at 24 h after incubation in normal mBEC media (Ctrl), in serum free media (SFM) or in SFM after a 1 h-incubation with 1 μg/ml cycloheximide (CHX). ***B***, Bar graph depicts the cell viability of mBEC, determined by MTT assay, at 48 h after incubation in normal mBEC media (Ctrl) or in SFM. **P<0.01, ***P<0.0001, using the unpaired Student *t* test. ***C*** and ***D,*** Representative transmission digital images showing mBEC and SVZ cells co-cultures. Scale bars, 100 μm.

as described in the protocol depicted in Figure 4.3.A. Briefly, mBEC were deposited on coverslips and left to grow for 24 h following an incubation with 1 µg/ml CHX in SFM for 1 h. SVZ neurospheres were placed on treated and untreated mBEC and allowed to develop for 24 h in SFM. In the last 4 h of the culture session, co-cultures were incubated with 10 µM BrdU. Cells were then fixed and processed for CD31 and BrdU double immunocytochemistry. As depicted in Figures 4.4.A and E, in control co-cultures, within the population of cells that contacts endothelial cells, the percentage of BrdU+ cells is 22.43 ± 1.11 % (n=8 coverslips, 572 pairs of cells counted) while in the carpet of differentiating cells, fewer SVZ cells are proliferating, as 15.45 ± 1.31 % of BrdU positive cells were counted (n=8 coverslips, 2391 cells counted, $p<0.05$). This indicates that contact with endothelial cells sustains SVZ cell proliferation. Following mBEC incubation with CHX (Figures 4.4.B and E), cell proliferation close to mBEC does not differ from BrdU levels found in the carpet of differentiating cells and it is significantly decreased as compared to control co-cultures (13.25 ± 2.61 %, n=6 coverslips, 661 pairs of cells counted, $p< 0.01$). This indicates that protein synthesis inhibition reduced the pro-proliferative effect of heterocellular contacts between mBEC and SVZ cells. These effects may be due to the slow-down of the membrane protein turnover.

To determine whether the observed effect in cell proliferation was in fact due to heterocellular contacts or due to endothelial cell-derived soluble factors, the protocol depicted in Figure 4.3.A was followed. Mouse BEC were incubated with 1 µg/ml CHX, in SFM, for 1 h. SVZ neurospheres were then deposited on treated mBEC and left to develop for 24 h in SFM or in 1:1 SFM and mBEC-conditioned media (CM). In some experiments, we exposed co-cultures performed with untreated mBEC to 5 µg/ml of anti-Tie-2 neutralizing antibody to test whether Ang-1, derived from mBEC, had any effect in the maintenance of cell proliferation. As depicted in Figure 4.4.F, in control co-cultures, while within the population of cells that contacts endothelial cells there is 13.32 ± 1.16 % BrdU positive nuclei (n=9 coverslips, 691

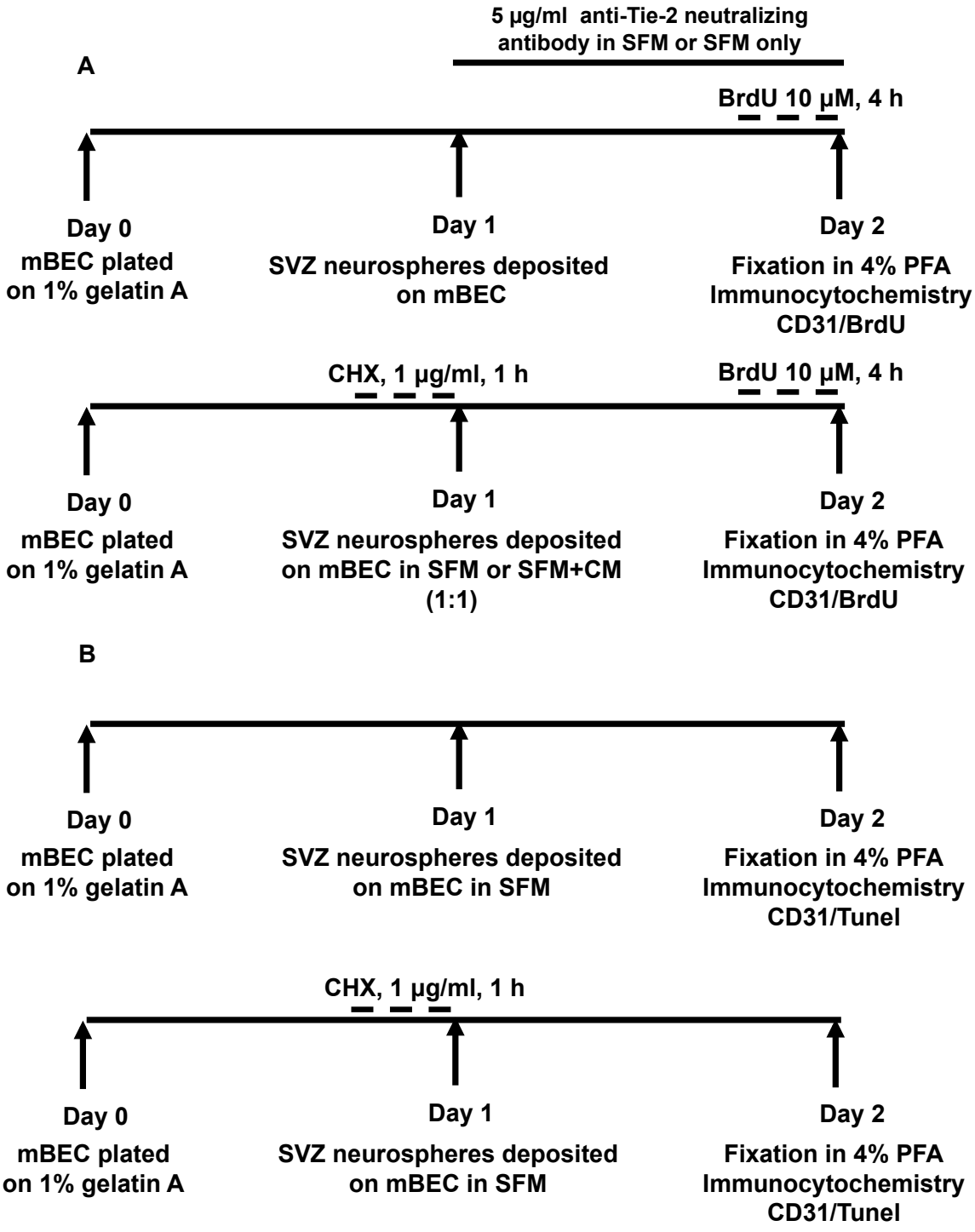
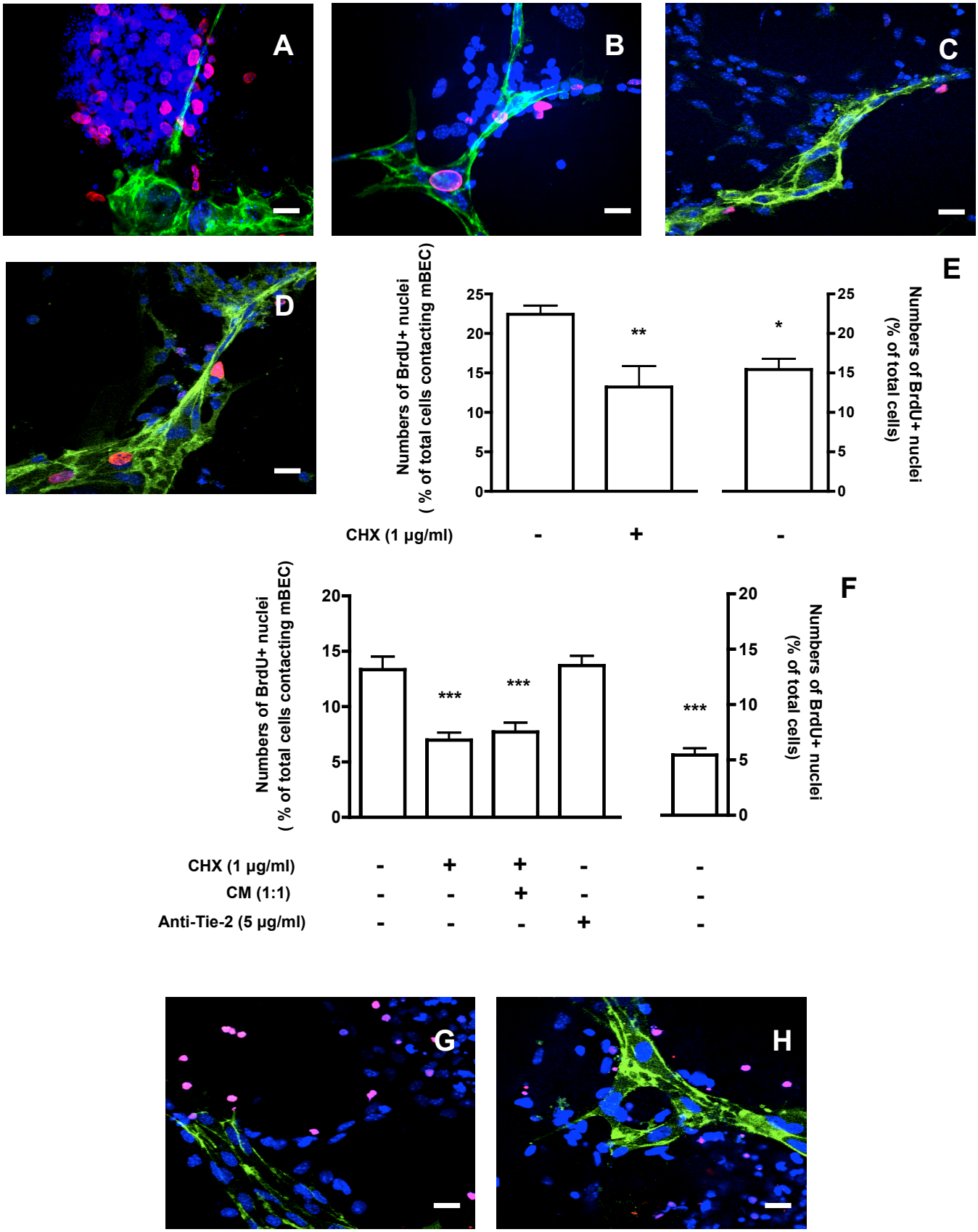


Figure 4.3. Subventricular zone (SVZ) cells and mouse brain endothelial cells (mBEC) co-culture protocol for proliferation and cell death by apoptosis studies. mBEC were deposited on 1% gelatin A-coated coverslips and left to grow for 24 h. SVZ neurospheres were then plated on 24 h mBEC layers and left to develop for another 24 h. SVZ neurospheres were also plated on mBEC treated 1 h with 1 µg/ml cycloheximide (CHX), an inhibitor of protein synthesis, in serum free media (SFM). To evaluate whether endothelial-derived soluble factors contribute to the observed effects in proliferation, SVZ neurospheres were plated on mBEC treated 1 h with 1 µg/ml CHX in SFM plus mBEC-conditioned media (CM) (1:1) or plated on untreated mBEC in 5 µg/ml anti-Tie-2 neutralizing antibody in SFM, and left to develop for 24 h. ***A***, For proliferation studies, in the last 4 h of the culture session, co-cultures were incubated with 10 µM BrdU (a marker of cell proliferation). After this, cells were fixed and processed for CD31 (a marker of endothelial cells)/BrdU double immunocytochemistry. ***B***, For cell death studies, cells were fixed and stained for CD31/ Tunel.


A
B
C
D
E
Numbers of BrdU+ nuclei
(% of total cells contacting mBEC)
Numbers of BrdU+ nuclei
(% of total cells)
25
20
15
10
5
0
**
*
CHX (1 µg/ml) - + -
F
Numbers of BrdU+ nuclei
(% of total cells contacting mBEC)
Numbers of BrdU+ nuclei
(% of total cells)
20
15
10
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0
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CHX (1 µg/ml) - + + - -
CM (1:1) - - + - -
Anti-Tie-2 (5 µg/ml) - - - + -
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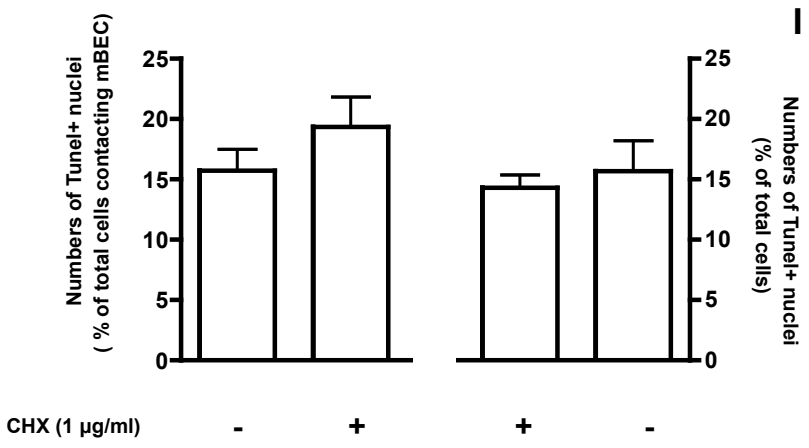

Figure 4.4. Contacts between subventricular zone (SVZ) cells and mouse brain endothelial cells (mBEC) promote SVZ cell proliferation and have no effect on cell death by apoptosis. ***A - D,*** Representative confocal digital images depicting proliferating cells in mBEC and SVZ cells co-cultures (green staining for CD31, an endothelial cell marker, red staining for BrdU, a marker of proliferation and Hoechst 33342 staining in blue). ***A***, Control co-cultures. ***B***, Co-cultures with 1 μg/ml cycloheximide (CHX) for 1 h-treated mBEC, in serum free media (SFM). ***C,*** Co-cultures with CHX-treated mBEC in SFM and mBEC-conditioned media (CM) (1:1) ***D***, Co-cultures grown in the presence of 5 µg/ml anti-Tie-2 neutralizing antibody in SFM. Scale bars, 20 μm. ***E and F***, Left: Bar graphs show the numbers of BrdU positive cells as a percentage (%) of the total cells contacting mBEC. Right: Graphs display the numbers of proliferating cells as a % of the total number of cells in the carpet of differentiation. *P<0.05, **P<0.01, ***P<0.001, using one-way ANOVA. ***G and H,*** Representative confocal digital images showing apoptotic cell death in mBEC and SVZ cells co-cultures. CD31 evidenced in green, Tunel positive (apoptotic) nuclei in red, total nuclei evidenced using Hoechst 33342 staining, in blue. ***G***, Control co-cultures, ***H***, Co-cultures with CHX-treated mBEC. Scale bars, 20 μm. ***I***, Left: Bar graph shows the numbers of apoptotic nuclei as a % of the total cells contacting mBEC in control and in co-cultures with CHX-treated mBEC. Right: Graph displays the numbers of apoptotic cells as a % of the total number of cells in the carpet of differentiation in control and in co-cultures with CHX-treated mBEC.

pairs of cells counted), in the carpet of differentiating cells there is a decrease in the numbers of proliferating cells: 5.43 ± 0.62 % of BrdU positive nuclei (n=9 coverslips, 1312 cells counted, p<0.001). Co-cultures performed with CHX-incubated mBEC exhibit numbers of mBEC-associated BrdU positive cells that do not differ from the ones found in the carpet of differentiating cells in the controls and are significantly decreased as compared to numbers of BrdU positive nuclei contacting mBEC in control co-cultures (6.96 ± 0.67 %, n=9 coverslips, 724 pairs of cells counted, p< 0.001). This indicates, as before, that contact with endothelial cells promotes the SVZ cell proliferation. Moreover, in co-cultures performed with CHX-treated mBEC and in the presence of mBEC-CM (Figures 4.4.C and F), numbers of BrdU + cells in contact with mBEC are also reduced as compared to the ones obtained in controls (7.69 ± 0.83 %, n=9 coverslips, 626 pairs of cells counted, p< 0.001) and do not differ statistically from values in the carpet of differentiating cells in the controls. Similarly there is no statistical difference from values of BrdU positive cells contacting mBEC in co-cultures where CHX-treated mBEC were used, even though there is a tendency for this value to be higher. These data strongly suggest that soluble factors are not critical to the observed effects in cell proliferation. Further reinforcing this idea, when co-cultures are incubated with 5 µg/ml anti-Tie 2 neutralizing antibody (Figures 4.4.D and F), which blocks the action of the endothelial-derived soluble factor Ang-1, no differences are seen in the numbers of BrdU positive cells contacting mBEC comparing to controls (13.71 ± 0.84 %, n=8 coverslips, 520 pairs of cells counted).

In order to verify whether contact with mBEC promotes SVZ survival, we evaluated cell death by Tunel assay in control co-cultures and in co-cultures where mBEC have been treated with 1 µg/ml CHX, for 1 h, as described in Figure 4.3.B. As shown in Figures 4.4.G and I, in controls, we did not find any differences in cell death between the population of cells contacting endothelial cells (15.74 ± 1.72 % apoptotic nuclei, n=9 coverslips, 1023 pairs of cells counted) and the carpet of differentiating cells (15.67 ± 2.51 % apoptotic nuclei, n=9 coverslips, 4330 cells counted). In co-

cultures performed with CHX-treated mBEC (Figures 4.4.H and I), results were similar: no differences were seen in apoptosis between the population of cells contacting endothelial cells (19.34 ± 2.48 % apoptotic nuclei, n=9 coverslips, pairs of 735 cells counted) and the carpet of cells (14.30± 1.03 % apoptotic nuclei, n=9 coverslips, 3498 cells counted). No differences were found either, when comparing CHX to control co-cultures values. We can therefore conclude that mBEC/SVZ cells heterocellular contacts do not seem to contribute to SVZ cell survival.

4.3.3. Contact between SVZ cells and mBEC sustains SVZ cell stemness

To assess whether the interaction between SVZ cells and mBEC affects SVZ cell stemness, co-cultures of SVZ cells and mBEC were performed as described in the protocol depicted in Figure 4.5. After this, cells were fixed and processed for CD31 and SOX-2 double immunocytochemistry. As depicted in Figures 4.6.A and E, in the carpet of differentiating cells of control co-cultures, we found less SOX-2 positive cells [23.43 ± 3.38 % (n=9 coverslips, 6536 cells counted, $p<0.01$)], while within the population of cells that contacts endothelial cells we identified 43.97 ± 2.96 % SOX-2 positive nuclei (n=8 coverslips, 1223 pairs of cells counted). This indicates that contact with endothelial cells plays a role in the maintenance of the SVZ stem cell state. In co-cultures performed in the presence of CHX-treated mBEC (Figures 4.6.B and E) the numbers of SOX-2 positive cells contacting mBEC do not differ from the ones found in the carpet of differentiating cells in the controls. Moreover, these numbers are significantly decreased as compared to numbers of SOX-2 positive nuclei contacting mBEC in control co-cultures, as seen in Figure 4.6.E (28.13 ± 4.55 %, n=9 coverslips, 1098 pairs of cells counted, $p< 0.05$). This indicates that CHX treatment reduces the stem state promoted by heterocellular contacts between mBEC and SVZ cells, an effect that may result from impairment of cell-to-cell contact *via* ECM proteins.

To determine whether the observed effect in cell stemness was in fact due to

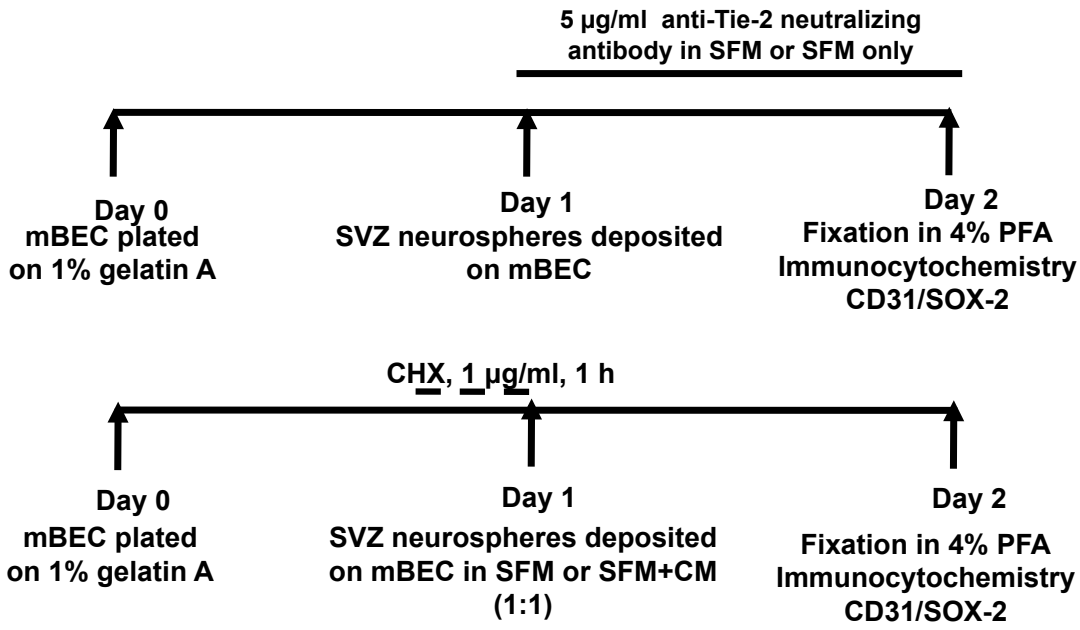

Figure 4.5. Subventricular zone (SVZ) cells and mouse brain endothelial cells (mBEC) co-culture protocol for stemness studies. mBEC were deposited on 1% gelatin A-coated coverslips and left to grow for 24 h. SVZ neurospheres were then plated on 24 h mBEC layers and left to develop for another 24 h. SVZ neurospheres were also plated on mBEC treated 1 h with 1 µg/ml cycloheximide (CHX), an inhibitor of protein synthesis, in serum free media (SFM). To evaluate whether endothelial-derived soluble factors contribute to the observed effects in stemness, SVZ neurospheres were plated on mBEC treated 1 h with 1 µg/ml CHX, in SFM plus mBEC-conditioned media (CM) (1:1) or plated on untreated mBEC with 5 µg/ml anti-Tie-2-neutralizing antibody in SFM, and left to develop for 24 h. After these incubations, co-cultures were fixed and processed for CD31 (a marker of endothelial cells) and SOX-2 (a marker of stem cells) double immunocytochemistry.

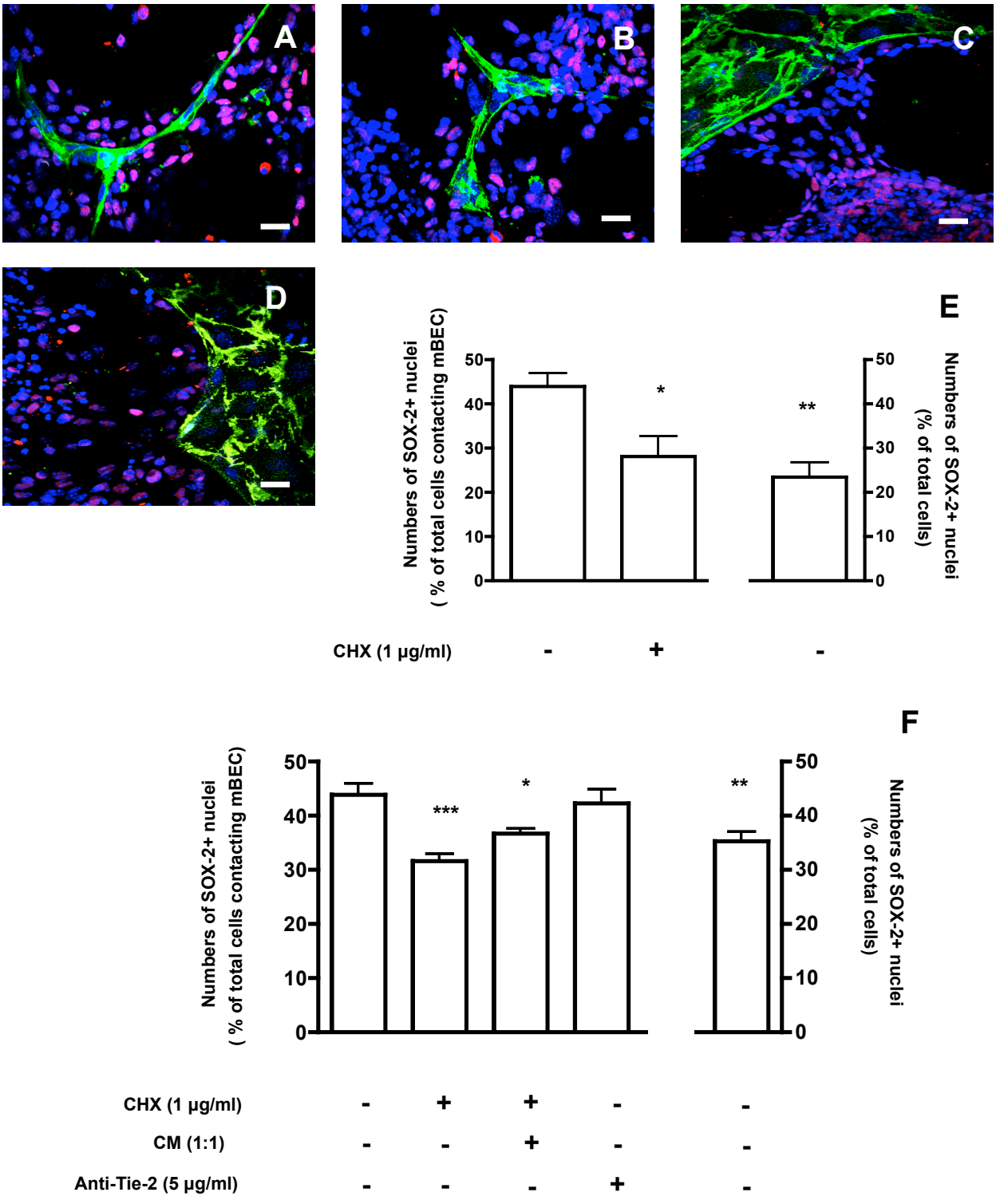

Figure 4.6. Contact between subventricular zone (SVZ) cells and mouse brain endothelial cells (mBEC) sustain stemness in SVZ cells. ***A*** - ***D***, Representative confocal digital images depicting SOX-2 positive cells in mBEC and SVZ cells co-cultures (green staining for CD31, an endothelial cell marker, red staining for SOX-2, a marker of stem cells, and Hoechst 33342 staining in blue). ***A***, Control co-cultures. ***B* and *C***, Co-cultures with 1 µg/ml cycloheximide (CHX) for 1 h-treated mBEC, in serum free medium (SFM) or SFM plus mBEC-conditioned media (CM) (1:1), respectively. ***D***, Co-cultures performed with 5 µg/ml anti-Tie-2 neutralizing antibody in SFM. Scale bars, 20 µm. ***E* and *F***, Left: Bar graphs show the numbers of SOX-2 positive nuclei as a percentage (%) of the total cells contacting mBEC. Right: Graphs show numbers of SOX-2 positive cells as a % of the total number of cells in the carpet of differentiation. *P<0.05, **P<0.01, *** P<0.001 using one-way ANOVA.

actual heterocellular contacts or due to endothelial cell-derived soluble factors, the protocol depicted in Figure 4.5. was followed. Mouse BEC were incubated with 1 µg/ml CHX, in SFM, for 1 h. SVZ neurospheres were then deposited on treated mBEC and left to develop for 24 h in SFM or in 1:1 SFM and mBEC-conditioned media (CM). In some experiments, we exposed co-cultures performed with untreated mBEC to 5 µg/ml of anti-Tie-2 neutralizing antibody to test whether Ang-1, derived for mBEC, had any effect in the maintenance of cell stemness. As depicted in Figure 4.6.F, in control co-cultures, while within the population of cells that contacts endothelial cells there was 43.87 ± 2.10 % SOX-2 positive nuclei (n=9 coverslips, 1790 pairs of cells counted), in the carpet of differentiating cells we found a decreased in the numbers of stem-like cells: 35.26 ± 1.76 % of SOX-2 positive nuclei (n=9 coverslips, 6116 cells counted, $p<0.01$). Co-cultures performed with CHX-incubated mBEC exhibit numbers of mBEC-associated SOX-2 positive cells that do not differ from the ones found in the carpet of differentiating cells in the controls and are significantly decreased as compared to numbers of SOX-2 positive nuclei contacting mBEC in control co-cultures (31.60 ± 1.39 %, n=9 coverslips, 887 pairs of cells counted, $p< 0.001$). This indicates, as before, that contact with endothelial cells promotes the maintenance of the SVZ stem cell state. Moreover, in co-cultures performed with CHX-treated and in the presence of mBEC-CM (Figures 4.6.C and F), numbers of SOX-2+ stem-like cells in contact with mBEC are also reduced as compared to the ones obtained in controls (36.71± 0.94 %, n=9 coverslips, 1070 pairs of cells counted, $p< 0.05$) and do not differ statistically from values in the carpet of differentiating cells in the controls. Similarly we found no statistical difference from values of SOX-2 positive cells contacting mBEC in co-cultures where CHX-treated mBEC were used, even though there was a tendency for this value to be higher. These data strongly suggest that soluble factors are not critical to the observed effects in cell stemness. Further reinforcing this idea, when co-cultures are incubated with 5 µg/ml anti-Tie 2 neutralizing antibody (Figures 4.6.D and F), which blocks the

action of the endothelial-derived soluble factor Ang-1, no differences were seen in the numbers of SOX-2 positive cells contacting mBEC, comparing to controls (42.29 ± 2.56 %, n=7 coverslips, 728 pairs of cells counted).

4.3.4. Contact between SVZ cells and mBEC does not affect SVZ neuronal differentiation

To clarify whether the interaction between SVZ cells and mBEC contributed to neuronal differentiation in co-cultures of SVZ cells and mBEC, the protocol depicted in Figure 4.7. was followed and co-cultures were processed for CD31/DCX double immunostaining. As shown in Figures 4.8.A-C, we found no difference in the numbers of DCX positive cell bodies or neurites contacting mBEC in control co-cultures (cell bodies: 12.18 ± 2.40 %, n=9 coverslips, 1221 pairs of cells counted; neurites: 15.56 ± 3.00 %, n=9 coverslips, 1221 pairs of cells counted) and in co-cultures where mBEC were treated with CHX (cell bodies: 9.26 ± 1.61 %, n=9 coverslips, 1461 pairs of cells counted; neurites: 14.82 ± 3.05 %, n=9 coverslips, 1461 pairs of cells counted). These data indicate that contacts with mBEC do not influence neuronal differentiation, at least, evaluated by DCX expression in SVZ neuroblasts.

4.3.5. SVZ cells and mBEC express α6β1 integrin and laminin-1, respectively

A recent study suggested that binding of endothelial-derived laminin-1 to $\alpha 6\beta 1$ integrin expressed by SVZ cells may promote stemness in SVZ cells (Shen *et al*., 2008). To ascertain that SVZ/mBEC heterocellular contacts could be mediated by α6β1 integrin-laminin-1 binding, the expression of $\alpha 6$ and $\beta 1$ integrins in SVZ neurospheres in proliferative conditions and laminin-1 in mBEC was investigated. Briefly, neurospheres were grown from single SVZ dissociated cells in SFM

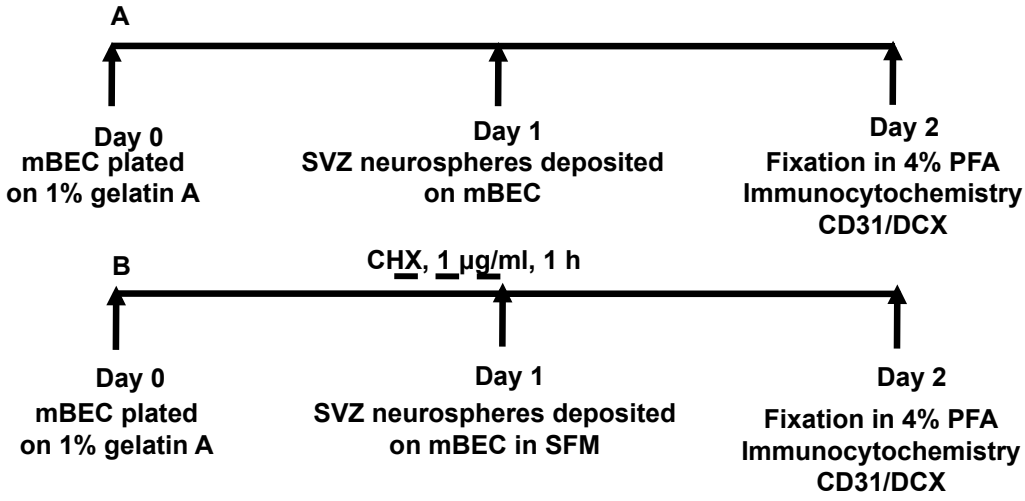

Figure 4.7. Subventricular zone (SVZ) cells and mouse brain endothelial cells (mBEC) co-culture protocol for neuronal differentiation studies. mBEC were deposited on 1% gelatin A-coated coverslips and left to grow for 24 h. SVZ neurospheres were then plated on 24-h mBEC layers and left to develop for another 24 h (***A***). SVZ neurospheres were also plated on mBEC treated 1 h with 1 µg/ml cycloheximide (CHX), an inhibitor of protein synthesis, in serum free media (SFM) (***B***). After these incubations, co-cultures were fixed and processed for CD31 (a marker of endothelial cells) and DCX (a marker of migrating neuroblasts) double immunocytochemistry.

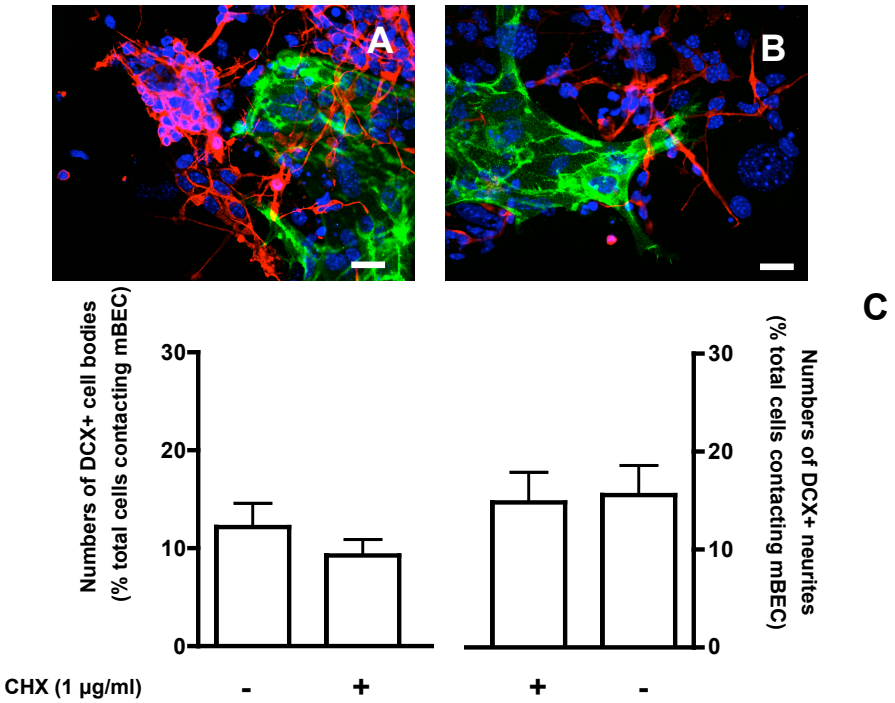

Figure 4.8. Contacts between subventricular zone (SVZ) cells and mouse brain endothelial cells (mBEC) do not affect SVZ-derived neuronal differentiation. ***A* and *B***, Representative confocal digital images depicting DCX positive cells in mBEC and SVZ cells co-cultures (green staining for CD31, an endothelial cell marker, red staining for DCX, a marker of migrating neuroblasts, and Hoechst 33342 staining in blue). ***A***, Control co-cultures, ***B***, Co-cultures with 1 µg/ml cycloheximide (CHX) for 1 h-treated mBEC. Scale bars, 20 µm. ***C***. Bar graph shows the numbers of DCX positive cell bodies (left portion of the graph) or neurites (right portion of the graph) as a percentage (%) of the total cells contacting mBEC in control and in CHX-treated co-cultures.

containing 10 ng/ml EGF and 5 ng/ml FGF-2. After 4–5 d, neurospheres were collected and processed for western blot, and immunocytochemistry. Concerning mBEC, western blotting and immunocytochemistry were performed on confluent cultures. Expression of α6 and β1 integrin protein was demonstrated in SVZ cells (Figures 4.9.A and B) and laminin-1 protein was detected in mBEC (Figure 4.9.C). Moreover, α6 and β1 integrins were detected by immunocytochemistry in Nestin-positive SVZ neurospheres (Figures 4.9.D and E, respectively) and laminin-1 was detected in CD31 positive mBEC (Figure 4.9.F). Negative controls were performed to confirm the specificity of the antibodies used (data not shown). Expression of α6 and β1 integrins in SVZ neurospheres and laminin-1 in mBEC suggests that the interaction between α6β1 integrin and laminin-1 is possible and that it may account for the observed modulatory effects.

4.3.6. SVZ cells and mBEC express Cx43

Gap junction intercellular communication (GJIC) has been shown to regulate homeostasis and differentiation of the stem/progenitor cells of the bone (Guillotin *et al*., 2008) as well as attachment of cancer cells to endothelial cells (Elzarrad *et al*., 2008). To disclose whether SVZ/mBEC heterocellular contacts could be in fact GJIC, expression of Cx43 was searched as aforementioned for integrins and laminin-1. By western blotting, expression of Cx43 protein was demonstrated in SVZ cells and in mBEC (Figure 4.10.A). Moreover, Cx43 was detected by immunocytochemistry in Nestin-positive SVZ neurospheres and in CD31 positive mBEC (Figures 4.10.B and C, respectively). Negative controls were performed to confirm the specificity of the antibodies used (data not shown). Expression of Cx43 in SVZ neurospheres and mBEC suggests that heterocellular contacts between SVZ and mBEC may include gap junctions, which may account for the effects observed. In fact, in control co-cultures, Cx43 clusters at the mBEC-SVZ cell interface can be observed

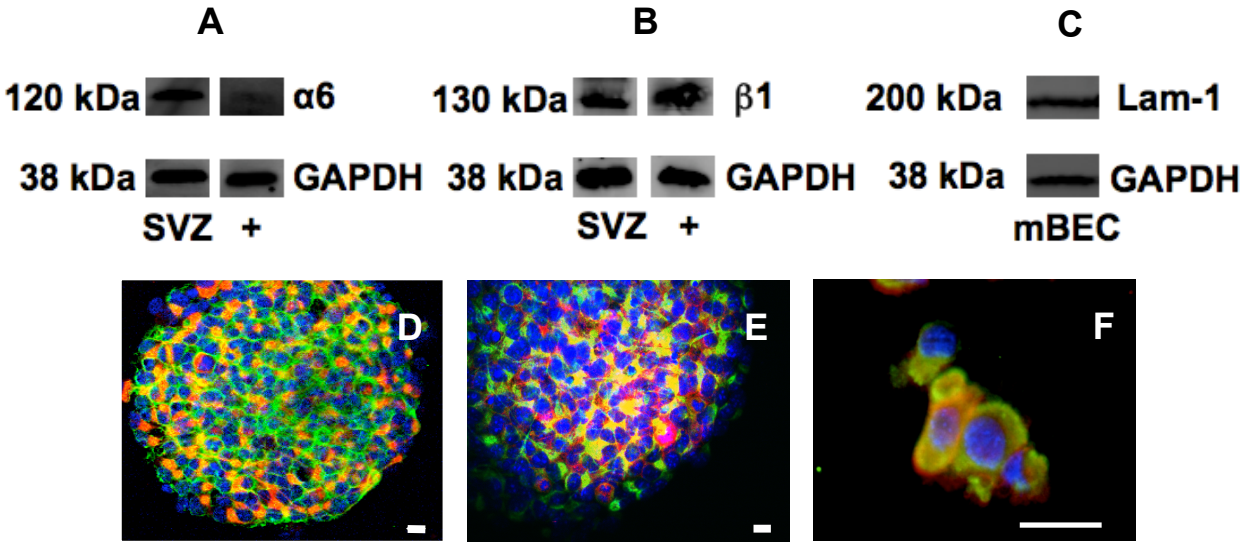

Figure 4.9. Subventricular zone (SVZ) cells express α6 and β1 integrins and mouse brain endothelial cells (mBEC) express laminin-1 (Lam-1). ***A*** - ***C***, Detection of α6 (***A***) and β1 (***B***) integrin proteins by western blotting in SVZ neurospheres and in whole brain extracts (positive control). ***C***, Detection of Lam-1 protein by western blotting in mBEC. GAPDH protein detection was used as a loading control. ***D* and *E*,** Representative confocal digital images depicting the presence of α6 (***D***) and β1 (***E)*** integrins in SVZ neurospheres (green staining for α6 and β1 integrins and red staining for Nestin, a neural stem cell marker). Scale bars, 20 μm. Nuclei evidenced by Hoechst 33342 staining in blue. ***F***, Fluorescence digital image shows the expression of Lam-1 in mBEC (green staining for Lam-1, red staining for CD31, an endothelial cell marker). Nuclei evidenced by Hoechst 33342 staining in blue. Scale bar, 20 μm.

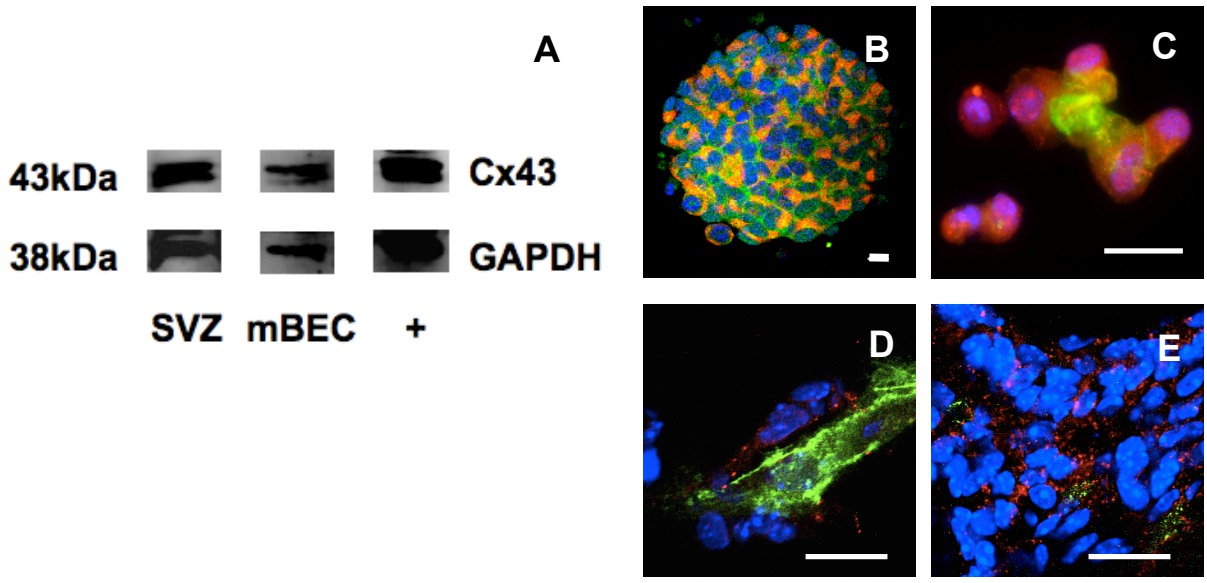

Figure 4.10. Subventricular zone (SVZ) cells and mouse brain endothelial cells (mBEC) express connexin 43 (Cx43). ***A*,** Detection of Cx43 proteins by western blotting in SVZ neurospheres, in mBEC and in whole brain extracts (positive control). GAPDH protein detection was used as a loading control. ***B,*** Representative confocal digital image depicting the presence of Cx43 in SVZ neurospheres (green staining for Cx43, red staining for Nestin, a neural stem cell marker, and Hoechst 33342 staining in blue). Scale bar, 20 μm. ***C***. Fluorescence digital image shows the expression of Cx43 in mBEC. CD31, an endothelial cell marker, is shown in green, Cx43 in red and nuclei are evidenced with Hoechst 33342 staining in blue. Scale bar, 20 μm; ***D and E***, Confocal fluorescence digital images depict Cx43 clusters in areas where SVZ cells interact with endothelial cells, in mBEC/SVZ cells co-cultures (***D)*** and in the SVZ *in vivo* ***(E)***. CD31 and Cx43 are shown in green and red, respectively, and nuclei are evidenced with Hoechst 33342 staining in blue. Scale bars, 20 μm.

(Figure 4.10.D). Also *in vivo*, in the SVZ, clusters of Cx43 labelling can be observed and some of these are present in regions where SVZ cells contact blood vessels as well (Figure 4.10.E).

4.3.7. Cycloheximide treatment decreases the turnover of laminin-1 and Cx43 in mBEC

We assumed that, by inhibiting protein synthesis in mBEC with CHX treatment, we could decrease the expression of ECM proteins involved in the interaction between mBEC and SVZ cells. To test this hypothesis, we evaluated the expression of laminin-1 and Cx43 in mBEC both in control and in 1 and 24 h-CHX-treated mBEC, by using immunocytochemistry and by western blotting. As depicted in Figures 4.11.A, a, B, b and E-G, CHX treatment decreases the expression of laminin-1 in mBEC after both 1 and 24 h of incubation. Because we did not see any differences in GAPDH expression between conditions we used this molecular marker to normalize our data. Other authors have done this as well (Lu *et al*., 2007). However, since it could be argued that GAPDH protein levels may be affected by CHX treatment, data were also normalized as a percentage of control protein levels. Indeed, when protein expression values are calculated as a percentage of GAPDH expression (Figure 4.11.F), while in controls Lam-1 levels are 15.29 ± 1.70% (n=3), after 1 and 24 h of CHX treatment values decrease to 11.81 ± 0.62 % (n=3) and 5.41 ± 2.12% (n=3, $p<0.05$), respectively. When Lam-1 protein levels are expressed as percentages of control Lam-1 values (Figure 4.11.G), after 1 h of CHX exposure, there is 85.84 ± 16.94 % Lam-1 while after 24 h of CHX treatment, there is 53.69 ± 2.91 % of the protein.

Figures 4.11.C, c, D, d, E, H and I illustrate the fact that CHX treatment decreases expression of Cx43 in mBEC after both 1 and 24 h of exposure. When protein expression values are shown as percentages of GAPDH expression (Figure 4.11.H), in controls, Cx43 levels are 21.65 ± 4.49 % (n=3), while after 1 and 24 h of

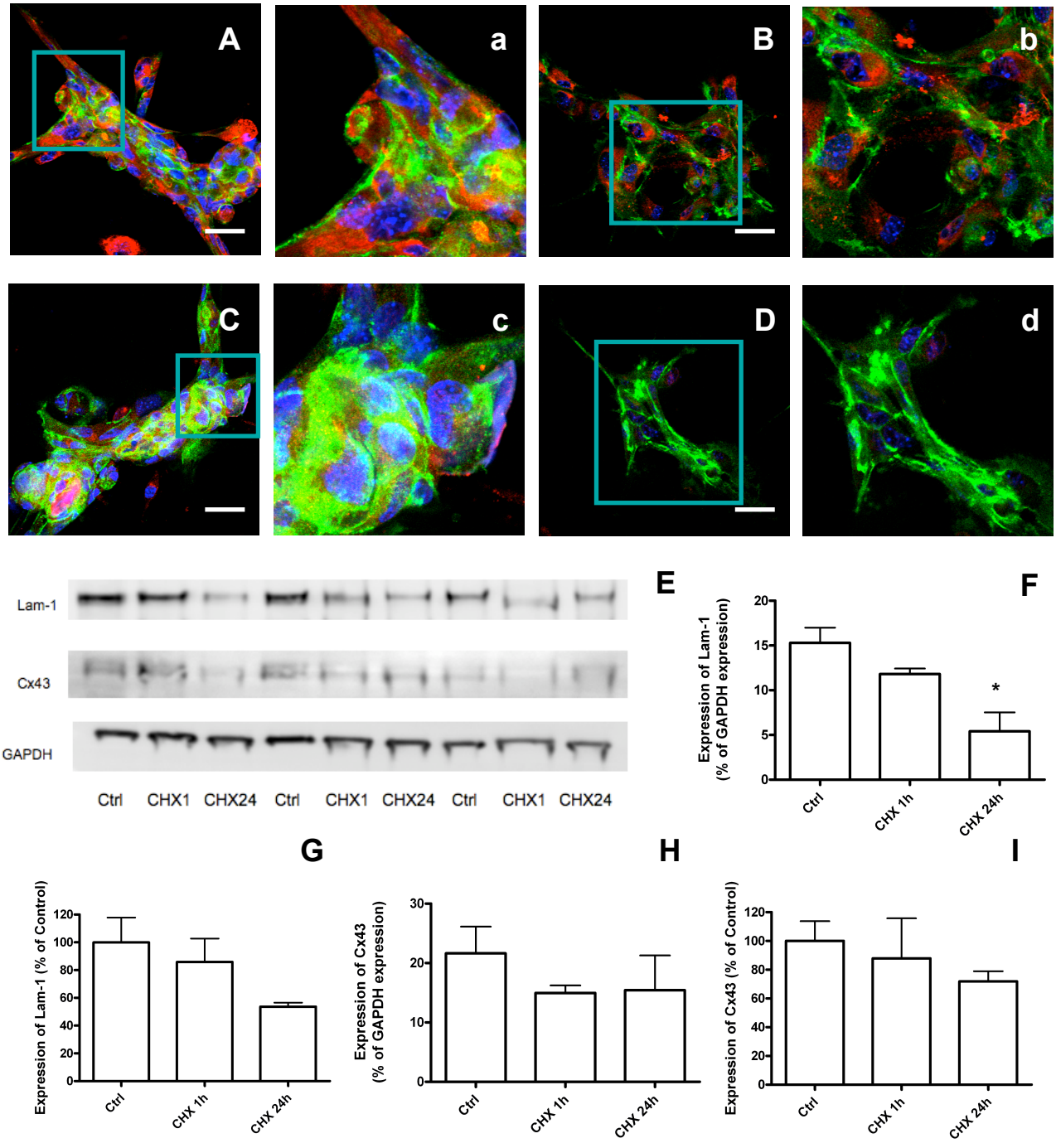

Figure 4.11. Cycloheximide (CHX) downregulates Laminin-1 (Lam-1) and Connexin43 (Cx43) in mouse brain endothelial cells (mBEC). ***A* and *a,*** Confocal fluorescence digital images showing Lam-1 expression in control mBEC. ***B* and *b,*** Detection of Lam-1 expression in CHX-treated cells (1 h incubation with 1 µg/ml CHX). ***C* and *c,*** Detection of Cx43 expression in control mBEC. ***D* and *d,*** Verification of Cx43 expression in CHX-treated cells. ***a***, ***b***, ***c* and *d*** are magnifications of **A**, ***B***, ***C* and *D***, respectively. Green staining indicates CD31, an endothelial cell marker, red staining shows Lam-1 or Cx43 expression and nuclei are evidenced with Hoechst 33342 staining in blue. Scale bars, 20 µm. ***E***, Western blot showing Lam-1 and Cx43 protein levels in 3 different mBEC cultures in controls and in mBEC treated with 1 µg/ml CHX for 1 or 24 h. GAPDH protein detection was used as a loading control. ***F* and *G,*** Bar graphs show the respective quantification of Lam-1 levels, as a percentage (%) of GAPDH expression (***F***) or as a % of control (***G***). ***H* and *I,*** Bar graphs show the quantification of Cx43 levels, as a % of GAPDH expression (***H***) or as a % of control (***I***). *P<0.05, using the unpaired Student *t* test.

CHX treatment values decrease to 14.96 ± 1.29 % (n=3) and 15.44 ± 5.843 % (n=3). When Cx43 levels are expressed as percentages of control Cx43 values (Figure 4.11.I), after 1 h of CHX exposure, we found 87.81 ± 27.94 % Cx43 while after 24 h of CHX treatment, this value decreased to 71.86 ± 7.06 % Cx43.

4.3.8. Contacts between SVZ cells and mBEC *via* α6β1 integrin/laminin-1 binding and gap junction intercellular communication positively regulate SVZ cell proliferation but exert no effect on cell death

In order to determine if heterocellular interactions *via* integrins-laminin-1 and gap junctions are involved in the observed pro-proliferative effect, co-cultures of SVZ cells and mBEC were performed as shown in the protocol depicted in Figure 4.12.A. Briefly, mBEC were deposited on coverslips and left to grow for 24 h. SVZ neurospheres were then placed on mBEC and left to develop for 24 h in SFM, with 5 μg/ml anti-α6 neutralizing antibody or 35 μM carbenoxolone (CBX) in SFM, to inhibit the putative α6β1 integrin-laminin-1 interaction or heterocellular GJICs, respectively. For the last 4 h of the experiments, co-cultures were incubated with 10 μM BrdU and its incorporation was evaluated. As depicted in Figures 4.13.A and D, while in control co-cultures we found 22.43 ± 1.11 % BrdU positive nuclei apposed to mBEC (n=8 coverslips, 572 pairs of cells counted), in co-cultures treated with the anti-α6 integrin neutralizing antibody (Figures 4.13.B and D) there was a decrease in the numbers of proliferating cells: 12.41 ± 1.50 % BrdU positive cells (n=9 coverslips, 1009 pairs of cells counted, $p<0.0001$). This indicates that the interaction between SVZ cell α6β1 integrin and mBEC laminin-1 contributes to SVZ cell proliferation. In the presence of the anti-α6 neutralizing antibody, as it can be observed in Figures 4.13.E, F and H no differences were found in the numbers of apoptotic SVZ cells either in the population that contacts mBEC or in the carpet, as compared to control levels (control, cells contacting mBEC: 15.74 ± 1.72 % Tunel+ nuclei, n=9, 1023 pairs of cells counted;

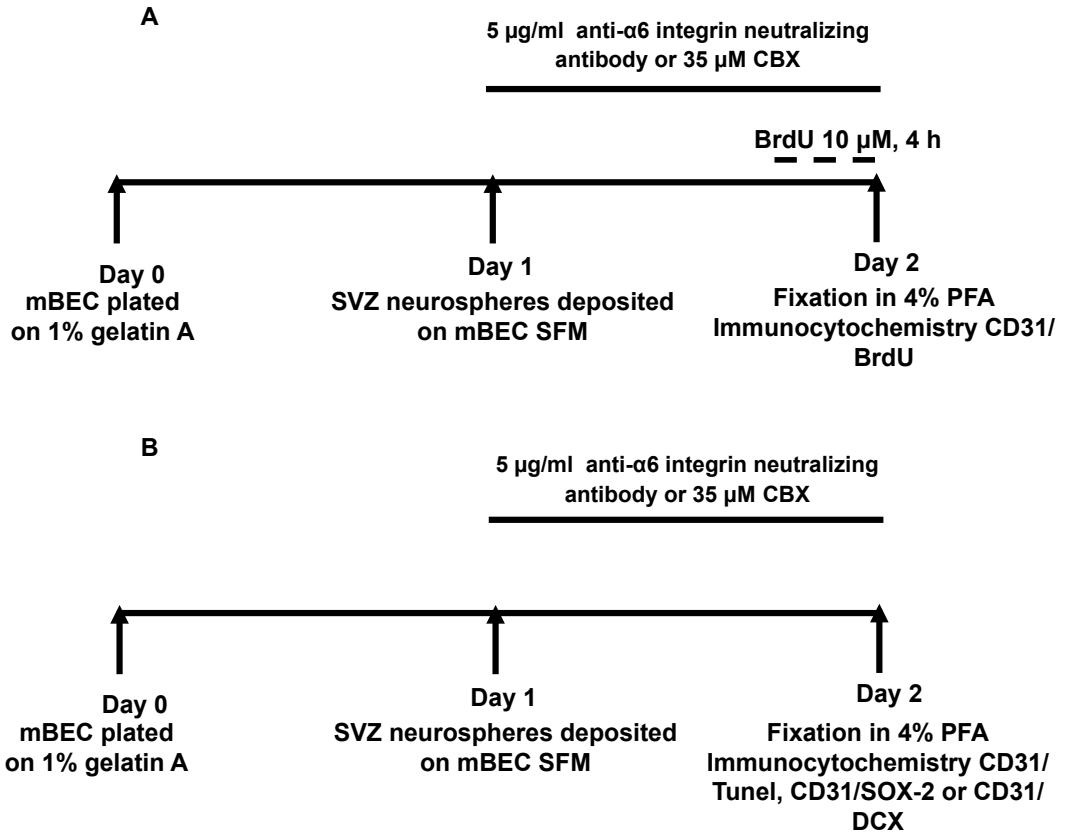

Figure 4.12. Subventricular zone (SVZ) cells and mouse brain endothelial cells (mBEC) co-culture protocol to study the role of endothelial laminin-1 and SVZ α6**β1** integrin interactions, as well as heterocellular gap junctions between mBEC and SVZ cells, on SVZ cell proliferation (***A***), cell death, stemness and cell differentiation (***B***). mBEC were deposited on 1% gelatin A-coated coverslips and left to grow for 24 h. SVZ neurospheres were then placed on mBEC and left to develop for 24 h in serum free media (SFM), in SFM with 5 µg/ml anti-α6 integrin neutralizing antibody, to inhibit the putative α6**β1** integrin-laminin-1 interactions, or in SFM with 35 µM carbenoxolone (CBX), an inhibitor of gap junction intercellular communications. For proliferation studies, in the last 4 h of the culture session, co-cultures were incubated with 10 µM BrdU (a marker of cell proliferation). After this, cells were fixed and processed for CD31 (a marker of endothelial cells) and BrdU double-immunocytochemistry. For cell death, stemness and neuronal differentiation studies, co-cultures were fixed and processed for CD31/Tunel, CD31/SOX-2 and CD31/DCX double immunocytochemistry, respectively.

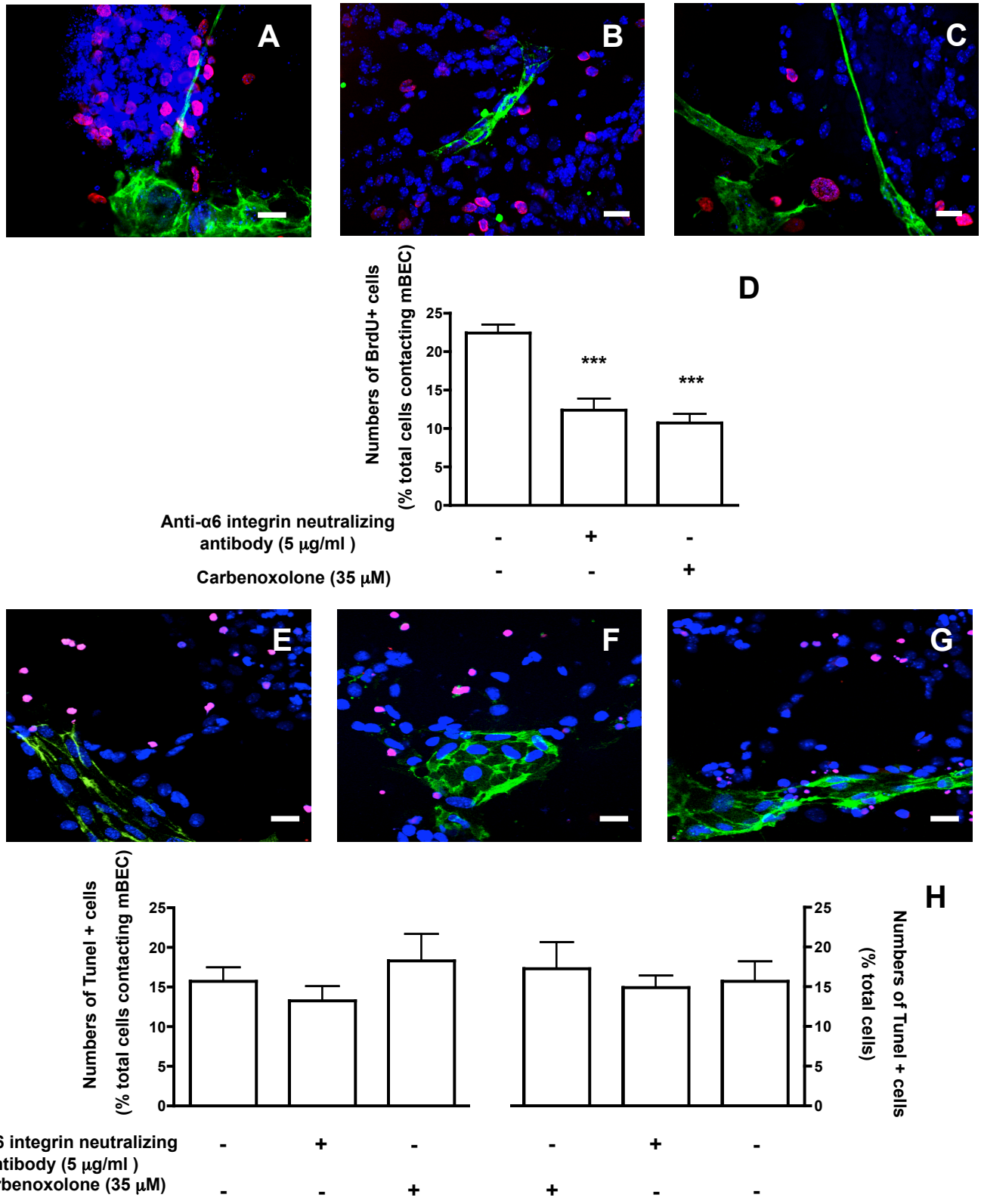

Figure 4.13. Contacts between subventricular zone (SVZ) cells α6β1 integrin and mouse brain endothelial cells (mBEC) laminin-1, as well as contacts mediated by gap junctions, promote SVZ cell proliferation and have no effect on apoptotic cell death. ***A*** - ***C***, Representative confocal digital images depicting proliferating cells in mBEC/SVZ cells co-cultures (green staining for CD31, an endothelial cell marker, red staining for BrdU, a marker of proliferation, and Hoechst 33342 staining in blue). ***A***, Control co-cultures. ***B***, Co-cultures incubated with 5 μg/ml anti-α6 integrin neutralizing antibody. ***C***, Co-cultures incubated with 35 μM carbenoxolone (CBX), a gap junction intercellular communication inhibitor. Scale bars, 20 μm. ***D***, Bar graph shows the numbers of BrdU positive cells as a percentage (%) of the total cells contacting mBEC in control co-cultures, in anti-α6 integrin neutralizing antibody-incubated co-cultures and in co-cultures incubated with CBX. *** P<0.0001 using the unpaired Student *t* test for comparison with control co-cultures. ***E*** - ***G***, Representative confocal digital images showing apoptotic cells in mBEC/SVZ cells co-cultures (green staining for CD31, apoptotic nuclei evidenced in red with Tunel staining, total nuclei evidenced in blue, with Hoechst 33342 staining). ***E***, Control co-cultures. ***F***, Co-cultures incubated with 5 μg/ml anti-α6 integrin neutralizing antibody. ***G***, Co-cultures incubated with 35 μM CBX. Scale bars, 20 μm. ***H***, Left: Bar graph shows the numbers of apoptotic nuclei as a % of the total cells contacting mBEC in control co-cultures, in anti-α6 integrin neutralizing antibody-incubated co-cultures and in co-cultures incubated with CBX. Right: Bar graph shows the numbers of apoptotic nuclei as a % of the total cells in the carpet of differentiation, in control co-cultures, in anti-α6 integrin neutralizing antibody-incubated co-cultures and in co-cultures incubated with CBX.

control, carpet: 15.67 ± 2.51 % Tunel+ nuclei, n=9, 4330 cells counted; anti-α6 neutralizing antibody, cells contacting mBEC: 13.25 ± 1.86 % Tunel+ nuclei, n=8, 679 pairs of cells counted; anti-α6 neutralizing antibody, carpet: 14.90 ± 1.52 % Tunel+ nuclei, n=8; 2572 cells counted). This demonstrates that heterocellular contacts mediated by α6β1 integrin and mBEC laminin-1 do no affect SVZ cell survival. In addition, incubation with the anti-α6 neutralizing antibody does not affect the viability of SVZ cells.

When co-cultures were treated with 35 μM carbenoxolone (Figures 4.13.C and D) there was a decrease in the numbers of proliferating cells contacting mBEC compared to control co-cultures: 10.71 ± 1.18 % BrdU positive cells (n=9 coverslips, 926 pairs of cells counted, $p<0.0001$). These data show that GJICs between SVZ cell and mBEC positively regulate cell proliferation.

When co-cultures were incubated with CBX, as depicted in Figures 4.13.E, G and H, no differences were observed in the numbers of apoptotic SVZ nuclei either in the population that contacts mBEC or in the carpet, as compared to control levels (control, cells contacting mBEC: 15.74 ± 1.72 % Tunel+ cells, n=9, 1023 pairs of cells counted; control, carpet: 15.67 ± 2.51% Tunel+ cells, n=9, 4330 cells counted; CBX, cells contacting mBEC: 18.33 ± 3.34 % Tunel+ cells, n=9, 567 pairs of cells counted; CBX, carpet: 17.29 ± 3.32 % Tunel+ cells, n=9; 3451 cells counted). This demonstrates that heterocellular contacts mediated by GJICs do not interfere with SVZ cell survival. Moreover, it shows that incubation with CBX does not affect the viability of SVZ cells.

4.3.9. Contacts between SVZ cells and mBEC *via* α6β1 integrin/ laminin-1 binding and GJIC promote stemness

To disclose whether the positive effects on stemness obtained in SVZ cells that contact mBEC are mediated *via* α6β1 integrin and laminin-1 binding and/or

through gap junctions, co-cultures, performed as described in the protocol depicted in Figure 4.12.B, were treated with either 5 μg/ml anti-α6 integrin neutralizing antibody, in order to impair endothelial-derived laminin-1 to bind to α6β1 integrin, or 35 μM of the GJIC inhibitor CBX. At the end of the co-culture session, cells were fixed and processed for CD31 and SOX-2 double immunocytochemistry. As shown in Figures 4.14.A and D, while in control co-cultures we found 43.97 ± 2.96 % SOX-2 positive nuclei apposed to mBEC (n=8 coverslips, 1223 pairs of cells counted), in co-cultures treated with the anti-α6 integrin neutralizing antibody (Figures 4.14.B and D) there was a decrease in the numbers of stem-like cells: 25.35 ± 2.76 % SOX-2 positive cells (n=9 coverslips, 1132 pairs of cells counted, $p<0.001$). This indicates that the interaction between SVZ cell α6β1 integrin and mBEC laminin-1 contributes to SVZ cell stemness.

In co-cultures treated with 35 μM CBX (Figures 4.14.C and D), there was a decrease in the numbers of stem-like cells contacting mBEC, compared to the control co-cultures: 27.46 ± 4.00 % SOX-2 positive cells (n=9 coverslips, 1098 pairs of cells counted, $p<0.001$). This suggests that GJICs between SVZ cell and mBEC positively regulate stemness.

4.3.10. Contact between SVZ cells and mBEC *via* α6β1 integrin/laminin-1 binding and GJIC do not contribute to neuronal differentiation

We previously showed that there was no difference in the numbers of DCX + cell bodies and/or neurites cells contacting mBEC in control co-cultures and in co-cultures where mBEC have been previously treated with CHX (section 4.3.4.) suggesting that heterocellular contacts do not influence neuronal differentiation. Consequently, and as expected, impairment of α6β1 binding to laminin-1 or blocking of GJIC (protocol depicted in Figure 4.12.B) does not modify the numbers of DCX positive cell bodies or neurites contacting mBEC obtained in tested conditions, as

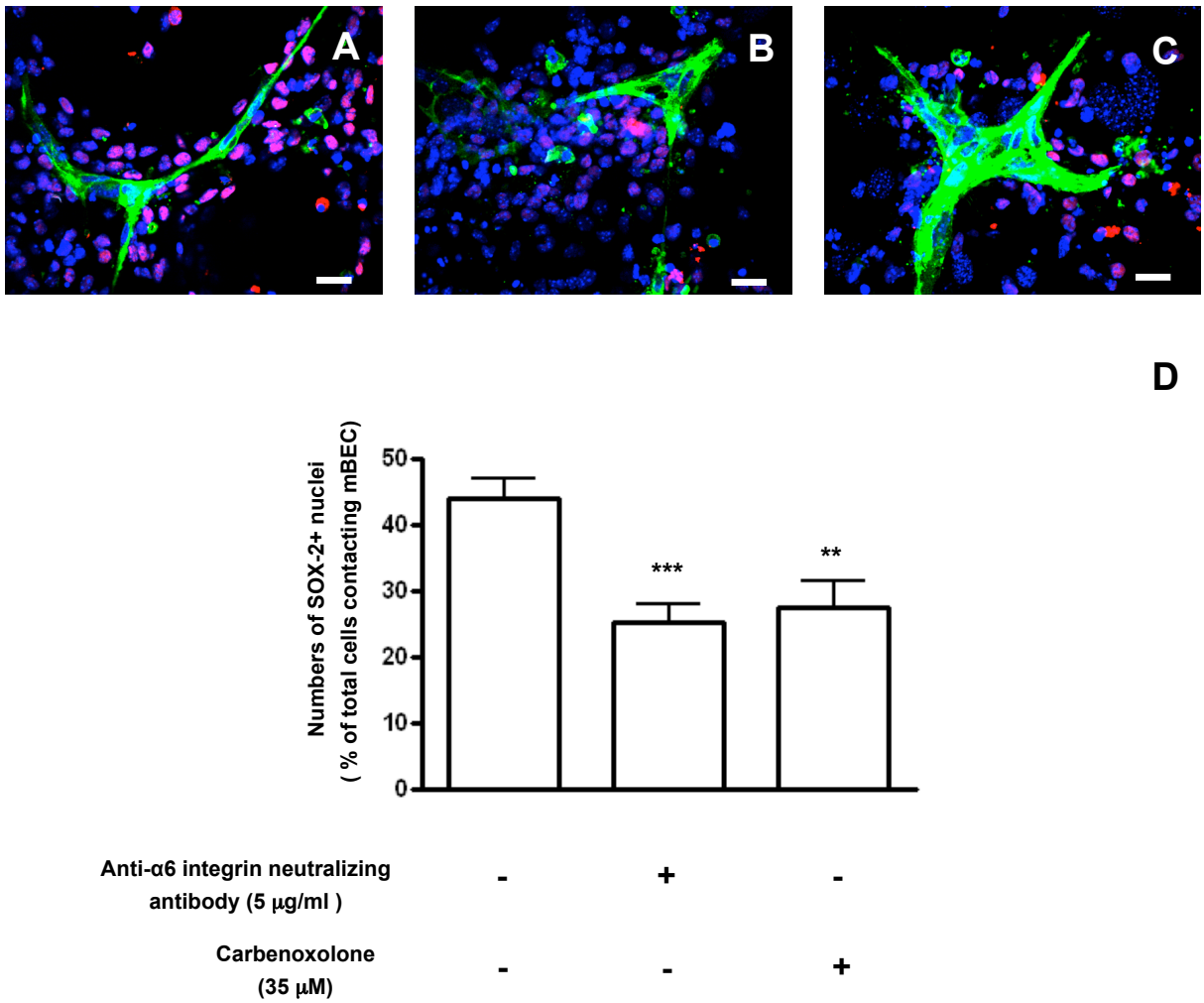

Figure 4.14. Contacts between subventricular zone (SVZ) cells α6β1 integrin and mouse brain endothelial cells (mBEC) laminin-1, as well as contacts mediated by gap junctions, sustain stemness in SVZ cells. ***A*** - ***C***, Representative confocal digital images depicting SOX-2 positive cells in mBEC and SVZ cells co-cultures (green staining for CD31, an endothelial cell marker, red staining for SOX-2, a marker of stem cells, and Hoechst 33342 staining in blue). ***A***, Control co-cultures. ***B***, Co-cultures incubated with 5 μg/ml anti-α6 integrin neutralizing antibody. ***C***, Co-cultures incubated with 35 μM carbenoxolone (CBX), an inhibitor of gap junction intercellular communications. Scale bars, 20 μm. ***D***, Bar graph shows the numbers of SOX-2 positive nuclei as a percentage (%) of the total cells contacting mBEC in control co-cultures, in anti-α6 integrin neutralizing antibody-incubated co-cultures and in co-cultures incubated with CBX. **P<0.01; ***P<0.001 using the unpaired Student *t* test for comparison to control co-cultures.

compared to control co-cultures (control co-cultures: 12.18 ± 2.40 % cell bodies, n=9 coverslips, 1221 pairs of cells counted; 15.56 ± 3.00 % neurites, n=9 coverslips, 1221 pairs of cells counted; co-cultures treated with the anti-α6 integrin neutralizing antibody: 10.82 ± 2.63 % cell bodies, n=9 coverslips, 1455 pairs of cells counted; 16.09 ± 3.62 % neurites, n=9 coverslips, 1455 pairs of cells counted; co-cultures treated with CBX: 8.58 ± 2.28 % cell bodies, n=9 coverslips, 1857 pairs of cells counted; 17.25 ± 3.48 % neurites, n=9 coverslips, 1857 pairs of cells counted; Figure 4.15.). These results further demonstrate that heterocellular contacts between SVZ cells and mBEC are not critically necessary to SVZ cell-derived neuronal differentiation.

4.3.11. SVZ cells communicate with mBEC through gap junctions: LAMP assay

As shown in section 4.3.6., both mBEC and SVZ cells express Cx43. In order to test whether endothelial and SVZ cells established functional gap junctions, LAMP assays were performed. The LAMP probe, NPE-HCCC2, is sensitive to UV photolysis and UV uncaging (100% laser power of 405nm 30mW diode laser) regenerates fluorescent HCCC2 that is blue. Because the molecular weight of HCCC2 (MW = 450 Da) is well below the molecular passage limit (1,000 Da) of connexin channels, HCCC2 diffuses between cells through gap junctions.

We performed a co-culture of mBEC from WT mice and SVZ cells from GFP mice. After 24 h, we chose putative communicating cell pairs consisting of a mBEC and a GFP SVZ cell. A region of interest (ROI) in a small fraction of the cytoplasm of the potential donor mBEC cell (Figures 4.16.A and B) was selected and then we proceeded to localized uncaging of NPE-HCCC2 (Figure 4.16.C). By locally photolysing caged HCCC2, we asymmetrically stained one cell of the coupled cell pair (Figure 4.16.D) - the mBEC cell was stained blue. We performed a time-lapse experiment by acquiring a sequence of images every 5 s. By sequence analysis of

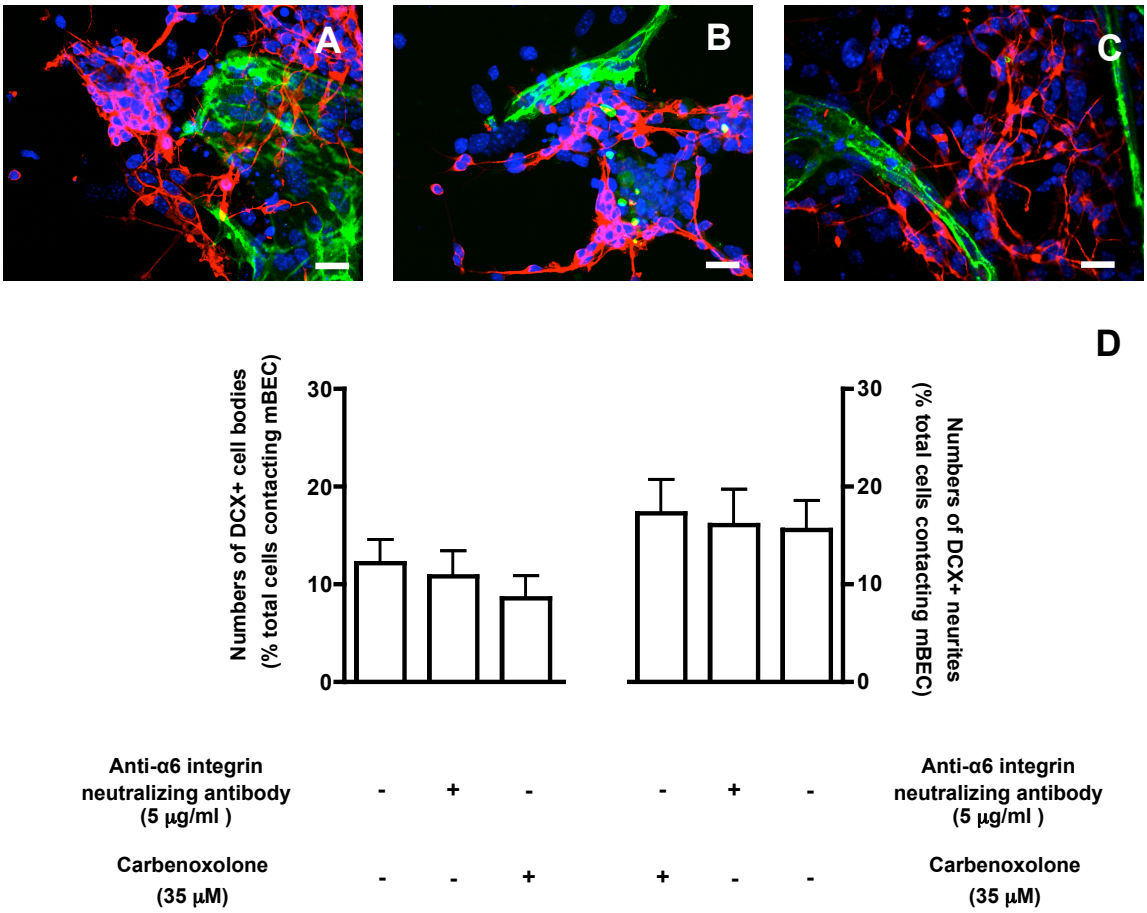

Figure 4.15. Contacts between subventricular zone (SVZ) cells α6β1 integrin and mouse brain endothelial cells (mBEC) laminin-1, as well as contacts mediated by gap junctions, do not affect SVZ-derived neuronal differentiation. ***A*** - ***C***, Representative confocal digital images depicting DCX positive cells in mBEC and SVZ cells co-cultures (green staining for CD31, an endothelial cell marker, red staining for DCX, a marker of migrating neuroblasts, and Hoechst 33342 staining in blue). ***A***, Control co-cultures. ***B***, Co-cultures incubated with 5 μg/ml anti-α6 integrin neutralizing antibody. ***C***, Co-cultures incubated with 35 μM carbenoxolone (CBX), an inhibitor of gap junction intercellular communications. Scale bars, 20 μm. ***D***, Bar graph shows the numbers of DCX positive cell bodies (left portion of the graph) or neurites (right portion of the graph) as a percentage (%) of the total cells contacting mBEC in control co-cultures, in anti-α6 integrin neutralizing antibody-incubated co-cultures and in co-cultures incubated with CBX.

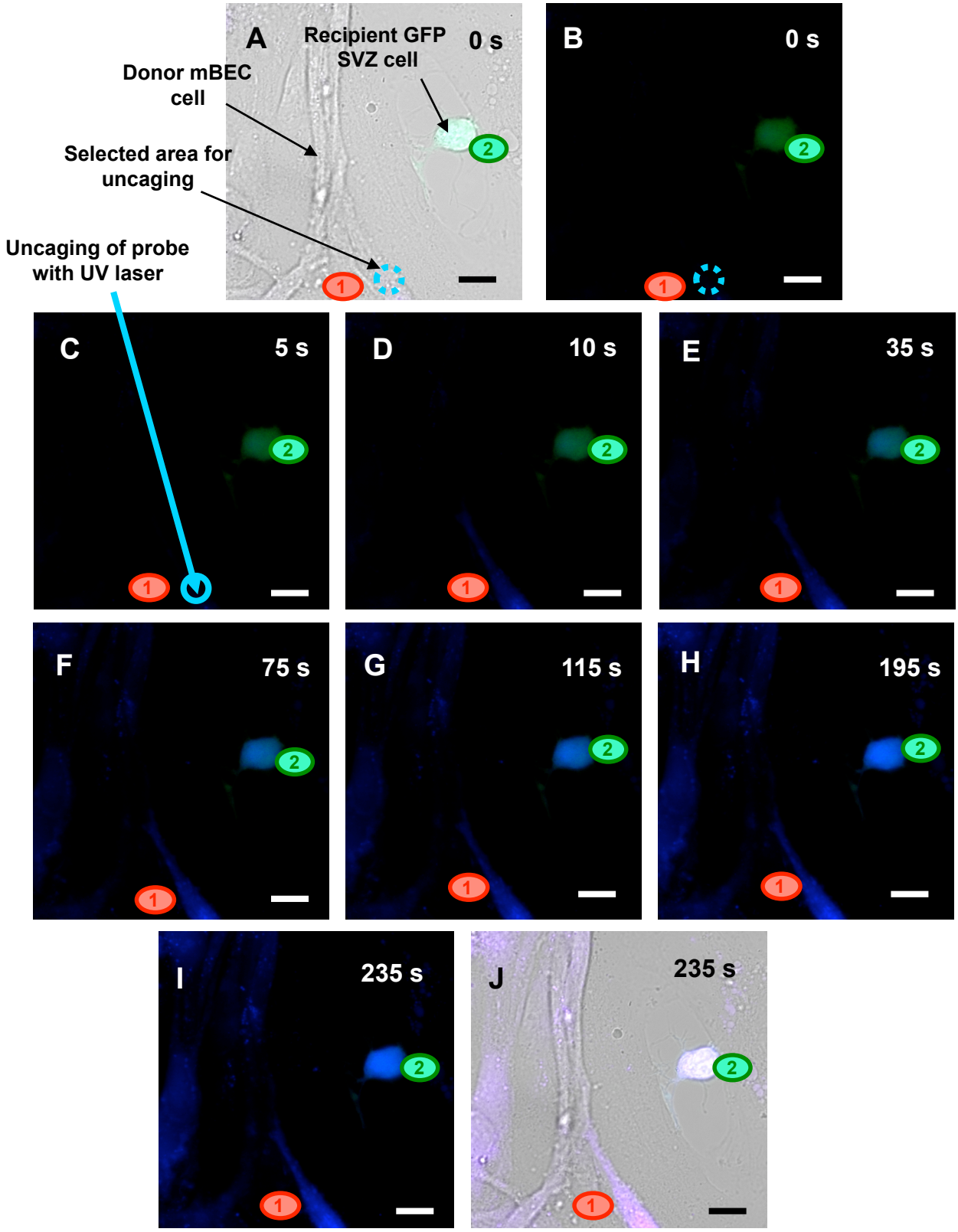

Figure 4.16. Confocal imaging sequence showing a mouse brain endothelial cell (mBEC) communicating with a subventricular zone (SVZ) cell through gap junctions, as shown by LAMP. ***A*** - ***J,*** Transmission and fluorescence confocal digital images showing a communicating cell pair consisting of a mBEC and a GFP SVZ cell. (***A*** and ***B***) Potential coupled pairs are chosen and a region of interest (ROI) is selected in the cytoplasm of the putative donor mBEC cell. Uncaging of the probe (***C***) is then processed using UV light, asymmetrically marking the donor cell (***D***). Fluorescence images of cells at different time points are then taken, illustrating asymmetric marking of the donor mBEC (cell 1) and subsequent probe transfer to the recipient SVZ cell (cell 2) (***E-I***). In the end of the experiment, a final transmission picture is taken to visualize residual activation of the probe in non-coupled cells (***J***). Scale bars, 10 μm. S, seconds; GFP, green fluorescence protein.

the acquired images we could follow the transfer of the fluorescent probe from the mBEC cell (cell 1) to the paired SVZ cell (cell 2) (Figures 4.16.E-J). We also plotted fluorescence intensities of HCCC2 from the different cells in the field (Figure 4.17.). In some cases, the HCCC2 blue probe did not pass from a mBEC to a contacting SVZ cell revealing there was no communication through gap junctions between the two cells (Figure 4.18.).

4.4. Discussion

4.4.1. Co-culture establishment

In this chapter, the establishment of a primary mBEC culture was described. The main challenge of performing such a culture is to obtain a high level of purity. The principle of using puromycin as a selection drug for endothelial cells was previously reported by Perrière and collaborators (2005) and it resides in the fact that endothelial cells express high levels of P-gp, efflux plasma membrane proteins, which are capable of excluding toxic substances such as puromycin. Therefore, by killing contaminant sensitive cells, puromycin selects P-gp high-expressing brain capillary endothelial cells. Obtained cultures were composed of more than 89 % of mBEC. The remaining cells were contaminant cells and included SMA positive pericytes and smooth muscle cells. It is possible that GFAP positive astrocytes and CD11b positive microglia cells were also present even though we did not find them in the performed immunocytochemistry studies. The obtained yield was considered acceptable for the usage of the obtained mBEC in co-cultures with SVZ cells. To perform such co-cultures, SVZ cells have to be cultured in SFM, as serum decreases proliferation and increases glial differentiation. Because endothelial cells grow in medium containing serum and undergo apoptosis after serum removal (Kwak *et al*., 1999) we had to test the mBEC viability at different time points. We demonstrated that SFM decreased endothelial cell viability at 48 h but not at 24 h culture period and, therefore, co-cultures were stopped after 24 h.

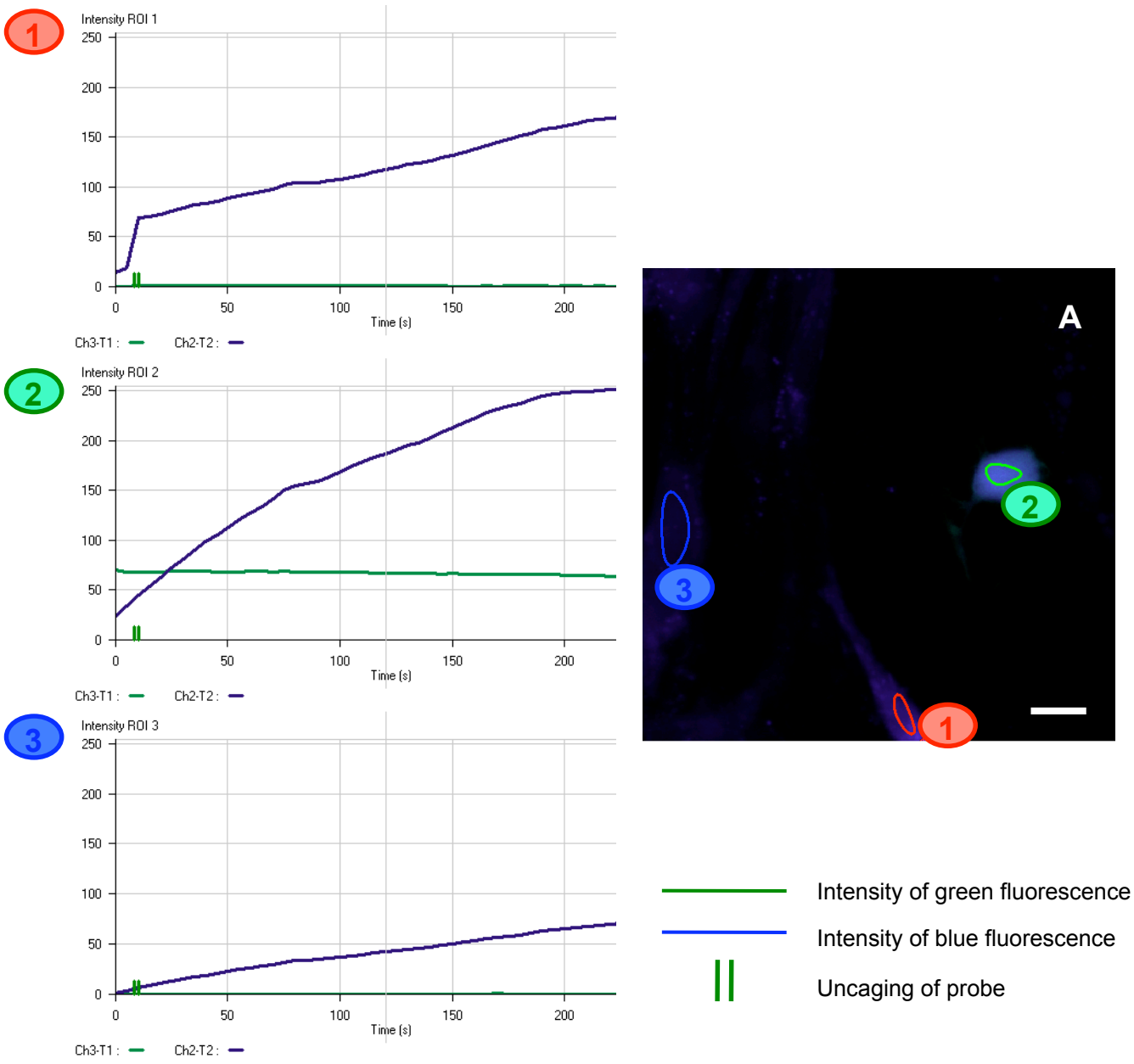

Figure 4.17. Fluorescence intensities of the LAMP probe (blue fluorescence) and of GFP (green fluorescence) from different cells in a microscopic field where a mouse brain endothelial cell (mBEC) and a subventricular zone (SVZ) cell communicate through gap junctions. ***A***: Confocal fluorescence digital image showing a donor mBEC cell (***1***), a SVZ GFP recipient cell (***2***) and an uncoupled cell (***3***). Graphs show the respective variation of fluorescence intensity during the time of the LAMP experiment. Upon uncaging (represented in graphs by 2 green lines at ~5 s), blue fluorescence increases rapidly in the donor cell (***1***). In the coupled cell (***2***), intensity of blue fluorescence increases more and more rapidly than in the uncoupled cell (***3***). Scale bars, 10 μm. S, seconds; GFP, green fluorescence protein.

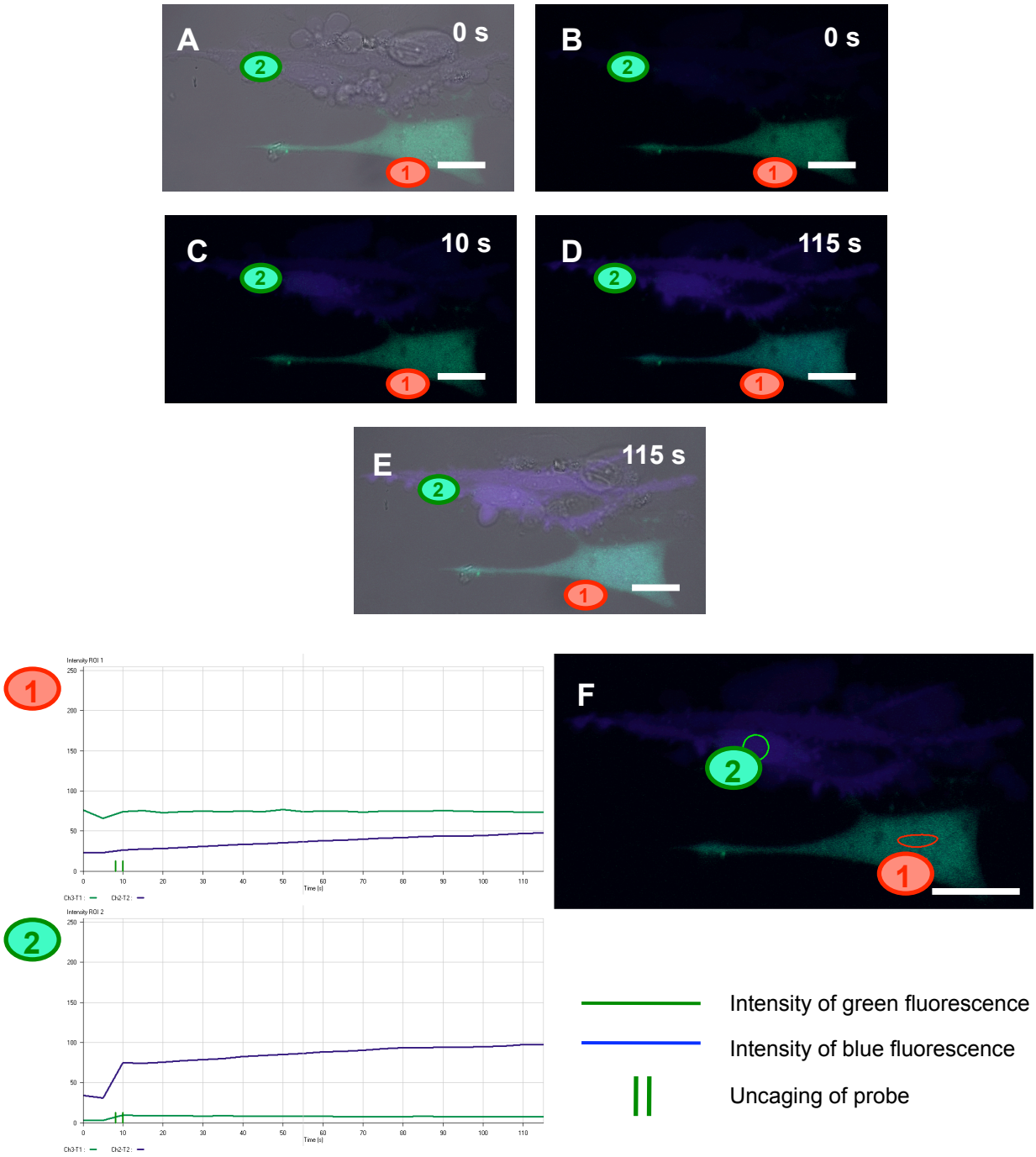

Figure 4.18. Not all mouse brain endothelial cell (mBEC)/subventricular zone (SVZ) cell pairs communicate through gap junctions, as evidenced by LAMP. ***A***-***E***, Confocal transmission and fluorescence digital pictures showing a mBEC/SVZ cell pair during the LAMP assay. ***A*** and ***B***, cells prior to uncaging; ***C***, cells after uncaging; ***D*** and ***E***, cells at the end of the assay. ***F***, Fluorescence intensities of the LAMP probe (blue fluorescence) and of GFP (green fluorescence) from cells in the shown confocal microscopic field. ***1***, Putative SVZ GFP recipient cell; ***2***, Donor mBEC cell. Upon uncaging (represented by 2 green lines at ~5 seconds), blue fluorescence increases rapidly in the mBEC (***2***) but does not increase in the neighbour SVZ cell (***2***), showing these cells do not communicate through gap junctions. Scale bars, 10 μm. S, seconds; GFP, green fluorescence protein.

Other models of endothelial cells and neural stem/progenitor cells have been previously described. These models are very diverse and include culture conditions allowing physical contact between both types of cells (Mathieu *et al*., 2006; Milner *et al*. 2007; Sosa *et al*., 2007; Roitbak *et al*., 2008; Chintawar *et al*. 2009), avoiding direct contact by using transwell co-cultures (Shen *et al*., 2004; Sosa *et al*., 2007; Weidenfeller *et al*., 2007; Roitbak *et al*., 2008; Plane *et al*., 2010), or even simply growing NSCs in the presence of endothelial cell conditioned-media (Plane *et al*., 2010). Most studies allowing physical contact between stem/progenitor neural stem cells use neurospheres obtained from the embryonic brain grown either on isolated brain endothelial cells (Sosa *et al*., 2007; Chintawar *et al*. 2009) or on endothelial cell lines of various origins such as the brain (B9V and bend), the heart (H5V) or the embryo (E10V) (Mathieu *et al*., 2006; Roitbak *et al*., 2008). Milner and collaborators describe a 3D co-culture in which isolated mouse brain endothelial cells are plated onto neurospheres obtained from the embryonic brain (Milner *et al*. 2007). In these models, it is conceivable that the usage of isolated brain endothelial cells mimics more accurately physiologic conditions than cell lines. Additionally, we must remember that endothelial cells from the brain have their particular characteristics and therefore, the use of cell lines from other sources may result in biased data. A recent study uses co-cultures of SVZ cells deposited on top of endothelial cell lines (Shen *et al*., 2008). Even though similar to our model, in our experiments we use primary mBEC which mimic more accurately the endothelial cells within the neurovascular niche and, to our knowledge, our model has never been described.

In the present work we aimed at studying cell-to-cell interactions between mBEC and SVZ cells and also to reveal the nature of the proteins involved in these physical interactions. To decrease the turnover of membrane proteins in mBEC and, thereby, impair normal cell-to-cell contacts between mBEC and SVZ cells, we used 1 µg/ml of the protein synthesis inhibitor CHX 1 h prior to the co-culture. CHX acts by inhibiting polysome aggregation and peptide chain elongation (Baliga *et al*., 1969)

and because it is not specific, it may affect vital proteins, essential for normal functioning and survival of the cell. Thus, to determine whether the CHX treatment affected mBEC survival, we performed MTT assays and showed that a 1 h-pulse of CHX only slightly decreases mBEC cellular viability. Therefore, we considered this treatment acceptable for usage in our mBEC/SVZ cell co-cultures.

4.4.2. Cell-to-cell contact between mBEC and SVZ cells sustain proliferation and stemness but not neuronal differentiation

The vasculature is a major component of the SVZ stem cell niche. In the SVZ, stem/progenitor cells are located close to blood vessels, frequently contacting them directly (Shen *et al*., 2008; Tavazoie *et al*., 2008). Additionally, NSCs from the SVZ have been described to project processes, which end on blood vessels and form specialized endfeet (Mirzadeh *et al*., 2008). This suggests that *in vivo* contacts with endothelial cells may contribute to SVZ cell properties, either *per se* or by allowing contact with endothelial or ECM-derived factors.

In our co-cultures, levels of BrdU positive SVZ nuclei in the carpet of differentiating cells were significantly lower than the ones found in SVZ cells immediately apposed to mBEC, clearly indicating that contact with mBEC contribute to SVZ cell proliferation. In another model, a recent study has shown that ECs promote proliferation and prevent exhaustion of long-term hematopoietic stem cells (LT-HSCs), contributing to clinical-scale expansion of LT-HSCs (Butler *et al*., 2010). However, a previous study (Shen *et al*., 2004) reported an increase in cell proliferation in a co-culture model that did not involve direct contact between NSC and mBEC. Thus, the differences we observed in cell proliferation could be due to the presence of endothelial cell-derived soluble factors rather than to cell-to-cell contact. Therefore we used CHX to inhibit the turnover of contact proteins in mBEC and showed that after this treatment, proliferation of SVZ cells contiguous to mBEC was reduced to levels similar to the ones in SVZ cells that do not contact mBEC. This

further indicates that heterocellular physical contacts are involved in SVZ cell proliferation.

CHX does not specifically inhibit the synthesis of contact proteins but also the synthesis of endothelial-derived soluble factors. Hence, it is plausible that the decrease in proliferation obtained in SVZ cells contiguous to CHX-treated mBEC was due to a decrease in mBEC-secreted factors rather than due to the impairment of cell contacts. To clarify this point, mBEC treated for 1 h with CHX were co-cultured with SVZ cells in the presence of mBEC-conditioned media (CM) to restore mBEC-derived soluble factors. In fact, endothelial-derived soluble factors do not seem to be involved in the pro-proliferative effects seen in SVZ cells contacting mBEC, as the CHX inhibiting effect in proliferation was still observed. Further reinforcing this idea, when co-cultures were grown in the presence of the anti-Tie-2 neutralizing antibody, which blocks the action of the endothelial-derived soluble factor Ang-1, no differences were seen in the numbers of proliferating cells in the population of cells contacting mBEC. This indicates that endogenous Ang-1 is not involved in the proliferation of this population of cells in basal conditions, despite the fact that Ang-1, when added to cell culture, is capable of inducing SVZ cell proliferation (Rosa *et al.*, 2010).

Heterocellular contacts between mBEC and SVZ cells do not seem to affect SVZ cell survival. Accordingly, no differences were seen in cell death when comparing the carpet of differentiating cells and the population of cells contacting endothelial cells, either in untreated co-cultures or in co-cultures in which mBEC had been treated with CHX. These data are in accordance with Plane *et al.* 2010, in which the presence of endothelial cells does not promote NSC cell survival. Our co-cultures were performed under basal conditions, however it is possible, that after submitting the co-cultures to injury paradigms, endothelial cells might secrete factors that promote SVZ cell survival such as Ang-1 (Valable et al., 2003).

Similarly to cell proliferation studies, we have shown lower levels of SOX-2

positive cells in the pseudomonolayer of differentiation than in cells directly apposed to mBEC. This observation suggests that stem/progenitor cells are in higher numbers within the population of cells in contact with mBEC. Our observation is in accordance with a previous study where direct contact of NPCs with endothelial cells was shown to promote an increase in stem cell numbers (Mathieu *et al*., 2006). In this study, the authors do not use SOX-2 to identify stem/progenitor cell but a functional assay based on the capacity of stem cells to exclude the DNA probe Hoechst 33342 *via* the multidrug transporter ABCG2 of the ATP-binding cassette family. The Hoechst 33342-negative population of cells is referred to as the side population and contains the stem cells.

Shen and collaborators in 2004 showed an increase in stem/progenitor cells in SVZ cultures incubated with endothelial-derived soluble factors. To verify if the observed effects were due to contact proteins, we performed experiments where mBEC had been incubated with CHX. In these experiments, the numbers of stem/progenitor cells in the carpet of differentiating cells did not differ from the values found in cells contacting with mBEC and, thus, heterocellular contacts between mBEC and SVZ cells seem to contribute to the maintenance of the stem state. Again, CHX lack of specificity could raise concerns about the observed effects in cell stemness. To clarify this, endothelial cells were treated for 1 h with CHX and then were used for co-cultures grown in SFM supplemented with mBEC-CM. CHX inhibiting effect in cell stemness was still observed, therefore cell-cell contacts between mBEC and SVZ cells sustain stemness. However, a partial restoration of the number of SOX-2 cells was observed and therefore diffusible factors account at least in part to stemness as well. Further reinforcing this idea, when co-cultures were grown in the presence of the anti-Tie-2 neutralizing antibody, which blocks the action of the endothelial-derived soluble factor Ang-1, no differences were seen in numbers of stem-like cells in the population of cells contacting mBEC. This suggests that Ang-1 does not contribute to the stem state of this population of cells. The Tie-2/Ang-1

signalling pathway plays a critical role in maintaining hematopoietic stem cells in the quiescent state in the bone marrow niche (Suda et al., 2005). However, this role has not been described in the neural stem cell niche.

As far as neuronal differentiation is concerned, in our co-cultures there was no difference in the numbers of DCX+ cell bodies and/or neurites cells contacting mBEC in control co-cultures and in co-cultures where mBEC have been previously treated with CHX. This points to the conclusion that neuronal differentiation does not need heterocellular contacts between mBEC and SVZ cells, at least under basal conditions. Nonetheless, it is possible that when deposited on mBEC, SVZ neurospheres are already partly committed and this may lead to a bias when we evaluate DCX expression. An earlier neuronal marker should then be tested, such as Mash1 (Casarosa *et al*., 1999; Helms *et al*., 2005). In fact, Mathieu and collaborators in 2006 showed that direct contact with endothelial cells stimulated the differentiation of NPC from the mouse telencephalon into astrocytes and into neurons, whereas it reduced differentiation into oligodendrocytes. Sosa and collaborators (2007) showed that direct physical interaction of endothelial cells from the E13.5-14.5 embryonic brain with neuroprogenitors from the E13.5-14.5 mouse cortex induced neuronal differentiation and maturation. Another recent study has shown that brain-derived endothelial cells induce neuronal differentiation from human NPCs from 18-weeks-old to 22-weeks-old fetal brain specimens when in direct physical contact (Chintawar *et al*., 2009). Contrary to these studies, in our work, we could not find a link between contact with mBEC and SVZ neuronal differentiation. However, mBEC and SVZ-derived DCX+ neuroblasts undoubtedly interact. This coupling is particularly interesting and worthwhile to further study, namely the involved molecules in such interaction, since blood vessels serve as migration scaffolds for SVZ neuroblasts *en route* to the OB (Bovetti *et al*., 2007; Whitman *et al.*, 2009; Snapyan *et al*., 2009) and to injured sites in disease models, such as stroke (Yamashita *et al*., 2006; Ohab *et al*., 2006; Thored *et al*., 2007; Kojima *et al.*, 2010).

4.4.3. Cell-to-cell contact between mBEC and SVZ stem cells mediated by α6β1 integrin/laminin is involved in the promotion of proliferation and stemness

Our results concerning integrin expression in SVZ neurospheres demonstrate that Nestin-positive SVZ cells express the α6β1 integrin receptor. This is in accordance with a recent study that shows the presence of both α6 and β1 integrins in SVZ cell cultures (Shen *et al*., 2008). Other studies demonstrate the presence of α6 and β1 integrins in spheres of neural precursors from the P0-P2 rat forebrain (Jacques *et al*., 1998; Campos *et al*., 2004). Recently, the α6β1 integrin receptor has been described in the SVZ *in vivo* as well (Shen *et al*., 2008). Because mBEC express laminin-1, it is conceivable that heterocellular contacts between mBEC and SVZ cells, mediated by endothelial laminin-1 and SVZ α6β1 integrins, occur. In fact, Shen and collaborators (2008) showed that SVZ cells *in vitro* attach to endothelial cell lines and this effect is decreased in the presence of anti-α6 or anti-β1 integrin neutralizing antibodies. SVZ cells and blood vessels of this region also physically interact *in vivo* (Shen *et al*., 2008; Tavazoie *et al*., 2008) and the α6β1 integrin has been associated with this interaction (Shen *et al*., 2008). Transplanted NPCs integrate into the SVZ and associate with the vasculature, a process directed by endothelial-derived stromal derived factor-1 (SDF1). SDF-1 may act through the upregulation of α6β1 integrin (Kokovay *et al.*, 2010).

Evidence points to the fact that α6β1 integrin interaction with endothelial laminin-1 maintains SVZ stem cells in the niche and adhesion to ECM components can ensure exposure to other extracellular cues responsible for SVZ cells characteristics (Kokovay et al., 2010). However, α6β1 integrin and laminin-1 may not simply hold SVZ stem cells in the niche but also play a more active role in controlling SVZ cell properties (Kazanis et al., 2010).

We showed that mBEC treatment with CHX reduces laminin-1, impairing the SVZ α6β1 integrin interaction with endothelial laminin-1. However, to selectively target this interaction, a neutralizing antibody against α6 integrin was used. Accordingly, when our co-cultures were incubated with the antibody, the pro-proliferative effect of the contact with mBEC was prevented, indicating that α6β1 integrin interactions with endothelial laminin-1 are relevant to SVZ-cell proliferation. Recently, *in vivo* ventricular injection of an anti-α6 integrin neutralizing antibody was described to result in progenitor SVZ cells moving away from vessels and, surprisingly, in an increase of proliferation. The authors of this study explain such apparently controversial proliferation with the influence of soluble factors derived from blood vessels, once the stem state control by direct contact with blood vessels is perturbed (Shen *et al*., 2008). Furthermore, laminin has been described to augment the proliferation/survival of human NSC (Hall *et al*., 2008).

It is relevant to notice that we did not find any differences between the numbers of apoptotic cells in the carpets of differentiating cells and the population of cells in contact with mBEC, nor between any of the conditions, suggesting that contacts with mBEC do not promote cell survival and that the anti-α6 integrin neutralizing antibody does not exert toxic effects in co-cultures.

Concerning stemness, we showed here that in the carpet of differentiating cells SVZ stem-like cells were in lower numbers than within the cells directly apposed to mBEC, indicating that contacts between mBEC and SVZ cells promote stemness. When cells were incubated with a neutralizing antibody for α6 integrin, the effect of the contact with mBEC, towards stemness, was lost, indicating that the α6β1 integrin interaction with endothelial laminin-1 is relevant to maintain the SVZ-cell stem state. Laminins are expressed very early in development and have been shown to maintain human ES cells in an undifferentiated state (Xu *et al*., 2001). Proximity to a basement membrane is a feature of germ cell niches, and transplantation experiments using

reconstitution of spermatogenesis reveal that stem cells can be selectively isolated from other testis cell populations following selection for expression of α6 or β1 integrin subunits or for laminin binding (Shinohara *et al*., 1999). In fact, a comparison of three independently derived lists of "stemness" genes showed only one common gene: integrin α6 (Fortunel *et al*., 2003).

We showed that in our co-cultures there was no difference in neuronal differentiation in cells contacting mBEC in control co-cultures and in co-cultures where mBEC have been previously treated with CHX. When cells were incubated with a neutralizing antibody for α6 integrin, no differences were found either, indicating that α6β1 integrin interaction with endothelial laminin-1 does not contribute to neuronal differentiation. This may be due to the fact that when deposited on mBEC, SVZ precursors may be already partly committed and, therefore, as mentioned above, an earlier marker such as Mash1 may be more adequate than DCX for neuronal differentiation studies. In fact, laminin has been shown to induce neuronal differentiation and neurite outgrowth from human embryonic stem cell lines and cell expansion was prevented by using neutralizing antibodies to α6 or β1 integrins (Ma *et al*., 2008). Integrin α6β1 and FAK-MEK/ERK signalling pathways also appear to be involved in laminin-1-induced neurite outgrowth in mesenchymal stem cells (Mruthyunjaya *et al*., 2009).

In vivo, in the RMS, migrating neuroblasts have been demonstrated to stain positive for α6β1 integrin and blockage of any of these integrins with local injection of adequate neutralizing antibodies results in the re-routing of these neuroblasts from their usual pathway, without observed toxic effects. Similarly, injection of a peptide containing the sequence of the binding site of laminin to this receptor, results in the migration of neuroblasts to the site of injection (Emsley and Hagg 2003). In neurospheres from the P0-P2 rat forebrain, migration of cells is also diminished in the presence of α6β1 integrin neutralizing antibodies. Conversely, the presence of a

peptide that binds α6β1 integrin stimulates migration (Jacques *et al*., 1998).

Studying heterocellular contacts mediated by SVZ α6β1 integrin and endothelial laminin-1 may be relevant for cancer therapy, since CSCs may derive from deregulated SVZ cells. In fact, glioblastoma stem cells express the laminin receptor integrin α6β1 and this receptor plays a role in self-renewal, proliferation and tumour formation (Lathia *et al*., 2010). Moreover, expression of α6β1 integrin enhances tumourigenesis in U87, a glioma cell line (Delamarre *et al*., 2009).

4.4.4. Cell-to-cell contact between mBEC and SVZ stem cells mediated by gap junctions is involved in the promotion of proliferation and stemness

When studying GJs between mBEC and SVZ cells, we chose to focus our study in Cx43 because in developing and mature astrocytes, Cx43 is the predominant GJ protein (Dermietzel *et al*., 1991; Giaume *et al*., 1991). During postnatal development Cx43 expression is primarily restricted to radial glial cells (Yamamoto *et al*., 1992), which give rise to both astrocytes and neurons (Anthony *et al*., 2004; Casper and McCarthy, 2006; Malatesta *et al*., 2003). In the SVZ, Cx43 GJs are involved in cell migration (Marins *et al*., 2009).

It has been described that stem/progenitor cells can form heterocellular contacts with endothelial cells through Cx43 (Villar *et al*., 2002). Results concerning GJ proteins expression in SVZ neurospheres demonstrate that Nestin-positive SVZ cells express Cx43. Cx43 is also present in hippocampal progenitor cells obtained from (P0–P2) mouse pups (Imbeault *et al*., 2009), in E14.5-15 embryonic mouse neural progenitor cells isolated from the mouse striatal germinal zone (Duval *et al*., 2002) and human neural stem cells obtained from the telencephalic ventricular zone (VZ) of 13 or 11-week human fetal cadavers (Jaderstad *et al*., 2010). In the SVZ *in vivo*, Cx43 has been described in GFAP positive cells, which establish Cx43 GJs (Liu *et al*., 2006).

Because mBEC also express Cx43, it is conceivable that heterocellular contacts between SVZ cells and mBEC occur. In fact, we have observed clusters of Cx43 staining in regions where endothelial cells and SVZ cells contact, both *in vitro* and *in vivo*. We have also performed preliminary LAMP assays, which allowed us to functionally visualize the passage of a UV-uncaged probe from a donor cell to a recipient cell, through GJs. However, not all mBEC/SVZ cells established GJs. Further experiments will be conducted in order to further dissect the specificity of the coupling using, for instance, carbenoxolone, and co-cultures performed with mBEC or SVZ cells knocked down for Cx43 by using specific si-RNAs.

GJIC has been shown to regulate homeostasis and differentiation of stem/progenitor cells of the bone (Villars *et al.*, 2002; Guillotin *et al*., 2008) as well as attachment of cancer cells to endothelial cells (Elzarrad *et al*., 2008). Published data, therefore, clearly indicate that GJIC plays a role in regulating stem/progenitor cells as well.

We showed that mBEC treatment with CHX reduces Cx43 protein levels in mBEC and thereby it should impair Cx43 GJs between mBEC and SVZ cells. However, to impair GJIC between mBEC and SVZ cells we used the general inhibitor of GJIC, CBX.

When co-cultures were incubated with CBX, the pro-proliferative effect of the contact with the mBEC was prevented, indicating that GJs between endothelial and SVZ cells are relevant to SVZ-cell proliferation. Accordingly, gap junctional communication through Cx43 has been described to maintain mouse cortical neural progenitor cells, obtained from E12 mice cortices, in a proliferative state, also promoting their survival (Cheng *et al*., 2004).

It is relevant to notice that we did not find any differences between the numbers of apoptotic cells in the carpets of differentiating cells and the population of cells in contact with mBEC, nor between any of the conditions, proving that contacts with mBEC do not promote cell survival and that CBX does not exert toxic effects in

co-cultures.

When GJIC were inhibited, the effect of the contact with mBEC, towards stemness, was prevented, indicating that GJs between mBEC and SVZ cells are important to maintain SVZ-cell stem state. Literature indicates that Cx43 plays a role in stemness: 6 of the 11 available human ES cell lines express it (Bhattacharya *et al*., 2004), as well as mouse ES cells (Parekkadan *et al*. 2008) and human neural crest cells (Thomas *et al*., 2008). Cx43 may promote stemness by maintaining stem/progenitor cells in the niche (Wagner *et al*., 2007).

No difference was found in neuronal differentiation in cells contacting mBEC in control co-cultures and in co-cultures where mBEC have been previously treated with CHX. When cells were incubated with a CBX, no differences were found either, indicating that gap junctions do not contribute to neuronal differentiation in our model. However, published data associate GJs with neuronal differentiation: treatment of NT2/D1 or P19 cells with GJ blockers, reduces the numbers of generated microtubule-associated protein 2 (MAP2) positive neurons, increasing expression of immaturity markers such as Nestin and Vimentin (Bani-Yaghoub *et al*., 1999 a and b). Moreover, neural crest-derived PC12 cells do not express appreciable levels of endogenous connexins, but when Cx43 is overexpressed during nerve growth factor-induced neural differentiation, neurite length is increased about twofold (Belliveau *et al*., 2006).

Recent evidence point to the fact that Cx43 is also important in neuronal migration: it is expressed at the contact points between radial fibers, the neuronal stem cells of the embryonic cerebral cortex, and migrating neurons. Acute downregulation of Cx43 impairs the migration of neurons to the cortical plate. More interesting is that GJs do not mediate neuronal migration by providing a channel for cell-cell communication but, instead, provide dynamic adhesive contacts (Elias *et al*., 2007). Furthermore, during mitotic division in the telencephalic proliferative ventricular zone (VZ), the nuclei of the neural precursors move basally away from the

ventricular surface for DNA synthesis, and return apically to the surface for mitotic division. This process is known as interkinetic migration during apical nuclear migration. VZ precursors display dynamic spontaneous Ca^{2+} transients, which depend on functional GJs/hemichannels *via* ATP release and Ca^{2+}-mobilizing messenger diffusion. Pharmacological blocking of GJs/hemichannels or short hairpin RNA-mediated knockdown of Cx43 retards the apically-directed interkinetic nuclear migration and promotes changes in nuclear morphology (Liu *et al*., 2010). Interestingly, GJs also seem to play a role in exogenous NSCs integration and protection or rescue of endogenous neurons at risk for degeneration. Mechanisms underlying these events have been only partially characterized, and although diffusible factors have typically been implicated, direct contact of NSCs with host cells is an important feature: in organotypic cultures, connexin-associated GJ formation and function is pivotal for ensuring host cell well-being. *In vivo*, NSC-mediated rescue of imperilled host neurons does not occur when GJ formation is suppressed (Jäderstad *et al*., 2010).

Interestingly, Cx43 may play a role in the process of metastasis formation and tumour invasion. In fact, several tumour cells have also been demonstrated to express Cx43 (Husøy *et al*., 2005, Tate *et al*., 2006, Udaka *et al*., 2007) and tumour cells interact with endothelial cells (Ito *et al*., 2000; Husøy *et al*., 2005; Tate *et al*., 2006; Elzarrad *et al*., 2008). Cx43 enhances the adhesion to astrocytes and mediates the invasion of malignant glioma cells (Lin *et al*., 2002). It also facilitates metastatic 'homing' by increasing adhesion of cancer cells to lung endothelial cells (Elzarrad *et al*., 2008).

4.4.5. Conclusion

We have shown in this work that the interaction between stem/progenitor cells α6β1 integrin and endothelial laminin-1 as well as GJIC between mBEC and SVZ cells contribute to the maintenance of stem/progenitor cells characteristics. In

the present chapter we show that interaction between mBEC and SVZ stem/progenitor cells, through SVZ α6β1 integrin and endothelial laminin-1, as well as heterocellular GJs, contribute to SVZ cell proliferation and stemness maintenance, while apoptotic cell death and neuronal differentiation remain unaffected, despite the fact that generated neuroblasts frequently contact endothelial cells. The study of these cell-to-cell contacts contributes to further understand the SVZ neurogenic niche and how SVZ cell characteristics are maintained. This knowledge is particular relevant in the cell therapy context, allowing the development of new tools for cell replacement, not only for the generation of desired cells, destroyed or damaged by pathologic conditions, but also allowing correct integration of newly-generated cells. In addition, targeting integrin α6β1 and Cx43 in tumours may prove to be advantageous, reducing tumourigenesis and contributing to patient overall survival.

Chapter 5 – Microvasculature in SVZ cell proliferation in the non-injured and epileptic brain

5.1. Abstract

In the adult mammalian brain, restricted neurogenic areas, including the SVZ of the lateral ventricle, host stem/progenitor cells and constantly give rise to new neurons. Neurogenic activity in the SVZ, as well as stem cell homeostasis, crucially depends on interaction between stem cells and vessels. Indeed, neuronal progenitors are closely associated with blood capillaries in the SVZ. We hypothesized that neurogenic areas may display a particular microvascular density, as compared to non-neurogenic areas, that supports stem cell activity. Moreover, upon brain injury, such as epilepsy, cell proliferation has been shown to increase in the SVZ and these newly-generated progenitors migrate towards lesion sites. According to the importance of heterocellular contacts between endothelial cells and cells from stem cell niches in the regulation of stem cells capacities, increase in proliferation may be correlated with changes in microvasculature.

To clarify these points, we compared the microvascular density in the neurogenic SVZ *versus* non-neurogenic areas (striatum and parietal cortex) in the intact and in kainic acid (KA)-induced epileptic brain of adult mice. Under basal conditions, we found no differences in blood vessel density between the SVZ and non-neurogenic areas. Upon epileptic seizures, microvascular density increased in all areas but much more prominently in the SVZ. Moreover, we found an increase in cell proliferation in the SVZ of KA-treated mice comparing to control animals, as evaluated by counting of BrdU+ cells at 7 days. We analysed the distribution of these BrdU + cells as compared to CD31 + vessels and found proliferating cells associated with the abluminal surface of endothelial cells, or at a short distance (< 50 µm) of blood vessels both in basal conditions and in KA-treated mice. In epileptic mice, the proportion of BrdU+ cells directly associated with vessels or at a short distance increased. This result is in accordance with literature reporting induction of proliferation following seizures. Our results suggest that proliferation of SVZ cells occurs close to vessels and that changes in microvasculature consequent to injury

may have a positive impact on SVZ proliferation. However, further studies are necessary to disclose the contribution of microvasculature in injury-induced neurogenesis.

Understanding the interplay between vasculature and neurogenesis in the intact and damaged brain may offer new perspectives for the use of stem cells in brain repair, by promoting neuronal repopulation in the injured brain.

5.2. Introduction

In the adult mammalian brain, neurogenesis occurs only in restricted areas, such as the SVZ of the lateral ventricle (Alvarez-Buylla and García-Verdugo, 2002). SVZ progenitors proliferate and generate immature neurons or neuroblasts that migrate long distances towards the OB (Luskin, 1993; Lois and Alvarez-Buylla, 1994; Lois *et al*., 1996) through the RMS using a peculiar form of tangential migration in chains (Kishi, 1987; Lois and Alvarez-Buylla, 1994; Doetsch and Alvarez-Buylla, 1996; Lois *et al*., 1996; Wichterle *et al*., 1997). Once in the OB, these precursors differentiate into interneurons (Lois and Alvarez-Buylla, 1994; Kornack and Rakic, 2001) and integrate in the existing circuitry, contributing to odour discrimination (Gheusi *et al*., 2000; Carleton *et al*., 2003). Several studies suggest that human SVZ cells also have neurogenic potential (Pincus *et al*., 1998; Roy *et al*., 2000; Sanai *et al*., 2004).

Following brain damage, such as ischemia and epileptic seizures, neurogenesis is increased in the SVZ, and neuronal precursors were observed to migrate outside the RMS towards sites of injury (Arvidsson *et al*., 2002; Parent, 2002; Parent *et al*., 2002a and b; Park *et al*., 2002; Imitola *et al*., 2004; Park *et al*., 2006; Li *et al.*, 2010). These observations suggest that a program of brain repair is settled down following injury by recruitment of the endogenous pool of stem cells.

Several studies point out a possible relationship between neurogenesis and angiogenesis, the generation of new blood vessels from pre-existing ones. For

instance, in the adult songbird, upon testosterone stimulation, proliferation of vascular endothelial cells is simultaneously observed with the increase in neurogenesis in the ventricular zone of the high vocal centre, which is involved in song elaboration (Louissaint *et al*., 2002). Moreover, angiogenesis and neurogenesis in the SVZ are regulated by common factors such as FGF-2 (Gritti *et al*., 1995; Kuhn *et al*., 1997), VEGF (Jin *et al*., 2002b) erythropoietin (EPO) (Shingo *et al*., 2001), Ang-1 (Bai *et al*., 2009a; Rosa *et al*., 2010), Ang-2 (Liu *et al*., 2009) and PEDF (Ramírez-Castillejo *et al*., 2006; Pumiglia and Temple, 2006), Upon cerebral injury, such as ischemia and epilepsy, angiogenesis is concomitantly increased with neurogenesis (Hellsten *et al*., 2004; Gotts and Chesselet, 2005), and both neurogenic and angiogenic factors - VEGF, EPO and FGF-2 - are overexpressed (Yoshimura *et al*., 2003; Wang *et al*., 2004; Gotts and Chesselet, 2005). In addition, endothelial cells secrete factors, such as Ang-1, that direct the migration of neuronal precursors towards lesion sites (Ohab *et al*., 2006). Not only do the processes of angiogenesis and neurogenesis seem to be linked, but also microvasculature itself appears to play a role in SVZ stem/progenitor cell dynamics. Indeed, the proximity between capillaries and stem cells seems to be a pre-requisite for neurogenesis in the SVZ, where progenitor/stem cells are closely apposed to blood vessels, which at some sites lack pericyte and astrocyte endfeet coverage (Tavazoie *et al*., 2008). This may allow not only the diffusion of endothelial- and blood-derived soluble factors but also cell-cell contacts between endothelial and stem-like cells, such as the ones mediated by endothelial cell laminin-1 and SVZ cell $\alpha 6\beta 1$ integrins (Shen *et al*., 2008). Furthermore, blood vessels serve as migration scaffolds, both in the normal brain (Whitman *et al*., 2009) and in disease paradigms (Kojima *et al*., 2010).

The present chapter aims at shedding light on impact of microvasculature in SVZ cell proliferation in the non-injured and epileptic brain. For this, we determined brain microvascular density and searched for signs of angiogenesis in control and KA-treated epileptic mice. We then tried to correlate SVZ cell proliferation with

changes in microvasculature upon injury and determined numbers of proliferating cells according to their distance to blood vessels both in control and KA-treated mice.

5.3. Results

5.3.1. Characterization of microvascular density in neurogenic *versus* non-neurogenic areas of the mouse brain

Brain microvessels were stained by revelation of the endogenous alkaline phosphatase activity displayed by endothelial cells. Digital images in Figure 5.1. depict alkaline phosphatase staining in the SVZ (Figure 5.1 A) and in non-neurogenic areas such as the striatum (Figure 5.1 B) and the cortex (Figure 5.1 C). As depicted in Figure 5.1 D, no differences were found in microvascular density, expressed as a percentage of the total analysed area, in the different brain regions (SVZ: 2.47 ± 0.10%, n=6 animals, 30 brain slices analysed; striatum: 2.10 ± 0.16%, n=6 animals, 30 brain slices analysed; cortex: 2.19 ± 0,16%, n=6 animals, 30 brain slices analysed).

5.3.2. Characterization of microvascular density in neurogenic *versus* non-neurogenic areas after kainic acid (KA)-induced epilepsy

Microvascular density was measured in epileptic animals. Epilepsy was triggered by ip injection of 30 mg/kg KA and alkaline phosphatase activity was revealed in the vessels 7 d after seizure induction. Control mice consist in animals injected with saline solution instead of KA. As seen in Figure 5.2 A-C, there is an increase in microvascular density, expressed as a percentage of the total analysed area, in the SVZ of KA-treated mice comparing to the SVZ of control mice. This percentage increases from 2.47 ± 0.10% (n=6 animals, 30 brain slices analysed), in controls, to 5.19 ± 0.22% (n=6 animals, 31 brain slices analysed), in KA-treated animals. An increase in microvascular density is also observed in non-neurogenic areas although the effect is not as pronounced as in the SVZ. Indeed, in the striatum,

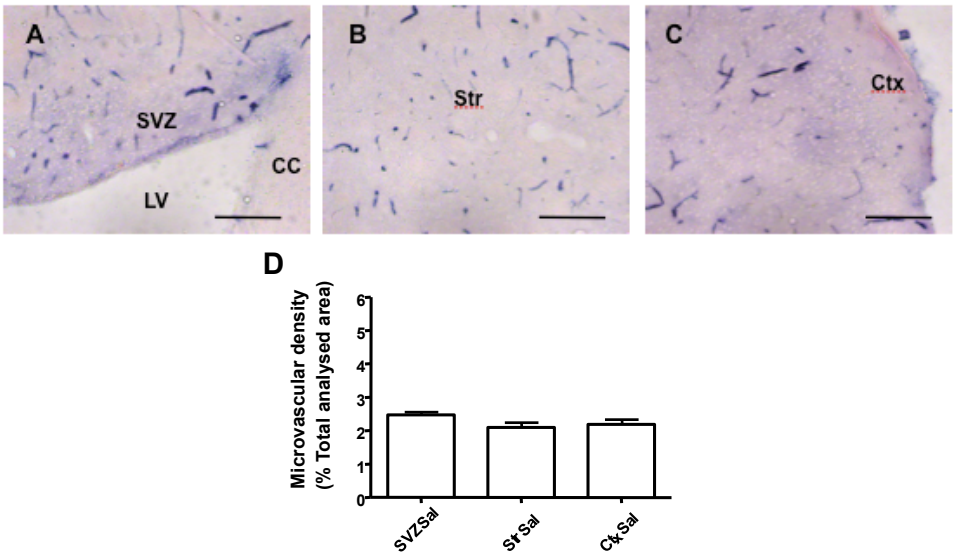

Figure 5.1. Characterization of microvascular density in the SVZ, striatum and cortex. ***A-C,*** Digital images depict the detection of brain blood vessels using alkaline phosphatase activity in the subventricular zone (SVZ, ***A***), striatum (Str, ***B***) and cortex (Ctx, ***C***). ***D,*** Bar graph shows the microvascular density of analysed brain regions, expressed as a percentage (%) of the total analysed area, in saline solution (Sal)-injected mice. Scale bars, 200 μm. LV, lateral ventricle; CC, *corpus callosum*.

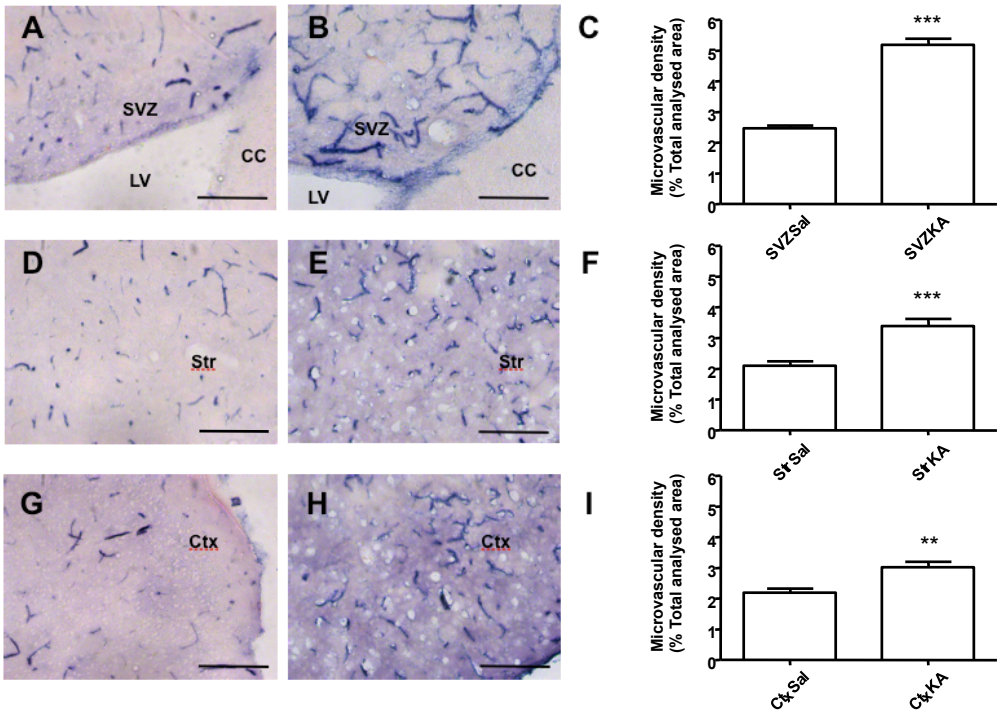

Figure 5.2. Characterization of microvascular density in the SVZ, striatum and cortex of the normal and kainic acid (KA)-treated mouse brain, 7 d after seizure induction. Digital images ***A*** and ***B*** depict the detection of brain blood vessels using alkaline phosphatase activity in the subventricular zone (SVZ) of saline solution (Sal)- and KA-injected mice, respectively. There is an increase in microvascular density, expressed as a percentage (%) of the total analysed area, in the KA-treated mice (***C***). Digital images ***D*** and ***E*** depict the vasculature network in the striatum (Str) of Sal- and KA-injected mice, respectively. There is an increase in the microvascular density, expressed as a % of the total analysed area in the KA-treated mice (***F***). Digital images ***G*** and ***H*** depict the vasculature network in the cortex (Ctx) of Sal- and KA-injected mice, respectively. There is an increase in the microvascular density, expressed as a % of the total analysed area in the KA-treated mice (***I***). Scale bars, 200 μm. LV, lateral ventricle; CC, *corpus callosum*. Data are expressed as mean ± SEM. ***$p < 0.001$, **$p < 0.01$, using the unpaired Student *t* test for comparison with controls.

microvascular density, expressed as a percentage of the total analysed area, increases from 2.10 ± 0.16% (n=6 animals, 30 brain slices analysed), in the control, to 3.39 ± 0.24%, (n=6 animals, 31 brain slices analysed), in KA-treated animals (Figure 5.2 D-F). In the cortex, microvascular density, expressed as a percentage of the total analysed area, increases from 2.19 ± 0.16%, (n=6 animals, 30 brain slices analysed), in the control, to 3.02 ± 0.20 (n=6 animals, 31 brain slices analysed), in KA-treated animals (Figure 5.2 G-I). Therefore, microvascular density in the SVZ of KA-treated mice increases 1.79 and 2.90 times more than in the striatum and cortex of KA-treated mice, respectively.

Results show that upon brain injury, such as KA-induced epileptic seizure, microvascular density is increased in the neurogenic SVZ as well as in the non-neurogenic cortex and striatum. Despite not determined here, the increase in the microvascular network could be due to angiogenesis and/or vascular remodelling. The increase in microvascular density following injury is more prominent in the neurogenic SVZ as compared to the non-neurogenic areas. According to the reported importance of heterocellular contacts between endothelial cells and cells from stem cell niches in the regulation of stem cells capacities, changes in the microvasculature may have an impact on stem cells' homeostasis and neurogenesis.

5.3.3. Correlation between increase in microvascular density and proliferation in the SVZ of KA-treated mice

We aimed at investigating whether there is a correlation between the increase in microvascular density and changes in SVZ cells dynamics. Reports have shown that proliferation in the rat SVZ increases following epileptic seizure induction (Parent *et al*., 2002a, Parent *et al*., 2006). In our model, we verified that KA treatment increased proliferation in the SVZ. Animals were treated with KA (or saline solution, in control animals), injected daily with BrdU and sacrificed 7 days after KA (or saline) injection as detailed in the "Material and Methods", section 2.1.3. Numbers of BrdU

positive cells were determined in the SVZ. In control animals, 511.60 ± 133.80 BrdU positive nuclei were obtained (n= 5 animals, 15 brain slices analysed). In KA-treated mice this number is almost twice as high, as 1008.00 ± 66.55 BrdU positive cells were counted (n= 4 animals, 12 brain slices analysed, Figure 5.3 A-D). Moreover, in some KA-treated mice, alteration of the structure of the ventricle was observed, indeed, ventricle walls were connected across the ventricle space by new blood vessels that abnormally grew in the ventricle lumen, as seen in Figure 5.3 C.

KA-induced injury increases both microvascular density and SVZ cell proliferation. Consequently, it is easy to speculate that there could be a correlation between the two phenomena. We determined the distribution of SVZ cells that incorporated BrdU according to the distance to the CD31+ micro-capillaries in KA-treated and saline-injected (control) mice. In control mice, 19.66 ± 5.77 % of the BrdU positive cells are closely apposed to vessels (n= 5 animals, 15 brain slices analysed) while, in treated animals (n= 4 animals, 12 brain slices analysed), this number increases to 45.30 ± 12.48%. There is also a tendency to increase within the population of BrdU cells located between 0-10 µm (28.50 ± 8.94% to 35.57 ± 7.40%) and 10-50 µm (49.73 ± 11.18% to 70.91± 20.90%) from blood vessels.

This points to the fact that SE induces the proliferation of SVZ cells and that this proliferation mainly occurs close to the blood vessels. It is important to notice that proliferating endothelial cells were not observed in control conditions and were only scarcely detected in treated animals (2.39± 0.54 %). Although other experiments are required to conclude undoubtedly, this result suggests that increase in vascular density observed in KA-treated mice mainly occurs through vascular remodelling instead of angiogenesis. Other possibility is that angiogenesis may occur shortly after injury and no longer be active 7 d after KA SE.

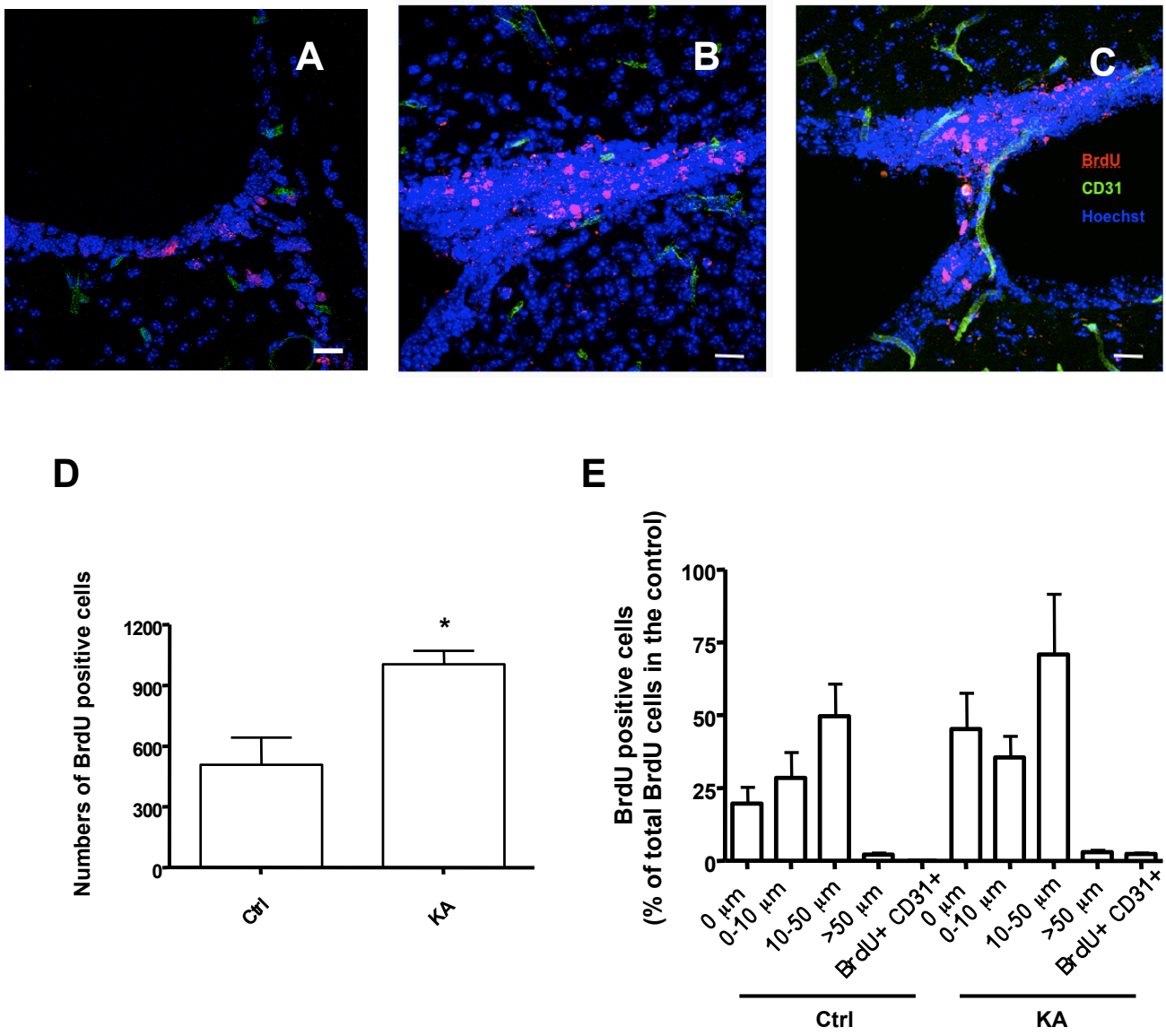

Figure 5.3. Correlation between increase in SVZ cell proliferation and vascular density in saline and epileptic mice 7 days after KA-injection. ***A***-***B***, Representative fluorescent confocal digital images of CD31 (green) positive blood vessels, BrdU (red nuclei) and Hoechst 33342 staining (blue nuclei) in the SVZ of a control (***A***) and a KA-injected mouse (***B*** and ***C***). Scale bars, 20 μm. ***D***. Absolute values of counted BrdU nuclei in the SVZ of control and KA-treated mice. Data are expressed as mean ± SEM. *$p < 0.05$, using unpaired Student *t* test for comparison with control. ***E***. Distribution of BrdU positive nuclei according to the distance from blood vessels, expressed as a percentage (%) of total BrdU cells in the control. Data are expressed as mean ± SEM.

5.3.4. Endostatin does not have an effect in SVZ dynamics *in vitro*

To determine if the anti-angiogenic peptide endostatin was a good candidate to block angiogenesis in the SVZ *in vivo,* and evaluate the impact of this process on SVZ dynamics, we had to discard endostatin's possible effects on SVZ cells *per se*. Thus, SVZ cells were incubated with 1 μg/ml endostatin and tested for cell death (Figure 5.4 A), proliferation (Figure 5.4 B) and neuronal differentiation (Figure 5.4 C). According to our observations, there are no differences in cell death between control and endostatin-treated cells (control: 18.57% ± 1.530% Tunel positive nuclei, n=10 coverslips, 5112 cells counted; endostatin: 22.33% ± 3.035% Tunel positive nuclei, n=8 coverslips, 5064 cells counted). Endostatin treatment does not alter cell proliferation (control: 8.132% ± 0.5160% BrdU positive nuclei, n=15 coverslips, 12912 cells counted; endostatin: 6.803% ± 0.3857% BrdU positive nuclei, n=8 coverslips, 7366 cells counted) nor neuronal differentiation (control: 11.64% ± 1.246% NeuN positive nuclei, n=12 coverslips, 9566 cells counted; endostatin: 11.75% ± 1.027% NeuN positive nuclei, n=4 coverslips, 2883 cells counted). Because endostatin does not affect neurogenesis *per se in vitro,* we can point this molecule as a potential candidate to block angiogenesis *in vivo* and study the effect of this process in SVZ dynamics.

5.4. Discussion

In the present chapter, we studied the impact of microvasculature in SVZ cell proliferation in the non-injured and epileptic brain. Because neurogenic activity is restricted in the brain to particular areas, such as the SVZ, and since interactions with endothelial cells are critical to support stem cell functions, we hypothesized that the vascular network would be particular within SVZ. In fact, while generally blood-brain barrier (BBB) occurs in capillaries of the brain and it is dependent on interactions between astrocytic endfeet, vascular smooth muscle cells, pericytes and endothelial cells, controlling the flux substances from the blood to the brain, (Rubin *et*

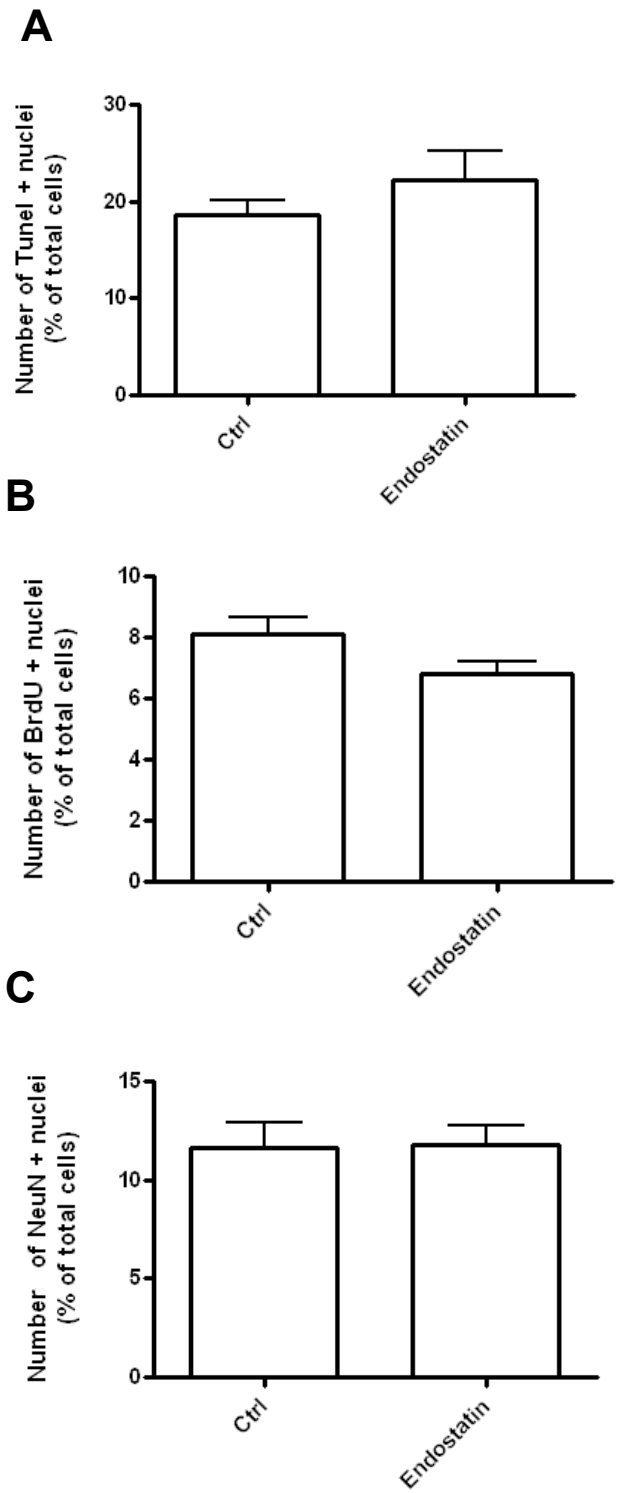

Figure 5.4. Endostatin 1μg/ml does not affect SVZ dynamics *in vitro*. Bar graphs depict that endostatin 1μg/ml does not induce cell death, indicated by Tunel staining (***A***), cell proliferation, indicated by BrdU staining (***B***) or neuronal differentiation, indicated by NeuN staining (***C***) in SVZ cell cultures.

al., 1999), in the SVZ, portions of the vasculature lack coverage both by astrocyte endfeet and pericytes. Stem cells and transit-amplifying cells directly contact the vasculature at these sites of altered BBB, allowing cell-to-cell contacts and contact with endothelial- and blood-derived molecules (Tavazoie *et al*., 2008). Moreover, unlike the adult hippocampus, where active angiogenesis accompanies neurogenesis (Palmer *et al*., 2000), the adult SVZ seems to possess a relatively stable vascular bed without angiogenic sprouting of endothelial cells or endothelial cell division in the SVZ (Tavazoie *et al*., 2008). Endothelial cell division was not found either in the present work, in the SVZ of control animals. Unexpectedly, we found that the vasculature density did not differ between the neurogenic SVZ and the non-neurogenic cortex and striatum. Nevertheless, upon SE, blood vessel density was incremented in all tested areas, particularly in the SVZ. Indeed, microvascular density in the SVZ of KA-treated mice increases 1.79 and 2.90 times more than in the striatum and cortex of KA-treated mice, respectively. In the SVZ, even though some BrdU/CD31 double-positive cells were observed in KA-treated mice, they were very rare. However, we did see an increase in vascular density. Two scenarios are capable of explaining this: the increase in vascular density is in fact due to endothelial cell proliferation but this occurs only very shortly after damage; or the increase of blood vessel density is due to vascular remodelling with increased permeability. To clarify this, BrdU+/CD31+ cell numbers should be evaluated at a shorter time point after KA-induced seizures (for instance, 24 h). Should no increase in BrdU+/CD31+ cells be observed at this shorter time point, one could conclude that increase in vascular density is achieved through vascular remodelling. In fact, it has been described that after SE blood vessel permeability is increased in the hippocampus (Rigau *et al*., 2007; Ndode-Ekane *et al*., 2010). If an increase in BrdU+/CD31+ cells was observed at this shorter time point, it could be concluded that increase in blood vessel density is due to angiogenesis. Studies in other paradigms show that angiogenesis occurs shortly after injury and, therefore, the 7-

day time point may not be adequate: after traumatic brain injury, the angiogenic factors VEGF and VEGF receptor 2 (VEGFR2) increase as well as capillary density and BrdU incorporation confirms the presence of newly-formed vessels up to 48 h post-injury (Morgan *et al*., 2007); In a study using pilocarpine-induced SE in rats, numbers of proliferating endothelial cells in the hippocampus peaked at 4 d post-SE and correlated with an increase in vessel length, parameter that recovered to control levels at 2 weeks post-SE (Ndode-Ekane *et al*., 2010). If angiogenesis, in fact, occurs in the SVZ after epileptic seizures, and because we found that the anti-angiogenic peptide endostatin does not alter SVZ cell characteristics *in vitro*, we hypothesize that it is a potential candidate to block angiogenesis *in vivo* by stereotaxic injection in the SVZ and, therefore, to study the effect of this process in SVZ dynamics. The option of stereotaxic injection relies in the fact that endostatin has a short half-life and does not cross the BBB (Bjerkvig *et al*., 2003).

In control and KA-treated animals BrdU positive cells in the SVZ were found in close association with blood vessels. Distances between proliferating cells and blood vessels in basal conditions are in accordance with distribution determined by Shen and collaborators (2008). In control animals, after BrdU injection for 7 days, we observed proliferating cells in the SVZ mainly at distances ranging from 10 to 50 μm from blood vessels. To verify whether proliferation firstly occurs close to blood vessels and whether cells migrate out of the vessels surface, BrdU immunohistochemistry studies should have been performed at an earlier time point (for instance at 24 h after an acute BrdU injection). If, in this case, more BrdU cells would be closer to blood vessels, we would conclude that in fact proliferation starts close to blood vessels, followed by subsequent cell migration. On the other hand, later time points would result in BrdU positive nuclei further away from blood vessels. This point will be investigated.

It is known that most brain insults increase SVZ proliferation. There was an increase of cell proliferation in the SVZ *in vivo*, 7 days after SE induction, comparing

to control animals, which is in accordance with previous studies in other models of epilepsy (Parent, 2002; Parent *et al*., 2002a). According to the importance of vasculature in stem cells functions, we hypothesized that a correlation between vasculature changes upon injury and the increase of proliferation may exist. In fact, when comparing control and KA-treated mice percentages of BrdU cells in contact with blood vessels, there was clearly an increase in BrdU positive nuclei in the later group, even though not significant, probably due to low sampling of the present study. This suggests that seizures induce cell proliferation close to blood vessels. The same happened with the percentages of BrdU cells located between 0 and 10 μm and 10 and 50 μm from blood vessels: KA-treated animals tend to possess more proliferating cells than control animals. One could argue that these proliferating cells are generated following seizure close to blood vessels and then migrate out of blood vessel surface. To verify this hypothesis, a shorter time point should be investigated.

Even though very preliminary, these data are indeed encouraging and, thus, an earlier time point should be studied to disclose: 1) Whether angiogenesis occurs and is responsible for the increase of blood vessel density after KA-induced SE or if, on the contrary, microvascular changes are due to vascular remodelling with an increase in permeability; 2) Whether in control animals and upon epileptic seizures, proliferation occurs close to blood vessels and later on, cells migrate away from blood vessels. Finally, it would be interesting to identify the particular phenotype of proliferating cells contacting blood vessels, as previously reported for the normal brain (Shen *et al*., 2008; Tavazoie *et al*., 2008). This would allow us to verify whether after epileptic seizure, cells closely associated with blood vessels are new stem-like cells enlarging the endogenous pool, or if these cells are newly-generated neuroblasts, intended for repair, already on a journey to brain damaged sites, using blood vessels as migrating scaffolds, as described in other injury paradigms (Ohab *et al*., 2006; Kojima *et al*., 2010).

Chapter 6 – General discussion and conclusions

6.1. General discussion

Three main pillars can be found in the core of the present thesis: 1) the role of the angiogenic factor Ang-1 in the SVZ neurogenesis *in vitro*; 2) the contribution, and nature, of cell-to-cell contacts between mBEC and SVZ stem/progenitor cells to SVZ cell proliferation and stemness, *in vitro*; 3) the role of microvasculature in SVZ cell proliferation in the non-injured and epileptic brain.

6.1.1. The role of the angiogenic factor Ang-1 in the subventricular zone neurogenesis *in vitro*

A lot of efforts have been devoted to the study of the so-called neurovascular niche where neural stem cells interact intimately with blood vessels (Shen *et al*., 2008; Tavazoie *et al*., 2008). This allows, for instance, diffusion of soluble factors, from endothelial cells or from the blood, contributing to stem cell properties. One of such factors is probably the angiogenic factor, Ang-1.

In the present work we showed that Nestin-positive SVZ cells express Ang-1 and its receptor Tie-2, suggesting a possible autocrine/paracrine regulation of the SVZ cell dynamics *via* Ang-1. Incubation of SVZ cells with 500 ng/ml Ang-1 did not affect SVZ cell death or survival but induced proliferation, an effect mediated *via* Tie-2 and ERK1/2 kinase activation. Activation of the PI3K/AKT kinases mediates the Ang-1-induced proliferation in endothelial cells (Kanda *et al*., 2005; Abdel-Malak *et al*., 2008). However, in SVZ cultures, ERK1/2 activation seems to prevail on PI3K/AKT pathway as total inhibition of Ang-1-induced proliferation was obtained in the presence of a MEK inhibitor. Exogenous administration of Ang-1 increased the numbers of neurons through the activation of Tie-2. Generated neurons were functional, as demonstrated by SCCI. Rapamycin-mediated inhibition of mTOR prevented neuronal differentiation induced by Ang-1. Several studies have implicated the ERK/MAP pathway in neuronal differentiation (Yang *et al*., 2008; Moon *et al*., 2009; Washio *et al*., 2009). In the present study, the involvement of ERK/MAP

kinases in neuronal differentiation was not assessed since mTOR inhibition completely blocked Ang-1/Tie-2-mediated neuronal differentiation, suggesting that ERK/MAP kinases may not be critical in our model. To assess whether Ang-1 triggers neurite outgrowth, we measured the numbers and length of neurites immunoreactive for P-SAPK/JNK in 6 h-treated cultures and these increased in Ang-1-treated cultures. Moreover, we also showed that Tie-2 expression occurs in neuronal progenitors and neurons along the SVZ-rostral migratory stream-OB system, *in vivo*.

The present observations indicate that Ang-1's local secretion may partly account for the basal neurogenic activity in the SVZ *in vivo* and identify this peptide as a component of the neurogenic niche. The pro-neurogenic effect of Ang-1 opens new perspectives for brain repair, as a deeper understanding of the neurovascular niche and of endothelial cell-derived soluble factors may be of extreme relevance to allow the development of new tools to enhance neuronal replacement using SVZ stem cells.

6.1.2. The contribution and nature of cell-to-cell contacts, between endothelial cells and SVZ stem/progenitor cells, to SVZ cell proliferation and stemness, *in vitro*

Proximity of stem/progenitor SVZ cells to blood vessels in the neurovascular niche allows not only the diffusion of endothelial-derived factors but also the establishment of direct heterocellular contacts, as well as cell-to-ECM contacts. A growing body of evidence shows that these contribute to the maintenance of stem cell properties as well (Shen *et al*., 2008; Tavazoie *et al*., 2008).

In our SVZ-mBEC co-cultures, the levels of BrdU positive SVZ nuclei in the carpet of differentiating cells were significantly lower than the ones found in SVZ cells immediately apposed to mBEC, indicating that contact with endothelial cells contributes to SVZ cell proliferation. When mBEC were incubated with CHX, an

inhibitor of protein synthesis used to reduce the turnover of contact proteins, proliferation levels detected close to mBEC were decreased to values similar to the ones found in the carpet. This demonstrates that indeed heterocellular physical contacts are involved in SVZ cell proliferation. No differences were observed concerning apoptotic cell death between the carpet of differentiating cells and the population of cells in contact with mBEC, nor between controls and CHX-treated co-cultures. This observation shows that contact with endothelial cells does not promote SVZ cell survival, at least in basal conditions. Similarly to proliferation studies, we found lower levels of SOX-2 positive cells in the pseudo-monolayer of differentiated cells than within the population of cells contacting mBEC. In co-cultures where mBEC had been incubated with CHX, the numbers of stem/progenitor cells in contact with mBEC did not differ from the values found in the carpet of differentiating cells and, thus, heterocellular contacts between mBEC and SVZ cells seem to sustain the stem state. In co-cultures, upon mBEC incubation with CHX, neuronal differentiation close to mBEC was not affected comparing to controls. This points to the conclusion that neuronal differentiation does not need direct heterocellular contacts between mBEC and SVZ cells, at least in a non-disease paradigm. Alternatively, it is possible that, when deposited on mBEC, SVZ neurospheres are already partly committed and it is too late to identify possible effects on neuronal differentiation using DCX staining. An earlier neuronal marker should then be tested, such as the transcription factor Mash1.

Interaction between α6ß1 integrin expressed by SVZ cells with vessel-derived laminin-1 may account for the maintenance of the stem cell state in SVZ cells (Shen *et al*, 2008). Furthermore, in the bone stem cell niche, junctional communication between endothelial cells and progenitor cells modulates osteoblast differentiation (Guillotin *et al*., 2008). We hypothesized that the interaction between endothelial cells and SVZ cells could be either through endothelial laminin-1 and SVZ cell α6β1 integrin receptor or through Cx43 mBEC/SVZ cells GJIC. The next step was to verify

whether these proteins were present in mBEC and SVZ cells. Our results concerning expression of contact proteins revealed that Nestin-positive SVZ neurospheres express the α6β1 integrin receptor. Because mBEC expressed laminin-1, and both cell types express Cx43, it is conceivable that heterocellular contacts between mBEC and SVZ cells, mediated by endothelial laminin-1 and SVZ α6β1 integrins, occur, as well as mBEC/SVZ cells GJIC. Concerning the study of GJIC, by LAMP, we observed the passage of a fluorescent probe through GJs from mBEC donor cells to SVZ recipient cells. By immunocytochemistry we identified Cx43 expression in areas of interaction between endothelial and SVZ cells, both *in vitro* and *in vivo*.

In this work we showed that proliferation in the carpet of differentiating cells was lower than in contact with mBEC, indicating that direct communication between mBEC and SVZ cells promotes proliferation. When cells were incubated with a neutralizing antibody for α6 integrin, the pro-proliferative effect of the contact with mBEC was prevented, indicating that α6β1 integrin interactions with endothelial laminin-1 were relevant to SVZ-cell proliferation. If co-cultures were incubated with CBX, an inhibitor of GJIC, the pro-proliferative effect of the contact with mBEC was prevented as well, indicating that GJIC also play a role in cell proliferation. When co-cultures were incubated with the anti-α6 integrin neutralizing antibody or with CBX, no differences were seen in apoptotic cell death between the carpet of differentiating cells and the cells contacting mBEC, nor comparing to controls. This indicates that α6β1 integrin interaction with endothelial laminin-1, as well as mBEC/SVZ cells GJIC, do not contribute to survival and that the anti-α6-neutralizing antibody and CBX do not exert any evident toxic effect. As described for proliferation, when co-cultures were incubated with a neutralizing antibody for α6 integrin or with CBX, the effect of the contact with the mBEC, regarding stemness, was prevented, indicating that the α6β1 integrin interaction with endothelial laminin-1 as well as mBEC/SVZ cells GJIC are relevant to maintain SVZ-cell stem state.

Concerning neuronal differentiation, our results depicted that the numbers of generated neurons close to mBEC did not differ from the ones obtained in co-cultures with CHX-incubated mBEC. When cells were incubated with a neutralizing antibody for α6 integrin, or with CBX, no differences were found either, indicating that α6β1 integrin interaction with endothelial laminin-1 and GJIC do not contribute to neuronal differentiation. Again, this may be due to the fact that when deposited on mBEC, SVZ precursors are already partly committed and DCX may not be the most adequate neuronal differentiation marker. As mentioned above, additional experiments should, thus, be repeated using an earlier neuronal marker such as Mash1.

Here, we showed that interaction between mBEC and SVZ stem/progenitor cells, endothelial laminin-1 and SVZ cell α6β1 integrin, or through GJIC, contribute to the maintenance of stem/progenitor cells characteristics, including proliferation and stemness. Apoptotic cell death and neuronal differentiation remain unaffected.

The present work contributes to further understand the SVZ neurovascular niche and also to reveal how SVZ cell characteristics are maintained, which is particularly relevant in the cell therapy context.

6.1.3. Impact of microvasculature in SVZ cell proliferation in the non-injured and epileptic brain

Neurogenic activity is restricted to specific areas in the brain, such as the SVZ. Here, stem cells and transit-amplifying cells interact with a specialized vasculature, with sites of altered BBB, which allow cell-to-cell contacts and diffusion of endothelial- and blood-derived molecules (Shen *et al*., 2008; Tavazoie *et al*., 2008). SVZ vasculature is also particular since no angiogenic sprouting of endothelial cells or endothelial cell division is observed (Tavazoie *et al*., 2008), unlike the adult hippocampus, where active angiogenesis accompanies neurogenesis (Palmer *et al*., 2000). In the present work, endothelial cell division was not found in the SVZ of

control animals. Because the SVZ possesses such a particular microvasculature and because stem/progenitor cells capacities are regulated by blood vessels, we hypothesized there would be differences between microvascular density in the SVZ and non-neurogenic areas. Unexpectedly, we found that this parameter did not differ between the neurogenic SVZ and the non-neurogenic cortex and striatum. Nevertheless, upon SE, microvascular density was incremented in all tested areas, with values in the SVZ of KA-treated mice increasing 1.79 and 2.90 times more than in the striatum and cortex of KA-treated mice, respectively. Some BrdU/CD31 double-positive cells were observed in KA-treated mice, but they were very scarce indicating that angiogenesis was not highly active. Because an increase in vascular density was observed, three hypothesis were raised: either the increase in vascular density was in fact due to endothelial cell proliferation but this occurred very shortly after damage, or the increase of blood vessel density was due to vascular remodelling, or both. To clarify this, BrdU+/CD31+ cell numbers should be evaluated again, in future studies, at a shorter time point after KA-induced seizures (for instance, 24 h). If angiogenesis, in fact, occurs in the SVZ after epileptic seizures, and because we found that the anti-angiogenic peptide endostatin does not alter SVZ cell characteristics *in vitro*, we can suspect that this compound might be a potential candidate to block angiogenesis *in vivo*.

According to the importance of vasculature in stem cells' functions, we hypothesized that a correlation between vasculature changes upon injury and the increase of proliferation may occur. There was, in fact, an increase of cell proliferation in the SVZ, 7 days after SE induction, comparing to control animals, and this is in accordance with previous studies in other epilepsy models (Parent, 2002; Parent *et al*., 2002a). In control and KA-treated animals, BrdU positive cells in the SVZ were found in close association with blood vessels. When comparing the percentages of BrdU cells in contact with blood vessels, in control and KA-treated mice, there was a clear increase in BrdU positive nuclei in the later group, even

though not significant, probably due to low sampling. This suggests that seizures induce cell proliferation close to blood vessels. One could argue that these proliferating cells are generated following seizure close to blood vessels and then migrate out of blood vessels surface. Again, to verify this hypothesis, experimental protocols using shorter time points should be investigated, in order to verify if more proliferating cells are found closer or in contact with blood vessels. This work shows a correlation between injury-induced SVZ proliferation and microvascular density changes. Even though preliminary, some these data are quite encouraging and deserve to be further investigated.

The present thesis expands the existing knowledge regarding the SVZ neurovascular niche and how SVZ stem cell characteristics are maintained, a knowledge that is particularly relevant in the cell therapy context since it may contribute to develop new strategies for cell replacement. The present work can also be looked at in the context of cancer therapy, as stem cell regulator molecules such as Ang-1, α6β1 integrin and Cx43 could be targeted in tumours, reducing tumourigenesis and contributing to patient overall survival.

6.2. Main conclusions

1. SVZ neurospheres express both Ang-1 and its receptor Tie-2, as shown by RT-PCR, western blotting and immunocytochemistry. Tie-2 is maintained upon differentiation in neuronal and glial lineages, even though to a lesser extent, as demonstrated by immunocytochemistry.

2. SVZ cell cultures exposure to 500 ng/ml Ang-1 results in an increase of cell proliferation with no effect on cell death. Ang-1-induced increase of proliferation is mediated by the Tie-2 receptor and the ERK/MAPK kinase pathway activation.

3. SVZ cell cultures exposure to 500 ng/ml Ang-1 results in an increase of neuronal differentiation through Tie-2 receptor activation and downstream mTOR pathway activation. Generated neurons are functional, as determined by single cell calcium imaging.

4. Ang-1 (500 ng/ml) promotes neuronal maturation of SVZ cell cultures *via* the activation of the SAPK/JNK pathway.

5. Tie-2 is expressed in neuronal progenitors and neurons along the SVZ-rostral migratory stream-OB system *in vivo*.

6. Direct contact between SVZ cells and mBEC contributes to SVZ cell proliferation and stemness but has no effect on cell survival or neuronal differentiation.

7. SVZ neurospheres and mBEC express α6β1 integrin and laminin-1, respectively, and both cell types express Cx43, as demonstrated by western blotting and immunocytochemistry. Therefore, it is possible that mBEC and SVZ cells communicate through endothelial laminin-1 and SVZ α6β1 integrin or through GJIC. In fact, concerning GJIC, by LAMP, we observed the passage of a fluorescent probe through GJs from mBEC donor cells to SVZ recipient cells. By immunocytochemistry we identified Cx43 expression in areas of interaction between endothelial and SVZ cells, both *in vitro* and *in vivo*.

8. Contacts between endothelial laminin-1 and SVZ α6β1 integrin as well as mBEC/SVZ cell GJIC contribute to SVZ cell proliferation and stemness, but have no effect on cell survival and neuronal differentiation.

9. We found no differences in microvasculature density between the neurogenic SVZ and the non-neurogenic cortex and striatum. However, 7 d after SE, microvasculature density increases in all areas, particularly in the SVZ. No proliferating endothelial cells were detected in controls and very few proliferating endothelial cells were seen in treated animals. Therefore we cannot assume that increased microvasculature density derives from angiogenesis. Earlier time points should be evaluated to clarify this point.

10. Increased proliferation in the SVZ can been observed in KA-treated mice 7 d after SE. This correlates with the observed increase in microvasculature density. Proliferating cells in the SVZ are located close or apposed to blood vessels, in the normal brain and, particularly, in the KA-injured brain (7 days after SE). To test if seizure activity stimulates cell proliferation close to blood vessels and then these cells migrate away from blood vessels surface, again, earlier time points after SE should be investigated.

11. The anti-angiogenic peptide endostatin (1 μg/ml) does not affect SVZ cell proliferation, neuronal differentiation or cell death *in vitro*, *per se*. Therefore, it is a good candidate to block angiogenesis in the SVZ and study the impact of this process in neurogenesis after SE.

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