UNIVERSIDADE DA BEIRA INTERIOR
Ciências da Saúde

The role of novel transcriptional regulation mechanisms in neurogenesis

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Dissertação para obtenção do Grau de Mestre em
Ciências Biomédicas
(2º ciclo de estudos)

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Covilhã, junho de 2018

Agradecimentos

Terminada mais uma etapa quero agradecer a todas as pessoas que de alguma forma me apoiaram e me acompanharam ao longo deste ano.

Em primeiro lugar, quero agradecer à minha orientadora, Profª. Doutora Liliana Bernardino, por ter aceite o meu pedido de orientação pois sem ele, não teria tido a oportunidade de realizar este trabalho. Obrigada por todos os conhecimentos partilhados, pela ajuda, pelas críticas construtivas que me ajudaram a crescer enquanto investigadora e por toda a motivação para este trabalho. O meu sincero obrigada.

À Profª. Doutora Raquel Ferreira e à Doutora Ana Clara Cristóvão, por todas as dicas e orientações úteis.

Agradeço também à Cláudia Saraiva por todos os conhecimentos transmitidos e por todos os momentos de boa disposição partilhados.

Um muito obrigado às minhas amigas e colegas do Brain Repair Group, Jéssica Nunes, Mariana Fiadeiro, Marta Esteves e Marta Pereira, pela constante ajuda, paciência, por todas as gargalhadas, por todos os momentos inesquecíveis. Sem vocês, este ano não teria sido o mesmo. Obrigada!

Quero agradecer também à Débora, à Emika e à Julieta por serem as melhores colegas de laboratório de sempre, pela ajuda, disponibilidade e por todos os bons momentos que passámos juntas.

A todos os meus amigos, em especial à Ana Rita, Carla, Dalinda, Diana, Jane e Marina, o meu obrigada pela vossa amizade, por todo o apoio e motivação, simpatia e por todos os momentos divertidos que passámos ao longo deste ano. Muito obrigada!

Por fim, mas não menos importante, quero agradecer à minha família, em especial aos meus pais e irmã, que me apoiaram e incentivaram ao longo deste ano e por terem sempre acreditado em mim. Obrigada!

Resumo

A Zona Subventricular (SVZ) é o maior nicho neurogénico no cérebro adulto de roedores. A neurogénese é regulada por mecanismos extracelulares e intracelulares, que incluem fatores de transcrição e modificações epigenéticas. As proteínas de ligação ao C-terminal (CtBPs) são corepressores transcricionais que, ao interagirem com fatores de transcrição, reprimem a transcrição. Além disso, estas proteínas são importantes na regulação da proliferação, diferenciação e sobrevivência celular. Estas evidências sugerem que as CtBPs podem ser alvos terapêuticos promissores para regular a neurogénese. Assim, o principal objetivo deste trabalho foi avaliar os efeitos das CtBPs na neurogénese da SVZ. Em particular, analisámos a expressão das CtBPs na SVZ *in vitro* e *in vivo* e os efeitos destas proteínas na sobrevivência, proliferação e diferenciação celular. A expressão das CtBPs foi analisada por imunomarcações em culturas de células da SVZ obtidas de murganhos C57BL/6J com 1-3 dias e em murganhos C57BL/6J com 8-10 semanas de idade *in vivo*. As CtBP1 e 2 são expressas em células proliferativas ($Ki67^+$), células imaturas ($Nestin^+$, $Sox2^+$), neuroblastos em proliferação ($Ki67^+$ e DCX^+), astrócitos ($GFAP^+$) e oligodendrócitos ($Olig2^+$), na SVZ *in vitro* e *in vivo*. Em seguida, o ácido 4-metiltio-2-oxobutírico (MTOB), um antagonista das CtBPs que pode ter um duplo efeito ao atuar como inibidor a altas concentrações, mas a baixas concentrações como substrato, foi utilizado para avaliar o efeito das CtBPs na neurogénese na SVZ *in vitro*. Os nossos resultados mostraram que o MTOB a 1 mM e 2.5 mM induzia morte celular, detetada pela incorporação de iodeto de propídio e pela análise da morfologia nuclear. Além disso, 5 µM, 25 µM e 50 µM de MTOB não afetaram o número total de células em proliferação, mas 25 µM de MTOB aumentou a percentagem de neuroblastos em proliferação. Relativamente à diferenciação celular, 5 µM, 25 µM e 50 µM de MTOB aumentaram a percentagem de neurónios maduros ($NeuN^+$) e oligodendrócitos ($Olig2^+$). Em suma, os nossos resultados sugerem que as CtBPs modulam a neurogénese na SVZ, ao promoverem a diferenciação em neurónios e oligodendrócitos, o que poderá ter relevância na reparação de doenças neurodegenerativas.

Palavras-chave

Neurogénese, Zona Subventricular, Proteínas de Ligação ao C-terminal, MTOB

Resumo Alargado

No cérebro adulto dos mamíferos, a neurogénese ocorre durante toda a vida em dois nichos neurogénicos principais: na zona subventricular (SVZ) e na zona subgranular (SGZ) no giro dentado do hipocampo. Nestes nichos existem células estaminais neurais (NSCs) com capacidade de autorrenovação, proliferação e multipotência, podendo diferenciar-se em neurónios, astrócitos ou oligodendrócitos. A diferenciação das NSCs pode ocorrer através de mecanismos extrínsecos, por exemplo através do contacto célula a célula, ou por mecanismos intrínsecos, como é o caso dos fatores de transcrição e das modificações epigenéticas, que incluem a metilação do ADN e as modificações nas histonas. As proteínas de ligação ao C-terminal (CtBPs) são corepressores da transcrição, importantes para o desenvolvimento do cérebro. Após a ligação aos fatores de transcrição, estas proteínas reprimem a transcrição principalmente através de um complexo corepressor. Vários estudos mostram que as CtBPs desempenham um papel importante na proliferação, sobrevivência e diferenciação celular.

Desta forma, o objetivo principal deste trabalho foi avaliar os efeitos das CtBPs na neurogénese na SVZ. Inicialmente fomos avaliar a expressão destas proteínas na SVZ *in vitro* e *in vivo*. O primeiro passo foi analisar a expressão quantitativa destas proteínas em culturas celulares da SVZ, em condições de proliferação, quando as células estão expostas a fatores de crescimento (EGF e FGF-2) e em condições de diferenciação, quando os fatores de crescimento são retirados e as células são expostas a um substrato. Verificámos que a expressão das CtBP1 e 2 analisada através de western blot é semelhante em ambas as condições, não se verificando diferença estatística. De seguida, analisámos a expressão das CtBPs nos diferentes fenótipos celulares da SVZ *in vitro* e *in vivo*. Para isso foram realizadas imunomarcações em culturas de células da SVZ obtidas de murganhos C57BL/6J com 1-3 dias e *in vivo* utilizando murganhos C57BL/6J adultos (8-10 semanas). De facto, observámos que ambas as CtBPs são expressas nos diferentes fenótipos celulares da SVZ, ou seja, ambas são expressas em células em proliferação ($Ki67^+$), em células imaturas ($Nestin^+$ e $Sox2^+$), neuroblastos em proliferação ($Ki67^+$ e DCX^+), astrócitos ($GFAP^+$) e oligodendrócitos ($Olig2^+$), na SVZ *in vitro* e *in vivo*. As CtBPs são também expressas em neurónios ($MAP2^+$ e $NeuN^+$) em culturas da SVZ *in vitro* enquanto que *in vivo* a co-localização é verificada na região do estriado, adjacente à região da SVZ. De seguida, avaliámos o efeito das CtBPs na neurogénese da SVZ *in vitro*. Para isso, as células da SVZ, em condições de diferenciação, foram tratadas com um antagonista das CtBPs, o ácido 4-metiltio-2-oxobutírico (MTOB), a diferentes concentrações (5 µM, 25 µM, 50 µM, 100 µM, 250 µM, 1 mM e 2.5 mM). Este composto pode ter um duplo efeito sobre as CtBPs, atua como inibidor a elevadas concentrações, enquanto que a baixas concentrações pode atuar como substrato. A viabilidade celular, avaliada 2 dias após os tratamentos, foi analisada através da incorporação de iodeto de propídio e por condensação nuclear. Verificou-se que as concentrações de 1 mM e 2.5 mM de MTOB induziam

morte celular e por esta razão, estas concentrações juntamente com a de 250 μM de MTOB, foram excluídas deste trabalho. A proliferação celular foi também avaliada 2 dias após os tratamentos através de imunomarcação para Ki67. Verificou-se que as concentrações de 5 μM, 25 μM e 50 μM de MTOB pareceram não afetar o número total de células proliferativas positivas para Ki67. De seguida, fomos avaliar a proliferação dos diferentes fenótipos celulares da SVZ. Estas concentrações pareceram não afetar a percentagem de células $Nestin^+$, $Olig2^+$ e $GFAP^+$ em proliferação, mas a concentração de 25 μM de MTOB aumentou a percentagem de neuroblastos em proliferação, sendo que 5 μM e 50 μM de MTOB também pareceram levar a um aumento desta população de células. Por fim, a diferenciação celular foi avaliada 7 dias após os tratamentos. Os nossos resultados mostraram que as concentrações de 5 μM, 25 μM e 50 μM de MTOB aumentaram a percentagem de neurónios maduros (NeuN) e de oligodendrócitos (Olig2). No entanto, estas concentrações pareceram não afetar a percentagem de astrócitos maduros.

O facto das baixas concentrações de MTOB usadas neste trabalho induzirem um aumento da diferenciação em neurónios e oligodendrócitos, pode ser benéfico para patologias onde ocorre a degeneração destes fenótipos celulares. O acidente vascular cerebral, a esclerose múltipla ou a contusão da medula espinhal são três possíveis exemplos de patologias caracterizadas pela desmielinização e morte neuronal. Concluindo, os nossos resultados sugerem que as CtBPs podem regular a neurogénese na SVZ, o que as torna um bom alvo de estudo em contextos de regeneração cerebral.

Abstract

Subventricular Zone (SVZ) is the main neurogenic niche in adult rodent brain. Neurogenesis is regulated by extracellular or intracellular mechanisms, which include transcriptional factors and epigenetic modifications. C-terminal Binding Proteins (CtBPs) are transcriptional corepressors that interact with transcriptional factors to repress the transcription. Moreover, these proteins are important in the regulation of cellular proliferation, differentiation, and survival. These findings suggest that CtBPs may play a role in the modulation of adult neurogenesis. Therefore, the main aim of this work was to evaluate the effects of CtBPs in SVZ neurogenesis. Herein, we analyze CtBPs expression in the SVZ *in vitro* and *in vivo* and its effects on SVZ neurogenesis *in vitro*. First, CtBPs expression was analyzed by immunostainings in SVZ cell cultures obtained from 1- to 3-day-old C57BL/6J mice and in 8-10-week-old mice *in vivo*. Both CtBP1 and 2 were expressed in proliferating cells ($Ki67^+$), immature cells ($Nestin^+$ and $Sox2^+$), proliferative neuroblasts ($Ki67^+$ and DCX^+), astrocytes ($GFAP^+$) and oligodendrocytes ($Olig2^+$), in the SVZ *in vitro* and *in vivo*. Then, a substrate-based inhibitor of CtBPs, the 4-methylthio 2-oxobutyric acid (MTOB) that may have a dual effect acting as an inhibitor at high concentrations but as substrate at low concentrations, was used to assess the effect of CtBPs on neurogenesis *in vitro*. Our results showed that 1 mM and 2.5 mM MTOB induced cell death as detected by propidium iodide incorporation and nuclear morphology analysis. Moreover, 5 µM, 25 µM and 50 µM of MTOB did not affect the total number of Ki67 proliferating cells while 25 µM MTOB increased the percentage of proliferating neuroblasts. Regarding cell differentiation, 5 µM, 25 µM and 50 µM of MTOB increased the percentage of NeuN-mature neurons and Olig2-oligodendrocytes. Altogether, our results suggest that CtBPs are a good target to regulate the transcriptional mechanisms in SVZ neurogenesis.

Keywords

Neurogenesis, Subventricular Zone, C-terminal Binding Proteins, MTOB

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List of Abbreviations

AD	Alzheimer's Disease
AMPK	AMP-Activated Protein Kinase
APC	Adenomatous Polyposis Coli
Ascl1	Achaete-scute homolog 1
BDNF	Brain-Derived Neurotrophic Factor
BMPR-I	Type 1 BMP receptor
BMPs	Bone Morphogenetic Proteins
BSA	Bovine Serum Albumin
CSF	Cerebrospinal Fluid
CtBP	C-terminal Binding Protein
DCX	Doublecortin
DG	Dentate Gyrus
Dlx2	Distal-less homeobox 2
DMEM/F-12	Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12
EGF	Epidermal Growth Factor
FGF-2	Fibroblast Growth Factor-2
GCL	Granule Cell Layer
GFAP	Glial Fibrillary Acid Protein
H_2O_2	Hydrogen Peroxide
HATs	Histones Acetyltransferases
HBSS	Hanks Balanced Salt Solution
HDACs	Histone Deacetylases
Hes1	Hairy and enhancer of split-1
HMTs	Histone Methyltransferases
IL	Interleukin
JNK	c-Jun N-terminal kinase
LPS	Lipopolysaccharide
MAP2	Microtubule-Associated Protein 2
MBD1	Methyl-CpG-Binding Domain Protein 1
MBP	Myelin Basic Protein
Mll1	Mixed-lineage leukemia 1
MTOB	4-methylthio-2-oxobutyric acid
NAD^+	Oxidized Nicotinamide Adenine Dinucleotide
NADH	Reduced Nicotinamide Adenine Dinucleotide
NeuN	Neuronal Nuclei
NeuroD	Neurogenic Differentiation
NLS	Nuclear Localization Signal
nNOS	Neuronal Nitric Oxide Synthase
NSCs	Neural Stem Cells
OB	Olfactory Bulb
Olig2	Oligodendrocyte Transcription Factor 2
PAK1	p21-Activated Kinase 1
PAK6	p21-Activated Kinase 6
Pax6	Paired Box 6

PBS	Phosphate Buffer Saline
PD	Parkinson's Disease
PEDF	Pigment Epithelium-Derived Factor
PERP	p53-effector related to pmp-22
PFA	Paraformaldehyde
PKA	Protein Kinase A
PI	Propidium iodide
PLP	Proteolipid Protein
PSA-NCAM	Polysialylated Neural-Cell-Adhesion Molecule
PTEN	Phosphatase and Tensin Homolog
PXDLS	Pro-X-Asp-Leu-Ser
RMS	Rostral Migratory Stream
RT	Room temperature
SGZ	Subgranular Zone
siRNA	Small Interfering RNA
Sox2	Sex Determining Region Y-box 2
SVZ	Subventricular Zone
TBI	Traumatic Brain Injury
TNF	Tumor Necrosis Factor
VEGF	Vascular Endothelial Growth Factor

Chapter 1

Introduction

1 Neurogenesis

The classic view of neurogenesis, the process that leads to the formation of new neurons, was that it only occurred during the embryonic and perinatal stages of the brain (1). However, this perception became outdated with the discovery of adult neural stem cells (NSCs), in the decade of 1960 (2). The first evidence for the presence of newborn neurons was made by Joseph Altman in 1962, which was posteriorly confirmed by Altman and Das in 1965 with the observation of hippocampal neurogenesis in the adult rat brain and in 1969 when Joseph Altman described the rostral migratory stream (RMS), through which the newly formed neurons migrate towards the olfactory bulb (OB) (3–5). It was only in the late 1990s, that the adult neurogenesis was discovered in humans (6,7).

1.1 Neural Stem Cells Niches

During the embryonic development, neuroepithelial cells acquire glial features, becoming radial glial cells that represent the NSCs in the developing brain. These radial glial cells present during development give rise to NSCs found in the adult brain. NSCs are defined by their ability to proliferate, self-replicate and differentiate into neurons and glial cells (8). Since neurogenesis remains active throughout the adult life, these cells can be found in specific regions of the adult mammalian brain, mainly in the two major neurogenic niches: the subventricular zone (SVZ) and the subgranular zone (SGZ) (Figure 1 A) (1).

1.1.1 Subventricular Zone

In rodents, SVZ is the largest neurogenic niche and is located along the lateral walls of the lateral ventricles. This niche contains four main type of cells: ependymal cells or type E cells, astrocyte-like NSCs or type B cells, transit amplifying progenitors or type C cells and neuroblasts or type A cells. Type E cells are multiciliate cells that separate SVZ from the ventricle cavity, function as a barrier between cerebrospinal fluid (CSF) and neural tissue, and have an important role in the regulation of SVZ neurogenesis. Unlike type E cells, type B cells possess a single cilium that contacts directly with the CSF and they are in direct contact with blood vessels (9). These cells can be in a quiescent or in an activated state and both co-exist in the neurogenic niches (10). The acquisition of quiescence, as well as their stable state in the niche, are perhaps the main characteristics that distinguish adult from the embryonic NSCs since embryonic NSCs are highly proliferative whereas adult NSCs can remain out of the cell cycle, in G0, for a long time, which is crucial to maintaining homeostasis (11).

So, NSCs in a quiescent state can receive signals either to maintain in G0 either to become activated (10). Activated NSCs or type B cells, which express several glial markers, such as the glial-fibrillary acidic protein (GFAP), and immatures markers like Nestin and sex determining region Y-box 2 (Sox2), divide asymmetrically to give rise to type C cells. (10,12). Type C cells, that also express Nestin as well as the transcriptional factor distal-less homeobox 2 (Dlx2), are the most proliferating cells in the SVZ. In turn, these cells differentiate into type A cells, which express doublecortin (DCX) and polysialylated neural-cell-adhesion molecule (PSA-NCAM) (9,12). These migrating neuroblasts form a chain along the RMS, ensheathed by GFAP-positive cells (13). Reaching the OB, type A cells differentiate into several subtypes of interneurons, mainly as GABAergic granule interneurons and GABAergic or dopaminergic periglomerular neurons, important for odor discrimination (Figure 1 B) (9).

In the human adult brain, astrocytic NSCs can also be found in SVZ, as well as type C and A cells. Nevertheless, the presence of RMS stills controversial. Although the human infant SVZ and RMS contain an elevated number of migrating neuroblasts that are destined not only to OB but also to the prefrontal cortex, it decreases drastically after 18 months of age. (14). However, in all ages, neuroblasts prevenient from SVZ can be found in the striatum of the human brain, revealing the generation of striatal neurons (15).

1.1.2 Subgranular Zone

The SGZ of the dentate gyrus (DG) in the hippocampus also contains astrocyte-like NSCs, known as type 1 radial glia-like cells or type B cells, that like type B cells in the SVZ, express the markers GFAP, Nestin, and Sox2. These cells are also in direct contact with the blood vessels (11). Through asymmetric division, type 1 cells give rise to transit-amplifying non-radial progenitors, also called type D cell or type 2 cells, which also express Nestin. These cells can be subdivided into type 2a and type 2b, which differs in their differentiated state. Subsequently, type 2 cells give rise to neuroblasts also called type G cells or type 3 cells, expressing DCX and PSA-NCAM (16,17). Unlike in SVZ, SGZ neuroblasts migrate a short distance towards the granule cell layer (GCL) of the DG, where they differentiate into glutamatergic granule cells, important for learning and memory (Figure 1 C) (16).

In humans, SGZ neurogenesis is similar to rodents. Hippocampal neurogenesis in rodents is maintained, although it decreases during aging. However, in the human adult brain, there are some controversies regarding SGZ neurogenesis. Some studies show that new neurons are added per day in the human hippocampus as well as human hippocampal neurogenesis is maintained during aging, although diminished (18,19). These reports are however contradicted by the study of Sorrells and colleagues that reveals that the number of proliferating progenitors and young neurons decreases quickly during the first years of life and that neurogenesis does not continue or is extremely rare, in adult humans brains (20).

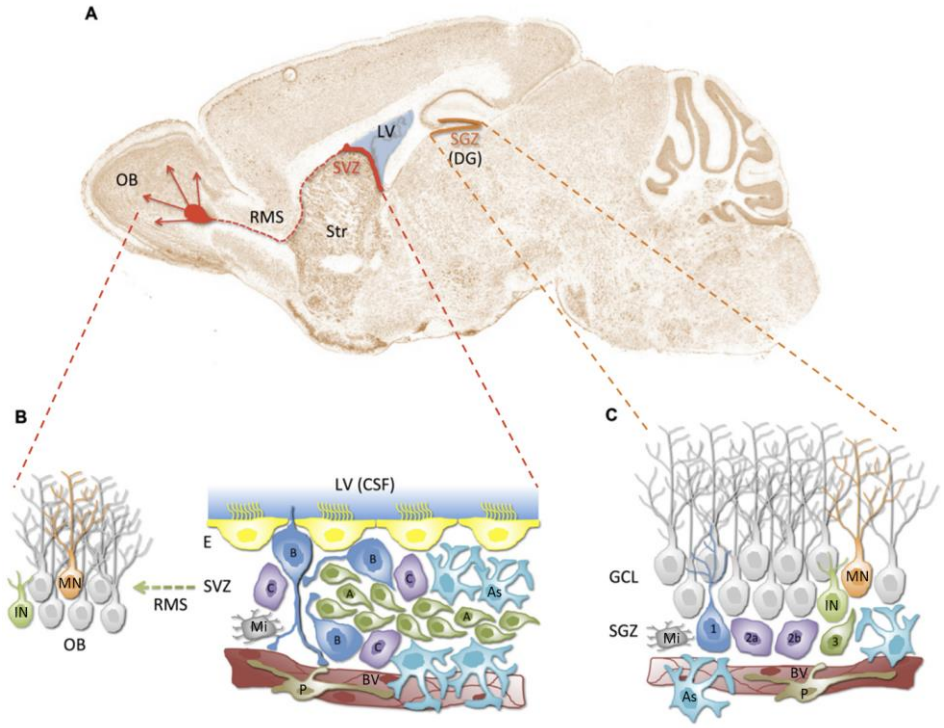

Figure 1 - Localization and composition of the two main neurogenic niches in the adult rodent brain. **(A)** Sagittal section of the adult rodent brain with the representation of the subventricular zone (SVZ, in red) of the lateral ventricles (LV), adjacent to the striatum (Str), from which the neuroblasts migrate towards the olfactory bulb (OB) through the rostral migratory stream (RMS), and of the subgranular zone (SGZ) of the dentate gyrus (DG). **(B)** The neurogenic niche of the SVZ: multiciliate E cells (E) separate the SVZ from the ventricle cavity. In this niche, there are the B cells (B), that when activated originate the C cells (C). In turn, C cells divide to give rise A cells (A), which migrate long distances through the RMS to the OB. In here, they become mature neurons (MN) from immature neurons (IM). **(C)** The neurogenic niche of the SGZ: Radial type 1 cells (1) corresponds to the NSCs that generate type 2a/b (2a,2b) cells, which differentiate into type 3 cells (3). Neuroblasts migrate helped by astrocytes, becoming MN into the granular cell layer (GCL). **(B,C)** There are also others components of the niche: astrocytes (As), microglia (Mi), pericytes (P) and blood vessels (BV). Adapted from (21).

Although SVZ and SGZ are the two main neurogenic niches in the adult brain, other regions of the central nervous system, commonly known as "non-canonical" neurogenic regions, were described to contain NSCs and neuroblasts. These regions are reviewed in Pino *et al*, and they are as follows: hypothalamus, striatum, olfactory epithelium, cerebral cortex, cerebellum, meninges and spinal cord (Figure 2) (8). Nonetheless, these "non-canonical" niches have been the object of many controversies. The neurogenic processes that may occur in these novel regions are considered a rare phenomenon in mammals and are dependent on several factors, including the regional location, progenitor cells origins as well as the animal species, age, and state (physiological/pathological). Even the cases documented are difficult to justify, for several reasons. First, there is an enormous heterogeneity associated to this "non-canonical" neurogenesis, for example, associated to the stages of development it occurs or in the exact origin and nature of the progenitor cells, the final outcome or the physiological function. Moreover, there are some cases reported by several research groups that are refuted by others, like the case of piriform cortex. Altogether, these evidences might mean that this

"non-canonical" neurogenesis is distinctively adapted to the brain anatomy of different mammals although it is not fully accepted (22).

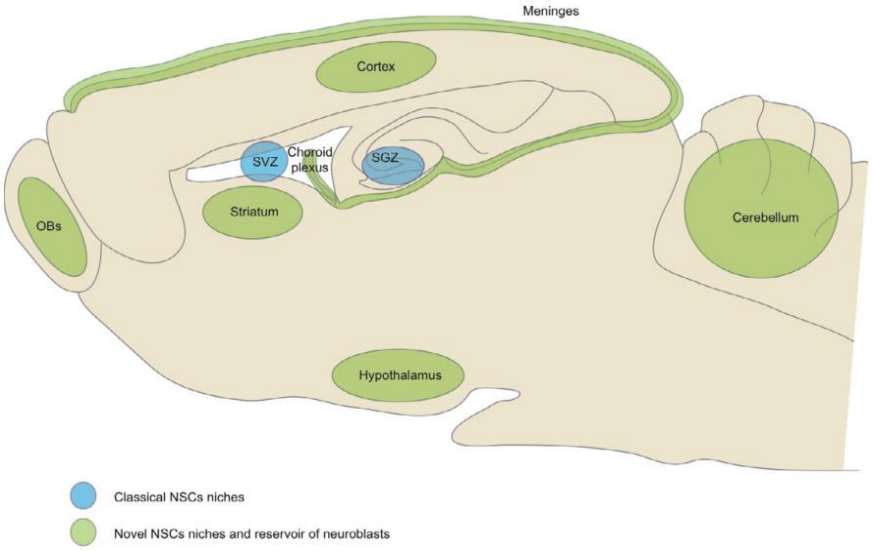

Figure 2 - Representation of the distribution of the two main neurogenic niches and other novel NSCs niches in the adult rodent brain. The main neurogenic niches are in blue and the novel ones, as well as the reservoir of neuroblasts, are in green. Adapted from (8).

1.1.3 Cellular Components of the Neurogenic Niches

The neurogenic niche is a microenvironment that allows the maintenance of self-renewal and multipotency abilities of adult NSCs, either to keep them in an undifferentiated state or to trigger neurogenesis in a precise timing (23). These niches, besides NSCs, are composed of cellular components, namely the ependymal cells, the blood vessels, microglia and astrocytes, important for the homeostasis of these niches. Cell to cell communication mechanisms, as well as, the molecular niches signals, which include bone morphogenetic proteins (BMPs), Notch, Wnt, growth factors and neurotrophins, cytokines and neurotransmitters, play an important role in the regulation of neurogenesis. Ependymal cells have motile cilia that contribute to the flow of CSF and secrete factors that regulate neurogenesis (24). For example, these cells secrete signaling factors, such as Noggin, that can promote neuronal differentiation of the NSCs in SVZ. Noggin is an antagonist of BMPs, which inhibit neurogenesis but increase the survival of neuroblasts. This suggests that BMPs in SVZ have distinct effects on cells at different stages of neurogenesis (25). Moreover, within these niches, there is a physical proximity between NSCs and blood vessels. Endothelial cells release factors that promote the migration, survival, and differentiation of neuroblasts, such as erythropoietin, vascular endothelial growth factor (VEGF) and brain-derived neurotrophic factor (BDNF). However, pigment epithelium-derived factor (PEDF), release by both ependymal and endothelial cells, contributes for self-renewal of NSCs, maintaining the pool of undifferentiated cells. Furthermore, pericytes, which are localized near the endothelial cells, can act as regulators of these factors. The interaction between pericytes, astrocytic endfeet and endothelial cells is important for the maintenance of the blood-brain barrier.

However, within SVZ seems to exist a modified blood-brain barrier. At certain regions of blood vessels, the lack of pericytes, as well as astrocyte endfeet, allows the contact of type B and C cells with vasculature, facilitating the direct passage of signaling molecules and then communication (26). The survival of the newborn cells is important for neurogenesis, being the removal of the apoptotic debris an important factor. In the neurogenic niche of SGZ, under basal conditions, the apoptotic newborn cells are phagocytosed by microglia, the brain innate immune cells (24). Interestingly, in the SVZ/RMS, there is a distinct activated microglia population essential for the survival and migration of neuroblasts. In SVZ, microglia have enlarged cell bodies and displays a more amoeboid morphology whereas along the RMS it presents a more ramified morphology but with few unbranched processes. Nevertheless, phagocytosis of neuroblasts within the SVZ and RMS by microglia is a rare phenomenon since markers of activated microglia were not detected in the SVZ/RMS. In addition, microglia express low levels of purinergic receptors, which transduce the purinergic "find me, eat me" signals, and it also displays little process motility in response to ATP. *In vivo,* the depletion of microglia in the SVZ reduces the number of neuroblasts reaching the OB with consequent accumulation in the SVZ/RMS. Also, the microglial cells differentially express the interleukin (IL)-4, which shows to promote neuroblast migration and neurogenesis, IL-6, that enhances self-renewal of NSCs *in vitro,* and IL-10. These results suggest that the survival and migration of neuroblasts are due to microglia possibly activated through their release of cytokines such as IL-4, IL-6, and IL-10 (27). Hence, microglia can regulate neurogenesis either in a positive or in a negative way through the secretion of inflammatory mediators, such as cytokines. For example, the stimulation of microglia by IL-4 and low levels of interferon-γ induce neuronal differentiation whereas the activation by lipopolysaccharide (LPS) inhibits it (28). Microglia also secretes tumor necrosis factor (TNF)-α and SVZ cells express its receptors. Besides TNF-α induce neurogenesis through the activation of the TNF receptor 1, it also positively regulates cell proliferation and survival in SVZ cells, in a concentration-dependent manner (29). Like microglia, astrocytes can have a dual role in neurogenesis (30). For example, astrocytes secrete Wnt3, promoting the differentiation of NSCs in SGZ (31), while on the other hand, may also express Jagged1, which maintains the NSCs in an undifferentiated state (32). Epidermal growth factor (EGF) and fibroblast growth factor-2 (FGF-2), released by astrocytes, are the principal growth factors responsible for proliferation and self-renewal of NSCs. *In vitro*, NSCs can be isolated and growth as neurospheres in the presence of these two growth factors. However, type C cells are the most responsive cells to EGF and about 70% of forming-neurospheres arise from this the type of cells (33,34). Additionally, astrocytes help in the migration of neuroblasts of the SVZ through the RMS and of the SGZ to reach the GCL (13,35). Finally, neurotransmitters can also regulate the neurogenesis in a positive or in a negative way, as reviewed by Lim *et al*. (9).

1.1.4 Internal Molecular Regulatory Mechanisms of Neurogenesis

In addition to extracellular mechanisms, several intrinsic mechanisms are implicated in the regulation of neurogenesis (Figure 3). In a precise spatial and temporal manner, the sequential activation of several transcriptional factors plays a critical role in adult neurogenesis (24). For example, the transcriptional factor Sox2, highly expressed by NSCs and early precursors in SVZ and SGZ niches, is involved in the regulation of the maintenance and proliferation of NSCs. However, its downregulation decreases the number of mature neurons produced by NSCs, suggesting that Sox2 is required for neuronal differentiation (36). Also, hairy and enhancer of split-1 (Hes1) expressed by NSCs maintains their ability of self-renewal, repressing neuronal differentiation (37). The transcriptional factor achaete-scute homolog 1 (Ascl1) is expressed by proliferating type B and C cells, in both neurogenic niches, and is essential for their activation, proliferation, and differentiation, either in neurons or oligodendrocytes (38). Moreover, oligodendrocyte transcription factor 2 (Olig2) and paired box 6 (Pax6) are examples of transcriptional factors that direct NSCs in a specific lineage. In the SVZ, Olig2 is expressed exclusively in type C cells whereas Pax6 is expressed in type A cells, not overlapping their expression. Olig2 promotes a transient amplifying precursor state and oligodendrogenesis whereas Pax6 promotes neurogenesis (39).

Epigenetic modifications, such as histone modifications or DNA methylation, also regulate the cell fate specification of NSCs. Epigenetics are heritable changes in phenotype or gene expression, without altering the DNA sequence. It is believed that histone modifications are the most important modifications in the differentiation of NSCs. Histone acetylation, by histones acetyltransferases (HATs), leads to a loose chromatin which is associated with an active transcription. However, the reverse reaction also occurs and is promoted by histones deacetylases (HDACs). Histone deacetylation forms a condensed chromatin, that prevents the binding of transcriptional activators, resulting in their repression (40). The pharmacologic application of Valproic acid, an inhibitor of HDACs, leads to NSCs from SGZ decreased their proliferation and leads to their differentiation into neurons, inhibiting the differentiation into astrocytes and oligodendrocytes. The neuronal differentiation is due to the overexpression of the transcriptional factor, neurogenic differentiation (NeuroD) (41). Importantly, the effects of HATs and HDACs is due not only to their intrinsic activity but also to their interaction with coactivators and corepressors, such C-terminal binding proteins (CtBPs), where they are the core of an enzymatic complex (42). Histone methylation is another modification that is catalyzed by histone methyltransferases (HMTs) and is linked to activation and repression of the transcription, depending on the methylated site. For example, histone 3 methylation at lysine 9 along with histone 4 methylation at lysine 9 represses transcription whereas the histone 3 methylation at lysine 4, activates transcription (40). Mixed-lineage leukemia 1 (Mll1) is an HMT responsible for the methylation of histone 3 methylation at lysine 4 and is expressed in SVZ NSCs. Mll1 targets Dlx2, a transcriptional factor important for the neurogenesis. The deletion of Mll1 from NSCs leads to the inactivation of Dlx2, leading to an

impaired neurogenesis but no gliogenesis. This suggests that Mll1 is necessary for neuronal, but not glial, differentiation in the SVZ, at least through its target Dlx2 (43). DNA methylation normally suppresses gene expression, which occurs mainly by the addition of methyl groups in cytosine residues at CpG dinucleotides and by the recruitment of methylated DNA-binding proteins such as the methyl-CpG-binding domain protein 1 (MBD1), that recruits corepressor proteins and enzymes responsible for histone modifications, resulting in an inactive chromatin that represses the transcription (40,44). MBD1 is highly expressed in the NSCs of the SGZ. One study reveals that adult mice lacking MBD1 have a decreased neuronal differentiation in the hippocampus and spatial learning but an increased genomic instability, which suggests that DNA methylation in NSCs is important for their genomic stability (44).

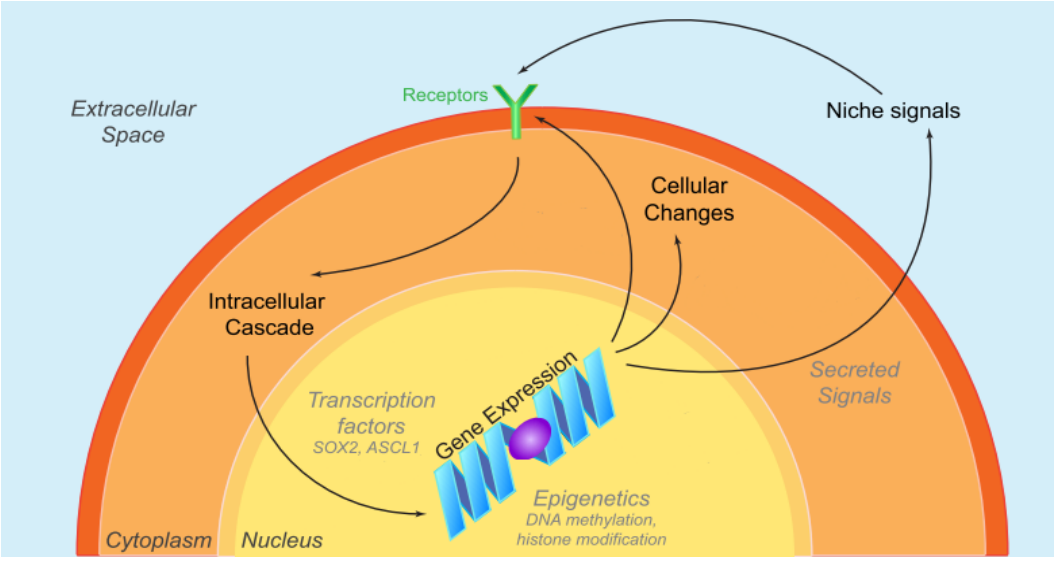

Figure 3 - Regulation of neurogenesis by intracellular and extracellular mechanisms. The receptors activated by extracellular niche signals trigger an intracellular signaling cascade that activates several mechanisms, including transcriptional factors and epigenetic modifications. This intracellular cascade leads to alterations in gene expression in NSCs, which can result in cellular changes, affecting NSCs behavior. Both intrinsic and extrinsic mechanisms that regulate NSCs are interconnected. Adapted from (45).

1.2 Regulation of Neurogenesis in Pathological Conditions

Under physiologic conditions, the regenerative process occurs in order to promote maintenance whereas under pathologic conditions it occurs to repair. There is no cure for neurological disorders, such as brain injuries or neurodegenerative diseases, but adult neurogenesis becomes interestingly since the adult brain can generate new neurons from NSCs. After a lesion, NSCs within the niches receive different factors and signals, as previously described. Despite SGZ neurogenesis is important, the SVZ neurogenic niche has a major potential for brain repair since it is the largest neurogenic niche in the adult brain and the SVZ neuroblasts can migrate for long distances even the migration processes have not yet been fully understood. Although the mechanisms of repairing in the adult central nervous system may be highly rare, the proliferation and differentiation of NSCs can be enhanced in

numerous injuries. However, the newly born neurons that are capable of brain repair are not enough for total recovery (8,46).

In traumatic brain injury (TBI) and stroke, it has been described that NSCs become activated, increase their proliferation and migrate to the injury location (8). TBI leads to neuronal death that produce many long-term and harmful symptoms, such as disturbances in memory, learning, headaches, among others. TBI is a complex process since it has a regional, molecular, and cellular variability. Despite the fact of TBI increases the NSCs proliferation and neurogenesis, the proliferation rate depends on the severity of injury (47). In a cortical injury model using mice, the study of Saha and colleagues revealed that the number of proliferating cells in the SVZ increased after the injury. However, one month after the lesion, the proliferating cells was lower than in control and after two months, the number of proliferative cells in SVZ return to the basal levels, suggesting that cortical injury leads to a transient increase in cell proliferation in the SVZ. Moreover, they showed that the number of proliferating neuroblasts also increased and although the majority of them continued to migrate towards OB, a large number of neuroblasts migrated to the cortical lesioned area, either in a chain or as individual cells, associated to the blood vessels and to astrocytes but in smaller percentage when compared to blood vessels. Finally, they also showed that the newly formed cells from SVZ differentiated into a few mature neurons ($NeuN^+$) and many astrocytes ($GFAP^+$) and oligodendrocytes ($Olig2^+$) (48). In adult human patients were found an increase in the number of DCX^+, PSA-$NCAM^+$, and $SOX2^+$ cells after a TBI, but in the cerebral cortex and not in the SVZ (47). Furthermore, ischemic stroke resultant from diverse causes like embolism or thrombosis results in neuronal damage and ultimately in necrosis and apoptosis of neurons and glial cells due to energy depletion (46). Nevertheless, one study showed that ischemic stroke caused by middle cerebral artery occlusion in rats increases the number of neuroblasts, apparently from SVZ, in the damaged striatum, which was continuously produced for at least four months after stroke. The number of neuroblasts was correlated with the volume of injury being that 30 minutes after the insult the number of DCX^+ cells were lower than after two hours when the damage was more extensive. However, only a few neuroblasts differentiated into mature neurons and many of them appeared to die after four months, maybe through a caspase-mediated apoptosis (49). In the human brain, neurogenesis also seems to increase after stroke. In the cortical ischemic penumbra of sections from human brain biopsies, there were cells that expressed markers associated with proliferation (Ki67) and with newborn neurons (DCX and βIII-tubulin) (50).

While acute brain injuries lead to an increase in cell proliferation of the NSCs, the neurogenic response in neurodegenerative diseases, like Alzheimer's disease (AD) or Parkinson's disease (PD), is not fully understood (8). AD is the most common form of dementia characterized by a progressive neuronal loss in the brain, where the hippocampus is one of the most vulnerable brain regions. However, the reports about the neurogenic activity in the AD are still not unanimous. For example, in an AD-like neurodegeneration mouse model, it has been

described that SGZ neurogenesis increases but is neurodegenerative stage-dependent which means that at early stages of AD, neurogenesis is enhanced, as shown by the significant increase of DCX and NeuN stainings, while at later stages the survival of newborn neurons declined dramatically, which may be associated with aging but it is not clear (51). On the other hand, others reported that in APPswe/PS1ΔE9 transgenic mice, neurogenesis is impaired in both niches in the early stages of the AD and these mice showed a significant reduction in proliferation and in neuronal differentiation (52). These contradictory results illustrate that the neurogenic response to neuronal loss may depend on the model used, the disease stage and severity (8). In AD post-mortem human brains, Jin, and colleagues observed an increase of neuronal cells expressing DCX, PSA-NCAM, and NeuroD, which is consistent with an enhanced hippocampal neurogenesis in the AD that might represent an attempt to replace the damaged or dead neurons (53). Contrarily to AD, neurogenesis in PD seems to be decreased. PD is a neurodegenerative disease characterized by the degeneration of dopaminergic neurons, leading to the depletion of dopamine. Dopamine seemed to modulate neurogenesis and its depletion reduced the proliferation of type C cells, which in turn reduces the number of neuroblasts, *in vitro* and *in vivo*. Furthermore, PD post-mortem human brains showed not only a reduced SVZ neurogenesis but also a reduced number of cells that expressed Nestin and βIII-tubulin markers in the SGZ, suggesting that adult neurogenesis is impaired in PD (54).

Altogether these reports show that neurogenesis can be modulated in pathological conditions. In acute brain injury neurogenesis is showed to be increased whereas in neurodegenerative diseases is still controversial, at least in AD. Therefore, more studies are needed to elucidate the role of neurogenesis in neurodegenerative diseases. As only a few neuroblasts differentiate into mature neurons and there is no significant neuronal replacement after an injury, it is necessary to boost neurogenesis through new efficient brain repair strategies.

2 C-terminal Binding Proteins

CtBP family proteins are transcriptional coregulators indispensable for the repression of pro-apoptotic genes and for brain development, where are expressed at high levels (55). These proteins act mainly as transcriptional corepressors that are recruited to promoters by DNA-binding transcriptional repressors (56). CtBP1 was the first member of CtBPs family to be discovered. This protein was identified as a 48 kDa phosphoprotein that was bound to the C-terminal region of the adenovirus E1a protein (57). Shortly after the discovery of CtBP1, a second CtBP major isoform called CtBP2 was discovered (58).

2.1 Structural Domain and Function of CtBPs

The CtBP family includes several protein isoforms. In mammals, these isoforms result from the two CtBP genes, *Ctbp1* and *Ctbp2* (56). The *Ctbp1* gene encodes for two isoforms of CtBP1, namely CtBP1-L and CtBP1-S (where L stands for longer and S stands for shorter) (59). These isoforms are almost identical and both act as transcriptional corepressors in the

nucleus and have cytoplasmic functions (60). On the other hand, the *Ctbp2* gene encodes for three proteins of CtBP2, namely CtBP2-L, CtBP2-S and RIBEYE (59). CtBP2-L and CtBP2-S are related to the isoforms of CtBP1. However, these isoforms have a localization preferably nuclear for which reason they act primarily as transcriptional co-regulators (61). The RIBEYE isoform, expressed from an alternative promoter, plays an important role in ribbon synapses and is located exclusively in the cytoplasm (59,62). The invertebrates, like *Drosophila*, own a single CtBP gene that codes for several isoforms. However, these isoforms apparently do not have cytosolic functions as in the vertebrates (62).

2.1.1 Structure of CtBPs

CtBP1 and CtBP2 exhibit a similar structure since all isoforms share three domains: a hydrophobic cleft or the Pro-X-Asp-Leu-Ser (PXDLS; X is a hydrophobic amino acid)-binding cleft, the RRT-binding cleft and the dehydrogenase domain (Figure 4) (55). The PXDLS-binding cleft allows the association of CtBPs with CtBP-interacting partners containing a PXDLS motif (59). This domain is important to the recruitment of the corepressor complex, which includes HDACs, HMTs, and transcriptional repressors. However, CtBPs can recruit members of the corepressor complex in a PXDLS-independent manner. The RRT-binding cleft is functionally redundant with the PXDLS-binding domain. Many CtBP-interacting partners with an RRT-binding motif also contain PXDLS-binding motifs (63). Considering that each CtBP monomer possesses the PXDLS-binding and the RRT-binding clefts, it is possible that these domains are simultaneously occupied by different members of the corepressor complex. The dehydrogenase domain of CtBPs share homology with D2-hydroxyacid dehydrogenases and contains not only the RRT-binding cleft but also a nucleotide-binding domain (55). The nucleotide-binding domain is capable of binding both oxidized nicotinamide adenine dinucleotide (NAD^+) or reduced nicotinamide adenine dinucleotide (NADH), affecting the conformation of CtBPs (64). Although CtBPs have more affinity for NADH than for NAD^+, the dimerization of CtBPs in a NAD(H)-dependent manner is required for these proteins exert transcriptional repression. A model has been suggested to show how the dimerization of CtBPs helps in the repression of transcription. In this model, it has been postulated that CtBP recruits DNA-binding transcription repressors through one of the two PXDLS-binding clefts of a CtBP dimer while recruiting the constituents containing several histone-modifying enzymes through the second cleft (62). Nonetheless, mutations in the NAD(H)-binding motif make a defective CtBPs dimerization although they can perform other functions (63).

Despite the high homology between all the CtBPs isoforms, there are however some differences responsible for the different functions of CtBP1 and CtBP2 (Figure 4). One of them is the presence of a nuclear localization signal (NLS) at the N-terminal of CtBP2, which contributes to its nuclear localization, which is absent in CtBP1. However, CtBP2 can shuffle between the cytoplasm and nucleus and there are isoforms, particularly CtBP2-S, that lack the NLS, which leads to retention of them in the cytoplasm (65). Unlike CtBP2, CtBP1

possesses in its C-terminal a PDZ-binding domain that through the interaction with proteins, such as the neuronal nitric oxide synthase (nNOS), maintains its cytoplasmic location (66).

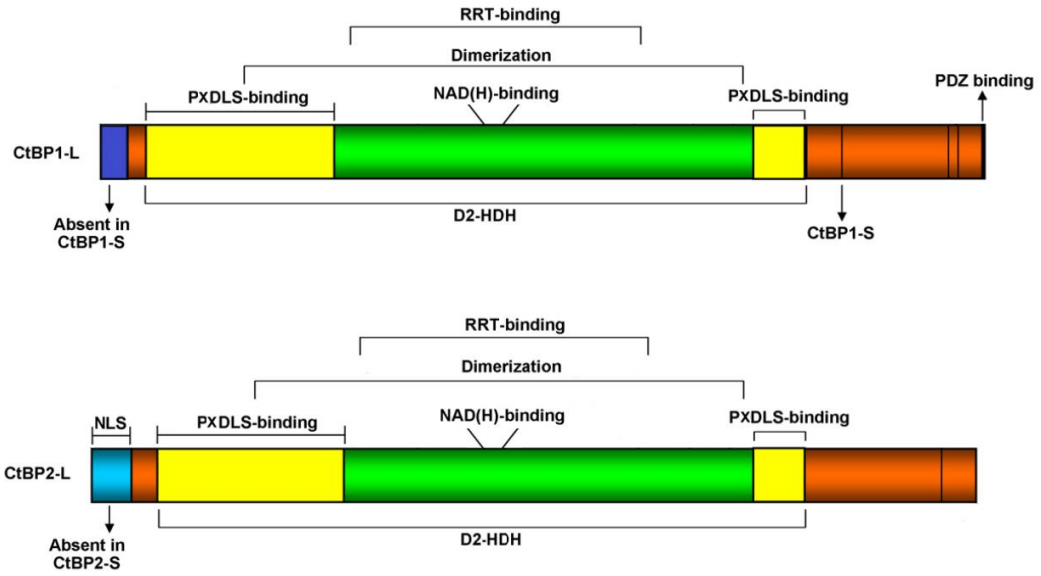

Figure 4 - Structure of CtBP1 and CtBP2. Both CtBPs have a PXDLS-binding cleft, an RRT-binding cleft, and the dehydrogenase domain. However, there are some slight differences between the two proteins: CtBP2 possesses an NLS-domain in its N-terminal while CtBP1 have a PDZ-binding domain in its C-terminal. Adapted from (62).

2.1.2 Nuclear and Cytoplasmic Functions of CtBPs

According to their location in the cell, CtBP family proteins can exert different functions. The main function of these proteins is to act as transcriptional corepressors in the nucleus, where they function as dimers. CtBP1 can heterodimerize with CtBP2 and due to the presence of the NLS in CtBP2, CtBP1 can shuffle its distribution from the cytoplasm to the nucleus (67). Because of CtBPs are not capable to bind directly to DNA, they are recruited by DNA-binding transcriptional repressors, where most of them interact with CtBPs through the PXDLS-binding motif. After binding to the transcriptional repressor, CtBPs recruit enzymes that are part of the corepressor complex and catalyze modifications on histones to repress transcription. Nevertheless, it is not known how CtBPs recruit these histones-modifying enzymes. The corepressor activity is facilitated mainly by the HDACs (63). Furthermore, transcriptional repression can be also regulated by the SUMOylation of transcription factors that unlike ubiquitination do not degrade them (62,66). However, CtBPs can repress transcription in a corepressor complex-independent manner, binding directly and inhibiting the function of the transcriptional coactivators, p300 and P/CAF (62).

In the cytoplasm, these proteins also perform important functions. CtBP1-S regulates membrane fission which is necessary for membrane trafficking and for Golgi complex partitioning during mitosis (Figure 5). Moreover, CtBP1-L is present in ribbon synapses although its function still unclear (67). In neurons, CtBP1 is highly expressed in the presynaptic compartment, where interacts with Piccolo and Bassoon, two presynaptic

proteins. Its distribution between the nucleus and the cytoplasm, which regulates its corepressor activity, depends on the neuronal activity. An elevated neuronal activity increases the synaptic retention of CtBP1 through Piccolo and Bassoon. The binding between CtBP1 and Bassoon is promoted by the increase of NADH concentrations that occurs after the elevation of neuronal activity. Contrarily, inactivity retains CtBP1 in the nucleus (60). In addition to CtBP1, CtBP2-S lacking NLS is also localized in the presynaptic compartment with Bassoon, in neurons. This might suggest that CtBP2-S might function as synapto-nuclear messenger proteins (68). As mentioned before, RIBEYE, an isoform of CtBP2, has a location exclusively cytoplasmic where has an important role in the ribbon synapses (69). CtBPs can be regulated by several processes, such as the binding of NAD(H) or post-translational modifications that together with protein-protein interactions can mediate the switch of the cellular location of CtBPs and their functions in the cell (67).

2.2 Regulation of CtBPs

CtBPs have a domain that allows the binding of NADH or NAD^+, promoting CtBP dimerization. In turn, the NAD(H)-binding contributes to transcriptional repression as it promotes the binding of transcriptional factors containing a PXDLS-binding motif (65). According to the *in vitro* results obtained by Zhang and colleagues, the levels of NADH in the nucleus increase under hypoxic conditions. This stimulates the interaction of CtBPs with the repressors, enhancing the transcriptional repression (70). For the contrary, the absence of the binding between NAD(H) and CtBPs abolish the ability for dimerization as well as their nuclear accumulation and therefore the corepressor activity of CtBPs is affected (67).

Post-translational modifications, especially phosphorylation and SUMOylation, can also regulate the distribution and the functions of CtBPs. These proteins are phosphorylated by several kinases that often target them for ubiquitination and therefore for proteasomal degradation (55). CtBP1 is more susceptible to these post-translational modifications than CtBP2 since the latter is particularly subject to acetylation by p300, which contribute to its nuclear retention, and to SUMOylation at lower levels when compared to CtBP1 (61,62). The phosphorylation of CtBP1-S by AMP-activated protein kinase (AMPK) and by the p21-activated kinases 1 and 6 (PAK1 and PAK6) results in its cytoplasmic location. However, phosphorylation of this isoform by protein kinase A (PKA) leads to its dimerization and retaining in the nucleus. SUMOylation also leads to nuclear retention of CtBP1-S but this modification is inhibited by nNOS, changing its location from the nucleus to the cytoplasm (Figure 5) (67).

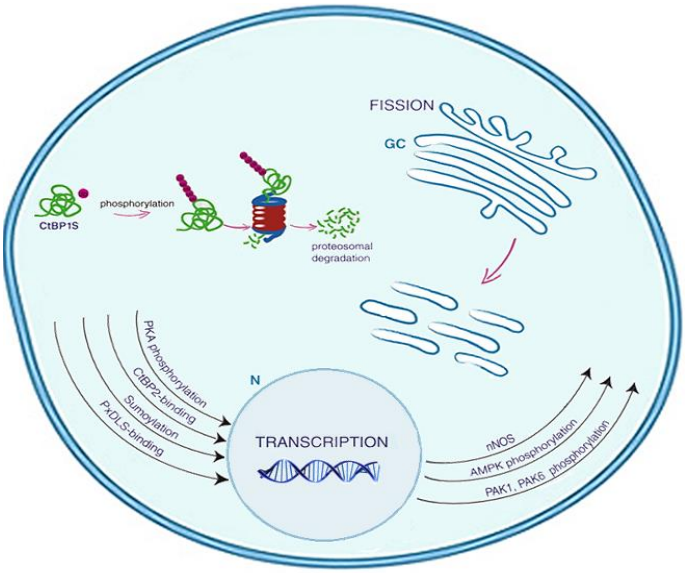

Figure 5 – Schematic representation of the mechanisms that mediate the location and functions of CtBP1-S in the cell. The location of CtBP1-S in the nucleus (N) might depend on its dimerization with CtBP2, binding to transcriptional factors containing the PXDLS-binding motif, PKA phosphorylation, and SUMOylation. On the other side, its cytoplasmic location maybe is due to the binding of nNOS, and to AMPK, PAK1, and PAK6 phosphorylation. In the cytoplasm, CtBP1-S is important for membrane fission required for the fragmentation of the Golgi complex (GC) during mitosis. After phosphorylation, CtBP1-S could be targeted for ubiquitylation followed by proteasome-mediated degradation. Adapted from (67).

2.3 CtBPs in Development, Cell Survival, Proliferation, and Differentiation

CtBPs are essential for normal embryonic development and adult lifespan (59). In mouse development, CtBP1 is expressed from embryo to adult while CtBP2 is primarily expressed during embryogenesis (71). The genetic elimination of these proteins results in severe developmental defects and embryonic lethality (55). Although *Ctbp1* knockout mice are viable and fertile, 30% are smaller and 23% die at postnatal day 20. In contrast, CtBP2 deletion is embryonic lethal and at embryonic day 10.5, the *Ctbp2*-null embryos have a small size and exhibit axial truncations and delayed development of the forebrain and midbrain. Furthermore, the complete elimination of *Ctbps* results in a more severe phenotype and an earlier embryonic death when compared with the other knockout mice (72).

The role of CtBPs in cell proliferation and survival was mostly evaluated in cancer conditions where they are often overexpressed. CtBPs overexpression is mostly protumorigenic not only because these proteins can negatively regulate some tumor suppressors genes as is the case of *phosphatase and tensin homolog* (PTEN), in which its repression promotes cell proliferation, but also because CtBPs promote cell survival and proliferation. Hyperactivation of CtBPs in cancer cells is also possible due to high levels of NADH in these cells, which result in their dimerization with a consequent increase in their activity (56). In parallel to their function as transcriptional corepressors, CtBPs can promote cell survival mainly through the repression of pro-apoptotic genes. The study performed by Frisch and colleagues using mouse embryonic fibroblasts revealed that CtBPs can downregulate pro-apoptotic genes such as *p53*-

effector related to pmp-22 (PERP), *p21*, *Bax,* and *Noxa*. Although these genes are targets of p53, their down-regulation is in a p53-independent manner (73). Another study realized by Zhang and colleagues demonstrated that in mouse embryonic fibroblasts null for *Ctbp1* and *Ctbp2* the activation of caspase-3, a signal that initiates apoptosis, was more prominent (74). CtBPs might also modulate the expression and activity of the Ink4 family tumor suppressors, coding for three cell cycle inhibitors, p16Ink4a, Ink4a/Arf and p15Ink4b. The repression of p16Ink4a and p15Ink4b by CtBPs enhances cell proliferation (75).

In addition to their role in tumorigenesis, these corepressors are involved in neuronal survival and differentiation, although these effects are poorly explored. In this context, one study reported that the expression of CtBP1 and CtBP2 is downregulated in a caspase-dependent manner in primary cerebellar granule neurons when these are exposed to several neurotoxins. Also, this study suggested that the downregulation of CtBPs might be associated with neurodegenerative diseases (76). CtBPs may also regulate the inflammatory response by astrocytes and microglia, with a dual function. The study made by Zhang and colleagues showed that CtBP2 was up-regulated after LPS injection in rat spinal cord as well as in astrocytes and microglia but its knock-down with small interfering RNA (siRNA) increased the activation of microglia, suggesting that CtBP2 may prevent inflammation by inhibiting the expression of pro-inflammatory genes (77). However, others indicate that these proteins, by repressing inhibitors of inflammatory response genes in microglia and astrocytes, can promote inflammation (78). Since CtBPs can act as sensors of the oxygen levels and oxygen regulate the state of NSCs, Dias and colleagues investigated the role of these proteins in NSCs. First, they found that the roof plate region presents high oxygen levels necessary for the expression of *Hes1*. The HES1 transcription factor maintains the self-renewal capacity of neural progenitors, inhibiting neurogenesis in the roof plate. Thus, high levels of oxygen and therefore low levels of NADH stimulates the depletion of CtBPs from the *Hes1* promoter. In this way, the high expression levels of *Hes1* are maintained, repressing neurogenesis in roof plate. Furthermore, the BMPs secreted by the neural tube induce the *Hes* family members in cortical NSCs. Altogether, this suggests that CtBPs repress neuronal differentiation in the presence of high oxygen levels and BMPs (79).

Nevertheless, it is unknown the cellular expression as well as the function of CtBP1 and CtBP2 in the neurogenic niches in the adult brain.

All these evidences suggest that CtBPs may be a good target to regulate the transcriptional mechanisms in neurogenesis.

Chapter 2

Objectives

Adult neurogenic niches are a source of new neurons throughout life and their differentiation can be regulated by extrinsic and by intrinsic factors, including transcriptional factors (80). CtBPs are transcriptional corepressors able to modulate cell proliferation, differentiation, and survival (55). However, the role of CtBPs in adult neurogenesis is unknown.

The main aim of this work is to study the effects of CtBPs in SVZ neurogenesis *in vitro*. For that, the specific tasks are as follows:

- Evaluate the expression of CtBPs in the SVZ *in vitro* and *in vivo*.

- Assess the effects of CtBPs on cell survival, proliferation and differentiation in SVZ cells *in vitro*.

Chapter 3

Materials and Methods

1 *In vivo* Studies

All experiments were performed in accordance with protocols approved by the national ethical requirements for animal research, and in accordance with the European Community guidelines (2010/63/EU). Wild-type C57BL/6J adult mice with 8- to 10-week-old were used for the *in vivo* experiments.

1.1 Brain Slices Preparation

Mice were anesthetized with an intraperitoneal injection of a mixture of ketamine (90 mg/kg of mouse weight) and xylazine (10 mg/kg of mouse weight). Then, an incision along the thoracic midline was made. In the left ventricle of the heart, a needle was insert and the right aorta was cut. The transcardial perfusion was made using 0,9% NaCl until the blood was totally clear, followed by perfusion with 4% paraformaldehyde (PFA; pH 7.4, Sigma-Aldrich). Brains were then removed and fixed overnight in 4% PFA, at 4ºC, followed by immersion in a 30% sucrose solution (Sigma-Aldrich), at 4ºC. Brains were frozen, embedded in optimal cutting temperature gel (Bio-Optica) and cut into coronal sections at a thickness of 40 µm, on a freezing cryostat-microtome (Leica CM 3050S, Leica Microsystems) at -20°C. The sections corresponding to the SVZ of each animal were collected sequentially in six wells of 24-well plate and were kept in anti-freeze solution (30% of ethylene glycol, 30% glycerol, 30% water and 10% phosphate buffer solution) until be used for immunohistochemistry.

2 Subventricular Zone Cell Culture

SVZ cells were isolated from 1- to 3-day-old C57BL/6J mice, as described by Agasse *et al.* (2008) (81). Animals were sacrificed by decapitation and brains were removed and placed into Hanks Balanced Salt Solution (HBSS; Gibco) supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin (all from Life Technologies). Meninges were removed and cerebellum was separated from the brain. SVZ fragments were dissected from 450 µm-thick coronal brain sections using a McIlwain tissue chopper. The fragments of SVZ were placed into HBSS supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin and digested in 0.025% trypsin and 0.265 mM EDTA (all from Life Technologies), for 20 minutes, at 37ºC, followed by mechanical dissociation with a P1000 pipette. Cell suspension was diluted in medium composed by Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12) [DMEM/F-12+GlutaMAX™-I; Gibco] supplemented with 100 U/ml penicillin, 100 µg/mL streptomycin, 1%

B27 supplement, 10 ng/mL EGF and 5 ng/mL basic FGF-2 (all from Life Technologies). Cells were plated on uncoated Petri dishes (Corning Life Science) and allowed to develop in an incubator with 5% CO_2 and 95% atmospheric air, at 37ºC, for six days.

3 SVZ Cell Treatments

Six-day-old neurospheres were collected from the uncoated Petri dishes and seeded onto 0.1 mg/mL PDL-coated 24-well plates in the presence of DMEM/F-12 devoid of growth factors. SVZ neurospheres were grown with 5% CO_2 and 95% atmospheric air, at 37ºC, for 48 h, until the experimental treatments were performed.

To investigate the effect of MTOB in neurogenesis, SVZ cells were treated with 5 µM, 25 µM, 50 µM, 100 µM, 250 µM, 1 mM and 2.5 mM of MTOB diluted in DMEM/F-12 devoid of growth factors. Controls were included in all experiments. Then, SVZ cells were placed in an incubator with 5% CO_2 and 95% atmospheric air, at 37ºC, for several timepoints, until fixation. For the evaluation of cell survival and proliferation, cells were fixed two days after treatments while for cell differentiation, cells were fixed seven days after treatments. Regarding the expression of CtBPs, cells were fixed two days after plating (Figure 6).

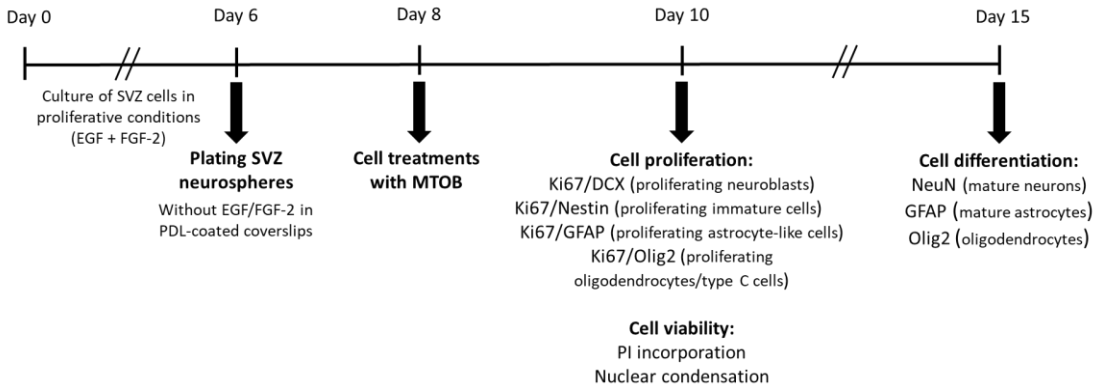

Figure 6 - Schematic representation of the experimental treatments and assays performed *in vitro*.

4 Propidium Iodide Incorporation

To evaluated cell death, propidium iodide (PI; 5 µg/mL; Sigma-Aldrich) was used. This cell death marker is incorporated by necrotic and late-apoptotic cells that have their membrane damaged. This allows the entrance of the dye in these cells and its bind directly to the DNA (82). 10 minutes before the end of the 48h treatments, PI was added to cell medium, at 37ºC. Then, cells were rinsed with phosphate buffer saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4; pH 7.4), fixed using 10% formalin solution, for 15 minutes, at RT and rinsed three times with PBS, 5 minutes each. Hereafter, cell nuclei were stained with Hoechst-33342 (2 µg/mL; Life Technologies), rinsed three times with PBS, 5 minutes each, and mounted in Fluoroshield Mounting Medium (Abcam). Five random microscopic fields were

acquired per replicate using an AxioImager microscope (Carl Zeiss) with a total magnification of 400x. The number of PI-positive cells and nuclear condensation was obtained using the ImageJ program.

5 Western Blot

For western blot experiments, SVZ cells cultivated under proliferation and differentiation conditions were used. Proliferation condition refers to neurospheres grown in the presence of growth factors (EGF, FGF-2) which are rich in neural and progenitor stem cells with abilities of self-renewal and proliferation. Differentiation condition refers to neurospheres that were seeded into 0.1 mg/mL poly-D-lysine (PDL, Sigma-Aldrich)-coated coverslips, in medium devoid of growth factors, to induce cell differentiation (81). To obtain SVZ cells under proliferation conditions, six-day-old neurospheres were collected from one uncoated Petri dish and were gently centrifuged at 300 rpm, for 1 minute, at RT. The supernatant was removed and cells were lysed on ice by adding 150 µL of RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Triton X- 100, 0.5% Sodium deoxycholate, 0.1% SDS, and a cocktail of protease inhibitors). For differentiation conditions, six-day-old neurospheres were allowed to adhere for 2 days onto 0.1 mg/mL PDL-coated 6-well plate, grown with DMEM/F-12 supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 1% B27 supplement, in other words, DMEM/F-12 devoid of growth factors, in an incubator with 5% CO_2 and 95% atmospheric air, at 37ºC. Then, SVZ cells were lysed using the RIPA buffer on the ice.

All the lysates were resuspended and sonicated. Then, a centrifugation at 14000 rpm, for 10 minutes, at 4ºC was made and the supernatant was collected. The total amount of protein was determined using the Pierce Bicinchoninic Acid Protein Assay Kit (Thermo Scientific). Then, samples were boiled for 5 minutes at 95ºC with Loading Buffer (6x concentrated: 350 mM Tris, 10% SDS, 30% glycerol, 0.6 M DTT, 0.06% bromophenol blue).

A total of 40 µg of protein was loaded into the 10% SDS polyacrylamide gels and proteins were separated by SDS-PAGE electrophoresis at 120V, using a running buffer solution (25 mM Tris, 190 mM glycine, 0.1% SDS; pH 8.3), at RT. Then, proteins were transferred to a polyvinylidene difluoride membrane (Millipore), through semi-dry transfer during 25 minutes at 1.0A, 25V, at RT, using transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol; pH 8.3). After transfer, membranes were blocked in Tris buffer saline solution–Tween 20 0.1% (20 mM Tris, 137 mM NaCl solution and 0.1% Tween 20) containing 0.1% gelatin (Fluka) for 1 hour at RT. Then, membranes were incubated with mouse anti-CtBP1 (1:2500; 48kDa; BD Bioscience) and anti-CtBP2 (1:2500; 48kDa; BD Bioscience) overnight at 4°C, or with mouse anti-actin (housekeeping; 1:5000; 42kDa; BD Bioscience) antibodies and further incubated with the goat anti-mouse antibody conjugated with horseradish peroxidase (1:5000; Santa Cruz Biotechnology), diluted in blocking solution, at RT for 1 hour. Immediately before the incubation with housekeeping, membranes were incubated with 10% hydrogen peroxide

(H_2O_2), in order to saturate the horseradish peroxidase, for 30 minutes, at RT. Then, membranes were incubated with Pierce™ ECL Western Blotting Substrate (Thermo Scientific) for 1 minute, in the dark. Protein lanes were detected using the ChemiDocTM MP Imaging System (Bio-Rad) and quantified using the Image Lab 5.1 software (Bio-Rad Laboratories).

6 Immunostainings and Imaging

6.1 Immunocytochemistry

Cells were fixed using 10% formalin solution, for 15 minutes, at RT. Then, cells were rinsed three times with PBS, 5 minutes each. The permeabilization and blocking step was then performed, to avoid non-specific binding sites, with 0.5% Triton X-100 (Fisher Scientific) and 3% bovine serum albumin (BSA; Amresco LLC), for 30 minutes, for cytoplasmic staining, or with 0.5% Triton X-100 and 6% BSA, for 1 hour, for nuclear staining, at RT. Cells were incubated overnight at 4ºC with the primary antibodies (Table 1) prepared in the blocking/permeabilization solution with 0.3% BSA and 0.1% Triton X-100. After three washes with PBS, 5 minutes each, cells were incubated for 1 hour, at RT, with the secondary antibodies (Table 1) together with Hoechst 33342 for nuclear staining, all diluted in PBS. Lastly, cells were rinsed three times with PBS and mounted in Fluoroshield Mounting Medium. Five random microscopic fields were acquired per replicate using a confocal microscope (LSM 710; Carl Zeiss) with a total magnification of 400x. The number of cells was obtained using the ImageJ program.

6.2 Immunohistochemistry

Brain slices were rinsed three times with PBS, 5 minutes each and once with PBS-Tween 20 0.1%, for 5 minutes. To prevent unspecific bindings, brain slices were incubated with a blocking solution containing 2% horse serum and 0.1% Triton X-100 in PBS, for 2 hours, at RT. Then, brain slices were incubated for 48 hours at 4ºC with the primary antibodies (Table 1) prepared in blocking solution. Thereafter, brain slices were rinsed three times with PBS, 5 minutes each. Next, brain slices were incubated for 2 hours, at RT, with the respective secondary antibodies (Table 1) together with Hoechst 33342, for nuclear staining, in PBS. Lastly, brain slices were rinsed three times with PBS, 5 minutes each and mounted in Fluoroshield Mounting Medium. All these steps were performed using an orbital shaker. Fluorescent images were acquired using a confocal microscope with a total magnification of 400x.

Table 1 - Primary and secondary antibodies used for immunostainings.

Primary Antibodies				
Reactivity	**Species**	**Dilution ICC**	**Dilution IHC**	**Manufacturer**
Ki67	Rabbit	1:50	1:1000	abcam
Ki67	Mouse	1:50	-	BD Bioscience
DCX	Goat	1:200	1:1000	Santa Cruz
Nestin	Mouse	1:100	-	abcam
Nestin	Goat	1:100	1:200	Santa Cruz
Sox2	Goat	1:200	1:500	Santa Cruz
GFAP	Rabbit	1:2000	1:2000	DAKO
GFAP	Mouse	1:500	-	BD Bioscience
NeuN	Mouse	1:100	-	Millipore
NeuN	Rabbit	-	1:500	Cell Signalling
MAP2	Rabbit	1:100	-	Santa Cruz
Olig2	Rabbit	1:200	1:500	Millipore
CtBP1	Mouse	1:200	1:1000	BD Bioscience
CtBP2	Mouse	1:200	1:500	BD Bioscience
Secondary Antibodies (Alexa-Conjugated)				
Reactivity	**Species**	**Dilution ICC**	**Dilution IHC**	**Manufacturer**
Anti-Rabbit	Donkey/Alexa 488	1:200	1:1000	Life Technologies
Anti-Rabbit	Donkey/Alexa 594	1:200	-	abcam
Anti-Goat	Donkey/Alexa 546	1:200	-	Life Technologies
Anti-Goat	Donkey/Alexa 647	1:200	1:1000	Life Technologies
Anti-Mouse	Donkey/Alexa 488	1:200	-	abcam
Anti-Mouse	Donkey/Alexa 594	1:200	1:1000	abcam
Anti-Mouse	Donkey/Alexa 647	1:200	-	Life Technologies

Legend: DCX: doublecortin; Sox2: sex determining region Y-box 2; GFAP: glial fibrillary acid protein; NeuN: neuronal nuclei; MAP2: Microtubule-associated protein 2; Olig2: oligodendrocyte transcription factor 2; CtBP1: C-terminal binding protein-1; CtBP2: C-terminal binding protein-2.

7 Statistical analysis

All experimental conditions were performed at least from three independent experiments, performed at least in duplicate (*in vitro*) or with two different animals (*in vivo*). Statistical analysis was performed using one-way ANOVA followed by the Dunnett's multiple comparisons test, except for western blot where statistical analysis was performed using an unpaired two-tailed Student's t-test. Values of $P<0.05$ were considered significant. All statistical analysis was made using the GraphPad Prism 7.0 Software (GraphPad Sotware Inc.).

Chapter 4

Results

4.1 Expression of CtBP1 and CtBP2 in the SVZ

The first task of this work was to analyze the expression of both CtBPs in SVZ cells. First, we evaluated the expression of CtBPs under proliferation and differentiation conditions, *in vitro* by western blot. As shown in figures 7A and B, no statistical differences were found in the protein expression levels of CtBP1 (Proliferation: 102.9±10.2%; n=6) and CtBP2 (Proliferation: 114.7±19.2%; n=4), when compared to differentiation (set to 100%).

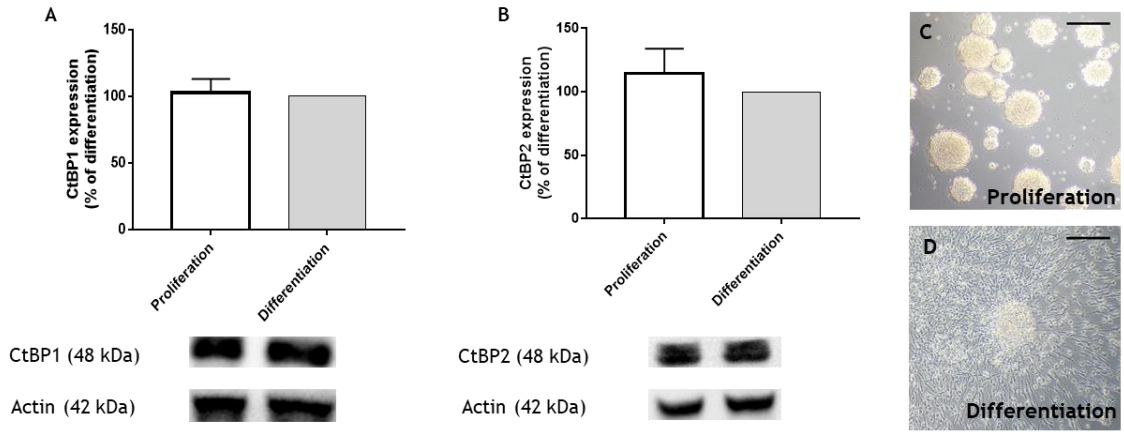

Figure 7 – Expression levels of CtBP1 and CtBP2 in SVZ, *in vitro*. Graphs depict the percentage of (A) CtBP1 and (B) CtBP2 in SVZ. Protein expression was normalized to actin. Data are expressed as a percentage of mean ± SEM (A: n=6; B: n=4). Expression of CtBPs in differentiation was normalized to 100%. Bellow each graph, a representative western blot of CtBP1 (48kDa), CtBP2 (48kDa) and actin (42kDa) is shown. Statistical analysis was performed using unpaired two-tailed Student's t-test. Representative images of SVZ cells under (C) proliferation and (D) differentiation conditions. Scale bar: 100 µm.

We then analyzed their expression in distinct cell SVZ phenotypes. For that, we performed co-stainings against CtBP1 or CtBP2 and markers for proliferating cells (Ki67), immature cells (Nestin and Sox2), neuroblasts (DCX), neurons (NeuN and microtubule-associated protein 2 (MAP2)), astrocytes (GFAP) and oligodendrocytes (Olig2). Our results show that CtBP1 is expressed in SVZ in almost every Ki67$^+$, Nestin$^+$, Sox2$^+$, DCX$^+$, MAP2$^+$, GFAP$^+$ and Olig2$^+$ cells, *in vitro* (Figure 8) and *in vivo* (Figure 9). CtBP1 also co-localized with NeuN$^+$ cells *in vitro* and in the striatum *in vivo*. As expected, NeuN$^+$ cells were not found in the SVZ niche *in vivo*.

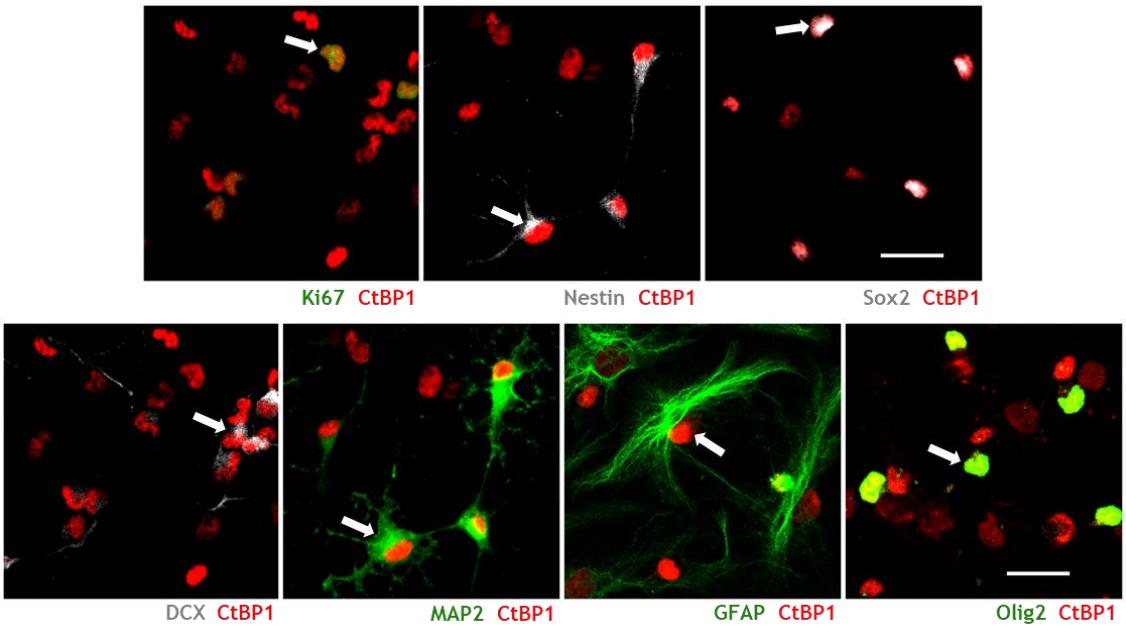

Figure 8 – Expression of CtBP1 in SVZ cells, *in vitro*. Representative confocal images of the expression of CtBP1 in proliferating (Ki67) and immature cells (Nestin, Sox2), neuroblasts (DCX), neurons (MAP2), astrocytes (GFAP), and oligodendrocytes (Olig2). White arrows highlight cells with double staining. Scale bar: 20 µm.

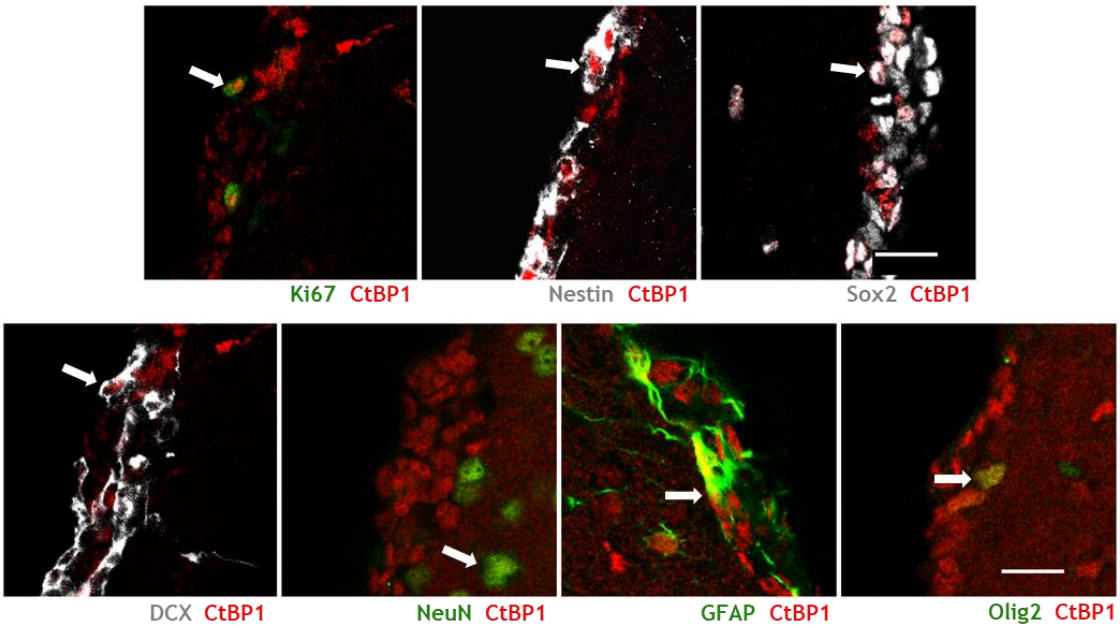

Figure 9 - Expression of CtBP1 in SVZ cells, *in vivo*. Representative confocal images of the expression of CtBP1 in proliferating (Ki67) and immature cells (Nestin, Sox2), neuroblasts (DCX), neurons (NeuN), astrocytes (GFAP), and oligodendrocytes (Olig2). White arrows highlight cells with double staining. Scale bar: 20 µm.

Likewise, the cellular expression of CtBP2 in the SVZ was also evaluated. CtBP2 is also expressed in Ki67$^+$, Nestin$^+$, Sox2$^+$, DCX$^+$, MAP2$^+$, GFAP$^+$ and Olig2$^+$ cells, *in vitro* (Figure 10) and *in vivo* (Figure 11). Like CtBP1, CtBP2 also co-localized with NeuN$^+$ cells *in vitro* and in the striatum *in vivo*. As expected, NeuN$^+$ cells were not found in the SVZ niche *in vivo*.

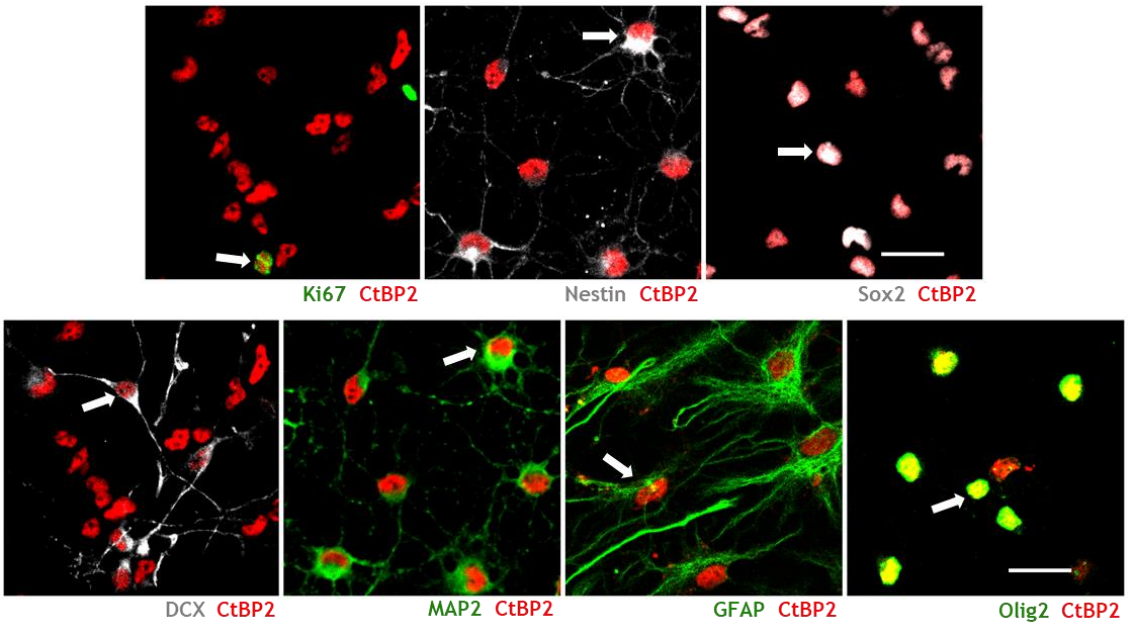

Figure 10 - Expression of CtBP2 in SVZ cells, *in vitro*. Representative confocal images of the expression of CtBP2 in proliferating (Ki67) and immature cells (Nestin, Sox2), neuroblasts (DCX), neurons (MAP2), astrocytes (GFAP), and oligodendrocytes (Olig2). White arrows highlight cells with double staining. Scale bar: 20 µm.

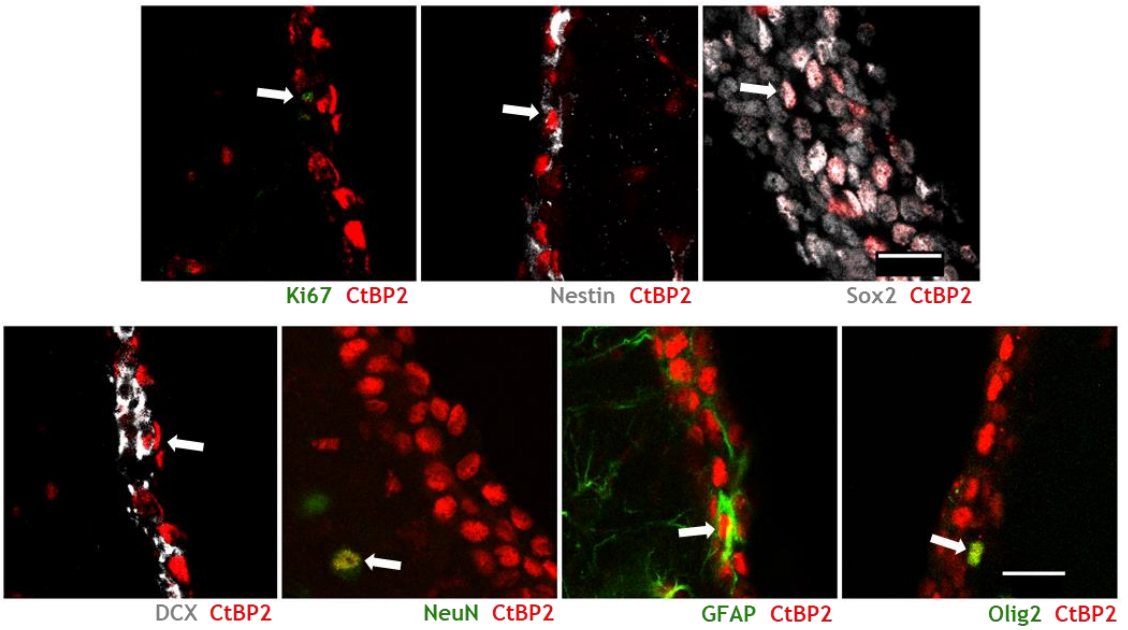

Figure 11 – Expression of CtBP2 in SVZ cells, *in vivo*. Representative confocal images of the expression of CtBP2 in proliferating (Ki67) and immature cells (Nestin, Sox2), neuroblasts (DCX), neurons (NeuN), astrocytes (GFAP), and oligodendrocytes (Olig2). White arrows highlight cells with double staining. Scale bar: 20 µm.

4.2 The effect of CtBPs in SVZ neurogenesis

Our second task was to assess the effect of CtBPs on SVZ neurogenesis *in vitro*. To achieve this goal, a substrate-based inhibitor of CtBPs, MTOB, was used. MTOB is a dehydrogenase substrate that displays a biphasic saturation curve, which means that at low concentrations

acts as a substrate, but at high concentrations acts as dehydrogenase inhibitor (59). To evaluate the effects of MTOB in neurogenesis, SVZ cells were treated with the concentrations of 5 µM, 25 µM, 50 µM, 100 µM, 250 µM, 1 mM and 2.5 mM of MTOB for two days (cell survival and proliferation) or seven days (cell differentiation) (Figure 6).

4.2.1 Effects of MTOB on cell viability

The effects of MTOB on cell survival were evaluated by nuclear condensation/fragmentation (Figure 12 A and B) and PI incorporation (Figure 12 C). First, we found that high concentrations of MTOB increased significantly the number of fragmented/condensed nuclei (control: 15.2±1.0; 1 mM: 22.9±3.8; 2.5 mM: 21.6±2.1; n=2-7). Based on these experiments, we then selected MTOB concentrations up to 250 µM to evaluated PI incorporation. We found the MTOB did not induce a significant increase in the number of PI^+ cells when used until 250 µM. Due to the toxic effect of the high concentrations of MTOB, we decided to use only the concentrations up to 100 µM of MTOB in the next experiments.

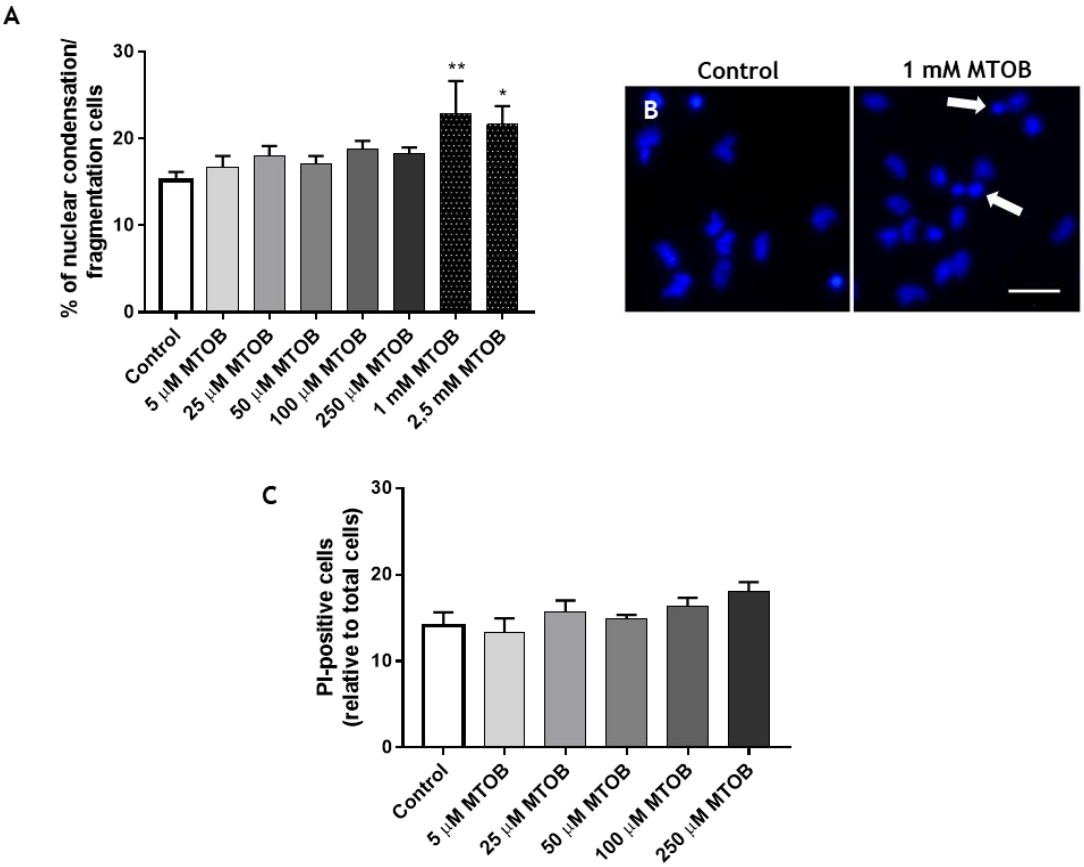

Figure 12 - MTOB induces cell death at high concentrations. After cell treatments with different concentrations of MTOB (5 µM, 25 µM, 50 µM, 100 µM, 250 µM, 1 mM, and 2.5 mM), cells were maintained in culture for 2 days. (A) Percentage of cells with nuclear condensation/fragmentation and (B) representative confocal digital images of Hoechst-labeled nuclei in control and cells treated with 1 mM of MTOB. White arrows highlight cells with nuclear condensation/fragmentation. Scale bar: 20 µm. (C) Percentage of PI^+ cells. Data are expressed as mean ± SEM (A: n=2-7; C: n=4). Statistical analysis was performed using one-way ANOVA, followed by the Dunnett's multiple comparison test. **P<0.01 and *P<0.05 when compared with control.

4.2.2 MTOB increased the number of proliferating neuroblasts

Next, we analyzed the effect of MTOB on cell proliferation by performing Ki67 staining, two days after cell treatments. Ki67 is a nuclear marker of dividing cells, that can be found in all phases of the cell cycle (83). As shown in Figure 13, total cell proliferation was not affected by MTOB as compared to control.

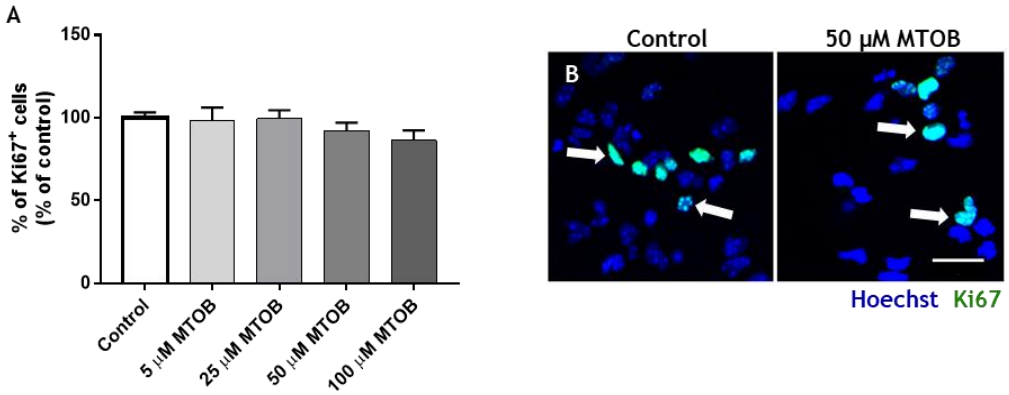

Figure 13 - MTOB did not affect the total number of Ki67 proliferating cells. Cell proliferation was assessed two days after cell treatments. (A) Bar graph depicts the percentage of Ki67$^+$ cells. (B) Representative confocal digital images of Ki67$^+$ cells in control and cells treated with 50 μM of MTOB; Nuclei are shown in blue (Hoechst). Control was set to 100%. Data are expressed as a percentage of control ± SEM (n=7-9). Statistical analysis was performed using one-way ANOVA, followed by the Dunnett's multiple comparison test. White arrows highlight cells with Ki67 staining. Scale bar: 20 μm.

Next, we evaluated the proliferation of specific SVZ cell phenotypes. For that, we performed co-stainings against Ki67 and markers for immature cells (Nestin), neuroblasts (DCX), type C cells/oligodendrocytes (Olig2) and astrocyte-like cells (GFAP).

SVZ cells treated with 25 μM of MTOB showed an increase in the number of proliferating neuroblasts (Ki67$^+$/DCX$^+$; Figure 14 A and B; control: 100±2.7; 25 μM: 146.1±12.4; n=3-4) and the concentrations of 5 μM and 50 μM of MTOB seem to increase it as well (5 μM: 127.4±10.0; 50 μM: 123.2±10.33; n=4). However, no statistical differences were found in the number of proliferating Nestin$^+$ (Ki67$^+$/Nestin$^+$; Figure 14 C), Olig2$^+$ (Ki67$^+$/Olig2$^+$; Figure 14 D) and astrocyte-like cells (Ki67$^+$/GFAP$^+$; Figure 14 E) when compared to control.

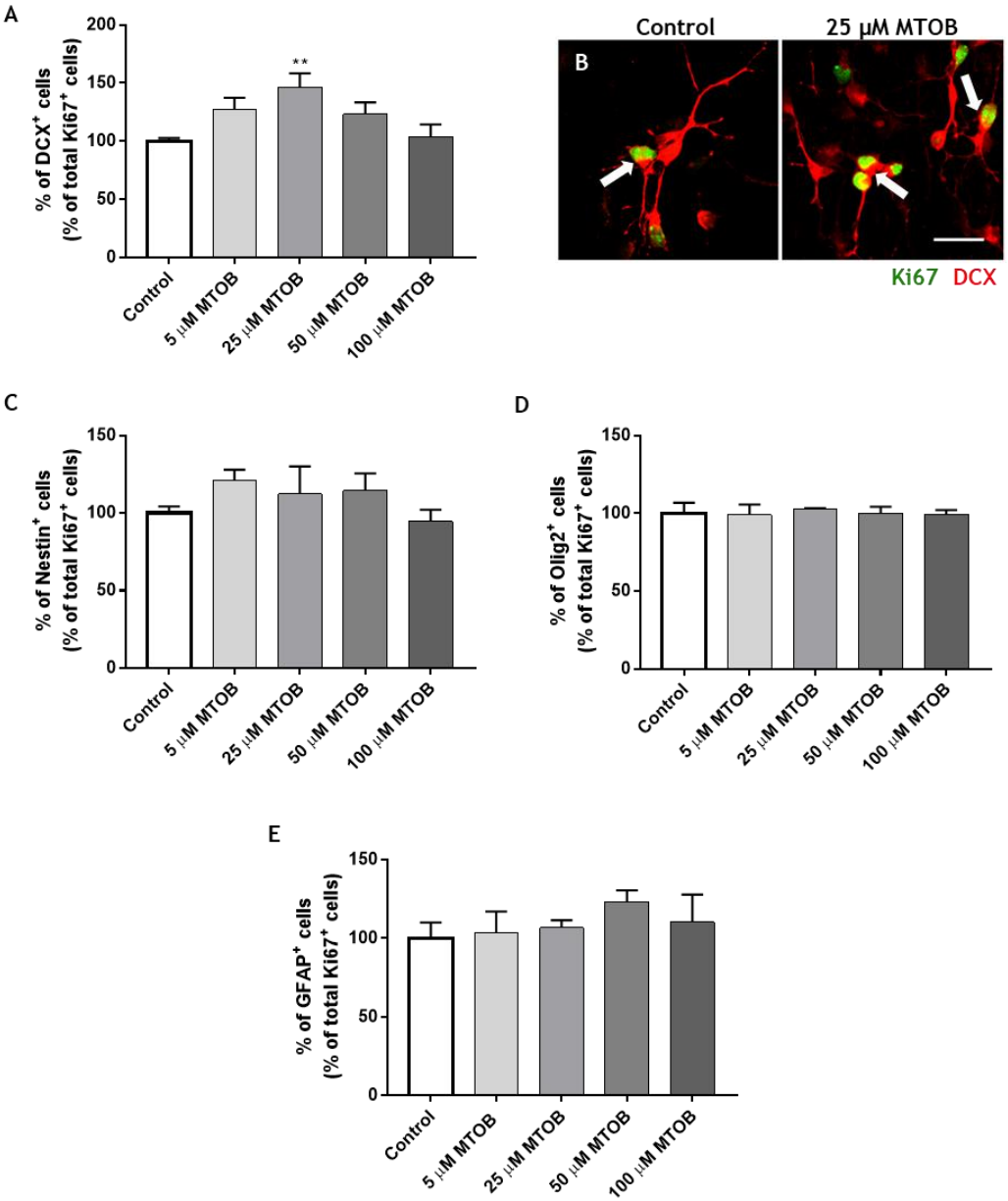

Figure 14 – MTOB increased the percentage of proliferating neuroblasts. (A) Percentage of DCX^+ cells co-stained with Ki67. (B) Representative confocal digital images of $Ki67^+/DCX^+$ cells in control and cells treated with 25 μM of MTOB. White arrows highlight cells with double staining. Scale bar: 20 μm. Percentage of (C) $Ki67^+/Nestin^+$, (D) $Ki67^+/Olig2^+$ and (E) $Ki67^+/GFAP^+$ cells. Control was set to 100%. Data are expressed as percentage of control ± SEM (A: n=3-4; C: n=5-6; D: n=3; E: n=2-3). Statistical analysis was performed using one-way ANOVA, followed by the Dunnett's multiple comparison test, **P<0.01 when compared with control.

4.2.3 MTOB increased neuronal and oligodendrocyte differentiation

Lastly, neuronal and glial differentiation was evaluated seven days after cell treatments. Our results showed that 5 μM, 25 μM and 50 μM of MTOB increased the percentage of NeuN-mature neurons ($NeuN^+$; Figure 15 A and B; control: 100±13.3; 5 μM: 171.8±19.9; 25 μM: 150.8±10.5; 50 μM: 171.2±16.6; n=4-5) as well as the percentage of oligodendrocytes ($Olig2^+$; Figure 15 C and D; control: 100±8.4; 5 μM: 174.1±16.9; 25 μM: 189.5±30.0; 50 μM: 184.3±10.9; n=2-3). Although Olig2 is necessary for oligodendrogenesis, this marker is also expressed by

progenitors cells (39). No statistical differences were found regarding astrocytes differentiation (GFAP$^+$; Figure 15 E) when compared to control.

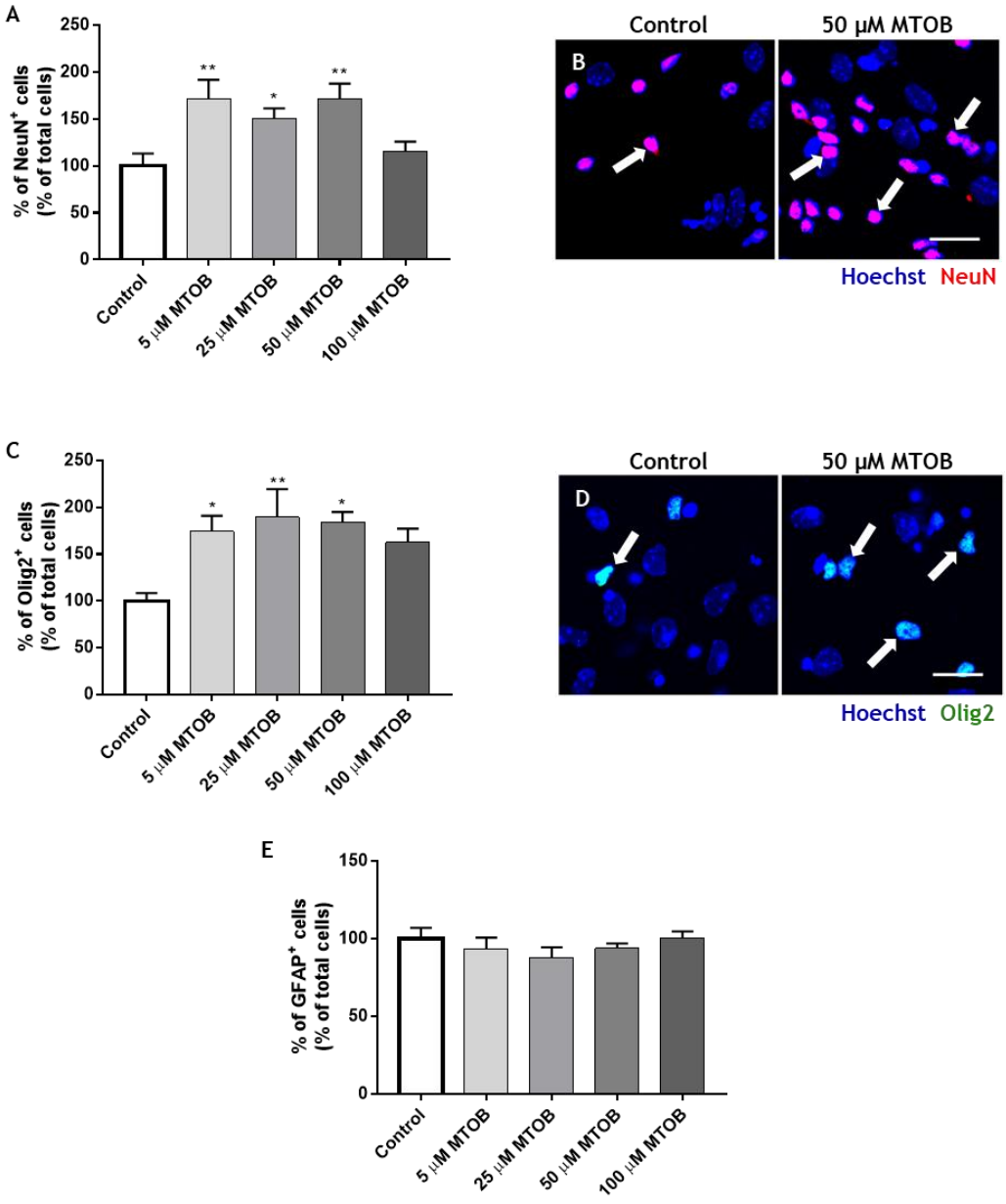

Figure 15 – MTOB promotes the differentiation of neurons and oligodendrocytes. SVZ cells were maintained in culture seven days after treatments. (A) Bar graph depicts the percentage of NeuN$^+$ cells. (B) Representative confocal digital images of NeuN$^+$ cells in the control and in cells treated with 50 μM of MTOB. Nuclei are shown in blue (Hoechst). White arrows highlight cells with NeuN staining. Scale bar: 20 μm. (C) Bar graph depicts the percentage of Olig2$^+$ cells. (D) Representative confocal digital images of Olig2$^+$ cells in the control and in cells treated with 50 μM of MTOB. Nuclei are shown in blue (Hoechst). White arrows highlight cells with Olig2 staining. Scale bar: 20 μm. (E) Bar graph depicts the percentage of GFAP$^+$ cells. Control was set to 100%. Data are expressed as percentage of control ± SEM (A: n=4-5; B: n=2-3; C: n=3). Statistical analysis was performed using one-way ANOVA, followed by the Dunnett's multiple comparison test. **P<0.01 and *P<0.05 when compared with control.

Chapter 5

Discussion

Extrinsic and intrinsic factors, which include transcriptional factors and post-translational modifications, are shown to regulate adult neurogenesis. Some of these intrinsic mechanisms interact with transcriptional coregulators like CtBPs to support their activity (42). Since there are no reports that associate CtBPs to adult neurogenesis, in this thesis we analyzed the effects of CtBPs in SVZ neurogenesis, under physiologic conditions.

First, we evaluated the expression of CtBP1 and CtBP2 at the cellular level and we found that both CtBPs are expressed in almost every Ki67$^+$, Nestin$^+$, Sox2$^+$, DCX$^+$, GFAP$^+$ and Olig2$^+$ cells in the SVZ, *in vitro* and *in vivo*. In the embryo, *in situ* hybridization studies demonstrated that CtBP1 and CtBP2 have a particularly strong expression in the nervous system (68). In the adult mouse, CtBP1 is expressed in the whole brain, either in the nucleus or in the cytoplasm interacting with Bassoon. Its location is found predominantly in the forebrain, cerebellum and in the substantia nigra. CtBP1 also has a stronger expression in the dorsal thalamus, in the diencephalon, and in the globus pallidus and the ventral pallidum of the basal ganglia. CtBP1 is highly expressed in the hippocampus, namely in the CA1 region and in the GCL of the DG, as well as in the cerebral cortex. However, this protein is less expressed in the CA2 and CA3 regions of the hippocampus as well as in the brainstem, except for substantia nigra, in white matter, like corpus callosum or the cerebral and cerebellar peduncles, in the subthalamus, and in the caudate, putamen and ventral striatum of the basal nuclei. Interestingly, the co-localization of CtBP1 with Bassoon in the CA1 and in the CA3 regions confirms its location in hippocampal synapses. In the case of CtBP2, it can be found in cell bodies throughout the brain, highlighting its nuclear location. CtBP2 is more expressed in OB, cerebellum, cerebral cortex and in the hippocampus, apart from CA1 region, where no immunoreactivity was detectable. Nevertheless, CtBP2 also showed to have a synaptic location in the cerebral cortex, hippocampus, and cerebellum (68). However, there are no reports about the expression of CtBPs in the SVZ. Thus, our results show that CtBP1 and CtBP2 are both expressed in the SVZ, *in vitro,* and *in vivo* in proliferating and immature cells, neuroblasts, astrocytes, and oligodendrocytes, in a preferably nuclear location. *In vitro*, under differentiation conditions, both CtBPs are also expressed in neurons.

Next, we evaluated the effects of CtBPs in SVZ neurogenesis *in vitro* using MTOB, a dual modulator of CtBPs activity. Since MTOB is a dehydrogenase substrate, it can affect both CtBPs. The first step was to analyze the effects of this antagonist on cell survival *in vitro*. Our results showed that 1 mM and 2.5 mM of MTOB induce cell death as detected by nuclear morphology analysis. Although MTOB has never been tested in SVZ cells, a previous study

showed that high concentrations of MTOB (in the order of mM) induce apoptosis in cultures of cerebellar granule neurons (76). Moreover, in different types of cancer cells, the use of MTOB concentrations above 1 mM lead to a significant amount of cell death (59). For example, the treatment of HCT116 colon cancer cells with 4 mM and 10 mM of MTOB prevents the recruitment of CtBPs to the Bik promoter, which is an apoptotic gene, inducing apoptosis (84). In accordance with these reports, we also observed that high concentrations of MTOB induce cell death and the low concentrations were not toxic to SVZ cells. As mentioned previously, CtBPs can repress proapoptotic genes such as *p21*, *Bax*, *Noxa*, *PERP*, promoting cell survival (73). The inhibition of these transcriptional corepressors with high concentrations of MTOB may be activating proapoptotic genes, reducing cell viability. On the other side, the lower concentrations may be leading MTOB to act as a substrate for CtBPs, leading to cell survival through the downregulation of the proapoptotic genes. Even though, the lower concentrations used in this work were not able to protect against the basal death occurring in these cultures. To avoid the toxic effects driven by high concentration of MTOB, the lower concentrations of MTOB (5 µM, 25 µM, 50 µM, 100 µM) were chosen for the subsequent experiments exploring the effects of MTOB in neurogenesis.

In cancer conditions, CtBPs are overexpressed. This overexpression can lead to the negative regulation of the expression of many tumor suppressor genes, such as *PTEN*, which increases cell proliferation, and proapoptotic genes (59). Another example of a tumor suppressor is the adenomatous polyposis coli (APC), which interacts with CtBPs to downregulate the transcription of Wnt target genes, like c-Myc (85). The Wnt signaling pathways, which are involved in cell proliferation and migration, or neuronal differentiation, are implicated in several diseases, including colon cancer (around 90% of the cases) and melanoma, as well as neurodegenerative diseases (86). Mutations on APC disconnect it from CtBPs, which might increase Wnt-dependent cell proliferation (85). One study using the human neuroblastoma cell line SHSY5Y showed that the downregulation of CtBP2 by RNA interference inhibited the cell proliferation and arrested the cell cycle. These results suggest that the downregulation of CtBP2 might be decreasing the expression of c-Myc, important for the cell cycle progression and proliferation of cancer cells, leading to a decrease in cell proliferation (87). Also, the downregulation of cell cycle inhibitors, such as p16Ink4a, by CtBP is shown to enhance cell proliferation (75). In our experiments, the use of MTOB at low concentrations do not affect the total number of proliferating cells in SVZ. These results might suggest that there is an accurate regulation of the ratio of the cellular proliferation and apoptosis by CtBPs, in physiologic conditions. Interestingly, we found that 25 µM of MTOB increased the number of proliferating neuroblasts being that the concentrations 5 µM and 50 µM of MTOB also seem to increase it.

Regarding cell differentiation, our results revealed that 5 µM, 25 µM and 50 µM of MTOB increased the number of NeuN-mature neurons, which agrees with the increased observed in the number of neuroblasts. Moreover, these concentrations also increased the number of

oligodendrocytes. Oligodendrocytes are responsible for the myelination of the axons, to allow the propagation of action potentials through the saltatory conduction (88). The increase found in the number of oligodendrocytes suggests that these cells are supporting the newly-formed neurons, that also increased. Our results also show that MTOB did not affect the $GFAP^+$ population, either the proliferating cells ($Ki67^+/GFAP^+$) or the mature astrocytes ($GFAP^+$). The increase in the differentiation of neurons and oligodendrocytes could be due to the inhibition of Jagged1, released by astrocytes, by CtBPs. Jagged1 increases the Notch signaling, which suppresses neuronal differentiation (32). The possible repression of Jagged1 by CtBPs may decrease the Notch signaling, promoting neuronal differentiation. Furthermore, several reports suggest that Notch signaling, for example through its ligand Jagged1, inhibits the differentiation of the oligodendrocytes progenitors (89). In the same way, the possible inhibition of this Notch ligand could be promoting oligodendrogenesis in our work.

BMP and Wnt signals have important functions in neurogenesis, being essential for NSCs maintenance and differentiation. BMPs can acts differently on neurogenesis due to the differential activity of the type 1 BMP receptor (BMPR-I). For example, in SGZ, BMPR-Ia is expressed by NSCs while BMPR-Ib is expressed in neuroblasts and neurons. When BMPs are released into the neurogenic niche, NSCs receive BMPs signal to maintain their undifferentiated state while neuroblasts receive it to differentiate (11). Although in the adult SVZ BMP signaling is less understood, these receptors are also expressed by the same cell type as in SGZ (25). Wnt family, which includes 19 different proteins, appear to be a key mechanism for SVZ neurogenesis, acting at distinct stages of neurogenesis (9). Wnt signals can induce directly neuronal differentiation by the transcription of neurogenic genes such as *NeuroD* (11). *Diversin*, a target of the Wnt signaling, is expressed in neuroblasts and its overexpression increase the proliferating neuroblasts (9). Considering the above, CtBPs may also act in the different phases of neurogenesis. On the one hand, in the early neurogenesis, CtBPs may repress genes to maintain the multipotency of NSCs, and on the other hand, in the middle and the late neurogenesis, CtBPs may act as transcriptional corepressors in order to promote differentiation. Since no effects were observed with the concentration of 100 μM of MTOB this suggest that this concentration can be the limit for MTOB to act as a substrate or as an inhibitor of CtBPs in SVZ cells.

Considering that all these signaling pathways act at different steps of neurogenesis, which make difficult to evaluate their specific contribution to the regulation of NSCs and given that the secretome of neurogenic niches can lead to ambiguity and dual effects, it is necessary more investigation for a better understanding of the role of CtBPs in the regulation of neurogenesis.

Chapter 6

Future Perspectives

Considering the possible role of CtBPs in cell survival, proliferation and differentiation of SVZ cells, the next step is to understand the role of each CTBP. This might be possible through the silencing of each CtBP using siRNAs or the CRISPR-Cas9 system.

For a better characterization of the population of oligodendrocytes, the differentiation of mature oligodendrocytes could be also analyzed by mature oligodendrocyte markers such as proteolipid protein (PLP) or myelin basic protein (MBP). Moreover, the characterization of axonal maturation by c-Jun N-terminal kinase (JNK) and tau, which is a microtubule-associated protein important for the stabilization of axonal microtubules (29), could be also done, as well as, the analysis of neuronal maturation through the evaluation of the dendritic complexity and the number of branches by DCX staining.

Also, for a better understanding of how CtBPs influence neurogenesis, it would also be interesting to evaluate the signaling pathways suppressed by the CTBPs as well as gene expression, such as Jagged1.

Our results showed an increase in the number of neurons and oligodendrocytes with low concentrations of MTOB, which seems promising for its application in pathologies. Stroke could be a possible therapy since occurs neuronal damage as well as demyelination, among others characteristics (88). Also in multiple sclerosis occurs a persistent demyelination leading to a parallel axonal damage and neuronal loss (89). CtBPs are sensitive to the levels of NADH, which increases in hypoxia. Considering this, it will be also interesting analyzed the role of CtBPs under hypoxia.

Chapter 7

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