Universidade de Lisboa

Faculdade de Farmácia

The potential therapeutic role of neural stem cells secretome in neurodegeneration: a bioenergetic approach

Catarina Moita Roxo

Dissertation supervised by Professor Susana Solá and co-supervised by Professor Joana Miranda

Masters in Biopharmaceutical Sciences

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Abstract

Adult neurogenesis, a process in which endogenous neural stem cells (NSCs) differentiate into a wide range of neural lineages of the adult brain, has been considered as a potential therapeutic strategy to arrest the progression of neurological disorders, including depression. Recently, a paradigm shift has emerged suggesting that the beneficial effects of NSCs may also rely on their paracrine activity, namely by releasing biomolecules (secretome). However, throughout life, NSCs decline and gradually lose their paracrine activity in response to chronological and replicative senescence, prompting urgency in exploring efficient strategies capable of enhancing the therapeutic role of NSC secretome and compensate neural degeneration. Interestingly, although mitochondria regulate neural stem cell fate and the secretome of all cell type, the underlying metabolic mechanisms responsible for the regenerative potential of NSC secretome are still largely unclear.

Therefore, we aimed to further explore the mitochondrial metabolic signature of NSC-producing a protective secretome, using different models of NSC stress. Further, we aimed to also test the influence of known metabolic regulators on the NSC secretome-associated therapeutic properties. Initially, hydrogen peroxide (H_2O_2)-treated differentiating NSCs were used as target models of neighbouring injured neuron-like differentiating cells. Our results showed that boosted NSCs acquire a more proliferative fate, exhibit increased mitochondrial fragmentation and diminish certain proteins associated with respiration and oxidative phosphorylation (OxPhos), as well as the mitochondrial DNA (mtDNA) copy number. The secretome derived from boosted NSCs (boosted CM), in turn, induced an increase in mtDNA copy number in target NSCs. Curiously, several mitochondrial boosters, including a cocktail of neurotrophin factors that mimic *in vitro* exercise-mediated effects, were also capable of improving the therapeutic properties of NSC-secretome (mitCM) since they abrogated H_2O_2-induced death of neuron-like differentiating NSCs. A deeper molecular characterization of these mitCM-producing NSCs showed that increased mitochondrial fission was also present in these cells but in this this context, was associated with an increased mtDNA copy number. Regarding the effect of mitochondrial boosters on OxPhos-associated proteins, we observed a significant increase of several OxPhos-associated proteins not related with ATP production. In agreement with what observed for the boosted CM, mitCM also induced an increase of mtDNA copy number in in target NSCs.

At last, to further understand the role of metabolism in NSC plasticity *in vivo*, NSCs were stimulated with serum derived from depressed mice, using the animal model of unpredictable chronic mild stress (uCMS). These NSCs were named uCMS-boosted NSCs

and their secretome, uCMS boosted-CM. Surprisingly, our results showed that uCMS boosted-CM was more efficient in preventing cell death of neuron-like differentiating cells, when compared with NSC stimulated with serum derived from healthy mice. More importantly, the metabolic characterization of uCMS-boosted NSCs revealed that these cells undergo to several metabolic changes when compared with NSC stimulated with serum derived from healthy mice, exhibiting a more proliferative phenotype and increased mitochondrial fission and biogenesis. Concomitantly, the uCMS boosted-CM appeared to also enhance NSC differentiation, associated with enhanced fatty acid oxidation (FAO) and OxPhos rates for energy production.

Collectively, these data demonstrate that profound metabolic alterations occur in NSCs exposed to external injury signals or metabolic boosters that go along with the production and delivery of a protective secretome to injured differentiating recipient cells.

Keywords: Mitochondrial metabolism; Neural stem cells; Neural stress; Neuroregeneration; Secretome.

Resumo

A neurogénese adulta, um processo em que as células estaminais neurais (NSCs) endógenas se diferenciam numa vasta gama de linhagens neurais do cérebro, tem sido considerada como uma potencial estratégia terapêutica para travar a progressão de doenças neurológicas, incluindo a depressão. Recentemente, uma alteração de paradigma, veio sugerir que os efeitos benéficos das NSCs possam, também, depender da sua atividade parácrina, nomeadamente através da libertação de biomoléculas (secretoma). No entanto, ao longo da vida, as NSCs diminuem em número e perdem gradualmente a sua atividade parácrina em resposta à senescência cronológica e replicativa, tornando-se imprescindível a descoberta de estratégias exequíveis e eficientes para reforçar o papel terapêutico do secretoma das NSCs e compensar a degeneração neural. Curiosamente, embora a mitocôndria regule o destino das NSCs e o secretoma de todos os tipos celulares, os mecanismos metabólicos subjacentes ao potencial regenerativo do secretoma das NSCs ainda não estão totalmente esclarecidos.

Neste trabalho pretendemos explorar o padrão metabólico mitocondrial das NSCs produtores de um secretoma protector, utilizando diferentes modelos de stress em NSCs. Além disso, o nosso objetivo foi, também, testar a influência de reguladores metabólicos (*mitochondrial boosters*) neste secretoma. Inicialmente, foram utilizadas NSCs em diferenciação tratadas com peróxido de hidrogénio (H_2O_2), como um modelo de células alvo e stress oxidativo. Os nossos resultados mostraram que as NSCs expostas a sinas de lesão *(boosted NSCs)* adquirem um fenótipo mais proliferativo, exibem maior fragmentação mitocondrial e diminuem a expressão de certas proteínas associadas à respiração e fosforilação oxidativa (OxPhos) e o número de cópias de DNA mitocondrial (mtDNA). O secretoma derivado das *boosted NSCs*, por sua vez, induziu um aumento do número de cópias de mtDNA nas NSCs alvo. Curiosamente, vários *mitochondrial boosters*, incluindo um cocktail de fatores neurotróficos que mimetiza algumas condições mediadas pelo exercício *in vitro*, foram também capazes de melhorar as propriedades terapêuticas do secretoma das NSCs (*mitCM*), uma vez que preveniram a morte celular induzida por H_2O_2 nas células alvo. Uma caracterização molecular mais profunda das NSCs produtoras de *mitCM* demonstrou que o aumento da fissão mitocondrial também estava presente nestas células, mas, neste contexto, encontrava-se associado a um aumento do número de cópias de mtDNA. No que diz respeito ao efeito dos *mitochondrial boosters* nas proteínas associadas a OxPhos, observámos um aumento significativo de várias proteínas associadas não relacionadas com a produção de ATP. Curiosamente, de acordo com o

previamente observado no *boostedCM*, o *mitCM* também induziu um aumento do número de cópias de mtDNA nas NCS alvo.

Finalmente, para compreender melhor o papel do metabolismo das NSCs na sua plasticidade *in vivo*, as NSCs foram estimuladas com soro proveniente de ratinhos deprimidos, utilizando um modelo animal de stress crónico e imprevisível ligeiro (uCMS), e o seu secretoma testado *in vitro* em céulas alvo em stress oxidativo. Estas NSCs foram denominadas de *uCMS-boosted NSCs* e o seu secretoma de *uCMS boosted-CM*. Surpreendentemente, os nossos resultados demonstraram que as propriedades neuroprotectoras do *uCMS boosted-CM* eram mais elevadas, quando comparado com o CM proveniente de NSCs estimuladas com soro derivado de ratinhos saudáveis. De realçar que, a caracterização metabólica das *uCMS-boosted NSCs* revelou que estas células sofreram também várias alterações metabólicas quando comparadas com as NSCs estimuladas com soro derivado de ratos saudáveis, exibindo um fenótipo mais proliferativo e um aumento da fissão mitocondrial e da sua biogénese. Concomitantemente, o soro de ratinhos deprimidos induziu a diferenciação de NSCs, que por sua vez, pareceu estar associada a elevadas as taxas de oxidação de ácidos gordos e OxPhos para a produção de energia.

Coletivamente, estes dados demonstram que NSC expostas a sinais externos de lesão ou de reforço metabólico sofrem alterações metabólicas profundas que acompanham a produção de um secretoma neuroprotector para células lesadas em processo de diferenciação.

Palavras-chave: Células estaminais neurais; Metabolismo mitocondrial; Neurorregeneração; Secretoma; Stress neural.

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List of abbreviations

ACC1	Acetil-Coenzyme A Carboxilase 1
AD	Alzheimer's disease
ASC	Adipose-derived stem cell
ATP	Adenosine triphosphate
BDNF	Brain-derived neurotrophic factor
CM	Conditioned media
CNS	Central nervous system
CoA	Coenzyme A
CPT1	Carnitine palmitoyltransferase 1
DA	Dopaminergic neuron
DG	Dentate gyrus
DNA	Deoxyribonucleic acid
DRP1	Dynamin-related protein 1
EGF	Epidermal growth factor
ESC	Embryonic stem cells
ETC	Electron transport chain
EV	Extracellular vesicle
FA	Fatty acid
FASN	Fatty acid synthase
FAO	Fatty acid oxidation
FGF	Fibroblast growth factor
GFAP	Glial fibrillary acidic protein
HD	Huntington's disease
hNSC	Human neural stem cell
HPRT	Hypoxanthine phosphoribosyltransferase
H_2O_2	Hydrogen peroxide
IGF-1	Insulin-like growth factor 1
IPC	Intremediate progenitor cell
iPSC	Induced pluripotent stem cell
LCAD	Long-chain acyl-CoA dehydrogenase
MCI	Mitochondrial complex I

MDD	Major depressive disorder
MFN2	Mitofusin 2
MPP^+	1-methyl-4-phenylpyridinium
MSC	Mensechymal stromal cell
mtDNA	Mitochondrial DNA
NADH	Nicotinamide adenine dinucleotide
NSC	Neural stem cell
NS-TGFP	tau-green fluorescent protein (GFP) mouse neural stem cell line
NPC	Neural progenital cell
OxPhos	Oxidative phosphorylation
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PD	Parkinson's disease
PGC-1α	Peroxisome proliferator-activated receptor (PPAR) γ coactivator 1 α
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Room temperature
RT-PCR	Real time polymerase chain reaction
SCFA	Short chain fatty acid
SGZ	Subgranular zone
SVZ	Subventricular zone
TC	Tissue culture
TFAM	Mitochondrial transcription factor A
TNT	Tunnelling nanotube
uCMS	Unpredictable chronic mild stress
TUDCA	Tauroursodeoxycholic acid
VEGF	Vascular endothelial growth factor
WB	Western blot
5-HT	5-Hidroxitriptamina, Serotonin
6-OHDA	6-Hydroxydopamine

1. Introduction

1.1 Neural Stem Cells

Over the years, stem cell technology has become an undeniably attractive option for cell therapy, tissue engineering and regenerative medicine along with pharmaceutical and biotechnological applications for the study and treatment of several human diseases (1). Stem cells have unlimited cell division potential, can transdifferentiate into other cell types, and have recently become an important source for regenerative medicine to repair abnormalities of tissues and organs caused by congenital defects, diseases, and age-related effects. Stem cells also form the basis for all tissue and organ systems of the body, playing multiple roles in disease progression, development and tissue repair processes in the host (2).

One of the seminal events of the last three decades in the field of neuroscience research has been the establishment of neural stem cells (NSCs) as a lifelong source of neurons, disproving the assumption that the nervous system has little regenerative capacity (3). NSCs are characterized by their ability to generate all neural lineages throughout the nervous system, including neurons, astrocytes, and oligodendrocytes to self-renew, and to give rise to different cell types in addition to themselves through asymmetric cell division (4).

Although a high proliferative capacity is a hallmark of stem cells, a unique property of NSCs compared to other cells of the central nervous system (CNS) is that they can remain inactive for very extensive periods of time, providing a reserve pool of cells available for tissue regeneration and cell replacement throughout life (5). Because of their quiescent state, adult NSCs are able to withstand metabolic stress and maintain their genomic integrity. Once activated, NSCs can undergo different division paths: symmetric division, which gives rise to two NSCs or two progenitor cells, or asymmetric division, which gives rise to one NSC and one progenitor cell. Then, neural progenitor cells (NPCs) can acquire a more committed state to give rise to a particular cell type, including neurons, astrocytes or oligodendrocytes (6,7).

NSCs can be derived *in vitro* from pluripotent stem cells (PSCs) or directly from fetal tissue or adult brain samples from germinative areas. There are two main types of PSCs: embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). NSCs can be generated from ESCs, derived from the inner cell mass of blastocysts, or from iPSCs, derived from somatic cells by reprogramming (8,9). The development of growth factor-based

protocols enabled the expansion of NSCs under both floating and adherent conditions and a better understanding of the biological and molecular properties of NSCs. Nevertheless, determining the best sources for *in vitro* derivation of NSCs and improving protocols for stable, clonal proliferation remain central goals of stem cell research (10).

NSCs can form neurospheres in culture (11). Although this culture system is an *in vitro* recapitulation of a niche-like structure, it has some serious limitations that do not make it a suitable model for neuronal regeneration. This is due to the fact that neurospheres are inherently heterogeneous and only a small percentage of cells within each sphere have the ability to form neurospheres (10,12). To address these issues, culturing NSCs as a monolayer has been investigated to precisely control the *in vitro* milieu of each cell and to obtain a fairly homogeneous, undifferentiated cell population suitable for studying the properties of cells at the single cell level (13).

1.1.1 Adult neurogenic niches

Neurogenesis, the process by which neurons are generated through NSCs, was traditionally believed to only occur in a limited period during development, as first presented by Santiago Ramón y Cajal (14). In the 1960s, proliferating cells were discovered in the adult brain and later confirmed to be neurons, while in 1998 adult-born neurons were discovered in the human hippocampus (15–17). Nowadays, with the development of increasingly better tools for labelling and tracking newborn neurons, studies in several species have shown that neurogenesis occurs in multiple regions of the adult mammalian brain (18).

The adult neurogenic niche defines a microenvironment in which NSCs are maintained after embryonic development for the production of new cells in the adult brain. These niches have many distinct features that allow the maintenance of the undifferentiated and self-renewing state of stem cells. There are heterologous cell-cell and cell-matrix interactions and associated molecules that create an advantageous microenvironment and architecture to support the physiology of NSCs, balance quiescence and proliferation, and regulate cell differentiation (19,20).

Adult neurogenesis occurs primarily in two discrete brain regions of the adult mammalian brain: the subgranular zone (SGZ) within the dentate gyrus (DG) and the subventricular zone (SVZ) of the lateral ventricles (**Figure 1.A**).

In the adult SGZ, radial glial cells (type 1 cells) are activated from quiescence and proliferate into non-radial cells that give rise to intermediate progenitor cells (IPCs, also

known as type 2 cells), which are transient amplifying cells. IPCs give rise to neural progenitor cells or neuroblasts (type 3 cells), which in turn give rise to immature neurons that migrate to the inner granule cell layer and differentiate into mature granule neurons in the DG, participate in learning, memory and behavioural function through synaptic integration into pre-existing neural networks of the hippocampus (20,21) (**Figure 1.C**).

In the adult SVZ, quiescent radial glial cells (type B cells) are activated and give rise to IPCs (type C cells), which in turn generate neuroblasts (type A cells) that can migrate along the rostral migratory stream (RMS) to the olfactory bulb, where they differentiate into mature neurons (20,22). Further, studies have suggest that SVZ neurogenesis also contributes to social behaviour such as mate recognition (23,24) (**Figure 1.D**).

Neurogenic niches create therefore a privileged environment for NSC expansion, maintenance, and neurogenic properties and are strongly regulated by both extrinsic and intrinsic factors. Intrinsic factors include neurotrophic factors, cytokines, growth factors, neurotransmitters, and hormones. Extrinsic factors include physical activity, ageing, stress, and dietary intake (25,26).

Microglia, in turn, are the resident macrophages and primary immune cells of the brain. They have a variety of functions ranging from phagocytosis to neuroprotection. Microglia contribution to the neurogenic niches appears to depend on the mode of activation, activated microglia can release proinflammatory or anti-inflammatory cytokines and thus modulate immune responses that support or suppress neurogenesis (27). Along with microglia, astrocytes are one of the major players in the neurogenic niche. Astrocytes from the adult hippocampus stimulate the differentiation of adult NSCs toward the neuronal lineage in co-culture (28), and populations of astrocytes have been reported to act as primary precursors for the new neurons. Therefore, astrocytes might be involved in the microenvironment that promotes neurogenesis in the germinal layers (29).

The anatomical relationship between SVZ-NSCs and blood vessels has been extensively studied. The vasculature represents a rich source of extrinsic factors that can modulate adult neurogenesis. The paracrine activity of the endothelial cells that form blood vessels can modulate neurogenesis and angiogenesis. Thus, contact between endothelial cells and NSCs lining the ventricles is critical for stem cell maintenance and activation of Notch signalling (25,27).

The hippocampus is considered the central brain area involved in regulating the stress response. Therefore, appropriate stress coping is associated with the processing of emotional and cognitive information in the hippocampus (30). Interestingly, acute and

chronic exposure to psychosocial stress can suppress neurogenesis in the DG in various animals (25).

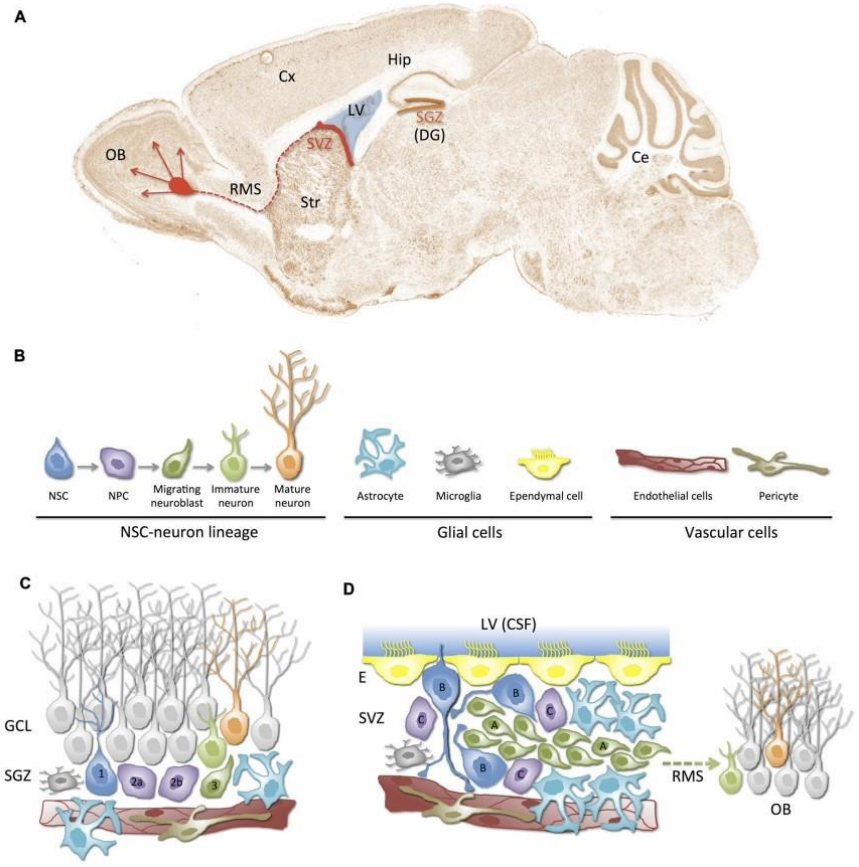

Figure 1 - Adult neurogenic niches in the mammalian brain. (**A**) Schematic representation of the neurogenic niches in a sagittal section of the adult mouse brain: the subgranular zone (SGZ, orange) in dentate gyrus (DG) of the hippocampus (Hip) and the subventricular zone (SVZ, red) in the lateral wall of the lateral ventricles (LV). Newborn neurons derived from the SVZ migrate to the olfactory bulb (OB) via the rostral migratory stream (RMS). (**B**) Cellular components of the neurogenic niches. (**C,D**) Illustration of the neurogenic niches of the SGZ and SVZ. The different cell types (colour and shape) correspond to those depicted in (B). Ce, cerebelum; Cx, cortex; Str, striatum (19).

Remarkably, physical exercise is known to improve learning and memory and counteract age-related mental decline. Indeed, in human subjects, physically fit individuals have been reported to perform better cognitively and memory-wise compared to their sedentary peers (31,32). Animal studies suggest that the positive effects of exercise on cognitive function may be related to increased neurogenesis in the hippocampus. For instances, pioneer studies by van Praag and co-workers have shown that physical running not only increases

neurogenesis in the hippocampus, but can also improve performance in the Morris water maze in mice (33,34). However, the underlying mechanisms responsible for the beneficial effects of exercise are still unclear. Neurotrophins such as brain-derived neurotrophic factor (BDNF), insulin-like growth factor 1 (IGF-1), and vascular endothelial growth factor (VEGF) are recognized as primary mediators of adult neurogenesis, and gene expression of BDNF and IGF-1 in hippocampal neurons has been demonstrated in response to exercise training (35–37). Blocking VEGF or IGF-1 has been shown to significantly reduce running-induced neurogenesis in rodent studies (38–40). Similarly, the knock-out of the BDNF receptor in hippocampal progenitor cells decreases the running-induced increase in neurogenesis in the hippocampus of mice (41). Thus, these neurotrophin factors are thought to interact in mediating exercise-induced neurogenesis in the hippocampus.

1.1.2 Therapeutic relevance of NSCs

The brain is essentially a "non-renewable organ," and brain cells slowly die as we age. However, given the ability to differentiate into multiple cell lineages, NSCs serve as internal repair systems that guarantee the CNS some ability to structurally reorganize and function according to intrinsic and environmental demands, thus representing promising tools for regenerative medicine and clinical applications. NSCs discovery has led to the potential use of intrinsic neurogenic activity to repress brain diseases and restore brain function after injury (42,43).

Parkinson's disease (PD) is a neurological disorder characterized by the presence of predominantly motor symptoms such as bradykinesia, rest tremor, rigidity, and postural disturbances, as well as the accumulation of Lewy bodies in the brain, loss of dopaminergic (DA) neurons in the *substantia nigra*, and decreased dopamine levels (44). Transplantation therapy with the use of dopamine neurons derived from ESCs turned out to have positive effects in PD animal models (45–48). In addition, another study reported the ability of NSC to restore nigrostriatal functionality after unilateral transplantation of NSCs into the *substantia nigra* of aged PD mice. They have also shown that NSC grafts trigger astrocyte-dependent Wnt1 signalling activation, leading to a range of neurotrophic and anti-inflammatory/antioxidant mechanisms that rejuvenate the aged inflammatory milieu and promote a neurorestorative programme for midbrain DA neurons (49).

Alzheimer's disease (AD) is the pathology most commonly associated with dementia and is the focus of intense research efforts to develop clinically useful therapies to address the

significant social, economic and medical impact of this disease (50). Injection of human undifferentiated NSCs into the brains of rats significantly improved the cognitive functions of the animals (51). Interestingly, Wu *et al.* found that human fetal brain-derived NSCs transplanted into adult rat brains were able to produce cholinergic neurons in specific regions (52). Recently, Zhu et al. showed that transplantation of NSC improved learning and memory functions in an AD mouse model. This treatment allowed repair of basal forebrain cholinergic neurons and increased the expression of cognition-related proteins: synaptophysin, postsynaptic density protein 95 and microtubule-associated protein (53).

Huntington's disease (HD) is a fatal neurodegenerative disorder characterized by the loss of neurons primarily in the caudate nucleus, putamen, and cerebral cortex. In later stages of the disease, other brain regions, such as the hippocampus and hypothalamus, are also affected. Degeneration of spiny neurons leads to motor dysfunction as well as cognitive and psychiatric dysfunction (54). Initial stem cell therapies focusing on ESC-derived NSCs transplanted into HD models have demonstrated motor neuron integration and circuit formation in the host (55). In addition, NSCs transplanted into rodent HD models were found to improve motor function, decrease aggregate formation, and extend lifespan (56,57).

Overall, NSC-based therapeutic strategies have great potential for developing treatments for neurodegenerative diseases by replacing dysfunctional neurons and providing neuroprotective and neurorestorative functions.

1.1.3. NSC secretome as a potential therapy

The secretome is a rich and complex set of molecules secreted from the cell surface by living cells. They are critical to regulate a wide range of cellular processes and thought to be encoded by approximately 10% of the human genome (58). The cell secretome includes a variety of serum proteins, cytokines, angiogenic factors, growth factors, hormones, extracellular matrix proteins, extracellular matrix proteases, and even, in small amounts, lipid mediators and genetic material released by mechanisms such as protein translocation, vesicle or exosome encapsulation, or exocytosis. The vesicles and soluble factors secreted by stem cells can then act directly in the injured cells by mediating intracellular signalling pathways or indirectly by inducing the secretion of functionally active products from adjacent tissues (59). Further, its therapeutic use offers several advantages over the use of cells, as secretomes are easier to produce, freeze-dry, package and transport. In addition, since the

secretome does not contain cells, rejection and immunological problems are not relevant between donor and recipient patients (60).

Proteins secreted by NSCs have been shown to play a key role coordinating various biological functions such as cell growth, division, differentiation, apoptosis, and other signalling pathways. They provide a supportive environment that enables injured cells to resist further degeneration, promote repair, reduce inflammation, and drive regeneration of injured tissue (50,61,62). Indeed, after NSC transplantation, neurotrophic growth factors, including nerve growth factor (NGF), BDNF, glial-derived neurotrophic factor (GDNF) and ciliary neurotrophic factor (CNTF) were found to be increased, being possibly responsible for the prevention of neuronal programmed cell death and glial scar formation in several neurological disorders, such as spinal cord injury (SCI), ischaemic stroke, HD, PD, and demyelinating disorders (63) (**Figure 2**).

Moreover, in an ischemic stroke animal model, it has been also shown that transplanted NSCs secrete VEGF and induce the reexpression of host guidance molecules, such as thrombospondins 1 and 2, enabling NSCs to regulate dendritic sprouting, axonal plasticity, and axonal transport *in vitro* (64).

Curiosly, McGinley *et al.* engineered a human cortical NSC (hNSC) line to produce increased levels of IGF-1, a key trophic factor for neuronal development and function that also exerts neuroprotective effects on neurons. In fact, *in vitro* experiments assessing amyloid-β toxicity in neurons co-cultured with the IGF-1 hNSCs revealed a neuroprotective paracrine mechanism compared to non-modified hNSCs co-cultures (65). Notably, other study demonstrated that transplanted hNSCs derived from fetal tissue had reduced tau phosphorylation and amyloid-β levels in a transgenic AD mouse model, which are usually highly upregulated in this animal model. *In vitro* work revealed that trophic factors secreted by hNSCs, including BDNF, NGF and VEGF, were increased in the transplanted mice and were probably related to the decrease in tau phosphorylation. Moreover, hNSC secretome treatment in human neuroblastoma cells expressing the mutated amyloid precursor protein (APP) gene was shown to decrease amyloid-β peptide production (66).

Remarkably, transplantation of human NSCs cloned by gene transfer into the 6-hydroxydopamine-lesioned (6-OHDA) striatum of rats (PD model) resulted in significant improvement in Parkinsonian behavioural symptoms compared with controls. Further experiments showed that the neuroprotective effect against the lack of DA both *in vitro* and *in vivo* relied on the secretion of trophic factors and neuronal differentiation (67). Additionally, Mendes-Pinheiro *et al.* analysed the secretome of hNSCs by proteomic analysis,

administering NSC-conditioned media to 6-OHDA rat model and comparing the treated animals with NSC-transplanted animals. Administration of NSC-conditioned media improved pathological features related to motor skills and protected *substantia nigra* and striatal neurons, which was more pronounced than in NSC-transplanted or control animals (68).

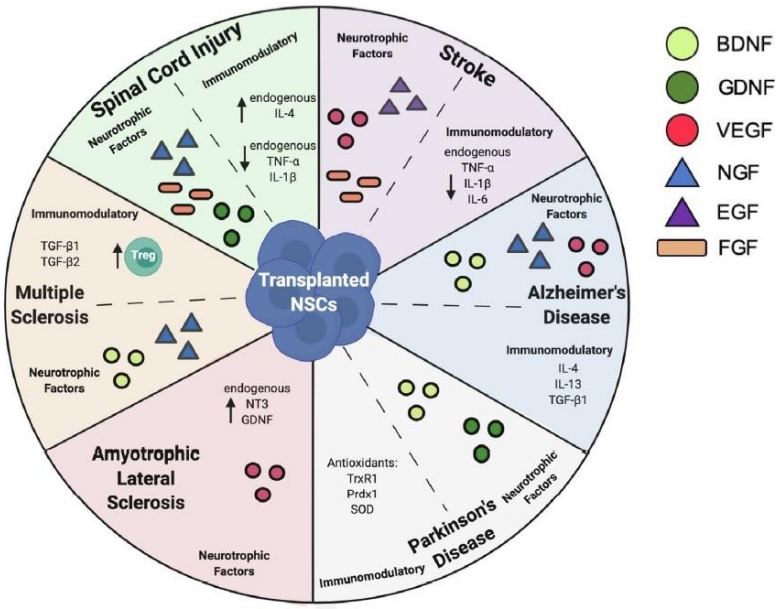

Figure 2 - Observed effects of hNSC secretome in rodent models of SCI and other NDs. Up arrow indicates an increase; down arrow indicates a decrease (63).

Extracellular vesicles (EVs) are nano-sized particles secreted by cells. EVs are exuded membrane-encircled “arrays” of cells holding cargoes such as cytosolic proteins, mRNAs, miRNAs, and even DNA, that play an important role in intercellular communication. EVs are important effectors of the therapeutic action of NSCs, and there are several advantages of using NSC-derived exosomes over NSCs themselves (69). Of note, Webb *et al.* suggested that intravenous administration of NSC-derived EVs after thromboembolic stroke can improve the cellular, tissue, and functional outcomes in middle aged mice. Using T2-weighted sequences and *ex vivo* Q-ball MRI, they also found a significant decrease in tissue loss in NSC-derived EV treated animals compared to controls. Furthermore, administration of EVs generated from NSCs had positive effects on motor function. Curiously, administration of NSC-derived EVs promoted a favourable environment for polarization of microglia into an M2-phenotype that most likely promoted debris clearance and reduced chronic inflammation. Overall, they showed that administration of NSC-derived EVs is an

efficient method to provide neuroprotection and alleviate motor and memory impairments and chronic inflammation (70).

These studies confirm the relevance of the interactions between NSCs and immune cells to reconfigure the deleterious inflammatory environment, promoting the healing and regeneration processes, and demonstrate that the ability of NSCs to secrete neuroprotective and immune modulatory factors is quite remarkable.

1.2 Mitochondrial regulation of NSC activity

1.2.1 General aspects of mitochondrial function and biogenesis

Mitochondria are intracellular organelles that control every aspect of cell function by providing a continuous supply of adenosine triphosphate (ATP), modulating Ca^{2+} signalling, influencing reactive oxygen species (ROS) levels and regulating redox control (71).

As a relic of their bacterial origin, mitochondria have their own small circular DNA, mitochondrial DNA (mtDNA), which are molecules of approximately 16.6 kilobases present in high copy number, ranging from tens to hundred thousand copies per cell (72,73).

Mitochondria have a specialised morphology that implements an efficient framework for oxidative phosphorylation (OxPhos) of adenosine diphosphate (ADP) to ATP. Mitochondria have a double membrane that divides the organelle into four distinct compartments - the outer membrane (OMM), the intermembrane space, the inner membrane (IMM) and the matrix. Each compartment performs different functions. The outer membrane contains a number of porins that allow free diffusion of molecules into the space between the outer and inner membranes. The intermembrane space contains proteins that play an important role in mitochondrial energetics and apoptosis. The inner membrane contains much of the total mitochondrial protein composition, including transporters for carrying proteins into the matrix and the enzymes of the electron transport chain (ETC), while the matrix contains most of the enzymes responsible for the reactions of the citric acid cycle (71,74). In addition, the inner membrane forms invaginations called cristae that extend deep into the matrix and contain most, if not all, of the fully assembled ETC and ATP synthase complexes, making it the major site of biological energy conversion in all non-photosynthetic eukaryotes (75).

The OxPhos consists of 5 protein complexes and 2 electron carriers embedded in the inner mitochondrial membrane. The production of high-energy phosphate is achieved by coupling electron transfer and proton translocation across the inner membrane. During respiration, electrons are first transferred from the products of the citric acid cycle, Nicotinamide adenine dinucleotide (NADH and succinate), to ubiquinone via complexes I and II, respectively. Then pass through the complex III and cytochrome c and end up in the complex IV. In this process, the complex IV reduces O_2 to H_2O. This leads to an electrochemical gradient that creates a driving motive force for ATP synthesis by the fifth complex, ATP synthase (complex V) (76).

In addition, mitochondria are involved in fatty acid oxidation (FAO) or β-oxidation, which requires the sequential activity of the mitochondrial outer membrane protein carnitine palmitoyltransferase-1 (CPT1) and the activity of the mitochondrial inner membrane protein carnitine palmitoyltransferase-2 (CPT2), to enable fatty acid (FA) transport across the double mitochondrial membrane and subsequent β-oxidation and ATP generation. In addition, oxidation of fatty acyl groups occurs within the mitochondrial matrix and the acetyl-CoA generated enters the tricarboxylic acid (TCA) cycle, again generating NADH and Flavin adenine dinucleotide ($FADH_2$), which are oxidised at ETC to generate OxPhos (77,78).

Mitochondrial biogenesis occurs constantly at basal levels and increases during cell renewal, proliferation, development and under stressful conditions such as oxidative stress, heat stress, exercise and caloric restriction. It requires synchronous expression of both the mitochondrial and nuclear genomes. The nuclear respiratory factors 1 and 2 (NRF1 and NRF2) and the estrogen related receptors (ERRs) are important transcription factors that regulate the expression of mitochondrial genes encoded in the nucleus. Interestingly, the peroxisome proliferator activated receptor gamma co-activator (PGC-1α) has been shown to increase the expression and/or coactivate these transcription factors (79,80). As a result of the activation of such transcription factors, there is increased expression of many mitochondrial proteins such as mitochondrial transcription factor A (TFAM). TFAM determines the abundance of the mitochondrial genome by regulating packaging, stability and replication. By regulating TFAM levels, PGC-1α can also control the expression of mtDNA-encoded proteins (81,82).

1.2.2 Mitochondrial dynamics and NSC fate

Mitochondria were first considered as static organelles. However, due to advances in molecular biotechnology, it has become apparent that mitochondria are in fact very dynamic structures. To maintain the required mitochondrial morphology in a dynamic environment, mitochondria continuously undergo tightly regulated and opposing remodelling processes called fusion and fission. The balance between these two opposing processes regulates the number, size and positioning of mitochondria in the cytoplasm and is referred to as 'mitochondrial dynamics'. In particular, the transient and rapid mitochondrial morphological adjustments are vital for many cellular processes such as cell cycle, apoptosis, immunity and mitochondrial quality control (83).

Mitochondrial fission ensures an even distribution of organelles among daughter cells and selects defective mitochondria for subsequent removal by mitophagy (84). Mitochondrial fission is mediated by dynamin-related protein 1 (Drp1), a large GTPase that, when activated, is recruited to the mitochondrial membrane in cooperation with accessory proteins such as mitochondrial fission factor (Mff) and mitochondrial fission protein 1 (Fis1) (85). On the other hand, mitochondrial fusion escapes autophagy-mediated destruction to preserve proper mitochondrial ultrastructure and elongation (84). Fusion is mediated by three GTPases, Mitofusin 1 and 2 (Mfn1 and 2) and optic atrophy 1 (Opa1). Mfn1 and 2 promote fusion of the OMM, while Opa1 promotes fusion of the IMM (86).

The mitochondrial dynamics vary between stem cells and differentiated cells. In stem cells, mitochondria are generally in an immature state and have increased fission events. They are localised perinuclearly, have a spherical, fragmented and punctate shape and have fewer cristae. During differentiation into terminal cell types, mitochondrial fusion events increase, which is accompanied by a change in the morphology of the mitochondria: the appearance of enlarged, elongated and tubular shapes (**Figure 3**) (87).

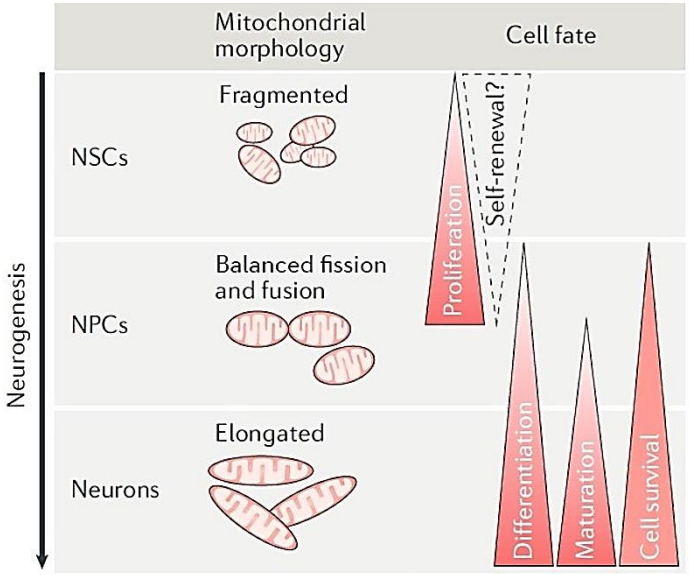

Figure 3 - Mitochondrial dynamics regulate NSC fate. Schematic representation of changes in mitochondrial structure during adult neurogenesis, when cells develop from NSCs to neural progenitor cells (NPCs) and to neurons. Solid line shapes indicate events based on published studies, while dotted shapes with a question mark indicate a speculative role. Adapted from (88).

Wang and colleagues demonstrated that Nestin knockdown, a marker of NSCs, reduced Drp1 phosphorylation and, consequently its activity in NSCs leading to stemness and self-renewal impairment (89). In another study, overexpression of Drp1 was shown to specifically increase mitochondrial fission, resulting in less organized and more fragmented mitochondria. These mitochondria can be remodelled during neuronal differentiation, increasing interconnectivity and branch length, but fails to elongate, thus impairing neuronal differentiation (90). Furthermore, Beckervordersandforth *et al*. have shown that mitochondria in adult NSCs appear small and fragmented and undergo progressive elongation and morphological maturation until neurons are formed (91).

1.2.3 Metabolic plasticty and NSC fate

Different cell states require specific metabolic programs to support the unique bioenergetic demands that underlie their specialized functions. Metabolic plasticity is essential for stem cells to meet their energy requirements in response to available nutrients and energy sources, thus maintaining a balance between anabolic processes to support the synthesis of cellular building blocks and catabolic processes to ensure adequate

bioenergetic resources (**Figure 4**). Currently, it has been established that beyond a role in energy support, mitochondrial metabolic shifts is an essential process in mediating stem cell fate decisions (92,93).

There is ample evidence to support the paradigm that glycolysis is preferentially maintained at high levels in rapidly proliferating stem cells, making glycolysis a critical factor in stemness. Glycolysis is inherently less efficient and generates much less ATP than OxPhos, but its kinetics are faster overall, allowing fast energy generation to support cell growth and proliferation (92,94). ESC and iPSCs, as well as many other multipotent stem cells, including hematopoietic stem cells (HSCs), mesenchymal stromal cells (MSCs) and NSCs, rely on glycolysis for ATP production (95–97). It is hypothesized that the lower energy requirements of undifferentiated NSCs help limit oxidative metabolism-dependent generation of ROS to limit ROS-induced cellular damage, thus ensuring lifelong NSC self-renewal and maintenance (98).

In contrast, differentiated cells no longer need to maintain high replication rates and therefore have lower anabolic demands, but they require large amounts of energy to support the processes of cellular homeostasis and the increasingly specialized functions of the progeny. Stem cell differentiation is thus accompanied by a switch in metabolism from one that is predominantly glycolytic to one that is highly dependent on mitochondrial OxPhos. OxPhos is the most efficient way for the cell to generate ATP and is therefore heavily used in differentiated cells with high energy demands, such as neurons (92,94). Indeed, OxPhos genes were shown to be required at the end of neurogenesis to reduce the size of NSCs and initiate their cell cycle exit toward the acquisition of neuronal differentiation in the *Drosophila* brain (99). Another study demonstrated that neuronal differentiation of NSCs derived from hESCs is accompanied by metabolic reprogramming from aerobic glycolysis to neuronal OxPhos (100). In addition, Cabello-Rivera *et al.* have shown that the differentiation of glia-like NSCs and subsequent maturation of oligodendrocytes or neurons is dependent on mitochondrial complex I activity (101).

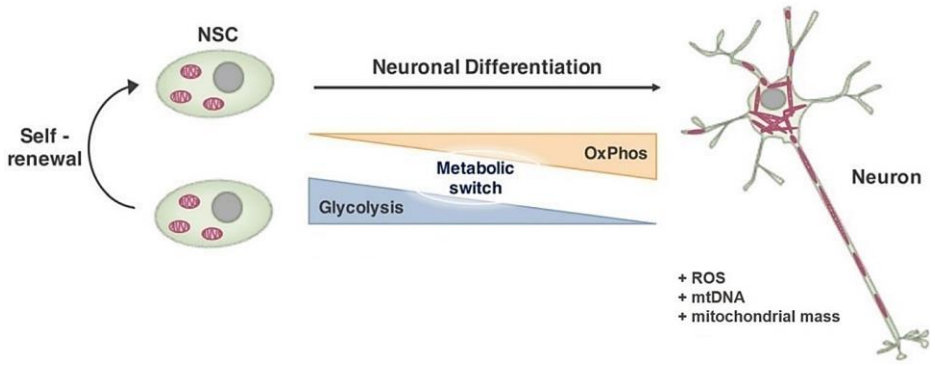

Figure 4 - Schematic representation of mitochondrial metabolic reprogramming during neuronal differentitation. Differentiated neurons, characterized by greater mitochondrial biomass and mtDNA as well as elongated mitochondria, depend mainly on oxidative metabolism from mitochondria (OxPhos), whereas NSCs are characterized by high activity of glycolytic enzymes accompanied by immature mitochondria, low mitochondrial biomass and DNA content. Adapted from (102).

These studies highlight the role of metabolic reprogramming as a driving force in stem cell fate. It precedes the establishment of nuclear identity, which is also regulated by mitochondrial homeostasis mechanisms that guarantee high metabolic plasticity when required (93).

1.2.4 The role of lipid metabolism in NSC fate

Lipids are a diverse group of organic compounds that are essential to higher organisms due to their central role as a structural component in any plasma membrane/organelle membrane and their high energy content. Also, lipids have the ability to act as signalling molecules, thus participating in the regulation of various biological processes, including cell proliferation, cell death, cell migration, gene expression or immune reactions such as inflammation (77,103).

FAs provide a source of energy because they can be stored in lipid droplets in the form of triglycerides. When FAs are required, triglycerides undergo lipolysis, and the resulting FAs can be broken down by β-oxidation in mitochondria or, in the case of very long-chain FAs, in peroxisomes to produce energy in the form of ATP and reducing power (77). This pathway is strongly regulated because FAs cannot enter mitochondria by diffusion. FAs must be actively transported via carnitine palmitoyl transferase enzymes (CPT), such as CPT1A, in a carnitine-dependent manner. Once in the mitochondria, fatty acyl-CoAs are

oxidized by FAO, producing NADH, $FADH_2$, and acetyl-CoAs, all of which can be used for ATP production. These products can be used directly for OxPhos in ETC, or indirectly in the case of acetyl-CoA via the TCA cycle (104).

Recently, it has been demonstrated that FAO plays a crucial role in the maintenance of several adult stem cell populations, including NSCs (77). Knobloch *et al.* demonstrated that quiescent NSCs have higher levels of Cpt1a-dependent FAO than proliferating NSCs. Manipulation of malonyl-CoA, the metabolite that repress FAO levels, was sufficient to terminate quiescence and increase NSC proliferation. Thus, this study reveals a shift in FAO metabolism that determines NSC behaviour and suggests that fatty acid metabolism plays an important role in regulating NSC activity (105). In addition, a link between the clinical association of congenital FAO deficiencies and neuropsychiatric developmental disorders has been established. Inhibition of FAO in embryonic cortex resulted in reduced NSCs and increased differentiation, suggesting that FAO may regulate the preservation of the embryonic NSC pool (106).

De novo lipogenesis, an alternative pathway for obtaining lipids, has also been shown to regulate stem cell behaviour. *De novo* lipogenesis is the formation of FAs from acetyl-CoA, malonyl-CoA, and NADPH. This process occurs in the cytoplasm and involves two key enzymes, acetyl-CoA carboxylase (ACC) and fatty acid synthase (FASN). FAs synthesised *de novo* may then serve as substrates for the production of membrane lipids, be stored in the form of triglycerides to generate energy later by β-oxidation, or provide metabolites involved in protein modifications and signalling networks (77). A study has shown that NSCs upregulate the lipogenic pathway during proliferation and that pharmacological and genetic inhibition of FASN leads to a significant reduction in proliferation of NSCs. Furthermore, knocking down Spot14, a molecule that acts as a regulator of FASN by reducing Malonyl-CoA levels, resulted in increased NSC activation, further confirming its regulatory role for NSC behavior (107). In addition, the endogenous neuroprotective bile acid tauroursodeoxycholic acid (TUDCA) has recently been shown to drive NSC proliferation by remodelling lipid metabolism, namely by causing a shift in metabolism from FAO to lipid synthesis (108,109).

Taken together, these studies on NSCs demonstrate that both FAO and *de novo* lipogenesis can directly influence NSC maintenance, proliferation, and differentiation.

1.2.5 Metabolic regulation of stem cell secretome

It is now clear that mitochondria not only generate ATP, but also plays a major role in regulating stem cell signalling events and epigenetics. To date, it has been demonstrated that mitochondrial components are present in cellular secretome inducing pro-inflammatory responses (110). A study has shown that impaired mitochondrial function of atherosclerotic-MSCs underlies their altered secretome and decreased immunopotency. It has been observed that atherosclerotic-MSCs had higher levels of both intracellular and mitochondrial ROS compared to non-atherosclerotic-MSCs, indicating altered mitochondrial function. The increased mitochondrial ROS levels of atherosclerotic-MSCs resulted in altered cytokine secretion. However, interventions aimed at restoring mitochondrial function of atherosclerotic-MSCs improve their immunosuppressive ability *in vitro* and decreased the secretion of proinflammatory cytokines (111). In addition, Lee *et al.* explored the therapeutic potential of the secretome released from adipose-derived stem cells (ASCs) transfected with PGC-1α (PGC-secretome). PGC-1α is a key mitochondrial biogenesis factor responsible for optimizing stem cell potential, by lowering ROS, compared with the normal ASC-derived secretome. In this study, they observed that the PGC-secretome exhibited higher antioxidant, anti-inflammatory and regenerative properties than the normal secretome, suggesting that mitochondria might play an important role not only in regulating stem cell functions, but also in regulating the stem cell secretome (112).

Notably, it has been also demonstrated that mitochondrial transfer occurs from adult stem cells and somatic cells, being capable of rescuing aerobic respiration in mammalian cells with non-functional mitochondria (113). Interestingly, Zhang *et al.* showed that transplantation of NSC into APP /PS1 mice, a model for AD, significantly increased cognitive performance, the number of functional mitochondria, and the expression of mitochondria-related proteins such as PGC-1α, NRF-1, and the cytochrome *c* oxidase subunit IV (Complex IV). Thus, this study suggests that transplanted NSCs play an important role in replenishing functional mitochondria and further alleviating the progression of AD to some extent (114).

Surprisingly, mitochondrial proteins, mtDNA, and fully functional mitochondria have been shown to be transferred between mammalian cells by EVs, including in stem cells (115). The transfer of mitochondria by MSC-derived EVs is responsible for enhancing phagocytosis and anti-inflammatory effects on macrophages by promoting OxPhos (116). Moreover, EVs from MSCs were shown to transfer partially depolarized mitochondria to macrophages, thereby enhancing bioenergetics in these cells in response to oxidative stress (117). In

particular, it has recently been shown that transport of functional mitochondria in NSCs also occurs via EVs. Peruzzotti-Jametti *et al.* demonstrated, for the first time, that transfer of mitochondria from NSC-derived EVs rescues mitochondrial function and cell survival in mtDNA-deficient target cells (118).

In addition, mitochondria can also be transferred from stem cells to recipient cells by tunneling nanotubules (TNTs). TNTs are nanotubular actin-based structures with a small diameter of 50 to 200 nm and a length of up to 100 µm. These nanostructures enable active transport of cargo and mitochondria between two or more cells (119). In particular, it has been demonstrated that neurons are able to establish intercellular contacts with bone marrow mesenchymal multipotent stromal cells (MMSCs) via TNTs to share their cytosolic contents. MMSCs were also shown to transfer their mitochondria to neurons or astrocytes, leading to the restoration of respiration and stimulation of cell proliferation in recipient cells, thus reducing ischemic damage (120,121). Finally, other recent study showed that mitochondrial transfer from MSCs via TNTs improves neuronal metabolism after oxidative injury *in vitro* (122). Although it is possible that NSCs also function as potential donors of mitochondria via this TNT pathway, there is no clear evidence for TNT-mediated mitochondrial transfer in NSCs.

To date, there is no clear evidence regarding the role of the mitochondria and bioenergetics in regulating the secretome of NSCs, either intracellularly or extracellularly. Hence, it is crucial to investigate the mechanisms by which mitochondria improve the paracrine activity of NSC to augment the benefits of NSC-based therapies in the CNS.

1.3 Major depressive disorder

Major depression disorder (MDD) is the most common mood disorder and the leading cause of disability worldwide, posing a massive burden in current society (123). The underlying cause of depression is difficult to elucidate because it is not a homogeneous disorder but a complex phenomenon based on cluster of symptoms attributable to more than one etiology. Furthermore, due to the lack of specific biomarkers, the early diagnosis is difficult and the treatment is often ineffective, with approximately one-third of patients not responding to conventional antidepressant therapy. This, in turn, underscore the relevance of innovation and discovery in this area (124).

There are currently several leading hypotheses that attempt to elucidate the pathophysiology of MDD.

The monoamine hypothesis was the first proposal for biological causes of depression to emerge in the 1950s. The monoamine hypothesis postulated a deficit of serotonin (5-HT), dopamine and nor-adrenaline as the major cause of MDD that could be reversed by antidepressants to restore normal function in depressed patients. Even though the understanding of the complexity of brain function and the etiology of MDD has greatly increased, a dysfunctional serotonergic system is still considered one of the major factors in the development of this disorder. This is the basis for the use of monoamine oxidase inhibitors as antidepressants in the treatment of depressive disorders to restore low levels of monoamine neurotransmitters (125,126). There are, however, some inconsistencies in the monoamine hypothesis. In particular, the most serious problem with this hypothesis is that it does not explain why antidepressants have a latency period. When antidepressants act on the basis of the monoamine hypothesis, they are thought to act quickly. Nevertheless, antidepressants commonly take 2-4 weeks to exert their therapeutic effects on depressive symptoms (127). Hence, the development of a monoamine-alternative hypothesis is strongly desired to clarify the pathophysiology of MDD.

A large body of evidence suggests that inflammation has central role in pathogenesis of MDD and, in recent years, “inflammatory hypothesis” has been proposed. Proinflammatory cytokines, acute phase response proteins, chemokines, and adhesion molecules have been shown to be elevated in individuals with MDD compared with healthy comparison subjects. High levels of inflammatory biomarkers have also been positively correlated with depression severity and negatively correlated with treatment resolution (128,129). There is also growing evidence that elevated levels of proinflammatory cytokines are a common factor underlying the bidirectional influence between major MDD and chronic inflammatory diseases such as cardiovascular disease, metabolic syndrome, and obesity (130). In addition, administration of inflammatory cytokines, e.g., interferon-α for the treatment of hepatitis C, induced symptoms of depression, including prominent irritability, anxiety, and dysphoria, in nondepressed subjects (131). Although cytokines appear to be implicated in the pathogenesis of MDD, only about one-third of MDD patients have higher levels of inflammation than the majority of non-depressed subjects, suggesting that inflammation is neither necessary nor sufficient to trigger or sustain MDD, but only plays a role in the pathophysiology of MDD (132,133).

The neurotrophic hypothesis of MDD postulates that reduced trophic factor support leads to selective vulnerability of specific neuronal populations, contributing to the pathophysiology of depression (134). It was reported that the expression of BDNF and its receptor TrkB was

decreased in postmortem brain samples, including in the hippocampus, as well as in serum derived from depressed patients (135–137). Since BDNF plays a central role in neuronal plasticity, a decrease in BDNF leads to dysfunction of synaptic plasticity, a decrease in excitatory neurons and glutamate, eventually leading to depression (138). Interestingly, a decrease in hippocampal volume has been observed in MDD patients (139). Chronic stress decreases the expression of BDNF, leading to negative morphological changes in hippocampal neurons. Antidepressants can restore stress-induced morphological changes by increasing the expression of BDNF. The fact that both the increased expression of trophic factors and the reversal of cellular damage were slow to occur and required chronic treatment with antidepressants equivalent to the time delay found for behavioural effects supports the idea that impaired trophic support may contribute to both the pathogenesis and treatment of depression (127,134).

Interestingly, a relationship between metabolism and MDD has been also suggested (140). Four longitudinal meta-analyses confirmed the existence of a bidirectional relationship: obesity increases the risk of developing MDD, and conversely, MDD increases the risk of subsequent obesity (141–144). Furthermore, there is evidence that this MDD-metabolism link is more pronounced in abdominal obesity, which is characterized by an accumulation of visceral fat, and closely related to metabolic dysregulation (140). Indeed, the association between depressed mood and abdominal visceral fat was shown to be stronger than MDD and changes in general obesity (145). Notably, obese individuals with a favourable metabolic profile had only a slightly increased risk of MDD compared to non-obese individuals. However, the risk of MDD was greater when obesity was associated with an unfavourable metabolic profile (146). The study of the MDD-metabolism link is still at an initial stage and needs to be further explored to further understand this disease and discover more effective therapeutic strategies.

Concisely, the current picture of the pathophysiology of MDD is largely incomplete, resulting in many potential hypotheses being proposed and tested that provide a fragmented neurobiological picture of depression and related mood disorders. Thus, continued research in this area is clearly warranted.

1.3.1 The link between adult neurogenesis and MDD

Stressful life events are known to contribute to an increased likelihood of developing MDD, with a higher risk of severe and chronic manifestation and unfavourable treatment

outcome (147). In fact, stress is the major cause of MDD and can produce several physiological responses, including in the nervous, endocrine, and immune systems, that could be harmful under certain conditions (148).

The hippocampus, an area of the brain important in memory, cognitive function, and mood regulation, is particularly vulnerable to chronic stress and mental disorders. Extensive studies have now demonstrated correlative changes in neurogenesis in the adult hippocampus under several pathophysiological conditions, including psychiatric disorders. Whether these changes represent adaptive responses to numerous pathophysiological conditions, are part of the pathophysiology contributing to the disease, or both remains largely unclear (149,150).

Remarkably, in most psychiatric disorders, such as MDD, a decrease in cell proliferation in the DG and reduced hippocampal volume have been reported, which is associated with impaired hippocampus-dependent functions, including memory and spatial pattern separation (150,151).

In contrast, administration of antidepressants, such as selective serotonin reuptake inhibitors (SSRIs), appeared to increase the number of NPCs in the hippocampus (152–155). Indeed, Malberg et al. demonstrated that antidepressants prompted hippocampal neurogenesis in rodents, and the time course for the upregulation is consistent with the time delay required for the therapeutic action of antidepressants (156), while Santarelli et al. showed that disrupting neurogenesis prevents behavioural responses to antidepressants (157). These and many other studies suggest that adult-generated hippocampal neurons are necessary for adequate mood control and for the efficacy of antidepressants. One possible explanation for antidepressant-induced neurogenesis is related to monoamine neurotransmitters, as they are known to influence neurogenesis in the adult brain. Thus, common antidepressants may promote neurogenesis in part by increasing synaptic levels of 5-HT and other monoaminergic neurotransmitters (158).

A number of factors or conditions that increase the rate of neurogenesis in the adult hippocampus have been described to be "resilience factors" or have antidepressant effects independent of neurogenesis, thus implying that they could mechanistically modulate adult neurogenesis to promote stress resilience (30). For instances, physical exercise is strongly associated with a broad spectrum of beneficial effects on the hippocampus, including boosting neurogenesis, and has been demonstrated to have a protective effect against the emergence of depression regardless of age and geographical region (159).

Moreover, in the last two decades, several studies have shown that depressive disorders are a risk factor for cognitive decline and dementia, especially AD, since adult hippocampal neurogenesis is affected (160). A postmortem analysis of human brain tissue suggests that treatment of MDD with SSRIs in patients with Lewy body dementia is associated with increased numbers of doublecortin (DCX^+) cells, representing progenitors or neuroblasts, suggesting increased neurogenic activity. Moreover, the patients receiving this treatment also exhibit less cognitive decline (161).

Using immunocytochemistry and immunohistochemistry analyses of human postmortem brain tissue, two recent studies investigated neurogenic changes in AD and MDD (**Figure 5**). Notably, many of the findings complement each other. DCX^+ cells are reduced, Nestin, labelling NSCs and early transiently amplifying NPCs, and neuronal nuclear antigen (NeuN) a marker for early postmitotic and more mature granule neurons are also reduced. Although the results of both studies indicate a decrease in the presented markers, supporting the hypothesis of adult hippocampal neurogenesis (AHN) dysregulation in neuropsychiatric disorders, neither study fully captured the entire neurogenic course. Furthermore, the use of different markers makes it difficult to compare the results of different studies, especially in quantitative terms. It might be useful to define different markers to label all stages of AHN so that the results of different studies are comparable (162–164).

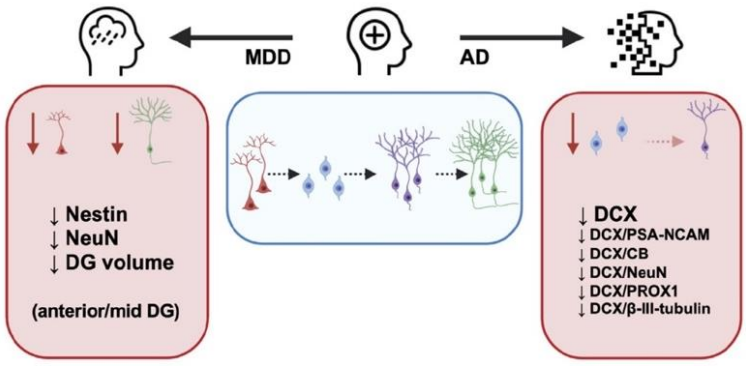

Figure 5 - Neurogenic changes in Alzheimer's Disease (AD) and Major Depressive Disorder (MDD). MDD is associated with decreased numbers of $Nestin^+$ cells and $NeuN^+$ cells, expressed by NSCs and mature neurons, respectively, and decreased DG volume. AD is associated with lower numbers of DCX^+ cells, both alone and in combination with second markers for postmitotic cells (NeuN, PSA-NCAM, βIII-tubulin, CB or PROX1), indicating impaired differentiation of neuroblasts into immature neurons. Transient amplyfing NPCs (red) give rise to neuroblasts (blue), that will form immature neurons (purple), that will eventually mature (green). Abbreviations: CB, calbindin; DCX,

doublecortin; NeuN, neuronal nuclear antigen; PCNA, proliferating cell nuclear antigen; Prospero homeobox protein 1, PROX1. Adapted from (164).

1.3.2 Unpredictable chronic mild stress animal model

Animal models of psychiatric conditions are procedures applied to laboratory animals that produce behavioural changes intended to resemble to aspects of psychiatric disorders. They can therefore be used as experimental tools to expand the understanding of human psychopathology (165).

The unpredictable chronic mild stress (uCMS) model consists of exposing rodents to a variety of socio-environmental stressors that have the following characteristics: [1] they are mild in terms of intensity (i.e., they never cause physical pain and none of the stressors can by themselves have lasting effects on the mouse phenotype); [2] they are chronic, since they are repeated over a period of weeks, whereas acute administration of such mild stressors would be expected to be ineffective; [3] they occur in an unpredictable manner in terms of timing (i.e., varying over days/weeks, duration of each stressor, timing of administration, etc.) (166).

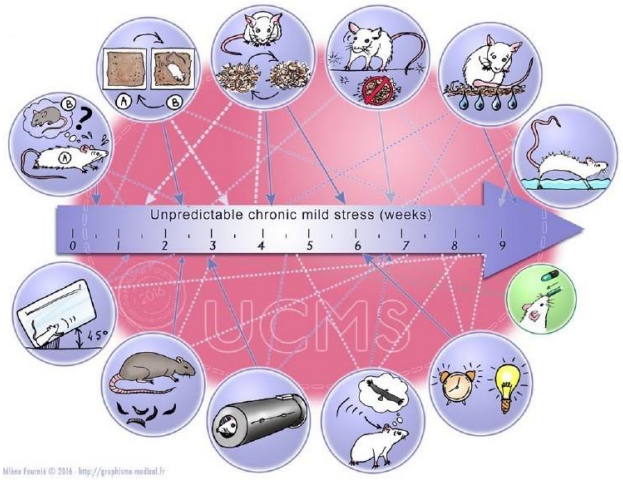

Figure 6 - The unpredictable chronic mild stress (uCMS) model of depression. Several stressors can be used, such as changes in lighting, spatial confinement, introduction of rats faeces, cage tilting, social stress, cage changes, sawdust changes, no sawdust, humid sawdust and water in the cage (166).

This model leads to the development of a number of behavioural changes in the vast majority of animals, including anhedonia (loss of pleasure) and apathy. Moreover, this model also leads to a variety of neurobiological effects that reflect the changes observed in MDD. These include decreased neurogenesis in the hippocampus, increased microglial activation, decreased 5-HT neurotransmission in the forebrain, decreased neurotrophins such as BDNF in the hippocampus, and decreased dendritic branching in the hippocampus (166). These behavioural alterations, as well as changes in certain endocrine and neuronal variables, are similar to those seen in humans with MDD and are also reversed by chronic but not acute treatment with antidepressants (167).

Altogether, mounting evidence suggests that NSC-driven neuroplasticity goes beyond their differentiation potential in the adult brain, being essential to unravel the molecular mechanisms, namely those related with mitochondrial metabolism, responsible for neuroprotection and/or regeneration of NSC secretome.

2. Motivation and Aims

Neurological disorders are a growing global public health challenge. Among the most common and challenging disorders, Alzheimer's disease (AD) and major depressive disorder (MDD) are interrelated. In fact, a depressive event early in life increases later AD risk and patients suffering from AD are more susceptible to develop depression. MDD has been associated with aberrations of the neurogenic niche, such as decreased hippocampal volume and consequent deficits in hippocampal function(164), however, an increased rate of SGZ neurogenesis induced by chronic treatment with antidepressants have been shown to improve psychiatric disorders, suggesting an important role of adult neurogenesis on antidepressant therapies (168,169). Moreover, adult neurogenesis promotes stress resilience by preventing the adverse effects of stress on cognition and mood in animal models of unpredictable chronic mild stress (uCMS) (170). Since the efficacy and tolerability advantages of newer drugs are quite modest and the limitations associated with their use persist in several important areas(171), there is growing clinical interest in the use of stem cells as therapy, particularly in patients resistant to conventional treatments (172). The paracrine activity of NSCs has been found to play a key role in neuroprotection, neuroplasticity and immune regulation of the nervous system (63), which has considerable therapeutic significance and prospects. Although it has been demonstrated that mitochondria regulate cell secretome (112), the underlying mechanisms responsible for the metabolic regulation of NSC-derived secretome are still largely unclear.

Our group have previously explored the therapeutical potential of NSC-derived secretome in different *in vitro* models of NSC damage. Our results showed that NSCs receiving inflammatory mediators or other “danger signals” from damaged neighbouring NSCs (boosted NSCS) were the ones capable of producing the most therapeutic secretome (boosted conditioned media – boosted CM) (**Figure 7**). In addition, boosted NSCs were shown to present several metabolic alterations, including increased expression levels of mitochondrial fragmentation, lipid biosynthesis- and oxidative protection-related genes and lower total ATP levels. In contrast, injured neuron-like differentiating cells receiving boosted CM were shown to present lower levels of cell death and increased ATP levels, when compared with those receiving non-boosted CM (CM).

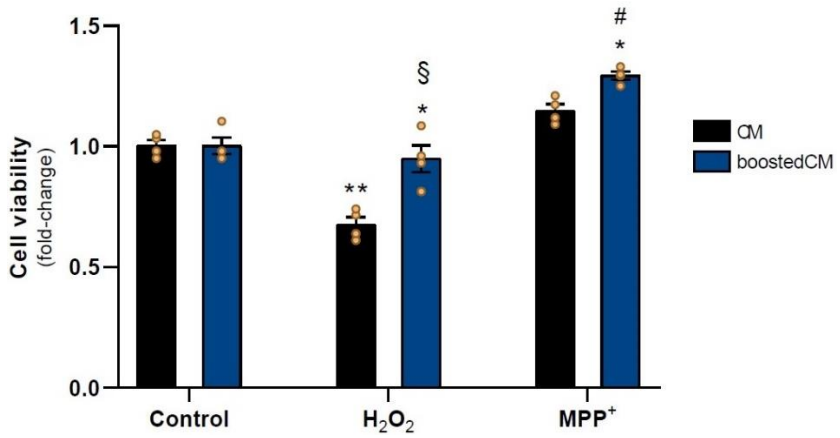

Figure 7 - BoostedCM rescues neuron-like differentiating NSCs from oxidative injury. NSCs were treated with hydrogen peroxide (H_2O_2) or 1-methyl-4-phenylpyridinium (MPP^+) and co-incubated with 25% of CM or boostedCM for 24h in differentiating conditions. Quantification of NSC viability was performed using Guava easyCyte 5HT flow cytometer. Data are expressed as fold change over non-injured NSCs treated with CM. Data represent mean values ± SEM for at least three independent experiments. $^*p < 0.05$ and $^{**}p < 0.01$ compared to non-injured NSCs with CM, $^{\S}p < 0.05$ compared to H_2O_2-treated NSCs with CM, $^{\#}p < 0.05$ compared MPP^+-treated NSCs with CM.

Thus, based on this previous data, we started to further characterizing the metabolic signature and alterations of boosted NSCs, evaluate the potential effect of key metabolic modulators in the paracrine activity of NSCs while also exploring the bioenergetic role of NSC secretome in a depressive-like behaviour animal model.

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

1. **Which type of metabolic alterations occur in NSCs to mediate neuroprotection of their secretome?**
2. **Can key metabolic regulators also impact on NSC-sourced secretome and induce similar neuroprotective effects?**
3. **Do depression-associated secreted factors stimulate NSC paracrine activity through metabolic changes?**

Unveiling the role of metabolism in NSC secretome-driven regeneration will certainly translate into important knowledge to design novel effective and less-invasive strategies to delay neural degeneration, confer stress resilience and improve life quality of people with neurological illnesses.

3. Materials and Methods

3.1 Ethic statement

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

3.2 Cell line handling, maintenance and differentiation

Tau-GFP mouse NSC (NS-TGFP) cells were derived from 14.5-dpc mouse fetal forebrain, and constitutively express the fusion protein tau-GFP (173,174). The mouse cell line was produced by a method that generates pure cultures of adherent bonafide NSCs that expand continuously by symmetric division and are capable of tripotential differentiation (8,175,176). NSCs were grown in monolayer as previously described (177) and routinely maintained in undifferentiating medium (self-renewal conditions), DMEM/F-12 + GlutaMAX medium (Gibco, Thermo Fisher Scientific, Inc., USA), supplemented with N-2 supplement (Gibco, Thermo Fisher Scientific, Inc.), penicillin-streptomycin (Pen-Strep; Gibco, Thermo Fisher Scientific, Inc.), epidermal growth factor (EGF; PeproTech EC, UK) and basic fibroblast growth factor (bFGF; PeproTech EC) (**see table 1 for resumed details**), in tissue culture (TC)-treated flasks (Falcon, Corning Inc., NY, USA) at 37ºC in a humidified atmosphere of 5% CO_2.

To prevent cells from detaching and degenerating due to nutrient deficiency and contact inhibition, cell culture was renewed every 2-3 days when the cells reached 70-80% confluence in the culture flask, which represents the critical point. The depleted medium was removed and then 1 mL of StemPro Accutase Cell Dissociation Reagent was added (Gibco, Thermo Fisher Scientific, Inc.) to gently help cell detachment. After incubation for 3 min in the incubator, PBS was added to dilute the Accutase. Cell suspension was collected and then centrifuged at 500 *g* for 5 min at room temperature (RT). After discarding the supernatant, the cell pellet was resuspended in freshly prepared medium.

Neural differentiation was induced by first plating NSCs in undifferentiating medium onto TC-treated 12-well plate with a density around 1.2×10^5 cells/mL. 24 h after plating, culture medium was changed to optimized neuronal differentiation-inducing medium, DMEM/F-12

+ GlutaMAX medium supplemented with Pen-Strep, N-2 supplement, B-27 supplement (Gibco, Thermo Fisher Scientific, Inc.), N-2 supplement, and bFGF (**see table 1 for resumed details**).

For CM collection, NSCs were maintained in self-renewal conditions and seeded with a density around 1 x 10^5 cells/cm^2 in 60 mm TC-treated cell culture dish.

Table 1 - Resumed details of culture media composition used to expand mouse NSCs

Undifferentiating growth medium % (v/v)	Differentiating growth medium % (v/v)
DMEM/F-12 + GlutaMAX;	DMEM/F-12 + GlutaMAX;
1% Pen-Strep;	1% Pen-Strep;
1% N-2;	0.5% N-2;
20 ng/mL bFGF;	10 ng/mL bFGF;
20 ng/mL EGF.	1% B-27.

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

3.3 Cellular treatments of secretome producing NSCs and conditioned medium collection

Undifferentiated NSCs were plated, and 24 h after treatment, or no treatment, with 50 µM of the injury molecule 1-methyl-4-phenylpyridinium (MPP^+) (D048-000; MERCK), the InjCM and CM (from untreated NSCs) were collected, respectively, and stored at - 80ºC (**Figure 8**).

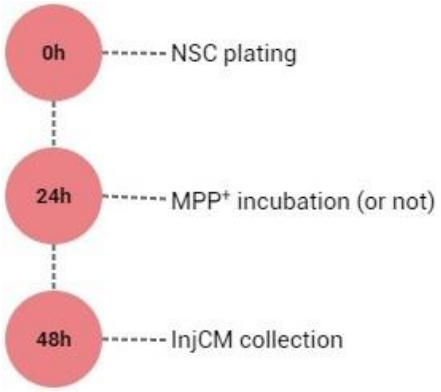

Figure 8 - Schematic step-by-step representation for InjCM collection in self-renewing NSCs.

For collection of secretome derived from injCM-stimulated NSCs (boosted CM), undifferentiated NSCs were treated with 25% of injCM or CM after 24 h of plating. NSCs and CMs were both collected 24 h later (**Figure 9**).

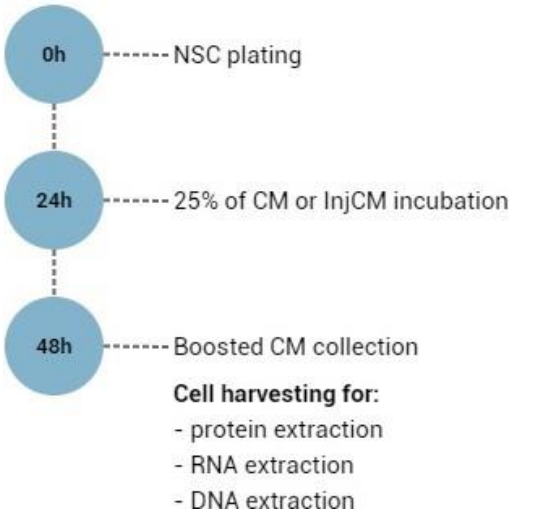

Figure 9 - Schematic step-by-step representation for boostedCM collection in self-renewing NSCs.

For the second part of this study, undifferentiated NSCs were treated with well-established mitochondrial/metabolic regulators, including 100 µM TUDCA (T0266; Sigma-Aldrich Corp.), 1mM of the short chain fatty acid propionate (P1880; Sigma-Aldrich Corp), and a cocktail of neurotrophin factors comprising 30 ng/mL VEGF (AF-100-20; PrepoTech), 30 ng/mL IGF-1 (I3769; Sigma-Aldrich Corp) and 30 ng/mL BDNF (kindly given by Regeneron) to mimic *in vitro* exercise-mediated effects, all 24 h after cell plating. CMs were collected 24 h after compound incubation and were named mitCMs (**Figure 10**).

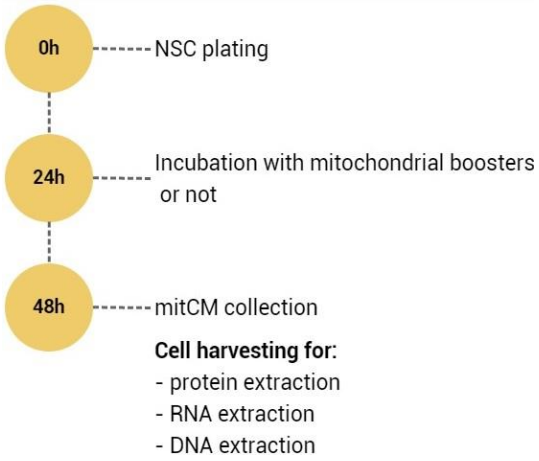

Figure 10 - Schematic step-by-step representation for mitCM collection in self-renewing NSCs.

For the third part of this study, NSCs were plated for 24 h and then exposed with 1% of either healthy or depressed mice-derived serum for additional 24 h. Healthy-boostedCM and uCMS-boostedCM were collected, respectively (**Figure 11**). Mice serums were provided by Dr. Sara Xapelli, iMM, University of Lisbon, Lisbon, Portugal.

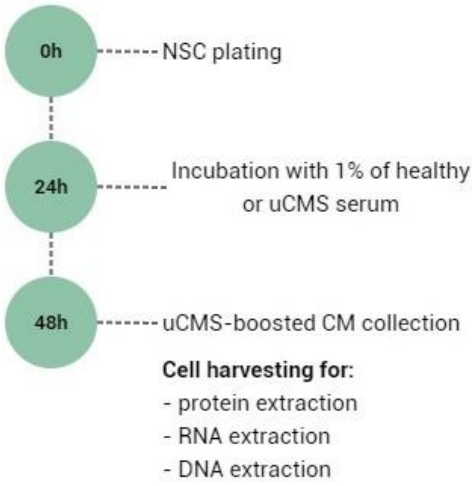

Figure 11 - Schematic step-by-step representation for uCMS-boostedCM collection in self-renewing NSCs.

3.4 Cellular treatments of target injury NSCs

NSCs were seeded in self-renewal conditions for 24 h. Media was replaced for neuron-like differentiating culture media. After 48 h in differentiating media, NSCs were treated with 100 µM Hydrogen peroxide (H_2O_2) and co-incubated with 25% of different CMs. After 24 h of incubations, differentiating NSCs were collected for cell viability assays. The schematic representation of the time course treatments of target injury differentiating NSCs is illustrated in **Figure 12**.

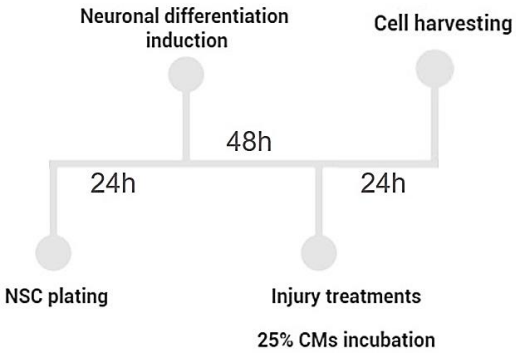

Figure 12 – Graphical scheme of differentiating NSC injury treatment course.

3.5 Cell viability assay

Cell death and viability were assessed by Guava® ViaCount™ reagent (Luminex Corp., Austin, Texas, USA) according to the manufacturer's instructions. After injury models and CM incubations in differentiating conditions, cell culture medium containing cell debris was collected together with adherent cells previously detached with StemPro Accutase (A11105-01; Gibco™). Neuron-like differentiating cells were centrifuged for 5 min at 500 *g* and resuspended in phosphate-buffered saline (PBS). Cell suspension (110 µL) was mixed with Guava® ViaCount™ reagent (40 µL) and incubated for 5 min at room temperature (RT). Sample acquisition was performed using Guava easyCyte 5HT flow cytometer (Luminex Corp., Austin, Texas, USA), according to the manufacturer's instructions. Guava soft (Luminex Corp. Austin, Texas, USA) was used for data analysis.

3.6 Total RNA extraction

Total cell RNA was extracted using 500 µL of TRIzol® Reagent (Invitrogen, Waltham, Massachusetts, USA). Cells were incubated for 5 min at RT to allow the complete dissociation of nucleoprotein complexes. Afterwards, 100 µL of chloroform was added and samples were vortexed for 15 sec and incubated for 3 min at RT. Samples then were centrifuged at 12,000 *g* for 15 min at 4ºC. The aqueous phase was transferred to a new tube. The RNA was precipitated from the aqueous phase using 250 µL of isopropyl alcohol. Samples were incubated at RT for 10 min and centrifuged at 12,000 g for 10 min at 4°C. The RNA pellet was washed with 75% ethanol and centrifuged at 7,500 g for 5 min at 4°C.

RNA pellets were air dried and resuspended in 50 µL of RNase-free water. RNA samples were incubated for 10 min at 55ºC to increase the solubilisation of the RNA.

3.7 Quantitative RT-PCR (qRT-PCR)

Complementary DNA (cDNA) synthesis of 1 µg of total RNA from each sample was performed using the NZY First-Strand cDNA Synthesis Kit (NZYTech, Lda., Lisbon, Portugal) according to the manufacturer instructions. mRNA gene expression was determined by real-time or quantitative polymerase chain reaction (qRT-PCR). Real time PCR analyses were performed in an Applied Biosystems® QuantStudio™ 7 Flex Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with SensiFAST™ SYBR® Hi-ROX Kit (bio-92020; Bioline), 600 nM of both forward and reverse primers (detailed in Table 2), 2 µL of cDNA template, and water to a final volume of 5 µL. Real time PCR was always performed in duplicate and melting point dissociation curves were performed between 60ºC and 95ºC to confirm that only a single product was amplified. To ensure quality of the measurements, each PCR experiment for each gene included a negative control. mRNA levels for the genes of interest were normalized to mRNA levels of hypoxanthine phosphoribosyltransferase (HPRT) and expressed as fold change from controls.

Table 2 - List of primers for RT-PCR experiments

Gene	Primer Fwd (5'-3')	Primer Rev (5'-3')
tfam	CACCCAGATGCAAAACTTTCAG	CTGCTCTTTATACTTGCTCACAG
gfap	CCAAACTGGCTGATGTCTACC	GCTTCATCTGCCTCCTGTCTA
βIII-tubulin	GCGCCTTTGGACACCTATTCA	TTCCGCACGACATCTAGGACTG
ki67	CCTTTGCTGTCCCCGAAGA	GGCTTCTCATCTGTTGCTTCCT
hprt1	GGTGAAAAGGACCTCTCGAAGTG	ATAGTCAAGGGCATATCCAACAACA

3.8 Total protein extraction

NSCs were collected, centrifuged, and the pellet resuspended in lysis buffer (1% NP-40, 20 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 10% Glycerol, 1 mM dithiothreitol (DTT), and 1X proteases and phosphatases inhibitors), and incubated for 30 min at 4ºC.

Cell lysates were sonicated for 30 sec and centrifuged at 3,200 g for 10 min at 4°C. Total protein extracts contained in the supernatants were recovered and stored at −80°C.

Protein was quantified using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA), according to the manufacturer's specifications. Bovine serum albumin was used as standard. Absorbance measurements were performed at 595 nm.

3.9 Western blot

Protein levels were determined by Western blot (WB) analysis. Briefly, 35 µg of total protein extracts were separated on (% or 12% (w/v) sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes. Uniform protein loading and transfer was assessed by staining with 0.2% Ponceau S (Merck, Darmstadt, Germany). Membranes were blocked with 5% milk solution in Tris-buffered saline (TBS) for 30 min and incubated overnight at 4°C with primary rabbit or mouse antibody reactive to the protein of interest (**Table 3**). After washing three times with TBS containing 0.2% Tween 20 (TBS-T), 5 min each, membranes were incubated with anti-rabbit or anti-mouse secondary antibodies conjugated with horseradish peroxidase (Bio-Rad; 1: 5,000) for 2 h at RT (**Table 4**). Finally, membranes were rinsed three times with TBS-T and processed for protein detection by chemiluminescence using Immobilon Western Chemiluminescent HRP Substracte (Millipore, Merk) in iBright^{TM} CL1500 Imaging System (Thermo Fisher Scientific). Densitometric analysis of images was performed with the iBright Analysis Software.

Table 3 - List of primary antibodies used for WB

Target	Host	Source	Dilution
MFN2	Mouse	Abcam PLC (ab56889)	1:1000
DRP1	Rabbit	Santa Cruz Biotechnology (sc-32898)	1:1000
Total OXPHOS	Mouse	Abcam PLC (ab110413)	1:1000
LCAD	Rabbit	ProteinTech (17526-i-ap)	1:10000
PGC-1α	Mouse	Calbiochem (St1202)	1:500

Table 4 - List of secondary antibodies used for WB

Target	Host	Source	Dilution
Goat Anti-Mouse IgG (H + L)-HRP Conjugate	Anti-mouse	Bio-Rad (1706516)	1:5000
Goat Anti-Rabbit IgG (H + L)-HRP Conjugate	Anti-rabbit	Bio-Rad (1706515)	1:5000

3.10 Total DNA extraction

Total DNA was extracted using QIAamp DNA Mini Kit (51304; Qiagen), according to manufacturer's protocols. This kit allowed the isolation of the circular mitochondrial DNA (mtDNA) together with the genomic DNA. DNA quantification was performed using Nanodrop analysis.

3.11 Quantification of mtDNA copy number

Quantitative PCR analysis was performed using SensiFAST™ SYBR® Hi-ROX Kit in Applied Biosystems 7300 System (both from Thermo Fisher Scientific Inc.), as previously described (178). Briefly, two independent reactions for mitochondrial and nuclear primer sets were run for each sample. The 12.5 µl reaction volumes contained 6.25 µl of SensiFAST™ SYBR® Hi-ROX Kit, 0.25 µl of a 16.6 µM stock of each primer, 0.75 µl of water, and 5 µl of template genomic DNA. All DNA samples were used at 15 ng/µL, and then diluted 2- to 500-fold to insure accurate sample profiling and linearity. The PCR protocol consisted of 50°C for 2 min and 95°C for 10 min, 40 cycles of 95°C for 15 sec and 60°C annealing/extension for 1 min, then 1 cycle of 95ºC for 15 sec, 60ºC for 30 sec and 95ºC for 15 sec.

Mitochondria encoded gene *mt-Co1* was used to determine mtDNA copy number while nuclear *Rn18s* gene to determine the number of cells. The relative amounts of each gene were calculated based on the standard curve. MtDNA copy number was determined by the ratio of *mt-Co1* gene amplification to *Rn18s* gene and expressed as fold change from controls.

Table 5 - List of primers used for qPCR experiments

Gene	Primer Fwd (5'-3')	Primer Rev (5'-3')
Rn18s	TAGAGGGACAAGTGGCGTTC	CGCTGAGCCAGTCAGTGT
mt-Co1	ATCTGTTCTGATTCTTTGGGCAC	AGCCTAGAAAGCCAATAGACATTA

3.12 Statistical analysis

Statistical analysis was performed using Student's t test and one-way ANOVA followed by Dunnet's post-test for multiple comparisons. Values of $p < 0.05$ were considered statistically significant. Statistical analysis was performed with GraphPad Prism 8.0.2 software (GraphPad Software, Inc., USA.)

4. Results and Discussion

4.1 Boosted NSCs sensing danger signals present a more protective secretory profile for neuron-like differentiating NSCs

4.1.1 NSCs producing the most protective secretome are more proliferative and present distinct patters of mitochondrial dynamics and activity

Lipid metabolism plays a major role in adult neurogenesis. In fact, lipids not only serve as building blocks for membranes and organelles, but also act as energy sources and signalling molecules involved in several biological processes such as proliferation and differentiation, including neurogenesis (77,103).

Our group has previously shown that boosted NSCs exhibit increased lipogenesis, as the expression of SREBP-1, a key positive regulator of lipid biosynthesis, and ACC1, a key enzyme for *de novo* lipogenesis that catalyses the production of malonyl-CoA, was significantly higher in boosted NSCs compared to unstimulated NSCs (**data not published**). On the other hand, it has been demonstrated that *de novo* lipogenesis is crucial for proliferating NSCs (107). Accordingly, our first data, regarding the biochemical characterization of boosted NSCs, showed that these cells present a significant increase in mRNA expression of the proliferation marker Ki67 ($p < 0.05$) (**Figure 13.A**).

Interestingly, a central role in regulating the transition from FAO to lipogenesis during stem cell differentiation, appears to be mediated by the levels of malonyl-CoA, which is a potent inhibitor of CPT1, the rate-limiting enzyme of mitochondrial FAO (179). Thus, we also investigated the differences in proteins levels of long chain acyl-CoA dehydrogenase (LCAD), a key enzyme for FAO and mitochondrial energy metabolism (180). Curiously, our results showed that proteins levels of LCAD are significantly decreased in boosted NSCs ($p < 0.01$), when compared with unstimulated NSCs (**Figure 13.B**). Indeed, this is consistent with the observation that quiescent NSCs require high levels of mitochondrial lipid degradation by FAO for energy production, and as an alternative carbon source, which in turn is downregulated in proliferating NPCs. Notably, it was already demonstrated that manipulation of malonyl-CoA levels results in quiescence exit of mouse NSCs and increased NSC proliferation (181,182).

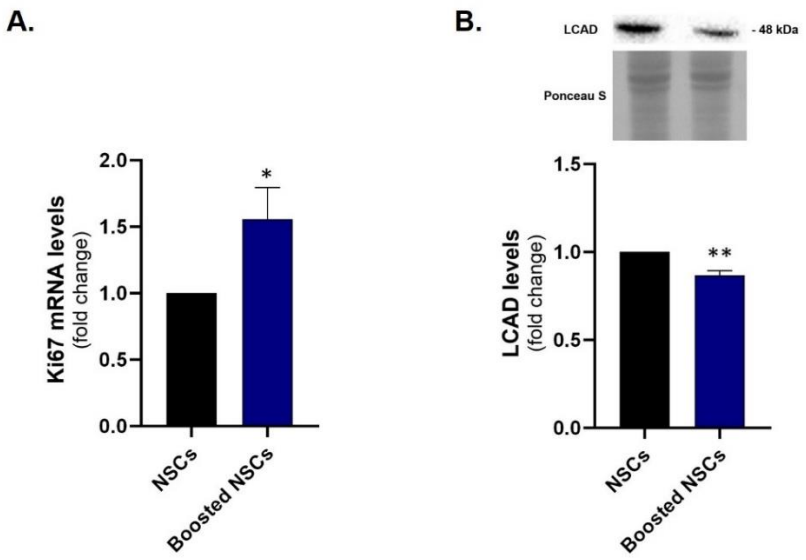

Figure 13 - Boosted NSCs are more proliferative and present decreased levels of a key FAO-related enzyme. NSCs were treated with either CM or InjCM for 24 h in self-renewal conditions, and then collected for RT-PCR and WB analysis to evaluate mRNA expression levels of the proliferation marker, Ki67 and the protein levels of a FAO-related enzyme, LCAD, respectively. (**A**) Effect of InjCM on Ki67 mRNA levels in NSCs. Values were normalized to the internal mRNA standard HPRT. (**B)** Effect of InjCM on LCAD protein levels in NSCs. Representative blot of LCAD levels (top) and corresponding densitometry analysis (bottom) in total protein extract. Values were normalized to Ponceau S. Data are represented as fold change over unstimulated NSCs. Data represent mean values ± SEM for at least three independent experiments. *$p < 0.05$ and **$p < 0.01$ compared to unstimulated NSCs (NSCs).

Mitochondrial fission and fusion are mediated by Drp1 and Mfn2, respectively. These mitochondrial dynamic processes are tightly controlled in living cells according to their energy and metabolic requirements. Thus, we decided to investigate potential changes in these protein levels in boosted NSCs (**Figure 14**). Although not significant, our results showed that the protein levels of Drp1 were slightly increased in boosted NSCs compared with unstimulated NSCs (**Figure 14.A**). On the other hand, the protein levels of Mfn2 were significantly decreased in boosted NSCs ($p < 0.05$) (**Figure 14.B**). More importantly, the ratio of Drp1/Mfn2 protein levels in NSCs increased after stimulation with InjCM (**Figure 14.C**), suggesting that mitochondrial fragmentation could be necessary for the metabolic remodelling of boosted NSCs.

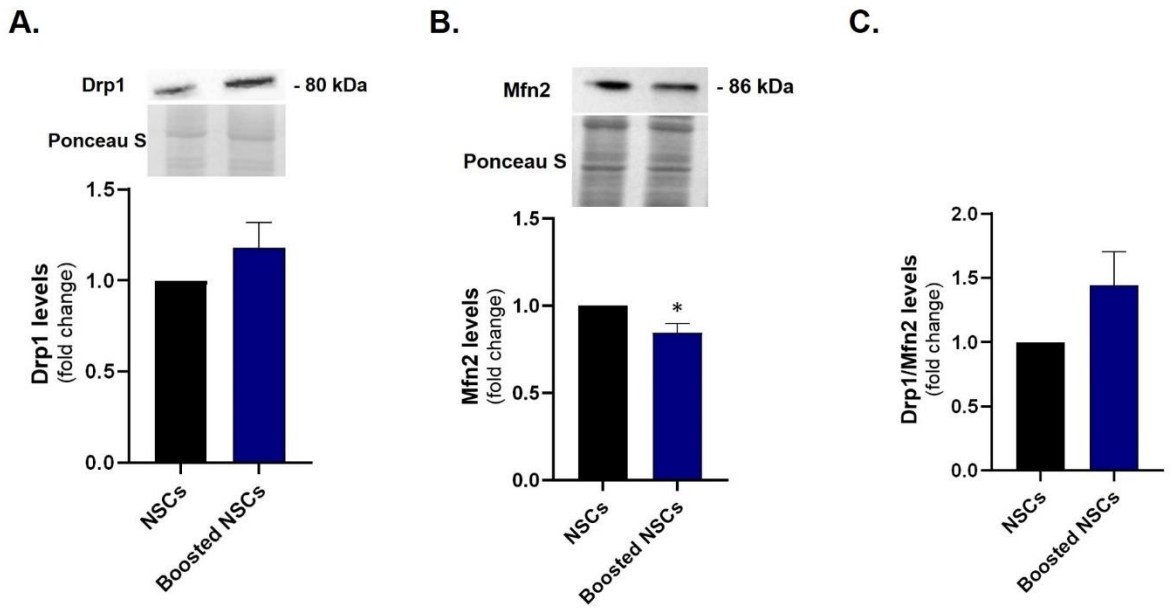

Figure 14 - Boosted CM appears to favor mitochondrial fission in NSCs. NSCs were treated with either CM or InjCM for 24 h in self-renewal conditions, and then collected for WB analysis to assess protein levels of mitochondrial fission and fusion markers, Drp1 and Mfn2, respectively. (**A**) Effect of InjCM on Drp1 levels. Representative blot of Drp1 levels (top) and corresponding densitometry analysis (bottom) in total protein extracts. (**B**) Effect of InjCM on Mfn2 levels. Representative blot of Mfn2 levels (top) and corresponding densitometry analysis (bottom) in total protein extracts. (**C**) Ratio between Drp1 and Mfn2 protein levels. Drp1 and Mfn2 protein levels were normalized to Ponceau S. Data are represented as fold changes over unstimulated NSCs. Data represent mean values ± SEM for at least three independent experiments. *$p < 0.05$ compared to unstimulated NSCs (NSCs).

Mitochondrial fission is essential for cell growth and division. It ensures an adequate number of mitochondria, maintains cell polarity, and helps to eliminate damaged mitochondria (183). Likewise, mitochondrial fission promotes stem cell self-renewal and pluripotency by stimulating glycolysis in MSCs (184,185).

Since it is commonly thought that cells with predominantly fragmented and immature mitochondria present lower levels of OxPhos (87), we then decided to also investigated possible changes in protein levels of OxPhos protein complexes I-V (**Figure 15**).

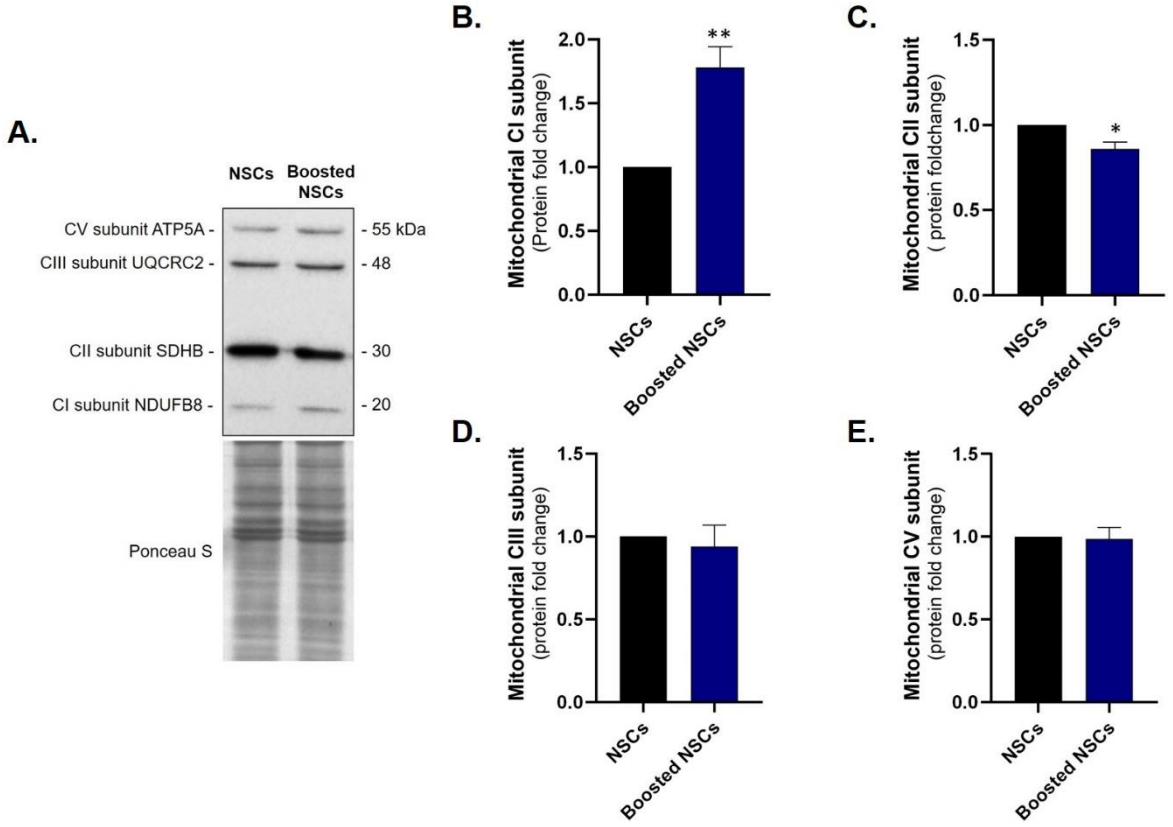

Figure 15 - A shift in mitochondrial complex subunits is observed in boosted NSCs. NSCs were expanded and treated with CM or either InjCM for 24 h in self-renewal conditions, and then collect for WB analysis. (**A)** Representative blot of mitochondrial respiratory chain complex I–V subunits in total protein extracts. Densitometry analysis of mitochondrial complex I (**B**), II (**C**), III (**D**) and V (**E**) subunits. in boosted NSCs. Protein levels were normalized to Ponceau S. Data are expressed as fold change over unstimulated NSCs. Data represent mean ± SEM for at least three independent experiments. *$p < 0.05$ and **$p < 0.01$ compared to unstimulated NSCs (NSCs).

Interestingly, when we compared unstimulated and boosted NSCs, we detected a significant increase in NADH-ubiquinone oxidoreductase (Complex I subunit) ($p < 0.01$, ~ 2 fold) (**Figure 15.B**), and a slight decrease in succinate dehydrogenase (Complex II subunit) levels ($p < 0.01$, ~ 0.25 fold) (**Figure 15.C**), while the ubiquinol-cytochrome *c* oxidoreductase (Complex III subunit) and ATP5A, the ATPase α chain (Complex V subunit) remained unchanged (**Figure 15.D** and **15.E**, respectively). We could not assess cytochrome *c* oxidase (Complex IV subunit) levels due to technical issues.

Curiously, Cabello-Rivera and colleagues have already shown that *in vitro* proliferation of postnatal SVZ-NSCs is highly dependent on the function of OxPhos Complex I (MC I). They

have reported that the number of SVZ-derived neurospheres was slightly lower in mice with MCI mutation compared with control animals, and exhibited a significant decrease in neurospheres diameter, suggesting that proliferation of NSCs was seriously affected by MCI dysfunction (101). On the other hand, conditional deletion of succinate dehydrogenase subunit D in cells of the astrocyte lineage, which includes adult SVZ and DG NSCs, impaired differentiation to neurons and oligodendrocytes in mice without affecting the generation, maintenance, and multipotency of adult NSCs (186). These studies and our results suggest that the increased proliferation state of boosted NSCs turn these cells more dependent on MC I for energy metabolism.

Since our team already demonstrated that boosted NSCs present decreased ATP levels and unchanged TFAM expression, a key factor for mtDNA maintenance and replication, we decided to investigate alterations in the levels of an important player in mitochondrial biogenesis, PGC-1α, as well as in mtDNA copy number in boosted NSCs (**Figure 16**). According with what was previously observed to TFAM mRNA levels (**data not published**), PGC-1α protein levels did not change in boosted NSCs (**Figure 16.A**). In contrast, in line with what observed for ATP levels, mtDNA copy number was shown to be significantly diminished in boosted NSCs, when comparing with unstimulated NSCs (**Figure 16.B**).

Regarding the role of mtDNA in stem cell fate determination, undifferentiated stem cells usually contain low mtDNA copy number, as the number of mitochondria and mtDNA content were shown to be significantly reduced in iPSCs and human ESCs compared to somatic fibroblasts, suggesting that reprogramming may indeed reduce the number of mitochondria within somatic cells to levels comparable to ESCs (15). Contrarily, NSC differentiation appears to be associated with a huge increase of mitochondrial mass and mtDNA copy number (16).

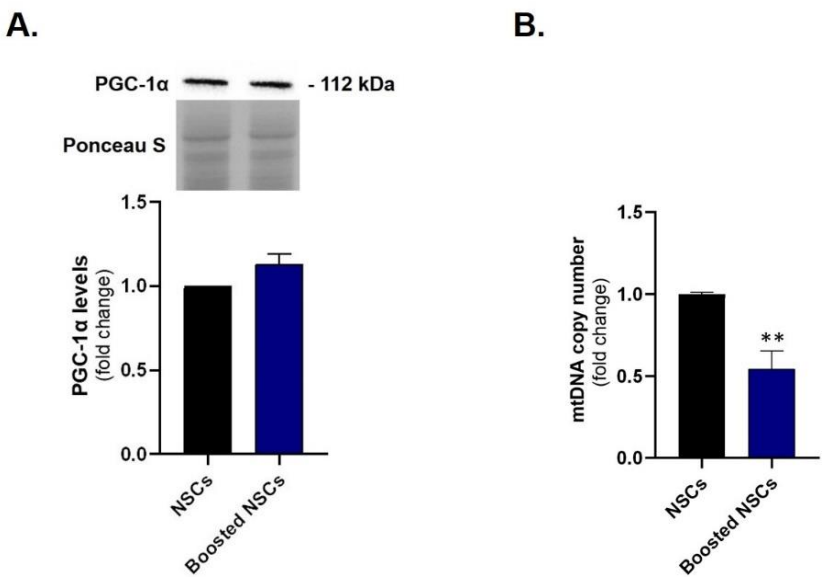

Figure 16 - Boosted NSCs have lower mtDNA copy number. NSCs were treated with CM or InjCM for 24 h in self-renewal conditions, and then collected for WB analysis to assess protein levels of the mitochondrial biogenesis marker, PGC-1α, and for qPCR analysis of mitochondrial and nuclear genes. (**A**) Effect of InjCM on PGC-1α levels. Representative blot of PGC-1α levels (top) and corresponding densitometry analysis (bottom) in total protein extracts. Levels were normalized to Ponceau S. (**B**) Effect of InjCM on mtDNA copy number in NSCs. Quantification of relative mtDNA copy number was assessed by qPCR analysis of mitochondria-encoded gene mt-Co1. Nuclear Rn 18s was used as loading control. Data are expressed as fold change over unstimulated NSCs. Data represent mean values ± SEM for at least three independent experiments, $^{**}p < 0.01$ compared to unstimulated NSCs (NSCs).

These results suggest that profound metabolic alterations occur in NSCs receiving external injury signals which go along with the production and delivery of a more protective secretome to target recipient neural cells.

4.1.2. Boosted CM increase mtDNA copy number in target NSCs

Since the secretome derived from boosted NSCs (boosted CM) was shown to be more efficient in protecting committed target cells from oxidative-mediated cell damage, we investigated possible changes in mtDNA copy number in cells receiving boosted CM. Our team had already demonstrated that, under normal and stress conditions, target neuron-like differentiating cells present a decreased NAD^+/NADH ratio and increased ATP levels when exposed to boosted CM (**data not published**).

In addition, we assessed mtDNA copy number and, according with our previous results cells receiving boosted CM presented an increase in mtDNA copy number, although not significantly (**Figure 17**).

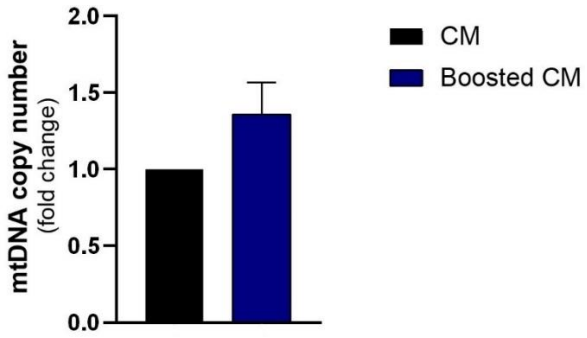

Figure 17 - Boosted CM appears to induce an increase of mtDNA copy number in target NSCs. NSCs were expanded and treated with CM or boosted CM in self-renewal conditions, and then collect for qPCR analysis of mitochondrial and nuclear genes. Quantification of relative mtDNA copy number was assessed by qPCR analysis of mitochondria-encoded gene mt-Co1. Nuclear Rn 18s was used as loading control. Data are expressed as fold change over NSCs treated with CM. Data represent mean values ± SEM for at least three independent experiments.

Notably, it has been recently reported that mitochondrial can be transferred from adult stem cells and somatic cells, being capable of rescuing aerobic respiration in recipient mammalian cells with non-functioning mitochondria (19). Indeed, mitochondrial proteins, mtDNA and fully functional mitochondria can be transferred between mammalian cells through EVs (115). For example, mitochondrial transfer via MSC-derived EVs is responsible for the anti-inflammatory and phagocytosis-enhancing effects of MSC on macrophages by promoting OxPhos (116). Finally, NSCs have been shown to release mitochondria into the extracellular space, wherein they continue to hold functional properties that can be transferred to target cells, thereby rescuing mitochondrial function and cell survival in mtDNA-deficient target cells (118).

Bearing in mind that differentiating NSCs rely more on mitochondria-dependent metabolism and that the injury insult chosen to produce injCM mediates oxidative damage by causing mitochondrial dysfunction, it is also possible that boosted CM contain mitochondria or several mitochondrial contents to enhance target cell survival. Although we did not use the same neuron-like differentiating cell model when assessing mtDNA copy

number, as we did to assess ATP and NADH levels, it is crucial to confirm these results in a near future on the same recipient cell model. Furthermore, it would be interesting to investigate whether mitochondria transfer also occur from boosted NSCs to protect target neuron-like differentiating cells from injury.

4.2. Role of mitochondrial boosters in NSC secretory profile

4.2.1 Mitochondrial boosters improve the neuroprotective effects of NSC secretome

It is now clear that mitochondria not only generate ATP but also play an important role in regulating stem cell signalling and epigenetic modulation. To date, mitochondrial components have been shown to be present in the cellular secretome inducing pro-inflammatory responses (110). In addition, the secretome of ASCs transfected with PGC-1α prsented higher antioxidant, anti-inflammatory, and regenerative properties when compared with the secretome derived from non-modulated ASCs. This suggests that mitochondria play an important role not only in regulating stem cell functions, such as self-renewal and differentiation, but also in regulating the stem cell secretome (112). Based on the fact that there is no clear evidence regarding the role of the mitochondria and bioenergetics in regulating the therapeutic properties of NSC secretome, we decided to test the effects of potent mitochondrial boosters in NSC-derived secretome for future improvement of their therapeutic properties.

The endogenous bile acid tauroursodeoxycholic acid (TUDCA), that acts as an anti-apoptotic and antioxidant molecule at the mitochondrial level, has been shown to be neuroprotective in several animal models of acute ischemic stroke (187) and neurodegenerative diseases (188–190). More importantly, TUDCA was shown to increase NSC pool and neurogenesis in adult rats by preventing mitochondrial dysfunction and increasing ATP levels (108,191). Recently, we also identified a fatty acid metabolic reprograming in NSCs treated with TUDCA (109). Propionate, in turn, is a short-chain fatty acid (SCFA), recently demonstrated to be capable of increasing mitochondrial bioenergetics and biogenesis in adult NSCs (192,193). Our group already demonstrated that both TUDCA and propionate treatments significantly increased ATP levels in NSCs, when compared to

untreated NSCs (**data not published**), thus suggesting that both compounds act as fine-tune mitochondrial boosters in NSCs.

In addition, the brain is known to be hightly reactive to exercise, which in turn has also been implicated in mitochondrial biogenesis and activity, ameliorating the electron transport system, and the antioxidant capacities in the mouse brain (194,195). Based on the fact that the growth factor VEGF, BDNF and IGF-1 are markedly elevated after physical exercise (196), we decided to use a cocktail of neurotrophic factors as described in *Material and Methods* to mimic physical exercise in brain *in vitro*.

To evaluate and establish comparisons of the potential therapeutic improvement of the secretome derived from bioenergetic-stimulated NSCs, NSCs were maintained in self-renewal medium with or without the above mentioned “mitochondrial boosters” and all CMs (mitCMs) were collected as described in *Material and Methods*. To test and compare the protective effects of CM and mitCMs, we assessed viability of neuron-like differentiating NSCs undergoing H_2O_2-induced oxidatve stress and simultaneously exposed to 25% of CM or mitCMs (**Figure 18**).

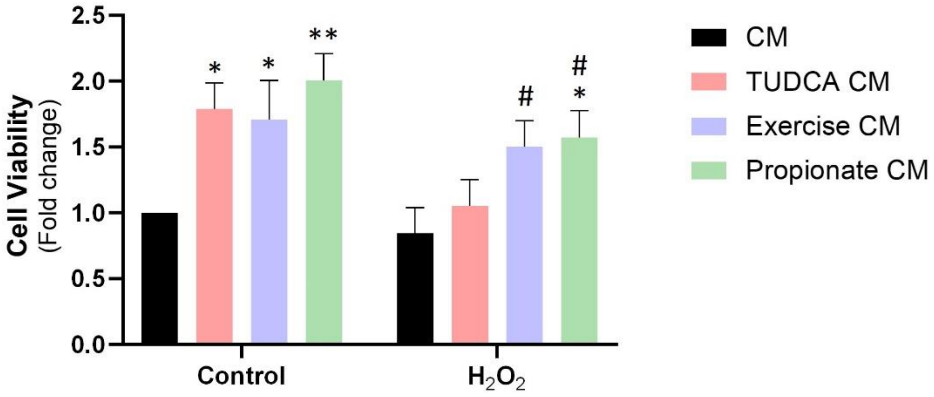

Figure 18 - MitCMs rescue viability of injured neuron-like differentiating cells. NSCs were expanded, treated with H_2O_2 and co-incubated with 25% of CM or mitCMs in differentiating conditions, and then collected for cell viability analysis. Cell viability was assessed using Guava easyCyte 5HT flow cytometer. Data are expressed as fold change over non-injured NSCs treated with CM. Data represent mean values ± SEM for at least three independent experiments. *$p < 0.05$ and **$p < 0.01$ compared to cells treated with CM.

Notably, our results showed that, in normal conditions, all mitCMs significantly increased cell viability in neuron-like differentiating target NSCs ($p < 0.01$, ~2 fold for all conditions), comparing to cells treated with CM. Further, in cell damage conditions, exercise-CM and propionate-CM showed a significant protective value in mediating protection of neuron-like differentiating NSCs against injury-induced cell death ($p < 0.05$, ~1.5 fold).

In fact, it has been suggested that physical exercise is an efficient way to regulate oxidative balance in the brain. Marosi and colleagues demonstrated that long-term physical exercise leads to decreased levels of ROS and protein carbonyls, and increased concentrations of antioxidant enzymes in the hippocampus of aging rats (197). Another study suggests that physical exercise attenuates motor and cognitive deterioration, oxidative stress, and neuroinflammation induced by 6-OHDA, an endogenous toxin found in the urine of PD patients, in the striatum of mice (198).

In addition of supplying cells with energy, SCFAs have been demonstrated to influence neuronal function (199). In fact, SCFAs help to improve cognitive performance in animal models of neurodegenerative diseases (200). Yang *et al.* (2020) demonstrated that SCFAs, at physiological levels, are able to induce the proliferation and mitosis of hNPCs (201). Moreover, other study demonstrated that *Clostridium butyricum*, a probiotic that produces the SCFA, butyrate, significantly improved neurological dysfunction, brain edema, neurodegeneration, and BBB impairment in a mouse model of traumatic brain injury (202).

These results suggest that external factors that enhance mitochondrial activity could be strategically used to enhance the therapeutic potentials of NSC-derived secretome.

4.2.2 Mitochondrial boosters decrease LCAD levels and increase mitochondrial fragmentation in NSCs

Since NSCs treated with TUDCA (TUDCA NSCs), an exercise cocktail (exercise NSCs), and propionate (propionate NSCs) produced a more competent secretome to protect committed target cells from oxidative-mediated cell damage, we decided to investigate what type of metabolic and mitochondrial changes occur in these bioenergetically stimulated NSCs.

Our group has already demonstrated that TUDCA (108,191) and propionate (193) promote the proliferation of NSCs *in vitro* and *in vivo* . Additionally, aerobic exercise, such as running, increases adult neurogenesis in the hippocampus of both male and female adult rodents (33,34,203,204), as well as in the SVZ neurogenic niche (205,206).

As already mentioned, cellular metabolism has been shown to be an important factor in the behaviour of NSCs, in which lipid metabolism plays a particular role in their regulation. Hence, we decided to investigate possible changes in LCAD protein levels in bioenergetically boosted NSCs (**Figure 19**).

Our results revealed that LCAD protein levels significantly decrease in NSCs treated with TUDCA ($p < 0.05$), exercise cocktail ($p < 0.01$), and propionate ($p < 0.05$), when compared with untreated NSCs.

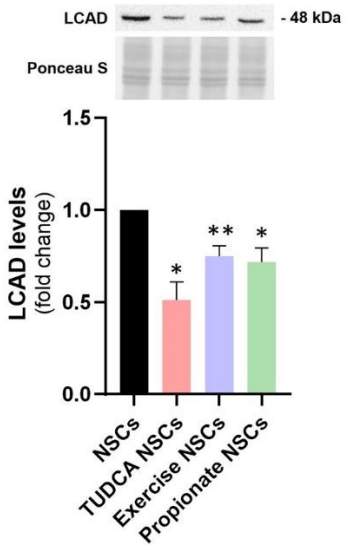

Figure 19 - Mitochondrial boosters decrease LCAD levels in NSCs. NSCs were treated with mitochondrial boosters, or not, for 24,h in self-renewal conditions, and then collected for WB analysis. Representative blot of the FAO related protein, LCAD (top) and corresponding densitometry analysis (bottom) in total protein extract. Values were normalized to Ponceau S. Data are represented as fold change over untreated NSCs (NSCs). Data represent mean values ± SEM for at least three independent experiments. *$p < 0.05$ and **$p < 0.01$ compared to untreated NSCs (NSCs).

These results obtained from TUDCA-treated NSCs are in accordance with our previous report, showing that TUDCA downregulates LCAD in NSCs, even in the SVZ neurogenic regions of adult rat brains(109). Moreover, the relevance of FASN, the key enzyme for *de novo* lipid synthesis, in proliferating NSCs was confirmed in a study of voluntary running in mice. In this study, physical exercise, also a strong inducer of AHN, increased lipogenesis, while the exercise-induced proliferation was prevented by chronic inhibition of FASN (207). This increased lipogenesis was also observed in a global metabolomics profiling of the

hippocampus and the frontal cortex in a voluntary running in mice study (208). In addition to their function as fuels for the oxidative generation of ATP, SCFAs also supply anabolic pathways, such as lipogenesis, and contribute to the regulation of cellular metabolism by activating signaling pathways. Thus, SCFAs play an important role in balancing lipogenesis and oxidative degradation of fatty acids (209).

These results suggest that mitochondrial boosters might favour anabolic lipogenesis, instead of lipid degradation, to enable the formation of lipid membranes, and ensure the maintenance of the high NSC proliferation rates.

We then decided to investigate possible changes in mitochondrial dynamics in these bioenergetically stimulated NSCs (**Figure 20**). Our results revealed that both TUDCA and exercise cocktail slightly decreased Drp1 protein levels, while propionate induced a more accentuated decrease in Drp1 levels, when compared to untreated NSCs (**Figure 20.A**). In addition, Mfn2 protein levels were demonstrated to be markedly diminished in NSCs treated with all mitochondrial boosters (~ 0.5 fold) (**Figure 20.B**). More importantly, the ratio of Drp1/Mfn2 protein levels increased in NCSs treated with all mitochondrial boosters (**Figure 20.C**), corroborating our previous observation in boosted NSCs, in which mitochondrial fission occurs to possibly promote mitochondrial metabolic adaptations in NSCs and induce a more protective secretome to neuron-like differentiating target cells.

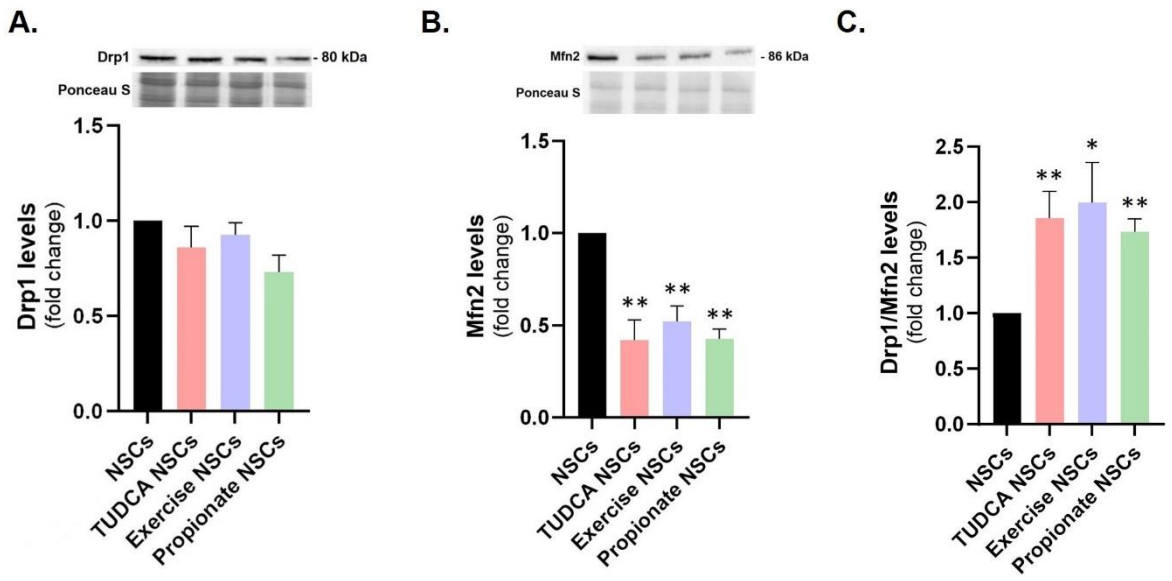

Figure 20 - Mitochondrial boosters induce mitochondrial fission in NSCs. NSCs were treated with several mitochondrial boosters, or not for 24 h, in self-renewal conditions, and then collected for WB analysis to assess protein levels of mitochondrial fission and fusion markers, Drp1 and Mfn2, respectively. (**A)** Effect of mitochondrial boosters in Drp1 levels. Representative blot of Drp1 levels (top) and corresponding densitometry analysis (bottom) in total protein extracts. (**B**) Effect of mitochondrial boosters in Mfn2 levels. Representative blot of Mfn2 levels (top) and corresponding densitometry analysis (bottom) in total protein extracts. Drp1 and Mfn2 levels were normalized to Ponceau S. (**C**) Ratio between Drp1 and Mfn2 protein levels. Data are represented as fold changes over untreated NSCs. Data represent mean values ± SEM for at least three independent experiments. *$p < 0.05$ and **$p < 0.01$ compared to untreated NSCs (NSCs).

Subsequently, potential changes in levels of OxPhos protein complexes I-V (**Figure 21**) were also investigated in bioenergetically-stimulated NSCs. Our results showed that NSCs stimulated with exercise cocktail exhibited a significant increase in Complex I subunit ($p < 0.05$), while NSCs treated with the other mitochondrial boosters exhibited unchanged levels for this protein (**Figure 21.B**). The levels of Complex II subunit was slightly increased in propionate-treated NSCs, while stimulation with other mitochondrial boosters did not induce any change (**Figure 21.C**). The protein levels of Complex III subunit, in turn, remained unchanged in each condition compared to untreated NSCs (**Figure 21.D**). Finally, NSCs stimulated with mitochondrial boosters showed a slight decrease in the levels of Complex V subunit, significant for the exercise cocktail boosted NSCs (**Figure 21.E**). In fact, in adipose tissue, physical exercise enhances expression of uncoupling protein 1 (UCP1), a protein

responsible for mitochondrial uncoupling and energy production in the form of heat instead of ATP production (210).

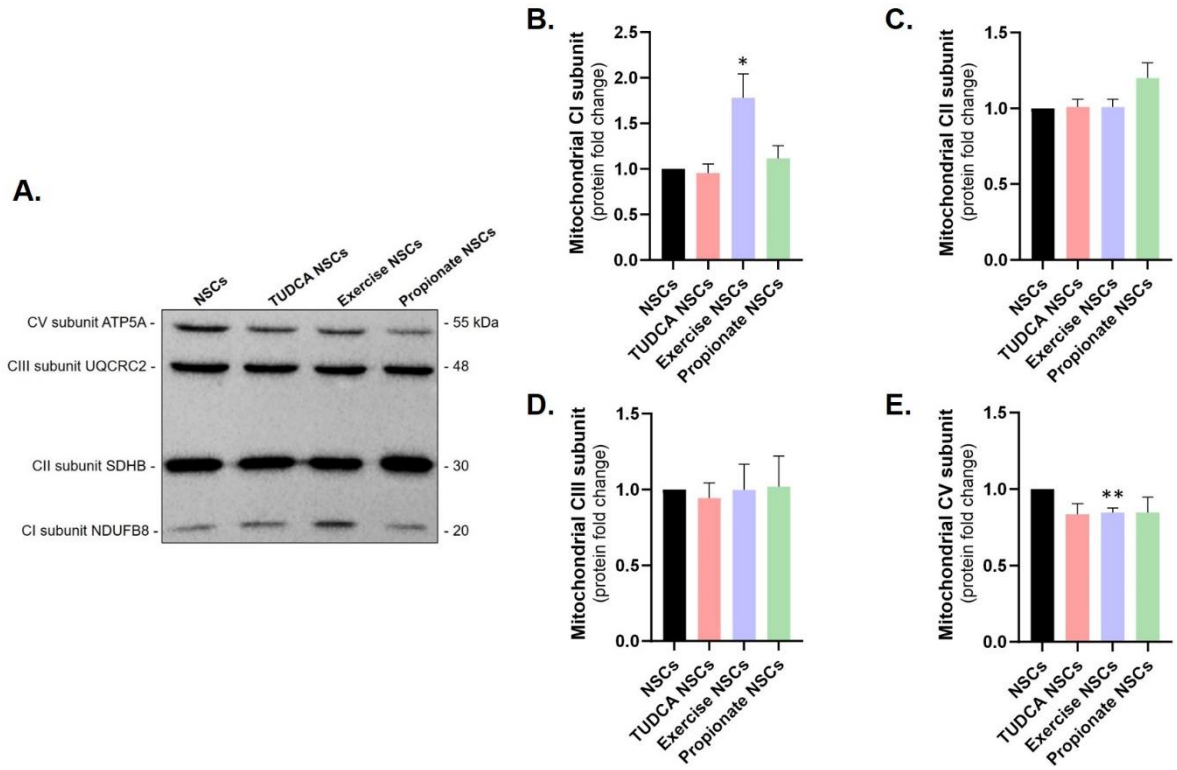

Figure 21 – Impact of mitochondrial boosters on the mitochondrial complex subunits in NSCs. NSCs were expanded and treated with mitochondrial boosters, or not, for 24 h in self-renewal conditions, and then collect for WB analysis. (**A)** Representative blot of mitochondrial respiratory chain complex I–V subunits in total protein extracts. Densitometry analysis of the effect of mitochondrial boosters in mitochondrial complex I (**B**), II (**C**), III (**D**) and V (**E**) subunits. Protein levels were normalized to Ponceau S. Data are expressed as fold change over untreated NSCs (NSCs). Data represent mean ± SEM for at least three independent experiments. *$p < 0.05$ and **$p < 0.01$ compared to untreated NSCs (NSCs).

It is also tempting to speculate that increased glycolysis might occur in NSCs stimulated with mitochondrial boosters to facilitate the production of a more effective and protective secretome. Although glycolysis has been strongly associated with proliferation of stem cells (93), future experiments need to be addressed to confirm this hypothesis.

As previously mentioned, these mitochondrial boosters are known to also induce mitochondrial biogenesis. Thus, potential changes in mitochondrial biogenesis-associated proteins, TFAM and PGC-1α, as well as in mtDNA copy number were investigated in bioenergetically regulated NSCs, by RT-PCR, WB, and PCR, respectively (**Figure 22**).

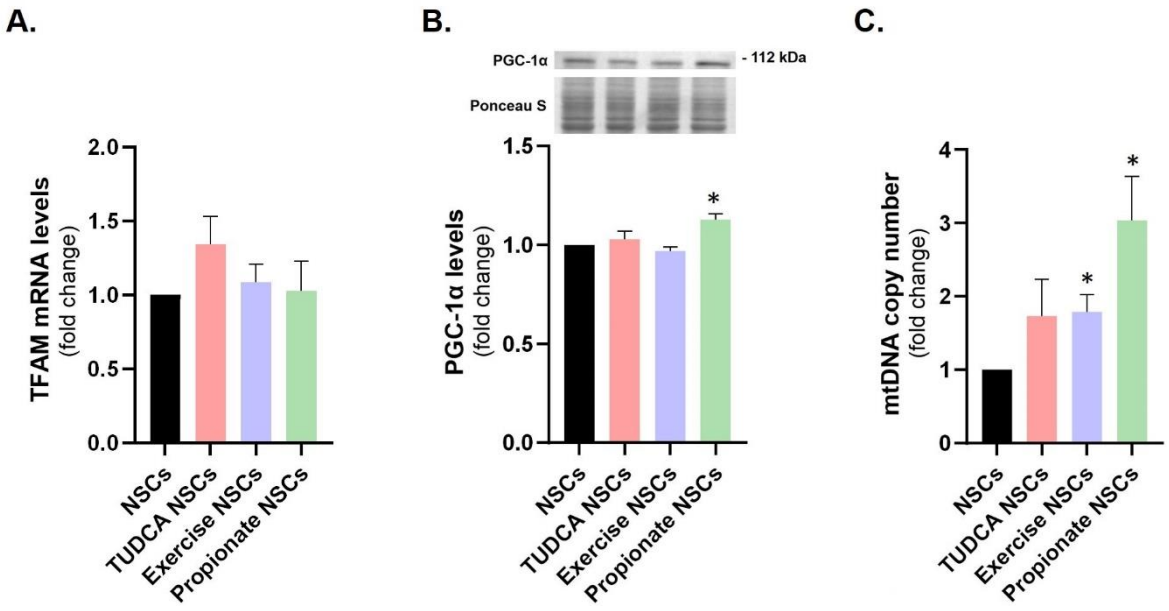

Figure 22 – Impact of mitochondrial boosters on NSC mitochondrial biogenesis. NSCs were treated with mitochondrial boosters, or not, for 24h in self-renewal conditions, and then collected for RT-PCR, WB or qPCR analysis. (**A**) Effect of mitochondrial boosters on TFAM mRNA expression levels. Levels were normalized to the internal mRNA standard HPRT. (**B**) Effect of mitochondrial boosters in PGC-1α levels. Representative blot of PGC-1α levels (top) and corresponding densitometry analysis (bottom) in total protein extracts. Levels were normalized to Ponceau S. (**C**) Effect of mitochondrial boosters in mtDNA copy number in NSCs. Quantification of relative mtDNA copy number was assessed by qPCR analysis of mitochondria-encoded gene mt-Co1. Nuclear Rn 18s was used as loading control. Data are expressed as fold change over untreated NSCs. Data represent mean values ± SEM for at least three independent experiments, *$p < 0.05$ compared to untreated NSCs (NSCs).

Curiously, TFAM mRNA levels remained unchanged in NSCs treated with the exercise cocktail and propionate, whereas TUDCA induced an increase, albeit not significant, in TFAM mRNA levels compared to untreated NSCs (**Figure 22.A**). Moreover, an increase in

PGC-1α protein levels was observed in NSCs treated with propionate, whereas levels remained unchanged in NSCs treated with TUDCA and exercise cocktail (**Figure 22.B**). Remarkably, all mitochondrial "boosters" induced an increase in mtDNA copy number compared to untreated NSCs, particularly propionate (~3 fold, $p < 0.05$) (**Figure 22.C**).

Regarding TUDCA, these results suggest that the induced increase in mitochondrial biogenesis in NSCs might involve up-regulation of TFAM. Moreover, Patki *et al*. (2011) have shown that in the striatal region of the brain of a chronic mouse model of PD, TFAM and PGC-1α are upregulated in response to repeated oxidative insults in a manner that may attempt to compensate for mitochondrial dysfunction, but both return to normal levels after exercise, including the associated oxidative stress, indicating that maintenance of normal mitochondrial function is essential to prevent neurodegeneration (211). Steiner and colleagues demonstrated that mtDNA was significantly increased after exercise training in several mice brain, including in the hippocampus (27). It would be important to confirm whether the changes in TFAM mRNA expression levels are also reflected on protein total levels. Lastly, our team had already demonstrated that propionate induces an increase in mtDNA copy number in NSCs (193).

Our next step was to investigate any alterations in NSC differentiation levels upon these specific mitochondrial regulators. For that, glial fibrillary acidic protein (GFAP), a marker for astrocytes, and βIII-tubulin, a marker for neuronal differentiation, were evaluated in bioenergetically regulated NSCs by RT-PCR (**Figure 23**). Our results revealed that NSCs treated with these mitochondrial boosters presented a slight decrease in mRNA expression levels of both differentiation markers, although not significant (**Figure 23**).

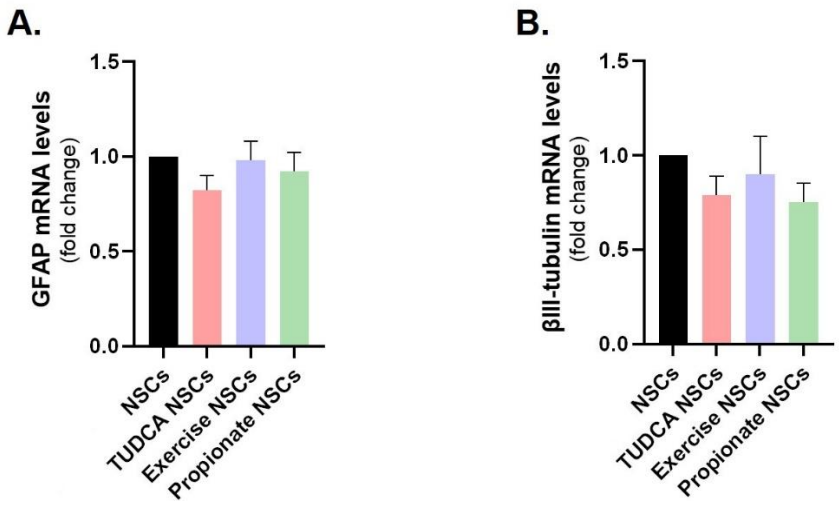

Figure 23 - Mitochondrial boosters do not alter the NSC stemness fate. NSCs were treated with mitochondrial boosters, or not, for 24 h in self-renewal conditions, and then collected for RT-PCR to assess mRNA expression levels of differentiation markers, GFAP and βIII-tubulin. Effect of mitochondrial boosters on GFAP (**A**) and βIII-tubulin (**B**) mRNA expression levels. Levels were normalized to the internal mRNA standard HPRT. Data represent mean ± SEM for at least three independent experiments.

4.2.3 MitCMs increase mtDNA copy number in target NSCs

Since secretome derived from bioenergetically stimulated NSCs have been shown to be more efficient in protecting committed target cells from oxidative-mediated cell damage, we investigated possible changes in mtDNA copy number in cells receiving mitCMs (**Figure 24**).

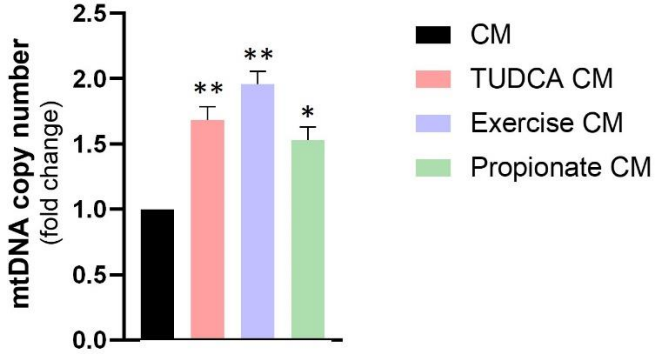

Figure 24 - NSCs receiving mitCM present an increase in mtDNA copy number. NSCs were expanded and treated with either CM or different mitCMs in self-renewal conditions, and then collect

for qPCR analysis of mitochondrial and nuclear genes. Quantification of relative mtDNA copy number was assessed by qPCR analysis of mitochondria-encoded gene mt-Co1. Nuclear Rn 18s was used as loading control. Data are expressed as fold change over NSCs treated with CM. Data represent mean values ± SEM for at least three independent experiments. *$p < 0.05$ and **$p < 0.01$ compared to NSCs treated with CM.

Notably, our results demonstrate that all target NSCs present a significant increase in mtDNA copy number when stimulate with the mitCMs ($p < 0.01$), particularly NSCs stimulated with Exercise CM (~ 2 fold). As mentioned earlier in chapter 4.1.2, stem cells have been shown to release mitochondrial proteins, mtDNA, and fully functional mitochondria into the extracellular space. As such, it is possible that NSCs stimulated with these mitochondrial boosters release mitochondria or mitochondrial components/metabolites that will increase mitochondrial number, or induce a mitochondrial biogenesis, in target cells. We did not assess mtDNA copy number in injury target neuron-like differentiating NSCs, as we did in cell viability assays, therefore it is vital to confirm these results using the same target model. Additionally, it would be interesting to investigate whether the transfer of mitochondria from boosted NSCs actually occurs in this *in vitro* model.

4.3 Depression-associated secreted factors induce NSCs to produce a more protective secretome to damaged target neural cells

4.3.1 NSCs stimulated with serum of depressed mice rescue neuron-like differentiating target cells from cell death

The composition of the stem cell secretome can vary according with several factors, including chemical and physical stimuli of the cell microenvironment. One of the most common approaches in cell secretome engineering involves the cell preconditioning, namely by exposing cells to inflammatory and damage stimuli (212,213).

In the unpredictable chronic mild stress (uCMS) animal model of depression, animals are exposed to a series of low-intensity stressors at unpredictable times over a period of several weeks, leading to the development of a number of behavioural changes in the vast majority of animals. These behavioural changes, along with alterations in certain endocrine and neural variables, are similar to those seen in patients with MDD (214). Concentrations of specific serum-associated molecules have been shown to vary in MDD patients, and peripheral biochemical changes are known to occur during antidepressant treatment (215). Patients with MDD had significantly higher levels of IL-1β, IL-10, IL-6 and TNF-α, but significantly lower levels of IL-8 compared to healthy controls (216,217). In addition, lower serum BDNF levels were found in patients with MDD (137). Moreover, the uCMS animal model induces a decrease in hippocampal neurogenesis and a decrease in neurotrophins such as BDNF (166). Thus, we decided to investigate whether exposure to serum derived from healthy or depressed mice would have a differential effect on NSCs-derived secretome.

For this purpose, NSCs were maintained in self-renewal medium and incubated with 1% serum derived from healthy or depressed mice (healthy-boosted NSCs and uCMS-boosted NSCs, respectively) to eventually boost the production of an adaptive protective response of these cells to neighbouring damaged cells.

To test the protective effect of the secretome derived from depressed serum-treated NSCs (uCMS boosted-CM), neuron-like differentiating NSCs and primary NSCs were co-incubated with the H_2O_2-insult with either healthy-boosted CM or uCMS boosted-CM and collected for cell viability analysis (**Figure 25**).

Although not significant, NSCs receiving depressed mice-derived serum produced a secretome that increased the viability of neighbouring neuron-like differentiating NSCs against injury-induced cell death, when compared to NSCs receiving healthy mice-derived serum (**Figure 25.A**). Indeed, this positive effect of depressed mice-derived serum on viability of neighbouring cells was shown to occur under both normal and neural stress conditions, being also validated in primary cultures of NSCs in one experiment (**Figure 25.B**).

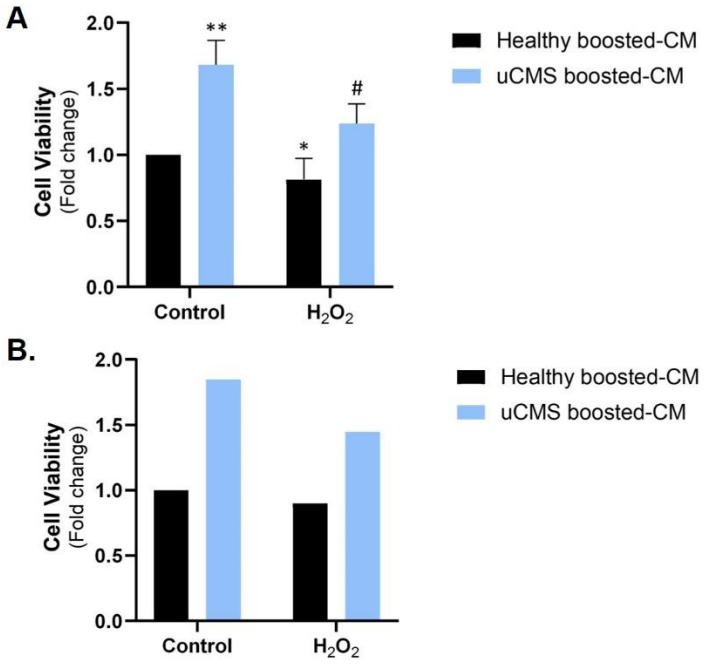

Figure 25 – The secretome derived from uCMS-boosted NSCs protects neuron-like differentiating NSCS from oxidative injury. Neuron-like differentiating NSCs were treated with H_2O_2 and co-incubated with 25% of healthy-boosted CM or uCMS-boosted CM produced by NSCs **(A)** or primary NSCs **(B)**, and then collected for cell viability analysis. NSC viability was assessed using Guava easyCyte 5HT flow cytometer. Data are expressed as fold change over non-injured NSCs treated with CM. (**A**) Data represent mean values ± SEM for at least three independent experiments. *$p < 0.05$ and **$p < 0.01$ compared to non-injured NSCs treated with healthy boosted-CM. #p < 0.05 compared to injured NSCs treated with healthy boosted-CM. (**B**) Data represent mean values for one independent experiment.

Recently, a paradigm shift has occurred suggesting that the beneficial effects of stem cells on damaged tissues may not be limited to their ability to differentiate into the required differentiated cells, but may also rely on their ability to secrete molecules capable of modulating and promoting the recovery of several target neural cells after acute or chronic cell damage (119,218). In this regard, it was shown that treatment with ASC-derived conditioned media (ASC-CM) prevented neuronal damage, tissue loss, and functional impairment in a neonatal rat model of hypoxic ischemic-induced encephalopathy, being already identified several neurotrophic factors in ASC-CM, particularly IGF-1 and BDNF, that might contribute for the protective effects of ASCs (219). Moreover, preconditioning NSCs from bilateral SVZ of fetal mouse brains with IL-6 successfully reprogrammed NSCs to tolerate ischemic injury. When NSCs were exposed to oxidative stress stimuli such as H_2O_2

and diethylenetriamine/nitric oxide, IL-6 preconditioned NSCs survived better compared to the non-preconditioned NSCs (220). Preconditioning of MSCs with proinflammatory cytokines such as IFN-γ, TNF-α and IL-17 has also been shown to enhance their immunomodulatory capacity, leading to increased therapeutic potential against autoimmune disorders (221).

Remarkedly, Anacker and colleagues have shown that neurogenesis confers resilience to chronic stress in mice. Inhibition of mature granule cell activity in the ventral DG, a subregion involved in mood regulation, was shown to promote vulnerability to stress from social defeat, whereas increasing neurogenesis confers resilience to chronic stress (222).Therefore, these results revealed that NSCs receiving stress mediators triggered by the uCMS model were the ones capable of producing the most therapeutic secretome.

4.3.2 NSC stimulated with depressed mice-derived serum are more proliferative and present distinct patters of mitochondrial dynamics and activity

Since NSCs treated with depressed mice-derived serum (uCMS- boosted NSCs) produced a more competent secretome to protect committed target cells from oxidative-mediated cell damage, we decided to explore what type of biochemical and metabolic changes occur in these secretome-producing NSCs. As previously mentioned, increased adult neurogenesis has been demonstrated to confer stress resilience in mice, therefore we began to explore changes in the proliferation marker Ki67 in uCMS-boosted NSCs. As expected, Ki67 mRNA levels were significantly increased in these cells when compared to healthy-boosted NSCs ($p < 0.05$) (**Figure 26**), reflecting the same response previous observed in boosted NSCs.

Lipid metabolism represents a carefully regulated balance between FAO and *de novo* biosynthesis that highly depends on the metabolic requirements of a specific cell state (97). Therefore, we examined the levels of LCAD in uCMS-boosted NSCs, through RT-PCR and WB, respectively. Surprisingly, NSC stimulated with depressed mice-derived serum exhibited significantly increased of both mRNA (**Figure 26.A**) and protein (**Figure 26.B**) levels of LCAD, when compared to healthy-boosted NSCs.

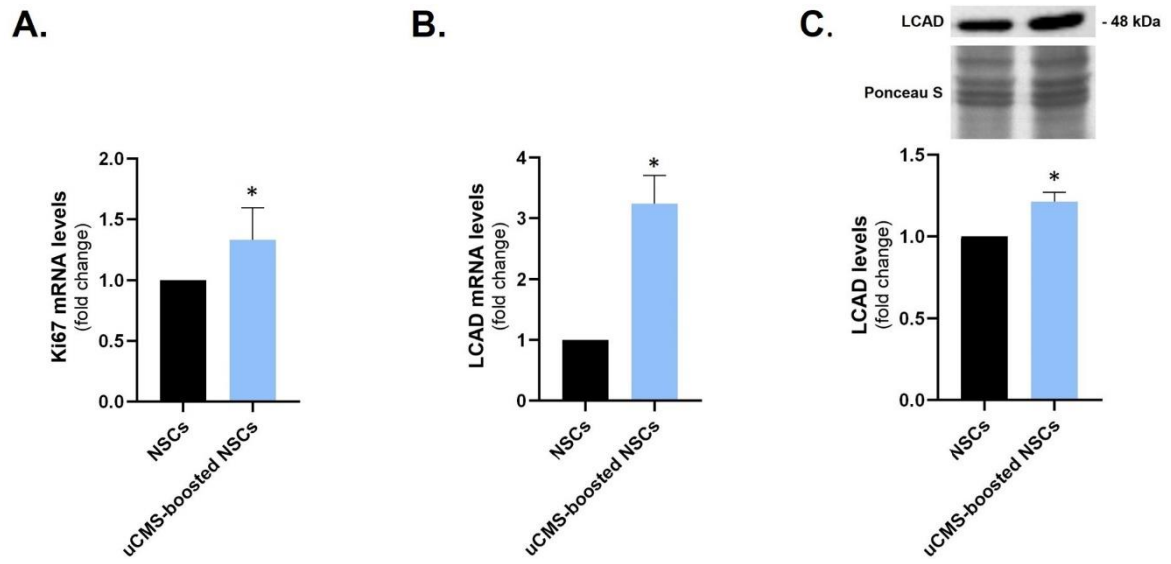

Figure 26 - NSCs stimulated with serum of depressed mice are more proliferative and present increased levels of LCAD. NSCs were treated with either serum derived from healthy or depressed mice for 24 h in self-renewal conditions, and then collected for RT-PCR and WB analysis to evaluate Ki67 and LCAD. (**A)** Effect of depressed mice-derived serum on Ki67 mRNA levels in NSCs. (**B**) Effect of depressed mice-derived serum on LCAD mRNA levels in NSCs. Values were normalized to the internal mRNA standard HPRT. (**C**) Effect of depressed mice-derived serum on LCAD protein levels in NSCs. Representative blot of LCAD levels (top) and corresponding densitometry analysis (bottom) in total protein extract. Values were normalized to Ponceau S. Data are represented as fold change over healthy-boosted NSCs. Data represent mean values ± SEM for at least three independent experiments. *$p < 0.05$ compared to healthy-boosted NSCs (NSCs).

FAs can directly enter into cells or be produced inside the cells to be used in mitochondria for energy production through β-oxidation. FAO consists of the active transport of medium- and long-chain FAs into mitochondria, to provide reducing equivalents directly to the ETC (223). The serum derived from depressed mice could induce cell proliferation, as we have seen in boosted NSCs, but concomitantly also induced premature differentiation of certain NSCs. In fact, our group has recently shown that both neural damage is capable of inducing premature differentiation (193) and increased LCAD levels are required for NSC differentiation (109).

Bearing in mind that NSC capable of producing the most efficient therapeutic secretome have been, so far, associated with increased mitochondrial fission, we then decided to also explore alterations in the levels of mitochondria dynamics-associated proteins in uCMS-boosted NSCs (**Figure 27**).

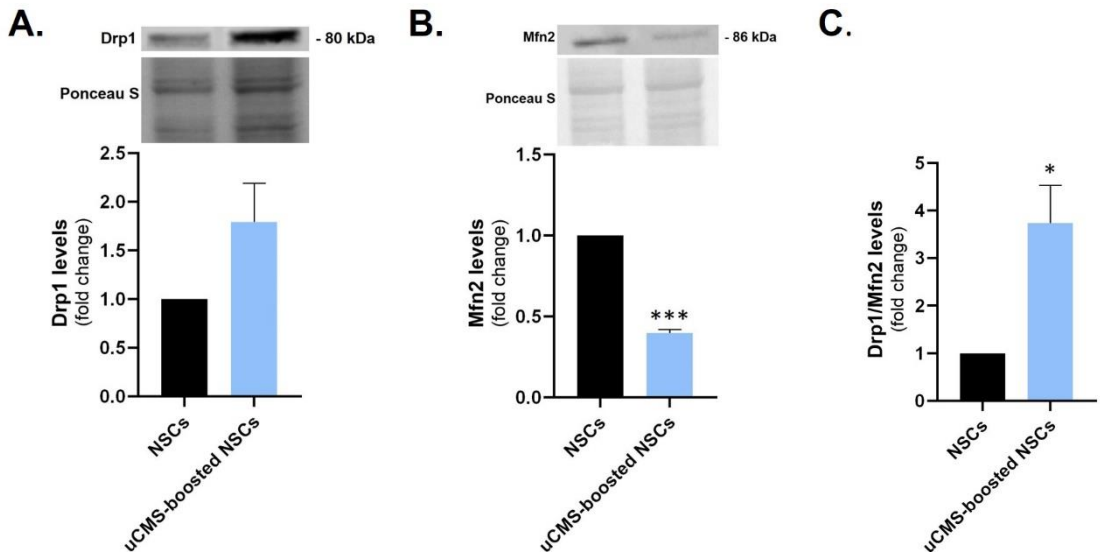

Figure 27 - NSCs stimulated with serum of depressed mice have higher levels of mitochondrial fission. NSCs, were treated with either serum derived from healthy or depressed mice for 24 h in self-renewal conditions, and then collected for WB analysis to assess protein levels of mitochondrial fission and fusion markers, Drp1 and Mfn2. (**A**) Effect of depressed mice-derived serum on Drp1 levels. Representative blot of Drp1 levels (top) and corresponding densitometry analysis (bottom) in total protein extract. (**B**) Effect of depressed mice-derived serum on Mfn2 levels. Representative blot of Mfn2 levels (top) and corresponding densitometry analysis (bottom) in total protein extract. Drp1 and Mfn2 levels were normalized to Ponceau S. (**C**) Ratio between Drp1 and Mfn2 protein levels. Data are represented as fold changes over healthy-boosted NSCs (NSCs). Data represent mean values ± SEM for at least three independent experiments. *$p < 0.05$ and ***$p < 0.001$ compared to healthy-boosted NSCs (NSCs).

Our results revealed that levels of Drp1 were increased (**Figure 27.A**), whereas Mfn2 levels were significantly decreased in uCMS-boosted NSCs (**Figure 27.B**). Additionally, the ratio of Drp1/Mfn2 protein levels was shown significantly increased in uCMS-boosted NSCs (**Figure 27.C**). These results suggest that mitochondrial fission occurs in NSCs stimulated with serum of depressed mice, being possibly responsible for metabolic adaptations and production of a more competent secretome to target neural cells.

Regarding potential variations in OxPhos complexes protein subunits in NSCs stimulated with serum of depressed mice, our results demonstrated that these cells present increased levels of Complex I subunit (~0.5 fold) (**Figure 28.B**), a slight increase in Complex II subunit

(**Figure 28.C**), and a significant increase in Complex V subunit ($p < 0.01$) (**Figure 28.E**), revealing a marked increase of ATP production in these cells. These results, together with LCAD increase (**Figure 26**) also suggest that depressed mice-derived serum may switch the metabolism of certain NSCs to use OxPhos and accommodate the changing metabolic demands associated with differentiation activation.

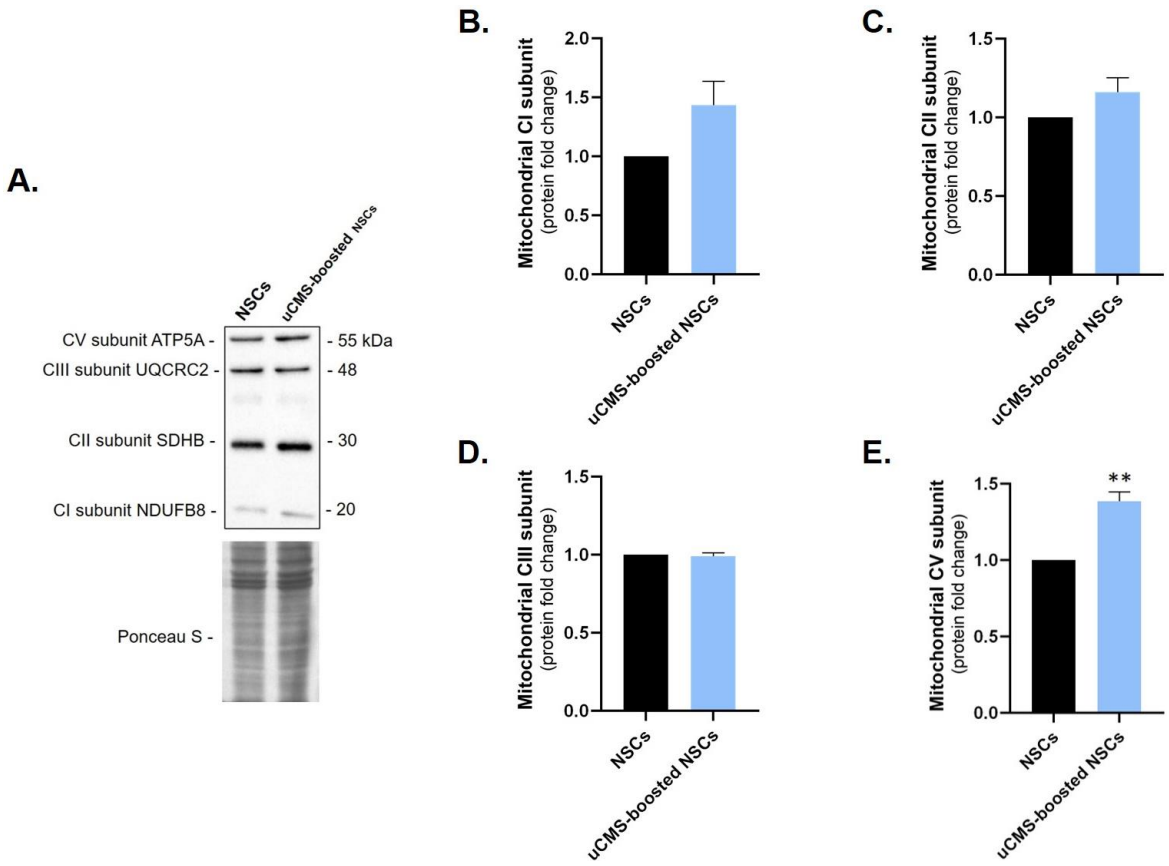

Figure 28 - NSCs stimulated with serum of depressed mice have alterations in mitochondrial complex subunits. NSCs were expanded and treated with either healthy or uCMS serum for 24h in self-renewal conditions, and then collect for WB analysis. (**A**) Representative blot of mitochondrial respiratory chain complex I–V subunits in total protein extracts. Densitometry analysis of the effect of uCMS serum on mitochondrial complex I (**B**) II (**C**) III (**D**) and V (**E**). Protein levels were normalized to Ponceau S. Data are expressed as fold change over NSCs treated with healthy mice-derived serum (NSCs). Data represent mean ± SEM for at least three independent experiments. $^{**}p < 0.01$ compared to healthy-boosted NSCs (NSCs).

Mitochondrial mass is controlled by the fission of pre-existing mitochondria as well as by the biogenesis of newly generated mitochondria (224). Therefore, we decided to investigate possible changes in TFAM and PGC-1α in uCMS-boosted NSCs by RT-PCR and WB, respetively (**Figure 29**).

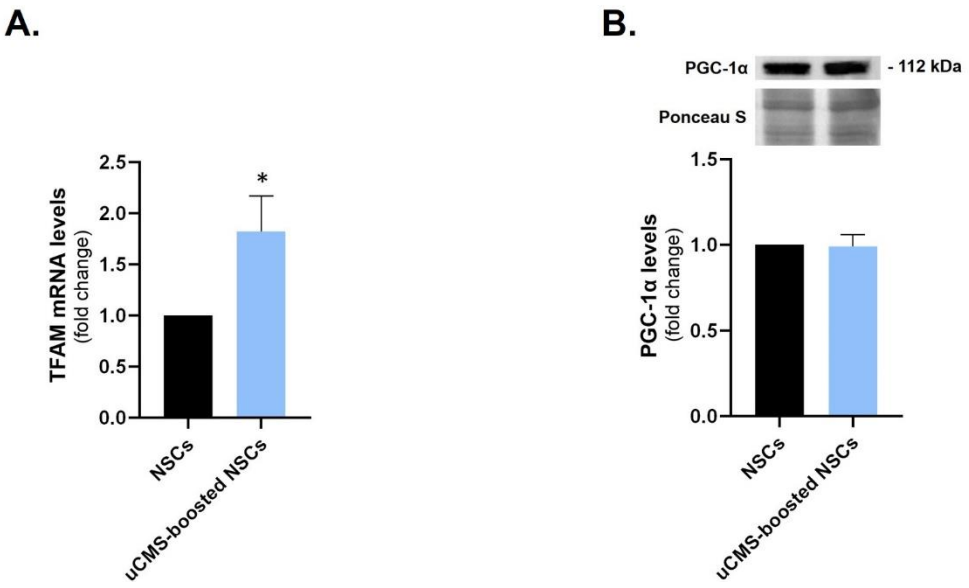

Figure 29 - NSCs stimulated with serum of depressed mice have increased expression of TFAM. NSCs were treated with either healthy or depressed mice-derived serum for 24 h in self-renewal conditions, and then collected for RT-PCR to assess mRNA expression levels of mitochondrial biogenesis marker, TFAM, and also for WB analysis to assess protein levels of mitochondrial biogenesis marker, PGC-1α. (**A**) Effect of depressed mice-derived serum on TFAM mRNA expression levels. Levels were normalized to the internal mRNA standard HPRT. (**B**) Effect of depressed mice derived serum on PGC-1α levels. Representative blot of PGC-1α levels (top) and corresponding densitometry analysis (bottom) in total protein extracts. Levels were normalized to Ponceau S. Data represent mean ± SEM for at least three independent experiments. *$p < 0.05$ compared to healthy-boosted NSCs (NSCs).

Interestingly, uCMS-boosted NSCs showed a significant increase in TFAM mRNA levels compared to healthy-boosted NSCs ($p < 0.05$) (**Figure 29.A**), while PGC-1α protein levels remained unchanged (**Figure 29.B**). Considering these results, uCMS-boosted NSCs would be expected to also present an increased mtDNA copy number, since their number is proportional to TFAM levels, as mentioned earlier. However, due to technical problems, we could not determine mtDNA copy number in these cells.

Since differentiating cells deeply depend on FAO and OxPhos for energy production, we finally decided to investigate potential changes in differentiation-related markers, namely in glial fibrillary acidic protein (GFAP), and βIII-tubulin, markers for glia and neuronal differentiation, respectively (**Figure 30**).

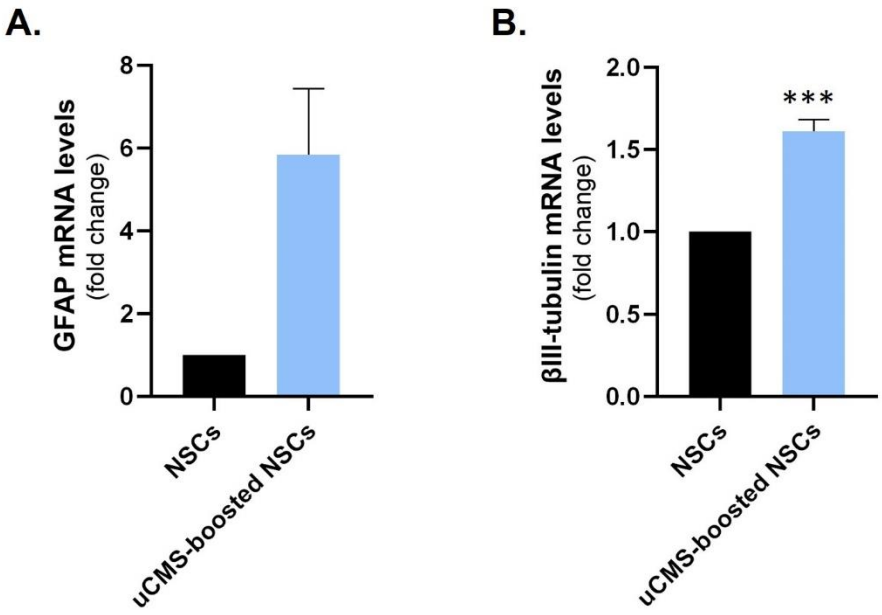

Figure 30 - NSCs stimulated with serum of depressed mice have higher levels of differentiation. NSCs were treated with either healthy or depressed mice-derived serum for 24 h in self-renewal conditions, and then collected for RT-PCR to assess mRNA expression levels of differentiation markers, GFAP and βIII-tubulin. (**A**) Effect of depressed mice-derived serum on GFAP mRNA expression levels. (**B**) Effect of depressed mice-derived serum on βIII-tubulin mRNA expression levels. Levels were normalized to the internal mRNA standard HPRT. Data represent mean ± SEM for at least three independent experiments. *$p < 0.05$, ***$p < 0.001$ compared to healthy-boosted NSCs (NSCs).

Interestingly, compared to healthy-boosted NSCs (NSCs), uCMS-boosted NSCs exhibited a marked increase in GFAP (~6-fold) ($p < 0.05$) and βIII-tubulin ($p < 0.001$) mRNA levels (**Figure 30**). The striking increase in GFAP levels might be associated with a NSC lineage shift favouring gliogenesis. Indeed, high levels of mitochondrial damage and ROS has been already shown with this NSC lineage shift (191).

Therefore, these results appear to suggest that, beside inducing NSC proliferation, the serum of depressed mice also induce NSC differentiation, being also in line with our previous findings on enhanced LCAD and OxPHOS-associated proteins in NSCs exposed to this serum. Our group has previously shown that the expression profile of LCAD increases during neural differentiation, in contrast to the reduced expression profile of SREBP-1 and ACC1, which are associated with lipid biosynthesis, suggesting that NSCs progressively rely more on lipid catabolic pathways to meet the energy demands of the differentiation process (109). In addition, astrocytes are thought to use β-oxidation as a fuel source, allowing glucose to be mainly used to support neurons (225). The most effective way for the cell to produce ATP is through OxPhos, which is therefore heavily used in differentiated cells with high energy demands such as neurons (94). Certainly, several studies have demonstrated that NSCs rely predominantly on glycolysis rather than OxPhos, whereas differentiated neurons exhibit higher mitochondrial respiration (100,226,227). Moreover, our group has previously shown that noxious stimuli, such as excessive amounts of SCFAs, trigger early premature differentiation of NSCs (193). This may also happen with the exposure of NSCs with serum derived from depressed mice, possibly enriched in stress mediators derived from the uCMS model.

Notably, mitochondrial biogenesis increases after the onset of differentiation of human NSCs into motor neurons (226), while mtDNA copy number and respiratory capacity are also significantly enhanced after differentiation of NSCs to support mitochondrial biogenesis (228). Furthermore, Steib and colleagues have demonstrated that during differentiation of newborn DG granule neurons, there is an extensive increase in mitochondrial biogenesis, associated with increased mitochondrial fission and distribution of mitochondria from the soma to dendrites of the newborn neurons, suggesting that mitochondrial biogenesis and fission are essential for neuron differentiation by supplying sufficient mitochondria to the growing dendrites of adult-born neurons (229). Thus, mitochondrial metabolism appears to be key in mediating NSC differentiation and/or inducing a NSC survival response through their secretome.

5. Conclusion and Future Perspectives

Stem cell therapy shows a great potential to alleviate the burden of neurological disorders (230). Remarkably, several studies have shown that NSCs not only possess the ability to self-renew and differentiate into all neural cell lineages, but are also capable of regulating the microenvironment through a paracrine mechanism (231). Therefore, the development of cell-free therapeutics using NSCs-derived secretome has emerged as a concept in regenerative medicine to achieve beneficial effects by modulating the function of tissue-resident CNS cells (63). Furthermore, mitochondria not only regulate stem cell fate (94), but have also been shown to regulate their secretome (112). Therefore, it is crucial to further investigate the mechanisms by which the metabolism enhance NSC paracrine activity to augment the benefits of NSC-based therapies in the CNS, namely of endogenous NSCs.

The work presented here has shown that NSCs receiving external injury signals and NSCs treated with compounds that augment the mitochondrial activity undergo profound metabolic changes, to produce and delivery a more protective secretome to target neuron-like differentiating cells. Altogether, our results clearly showed that activation of NSC necessary to trigger a protective and regenerative secretome is associated with an increase of the proliferation rate and mitochondrial fission in these cells. Therefore, it would be interesting to modulate this process in NSCs, namely by forcing mitochondrial fusion or repression mitochondrial fission and reassess the beneficial properties of their secretome.

In addition, analysis of other markers of metabolic pathways such as glycolysis and lipogenesis should also be performed to complement the metabolic characterization of these secretome producing NSCs. Moreover, since boosted and uCMS NSCs are exposed to external injury signals, it would be interesting to perform a robust characterization of the InjCM and serum of depressed mice to understand what signals trigger these metabolic changes. We could analyse the presence of proinflammatory mediators by flow cytometry, but also other metabolic components in this cell medium, such as ATP. In addition, it would also be interesting to further characterize boostedCM, mitCM and uCMS-boosted CMs by performing a metabolomic analysis using GC-MS/MS.

Further, the secretome of boosted NSCs and mitochondrial boosters-treated NSCs was able to induce metabolic changes in target cells by increasing their mtDNA copy number. Since we did not use the same model for target cells as we did to assess cell viability, it would be important to validate these results in the same target model of differentiating cells. We also need to perform ATP and NAD^+/NADH quantification analysis in secretome-producing NSCs as well as in differentiating target NSCs, as it was performed in boosted

NSCs. Since the majority of NSC secretome were shown to induce an increase in mtDNA copy number in target cells, it would also be important to investigate whether mitochondrial transfer also occurs from secretome-producing NSCs to the extracellular milieu by using fluorescence confocal microscopy to assess the integration of MitoTracker-labelled mitochondria. Namely, to discover in which situations this transfer occurs and how could we strategically modulate it.

As a final note, further studies are needed to understand which individual lifestyle has a positive effect on the endogenous NSC secretome. We have studied the effect of some features associated with physical exercise *in vitro*, but it would be remarkable to validate these results *in vivo* as well. Moreover, it would be extremely important to investigate the possible beneficial effects of diet on the endogenous secretome of NSCs.

In conclusion, our results suggest that regulation of NSC metabolism may be an effective and ground-breaking strategy to improve neuroplasticity and brain regeneration to arrest neurodegeneration in several neurological disorders. This work provides a new framework to further explore its use and develop less invasive pharmacological strategies to confer stress resilience and improve life quality of people with neurological illness.

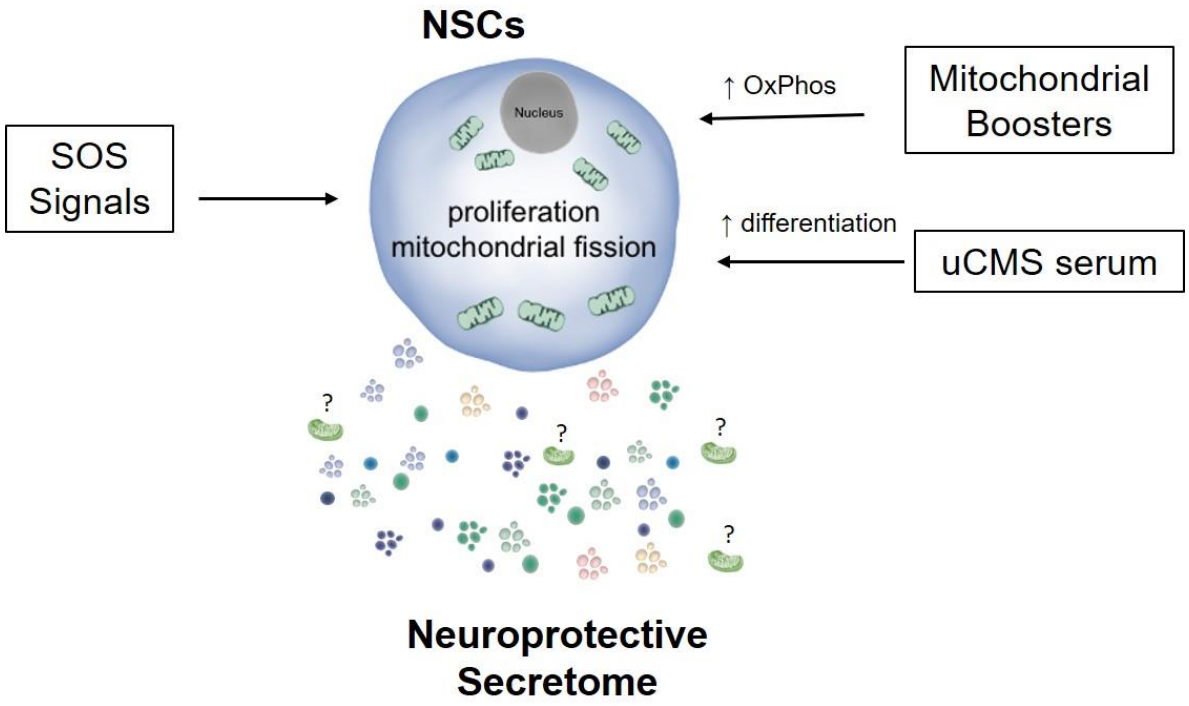

Figure 31 - Graphical abstract. Schematic representation of the main effects induced by SOS signals, mitochondrial boosters and uCMS mice-derived serum in NSCs, namely in their ability to produce a more neuroprotective secretome for oxidative-damaged neuron-like differentiating cells.

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