Marta Sofia Barbosa Fernandes

Licenciada em Bioquímica

Tauroursodeoxycholic Acid Drives Mitochondrial Bioenergetics Toward Neural Stem Cell Proliferation

Dissertação para obtenção do Grau de Mestre em
Genética Molecular e Biomedicina

Orientador: Susana Solá, Professora Doutora, Faculdade de Farmácia, Universidade de Lisboa

Co-orientador: Cecília M. P. Rodrigues, Professora Doutora, Faculdade de Farmácia, Universidade de Lisboa

Júri:

Presidente: Doutora Paula Maria Theriaga Mendes Bernardes Gonçalves
Arguente: Doutora Joana Paiva Gomes Miranda
Vogal: Doutora Susana Zeferino Solá da Cruz

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

Setembro 2017

Marta Sofia Barbosa Fernandes

Licenciada em Bioquímica

Tauroursodeoxycholic Acid Drives Mitochondrial Bioenergetics Toward Neural Stem Cell Proliferation

Dissertação para obtenção do Grau de Mestre em
Genética Molecular e Biomedicina

Orientador: Susana Solá, Professora Doutora, Faculdade de Farmácia, Universidade de Lisboa

Co-orientador: Cecília M. P. Rodrigues, Professora Doutora, Faculdade de Farmácia, Universidade de Lisboa

Júri:

Presidente: Doutora Paula Maria Theriaga Mendes Bernardes Gonçalves
Arguente: Doutora Joana Paiva Gomes Miranda
Vogal: Doutora Susana Zeferino Solá da Cruz

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

Setembro 2017

Tauroursodeoxycholic Acid Drives Mitochondrial Bioenergetics Toward Neural Stem Cell Proliferation

Part of the results discussed in this dissertation were presented in the following meetings:

Marta B. Fernandes, Maria F. Ribeiro, Tânia Genebra, Cecília M.P. Rodrigues, Susana Solá. Driving Mitochondrial Proteins Toward Neural Differentiation. Jornadas Intercalares das Dissertações Anuais dos Mestrados, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, 2nd February 2017, Caparica, Portugal. [Oral Communication]

Marta B. Fernandes, Maria F. Ribeiro, Tânia Genebra, Cecília M.P. Rodrigues, [Susana Solá]. Driving Mitochondrial Bioenergetics Toward Neural Proliferation and Differentiation. MitoPorto – Advances in mitochondrial research International Symposium, 26th May 2017 Porto, Portugal. [Abstract and Oral Communication]

Marta B. Fernandes, Maria F. Ribeiro, Tânia Genebra, Cecília M.P. Rodrigues, Susana Solá. Driving Mitochondrial Bioenergetics Toward Neural Stem Cell Proliferation. 9th iMed.ULisboa Postgraduate Students Meeting and 2nd i3DU Meeting, 13-14th July 2017, Lisbon, Portugal. [Abstract and Poster]

Marta B. Fernandes, Maria F. Ribeiro, Tânia Genebra, Joana Martins, Ana Varela, Cecília M.P. Rodrigues, Susana Solá. Tauroursodeoxycholic Acid Drives Mitochondrial Bioenergetics Toward Neural Stem Cell Proliferation. 10th International Meeting of the Portuguese Society for Stem Cells and Cell Therapies, 12-14th October 2017, Covilhã, Portugal. [Abstract and Poster]

This work was developed at the Research Institute for Medicines (iMed.ULisboa), Faculty of Pharmacy, Universidade de Lisboa and received funding support from Fundação para a Ciência e a Tecnologia (FCT) (UID/DTP/04138/2013), and from European Structural and Investment Funds through the COMPETE Programme and from National Funds through FCT (SAICTPAC/0019/2015).

Agradecimentos

As minhas primeiras palavras de agradecimento são dirigidas à Professora Doutora Cecília Rodrigues pela oportunidade que me concedeu em realizar a dissertação de mestrado no seu laboratório de investigação, por todo o apoio e pelos conselhos cruciais que conduziram ao progresso deste trabalho.

Um agradecimento especial à Professora Doutora Susana Solá que me acompanhou durante a realização deste trabalho. Por todos os conhecimentos transmitidos e por toda a atenção quando tudo parecia mais complicado. Igualmente pela simpatia, confiança e entusiasmo contagiante mas acima de tudo pelo apoio constante.

Quero agradecer à Professora Doutora Margarida Silva e ao Doutor Marco Moedas por toda disponibilidade na obtenção de resultados de grande relevância contribuindo para o enriquecimento deste trabalho.

À Professora Doutora Elsa Rodrigues e à Professora Doutora Maria João Nunes pelo interesse, conselhos e disponibilidade em ajudar.

Às minhas irmãs das NSCs pelo companheirismo, pelos conhecimentos transmitidos e por toda a ajuda fulcral. À Tânia Genebra por toda disponibilidade, pela boa energia e pela simpatia constante. À Maria Ribeiro, pela troca de ideias e cumplicidade, também pela paciência, mas acima de tudo, pelo apoio e alegria essenciais em muitas ocasiões. Um obrigada do tamanho de todos os bons momentos que partilhámos.

A todos os meus colegas do laboratório, ao Pedro Rodrigues, à Marta Afonso, à Diane Pereira, à Sofia Gomes, ao André Simão, ao Pedro Dionísio, ao Hugo Brito, à Sara Oliveira, à Vanda Marques, também aos Doutorados Joana Amaral, Rui Castro, André Santos e Alexandra Rosa pelo grande espírito de entreajuda que senti, por toda a simpatia e por todos os momentos de boa disposição que são uma constante neste grupo. Foi uma excelente experiência, por tudo o que aprendi convosco tanto a nível profissional como pessoal, muito obrigada!

Aos meus colegas quase mestres. Ana Poim, desejo-te muita sorte e agradeço a ajuda em várias ocasiões.

Carolina Tavares, que boa surpresa e que seja o começo de uma boa amizade, obrigada por tudo!

Mariana Mendes, que feliz coincidência esta de termos passado este ano juntas. Muito

obrigada por me teres aturado! Já tinhas um lugarzinho reservado há muito tempo mas nunca é demais dizer que prezo muito a nossa amizade.

Dário Valezim, também gostei de ter novamente por perto amigo, muito obrigada por todos os momentos de boa disposição!

À Teresa Duarte, a companhia e alegria lá de casa, todo o apoio por entre desabafos e gargalhadas.

Às minhas amigas Margarida Pita, Rita Garcia Marques e Andreia Henriques pela força, preocupação e compreensão pela ausência.

Um enorme obrigada ao Miguel Palhas por acreditar sempre em mim e me transmitir sempre o melhor. Toda a paciência e amor. Obrigada por seres uma fonte de inspiração e por teres a capacidade de tornar tudo sempre tão mais simples.

À minha família, estou-vos eternamente grata por todo o amor e apoio incondicional. Aos meus queridos avós toda a preocupação e por me encorajarem sempre. Finalmente, aos meus pais e ao meu irmão Guilherme que são o meu suporte e que nunca me deixam vacilar, o maior dos obrigadas!

Resumo

A neurogénese ocorre ao longo da vida em áreas específicas do cérebro de mamíferos adultos. Infelizmente, não existe uma regeneração eficaz durante o envelhecimento ou após lesão. Deste modo, potenciais estratégias responsáveis por um aumento da neurogénese endógena, poderão constituir um importante contributo. Recentemente, diversos estudos têm demonstrado que a bioenergética mitocondrial e a lipogénese de ácidos gordos (FA) determinam o potencial de proliferação e diferenciação das células estaminais neurais (NSCs).

Curiosamente, o ácido tauroursodesoxicólico (TUDCA), um ácido biliar neuroprotector endógeno, é um regulador do metabolismo energético e um inibidor dos eventos apoptóticos que ocorrem no início da diferenciação das NSCs, estimulando a proliferação e a conversão neuronal destas células.

Neste trabalho pretendemos esclarecer o impacto do TUDCA no proteoma mitocondrial de NSCs de ratinho em autorrenovação e/ou em diferenciação, recorrendo à proteómica de expressão diferencial por cromatografia líquida acoplada a espectrometria de massa (LC-MS). A validação da análise do perfil proteómico mitocondrial foi realizada através de *Western blot* em duas linhas de NSCs e revelou que o TUDCA diminui significativamente os níveis mitocondriais da proteína acil-coA desidrogenase de cadeia longa (LCAD), uma enzima crucial na β-oxidação de FAs de cadeia longa, em NSCs em diferenciação. Por outro lado, os níveis nucleares da proteína de ligação a elementos reguladores de esteróis (SREBP-1), um fator de transcrição crucial para biossíntese de lípidos, encontraram-se aumentados, tal como, os níveis de FAs, como o ácido palmítico e esteárico.

Curiosamente, os níveis mitocondriais da subunidade E1-α da enzima piruvato desidrogenase (PDHE1-α), a qual pertence ao metabolismo da glucose, também se encontraram significativamente aumentados pelo TUDCA. Além disso, o TUDCA promoveu a translocação da PDHE1-α para o núcleo das NSCs. Assim, o papel proliferativo deste ácido biliar poderá dever-se, em parte, ao aumento das reservas de acetil-CoA mitocondrial e/ou nuclear para garantir a progressão do ciclo celular das NSCs. Os perfis de expressão das proteínas LCAD, SREBP-1 e PDHE1-α nos primeiros estadios de diferenciação neural foram, também, avaliados, revelando escolhas metabólicas das NSCs durante o processo de diferenciação.

Os nossos resultados esclarecem o mecanismo de ação do TUDCA no destino das NSCs, demonstrando que este ácido biliar não só induz condições mitocondriais mais vantajosas, mas é também um importante regulador da plasticidade metabólica.

Palavras-chave: Ácido tauroursodesoxicólico; Células estaminais neurais; Diferenciação; Metabolismo lipídico; Mitocôndria; Proliferação.

Abstract

Neurogenesis occurs throughout life in discrete areas of the adult mammalian brain. Unfortunately, there is a lack of effective regeneration during aging or after injury. Therefore, life-long potentiation of endogenous neurogenesis represents a major issue. Curiously, proliferation and differentiation potential of neural stem cells (NSCs) were recently shown to be highly dependent on mitochondrial bioenergetics and fatty acid (FA) lipogenesis.

Furthermore, tauroursodeoxycholic acid (TUDCA), an endogenous neuroprotective bile acid, considered a regulator of energy metabolism and an inhibitor of early differentiation-associated apoptosis events in NSCs, stimulates proliferation and neuronal conversion of these cells.

We aimed to clarify the impact of TUDCA on the mitochondrial proteome in self-renewing or differentiating mouse NSCs, using liquid chromatography coupled with mass spectrometry (LC-MS) based detection of differential proteomics. Validation of mitochondrial proteomic analysis by Western blot in two different NSC lines revealed that TUDCA significantly decreases the mitochondrial levels of long-chain acyl-CoA dehydrogenase (LCAD) protein upon differentiation, an enzyme crucial for β-oxidation of long-chain FAs. Further, nuclear levels of sterol regulatory element-binding protein (SREBP-1), a major transcription factor of lipid biosynthesis, were also found significantly increased, as the levels of palmitic and stearic FAs raise up. Interestingly, mitochondrial levels of pyruvate dehydrogenase E1-α (PDHE1-α), an enzymatic subunit belonging to glucose metabolism, were also markedly enhanced by TUDCA. Of note, TUDCA promoted mitochondria-nucleus translocation of PDHE1-α. Therefore, the proliferative role of this bile acid may rely, in part, in increasing the pool of mitochondrial and/or nuclear acetyl-CoA to assure NSC cycle progression. Finally, LCAD, SREBP-1, and PDHE1-α expression profiles were also assessed during early stages of neural differentiation bringing novel insights to NSC metabolic choices throughout differentiation.

Altogether, our results unravel the metabolic impact of TUDCA in controlling NSC fate, demonstrating that this bile acid not only induces mitochondrial advantageous conditions but also metabolic plasticity.

Keywords: Differentiation; Lipid metabolism; Mitochondria; Neural stem cells; Proliferation; Tauroursodeoxycholic acid.

Table of Contents

Figure Index

Table Index

List of Abbreviations

ACC	Acetyl-CoA carboxylase
Acetyl-H3	Acetylated histone 3
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BA	Bile acid
BBB	Blood brain barrier
bFGF	Basic fibroblast growth factor
BSA	Bovine serum albumin
CNS	Central nervous system
CoA	Coenzyme A
DG	Dentate gyrus
DNA	Deoxyribonucleic acid
Drp1	Dynamin-related protein 1
EGF	Epidermal growth factor
ER	Endoplasmic reticulum
ERR α	Estrogen-related receptor α
ESC	Embryonic stem cell
ETC	Electron transport chain
FA	Fatty acid
FAO	Fatty acid oxidation
FASN	Fatty acid synthase
FGF	Fibroblast growth factor
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GC-MS	Gas chromatography-mass spectrometry
Gβ2	G-protein β2 subunit
H3	Histone 3
hnRNP A2/B1	Heterogeneous nuclear ribonucleoprotein A2/B1
Hsp70	Heat shock protein 70
iPSC	Induced pluripotent stem cells
LCAD	Long-chain acyl-CoA dehydrogenase
LC-MS	Liquid chromatography-mass spectrometry
Mfn 2	Mitofusin 2
mPTP	Mitochondrial permeability transition pore
mRNA	Messenger ribonucleic acid (RNA)

NADH	Nicotinamide adenine dinucleotide (reduced form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
ND	Neurodegenerative disease
NPC	Neural progenitor cell
NSC	Neural stem cell
NSPC	Neural stem/progenitor cell
NS-TGFP	tau-green fluorescent protein (GFP) mouse neural stem cell line
OMM	Outer mitochondrial membrane
ORO	Oil-Red-O
OXPHOS	Oxidative phosphorylation
PDC	Pyruvate dehydrogenase complex
PDH	Pyruvate dehydrogenase
PDHE1-α	Pyruvate dehydrogenase E1-α subunit
PDK	Pyruvate dehydrogenase kinase
PGC-1α	Proliferator-activated receptor gamma coactivator 1 α
PPAR	Peroxisome proliferator-activated receptor
PUFA	Polyunsaturated fatty acid
qNSC	Quiescent neural stem cell
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Room temperature
SC	Stem cell
SGZ	Subgranular zone
SREBP1	Sterol regulatory element-binding protein 1
SVZ	Subventricular zone
TCA	Tricarboxylic acid
TUDCA	Tauroursodeoxycholic acid
UDCA	Ursodeoxycholic acid
VDAC	Voltage-dependent anion channel
β-F1-ATPase	ATP synthase β subunit

1. Introduction

1.1 Neural Stem Cells

Since the second half of the XX century, neural stem cells (NSCs) in the adult mammalian brain have been playing a major role in enriching our understanding about the plastic nature of the mammalian brain (Ma *et al.*, 2009), opening gates to investigate future therapeutic approaches in the field of neuroscience. The discovery of these powerful cells fostered whole new perspectives to treat many neurodegenerative diseases (NDs) due to their identity and pertinent properties. (Filippis & Binda, 2012)

NSCs are the most primitive cells residing in the central nervous system (CNS). They have a huge relevance in sustaining the development and homeostasis of nervous tissue by giving the possibility to create a dynamic equilibrium between self-renewal and differentiation without depleting stem cell (SC) pool and to replace the most mature neural cells. This potential neuroregeneration is progressively and temporally restricted and it has been difficult to understand the temporal control of NSCs output. Under physiological conditions, *in vivo*, adult NSCs basically rest in a state of quiescence, which allow them to bear up metabolic stress and to preserve genome integrity during their lifetime. However, once activated to entering the cell cycle, NSCs must choose between its self-renewal and a mode of dividing. According to this idea, a NSC can divide symmetrically, yielding two progenitors or two NSCs, or asymmetrically, generating one NSC and one progenitor cell. Then, neural progenitor cells (NPCs) can acquire a more committed state to give rise to a specific cell type, including neurons, or glial cells, such as astrocytes or oligodendrocytes. Nevertheless, in this last scenario, they lose their capacity to self-renewal or proliferate (Figure 1.1). (Filippis & Binda, 2012; Bond *et al.*, 2015)

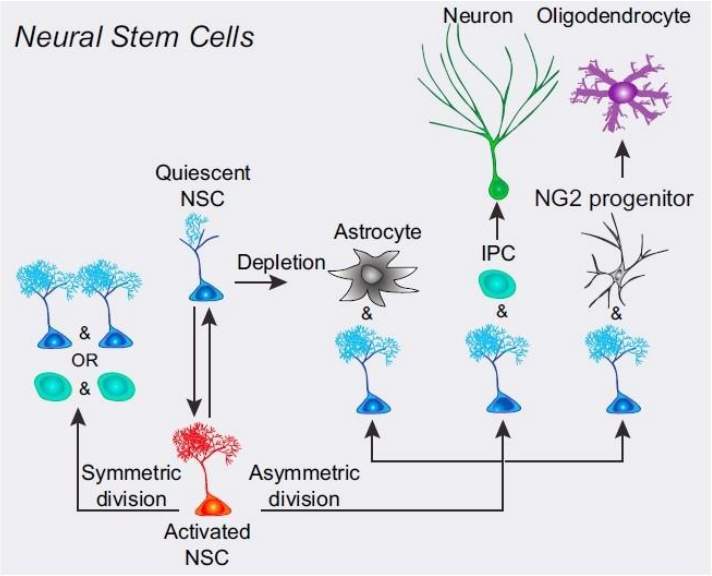


Figure 1.1 - Potential behavior of adult NSCs over cell life cycle. NSCs remain quiescent

throughout life but once activated they choose between symmetric or asymmetric division. Both modes of dividing can give rise to progenitor cells, which differentiate into a specific cell type. Intermediate progenitor cells (IPCs) are a type of neurogenic transient amplifying cells that finally become neurons. NG-2 oligodendrocyte precursor cells become oligodendrocytes. Neural progenitor cells can generate astrocytes as well. Adapted from Bond *et al*., 2015.

Therefore, adult NSCs are self-renewing and multipotent progenitors, also differentiating into a wide-range of cell types. Meanwhile, the identification of trophic and mitogenic actions of growth factors, including epidermal growth factor (EGF) (Morrison *et al*., 1987) and fibroblast growth factor (FGF) (Nurcombe *et al*., 1993) family proteins enabled to culture and maintain NSC expansion in both floating and adherent conditions. (Ma *et al*., 2009) These two *in vitro* culture systems have several controversies, in terms of conservation of the molecular and biological properties of genuine NSCs. NSCs can form neurospheres in culture (Reynolds & Weiss, 1992), neurospheres mimic physiological conditions because of their three-dimensional structure, although they are not considered a good model for efficient neuronal generation due to its highly-associated heterogeneity. In contrast, expanding NSC cultures in monolayer conditions were shown to assure almost pure NSC populations, with a negligible differentiated component and a higher neurogenic potential in comparison to neurosphere models. (Conti & Cattaneo, 2010)

NSCs can be derived *in vitro* from embryonic stem cells (ESCs), derived from the inner cell mass of blastocysts; as from induced pluripotent stem cells (iPSCs), acquired by reprogramming from somatic cell; and also, isolated from fetal tissue and adult brain samples of germinative areas. Current NSC systems are not perfect. Therefore, determining the best sources for the *in vitro* derivation of NSCs and optimizing protocols for stable and clonal proliferation are still central goals of SC research. (Conti & Cattaneo, 2010) Cell propagation sets close to homogeneity in appropriate models for studying the neurogenesis process.

1.1.1 Adult neurogenesis

Neurogenesis was only believed to occur during embryonic stages in the CNS of mammalians. (Ramon y Cajal, 1913) The very first evidence for the existence of adult NSCs in mammalian brain was provided by pioneering studies with tritiated thymidine (thymidine-H^3), a thymidine analogue that has been used as a label of proliferating cells. (Altman & Das, 1965) Other progresses were made but, for decades, the concept of adult neurogenesis has been ignored until it was rediscovered in the early 1990s. The introduction of 5-bromo-2-deoxyuridine (BrdU), other thymidine analogue which incorporates into the nuclear DNA during the S-phase of the cell cycle, demonstrated that in aged mammals, including humans, many newly generated cells were in fact neurons, and the field of adult neurogenesis took off. (Eriksson *et al*., 1998; Ma *et al*., 2009)

In most regions of the mammalian brain, the production of neurons is largely confined to the prenatal period as adult neurogenesis shows an age-dependent decline. Although it recapitulates the complete process of neuronal development, adult neurogenesis takes place in unique local niches that

support distinct NSCs behaviors, including their maintenance, self-renewal, fate specification and development. (Kuhn *et al*., 1996; Ming & Song, 2011; Gage & Temple, 2013) There are four processes critical to the overall levels of neurogenesis, cell survival, proliferation, neuronal differentiation, and migration. Moreover, quiescent neural SCs (qNSCs) can be localized in several regions across the adult brain. There is evidence to suggest the presence of neurogenesis in other regions of the adult brain. But neurogenesis is known to happen mainly in restricted zones with a distinct microenvironment. These germinative zones are evolutionarily conserved in mammals and have been identified in the subventricular zone (SVZ) lining the lateral ventricles and the subgranular zone (SGZ) within the dentate gyrus of the hippocampus (Figure 1.2 (A)). (Bond *et al*., 2015; Ernst & Frisén, 2015) Several cellular components integrate these neurogenic niches underlining, mature neurons, NSC progeny, astrocytes, endothelial cells, microglia and the blood vascular system itself (Figure 1.2 (B)). (Ma *et al*., 2009; Gage & Temple, 2013)

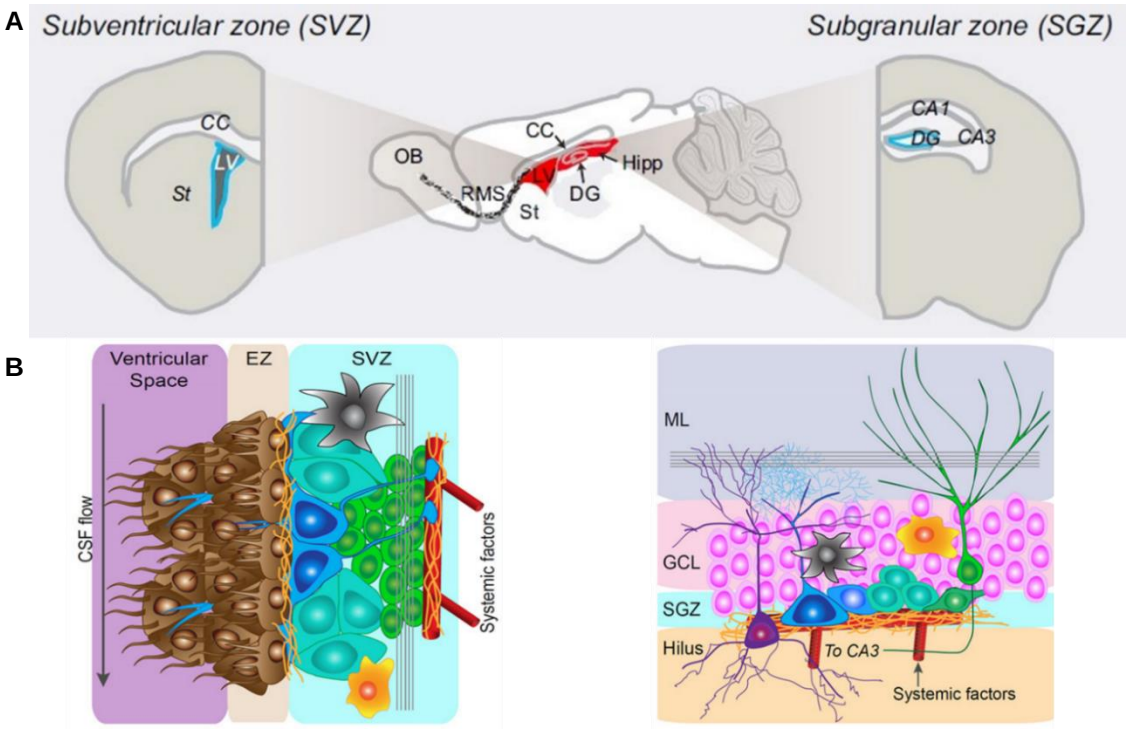


Figure 1.2 - Adult NSC niches. (A) A sagittal plane of the adult rodent brain, highlighting the two major niches where adult neurogenesis occurs, the lateral ventricle (LV) in the subventricular zone (SVZ) to the rostral migratory stream (RMS) to the olfactory bulb (OB) and the dentate gyrus (DG) in the subgranular zone (SGZ) of the hippocampal formation (Hipp). There are also signalized the brain areas of the corpus callosum (CC) and the striatum (St). (B) A schematic diagram depicting cellular and molecular components of the SVZ and SGZ niches. CSF, cerebrospinal fluid; ECM, extracellular matrix; EZ, ependymal zone; GCL, granule cell layer; ML, molecular layer. Adapted from Bond *et al*., 2015.

Adult SVZ neurogenesis comprises different developmental stages, starting with the activation of radial glia-like cells (Type B cells) in the subventricular zone which gives rise to rapidly dividing

transient amplifying cells (Type C cells) and leads to the generation of neuronal progenitor cells (Type A cells or neuroblasts). Then, these neuroblasts tangentially migrate along the rostral migratory stream (RMS) to the olfactory bulb. During this migration, these cells continue to divide, but the cell cycle is lengthened and the synaptic integration and maturation of granule cells and periglomerular neurons only occur when these cells reside in the core of olfactory bulb (OB). (Li *et al*., 2008; Ming & Song, 2011) It is important to note that the direction of neuroblast migration is the OB in most mammals, but in adult humans striatal neuroblast migration is most pronounced. Further, neuroblasts are not restricted to the lateral ventricles, but are also present beyond the neurogenic niche. (Ernst & Frisén, 2015)

In the SGZ of the hippocampus, NSCs migrate into the granule cell layer, a shorter distance when compared with the extensive migration undertaken by OB neurons. These cells give rise to mature granule neurons in the dentate gyrus. Analogous to the type B cells in the SVZ, they generate transient amplifying progenitor cells (Type IIa, IIb, and III cells) that then differentiate into immature neurons. At last, immature neurons become mature granule neurons and the sequential process of synaptic integration takes place. From the beginning of NSC differentiation to the synaptic integration of newly generated neurons takes approximately 2-4 weeks. Throughout the neurogenesis process, both in SVZ and SGZ regions, different cell stages and cell lineages can be detected by stage-specific markers, as illustrated in Figure 1.3. (Li *et al*., 2008; Ming & Song, 2011)

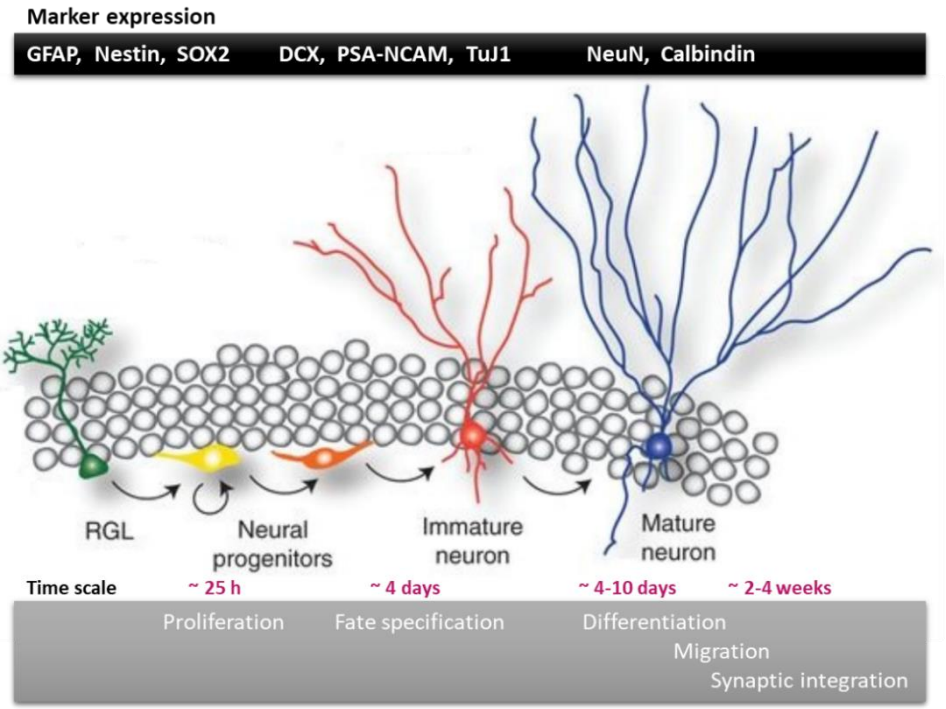


Figure 1.3 - Schematic diagram illustrating specific stages of adult hippocampal neurogenesis. The neuronal differentiation cascade, from radial glia-like cells (RGL) to mature neurons, is represented by the major stage-specific markers: GFAP, glial fibrillary acidic protein; Sox2, sex determining region Y (SRY)-box 2; DCX, doublecortin; PSA-NCAM, polysialylated form of the

neural cell adhesion molecule; TuJ1, β-tubulin III; NeuN, neuronal nuclear antigen. Adapted from Lucassen *et al*., 2010; Kang *et al*., 2016.

1.1.2 Therapeutic relevance of NSCs

The discovery of NSCs and neurogenesis in the adult mammalian brain has changed our view about the plasticity and function of the human brain bringing up new expectations for rescuing brain function after injury or NDs. (Casarosa *et al.*, 2014)

Following brain injury adjacent NSCs have the capacity to proliferate and to migrate to the local lesion. Indeed, NSCs in the SVZ and DG are stimulated after traumatic brain injury or seizures, revealing that adult neurogenesis may be relevant in self-recovery mechanisms of the brain. However, far from obtaining regenerative effects, the amount of produced neuroblasts after brain injury as well as their survival and differentiation into mature neurons is highly limited. (Casarosa *et al.*, 2014)

Aging is the primary risk factor for prevalent NDs. (Mattson & Magnus, 2006) However, irrespective of their specific etiology, NDs eventually lead to loss or functional alteration of neurons and glia in the brain or spinal cord either in acute or chronic cases. In acute cases, like ischemic stroke or spinal cord injury, different types of neurons and glial cells die within a restricted area over a short time period. Whereas in chronic cases, usually there is a progressive loss of a specific population of cells, such as motor neurons in amyotrophic lateral sclerosis (ALS) and dopamine neurons in Parkinson disease (PD), or an extensive degeneration of many types of neurons in Alzheimer disease (AD). NDs currently lack effective treatments and SC research could lead to the development of new powerful therapies. (Lindvall & Kokaia, 2010)

Over the past few years, and based on the pathology of each disease, there has been a continuous progress in developing approaches to generate the types of human-derived neurons and glial cells for cell replacement therapy. Indeed, there are three principal mechanisms by which SC-based therapies could be helpful: cell replacement, where cell transplants are given to directly replace cells that are damaged or lost; trophic support, where transplanted cells are used to promote endogenous repair of specific diseased brain areas; and, modulation of inflammation, frequently involved in the disease process and where transplanted cells are used to release anti-inflammatory agents. (Lindvall & Kokaia, 2010)

Different sources of SCs have been proposed to be used for the recovery of CNS injuries. (Lodi *et al.*, 2011) Recently, the discovery iPSCs, by Yamanaka in 2006, opened new possibilities for autologous transplantation. (Takahashi & Yamanaka, 2006) Human iPSC-derived neural precursor cells can be transplanted into a rat model of PD and ameliorate the motor-sensory behavior by increasing the number of midbrain dopamine neurons. (Wernig *et al*., 2008) Although, the major drawback of these genetically manipulated cells is the high risk of cancer formation, mainly due to possible uncontrolled integration of viral vectors and non-specific recombination events. In fact, due to their high proliferative potential, iPSCs were shown to induce tumor formation and impair neural

outgrowth and their use in the clinical therapy must be very cautious. (Lodi *et al.*, 2011) ESCs have also indefinite self-renewal capabilities as well as the ability to differentiate into all cell types derived from the three embryonic germ layers. However, there are major challenges for the safety of using them in any kind of transplantation therapy, also involving the risk for unregulated cell growth. Therefore, the most suitable candidates for SC therapies in the context of neurological disorders are adult SCs, particularly NSCs. NSCs can potentially be an autologous SC source for transplantation with minimal risk of tumor formation. (Yu *et al*., 2008)

The therapeutic use of NSCs is being actively investigated, once they show considerable promise for regenerative repair of CNS and there are present in a considerable number of ongoing clinical trials. (Trounson & McDonald, 2015) Plus, for a realistic exploitation of NSCs in cell therapies, they must have a reproducible safe behavior as well as long-term survival and ability to differentiate. The underlying problem in NSC use is the short time window following neural induction during which the cells can be directed toward specific neuronal lineages. Ultimately, increasing the number of cells capable of differentiating might prove useful for *in vitro* or *in vivo* expansion of NSCs. (Yu *et al*., 2008)

Collectively, a more comprehensive understanding of the regulatory mechanisms involved in self-renewal, proliferation and differentiation of NSCs will certainly help to ensure well-established methods to manipulate these cells *in vitro* and *in vivo* and improve the successful use of NSCs in clinics.

1.2 Mitochondrial control of NSC activity

The relevance of mitochondrial function and energy metabolism in determinating NSC fate is being currently widely discussed. In fact, the whole integration of different mitochondrial events is yet to be revealed as the metabolic force driving NSC identity. In this regard, it might be worth increasing the knowledge on the influence of cell metabolism on NSC behavior during CNS diseases. (Wanet *et al*., 2015; Xavier *et al*., 2015; Ottoboni *et al*., 2017)

Mitochondria are classically known to be the powerhouse of eukaryotic cells. Despite their major function as cell energy producer, mitochondria regulate intracellular calcium and redox homeostasis, playing important roles in biosynthetic processes, including lipid, cholesterol, nucleotide, heme, and steroid synthesis, as in amino acid metabolism and ion homeostasis. This dynamic organelle has even gained more attention over the years by being implicated in signaling pathways of cell death and survival. (Hock & Kralli, 2009; Murphy, 2009)

The CNS has an extraordinary high metabolic rate. It consumes about 20% of oxygen inspired at rest, whereas accounting for only 2% of the body weight. CNS depends upon glucose as its main source of energy and most of neuronal adenosine triphosphate (ATP) is generated through oxidative phosphorylation (OXPHOS) in mitochondria. Neurons, in turn, are highly differentiated cells, consuming much of this energy to sustain cell membrane ionic gradients and neurotransmission.

Notably, they display a complex morphology and are highly compartmentalized in a complex network, where mitochondrial trafficking matches the energy demand throughout the long neuronal segments. (Kann & Kovács, 2007)

Cellular specialization relies on specific mitochondrial specialization and maturation. Indeed, several neural development processes rely on mitochondrial regulation: self-renewal and differentiation of NSCs, as neurogenesis it-self within axonal and dendritic growth, synaptic formation, and reorganization. Moreover, mitochondrial dynamics and bioenergetics are closely related to NSC fate and behavior also, intriguingly, providing signal to the nucleus to modulate differentiation and development. (Kann & Kovács, 2007; Wanet *et al.*, 2015; Xavier *et al*., 2015) In this regard, mitochondrial dysfunction can be an underlying problem, namely in the eventual depletion of the SC pool and impaired neurogenesis, as observed during aging and degenerative or metabolic diseases. (Wallace, 2005; Khacho *et al*., 2017) In addition, mitochondria are responsible for long-term survival, differentiation and synaptic integration of newborn neural cells. (Xavier *et al*., 2015) Therefore, it is likely that mitochondria and its regulatory network will have major implications toward a more efficient use of neural replacement therapies.

1.2.1 General aspects of mitochondrial structure, function, and biogenesis

Mitochondria have two membranes, an intermembrane space, where there are important enzymes, and an internal matrix. The outer mitochondrial membrane (OMM) is permeable to ions and small molecules. The inner mitochondrial membrane (IMM), in turn, is almost impermeable and forms a tight barrier between the mitochondrial matrix and the cytoplasm, being composed by different ion channels and transporters, such as the Ca^{2+} uniporter, K^{+}_{ATP} channels, and Na^{+}/Ca^{2+} exchanger, and mitochondrial enzyme systems, such as the molecular machinery for energy production, the electron transport chain (ETC). The ETC consists of five protein complexes. Three of the complexes (I, III, and IV) pump protons (H^{+}) across the inner membrane to establish a H^{+} gradient with a potential difference of 150–180 mV (negative with respect to cytosol). This potential difference sets the driving force for protons (along with ΔpH) that actuate F1F0-ATP synthase (complex V) to generate ATP and for cytosolic Ca^{2+} ions to accumulate in the matrix via the mitochondrial Ca^{2+} uniporter. Nicotinamide adenine dinucleotides (NADH) and flavin adenine dinucleotides ($FADH_2$) serve in energy transfer to the ETC, via the complexes I (NADH-ubiquinone oxidoreductase), II (succinate dehydrogenase), III (ubiquinol cytochrome c oxidoreductase) and IV (cytochrome c oxidase), electrons are transferred from NADH and $FADH_2$ to O_2. During electron transport, the free radical superoxide ($O_2^{-\cdot}$) is also generated. (Kann & Kovács, 2007; Mattson *et al*., 2008)

Importantly, voltage-dependent anion channel (VDAC) is a key protein complex involved in ATP rationing, Ca^{2+} homeostasis, protection against oxidative stress and regulation of apoptosis. VDAC is located at OMM and helps to form the mitochondrial permeability transition pore (mPTP) where mitochondrial metabolites and ions enter and exit. During apoptosis, the opening of mPTP

results in release of cytochrome *c* into the cytoplasm. (Shoshan-Barmatz *et al.*, 2010)

Recently, it has become clear that Bcl-2 family members are implicated in mitochondria-mediated death during neurogenesis and neuronal differentiation processes. Direct interactions between mitochondria and the endoplasmic reticulum (ER) have also a role to play. For example, during apoptosis, cytochrome *c* released from mitochondria can trigger the release of Ca^{2+} from the ER. (Mattson *et al*., 2008)

Undouthbly, mitochondrial function is connected to a wide range of cell signaling pathways through changes in redox and phosphate pairs (NAD/NADH; ATP/adenosine diphosphate (ADP) vs. adenosine monophosphate (AMP)), reactive oxygen species (ROS) production and critical metabolite concentrations, such as acetyl-CoA. (Cagin & Enriquez, 2015)

Interestingly, mitochondrial biogenesis is regulated to respond to energetic and metabolic demands of the cell and is influenced by environmental stress, such as exercise and caloric restriction, cell division, renewal, and differentiation. In fact, throughout these processes, mitochondria variations in number and size and mass occur. Thus, mitochondrial dynamic is a complex process that requires the synthesis, import, and incorporation of new proteins and lipids to the existing mitochondrial reticulum. Intriguingly, mitochondria have their own genome and can autoreplicate, which is related to their bacterial origin. The mitochondrial proteome comprises ∼1100 to 1500 proteins and the vast majority of them are encoded by nuclear genes. However, the mitochondrial genome encodes 13 essential proteins that are components of OXPHOS. Indeed, mitochondrial biogenesis is regulated by a transcriptional network in between the coordination of the two genomes. (Hock & Kralli, 2009; Jornayvaz & Shulman, 2010)

1.2.2 Mitochondria nucleus cross-talk

Mitochondrial function and activity are under strong nuclear control and several signaling pathways coordinate the communication between mitochondria and the nucleus. The so called ‘anterograde regulation’ is based mainly on the expression of several nuclear-encoded mitochondrial proteins, transcription factors, and co-regulators. (Quirós *et al*., 2016) The expression of nuclear-encoded mitochondrial proteins is principally mediated by nuclear transcription factors, such as nuclear respiratory factor 1 (NRF-1), which controls the expression of the vast majority of nuclear-encoded subunits that are involved in OXPHOS. In fact, Dhar *et al*. demonstrated that NRF-1 regulates all ten nuclear-encoded subunits of cytochrome *c* oxidase in neurons. (Dhar *et al*., 2008) Moreover, nuclear receptor factors, which include peroxisome proliferator-activated receptors (PPARs), are nuclear receptors that sense lipids and control lipid homeostasis. PPARα and PPARδ are primary regulators of lipid oxidation, whereas PPARγ promotes lipid synthesis and storage. The estrogen-related receptors (ERRs) - ERRα, ERRβ, and ERRγ, in turn, are associated with the expression of nuclear-encoded mitochondrial proteins that are involved in the tricarboxylic acid (TCA) cycle, also in OXPHOS and fatty acid oxidation (FAO). Importantly, proliferator-activated receptor

gamma coactivators 1 α and β (PGC-1α/β) are master regulators of mitochondrial biogenesis known to activate the NRF, ERR and PPAR factors, which may lead to induced mitochondrial biogenesis by increasing the expression of mitochondrial proteins encoded by nuclear DNA, such as the mitochondrial transcription factor A (TFAM). Upregulation of TFAM drives an increase in replication and expression of mtDNA. (Picca & Lezza, 2015; Quirós *et al*., 2016)

Conversely, mitochondria can create a 'retrograde response' signaling the nucleus to alter the expression of nuclear genes in order to modify cell function or/and metabolism. Retrograde signals are generally classified as energetic stress responses. This retrograde response has been classically linked to mammalian target of rapamycin (mTOR) and AMP-activated protein kinase (AMPK). A decrease in mitochondrial ATP synthesis stimulates AMPK which, in turn, promotes the activation of PGC-1α. Additionally, AMPK triggers the mitochondrial quality control system, interfering with mitochondrial dynamics to induce mitophagy, leading to either an elimination of mitochondrial content or damaged mitochondria. (Ashrafi & Schwarz, 2013; Quirós *et al*., 2016) Similarly, stress reduced mTOR activity facilitates mitochondrial retrograde signaling, while its activation inhibits retrograde response. (Quirós *et al*., 2016) Furthermore, this organelle has also been implicated in the regulation of cell cycle progression in NSCs through this kind of mechanism. (Owusu-Ansah *et al*., 2008; Xavier *et al*., 2014)

Apart from complex signaling pathways responsible for mediating the communication between nucleus and mitochondria a more direct way of interorganellar coordination has emerged, the redistribution of nuclear or mitochondrial proteins between these two compartments. (Lionaki *et al.*, 2016) Of the dual-localized proteins, a good number of predominantly nuclear proteins are now known to be localized to and function at the mitochondria. Prominent among these are those whose activities are related to cell death and survival, as well as senescence and aging. For example, the tumor suppressor p53, ERR *α* and *β*, Sirtuin 1 (Sirt1) and PGC-1*α*, among others with or without mitochondrial defined activities. (Tang, 2015; Lionaki *et al.*, 2016)

On the other hand, several recent findings have indicated that a small number of classically mitochondrial-localized proteins could be also found in the nucleus, performing non-transcriptional activities. (Tang, 2015; Lionaki *et al.*, 2016) Surprisingly, one of the largest multiprotein complexes known, the pyruvate dehydrogenase complex (PDC), is one of them. (Sutendra *et al*., 2014) In mitochondria matrix, the PDC catalyzes multiple enzymatic steps to generate acetyl-CoA from glycolysis-derived pyruvate that, then, fuels the TCA cycle. The mammalian PDC is about 8-10 MDa in size and has 3 enzyme activities (E1, E2, and E3), each comprising of multiple polypeptide subunits, which makes PDC even larger than the largest complex in the mitochondrial ETC. (Tang, 2015) Interestingly, nuclear PDC is functional in generating acetyl-CoA from pyruvate (Figure 1.4). (Sutendra *et al*., 2014) Another interesting example of bi-organellar protein distribution is fumarase, a TCA cycle enzyme, now known to be also important for DNA repair in the nucleus. There are other examples of proteins whose nuclear function and mechanism driving nuclear localization remains elusive. (Tang, 2015; Lionaki *et al.*, 2016)

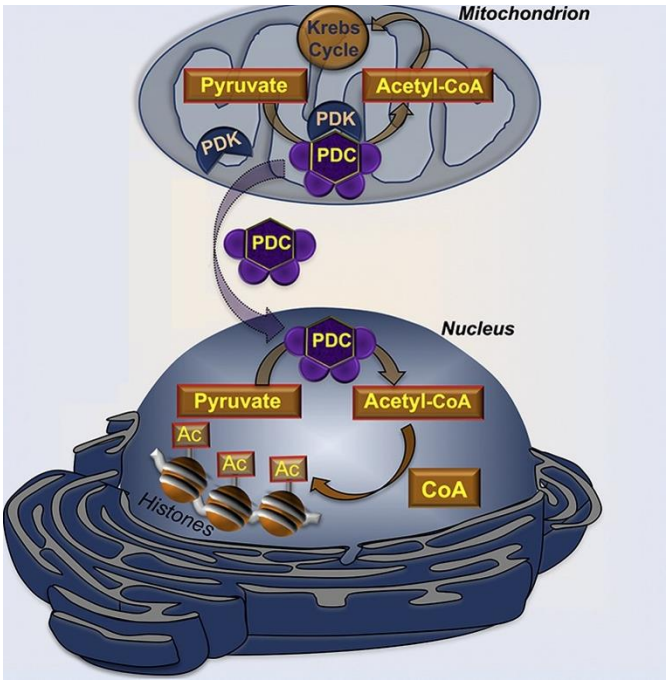


Figure 1.4 – Dynamic translocation of mitochondrial PDC to the nucleus. The PDC plays a central role in cellular metabolism by catalyzing the irreversible conversion of pyruvate into acetyl-CoA in mitochondria. The activity of PDC is tightly controlled via reversible inactivating phosphorylation due to the activity of specific pyruvate dehydrogenase kinases (PDKs). Mitochondrial PDC translocates to the nucleus of mammalian cells, where is functional, providing a novel pathway for nuclear acetyl-CoA synthesis required for histone acetylation and epigenetic regulation. Adapted from Sutendra *et al*., 2014.

Ultimately, to a great extent, the integration of anterograde (from nucleus to mitochondria) and retrograde (from mitochondria to nucleus) signals and this dual localization of proteins is a level of interorganellar communication and coordination that allows for direct and finely tuned responses of both organelles, leading to an enhanced and precise defense of cell homeostasis. (Lionaki *et al.*, 2016; Quirós *et al*., 2016)

1.2.3 Mitochondrial dynamics

The ultrastructure, morphology and intracellular distribution of mitochondria undergo significant changes during cellular life. Somatic cells exhibit mature elongated mitochondria, with numerous cristae and an electron-dense matrix. (Varum *et al*., 2011) However, the first observations of mitochondria in mouse and human ESCs (mESCs and hESCs) using transmission electron microscopy surprisingly showed characteristics that are not related to the well-known ultrastructure of mitochondria. Indeed, in these cases, mitochondria morphology appeared globular displaying a perinuclear localization and containing poorly developed cristae, along with an electron-lucid matrix. (Baharvand & Matthaei, 2003; Prowse *et al*., 2012; Wanet *et al*., 2015)

Mitochondria are constantly undergoing cycles of fission and fusion, therefore the balance of

these two opposing processes dictates the connectivity and the overall length of mitochondria. Mitochondrial elongation requires the coordinated process of outer and inner membrane fusion. Mitochondrial fusion is mainly mediated by three dynamin-related large GTPases. Mitofusin 1 (Mfn1) and 2 (Mfn2) mediate the fusion of the OMM, and the optic atrophy 1 (Opa1) protein and Mfn1 mediate the fusion of the IMM. The fission process, on the other hand, involves the division of mitochondria and is regulated by the cytosolic dynamin-related protein 1 (Drp1). (Khacho & Slack, 2015)

Notably, changes in mitochondrial dynamics regulate NSC fate decisions by interfering in physiological levels of ROS, which in turn, suppressed self-renewal and promoted differentiation through transcriptional programming. Recently, the view about the classical mitochondrial dynamics regulation has been modified. Mitochondrial fragmentation has been demonstrated to be required for the transient passage of NSCs to committed progenitors. (Figure 1.5) (Khacho *et al*., 2016) Although, it is generally perceived as a sign of mitochondrial dysfunction toward a chronical imbalance that is observed during aging and many NDs. (Khacho & Slack, 2015)

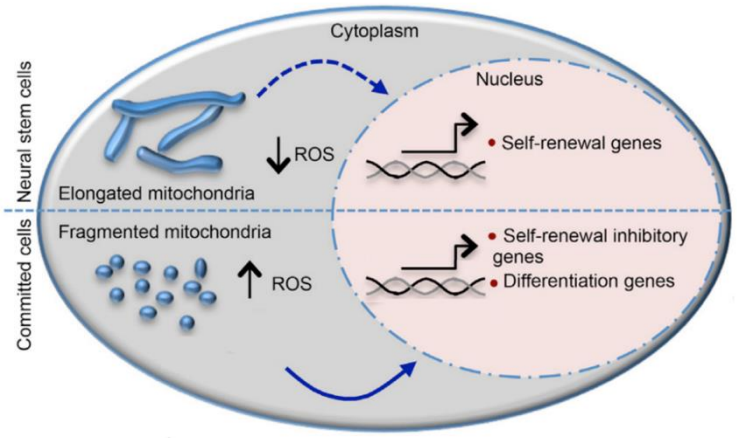


Figure 1.5 - Schematic diagram of mitochondrial dynamic changes occurring as NSCs differentiate. In NSCs, elongated mitochondria maintain low ROS levels and promote self-renewal, while a more fragmented state of mitochondria is normally associated with increase of ROS levels, inhibition of self-renewal and induction of cell differentiation. Adapted from Khacho *et al*., 2016.

Fission and fusion events do not only dictate the structural morphology of mitochondria but also regulate cell metabolism, playing a major role in NSC differentiation and survival.

1.3 Metabolic regulation of NSC fate

Increasing evidence suggests that metabolic plasticity is essential in the transition between stemness maintenance and lineage specification. Indeed, adult NSCs may rely on different metabolic pathways (Figure 1.6) to keep up with cell-specific bioenergetic demands. Energy generation is always fundamental to cell homeostasis, although SCs must fine-tune the balance between catabolism to

generate ATP, and anabolism to create biomass. (Folmes *et al*., 2013; Knobloch & Jessberger, 2017)

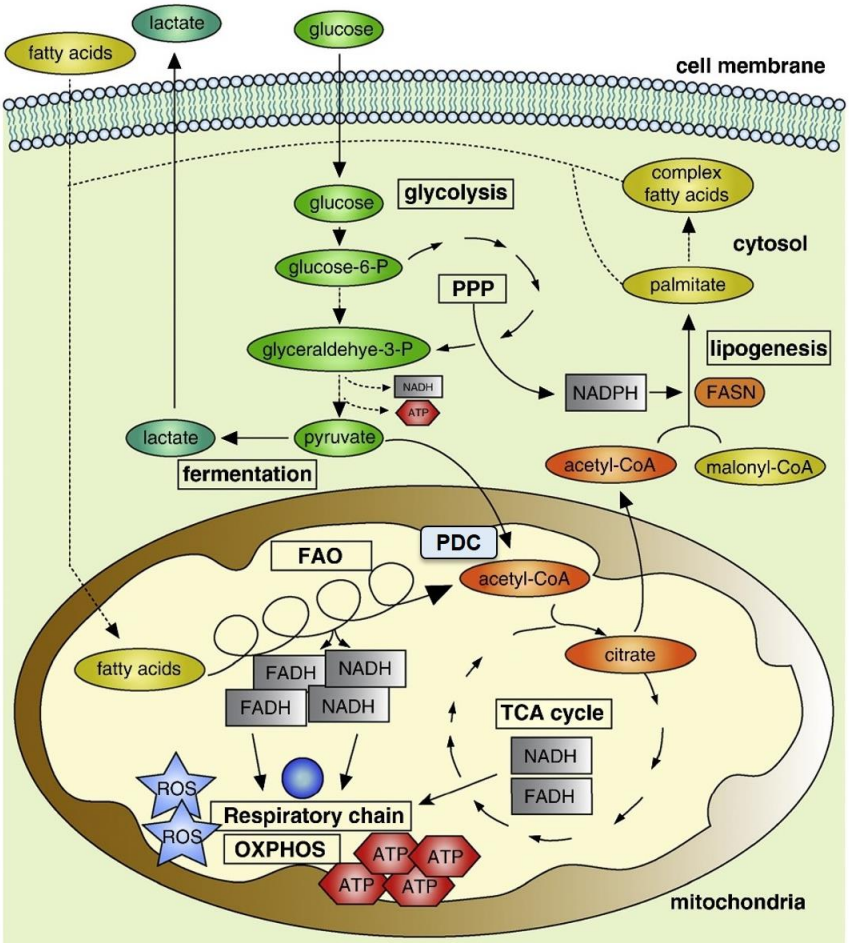


Figure 1.6 - Major cellular metabolic pathways. Glucose is taken up and metabolized through glycolysis. The final product, pyruvate, can be either fermented into lactate, which is secreted by the cell or can be shuttled into the mitochondria and converted into acetyl-CoA by PDC. This acetyl-CoA is subsequently used in the TCA cycle to generate NADH and reduced FADH, which are necessary for the OXPHOS process. Overall, this results in ATP generation. As a side product, ROS can also be produced. NADH and FADH are also generated in large amounts by FAO. Resulting acetyl-CoA levels enter into the TCA cycle for further energy production, and as a carbon source, or even be exported from mitochondria via citrate for other use, like lipid biosynthesis. Fatty acid synthase (FASN) is involved in this process, yielding palmitate, which can be used to generate more complex fatty acids. The reduced nicotinamide adenine dinucleotide phosphate (NADPH) required for lipid synthesis can be generated during the pentose phosphate pathway (PPP), a metabolic pathway parallel to glycolysis. Adapted from Knobloch & Jessberger, 2017.

NSCs reside in hypoxic niches where low oxygen tensions contribute to the maintenance of an undifferentiated state, influencing cell fate and proliferation. (Ito & Suda, 2014) Numerous studies show that mouse and human ESCs and iPSCs rely principally on glycolysis under aerobic conditions. (Folmes *et al*., 2011; Varum *et al*., 2011; Panopoulos *et al*., 2012) Glycolysis generates reducing equivalents through the pentose phosphate cycle and, by attenuating mitochondrial activity, directly diminishes the generation of ROS, thus, favoring NSC self-renewal and long-term maintenance.

(Wanet *et al*., 2015)

Metabolic changes between SCs and progeny suggest that mitochondrial mass and activity, as well as, OXPHOS increase with lineage progression whereas elevated anaerobic glycolytic activity is rather considered a metabolic hallmark of cell stemness. (Wanet *et al*., 2015) Although, it has been demonstrated, in NSCs, that inhibition of glycolytic pathways, even when oxidizable substrate was provided, greatly impaired their survival. (Candelario *et al*., 2013) An interesting study in Drosophila during metamorphosis suggests a direct regulation of NSC differentiation via OXPHOS. (Homem *et al*., 2014) Also, differentiation of hNSCs into motor neurons stimulated mitochondrial biogenesis and decreased glycolytic flux. (O'Brien *et al*., 2015) Being now well-stabilized that the metabolic transition profile of NSCs to neurons relies on a switch from glycolysis to mitochondrial OXPHOS to meet the robust energy demands associated with differentiation. (Hu *et al*., 2016)

The identity of stage-specific metabolic programs and their impact on adult neurogenesis are largely unknown, we are now starting to understand mitochondria and its stage-specific molecular program adaptation of metabolic circuits under this scenario. The adult hippocampal neurogenic lineage is critically dependent on the mitochondrial ETC and OXPHOS machinery at the stage of the fast proliferating neural stem/progenitor cell (NSPC). (Beckervordersandforth *et al*, 2016)

As mentioned above, ROS are naturally produced by OXPHOS in mitochondria under physiological conditions. (Murphy, 2009) In a mouse model of adult hippocampal neurogenesis, a peak in mitochondria number and ROS levels occurred immediately after inducing differentiation, in a highly proliferative, intermediate progenitor state, but not in undifferentiated NSCs or postmitotic neurons. (Walton *et al*., 2012) Notably, ROS-mediated process triggers a dual program to suppress self-renewal and promote differentiation via retrograde signaling in NSCs. ROS force SCs out of hypoxia-dependent quiescence into a more proliferative state, promoting cellular commitment and differentiation. (Ito & Suda, 2014; Khacho *et al*., 2016)

Under the road of cellular metabolic pathways, lipid metabolism has also been largely neglected for the role it may play in neurogenesis process. Lipids emerge in NSC life as building blocks of membranes, an alternative energy source and as signaling entities. (Knobloch, 2016) The current knowledge of lipid metabolism in NSC regulation and neurogenesis will be discussed ahead.

1.3.1 The emerging role of lipid metabolism in NSC behavior

Lipid metabolism plays a crucial role in tissue physiology and cell signaling, being important for the CNS, as lipids make up roughly 50% of brain dry weight. In fact, brain is the organ with the second highest lipid content next to adipose tissue. (Mitchell & Hatch, 2011) On the other hand, altered lipid metabolism is believed to contribute to CNS injury and is linked to many NDs. Lipids are classified into eight categories (fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids, prenol lipids, saccharolipids, and polyketides) comprising a large number of chemically distinct molecules that arise from combinations of fatty acids (FAs) with different backbone structures. These

FAs can be used as raw materials to build cell membranes, be metabolized into bioactive agents or be degraded. (Adibhatla & Hatcher, 2008) FAs are present in the bloodstream of adult mammals, commonly bound to serum albumin. For a long period of time, it was thought that FAs do not penetrate the blood brain barrier (BBB); however, there is a slow speed passage of FAs across the BBB. Although FA import mechanism into the BBB is still uncertain (Hamilton & Brunaldi 2007; Mitchell & Hatch, 2011), during brain development, FAs are necessarily taken up because they are critically involved in neurodevelopment, neurotransmission and repair processes. (Schönfeld & Reiser, 2013)

FA import into cells has also been a source of constant debate. FAs can enter a cell through protein-mediated mechanisms involving "FAs transporters" or, much like other hydrophobic molecules, can cross lipid bilayers by passive diffusion using a 'flip-flop' mechanism independent of proteins. (Mitchell & Hatch, 2011) After entry into the cell, FAs are activated to acyl-CoA esters by acyl-CoA synthetases and can be targeted to esterification to be stored as triglycerides or be an alternative source of energy when submitted to a mitochondrial degradation process called β-oxidation. Mitochondrial β-oxidation, in turn, can be conceptually divided into two major phases: the process of getting acyl groups into the mitochondria for oxidation and the intramitochondrial chain shortening. Indeed, medium- and short-chain FAs can cross the mitochondrial membrane directly and be oxidized as their CoA (coenzyme A) esters. In contrast, long-chain FAs are imported into the mitochondria in a carnitine palmitoyl transferase (CPT)-1 dependent manner, through a "carnitine shuttle". Once inside mitochondria, FAs are then oxidized to acetyl-CoA units. Chain length is reduced by two carbons in every cycle of β-oxidation and the acetyl-CoA produced from FAO enters the TCA cycle. At the same time, there is production of reducing equivalents ($FADH_2$ and NADH), which are utilized in the ETC contributing toward the production of ATP. Subsequent rounds of β-oxidation generate successively shorter fatty acids. The first step is catalysed by acyl-CoA dehydrogenase enzymes, like long-chain acyl-CoA dehydrogenase (LCAD) that have preference for acyl-CoA substrates of long-chain length. (Bartlett & Eaton, 2004; Kompare & Rizzo, 2008)

The relation between FAO and SC identity was initially suggested by the observation that, in mice, disruption of promyelocytic leukemia gene (PML) (which regulates FAO), or direct inhibition of FAO, decreases the hematopoietic SC pool. (Ito *et al*., 2012) Consistently, congenital defects in mitochondrial FAO in NSCs promote symmetric differentiation division at the expense of NSC self-renewal in development of neocortical mouse brain. (Xie *et al*., 2016) It seems that this pathway is specifically up-regulated in qNSCs. Notice that, upon its activation, NSCs increase translational capacity, followed by cell-cycle entry with G1 to S transition. Furthermore, new data from single-cell transcriptomes of adult hippocampal qNSCs and their immediate progeny revealed that oxidative metabolism through FAO in qNSC may represent an alternative energy fuel to glucose. During the transition from quiescent to active NSCs, glycolysis and FAO tend to gradually decrease while there is an increased dependence on glucose to supply OXPHOS for energy generation. (Shin *et al*., 2015;

Fidaleo *et al*., 2017)

FAs can also be produced by the cell itself. In fact, FAs have been shown to be produced endogenously in adult NSCs; a novel mechanism governing adult neurogenesis has been identified, where lipogenesis determines the proliferative activity of NSPCs. Lipogenesis is dependent upon the rate-limiting enzyme for FA synthesis, fatty acid synthase (FASN), as well as on the availability of acetyl-CoA and NADPH and, consequently, on acetyl-CoA carboxylase (ACC) for the synthesis of malonyl-CoA (Figure 1.6). (Folmes *et al*., 2013) Importantly, sterol regulatory element binding protein-1c (SREBP-1c) exerts a positive transcriptional regulation on FASN and ACC expression, the sterol regulatory elements binding protein (SREBP), a family of transcriptional activators plays a crucial role in both FA and cholesterol homeostasis in the brain. (Kim *et al*., 2007) Curiously, it has been noticed FASN is preferably distributed in major areas of neurogenesis (SVZ and DG) within the adult murine brain. Expression is high in proliferating NSPCs and reduced in differentiated progeny. Accordingly, inhibition or deletion of FASN reduces proliferation of NSCs. (Knobloch *et al*., 2012) FASN expression in support of anabolic lipogenesis enables the production of lipid membranes required to sustain high SC proliferation. (Folmes *et al*., 2013) This mechanism is very similar to that of cancer cells, which produce the majority of their lipids *de novo*. (Menendez & Lupu, 2007)

Indeed, lipids are synthesized by *de novo* lipogenesis with the exceptions of two essential FAs, α-linolenic acid (ALA, an omega-3 FA) and linoleic acid (LA, an omega-6 FA), which are polyunsaturated fatty acids (PUFAs) and must be obtained via nutrition. Long-term culture of neurons *in vitro*, in turn, requires serum-free neurobasal medium with B-27 supplement, which includes lipids such as ALA and LA. In addition, many studies have demonstrated a beneficial role of FA precursors for neurogenesis both during development and in the adult. These are examples of bioactive lipid signaling playing a key role in neurogenesis. Bioactive lipid signaling, for instance, has recently emerged, as a lipid second messenger to regulate the energy and redox balance of differentiating NSCs. (Bieberich, 2012; Knobloch, 2016) Furthermore, recently, the receptor 1 for lysophosphatidic acid (LPA), a phospholipid, has been suggested as a novel NSC marker. LPA plays signaling functions, is important for brain development and neurogenesis, and shows an interesting expression pattern in the adult DG. (Walker *et al*., 2016) This example illustrates an emerging and complex topic bringing also the question if lipids can be novel NSC markers. (Knobloch, 2016)

Not less important, mechanistic studies have recently demonstrated that lipid accumulation perturbs the neurogenic niche microenvironment and restrains neurogenesis in diseased brains. For example, a FA-mediated mechanism suppressed NSC activity, showing potential relevance in cases of AD risk and cognitive decline in obesity and type 2 diabetes. (Hamilton *et al*., 2015; Fidaleo *et al*., 2017)

Cell metabolism has undoubtedly a central role in determining NSC physiology and behavior on multiple levels either by changing energy state, fuel source, biomass production, or epigenetics. (Knobloch & Jessberger, 2017) Figure 1.7 attempts to summarize the current knowledge on the

metabolic switches ruling NSC transformation into immature neurons.

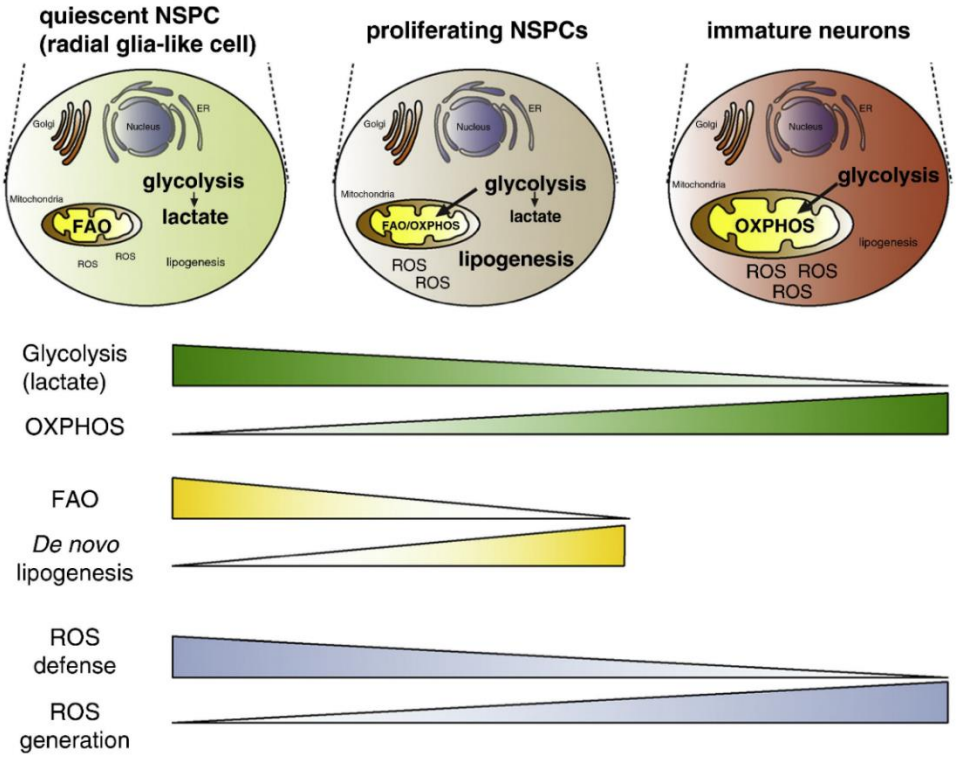


Figure 1.7 - Schematic representation of the major changes in metabolic pathways from qNSPCs to their neuronal progeny. Fateful metabolic shifts, occurring in quiescent and proliferating NSPCs as well as in immature neurons, control NSC identity through glycolysis *versus* OXPHOS, FAO *versus* lipogenesis, and ROS defense *versus* generation. Adapted from Knobloch & Jessberger, 2017.

Specific modulation of metabolic pathways might be awaiting future discovery, with the potential to improve adult neurogenesis and revolutionize regenerative medicine.

1.4 The potential of tauroursodeoxycholic acid and its unexpected effects

1.4.1 Bile acids and cell function

Bile acids (BAs), the major constituents of bile, are mainly produced in the liver. Bile acid synthesis is the primary pathway for cholesterol catabolism. Thus, BAs are end products of cholesterol, water-soluble as result of their amphipathic structure, helping to balance cholesterol levels in the body. They are usually conjugated with glycine or taurine groups and, then, secreted into the small intestine, where they assume an important role in the solubilization of lipids. They act as detergents forming mixed micelles with dietary lipids, thereby promoting their uptake. (Russell & Setchells, 1992) Minor changes in the chemical structure of bile acids determine their mechanism of action either by increasing or decreasing their hydrophobicity. Hydrophobic bile acids can not only destabilize biological membranes but they can also activate cell death pathways, while hydrophilic bile acids may be cytoprotective triggering survival pathways and inhibiting induced cellular toxicity.

(Amaral *et al*., 2009)

Importantly, BAs are increasingly being comprehended not just as lipid solubilizers and simple regulators of BA homeostasis but as complex metabolic integrators and signaling factors. BAs are ligands for G-protein-coupled receptors (GPCRs), such as TGR5, as well as for nuclear hormone receptors, such as farnesoid X receptor-α (FXR-α), also known as the 'nuclear bile-acid receptor', because BAs are the best-characterized ligands. Through activation of signaling pathways, BAs have been shown to regulate triglyceride, cholesterol, energy and glucose homeostasis, thus becoming attractive therapies for metabolic disorders, such as obesity and type 2 diabetes, as well as other associated chronic diseases, including non-alcoholic steatohepatitis. (Thomas *et al*., 2008)

Ursodeoxycholic acid (UDCA) is an endogenous hydrophilic bile acid US Food and Drug Administration (FDA)–approved for the treatment of certain cholestatic liver diseases. Curiously, UDCA is one of the major components of bear bile and has been used in Chinese medicine for centuries to treat numerous health problems. (Vang *et al*., 2014) In humans, it is also produced endogenously but at very low concentrations. (Bentayeb *et al*., 2008) It is widely known to be a cytoprotective agent that strongly detain programmed cell death through modulation of classical mitochondrial pathways, also preventing unfolded protein response dysfunction and ameliorating ER stress. (Amaral *et al.*, 2009; Vang *et al.*, 2014) Accordingly, UDCA inhibits several typical apoptotic events by stabilizing the mitochondrial membrane. (Rodrigues *et al*., 1998a; Rodrigues *et al*., 1998b; Rodrigues *et al*., 1999) Moreover, 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, proliferation and cell metabolism, in primary rat hepatocytes. (Castro *et al*., 2005) Tauroursodeoxycholic acid (TUDCA) is the taurine-conjugated form of UDCA. Importantly, after conjugation with taurine, UDCA administrated in high doses can be delivered to other tissues, including the brain. Thus, TUDCA is orally bioavailable and able to penetrate the CNS through systemic circulation and, by crossing the BBB. (Keene *et al*., 2002) This is possible due to the small size and relative hydrophobicity of TUDCA, and probably because of the existence of transporters for the taurine-conjugated molecule in the brain. (Pow *et al*., 2002) Gene expression microarray analysis demonstrated that TUDCA specifically modulates transcripts for proteins with kinase activity, diverse transcription factors and several enzymes involved in FA metabolism in primary rat hepatocytes. (Castro *et al*., 2005) Importantly, TUDCA has potential therapeutic effects on a wide variety of non-liver diseases (Vang *et al.*, 2014), which will be described ahead.

1.4.2 TUDCA affords neuroprotection

Neurobiological disorders are caused by many factors directly related to mitochondrial dysfunction, such as oxidative stress, misfolded proteins, impairment of ECT complexes and Ca^{2+} imbalance. (Bredesen *et al*., 2006) Over the past few years, the protective role of TUDCA has been demonstrated in a wide range of models of neurological disorders, including Alzheimer's (Dionísio *et*

al., 2015), Parkinson's (Castro-Caldas *et al*., 2012) and Huntington's (Keene *et al*., 2002) diseases and ischemic (Rodrigues *et al*., 2002) and hemorrhagic (Rodrigues *et al*., 2003) stroke. More recently, a study conducted with TUDCA treatment demonstrated beneficial effects on amyotrophic lateral sclerosis patients. (Elia *et al*., 2016) In addition, TUDCA has been implicated in favoring the resolution of the inflammatory process in the CNS. (Romero-Ramírez *et al*., 2017)

In summary, in the brain, TUDCA acts mainly as a mitochondrial stabilizer, inhibits apoptosis induced mechanisms and upregulates cell survival pathways. Thus, exhibiting anti-inflammatory effects and attenuating neuronal loss in NDs. (Gronbeck *et al*., 2016)

This bile acid is definitely a strong candidate to treat several NDs, and it would be interesting to explore its potential in other experimental models, such as aging and other impaired neurogenesis conditions.

1.4.3 Regenerative potential of TUDCA

Given the established beneficial role of TUDCA in neuropathological context, the effects of this bile acid on neurogenesis would open a new window of opportunities for SC-based therapies.

Notably, TUDCA is capable to trigger hepatic differentiation of mesenchymal SCs *via* BA receptors, FXR and TGR5, as well as other signaling pathways known to control SC development. This may represent a novel tool to generate hepatocytes *in vitro*, facilitating the generation of patient-specific hepatocytes from SCs. (Sawitza *et al*., 2015) TUDCA was also shown to promote *in vivo* bone tissue regeneration and markedly increase osteogenic differentiation of bone marrow mesenchymal SCs *in vitro*. (Cha *et al*., 2016) Moreover, TUDCA improved blood vessel regeneration by enhancing the mobilization of stem/progenitor cells from bone marrow, differentiation into endothelial progenitor cells, and integration with pre-existing endothelial cells. (Cho *et al*., 2015) TUDCA has also been implicated in erythropoiesis process and markedly enhanced red blood cell viability. (Hong *et al*., 2016) Curiously, TUDCA also regulates energy metabolism, acting as a chemical chaperone stabilizing protein conformation. It was demonstrated that this specific bile acid also reduces ER stress and improves impaired insulin signaling, thus restoring glucose homeostasis in obese and diabetic mice models. (Ozcan *et al*., 2006; Guo *et al*., 2015)

This singular bile acid, previously described as a potent inhibitor of apoptosis mediated mitochondrial perturbations in neurons, may also have an important role in neurogenesis through mitochondrial regulation. (Rodrigues *et al*., 2000; Xavier *et al*., 2014). In fact, it has been shown that TUDCA modulates apoptotic events typical of the early stages of NSC differentiation in a mouse NSC line. In this context, TUDCA does not affect NSC survival but prevents early differentiation induced mitochondrial alterations, including cytochrome *c* release, membrane depolarization, and p53 mitochondrial translocation, mainly through diminished mitochondrial ROS (mtROS) levels and reverse ATP depletion. This bile acid elicits a marked increase in NSC population at S phase with a subsequent reduction at G1 phase, indicating a short G1 phase, which is typical of cells in expansion.

Thus, TUDCA is able to mediate mitochondria-cell cycle retrograde signals to regulate NSC fate. Interestingly, TUDCA also directs differentiating NSCs toward the neuronal lineage. TUDCA-induced effects in increasing NSC pool and determining lineage specification occur in a mitochondrial redox state and ATP-dependent manner. (Figure 1.8) (Xavier *et al*., 2014)

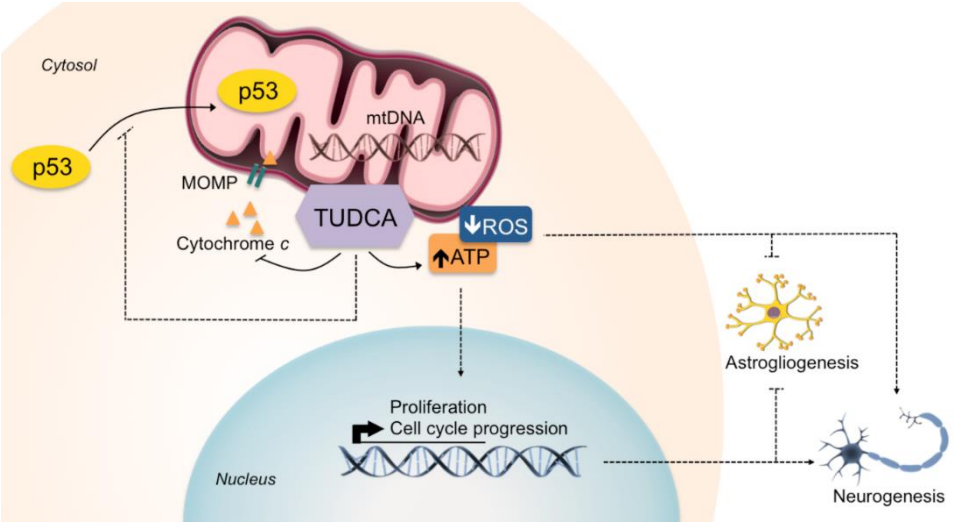


Figure 1.8 - The role of the bile acid TUDCA in early neural differentiation. TUDCA prevents early differentiation-induced mitochondrial apoptotic events while enhancing self-renewal potential and accelerating cell cycle exit of NSCs, through the regulation of redox state and ATP levels. As a result, TUDCA favors neuronal rather than astroglial conversion of differentiating NSCs. (Xavier *et al*., 2014)

Recently, the influence of TUDCA on NSC proliferation, self-renewal, and neurogenesis was explored in postnatal NSCs. Further, the maintenance of its beneficial effects was tested in neurogenic niches of adult rats. DG-derived NSCs were not sensitive to the proliferative effects of TUDCA. Intrinsic characteristics of this niche or the method used for bile acid delivery may explain this outcome. However, the impact of TUDCA on proliferation and differentiation of SVZ-derived NSCs was corroborated. Additionally, TUDCA also modulated mitochondrial biogenesis- and dynamics-associated proteins in differentiating NSCs, thus, starting to unveil TUDCA acting mechanisms. TUDCA increased the expression of PGC-1α, as well as the expression of ERRα and Mfn2, direct targets of PGC-1α. Accordingly, Mfn2 may be a mediator of TUDCA-induced PGC-1α to promote mitochondrial biogenesis and oxidative metabolism. Moreover, this bile acid significantly reduces mtROS, while increasing ROS detoxifier MnSOD. In this study, TUDCA appears again to improve mitochondria function of NSCs. (Soares *et al*., 2017) But more importantly, the potential therapeutic effect of TUDCA in increasing the NSC pool and early neuronal differentiation *in vivo* was uncovered, reinforcing the proliferative and pro-neurogenic effects of this singular bile acid in the SVZ region. (Soares *et al*., 2017)

Altogether, mounting evidence suggests that the neural protective role of TUDCA goes beyond its antiapoptotic and antioxidant properties being essential to unravel the mitochondrial

modulatory mechanisms by which TUDCA enhances neurogenesis.

1.5 Motivation and Aims

Given the lack of effective regeneration in the mammalian brain, it is important to invest in NSCs, the main characters in adult neurogenesis. Over the past few years, our perception of NSC potential has greatly increased, although we are only beginning to understand molecular, metabolic, and epigenetic profile in physiological and pathological context. (Ottoboni *et al*., 2017) A more comprehensive understanding of the behavior of endogenous NSCs will contribute to tune or model them toward the desired response. Thereby, having the opportunity to therapeutically address aging and complex NDs, as age-related metabolic and degenerative diseases increase to epidemic proportions in modern society (Wallace, 2005), we have decided to further understand the mechanisms underlining TUDCA control of NCS fate and exploit its potential effect in enhancing NSC pool and neurogenesis.

In our laboratory, the impact of TUDCA on the mitochondrial proteome in self-renewing or differentiating mouse NSCs was evaluated by high-throughput proteomics analysis using liquid chromatography coupled with mass spectrometry (LC-MS) based detection of differential proteomics from mitochondria purified extracts. (Figure 1.9) This experimental work was performed in Instituto de Tecnologia Química e Biológica – ITQB by Ana Varela Coelho's Proteomics of Non-Model Organisms research group. Of notice, some proteins from the mitochondrial proteome up-regulated or down-regulated by TUDCA were unable to be identified during proteomic analysis.

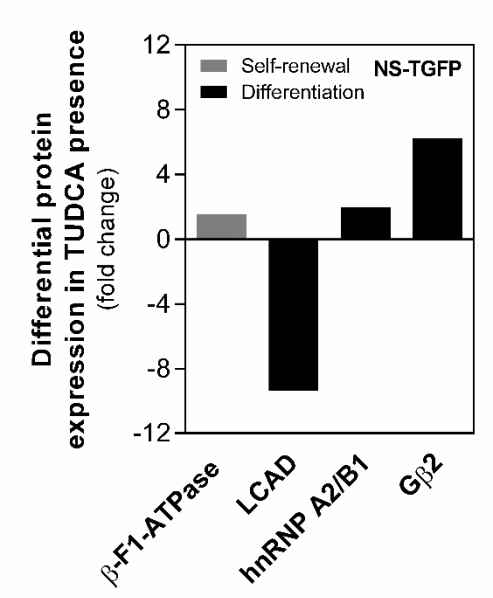


Figure 1.9 – Representative results of TUDCA impact on the mitochondrial proteome in self-renewing or differentiating NSCs. The mouse NSC line NS-TGFP was expanded and induced to differentiate, or not, for 24 h in the presence of TUDCA. Mitochondrial protein purified extracts were obtained for liquid chromatography mass spectrometry-based proteomics analysis. TUDCA modulates the expression of four distinct intriguing proteins: ATP synthetase subunit β (β-F1-ATPase) in self-renewing conditions and long-chain acyl-CoA dehydrogenase (LCAD), heterogeneous nuclear ribonucleoprotein A2/B1 (hnRNP A2/B1) and G-protein subunit β2 (Gβ2) in differentiating

conditions.

Based on this previous data, we started to validate TUDCA-regulated mitochondrial proteins and investigate their relevance in NSC fate. We also aimed to explore the possible metabolic impact of TUDCA treatment in differentiating NSCs. The underlying proteins related to distinct metabolic pathways were also investigated during early stages of neural differentiation.

Specifically, the most relevant questions addressed in this study were:

1. **Does TUDCA drive a metabolic shift in NSCs?**
2. **How does NSC metabolism occur in early stages of neural differentiation?**

Dissecting TUDCA impact on mitochondrial proteomics in NSCs led us to discuss the interplay between nuclear and mitochondrial protein expression regulation toward a defined metabolic state. This study may substantially improve our understanding of how NSCs orchestrate adult neurogenesis in a metabolic-dependent manner suggest TUDCA as an efficient molecule in the maintenance and expansion of these promising cells.

2. Materials and Methods

2.1 Ethics statement

The two mouse NSC lines, NS-TGFP and CGR8, used in this study were both obtained from Dr. Smith's Laboratory, University of Cambridge, Cambridge, UK. The NS-TGFP line was provided by Dr. Henrique, University of Lisbon, Lisbon, Portugal and the CGR8 line was provided by Dr. Margarida Diogo, University of Lisbon, Lisbon, Portugal. The Animal Ethical Committee at the Faculty of Pharmacy, University of Lisbon, Portugal waived the need for approval.

2.2 Mouse NSC lines

The CGR8 competent cell line was established from the inner cell mass of a 3.5-day male pre-implantation mouse embryo (ECACC 07032901). (Nichols *et al*., 1990; Smith, 1991; Mountford *et al*., 1994) Tau-GFP mouse NSC (NS-TGFP) cells were derived from 14.5-dpc mouse foetal forebrain, and constitutively express the fusion protein tau-green fluorescent protein (GFP). (Pratt *et al*., 2000; Silva *et al*., 2006)

2.2.1 Cell line handling, maintenance, and differentiation

Mouse cell lines were established using a method that produces pure cultures of adherent NSCs, which continuously expand by symmetrical division and are both capable of tripotential differentiation. (Conti *et al*., 2005; Pollard *et al*., 2006; Glaser *et al*., 2007) NSCs were grown in monolayer as previously described (Spiliotopoulos *et al*., 2009) and routinely maintained in undifferentiating medium (self-renewal conditions), Euromed-N medium (EuroClone S.p.A., Pavia, Italy), supplemented with N-2 supplement (Gibco, Thermo Fisher Scientific, Inc., USA), epidermal growth factor (EGF; PeproTech EC, UK), basic fibroblast growth factor (bFGF; PeproTech EC) and penicillin-streptomycin (Pen-Strep; Gibco, Thermo Fisher Scientific, Inc.) (see table 2.1 for resumed details), in 75 cm^2 tissue culture (TC) treated flasks (Falcon, Corning Inc., NY, USA) at 37ºC in a humidified atmosphere of 5% CO_2.

Culture cell renewal was performed when NSCs reached approximately 80% of confluence in the culture flask, which represents the critical point when cells start to detach and to degenerate due to lack of nutrients and contact inhibition. The depleted medium was removed and followed by the addition of 1 mL of StemPro Accutase Cell Dissociation Reagent (Gibco, Thermo Fisher Scientific, Inc.) to gently help cell detachment. After an incubation period of 3 min in the incubator, accutase activity was blocked by the addition of 4 mL cell medium. The cell suspension solution was transferred to tubes of 15 mL and centrifuged at 500 *g* for 4 min at room temperature (RT). The supernatant was then discarded and the remaining pellet was resuspended in fresh medium. For each

75 cm^2 cell culture flask, carrying 12 mL of fresh medium it was added 1:4 of the cell suspension, being subsequently incubated in the described conditions. This procedure was performed every 2/3 days.

Neural differentiation was performed by first plating NSCs in undifferentiating medium onto TC-treated cell culture dishes at 5.4×10^6 cells/cm^2 for 24 h. To count cells, a hemocytometer was used. Trypan blue (T8154; Sigma-Aldrich Corp., USA) stained damaged or dead cells (an exclusion dye method), therefore enabling to distinguish between viable and non-viable cells. After 24 h of cell stabilization, the medium was changed to optimized neuronal differentiation-inducing medium, Euromed-N medium supplemented with N-2 supplement, B-27 supplement (Gibco, Thermo Fisher Scientific, Inc.), bFGF, and Pen-Strep (see table 2.1 for resumed details). For NSCs that remained undifferentiated, the medium was not changed. Differentiating NS-TGFP cells at 8×10^5 cells/cm^2 were at fixed at 0, 3, 6, 24, 48 and 72 h and processed for immunoblotting. For cellular treatments, cells were maintained in differentiating and undifferentiating medium for 24 h. Then, cells were collected and processed for immunoblotting, differentiated cells were also processed for Oil-Red-O staining, gas chromatography-mass spectrometry (GC-MS) analysis, and immunocytochemistry.

Table 2.1 – Resumed details and characteristics of mouse NSC lines used in this study.

Cell designation	Cell type	Growth mode	Undifferentiating growth medium % in (v/v)	Differentiating growth medium % in (v/v)
CGR8	Mouse NSC	Adherent	Euromed-N; 20 ng/µL EGF; 20 ng/µL bFGF; 1 % Pen-Strep; 1 % N-2	Euromed-N; 10 ng/µL bFGF; 1 % B-27; 1 % Pen-Strep; 0.5 % N-2
NS-TGFP	Mouse NSC	Adherent		

(EGF, epidermal growth factor; bFGF, basic fibroblast growth factor; Pen-Strep, Penicillin-Streptomycin).

2.2.2 Cellular treatments

Cells were treated with 100 µM of TUDCA (T0266; Sigma-Aldrich Corp.) after 24 h of plating and/or upon medium change for differentiation, and then collected after 24 h for protein extraction and other analyses. The schematic representation of time course treatment is illustrated in Figure 2.1.

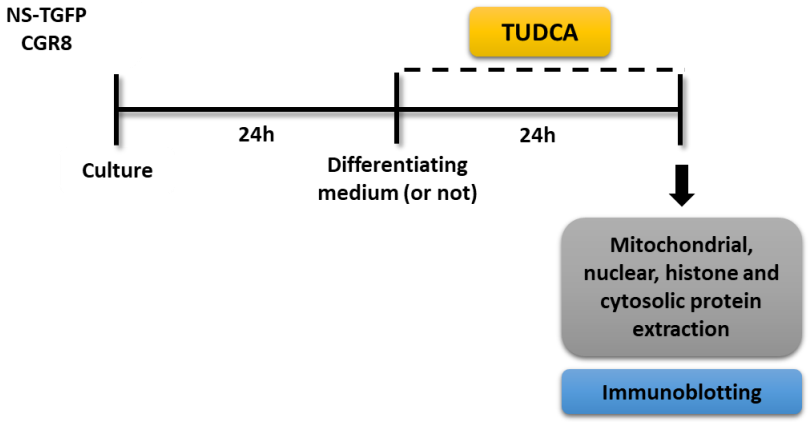


Figure 2.1 – Graphical scheme of NS-TGFP and CGR8 cells treatment course.

2.3 Mitochondrial, cytosolic, and nuclear protein extraction

Cells were collected after 24 h of incubation with TUDCA and processed for extraction of mitochondrial and nuclear fractions. Both protocols allow also the extraction of the cytosolic fraction. Cell pellet was firstly washed with phosphate-buffered saline (PBS) and centrifuged at 600 *g* for 5 min at 4ºC. For isolation of mitochondrial protein extracts, cells were lysed upon incubation in ice for 20 min with an isolation buffer (20 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2.6H_2O$, 1 mM Na_2EDTA, 1 mM EGTA, 250 mM Sucrose), supplemented with 1 mM dithiothreitol (DTT) and Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific, Inc.) and, then, disrupted by 40 strokes in a Dounce homogenizer. The homogenates were centrifuged at 2,500 *g* for 10 min at 4ºC, then, the supernatant was saved and a second centrifugation was necessary to increase the product yield, so the pellet obtained from the homogenates was resuspended again in a minimum volume of isolation buffer. The total homogenate recovered was, then, centrifuged at 12,000 *g* for 30 min at 4ºC to remove unbroken cells and nuclei. Finally, the mitochondrial fraction was obtained, the respective pellet resuspended in the isolation buffer and frozen at -80ºC. The supernatant was removed and filtered through 0.2 µm and, then, 0.1 µm Ultrafree MC filters (Merck Millipore Corp., Germany) by centrifugation at 12,000 *g* for 20 min at 4ºC to obtain cytosolic proteins.

For nuclear extracts, cells were lysed with hypotonic buffer (10 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 1.5 mM potassium acetate, 2 mM DTT, and protease inhibitors), homogenized with 20 strokes in a loose fitting Dounce homogenizer, and centrifuged twice at 500 *g* for 10 min at 4ºC. Cytosolic proteins were recovered in the supernatant and centrifuged at 3,160 *g* for 10 min at 4ºC saving the supernatant again, while the nuclear pellet was washed in a buffer composed of 10 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 0.25 M sucrose, 0.5% Triton X-100, and protease inhibitors through centrifugation at 500 *g* for 5 min at 4ºC. Then, the nuclear pellet was resuspended and sonicated for four cycles of 10 sec in buffer composed by 10 mM Tris-HCl, pH 7.6, 0.25 M sucrose and protease inhibitors. Finally,

the suspension was centrifuged through 0.88 M sucrose at 2,000 *g* for 20 min at 4ºC, and nuclear proteins were recovered in the supernatant.

All protein content was measured by the Bio-Rad protein assay kit (Bio-Rad Laboratories, USA), according to the manufacturer's specifications, using bovine serum albumin (BSA) as standard.

2.4 Histone purification

Histone purification was performed using an acid extraction protocol. Cells were collected at 24 h of TUDCA treatment, washed in PBS and lysed with hypotonic lysis buffer (10 mM Tris-HCl, pH 8, 1 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT and protease inhibitors) for 30 min at 4ºC. Intact nuclei were recovered by centrifuging at 10,000 *g* for 10 min at 4ºC, and the supernatant discarded. Nuclei were resuspended in 0.4 N H_2SO_4 and incubated on a rotator at 4ºC overnight. Nuclear debris were removed by centrifuging at 16,000 *g* for 10 min at 4ºC, and the supernatant containing histones was transferred into a fresh tube. Trichloroacetic acid (100%) was added drop by drop to histone solution, mixed and incubated on ice for 30 min. Histones were recovered after centrifugation at 16,000 *g* for 10 min at 4ºC. The histone pellet was washed twice with ice-cold acetone and air-dried for 20 min at RT. Histones were finally dissolved in 100 µL of Milli-Q water and transferred into a new tube. Histone protein content was measured by the Bio-Rad protein assay kit, according to the manufacturer's specifications, using BSA as standard.

2.5 Total protein extraction

Differentiating NSCs from NS-TGFP cell line were processed for isolation of total protein extracts. NSCs were fixed at 0, 3, 6, 24, 48 and 72 h with or without 24 h of TUDCA treatment. NSCs were lysed using an ice-cold lysis buffer (10 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 1.5 mM potassium acetate, 1% Nonidet P-40, 2 mM DTT, and protease inhibitors) for 30 min in ice. Samples were then sonicated for 30 sec in ultrasounds, the lysate was centrifuged at 10,000 *g* for 10 min at 4ºC, and the supernatant recovered. Protein content was also measured by the Bio-Rad protein assay kit, according to the manufacturer's specifications, using BSA as standard.

2.6 Immunoblotting

Protein levels of interest (β-F1-ATPase, GAPDH, Gβ2, acetyl-H3, histone 3, hnRNP A2/B1, Hsp70, lamin B1, LCAD, PDHE1-α, SREBP1 and VDAC) were determined by Western blot analysis. Briefly, 20 µg of protein from mitochondrial, nuclear, and histone protein extracts and 40 µg of cytoplasm protein fraction and total protein extracts were separated in a discontinuous gel system of a 4% stacking gel and a 12% sodium dodecyl sulphate-polyacrylamide electrophoresis gel (SDS-PAGE), comprised by 30% (w/v) protogel, mili-Q water, upper/lower gel buffers, ammonium

persulfate (APS) and tetramethylethylenediamine (TEMED). The run was performed at a constant voltage of 140 V for 1 h.

Gel transfer to blot was performed by using a transfer buffer (250 ml 4X transfer buffer, 200 ml of methanol and H_2O to 1 L) upon hydration of sponges, filter paper, and 8.5 x 6.5 cm hybond-C nitrocellulose membrane. The transference occurred in cold room packed ice at a constant amperage of 0.3 A for 90 min. To proceed to immunolabeling of the blots the membrane was previously incubated with milk 5% (w/v) at least 30 min at RT, then it followed an overnight incubation at 4ºC with the primary monoclonal antibodies. (Table 2.2) After washing, blots were subsequently incubated with secondary antibodies (Table 2.3) conjugated with anti-mouse or anti-rabbit IgG, and with horseradish peroxidase (HRP) for 2 h at RT. Finally, membranes were processed for protein detection using Immobilon™ Western Chemiluminescent HRP Substrate (Millipore Corp.) or Super Signal™ West Femto substrate (Thermo Fisher Scientific, Inc.) in a ChemiDoc™ MP System (Bio-Rad Laboratories) with approximately 1 min of exposure. Ponceau S (P7170; Sigma-Aldrich Corp.), a sodium salt of a diazo dye of a light red color, was used to reversible stain total protein bands. Ponceau S was used to confirm the purity of histone extracts (check annex A - figure A.1). GAPDH, Lamin B1, VDAC were used as loading control and/or to certify the purity of the cytosolic, nuclear and mitochondrial fractionation, respectively (check for the purity of protein extracts in annex A - figures A.2 and A.3).

Table 2.2 – Details of the primary antibodies used for Western blot.

Antigen	Host	Clonality	Company	Catalog number	Dilution
β-F1-ATPase	Mouse	Monoclonal	Santa Cruz Biotec.	sc-166443	1:1000
GAPDH	Mouse	Monoclonal	Santa Cruz Biotec.	sc-32233	1:2500
Gβ2	Mouse	Monoclonal	Santa Cruz Biotec.	sc-166123	1:10000
Acetyl-H3	Rabbit	Polyclonal	Abcam plc	ab47915	1:1000
Total H3	Rabbit	Polyclonal	Millipore Corp.	06-755	1:500
hnRNP A2/B1	Mouse	Monoclonal	Santa Cruz Biotec.	sc-53531	1:200
Hsp70	Mouse	Monoclonal	ReD Systems	242707	1:10000
Lamin B1	Rabbit	Polyclonal	Abcam plc	ab16048	1:10000
ACADL	Rabbit	Monoclonal	Abcam plc	ab196655	1:10000
PDHE1-α	Mouse	Monoclonal	Abcam plc	ab110330	1:1250
SREBP-1	Mouse	Monoclonal	Abcam plc	ab3259	1:400
VDAC	Rabbit	Polyclonal	Cell Signaling Tech.	4866	1:1000

Table 2.3 – Details of the secondary antibodies used for Western blot.

Secondary Antibody	Host	Company	Catalog number	Dilution
HRP conjugated anti-mouse	Goat	Bio-Rad Lab.	1706516	1:5000
HRP conjugated anti-rabbit	Goat	Bio-Rad Lab.	1706515	1:5000

2.7 Oil-Red-O staining

The possible intracellular accumulation of lipids in NSCs was evaluated by Oil-Red-O (ORO)/isopropanol method (Fukumoto & Fujimoto, 2002). NSCs, from mouse NS-TGFP cell line,

were plated onto TC-treated dishes at 3 × 10^5 cells/cm^2 for 24 h followed by differentiation and TUDCA cellular treatment, as previously described. Free FAs mixture (2:1, oleate: BSA-palmitate) in a concentration of 500 μM was added to NSCs for positive control, also 500 μM of BSA for endogenous control. The work solution of ORO (O-0625; Sigma-Aldrich Corp.) was prepared, ORO was dissolved in ≥ 99.8% isopropanol (109634; Merck Millipore Corp) and left overnight at RT. The solution was filtered with cellulose acetate membrane syringe filter, pore size 0.45 μm (1520014; Frilabo). Three parts of ORO solution were added to 2 parts of bidistilled H_2O. The solution was then filtered, left to stand for 30 min, and filtered again before use. Before staining, the medium was removed from the dishes with a Pasteur pipette, and the dishes were then gently rinsed with PBS. One milliliter of 4% (w/v) paraformaldehyde was added to each dish for 20 min to fix the cells at RT. Each dish was then rinsed twice with PBS and once with 60% (v/v) of isopropanol. 1 ml of ORO stain was applied per dish for 5 min and removed. Next, the dishes were rinsed with PBS, until no excess stain was observed, followed by the addition of 1 ml per dish of Mayer's hematoxylin solution (MHS-32; Sigma-Aldrich Corp.) for 2 min. Cells were then washed twice with PBS to remove the excess of the staining of the nuclei. The dishes were air dried and mounted under a glass coverslip with 30 μL PBS/glycerol (3:1, v/v). The staining effectiveness was evaluated with Zeiss Axioskop 50 microscope (Carl Zeiss Corp., Germany) inverted research microscope equipped with Axiocam 105 color (Carl Zeiss Corp.). Images (400x) were captured and enhanced using ImageJ.

2.8 Immunocytochemistry

Cellular distribution of PDHE1-α was evaluated in mouse NS-TGFP cell line. Briefly, NSCs were plated onto TC-treated dishes at 3 × 10^5 cells/cm^2 for 24 h followed by differentiation and TUDCA cellular treatment, as previously described. Cells were incubated with 0.5 μM MitoTracker® Red CMXRos (M-7512; Molecular Probes, Life Technologies Corp.), which preferentially accumulates in mitochondria in live cells, for 30 min at 37ºC before cell harvesting. Cells were washed twice, fixed with 4% paraformaldehyde in PBS, washed three times and then blocked for 1 h at RT in PBS, containing 0.1% Triton-X-100, 1% FBS, and 10% normal donkey serum (Jackson ImmunoResearch Laboratories, Inc., USA). Cells were incubated with a primary mouse monoclonal antibody reactive to PDHE1-α (table 2.4) overnight at 4ºC. After three washes, the secondary DyLight 488-conjugated anti-mouse (table 2.5) was added to cells for 2 h at RT. Mouse NSC nuclei were stained with Hoechst 33258 (861405; Sigma-Aldrich Corp.) at 5 μg/ml in PBS, for 3 min at RT and, then, washed three times. Samples were mounted using Mowiol® 4-88 (81381; Sigma-Aldrich Corp.). The resulting fluorescent signals were imaged using fluorescence microscopy assessments performed with a Zeiss AX10 microscope (Carl Zeiss Corp.), equipped with a 63x/1.4 oil plan-apochromat objective and an AxioCam HRm camera (Carl Zeiss Corp.). Images were processed using ImageJ.

Table 2.4 – Details of the primary antibody used for immunocytochemistry.

Antigen	Host	Clonality	Company	Catalog number	Dilution
PDHE1-α	Mouse	Monoclonal	Abcam plc	ab110330	1:200

Table 2.5 – Details of the secondary antibody used for immunocytochemistry.

Secondary Antibody	Host	Company	Catalog number	Dilution
DyLight 488-conjugated anti-mouse	Goat	Jackson ImmunoResearch	211-482-171	1:200

2.9 GC-MS determination of free-FAs

Analysis of individual FFA in NSCs was performed by GC-MS. NSCs, from mouse NS-TGFP cell line, were plated onto TC-treated dishes at 5.4×10^6 cells/cm^2 for 24 h followed by differentiation and TUDCA cellular treatment, as previously described. Cells were collected in ice-cold 80% (v/v) of methanol (106009; Merck Millipore Corp). Cellular material was subsequently collected by scraping. The respective homogenate was washed three times and centrifuged at 12,000 *g* for 10 min at 4ºC for isolation of total protein extracts and, then, to quantify total protein amount. The supernatant containing free-FA was processed for the qualitative and quantitative analysis of individual FFA using GC-MS (Shimadzu QP2010 Plus) in single ion monitoring detection mode (SIM), based upon a published procedure (Costa *et al*., 1998). This part of experimental work was performed by Marco Moedas, Ph.D. and Professor Margarida Silva from the Metabolism and Genetics research group at iMed.ULisboa. The supernatant was evaporated under a N2 stream and derivatized with a N-methyl-N-(tert-butyldimethylsilyl)-trifluoroacetamide (MTBSTFA): methyl-bis(trifluoroacetamide) (MBTFA): Pyridine mix (4:5:1) (Pierce-Thermo Scientific, USA) at 60ºC for 1 h. Samples were injected with an automatic injector (AOC 20i), with split injection mode (1/50 ratio) and free-FAs were detected in SIM mode. The obtained mass spectra were interpreted using internal FA standards (Sigma Chemical Co., USA). Data are expressed as the peak-area ratios (sample/internal standard) over total protein content (mg). Total protein extracts were measured by the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's specifications, using BSA as standard.

2.10 Densitometry and statistical analysis

The relative intensities of protein bands were analyzed using the Image Lab Version 5.0 densitometric analysis program (Bio-Rad Laboratories). Results were compared using an unpaired Student's t test. Values of $p < 0.05$ were considered statistically significant. All statistical analysis was performed with GraphPad Prism 6.1 software (GraphPad Software, Inc., USA.

3. Results and Discussion

3.1 Validation of the TUDCA impact on the mitochondrial proteome in NSCs

TUDCA is a well-known mitochondria protecting bile acid in neural differentiation, awaiting for further exploration in regulating the expression of mitochondria regulators and dynamic proteins. (Xavier *et al*., 2015) In fact, proteomics data should reveal possible novel and unexpected mitochondria interacting partners and regulators possibly modulated by TUDCA in NSCs. Here, the validation of proteomics data was performed by Western blot analysis in mouse NSCs treated, or untreated, with the bile acid in the same experimental conditions. TUDCA potential effects in NSCs will be discussed along for each protein. Importantly, the purity of mitochondrial extracts was certified (annex A).

3.1.1 ATP synthase β subunit

Proteomic analysis showed that TUDCA increases mitochondrial ATP synthase β subunit (β-F1-ATPase) levels in undifferentiated NSCs. β-F1-ATPase is the major component of the catalytic centre of ATP synthase complex, the complex V of mitochondrial ETC, which is encoded in the nuclear genome. β-F1-ATPase mRNA localizes at mitochondria and the regulation β-F1-ATPase expression is exerted at the level of translation. The accumulation of β-F1-ATPase has shown to occur throughout the cell cycle with a significant increase at G2/M when compared to cells in S phase. Further, oligomerization of the ATP synthase complex plays a critical role in the folding of the inner membrane into cristae morphology. Thus, the synthesis of β-F1-ATPase, driven at G2/M, could rate-limit the development of a functional organelle. (Martínez-Diez *et al*., 2006) In fact, TUDCA treatment has shown to increase ATP levels dependent on F1F0-ATP synthase complex activity at 24 h of differentiation of NSCs. (Xavier *et al*., 2014)

At odds with that previously shown in proteomic analysis, Western blot experiment did not demonstrate any increase of β-F1-ATPase in the presence of TUDCA in self-renewing NSCs. (Figure 3.1) Thus, we were not able to validate this proteomic result. Given the fact that β-F1-ATPase translation is regulated at the cell cycle level and TUDCA elicits a marked increase in NSC population at S/G2-M phase (Xavier et al., 2014), it would be interesting, in the future, to clarify the mechanisms by which cell cycle regulates changes in the translational efficiency of β-F1-ATPase in NSCs. For that, we could synchronize the cell cycle of NSCs and investigate how β-F1-ATPase levels vary over cell cycle progression. Furthermore, we could assess whether TUDCA has any effect on β-F1-ATPase mitochondrial mRNA levels. Indeed, TUDCA upregulates PGC-1α and ERRα in SVZ-derived NSCs (Soares *et al*., 2017), which were shown to promote the transcription of β-F1-ATPase. (Scarpulla, 2011) Importantly, this protein might have a prominent role in mitochondrial biogenesis.

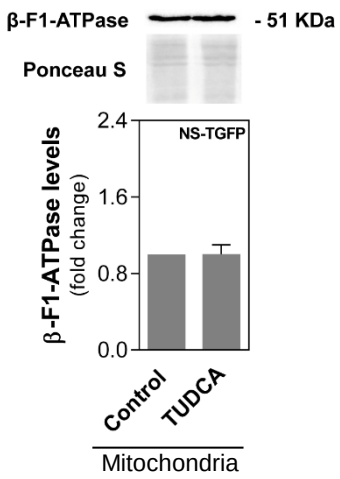


Figure 3.1 – TUDCA does not modulate the expression of β-F1-ATPase in self-renewing NSCs. The mouse NSC line NS-TGFP was expanded and treated with TUDCA for 24 h in self-renewal conditions, and then collected for immunoblotting analysis. Representative immunoblots of β-F1-ATPase levels (top) and corresponding densitometry analysis (bottom) in mitochondrial protein extracts. β-F1-ATPase levels were normalized to Ponceau S. Data are expressed as mean ± SEM fold-change for at least three independent experiments. Non-treated undifferentiated cells served as control.

Interestingly, β-F1-ATPase expression was shown to also interfere with mitochondrial dynamics. β-F1-ATPase overexpression enhances elongation of mitochondria in human embryonic kidney cells (Seo *et al*., 2016), a mitochondrial event that has been related to self-renewal in NSCs. (Khacho *et al*., 2016) β-F1-ATPase was also implicated in cancer, in which the overexpression of β-F1-ATPase mRNA transduces an attempt to meet the high demand for ATP in highly proliferative tumors. (Cuezva *et al*., 1997; Xu & Li, 2016) Most notably, the *ATP5B* gene, which encodes for β-F1-ATPase, showed consistent upregulation during early lineage progression of adult NSCs. (Beckervordersandforth *et al*., 2016) In fact, in differentiation context, there is an upregulation of components of the mitochondrial complexes, paralleled by upregulation of enzymes of the TCA cycle. These changes result in an increase in the activity of the ETC, thus, in OXPHOS, to sustain cellular specialization. (Sánchez-Aragó *et al*., 2013; Beckervordersandforth *et al*., 2016)

Taking this into account, the increased effect in β-F1-ATPase levels by TUDCA should be further confirmed by other techniques and/or other time-points, since TUDCA proliferative and neurogenic effects in NSCs might be justified, in part, by β-F1-ATPase-induced mitochondria fusion and metabolic shift.

3.1.2 G protein β2 subunit

Guanine nucleotide binding protein-β subunit 2 (Gβ2) was also found to be increased in mitochondria in the presence of TUDCA in proteomic analysis upon 24 h of NSC differentiation. Gβ2 is a member of the β-subunits of heterotrimeric G proteins, and it has been demonstrated to have a

crucial function in mitochondrial fusion. Gβ2 is partially found on the surface of mitochondria. It physically interacts with Mfn1 and its depletion perturbs mitochondrial morphology, decreasing mitochondrial fusion rate. Thereby, Gβ2 acts by limiting the membrane mobility of Mfn1 at the mitochondrial surface to regulate the process of mitochondrial fusion. (Zhang *et al*., 2010) Of note, this is a non-canonical localization of heterotrimeric G proteins that typically are located at the cytoplasmic surface of the plasma membrane and transduce signals from G protein-coupled receptors to effector proteins, also deeply implicated in adult neurogenesis. (Doze & Perez, 2012; Hewavitharana & Wedegaertner, 2012) Intriguingly, TUDCA has already been implicated in the regulation of mitochondrial dynamic proteins in SVZ-derived NSCs. (Soares *et al*., 2017) However, Western blot analysis did not reveal any increase the expression of Gβ2 in mitochondria with TUDCA. (Figure 3.2)

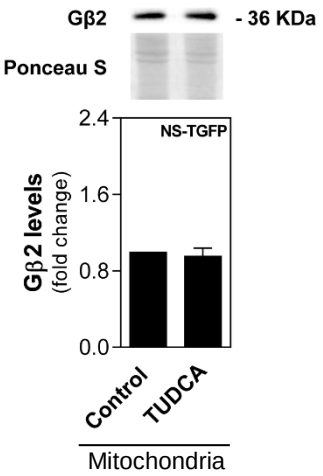


Figure 3.2 – TUDCA does not modulate the expression of Gβ2 in differentiating NSCs. NSCs, from the NS-TGFP cell line, were expanded and induced to differentiate for 24 h, in the presence or absence of TUDCA, and then collected for immunoblotting analysis. Representative immunoblots of Gβ2 levels (top) and corresponding densitometry analysis (bottom) in mitochondrial protein extracts. Gβ2 levels were normalized to Ponceau S. Data are expressed as mean ± SEM fold-change for at least three independent experiments. Non-treated differentiating cells served as control.

Mitochondrial fission events are required for the transient passage of NSCs to committed progenitors. (Khacho *et al*., 2016) Nevertheless, the precise balance of mitochondrial dynamics is among the most critical features for the juxtaposed processes of cell death and survival typically described in aging and in many NDs. Exceeding mitochondria fragmentation can lead to mitochondrial dysfunction. (Khacho & Slack, 2015) Increased levels of Gβ2, therefore, could be an additional force for bringing mitochondria in proximity for tethering and docking, leading to initiation of fusion and balance of mitochondrial dynamics in the following stages of neurogenesis. Thus, it would be interesting to confirm if TUDCA causes an increase in Gβ2 levels in a later time-point of neural differentiation.

3.1.3 Heterogeneous nuclear ribonucleoprotein A2/B1

Proteomic results revealed an increase of heterogeneous nuclear ribonucleoprotein (hnRNP) A2 and its longer B1 isoform levels in mitochondria of differentiating NSCs treated with TUDCA. hnRNPs are a family of abundant and multitasking proteins that play a central role in RNA metabolism, within cytoplasmic trafficking of mRNAs and mRNA processing, stability, and turnover. They also participate in splicing regulation and telomerase biogenesis, as well as in cell proliferation and carcinogenesis processes. hnRNP A2/B1 is a major component of the hnRNP core complex in mammalian cell nuclei. (He *et al*., 2005; Choi *et al*., 2013)

The levels of hnRNP A2 isoform increased in a similar way in mitochondrial extracts of two different NSC lines (Figure 3.3 (A and B)), corroborating proteomic results. However, hnRNP B1 protein was not detectable by Western blot in mitochondrial extracts either in the presence or absence of TUDCA. Many studies do not distinguished hnRNP A2/B1 isoforms are focused only on A2, which is the major isoform in most tissues. (Han *et al.*, 2010) Surprisingly, there is no literature describing hnRNP A2/B1 function or even its localization at mitochondria. But, it is known that cytosolic ribosomes are closely associated with the OMM, so a strong enrichment of nuclear-encoded RNAs commonly co-purify with mitochondria. (Mercer *et al*., 2011) Given hnRNP A2/B1 tight association with mRNAs, we suspect that our mitochondrial extracts have co-purified mRNA contaminants within at least, for hnRNP A2 isoform. Therefore, it would be important to proceed with mitochondrial preparations where OMM is stripped (mitoplasts), thus providing a selective depletion of nuclear-encoded RNAs. (Mercer *et al*., 2011)

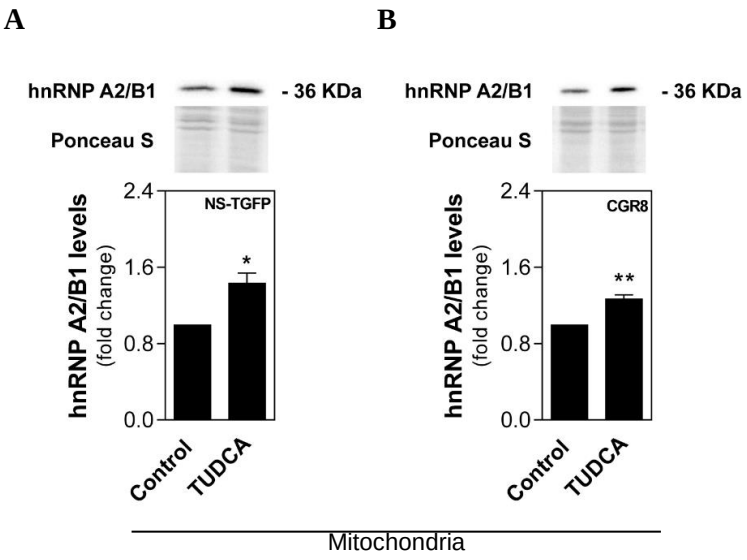


Figure 3.3 – TUDCA increases hnRNP A2/B1 expression in mitochondria of differentiating NSCs. NSCs, from NS-TGFP and CGR8 cell lines, were expanded and induced to differentiate for 24 h, in the presence or absence of TUDCA, and then collected for immunoblotting analysis. Representative immunoblots of hnRNP A2/B1 levels (top) and corresponding densitometry analysis (bottom) in mitochondrial protein extracts of NS-TGFP **(A)** and CGR8 **(B)** cell lines. hnRNP A2/B1

levels were normalized to Ponceau S. Data are expressed as mean ± SEM fold-change for at least three independent experiments. *$p < 0.05$ and **$p < 0.01$ from non-treated differentiating cells (control).

hnRNP A2/B1 was the first trans-acting factor to be described in neural mRNA trafficking, necessary for dendritic delivery of various mRNAs that are involved in myelination and synaptic regulation. hnRNP A2/B1 recognizes the A2 response element (A2RE), a cis-acting signal present in certain trafficked mRNAs. In fact, mRNA trafficking in neural cells is a highly dynamic and regulated process in which a subset of mRNAs is transported from the nucleus to distal cellular zones, where translation leads to localized protein expression. (Smith, 2004; Han *et al*., 2010) Nuclear hnRNP A2 participates in mitochondrial respiratory stress retrograde response as a transcriptional activator of target genes and as a novel histone acetyltransferase. (Guha *et al*., 2009; Guha *et al*., 2016) hnRNP A2/B1 has also shown to positively regulate the self-renewal and pluripotency of hESCs and its levels gradually decrease during differentiation. (Choi *et al*., 2013) Consistent with this model, during neuronal differentiation hnRNP A2 RNA levels decreased compared to NPCs. The decreased expression of hnRNP proteins, such as hnRNP A2, induces a switch in pyruvate kinase gene splicing from PKM2 to PKM1. Pyruvate kinase is responsible for catalyzing the final step of glycolysis. This nuclear event marks the transition from glycolysis in NPCs to neuronal OXPHOS, although it is still unclear how PKM1/M2 isoforms operate on cell metabolism. (Zheng *et al*., 2016) Regarding these notable nucleocytoplasmic functions of hnRNP A2/B1, we decided to see how TUDCA modulates hnRNP A2/B1 levels in the nucleus and cytoplasm. Notably, we found that, in differentiating NSCs, TUDCA downregulated hnRNP A2 expression in the nucleus (Figure 3.4 A) while almost doubled cytoplasmic levels of hnRNP A2 when compared with control. (Figure 3.4 B) Importantly, the purity of nuclear and cytosolic fractionation was carefully certified (annex A).

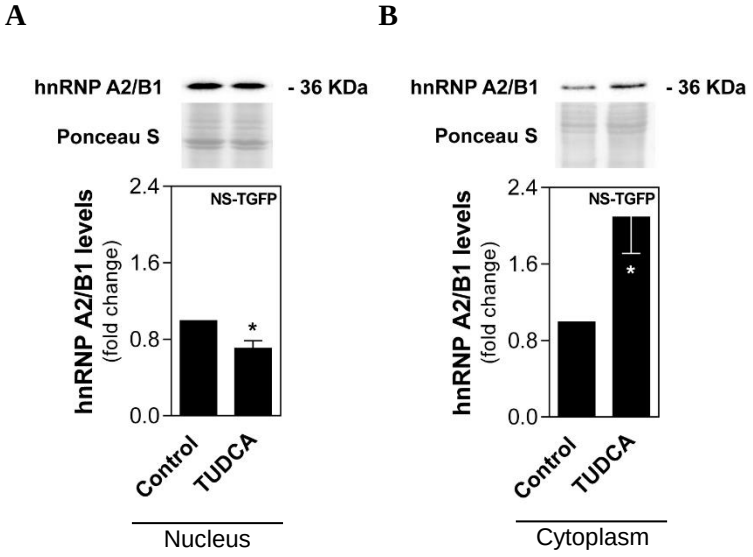


Figure 3.4 – TUDCA modulates hnRNP A2/B1 expression in different cellular compartments of NSCs. NSCs, from the NS-TGFP cell line, were expanded and induced to differentiate for 24 h, in the presence or absence of TUDCA, and then collected for immunoblotting analysis. Representative immunoblots of hnRNP A2/B1 levels (top) and corresponding densitometry analysis (bottom) in both

nuclear **(A)** and cytosolic **(B)** protein extracts. hnRNP A2/B1 levels were normalized to Ponceau S. Data are expressed as mean ± SEM fold-change for at least three independent experiments. *$p < 0.05$ and **$p < 0.01$ from non-treated differentiating cells (control).

Therefore, TUDCA appears to promote the translocation of hnRNP A2 from the nucleus to the cytoplasm where it might have important functions mainly in dendritogenesis and in synaptic regulation. TUDCA does not affect *in vitro* neurite outgrowth output after 2 days of postnatal NSC differentiation. (Soares *et al*., 2017) Nevertheless, the downregulation of hnRNP A2 nuclear levels appears to be more important for early development stages of NSCs. It might intercede for the metabolic shift driving NSC differentiation, in which, NSCs start to rely on mitochondrial OXPHOS to meet energy demands. In fact, reduced nuclear levels of hnRNP A2 probably support a molecular program adaptation to cellular differentiation. hnRNP A2/B1 levels were indeed shown to be decreased during cellular differentiation along with pluripotency and self-renewal features. hnRNP B1 levels are perhaps too low for Western blot detection in NSCs. However, hnRNP A2 seems to carry out major functions.

3.1.4 Long-chain acyl-CoA dehydrogenase

Proteomic analysis showed that LCAD levels were significantly decreased in the presence of TUDCA in differentiating NSCs. LCAD is one of five enzymes from the initial step of mitochondrial β-oxidation, acting on C8-C20 FAs. (Kompare & Rizzo, 2008) In mice, LCAD plays an essential role in the degradation of PUFAs but it seems more redundant in the oxidation of long-chain saturated FAs. (van Vlies *et al*., 2005; Chegary *et al*., 2009) Again, we were able to validate proteomic results in two different mouse NSC lines by Western blot. TUDCA significantly decreased LCAD protein levels by approximately 40%, when compared to the respective control in the NS-TGFP cell line (Figure 3.3 (A)). In CGR8 cells, this decrease was approximately 50% (Figure 3.3 (B)).

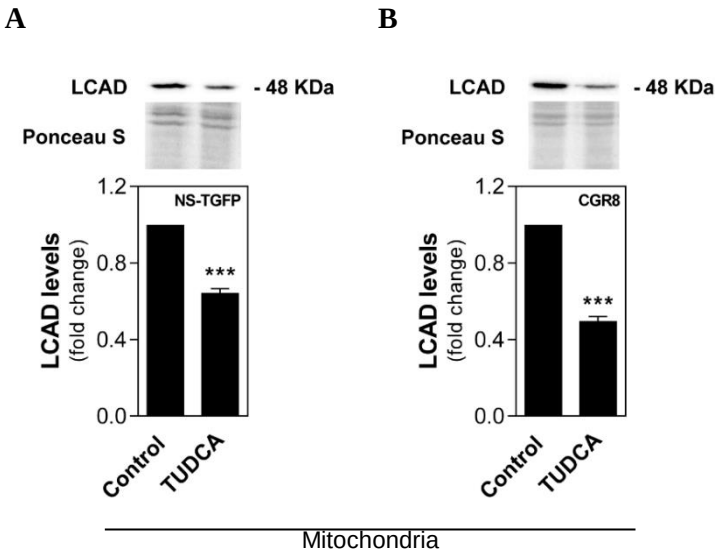


Figure 3.5 – TUDCA decreases LCAD expression in differentiating NSCs. NSCs, from NS-TGFP

and CGR8 cell lines, were expanded and induced to differentiate for 24 h, in the presence or absence of TUDCA, and collected then for immunoblotting analysis. Representative immunoblots of LCAD levels (top) and corresponding densitometry analysis (bottom) in mitochondrial protein extracts of NS-TGFP **(A)** and CGR8 **(B)** cell lines. LCAD levels were normalized to Ponceau S. Data are expressed as mean ± SEM fold-change for at least three independent experiments. ***$p < 0.001$ from non-treated differentiating cells (control).

No human deficiency of LCAD has been described so far, it is only hypothesized that LCAD deficiency may be a cause of autism. (Clark-Taylor & Clark-Taylor, 2004; Chegary *et al*., 2009) Interestingly, LCAD is highly expressed in the fetal brain, having a relative expression in the CA1 and CA4 areas and granular layer region of human hippocampus, regions of adult neurogenesis and with proximal spatial relationship. (Sohur *et al*., 2006; He *et al*., 2007) Several mouse models for mitochondrial β-oxidation defects have been generated including the $LCAD^{-/-}$ (LCAD-deficient) mouse. (Kurtz *et al*., 1998; van Vlies *et al*., 2005) Furthermore, the phenotype of the $LCAD^{-/-}$ mouse is most similar to human long-chain acyl-CoA dehydrogenase (VLCAD) deficiency. In mice, LCAD and VLCAD have overlapping and distinct roles in FAO but the absence of VLCAD is apparently fully compensated, whereas LCAD deficiency is not. (van Vlies *et al*., 2005; Chegary *et al*., 2009) Therefore, TUDCA is unequivocally inhibiting mitochondrial β-oxidation of long-chain FAs in mouse NSCs lines, exerting a fine-tune regulation on long-chain FAs degradation. Notice that PGC-1α and ERRα were found to be upregulated by TUDCA in SVZ-derived NSCs (Soares *et al*. 2017), possibly having a positive impact on mitochondrial biogenesis, as well as on upregulation of medium-chain acyl-CoA dehydrogenase (MCAD) promoter and FAO of medium-chain FAs. (Scarpulla, 2011)

Recently, lipid accumulation of oleic acid decreased NSC proliferation in an Alzheimer's disease mouse model, but more importantly, this was mimicked in wildtype mice by a local increase and subsequent accumulation of lipids, suggesting that aberrant lipid metabolism in disease may be directly influencing NSC behavior. (Hamilton *et al*., 2015) Thus, the possible intracellular lipid accumulation by TUDCA would be a major concern. To answer this question, differentiating NSCs were stained with ORO and the presence of lipid droplets was evaluated in NSCs treated or untreated with TUDCA. Interestingly, NSCs did not show any sign of lipid accumulation after 24 h of TUDCA treatment, having a similar appearance compared to control. In contrast, NSCs treated with long-chain FAs, oleate: BSA-palmitate, showed intracellular major lipid droplets around the nucleus whereas the relative control was clear of lipid accumulation. (Figure 3.6) FAO disruption may also lead to carnitine derivatives accumulation that affects mitochondria homeostasis. (van Vlies *et al*., 2005; Wajner *et al*., 2016) Nevertheless, this bile acid has been shown to preserve mitochondrial integrity and function in NSCs using similar experimental conditions. (Xavier *et al*., 2014)

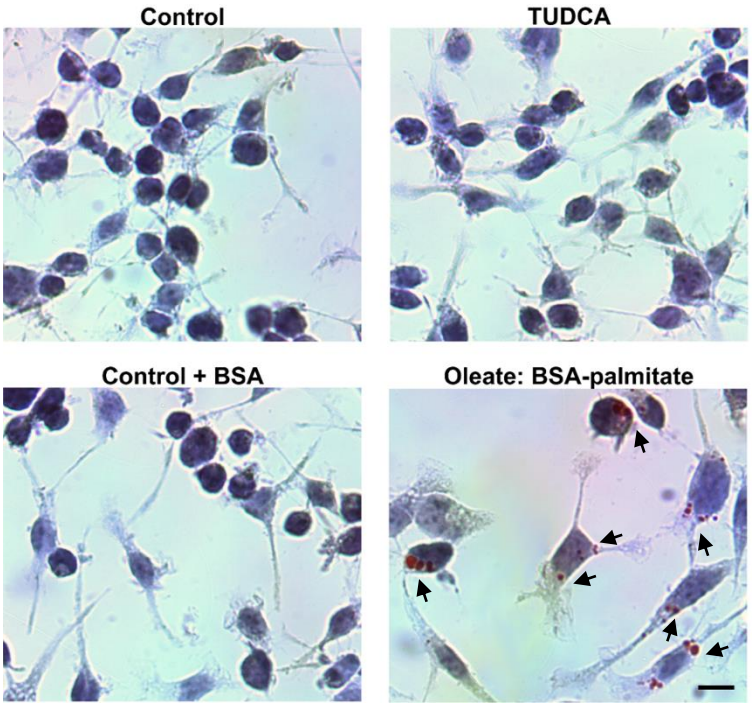


Figure 3.6 - TUDCA does not cause intracellular lipid accumulation in differentiating NSCs. Oil-Red-O/isopropanol method was performed in differentiating NSCs, from the NS-TGFP cell line, untreated or treated with TUDCA for 24 h, as described in *Materials and Methods*. Free FAs mixture (oleate: BSA-palmitate) was added to NSCs for positive control. BSA was used for endogenous control. Representative images of NSCs with nuclei stained with hematoxylin (blue). Lipid droplets appear orange/red and are indicated by arrows. Scale bar, 25 μm.

The energy yield per molecule of palmitic acid is three times higher than per glucose molecule. (Knobloch, 2016) NSCs may develop strategies to compensate the deficiency of LCAD either by induction of other mitochondrial acyl-CoA dehydrogenases or by enhancement of glucose oxidation for energy production. (Tucci *et al*., 2012; Bakermans *et al*., 2013) Thus, a decreased β-oxidation of long-chain FAs might ignite a metabolic shift in differentiating NSCs. Western blot validation of TUDCA-induced downregulation of LCAD was a turning point in this work, regarding the emerging need to clarify the metabolic paradigm of NSC fate control. Therefore, we decided to explore how TUDCA modulates the balance between lipid oxidation and biosynthesis.

3.2 TUDCA promotes *de novo* lipogenesis in early differentiation of NSCs

3.2.1 TUDCA increases SREBP-1 expression

SREBPs are basic-helix-loop-helix-leucine zipper transcription factors synthesized as inactive precursors bound to the membranes of the ER. Upon activation, the ER-anchored SREBP precursor

undergoes a cleavage process and stays as a nuclear active form of SREBP. (Eberlé *et al*., 2004) SREBP-1 has two different isoforms, SREBP-1a and 1c, both produced from a single gene through alternative splicing. SREBP-1c is the predominant isoform expressed in most tissues of mice and humans, with high levels in the brain. In differentiating NSCs, TUDCA significantly increased the levels of nuclear SREBP-1 constitutively active form, when compared to control. (Figure 3.7)

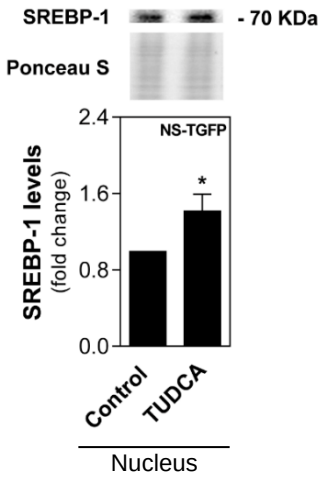


Figure 3.7 – TUDCA increases SREBP-1 nuclear levels in differentiating NSCs. NSCs, from the NS-TGFP cell line, were expanded and induced to differentiate for 24 h, in the presence or absence of TUDCA, and then collected for immunoblotting analysis. Representative immunoblots of SREBP-1 levels (top) and corresponding densitometry analysis (bottom) in nuclear protein extracts. SREBP-1 levels were normalized to Ponceau S. Data are expressed as mean ± SEM fold-change for at least three independent experiments. *$p < 0.05$ from non-treated differentiating cells (control).

SREBP-1c promotes the expression of two key enzymes, ACC and FASN, for *de novo* FA synthesis. Intriguingly, insulin may represent a clue for TUDCA induction of SREBP-1 expression. Among distinct mechanisms, independent of any effect on SREBP-1c transcription or glucose cellular uptake, insulin *per se* is able to rapidly modulate SREBP-1c concentration in the nucleus by proteolytic cleavage activation. (Eberlé *et al*., 2004) Also, TUDCA was shown to improve insulin signaling and sensitivity. (Ozcan *et al*., 2006; Guo *et al*., 2015) Therefore, TUDCA might increase SREBP-1c expression *via* insulin in NSCs, which in turn could be further investigated in our model. In fact, insulin is a critical component in N-2 and B-27 supplements present in the serum-free medium of NSCs, as mentioned in *Materials and Methods*. (Bieberich, 2012) Insulin and the insulin-like growth factors (IGFs) support diverse essential roles in neurogenesis. Moreover, it is well-known that insulin and/or IGFs potentiate the proliferation of NPCs. (Ziegler *et al*., 2015) However, it would be important to first quantify the levels of SREBP-1c precursor or measure SREBP-1c mRNA levels to check other mechanisms by which insulin signaling may modulate SREBP-1c levels in NSCs, even because SREBP-1c is mainly regulated by insulin at the transcriptional level. (Eberlé *et al*., 2004)

Therefore, TUDCA appears to regulate a major upstream regulator of lipogenesis, which can be important for proliferation of NSCs at an early stage of differentiation. Indeed, this bile acid has

been shown to increase NSC proliferation (Xavier *et al*., 2014; Soares *et al*., 2017). In concordance, chronic administration of both TUDCA and UDCA in rats significantly increased the overall hepatic microsomal lipid content and composition. This effect was particularly evident for phospholipids and free FAs. (Bellentani *et al*., 1996) In conclusion, TUDCA might privilege anabolic lipogenesis to enable generation of lipid membranes, required to sustain high SC proliferation.

3.2.2 TUDCA increases the expression of palmitic and stearic acid

Palmitic acid is a saturated 16-carbon FA while stearic acid is a saturated 18-carbon FA, two long-chain FAs. Palmitate is the predominant product of FASN, synthesized from three substrates, acetyl-CoA, malonyl-CoA, and NADPH, and can also be elongated by FASN to stearate. (Chorna *et al*., 2013) Remarkably, TUDCA appeared to increase the levels of these two FAs at 24 h of NSC differentiation. (Figure 3.8) Palmitate and stearate serve as building blocks and can be elongated and unsaturated to form more complex lipids who may have critical cell signaling functions. (Bieberich, 2012; Knobloch *et al*., 2016)

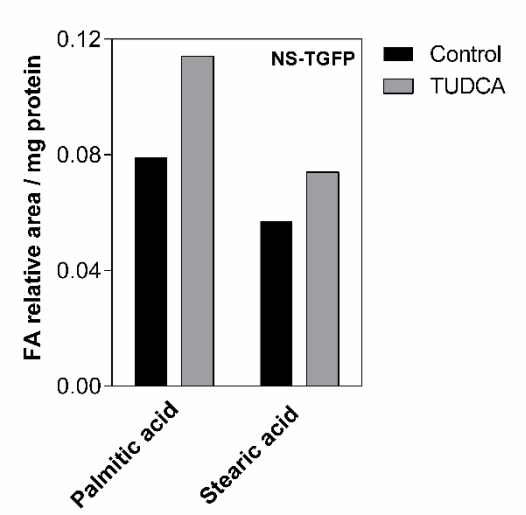


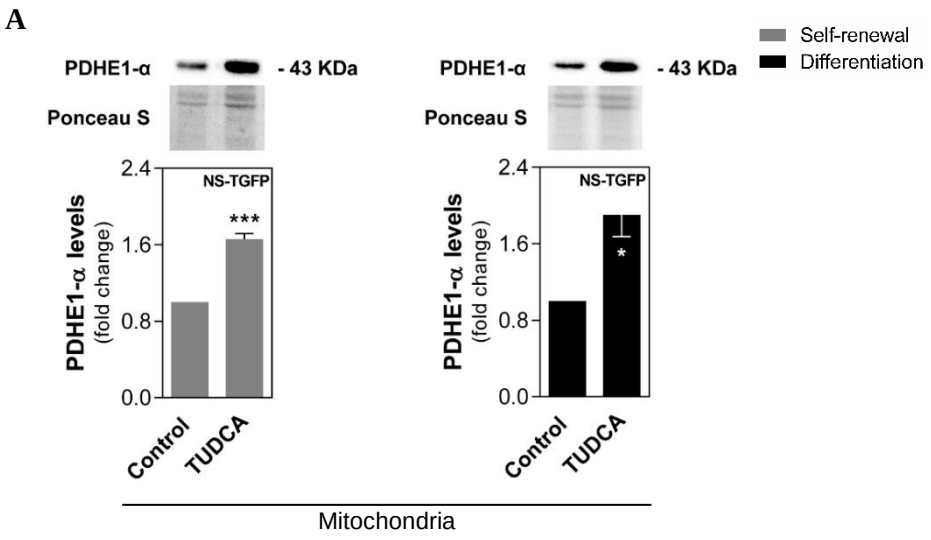
Figure 3.8 – TUDCA increases the cellular amount of palmitic and stearic acid in differentiating NSCs. NSCs, from the NS-TGFP cell line, were untreated or treated with TUDCA for 24 h, in differentiating conditions and then collected for GC-MS analysis. Saturated FAs levels were normalized to total mg of cellular protein. Data are expressed as FA relative area over mg of total protein for one single experiment.

Interestingly, increased FASN mRNA levels was associated with increased proliferation of NSCs in the hippocampus of adult mice upon running. Subsequent FA profiling revealed higher hippocampal-specific accumulation of palmitate and stearate FAs. (Chorna *et al*., 2013) Notice that these are preliminary results, however, they confirm TUDCA-induced *de novo* lipogenesis through transcriptional activation of FASN by SREBP-1 increased expression in early stage of NSC differentiation. In support of this view, conditional knockout of FASN leads to the loss of lineage-traced NSCs and to a dramatic loss of newly generated neurons, both in the SVZ and in the DG,

demonstrating the crucial role of lipid metabolism in adult neurogenesis. (Knobloch *et al*., 2012) Thus, FASN expression should be further evaluated in the presence of TUDCA. We formally hypothesize that TUDCA increases NSC proliferation through *de novo* biosynthesis of FAs. Next, we attempt to unravel the possible impact of TUDCA in cellular energy production of differentiating NSCs through glucose metabolism.

3.3 TUDCA increases PDHE1-α expression in NSCs

Pyruvate dehydrogenase E1 component subunit alpha (PDHE1-α) contains the E1 active site from PDC, therefore playing a key role in its function. The PDC provides the primary link between glycolysis and the TCA cycle by catalyzing the irreversible conversion of pyruvate into acetyl-CoA. (Varum *et al*., 2011) Our results demonstrated that TUDCA increases the mitochondria levels of PDHE1-α. In NS-TGFP cell line, PDHE1-α levels increased up to 1.7-fold in undifferentiated NSCs and 1.9-fold in differentiating NSCs, when compared to the respective controls. (Figure 3.9 (A)) Similarly, in CGR8 cells, PDHE1-α levels increased up to 1.5- and 1.8-folds in undifferentiated and differentiating NSCs, respectively. (Figure 3.9 (B))



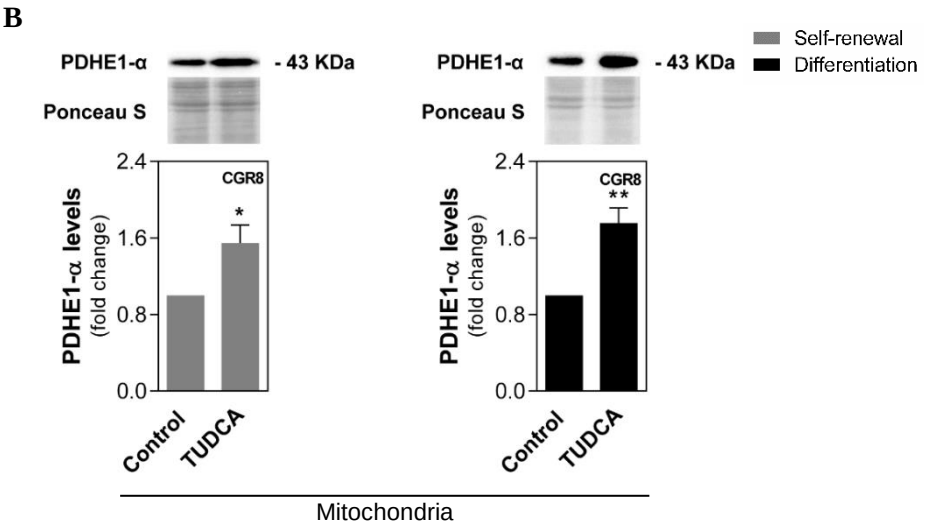


Figure 3.9 – TUDCA increases mitochondrial levels of PDHE1-α either during self-renewal or differentiation of NSCs in two different mouse NSC lines. NSCs, from NS-TGFP and CGR8 cell lines, were untreated or treated with TUDCA for 24 h in self-renewal or differentiating conditions and then collected for immunoblotting analysis. Representative immunoblots of PDHE1-α levels (top) and corresponding densitometry analysis (bottom) in mitochondrial protein extracts of NS-TGFP **(A)** and CGR8 **(B)** cell lines. PDHE1-α levels were normalized to Ponceau S. Data are expressed as mean ± SEM fold-change for at least three independent experiments. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ from non-treated undifferentiated or differentiating cells (control).

The upregulation of PDHE1-α might indicate that TUDCA privileges the conversion of pyruvate into mitochondrial acetyl-CoA rather than lactate. Nevertheless, it may not necessarily lead to an increase in PDC activity because PDC is tightly controlled *via* reversible inactivating phosphorylation. (Boer & Hounten, 2014) This should be further investigated in future experiments. Interestingly, human pluripotent SCs display more phosphorylated PDHE1-α, when compared with differentiated cells, suggesting inactivation of the PDC and lower levels of substrate entering the TCA cycle in undifferentiated cells. (Varum *et al*., 2011) Consistently, the expression of PDK 2 and 4, favors glycolysis, which is important for the quiescence and function of mouse hematopoietic SCs. (Takubo *et al*., 2013) mRNA levels of PDK 1, 3 and 4 were also found significantly declined during neuronal differentiation as the overall changes in PDC favor increased PDC complex activity in neurons. (Zheng *et al*., 2016) In this cellular context, it is possible that TUDCA might increase PDHE1-α levels through PCG1-α upregulation. (Soares *et al*., 2017) It has been shown that PCG1-α increases PDHE1-α protein content without influencing pyruvate dehydrogenase regulation. (Kiilerich *et al*., 2010) Nevertheless, these findings bring insightful signs that TUDCA-induced expression of PDHE1-α might really favor PDC activity and, consequently, modulate intracellular levels of acetyl-CoA at least in differentiating NSCs.

Importantly, acetyl-CoA is a central metabolite that interconnects multiple metabolic pathways. From a biosynthetic point of view, acetyl-CoA is the building block for the biosynthesis of

lipids, requiring the active export of 'acetyl-CoA' equivalents in the form of citrate into the cytosol. (Frezza, 2017) Nevertheless, the major fate of acetyl-CoA is its oxidation. (Boer & Hounten, 2014) Therefore, PDHE1-α upregulation might prove useful to fuel OXPHOS for cellular energy production. Of note, TUDCA increases ATP levels dependent on F1F0-ATP synthase complex upon 24 h of differentiation of NSCs. (Xavier *et al*., 2014) Acetyl-CoA is also the obligatory acetyl donor for lysine acetylation reactions, possibly linking metabolic activity to epigenetics. (Boer & Hounten, 2014) In support of this view, we decided to further investigate the involvement of a new described mechanism for mitochondria-nucleus PDC communication in TUDCA mechanism of action.

3.4 TUDCA potentiates a novel mechanism for mitochondria-nucleus PDC cross-talk in early differentiating NSCs

3.4.1 TUDCA induces nuclear translocation of PDHE1-α

Sutendra *et al.* recently demonstrated that nuclear PDHE1-α exists in human normal and cancer cells. Better characterized in cancer cells, the levels of functional nuclear PDC are elevated in a cell cycle-dependent manner, particularly upon mitogenic stimulation (during S phase), and chemical stress, concomitant with a reduction in mitochondrial PDC levels. (Sutendra *et al*., 2014) Surprisingly, our results showed that PDHE1-α was indeed present in the nucleus of NSCs and being further accumulated by TUDCA in this cellular compartment. During self-renewal of NSCs, TUDCA slightly induced the nuclear expression of PDHE1-α while, in differentiating NSCs, this effect was markedly evident, increasing PDHE1-α nuclear levels in 70%, when compared to control. (Figure 3.10 (A)) Although the mechanism of PDC mitochondrial-nuclear translocation remains unclear, it appears to be dependent on the chaperone Hsp70, which facilitates its nuclear import through association with E1 and E2, two of the three enzymes of PDC. Hsp70, in turn, is involved in the nuclear translocation of several proteins and is known to activate mitochondrial PDC. Induction of Hsp70 is cell cycle-dependent, with its highest expression also observed during S phase. (Sutendra *et al*., 2014; Tang, 2015) In agreement, our results demonstrated that Hsp70 is expressed at mitochondria. Moreover, its expression is increased in differentiating NSCs treated with TUDCA. (Figure 3.10 (B)) Importantly, the purity of mitochondrial and nuclear fractionation was certified (annex A).

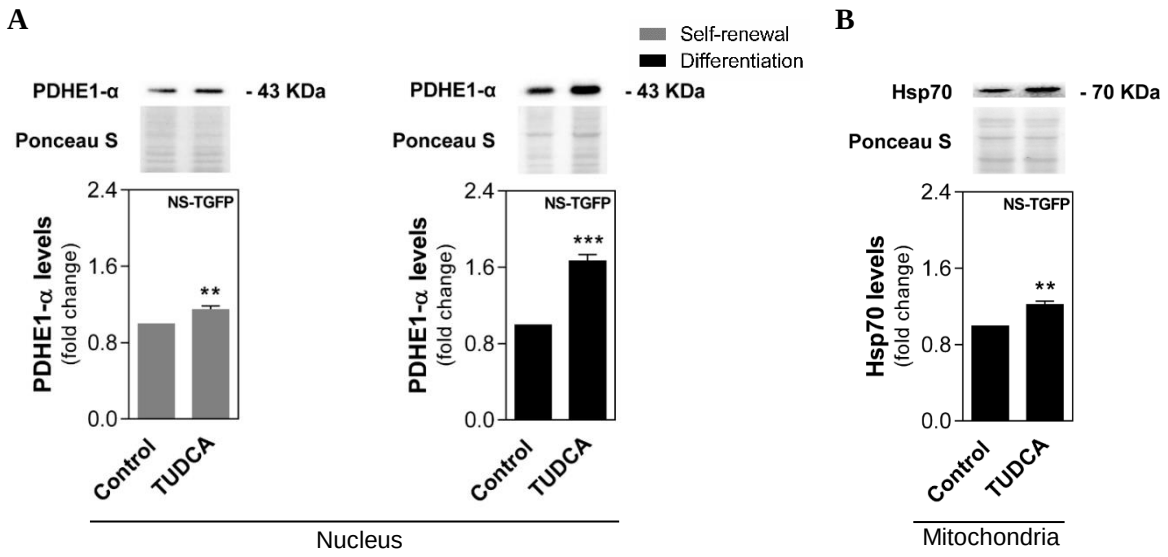


Figure 3.10 – TUDCA promotes nuclear traffic of PDHE1-α and increased mitochondrial expression of Hsp70 in NSCs. NCSs, from the NS-TGFP cell line, were untreated or treated with TUDCA for 24 h in self-renewal or differentiating conditions and then collected for immunoblotting analysis. Representative immunoblots of PDHE1-α **(A)** and Hsp70 **(B)** levels (top) and corresponding densitometry analysis (bottom) in both nuclear and mitochondrial protein extracts, respectively. PDHE1-α and Hsp70 levels were normalized to Ponceau S. Data are expressed as mean ± SEM fold-change for at least three independent experiments. $^{**}p < 0.01$ and $^{***}p < 0.001$ from non-treated undifferentiated or differentiating cells (control).

The cellular distribution of PDHE1-α in our model was also evaluated by immunocytochemistry, colocalizing with mitochondria organelles. In fact, PDHE1-α was detectable in the nucleus of differentiating NSCs untreated or treated with TUDCA for 24 h. Corroborating our previous results, TUDCA-induced upregulation of PDHE1-α can be observed either in mitochondria or in the nucleus, when compared to control. (Figure 3.11) This experiment should, however, be repeated and performed using confocal microscopy to acquire images with higher resolution.

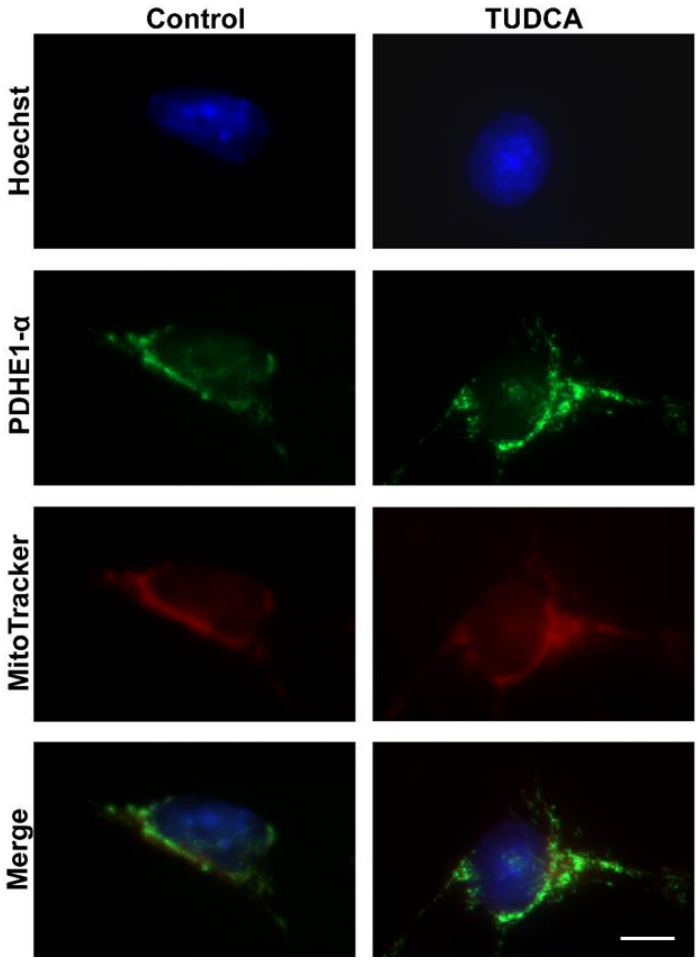


Figure 3.11 - TUDCA increases mitochondrial and nuclear levels of PDHE1-α in differentiating NSCs. Immunohistochemistry against the enzymatic subunit for glucose metabolism, PDHE1-α, was performed in NSCs, from the NS-TGFP cell line, treated with or without TUDCA for 24 h, as described in *Materials and Methods*. Representative images of immunofluorescence detection of cells labeled with an anti-PDHE1-α antibody (green). Nuclei were stained with Hoechst 33258 (blue) and mitochondria with Mitotracker (red). Scale bar, 20 μm.

Since TUDCA-induced nuclear levels, PDHE1-α did not result in concomitant mitochondrial PDHE1-α decrease, we presume that TUDCA increases the total cellular levels of PDHE1-α. It would be important to look for other nuclear subunits of PDC as well as to confirm that PDC is functional in the nucleus of NSCs. Notably, unlike mitochondrial PDC, nuclear PDC is not subject to PDK phosphorylation. The acetyl-CoA generated by PDC in the nucleus is apparently important for histone acetylation, as required for S phase entry and cell cycle progression. (Tang, 2015) In fact, TUDCA enhances NSC proliferation by modulating cell cycle progression, decreasing cells in G0-G1 phase and markedly increasing S/G2-M phases 24 h after the induction of NSC differentiation. (Xavier *et al*., 2014) In this regard, we decided to measure acetylation levels of H3 in differentiating NSCs.

3.4.2 TUDCA significantly increases histone 3 content and acetylation levels

Histone modifications may determine different neural cell phenotypes through a complex interplay of specific regulators and multiple cellular factors. (Podobinska *et al*., 2017) Our results demonstrated that histone 3 (H3) total levels slightly increased in the presence of TUDCA. (Figure 3.12 (A)) In addition, the levels of histone 3 acetylation were also similarly increased by TUDCA. (Figure 3.12 (B)) This indicates that TUDCA-induced H3 expression is accompanied by an increase in H3 acetylation levels. However, when acetyl-H3 levels were normalized to total levels of H3 (data not shown), we did not detect any significant difference in acetyl-H3 levels by TUDCA treatment, indicating that the increase in acetylation process is, indeed, dependent on H3 total expression levels. Importantly, the purity of histone extracts was carefully certified (annex A).

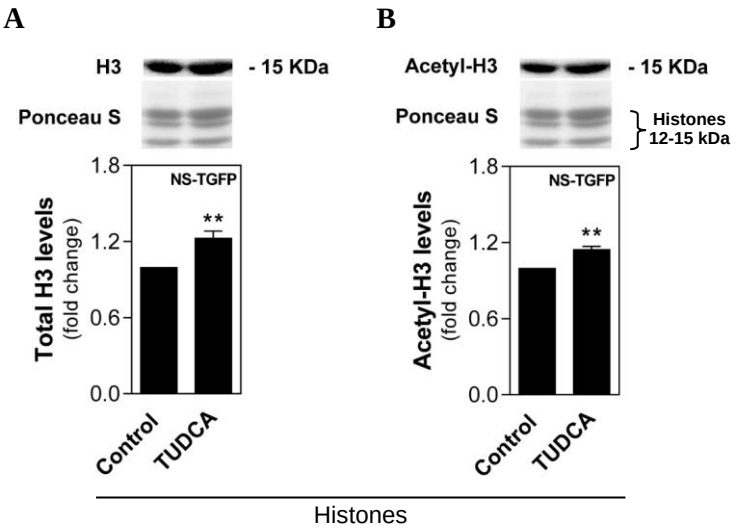


Figure 3.12 – TUDCA increases total histone 3 their acetylation levels in differentiating NSCs. NSCs, from the NS-TGFP cell line, were expanded and induced to differentiate for 24 h in the presence or absence of TUDCA, and then collected for immunoblotting analysis**.** Representative immunoblots of H3 total **(A)** and acetyl-H3 **(B)** levels (top) and corresponding densitometry analysis (bottom) in purified histone extracts. Acetyl-H3 and H3 total levels were normalized to Ponceau S. Data are expressed as mean ± SEM fold-change for at least three independent experiments. $^{**}p < 0.01$ and from non-treated differentiating cells (control).

TUDCA-induced H3 supply might be important for the S phase of cell cycle. Lack of *de novo* histone content can extend S phase and cause cell cycle arrest during G2 phase, preventing cells from mitosis. (Günesdogan *et al*., 2014)

The availability or proximity of acetyl-transferases or deacetylases with acetyl-CoA-generating enzymes, like PDC, defines the acetylation rates of targets in the nucleus. Curiously, there is a rapid turnover of acetyl groups in histones, compared to those of other proteins. This, in turn, may explain why we were not able to detect a marked increase on histone acetylation. To confirm whether the translocation of PDHE1-α really affects histone acetylation in NSCs we should perhaps adopt a more complex strategy, by firstly synchronizing NSC acetylation, silencing PDHE1-α through siRNA

in pyruvate-treated cells, and measuring histone acetylation, as described in cancer cells. (Sutendra *et al*., 2014)

Histone acetylation usually depends on glucose rather than FA metabolism. There are mechanisms capable of generating acetyl-CoA within the nucleus, such as the nuclear form of ATP citrate lyase using citrate. (Wellen *et al*., 2009) Interestingly, TUDCA was found to up-regulate ATP citrate lyase transcript in primary rat hepatocytes. (Castro *et al*., 2005) Indeed, nuclear PDC ability to generate acetyl-CoA may become important when the citrate pool is depleted or shifted toward lipid synthesis. (Tang, 2015) Nucleocytosolic amounts of acetyl-CoA, in turn, can facilitate lipid synthesis and histone acetylation processes. (Shi & Tu, 2015) In fact, it was hypothesized that acetyl-CoA generated by nuclear PDC may also be useful for certain biochemical processes, such as lipid synthesis. (Boer & Houten, 2014) In this regard, it is possible that increased acetyl-CoA generation by TUDCA-induced PDC in the nucleus may also be required for lipid synthesis, in addition to histone acetylation.

Overall, it would be interesting to further explore this novel metabolic cross-talk triggered by TUDCA.

3.5 TUDCA increases VDAC expression in differentiating NSCs

VDAC has an active role in the cross-talk between mitochondria and the rest of the cell. VDAC is known to be responsible for ATP/ADP exchange as well as for the flux of several metabolites across OMM, controlling the switch between open and closed states. (Lemasters & Holmuhamedov, 2006)

During self-renewal of NSCs, VDAC levels did not change in the presence of TUDCA (Figure 3.13 (A) and (B)), while upon 24 h of differentiation, mitochondrial VDAC levels markedly increased up to 2.3-and 1.4-folds in NSCs from the NS-TGFP cell line (Figure 3.13 (A)) and the CGR8 cell line (Figure 3.13 (B)), respectively, when compared to respective controls. Since TUDCA was shown to modulate the levels of VDAC in our study, the Western blot data from mitochondrial extracts could not be normalized to endogenous levels of VDAC.

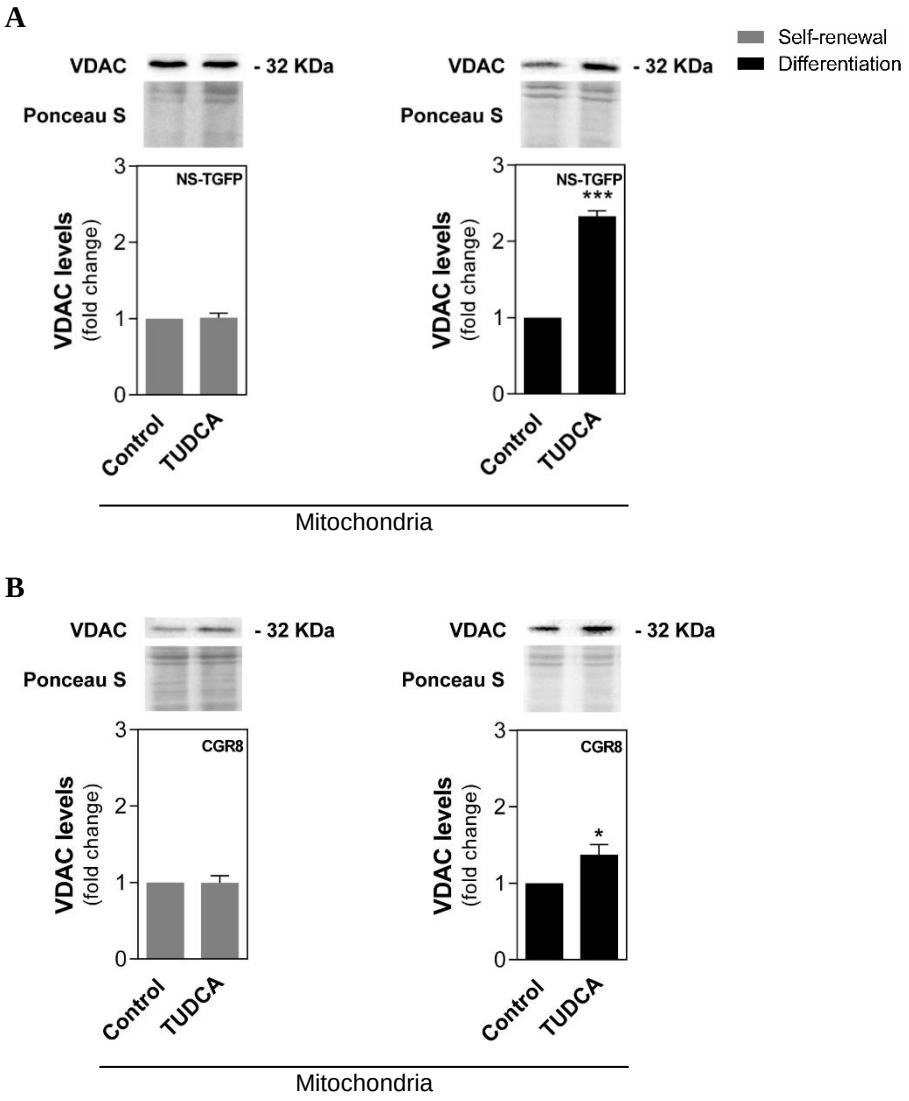


Figure 3.13 – TUDCA increases VDAC levels in two different mouse NSCs lines. NSCs, from NS-TGFP and CGR8 cell lines, were treated or untreated with TUDCA for 24 h in self-renewal or differentiating conditions, and then collected for immunoblotting analysis. Representative immunoblots of VDAC levels (top) and corresponding densitometry analysis (bottom) in mitochondrial protein extracts of NS-TGFP **(A)** and CGR8 **(B)** cell lines. VDAC levels were normalized to Ponceau S. Data are expressed as mean ± SEM fold-change for at least three independent experiments. *$p < 0.05$ and ***$p < 0.001$ from non-treated undifferentiated or differentiating cells (control).

VDAC overexpression may also serve as a crucial factor in the process of mitochondria-mediated apoptosis. However, as previously described, TUDCA acts as an anti-apoptotic and antioxidant molecule at the mitochondrial level. In fact, it prevents mitochondrial apoptotic events in early-stage of NSC differentiation. (Xavier *et al*., 2014) TUDCA also reverted the decrease in mtDNA copy number in NSCs and increased the mRNA levels of PGC-1α and ERRα in postnatal NSCs at 24 h of differentiation, suggesting that this bile acid enhances mitochondrial biogenesis to assure NSC proliferation and differentiation potential. (Xavier *et al*., 2014; Soares *et al*., 2017) Our results, support

this view, in which TUDCA induces VDAC expression upon 24 h of NSC differentiation, and thus mitochondrial biogenesis. Despite the fact that VDAC is a nuclear-encoded protein, this protein is absolutely essential for mitochondrial homeostasis. Except for relatively few membrane-permeant lipophilic compounds, all metabolites must cross the OMM through VDAC. VDAC is even essential for the mitochondrial import of long-chain FAs. Further, generation of ATP in the mitochondrial matrix from F1F0-ATPase requires efficient transport of metabolites, including cytosolic ADP, ATP, and inorganic phosphate, across both mitochondrial membranes for OXPHOS. (Lemasters & Holmuhamedov, 2006) Notably, the inhibition of the mPTP has been shown to suppress neuronal differentiation in cortical neural progenitors. (Hou *et al*., 2013) Furthermore, Levy *et al*. demonstrated that VDAC deficient mice presented learning and hippocampal synaptic plasticity impairment, indicating that the activation of mPTP is crucial for neuroplasticity, in particular for synaptic Ca^{2+} buffering. (Levy *et al*., 2003) Indeed, VDAC is an important regulator of Ca^{2+} transport in and out of mitochondria. Overexpression of VDAC enhances the mitochondrial matrix Ca^{2+} concentration by allowing fast diffusion of Ca^{2+} from the ER to the IMM. Since Ca^{2+} represents a cofactor for metabolic enzymes, including pyruvate dehydrogenase, VDAC does participate in the control of energy production. (Shoshan-Barmatz & Gincel, 2003) Thus, VDAC overexpression possible provides plenty of Ca^{2+} to match TUDCA-induced expression of PDC with its activity.

VDAC can exert a complex control over mitochondrial metabolism and, therefore, over cell fate. (Lemasters & Holmuhamedov, 2006) Thus, it would be useful to understand the full impact of VDAC upregulation by TUDCA.

3.6 Metabolic protein expression profile in early stages of neural differentiation

How metabolism is reprogrammed during neural differentiation remains practically unknown. LCAD, SREBP-1, and PDHE1-α proteins play pivotal roles in cellular metabolism and were modulated by TUDCA. Therefore, the final step in this project was to evaluate the expression of these proteins throughout early stages of NSC differentiation.

Neural differentiation is a multistep process that shapes and re-shapes NSCs by progressing through several typical stages. LCAD and SREBP-1 (active form) exhibited differential expression during early neural differentiation. Our results showed that LCAD protein levels gradually increased up to 2.5-fold (Figure 3.14 (A)), while SREBP-1 protein levels decreased by 0.5-fold (Figure 3.14 (B)) after 3 days of differentiation, both when compared to undifferentiated cells (0 h).

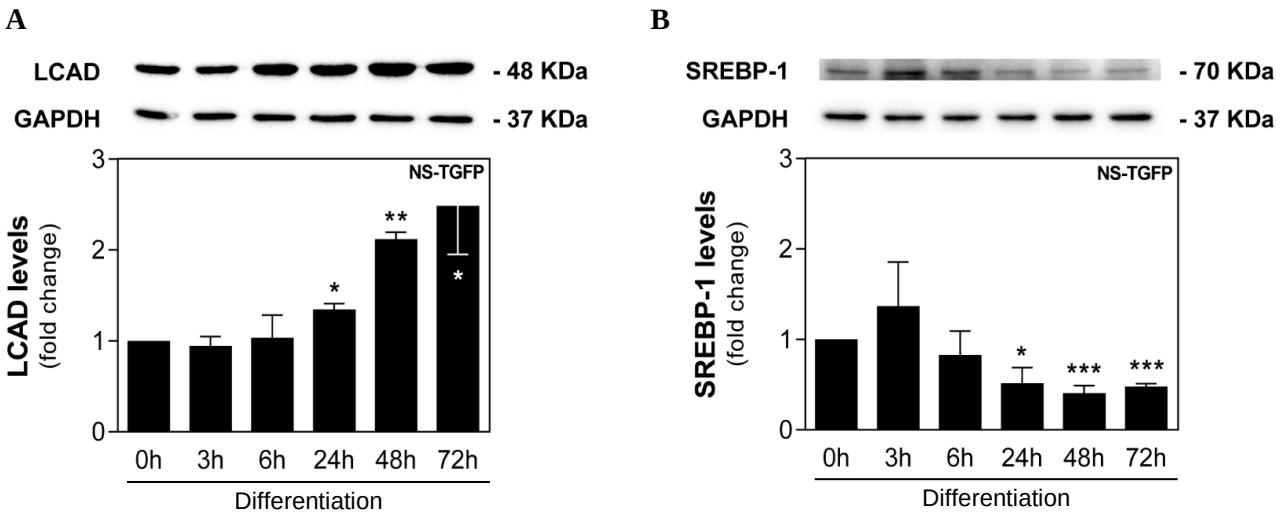


Figure 3.14 – NSC differentiation is associated with increased LCAD and decreased SREBP-1 levels. NSCs, from the NS-TGFP cell line, were induced to differentiate up to 72 h and collected for immunoblotting analysis, as described in *Materials and Methods*. Representative immunoblots of LCAD **(A)** and SREBP-1 **(B)** levels (top) and corresponding densitometry analysis (bottom) in total protein extracts. LCAD and SREBP-1 levels were normalized to GAPDH protein levels. Data are expressed as mean ± SEM fold-change for at least three independent experiments. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ from undifferentiated cells.

It is estimated that NSCs give rise to a population of amplifying neural progenitors during the first 24 h of differentiation. In turn, following days are pivotal for cell fate specification, when they can differentiate and transit to new immature neurons (check figure 1.3). It is now known that during the transition from quiescent to active NSC, both glycolysis and FAO gradually decrease, while OXPHOS of glucose overcomes NSC metabolism. (Shin *et al*., 2015; Fidaleo *et al*., 2017) Our results suggest that β-oxidation of long-chain FAs takes over along fate specification, while lipogenesis tends to be diminished due to lower levels of SREBP-1. Indeed, these events reflect how much the β-oxidation process is important to provide energy for differentiation. Neurons avoid extensive FA oxidation and favor glucose oxidation in the brain. (Schönfeld & Reiser, 2013) Although, at this stage of NSC development, high rate of oxidative metabolism through oxidation of long-chain FAs no longer represents an alternative energy fuel, it might support OXPHOS for energy production.

On the other hand, the total levels of PDHE1-α did not show any substantial differences during neural differentiation, remaining basal. (Figure 3.15) Perhaps because the required metabolic shift toward OXPHOS does not depend on PDHE1-α increased expression. Indeed, it is possible that the impact of differentiation depends on regulation of other changes, such as PDC kinase activity and phosphatase levels.

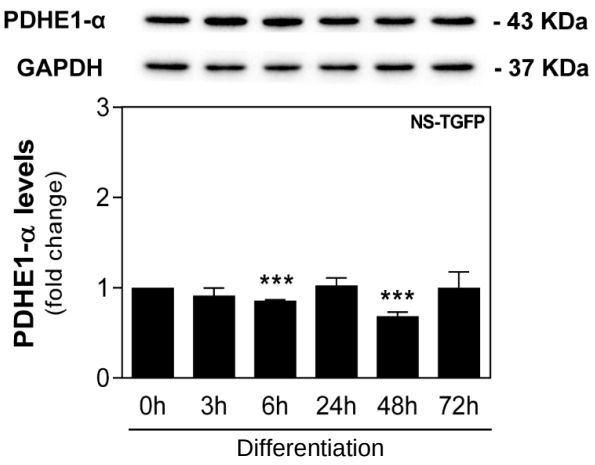


Figure 3.15 – PDHE1-α protein expression is not affected during neural differentiation. NSCs, from the NS-TGFP cell line, were induced to differentiate up to 72 h, and collected for immunoblotting analysis, as described in *Materials and Methods*. Representative immunoblots of PDHE1-α levels (top) and corresponding densitometry analysis (bottom) in total protein extracts. PDHE1-α levels were normalized to GAPDH protein levels. Data are expressed as mean ± SEM fold-change for at least three independent experiments. ***$p < 0.001$ from undifferentiated cells.

Pyruvate dehydrogenase activity is inhibited in response to site-specific phosphorylation at three different serine residues on PDHE1-α with different affinities. Curiously, PDH Ser300 phosphorylation, a major inhibitory site, decreases considerably during neuronal differentiation which is consistent with a more active PDC. As previously mentioned, neural activity is tightly coupled to glucose oxidation. (Zheng *et al*., 2016) Thus, PDC activity certainly has an important role in neuronal differentiation through post-translational modifications.

4. Conclusions and Future Perspectives

Adult neurogenesis may well contribute to the capacity of the brain to maintain physiological tissue homeostasis required for the regeneration of damaged tissue. (Ma *et al*., 2009) During development, neurogenesis must be coordinated at the temporal and spatial level to generate a functioning CNS. Here, an extensive proliferation is demanded to produce the required number of progenitor cells for correct tissue and organ formation, followed by neuronal differentiation. (Hardwick *et al*., 2015) Further, the decrease in proliferation of NSC precursors is a primary factor for neurogenesis decline throughout life. (Kuhn *et al*., 1996) Thus, a better understanding of the mechanisms that regulate proliferation and differentiation potential of adult NSCs proves to be pertinent for the discovery of novel competitive clinical approaches leading to enhanced brain capacity for self-repair.

The presented work revealed that the endogenous neuroprotective bile acid, TUDCA, drives NSC fate through metabolic remodeling. TUDCA was previously described as an antiapoptotic and antioxidant mitochondrial acting agent in NSCs and in mature neurons. (Rodrigues *et al*., 2000; Xavier *et al*., 2014) Furthermore, TUDCA has been shown to enhance NSC proliferation and neuronal differentiation, both *in vitro* and *in vivo*. (Xavier *et al*., 2014; Soares *et al*., 2017)

Our work contributed to deciphering TUDCA mitochondrial proteomic signature, demonstrating that TUDCA also regulates metabolic-related proteins in this context. β-F1-ATPase and Gβ2 proteins were not validated as targets of TUDCA, although they could have major implications in mitochondria function, and therefore in NSCs behavior. Surprisingly, hnRNP A2/B1 modulation might support a metabolic molecular program adaptation to NSC differentiation regarding its translocation from nucleus to the cytoplasm. However, the downregulation of LCAD by TUDCA appeared as a major finding, possibly leading to a fine-tune reduction of mitochondrial β-oxidation of long-chain FAs in differentiating NSCs. Importantly, we have shown that TUDCA does not generate intracellular lipid accumulation in NSCs. In fact, alterations in lipid metabolism are associated with many cognitive disorders, including Huntington's, Parkinson's and Alzheimer's diseases, and were already shown to directly interfere with NSC behavior, decreasing NSC proliferation. (Hamilton *et al*., 2015) In contrast, TUDCA seems to trigger a metabolic shift toward lipid synthesis, which is known to determine the proliferative activity of NSCs (Knobloch *et al*., 2012), in detriment of long-chain FA degradation. (Figure 4.1 (A)) TUDCA-induced SREBP-1 upregulation, perhaps *via* insulin, resulted in increased palmitic and stearic acid content probably relevant for the generation of lipid membranes to sustain NSC proliferation and/or to give rise to bioactive lipids. This may also support the neurogenesis process.

Importantly, NSCs live in niches that separate them from an unrestricted supply of nutrients. It is believed that NSCs reside in a metabolic defined environment. However, little is known about the

metabolic compartmentalization of developmental niches. In this work, the NSC differentiating growth medium has been supplemented with B-27, a mixture of ingredients including lipids such as LA, essential to NSC nutrition. (Bieberich, 2012) Thus, we are at least exposing NSCs to a vital form of lipid content that has to reach the neurogenic niche *in vivo*.

In addition, we also revealed that TUDCA upregulates PDHE1-α expression independently of NSC status, suggesting an increase in the amount of substrate entering the TCA cycle. The consequences of PDHE1-α increased expression by TUDCA in self-renewing NSCs remain completely to be explored. However, we think that this would help triggering the proliferation process due to an improvement in mitochondrial biomass and activity. In differentiating NSCs, TUDCA enhanced a novel signaling pathway of mitochondrial-nuclear communication boosting nuclear PDHE1-α as a mitochondrial expatriate and increased H3 content *per se*. Importantly, the acetylation levels of H3 accompanied the increase in total H3 levels, possibly due to a nuclear functional PDC. (Figure 4.1 (B)) This, in turn, might assure NSC cycle progression and differentiation-related epigenetic alterations. Curiously, VDAC expression is modulated by TUDCA only in differentiating NSCs, probably to respond to high mitochondrial metabolic turnover, as previously mentioned.

Lipid metabolic pathways have been known to play a major role in cancer, providing proliferation and survival advantages. (Menendez & Lupu, 2007) Proliferating tumor cells critically rely on cytosolic acetyl-CoA for membrane generation. (Lunt & Vander Heiden, 2011) Interestingly, similar lipid metabolic pathways are important for adult neurogenesis. (Knobloch, 2016) Similarly, TUDCA seems to drive NSC lipid metabolic choices into a 'lipogenic phenotype' well-known in the carcinogenic context. However, we should not forget that TUDCA properties in preserving mitochondrial integrity and function, and thus, avoiding its impairment, are also relevant for tumor-forming capacity. In fact, a tight metabolic control is crucial to prevent uncontrollable growth, as a certain level of OXPHOS might even be necessary to prevent oncologic transformation of NSCs. (Knobloch & Jessberger, 2017)

Nowadays the molecular events involved in neuronal differentiation are relatively well studied. Although we do not know much about their bioenergetic demands and how strictly the energy metabolism is ruled by a genetic developmental programme. Our results showed that the expression profile of LCAD increases during neural differentiation contrasting with the expression profile of SREBP-1, suggesting that NSCs gradually rely more on lipid catabolic pathways to meet energy demands of the differentiation process. Remarkably, our results appear to suggest that this endogenous bile acid causes an inverse shift in the expression of these two proteins, driving the cell into a more proliferative state and probably delaying fate specification of NSCs. Xavier *et al*. and Soares *et al*. have demonstrated that TUDCA favors neuronal rather than glial differentiation of NSCs. (Xavier *et al*., 2014; Soares *et al*., 2017) Indeed, changes in the metabolic profile of NSCs may also have a substantial impact on lineage choice even by the interchange of mitochondrial retrograde signals and its impact on cell cycle progression of NSCs. (Xavier *et al*., 2014)

For future work, it would be interesting to perform an efficient knockdown of LCAD and understand the impact of LCAD during NSC differentiation. In addition, the ratio of acetyl-CoA/CoA levels could also be measured in this cellular scenario, as an indication of NSC metabolic state. Another important aspect would be to also measure mitochondrial PDC activity in NSCs treated with TUDCA. Given the well-established survival role of taurine in several biological processes including in NSC proliferation (Hernández-Benítez *et al*., 2012; Ramos-Mandujano *et al*., 2014), we should also confirm the involvement of taurine itself in modulating these TUDCA-induced metabolic alterations.

In conclusion, our results unravel a novel TUDCA acting mechanism by which this bile acid promote NSC proliferation. (Figure 4.1 (A and B)) Furthermore, a coordinated regulation of lipid turnover through lipid biosynthesis *versus* lipid oxidation might be an effective way to increase NSC survival. In fact, the latest findings support our view, indicating that manipulation FAO is sufficient to instruct qNSCs to enter the cell cycle and to proliferate *in vitro*. (Knobloch *et al*., 2017) Our results reinforce that the neural role of TUDCA goes beyond its antiapoptotic and antioxidant capacities and may well become an efficient molecule in modulating NSC metabolism. This information together with the fact that this bile acid is non-toxic, safe and tolerable provides a new framework to keep exploring its use in NSC maintenance and expansion. We are now capable of moving towards a more comprehensive understanding of the metabolic machinery linking the processes of cell proliferation and neuronal differentiation. Finally, this work should be a useful resource for further analyses of metabolic proteins involved in neurodevelopment of adult NSCs toward a more efficient use of neural replacement therapies.

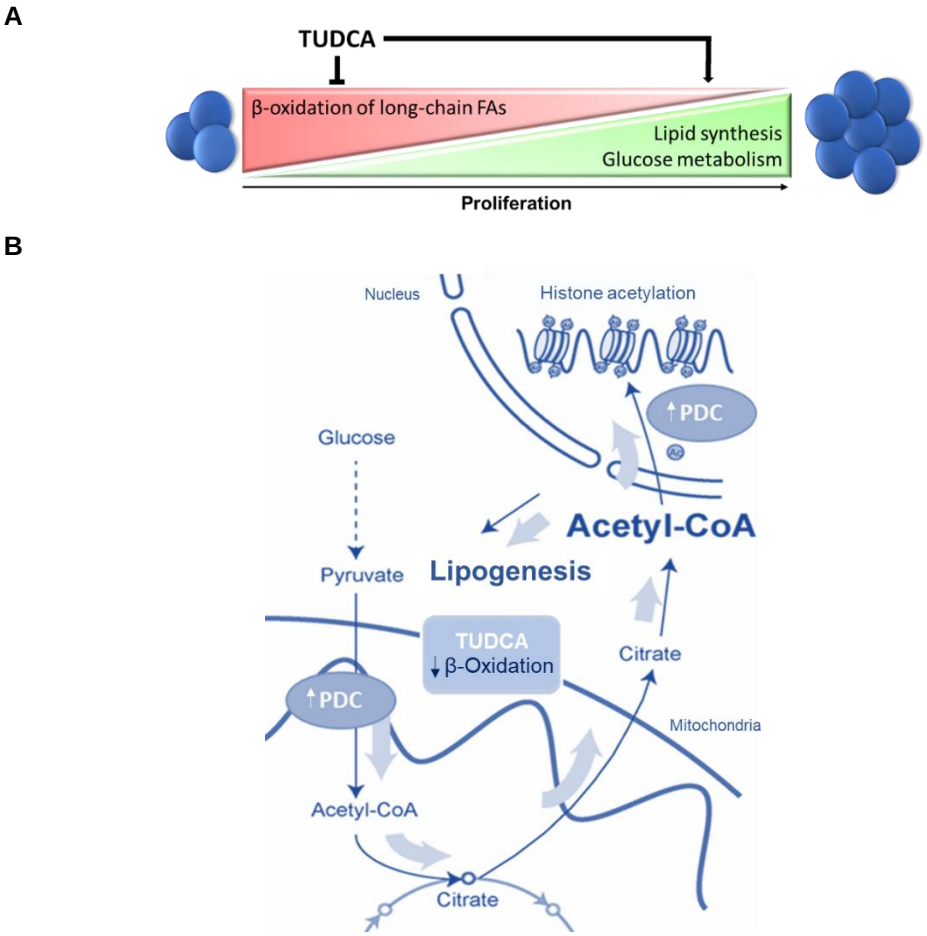


Figure 4.1 – Graphical abstract. (A) Schematic representation of TUDCA proposed modulation of NSC fate through a metabolic shift culminating in the increase of NSC proliferation. **(B)** Schematic diagram of TUDCA-induced events in differentiating NSCs toward a 'lipogenic phenotype' accompanied by the enhancement of a novel signaling pathway of mitochondrial-nuclear communication. Adapted from Shi & Tu, 2015.

5. References

Altman, J. & Das, G. D. (1965). Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. J Comp Neurol. 124(3): 319-335.

Amaral, J. D., Viana, R. J. S., Ramalho, R. M., Steer, C. J., & Rodrigues, C. M. P. (2009). Bile acids: regulation of apoptosis by ursodeoxycholic acid. J Lipid Res. 50(9): 1721-1734.

Ashrafi, G., & Schwarz, T. L. (2013). The pathways of mitophagy for quality control and clearance of mitochondria. Cell Death Differ. 20(1): 31-42.

Baharvand, H., & Matthaei, K. I. (2003). The ultrastructure of mouse embryonic stem cells. Reprod Biomed Online 7(3): 330-335.

Bakermans, A. J., Dodd, M. S., Nicolay, K., Prompers, J. J., Tyler, D. J., & Houten, S. M. (2013). Myocardial energy shortage and unmet anaplerotic needs in the fasted long-chain acyl-CoA dehydrogenase knockout mouse. Cardiovasc Res. 100(3): 441-449.

Bartlett, K., & Eaton, S. (2004). Mitochondrial β-oxidation. Eur. J. Biochem. 271: 462-469.

Beckervordersandforth, R., Ebert, B., Schäffner, I., Moss, J., Fiebig, C., Shin, J., … Lie, D. C. (2017). Role of Mitochondrial Metabolism in the Control of Early Lineage Progression and Aging Phenotypes in Adult Hippocampal Neurogenesis. Neuron 93(3): 560-573.e6.

Bellentani, S., Chao, Y. C., Ferretti, I., Panini, R., & Tiribelli, C. (1996). Chronic administration of ursodeoxycholic and tauroursodeoxycholic acid changes microsomal membrane lipid content and fatty acid compositions in rats. Biochem Biophys Res Commun. 220(3): 479–483.

Bentayeb, K., Batlle, R., Sánchez, C., Nerín, C., & Domeño, C. (2008). Determination of bile acids in human serum by on-line restricted access material-ultra high-performance liquid chromatography-mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci. 869(1-2): 1-8.

Bieberich, E. (2012). It's a lipid's world: bioactive lipid metabolism and signaling in neural stem cell differentiation. Neurochem Res. 37(6): 1208-1229.

Boer, V. C. J. De, & Houten, S. M. (2014). A Mitochondrial Expatriate: Nuclear Pyruvate Dehydrogenase. Cell 158(1): 9-10.

Bond, A. M., Ming, G. L., & Song, H. (2015). Adult Mammalian Neural Stem Cells and Neurogenesis: Five Decades Later. Cell Stem Cell 17(4): 385-395.

Bredesen, D. E., Rao, R. V, & Mehlen, P. (2006). Cell death in the nervous system. Nature 443(7113): 796-802.

Cagin, U., & Enriquez, J. A. (2015). The complex crosstalk between mitochondria and the nucleus: What goes in between? Int J Biochem Cell Biol., 63: 10-5.

Candelario, K. M., Shuttleworth, C. W., & Cunningham, L. A. (2014). Neural stem/progenitor cells display a low requirement for oxidative metabolism independent of hypoxia inducible factor-1 alpha expression. J Neurochem. 125(3): 420-429.

Casarosa, S., Bozzi, Y., & Conti, L. (2014). Neural stem cells: ready for therapeutic applications? Mol Cell Ther. 2: 31.

Castro, R. E., Solá, S., Ma, X., Ramalho, R. M., Kren, B. T., Steer, C. J., & Rodrigues, C. M. P. (2005). A distinct microarray gene expression profile in primary rat hepatocytes incubated with ursodeoxycholic acid. J Hepatol. 42(6): 897-906.

Castro-Caldas, M., Carvalho, A. N., Rodrigues, E., Henderson, C. J., Wolf, C.R., Rodrigues, C. M., & Gama, M. J. (2012). Tauroursodeoxycholic acid prevents MPTP-induced dopaminergic cell death in a mouse model of Parkinson's disease. Mol Neurobiol. 46(2): 475-486.

Cha, B. H., Jung, M. J., Moon, B. K., Kim, J. S., Ma, Y., Arai, Y., … Lee, S. H. (2016). Administration of tauroursodeoxycholic acid enhances osteogenic differentiation of bone

marrow-derived mesenchymal stem cells and bone regeneration. Bone 83: 73-81.
Chegary, M., Brinke, Ht., Ruiter, J. P., Wijburg, F. A., Stoll, M. S., Minkler, P. E., … Houten, S. M. (2009). Mitochondrial long chain fatty acid β-oxidation in man and mouse. Biochim Biophys Acta. 1791(8): 806-815.
Cho, J. G., Lee, J. H., Hong, S. H., Lee, H. N., Kim, C. M., Kim, S. Y., … Park, S. G. (2015). Tauroursodeoxycholic acid, a bile acid, promotes blood vessel repair by recruiting vasculogenic progenitor cells. Stem Cells 33(3): 792-805.
Choi, H. S., Lee, H. M., Jang, Y. J., Kim, C. H., & Ryu, C. J. (2013). Heterogeneous nuclear ribonucleoprotein A2/B1 regulates the self-renewal and pluripotency of human embryonic stem cells via the control of the G1/S transition. Stem Cells 31(12): 2647-2658.
Chorna, N. E., Santos-Soto, I. J., Carballeira, N. M., Morales, J. L., De La Nuez, J., Cátala-Valentin, A., … De Ortiz, S. P. (2013). Fatty acid synthase as a factor required for exercise-induced cognitive enhancement and dentate gyrus cellular proliferation. PLoS ONE 8(11): e77845.
Clark-Taylor, T. & Clark-Taylor, B. E. (2004). Is autism a disorder of fatty acid metabolism? Possible dysfunction of mitochondrial beta-oxidation by long chain acyl-CoA dehydrogenase. Med Hypotheses. 62(6): 970-975.
Conti, L., & Cattaneo, E. (2010). Neural stem cell systems: physiological players or in vitro entities? Nat Rev Neurosci. 11(3): 176-187.
Conti, L., Pollard, S. M., Gorba, T., Reitano, E., Toselli, M., Biella, G., Sun, Y., … Smith. A. (2005). Niche-independent symmetrical self-renewal of a mammalian tissue stem cell. PLoS Biol. 3(9): e283.
Costa, C. G., Dorland, L., Holwerda, U., de Almeida, I. T., Poll-The, B. T., Jakobs, C., & Duran, M. (1998). Simultaneous analysis of plasma free fatty acids and their 3-hydroxy analogs in fatty acid beta-oxidation disorders. Clin. Chem. 44(3), 463-471.
Cuezva, J. M., Ostronoff, L. K., Ricart, J., Heredia, M. L. De, Liegro, C. M. Di, & Izquierdo, J. M. (1997). Mitochondrial Biogenesis in the Liver during Development and Ontogenesis. J Bioenerg Biomembr. 29(4): 365-377.
Dhar, S. S., Ongwijitwat, S., & Wong-riley, M. T. T. (2008). Nuclear Respiratory Factor 1 Regulates All Ten Nuclear-encoded Subunits of Cytochrome c Oxidase in Neurons. J Biol Chem. 283(6): 3120-3129.
Dionísio, P. A., Amaral, J. D., Ribeiro, M. F., Lo, A. C., D'Hooge, R., & Rodrigues, C. M. P. (2015). Amyloid-β pathology is attenuated by tauroursodeoxycholic acid treatment in APP/PS1 mice after disease onset. Neurobiol Aging 36(1): 228-240.
Doze, V. A., & Perez, D. M. (2012). G-protein-coupled receptors in adult neurogenesis. Pharmacol.Rev. 64(3): 645-675.
Eberlé, D., Hegarty, B., Bossard, P., Ferré, P., & Foufelle, F. (2004). SREBP transcription factors: Master regulators of lipid homeostasis. Biochimie 86(11): 839-848.
Elia, A. E., Lalli, S., Monsurrò, M. R., Sagnelli, A., Taiello, A. C., Reggiori, B., … Albanese, A. (2016). Tauroursodeoxycholic acid in the treatment of patients with amyotrophic lateral sclerosis. Eur J Neurol. 23(1): 45-52.
Eriksson, P. S., Perfilieva, E., Björk-Eriksson, T., Alborn, A. M., Nordborg, C., Peterson, D. A., & Cage, F. H. (1998). Neurogenesis in the adult human hippocampus. Nat Med. 4(11): 1313–1317.
Ernst, A., & Frisén, J. (2015). Adult Neurogenesis in Humans- Common and Unique Traits in Mammals. PLoS Biol. 13(1): e1002045.
Fidaleo, M., Cavallucci, V., & Pani, G. (2017). Nutrients, neurogenesis and brain ageing: from disease mechanisms to therapeutic opportunities. Biochem Pharmacol S0006-2952(17): 30283-30286.
Filippis, L. De, & Binda, E. (2012). Concise Review: Self-Renewal in the Central Nervous System: Neural Stem Cells from Embryo to Adult. Stem Cells Transl Med. 1(4): 298-308.

Folmes, C. D. L., Nelson, T. J., Martinez-Fernandez, A., Arrell, D. K., Lindor, J. Z., Dzeja, … Terzic, A. (2011). Somatic Oxidative Bioenergetics Transitions into Pluripotency-Dependent Glycolysis to Facilitate Nuclear Reprogramming. Cell Metab. 14(2): 264-271.

Folmes, C. D. L., Park, S., & Terzic, A. (2013). Lipid Metabolism Greases the Stem Cell Engine. Cell Metab. 17(2): 153-155.

Frezza, C. (2017). Mitochondrial metabolites: undercover signalling molecules. Interface Focus 7(2): 20160100.

Fukumoto, S., & Fujimoto, T. (2002). Deformation of lipid droplets in fixed samples. Histochem Cell Biol. 118(5): 423-428.

Gage, F. H., & Temple, S. (2013). Neural Stem Cells: Generating and Regenerating the Brain. Neuron. 80(3): 588-601.

Glaser, T., Pollard, S. M., Smith, A., & Brustle, O. (2007). Tripotential differentiation of adherently expandable neural stem (NS) cells. PLoS One. 2(3): e298.

Gronbeck, K. R., Rodrigues, C. M. P., Mahmoudi, J., Bershad, E. M., Ling, G., Bachour, S. P., & Divani, A. A. (2016). Application of Tauroursodeoxycholic Acid for Treatment of Neurological and Non-neurological Diseases: Is There a Potential for Treating Traumatic Brain Injury? Neurocrit Care 25(1): 153-166.

Guha, M., Pan, H., Fang, J.-K., & Avadhani, N. G. (2009). Heterogeneous Nuclear Ribonucleoprotein A2 Is a Common Transcriptional Coactivator in the Nuclear Transcription Response to Mitochondrial Respiratory Stress. Mol Biol Cell. 20(18): 4107-4119.

Guha, M., Srinivasan, S., Guja, K., Mejia, E., Garcia-Diaz, M., Johnson, F. B., … Avadhani, N. G. (2016). HnRNPA2 is a novel histone acetyltransferase that mediates mitochondrial stress-induced nuclear gene expression. Cell Discov. 2: 16045.

Günesdogan, U., Jäckle, H., & Herzig, A. (2014). Histone supply regulates S phase timing and cell cycle progression. Elife 3: e02443.

Guo, Q., Shi, Q., Li, H., Liu, J., Wu, S., Sun, H., & Zhou, B. (2015). Glycolipid metabolism disorder in the liver of obese mice is improved by TUDCA via the restoration of defective hepatic autophagy. Int J Endocrinol. 2015: 687938.

Hamilton, J. A., & Brunaldi, K. (2007). A model for fatty acid transport into the brain. J Mol Neurosci. 33(1): 12-17.

Hamilton, L. K., Dufresne, M., Joppé, S. E., Petryszyn, S., Aumont, A., Calon, F., … Fernandes, K. J. (2015). Aberrant Lipid Metabolism in the Forebrain Niche Suppresses Adult Neural Stem Cell Proliferation in an Animal Model of Alzheimer's Disease. Cell Stem Cell 17(4): 397-411.

Han, S. P., Friend, L. R., Carson, J. H., Korza, G., Barbarese, E., Maggipinto, M., … Smith, R. (2010). Differential subcellular distributions and trafficking functions of hnRNP A2/B1 spliceoforms. Traffic. 11(7): 886-898.

Hardwick, L. J., Ali, F. R., Azzarelli, R. & Philpott, A. (2015). Cell cycle regulation of proliferation versus differentiation in the central nervous system. Cell Tissue Res. 359(1): 187-200.

He, M., Rutledge, S. L., Kelly, D. R., Palmer, C. A., Murdoch, G., Majumder, N., … Vockley, J. (2007). A New Genetic Disorder in Mitochondrial Fatty Acid β-Oxidation: ACAD9 Deficiency. Am J Hum Genet. 81(1): 87-103.

He, Y., Brown, M. A., Rothnagel, J. A., Saunders, N. A., & Smith, R. (2005). Roles of heterogeneous nuclear ribonucleoproteins A and B in cell proliferation. J Cell Sci. 118(Pt 14): 3173-3183.

Hernández-Benítez, R., Ramos-Mandujano, G., & Pasantes-Morales, H. (2012). Taurine stimulates proliferation and promotes neurogenesis of mouse adult cultured neural stem/progenitor cells. Stem Cell Res. 9(1): 24-34.

Hewavitharana, T., & Wedegaertner, P. B. (2012). Non-canonical signaling and localizations of heterotrimeric G proteins. Cell Signal. 24(1): 25-34.

Hock, M. B., & Kralli, A. (2009). Transcriptional Control of Mitochondrial Biogenesis and Function. Annu Rev Physiol. 71: 177-203.

Homem, C. C. F., Steinmann, V., Burkard, T. R., Jais, A., Esterbauer, H. & Knoblich, J. A. (2014). Ecdysone and Mediator Change Energy Metabolism to Terminate Proliferation in Drosophila Neural Stem Cells. Cell 158(4): 874–888.

Hong, S. H., Yoon, K. J., Lim, K. H., Um, Y. J., Cho, J. G., Jo, Y. J., & Park, S. G. (2016). Tauroursodeoxycholic acid improves viability of artificial RBCs. Biochem Biophys Res Commun. 478(4): 1682-1687.

Hou, Y., Mattson, M. P., & Cheng, A. (2013). Permeability Transition Pore-Mediated Mitochondrial Superoxide Flashes Regulate Cortical Neural Progenitor Differentiation. PLoS ONE 8(10): e76721.

Hu, C., Fan, L., Cen, P., Chen, E., Jiang, Z., & Li, L. (2016). Energy Metabolism Plays a Critical Role in Stem Cell Maintenance and Differentiation. Int J Mol Sci. 17(2): 253.

Ito, K., & Suda, T. (2014). Metabolic requirements for the maintenance of self-renewing stem cells. Nat Rev Mol Cell Biol. 15(4): 243-256.

Ito, K., Carracedo, A., Weiss, D., Arai, F., Ala, U., Avigan, D. E., Schafer, Z. T., Evans, R. M., Suda, T., Lee, C. H., & Pandolfi, P. P. (2012). A PML–PPAR-δ pathway for fatty acid oxidation regulates hematopoietic stem cell maintenance. Nat Med. 18(9): 1350–1358.

Jornayvaz, F. R., & Shulman, G. I. (2010). Regulation of mitochondrial biogenesis. Essays Biochem. 47: 69-84.

Kang, E., Wen, Z., Song, H., Christian, K. M., & Ming, G. L. (2016). Adult neurogenesis and psychiatric disorders. Cold Spring Harb Perspect Biol. 8(9): 1-28.

Kann, O., & Kovács, R. (2007). Mitochondria and neuronal activity. Am J Physiol Cell Physiol. 292(2): C641-657.

Keene, D. C., Rodrigues, C. M., Eich, T., Chhabra, M. S., Steer, C. J., & Low, W. C. (2002). Tauroursodeoxycholic acid, a bile acid, is neuroprotective in a transgenic animal model of Huntington's disease. Proc Natl Acad Sci U S A. 99(16): 10671-10676.

Khacho, M., & Slack, R. S. (2015). Mitochondrial dynamics in neurodegeneration: from cell death to energetic states. AIMS Molecular Science. 2(2): 161-174.

Khacho, M., Clark, A., Svoboda, D. S., Azzi, J., MacLaurin, J. G., Meghaizel, C., Sesaki, H., … Slack, R. S. (2016). Mitochondrial Dynamics Impacts Stem Cell Identity and Fate Decisions by Regulating a Nuclear Transcriptional Program. Cell Stem Cell 19(2): 232-247.

Khacho, M., Clark, A., Svoboda, D. S., MacLaurin, J. G., Lagace, D.C., Park, D. S., & Slack, R. S. (2017) Mitochondrial dysfunction underlies cognitive defects as a result of neural stem cell depletion and impaired neurogenesis. Hum Mol Genet. 26(17): 3327-3341.

Kiilerich, K., Adser, H., Jakobsen, A. H., Pedersen, P. A., Hardie, D. G., Wojtaszewski, J. F. P., Pilegaard, H. (2010). PGC-1α increases PDH content but does not change acute PDH regulation in mouse skeletal muscle. Am J Physiol Regul Integr Comp Physiol. 299(5): 1350-1359.

Kim, E. K., Kleman, A. M., & Ronnett, G. V. (2007). Fatty acid synthase gene regulation in primary hypothalamic neurons. Neurosci Lett. 423(3): 200-204.

Knobloch, M. (2016). The Role of Lipid Metabolism for Neural Stem Cell Regulation. Brain Plasticity. 1-11.

Knobloch, M., & Jessberger, S. (2017). Metabolism and neurogenesis. Curr Opin in Neurobiol. 42: 45-52.

Knobloch, M., Braun, S. M., Zurkirchen, L., von Schoultz, C., Zamboni, N., Araúzo-Bravo, M. J., … Jessberger, S. (2012). Metabolic control of adult neural stem cell activity by Fasn-dependent lipogenesis. Nature 493(7431): 226-230.

Knobloch, M., Pilz, G. A., Ghesquière, B., Kovacs, W. J., Wegleiter, T., Moore, D. L., … Jessberger,

S. (2017). A Fatty Acid Oxidation-Dependent Metabolic Shift Regulates Adult Neural Stem Cell Activity. Cell Rep. 20(9): 2144-2155.

Kompare, M., & Rizzo, W. B. (2008). Mitochondrial fatty-acid oxidation disorders. Semin Pediatr Neurol. 15(3): 140-149.

Kuhn, H. G., Dickinson-Anson, H., & Gage, F.H. (1996). Neurogenesis in the Dentate Gyrus of the Adult Decrease of Neuronal Progenitor Proliferation. J Neurosci. 16(6): 2027-2033.

Kurtz, D. M., Rinaldo, P., Rhead, W. J., Tian, L., Millington, D. S., Vockley, J., … Wood, P. A. (1998). Targeted disruption of mouse long-chain acyl-CoA dehydrogenase gene reveals crucial roles for fatty acid oxidation. Proc Natl Acad Sci U S A. 95(26): 15592-15597.

Lemasters, J. J., & Holmuhamedov, E. (2006). Voltage-dependent anion channel (VDAC) as mitochondrial governator - Thinking outside the box. Biochim Biophys Acta. 1762(2): 181-190.

Levy, M., Faas G. C., Saggau P., Craigen W. J., & Sweatt J. D. (2003). Mitochondrial regulation of synaptic plasticity in the hippocampus. J Biol Chem 278: 17727-17734.

Li, X., Barkho B. Z., & Zhao X. (2008). Neural Stem Cells and Neurogenic Niche in the Adult Brain. *In* Stem Cell Research and Therapeutics (Y. Shi & D. O. Clegg eds), Advances in Biomedical Research, vol 1, pp 83-103, Springer, Dordrecht.

Lindvall, O., & Kokaia, Z. (2010). Stem cells in human neurodegenerative disorders — time for clinical translation? J Clin Invest. 120(1): 29-40.

Lionaki, E., Gkikas, I., & Tavernarakis, N. (2016). Differential Protein Distribution between the Nucleus and Mitochondria: Implications in Aging. Front Genet. 7:162.

Lodi, D., Iannitti, T., & Palmieri, B. (2011). Stem cells in clinical practice: applications and warnings. J Exp Clin Cancer Res. 30:9.

Lucassen, P. J., Meerlo, P., Naylor, A. S., van Dam, A. M., Dayer, A. G., … Czéh, B. (2010). Regulation of adult neurogenesis by stress, sleep disruption, exercise and inflammation: Implications for depression and antidepressant action. Eur Neuropsychopharmacol. 20(1): 1-17.

Lunt, S. Y., & Vander Heiden, M. G. (2011). Aerobic Glycolysis: Meeting the Metabolic Requirements of Cell Proliferation. Annual Review of Cell and Developmental Biology. 27(1): 441–464.

Ma, D. K., Bonaguidi, M. A., Ming, G. L., & Song, H. (2009). Adult neural stem cells in the mammalian central nervous system. Cell Res. 19(6): 672-682.

Martínez-Diez, M., Santamaría, G., Ortega, A. D., & Cuezva, J. M. (2006). Biogenesis and dynamic of mitochondria during the cell cycle: Significance of 3'UTRs. PLoS ONE 1: e107.

Mattson, M. P., & Magnus, T. (2006). Ageing and neuronal vulnerability. Nat Rev Neurosci. 7(4): 278–294.

Mattson, M. P., Gleichmann, M., & Cheng, A. (2008). Mitochondria in Neuroplasticity and Neurological Disorders. Neuron. 60(5): 748–766.

Menendez, J. A., & Lupu, R. (2007). Fatty acid synthase and the lipogenic phenotype in cancer pathogenesis. Nat Rev Cancer. 7(10): 763-77.

Mercer, T. R., Neph, S., Dinger, M. E., Crawford, J., Smith, M. A., Shearwood, A. J., … Mattick, J. S. The human mitochondrial transcriptome. (2011). Cell 146(4): 645-658.

Ming, G., & Song, H. (2011). Adult Neurogenesis in the Mammalian Brain: Significant Answers and Significant Questions. Neuron. 70(4): 687-702.

Mitchell, R. W., & Hatch, G. M. (2011). Fatty acid transport into the brain: Of fatty acid fables and lipid tails. Prostaglandins Leukot Essent Fatty Acids. 85(5): 293-302.

Mountford, P., Zevnik, B., Dowel, A., Nichols, J., Li, M., Dani, C., … Smith, A. (1994). Dicistronic targeting constructs: reporters and modifiers of mammalian gene expression. Proc Natl Acad Sci U S A. 91(10): 4303-4307.

Murphy, M. P. (2009). How mitochondria produce reactive oxygen species. Biochem J. 417(Pt 1): 1-

13.
Nichols, J., Evans, E. P., & Smith, A. G. (1990). Establishment of germ-line-competent embryonic stem (ES) cells using differentiation inhibiting activity. Development (Cambridge, England), 110(4), 1341–1348.
O'Brien, L. C., Keeney, P. M., & Bennett, J. P. Jr. (2015) Differentiation of Human Neural Stem Cells into Motor Neurons Stimulates Mitochondrial Biogenesis and Decreases Glycolytic Flux. Stem Cells Dev 24(17): 1984-1994.
Ottoboni, L., Merlini, A., & Martino, G. (2017). Neural Stem Cell Plasticity: Advantages in Therapy for the Injured Central Nervous System. Front Cell Dev Biol. 5: 52.
Owusu-Ansah, E., Yavari, A., Mandal, S., & Banerjee, U. (2008). Distinct mitochondrial retrograde signals control the G1-S cell cycle checkpoint. Nat Genet. 40(3): 356-361.
Ozcan, U., Yilmaz, E., Ozcan, L., Furuhashi, M., Vaillancourt, E., Smith, R. O., … Hotamisligil, G. S. (2006). Chemical chaperones reduce ER stress and restore glucose homeostasis in a mouse model of type 2 diabetes. Science 313(5790): 1137-1140.
Panopoulos, A. D., Yanes, O., Ruiz, S., Kida, Y. S., Diep, D., Tautenhahn, R., … Izpisua Belmonte, J. C. (2012). The metabolome of induced pluripotent stem cells reveals metabolic changes occurring in somatic cell reprogramming. Cell Res. 22(1): 168-177.
Picca, A., & Lezza, A. M. (2015). Regulation of mitochondrial biogenesis through TFAM – mitochondrial DNA interactions: Useful insights from aging and calorie restriction studies. Mitochondrion. 25: 67-75.
Podobinska, M., Szablowska-Gadomska, I., Augustyniak, J., Sandvig, I., Sandvig, A., & Buzanska, L. (2017). Epigenetic Modulation of Stem Cells in Neurodevelopment: The Role of Methylation and Acetylation. Front Cell Neurosci. 11: 23.
Pollard, S. M., Conti, L., Sun, Y., Goffredo, D., & Smith, A. (2006). Adherent neural stem (NS) cells from fetal and adult forebrain. Cereb Cortex. 1: i112-120.
Pow, D. V., Sullivan, R., Reye, P. & Hermanussen, S. (2002). Localization of taurine transporters, taurine, and ^{3}H taurine accumulation in the rat retina, pituitary, and brain. Glia. 37: 153-168.
Pratt, T., Sharp, L., Nichols, J., Price, D. J., & Mason, J. O. (2000). Embryonic stem cells and transgenic mice ubiquitously expressing a tau-tagged green fluorescent protein. Dev Biol. 228(1): 19-28.
Prowse, A. B., Chong, F., Elliott, D. A., Elefanty, A. G., Stanley, E. G., Gray, P. P., Munro T. P., & Osborne, G. W. (2012). Analysis of Mitochondrial Function and Localisation during Human Embryonic Stem Cell Differentiation In Vitro. PLoS ONE 7(12): e52214.
Quirós, P. M., Mottis, A., & Auwerx, J. (2016). Mitonuclear communication in homeostasis and stress. Nat Rev Mol Cell Biol. 17(4): 213-226.
Ramon y Cajal, S. (1913). Degeneration and Regeneration of the Nervous System. London: Oxford Univ. Press.
Ramos-Mandujano, G., Hernández-Benítez, R., & Pasantes-Morales, H. (2014). Multiple mechanisms mediate the taurine-induced proliferation of neural stem/progenitor cells from the subventricular zone of the adult mouse. Stem Cell Res. 12(3): 690-702.
Reynolds, B. A. & Weiss, S. (1992). Generation of Neurons and Astrocytes from Isolated Cells of the Adult Mammalian Central Nervous System. Science 255(5052): 1707-1710.
Rodrigues, C. M. P., Keene, C. D., Linehan-Stieers, C., Ma, X., Low, W., & Steer, C. J. (2000). Tauroursodeoxycholic acid prevents apoptosis induced by 3-nitropropionic acid: Evidence for a mitochondrial-dependent pathway independent of the permeability transition. J Neurochem. 7(6): 2368-2379.
Rodrigues, C. M. P., Solá, S., Nan, Z., Castro, R. E., Ribeiro, P. S., Low, W. C., & Steer, C. J. (2003). Tauroursodeoxycholic acid reduces apoptosis and protects against neurological injury after acute

hemorrhagic stroke in rats. Proc Natl Acad Sci U S A. 100(10): 6087-6092.

Rodrigues, C. M., Fan, G., Ma, X., Kren, B. T., & Steer, C. J. (1998a). A novel role for ursodeoxycholic acid in inhibiting apoptosis by modulating mitochondrial membrane perturbation. J Clin Invest. 101(12): 2790-2799.

Rodrigues, C. M., Fan, G., Wong, P. Y., Kren, B. T., & Steer, C. J. (1998b). Ursodeoxycholic acid may inhibit deoxycholic acid-induced apoptosis by modulating mitochondrial transmembrane potential and reactive oxygen species production. Mol Med. 4(7): 165-178.

Rodrigues, C. M., Ma, X., Linehan-Stieers, C., Fan, G., Kren, B. T., & Steer, C. J. (1999). Ursodeoxycholic acid prevents cytochrome c release in apoptosis by inhibiting mitochondrial membrane depolarization and channel formation. Cell Death Differ. 6(9): 842-854.

Rodrigues, C.M., Spellman, S.R., Solá, S., Grande, A.W., Linehan-Stieers, C., L., & W.C., Steer, C. J. (2002). Neuroprotection by a bile acid in an acute stroke model in the rat. J Cereb Blood Flow Metab 22(4): 463-471.

Romero-Ramírez, L., Nieto-Sampedro, M., & Yanguas-Casás, N. (2017). Tauroursodeoxycholic acid: more than just a neuroprotective bile conjugate. Neural Regen Res. 12(1): 62–63.

Russell, D. W., & Setchell, K. D. (1992). Bile acid biosynthesis. Biochemistry 31(20): 4737-49.

Sánchez-Aragó, M., García-Bermúdez, J., Martínez-Reyes, I., Santacatterina, F., & Cuezva, J. M. (2013). Degradation of IF1 controls energy metabolism during osteogenic differentiation of stem cells. EMBO Rep. 14(7): 638-644.

Sawitza, I., Kordes, C., Götze, S., Herebian, D., & Häussinger, D. (2015). Bile acids induce hepatic differentiation of mesenchymal stem cells. Sci Rep. 5: 13320.

Scarpulla, R. C. (2011) Metabolic control of mitochondrial biogenesis through the PGC-1 family regulatory network. Biochim Biophys Acta. 1813(7): 1269-1278.

Schönfeld, P., & Reiser, G. (2013). Why does brain metabolism not favor burning of fatty acids to provide energy? - Reflections on disadvantages of the use of free fatty acids as fuel for brain. J Cereb Blood Flow Metab. 33(10): 1493-1499.

Seo, H., Lee, I., Chung, H. S., Bae, G.-U., Chang, M., Song, E., & Kim, M. J. (2016). ATP5B regulates mitochondrial fission and fusion in mammalian cells. Anim Cells Syst. 20(3): 157–164.

Shi, L., & Tu, B. P. (2015). Acetyl-CoA and the Regulation of Metabolism: Mechanisms and Consequences. Curr Opin Cell Biol. 33: 125-131.

Shin, J., Berg, D. A., Zhu, Y., … Christian, K. M., Ming, G. L., Song, H. (2015). Single-Cell RNA-Seq with Waterfall Reveals Molecular Cascades underlying Adult Neurogenesis. Cell Stem Cell 17(3): 360-372.

Shoshan-Barmatz, V., & Gincel, D. (2003). The voltage-dependent anion channel: characterization, modulation, and role in mitochondrial function in cell life and death. Cell Biochem Biophys. 39(3): 279-292.

Shoshan-Barmatz, V., De Pinto, V., Zweckstetter, M., Raviv, Z., Keinan, N., & Arbel, N. (2010). VDAC, a multi-functional mitochondrial protein regulating cell life and death. Mol Aspects Med. 31(3): 227-285.

Silva, J., Chambers, I., Pollard, S., & Smith, A. (2006). Nanog promotes transfer of pluripotency after cell fusion. Nature 441(7096): 997-1001.

Smith, A. G. (1991). Culture and differentiation of embryonic stem cells. J Tissue Cult Methods. 13(2): 89-94.

Smith, R. (2004). Moving molecules: mRNA trafficking in Mammalian oligodendrocytes and neurons. Neuroscientist. 10(6): 495-500.

Soares, R., Ribeiro, F. F., Xapelli, S., Genebra, T., Ribeiro, M. F., Sebastião, A. M., Rodrigues, C. P. M., & Solá, S. (2017). Tauroursodeoxycholic Acid Enhances Mitochondrial Biogenesis, Neural Stem Cell Pool, and Early Neurogenesis in Adult Rats. Mol Neurobiol. [Epub ahead of print]

Sohur, U. S., Emsley, J. G., Mitchell, B. D., & Macklis, J. D. (2006). Adult neurogenesis and cellular brain repair with neural progenitors, precursors and stem cells. Philos Trans R Soc Lond B Biol Sci. 361(1473): 1477-1497.

Spiliotopoulos, D., Goffredo, D., Conti, L., Di Febo, F., Biella, G., Toselli, M., & Cattaneo, E. (2009). An optimized experimental strategy for efficient conversion of embryonic stem (ES)-derived mouse neural stem (NS) cells into a nearly homogeneous mature neuronal population. Neurobiol Dis. 34(2): 320-331.

Sutendra, G., Kinnaird, A., Dromparis, P., Paulin, R., Stenson, T. H., Haromy, A., … Michelakis, E. D. (2014). A Nuclear Pyruvate Dehydrogenase Complex Is Important for the Generation of Acetyl-CoA and Histone Acetylation. Cell 158(1): 84-97.

Takahashi, K., & Yamanaka, S. (2006). Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors. Cell 126(4): 663–676.

Takubo, K., Nagamatsu, G., Kobayashi, C. I., Nakamura-ishizu, A., Kobayashi, H., Ikeda, E., … Suematsu, M., & Suda, T. (2013). Regulation of Glycolysis by Pdk Functions as a Metabolic Checkpoint for Cell Cycle Quiescence in Hematopoietic Stem Cells. Cell Stem Cell 12(1): 49-61.

Tang, B. L. (2015). Mitochondrial Protein in the Nucleus. CellBio. 4(2): 23-29.

Thomas, C., Pellicciari, R., Pruzanski, M., Auwerx, J., & Schoonjans, K. (2008). Targeting bile-acid signalling for metabolic diseases. Nat Rev Drug Discov. 7(8): 678-693.

Trounson, A., & Mcdonald, C. (2015). Stem Cell Therapies in Clinical Trials: Progress and Challenges. Cell Stem Cell 17(1): 11-22.

Tucci, S., Herebian, D., Sturm, M., Seibt, A., & Spiekerkoetter, U. (2012). Tissue-Specific Strategies of the Very-Long Chain Acyl-CoA Dehydrogenase-Deficient (VLCAD-/-) Mouse to Compensate a Defective Fatty Acid β-Oxidation. PLoS ONE 7(9): e45429.

van Vlies, N., Tian, L., Overmars, H., Bootsma, A. H., Kulik, W., Wanders, R. J., … Vaz, F. M. (2005). Characterization of carnitine and fatty acid metabolism in the long-chain acyl-CoA dehydrogenase-deficient mouse. Biochem J. 387(Pt 1): 185-193.

Vang, S., Longley, K., Steer, C. J., & Low, W. C. (2014). The Unexpected Uses of Urso- and Tauroursodeoxycholic Acid in the Treatment of Non-liver Diseases. Glob Adv Health Med. 3(3): 58-69.

Varum, S., Rodrigues, A. S., Moura, M. B., Momcilovic, O., Easley, C. A., Ramalho-Santos, J., Houten, B. Van, & Schatten, G. (2011). Energy Metabolism in Human Pluripotent Stem Cells and Their Differentiated Counterparts. PLoS ONE 6(6): e20914.

Wajner, M., & Amaral, A. U. (2016). Mitochondrial dysfunction in fatty acid oxidation disorders: insights from human and animal studies. Biosci Rep. 36(1): e00281.

Walker, T. L., Overall, R. W., Vogler, S., Sykes, A. M., Ruhwald, S., Lasse, D., … Kempermann, G. Lysophosphatidic Acid Receptor Is a Functional Marker of Adult Hippocampal Precursor. (2016). Stem Cell Reports 6(4): 552-565.

Wallace, D. C. (2005). A Mitochondrial Paradigm of Metabolic and Degenerative Diseases, Aging, and Cancer: A Dawn for Evolutionary Medicine. Annu Rev Genet. 39: 359-407.

Walton, N. M., Shin, R., Tajinda, K., Heusner, C. L., Kogan, J. H., Miyake, S., … Matsumoto, M. (2012). Adult Neurogenesis Transiently Generates Oxidative Stress. PLoS ONE 7(4): e35264.

Wanet, A., Arnould, T., Najimi, M., & Renard, P. (2015). Connecting Mitochondria, Metabolism, and Stem Cell Fate. Stem Cells Dev. 24(17): 1957-1971.

Wellen, K. E., Hatzivassiliou, G., Sachdeva, U. M., Bui, T. V, Justin, R., & Thompson, C. B. (2009). ATP-Citrate Lyase Links Cellular Metabolism to Histone Acetylation. Science 324(5930): 1076-1080.

Wernig, M., Zhao, J., Pruszak, J., Hedlund, E., Fu, D., Soldner, F., … Jaenisch, R. (2008). Neurons

derived from reprogrammed fibroblasts functionally integrate into the fetal brain and improve symptoms of rats with Parkinson's disease. Proc Natl Acad Sci USA. 105(15): 5856-5861.

Xavier, J. M., Morgado, A. L., Rodrigues, C. M. P., & Solá, S. (2014). Tauroursodeoxycholic acid increases neural stem cell pool and neuronal conversion by regulating mitochondria-cell cycle retrograde signaling. Cell Cycle 13(22): 3576-3589.

Xavier, J. M., Rodrigues, C. M. P., & Solá, S. (2015). Mitochondria: Major Regulators of Neural Development. Neuroscientist. 22(4): 346-358.

Xie, Z., Jones, A., Deeney, J. T., Hur, S. K., & Bankaitis, V. A. (2016). Inborn Errors of Long Chain Fatty Acid β-Oxidation Link Neural Stem Cell Self-Renewal to Autism. Cell Rep. 14(5): 991-999.

Xu, G., & Li, J. Y. (2016). ATP5A1 and ATP5B are highly expressed in glioblastoma tumor cells and endothelial cells of microvascular proliferation. J Neurooncol. 126(3): 405-413.

Yu, D., & Silva, G.A. (2008). Stem cell sources and therapeutic approaches for central nervous system and neural retinal disorders. Neurosurg Focus. 24(3-4): E11.

Zhang, J., Liu, W., Liu, J., Xiao, W., Liu, L., Jiang, C., … Chen, Q. (2010). G-protein β2 subunit interacts with mitofusin 1 to regulate mitochondrial fusion. Nat Commun. 1(7): 101.

Zheng, X., Boyer, L., Jin, M., Mertens, J., Kim, Y., Ma, L., … Hunter, T. (2016). Metabolic reprogramming during neuronal differentiation from aerobic glycolysis to neuronal oxidative phosphorylation. Elife 5: e13374.

Ziegler, A. N., Levison, S. W., & Wood, T. L. (2015). Insulin and IGF receptor signalling in neural-stem-cell homeostasis. Nat Rev Endocrinol. 11(3): 161-170.

6. Annexes

Annex A

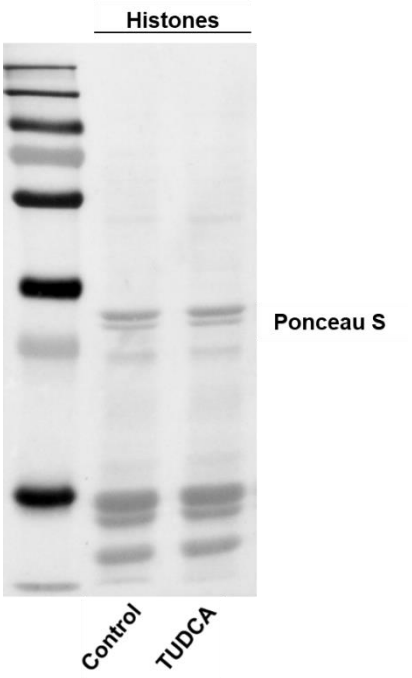


Figure A.1 –Total protein profile of histone purified extracts of NSCs.

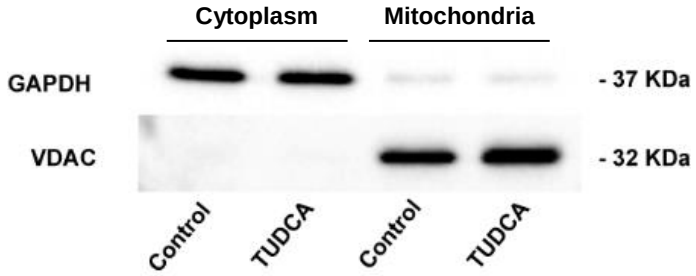


Figure A.2 - Immunoblots showing isolated pure mitochondria of NSCs.

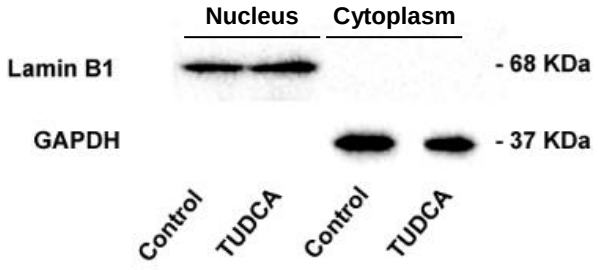


Figure A.3 - Immunoblots showing isolated pure nuclei of NSCs.