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Control of migration of neural stem cells by calpain signaling

Maria Manuela Coutinho Alves de Azevedo

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Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Celular e Molecular, realizada sob a orientação científica da Professora Doutora Inês Araújo (Universidade do Algarve) e do Professor Doutor Paulo Santos (Universidade de Coimbra)

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Abbreviations

ANOVA	Analysis of variance
AKT	Protein kinase B, also referred as PKB
bFGF	Basic fibroblast growth factor
BCA	Bicinchoninic acid
BDNF	Brain-derived neurotrophic factor
BSA	Bovine serum albumin
BrdU	5-bromo-2'-deoxyuridine
CAPS	*N*-cyclohexyl-3-aminopropanesulfonic acid
Cdc42	Cell division control protein 42
CLAP	Chymostatin, leupeptin, antiparin, pepstatin A – cocktail of protease inhibitors
CSF	Cerebrospinal fluid
CNS	Central nervous system
CSTN	Calpastatin knock-out mice
DCX	Doublecortin
DG	Dentate gyrus
DMEM/F12	Dulbecco's modified eagle medium: nutrient mixture F-12
DNA	Deoxyribonucleic acid
ECF	Enhanced chemifluorescence
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EdU	5-ethynyl-2'-deoxyuridine
EGF	Epidermal growth factor
Eph	Ephrin receptor
ERK	Extracellular signal-regulated kinase
GAPs	GTPase-activating proteins
GDIs	Guanine nucleotide dissociation inhibitors
GDP	Guanosine diphosphate
GEFs	Guanine nucleotide exchange factors
GTP	Guanosine-5'-triphosphate
GZ	Granular zone

HBSS	Hank's balanced salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
LV	Lateral ventricle
MAPK	Mitogen-activated protein kinase
MCAO	Middle cerebral artery occlusion
MDL28170	*N*-[*N*-[Phenylmethoxy)carbonyl]-*L*-valyl]-phenylalaninal
NGF	Nerve growth factor
NHS	Normal horse serum
NSC	Neural stem cells
OB	Olfactory bulb
PBS	Phosphate buffered saline
PSA-NCAM	Polysialic acid-neural cell adhesion molecule
PVDF	Polyvinylidene difluoride
PKA	Protein kinase A
Rac1	Ras-related C3 botulinum toxin substrate 1
RMS	Rostral migratory stream
RhoA	Ras homolog gene family, member A
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrilamide gel electrophoresis
SEM	Standard error of the mean
SGZ	Subgranular zone
SVZ	Subventricular zone
TBS-T	Tris buffered saline with Tween 20
TEMED	Tetramethylethylenediamine
U0126	1,4-Diamino-2,3-dicyano-1,4-bis(o-aminophenylmercapto)butadiene monoethanolate
WT	Wild-type

Abstract

Calpains are ubiquitous calcium-dependent cysteine proteases. Calpains are best known for the cleavage of proteins during excitotoxic cell death (del Cerro *et al.* 1994, Bednarski *et al.* 1995), which translates into a strong participation of calpains in the loss of brain tissue in either acute situations, like stroke, traumatic brain injury or seizures (Araujo *et al.* 2008), or in slow progressing disorders like Alzheimer's, Parkinson's or Huntington's disease (Vanderklish & Bahr 2000, Wu *et al.* 2004). One function described for calpains is their role in cell adhesion, motility and migration (Stifanese *et al.* 2006). Rho GTPases are described as targets of calpains and are involved in the regulation of cytoskeleton modification. Recently, Rho GTPases were linked to the enhanced spreading of platelets in μ-calpain$^{(-/-)}$ mice (Kuchay *et al.* 2012). In turn, extracellular signal-regulated kinase (ERK) is part of the mitogen-activated protein kinase (MAPK) signaling pathway and is also described as involved in cell migration (Anand-Apte *et al.* 1997, Klemke *et al.* 1997, Webb *et al.* 2000). However, the involvement of calpains, Rho GTPases and ERK in the proliferation and migration of neural stem cells (NSC) during neurogenesis in the adult central nervous system remains elusive. Another important factor is the possible involvement of integrins in NSC migration. Integrins appear described as being involved in the process of remodeling the cytoskeleton (Hynes 1992), and on this way they could be involved in NSC migration.

Previous work by our group demonstrated that inhibition of calpains increases migration rates of cultured NSC and subventricular zone (SVZ) explants. However, the signaling pathways involved in this process remain to be identified. Based on these assumptions, we investigated the signaling pathways by which calpains modulate adult neural stem cell migration, in cultures of NSC isolated

from the SVZ. For this purpose, we assessed whether calpain modulation of the activity of the Rho GTPases Cdc42, Rac1 and RhoA, ERK signaling pathway and integrin signaling is involved in the regulation of SVZ-derived NSC migration. On the other hand, we also analyzed cell migration in the rostral migratory stream (RMS) of brain sections from wild-type and calpastatin-deficient mice (CSTN).

Our data suggests that Cdc42 and Rac1, but not RhoA or the ERK signaling pathway, are involved in the migration of NSC. Moreover, we show that integrins are important for the migration of these cells, as well as laminin. On the other hand, calpains seem to be modulating Rho GTPases as well as integrins signaling. In addition to that, our *in vivo* studies show that the absence of calpastatin leads to a decrease in doublecortin (DCX) immunoreactivity along the RMS, which is translated to an impairment of cell migration from the SVZ to the olfactory bulbs. This suggests that calpains negatively regulate the migration of NSC.

Keywords: calpains; intracellular signaling; migration; neural stem cells; neurogenesis.

Resumo

As calpaínas são proteases cisteínicas ubíquas dependentes de cálcio. Estas proteases estão amplamente descritas como envolvidas na clivagem de proteínas durante a morte celular por excitotoxicidade (del Cerro et al. 1994, Bednarski et al. 1995), o que se traduz numa participação clara das calpaínas em processos de perda de tecido cerebral, como no caso de situações agudas, tais como isquémia cerebral, traumatismo crânio-encefálico ou convulsões (Araujo *et al.* 2008), ou em patologias de progressão lenta, como o caso das doenças de Alzheimer, Parkinson ou Huntington (Vanderklish & Bahr 2000, Wu et al. 2004). Uma função descrita para as calpaínas é o seu papel na adesão, motilidade e migração das células (Stifanese et al. 2006). As Rho GTPases estão descritas como alvos das calpaínas, e estão envolvidas na regulação das alterações que ocorrem a nível do citoesqueleto. Recentemente, as Rho GTPases foram identificadas como intervenientes no aumento do *spreading* das plaquetas em murganhos knock-out para as μ-calpaínas (Kuchay et al. 2012). Por sua vez, a via de sinalização da proteína cinase regulada por sinais extracelulares (ERK), que faz parte da via de sinalização das proteína-cinases activadas por mitogénios (MAPK), também se encontra descrita como envolvida na migração celular (Anand-Apte et al. 1997, Klemke et al. 1997, Webb et al. 2000). No entanto, o envolvimento das calpaínas, Rho GTPases e da ERK na proliferação e migração das células estaminais neurais (NSC) durante o processo de neurogénese no sistema nervoso central adulto permanece por esclarecer. Um outro facto que permanece por elucidar é o envolvimento das integrinas na migração das NSC. As integrinas estão descritas como envolvidas em processos de remodelação do

citoesqueleto (Hynes 1992), podendo estar a intervir no processo de migração das NSC.

Resultados preliminares do nosso grupo mostraram que a inibição das calpaínas leva a um aumento da migração das NSC em cultura e em explantes da região subventricular (SVZ). Contudo, as vias de sinalização envolvidas neste processo permanecem por ser identificadas. Com base nestes pressupostos, investigámos as vias de sinalização através das quais as calpaínas modulam a migração das NSC, em culturas de NSC isoladas da SVZ. Neste sentido, fomos avaliar se a modulação pelas calpaínas da actividade das Rho GTPases Cdc42, Rac1 e RhoA, da via de sinalização ERK e das integrinas está envolvida na regulação da migração das NSC derivadas da SVZ. Foram feitos ainda estudos de migração celular ao longo da via migratória rostral (RMS) *in vivo*, usando animais *wild-type* e animais geneticamente modificados (CSTN) que não possuem o inibidor endógeno das calpaínas, a calpastatina.

Os nossos resultados sugerem que a Cdc42 e a Rac1 estão envolvidas na migração das NSC, ao contrário do que acontece no caso da RhoA e da via da ERK. Para além disso, também mostramos que as integrinas são importantes para que a migração destas células ocorra, assim como a laminina. Por outro lado, as calpaínas parecem estar a actuar sobre a sinalização das Rho GTPases e sobre a sinalização pelas integrinas. Além disso, os nossos estudos *in vivo* mostraram que na ausência do inibidor endógeno das calpaínas ocorre uma diminuição na imunoreactividade da *doublecortin* (DCX) ao longo da RMS, o que se traduz numa diminuição da migração celular da SVZ até aos bolbos olfactivos. Isto sugere que as calpaínas exercem um efeito negativo na regulação da migração das NSC.

Palavras-chave: Calpaínas, sinalização intracelular, migração, células estaminais neurais, neurogénese

Chapter I

Introduction

1. Neurogenesis

Neurogenesis is defined as the process of generating new neurons from progenitor cells and over many years it was thought that this process occurred exclusively during embryonic development in the mammalian central nervous system (CNS). Altman was the major contributor to show that neurogenesis also occurs in the adult rat brain. Altman used in his study ([^{3}H])-thymidine that was incorporated by dividing cells and showed a constitutive production of new neurons in the hippocampus (Altman & Das 1965) and olfactory bulb (OB) (Altman 1969a). However, this finding was not accepted by the majority of biologists, because the method used in the study of Altman was not able to prove that the newborn cells would become neurons with the capacity to integrate in the CNS. In 1984, Paton and Nottebohm showed a functional integration of new neurons in the adult CNS of songbirds (Paton & Nottebohm 1984). However, the method used in the study was the same that Altman used (incorporation of ([^{3}H])-thymidine by dividing cells) combined with electron microscopy, which still raised some issues with the fate of the newborn cells. In 1996, Kuhn and colleagues showed the occurrence of neurogenesis in the dentate gyrus (DG) of the adult rat, using another thymidine analogue, 5-bromo-2'-deoxyruridine (BrdU), as a proliferation marker. This new marker allows the phenotypic analysis and stereological quantification of new cells through immunocyto- or immunohistochemistry, leaving behind the need to resort the autoradiographic detection of ([^{3}H])-thymidine (Sidman *et al.* 1959, Gratzner 1982). In fact, with the evolution of the research techniques, it was possible to prove the existence of adult neurogenesis, which has been demonstrated to occur in the brain of almost all mammalians, including humans (Eriksson *et al.* 1998).

The paradigm of the adult brain as a static structure was abandoned and regions where neurogenesis can occur were identified and designated as neurogenic areas.

1.1. Neurogenic niches in the adult mammalian brain

Two main regions of the adult mammalian brain were identified as the local of occurence of neurogenesis: the subgranular zone (SGZ) of the DG in the hippocampus and the subventricular zone (SVZ), in the wall of the lateral ventricles (Altman & Das 1965, Altman 1969a, Reynolds *et al.* 1992, Lois & Alvarez-Buylla 1993, Palmer *et al.* 1995). Other regions in the adult mammalian brain were also suggested as places where neurogenesis occurs, such as the cortex (Gould *et al.* 1999, Dayer *et al.* 2005) and the substantia nigra (Zhao *et al.* 2003), but this is still an issue with some controversy and further experimental support is needed (Rakic 2002, Gould 2007). Basically, the neurogenic niches provide a specific microenvironment that allows the maintenance of self-renewal and/or multipotency of neural stem cells (NSC). NSC have been extensively characterized and present two major characteristics: capacity of self-renewal through cell division and the ability to differentiate into specialized cell types such as neurons, astrocytes and oligodendrocytes (for more details see Gage 2000 and Reynolds et al. 1992). Adult neurogenesis occurs in several stages: the proliferation of adult NSC or progenitor cells, fate specification of progenitor cells, migration of immature neurons of the SGZ or the SVZ into the granule cell layer of the dentate gyrus or through the rostral migratory stream (RMS) towards the OB, respectively, differentiation and, finally, integration of newborn neurons in the

existing neuronal network and their survival and maturation into fully functional neurons (described in more detail in the next section).

1.2. Stages of neurogenesis

The introduction of new techniques such as detection of BrdU incorporation (Kuhn *et al.* 1996) or retroviral labeling methods (van Praag *et al.* 2002) allowed the characterization of the different steps of the neurogenic process.

The SVZ region of the adult brain has ependymal cells, quiescent radial glia-like cells, also known as B cells, which give rise to transient amplifying cells (or C cells) with high proliferative capacity. C cells in turn give rise to migrating neuroblasts (A cells) (Doetsch *et al.* 1999) that migrate through the RMS, in a chain along a tube formed by astrocytes, into the olfactory bulbs (OB) (Lois *et al.* 1996). Once in the OB, neuroblasts detach from the RMS and migrate radially, towards the granule cell layer and glomerular layer, where they differentiate into mature neurons and integrate into the neuronal circuits (Figure 1) (Ming & Song 2011).

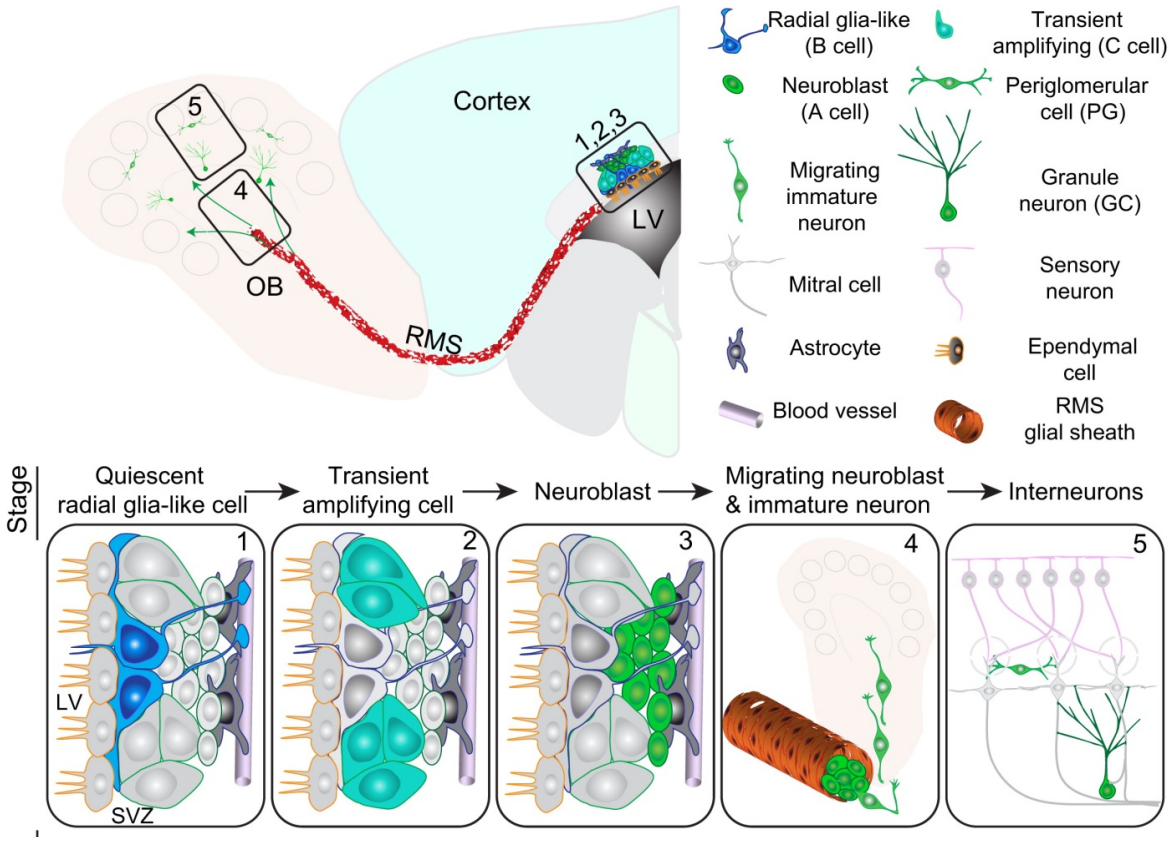

Figure 1 - Schematic representation of adult neurogenesis in the SVZ. Five developmental stages: **(1)** activation of radial glia-like cells in the wall of the lateral ventricles (LV); **(2)** quiescent radial glia-like cells give rise to C cells which proliferate; **(3)** C cells give rise to migrating neuroblasts; **(4)** chain migration of neuroblasts allong the RMS, through a tube formed by astrocytes; and **(5)** synaptic integration, survival and maturation of immature neurons in the OB. Adapted from (Ming & Song 2011).

In the SGZ of the DG of the adult brain, immature neurons are derived from neuroblasts, which, in turn, derived from progenitor cells. In this case, migration of newly formed cells takes place within a smaller area, compared to the SVZ. Thus, immature neurons from the SGZ migrate into the inner granule cell layer, where they differentiate into granule neurons. The newborn neurons extend their axons to the CA3 region of the hippocampus, being integrated in the hippocampal neural circuitry (Figure 2) (Zhao *et al.* 2006).

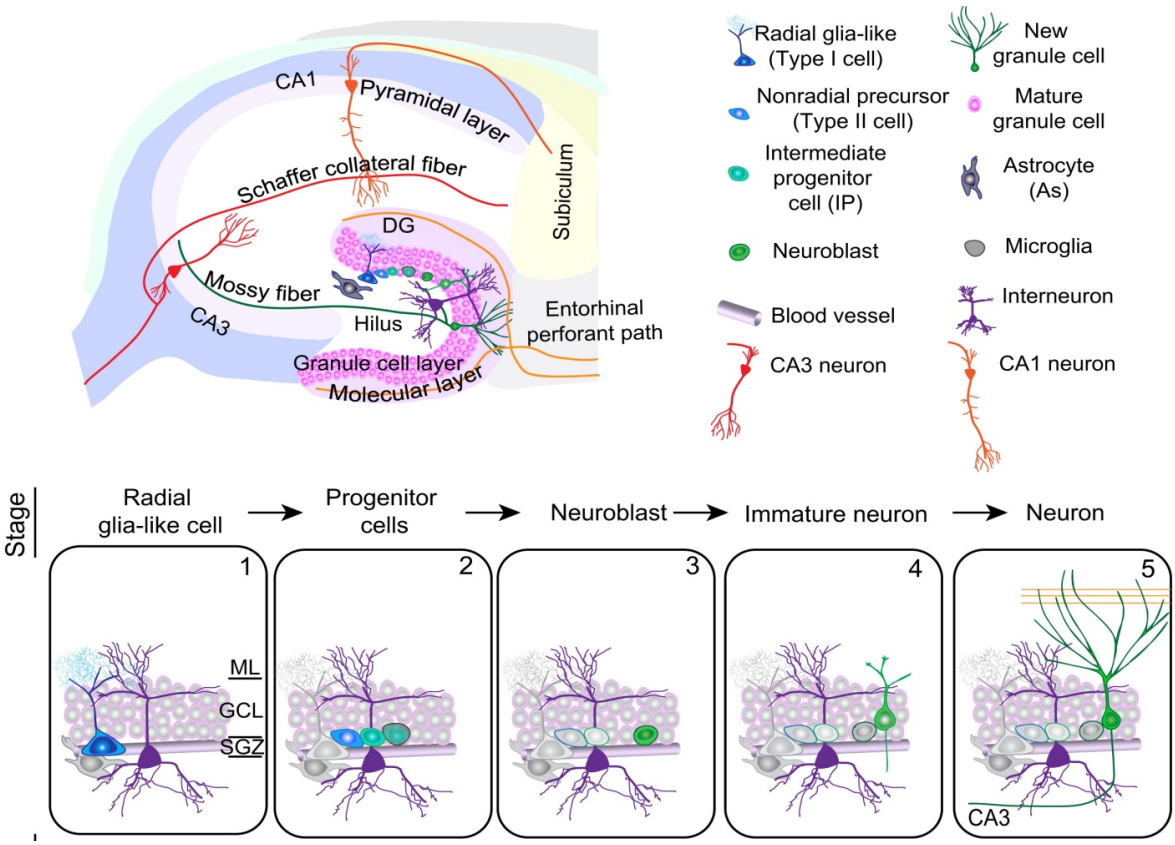

Figure 2 - Schematic representation of adult neurogenesis in the DG. Five developmental stages: **(1)** activation of quiescent radial glia-like cell in the SGZ; **(2)** proliferation of non-radial precursors and intermediate progenitors in the SGZ molecular layer; **(3)** progenitor cells give rise to neuroblasts; **(4)** immature neurons from the SGZ migrate into the inner granule cell layer and differentiate in granule neurons; and **(5)** mature neurons integrate the neural network and suffer maturation becoming totally functional neuronal cells, receiving inputs from the entorhinal cortex and extending axonal projections to the CA3 region. Adapted from (Ming & Song 2011).

1.3. Regulation of adult neurogenesis

Adult neurogenesis is regulated in several ways by intrinsic as well as extrinsic mechanisms. Numerous factors have been described to be involved in the regulation of the neurogenic process: signaling pathways, factors/receptors in neurogenic niches, cytoplasmatic and transcription factors. For example, cell-cycle inhibitors, such as p16, p21 and p53, play an important role in maintaining the quiescent state of adult neural precursors since the deletion of these factors

results in transient activation and, consequently, loss of the precursor pool (Ming & Song 2011). The Notch signaling pathway has been described as another controller of the maintenance of the NSC pools, since the ablation of Notch1 during the early post-natal period and along adulthood leads to an increase in neuronal fate specification of NSC and, consequently, to a decrease in the proliferation of these cells (Chojnacki *et al.* 2003, Breunig *et al.* 2007).

Hormones are other regulators of adult neurogenesis. Estrogens, for example, present a proliferative effect over progenitor cells of the DG (Tanapat *et al.* 1999). However, this hormone does not seem to affect neurogenesis in the SVZ of adult rats, although these cells express specific receptors for estrogen (Brannvall *et al.* 2002, Isgor & Watson 2005). Stress hormones, such as corticosteroids and particularly glucocorticoids, on the other hand, appear described as decreasing neurogenesis in the DG of young rats and primates (Gould *et al.* 1998, Kippin *et al.* 2004).

Another group of regulators of adult neurogenesis is composed by trophic factors. EGF and bFGF are described as potent factors in promoting NSC proliferation in the SVZ *in vivo*. However, only bFGF leads to an increase in the number of newborn neurons in the OB (Kuhn *et al.* 1997). Brain-derived neurotrophic factor (BDNF) is also of the growth factor family and appears described as preventing neural death during development (Hempstead 2006), and also as increasing cell proliferation in the granule cell layer of the OB and in the DG of rodents (Benraiss *et al.* 2001, Lee *et al.* 2002).

Classic neurotransmitters, such as glutamate and gamma-aminobutyric acid (GABA), are also described as increasing the differentiation of hippocampal progenitors (Tozuka *et al.* 2005).

The migration that occurs during neurogenesis is also controlled by innumerous factors. In the SVZ, migrating neuroblasts are normally bipolar, presenting extensions that allow the connection with adjacent cells forming "chains" (Lledo & Saghatelyan 2005). These chains allow the sliding of migrating cells and are coated by astrocytes, being this structure also called glial tube. The SVZ is characterized as being a thin cell layer founded next to the ependyma of the telencephalic lateral walls of the lateral ventricles (Altman 1969b, Zhao et al. 2008) (Figure 3). Extracellular signals from neighboring cells, humoral factors and the extracellular matrix are other important cues involved in the regulation of neuronal migration, axon/dendritic development and synapse formation, during adult neurogenesis. The close proximity between stem cells and ependymal cells in the SVZ, blood vessels and the direct contact with the cerebrospinal fluid (CSF) are responsible for controlling the migration of NSC (Kazanis & ffrench-Constant 2011, Kazanis *et al.* 2008). Chemorepellent signals such as Slit1 and Slit2, cyclin-dependent kinase 5 (Hirota *et al.* 2007), EphB2-ephrin-B2 and neuroregulin-ErbB4 pathways, polysialic acid-neural cell adhesion molecule (PSA-NCAM) protein, β1-integrin, proteoglicans and laminins appear described as important for the regulation of the migration of SVZ neuroblasts (Lledo & Saghatelyan 2005) (discussed in more detail in section 3.1).

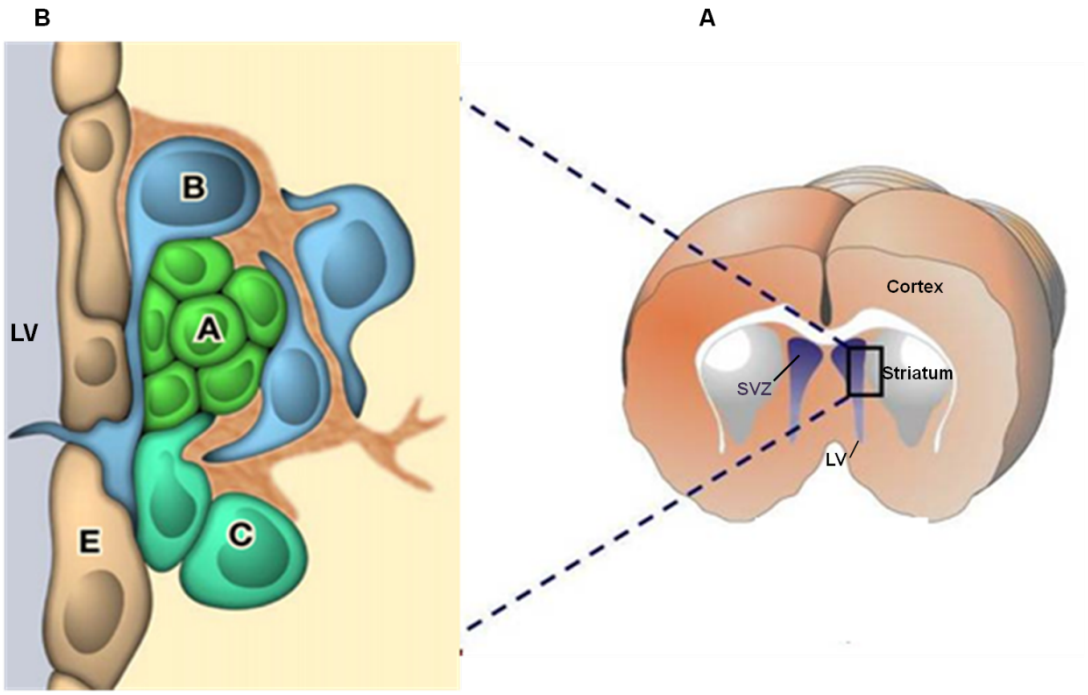

Figure 3 – The neurogenic niche in the SVZ. (A) Cross section of the adult mouse brain showing the subventricular zone (SVZ, purple), adjacent lateral ventricle (LV, light purple), cortex and the striatum. **(B)** Schematic illustration of the SVZ architecture and cell types. Multiciliated ependymal cells (E, brown), SVZ astrocytes or B cells (B, blue), C cells (C, light blue) that give rise to A cells or neuroblasts (A, green). Adapted from (Zhao *et al.* 2008).

1.4. Role of adult neurogenesis

Neurogenesis in the adulthood contributes to improve adult brain function in terms of neuronal network, under physiological conditions (Kempermann *et al.* 2004). In the last decades, a lot of research regarding adult neurogenesis has been done. However, only in 2002 was proved that newborn neurons are functional in the adult brain (Carlen *et al.* 2002). According to the region, the newborn cells can have different contributions to the CNS. In the adult DG, newly formed granule cells demonstrated a greater predisposition for synaptic plasticity when compared with existing granule cells (Wang *et al.* 2000, Snyder *et al.* 2001, Schmidt-Hieber *et al.* 2004), whereas in the OB newborn granular and

periglomerular cells presented different membrane properties compared with existing neurons surrounding them (Belluzzi *et al.* 2003, Carleton *et al.* 2003). Neurogenesis in the SGZ presents another important role, since it provides a substrate for additional brain plasticity and is crucial for spatial learning and memory in adult mice (Imayoshi *et al.* 2008). One proof of this, is the significantly decrease in learning capacity in rats when neurogenesis in the hippocampus is inhibited (Shors *et al.* 2001). In the OB, neurogenesis showed to be as important as in the hippocampus. In fact, when adult neurogenesis is suppressed olfactory learning and memory, in adult crickets, is impaired (Scotto-Lomassese *et al.* 2003). Similar results were obtained in the OB of rodents, where adult neurogenesis was also associated to the learning process (Alonso *et al.* 2006, Mouret *et al.* 2008, Mandairon *et al.* 2006).

Moreover, neurogenesis seems to be closely involved with tissue repair in pathological conditions (Lowenstein & Parent 1999). Brain injury, such as ischemic brain events (Kokaia & Lindvall 2003), traumatic brain injury (Dash *et al.* 2001, Rice *et al.* 2003), epileptic seizures (Parent *et al.* 1997, Gray & Sundstrom 1998) and neurodegenerative diseases (such as Huntington's disease (Lazic *et al.* 2004) and Alzheimer's disease (Jin *et al.* 2004a, Jin *et al.* 2004b)) have already been shown to present increased neurogenesis. In other words, these pathologies induce proliferation of NSC and migration of newborn cells into the lesioned areas, where they may potentially differentiate and integrate the neuronal network to substitute the lost cells (Arvidsson *et al.* 2002, Magavi *et al.* 2000).

However, the regulatory mechanisms by which NSC migrate into lesioned areas remain to be clarified.

2. Calpains

First described by Guroff in the rat brain (Guroff 1964), calpains are ubiquitous calcium-dependent cysteine proteases with maximum activation at neutral pH. Calpains belong to the papain superfamily of cysteine proteases. Fifteen different isoforms of calpains have been described, six of which are tissue-specific, while the other nine are ubiquitously expressed (Sorimachi *et al.* 2011). Two of the latter ones are the most extensively studied and well characterized, namely μ- and m-calpains, also known as calpain I or calpain II, respectively (Suzuki & Sorimachi 1998). This designation is closely linked to the calcium concentration required for their activation *in vitro*. For μ-calpain activation, calcium concentration is approximately 2-8 μM and, for m-calpain, 0.2-0.8 mM (Cong *et al.* 1989). μ-calpain and m-calpain are commonly designated as 'conventional' calpains, because their study was on the basis for the creation of the calpain superfamily (Suzuki *et al.* 1995). Meanwhile, all the other calpains are called "unconventional".

2.1. Structure of calpains

Calpain isoforms μ- and m- present a heterodimeric structure (Figure 4). They are composed by a large catalytic subunit, with 80 kDa (Capn1 in μ-calpain and Capn2 in m-calpain, which are not identical) and a small regulatory subunit with 28 kDa, identical for both isoforms. The large catalytic subunits are encoded by two different genes, *capn*1 and *capn*2 (for μ- and m-calpain, respectively) (Ohno *et al.* 1990). The small subunit is encoded by *capn*4 (Franco & Huttenlocher 2005).

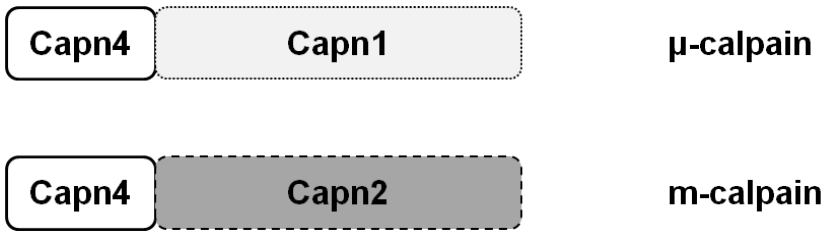

Figure 4 - Schematic representation of μ-calpain and m-calpain structure.

The large catalytic subunit has a cysteine protease domain and a calcium-binding domain with five helix-loop-helix (EF-hand) structures and the small regulatory subunit has also five EF-hand structures. It is through the fifth EF-hand domain of each subunit that the assembly of the heterodimers occurs (Blanchard *et al.* 1997, Lin *et al.* 1997, Hosfield *et al.* 1999).

According to the domain structure of the catalytic subunit, calpains are classified as classical (with a structure for the catalytic domain similar to μ- and m-calpains) or non-classical (with a different catalytic domain from μ- and m-calpains) (Ono & Sorimachi 2012). At the moment, fifteen genes for calpains were identified in humans, nine of them encoding the classical calpains (calpains 1, 2, 3, 8, 9, 11 and 14). They are distributed in a wide range of living organisms, such as prokaryotes and almost all eukaryotes (Croall & Ersfeld 2007).

2.2. Expression and localization of calpains

In the cell, patterns of calpain localization present a wide complexity and variability (Beckerle *et al.* 1987, Gil-Parrado *et al.* 2003, Kifor *et al.* 2003, Hood *et al.* 2004, Raynaud *et al.* 2004), which suggests that the subcellular localization of calpains may be an important factor in the modulation of their functions. In fact, calpains are typically described as intracellular proteases (Nishihara *et al.* 2001,

Xu & Deng 2004). Calpains are localized mostly in the cytoplasm of cells, but can also act outside the cell in some circumstances, as suggested by Adachi and collaborators (Adachi *et al.* 1990). Another consideration about the localization of calpains is related to their activation, because it is speculated that, when active, they associate to the cell membrane (Croall & DeMartino 1991).

As the name suggests, ubiquitous calpains play a fundamental role in all cells, while tissue-specific calpains have more specified cellular functions. A clear example of this is that defects in ubiquitous calpains (e.g. *capn2*$^{-/-}$) can be lethal (Dutt *et al.* 2006), while defects in tissue-specific calpains may result in different phenotypes (e.g. Capn3 mutation results in muscular dystrophy) (Richard *et al.* 1995).

μ-calpain and m-calpain, as mentioned before, were first identified in the rat brain (Guroff 1964) and, despite their wide distribution, they have a particular expression in CNS. Calpains were found in the axoplasm of giant axon of the *Myxicola* (Gilbert & Newby 1975), in squid (Pant *et al.* 1979) and in the rat peripheral nerve (Schlaepfer & Freeman 1980, Kamakura *et al.* 1983), and their localization is exclusively intracellular (Hamakubo *et al.* 1986).

On the other hand, there are at least six tissue-specific calpains identified until now. Capn3 was identified in skeletal muscle (Sorimachi *et al.* 1989), Capn6 in the placenta and embryonic striated muscle (Dear & Boehm 1999, Dear *et al.* 1997), Capn8 and Capn9 in the gastrointestinal tract (Sorimachi *et al.* 1993), Capn11 in testis (Dear *et al.* 1999, Dear & Boehm 1999) and, finally, Capn12 was found in hair follicles (Dear *et al.* 2000). The discovery of tissue-specificity of calpains has been described to be linked to specific calpain functions in the respective tissues.

2.3. Regulation of calpain activity

There are two ways of regulating calpains: through the small regulatory subunit or via calpastatin.

The small regulatory subunit acts as a chaperone-like component for the catalytic subunits of the conventional calpains, stabilizing them (Yoshizawa *et al.* 1995). This subunit is extremely important, as shown by the embryonic lethality of *capn4*$^{-/-}$ mice, indicating that the small regulatory subunit is required for the stability of catalytic subunits of μ- and m-calpain *in vivo* (Arthur *et al.* 2000, Zimmerman *et al.* 2000).

Calpastatin, a protein encoded by the *CAST* gene (Ono & Sorimachi 2012), appears described as ubiquitously expressed and as a selective and reversible endogenous inhibitor for both calpains (Murachi 1984). Calpastatin acts through the prevention of the autolysis of calpains, necessary for their activation, and by competing with the native protein during its binding to the cell membrane (Melloni *et al.* 1992, Kawasaki & Kawashima 1996). Calpastatin exert its inhibitory effect through four repeats calpain inhibitory domains (CID) (Kawasaki *et al.* 1993, Nishimura & Goll 1991, Takano *et al.* 1995, Yang *et al.* 1994). After an increase in the intracellular concentration of calcium, it binds to calpains and they suffer conformational alterations, and autolysis can occur. Through this mechanism, calpains active center becomes available for calpastatin binding (Cottin *et al.* 1981, Shigeta *et al.* 1984, Pal *et al.* 2001).

Synthetic calpain inhibitors have also been developed. MDL28170 and calpeptin are two examples of peptidyl aldehydes, which reversibly inhibit calpains (Figure 5) (Mehdi *et al.* 1988, Yano *et al.* 1993).

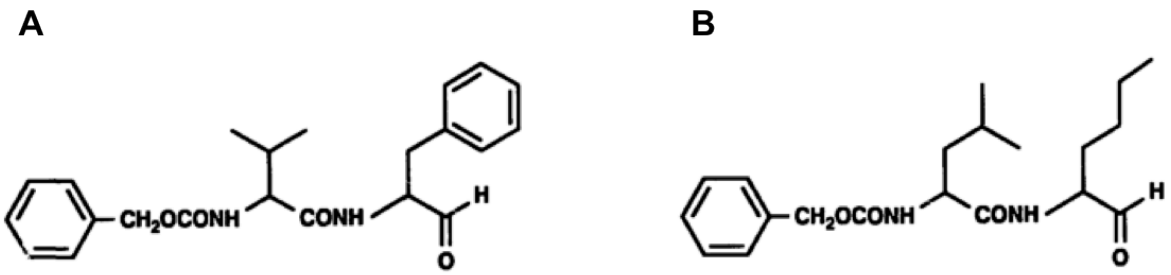

Figure 5 - Chemical structures of synthetic inhibitors of calpains. (A) Chemical structure of *N*-protected dipeptide aldehyde MDL28170 (CbzValPheH); **(B)** Chemical structure of calpeptin, synthetic aldehyde CbzLeu-nLeuH. Calpeptin and MDL28170 are similar compounds. Both are short and are hydrophobic *N*-blocked dipeptidyl aldehydes lacking charged residues. They have the ability to penetrate membranes by passive diffusion. When compared in terms of selectivity, MDL28170 and calpeptin present a similar spectrum of inhibition of calpains. Adapted from (Mehdi 1991).

There are also irreversible peptide and non-peptide inhibitors for calpains, such as leupeptin (Mehdi 1991) and α-mercaptoacrylates (as PD150606 and PD151746) (Wang *et al.* 1996), respectively. Calpain inhibitors vary in their chemical structure, selectivity and membrane permeability, thus presenting different efficacies (Donkor 2000, Mehdi 1991).

Calpains are also physiologically regulated by other mechanisms, such as elevations in the intracellular calcium concentration (Figure 6). In fact, the calcium concentration necessary for calpain activation *in vitro* is too high to be supported *in vivo*, which suggests the existence of some mechanisms capable of decreasing these calcium requirements (Franco & Huttenlocher 2005). Thus, autolysis of the large subunit (Suzuki *et al.* 1981, Zimmerman & Schlaepfer 1991, Brown & Crawford 1993) and connection to phospholipids (Coolican & Hathaway 1984, Saido *et al.* 1992) have been described to be important events that lead to a decreased requirement of calcium for calpain activation. Moreover, μ- and m-calpains can be regulated by the phosphorylation of specific residues, such as Tyr,

Ser and Thr, according to the isoform (Goll *et al.* 2003). For example, m-calpain can be activated by phosphorylation of the Ser50 residue through different mechanisms, which may involve the activation of the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) signaling pathway and/or EGF increased signaling (Glading *et al.* 2000, Glading *et al.* 2004). On the other hand, m-calpain activity can also be inhibited by phosphorylation of Ser369/Thr370, namely through protein kinase A (PKA) increased signaling (Shiraha *et al.* 2002).

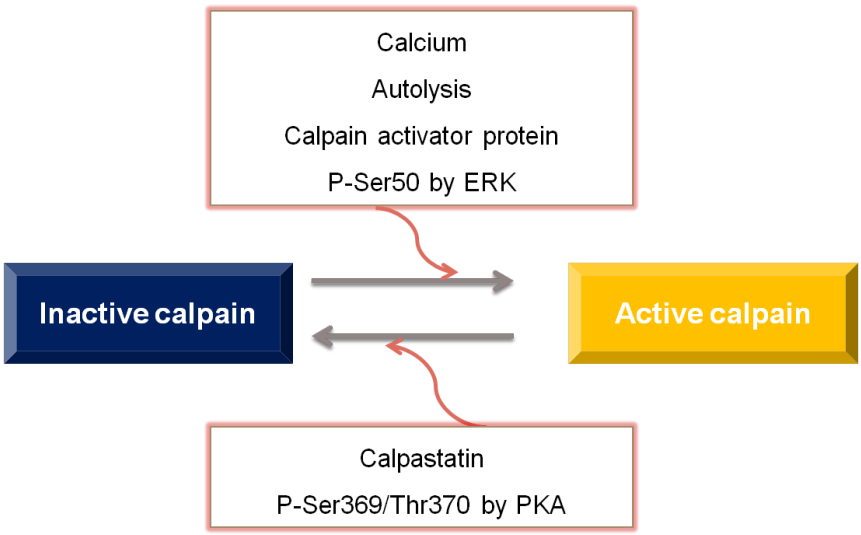

Figure 6 - Mechanisms of control of calpain activity. Calpain can be activated by calcium, the best-studied mechanism. When occurs an increase in the intracellular calcium concentration, calpains suffer a conformational change; autolysis is another process that leads to calpain activation, by decreasing the levels of calcium necessary, but it is a process that occurs more in the progression of activation than in its initiation; protein-protein interactions change the calcium requirements of calpains, facilitating the activation process; phosphorylation of some specific sites can also control calpain activation. On the other hand, calpain can be inhibited by their endogenous inhibitor, calpastatin, attenuating the activity of calpains, and by phosphorylation through PKA.

2.4. Targets of calpains

Calpains are proteases with a broad range of substrates, such as transcription factors, transmembranar receptors, signaling enzymes and cytoskeleton proteins (as reviewed in Table I) (Chan & Mattson 1999, Goll et al. 2003, Wang & Yuen 1997). However, calpain substrates *in vitro* may not be substrates *in vivo*, and the degradation of a specific substrate is dependent on external/internal signals, the presence/absence of calpains within the cell, among other factors (Goll et al. 2003). Calpains can cleave their substrates in a limited number of sites, creating large stable fragments rather than small peptides or amino acids (Carragher & Frame 2002). Brain α-spectrin is cleaved by both calpain isoforms in the same manner resulting in two fragments of approximately 145 and 150 kDa, which can serve as a mean of identifying calpain activity in lysates or via fragment-specific antibodies visualized by immunofluorescence (Wang 2000).

Table I - Examples of some calpain targets. This table is a sampling of some reported substrates for calpains; not all classes of calpain substrates are included.

Class	Target proteins
Cytoskeletal / Secretory pathway	SNAP-23, ICA512, L-type, calcium-channel, α-actinin, αII-spectrin/α-fodrin, talin, ezrin, paxillin, vinculin, desmin, γ-filamin, titin, focal adhesion kinase, integrin β3, RhoA, tubulin, tau, microtubule-associated protein 1, microtubule-associated protein 2, myosin light chain 1, filamin C, SNAP-25
Membrane-bound receptors and proteins	EGF receptor, AMPA receptor, anion channel, calcium release channel, L-type calcium channel, G proteins
Calmodulin-binding proteins	Calcium pump, calcineurin, calmodulin-dependent kinase II, myosin light chain kinase, neuromodulin, connexins, inositol triphosphate kinase
Signaling enzymes	Protein kinase C, HMG-CoA reductase, cyclic-AMP-depend kinase, pyruvate kinase, phosphorylase kinase
Myofibrillar proteins	Troponin I, troponin T, tropomyosin, myosin
Transcription factors	c-fos, c-jun, Pit-1, Oct-1, CP1a, CP1b, c-Myc, p53

As shown in Table I, calpains can act in the “remodeling” of the cytoskeleton, particularly during motility and cell migration through a rapid cleavage of cytoskeleton elements. The first implication of calpains in cell migration was showed when pharmacological inhibition of calpains lead to a decrease in integrin-mediated cell migration (Huttenlocher *et al.* 1997, Palecek *et al.* 1998). These studies showed that calpain inhibition resulted in the stabilization of adhesion complexes, and for that reason, an increase in adhesiveness was verified, which caused a reduction in the rate of detachment of the rear of the cell, consequently decreasing cell migration. Calpains are also involved in cell spreading and Croce *et. al* showed that inhibition of μ-calpain in platelets reduce their ability to spread (Croce *et al.* 1999). Basically, cell spreading and cell motility require degradation of focal adhesions at the attachment sites in both the leading and rear edges of the cells, by calpains (Perrin & Huttenlocher 2002, Glading *et al.* 2002). The membrane protrusions are also regulated by calpains, since whereas calpain inhibition by calpastatin results in abnormal lamellipodia and filopodia (Potter *et al.* 1998). On the other hand, calpains can exert a negative effect in cell migration being neutrophils an example of that. In resting neutrophils, high levels of calpain activity were detected and inhibition of this protease resulted in membrane protrusion formation and rapid chemokinesis (Lokuta *et al.* 2003), which is the opposite of what has been seen in other cell types.

The different effects of calpains according to the cell type can be linked with the signaling mechanisms that calpains are regulating. In the case where it was observed an increase in cell migration when calpains were inhibited, this effect may be related with the possible negative regulation of the Rho GTPases, as showed in neutrophils (Lokuta et al. 2003).

2.5. Physiological and pathological roles of calpains

Calpains have also been linked to numerous physiological roles in cells, such as exocytosis, cell fusion, differentiation, apoptosis, proliferation, migration and other important cellular processes (Goll et al. 2003). The involvement of calpains in the rearrangement of the cytoskeleton is the most referenced process. The detachment of the cell from the extracellular matrix, which allows cell migration, needs a coordinated mechanism between the focal adhesion assembly in the leading edge of the cell and the disassembly at its rear. Since proteins involved in this process are targets of calpains, these proteases have been linked to important events occurring during cell migration, such as adhesion, spreading, membrane protrusion and detachment of the cell, but also to the regulation of signaling mediators of cell migration such as integrins, Rho GTPases and growth-factor-mediated signaling (Franco & Huttenlocher 2005).

Some studies have addressed the effect of calpain inhibition in different cell types, and distinct results were reported. A decrease in the spreading ability was observed in platelets, T-cells, vascular smooth muscle cells, pancreatic cells and fibroblasts when calpains were inhibited (Croce *et al.* 1999, Parnaud *et al.* 2005, Paulhe *et al.* 2001, Potter *et al.* 1998, Rock *et al.* 2000). In contrast, calpain inhibition in neutrophils leads to an increased capacity of cells to spread (Lokuta *et al.* 2003). The physiological roles of calpains in the CNS are poorly described, but preliminary results from our group suggested that the inhibition of calpains in NSC leads to an increased cell migration (Inês Araújo, unpublished results). However, the mechanisms involved in this process are not known yet. It is necessary to identify the signaling pathways involved in the modulation of cell migration by calpains.

Calpains have been reported to be involved in several pathological conditions. Although they are not linked to a genetic cause for the diseases, in almost all pathologies the deregulation of their activity seems to be the leading cause for their involvement in these conditions. The loss of calcium homeostasis, particularly increased calcium influx into the cell, seems to be a major cause for calpains increased proteolytic activity, which has been associated with the following diseases: Alzheimer's disease, cataract formation, muscular dystrophies, myocardial infarcts, multiple sclerosis, brain ischemia (stroke), obsessive-compulsive disorders and traumatic spinal cord (brain) injury. However, some authors suggested other mechanisms to be the leading cause for the increased activation for calpains, such as failure on the activation of calpastatin and/or phosphorylation of Ser369/Thr370 by PKA (Goll et al. 2003).

3. Cell migration

3.1. Cell migration during neurogenesis

Cell migration is described as a key point of normal and abnormal processes, such as embryonic development, defense against infections, wound healing, development of tumor cell metastasis, atherosclerosis and chronic inflammatory diseases (e.g. rheumatoid arthritis) (for more detailed information, see Martin 1997, Ridley 2001). As previously mentioned, cell migration is one of the five different processes occurring during neurogenesis. However, the mechanisms beyond migration of NSC are not fully understood.

As described in section 1.3, the close proximity between stem cells and ependymal cells in the SVZ, blood vessels and the direct contact with the CSF are

responsible for controlling the migration of NSC (Kazanis & ffrench-Constant 2011, Kazanis *et al.* 2008). Several studies referred the involvement of different molecules in migration of neuroblasts and referred that migration through RMS occurs in parallel with the directional flow of CSF, in the lateral ventricle (LV) (Sawamoto *et al.* 2006). The physical adjacent position of the NSC to the ependymal cells implies an interaction between them. Ependymal cells are described as exerting a supporting/regulatory function in the niche, since they can modulate the transport of ions and other factors from the CSF (Bruni 1998). The choroid plexus is responsible to the secretion of chemorepulsive factors, being created a concentration gradient of these factors in CSF flow. Ependymal cells allow the access of CSF proteins or exogenous tracers through the ciliary beating (Sawamoto et al. 2006), being indispensable for the formation of chemorepulsive-factor gradients that conduct neuroblasts migration in the adult brain. These chemorepulsive factors may contain axon guidance molecules such as semaphorins (Kolodkin 1996a, Kolodkin 1996b), ephrins (Flanagan & Vanderhaeghen 1998) and Slits, which help guiding the migration of the neuroblasts through RMS.

Semaphorins are described as axonal guidance molecules with attractant or repellent activity. They participate in processes like early development, angiogenesis and cell migration (Tamagnone & Comoglio 2000). The presence of semaphorin-3A and its homodimer receptor NP1 was reported along the entire RMS in the adult brain, which suggests that they are involved in the regulation of neuroblast migration (Melendez-Herrera *et al.* 2008). Ephrins are transmembrane-associated proteins that need to be linked to Ephrin (Eph) receptors to exert their action. Axonal growth and cell guidance are referred to as being processes where

ephrins-Eph interaction is involved, during development (Wilkinson 2001), and during neurogenesis, this interaction has also been implicated (Conover *et al.* 2000, Holmberg *et al.* 2005, Theus *et al.* 2010). Eph-B1-3 and EphA4 receptors are expressed in the SVZ and RMS (Conover et al. 2000, Theus et al. 2010), and EphB2 receptor seems to be surrounding chains of migrating neuroblasts, in the RMS (Conover et al. 2000). The ephrin/EphB interaction controls cell spatial organization in the SVZ (Conover et al. 2000). These findings indicate that ephrin signaling is involved in the regulation of the neuroblast guidance into chain migration during adult neurogenesis. Slit is a secreted protein that binds directly to it receptor, the Roundabout (Robo), and acts as a chemorepulsive for OB axons (Li *et al.* 1999). In fact, in mammals Slit1-3 and Robo1-3 have been identified and implicated in axonal repulsion as in cell guidance (Brose & Tessier-Lavigne 2000). Studies with brain explants showed that Slit1 and Slit2 are expressed in the septum, where they act repelling progenitor cells rising from SVZ and maintained along the RMS (Wu *et al.* 1999). Another study using knockout Slit1 mice showed that Slit1 is important in directing migration since neuroblasts raised from the SVZ migrate caudally to the corpus callosum, rather than to the RMS, in this mice (Nguyen-Ba-Charvet *et al.* 2004). Based on this, Slit1 and Slit2 are suggesting as being involved in cell migration from the SVZ towards the OB.

PSA-NCAM is another important factor that regulates the migration of SVZ neuroblasts. NCAM is a cell surface glycoprotein, while PSA is a polymer of neuroaminic acid added to the NCAM molecule. The expression of this molecule has been reported in neurogenic niches in the adult brain (Seki & Arai 1993), and that neuroblasts in the chain migration in the RMS also express PSA-NCAM (Rousselot *et al.* 1995). It was also showed that PSA-NCAM deficient mice have

an altered RMS, and present an altered morphology due to the accumulation of migrating cells (Chazal *et al.* 2000).

The extracellular matrix is an important controlling element of neurogenesis, being laminin its best-known constituent. Laminin is a heterodimeric glycoprotein, which can present a wide range of possible combinations of its subunits (in mammals - five alpha, three beta and three gamma subunits) (Colognato & Yurchenco 2000). Besides the involvement of laminin in the transduction of different signals in NSC, members of laminin-like secreted molecules, nominated neutrin and reelin, have also been described as being involved in the regulation of the migration of newly generated cells along the RMS. Neutrin and reelin are known to be involved in the regulation of axon guidance during development, being expressed by astrocytes that surround the RMS (Staquicini *et al.* 2009). Moreover, these molecules have also been described to control the positioning of newborn neurons during embryonic cortical development (Frotscher 2010, Gaiano 2008), and as regulator elements of the migration of neuroblasts from the RMS into the olfactory bulb (Hack *et al.* 2002).

Cell migration requires changes in the cytoskeleton, cell-substrate adhesion and in the extracellular matrix (ECM). The first step of this process consists in the release of extracellular cues, like diffusible factors and/or signals coming directly from the ECM. The intracellular machinery triggered by transmembranar receptors, which are activated by extracellular stimuli. Depending on the trigger, different intracellular signaling molecules can be recruited to participate in cell migration. These molecules include small GTPases, calcium-regulated proteins, MAPK pathway, protein kinase C, phosphatidylinositide kinases, phospholipases C and D, and tyrosine kinases (Ridley 2001). Integrins have been described as

having an important role in cell spreading and migration (Lafrenie & Yamada 1996, Meredith *et al.* 1996). This large-range of different intracellular signaling molecules is connected with a wide diversity of extracellular signals that can induce cell migration and also with the huge number of cellular responses that have to be synchronized along the process.

3.2. Integrins in cell migration

Integrins are cell surface glycoproteins composed of one α and one β subunit that allow the binding to the ECM components. They are the major group of cell-surface receptors for the ECM and cell-surface molecules (Montgomery *et al.* 1996, Ruppert *et al.* 1995). Both of the non-covalent transmembrane glycoproteins (α and β subunits) have been described as being involved in inside-out signaling and in processes such as the coordination of the actin cytoskeleton and the cellular response to growth factors (Hynes 1992). It has also been described the clustering of integrins into small complexes with cytoskeleton proteins (e.g. vinculin and talin (Jockusch & Rudiger 1996)) and signaling molecules (e.g. Rho GTPases), following integrin-ligand interaction. These clusters are known as focal adhesions. Calpains have been indentified in focal adhesions, thus suggesting cell migration as being a calcium-mediated event (Beckerle et al. 1987, Du *et al.* 1995, Cooray *et al.* 1996). Moreover, focal adhesions allow integrin signal transduction, which ultimately leads to changes in cell morphology and physiology (Clark & Brugge 1995, Schwartz *et al.* 1995, Yamada & Miyamoto 1995). The signals transmitted from this clusters include activation of tyrosine kinases, such as focal adhesion kinase (FAK) or Src, and

activation of serine/threonine kinases, such as ERK or AKT (Chen *et al.* 1994, Schlaepfer *et al.* 1994, King *et al.* 1997).

Rho GTPases also seem to contribute to integrin signaling transduction, and their activation induces actin cytoskeleton alterations. An important example of the involvement of integrins in cell migration is the presence of β1 integrin in the RMS, as described by Jacques and collaborators (Jacques *et al.* 1998). Furthermore, it has been shown that absence of β1 integrin perturbs the RMS, which appears less compact and with cells no longer assembled into chains. It was also founded defects in the size of the OB and in cell migration in this animal model. The OB was significantly reduced in size in the absence of β1 integrin, and the migration was also impaired in this case (Belvindrah *et al.* 2007).

3.3. Rho GTPases in cell migration

The Rho family of GTPases has been shown to regulate many intracellular events, such as morphogenesis, polarity, movement and cell division. These GTPases are present in all eukaryotic cells and are part of the Ras superfamily of small (21 kDa) signaling G proteins. There are at least twenty two different Rho GTPases, being the most important for cell migration the Ras homolog gene family-member A (RhoA), cell division control protein 42 (Cdc42) and Ras-related C3 botulinum toxin substrate 1 (Rac1) (Jaffe & Hall 2005). Rho GTPases present a cycling switch between their active and inactive states. In their active state, they are bound to guanosine triphosphate (GTP), and in the inactive state to guanosine diphosphate (GDP). It is their intrinsic phosphatase activity that hydrolyses GTP to GDP that leads to the turning "off" of the protein. The transition between this two states is controlled by: a) guanine nucleotide exchange factors (GEFs) that allow

the exchange of GDP to GTP, activating the switch (Schmidt & Hall 2002), b) GTPase-activating proteins (GAPs), which inactivate the switch under the stimulation of intrinsic GTPase activity (Bernards 2003), and by c) guanine nucleotide dissociation inhibitors (GDIs), which seem to block spontaneous activation of Rho GTPases (Olofsson 1999) (Figure 7). Thus, Rho GTPases are known as "molecular switches" playing their cellular functions through the activation of numerous downstream effectors. There are many effector proteins described for Rho, Rac and Cdc42. These include serine/threonine kinases, tyrosine kinases, formins, families of WASp proteins, as example (Bishop & Hall 2000).

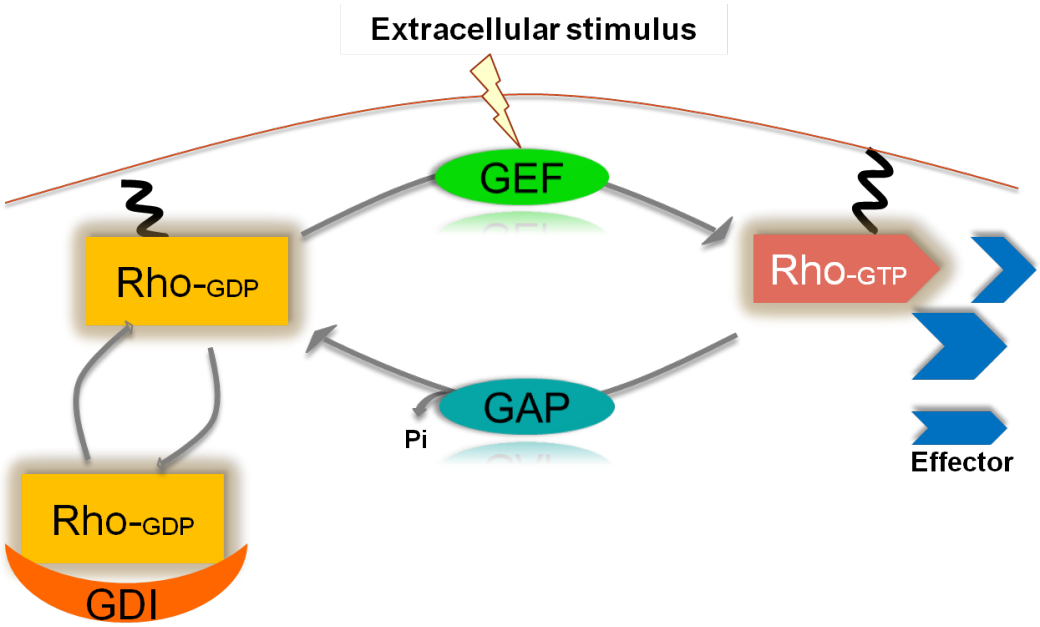

Figure 7 - Switch model for Rho GTPases activation. Rho GTPases cycle between an inactive state - GDP-bound form - and an active state - GTP-bound form. This GTPases are regulated by GEFs, GAPs and GDIs. When active, GTPases interact with effector proteins to mediate a response. Adapted from (Jaffe & Hall 2005).

Rho GTPases have been linked to the control of cytoskeletal organization, and are also involved cell morphology changes. RhoA has an active function in stimulating microfilament bundling, originating stress fibers, and is also essential for the formation of focal adhesion complexes that allow the generation of contractile forces at the rear of the cell, which make the cell body move forward (Hotchin & Hall 1995, Riento & Ridley 2003). RhoA also seems to have a controlling function, since it can inhibit inappropriate lateral protrusion, through the restriction of the formation of new integrin adhesion complexes (Worthylake & Burridge 2003). Rac1 is required to induce actin polymerization in the mobile edge of cells during migration and is responsible for controlling the formation of lamellipodia, which is a sheet-like structure consisting in a cross-linked meshwork of actin filaments (Ridley *et al.* 1992). Cdc42 mediates the formation of long, thin, actin-dependent extensions beyond lamellipodia, also known as filopodia, that allows the pushing forward of the leading edge of migrating cells (Nobes & Hall 1995). Filopodia and lamellipodia, represent two protrusive structures found in the leading edge of cells during migration. At the rear of the cell, it is necessary the contraction of the actin:myosin filaments that drive the forces for migration (Figure 8).

There is a connection between integrins, calpains and Rho GTPases. It has been shown that the presence of calpains is necessary for the formation of specific types of integrin clusters (Fox *et al.* 1993). Moreover, the activation of Rac1 seems to occur in integrin clusters, immediately after integrin-ligand interactions. Since calpains are located in integrin clusters, it has been suggested that they might be involved in the regulation Rho GTPases activity (Bialkowska *et al.* 2000).

Rho GTPases have an important role in controlling cell movement, as mentioned before. Two different types of migration have been described. Some cells present an isolated movement, as leukocytes that migrate towards chemotactic factors, while others present a coordinated migration, as NSC during neurogenesis (Lois et al. 1996). The dynamic of the cytoskeleton is extremely important to allow these different kinds of migration.

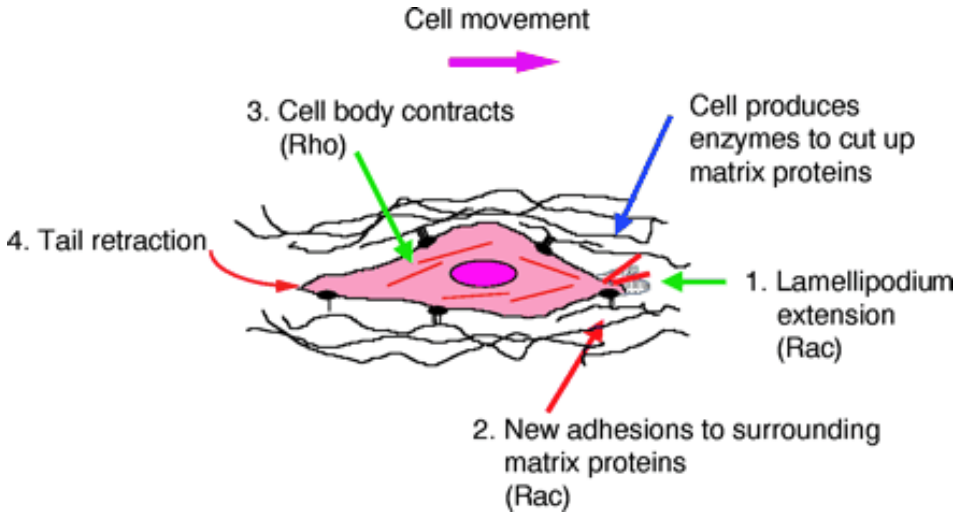

Figure 8 - Representative scheme of migration steps. Extension of lamellipodium at the front of the cell; stabilization of the structure through the formation of new adhesions with the extracellular matrix; forward movement of the cell body through actin:myosin contraction; retraction of the tail of the cell after cell detachment. Adapted from (Ridley 2001).

3.4. ERK in cell migration

ERK is part of the MAPK signaling pathway. There are two isoforms of ERK, p44 (ERK-1) and p42 (ERK-2), and both present a Thr-Glu-Tyr motif within the activation loop of the kinase domain. ERK activity can be stimulated by numerous growth factors and mitogens (Johnson & Lapadat 2002). ERK has been described as being involved in the migration of various cell types. The inhibition of

ERK, for example, inhibits the migration of platelets, in response to fibronectin stimuli (Anand-Apte *et al.* 1997), even in the presence of vitronectin. Moreover, collagen migration does not occur when ERK is inhibited (Klemke *et al.* 1997, Webb *et al.* 2000), which indicates that ERK has an important role in the regulation of cell migration. Different substrates of ERK have been identified, such as nuclear proteins, transcription factors, paxilin and calpains. Furthermore, the activity of m-calpain can be stimulated by ERK, which seems to be required for adhesion turnover and cell migration (Glading et al. 2004). However, the relationship between calpain and ERK in regulation cell migration is still unclear.

4. Objectives

Although there is no information regarding the involvement of calpain signaling in the proliferation and migration of NSC during neurogenesis in the adult CNS, calpains have been already described as being involved in the proliferation and migration of cells in other systems. Moreover, previous results of our group demonstrated that inhibition of calpains increases the migration rates of cultured NSC and SVZ explants. However, the signaling pathways involved in this process remain to be identified. Therefore, in this work our aim was to analyze the effect of calpain modulation in adult NSC migration and the signaling pathways by which calpains modulate this process, using NSC isolated from the SVZ and treated with calpain inhibitors and/or different signaling pathways inhibitors. Furthermore, cell migration will be analyzed in brain sections from wild-type (WT) and calpastatin-deficient mice (CSTN), by looking at the immunoreactivity of DCX.

Chapter II

Material and Methods

1. Materials

Dulbecco's Modified Eagle's Medium: F-12 nutrient mixture, (DMEM/F12 with GlutaMAX™ I), B27 supplement, antibiotic (10,000 units/ml penicillin, 10 mg/ml stretomycin), gentamicin, trypsin-EDTA, were purchased from GIBCO BRL, Life Technologies (Invitrogen, Paisley, UK). Phenylmethylsufonyl fluoride (PMSF), dithiothreitol, orthovanadate, chymostatin, leupeptin, leuptin, antiparin, pepstatin A, Tween-20, tetramethylethylenediamine (TEMED) and trypan blue were purchased from Sigma Chemical (St Louis, MO, USA). Epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) were from PeproTech Inc. (London, UK) and the matrix from Stoelting Co. (Wood Dale, IL, USA). Slides, coverslips and Tris base were obtained from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Calpeptin, MDL28170, NCS23766, EHT1864 and ML141 were purchased from Tocris Bioscience (Bristol, UK), Echistatin from Sigma Aldrich (St. Louis, MO, USA). Laminin was obtained from Roche (Mannheim, Germany), U0126 from Cell Signaling Technology, Inc. (Danvers, MA, USA) and DAKO fluorescence mounting medium was obtained from DAKO (Glostrup, Denmark). Bovine serum albumin (BSA) was purchased from Calbiochem (San Diego, CA, USA). Hoechst 33342 dye and phalloidin were obtained from Molecular Probes (Leiden, The Netherlands). Goat anti-DCX was acquired from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit anti-goat IgG conjugated with Alexa Fluor 594 secondary antibody was purchased from Molecular Probes (Leiden, Netherlands). G-LISA™ Rac1 Activation Assay Biochem Kit™ was purchased from Cytoskeleton (Denver, CO, USA). Bicinchoninic acid (BCA) Protein Assay kit was obtained from Pierce (Rockford, IL, USA). Polyvinylidene difluoride (PVDF) membranes and enhanced chemifluorescence (ECF) were from Amersham Pharmacia Biotech

(Buckinghamshire, UK). Bis-acrilamide and other reagents used in immunoblotting were purchased from Bio-Rad Laboratories Inc. (Hercules, CA, USA). All the primary and secondary antibodies used for Western blot analysis are described in Table II.

Table II - Primary and secondary antibodies used in Western blot experiments.

Antibody	Host	Dilution	Origin
Anti-Erk 1/2	Rabbit	1:1 000	Cell signaling Technology, Inc. (Danvers, MA, USA)
Anti-phospho-Erk 1/2	Rabbit	1:1 000	Cell signaling Technology, Inc. (Danvers, MA, USA)
Anti β-tubulin	Mouse	1:10 000	Signal Chemical (St Louis, MO, USA)
Anti-calpain 1	Rabbit	1:1 000	Santa Cruz Biotechnology (Santa Cruz, CA, USA)
Anti-calpain 2	Rabbit	1:1 000	Cell signaling Technology, Inc. (Danvers, MA, USA)
Alkaline phosphatase-conjugated anti-rabbit	Mouse	1:20 000	GE Healthcare Life Sciences (Buckinghamshire, UK)
Alkaline phosphatase-conjugated anti-mouse	Mouse	1:20 000	GE Healthcare Life Sciences (Buckinghamshire, UK)

2. Methods

2.1. *In vitro* experiments

2.1.1. Animals

C57BL/6J mice were obtained from Charles River (Barcelona, Spain) and kept in our animal facilities with food and water *ad libitum* in a 12 hours dark:light

cycle. All experiments were performed in accordance with institutional and European guidelines (86/609/EEC) for the care and use of laboratory animals.

2.1.2. Subventricular zone cultures

Neural stem cell cultures were obtained from C57BL/6J mice (0-3 days old), as previously described (Carreira *et al.* 2010). Brains were removed from the skull, following decapitation, and placed in dissection medium composed of Ca^{2+} and Mg^{2+}-free Hank's balanced salt solution (HBSS) (137 mM NaCl, 5.36 mM KCl, 0.44 mM KH_2PO_4, 4.16 mM $NaHCO_3$, 0.34 mM $Na_2HPO_4.2H_2O$ and 5 mM glucose, supplemented with 0.001% phenol red, 1 mM sodium pyruvate and 10 mM HEPES, pH 7.4), supplemented with 0.25% gentamicin. Then, the meninges were removed and brains were sectioned in 1 mm thickness coronal slices with a mouse brain matrix, from which the SVZ was excised. The sections were kept in 0.24% gentamicin/HBSS and the SVZ was isolated from each section. The fragments of SVZ in 0.24% gentamicin/HBSS were then digested in 0.025% trypsin/0.265 mM EDTA, for 15-20 min at 37ºC, washed with 0.24% gentamicin/HBSS, and then mechanically dissociated by gentle dissociation with a pipette tip. The cells were ressuspendend in D-MEM/F12 with GlutaMAX™-I, supplemented with 1% B27, 1% antibiotic (Pen/Strep, 10,000 units/ml of penicillin, 10 mg/ml streptomycin), 10 ng/ml EGF and 5 ng/ml basic FGF, and plated on uncoated flasks with filter caps, at a density of 100,000 cells/ml. Cell viability was evaluated by 0.1% Trypan Blue exclusion assay. SVZ-derived neural stem cells were grown as floating aggregates in a 95% air/5% CO_2 humidified atmosphere at 37ºC, for about 7 days. Then, the primary neurospheres were harvested, centrifuged and mechanically dissociated as single cells. Cells were replated as

above and allowed to grow as secondary neurospheres. Six-seven days later, the floating neurospheres were collected and plated on 0.1 mg/ml poly-*L*-lysine and 10 µg/ml laminin-coated 16-mm diameter glass coverslips, for migration assays, or on 12-well plates for preparation of lysates and maintained with the same medium as before.

2.1.3. Experimental treatments

SVZ cells were left for 30 min and then treated with different inhibitors as follows. For the migration assays, SVZ-derived NSC were treated for 4 h with different stimuli, described as follows: calpain inhibitors, MDL28170 10 and 25 µM and calpeptin 10 and 25 µM; RhoA inhibitor, Y27632 10 µM; Rac1 inhibitors, EHT1864 1, 10 and 20 µM and NCS23766 50, 100 and 200 µM; Cdc42 inhibitor, ML141 1, 10 and 50 µM; MEK 1/2 inhibitor, U0126 1 µM; or β1 and β3 integrins inhibitor, Echistatin 10 nM. The calpain inhibitors were applied alone or together with the different Rho GTPase inhibitors or the MEK 1/2 inhibitor.

To analyze the activity of Rac1 and Cdc42 GTPases, EHT1864 10 µM or ML141 1 µM, respectively, were applied alone or together with the calpain inhibitors MDL28170 10 µM or calpeptin 10 µM, for 30 min.

All the experiments were performed together with the respective controls (untreated cells).

2.1.4. Migration assay

After treatment, cells were washed with phosphate-buffered saline 0.01 M (PBS, 7.8 mM $Na_2HPO_4.2H_2O$, 2.7 mM $NaH_2PO_4.H_2O$, 154 mM NaCl, pH 7.2),

and fixed with 4% paraformaldehyde/4% sucrose in PBS 0.01 M, for 20 min, and washed again with PBS 0.01 M. Nuclei were stained with Hoechst 33342 (2 µg/ml) for 3 min, protected from light. Cells were washed with PBS 0.01 M and mounted in uncoated glass slides with DAKO fluorescence mounting medium. The migration radia of SVZ-derived NSC were measured in images acquired by phase contrast microscopy (Axioskop 2 Plus, Zeiss, Jena, Germany) with the AxioVision software Rel. 4.8. The migration distance of five different cells was measured and the average value was used (Figure 9). Each experimental condition was evaluated by the analysis of 20 neurospheres.

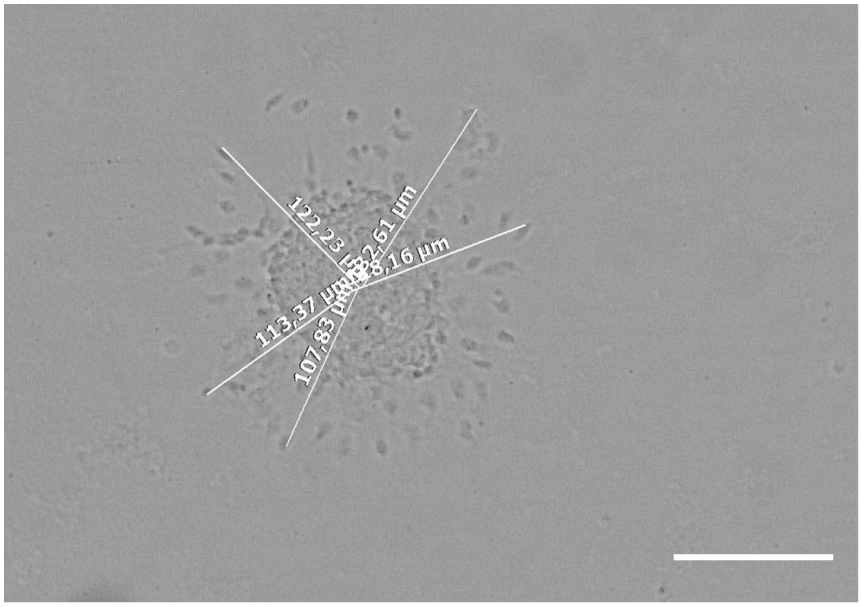

Figure 9 - Representative image, acquired by phase contrast microscopy, of the determination of migration distance of five different cells. Scale bar: 100 µm.

2.1.5. Preparation of lysates

SVZ cells were washed with PBS 0.01 M and whole cell extracts were prepared by lysing the cells in 100 mM Tris-HCl, 10 mM ethylene glycol tetraacetic

acid, 1% Triton X-100 and 2 mM $MgCl_2$, supplemented with 200 µM phenylmethanesulphonyl fluoride, 1 mM dithiothreitol, 1 µg/ml CLAP (chymostatin, pepstatin, antipain and leupeptin), 1 µM sodium orthovanadate and 5 mM sodium fluoride, pH 7.4, at 4ºC. Three freezing/thawing cycles followed by 5 seconds sonication cycles were applied, separated by 2 seconds. Protein concentration was determined by the BCA method, according to the manufacturer's instructions. Cell lysates were then used for Rho GTPase activity assays. For Western blot experiments, 6X concentrated sample buffer (0.5 M Tris-HCl/0.4% SDS pH 6.8, 30% glycerol, 10% SDS, 0.6 M dithiothreitol, 0.012% bromophenol blue) was added and samples were denatured at 95ºC for 5 min.

2.1.6. Rho GTPase activity

Rac1 activity was determined using a commercially available kit (G-LISA™ Rac1 Activation Assay Biochem Kit™). This method is based on interactions of Rac-GTP-binding protein with active Rac1, while inactive Rac1 is removed by washing. Detection of active Rac1 was performed by using a Rac1 specific antibody. The protein concentration was determined by the bicinchoninic acid method, using the BCA protein kit, according to the manufacturer's instructions.

2.1.7. Western blot analysis

Samples for Western blot analysis were electrophoresed in SDS-polyacrylamide gels using MiniPROTEAN® 3 systems. Resolving gels were composed by 12% bis-acrylamide, 25% Tris-HCl 1.5 M pH 8.0, 0.1% SDS, 0.05% TEMED and 0.05% ammonium persulfate, in milliQ water, for all Western blot

experiments. Stacking gels were composed by 4% bis-acrylamide, 25% Tris-HCl 0.5 M pH 6.8, 0.1% SDS, 0.05% TEMED and 0.05% ammonium persulfate, in milliQ water. Equal amounts of protein were applied on each lane of the SDS-polyacrilamide gels submerged in a running buffer (25 mM Tris, 25 mM bicine and 0.1% SDS, in milliQ water). Proteins were separated by electrophoresis, firstly at 60 V for 10 min, followed by 120 V until proper bands separation were reached. The polyvinylidene difluoride membranes were activated, first in 100% methanol (2.5 to 5 min), followed by water (2.5 to 5 min) and, finally, 15 to 30 min in electrotransference buffer (CAPS 10 mM, methanol 10%, pH 11.0). Proteins were electrophoretically transferred to the activated membranes, submerged in electrotransference buffer at 750 mA, for 90 min, at 4ºC, using the Trans-Blot Cell apparatus. Membranes were blocked for 1 h, at room temperature, with Tris-buffered saline (137 mM NaCl, 20 mM Tris-HCl, pH 7.6) containing 0.1% Tween-20 (TBS-T) and 3% BSA. Incubations with the primary antibodies (rabbit anti-phospho-ERK 1/2; rabbit anti-calpain 1 (sc-7531) and rabbit anti-calpain 2, 1:1000) in TBS-T containing 1% blocking solution were performed overnight, at 4ºC. After rinsing with TBS-T (20 min, with 2 quick washes before and after), incubation with the appropriated alkaline phosphatase-linked secondary antibodies (anti-rabbit or anti-mouse, 1:20 000 in TBS-T containing 1% blocking solution) was performed at room temperature, during 1 h. After extensive washing in TBS-T (for 1 h, changing into new TBS-T every 20 min), followed by the incubation of the membranes with the enhanced chemifluorescence reagent for the maximum of 5 min, immunoreactive bands were visualized in the VersaDoc 3000 imaging system (Bio-Rad Laboratories Inc., Hercules, CA, USA). Data were analyzed with the Quantity One software version 4.6.9 (Bio-Rad Laboratories Inc., Hercules, CA,

USA). Protein control loadings were performed after membranes reactivation (5-10 seconds in 100% methanol and 20 min in TBS-T), using primary antibodies against rabbit ERK 1/2 (1:1000) or mouse β-tubulin (1:10 000). The protocol used was the same as explained above.

2.1.8. Actin cytoskeleton labeling with phalloidin

Following fixation and permeabilization, non-specific binding was blocked with 3% BSA. After this process, nuclei were stained with Hoechst 33342 (2 μg/ml) and actin cytoskeleton with phalloidin (1:50) for 1 h, protected from light. Then, coverslips were mounted on glass slides with DAKO fluorescence mounting medium. Images were acquired in a laser scanning microscope LSM 510 META (Zeiss, Jena, Germany).

2.1.9. Statistical analysis

Data are expressed as means ± SEM. Statistical significance was determined using two-tailed t-test or one-factor analysis of variance (ANOVA) as appropriate, followed by post hoc Bonferroni's or Dunnett's test, as indicated in the figure legends and in the text. Differences were considered significant when $p<0.05$. The software used was GraphPad Prism 5.0 (GraphPad Software, La Jola, CA, USA).

2.2. *In vivo* experiments

2.2.1. Animal models

C57BL/6J male WT and calpastatin knockout (CSTN) (Takano *et al.* 2005) mice with 6 months were used in this study. Takano and colleagues (Takano et al. 2005) used a targeting vector for the CSTN animals, containing the following deoxyribonucleic acid (DNA) fragments: a 1.5-kb SphI fragment from intron 4 to exon 6, three tandem repeats of 250-bp SV40 early mRNA polyadenylation signals to terminate transcription, a 5-5-kb SphI-ScaI calpastatin gene fragment from intron 6 to intron 8, a 2.0-kb *pgk-neo* gene cassette (for positive selection), and a 1.0-kb SacI-NotI diphtheria toxin A fragment cassette from pMC1DT-A (for negative selection). Polyadenylation signals were placed in exon 6 to inhibit transcription of the calpain-inhibitory domains (Takano et al. 2005). C57BL/6J WT mice were obtained from Charles River (Barcelona, Spain) and kept in our facilities. All animals were kept with food and water *ad libitum* in a 12 hours dark:light cycle. All experiments were performed in accordance with institutional and European guidelines (86/609/EEC) for the care and use of laboratory animals.

2.2.2. Experimental treatments

The animals were perfused transcardially on day 12, with 0.9% NaCl followed by 4% paraformaldehyde in PBS, following deep anesthesia with Eutasil (20% sodium pentobarbital). Brains were removed and kept overnight in 4% paraformaldehyde for further fixation, and then dehydrated in 20% sucrose/0.2 M phosphate buffer (PB, 48 mM $NaH_2PO_4.H_2O$, 152 mM $Na_2HPO_4.2H_2O$, pH 7.2), at 4ºC. Sagittal sections from the striatum region were cryosectionated (30 µm thick,

in 6-series) and stored in an antifreeze solution (0.05 M PB, 30% ethylene glycol, 30% glycerol), at 4ºC.

2.2.3. Immunohistochemistry

Free-floating sagittal sections from the striatum region were processed for immunohistochemistry against DCX. Brain sections were blocked for 1 h with 5% normal horse serum (NHS) in 0.25% Triton X-100 in PBS. Slices were then incubated with the primary antibody goat anti-DCX (1:400), overnight at room temperature or 48 h at 4ºC. After rinsing with 0.25% Triton X-100 in PBS and with 2% block solution (NHS), the sections were incubated with the correspondent secondary antibody (1:200), in 2% block (NHS), conjugated with Alexa Fluor 594, for 2 h in the dark, at room temperature. After rinsing with 0.25% Triton X-100 in PBS and with PBS alone, the sections were mounted in 2% gelatin-coated slides with DAKO fluorescence mounting medium.

2.2.4. Doublecortin immunoreactivity

The immunoreactivity of doublecortin in the RMS was quantified using an image analysis software (ImageJ, version 1.43u). The images were acquired in an inverted research microscope (Zeiss ObserverZ1, Zeiss, Jena, Germany), with the Axio CAM HRm and with the AxioVision software Rel. 4.8.2 through module Mosaix. Immunopositive staining was measured for each image, in 3 boxes with 250 µm x 250 µm, randomly placed along the length of the RMS, as previously described by Kuhn and collaborators (Kuhn *et al.* 1997) (Figure 10). Data are reported in immunopositive area (μm^2).

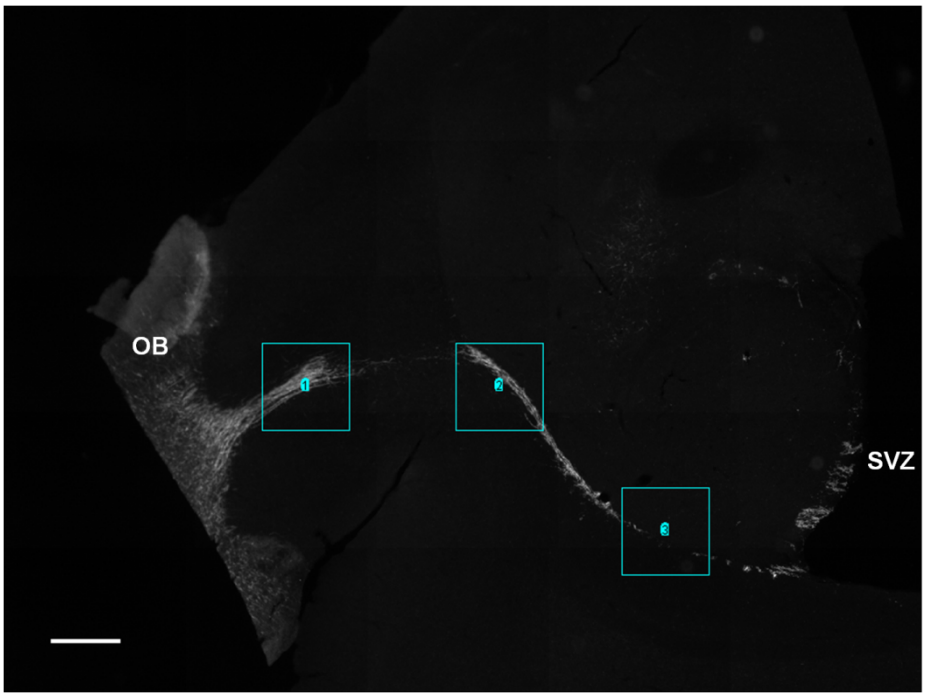

Figure 10 – DCX immunoreactivity determination. Representative image of DCX immunoreactivity along RMS. Three boxes with 250 µm x 250 µm were distributed along the RMS and the immunoreactivity of those areas was determined using image analysis software (ImageJ, version 1.43u). The immunoreactivity is reported in immunopositive area (μm^2). Scale bar: 200 µM.

2.2.5. Statistical analysis

Data are expressed as means ± SEM. Statistical significance was determined using two-tailed t-test, as indicated in the figure legends and in the text. Differences were considered significant when $p<0.05$. The software used was GraphPad Prism 5.0 (GraphPad Software, La Jola, CA, USA).

Chapter III

Results

1. *In vitro* modulation of SVZ-derived NSC migration by calpains

1.1. µ-calpain and m-calpain are present in NSC isolated from the SVZ

The presence of both µ-calpain and m-calpain isoforms in SVZ cell cultures was evaluated by Western blot analysis (Figure 11). We observed that both calpain isoforms were present in SVZ-derived NSC lysates.

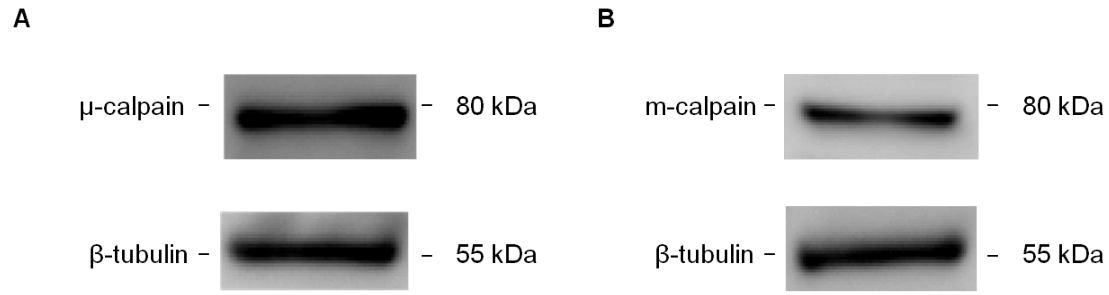

Figure 11 - µ-calpain and m-calpain are present in NSC from the SVZ. µ-calpain **(A)** and m-calpain **(B)**, respectively, were detected in SVZ cultures by Western blot, using 15 µg of protein. β-tubulin was used as a loading control.

1.2. Laminin is essential for SVZ-derived NSC migration

To assess the involvement of laminin in SVZ-derived NSC migration, SVZ cells were plated in 16-mm glass coverslips coated with poly-*L*-lysine with or without laminin, for 4 h.

In the absence of laminin, migration of NSC was significantly decreased (70.72 ± 4.08 µm, $p<0.001$) after 4 h, when comparing to cells plated in 16-mm glass coverslips coated with poly-*L*-lysine and laminin (181.80 ± 7.46 µm) (Figure 12C).

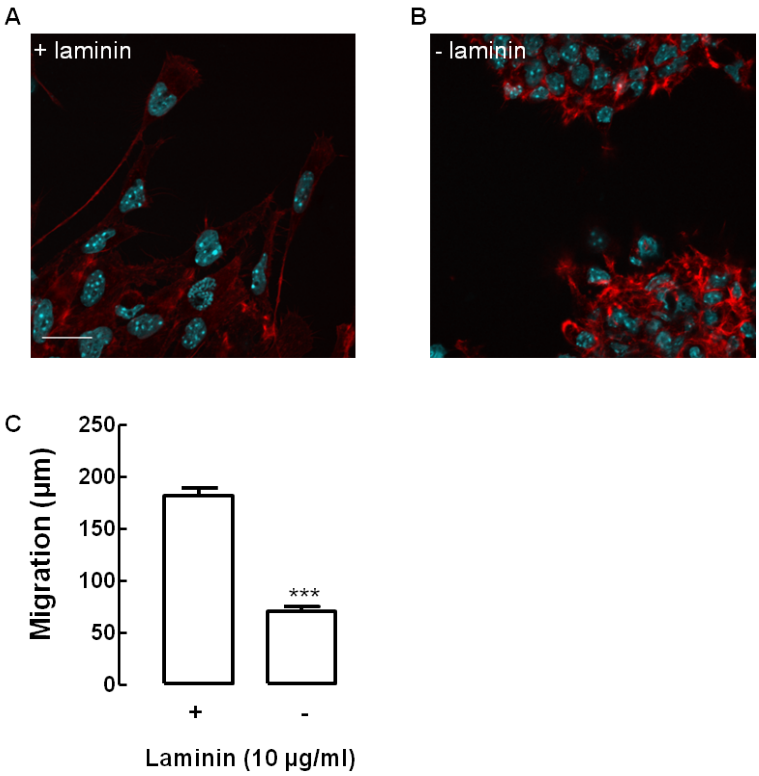

Figure 12 - Laminin is essential for SVZ-derived NSC migration. Representative laser scanning confocal images of actin cytoskeleton of SVZ NSC cells labeled against phalloidin, in red, after 4 h in the presence **(A)** and absence **(B)** of laminin. Nuclei, in blue, were labeled with Hoechst 33342. Cells were left for 4 h in the presence or absence of laminin, and the migration radia were measured in images acquired by phase contrast microscopy. The means ± SEM of four independent experiments were analyzed by two-tailed t-test, ***p<0.001 (significantly different from cells in contact with laminin) **(C)**. Scale bar: 20 µm.

1.3. SVZ-derived NSC migration is increased by calpain inhibition

With the purpose of studying the effect of calpain inhibition on NSC migration, SVZ-derived NSC were treated with the inhibitors MDL28170 and calpeptin, for 4 h. Two different concentrations were tested for both inhibitors (10 µM and 25 µM). Cell migration was analyzed in images acquired by phase contrast microscopy (Figure 13A, B and C).

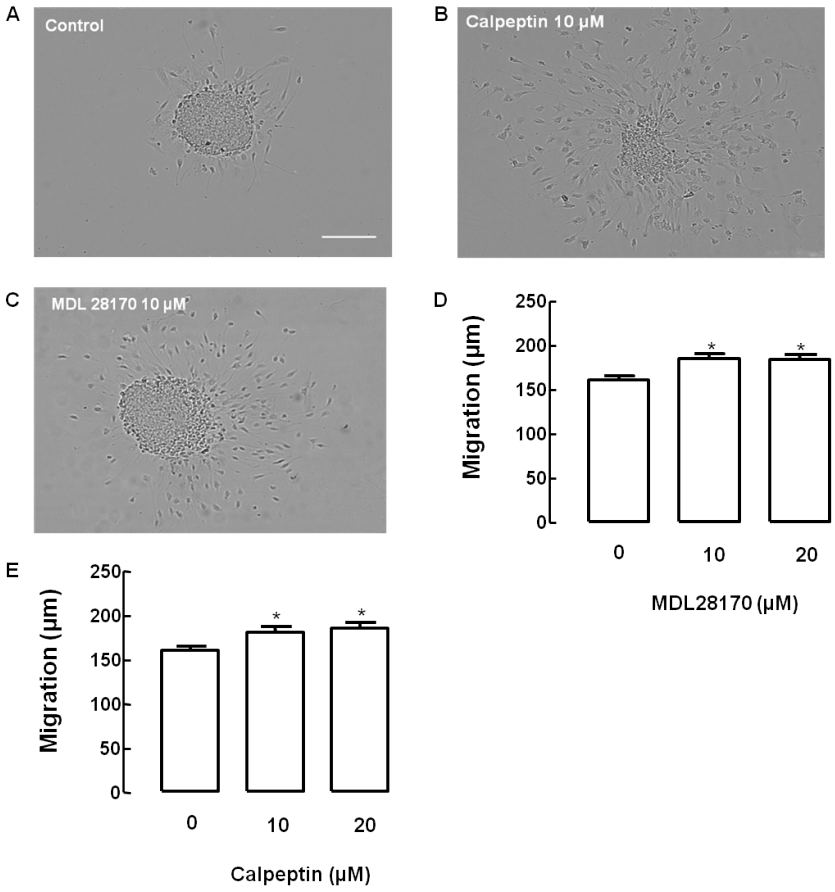

Figure 13 - SVZ-derived NSC migration is increased by calpain inhibition. (A), **(B)** and **(C)** - Representative images showing the migration of SVZ-derived NSC for the different conditions. Cells were treated for 4 h with the calpain inhibitors MDL 28170 **(D)** and calpeptin **(E)**. The means ± SEM of at least five independent experiments were analyzed by one-way ANOVA (Dunnett's post-test), *p<0.05 (significantly different from control). Scale bar: 100 µm.

Both concentrations tested of MDL28170 (10 µM, 184.79 ± 6.03 µm, *p<0.05; 25 µM, 184.18 ± 5.99 µm, *p<0.05; Figure 13D) and calpeptin (10 µM, 182.42 ± 7.12 µm, *p<0.05; 25 µM, 191.50 ± 2.23 µm, **p<0.01; Figure 13E), significantly increased cell migration when compared to control (untreated cells) (161.08 ± 4.55 µm). Neither MDL28170 (10 µM, 17.68 ± 2.77%; 25 µM, 17.99 ± 3.16%, p>0.05) nor calpeptin (10 µM, 17.47 ± 2.60%; 25 µM, 17.53 ± 2.079%, p>0.05) had a cytotoxic effect in SVZ-derived NSC under the conditions tested, as assessed by the evaluation of the percentage of condensed nuclei, as compared

to untreated cells (16.59 ± 1.198%) (see Table III). According to these results, MDL28170 10 μM and calpeptin 10 μM were selected for all of the following experiments.

1.4. Integrins are involved in SVZ-derived NSC migration

Integrins are the major laminin receptors, being the α6β1 integrin receptor present at high levels on embryonic NSC and proliferating adult NSC (Kazanis *et al.* 2010, Staquicini et al. 2009). With the purpose of evaluating the involvement of integrins in the migration of SVZ-derived NSC, cultures were exposed to echistatin, a polypeptide disintegrin isolated from the venom of *Echis carinatus* (Gould *et al.* 1990). Disintegrins are the most potent inhibitors of integrin function. We observed that 10 nM echistatin decreased SVZ-derived NSC migration (118.01 ± 9.00 μm, **$p<0.01$) following 4 h of exposure, as compared to control (166.26 ± 5.45 μm) (Figure 14C).

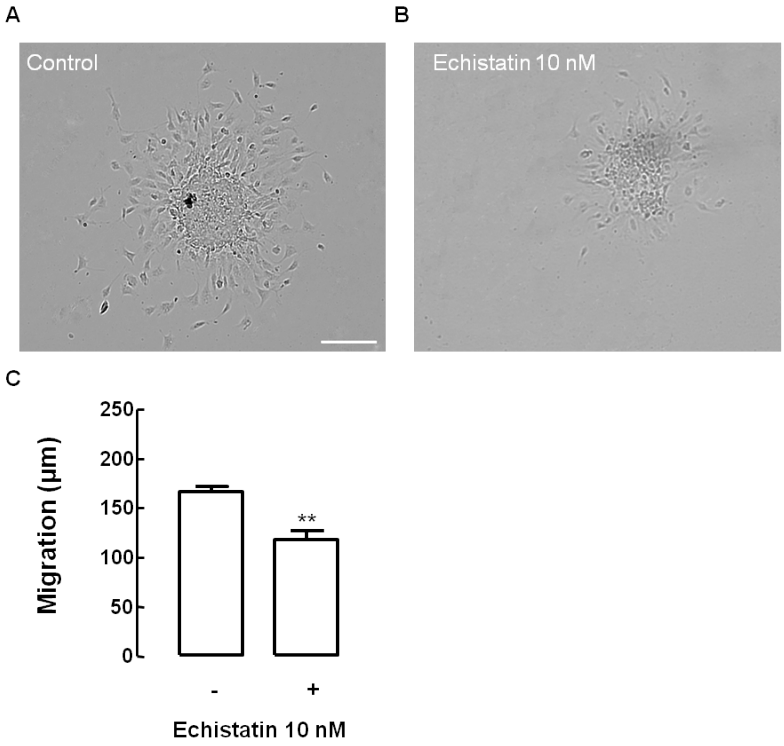

Figure 14 - Integrins are involved in SVZ-derived NSC migration. Cells were treated for 4 h with integrins inhibitor. Migration radia were measured in images acquired by phase contrast microscopy in the presence **(A)** and in the absence of integrins inhibitor **(B)**. The means ± SEM of at least four independent experiments were analyzed by two-tailed t-test and one-way ANOVA (Bonferroni's post-test), **p<0.01 (significantly different from control) **(C)**. Scale bar: 100 µm.

Furthermore, echistatin prevented the increased migration observed following calpain inhibition to MDL28170 (162.60 ± 5.85 µm, $^{++}$p<0.01), as compared to MDL28170 alone (184.79 ± 6.03 µm) (Figure 15F). Similar results were observed in the experiments where calpains were inhibited by calpeptin. Although echistatin did significantly prevent the increased migration observed following calpain inhibition with calpeptin, a clear tendency for a decrease in cell migration in the presence of both inhibitors (163.58 ± 5.33 µm, p>0.05) can be observed, when compared to calpeptin alone (181.25 ± 5.93 µm) (Figure 15G).

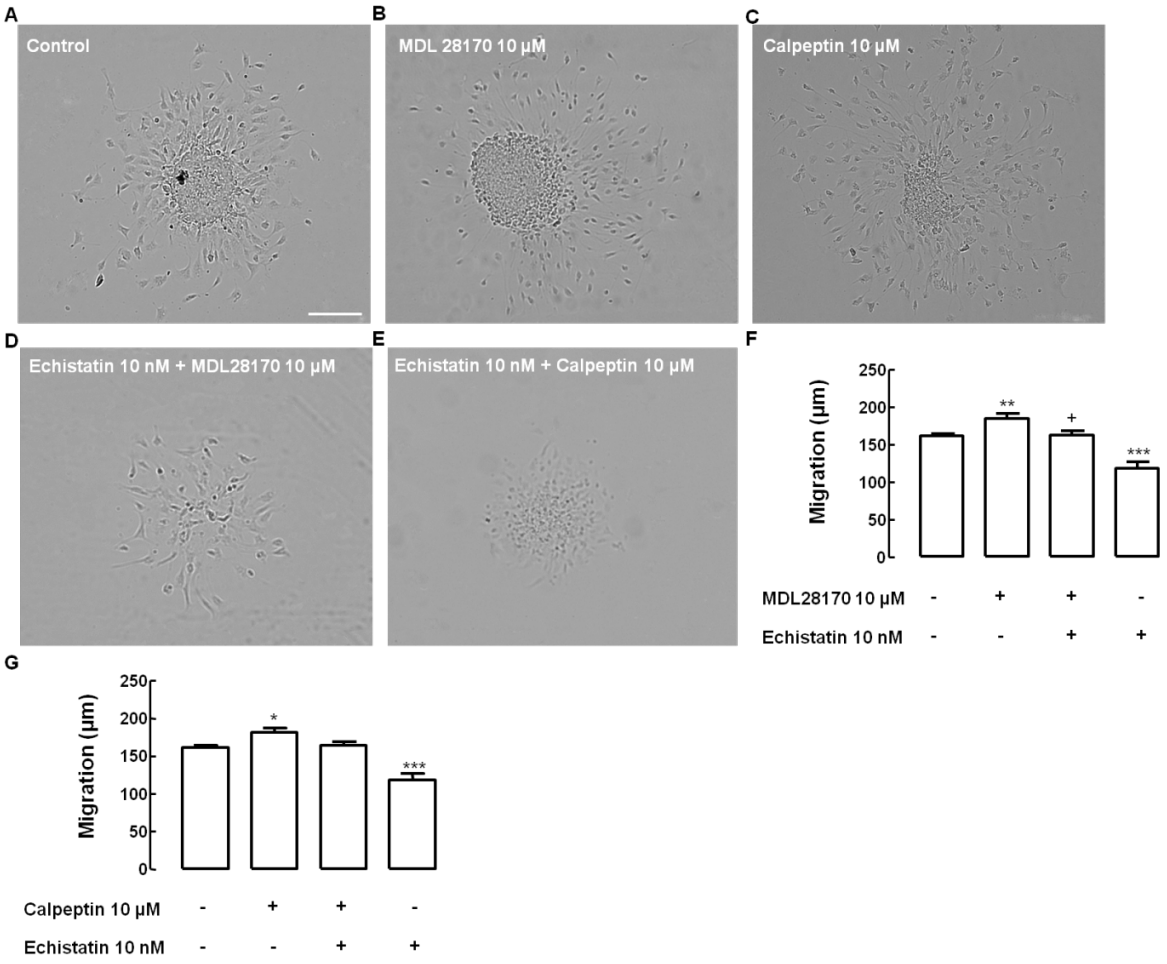

Figure 15 - Calpains acting through integrins. (A), **(B)**, **(C)**, **(D)** and **(E)** - Representative images showing the migration of SVZ-derived NSC for the different conditions. Cells were treated for 4 h with integrins inhibitor, or with calpain inhibitor, or with both. Migration radia were measured in images acquired by phase contrast microscopy. The means ± SEM of at least four independent experiments were analyzed by two-tailed t-test and one-way ANOVA (Bonferroni's post-test), *p<0.05, **p<0.01 and ***p<0.001 (significantly different from control), and $^{++}$p<0.01 (significantly different from MDL28170) **(F and G)**. Scale bar: 100 µm.

1.5. Involvement of Rho GTPases in SVZ-derived NSC migration

In order to study the involvement of Rho GTPases in SVZ-derived NSC migration, SVZ cells were treated with four different Rho GTPase inhibitors: the selective inhibitor of Cdc42 ML141 (1, 10 and 50 µM), the Rac1 inhibitors EHT1864 (high affinity) (1, 10 and 20 µM) and NSC23766 (low affinity) (50,100

and 200 µM) (Figure 16F, G and H) or the RhoA inhibitor Y27632 (Figure 16I). The effect of Rho GTPase inhibitors on the cell migration was evaluated by determining the migration radia in images acquired by phase contrast microscopy.

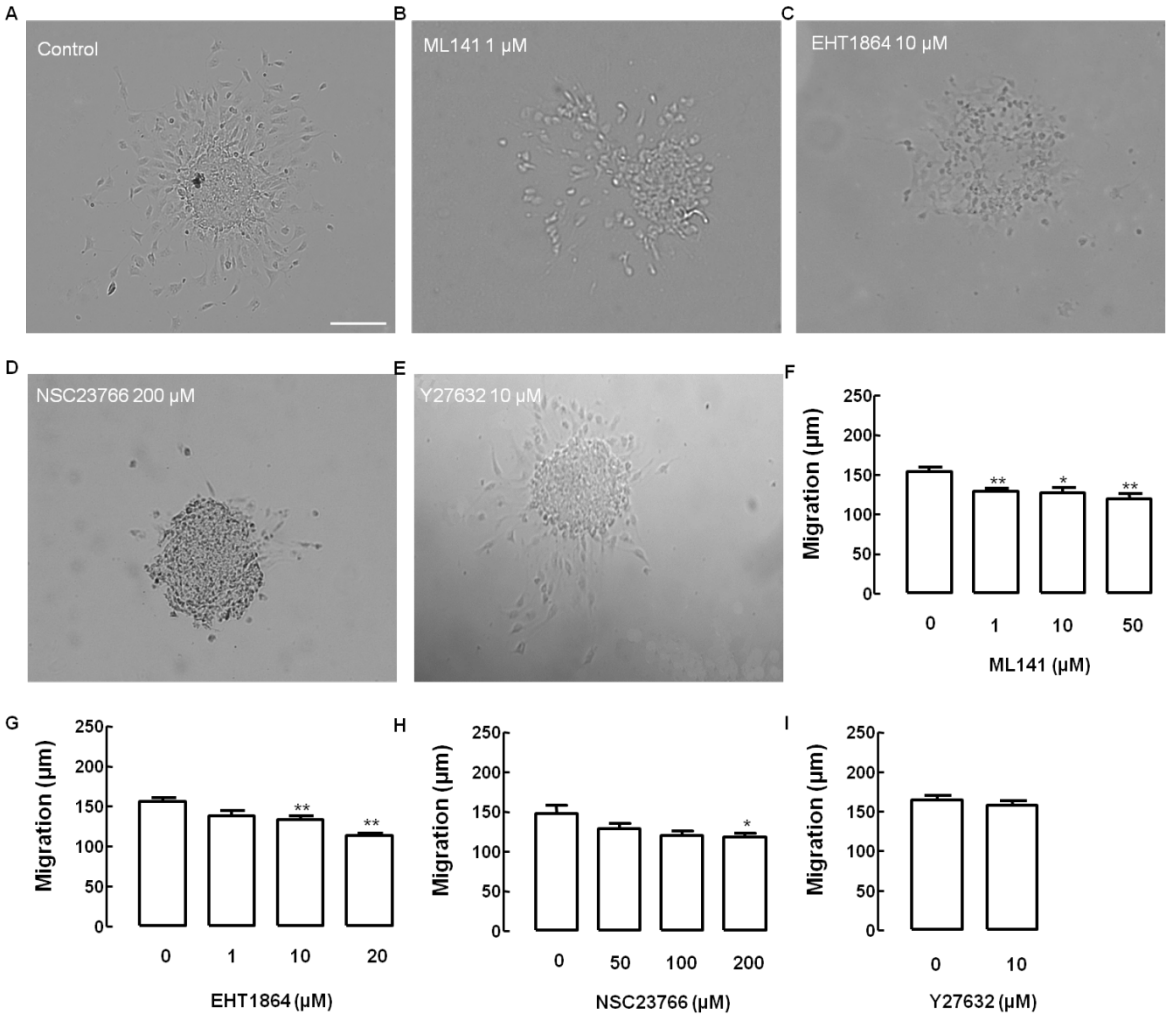

Figure 16 - Involvement of Rho GTPases in SVZ-derived NSC migration. (A), **(B)**, **(C)**, **(D)** and **(E)** - Representative images showing the migration of SVZ-derived NSC for the different conditions. Cells were treated for 4 h with 1 µM, 10 µM or 50 µM ML141 **(F)**, 1 µM, 10 µM or 20 µM EHT1864 **(G)**, 50 µM, 100 µM or 200 µM NSC23766 **(H)**, or 10 µM Y27632 **(I)**, and the migration radia were measured in images acquired by phase contrast microscopy. Data are expressed as means ± SEM of at least three independent experiments. Two-tailed t-test (**I**) and one-way ANOVA (**F, G** and **H** Dunnett's post-test), *p<0.05 and **p<0.01 (significantly different from control). Scale bar: 100 µm.

All concentrations of ML141 showed a significant decrease in cell migration (1 μM, 128.27 ± 4.32 μm, **p<0.01; 10 μM, 127.27 ± 6.44 μm, *p<0.05; 50 μM, 119.59 ± 6.33 μm, **p<0.01; Figure 16F), as compared to 153.52 ± 5.74 μm in untreated cells. Concerning Rac1, just two of the three concentrations tested for EHT1864, showed a significant decrease in cell migration (10 μM, 132.87 ± 5.37 μm; 20 μM, 113.34 ± 3.27 μm, **p<0.01; Figure 16G), when compared to untreated cells (155.91 ± 5.05 μm). When using NSC23766, only the higher concentration showed a significant decrease in cell migration (200 μM, 118.24 ± 4.80 μm; *p<0.01; Figure 16H).

None of the Rho GTPase inhibitors showed a cytotoxic effect as evaluated by assessing the percentage of condensed nuclei (see Table III). Based on these results, ML141 1 μM, EHT1864 10 μM and NSC23766 200 μM were chosen for all the following experiments. No changes in cell migration were observed following RhoA inhibition by Y27632 (157.9 ± 5.60 μm, p>0.05; Figure 16I), comparing to untreated cells (164.16 ± 6.09 μm).

Table III - Cell viability of NSC after treatment with different pharmacological inhibitors.

Treatment		% dead cells	% live cells
Control		29.239 ± 1.259 %	70.761 ± 1.259 %
MDL28170	10 μM	17.678 ± 2.767 % (n.s.)	82.321 ± 2.767 % (n.s.)
	25 μM	17.987 ± 3.157 % (n.s.)	82.013 ± 3.157 % (n.s.)
Calpeptin	10 μM	17.468 ± 2.596 % (n.s.)	82.531 ± 2.596 % (n.s.)
	25 μM	17.534 ± 2.079 % (n.s.)	82.466 ± 2.079 % (n.s.)
ML141	1 μM	27.725 ± 1.410 % (n.s.)	72.275 ± 1.409 % (n.s.)
	10 μM	28.484 ± 2.732 % (n.s.)	71.516 ± 2.732 % (n.s.)
	50 μM	29.672 ± 3.896 % (n.s.)	70.328 ± 3.896 % (n.s.)
EHT1864	1 μM	24.143 ± 1.184 % (n.s.)	75.857 ± 1.184 % (n.s.)
	10 μM	25.633 ± 1.369 % (n.s.)	74.367 ± 1.369 % (n.s.)
	20 μM	32.368 ± 1.897 % (n.s.)	67.632 ± 1.897 % (n.s.)
NSC23766	50 μM	23.108 ± 1.755 % (n.s.)	76.892 ± 1.755 % (n.s.)
	100 μM	25.549 ± 1.264 % (n.s.)	74.451 ± 1.264 (n.s.)
	200 μM	27.461 ± 1.476 % (n.s.)	72.539 ± 1.476 % (n.s.)
U0126	1 μM	27.534 ± 2.221 % (n.s.)	72.476 ± 2.221 % (n.s.)

Cell viability was assessed through analysis of nuclear morphology after treatment with two different calpain inhibitors (MDL28174 and calpeptin) and with three different Rho GTPase inhibitors (ML141, EHT1864 and NSC23766), at different concentrations, and after treatment with 1 μM of MEK 1/2 inhibitor (U0126). When nuclei were condensed/fragmented and brightly stained with Hoechst 33342, cells were considered dead. In the presence of a cell regular nuclear morphology and a light nuclear stain with bright nucleoli, cells were considered as live cells. The means ± SEM of at least five independent experiments were analyzed by one-way ANOVA, Dunnett's post-test. n.s. (non-significant) $p > 0.05$, not different from the control.

1.6. SVZ-derived NSC migration is regulated by Rho GTPases and calpains

We next investigated if Rho GTPases were involved in the migratory effect observed with calpain inhibition in SVZ-derived NSC. For that purpose, cell migration was evaluated following exposure to Rho GTPase inhibitors (ML141,

EHT1864 or NSC23766) in the presence of calpain inhibitors (MDL28170 or calpeptin), for 4 h.

We observed that both calpain inhibitors MDL28170 (184.79 ± 6.03 µm, **p<0.01) and calpeptin (184.87 ± 5.76 µm, **p<0.01) increased cell migration in comparison to control (154.98 ± 4.44 µm). On the other hand, the Cdc42 inhibitor ML141, alone, decreased cell migration (127.65 ± 4.95 µm, **p<0.01) as compared to control. Furthermore, the increased migration rates observed following calpain inhibition were prevented when ML141 was added to cultures (128.74 ± 4.62 µm; 124.70 ± 5.49 µm, $^{+++}$p<0.001, respectively), being the migration distances similar to those observed when Cdc42 is inhibited (ML141 alone), and significantly different from control (**p<0.01 and ***p<0.001, respectively) (Figure 17F and Figure 18F). An identical approach was made to study the involved of the Rac1 signaling pathway in the migratory effect observed with calpain inhibition in SVZ-derived NSC. Similar to what was described for Cdc42, calpain inhibitors MDL28170 (184.79 ± 6.03 µm, ***p<0.001) and calpeptin (184.87 ± 5.76 µm, **p<0.01) increased cell migration, in comparison to control. Furthermore, inhibition of calpains (with either MDL28170 or calpeptin) and Rac1 (with EHT 1864) prevented the increased migration following calpain inhibition (130.94 ± 2.70 µm; 115.09 ± 1.85 µm, $^{+++}$p<0.001, respectively) being the migration distances similar to those observed when Rac1 is inhibited (132.87 ± 5.37 µm, **p<0.01), and significantly different from control (**p<0.01 and ***p<0.001, respectively), (Figure 17G and Figure 18G). Similar results were obtained with the other Rac1 inhibitor, NSC23766 (Figure 17H and Figure 18H). In the presence of MDL28170 and NSC23766 together the increased migration following calpain inhibition was also inhibited (122.78 ± 5.68 µm; 123.96 ± 4.57

μm, $^{+++}$p<0.001, respectively), being the migration distance similar to those observed when Rac1 is inhibited 132.87 ± 5.37 μm, **p<0.01), and significantly different from control (***p<0.001).

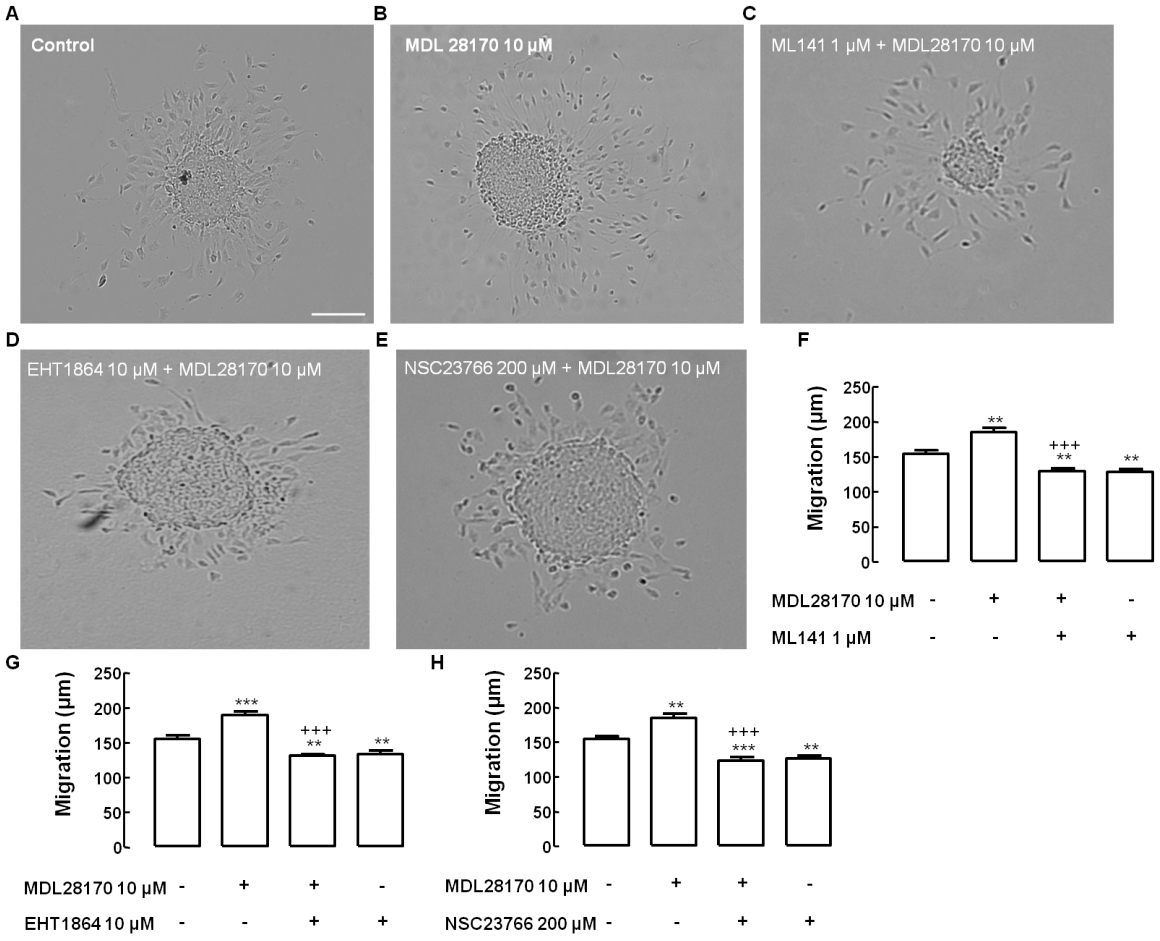

Figure 17 - SVZ-derived NSC migration in the presence of Rho GTPases and MDL28170. (A), **(B)**, **(C)**, **(D)** and **(E)** - Representative images showing the migration of SVZ-derived NSC for the different conditions. Cells were treated for 4 h with MDL28170 or with Rho GTPases inhibitors and with both. Migration radia were measured in images acquired by phase contrast microscopy. The means ± SEM of at least five independent experiments were analyzed by one-way ANOVA (Bonferroni's post-test), **p<0.01 and ***p<0.0019 (significantly different from control) and $^{+++}$p<0.001 (significantly different from MDL28170) **(F)**, **(G)** and **(H)**. Scale bar: 100 μm.

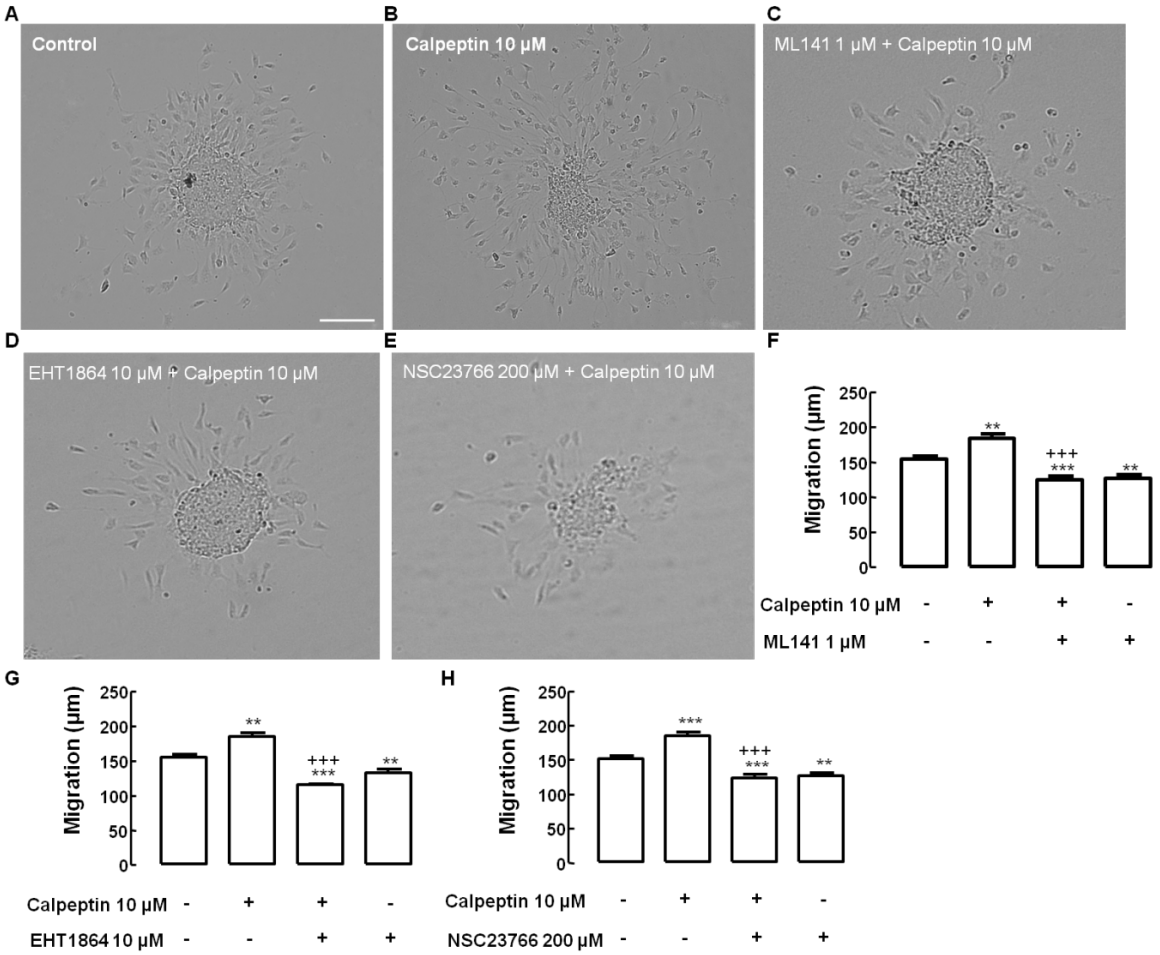

Figure 18 - SVZ-derived NSC migration in the presence of Rho GTPases and calpeptin. (A), **(B)**, **(C)**, **(D)** and **(E)** - Representative images showing the migration of SVZ-derived NSC for the different conditions. Cells were treated for 4 h with calpeptin or with Rho GTPases inhibitors and with both. Migration radia were measured in images acquired by phase contrast microscopy. The means ± SEM of at least five independent experiments were analyzed by one-way ANOVA (Bonferroni's post-test), **p<0.01 and ***p<0.001 (significantly different from control) and $^{+++}$p<0.001 (significantly different from calpeptin) **(E)**, **(F)**, **(G)** and **(H)**. Scale bar: 100 µm.

1.7. Rac1 activity is not altered in the presence of calpain inhibitors

Next, we studied of the involvement of Rac1 GTPase in the migratory effect observed with calpain inhibition in SVZ-derived NSC by assessing Rac1 activity by G-Lisa assay. We observed an increased activity in Rac1 activity 30 min following plating of SVZ-derived NSC (150.41 ± 16.86%, p>0.05), when compared to the

control (0 min). In the presence of EHT1864 or calpeptin, we observed a decreased activation of Rac1, although the differences observed were not significantly different when compared to migration rates observed 30 min following plating (125.36 ± 21.90% and 124.13 ± 29.31%, respectively, p>0.05) (Figure 19A and C). Moreover, calpain inhibition by MDL28170 does not alter Rac1 activity (154.58 ± 26.70%, p>0.05), when compared to the 30 min following plating (150.41 ± 16.86%, p>0.05) (Figure 19B).

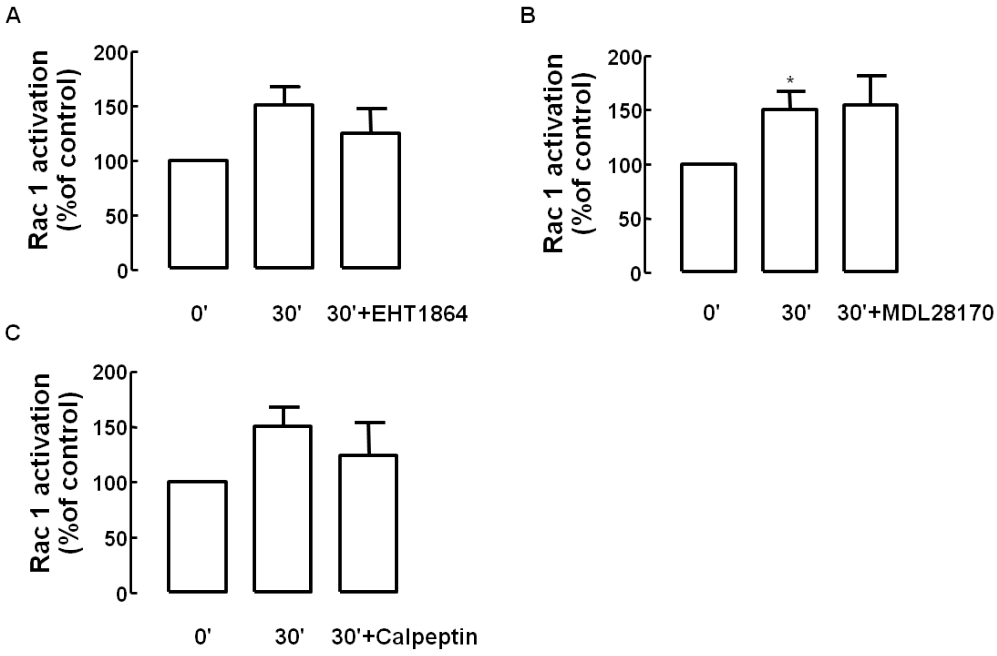

Figure 19 - Rac1 activity following 30 min of treatment with Rac1 inhibitor and calpain inhibitors. Rac1 activation levels following treatment with EHT1864 **(A)**, MDL28170 **(B)** or calpeptin **(C)** for 30 min were assessed by G-Lisa assay. The means ± SEM of at least five independent experiments were analyzed by one-way ANOVA (Bonferroni's post-test), *p<0.05 (significantly different from control).

1.8. ERK 1/2 pathway is not involved in SVZ-derived NSC migration

To evaluate the involvement of the MAPK/ERK 1/2 pathway in SVZ-derived NSC migration, cultures were treated with 1 μM U0126, a selective inhibitor of MEK 1/2, the kinase immediately upstream of ERK 1/2. In the presence of U0126, cell migration was not significantly altered (159.17 ± 8.95 μm, $p>0.05$) when compared to untreated cells (180.95 ± 5.84 μm) (Figure 20G). Moreover, no changes were observed in cell migration following inhibition of calpains (with either MDL 28170 or calpeptin) and MEK 1/2 (with U0126) (Figure 20H and I).

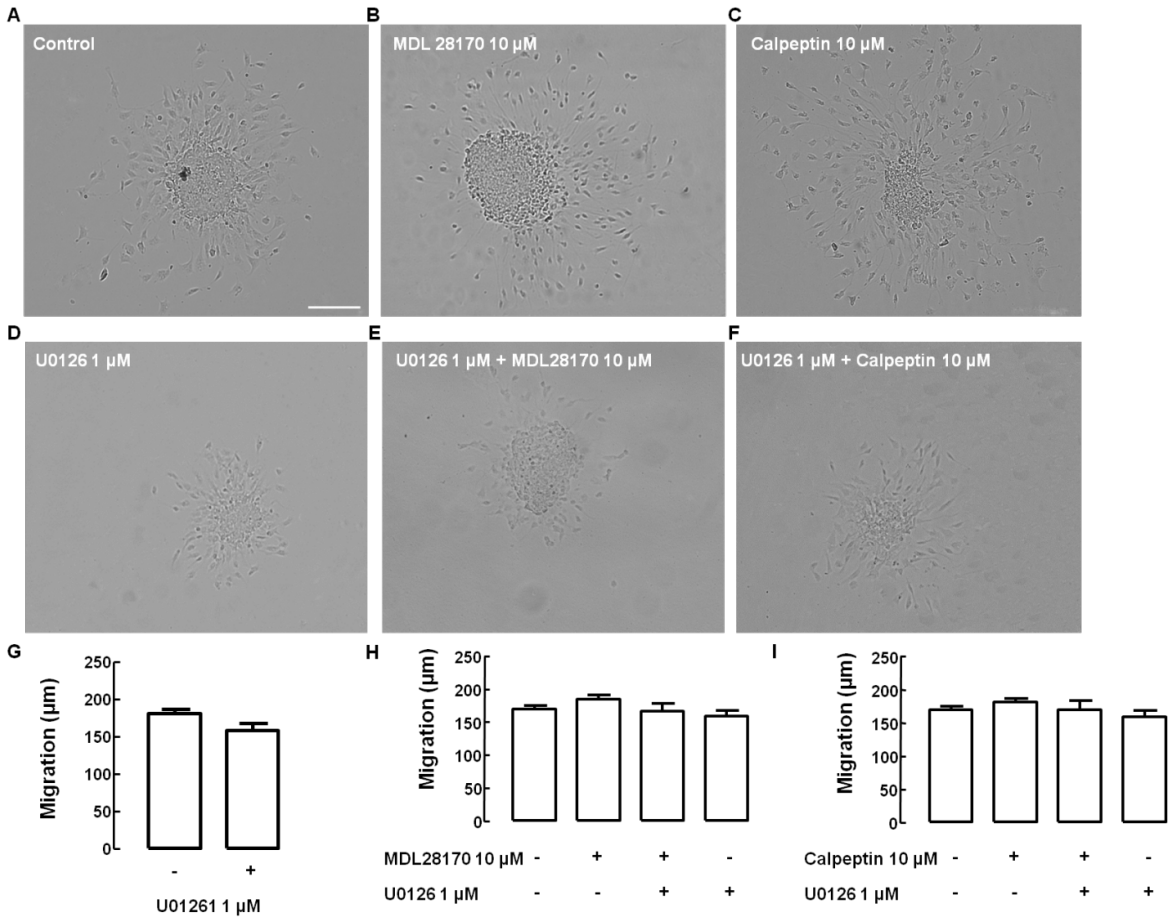

Figure 20 – ERK 1/2 pathway is not involved in SVZ-derived NSC migration. (A), **(B)**, **(C)**, **(D)**, **(E)** and **(F)** - Representative images showing the migration of SVZ-derived NSC for the different conditions. Cells were treated for 4 h with an MEK 1/2 inhibitor, with calpain inhibitors, or with both. Migration radia were measured in images acquired by phase contrast microscopy. The means ± SEM of at least four independent experiments were analyzed by two-tailed t-test or one-way ANOVA (Bonferroni's post-test). Scale bar: 100 μm.

We also analyzed the phosphorylation of ERK 1/2 by Western blot. Thirty min following plating, there is an increased activation of ERK 1/2 (335.51 ± 60.24%, *p<0.05), when compared to control (0 min) (Figure 21A). Calpain inhibition (either by MDL28170 or calpeptin) significantly increased ERK 1/2 phosphorylation (calpeptin: 422.04 ± 92.18%, Figure 21B; MDL28170: 362.79 ± 65.02%, Figure 21C; *p<0.05).

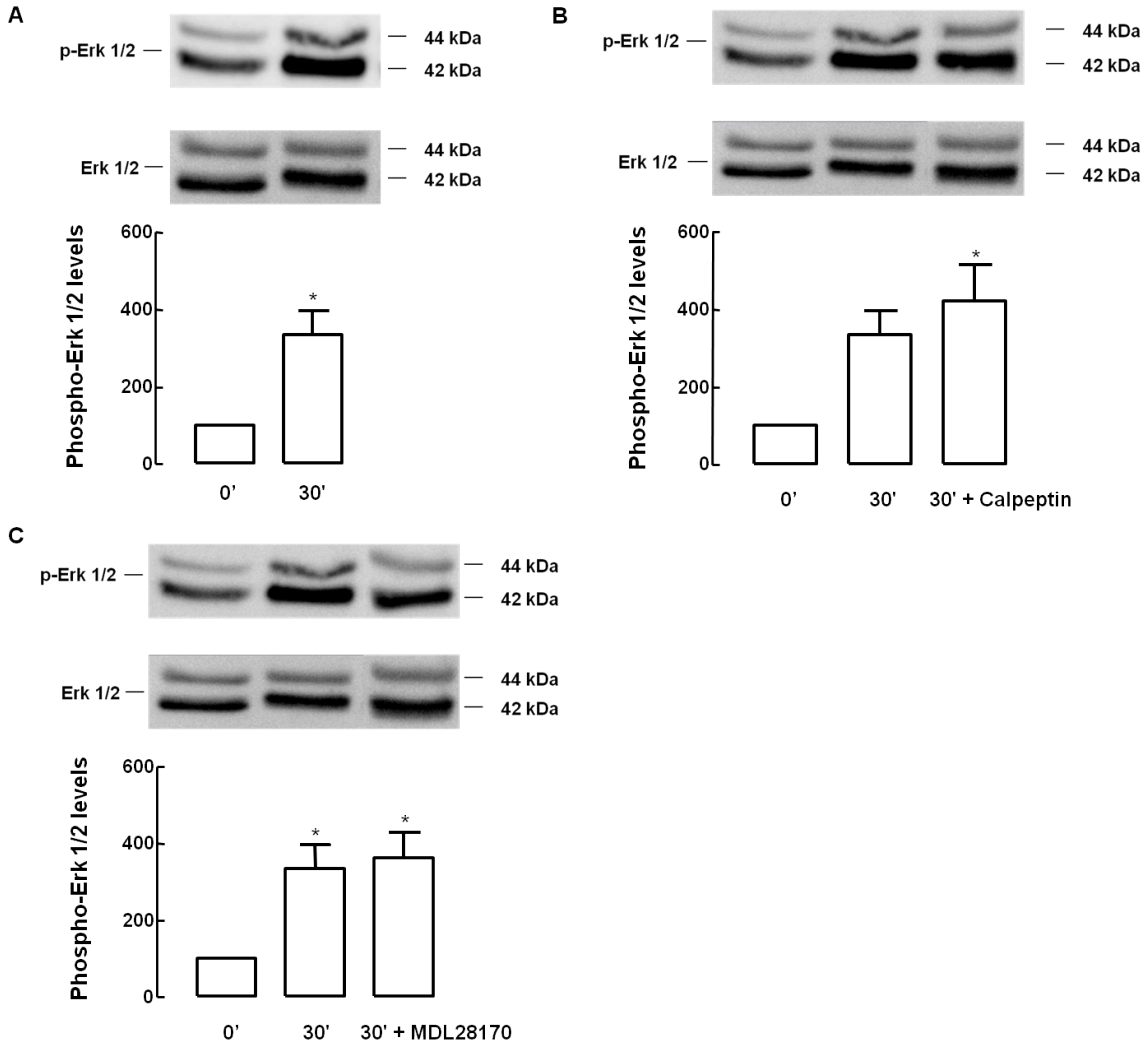

Figure 21 - ERK 1/2 phophorylation following 30 min of treatment with MEK 1/2 inhibitor and calpain inhibitors. Phospho-ERK 1/2 levels following plating **(A)**, calpeptin **(B)** or MDL28170 **(C)**, for 30 min were assessed by Western blot. The means ± SEM of five independent experiments were analyzed by one-way ANOVA (Bonferroni's post-test), *p<0.05 (significantly different from control).

2. *In vivo* modulation of SVZ-derived neuroblast migration, in the subventricular zone

2.1. Calpastatin deletion decreases neuroblast migration in the RMS

SVZ-derived neuroblast migration was also evaluated *in vivo* by assessing DCX immunoreactivity, which is a migrating neuroblast marker, along the RMS.

We observed a decreased immunoreactivity in CSTN animals (1899.90 ± 575.26 μm^2, $p>0.05$) when compared to WT (4259.21 ± 764.81 μm^2) (Figure 22C), which suggests a decreased migration of neuroblasts along the RMS in those animals.

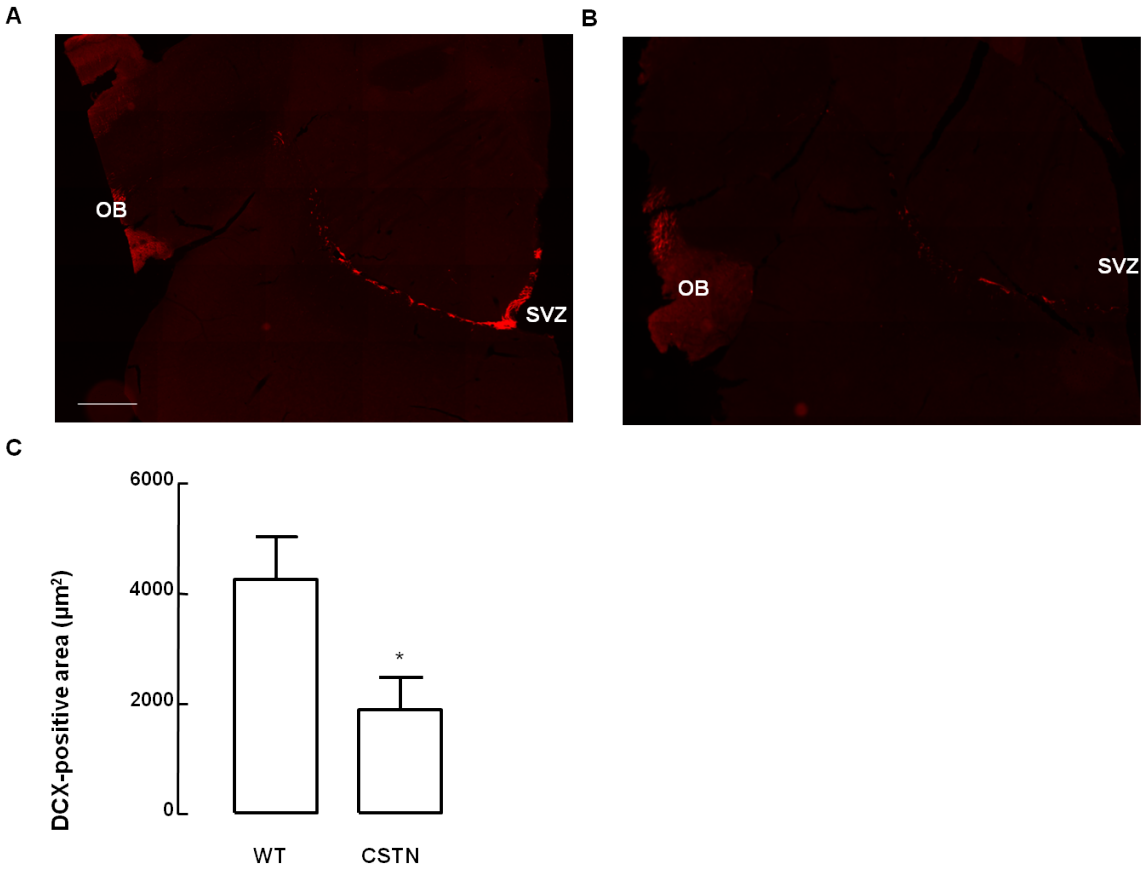

Figure 22 - Calpastatin deletion results in impaired neuroblast migration in the RMS. Representative images of WT **(A)** and CSTN **(B)** mice, showing migrating cells in red, labeled for DCX. Brain slices were stained for DCX and its immunoreactivity in the RMS was measured in sagittal sections of the striatum for each animal **(C)**. The means ± SEM of five independent experiments were analyzed by two-tailed t-test. Scale bar: 300 µm

Chapter IV

Discussion

In this work, we show that calpains have a negative effect in the controlling of migration of SVZ-derived NSC since the inhibition of calpains leads an increase in cell migration. We also show that Rho GTPases are involved in migration of SVZ-derived NSC, since in the presence of inhibitors of the different molecules, cell migration is like abolished. Interestingly, we show in this work, for the first time that calpains are modulating SVZ-derived NSC through Rho GTPases and integrins because in the presence of calpain inhibitors and Rho GTPases or integrin inhibitors, as result was observed a decrease in cell migration. On the other hand, ERK signaling pathway showed be active in migrating NSC, but was not mandatory for cell migration as showed in the lack of effect of the MEK 1/2 inhibitor on migration. Additionally, in an *in vivo* model without the endogenous calpain inhibitor (calpastatin), we show that the absence of calpastatin results in a decrease in the migration of neuroblasts along the RMS.

1. Calpains modulate SVZ-derived NSC migration

SVZ-derived NSC shows the ability to migrate from the SVZ to the OB (Carleton et al. 2003) or in direction of a lesioned area of the brain in case of injury (Kaneko & Sawamoto 2009). Our results strongly suggest that calpains are regulating the migration of NSC since we showed a significant increase in cell migration, following 4 h of treatment with 10 μM MDL28170 and calpeptin. These suggest that calpains, when active, regulate SVZ-derived NSC migration, resulting in a lower migration rate than what occurs when they are inactive. Similar results were obtained when μ-calpain was blocked in human neutrophils, where an increase in cell polarization and migration were registered (Lokuta et al. 2003).

More recently, was showed that platelets from μ-calpain$^{(-/-)}$ mice present an enhanced spreading (Kuchay et al. 2012).

On the other hand, laminin, an ECM component of the neurogenic niche, showed to be important to the migration of these cells, since in the absence of this element the migration observed is significantly lower, in comparison with the situation in which laminin is present, which strongly suggests that laminin is essential for cell migration to occur. In fact, it is described that lack of response to laminin-1 is associated with downregulation of the α6β1 integrin (the major laminin receptor), which leads a blocked in migration of neural precursor cells (Jacques *et al.* 1998).

As introduced in chapter I, integrins are described as being involved in the regulation of some processes, such as cell proliferation, migration and differentiation, in a wide range of tissues (Danen & Sonnenberg 2003). β1 and β3 integrins are the most expressed in the brain, and as refereed in the previous section integrins composed by β1 subunit appear involved in the regulation of some process in the CNS as migration of neural precursor cells (Jacques et al. 1998).

We demonstrated that integrins are involved in the migration of NSC since the presence of echistatin (β1 and β3 integrins inhibitor) induced a decrease in cell migration following 4 h of exposure to the inhibitor. This could be associated with the loss of the laminin receptor α6β1 since we bloqued the possible dimerization between α and β subunit being unable to signaling, which was previously described as leading a decrease in neural precursor cells migration (Jacques et al. 1998).

To further demonstrate the involvement of calpain modulation in the integrins signaling in NSC migration, cells were treated with calpain inhibitors, echistatin, or both. In the presence of both inhibitors our results show that both calpains and integrins are involved in NSC migration. The migration radia obtained in the presence of both inhibitors was similar to the levels obtained in the control, which suggest that the inhibition of calpains leads another signaling pathway to be able to induce migration.

2. Rho GTPases are involved in the migration of SVZ-derived NSC

Since Rho GTPases are described as being involved in cytoskeleton reorganization during spreading, migration, proliferation and differentiation in many types of cells (Hall 1998, Lauffenburger & Horwitz 1996), we studied the involvement of these GTPases in migration of NSC. Our results showed a decrease in cell migration distance in the presence of Cdc42 (1 μM ML141) and Rac1 (10 μM EHT1864 and 200 μM NSC23677) inhibitors. However, in the presence of the RhoA inhibitor (10 μM Y27632), no alteration was observed in cell migration, in comparison to the control. This strongly suggests that Cdc42 and Rac1 are involved in the migration of SVZ-derived NSC, whereas RhoA is not. We cannot say that RhoA is not a signaling pathway important to this type of cells because this signaling pathway can be involved in another important process to the cells besides migration.

To understand if Rho GTPase were involved in the migratory effect observed with calpain inhibition in SVZ-derived NSC, since calpain inhibition increases NSC migration, we evaluated the migration radia in the presence of both, calpain and Rho GTPases inhibitors (for Cdc42 and Rac1). We observed

that, in the presence of any of the Rho GTPases in combination with any of the calpain inhibitors, the migration radia decreased to levels similar to those obtained when cells were treated with the Rho GTPases inhibitors alone. This strongly suggests that the migratory effect observed with calpain inhibition in NSC has the participation of Rho GTPases. Some studies reported the modulation of Rho GTPases signaling by calpains, like the recent study of Kuchay and collaborators (Kuchay et al. 2012).

The activity of Rac1 was studied, in order to understand if the effect observed in the migration was directly linked to the activity of these signaling pathways. So, it was expected that, in the presence of calpain inhibitors, Rac1 activity would be increased. In fact, this signaling pathway presented a higher activation in untreated NSC after 30 min, and this activation is slightly inhibited in the presence of EHT1864, its selective inhibitor. However, in the presence of calpain inhibitors, the activation of Rac1 is not altered. Here we showed that Rac1 is a signaling pathway important for the occurrence of migration in NSC, although calpains are not involved in the modulation of this signaling pathway.

Altogether, our results show that Cdc42 and Rac1 are involved in migration of SVZ-derived NSC and calpains are involved in this process modulating by some way the Cdc42 Rho GTPases since Rac1 is not its target. However, the precise mechanisms involved on this process remain to be addressed.

3. ERK 1/2 is active in SVZ-derived NSC but is not involved in the migration of these cells

The study of Noma *et al.* reported that ERK 1/2 signaling pathway is involved in the migration of monocytes, in the presence of calpain inhibitors (Noma

et al. 2009). Based on this report we decided to investigate if this signaling pathway is also involved in the migration of NSC and if so, whether it is modulated through calpains.

Our results showed a no effect in NSC migration in the presence of MEK 1/2 inhibitor, which indicate that this signaling pathway is not involvement in NSC migration. In order to understand if calpains are involved in regulation of this signaling pathway, cells were treated with calpain inhibitors or MEK 1/2 inhibitor or with both. Our results in this situation were clear because in the presence of both inhibitors, the migratory effect of the calpain inhibitor was not changed by U0126. Our data suggests that this signaling pathway is not involved in NSC migration nor participate in the migratory effect of calpain inhibitors.

By Western blot analysis, we investigated the activation of this signaling pathway in NSC. After 30 min, ERK 1/2 presented a significantly increased activation. In the presence of calpain inhibitors, it was not observed an alteration of ERK 1/2 activation, which in fact is in agreement with the results that we have obtained in migration assay.

This study suggests that calpain inhibition-mediated NSC migration is independent from the activation of ERK 1/2. Despite the fact that the ERK 1/2 signaling pathway is not involved in the migration of NSC, this kinase can still be involved in other important processes, such as proliferation, since it is already showed by our group that this signaling pathway is involved in proliferation of this type of cells (Carreira et al. 2010).

4. Calpastatin depletion in an *in vivo* model leads to a decrease in the migration of neuroblasts along the RMS

To evaluate the effect of calpains in migration of NSC *in vivo*, we examined migration of neuroblasts in a mouse model with calpastatin deficiency (CSTN), in comparison to WT mice. We observed that migration was greatly decreased in the RMS, in comparison to WT mice since DCX immunoreactivity was significantly lower in CSTN mice. This data strongly suggests that the lack of the endogenous inhibitor of calpains leads calpains negatively regulate the migration of these cells. These results are consistent with the results that we have obtained in the *in vitro* studies since we observed that calpain inhibition enhances migration of NSC.

Similar results were obtained by our group in the DG (Vanessa Machado and Inês Araújo, data not shown). Significantly less migrating neuroblasts were observed in the DG of CSTN mice, and the new cells (that incorporated BrdU) also presented a shorter distance of migration into the SGZ (data not shown), which suggest that calpains exert their negative effect in the migration of NSC from the SVZ, as well as in NSC from the SGZ.

Chapter V

Conclusion

1. Conclusion

During this work, we came to the following conclusions:

- Calpain inhibition was shown to lead to an increase in NSC migration.
- Cdc42 and Rac1 signaling pathways are involved in NSC migration. Although RhoA signaling pathway was not involved in the migration of these cells.
- The migratory effect observed with calpain inhibition in SVZ-derived NSC occurs via Rho GTPases and integrins.
- ERK 1/2 signaling pathway is active in NSC but is not involved in migration of these cells.
- Depletion of calpastatin, in an *in vivo* model, results in an impairment of cell migration from the SVZ to the OB, through the RMS.

There are still many open questions to answer before determining the best way to modulate calpain activity in order to improve brain injury repair. However, the observation that calpain inhibition enhances NSC migration opens many doors to this investigation.

2. Future perspectives

In our *in vitro* study about the involvement of Rho GTPases in NSC migration we just studied the activation of Rac1. Therefore, it would also be interesting to investigate the activation of Cdc42, as well as the activation of RhoA in a normal situation as either in the presence of calpain inhibitors. The use of other tools would be useful, in order to verify if what we saw with the pharmacological inhibitors used also happens with, for example, dominant negative mutants, or with siRNAs.

Investigating the effect of calpain inhibitors in neurogenesis following brain ischemia is another step of interest in this study. For that, we can use the genetically modified animals as tools in an experimental model of brain stroke, the middle cerebral artery occlusion (MCAO) model. Associated with this model we can apply blood-brain-barrier permeable calpain inhibitors, to show that calpain inhibition not only affords neuroprotection against stroke, but also possibly enhances migration of neuroblasts to the injured areas.

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