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Regulation of mitochondrial biogenesis by nitric oxide during neural stem cell proliferation

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

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Abbreviations

ΔΨm (Mitochondrial membrane potential)

8-Br-cGMP (8-bromoguanosine 3',5'-cyclic monophosphate)

AMP (Adenosine monophosphate)

ANOVA (Analysis of variance)

ATP (Adenosine triphosphate)

BCA (Bicinchoninic acid)

bFGF (Basic fibroblast growth factor)

CAPS (N-cyclohexyl-3-aminopropanesulfonic acid)

cGMP (Cyclic guanosine monophosphate)

CNS (Central nervous system)

COX I (Subunit I of Complex IV)

COX III (Subunit III of Complex IV)

COX IV (Subunit IV of Complex IV)

DG (Dentate gyrus)

DMEM/F12 (Dulbecco's modified eagle medium: nutrient mixture F-12)

EDTA (Ethylenediaminetetraacetic acid)

EdU (5-ethynyl-2'-deoxyuridine)

EGF (Epidermal growth factor)

eNOS (Endothelial nitric oxide synthase)

ERK 1/2 (Extracellular signal-regulated kinases 1 and 2)

EX527 (6-chloro-2,3,4,9-tetrahydro-1*H*-carbazole-1-carboxamide)

HDAC (Histone deacetylase)

HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)

HSC (Hematopoietic stem cell)

HBSS (Hank's balanced salt solution)

iNOS (Inducible nitric oxide synthase)

MAPK (Mitogen-activated protein kinase)

MEK 1/2 (MAPK kinases 1 and 2)

mtDNA (Mitochondrial DNA)

NAD^+ (Nicotinamide adenine dinucleotide)

NADH (Nicotinamide adenine dinucleotide hydride)

nDNA (Nuclear DNA)

nNOS (Neuronal nitric oxide synthase)

NOS (Nitric oxide synthase)

$NO^{\bullet}$ (Nitric oxide)

NOC-18 (DETA-NONOate)

NSC (Neural stem cells)

ODQ (1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one)

PBS (Phosphate buffer saline)

PCR (Polymerase chain reaction)

PGC-1α (Peroxisome proliferator-activated receptor γ coactivator-1α)

PK (Pyruvate kinase)

ROS (Reactive oxygen species)

SDS (Sodium dodecyl sulfate)

SEM (Standard error of the mean)

sGC (Soluble guanylyl cyclase)

SGZ (Subgranular zone)

SIRT1 (Sirtuin 1)

SVZ (Subventricular zone)

TBS-T (Tris buffer saline with 0.1% Tween 20)

TMRM (Tetramethylrhodamine methyl ester)

U0126 (1,4-Diamino-2,3-dicyano-1,4-bis-(o-amino-phenylmercapto)butadiene ethanolate)

Abstract

The presence of neural stem cells (NSC) in the mammalian brain allows the formation of new neurons (neurogenesis) during adult life. Following injury, neurogenesis may increase in an attempt to repair the lesioned area. However, the resulting neuroinflammation, characterized by activation of microglia, can be detrimental to neurogenesis. Nitric oxide ($NO^{\bullet}$) is released by microglia in these conditions, and our group showed that $NO^{\bullet}$ stimulates the proliferation of NSC by the MAPK pathway. Given the importance of the identification of new targets to enhance endogenous neurogenesis, it would be useful to identify the mechanism by which cells acquire the energy necessary to proliferate, as it could support the evidences of the proliferative role of $NO^{\bullet}$. Several studies report that $NO^{\bullet}$ can induce mitochondrial biogenesis, a complex process that results in the increase in the number and/or functionality of mitochondria, in a cGMP-dependent manner. In this work, we investigated whether $NO^{\bullet}$ induced proliferation on NSC, and studied two of the signaling pathways that could be involved in the proliferative effect of $NO^{\bullet}$. We also evaluated whether mitochondrial biogenesis would be the mechanism supportive of the effect of $NO^{\bullet}$ on the proliferation of NSC. Our results showed an increase in the proliferation of NSC induced by $NO^{\bullet}$, mediated by the MAPK pathway. Moreover, we identified the sGC/cGMP pathway as another mechanism by which $NO^{\bullet}$ increases the proliferation of these cells. We also found that mitochondrial biogenesis may not be the process used by cells to increase its energy gain. Thus, $NO^{\bullet}$ may be a promising target to stimulate the proliferation of NSC, but alterations in mitochondrial biogenesis are not involved.

Keywords: adult neurogenesis, nitric oxide, mitochondrial biogenesis, cGMP

Resumo

A presença de células estaminais neurais (NSC) no cérebro dos mamíferos permite a formação de novos neurónios (neurogénese) durante a vida adulta. Após lesão, a neurogénese pode aumentar numa tentativa de reparar a zona danificada. No entanto, a neuroinflamação resultante, caracterizada pela activação da microglia, pode ser prejudicial para a neurogénese. O óxido nítrico ($NO^{\bullet}$) é libertado pela microglia nestas condições, e o nosso grupo mostrou que o $NO^{\bullet}$ estimula a proliferação de NSC pela via da MAPK. Dada a importância da identificação de novos alvos para aumentar a neurogénese endógena, seria útil identificar o mecanismo pelo qual as células adquirem a energia necessária para proliferarem, pois poderia apoiar as evidências da função proliferativa do $NO^{\bullet}$. Vários estudos referem que o $NO^{\bullet}$ pode induzir biogénese mitocondrial, um processo complexo que resulta no aumento do número e/ou funcionalidade das mitocôndrias, de uma maneira dependente de cGMP. Neste trabalho, investigámos se o $NO^{\bullet}$ induziria proliferação em NSC, e estudámos duas das vias de sinalização que poderiam estar envolvidas no efeito proliferativo do $NO^{\bullet}$. Também avaliámos se a biogénese mitocondrial seria o processo que suporta o efeito do $NO^{\bullet}$ na proliferação de NSC. Os nossos resultados demonstraram um aumento na proliferação de NSC induzido por $NO^{\bullet}$, mediado pela via da MAPK. Além disso, identificámos a via sGC/cGMP como outro mecanismo pelo qual o $NO^{\bullet}$ aumenta a proliferação destas células. Verificámos também que a biogénese mitocondrial poderá não ser o processo utilizado pelas células para aumentar o seu ganho energético. Assim, o $NO^{\bullet}$ poderá ser um alvo promissor para estimular a proliferação de NSC, mas não estão envolvidas alterações na biogénese mitocondrial.

Palavras-chave: neurogénese, óxido nítrico, biogénese mitocondrial, cGMP

Chapter 1 Introduction

1.1. Adult neurogenesis

Neurogenesis, the formation of new neurons, involves cell proliferation, migration, differentiation and integration into the neuronal circuits. This process starts during embryonic life and continues throughout the adult life of mammals, due to the existence of neural stem cells (NSC) mainly in two brain regions: the subventricular zone (SVZ) at the walls of the lateral ventricles, and the subgranular zone (SGZ) at the dentate gyrus (DG) of the hippocampus (Fig. 1) (Altman 1969, Kaplan & Hinds 1977, Cameron *et al.* 1993, Doetsch & Alvarez-Buylla 1996, Eriksson *et al.* 1998). Both the SGZ and the SVZ are located close to a wide vascular niche (Palmer *et al.* 2000, Mercier *et al.* 2002), which suggests that NSC behavior may be influenced by factors released from blood vessels.

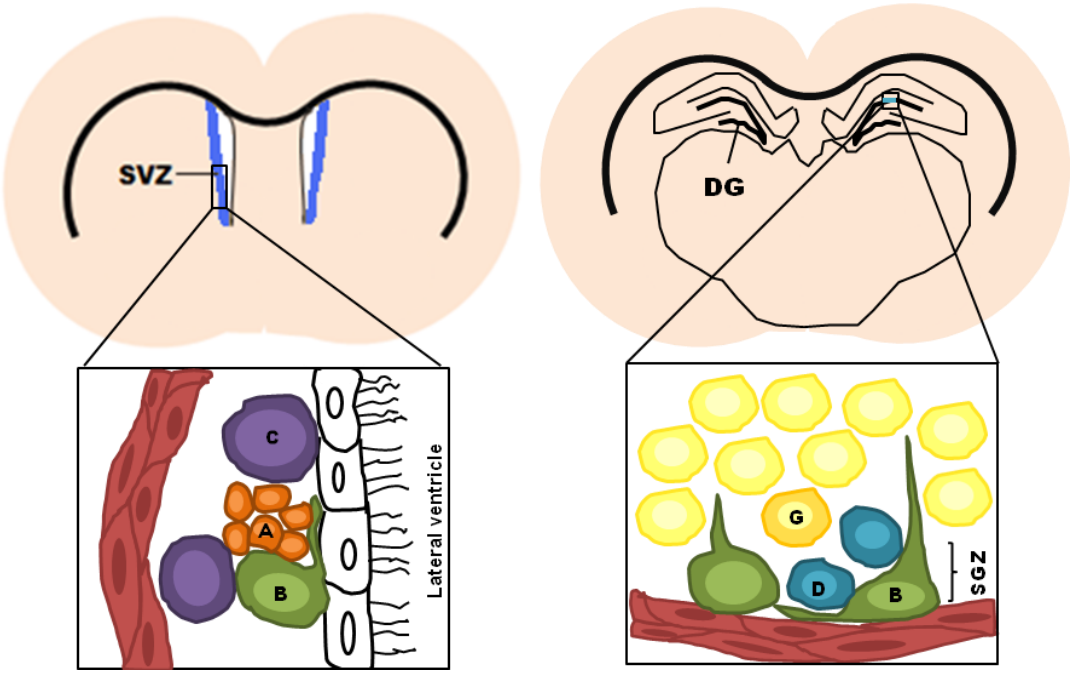

Figure 1. Schematic representation of the two neurogenic niches in the adult rodent brain (coronal view). Left panel: ependymal cells (white), B cells (B), C cells (C), A cells (A), blood vessel (red). Right panel: blood vessel (red), SGZ B cells (B), precursor cells (D), differentiated granule cells (G).

The SGZ stem cells migrate from the SGZ into the granule cell layer of the DG (van Praag *et al.* 2002), where they differentiate into neuronal and glial cells (Cameron *et al.* 1993). The new neurons extend their axons into the CA3 region of the hippocampus (Hastings & Gould 1999, Markakis & Gage 1999), and are important to maintain the normal hippocampal functions such as learning and memory (Gould *et al.* 1999, Shors *et al.* 2001). The SVZ is composed of different types of cells. The B cells are resting NSC and originate the highly proliferative C cells (Doetsch *et al.* 1999). These, in turn, give rise to immature neuroblasts (A cells) that migrate to the olfactory bulbs via the rostral migratory stream (Lois & Alvarez-Buylla 1994, Kornack & Rakic 2001). Once in the olfactory bulbs, these cells differentiate into interneurons (Belluzzi *et al.* 2003, Carleton *et al.* 2003).

1.2. Neurogenesis in pathological conditions

The mechanisms that regulate the NSC niches are not fully characterized, but several factors affect neurogenesis: genetics (Kempermann & Gage 2002), aging (Kuhn *et al.* 1996, Enwere *et al.* 2004), hormones (Cameron & Gould 1994), neurotransmitters (Kempermann 2002), stress (Duman *et al.* 2001), growth factors (Yoshimura *et al.* 2001, Okano *et al.* 1996), and pathological conditions, which will next be addressed.

NSC are mobilized in response to pathological conditions, such as ischemic stroke, epilepsy, and neurodegenerative diseases.

Ischemic brain insults are known to stimulate the proliferation of NSC in both the SGZ and SVZ of adult rodents (reviewed in Kokaia & Lindvall 2003). In a model of middle cerebral artery occlusion, it was shown that despite the ability of SVZ-derived neuroblasts to migrate to the lesioned area to replace the dead neurons, the majority of them did not survive, thus not being able to differentiate into mature neurons

(Arvidsson *et al.* 2002). Contrarily, in a study with a model for transient global ischemia, there was regeneration of the injured area with the proper neurons, associated with an improvement of the brain function (Nakatomi *et al.* 2002). Therefore, it is important not only to trigger the migration and differentiation of the new neurons, but also to allow their survival.

In epilepsy animal models, neurogenesis in the SGZ and the SVZ is enhanced after induction of seizures (Parent 2003). The proliferation of NSC in the SGZ is highly stimulated by epilepsy, after a latent period (Parent *et al.* 1997). Many of these new cells then differentiate into granule neurons, and some can end, aberrantly, in the hilus region (Walter *et al.* 2007), where they exhibit abnormal functional behaviors (Scharfman *et al.* 2000). Regarding the NSC of the SVZ, epilepsy increases their proliferation and some of the resulting neuroblasts reach the olfactory bulb in a faster way, while others leave the ordinary route to reach the lesioned areas in the forebrain (Parent *et al.* 2002).

In a rat model of Huntington's disease, the proliferation of NSC in the SVZ was also increased, and some neuroblasts migrated to the lesioned striatum (Tattersfield *et al.* 2004). In addition, in a transgenic mouse model of Alzheimer's disease, there was increased proliferation, evaluated by incorporation of the thymidine analogue BrdU, as well as presence of immature neuronal markers in cells from the SGZ and SVZ (Jin *et al.* 2004). However, in relation to Parkinson's disease, rodents with reduction of dopamine present impaired proliferation in both SVZ and SGZ (Baker *et al.* 2004, Hoglinger *et al.* 2004).

1.2.1. Neuroinflammation and neurogenesis

In conditions of stress, injury or infection, the organism reacts by trying to restore its equilibrium, triggering actions that lead to inflammation. In the central

nervous system (CNS), neuroinflammation is an important factor in a pathological environment that can range from mild acute to uncontrolled chronic inflammation, which leads to opposing effects on neurogenesis (Whitney *et al.* 2009, Ekdahl *et al.* 2009, Russo *et al.* 2011) (Fig. 2). Activation of microglia in rodents, and consequent inflammation, can either enhance the integration of new neurons in the neuronal hippocampal circuits (Jakubs *et al.* 2008), or substantially impair SGZ neurogenesis (Ekdahl *et al.* 2003). The current knowledge suggests that microglia has a dual role on adult neurogenesis, being able to either enhance or impair it, both at physiological or pathological conditions, during all phases of neurogenesis (topic reviewed in Ekdahl *et al.* 2009). Different microglial activation manners may influence the set of inflammatory factors characteristic of the two contrary effects on neurogenesis. (Whitney *et al.* 2009).

In conditions that trigger neuroinflammation, activation of microglia contributes with the release of factors, such as inflammatory cytokines or neurotransmitters, to attract more immune cells to the affected area. Reactive oxygen and nitrogen species are important factors released in inflammation mediated by microglia (Rock *et al.* 2004), of which nitric oxide ($NO^{\bullet}$) is of particular interest.

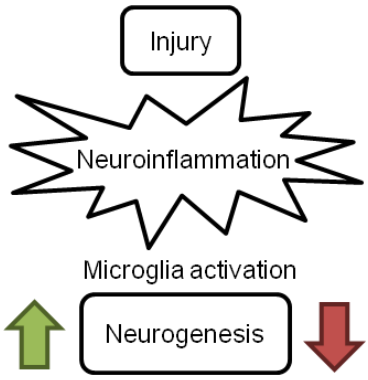

Figure 2. Effect of neuroinflammation on neurogenesis.

1.2.2. Role of nitric oxide in neurogenesis

$NO^{\bullet}$ is a free radical diffusible gas that is synthesized by nitric oxide synthase (NOS) from L-arginine and oxygen (Fig. 3). There are three different isoforms of NOS,

all constitutively expressed in certain tissues, and transcriptionally and post-transcriptionally regulated. The endothelial (eNOS) and neuronal (nNOS) isoforms are activated by calcium binding, while inducible NOS (iNOS) is tonically active once it is induced by cytokines or bacterial components (Moncada *et al.* 1991, Alderton *et al.* 2001). The main isoform present in the brain is nNOS, expressed mostly in neurons and muscle (Schild *et al.* 2006). The $NO^{\bullet}$ synthesized by nNOS modulates synaptic activity (Moreno-Lopez *et al.* 1996) and is important for neuronal differentiation, survival, and synaptic plasticity (reviewed in Holscher 1997). There are nitrergic neurons located close to the NSC of the SVZ and the SGZ (Moreno-Lopez *et al.* 2000), which leads to the hypothesis of a putative role for $NO^{\bullet}$ in the regulation of adult neurogenesis.

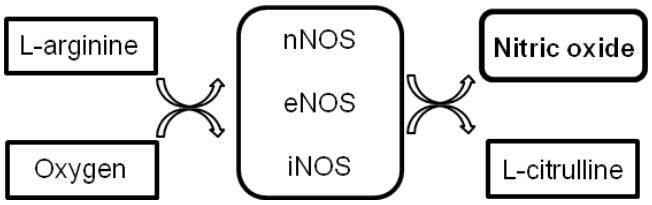

Figure 3. Production of $NO^{\bullet}$ by the 3 isoforms of NOS.

It has been demonstrated that endogenous $NO^{\bullet}$ functions as a negative regulator of adult neurogenesis in physiological conditions (Cheng *et al.* 2003, Packer *et al.* 2003, Moreno-Lopez *et al.* 2004, Matarredona *et al*. 2005). This physiologic antiproliferative effect of $NO^{\bullet}$ may be related to the disruption of epidermal growth factor receptor signaling, since it was observed that an increase of proliferation in the SVZ, due to inhibition of NOS, was restricted to cells that expressed this receptor (Romero-Grimaldi *et al.* 2006). Furthermore, it was suggested that, at supraphysiological concentrations of $NO^{\bullet}$, the decrease in proliferation may be due to an impairment of the tyrosine kinase activity of the EGF receptor, interfering with its activation of the phosphoinositide-3-kinase/Akt pathway (Torroglosa *et al.* 2007). However, $NO^{\bullet}$ from iNOS stimulates neurogenesis following ischemic brain damage

(Zhu *et al.* 2003), and treatment with a NO• donor increases cell proliferation, neurogenesis, and functional recovery following middle cerebral artery occlusion (Zhang *et al.* 2001). In addition, in a recent study by Carreira *et al.*, a NO• donor (NOC-18) was used to stimulate SVZ cell proliferation. An increase in cell proliferation was observed at lower concentrations of NOC-18 (10 µM), and an antiproliferative effect observed for higher concentrations (100 µM), suggesting a dual role for NO• in proliferation (Carreira *et al.* 2010) (Fig. 4). The mechanism responsible for this proliferative effect of NO• was described to be the bypass of the EGF receptor and activation of p21Ras and MAPK pathway (Carreira *et al*. 2010). This work also showed that, under pathophysiological conditions in vivo, NO• released by iNOS increases proliferation in the hippocampus (Carreira *et al.* 2010). Moreover, it is known that NO• interacts allosterically with soluble guanylyl cyclase (sGC), increasing cGMP concentrations, which leads to cGMP-dependent responses (Arnold *et al.* 1977). Thus, in another study by Carreira *et al.*, the involvement of cGMP in the proliferative effect of NO• was investigated in NSC, and NO• was shown to increase proliferation of NSC via the sGC/cGMP pathway (Carreira *et al.* submitted).

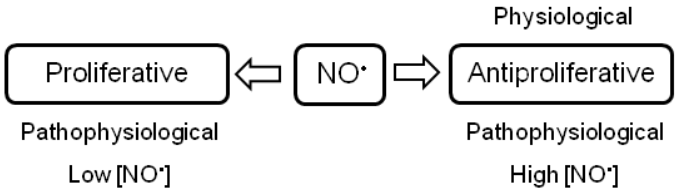

Figure 4. Effect of NO• on the proliferation of NSC.

1.3. Different energy requirements for proliferation and differentiation of stem cells: role of mitochondria

To be able to proliferate, cells require energy that is mostly provided by mitochondria. In mammalian cells, mitochondria are intracellular organelles composed of two layers of membranes that divide them into four compartments: outer membrane,

intermembrane space, inner membrane and matrix. There are various essential biochemical reactions that take place in the matrix, such as synthesis of steroid hormones, β-oxidation of fatty acids, tricarboxylic acid cycle and Ca^{2+} homeostasis. The mitochondrial respiratory chain enzyme complexes are located in the inner membrane, and perform respiration and oxidative phosphorylation, in order to produce the ATP necessary to support the processes that need energy (Chen *et al.* 2010). In oxidative phosphorylation, NADH and $FADH_2$ are oxidized by the enzyme complexes I and II, respectively. The electrons obtained by these complexes are then transferred along the respiratory chain, until the acceptor of electrons (O_2) is reduced. The energy of the passing of electrons allows the complexes I, III and IV to pump protons to the intermembrane space, creating a gradient. The ATP synthase (complex V) tries to compensate this gradient, by diffusing the protons into the matrix. This process releases energy that is used by the ATP synthase to phosphorylate ADP into ATP (Fig. 5). Complex IV is an essential component of oxidative phosphorylation, composed by thirteen different subunits (Kadenbach *et al.* 1983), three of which (COX I, COX II and COX III) are encoded by mitochondrial DNA (mtDNA), while the other subunits are nuclear-encoded.

Given its central energy control function, mitochondria may have a crucial role in regulating proliferation and differentiation of stem cells. It is well established that mitochondrial density and activity are different between cell types, being related to their energetic needs (Williams 1986). Also, mitochondria seems to be important for the differentiation of different cell types (Kaneko *et al.* 1988, Herzberg *et al.* 1993, Moyes *et al.* 1997, Komarova *et al.* 2000), including neurons (Vayssiere *et al.* 1992). Some studies indicate that, in the process of differentiation of somatic cells, the activation of mitochondria is associated with an increase in the activity of the respiratory chain enzyme complexes (Leary *et al.* 1998, Spitkovsky *et al.* 2004), an increase in the levels of protein and lipid constituents essential for the creation of mitochondria (Kanamura *et*

al. 1990), a maturation of mitochondrial compartments (Moyes & Battersby 1998) and an increase on the regulation of proteins or factors crucial for mitochondrial biogenesis (May-Panloup *et al.* 2005). However, in contrast to its high activity in differentiated cells, mitochondria do not seem to display much activity in stem cells (Siggins *et al.* 2008).

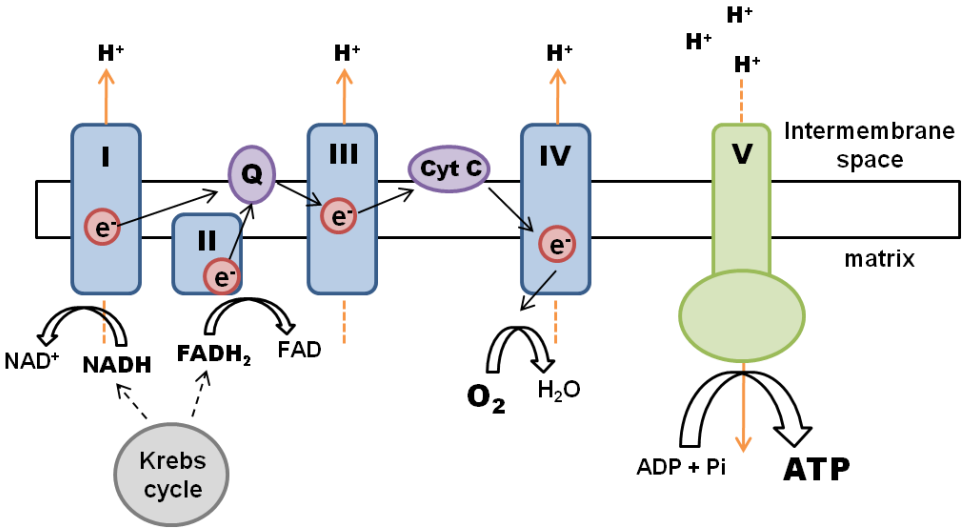

Figure 5. Schematic illustration of mitochondrial electron transport chain and oxidative phosphorylation. I (Complex I), II (Complex II), III (Complex III), IV (Complex IV), V (ATP synthase), Q (coenzyme Q), Cyt C (cytochrome *c*).

Mitochondrial number and function determine whether cells can regulate both reactive oxygen species (ROS) levels and oxidative stress (Parker *et al.* 2009), being a useful strategy to assess hematopoietic stem cell (HSC) proliferation and fate decisions (Lyu *et al.* 2008). HSC progeny seem to have higher ROS levels, compared to HSC (Ito *et al.* 2004, Tothova *et al.* 2007), which is related to self-renewal decrease, increased cell cycling, and reduced HSC survival (Ghaffari 2008, Naka *et al.* 2008). Likewise, NSC seem to have lower ROS levels than neurons that are more mature (Tsatmali *et al.* 2005, Madhavan *et al.* 2006). This reduction in the levels of ROS is thought to be caused by an increase in the levels of antioxidant enzymes (Madhavan *et al.* 2006). There is evidence that cells of the postnatal SVZ, rostral migratory stream, and SGZ

express antioxidant enzymes (Faiz *et al.* 2006), and that oxidative stress increases their expression, in embryonic NSC (Madhavan *et al.* 2008), attributing a crucial role to these enzymes in the control of ROS levels in NSC.

Regarding the intracellular ATP content, an increase in ATP levels may be associated to loss of characteristics associated with stem cells, and therefore to the beginning of differentiation (Lonergan *et al.* 2007). This is based on the fact that, in order to differentiate, cells need to produce ATP more efficiently, which results in a metabolic shift from glycolysis to oxidative phosphorylation (Chen *et al.* 2010). A recent study showed that human pluripotent stem cells depend mainly of glycolysis, based on the analysis of lactate and ATP levels, when comparing to more differentiated cells (Varum *et al.* 2011). Regarding the oxygen consumption rates, the results are still not consensual. Studies showed that human pre-adipocytes and HCS consume oxygen at lower rates than more mature cells (von Heimburg *et al.* 2005, Piccoli *et al.* 2005). Moreover, younger and smaller neurospheres from cultured NSC presented higher mitochondrial membrane potential than older and larger neurospheres (Plotnikov *et al.* 2006).

The role of mitochondria in the regulation of proliferation and differentiation of stem cells is not well understood and needs further investigation. Most of the studies are mainly focused on stem cell differentiation, which results in poor knowledge about the energetic requirements during stem cell proliferation.

1.4. Mitochondrial biogenesis

Mitochondria are crucial to the good functioning of the cells, so it is essential that the regulation of their number is done properly. Cells in need for more energy may respond by activating the production of new mitochondria. Mitochondrial biogenesis is a complex process involving both protein and lipid formation and transportation,

replication of mtDNA and increased mitochondrial function (Hock & Kralli 2009). Most of the 1100-1500 proteins of mitochondria (Pagliarini *et al.* 2008) are encoded in the nDNA, while the mtDNA only encodes 13 proteins that are necessary for the constitution of oxidative phosphorylation components (Hock & Kralli 2009). In order to increase their number, mitochondria need the cooperation of gene transcription from the nDNA and the mtDNA. This process is regulated by co-regulators, which regulate the transcription factors acting upon the mitochondrial genes (Hock & Kralli 2009).

PGC-1α (peroxisome proliferator-activated receptor γ coactivator-1α) is very important for the regulation of mitochondrial functions (Puigserver *et al.* 1998, Wu *et al.* 1999, Lehman *et al.* 2000, Lin *et al.* 2002, Kelly & Scarpulla 2004), being involved in metabolic responses by the cell. For instance, it regulates adaptive thermogenesis in brown adipose tissue (Puigserver *et al*. 1998), fibre-type conversion in skeletal muscle (Lin *et al.* 2002), and induces β-oxidation of fatty acids and gluconeogenesis in the liver (Herzig *et al.* 2001, Yoon *et al.* 2001, Puigserver *et al.* 2003, Rhee *et al.* 2003). PGC-1α is one of the members of the PGC-1 family, a group of molecules that interact with transcription factors, resulting in an increase of transcription or elongation. The regulation of PGC-1α activity is made post-transcriptionally by different mechanisms, such as inhibition by acetylation and activation by arginine methylation (Feige & Auwerx 2007).

SIRT1 (Sirtuin 1) belongs to the sirtuin family of NAD^+-dependent class III histone deacetylases (HDAC) (Imai *et al.* 2000, Landry *et al.* 2000, Tanner *et al.* 2000, Schmidt *et al.* 2004) and is dependent on the NAD^+/NADH ratio, being activated upon its elevation (Lin *et al.* 2004, Feige & Auwerx 2007). SIRT1 interacts with PGC-1α and causes its activation by deacetylation, causing mitochondria to replicate (Lagouge *et al.* 2006). When interacting with PGC-1α, SIRT1 is found in the nucleus, but there are studies that show that, as a response to oxidative stress, it can also be found in the cytosol (Tanno *et al.* 2007, Hisahara *et al.* 2008). Moreover, a recent study has shown

that SIRT1, as well as PGC-1α, can be found localized inside mitochondria (Aquilano *et al.* 2010). SIRT1 is a major factor involved in NSC differentiation. Prozorovski *et al.* showed an increase in SIRT1 levels upon NSC oxidative conditions, which appears to be necessary for NSC to differentiate into astrocytes, instead of neurons (Prozorovski *et al.* 2008). On the other hand, another group observed that, in non-oxidative conditions, SIRT1 preferentially induced differentiation of NSC into neurons (Hisahara *et al.* 2008). Given this difference in the resulting cell types, it appears that the effect of SIRT1 on differentiation depends on the oxidative status of the cells (Rafalski & Brunet 2011). PGC-1α is regulated by transcription factors other than SIRT1, reviewed in Lopez-Lluch *et al.* 2008, Hock & Kralli 2009 and Onyango *et al.* 2010. The nuclear respiratory factors 1 (NRF1) and 2 (NRF2) are transcription factors regulated by PGC-1α, and are involved in the regulation of the mitochondrial transcription factors A (TFAM) and B1/B2 (TFB1M/TFB2M), which are activated as a response to signals that induce mitochondrial biogenesis (Chow *et al.* 2007, Civitarese *et al.* 2007, Scarpulla 2008), and increase the transcription and replication of mtDNA (Kang & Hamasaki 2005, Scarpulla 2006) (Fig. 6).

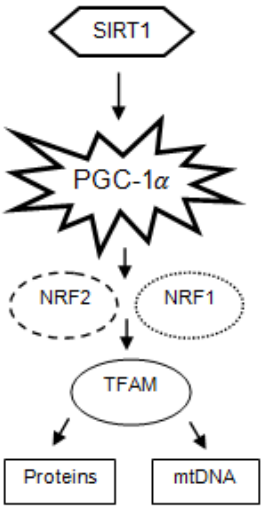

Figure 6. Mechanism of mitochondrial biogenesis.

1.4.1. Regulatory factors

Mitochondrial biogenesis is enhanced by different factors, such as caloric restriction (Nisoli *et al.* 2005) and chronic treatment with PPARγ activators (Strum *et al.* 2007). Exercise increases the biogenesis of mitochondria through activation of AMPK resultant from an increase in the AMP/ATP ratio (Holloszy & Booth 1976, Chabi *et al.* 2005). Moreover, during exercise, ROS usually increase in skeletal muscle, which is necessary for the stimulation of mitochondrial biogenesis after exercise, an effect that can be prevented by antioxidants (Gomez-Cabrera *et al.* 2008). However, mitochondrial biogenesis can also be increased by the antioxidant polyphenol resveratrol (Baur & Sinclair 2006), which binds allosterically to SIRT1, and induces a higher affinity of SIRT1 for acetylated substrates resulting in an enhancement of SIRT1 activity (Howitz *et al.* 2003). Pyruvate also stimulates mitochondrial biogenesis, either indirectly due to oxidation of NADH to NAD^+ that increases activity of SIRT1, and therefore of PGC-1α (Rodgers *et al.* 2005); or in a more direct way at high concentrations, independently of PGC-1α (Wilson *et al.* 2007). Finally, $NO^{\bullet}$ also regulates mitochondrial biogenesis, as discussed next.

$NO^{\bullet}$ and mitochondria interact in various ways. $NO^{\bullet}$ can provide respiratory substrates to mitochondria, distribute heat produced by mitochondrial respiration and regulate the provision of oxygen to mitochondria (Nisoli *et al.* 2008). Also, $NO^{\bullet}$ binds to Complex IV in competition with oxygen, which leads to inactivation of this complex (Brown & Cooper 1994), and therefore impairing mitochondrial oxidative phosphorylation, mostly at low oxygen concentrations (Clementi *et al.* 1999). Moreover, in neurons and endothelial cells, the outer membrane of mitochondria present eNOS attached (Gao *et al.* 2004). This suggests that the activity of NOS is regulated by mitochondria, and vice-versa, eNOS may regulate mitochondrial function (Nisoli *et al.* 2008).

Some studies have shown that $NO^{\bullet}$ regulates mitochondrial biogenesis through activation of PGC-1α. Nisoli *et al.* first observed that cells exposed to $NO^{\bullet}$ donors showed increased mtDNA content, caused by higher expression of PGC-1α, with the involvement of cGMP, and that this increase was prevented by removing $NO^{\bullet}$, using a $NO^{\bullet}$ scavenger (Nisoli *et al.* 2003). They also found that $NO^{\bullet}$ from eNOS origin activated sGC, increasing the levels of cGMP, which in turn induced transcription of PGC-1α and thus, mitochondrial biogenesis (Nisoli *et al.* 2003). This originated functionally active mitochondria that were able to produce ATP using oxidative phosphorylation (Nisoli *et al.* 2004). Moreover, caloric restriction was shown to increase cGMP levels, which induced the expression of SIRT1 (Nisoli *et al.* 2005), enhancing the activity of PGC-1α by deacetylation (Nisoli & Carruba 2006).

1.5. Objectives

The modulation of endogenous neurogenesis is a promising strategy to limit the adverse effects of brain injury. In that context, the study of the effects of $NO^{\bullet}$ on the proliferation of NSC, and its mechanisms, are of great interest. In order to extend the knowledge regarding the proliferative effect of $NO^{\bullet}$, we proposed to analyze the proliferation of NSC derived from the SVZ, after exposure to a $NO^{\bullet}$ donor, in a concentration previously described as proliferative (Carreira *et al.* 2010). As cells in proliferation will need more energy, one of the ways through which they may respond is by increasing the functionality and number of mitochondria. Therefore, we also proposed to investigate the role of mitochondrial biogenesis as an energetic platform for the proliferation induced by $NO^{\bullet}$, by analyzing mitochondrial function and number, in those proliferative conditions.

Chapter 2 Materials and methods

2.1. Methods

2.1.1. SVZ cell cultures

SVZ primary cultures were obtained from 0-3 days C57BL/6J mice, as described previously (Carreira *et al.* 2010). The animals were decapitated, and the brains were removed and submersed in Hank's balanced salt solution (HBSS, 137 mM NaCl, 5.36 mM KCl, 0.44 mM KH_2PO_4, 4.16 mM $NaHCO_3$, 0.34 mM $Na_2HPO_4.2H_2O$ and 5 mM glucose, supplemented with 0.001 % phenol red, 1 mM sodium pyruvate and 10 mM HEPES, pH 7.2) with 0.24 % gentamicin. Following the removal of the meninges, the brains were sliced coronally and the subventricular zone of each slice (1 mm thick) was dissected. That tissue was then enzymatically digested with 0.025 % trypsin/0.265 mM EDTA, during 15 minutes at 37°C, and after washing 3 times with 0.24 % gentamicin/HBSS, it was mechanically dissociated. Single cells were counted using 0.1 % trypan blue exclusion assay, and plated in uncoated flasks, at a density of 100,000 cells/ml, in warm Dulbecco's modified eagle medium: nutrient mixture F-12 (D-MEM/F-12) with 2 mM GlutaMAX^{TM}-I (L-Ala-L-Gln), supplemented with 1 % B27, 1 % antibiotic (Pen/Strep, 10,000 units/ml of penicillin, 10 mg/ml streptomycin), 10 ng/ml epidermal growth factor (EGF) and 5 ng/ml basic fibroblast growth factor (bFGF). Cells were grown as floating aggregates (neurospheres) in a 95 % air/5 % CO_2 humidified atmosphere at 37ºC. After approximately 7 days, neurospheres were dissociated and resuspended in fresh supplemented D-MEM/F-12 with GlutaMAX^{TM}-I medium (passage). This process was repeated after 7 days and the neurospheres were left to grow. Then, they were plated in 0.1 mg/ml poly-L-lysine-coated multiwells or coverslips, and maintained with supplemented D-MEM/F-12 with GlutaMAX^{TM}-I medium for 3 days, until the desired confluency was achieved.

2.1.2. Experimental treatments

Neural stem cells were left 24 hours without growth factors (EGF and bFGF), and then were exposed to the NO• donor DETA-NONOate (NOC-18, 10 μM) or the cGMP analogue 8-bromoguanosine 3',5'-cyclic monophosphate (8-Br-cGMP, 20 μM), during 6 hours, 12 hours and 24 hours. The inhibitors were added 30 minutes before the treatments with NOC-18 or 8-Br-cGMP. Guanylyl cyclase was blocked using 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ, 50 μM), ERK 1/2 activation was blocked by the MEK 1/2 inhibitor 1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio] butadiene (U0126, 1 μM) and SIRT1 was inhibited by 6-chloro-2,3,4,9-tetrahydro-1*H*-carbazole-1-carboxamide (EX527, 1 μM). The cells without any treatment were considered the controls.

2.1.3. Analysis of cell proliferation by flow cytometry

NSC proliferation was evaluated by incorporation of the thymidine analogue 5-ethynyl-2'-deoxyuridine (EdU), detected by flow cytometry. Cells were exposed to EdU 10 μM during the last 4 hours of treatment. After washing with sterile 0.01 M phosphate-buffered saline (PBS, 7.8 mM $Na_2HPO_4.2H_2O$, 2.7 mM $NaH_2PO_4.H_2O$, 154 mM NaCl, pH 7.2), cells were detached using sterile StemPro® Accutase®, incubated for 20 minutes, at 37ºC. Cells were harvested into flow tubes that were centrifuged, the supernatant was discarded and the pellet resuspended in 70 % ethanol, as described previously (Carreira *et al.* 2010). The samples were kept at 4ºC during one to four days. EdU detection was based on a click-chemistry reaction, a copper catalyzed covalent reaction between an azide (Alexa Fluor® 488 dye) and an alkyne (EdU), using a Click-iT® EdU Alexa Fluor® 488 Flow Cytometry Assay Kit, following the instructions of the manufacturer. Cell cycle analysis was performed using the cell cycle dye 7-amino-actinomycin D (7-AAD), also available from the kit. The analysis of the cell cycle and incorporation of EdU was performed by a BD FACSCalibur™ Flow Cytometer,

using the BD CellQuest Pro software (version 0.3.efab). Thirty thousand events were acquired, per experiment, in the region of interest, which included apoptosis, G0/G1, S and G2/M. The data was analyzed using WinMDI2.9 software, and is presented as means ± SEM of the number of live cells that incorporated EdU (% of control).

2.1.4. Western blot analysis

To evaluate the protein levels of COX I and SIRT1, cells were exposed to either NOC-18 or 8-Br-cGMP during 24 h. To obtain whole cell lysates, after washing with 0.01 M PBS, cells were lysed in 100 mM Tris-HCl, 10 mM ethylene glycol tetraacetic acid, 1% Triton X-100 and 2 mM $MgCl_2$ (lysis buffer), supplemented with 200 µM phenylmethanesulphonyl fluoride, 1 µM dithiothreitol, 1 µg/ml CLAP (chymostatin, pepstatin, antipain and leupeptin), 1 µM sodium orthovanadate, 5 mM sodium fluoride, 5 mM nicotinamide and 300 nM trichostatin A, pH 7.4, at 4ºC.Then, the lysates were freeze/thawed 3 times in liquid nitrogen and sonicated 10 times with 5-second pulses separated by 5 seconds. The protein concentration was determined by the bicinchoninic acid (BCA) method, using the BCA protein kit, following the manufacturer's instructions. Sample buffer 6-times concentrated was added (0.5 M Tris-HCl/0.4 % sodium dodecyl sulfate (SDS) pH 6.8, 30 % glycerol, 10 % SDS, 0.6 M dithiothreitol, 0.012 % bromophenol blue), and the lysates were denatured at 95ºC for 5 min. Equal amounts of protein (50 µg) were separated by electrophoresis on SDS-polyacrylamide gels, using MiniPROTEAN® 3 systems. Electrophoresis gels composition was 15 % (COX I) or 8 % (SIRT1) bis-acrilamide and 1.5 M Tris-HCl pH 8.0 (for the resolving gels) or 4 % bis-acrilamide and 0.5 M Tris-HCl pH 6.8 (for the stacking gel); plus 0.1 % SDS, 0.05 % tetramethylethylenediamine, 0.05 % ammonium persulfate, in ultrapure water. The electrophoresis, in running buffer (25 mM Tris, 25 mM bicine, 0.1 % SDS, pH 8.3), started at 60 V for 10 min, followed by 160 V (COX I) or 140 V (SIRT1) until the desired band separation was reached (about 90 min).

Polyvinylidene difluoride membranes were activated in 100 % methanol during 5 min and during 15-30 min in electrotransference buffer (CAPS 10 mM, methanol 10 %, pH 11.0). Then, the proteins were transferred electrophoretically (750 mA for 90-120 min) to the membranes, submerged in electrotransference buffer, using Trans-Blot Cell apparatus. To simultaneously analyze the loading control (α-tubulin), the membrane designed to the detection of SIRT1 was cut by the band corresponding to 75 kDa. Membranes were blocked for 1 h at room temperature, in Tris-buffered saline (137 mM NaCl, 20 mM Tris-HCl, pH 7.6) containing 0.1 % Tween-20 (TBS-T) and 5 % low-fat dry milk, and incubations with the primary antibodies (mouse anti-COX I 1:500, rabbit anti-SIRT1 1:1,000, and mouse anti-α-tubulin 1:20,000 in TBS-T 1 % low-fat dry milk) were performed overnight, at 4ºC. After washing in TBS-T, the membranes were incubated for 1 h, at room temperature, with the appropriated alkaline phosphatase-conjugated secondary antibody (anti-rabbit or anti-mouse 1:20,000 in TBS-T 1 % low-fat dry milk). Membranes were washed again in TBS-T and dried before incubation, for 5 min maximum, with Enhanced Chemifluorescence substrate, after which immunoreactive bands were visualized in a VersaDoc imaging system. The data from at least 4 independent experiments was analyzed with the QuantityOne software (version 4.6.9) from Bio-Rad, and is presented as means ± SEM (% of control).

2.1.5. Immunocytochemistry

NSC plated on coverslips were washed with 0.01 M PBS, fixed in 4 % paraformaldehyde with 4 % sucrose in PBS, for 20 min at room temperature, and washed again in PBS. Then, cells were permeabilized in 1 % Triton X-100 in PBS during 5 min at room temperature, and washed again in PBS. They were blocked in 3 % bovine serum albumin (BSA) with Tween 20 in PBS, for 1 h at room temperature, and incubated with the primary antibody (mouse anti-COX I 1:200 in 3 % BSA in PBS) overnight at 4ºC. After washing the cells, the secondary antibody (Alexa-Fluor 488 anti-

mouse 1:200 in 3 % BSA in PBS) and the rhodamine phalloidin 1:100 were incubated, during 90 min at room temperature. Hoechst 2 µg/ml was incubated for 10 min at room temperature, between rinses with 0.01 M PBS. Finally, the coverslips were mounted in slides with DAKO fluorescence mounting medium. They were observed in a laser scanning microscope (LSM 510 Meta, Zeiss, Jena, Germany) and representative images were acquired.

2.1.6. Labeling of mitochondria with MitoTracker® Green FM

The label of active mitochondria was performed using the cell-permeant probe MitoTracker® Green FM. Cells plated on coverslips were treated with 100 nM MitoTracker® Green FM in prewarmed Krebs buffer (132 mM NaCl, 4 mM KCl, 1.4 mM $MgCl_2$, 1mM $CaCl_2$, 10 mM glucose, 10 mM HEPES, pH 7.4) for 30 minutes at 37°C. After adding fresh Krebs, the coverslips were mounted in appropriate slides, immediately observed in a laser scanning microscope (LSM 510 Meta, Zeiss, Jena, Germany) and representative images were acquired.

2.1.7. Evaluation of mitochondrial membrane potential

Mitochondrial membrane potential (ΔΨm) was measured using tetramethylrhodamine methyl ester (TMRM), a cationic fluorophore that is membrane-permeable and accumulates in mitochondria proportionally to their membrane potential (Ehrenberg *et al.* 1988). After the treatments, the cells were maintained with 6.6 µM of TMRM in D-MEM/F-12 supplemented with 5.5 mM glucose, during 15 minutes, at 37ºC. After this time of incubation, fresh glucose supplemented D-MEM/F-12 was added, and the fluorescence (excitation 485 nm, emission 590 nm) was measured on a VICTOR^3 plate reader (Perkin-Elmer, Massachusetts, USA), using Wallac 1420 manager software. ΔΨm was calculated by the slope between basal fluorescence, and the fluorescence upon mitochondrial depolarization caused by addition of 2,4-

Dinitrophenol (DNP, 75 µM). Cells were scraped with lysis buffer supplemented with proteases inhibitors, for protein quantification by the BCA method. All results were normalized by mg of protein. Data is presented as means ± SEM (% of control) of at least 3 independent experiments.

2.1.8. Measurement of intracellular ATP levels

The intracellular ATP levels were measured by bioluminescence (Tsujimoto *et al.* 1970). During all the protocol, the samples were kept at 4ºC. Following the treatments, the cells were harvested with 2.5 M KOH in 1.5 M K_2HPO_4 diluted 4-times in distilled water. After vortexed and a centrifugation of 14,000 rpm during 5 min, the supernatants were collected to new eppendorfs. The pH of all samples was neutralized (pH 7.0) with 1 M KH_2PO_4. To determine protein concentration (BCA method), the pellets were resuspended in radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.4, 5 mM ethylene glycol tetraacetic acid pH 7.4, 1 % Triton X-100, 0.5 % sodium deoxycholate and 0.1 % SDS) supplemented with 200 µM phenylmethanesulphonyl fluoride, 1 µM dithiothreitol, 1 µg/ml CLAP. The ATP levels were determined using the Adenosine 5'-triphosphate (ATP) Bioluminescent Assay Kit, due to a reaction catalyzed by the enzyme firefly luciferase, in which ATP is consumed and light is emitted proportionally to the amount of ATP present. The ATP assay mix (containing the enzyme) was used diluted 25-times; and each sample was loaded into a 96-well plate (50 µl), diluted 4-times. In each well, 100 µl of the ATP assay mix were injected and after 20 seconds the light produced was measured on a VICTOR^3 plate reader (Perkin-Elmer, Massachusetts, USA), using Wallac 1420 manager software. A calibration curve was obtained from a series of dilutions of the ATP standard, from which the levels of ATP in each sample were calculated. Data is presented as means ± SEM (pmol ATP/mg of protein) of at least 4 independent experiments.

2.1.9. Analysis of mitochondrial copy number

The mitochondrial copy number was evaluated by semi-quantitative real-time PCR, using the MiniOpticon™ Two-Color Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The cells were exposed to the treatments, and DNA was extracted using QIAamp DNA mini kit, according to the manufacturer's protocol. The DNA samples were run against a mitochondrial DNA-encoded and nuclear DNA-encoded mitochondrial proteins. The primer sequences used were pyruvate kinase (PK): Upper - 5'-CTT CAG TGG AAA TTA AGG GAG AAA-3'; Lower - 5'-CCA TTC AAT TCA GCA CTT TAT GAG-3', and the PCR was run with SsoFast EvaGreen Supermix. For each sample, the threshold cycle, C(t), of the mitochondrial-encoded gene (subunit III of Complex IV, COX III) was divided by the C(t) of the nuclear-encoded gene (PK). This ratio evaluates the number of copies of mitochondrial genome per cell; the lower the ratio, the higher the copy number. Data is presented as means ± SEM (mtDNA/nDNA ratio) of at least 4 independent experiments.

2.1.10. Statistical analysis

Statistical significance was determined by a one-way analysis of variance (ANOVA) followed by Dunnett's or Bonferroni's post-tests, or a two-tailed t-test, using GraphPad Prism 5. Differences were considered significant when $p<0.05$.

2.2. Materials

DMEM/F12 GlutaMAX^{TM} I, B27, Click-iT® EdU Alexa Fluor® 488 Flow Cytometry Assay Kit, StemPro® Accutase®, Pen/Strep, Gentamicin, Trypsin-EDTA, TMRM and MitoTracker® Green FM were purchased to Invitrogen (Paisley, UK); and EGF and bFGF were from PeproTech Inc. (London, UK). EX527 and ODQ were from Tocris Bioscience (Bristol, UK), and NOC-18 was from Alexis Biochemicals (San

Diego, CA, USA). Slides and coverslips were obtained from Thermo Fisher Scientific Inc. (Waltham, MA, USA) and DAKO fluorescence mounting medium from DAKO (Glostrup, Denmark). QIAamp DNA mini kit was from Qiagen (Iberia, S.L., Madrid, Spain). BCA Protein Assay kit was from Pierce (Rockford, IL, USA); SDS, ammonium persulfate, bis-acrilamide, MiniPROTEAN® 3 systems, Trans-Blot Cell apparatus and SsoFast EvaGreen Supermix were all acquired from Bio-Rad Laboratories Inc. (Hercules, CA, USA), polyvinylidene difluoride membranes were from Millipore (Billerica, MA, USA), low-fat dry milk from Nestlé (Vevey, Switzerland) and Enhanced Chemifluorescence substrate from GE Healthcare Life Sciences (Buckinghamshire, UK). The primary antibodies used for Western blot, rabbit anti-SiRT1 and mouse anti-COX I, were purchased from Cell Signaling Technology (Danvers, MA, USA) and MitoSciences (Eugene, OR, USA) respectively, and the alkaline phosphatase-conjugated secondary antibodies, anti-rabbit and anti-mouse, were acquired from GE Healthcare Life Sciences (Buckinghamshire, UK). The other reagents were from Sigma Aldrich (St Louis, MO, USA) or Merck KgaA (Darmstadt, Germany).

Chapter 3 Results

3.1. NO• stimulates the proliferation of NSC in a biphasic way

To study the effect of NO• on NSC proliferation, we exposed SVZ cultures to a NO• donor (NOC-18, 10 µM), during 6 h, 12 h and 24 h, and detected EdU (10 µM, 4 h) incorporation by flow cytometry (Salic & Mitchison 2008). We observed that (Fig. 7) NO• significantly increased proliferation of cells treated for 6 h (133.4 ± 5.2 %, $p<0.05$) and significantly increased even more the proliferation of cells exposed for 24 h (157.3 ± 12.4 %, $p<0.001$), while there was no change in the proliferation of cells exposed to NO• during 12 h (80.3 ± 8.9 %, $p>0.05$), when comparing with untreated cells (control, 100 %).

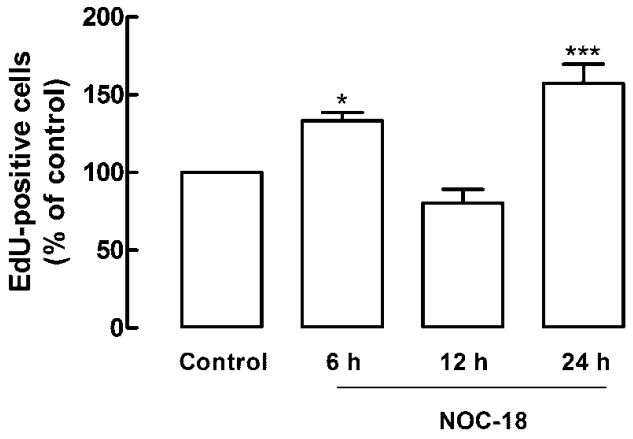

Figure 7. Exposure to NOC-18 for 6 h and 24 h increases NSC proliferation. Cells were treated with 10 µM NOC-18 during 6 h, 12 h and 24 h and the incorporation of EdU was detected by flow cytometry. Data from 2-13 independent experiments is presented as means ± SEM. One-way ANOVA (Dunnett's post-test), *$p<0.05$ and ***$p<0.001$ significantly different from control.

3.2. MAPK pathway is involved in the proliferative effects of NO•

We next investigated whether MAPK pathway was involved in the early (6 h) or late (24 h) NO•-induced proliferation of NSC, using MEK 1/2 inhibitor (U0126, 1 µM) to block ERK 1/2 activation. Cells that incorporated EdU were detected by flow cytometry.

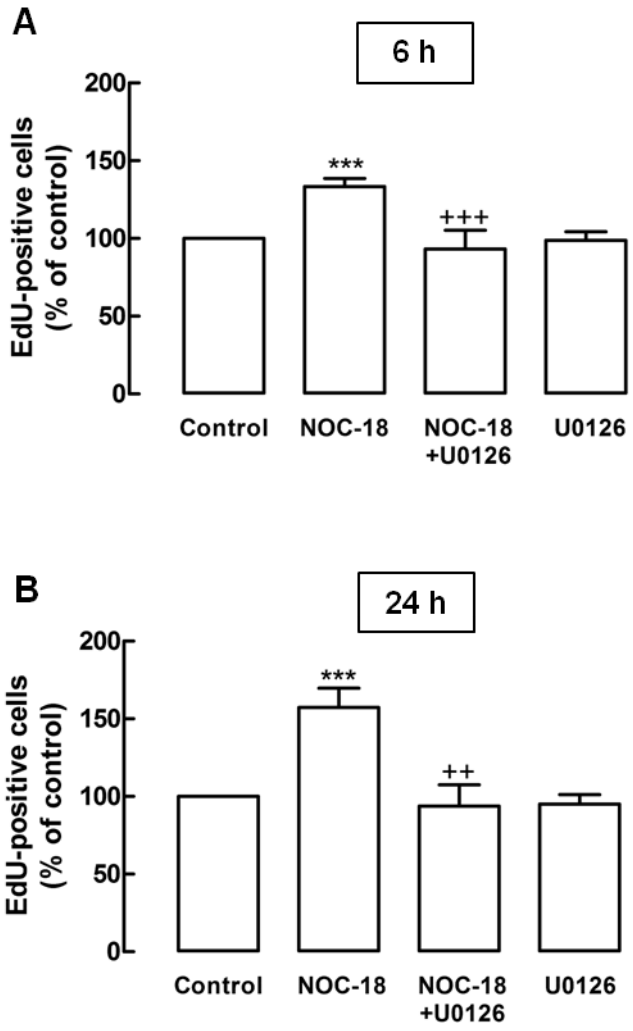

Figure 8. Blocking ERK 1/2 activation prevents the proliferative effects of $NO^{\bullet}$. Cultures were exposed to U0126, alone and with NOC-18, during 6 h (**A**) and 24 h (**B**). EdU incorporation was evaluated by flow cytometry. Data from 4-13 independent experiments is presented as means ± SEM. One-way ANOVA (Bonferroni's post-test), ***p<0.001 significantly different from control, ++p<0.01 and +++p<0.001 significantly different from NOC-18.

As already explained in the previous section, $NO^{\bullet}$ significantly increased NSC proliferation following exposure to NOC-18 for 6 h (133.4 ± 5.2 %, p<0.001, Fig. 8 A) and 24 h (157.3 ± 12.4 %, p<0.001, Fig. 8 B), compared to control (100 %). Moreover, treatment with NOC-18 and U0126 significantly prevented early (93.2 ± 12.1 %, p<0.01, Fig. 8 A) and late (93.8 ± 13.8 %, p<0.001, Fig. 8 B) proliferative effects of $NO^{\bullet}$.

U0126 alone did not significantly affect proliferation in either cases (98.7 ± 5.7 %, p>0.05, Fig. 8 A; 95.2 ± 6.1 %, p>0.05, Fig. 8 B), compared to control (100 %).

3.3. The late proliferative effect of NO• is dependent on cGMP

Then we investigated a possible role for sGC/cGMP pathway in the early (6 h) or late (24 h) proliferative effects of NO•, by inhibiting the sGC (ODQ, 50 μM), and using flow cytometry to detect EdU incorporation.

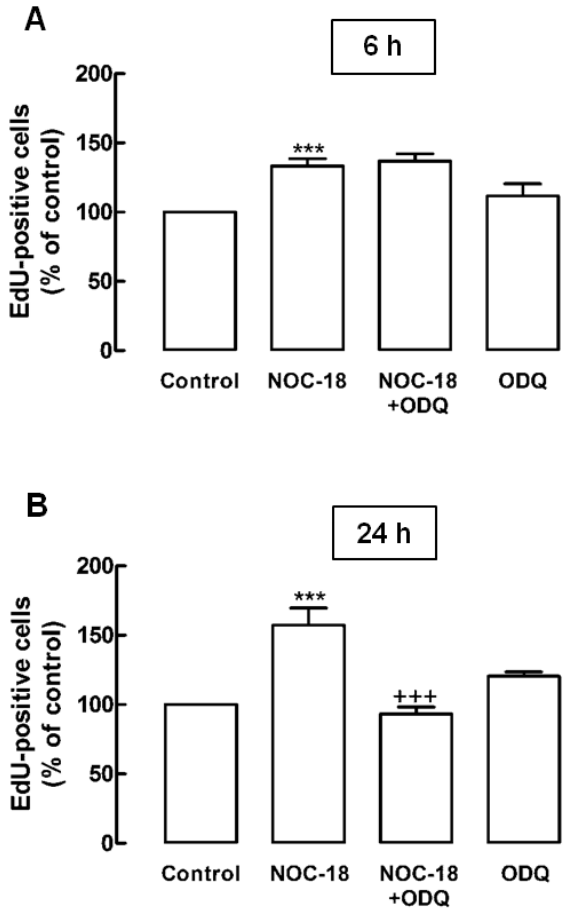

Figure 9. Inhibition of sGC prevents late (24 h) but not early (6 h) proliferative effects induced by NOC-18. Cultures were exposed to ODQ, alone and with NOC-18, for 6 h (**A**) and 24 h (**B**). EdU incorporation was detected by flow cytometry. Data from 6-13 independent experiments is presented as means ± SEM. One-way ANOVA (Bonferroni's post-test), ***p<0.001 significantly different from control, +++p<0.001 significantly different from NOC-18.

We observed that the late proliferative effect of NO• (157.3 ± 12.4 %, p<0.001, Fig. 9 B) was significantly prevented by the treatment with NOC-18 and ODQ (93.2 ± 5.1 %, p<0.001, Fig. 9 B), while the early proliferative of NO• (133.4 ± 5.2 %, p<0.001, Fig. 9 A) was not significantly affected by the treatment with NOC-18 and ODQ (136.9 ± 5.3 %, p>0.05, Fig. 9 A). ODQ alone (120.4 ± 3.3 %, p>0.05, Fig. 9 B; 111.6 ± 8.8 %, p>0.05, Fig. 9 A) did not significantly affect proliferation, compared to control (100 %).

3.4. 8-Br-cGMP increases the proliferation of NSC

To evaluate the direct effect of cGMP on NSC proliferation, we exposed the cultures to a cGMP analogue (8-Br-cGMP, 20 µM) for 6 h, 12 h and 24 h, and detected EdU incorporation using flow cytometry.

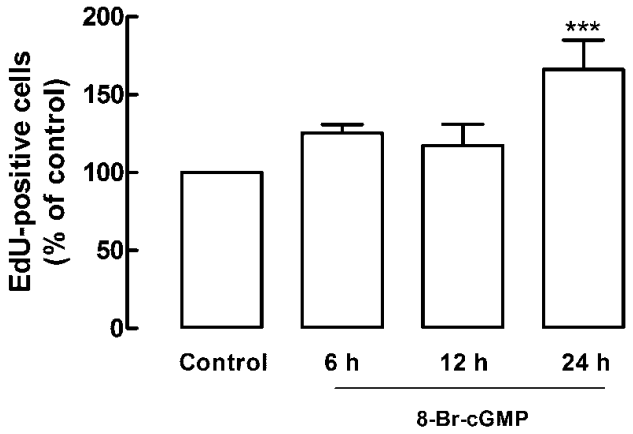

Figure 10. Treatment with 8-Br-cGMP during 6 h and 24 h increases NSC proliferation. Cells were exposed to 8-Br-cGMP for 6 h, 12 h, and 24 h, and EdU incorporation was evaluated by flow cytometry. Data from 2-12 independent experiments is presented as means ± SEM. One-way ANOVA (Dunnett's post-test), ***p<0.001 significantly different from control.

We observed (Fig. 10) that exposure to 8-Br-cGMP for 6 h (125.4 ± 5.5 %, p>0.05) and 12 h (117.3 ± 13.6 %, p>0.05) did not significantly increase the proliferation of cells, but showed a tendency to increase. However, treatment with 8-Br-

cGMP for 24 h (166.1 ± 19.0 %, p<0.001) significantly increased the proliferation of cells compared to control (100 %).

3.5. Activation of MAPK pathway is essential to the 8-Br-cGMP proliferative effect

To investigate a possible involvement of the MAPK pathway on the proliferative effect induced by 8-Br-cGMP, we blocked ERK 1/2 activation by inhibiting MEK 1/2 with U0126. EdU incorporation was detected by flow cytometry.

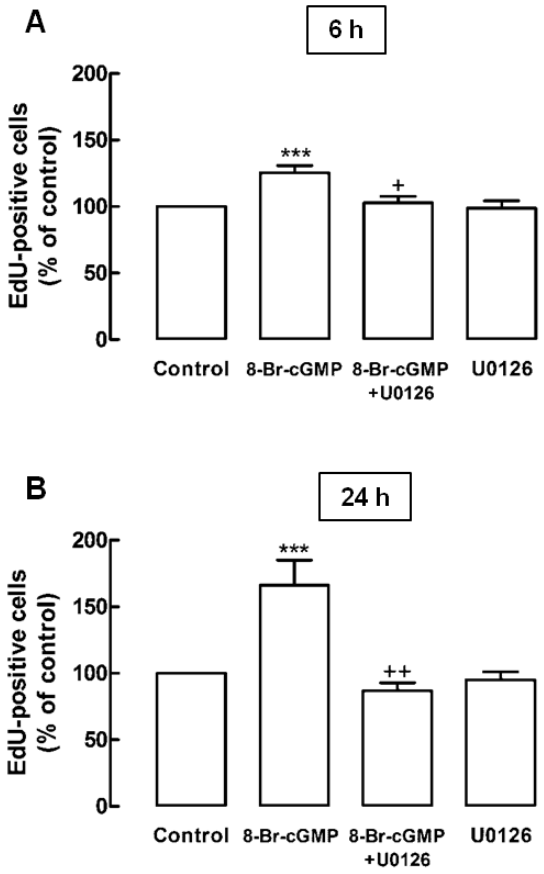

Figure 11. Blocking activation of ERK 1/2 prevents the proliferative effect of 8-Br-cGMP. Cells were exposed to U0126, alone and with 8-Br-cGMP, during 6 h (**A**) and 24 h (**B**). Incorporation of EdU was detected by flow cytometry. Data from 5-12 independent experiments is presented as means ± SEM. One-way ANOVA (Bonferroni's post-test), ***p<0.001 significantly different from control, ++p<0.01 and +p<0.05 significantly different from 8-Br-cGMP.

We observed that treatment with 8-Br-cGMP and U0126 during 6 h (102.9 ± 4.8 %, $p<0.05$, Fig. 11 A) and 24 h (87.0 ± 5.8 %, $p<0.01$, Fig. 11 B) significantly prevented the increase on proliferation caused by 8-Br-cGMP (125.4 ± 5.5 %, $p<0.001$, Fig. 11 A; 166.1 ± 19.0 %, $p<0.001$, Fig. 11 B). U0126 alone had no effect on proliferation (98.7 ± 5.7 %, $p>0.05$, Fig. 11 A; 95.2 ± 6.1 %, $p>0.05$, Fig. 11 B), compared to control (100 %).

3.6. SIRT1 is not involved in the proliferation of NSC

In order to study the role of SIRT1 on NSC proliferation, the cultures were exposed, during 24 h, to a selective SIRT1 inhibitor (EX527, 1 μM), and its effect on proliferation stimulated by NOC-18 and 8-Br-cGMP was evaluated. EdU incorporation was detected by flow cytometry. Furthermore, to evaluate whether SIRT1 was influenced by NSC proliferation, we analyzed the levels of this protein, by Western blot, on cells treated with NOC-18 and 8-Br-cGMP, during 24 h.

Regarding the proliferation induced by $NO^{\bullet}$ (Fig. 12), we observed that treatment with NOC-18 and EX527 (134.6 ± 14.7 %, $p>0.05$) did not prevent the proliferation stimulated by NOC-18 alone (157.3 ± 12.4 %, $p<0.001$). On the other hand (Fig. 13), exposure to 8-Br-cGMP and EX527 (134.6 ± 14.7 %, $p>0.05$) showed a tendency to decrease the proliferation caused by 8-Br-cGMP (166.1 ± 19.0 %, $p<0.01$). EX527 alone increased proliferation (121.0 ± 11.5 %, $p>0.05$, Fig. 12 and Fig. 13), compared to control (100 %), despite the lack of statistic significance.

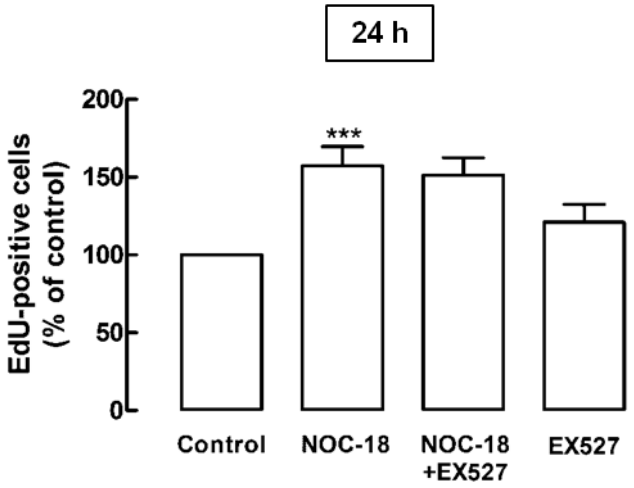

Figure 12. SIRT1 inhibition does not prevent the proliferative effect of $NO^{\bullet}$. Cultures were exposed to EX527, alone and with NOC-18, during 24 h. EdU incorporation was detected by flow cytometry. Data from 5-13 independent experiments is presented as means ± SEM. One-way ANOVA (Bonferroni's post-test), ***p<0.001 significantly different from control.

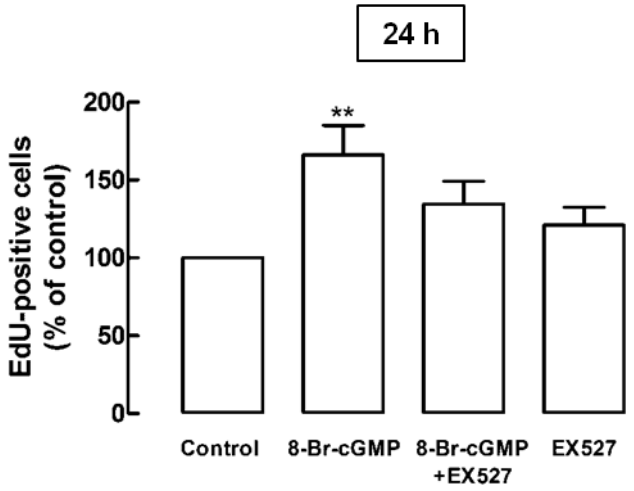

Figure 13. SIRT1 inhibition slightly decreases the proliferation induced by 8-Br-cGMP. Cells were treated with EX527 alone and with 8-Br-cGMP, during 24 h. Incorporation of EdU was evaluated by flow cytometry. Data from 5-12 independent experiments is presented as means ± SEM. One-way ANOVA (Bonferroni's post-test), **p<0.01 significantly different from control.

In relation to the protein content, our results showed no changes on SIRT1 levels during proliferation induced by either $NO^{\bullet}$ (105.4 ± 11.1 %, p>0.05, Fig. 14 A) or 8-Br-cGMP (95.6 ± 7.4 %, p>0.05, Fig. 14 B), compared to control (100 %).

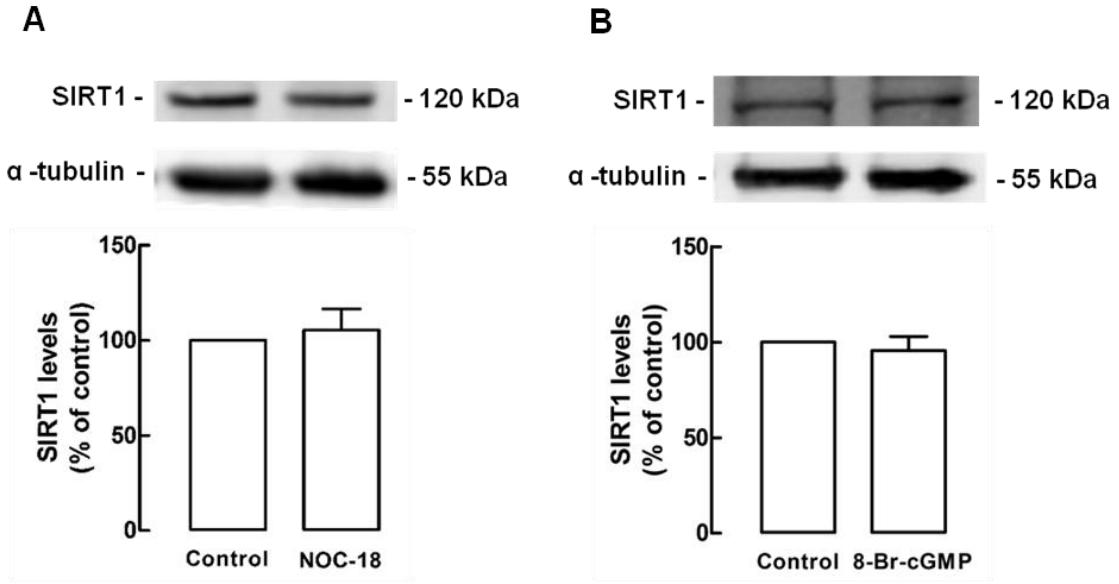

Figure 14. SIRT1 levels are unchanged during NSC proliferation. Cells were treated with NOC-18 and 8-Br-cGMP for 24 h. Protein content was analyzed by Western blot and α-tubulin was used as a loading control. Data from 4-5 independent experiments is presented as means ± SEM. Two-tailed t-test, p>0.05.

3.7. Proliferating NSC do not present significant alterations in COX I levels

To study whether there would be alterations in the levels of functionally important mitochondrial proteins during NSC proliferation, we analyzed the levels of COX I, one of the mitochondrial respiratory chain Complex IV subunits, by Western blot, after treatment with NOC-18 and 8-Br-cGMP during 24 h. Our results showed that treatment with NOC-18 did not affect the levels of COX I (97.1 ± 4.8 %, p>0.05, Fig. 15), compared to control (100 %). Nevertheless, cells exposed to 8-Br-cGMP showed a tendency to have higher levels of COX I (129.4 ± 21.2 %, p>0.05, Fig. 16), when comparing to control (100 %), although this difference was not significant.

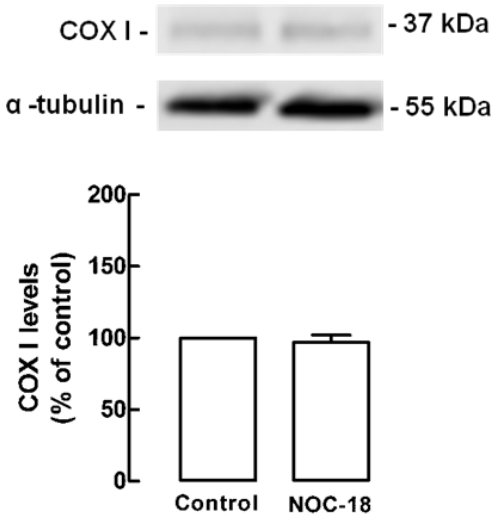

Figure 15. COX I levels do not change during NSC proliferation induced by $NO^{\bullet}$. Cells were treated for 24 h with NOC-18 and the protein levels analyzed by Western blot, using α-tubulin as a loading control. Data from 4-5 independent experiments is presented as means ± SEM. Two-tailed t-test, p>0.05.

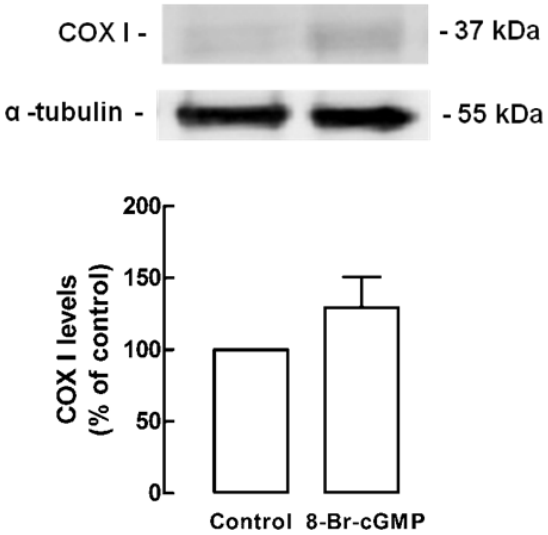

Figure 16. COX I levels increase slightly during NSC proliferation induced by 8-Br-cGMP. Cells were exposed to 8-Br-cGMP for 24 h and the protein levels were analyzed by Western blot, using α-tubulin as a loading control. Data from 4-5 independent experiments is presented as means ± SEM. Two-tailed t-test, p>0.05.

3.8. NSC present active mitochondria

To investigate the presence of mitochondria in NSC, we analyzed the distribution of a mitochondrial protein (COX I), in SVZ cultures, by immunocytochemistry. Moreover, we used MitoTracker® Green FM probe to evaluate the functionality of mitochondria.

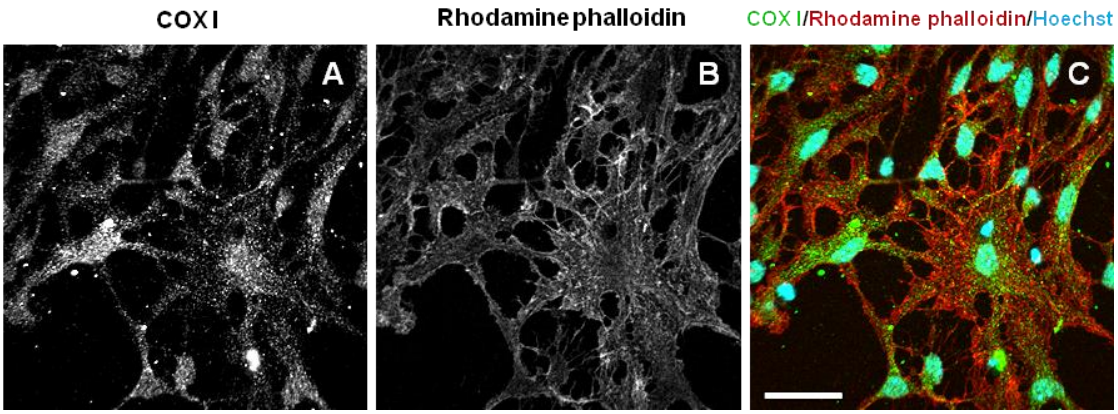

Figure 17. NSC present mitochondria widely distributed in the cell. An immunocytochemistry was performed with SVZ cultures. Representative image of COX I (**A**), Rhodamine phalloidin (**B**) and the merge (**C**; COX I, green; Rhodamine phalloidin, red; nuclei labeled with Hoechst 33342, blue). Scale bar: 30 µm.

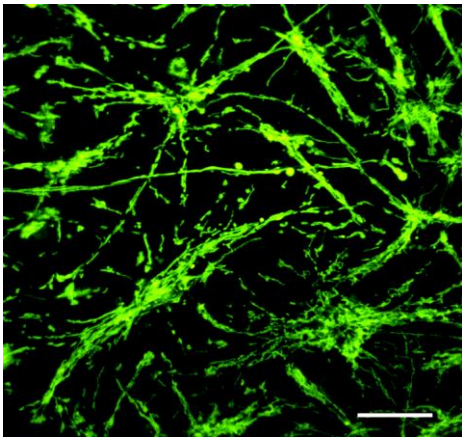

Figure 18. NSC present a mitochondrial network of active mitochondria. Representative image of cells incubated with MitoTracker® Green FM probe. Scale bar: 20 µm.

Our results showed that NSC present mitochondria distributed all over the cell (Fig. 17). In addition, we confirmed that the mitochondrial network present in NSC was composed by polarized mitochondria that took up the potential-dependent Mitotracker probe (Fig. 18).

3.9. ΔΨm and ATP levels are differently affected by NO• and 8-Br-cGMP

To evaluate whether NSC proliferation induced by NO• and 8-Br-cGMP would affect mitochondrial function, we then analyzed ΔΨm (incubation with TMRM) and intracellular ATP levels (detection of luminescence) of cells treated with NOC-18 (alone or with the MEK 1/2 and sGC inhibitors) and 8-Br-cGMP (alone or with MEK 1/2 inhibitor) for 24 h.

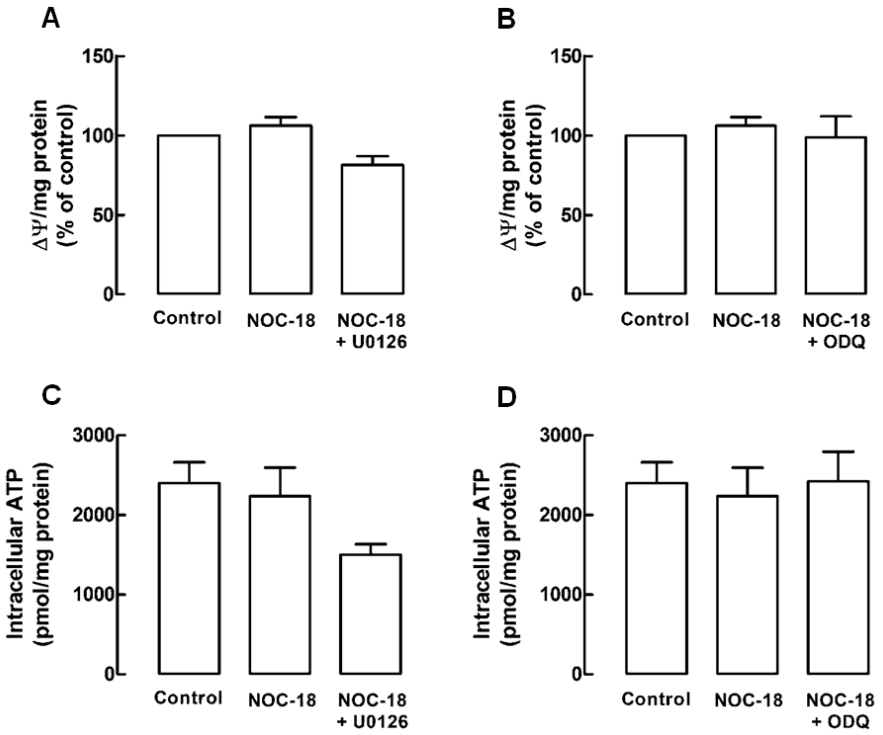

Figure 19. ΔΨm and ATP levels are not affected by NOC-18. Cells were treated during 24 h with NOC-18 (alone, with U0126, and with ODQ). ΔΨm (**A** and **B**) was analyzed after incubation of TMRM, and ATP content was measured by luminescence (**C** and **D**). Data from 3-6 independent experiments is presented as means ± SEM. One-way ANOVA (Bonferroni's post-test), $p>0.05$.

The results showed that NOC-18 alone (106.2 ± 5.5 %, p>0.05, Fig. 19 A and B), as well as with the inhibitors (NOC-18 and U0126 81.5 ± 5.5 %, p>0.05, Fig. 19 A; NOC-18 and ODQ 99.0 ± 13.1 %, p>0.05, Fig. 19 B), did not affect ΔΨm, when comparing with control (100 %). Regarding the ATP levels, NOC-18 alone (2236 ± 358 pmol/mg, p>0.05, Fig. 19 C and D) and with ODQ (2422 ± 373 pmol/mg, p>0.05, Fig. 19 D) did not change ATP content relatively to control (2400 ± 263 pmol/mg), but NOC-18 with U0126 showed a tendency to a small decrease (1505 ± 131 pmol/mg, p>0.05, Fig. 19 C).

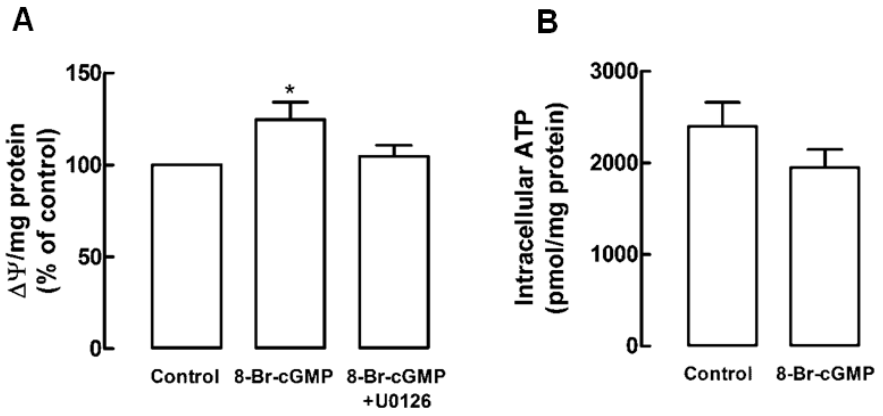

Figure 20. 8-Br-cGMP increases ΔΨm but decreases ATP levels. Cells were exposed to 8-Br-cGMP during 24 h (alone or with U0126). ΔΨm (**A**) was analyzed after incubation of TMRM, and ATP content was measured by luminescence (**B**). Data from 3-5 independent experiments is presented as means ± SEM. (**A**) One-way ANOVA (Bonferroni's post-test), *p<0.05 significantly different from control; (**B**) Two-tailed t-test, p>0.05.

As for the effect of 8-Br-cGMP, our results showed a significant increase in the ΔΨm in cells treated with 8-Br-cGMP alone (124.7 ± 9.5 %, p<0.05, Fig. 20 A), compared to control (100 %), that appears to be prevented by the treatment with U0126 (104.6 ± 6.1 %, p>0.05, Fig. 20 A), although not significantly. However, the treatment with 8-Br-cGMP suggested a decrease in the ATP levels (1951 ± 197

pmol/mg, p>0.05, Fig. 20 B), when comparing with control (2400 ± 263 pmol/mg), despite not being significant.

3.10. Mitochondrial copy number is maintained during NSC proliferation

In order to investigate the existence of mitochondrial mass alterations during NSC proliferation, we analyzed the mitochondrial copy number of cells treated with NOC-18 (alone, with U0126, and with ODQ) and 8-Br-cGMP for 24 h. The expression of one mitochondrial gene (COX III) and one nuclear gene (PK) was analyzed by semi-quantitative Real-Time PCR, and their ratio calculated.

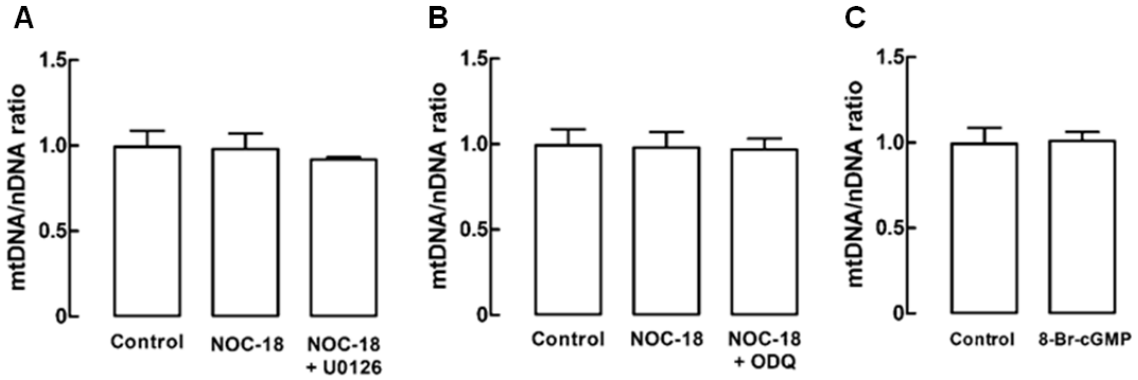

Figure 21. Mitochondrial copy number is unchanged during NSC proliferation. Cells were treated during 24 h with NOC-18, alone, with U0126 (**A**), and with ODQ (**B**); and 8-Br-cGMP **(C)**. COX III and PK genes were analyzed by semi-quantitative Real-Time PCR and the ratio COX III/PK was calculated. Data from 4 independent experiments is presented as means ± SEM. (**A** and **B**) One-way ANOVA (Bonferroni's post-test), p>0.05, (**C**) Two-tailed t-test, p>0.05.

We observed that the ratio was not modified by any treatment, NOC-18 (0.98 ± 0.09, p>0.05, Fig. 21 A and B), NOC-18 and U0126 (0.92 ± 0.02, p>0.05, Fig. 21 A), NOC-18 and ODQ (0.97 ± 0.06, p>0.05, Fig. 21 B), and 8-Br-cGMP (1.01 ± 0.05, p>0.05, Fig. 21 C), when comparing to control (0.99 ± 0.09).

Chapter 4 Discussion

4.1. NO• and the proliferation of NSC

To be able to address the question of whether there would be mitochondrial biogenesis during NSC proliferation, we first studied the effect of NO• in the proliferation of these cells.

Our group has already shown that treatment with 10 µM of NOC-18 stimulates NSC proliferation (Carreira *et al.* 2010). Indeed, in this work, this concentration of the NO• donor increased the proliferation of NSC in a biphasic way. There was a significant increase in the proliferation of NSC following 6 h, and an even higher increase after 24 h of treatment, but no effect during 12 h exposure. So, we next investigated the pathways that could be involved in these different stages of the proliferation induced by NO•. The MAPK pathway activation is described as one of the mechanisms by which NO• can stimulate proliferation (Carreira *et al.* 2010). In fact, we observed that inhibition of MEK 1/2 significantly prevented both the early (6 h) and the late (24 h) proliferative effects of NO•, which indicates that the activation of this pathway is involved in these two effects. Additionally, since one of the ways of NO• action is by binding to sGC originating cGMP (Arnold *et al.* 1977), we assessed whether this mechanism was also involved in the proliferation mediated by NO•. Our results showed that inhibition of sGC significantly prevented the late, but not the early proliferative effects of NO•, suggesting that cGMP is necessary for the late proliferation induced by NO•. To directly confirm the involvement of cGMP, we used its analogue 8-Br-cGMP, during the same incubation times used for NOC-18. We observed a significant increase in the proliferation of cells treated for 6 h and 24 h, which was higher at 24 h, as with NOC-18. The treatment of 12 h did not show any effect, probably because of the low number of experiments, as it would be expected to be higher than that of 6 h, but lower than that of 24 h. Interestingly, the proliferative effect induced by 8-Br-cGMP was significantly prevented by inhibition of MEK 1/2, indicating that activation of MAPK pathway is essential to the effect of 8-Br-cGMP on the proliferation of NSC. Overall, these results suggest that NO•

induces proliferation of NSC, first (6 h) because of the activation of MAPK pathway (rapid phosphorylation of ERK 1/2 by MEK 1/2 (Carreira *et al.* 2010)); and then (24 h) due to the activation of the sGC pathway and accumulation of cGMP, with involvement of the MAPK pathway. In the treatment that did not have any effect by $NO^{\bullet}$ (12 h), the most likely explanation is that the activation of MAPK was already over, but there was still not activation of sGC and production of cGMP.

4.2. $NO^{\bullet}$ and mitochondrial biogenesis during the proliferation of NSC

In conditions of proliferation, cells would require higher amounts of energy. One of its possible sources could be the increase in the number and functionality of mitochondria: mitochondrial biogenesis. It has been described that $NO^{\bullet}$ induces mitochondrial biogenesis in certain cell types by means of cGMP (Nisoli *et al.* 2003). Given that our results showed that the cGMP is involved in the proliferation induced by the treatment with NOC-18 for 24 h, we conducted the remaining experiments likewise, and having as a positive control the cGMP analogue 8-Br-cGMP.

Mitochondrial biogenesis results from the activation of transcription factors, such as PGC-1α (Nisoli *et al*. 2003), which can be activated by deacetylation by SIRT1 (Nemoto *et al.* 2005). In order to investigate whether a possible mitochondrial biogenesis would be mediated by SIRT1, we evaluated the effect of inhibiting this protein on the proliferation induced by $NO^{\bullet}$ and 8-Br-cGMP. We observed that, in the case of $NO^{\bullet}$, the inhibition of SIRT1 did not prevent proliferation; but the proliferation induced by 8-Br-cGMP appeared to have a tendency to be slightly impaired, although not significantly. Moreover, treatment with the inhibitor alone slightly increased the proliferation of NSC, but not in a significant way. This may be explained due to the role of SIRT1 in the differentiation of NSC (Hisahara *et al.* 2008, Prozorovski *et al.* 2008), such that its inhibition could lead NSC for a more proliferative state. Furthermore, we also analyzed the levels of SIRT1 during the proliferation caused by $NO^{\bullet}$ and 8-Br-

cGMP and observed no alterations in either cases. These results indicate that SIRT1 is not involved in the proliferative effects of $NO^{\bullet}$ and 8-Br-cGMP, so in the case of occurring mitochondrial biogenesis in these conditions, it would not be mediated by this protein.

Our next approach was to evaluate whether the levels of important proteins for mitochondrial functionality and biogenesis would be affected during proliferation. We observed no alterations on the levels of COX I in cells treated with NOC-18. However, 8-Br-cGMP appeared to increase the levels of COX I, although not significantly, since the SEM is high. With this result it appears that the functionality of mitochondria is not altered during proliferation induced by $NO^{\bullet}$. We also tried to evaluate the levels of COX IV and PGC-1α, unsuccessfully, since we could not detect these proteins in the Western blots performed.

Given our difficulty in detecting those proteins, we investigated whether SVZ-derived NSC had functional mitochondria. The immunocytochemistry for COX I showed the presence of mitochondria, which were distributed all over the cell. Moreover, that was confirmed by MitoTracker® Green FM. It showed the existence of a mitochondrial network, proving that the mitochondria were functional, since this probe only diffuses into active mitochondria.

Knowing that the NSC presented mitochondria, we evaluated whether there would be changes in its function during proliferation. $NO^{\bullet}$ did not change either the $\Delta\Psi m$ or the ATP levels. There was no significant effect on the inhibition of the two pathways, except for the inhibition of MEK 1/2, which showed a tendency to decrease the levels of ATP, but not in a significant way. The 8-Br-cGMP significantly increased the $\Delta\Psi m$, and that appeared to be prevented by inhibition of MEK 1/2, although not significantly. However, treatment with 8-Br-cGMP also decreased not significantly the levels of ATP. An increase in the $\Delta\Psi m$ and decrease in the ATP levels usually occurs

when the ATP synthase is not working (Masini *et al.* 1984, Macouillard-Poulletier de *et al.* 1998), so there is no formation of ATP and the ΔΨm is not dissipated. However, this process could also be explained by the fact that cells could be using glycolysis instead of oxidative phosphorylation. If that was the case, since the ATP would not be formed by oxidative phosphorylation, which has a higher energetic yield, the ΔΨm would not dissipate and the ATP gain could not equal that of the control. We also tried to study the oxygen consumption, but we were not able to detect any signal, as the cells did not react very well to being detached and this assay has to be performed in suspension cells. In summary, the $NO^{\bullet}$ caused no effect on mitochondrial function, while the 8-Br-cGMP induced alterations. This difference may be due to the fact that, with 8-Br-cGMP, cGMP is directly added to the cultures during all the time of exposure, whereas with the $NO^{\bullet}$ the cGMP is accumulated (Arnold *et al.* 1977).

Finally, we investigated whether there would be changes in the mitochondrial mass during proliferation. Our results showed no alteration in the number of mitochondria, after treatment with either $NO^{\bullet}$ or 8-Br-cGMP. Mitochondrial biogenesis can occur without an increase in the number of mitochondria. However, we also did not observe an enhancement of mitochondrial function, which suggests that the energetic support for NSC proliferation induced by $NO^{\bullet}$ is not given by mitochondria.

Chapter 5 Conclusion

5. Conclusion

In order to investigate whether there would be mitochondrial biogenesis during the proliferation of NSC induced by $NO^{\bullet}$, we first studied the role of $NO^{\bullet}$ in the proliferation of NSC. Our findings suggest that $NO^{\bullet}$ stimulates the proliferation of NSC by two mechanisms. The MAPK pathway is involved in both the early and the late proliferative effects of $NO^{\bullet}$; while the cGMP is involved only in the late proliferation induced by $NO^{\bullet}$, but is dependent on the MAPK pathway.

Secondly, we analyzed a set of parameters to evaluate the mitochondrial biogenesis. We found that SIRT1 is not involved in the proliferation caused by $NO^{\bullet}$, and also that the mitochondrial number and function are not changed.

These results suggest that there is no increase in mitochondrial biogenesis during the stimulation of the proliferation of NSC by $NO^{\bullet}$. Therefore, it still remains to understand the energetic platform that supports the proliferative effect of $NO^{\bullet}$ in NSC.

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