Role of a Bile Acid in Improving Survival and Function of Neural Stem Cells

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Thesis to obtain the Master of Science Degree in Biomedical Engineering

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To my family and friends
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Resumo

Embora a neurogênese possa ocorrer em áreas específicas do cérebro de mamíferos adultos, as células estaminais neurais (NSCs) originam muito poucos neurônios durante o envelhecimento ou após lesão. Recentemente, sugeriu-se que a mitocôndria desempenhe um papel determinante nos processos de diferenciação e manutenção das NSCs. O nosso grupo descobriu que o ácido biliar endógeno tauroursodesoxicólico (TUDCA) é um agente anti-apoptótico e neuroprotector em modelos animais de doenças neurodegenerativas (NDs). Recentemente, numa linha de NSC de murganho, o TUDCA mostrou prevenir eventos apoptóticos mitocondriais na fase inicial de diferenciação das NSCs, aumentando o seu potencial prolifero e facilitando a diferenciação neuronal destas células.

Neste trabalho investigámos se o TUDCA induz efeitos semelhantes em culturas primárias de NSC e in vivo. Nos estudos in vitro, utilizámos células derivadas do giro dentado (DG) do hipocampo e da zona subventricular (SVZ) dos ventrículos laterais. Os resultados obtidos mostraram que o TUDCA duplica a proliferação das NSC derivadas do SVZ e DG, aumentando, também a diferenciação neuronal para 2 e 1,5 vezes mais, respectivamente. Contudo, o TUDCA não afecta a neuritogênese destas células. Relativamente à expressão de alguns reguladores mitocondriais, não detectámos também nenhuma diferença significativa após tratamento com este ácido biliar. De realçar que o TUDCA aumenta a neurogênese endógena e o número de NSC em proliferação in vivo. Em suma, estes resultados realçam o papel do TUDCA no destino das NSC, sublinhando o seu potencial efeito terapêutico no envelhecimento e noutras doenças associadas a um défice de neurogênese.

Palavras-chave: Ácido tauroursodesoxicólico; Células estaminais neurais; Mitocôndria; Neurogênese; Proliferação.
Abstract

Although neurogenesis occurs in restricted regions of the adult mammal brain, neural stem cells (NSCs) produce very few neurons during ageing or after injury. Thus, the identification of novel molecules responsible for life-long activity of NSC represents a major key issue. Recent evidence suggests that mitochondria affect the proliferative and differentiation potential of NSCs. Our group has discovered that the endogenous bile acid tauroursodeoxycholic acid (TUDCA) is an anti-apoptotic agent and neuroprotective in several animal models of neurodegenerative diseases (NDs). Recently, in a mouse NSC line, TUDCA was shown to prevent mitochondrial apoptotic events at early-stages of neural differentiation and enhance NSC proliferation and self-renewal potential.

Here, we aimed to further explore whether TUDCA induce similar effects in primary rat cultured NSCs, as well as in vivo. In vitro studies, we used cells derived from two neurogenic regions: the dentate gyrus (DG) of the hippocampus and the subventricular zone (SVZ) of the lateral ventricles. Our results showed that TUDCA treatment doubled NSC proliferation of both SVZ- and DG-derived NSCs, while increased neuronal differentiation in both niches by 2- and 1.5- folds, respectively. However, TUDCA did not affect neuritogenesis of NSCs. Regarding the expression of specific mitochondrial regulators, we did not detect any significant difference after bile acid treatment. More importantly, TUDCA induced endogenous neurogenesis and enhanced in vivo NSC pool. Collectively, these data extend the role of this bile acid in regulating NSC fate, underlining the potential therapeutic effect of TUDCA for ageing and other impaired neurogenesis diseases.

Key words:Mitochondria; Neural stem cells; Neurogenesis; Proliferation; Tauroursodeoxycholic acid.
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List of Abbreviations

3-NP 3-nitropropionic acid
aCSF Artificial cerebrospinal fluid
AD Alzheimer disease
ALS Amyotrophic lateral sclerosis
AP Anterior-posterior
APP Amyloid precursor protein
ASC Adult stem cell
ATP Adenosine triphosphate
BA Bile acid
Bad B-cell lymphoma 2 (Bcl-2)-associated death promoter
Bcl-2 B-cell lymphoma 2
Bcl-xl B-cell lymphoma-extra large
BDNF Brain-derived neurotrophic factor
BMP Bone morphogenetic protein
BrdU Bromodeoxyuridine
BSA Bovine serum albumin
Cdh 1 Cadherin 1
CNS Central nervous system
CsA Cyclosporin A
CSF Cerebrospinal fluid
DCA Deoxycholic acid
DCX Doublecortin
DG Dentate gyrus
DNA Deoxyribonucleic acid
Drp1 Dynamin-related protein 1
ECM Extracellular matrix
EDTA Ethylenediamine tetraacetic acid
EGF Epidermal growth factor
ESC Embryonic stem cells
ETC Electron transport chain
FDA Food and drug administration
FGF2 Fibroblast growth factor 2
Fis1 Fission protein 1
Foxo3 Forkhead box O3
GABA Gamma-AminoButyric Acid
GAPDH Glyceraldehyde-3-phosphate dehydrogenase
GCL Granule cell layer
<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
</tr>
<tr>
<td>GR</td>
<td>Glucocorticoid receptor</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
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<tr>
<td>GUDCA</td>
<td>Glycoursodeoxycholic acid</td>
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<tr>
<td>HD</td>
<td>Huntington disease</td>
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<tr>
<td>Hsp90</td>
<td>Heat shock protein 90</td>
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<tr>
<td>IMM</td>
<td>Inner mitochondrial membrane</td>
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<tr>
<td>iPSC</td>
<td>Induced pluripotent stem cells</td>
</tr>
<tr>
<td>Mfn</td>
<td>Mitofusin</td>
</tr>
<tr>
<td>MnSOD</td>
<td>Manganese superoxide dismutase</td>
</tr>
<tr>
<td>MR</td>
<td>Mineralocorticoid receptor</td>
</tr>
<tr>
<td>mtDNA</td>
<td>Mitochondrial deoxyribonucleic acid (DNA)</td>
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<tr>
<td>mtUPR</td>
<td>Mitochondrial unfolded protein response</td>
</tr>
<tr>
<td>ND</td>
<td>Neurodegenerative disease</td>
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<tr>
<td>NeuN</td>
<td>Neuronal nuclei</td>
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<tr>
<td>NIH</td>
<td>National institutes of health</td>
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<tr>
<td>NSC</td>
<td>Neural stem cell</td>
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<tr>
<td>OB</td>
<td>Olfactory bulb</td>
</tr>
<tr>
<td>OGG1</td>
<td>Eight-oxoguanine deoxyribonucleic acid (DNA) glycosylase</td>
</tr>
<tr>
<td>OligoA</td>
<td>Oligomycin A</td>
</tr>
<tr>
<td>OMM</td>
<td>Outer mitochondrial membrane</td>
</tr>
<tr>
<td>OPA1</td>
<td>Optic Atrophy 1</td>
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<tr>
<td>OXPHOS</td>
<td>Oxidative phosphorylation</td>
</tr>
<tr>
<td>PARP</td>
<td>Poly(ADPribose) polymerase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson's disease</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
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<tr>
<td>PGC1</td>
<td>Peroxisome proliferator-activated receptor (PPAR) γ coactivator 1</td>
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<td>Putative kinase 1</td>
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<tr>
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<td>PGC related coactivator</td>
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<td>PS1</td>
<td>Presenilin 1</td>
</tr>
<tr>
<td>QC</td>
<td>Quality control</td>
</tr>
<tr>
<td>RMS</td>
<td>Rostral migratory stream</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SC</td>
<td>Stem cell</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate-polyacrylamide electrophoresis gels</td>
</tr>
<tr>
<td>SFM</td>
<td>Serum-free culture medium</td>
</tr>
<tr>
<td>SGZ</td>
<td>Subgranular zone</td>
</tr>
<tr>
<td>Shh</td>
<td>Sonic Hedgehog</td>
</tr>
<tr>
<td>Sox2</td>
<td>Sex determining region Y-box 2</td>
</tr>
<tr>
<td>SRY</td>
<td>Sex determining region Y</td>
</tr>
<tr>
<td>SVZ</td>
<td>Subventricular zone</td>
</tr>
<tr>
<td>TFAM</td>
<td>Mitochondrial transcription factor A</td>
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<td>Transforming growth factor β1</td>
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<td>TUDCA</td>
<td>Tauroursodeoxycholic acid</td>
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<td>UDCA</td>
<td>Ursodeoxycholic acid</td>
</tr>
<tr>
<td>VZ</td>
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I. Introduction

I.1. Neural stem cells (NSCs)

Stem cells (SCs) unquestionably offer remarkable potential to treat many human diseases and to repair tissue damage resulting from aging and injury (Mimeault & Batra, 2006). The definition of a SC and its features has been a matter of discussion since the beginning of their study (Becker, et al., 1963) (Watt & Driskell, 2010). Nevertheless, the set of criteria usually accepted to define a cell as being a SC are the following: self-maintenance which includes cell division with maintenance of the functionality of the cell and the number of SCs in a specific tissue; self-renewal is a process by which SCs divide to make more SCs; clonogenicity which regards the capacity of the cell undergoing a mitotic process to originate a large number of progeny, also called clone of cells; and potency or the ability to produce differentiated cells, which are cells that acquired a certain gene expression pattern that makes them morphologically distinct and functionally competent (Potten & Loeffler, 1990) (Fuchs & Chen, 2013). Regarding to the potency, SCs are divided in different groups: the cells derived from an early progeny of the zygote up to the eight cell stage of the morula, are characterized as totipotent, owing to their ability to generate a complete organism. The pluripotent cells can form the tissues of all embryonic germ layers (endoderm, mesoderm, and ectoderm), while multipotent cells, such as Adult stem cells (ASC), can differentiate into a specific number of cell types (Nichols & Smith, 2011) (Ratajczak, et al., 2008).

The first evidence to support the existence of new neurons residing in the brain of adult mammals dates from 1962, when Joseph Altman found a set of labeled neurons suggesting that they were formed from undifferentiated cells which divided mitotically (Altman, 1962). Altman continued to study the neurogenesis process and after one year, he demonstrated the existence of adult neurogenesis in dentate gyrus (DG) of the hippocampus (Altman, 1963). In 1969, he discovered and named the rostral migratory stream (RMS) as a proliferative and migratory region of NSCs (Altman, 1969). Since then, several research groups have explored NSCs and their properties (Zhao, et al., 2003) (Falcão, et al., 2012) (Arvidsson, et al., 2015) in different species (Kizil, et al., 2012) (Bunk, et al., 2011), because they could demonstrate to be useful for the development of novel therapeutic tools for damaged adult Central nervous system (CNS), after neurodegenerative disorders or injury.

NSCs are the most primordial cells of the nervous system capable to generate all type of neurons of the mammalian CNS during development and throughout adult life, also being the source of glial cells, such as astrocytes and oligodendrocytes (Götz & Huttner, 2005). Therefore, they are considered as multipotent stem cells (Ratajczak, et al., 2008). Neurons, functional components of the nervous system, are responsible for information processing and transmission, while glial cells support neurotransmission and insulate axons to speed up electrical communication (Vescovi, et al., 2006). In the adult brain, the primary precursors of neurons are radial glia-like cells (Ming & Song, 2011). Glial fibrillary acidic protein (GFAP)-expressing radial glia-like cells characterize quiescent NSCs that generate neurons in olfactory bulb (OB) and hippocampus (Ming & Song, 2011) (Conti & Cattaneo,
In contrast with embryonic NSCs that have a high proliferative potential, adult NSCs remain for long periods in G0 (Orford & Scadden, 2008). This feature is similar with those found in many adult SCs present in other mature tissues, and is crucial to maintain tissue homeostasis and avoid exhaustion of stem cell function (Simons & H, 2011).

Numerous protocols have been improved to allow derivation, expansion and differentiation of NSCs, *in vitro*, from different cell sources.

### I.1.1. Sources and culture systems of NSCs

NSCs and progenitor cells, unlike a SC, have a limited dividing capacity. They can be derived *in vitro* from embryonic stem cells (ESCs), that can be isolated from the inner cell mass of blastocysts; as well as from induced pluripotent stem cells (iPSCs), obtained by reprogramming from somatic cell; and can also be isolated from fetal tissue and adult brain samples. It is important to refer that both ESCs and iPSCs can be expanded indeterminately in culture since they express telomerase avoiding chromosome aging. *In vitro*, numerous protocols have been optimized to allow derivation, expansion and differentiation of NSCs (Conti & Cattaneo, 2010).

Reynolds and Weiss (1992) demonstrated that NSCs are able to form neurospheres in culture, which consist in floating clusters of committed progenitors, differentiated astrocytes, neurons, and very few NSCs (Reynolds & Weiss, 1992) (Gil-Perotin, et al., 2013). Being heterogeneous, neurospheres are not considered a good model for efficient neuronal generation, leading to different cells in the sphere which are exposed to suboptimal conditions. This fact is observed by the tendency of neurospheres to have more differentiated cells in their innermost part. Posterior studies have revealed the possibility of expanding NSC cultures in monolayer conditions from several NSCs sources, which can maintain almost pure NSC populations, with insignificant differentiated component in comparison to neurosphere models (Figure 1) (Conti & Cattaneo, 2010) (Conti, et al., 2005) (Johe, et al., 1996) (Bonnamain, et al., 2012).

There are many controversies around these two culture systems, in terms of conservation of the molecular and biological properties of genuine NSCs. Despite the heterogeneity associated with neurospheres, they are able to mimic physiological conditions due to their three-dimensional structure. This culture system is also technically easier to culture, compared with the monolayer culture system (Weinberg, et al., 2015). For example, with the neurosphere culture system, it is easy to control the exposed extrinsic cues during the cell development, either during the expansion or the differentiation phase, by adding precise and variable amounts of factors of interest to the media or by culturing the neurospheres with other type of cells (Jesen & Parmar, 2006). Neurospheres cultures can be expanded to acquire numerous cells that can be cryopreserved (Casarosa, et al., 2013). Moreover, in this culture system the density of cells, in a small surface area, is higher in comparison with the monolayer culture system (Weinberg, et al., 2015).
Researche have tried to reproduce, in vitro, NSC niches, by recreating the complex interactions between NSCs and other cells, extracellular matrix, physical factors and gradients of regulatory molecules (Casarosa, et al., 2013).

Figure 1. Sources and different culture systems of NSCs: neurospheres and monolayer. Neurospheres and monolayer NSC lines can be derived from ESCs, iPSCs and from the germinative areas of the fetal and adult brain. NSCs are able to generate the three main cell types of the brain: neurons, astrocytes and oligodendrocytes. Neurospheres cultures present a heterogeneous composition resulting in a low neurogenic potential, while monolayer culture systems present a more homogeneous composition leading to a high neurogenic potential (Conti & Cattaneo, 2010).

I.1.2. Neurogenic niches

The niche is basically defined as the microenvironment that anatomically houses SCs and functionally controls their development in vivo (Rezza, et al., 2014). In order to provide structural support and molecular signals, the niche must regulate SC quiescence, self-renewal, as well as the activation for tissue maintenance. The niche is composed by multiple components: SCs themselves and their progeny; neighboring mesenchymal or stromal cells; extracellular matrix (ECM) or cell-cell contacts involving adhesion molecules; and external cues from distant sources within the tissue or outside the tissue (Rezza, et al., 2014).

The major cellular components of the adult neurogenic niche are endothelial cells, astrocytes,
ependymal cells, microglia, mature neurons, and progeny of adult neural precursors (Ming & Song, 2011). Astrocytes produce morphogens, which are signal molecules that directly act on cells to produce specific cellular responses in a concentration-dependent manner (Tabata & Takei, 2004). They also provide direct cell-cell contacts with NSCs to control proliferation, differentiation, migration and synapse formation. The ependymal cells use beating cilia to generate gradients of morphogens that specify particular cell fates during differentiation (Sawamoto, et al., 2006). Like these cells, microglia also actively regulates adult neurogenesis (Ziv, et al., 2006). Vascular cells, extending around the neural tissue, can also influence the progenitor cell fate choice since they bring blood-derived signals near the target cells and provide paths along which the progenitors migrate to reach different regions of the brain (Rezza, et al., 2014).

Neurogenesis is as a complex neurobiological process by which functional neurons are generated from NSCs. According to the existent data, it is estimated that around 700 new neurons are added to the adult human hippocampus daily, however they are preferentially lost and do not survive as long as the neurons generated during development (Spalding, et al., 2013). It was shown that the neurogenesis declines 4-fold during the entire adult lifespan in humans (Spalding, et al., 2013). In addition, the number of synapses was also demonstrated to decrease throughout adult life (Doll & Broadie, 2014). Another reason responsible for the minimal success of mammalian neuronal regeneration rely on an unstable integration of newborn cells into the pre-existing network (Doll & Broadie, 2014).

Adult neurogenesis has been regularly observed in two main regions of the adult brain, represented in Figure 2: the subventricular zone (SVZ) of the lateral wall in the lateral ventricles, where new neurons are generated and migrate through the RMS to become interneurons in the OB; and the subgranular zone (SGZ) of the DG in the hippocampus, a NSC pool much lower than that found in SVZ, where new dentate granule cells are generated and integrated (Deng, et al., 2010) (Ming & Song, 2011).

In the adult SVZ, NSC populations are located in distinct regions, having also different properties acquired during development. Proliferating radial glia-like cells, also known as B cells, give rise to transient amplifying cells, also called C cells, which are then responsible to generate neuroblasts (i.e. immature neurons). In RMS, the neuroblasts also known as A cells form a chain and migrate toward the OB through a tube composed by astrocytes (Lois, et al., 1996). After reaching the core of OB, immature neurons detach from the RMS and migrate radially, differentiating into two main types of interneurons. The majority of these cells give rise to Gamma-Aminobutyric Acid (GABA)ergic granule neurons and GABAergic periglomerular cells (Lledo, et al., 2006). Recent work have demonstrated that NSCs, from SVZ niche, present a stereotypic architecture that allow them to interact with the cerebrospinal fluid (CSF) and blood vessels, revealing that this adult germinal niche not only includes the SVZ, but also the ventricular zone (VZ), from now referred as VZ-SVZ (Ihrie & Alvarez-Buylla, 2011).

In the adult SGZ, proliferating radial and nonradial precursors also known as type 1 and type 2 progenitor cells respectively, generate intermediate progenitors, which successively give rise to neuroblasts. Immature neurons migrate through the inner granule cell layer and differentiate into dentate granule cells. After a few days, newborn neurons extend dendrites in the direction of the molecular layer and project axons through the hilus toward the CA3 area of the hippocampus. New neurons are
integrated into the existing circuit in the granule cell layer (GCL) located just above the molecular layer (Zhao, et al., 2006).

Although several differences between adult NSCs exist in the two neurogenic niches, many characteristics are similar including the niche composition, such as direct contact blood vessels, and temporal sequence of new neuron integration (Ming & Song, 2011).

Figure 2. The anatomy and functioning of the SVZ and SGZ in rodents and humans. a) A sagittal section through the lateral ventricle shows the SVZ, one neurogenic region. b) Another neurogenic region is found in the SGZ, located in the DG of the hippocampus. c) In the adult human brain, a population of astrocytes has been identifies as comprising NSCs. d) The germinal zone of the adult human hippocampus is located in the DG (Vescovi, et al., 2006).

Both extrinsic and intrinsic mechanisms are involving in the regulation of neurogenesis. Regarding extrinsic players, an amount of different morphogens operate as niche signals, namely Notch, Sonic Hedgehog (Shh), Wnts, and bone morphogenetic proteins (BMPs). The action of BMPs can be antagonized by Noggin, expressed by ependymal niche cells. This action, combined with the production of BMP ligands by NSCs, is involved in a specific balance of BMP signaling that occurs in SVZ to maintain NSCs in a quiescence stage (Lim, et al., 2000). Several studies have identified the Notch
signaling in maintenance of adult NSCs. Curiously, Notch1 signaling develops distinctive functions in DG when compared to SVZ. Basak O. et al observed that Notch 1 deletion in the SVZ only affects the proliferation process of NSCs without changing their total number (Basak, et al., 2012), while Ables J. et al demonstrated that Notch1 loss in DG leads to a substantial decrease in NSC pool (Ables, et al., 2010). Wnt signaling, in turn, plays an important role in postnatal and adult neurogenesis (Matamoros, et al., 2013). It has been shown that the overexpression of Wnt3 was sufficient to increase neurogenesis from adult hippocampal stem/progenitor cells in vivo and in vitro (Lie, et al., 2005). At the end, Shh signaling is only activated in NSCs and their immediate precursor cells (Ahn & Joyner, 2005), being required for their establishment and maintenance in the adult SGZ and SVZ (Balordi & Fishell, 2007).

Cytokines, growth factors, neurotrophins and hormones are also important in regulation of adult neurogenesis. Several neurotransmitter systems, such as GABA and glutamate, are frequently used, as pharmacological manipulations, to regulate different stages of neurogenesis. For example, the neurons of DG receive a set of inputs from many brain regions through the release of distinct neurotransmitters and neuropeptides (Zhao, et al., 2008). Adult neural progenitors are also regulated by growth factors such as epidermal growth factor (EGF) and fibroblast growth factor 2 (FGF2). These are potent factors that assure the maintenance of adult NSCs in vitro, while in vivo, they induce proliferation in SVZ, despite FGF-2 induces an increase in the amount of newborn neurons in the OB (Kuhn, et al., 1997).

The main intrinsic regulators of adult neurogenesis are transcription factors, cell-cycle regulators, and epigenetic factors (Zhao, et al., 2008). Cell-cycle inhibitors, such as p16, p21, p27 and p53 are crucial for maintenance of adult NSC quiescence. In fact, deletion of these regulators leads to a temporary activation and subsequent reduction of the precursor cell pool. Sun G. et al, Favaro R. et al and Renault V. et al observed that the nuclear orphan receptor Tlx, the transcription factor Sex determining region Y (SRY)-box 2 (Sox2), and the longevity-associated factor Forkhead box O3 (Foxo3) respectively, play an important role as regulators of NSC quiescence (Sun, et al., 2007) (Favaro, et al., 2009) (Renault, et al., 2009). According to Favaro R. et al, Shh is a direct target of Sox2 in neural precursors and deletion of this transcription factor in adult mice results in loss of hippocampal neurogenesis (Favaro, et al., 2009). In addition, several epigenetics mechanisms are important in coordinating gene expression as well as fine-tuning during adult neurogenesis. These mechanisms involve Deoxyribonucleic acid (DNA) methylation, histone modification and non-coding Ribonucleic acids (RNAs) (Sun, et al., 2011). Figure 3 represents the SVZ and DG niches of NSCs, demonstrating the proximity of the NSC population to the vasculature, and also highlighting different soluble and insoluble interactions.
I.1.3. Neural replacement therapies

Over the past few years, several studies have been developed with the aim of generating the types of human-derived neurons required for cell replacement therapy based on the pathology of specific diseases. Many studies have confirmed that human and rodent NSC progenitors derived from ESC, fetal brain, and/or adult stem, and progenitor cells can be expanded in vitro or ex vivo, having a huge potential for the development of novel cellular and gene therapies (Lindvall & Kokaia, 2010) (Mimeault & Batra, 2006).

Indeed, a wide-range of efforts have been done to understand the adult neurogenesis process in terms of stem cell regulation, neuronal development, molecular mechanisms and functional contributions. Since the discovery of NSCs in the adult CNS as well as their potential to give rise to a range of mature cells types, these cells have been an interesting source for neural repair after disease or injury. There are three distinct ways where stem cell-based approaches could be used to give therapeutic benefits. First, by transplanting stem cell-derived neuron precursors/neuroblasts after their expansion in vitro; second, by infusing compounds that would promote neurogenesis from endogenous stem/progenitor cells; and finally, by injecting systemically SCs for neuroprotection and modulation of inflammation.

Neurodegenerative diseases (NDs) are associated with the lost of neurons and glial cells in the brain or spinal cord. As an example, in acute cases like in ischemic stroke or spinal cord injury, different neuronal cells die rapidly, within a restricted brain area. Whereas in chronic cases, such as Parkinson´s disease (PD) and Amyotrophic lateral sclerosis (ALS), there is a selective loss of a specific cell population and in Alzheimer disease (AD) there is an extensive degeneration of many types of neurons.
However, a remarkably weak capacity of the CNS for endogenous cell replacement has been observed through different studies. Indeed, a small fraction of physiological regeneration occurs, although it is not enough for a significant functional recovery (Bonnamain, et al., 2012) (Lindvall & Kokaia, 2010) (Williams, 2014).

In the last years, a huge number of studies related to neurological disorders, have been performed using different sources of NSCs are used. Importantly, the discovery of iPSCs, by Yamanaka in 2006, has opened a new field of possibilities in SC-based therapies (Takahashi & Yamanaka, 2006). In 2008, Wernig et al demonstrated that neural precursor cells, differentiated from human iPSCs, could be transplanted into a rat model of PD, improving the motor-sensory behavior by increasing the number of midbrain dopamine neuron (Wernig, et al., 2008). However, this type of cells presents many associated problems namely the used of viral vectors for their programming avoiding its use in clinical therapy. Moreover, because of their increased proliferative potential, iPSCs were also shown to induce tumor formation and impaired neural outgrowth (Lodi, et al., 2011).

NSCs transplantation was also shown to represent an approach for treating AD. Blurrton-Jones et al used aged triple transgenic mice that express pathogenic forms of amyloid precursor protein, presenilin, and tau to investigate the effect of NSC (harvested from postnatal day 1) transplantation on AD (Blurrton-Jones, et al., 2009). They showed that transplantation of such cells in the brain of animals with advanced AD-related neuropathology rescues memory and learning deficits. Regarding to ALS, which is caused by the lost of central and peripheral motor neurons (Evely, 2013), many preclinical experiments have been done with the aim to reach the clinic (Lindvall & Kokaia, 2010). Xu L. et al showed that human fetal NSCs transplantation into the spinal cord of the rat model of ALS protects motor neurons avoiding disease progression (Xu, et al., 2006).

To improve neuroreplacement therapies, it is necessary to take into account many issues, namely: differences between the microenvironment of the mature CNS and those present during development, because the first may not provide the correct conditions, essential for survival and differentiation of the newly formed adult neural cells, affecting the recovery of neuronal activities. On the other hand, the incapacity of cells to migrate to the lesions, which are far away from the injection site, is other major problem (Bonnamain, et al., 2012).

Over the last few years, it has been demonstrated that mitochondrial modulation of metabolism and morphology represents an important step in the efficiency of neural differentiation in vitro and in vivo (Kasahara & Scorrano, 2014).

**I.2. Mitochondrial in neural activity**

Several neural development processes, including neurogenesis, self-renewal and differentiation of NSCs, depend on mitochondrial regulation (Xavier, et al., 2015). Moreover, it has been recently demonstrated that this organelle is highly associated with the regulation of cell cycle progression of NSCs through mechanisms of retrograde signaling (Butow & Avadhani, 2004) (Owusu-Ansah, et al.,
Other reason why neurons rely on mitochondrial function is due to the fact that they require large amounts of oxygen, which is predominantly generated by oxidative metabolism (Silver & Erecinska, 1998). Indeed, since these cells are highly differentiated, they need great amounts of adenosine triphosphate (ATP) for conservation of cell membrane gradients and neurotransmission (Ames, 2000). Interestingly, mitochondria also coordinate neural positioning. In fact, mitochondria are deeply involved in the regulation of both dendritogenesis and axon branching, where axonal mitochondria contribute to axonal protein production, as well as to sites of highest synthesis along neurons (Spillane, et al., 2013). The neuronal demand for mitochondria is indeed observed by the high numbers of mitochondria at presynaptic endings, pos-synaptic densities, nodes of Ranvier, and in growth cones, where mitochondrial function is essential to support neuronal activity (Ashrafi & Schwarz, 2013).

Mitochondrial and metabolic dysfunction have been increasingly revealed be associated with neurogenesis in age- and disease-related neural loss and impaired neuroplasticity (Stoll, et al., 2011). Therefore, it is possible that modulation of mitochondrial function, might contribute to an increase of neurogenic potential in aging and other impaired neurogenesis models (Xavier, et al., 2015). Among ND associated with mitochondrial impairment are PD, AD, ALS and Huntington disease (HD).

I.2.1. Mitochondrial function

Mitochondria are organelles involved in many important cellular processes, namely ATP production by oxidative phosphorylation (OXPHOS); fatty acid oxidation; apoptosis; intracellular Ca$^{2+}$ homeostasis; generation of reactive oxygen species (ROS) as well as cellular specialization in a diversity of tissues. Nowadays, theses organelles are the only containing circular and non-nuclear DNA in eukaryotic cells (Wang, et al., 2011). Mitochondrial DNA (mtDNA) is composed by 16 Kb, responsible for the control of 67 proteins, including 13 essential proteins of the electron transport chain (ETC) (Kurland & Andersson, 2000). This chain consist in five complexes, from complex I to V, in which four of them are encoded by mtDNA, including the terminal complex of electron transport chain, where 90% of the oxygen is consumed.

The more recent findings on mitochondrial function and energy metabolism, in the context of neuronal development and activity, will be explored below.

I.2.1.1. Role of Mitochondrial in neural differentiation

Over the past few years, it has been investigated the role of ROS production in NSC fate control. Clear consensus with respect to the quantity of ROS required by NSCs, for efficient neuronal, oligodendrocyte and astroglial activity and development, need to be reached. In the presence of a higher rate of ROS production, an imbalance between mtROS production and detoxification can be caused by dysfunction in mitochondrial oxidative protection mechanisms (Andreyev, et al., 2005).
Different cellular sites are responsible for the production of such reactive species like cytosol, peroxisomes, plasma and endoplasmic reticulum membranes, and matrix and membranes of mitochondria, however the ETC is the most important site of ROS production under physiological conditions. Superoxide ($O_2^-$) is considered the primary product and the hydrogen peroxide as a secondary product (Venditti, et al., 2013). Nevertheless, there are several mitochondrial enzymes responsible for ROS detoxifier, namely superoxide dismutases (e.g. manganese superoxide dismutase (MnSOD)) that eliminate the superoxide radicals, converting them into molecular oxygen and water.

During the undifferentiated stages, the cells do not require large amounts of energy to maintain their proliferative capacity. Thus, there is lower mitochondrial oxygen consumption (Facucho-Oliveira, et al., 2007). In contrast, throughout differentiation process, the cells begin to use the aerobic and oxidative metabolism, associated with an increased of ROS production (Wang, et al., 2010).

It has been shown that ROS are involved in oxidative mitochondrial and nuclear DNA damage in several neurological disorders such as PD (Alam, et al., 1997) and AD (Markesbery, 1997). ROS are also responsible for lipid peroxidation and oxidative protein modifications. Interestingly, ROS have been recently implicated in NSC lineage determination. In other words, they influence the choice between neuronal and astroglial differentiation (Xavier, et al., 2014b). Xavier and others, have also revealed that the tumor suppressor protein p53, with multiple roles in cell control, prevents mitochondrial ROS production at early stages of NSC differentiation, favoring neuronal fate (Xavier, et al., 2014a). Moreover, mitochondrial levels of both ATP and ROS have been shown to interfere with cell cycle progression since differentiation implies cell cycle exit and irreversible proliferative arrest (Xavier, et al., 2014b).

### I.2.1.2. Mitochondrial quality control systems in neural differentiation

Different pathways of mitochondrial Quality control (QC) are involved in detection and elimination of adverse effects resulting from molecular damage. These mechanisms, represented in the Figure 4, allow mitochondria to sense damage and delete dysfunctional organelles or proteins during stress conditions, such as those present in differentiation. There are two main QC pathways: the intra-mitochondrial and organellar pathways (Fischer, et al., 2012).

Regarding to the first QC pathway (Figure 4A), its main role is the maintenance of mtDNA integrity. Recently, it has been discuss the relationship between the mtDNA integrity and modulation of neural differentiation. The presence of oxidative stress and increase levels of ROS induce mtDNA damage in NSCs, impairing ECT activity and, thus, compromising the differentiation and maturation process. Like ROS, mtDNA damage also favors astroglial differentiation of NSC. Moreover, it interferes in mitochondrial transcription and replication processes. In this regard, Kang and others showed the relevance of the mitochondrial transcription factor A (TFAM) in increasing mtDNA content (Kang, et al., 2007). TFAM directly interacts with mtDNA, promoting their stabilization. Another mechanism included...
in intra-mitochondrial QC pathway is the regulation by chaperones and proteases, as well as by a multienzymatic cascade (Xavier, et al., 2015).

Eight-oxoguanine DNA glycosylase (OGG1), responsible by the triggering of the multienzymatic cascade, has been shown to protect cells from mitochondrial oxidative stress as well as to protect mtDNA from differentiation-mediated oxidation damage. Wang et al detected that mtDNA suffers from damage insults after the first day of differentiation. Nonetheless, they also demonstrated that OGG1 is essential to remove such damage (Wang, et al., 2011). Actually, the role of OGG1 has been associated with increased neurogenesis and its inhibition results in the shift of NSC differentiation direction toward an astrocytic lineage, via Sirt1 signalling (Wang, et al., 2011).

Regarding to the organellar QC system (Figure 4B), this involves the control of mitochondrial integrity and dynamics including mitochondrial fusion and fission, and mitophagy. Mitochondrial fusion can be described as a compensatory mechanism that ensure the unifying and mixing of mitochondrial components, while mitochondrial fission is an elimination process where it is segregated morphologically and functionally deleterious organelles from health ones. The balance between these processes shapes the complex mitochondrial network in cells and are mechanically regulated by key dynamin-related fission and fusion gene products, which are represented in a Figure 5 (Kasahara & Scorrrano, 2014). Mitophagy, in turn, is the selective degradation of mitochondria by autophagy leading to either an elimination of mitochondria content or damaged mitochondria (Ashrafi & Schwarz, 2013).

![Figure 4. Mitochondria quality control systems. A) Intra-mitochondrial QC mechanism, where the OGG1 repair cascade is responsible for the maintenance of mtDNA integrity by activation of the mtDNA polymerase (Pol γ), and its interaction with the mitochondrial ROS scavenger enzyme, MnSOD. Schematic overview of the mitochondrial unfolded protein response (mtUPR), responsible for the detection and degradation of misfolded proteins. B) The](image-url)
organellar QC comprises mitochondrial membrane fusion and fission events, which include the activity of several proteins, namely the fission protein 1 (Fis1), the dynamin-related protein 1 (Drp1), the putative kinase 1 (PINK1) and Parkin, thus resulting in selective mitophagy. Adapted from (Xavier, et al., 2015).

Mitochondrial fusion is mediated by dynamin-related GTPases at the outer mitochondrial membrane (OMM), Mitofusin (Mfn) 1 and 2 and by the Optic Atrophy 1 (OPA1) at the inner mitochondrial membrane (IMM). Inhibition or loss of such proteins leads to increase mitochondrial fragmentation (Chen, et al., 2005). Additionally, mutations in Mfn2 and OPA1 cause severe ND namely Charcot–Marie–Tooth type 2A (Zuchner, et al., 2004) and dominant optic atrophy (Alexander, et al., 2000), respectively. Under cellular stress conditions, it is formed a hyperfused complex resulting an increased of ATP, preventing damage and confers stress resistance on cells (Tondera, et al., 2009).

Mitochondria fission is mediated by the dynamin-related protein 1 (Drp1) and the fission protein 1 (Fis1). These proteins are presented in the cytosol, being recruited to form spirals around mitochondria to break this organelle in two. After that, the mitophagy pathway also seems to depend on fusion blockage or fission activation leading to the destruction of the mitochondrial network and thereby, to isolation of the mitochondrion labeled for mitophagy, from the rest of the mitochondrial network (Ashrafi & Schwarz, 2013). The fission process also occurs when cell undergo apoptosis. Despite of, under critical stress conditions, organellar QC system activates the intrinsic mitochondrial network.

Figure 5. Mitochondria fusion and fission processes. A) Fusion involves the interaction of OMM and IMM. Fusion of the OMM needs low Guanosine triphosphate (GTP) levels, while fusion of the IMM demands high GTP levels. In mammalian cells, this process involves the outer membrane proteins mitofusins Mfn1 and Mfn2, which have a cytosolic GTPase domain and two coiled-coil regions, and the intermembrane space proteins GTPases OPA1. B) Models and molecules of mitochondrial fission. Drp1 is localized to the cytosol and punctate spots on mitochondria. Some of these spots are constriction sites that lead to mitochondrial fission. Fis1 is located equally to the OMM. Adapted from (Nisoli & Carruba, 2006).
A huge number of NDs are related with genes involved in the mitochondrial QC system. Consequently, the study of the mechanisms and components involved in this system contribute for the establishment of novel therapeutic interventions faced to the enrichment of neuronal function and activity, as well as plasticity of new born and vulnerable neurons (Alam, et al., 1997) (Markesbery, 1997) (Zuchner, et al., 2004).

I.2.1.3. **PGC1 α: a master regulator of mitochondria biogenesis**

Peroxisome proliferator-activated receptor (PPAR) γ coactivator 1 (PGC1) family has appeared as central regulator of metabolism. This family compromised PGC1α, PGC1β and the PGC related coactivator (PRC), which interacts with nuclear receptor and transcription factors to exert their biological functions (Handschin & Spiegelman, 2006).

PGC1α is involved in multiple biological responses, promoting mitochondrial biogenesis in cells. It also regulates the global oxidative metabolism, by controlling both organelle biogenesis and remodeling (Handschin & Spiegelman, 2006). Moreover, PGC1α elevates oxidative metabolism and at the same time controls ROS removal by regulating the expression of several ROS-detoxifying enzymes. Therefore, it increases mitochondrial functions and reduces the buildup of its by-products, contributing to a positive impact on oxidative metabolism (Austin & St-Pierre, 2012). Further, PGC1α has been already revealed to interfere in the formation and maintenance of neuronal dendritic spines (Cheng, et al., 2012), and to have a beneficial impact in aging, NDs and depression (Austin & St-Pierre, 2012) (Agudelo, et al., 2014). However, its influence in neuronal differentiation has never been investigated.

Wrann et al demonstrated that PGC1α increases the brain-derived neurotrophic factor (BDNF) levels, in response to exercise (Wrann, et al., 2013). BDNF is responsible to promote many aspects of brain development namely neuronal cell survival and differentiation (Wrann, et al., 2013). In addition to this, it was also shown that the expression of PGC1α increases with exercise (Agudelo, et al., 2014) (Wrann, et al., 2013). Curiously, the exercise promotes de novo neurogenesis and synaptic plasticity (Cotman, et al., 2007). Therefore, it is possible to think that PGC1α could increase neurogenesis, being an attractive target to explore in the future.

I.3. **Regenerative potential of Tauroursodeoxycholic acid**

I.3.1. **Bile acids and cell function**

Bile Acids (BAs) are synthetized, in human and majority of animals, from cholesterol in the liver through a chain of chemical reactions and excreted by bile and intestine (Russell & Setchell, 1992). BAs, the main constituents of bile, are responsible for the removal of cholesterol from the body and
represent the major pathway of metabolism. These product ends of cholesterol are water-soluble compounds enable to associate with other molecules namely proteins (Russell & Setchell, 1992) (Amaral, et al., 2009).

Ursodeoxycholic acid (UDCA), an endogenous hydrophilic bile acid, represents up to 4% of total bile acids present in human bile (Lazaridis, et al., 2001). However, in American black bears, UDCA is the major biliary bile acid. Bear bile has been used, for many centuries, in traditional Chinese medicine to treat liver disorders (Hagey, et al., 1993) (Lazaridis, et al., 2001). It has been also used, in the Western world, as a therapeutic agent for chronic cholestatic liver diseases (Lazaridis, et al., 2001). In fact, unlike hydrophobic BAs that have a toxic effect and induce cell death, UDCA was shown to have cytoprotective effects (Heuman, et al., 1991). Moreover, UDCA has been extensively studied in nonliver diseases related to increased levels of apoptosis, namely NDs (Amaral, et al., 2009). After oral administration, UDCA is directly conjugated with taurine or glycine, originating tauroursodeoxycholic acid (TUDCA) and glycoursoodeoxycholic acid (GUDCA), respectively.

UDCA conjugated as a more hydrophilic molecule tolerates an administration of higher doses of this bile acid and a targeting of other tissues, such as the brain (Keene, et al., 2002). TUDCA is a Food and Drug Administration (FDA)-approved molecule, able to cross the blood-brain barrier (Keene, et al., 2002), probably due to its small size and the presence of transporters for the taurine-conjugated molecule in the brain (Xavier, et al., 2015). TUDCA has been revealed to play a unique role in modulating apoptosis in distinctive cell types, in response to a diversity of agents, involving different apoptotic pathways (Rodrigues, et al., 1998a).

I.3.2. Anti-apoptotic effects of TUDCA

Over the past few years, UDCA and its amidated conjugates TUDCA and GUDCA have been described as strong inhibitors of classic pathways of apoptosis (Amaral, et al., 2009). These hydrophilic bile acids were shown to prevent depolarization of mitochondrial membrane, pore creation, Bax channel formation, cytochrome c release (Rodrigues, et al., 1998a) (Rodrigues, et al., 1998b), caspase-3 activation, and subsequent cleavage of nuclear enzyme substrate poly(ADPribose) polymerase (PARP), induced by hydrophobic deoxycholic acid (DCA) (Rodrigues, et al., 1998a) (Rodrigues, et al., 2000b) (Keene, et al., 2002). Particularly, UDCA is able to revert the effect of DCA-induced mitochondria alterations in vivo, including pro-apoptotic Bax translocation to the mitochondria (Rodrigues, et al., 1998b). In addition to DCA, UDCA and its amidated conjugates were also shown to inhibit the toxicity of different cytotoxic agents, namely transforming growth factor (TGF)-β1, the Fas ligand, okadaic acid and other hydrophobic BAs (Rodrigues, et al., 1999) (Rodrigues, et al., 1998a).

DNA microarray analysis revealed that UDCA could significantly regulate the expression of 96 different genes, involved not only in apoptosis but also in cell cycle regulation and proliferation (Castro, et al., 2005). Apaf-1 was shown to be downregulated in response to UDCA, in rat hepatocytes (Castro, et al., 2005). In addition, this bile acid inhibits TGF-β1-induced E2F-1 transcriptional activation, p53
stabilization and p53-associated Bax expression (Solà, et al., 2003b). Moreover, these bile acids act against apoptosis through a pathway, involving nuclear steroid receptors: glucocorticoid (GR) and mineralocorticoid (MR) receptor (Solà, et al., 2004) (Solà, et al., 2005). Furthermore, the modulation neuronal apoptosis by TUDCA involves an interaction with MR (Solà, et al., 2006).

Moreover, Castro et al demonstrate that TUDCA reduced the apoptotic threshold caused by glutamate. It occurs since TUDCA promotes the phosphorylation of B-cell lymphoma 2 (Bcl-2)-associated death promoter (Bad), preventing its interaction with anti-apoptotic protein Bcl-2 and B-cell lymphoma-extra large (Bcl-xL) and hence, allowing them to promote cell survival. Cell death induced by this neurotransmitter is associated with several acute and chronic ND, namely AD and PD (Castro, et al., 2004).

Interestingly, a recent study reported that TUDCA increases NSC pool and favors neuronal rather than astroglial conversion by preserving mitochondrial integrity and function, while enhancing self-renewal potential. This last property of the bile acid appears to be mediated by inhibiting the increase of ROS and the reduction of ATP, as well as, the p53 mitochondria translocation, caused by TUDCA (Figure 6) (Xavier, et al., 2014b). Interestingly, some preliminary results also showed that a diet containing TUDCA partially reverted the significant reduction of PGC1α levels in animal models of impaired neurogenesis (data not shown), suggesting the involvement of mitochondrial biogenesis and bioenergetics in TUDCA effect. This holds great promise for the use of TUDCA as useful therapeutic tool, improving the long term-survival and differentiation of NSCs.

**Figure 6. The role of TUDCA in neuronal differentiation.** TUDCA inhibits differentiation-induced mitochondrial apoptotic events, while enhancing self-renewal potential and accelerating cell cycle exit of NSCs. This contributes for the enhancement of neuronal rather than astroglial conversion of differentiating NSCs (Xavier, et al., 2014b).

Finally, the potential role of TUDCA in the treatment of apoptosis associated neurological disorders has been extensively studied and it will be described ahead.
I.3.3. Neuroprotective effects of TUDCA

Several neurobiological disorders, namely acute stroke and chronic ND, are caused by many factors such as oxidative stress, misfolded proteins, impairment of ECT complexes and Ca$^{2+}$ imbalance (Bredesen, et al., 2006).

The protective role of TUDCA has been tested in a wide range of models of neurological disorders, including HD (Keene, et al., 2002) (Keene, et al., 2001), PD (Duan, et al., 2002), AD (Ramalho, et al., 2008), and ischemic (Rodrigues, et al., 2002) and hemorrhagic stroke (Rodrigues, et al., 2003b). A recent clinical study demonstrated that UDCA is safe and well tolerated in ALS, showing affective and dose-dependent cerebrospinal fluid penetration (Parry, et al., 2010).

Regarding to HD, which is caused by specific neuronal loss and dysfunction in the striatum and cortex, the systemic administration of TUDCA in a pharmacological induced HD model was able to reduce the associated morphologic striatal lesions (Keene, et al., 2001). This nontoxic bile acid also clearly reduced the mitochondria perturbations in cultures neuronal cells associated with apoptosis induction by 3-nitropropionic acid (3-NP), a fungal toxin that produce HD-like symptoms (Rodrigues, et al., 2000b). Additionally, TUDCA improved the locomotor and sensoriomotor deficits in a genetic mouse model of HD (Keene, et al., 2002).

Interestingly, the treatment of a cell suspension with TUDCA, following by its transplantation, exhibited a reduction of apoptosis and an increasing of survival of nigral grafts cells, in a rat model of PD (Duan, et al., 2002). The results of this study showed that the incubation of ventral mesencephalic-derived cells with TUDCA reduced the number of cultured cells undergoing apoptosis, increasing the number of tyrosine-hydroxylase-positive neurons. This demonstrates that TUDCA is able to enlarge the life-long activity of SCs, and therefore guarantee efficient differentiation. Another study demonstrates that TUDCA partially rescue a PD model of C. elegans from mitochondrial dysfunction (Ved, et al., 2005).

Many studies related to AD, have been also performed in order to test the potential clinical applications of TUDCA in such disease. In 2000, TUDCA showed to prevent Aβ-induced apoptosis in primary rat neuron and astrocyte cultures (Rodrigues, et al., 2000a) as well as in PC12 cells (Ramalho, et al., 2004). In the last model, TUDCA was able to inhibit the apoptotic E2F-1/p53/Bax pathway (Ramalho, et al., 2004). The same results were obtained in PC12 cells expressing amyloid precursor protein (APP) with the swedish mutation or double-mutated human for APP and presenilin 1 (PS1) (Ramalho, et al., 2006), as well as in cultured cortical neurons incubated with fibrillar Aβ (Ramalho, et al., 2008) (Solá, et al., 2003a). The neuroprotective role of TUDCA was extended to the synaptic level, where this endogenous bile acid revealed to increase the capacity of neurons to tolerate the toxic effects of Aβ, both in in vitro and in vivo (Ramalho, et al., 2013). Finally, TUDCA was shown to inhibit the accumulation of Aβ deposits in APP/PS1 mice by reducing Aβ production and also prevented the neuronal integrity loss (Nunes, et al., 2012).

In addition to the previous studies, the anti-apoptotic role of TUDCA was expanded to acute conditions. In a rat model of transient focal cerebral ischemia, intravenous administration of TUDCA
showed to reduce infarct volumes, the number of apoptotic cells, mitochondria swelling and partially inhibit downstream caspase activation and substrate cleavage associated with apoptosis while also improved neurological functions (Rodrigues, et al., 2002). Moreover, in a collagenase-induced hemorrhagic model of stroke, TUDCA also reduced the levels of apoptosis by maintaining the membrane stability and inhibiting caspase activation (Rodrigues, et al., 2003b). Also, in a murine model of spinal cord injury, TUDCA was shown to reduce the infarct size and improve the neurological performance (Colak, et al., 2008).

Taken together, these studies provide evidence for the anti-apoptotic role of UDCA and TUDCA. Being a hydrophilic molecule and nontoxic, TUDCA can be administrated both intravenously and orally. These features make TUDCA a strong candidate in the treatment of many NDs. It would be interesting to expand its clinical potential in other models, of apoptotic pathways where this bile acid can interfere, such as aging and other impaired neurogenesis conditions.
I.4. Motivation and Aims

Neurological diseases, namely NDs, congenital disorders, and cancers, affect millions of people of all ages (Winslow, 2006). NDs are related with a wide range of acute and chronic conditions leading to either sensory dysfunction (dementia) or functional loss (ataxia) (Winslow, 2006) (Lindvall & Kokaia, 2010) (Uttara, et al., 2009). Like in NDs, ageing is characterized by a decrease of neural cells, frequently combined with disturbances in mitochondrial function and signaling (López-Otín, et al., 2013). Unfortunately, the nervous system has a limited capacity for self-repair. Mature nerve cells lack the ability to regenerate and NSCs has a limited capability to generate new functional neurons in response to injury (Stichel & Müller, 1998) (Bjorklund & Lindvall, 2000).

In 2015, the number of people aged 60 years and over, living worldwide was estimated for approximately 900 million (Prince, et al., 2015). Among NDs, nearly 44 million people have AD, worldwide (Prince, et al., 2015). Although these numbers are already remarkable, they are expected to increase in the future (Prince, et al., 2015) (Brookmeyer, et al., 2007). This, in turn, highlights the importance of improving the current treatments of the age- and disease-related neural loss in a joint action between clinician and researchers in order to minimize the associated mortality and maximize the quality of life of patients. While many treatments aim to reduce the damage, in some cases scientists believe that the lost of neuronal cells can be restored by expansion and maintenance of functional NSC pools (Winslow, 2006).

Previous reported data from Xavier et al. showed that the specific bile acid TUDCA increases the proliferation and self-renewal potential of NSC by accelerating cell cycle exit of NSCs in a mouse NSC line (Xavier, et al., 2014b). In this study, it was also demonstrated that TUDCA effect on NSC proliferation and lineage determination is dependent on its role in modulating mitochondria integrity and function at early-stages of mouse NSC differentiation (Figure 7) (Xavier, et al., 2014b).

![Figure 7. TUDCA induces NSC proliferation and enhances neuronal differentiation in a mouse cell line. NSCs were expanded and induced to differentiate for 24 h in the presence or absence of TUDCA and/or CsA (an inhibitor of the MnSOD) and/or OligoA (an inhibitor of ATPase). Cells were then collected for flow cytometry analysis. A) BrdU experiments showed that TUDCA modulates the NSC proliferation by regulating mitochondrial](image-url)
ROS and ATP levels. B) TUDCA enhances neuronal rather than astroglial conversion of NSCs by regulating mitochondrial ROS and ATP levels. Adapted from (Xavier, et al., 2014b).

Based on these previous data, we decided to explore whether TUDCA would present same properties in another in vitro model as well as in vivo. In addition, we also intended to better understand the influence of this bile acid in modulating the expression of certain mitochondrial regulators. To accomplish this aim, we defined three major objectives. First, our goal was to further elucidate the potential involvement of TUDCA in proliferation, self-renewal, differentiation and neuritogenesis processes using rat primary cultured NSCs derived from SVZ and DG. Next, we aimed to investigate the potential effect of TUDCA in regulating the expression levels of PGC1α, Mfn2 and Drp1 at latter time points of neural differentiation in this model. Finally, we sought to explore the influence of TUDCA in endogenous proliferation and differentiation of NSCs, in vivo.

The specific questions addressed in this thesis are:
1. Does TUDCA also increase proliferation and neurogenesis in rat primary cultured NSCs?
2. Are PGC1α, Mfn2 and Drp1 expression modulated by TUDCA after 7 days of neural differentiation?
3. Does TUDCA also increase proliferation and differentiation in vivo?

The overarching goal of the research presented in this thesis is to extend our knowledge on the role of the bile acid TUDCA in promoting the expansion of functional NSC pools as well as in increasing the number of new-born neurons. In addition, the results may also have implications in advancing our understanding on the role of this bile acid in mitochondria biogenesis, through their interplay with mitochondria regulators. Ultimately, our findings may prove useful in the development of novel stem cell-based therapeutic strategies, whose focus will be the expansion and maintenance of NSCs.
II. Material and Methods

II.1. Ethics statement

All animals used in the present study received humane care according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals prepared by the national Academy of Sciences and published by the National institutes of health (NIH) (NIH publication 86–23, revised 1985).

II.2. Neurosphere cultures

SVZ and DG stem/progenitor cell cultures were obtained from postnatal (P1-3) Sprague-Dawley rats. This model is appropriate to mimic posnatal neurogenesis. Briefly, fragments of SVZ and DG were dissected out from the other brain sections and single cells were seeded and expanded in serum-free culture medium (SFM) composed of Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium with GlutaMAX-I supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 1% B27, 20 ng/ml EGF (Invitrogen Corp., Carlsbad, CA, USA) (proliferative conditions) for SVZ, and an additional supplement of 10ng/ml FGF2 (Invitrogen Corp.) to DG cultures. Cells were plated on uncoated 6mm-Petri dishes and were allowed to develop in an incubator with a humidified (5% CO₂) and 95% atmospheric air at 37°C.

Six to 7 days or 8 to 10 days in culture, the initial SVZ or DG cells, respectively, divided and formed spheres of precursor cells in suspension (Figure 8). Neurospheres were collected and seeded on glass coverslips coated with 0.1mg/mL poly-D-lysine in SFM medium devoid of growth factors, for immunocytochemistry or immunoblotting (see annex A to check for medium composition used in neurosphere culture).

Figure 8. Time lines for differentiation experiments in SVZ- (A) and DG- (B) derived NSCs.
To investigate the effect of TUDCA (T0266; Sigma-Aldrich Corporation, St. Louis, MO, USA) on cell proliferation, differentiation, neuritogenesis and expression of mitochondrial proteins, 100 μM of TUDCA was added or not (control) to the medium. Neurospheres were allowed to develop for 48 hours or 7 days with 5% CO₂ and 95% atmospheric air at 37°C before in vitro assays. Regarding the analysis of neuritogenesis, before the differentiation process, the cells were dissociated with dissociation Phosphate-buffered saline (PBS) (PBS without Mg²⁺/Ca²⁺ with Ethylenediamine tetraacetic acid (EDTA)).

II.2.1. Cell proliferation assay

To investigate the effect of TUDCA on NSC proliferation, cells were exposed to 10 μM Bromodeoxyuridine (BrdU) (Sigma-Aldrich), a synthetic thymidine analogue able to substitute thymidine in the DNA double chain synthesis occurring in dividing cells (Wojtowicz & Kee, 2006), for the last 4 hours of 24 hours of TUDCA treatment. Then, cells were fixed for 30 minutes in Paraformaldehyde (PFA) 4%, which was washed with PBS, at room temperature (RT).

After this process, the immunocytochemistry for BrdU labeling was performed. Fixed cells were incubated in PBS 1% Triton X-100 to permeabilize the cellular membrane and, blocked for non-biding sites for 1 hour with 0.5% Triton X-100 (BDH Chemicals Limited, London, UK) and 3% bovine serum albumin (BSA, NZYTech, Lisbon) dissolved in PBS. Cells were then subsequently incubated overnight at 4°C with the conjugate anti-BrdU-Alexa Fluor-594 (1:100 and from Life Technologies Ltd., Carlsbad, CA, USA). Nuclei were stained with Hoechst 33342 incubation (12 μg/ml in PBS) (Invitrogen Corp.) and the preparations were mounted using Mowiol. The resulting fluorescent signals were imaged using fluorescence microscopy assessments performed with a Zeiss AX10 microscope (Carl Zeiss, Corp., Jena, Germany), equipped with a 40x plan-apochromat objective and a Leica DFC490 camera (Leica Microsystems, Germany). In each experiment, the number of cells was calculated through the average obtained in three random fields (0.09 mm²) per chamber in triplicates, using the ImageJ software.

II.2.2. Neuronal differentiation assay

To investigate the effect of TUDCA on neuronal differentiation, cells remained for 7 days at 37°C in a 5% CO₂ and 95% air humidified atmosphere after treatment. At day 7 the medium was removed and the cells were fixed and washed, as mentioned before. Plates were saved in the fridge at 4°C. Following this procedure, the immunocytochemistry assay was performed against Neuronal Nuclei (NeuN). NeuN is a neuron-specific nuclear protein, whose expression is commonly presented in most neuronal cell types throughout the adult nervous system (Mullen, et al., 1992). Then, the fixed cells were incubated in 0.5% Triton X-100 and 6% BSA to permeabilize the cellular membrane and blocked for non-biding sites. Cells were then subsequently incubated overnight at 4°C with the conjugate rabbit anti-
NeuN (1:200) (Cell Signalling Technology, Danvers, USA) and for 1h at RT with the appropriate secondary antibody donkey anti-rabbit Alexa Fluor 568 antibody (1:200) (Life Technologies Ltd). Nuclei were stained with Hoechst dye and the preparations were mounted using Mowiol. Cell count was determined by the aforementioned method.

II.2.3. Analysis of neurite outgrowth and dendritic spines

After 2 days in differentiation medium, the cells were fixed using the same procedure already described. Then they were blocked and permeabilize with 0.5% Triton X-100 and 3% BSA in PBS. For evaluation of NSC differentiation, namely the total neurite output, length of the longest neurite and number of neuritis per cell, fixed cells were washed and incubated overnight at 4°C with mouse primary antibody reactive to βIII-tubulin (1:200) (Cell Signalling Technology). βIII-tubulin is a protein present in throughout the neuronal cell, including soma, dendrites and axon (Cáceres, et al., 1986). Cells were then washed and incubated with the appropriate secondary antibody donkey anti-mouse 568 (1:200) (Invitrogen Corp.). In this assay, the images were obtained through the 63x/1.4 oil plan-apochromat objective. The number of cells of interest and the lengths of the neurites were manually obtained per field (10 fields with 0.03 mm² per chamber in triplicates), using ImageJ. An average was acquired per chamber for each experiment.

II.2.4. Self-renewal assay

To address self-renewal capacity of progenitor cells, SVZ dissociated cells were seeded and grown in SFM containing 10 ng/ml EGF (low EGF), with TUDCA or without (control). For progenitors cells of DG dissociated cells, it was used 10 ng/ml EGF and 5 ng/ml FGF-2 (low EGF/FGF-2) with TUDCA or without (control). After 7 or 11 days, primary neurospheres were counted, for SVZ or DG, respectively. These were collected, dissociated as single cells, seeded and grown in low EGF for SVZ cells or low EGF/FGF-2 for DG cells. After additional 7 or 11 days, secondary neurospheres were counted, for SVZ and DG, respectively. In this assay, it was used a low concentration of growth factors in order to accentuate the differences observed in the proliferation process, in the presence of TUDCA. Like in BrdU assay, the number of primary neurospheres reflects the proliferative potential of NSCs and neural progenitors, whereas the number of secondary neurospheres is proportional to NSC to self-renewal potential, the ability to generate neurospheres from a single cell. The experimental protocol is described in the Figure 9.
II.3. Western blot analysis

Steady-state levels of mitochondrial proteins, PGC1α, Mfn2 and Drp1, were determined by Western blot analysis. The protein extracts were obtained from SVZ neurosphere cultures after 7 days in differentiation medium. Cells were collected and lysed for isolation of total protein extracts using ice-cold lysis buffer (10mM Tris-HCl, pH7.6, 5mM MgCl₂, 1.5mM KAc, 1% Nonidet P-40, 2mM dithiothreitol) supplemented with protease and phosphatase inhibitors (Thermo Fisher Scientific, Waltham, MA, USA.) for 30 min. After sonication, samples were then centrifuged at 3,200 g at 4°C for 10 min. Protein content was measured by the Bradford Method using the BioRad protein assay kit (BioRad Laboratories, Hercules, CA, USA) accordingly to the manufacturer’s specifications. BSA was used as a control sample to perform a calibration curve. Samples were denatured in the presence of a 5x denaturing/loading buffer (0.2M Tris-HCl, pH6.8, 20% glycerol, 2% SDS, 10mM β-mercaptoethanol, H₂O, bromophenol blue) at 95°C during 5 min. Eighty µg of protein extracts and the protein size marker (Precision Plus Protein™ Standards Dual Color, BioRad) were separated on 7.5% sodium dodecyl sulfate-polyacrylamide electrophoresis gels (SDS-PAGE). After electrophoresis, samples were transferred to a Hybond-C nitrocellulose membrane 8.5 cm x 6.5 cm by electroblotting and blocked with 5% milk during 1 hour at RT. Immunolabeling of the blots occurred overnight at 4°C with the primary antibodies (Table 1). After washing, membranes were incubated with the secondary antibodies anti-mouse and anti-rabbit (1:5000, BioRad) for 2 hours at RT. Finally, membranes were processed for protein detection using Immobilon (Millipore Corp.). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (sc-32233; Santa Cruz Biotechnology, Inc.) and heat shock protein (Hsp) 90 (sc-13119; Santa Cruz Biotechnology, Inc) were used to as loading control.
Table 1. Primary antibodies used for Western Blotting

<table>
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<tr>
<th>Antigen</th>
<th>Company</th>
<th>Catalog number</th>
<th>Host</th>
<th>Clonality</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGC1α</td>
<td>Calbiochem</td>
<td>ST1202</td>
<td>Mouse</td>
<td>monoclonal</td>
<td>1:1000</td>
</tr>
<tr>
<td>Mfn 2</td>
<td>Abcam</td>
<td>ab56889</td>
<td>Mouse</td>
<td>monoclonal</td>
<td>1:1000</td>
</tr>
<tr>
<td>Drp1</td>
<td>Santa Cruz, Tec.</td>
<td>Sc-32898</td>
<td>Rabbit</td>
<td>polyclonal</td>
<td>1:200</td>
</tr>
</tbody>
</table>

II.4. Animal models and TUDCA delivery

Miniosmotic pumps (Alzet, Cupertino, CA, USA) were implanted in 6-week-old male Wistar rats for intraventricular infusion of 300 µM TUDCA dissolved in artificial cerebrospinal fluid (aCSF) or aCSF alone, as vehicle, for continuous dosing of unrestrained laboratory animals. During this process the rats were anesthetized with isoflurane (Esteve Farma Ltd, Lisbon). The delivery rate was 0.25 µl/hour for 28 days. To evaluate cell migration and differentiation, the animals were injected intraperitoneally with BrdU two times per day during the first three days of the experience. To assess proliferation, the rats were injected two times, 4 and 2 hours before scarification. Animals were perfused with saline solution (NaCl 0.9%) and fixed with PFA 4% in PBS. Therefore, they were killed by exsanguination, under isoflurane anesthesia. The scarification of the anesthetized animals was made by decapitation and the brains immediately removed. Contralateral hemisphere was submitted to the sectioning process (Figure 10) to performed immunohistochemistry assays.
II.4.1. Tissue processing and immunohistochemistry

Brains were postfixed for 1 day in 4% PFA, and then cryopreserved in 30% sucrose. For immunohistochemistry of NSC markers, brains were coronally processed in 30-μm-thick cryostat sections that were collected in series of 10 slides. Each series contained an anterior-posterior (AP) reconstruction of four brains sections separated 300 μm between them. For doublecortin (DCX) quantification, a microtubule-associated protein expressed by neuronal precursor cells and immature neurons in embryonic and adult brain structures (Vukovic, et al., 2013), were used the goat primary antibody (1:500) (Santa Cruz Biotechnology, Dallas, Texas, USA) and subsequently the secondary antibody donkey anti-goat 488 (1:500, Invitrogen Corp.). For ki67 labeling, a nuclear protein that is
associated with cellular proliferation (Scholzen & Gerdes, 2000), tissue sections were incubated with the primary monoclonal rabbit antibody anti-ki67 (1:200) (Abcam, Cambridge), for 2h at RT. Then, slices were incubated with Dako EnVision conjugated w/ HRP (anti-rabbit) for 1 hour at RT. Thymus slice was used as positive control. For negative controls, the primary antibody was omitted during the staining. The immunohistochemistry of ki67 was performed by Histology and Comparative Pathology Laboratory, a technical facility of iMM. The quantification of DCX- and ki67-positive cells was performed in every series of the reconstruction. Thus, the distance between the sections analyzed was 300 μm. At least six SVZ and eight DG consecutive sections per animal were included in the analysis. The distribution of DCX- and ki67-positive cells in SVZ and DG was calculated as the average number of positive cells in two random fields (0.02 mm² for DCX and 0.20 mm² for ki67) per slide (n=3 animals). In the case of DCX, to better visualize the cells for quantification, nuclei were counterstained with Hoechst 33342 (12 μg/ml in PBS) (Invitrogen Corp.). Tissue sections labeled with DCX were captured in Zeiss LSM 510 META, a Confocal Laser Point-Scanning Microscope, equipped with a 63x/1.4 oil DIC plan-apochromat objective. For ki67 labeling, the slides were scanned and magnified with a 20X objective lens, using the viewer software NDP.view2. In both cases, positive cells were counted using ImageJ software.

II.5. Densitometry and statistical analysis

The relative intensities of protein bands were analyzed using the Quantity One Version 4.6.3 densitometric analysis program (Bio-Rad Laboratories). Results from different groups were compared using the Student’s t test, 2-way ANOVA or one-way ANOVA followed by Bonferroni’s or Dunnett’s multiple comparison tests. Values of p < 0.05 were considered statistically significant. All statistical analysis was performed with GraphPad Prism 5 software (GraphPad Software, Inc.).
III. Results and Discussion

III.1. TUDCA increases proliferation and enhances neuronal fate of SVZ- and DG-derived NSCs

Recent evidences suggest that mitochondrial events influence the proliferative and differentiation potential of NSCs (Xavier, et al., 2014b). In addition, the endogenous bile acid, TUDCA, has already been described as a strong inhibitor of mitochondrial apoptotic events in different cell types. Interestingly, in a mouse NSC line, this bile acid was shown to modulate NSC proliferation and differentiation through mechanisms dependent on mitochondrial retrograde signals (Xavier, et al., 2014b). In the present work, we began to clarify whether TUDCA would have a similar effect in rat primary cultured NSCs, including SVZ- and DG-derived NSCs. Cell proliferation was evaluated by counting the number of BrdU-positive NSCs and neural progenitors, treated or untreated with TUDCA. In fact, immunocytochemistry analysis revealed that, in both neurogenic niches, treatment with TUDCA significantly increased BrdU incorporation (p < 0.05), when compared with untreated cells, (Figure 11). Our results are in accordance with the previous study on NSC lines showing that TUDCA increases proliferation by retarding cell cycle arrest, namely by decreasing the expression of p21 and p27. In that work, the authors have also demonstrated that treatment with TUDCA decreases NSCs in G0-G1 phase, while increases S/G2-M phase (Xavier, et al., 2014b). More importantly, TUDCA effect was shown to be dependent on mitochondrial ROS and ATP levels (Xavier, et al., 2014b). Moreover, Castro et al showed that the unconjugated form of this bile acid promotes cell proliferation in liver cells by upregulation of the microRNA-21 (Castro, et al., 2010). In agreement, negative regulators of cell proliferation, such as cadherin 1 (Cdh 1) and E-cadherin, decreased after UDCA exposure (Castro, et al., 2005). Therefore, it is possible that the effect of TUDCA observed in primary cultured NSCs might be dependent on both mitochondria and microRNA-21 expression.
Figure 11. TUDCA increases proliferation of NSCs in both SVZ and DG niches. NSCs were expanded, induced to differentiate in the presence or absence of TUDCA, and then collected for immunocytochemistry to detect BrdU incorporation, as described in Methods. A) Representative quantification data of BrdU positive cells after 24h under differentiation medium, for SVZ (left) and DG (right) niches. B) Representative images of immunofluorescence detection of cells labeled with anti-BrdU antibody. Nuclei were staining with Hoechst 33342. Results are expressed as mean ± SEM fold change for three different experiments. *p < 0.05. Scale bar, 10μm.

To further explore the impact of TUDCA in regulating NSC differentiation in our cell model, neurospheres were cultured and allowed to differentiate in an optimized neuronal differentiation-inducing medium, as described in the Methods. Our results showed an increase in the percentage of NeuN-labeling cells after TUDCA treatment, indicating that this endogenous bile acid is able to induce a significant increase of neuronal population, in SVZ (p < 0.05) and DG (p < 0.01) niches compared to controls (Figure 12). However, its effect was more evident in SVZ than in DG NSC populations. In fact, TUDCA doubled the number of NeuN-positive cells in SVZ-derived NSCs, while in DG-derived NSC culture, the increase was by approximately 1.5-fold, compared with respective controls. The fact that TUDCA was able to increase both proliferation and neurogenesis in our cell model was not surprising. Indeed, it has already been shown that, by regulating mitochondrial stress and function, TUDCA increases the ratio of neurons versus glial cells (Xavier, et al., 2014b). It has been reported that cyclosporin A (CsA), an inhibitor of mtROS scavenger MnSOD, and oligomycin A (OligoA), an inhibitor of F1F0-ATP synthase complex, were able to revert TUDCA effects in increasing the proportion of neuronal vs glial-differentiating NSCs (Xavier, et al., 2014b). In fact, mtDNA damage has been largely associated with the elevated of astrogliogenesis and lack of neurogenesis during repair of neuronal injury (Wang, et al., 2011). Although gliogenesis was not evaluated in our model yet, we would also expect to observe a significant reduction of glial markers in both SVZ- and DG-derived cultures treated with TUDCA.
Figure 12. TUDCA increases neuronal differentiation of NSCs. Rat NSCs were expanded, induced to differentiate in the presence or absence of TUDCA, and then collected for immunocytochemistry to detect NeuN expression, as described in Methods. A) Representative quantification data of NeuN-positive cells at 7 days of differentiation in SVZ- (left) and DG- (right) derived NSCs. B) Representative images of immunofluorescence detection in cells labeled with anti-NeuN antibody. Nuclei were staining with Hoechst 33342. Results are expressed as mean ± SEM fold change for at least three different experiments. *p < 0.05 and **p < 0.01. Scale bar, 10μm.

It is important to refer that the length of G1 phase of the cell cycle also influences cell lineage determination of neural precursors (Salomoni & Calegari, 2010). In this regard, Quian et al showed that glial cells are formed after neurons because glioblasts are generated after the neuroblasts and undergo a sequence of proliferative divisions that effectively delay the appearance of differentiated progeny (Quian, et al., 2000). They also demonstrated that glial cells take more time to re-enter cell cycle than neurons, having a more prolonged G1 phase (Quian, et al., 2000). Taking together, we think that the effect of TUDCA in increasing neurogenesis might not rely in increasing differentiation process per se, but rather in modulating NSC-lineage shift toward neurogenesis. Of note, previous experiments in the
laboratory using flow cytometry revealed that TUDCA does not change the percentage of astrocyte-positive cells in both SVZ- and DG-cultured NSCs, but rather significantly decreases the percentage of oligodendrocyte markers (data not shown). In the future, it would be interesting to confirm the effect of TUDCA on oligodendrocyte cells by other techniques. Nevertheless, these findings corroborate the potential therapeutic relevance of TUDCA in augmenting NSC proliferation and neuronal fate.

### III.2. TUDCA induces proliferation but not self-renewal of SVZ- and DG-derived NSCs

During the adult life of mammalians, the self-renewal capacity and the regenerative potential of NSCs are progressively deteriorated, leading to an inefficient tissue repair and maintenance (Piccin & Morshead, 2010) (Lledo, et al., 2006). Thus, developing methods to expand adult NSC population, either by promoting NSC proliferation or enhancing self-renewal divisions are pivotal to bring SCs one step closer to its successful application in neuro-replacement therapies.

Based on the previous findings showing the proliferative and self-renewal potential of TUDCA in NSC lines (Xavier, et al., 2014b), we decided to explore these TUDCA properties in our *in vitro* model. Applying the method described on the Methods section II.2.4, we evaluated the number of primary and secondary neurospheres in both SVZ and DG. A schematic representation of the primary and secondary neurosphere formation is shown in Figure 13A.

Our results revealed that TUDCA significantly increased the number of primary neurospheres in both SVZ- and DG-derived NSCs, ($p < 0.05$ and $0.01$, respectively) (Figure 13 B and C left). This is in accordance with the literature (Xavier, et al., 2014b) as well as with our previous results obtained with BrdU assay. In contrast, the number of secondary neurospheres was not significantly increased by the bile acid (Figure 13B and C right). The trends observed in the number of secondary neurospheres with TUDCA treatment when compared to controls cannot be totally accepted. Further, a recent study reported in NSC lines contradicts that tendency (Xavier, et al., 2014b). Hence, in the near future, it would be important to repeat these experiments, to have a better understanding on the effect of TUDCA in self-renewal of SVZ- and DG-derived NSCs. In fact, after 24h of TUDCA incubation, mouse NSC lines presented higher levels of symmetrical divisions (self-renewal), as detected by the Sox2 cell pair assay (Xavier, et al., 2014b). Of note, the already reported experiments were performed using the same conditions (low percentage of growth factors) that we used. This type of division is described as the generation of daughter cells that are destined to acquire the equal fate. Whereas, asymmetrical division occurs when SCs are capable to simultaneously perpetuate themselves (self-renewal) and generate differentiated progeny (Morrison & Kimble, 2006).
Figure 13. TUDCA induces proliferation but not self-renewal of SVZ- and DG-derived NSCs. NCSs were expanded in low proliferative conditions, in the presence or absence of TUDCA. The number of primary and secondary neurospheres was counted, as described in Methods. (A) Schematic illustration of the generation of primary and secondary neurospheres. (B) Representative quantification data of primary neurospheres (left) and secondary neurospheres (right) in SVZ-derived NSCs with or without TUDCA. (C) Quantification of the number of primary neurospheres (left) and secondary neurospheres (right) in DG, in the presence or absence of TUDCA. Results are expressed as mean ± SEM fold change for at least six different experiments for SVZ and three for DG regions. *p < 0.05 and **p < 0.01. Gen, generation. 

Importantly, cell pair assays previously performed in the laboratory by other students revealed that TUDCA significant increases NSC symmetric divisions in SVZ- and DG-derived cultures (data not shown). Thus, these data are still elusive and additional studies are in course and are expected to provide a better understanding of the role of TUDCA on NSC self-renewal in both SVZ and DG niches.
III.3. Neuritogenesis of SVZ- and DG-derived NSCs is not affected by TUDCA

The branching of axons is an essential aspect of nervous system development and neuroplasticity (Spillane, et al., 2013). This process was described to be dependent on mitochondria, through ATP generation and the determination of localized hot spots of vigorous axonal mRNA translation (Spillane, et al., 2013). Interestingly, the major mitochondrial regulator PGC1α was also shown to increase the formation of neuronal dendritic spines, in hippocampal dentate granule neurons in vivo (Cheng, et al., 2012). Here, we decided to evaluate the role of TUDCA in modulation of neurite elongation and branching of differentiating NSCs. For that, three different parameters, including number of neurites per cell, total neurite output and longest neurite length, were analyzed after 2 days of NSC differentiation. However, TUDCA did not induce significant alterations in the three analyzed parameters in both niches (Figure 14).
Figure 14. TUDCA does not affect neuritogenesis of both SVZ- and DG-derived NSCs. NSCs were induced to differentiate for 2 days, and collected for immunocytochemistry analysis of βIII-tubulin, as described in Methods. A) Number of neurites per cell, total neurite output and length of the longest neurite. B) Representative images of immunofluorescence detection of cells labeled with anti-βIII-tubulin antibody. Nuclei were staining with Hoechst 33342. Results are expressed as mean ± SEM for four different experiments. Scale bar, 10μm.

In fact, it is possible that TUDCA does not interfere with pathways involved in neurite outgrowth or neuritogenesis. It is important to note that the TUDCA effect on the mitochondria during differentiation was observed at early stages of NSC differentiation, probably only affecting NSC-lineage commitment and not the terminal neuronal differentiation process itself (Xavier, et al., 2014b). Curiously, it has been demonstrated that p53 mitochondrial translocation increases not only the mitochondrial protection throughout NSC differentiation, but also the neurite elongation and branching of NSCs (Xavier, et al.,...
On the other hand, by preventing differentiation-mediated mitochondrial damage, TUDCA treatment was shown to diminish p53 mitochondrial translocation, as an endogenous mechanism of cellular stress protection (Xavier, et al., 2014b). Taking into account these data, it remains to be determined whether the absence of TUDCA effect on neuritogenesis is due to a p53-dependent mechanism or not.

### III.4. TUDCA does not affect the expression of mitochondrial regulators

Several processes, namely neural differentiation and survival largely relies on mitochondria biogenesis, integrity and function (Xavier, et al., 2015). Therefore, the number of mitochondria should be preserved existing a balance between elimination of dysfunctional mitochondria and its synthesis. To further explore the role of TUDCA in modulating the expression of mitochondrial dynamic proteins and other regulators, we performed Western blot analysis to detect the total levels of PGC1α, Mfn2 and Drp1 in SVZ-derived NSC culture. Nevertheless, we did not observe any significant change in the expression of these three proteins by TUDCA incubation (Figure 15). These unexpected results were obtained after 7 day of NSC differentiation. Indeed, the absence of alterations by the bile acid does not exclude a potential effect of TUDCA in the total levels of these mitochondrial-related proteins. As already stated, TUDCA was shown to prevent mitochondria apoptotic events at early stages of neural differentiation, such as at 6h in differentiation medium (Xavier, et al., 2014b). Therefore, in the near future, similar Western blot experiments should be performed at early time-points, such as at 6h of SVZ- and DG-derived NSC differentiation.

![Western Blot Analysis](image)

**Figure 15. TUDCA does not change the expression of mitochondrial-related proteins in SVZ-derived NSCs.** NSCs were induced to differentiate up to 7 days and collected for immunoblotting analysis. Representative
immunoblots of PGC1α, Mfn2 and Drp1 levels in total protein extracts and respective quantification data, at 7 days of NSC differentiation. Results were normalized to GAPDH protein levels. Results are expressed as mean ± SEM fold-change for three different experiments for PGC1α and Mfn2 and two different experiments for Drp1.

Moreover, it is also possible that TUDCA might only affect the subcellular distribution of Mfn2 and Drp1, not influencing the total expression levels of these mitochondrial-related proteins.

**III.5. TUDCA stimulates NSC proliferation in vivo**

Our *in vitro* experiments have indicated that TUDCA promotes the proliferation of SVZ- and DG-derived NSCs, contributing to an increase of the NSC pool. Moreover, as previously mentioned, this effect was also observed in a NSC line (Xavier, et al., 2014b). Hence, we decided to clarify whether this bile acid would have the same impact *in vivo* NSC proliferation. For that, we have used a rat animal model to access endogenous neurogenesis. After 28 days of TUDCA administration in rat brains by mini-osmotic pumps, the animals were sacrificed and the brains collected for cryostat sectioning. Brain slices were then used to immunohistochemistry analysis against ki67. It is important to refer, that although BrdU was administrated at different time points in all animals, in the present work we could only assess TUDCA effect on NSC proliferation at the exact time of animal scarification, by counting ki67-positive cells. Interestingly, analysis of SVZ and DG sections revealed an increased number of ki67-positive cells in the presence of TUDCA, being significantly higher in DG region (p<0.05) (Figure 16).
Figure 16. TUDCA promotes NSC proliferation in vivo. Immunohistochemistry against the cell proliferation marker ki67 was performed in both SVZ and DG regions of rat brains treated with or without TUDCA as described in Methods. Sagittal diagram of the rat brain exhibiting a frontal plane that represents the tissue section in SVZ (A) and DG (D) regions. Frontal views of the SVZ (B) and DG (E) brain regions (left). Representative images of ki67-positive cells, in the absence (control) and presence of TUDCA in a frontal section of SVZ and DG are also represented (right). Quantitative analysis of ki67-positive cells with and without TUDCA treatment in SVZ (C) and DG (F) regions. Nuclei were staining with Hoechst 33342. Results are expressed as mean ± SEM per field for two different experiments for SVZ and three for DG. *p < 0.05. Scale bars = 100 μm in (B) left; 50 μm in (E) left; and 20 μm in (B) right and (E) right.

Comparing the proliferation levels in both niches, the rise seemed to be more evident in DG region where the number of ki67-positive cells almost doubled by TUDCA. These results corroborate those obtained from our in vitro experiments, indicating that TUDCA is able to enhance the NSC pool. Future work is now required to assess the impact of TUDCA on BrdU incorporation in these in vivo experiments and also to establish the time window in which this acid acts. Nevertheless, these results demonstrate the potential relevance of TUDCA in increasing the number of NSCs in vivo, during long periods of time, reinforcing its promising therapeutic role for ageing and other impaired neurogenesis diseases.

III.6. TUDCA increases early NSC differentiation in vivo

In addition to the ability to increase proliferation in vivo, we also investigated the effect TUDCA in neural differentiation in vivo. In fact, our previous in vitro studies have demonstrated that this bile acid increases neurogenesis, specifically the mature neurons generated from primary culture of SVZ- and DG-derived NSCs. In addition, the enhancement of neurogenesis was also observed in other in vitro model (Xavier, et al., 2014b). Therefore, to clarify the ability of this endogenous bile acid to enhance
neurogenesis in vivo, we have also used rat animal models treated for 28 days with TUDCA by mini-osmotic pumps. The animals were sacrificed and the brains collected for cryostat sectioning as previously described above. Brain slices were then used to performed immunohistochemistry against DCX. Notably, our results showed an increase of newly generated DCX-positive immature neurons and neuroblasts, in both neurogenic regions (Figure 17). In SVZ region, the increase was significant (p < 0.05) and approximately doubled with TUDCA exposure, while in DG, the effect of this bile acid resulted in an increase of 1.3-fold in DCX-positive cells, when compared with control rats.

Figure 17. TUDCA treatment increases early NSC differentiation in rat models. Immunohistochemistry against the NSC early differentiation marker DCX was performed as described in Methods. Sagittal diagram of the rat brain exhibiting a frontal plane that represents the tissue section in SVZ (A) and DG (D) regions. Frontal views of the SVZ (B) and DG (E) brain regions (left). Representative confocal images of DCX-positive cells, in the absence
(control) and presence of TUDCA in a frontal section of SVZ and DG are also represented (right). Quantitative analysis of DCX-positive cells with and without TUDCA treatment in SVZ (C) and DG (F) regions. Nuclei were staining with Hoechst 33342. Results are expressed as mean ± SEM per field for three different experiments. *p < 0.05. Scale bars = 100 μm in (B) left and (E) left; 10 μm in (B) right and (E) right.

Curiously, the rise rates observed after TUDCA treatment have a similar pattern to those observed in our in vitro model where we assessed the mature neuronal marker NeuN. Thus, these results corroborate our previous data showing an enhancement of differentiating cells into neuronal lineage by TUDCA. The protective effect of this bile acid was already studied in different animal models of ND (Keene, et al., 2002) (Duan, et al., 2002) (Ramalho, et al., 2008). However the contribution of TUDCA in enhancing endogenous neurogenesis has never been explored yet. These studies add significant new information on the role of TUDCA in increasing NSC pool and neurogenesis in both primary cultured NSCs and in vivo, which may prove useful in the development of NSC-based therapeutic strategies.
IV. Conclusions and Future Perspectives

During development of the CNS, an extensive proliferation is demanded to produce the required number of progenitor cells for correct tissue and organ formation, followed by differentiation in order to generate functional neuronal cells at the correct time and place (Hardwick, et al., 2015). During adulthood, NSC proliferation markedly diminishes and neurogenesis declines. Indeed, the production of neurons, in the adult mammalian brain, is restricted to only a few brain regions (Lledo, et al., 2006). The balance between proliferation and neurogenesis gives rise to differential growth as well as cellular diversity (Hardwick, et al. 2015). Thus, it is crucial to have a solid scientific understanding about the mechanisms that regulate proliferation and differentiation potential, as well as survival and function of NSCs, to efficiently control and deliver clinically competitive therapies for neurological disorders.

The work presented in this thesis showed that the endogenous bile acid TUDCA, previously described as a potent inhibitor of apoptosis-mediated mitochondrial perturbations in neurons (Rodrigues, et al., 2000b), also raises proliferation of SVZ- and DG-derived NSCs, contributing for the enhancement of NSC pool, in vitro. Notably, our results also revealed that TUDCA increases the number of new-born neurons, highlighting its possible application in NSC-based therapies. Given these findings, we decided to investigate whether these effects are also maintained in vivo. Indeed, TUDCA appeared to increase the NSC pool and neurogenesis in vivo. Moreover, this bile acid demonstrated to retain its proliferative effect for long periods of time. In the near future we intend to confirm the impact of TUDCA in NSC proliferation by evaluating BrdU incorporation in the same rat brain slices. Notably, these effects of TUDCA open a new window of opportunities for therapeutic intervention in aging and other impaired neurogenesis diseases. Indeed, since NSCs self-renewal potential decreases during the adulthood of mammals, compromising the regenerative capacity of the brain in ways that tissue repair and maintenance become inefficient (Gage, 2000), novel strategies must focus not only in enhancing NSC commitment and differentiation, but also on the expansion and maintenance of functional NSC pools.

As future work, it would be interesting to characterize the impact of TUDCA in increasing the NSC pool and function of new-born neurons in animal models of neuronal loss, namely in ageing and AD, the major cause of dementia (Association, 2014). Further, similar in vitro studies should be performed in primary NSCs isolated from aged rat.

Regarding to self-renewal analysis, our results were unclear about the impact of TUDCA in the formation of secondary neurospheres. In this in vitro assay, many factors must be tightly regulated, including culture conditions, number of cell passages, initial cell density as well as age, since they have a huge impact on the homogeneity or heterogeneity of cell neurospheres (Coles-Takabe, et al. 2008) (Gil-Perotin, et al. 2013). Beside all these obstacles, several laboratories have used this method to study NSC proliferation and self-renewal as well as to identify NSCs in the brain (Reynolds & Weiss, 1992) (Alagappan, et al. 2014) (Craig, et al. 1996). Nevertheless, the number of the experiments should be elevated to minimize the effect of heterogeneity. In the future, it could be attractive to study not only the number of neurospheres in the first two generations but also in the following ones, in both niches.
Additionally, it would be interesting to study the size of neurospheres (usually ranging between 100 and 200 mm of diameter) (Gil-Perotin, et al. 2013) in vitro.

It is important to note that, it remains to be determined whether the absence of TUDCA effect on neuritogenesis is due, or not, to its interference with p53-related mechanisms. Interestingly, different localization of p53 at the mitochondria compartment completely changes the effect of this protein on mitochondrial protection. OMM localization of p53 appears to be related with mitochondrial apoptosis induction (Wolff, et al. 2008), while IMM localization of p53 has been more associated with maintenance of mtDNA integrity and repair (Achanta, et al. 2005). In this regard, it would be also important to investigate the precise mitochondria localization of p53 at different stages of neuronal differentiation, in order to better understand the possible different roles that this protein can have during this process. On the other hand, being TUDCA a cholesterol-derived molecule presenting high affinity to lipid membranes (Solà, et al., 2006), it would be attractive to further explore whether this bile acid interferes with p53 sub-mitochondrial localization.

Given our interest in exploring the role of TUDCA in regulating the expression of mitochondria regulators and dynamic proteins, in the future, we should explore the possible effect of TUDCA in the total levels of mitochondrial regulators at early stages of neuronal differentiation since this bile acid was shown to prevent apoptosis-associated events at that time points (Xavier, et al., 2014b). In fact, preserving the number of mitochondria is crucial to arrest degenerative processes (Fischer, et al., 2012). Accumulation of dysfunctional mitochondria accelerates ageing as well as ND progression. Addressing these questions and understanding the potential impact of TUDCA on mitochondrial biogenesis, function and bioenergetics, can have a huge impact in age-associated impaired neurogenesis and cognitive decline.

As a final note, it remains to be investigated whether the new generated neurons by TUDCA can efficiently restore brain functions. In fact, the low synaptic integrity of the new-born neurons observed in the adult human brain has been the main obstacle for the success of NSC-based therapies.

In conclusion, our results confirm that the neural protective role of TUDCA goes beyond its anti-apoptotic and antioxidant capacities. In fact, this bile acid is able to increase the NSC pool as well as neuronal differentiation, in both neurogenic regions of adult mammalian brain. This information together with the fact that this bile acid is non-toxic, safe and tolerable provides a new framework to further explore its use in the treatment of neurological disorders with neurogenic deficit as well as in ageing.
V. References


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VI. Annexes A

VI.1. Media Composition

Table A.1: Details of Dissection medium, used in rat primary cultured NSCs.

<table>
<thead>
<tr>
<th>Composition</th>
<th>Volumes</th>
</tr>
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<tbody>
<tr>
<td>Hanks’ balanced saline solution (HBSS) (Gibco)</td>
<td>50mL</td>
</tr>
<tr>
<td>Penicillin/Streptomycin (Pen/Strep) (Gibco)</td>
<td>500µL</td>
</tr>
</tbody>
</table>

Table A.2: Details of Proliferative medium, used to expand SVZ- and DG-derived NSCs.

<table>
<thead>
<tr>
<th>Composition</th>
<th>Volumes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium with GlutaMAX-I supplemented (DMEM/F-12 + GlutaMAX) (Gibco)</td>
<td>50mL</td>
</tr>
<tr>
<td>Pen/Strep (Gibco)</td>
<td>500µL</td>
</tr>
<tr>
<td>B27 (Gibco)</td>
<td>500µL</td>
</tr>
<tr>
<td>Epidermal growth factor/ Fibroblast growth factor 2 (EGF/FGF) (Invitrogen Corp)</td>
<td>50µL</td>
</tr>
</tbody>
</table>

Table A.3: Details of Differentiation medium, used to promote differentiation of SVZ- and DG-derived NSCs.

<table>
<thead>
<tr>
<th>Composition</th>
<th>Volumes</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM/F-12 + GlutaMAX (Gibco)</td>
<td>50mL</td>
</tr>
<tr>
<td>Pen/Strep (Gibco)</td>
<td>500µL</td>
</tr>
<tr>
<td>B27 (Gibco)</td>
<td>500µL</td>
</tr>
</tbody>
</table>