DEPARTAMENTO DE CIÊNCIAS DA VIDA

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE DE COIMBRA

Stimulation of Neural Stem Cell Proliferation by Inhibition of Phosphodiesterase 5

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)

Ana Isabel Reis dos Santos

2011

ESTE TRABALHO FOI REALIZADO NO CENTRO DE NEUROCIÊNCIAS E BIOLOGIA CELULAR – CNC, UNIVERSIDADE DE COIMBRA

Agradecimentos

No final de mais uma etapa, é com o sentimento de missão cumprida e com todo o coração, que me sinto no dever de agradecer a todos os que contribuíram e me apoiaram incondicionalmente ao longo de todo este percurso.

Em primeiro lugar, gostaria de agradecer à Doutora Inês Araújo e à Professora Doutora Caetana Carvalho por me terem acolhido no seu grupo. Obrigada por me orientarem exemplarmente, por me fazerem encarar cada adversidade como um desafio, por todo o apoio e paciência disponibilizados e por toda a confiança depositada em mim. Agradeço ainda pelo tempo disponibilizado para a revisão desta tese. Guardo em mim os vossos ensinamentos e conselhos.

Seguidamente, mas não menos importantes, estão o Bruno e a Inês, a quem devo todos os ensinamentos laboratoriais, os conselhos e os bons momentos proporcionados. Agradeço toda a paciência e o facto de estarem sempre dispostos a dispensar parte do seu tempo para as minhas dúvidas, por me fazerem levantar a cabeça e olhar em frente nos momentos menos bons e por nunca me deixarem desmotivar. Obrigada por terem sido incansáveis e por toda a vossa boa disposição e entusiasmo.

Devo igualmente muito à Sofia e à Vanessa. Creio que o termo “colegas” é insuficiente para explicar o que foram para mim durante este ano. Apesar de todas as nossas diferenças, a verdade é que formamos uma excelente equipa. Tenho a agradecer-lhes por toda a ajuda laboratorial naqueles dias “loucos” e por toda a solidariedade e compreensão sempre disponibilizadas. Obrigada ainda por todos os momentos de galhofa, por encararem sempre tudo com boa disposição, mesmo aos fins-de-semana, feriados e, sobretudo, às míticas quintas-feiras.

Agradeço aos restantes membros do grupo de neurogénese e neuroendocrinologia por estarem sempre prontos a ajudar no que fosse necessário e, principalmente, à Professora Doutora Cláudia Cavadas por ser uma verdadeira líder do nosso laboratório. Agradeço ainda a todos os investigadores, técnicos e funcionários do CNC que, de alguma forma, contribuíram para um melhor desempenho do meu trabalho. Devo também um agradecimento ao Luís Ribeiro pela ajuda prestada e pelas conversas sempre bem-humoradas.

Agradeço a todos os meus amigos por preencherem a minha vida de bons momentos e por me ajudarem a ultrapassar os menos bons. Aos que estão perto e aos que, por circunstâncias do destino, estão longe, agradeço toda a vossa energia, preocupação e apoio. Agradeço em especial à Rita, por estar sempre perto, apesar de fisicamente distante, por ser uma fonte incansável de motivação em todas as situações. Obrigada a todos por me fazerem rir, pelas conversas, mesmo que sem fundamento, e por serem aquele “ombro amigo” sempre que precisei. Obrigada por serem simplesmente fantásticos!

Finalizo com um agradecimento especial aos meus pais e irmão, por sempre me apoiarem em todos os aspectos ao longo da minha vida, sem questionarem as minhas decisões. Agradeço toda a compreensão, principalmente nos meus momentos de mau humor. Obrigada por serem os melhores!

Table of contents

Abbreviations

7-AAD (7-actinomycin D)

AMPA (α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate)

ANOVA (Analysis of variance)

BBB (Blood-brain barrier)

BCA (Bicinchoninic acid)

BSA (Bovine serum albumin)

cAMP (Cyclic adenosine monophosphate)

CAPS (N-cyclohexyl-3-aminopropanesulfonic acid)

CLAP (chymostatin, leupeptin, antiparin, pepstatin A)

cGMP (Cyclic guanosine monophosphate)

CNS (Central nervous system)

CSF (Cerebrospinal fluid)

DG (Dentate gyrus)

D-MEM/F-12 (Dulbecco's Modified Eagle's Medium: F-12 nutrient mixture)

DNA (Deoxyribonucleic acid)

EDTA (Ethylenediaminetetraacetic acid)

EdU (Ethynyl-2'-deoxyuridine)

EGF (Epidermal growth factor)

EGFR (Epidermal growth factor receptor)

ERK (Extracellular signal-regulated kinase)

FGF (Fibroblast growth factor)

eNOS (Endothelial nitric oxide synthase)

GFAP (Glial fibrillary acidic protein)

GMP (Guanosine monophosphate)

GTP (Guanosine triphosphate)

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

HBSS (Hank's balanced salt solution)

IC (Immunocytochemistry)

IC_{50} (50 % inhibitory concentration)

iNOS (Inducible nitric oxide synthase)

MAPK (Mitogen-activated protein kinase)

NMDA (N-methyl-D-aspartic acid)

nNOS (Neuronal nitric oxide synthase)

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

NOS (Nitric oxide synthase)

NSC (Neural stem cells)

OB (Olfactory bulb)

PBS (Phosphate-buffered saline)

PDE (Phosphodiesterases)

PI3-K (Phosphatidylinositol 3-kinase)

PKG (Protein kinase G)

PMSF (Phenylmethylsulfonyl fluoride)

RMS (Rostral migratory stream)

SDS (Sodium dodecyl sulfate)

SEM (Standard error of the mean)

sGC (Soluble guanylyl cyclase)

SGZ (Subgranular zone)

SVZ (Subventricular zone)

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

TEMED (Tetramethylethylenediamine)

TUBEs (Tandem Ubiquitin Binding Entities)

VEGF (Vascular endothelial growth factor)

WB (Western blot)

Abstract

cGMP is a second messenger signaling molecule, whose levels are regulated by an equilibrium between its production and hydrolysis. cGMP is produced in a reaction catalyzed by soluble guanylyl cyclase (sGC) and this enzyme can be activated by factors such as nitric oxide ($NO^{\bullet}$). $NO^{\bullet}$ and cGMP are important in a wide array of biological processes, and in the last years increasing attention has been given to their involvement in the formation of new neurons – neurogenesis. Neurogenesis occurs during the entire adult life in the mammalian brain, and is affected by several agents, including $NO^{\bullet}$. Phosphodiesterases are the enzymes responsible for the degradation of cGMP. In several conditions, such as aging, cGMP levels are decreased and may be involved in age-related neurodegeneration, decreased neurogenesis and cognitive decline. The inhibition of cGMP hydrolysis could be a strategy to increasing the levels of cGMP and, consequently, reverse these effects. Phosphodiesterase type 5 (PDE5) is specific for cGMP degradation and is present in the brain. Interestingly, there are a few studies reporting the enhancement of neurogenesis by the use of PDE5 inhibitors. Within this background, we tested the effect of three inhibitors with different selectivity and potency for PDE5, T0156, sildenafil and zaprinast, in the proliferation of subventricular zone cells. With this work we show that PDE5 inhibitors increase cell proliferation, an effect that appears to involve the activation of the sGC and MAPK pathways. However, these do not seem to be the only pathways activated and further studies are needed in order to clarify the mechanisms involved. In agreement with the present results, PDE5 inhibitors may be an interesting therapeutic approach for enhancing adult neurogenesis.

Keywords: Adult neurogenesis, cGMP, phosphodiesterases, PDE5 inhibitors.

Resumo

O cGMP é uma molécula sinalizadora que actua como segundo mensageiro e os seus níveis são regulados por um equilíbrio entre a sua produção e a sua hidrólise. O cGMP é sintetizado numa reacção catalisada pela guanilil ciclase solúvel (sGC), cuja activação depende de alguns factores, em particular o óxido nítrico ($NO^{\bullet}$). O $NO^{\bullet}$ e o cGMP são importantes numa grande variedade de processos biológicos e, nos últimos anos, tem sido dada atenção ao seu envolvimento na formação de novos neurónios – neurogénese. A neurogénese ocorre ao longo da vida adulta no cérebro dos mamíferos e é afectada por vários factores, incluindo o $NO^{\bullet}$. As fosfodiesterases são enzimas responsáveis pela degradação do cGMP. Em algumas condições, como o envelhecimento, os níveis de cGMP estão diminuídos, podendo estar envolvidos na neurodegeneração relacionada com a idade, na diminuição da neurogénese e no declínio cognitivo. A inibição da hidrólise de cGMP poderá ser uma estratégia para aumentar os níveis de cGMP e, consequentemente, reverter estes efeitos. A fosfodiesterase tipo 5 (PDE5) é específica para a degradação de cGMP e está presente no cérebro. Curiosamente, existem alguns estudos sobre o aumento da neurogénese pelo uso de inibidores da PDE5. Nesta perspectiva, testámos o efeito de três inibidores com diferentes selectividades e potências para a PDE5, o T0156, o sildenafil e o zaprinast, na proliferação de células da zona subventricular. Com este trabalho mostrámos que os inibidores da PDE5 aumentam a proliferação celular, um efeito que parece estar envolvido na activação das vias das MAPK e da sGC. Contudo, estas não parecem ser as únicas vias activadas, sendo necessários mais estudos de modo a esclarecer os possíveis mecanismos envolvidos. De acordo com os resultados obtidos, os inibidores da PDE5 podem tornar-se numa estratégia terapêutica interessante para estimular a neurogénese adulta.

Palavras-chave: Neurogénese, cGMP, fosfodiesterases, inibidores da PDE5.

Chapter 1 Introduction

1.1. Neurodegeneration

Neurodegeneration is characterized by progressive neuronal death and loss of synaptic function in vulnerable areas of the central nervous system (CNS). Neurodegeneration can be a result of acute neuronal lesions or due to idiopathic or genetic slow-progressing disorders in the CNS.

1.1.1. Acute brain injury

Acute neuronal lesions, such as stroke, spinal cord injury, brain trauma and seizures, have a strong participation on excitotoxicity of the neuronal damage, which may contribute to cause inflammation and activate local signals that induce scar formation by reactive gliosis.

In pathological conditions, the excitatory neurotransmitter glutamate is excessively released into the lesioned areas, which causes an overactivation of the ionotropic glutamate receptors, AMPA and NMDA. When overactivated, glutamate receptors trigger calcium and sodium influx into the cells. Excitotoxicity causes cell death by necrosis or apoptosis. The latest research in excitotoxicity mechanisms was recently reviewed by Dong *et al.* 2009 and Lau *et al.* 2010.

Neuroinflammation is a response in which the brain attempts to defend against insults, such as injuries, diseases or infections, in an effort to return the affected area to its normal state. In physiological conditions, the brain is protected by the blood-brain barrier (BBB), which provides a high selectivity, only allowing the passage of certain specific molecules to and from the brain. However, after a brain insult, the inflammatory process leads to astrocyte and resident microglia activation and cell migration (peripheral macrophages and lymphocytes) from the hematopoietic system to the injured site. The inflammatory response causes the release of several regulatory molecules, such as anti- and pro-inflammatory cytokines, chemokines,

neurotransmitters and reactive oxygen and nitrogen species (e.g. nitric oxide, NO•), which contribute to the disruption of the BBB and to the recruitment of monocytes and lymphocytes to cross the BBB to inflammation site (extensively reviewed in Taupin 2008 and Lossinsky *et al.* 2004). Scar formation is thought to inhibit or compete with neuronal differentiation of endogenous stem cells, which means that glial cells are crucial to maintain a suitable environment for neuron maturation, survival and function, being imperative the complete restitution of cellular environment (reviewed in Lie *et al.* 2004).

1.1.2. Slow-progressive neurodegenerative disorders

Most neurodegenerative disorders are known as “protein misfolding diseases” or “proteinopathies”, due to the involvement of protein conformational changes that result in intra- and/or extracellular accumulation of misfolded proteins that self-aggregate and form high-ordered insoluble fibrils. Protein aggregates, together with gliosis, are hallmarks of many neurodegenerative diseases (Jellinger 2009, Woulfe 2007, Herczenik *et al.* 2008). The protein aggregation process occurs slowly over time, contributing to a gradual appearance of symptoms. Neurodegenerative diseases such as Alzheimer’s, Huntington’s, Parkinson’s diseases or amyotrophic lateral sclerosis are classified according to their protein deposits. They all have in common the feature that when enough protein is accumulated, a cascade of symptoms occurs and can last various years (from 2 to 20), with increasing cognitive and/or motor disability and ultimately resulting in death. There are many factors that contribute to induce neurodegenerative diseases, i.e., genetic, environmental and endogenous factors, such as factors related to aging. However, their pathogenic role and the molecular mechanisms behind most neurodegenerative diseases are not yet completely understood (Jellinger 2009, Forman *et al.* 2004, Jellinger 2003, Selkoe 2004, Skovronsky *et al.* 2006), despite the major advances in this area over the last few

decades. The main pathological mechanisms underlying these diseases are abnormal protein dynamics, oxidative stress and formation of free radicals, impaired bioenergetics, mitochondrial dysfunctions and DNA damage, fragmentation of neuronal Golgi apparatus, disruption of cellular/axonal transport, calcium entry, excitotoxicity, among others (Jellinger 2009, Bredesen *et al.* 2006). Although the pathways behind these mechanisms are not entirely known, they seem to trigger vicious cycles of aberrant neuronal activity and compensatory alterations in neurotransmitter receptors and related signaling pathways that lead to synaptic deficits, disintegration of neural networks and, ultimately, failure of neurological functions.

The development of some neurodegenerative disorders is directly related to aging. One common feature of aging is the decrease in the levels of second messengers, namely $NO^{\bullet}$ and cyclic guanosine monophosphate (cGMP) (Chalimoniuk *et al.* 1998). This effect is responsible for the loss of cognitive functions and synaptic plasticity, which occur in this type of pathologies, such as Alzheimer's disease (Chalimoniuk *et al.* 1998, Sabayan *et al.* 2010). Therefore, alterations in some signaling pathways are closely related to the emergence of these diseases.

Understanding the mechanisms underlying the evolution of these diseases, the factors that lead to their initiation and the biology of neuronal injury is of extreme importance, providing a therapeutic rationale to treat these diseases, as well as allowing to act on risk groups in order to prevent their occurrence. However, the major limitation that researchers face is the restricted regeneration ability of the CNS. Within this scenario, effective treatments must be able to limit the progression of degeneration in patients after the injury is detected (Yanamadala *et al.* 2010), and new therapies to promote regeneration or graft survival are needed.

1.2. Neurogenesis in the adult brain

Neurogenesis is the biological process of generating new neurons from progenitor cells or neural stem cells (NSC). Since the early 1900s, it was believed that the CNS was not able to form new brain cells, unlike what happens during embryonic life, during which neurogenesis occurs to form the nervous system. This paradigm was challenged approximately 40 years ago, by the pioneer work of Altman and Das suggesting, for the first time, that the adult brain has the ability of creating new neurons – neurogenesis (Lewis 1968, Privat *et al.* 1972, Altman 1969, Altman *et al.* 1965). In fact, all mammals, including nonhuman primates and humans, have the distinctive feature of creating new neurons during adulthood that can be integrated into its complex circuit (Eriksson *et al.* 1998, Curtis *et al.* 2007).

NSC are a type of precursor cells derived from the nervous system that can originate the several cell types of neural tissue. NSC exhibit two main features: the ability to self-renew, producing progeny similar to themselves, and they are multipotent cells for the different neuroectodermal lineages of the CNS (Emsley *et al.* 2005). For this last reason, NSC give rise to the main cell types of the mammalian CNS: neurons and glial cells, which include astrocytes and oligodendrocytes.

In the CNS, the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of dentate gyrus (DG) of the hippocampus are the two main regions where NSC are found to proliferate lifelong (Fig. **1**). Anatomically, the SVZ is a thin layer extended along the length of the lateral walls of the lateral ventricles, separated from the cerebrospinal fluid (CSF) by a layer of ciliated ependymal cells. The SGZ, in turn, is located between the hilus and the granule cell layer of the DG. Nevertheless, NSC were found in many other regions of the CNS, like the neocortex (Magavi *et al.* 2000), spinal cord (Yamamoto *et al.* 2001a, Yamamoto *et al.* 2001b, Chen *et al.* 2004), tegmentum (Hermann *et al.* 2006), substantia nigra (Zhao *et al.* 2003), amygdala (Bernier *et al.* 2002) and brainstem (St-John 1998), but some of these

findings are not consensual among the scientific community and the number of cells found is very small, in comparison to the SVZ and SGZ. Although neurogenesis in these regions does not occur in the adult brain, neurogenesis in non-neurogenic regions could be induced by stimulating local NSC or by recruiting NSC from neurogenic areas to other regions (Whitney *et al.* 2009).

Studies in the SVZ and in the SGZ of rodents showed that new neurons formed from NSC are physiologically mature by their ability to fire action potentials and receive synaptic inputs (Carlén *et al.* 2002). The integration of functional neurons in the neural networks is constituted by several sequential steps, equivalent to those occurring in developmental neurogenesis as shown in Fig. **1**. The main neurogenesis steps are, firstly, the proliferation and fate determination, in which stem cells in the SGZ and SVZ give rise to transit amplifying cells that differentiate into immature neurons. Secondly, immature neurons of the SGZ and the SVZ migrate into the granule cell layer of the dentate gyrus and through the rostral migratory stream (RMS), respectively. Thirdly, differentiation occurs, along with growth of axon and dendrites, formation of synapses with other neurons in the circuits and, finally, integration of the newborn neuron in the neural network, ending with the maturation into a fully functional neuron.

1.2.1. Neurogenesis following brain injury

Adult neurogenesis is modulated by pathological (Kee *et al.* 2001, Parent *et al.* 1997, Parent *et al.* 2002) and physiological stimuli (Gould *et al.* 1999). Several types of brain injury appear to stimulate neurogenesis in the adult brain, mainly in the SVZ and SGZ. Some evidence suggests that newborn neurons are able to replace lost neurons and, thus, contribute to brain function recovery (Nakatomi *et al.* 2002, Blaiss *et al.* 2011, Im *et al.* 2010). Several types of brain injuries, such as ischemic brain injury, traumatic brain injury and epileptic seizures, are able to induce NSC proliferation and to stimulate migration of newborn cells to the lesion sites.

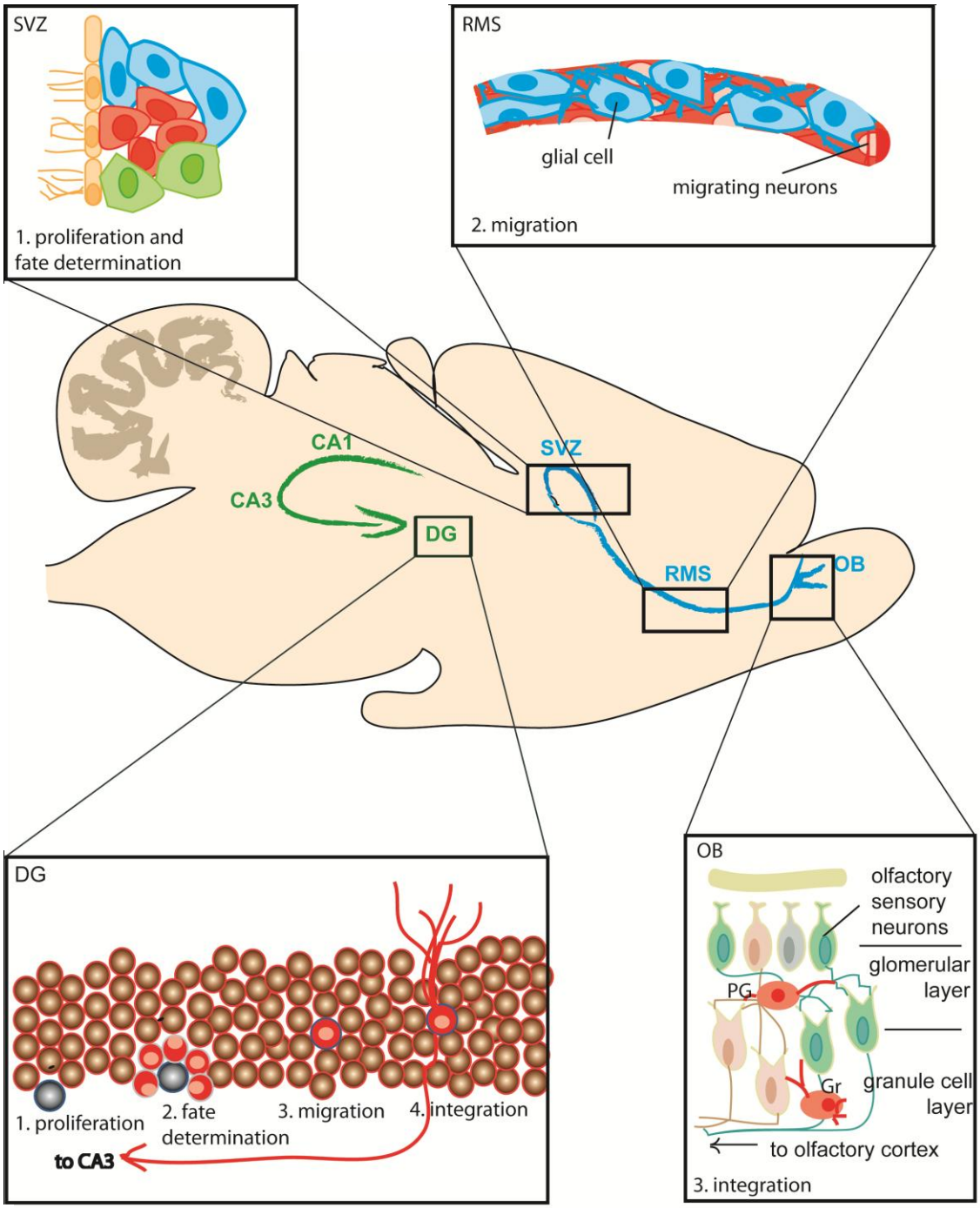

Figure 1. Schematic sagittal view of the adult rodent brain showing the two main regions of persistent neurogenesis: the SGZ of the hippocampal DG and the SVZ of the lateral ventricles. In the DG: 1. Proliferation: neural stem cells (*gray*) in the SGZ molecular layer; 2. Fate determination: NSC give origin to transit amplifying cells (*not shown*) that become immature neurons (*red*); 3. Migration: immature neurons migrate

into the granule cell layer; 4. Integration: immature neurons differentiate in mature neurons and integrate the neural network, becoming totally functional neuronal cells, receiving inputs from the entorhinal cortex and extending axonal projections to the CA3 region. In the olfactory bulb (OB) system: 1. Proliferation and fate determination: neural stem cells give rise to transit amplifying cells (*green*) in the SVZ of the lateral ventricle, differentiating into immature neurons (*red*); 2. Migration: immature neurons (*red*) ensheathed by astrocytes (*blue*) migrate along the RMS; and, 3. Integration: in the OB, immature neurons differentiate in local interneurons (*red*) in the granule layer (granule neurons, Gr) and in the periglomerular layer (periglomerular neurons, PG). (Adapted from Lie *et al.* 2004).

1.2.2. Regulation of neurogenesis

Adult neurogenesis seems to be modulated by various factors. Factors such as aging (Kuhn *et al.* 1996, Enwere *et al.* 2004, Jin *et al.* 2003) and stress (Duman *et al.* 2001) appear to decrease neurogenesis, mainly by activation of the hypothalamic-pituitary-adrenal axis that increases corticosteroids levels (Cameron *et al.* 1994). On the other hand, factors such as environmental enrichment (Brown *et al.* 2003, Kempermann *et al.* 1997, Nilsson *et al.* 1999), physical exercise (van Praag *et al.* 1999a, van Praag *et al.* 1999b) and dietary restrictions seem to stimulate neurogenesis in the adult DG and SVZ (reviewed in Ming *et al.* 2005, Parent 2003).

Growth factors, neurotransmitters and hormones appear to influence adult NSC proliferation. $NO^{\bullet}$ is a neuromodulator that can either stimulate (Carreira *et al.* 2010) or decrease (Matarredona *et al.* 2004, Matarredona *et al.* 2005, Moreno-Lopez *et al.* 2004, Torroglosa *et al.* 2007) the proliferation of NSC in both the SVZ and the SGZ. In the brain, $NO^{\bullet}$ modulates neurotransmitter release (Kahn *et al.* 1995), contributes to synaptic formation and remodeling (Cserep *et al.* 2011, Tegenge *et al.* 2009) and participates in the control of cerebral blood flow (Zhang *et al.* 1994). Moreover, Reif *et*

al. showed that vascular endothelial growth factor (VEGF) could mediate the positive effect of NO• in adult neurogenesis (Reif *et al.* 2004). The effect of VEGF is probably via activation of the kinase Akt and the subsequent downstream effectors (Conover *et al.* 2000). In addition, NO• stimulates the secretion of VEGF (Zhang *et al.* 2003) and, thus, further modulates VEGF induction of neurogenesis. This positive reciprocal modulation is an important neurogenic control.

1.3. Nitric oxide and cGMP signaling in adult neurogenesis

1.3.1. Nitric oxide

NO• is a gaseous free radical that acts as an important second messenger, having a crucial role in intercellular communication and in intracellular signaling in many tissues (Kerwin *et al.* 1995, Murad 1994a, Moncada *et al.* 1989), including the brain (Garthwaite *et al.* 1988). NO• can be produced by three nitric oxide synthase (NOS) genetically different isoforms: the neuronal NOS (nNOS) is constitutively expressed in neuronal tissues, constituting the predominant source of NO• in neurons, the endothelial isoform (eNOS) is constitutively expressed in endothelial cells from all vessel types and, the inducible NOS (iNOS) is expressed in microglia and cells from the immune system, leading to a production of large amounts of NO• that may be cytotoxic.

NO• mediates proliferative signaling in NSC through two main pathways: the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) 1/2 pathway and the soluble guanylyl cyclase (sGC) pathway (Fig. **2**). The activation of these pathways seems to be biphasic, depending on the time of exposure to NO• (Carreira *et al.* 2011).

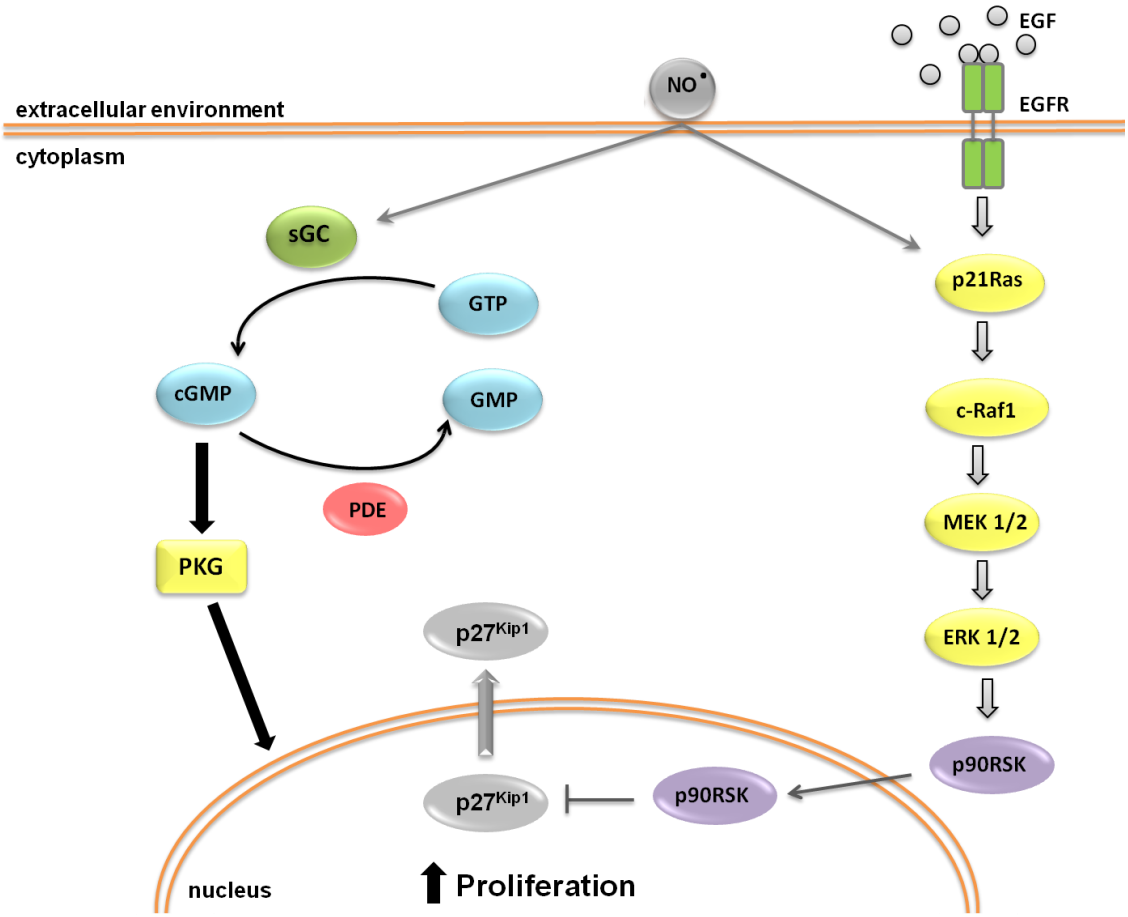

Figure 2. NO• enhances SVZ cell proliferation by two main pathways: the MAPK and the sGC pathways. On the one hand, the activation of the elements of the ERK 1/2 pathway, bypassing the epidermal growth factor receptor (EGFR) activation, stimulates cell proliferation by the inhibition of $p27^{Kip1}$, which is translocated to the cytosol and degraded. On the other hand, NO• activates sGC, thus, increasing the levels of the second messenger cGMP. cGMP activates protein kinase G (PKG), which signal to the nucleus and increase cell proliferation. cGMP is maintained at basal levels by the action of phosphodiesterases (PDE), responsible for the formation of guanosine monophosphate (GMP) from cGMP.

On the one hand, NO• signals via the MAPK pathway, triggering cell proliferation downstream of the EGFR (Carreira *et al.* 2010). Studies from our group suggest that NO• induces cell proliferation by activation of p21Ras, by S-nitrosylation, a

post-translational modification on cysteine residues. Moreover, exposure to a $NO^{\bullet}$ donor, NOC-18, showed to have a direct effect on p21Ras and also phosphorylated other elements of this pathway, namely p90RSK, which is able to phosphorylate its substrate $p27^{Kip1}$ (Carreira *et al.* 2010). $p27^{Kip1}$, an element that prevents cell cycle progression, is then translocated to the cytosol, where it may be tagged with ubiquitin for subsequent degradation in the proteosome. On the other hand, $NO^{\bullet}$ binds to the heme center of the sGC, changing the conformation of the catalytic domain and thus, activating the production of the second messenger cGMP and subsequently, activation of protein kinase G (PKG) (Ignarro 1991, Murad 1994b). It is via this mechanism that $NO^{\bullet}$ is responsible, for example, for blood vessel relaxation, inducing vasodilatation, and for the increase in cell proliferation, stimulating adult neurogenesis.

1.3.2. cGMP synthesis

There are two types of guanylyl cyclases that are classically distinguished by their subcellular location. Soluble guanylyl cyclase is mainly located in the cytosol and is activated by $NO^{\bullet}$ and membrane guanylyl cyclases are integral proteins of the cell membrane that are activated by specific peptides, such as the atrial natriuretic peptide. sGC is an heterodimer composed of two different subunits: α and β. For each subunit, there are known three different isoforms. The most found combinations are the heterodimer α1β1, present in all the tissues, and the heterodimer α2β1, present in large amounts in the brain and in some fetal tissues.

Figure 3. cGMP production by sGC and degradation by PDE.

sGC is activated by $NO^{\bullet}$ and acts on guanosine triphosphate (GTP), converting it into the second messenger, cGMP, as illustrated in Fig. **3**. In physiological conditions, intracellular cGMP is maintained at basal levels by phosphodiesterases. Cyclic nucleotide phosphodiesterases (PDE) are enzymes that hydrolyze the 3'-phosphodiester bound of cyclic adenosine monophosphate (cAMP) or cGMP, originating their respective monophosphates, 5'-AMP or 5'-GMP, respectively.

1.3.3. Phosphodiesterases

There are 11 known PDE families. Each family encompasses 1 to 4 distinct genes and each gene encodes multiple protein products, existing more than 50 different PDE proteins in mammalian cells. PDE activity is found in every cell in the body, presenting different substrate specificity, kinetic properties and cellular and subcellular distribution of the 11 isoenzymes (Table **I**). As different PDE families present such a wide distribution among the tissues, including the brain, inhibition of one or more PDE is an option to treat several diseases, by controlling the levels of the respective second messengers. cAMP and cGMP levels can be altered in several pathologies, such as cancer, inflammation, neurodegeneration and oxidative stress. As shown in Table **I**, the PDE isoenzymes display different affinities for either cAMP or cGMP, being that some are selective for cGMP or for cAMP and others are able to hydrolyze both substrates with equal or different specificity. Among these various types, only two selectively hydrolyze cGMP. Here, we focused our studies on PDE5, because of its involvement in the regulation of cGMP signaling in the brain.

Table I. Human cyclic nucleotide PDE superfamily (adapted from Boswell-Smith *et al.* 2006, Essayan 2001 and Bischoff 2004).

PDE family	Substrate specificity	Main tissue distribution
1	cGMP>cAMP Ca^{2+}/calmodulin-stimulated	Heart, brain, lung, smooth muscle
2	cGMP=cAMP	Adrenal gland, heart, lung, liver, platelets
3	cAMP>cGMP	Heart, liver, lung, platelets, adipose tissue, inflammatory cells
4	cAMP	Sertolli cells, kidney, brain, liver, lung, inflammatory cells
5	cGMP	Lung, brain, vascular smooth muscle, platelets
6	cGMP>cAMP	Photoreceptors
7	cAMP>>cGMP (cAMP high affinity)	Skeletal muscle, T lymphocytes, heart, kidney, brain, pancreas
8	cAMP	Testis, eye, liver, skeletal muscle, heart, kidney, ovary, brain, T lymphocytes
9	cGMP	Kidney, liver, lung, brain
10	cGMP<cAMP	Brain, testis
11	cGMP=cAMP	Skeletal muscle, prostate, liver, kidney, pituitary and salivary glands, testis

1.3.3.1. cGMP-specific phosphodiesterases

Phosphodiesterases type 5 are enzymes that specifically hydrolyze cGMP. So far, only one gene of PDE5 was discovered, PDE5A, and three variants of this gene have been identified: PDE5A1 and PDE5A2 that are widely expressed, and PDE5A3, which is specific to vascular smooth muscle (Loughney *et al.* 1998, Lin *et al.* 2000, Kotera *et al.* 1999). PDE5A is considered a cytosolic enzyme and its protein activity was found in the lung, vascular and tracheal smooth muscle, spleen, platelets, corpus cavernosum (Lincoln *et al.* 1976, Coquil *et al.* 1985, Wallis *et al.* 1999, Wang *et al.* 2005, Bender *et al.* 2004) and in several brain regions, being particularly abundant in Purkinje cells (Shimizu-Albergine *et al.* 2003, Bender *et al.* 2004). Therefore, cGMP-related physiological functions can be regulated by controlling the levels of PDE5 in these tissues.

Moreover, there are other enzymes with high specificity for the cGMP present in the brain. For example, PDE9 is an enzyme that specifically hydrolyzes cGMP. Until now there is only one isoform identified, PDE9A. It is one of the more recently discovered PDE families and very little is known about this enzyme. However, it was suggested that PDE9A might be a regulator of cGMP in the brain (van Staveren *et al.* 2002) but more studies are required to understand PDE9 functions in its targets.

PDE10 was also recently discovered and to date, only one gene is known, PDE10A. PDE10 has higher specificity to cAMP rather than cGMP. This PDE is highly expressed in the brain, mainly in the striatum, but also in the cerebellum, thalamus, hippocampus and spinal cord. Furthermore, inhibitors of PDE10 have been developed for the treatment of schizophrenia.

PDE1 was the first family identified (Cheung 1970) and is a calcium- and calmodulin-dependent PDE. There are three isoforms of this PDE and its substrate specificity is different for each isoform, being mainly specific to cGMP. All the isoforms are present in several brain regions. Therefore, there are few definitive studies on the functional roles of the various PDE1 isoenzymes.

Although not expressed in the brain, PDE6, is structurally highly homologous to PDE5 but, contrarily to PDE5, is able to hydrolyze cGMP and cAMP, with higher specificity to cGMP (Lugnier 2006). There are three genes in the family of this PDE. PDE6 is highly expressed in the photoreceptors of the mammalian retina, where it mediates the conversion of a light signal into a photoresponse (Bender *et al.* 2006).

Taking into account the existing background on the therapeutic effects of the specific cGMP-hydrolyzing PDE5, namely at the brain level, we decided to focus our studies on this PDE.

Many PDE5 inhibitors have been developed, being very useful in the study of PDE5 distribution among tissues and, thus, essential in the treatment of several pathologies in which the levels of cGMP are altered. The most characterized inhibitor of

PDE5 is sildenafil. PDE5A is, mostly, a regulator of vascular smooth muscle contraction through regulation of cGMP in two main tissues: the penis and the lung. PDE5 inhibition enhances relaxation of the cavernal smooth muscle by nitric oxide and cGMP, stimulating penile erection (Rosen *et al.* 2003, Corbin 2004). Therefore, PDE5 inhibitors started to be used for the treatment of erectile dysfunction. In the lung, PDE5 inhibitors act as vasodilators, increasing the blood supply, antagonizing the vasoconstriction of smooth muscle and decreasing pulmonary arterial resistance. Therefore, PDE5 inhibitors have been used to treat pulmonary hypertension (extensively reviewed in Lewis *et al.* 2004, Patel *et al.* 2005 and Steiner *et al.* 2005). Similarly to pulmonary hypertension, some studies suggest that PDE5 inhibition by sildenafil prevents the pressure-induced cardiac hypertrophy, even though the levels of PDE5 in cardiomyocytes are rather low (Takimoto *et al.* 2005). In the CNS, recent reports show that sildenafil has a neuroprotective role, being effective in improving the symptoms in a model of multiple sclerosis, suggesting that PDE5 can be a target for the therapy against this disease (Pifarre *et al.* 2011). The various pharmacological effects of sildenafil are reviewed in Uthayathas *et al.* 2007.

By its presence in the brain, PDE5 was reported to have a role in learning and memory. For example, in Alzheimer's disease, the progressive neurodegeneration results in a cognitive dysfunction, with memory loss and motoneural impairment. The administration of PDE5 inhibitors can be a possible therapy for this disease, due to their ability to reverse deficits in long-term memory caused by pharmacological agents or aging. It has also been described that the administration of sildenafil enhances memory and restores learning ability in animal models (Baratti *et al.* 1999, Devan *et al.* 2006, Erceg *et al.* 2005, Prickaerts *et al.* 2005, Prickaerts *et al.* 2004, Prickaerts *et al.* 2002b, Prickaerts *et al.* 2002a, Rutten *et al.* 2005, Singh *et al.* 2003, Devan *et al.* 2004). Beyond this important role in memory and cognition, PDE5 inhibitors also

appear to stimulate neuronal plasticity and to be neuroprotective, through the enhancement of endogenous neurogenesis in the adult brain.

1.3.3.2. Inhibition of PDE5 as a therapeutic strategy

PDE5 inhibitors can be classified by their potency and selectivity in relation to other PDE. The potency is indicated by the value of 50 % inhibitory concentration (IC_{50}) and represents the drug concentration required to reduce the activity of the tested PDE by 50% (Table **II**). The lower the IC_{50}, the more potent the inhibitor is.

Table II. IC_{50} of some PDE inhibitors (Tocris Bioscience).

PDE	T0156 (nM)	Sildenafil (nM)	Zaprinast (nM)
1-4	>63,000	>270	-
5	0.23	3.6	760
6	26.0	29.0	150
9	-	-	12,000
11	-	-	29,000

Selectivity is a parameter that determines the clinical relevance and the therapeutic efficacy of a drug. In this case, selectivity is defined as the ratio between the IC_{50} for a comparative PDE (e.g. PDE6) and the IC_{50} for PDE5 (Table **III**). The greater the ratio, the more selective the inhibitor is for PDE5 compared to other PDE (reviewed in Bischoff 2004).

Table III. Selectivity for PDE5 (ratio PDEx/PDE5).

PDE	T0156	Sildenafil	Zaprinast
1-4	>270,000	>75	-
5	1	1	1
6	113.0	8.1	0.2
9	-	-	15.8
11	-	-	38.2

Zaprinast (Fig. **4**) was the first PDE inhibitor to be used in the studies of PDE5 functions. However, zaprinast proved to be a nonpotent and nonselective inhibitor. Its inhibitory potency is higher for PDE6 than for PDE5, and it also inhibits other PDE such as PDE9 and PDE11. Some years later, a more specific PDE5 inhibitor was developed, sildenafil, commercially known as Viagra. Sildenafil (Fig. **4**) was the first oral drug to be approved by the United States Food and Drug Administration, in 1998, for the treatment of erectile dysfunction. Sildenafil showed to inhibit PDE5 with concentrations in the nanomolar range, about 200-fold lower than the concentration of zaprinast required to inhibit PDE5. Sildenafil is typically considered a PDE5 inhibitor, with an IC_{50} of approximately 4 nM (Ballard *et al.* 1998). However, it also inhibits PDE1 and PDE6 (IC_{50} values of 281 and 29 nM, respectively, (Ballard *et al.* 1998), Table **II**), indicating that sildenafil is not the ideal compound for the selective inhibition of PDE5. Similarly to sildenafil, other two inhibitors were developed for the treatment of erectile dysfunction: tadalafil (Cialis) and vardenafil (Levitra). These inhibitors have higher selectivity for PDE5 than sildenafil, and tadalafil has a much longer period of responsiveness than sildenafil and vardenafil.

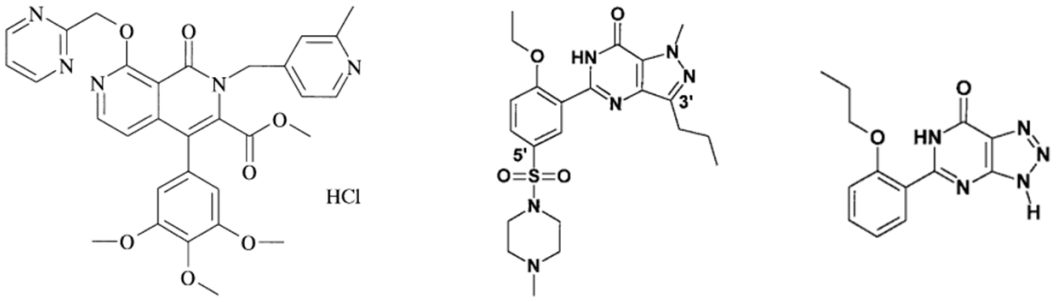

Figure 4. Chemical structures of T0156, sildenafil and zaprinast. Adapted from Mochida *et al.* 2002 and Turko *et al.* 1999.

More recently, a new compound was developed, T0156 (Fig. **4**), which potently inhibits PDE5 (Mochida *et al.* 2002). In fact, T0156 has a much lower IC_{50} for PDE5 (0.23 nM), thus, inhibiting it with higher potency than sildenafil (IC_{50} 3.6 nM) and also presenting higher selectivity for PDE5 (113.0) in comparison to PDE6, than sildenafil (8.1) as shown in Tables **II** and **III**, respectively. Therefore, the rank order of potency and, thus, of selectivity for PDE5 is T0156 > sildenafil > zaprinast.

The effects of these inhibitors on the brain are dependent on their permeability to the BBB. For instance, zaprinast is unable to cross the BBB and, thus, it needs to be added directly into the brain in order to exert its effects in this organ. On the contrary, sildenafil crosses the BBB to the brain and can be easily administered (e.g. orally). Unfortunately, there is no information about the BBB permeability of T0156 but the use of PDE5 inhibitors as an effective therapy for neurodegenerative diseases is dependent on this factor.

1.3.3.3. cGMP and neurogenesis

Neurogenesis generally declines with aging and is correlated with the emergence of neurodegenerative diseases. With the increasing age, the levels of $NO^{\bullet}$ gradually decrease, leading to a subsequent decrease in the levels of cGMP and an abolishment of cell proliferation and impairments in learning and memory. Moreover, in aged rats, levels of cGMP are decreased by the increasing phosphodiesterase activity comparatively to the adult brain (Chalimoniuk *et al.* 1998). Therefore, targeting an enzyme specific for the hydrolysis of cGMP, such as PDE5, is a good strategy to reverse this process and, thus, enhance neurogenesis.

There are some reports about the effect of sildenafil in the stimulation of endogenous neurogenesis. Sildenafil was shown to induce neurogenesis in the SVZ and DG (Zhang *et al.* 2002) and neuronal function recovery in rats following a stroke (Zhang *et al.* 2005) and an ischemic injury either in adult young rats as in aged rats

(Zhang *et al.* 2006b). Furthermore, PDE5 inhibition by sildenafil stimulated cell proliferation in rat SVZ cultures, an effect that appears to be associated with the activation and phosphorylation of Akt in the phosphatidylinositol 3-kinase (PI3-K)/Akt pathway (Wang *et al.* 2005). Another study has demonstrated that SVZ cell proliferation was enhanced in a rat model of ischemia following the administration of tadalafil, another PDE5 inhibitor (Zhang *et al.* 2006a).

Thus, the use of inhibitors for PDE5 deserves further investigation in order to clarify their role on neurogenesis and to understand the mechanisms behind this effect.

1.4. Objectives

Within the background of the potentially neurogenic effect of sildenafil in the subventricular zone, our first aim was to study the PDE inhibitors with different selectivities for PDE5 on the proliferation of SVZ neural stem cells, the first step of neurogenesis. To address this question, we compared the proliferation of SVZ cells exposed to T0156, sildenafil or zaprinast.

The pathways that mediate this proliferative effect of PDE5 inhibitors have not been characterized. As recently described by our group, nitric oxide is known to stimulate SVZ cell proliferation and this effect is mediated by two main pathways, the MAPK and the sGC pathways (Carreira *et al.* 2010, Carreira *et al.* 2011). Within this background, our second aim was to address whether these pathways are activated during cell proliferation triggered by PDE5 inhibition.

Chapter 2 Materials and Methods

2.1. Materials

Dulbecco's Modified Eagle's Medium: F-12 nutrient mixture, (D-MEM/F-12, with GlutaMAX™ I), B27 supplement, gentamicin, trypsin-ethylenediaminetetraacetic acid (EDTA) solution and antibiotics (10,000 units/ml of penicillin, 10 mg/ml streptomycin), Hoechst 33342, Click-iT® EdU Alexa Fluor® 488 HCS Assay kit and StemPro® Accutase® cell dissociation reagent were purchased from Invitrogen (Paisley, UK). Epidermal growth factor (EGF) and fibroblast growth factor (FGF) were from PeproTech Inc. (London, UK). The Matrix used was from Stoelting Co. (Wood Dale, IL, USA). Bicinchoninic acid (BCA) Protein Assay kit was from Pierce (Rockford, IL, USA). Phenylmethylsulfonyl fluoride (PMSF), orthovanadate, chymostatin, leuptin, antiparin, pepstatin A, trypan blue, Tween-20, tetramethylethylenediamine (TEMED), dimethyl sulfoxide and KT5823 were purchased from Sigma Chemical (St Louis, MO, USA). T0156 hydrochloride, ODQ, sildenafil citrate and zaprinast were obtained from Tocris Bioscience (Bristol, UK) and NOC-18 from Alexis Biochemicals (San Diego, CA, USA). U0126 was obtained from Cell Signaling (Danvers, MA, USA) and YC-1 from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Agarose-TUBEs and PR-619 were obtained from LifeSensors (Malvern, PA, USA). DAKO fluorescent mounting medium was purchased from DAKO (Glostrup, Denmark). Bovine serum albumin (BSA) was purchased from Calbiochem (San Diego, CA, USA) and low-fat dry milk from Nestlé (Vevey, Switzerland). Polyvinylidene difluoride membranes were purchased from Millipore (Madrid, Spain). Amersham cGMP Enzymeimmunoassay Biotrak System, enhanced chemifluorescence reagent and anti-rabbit and anti-mouse alkaline phosphatase-conjugated antibodies were from GE Healthcare Life Sciences (Buckinghamshire, UK). Other reagents used in immunoblotting experiments were purchased from Bio-Rad (Hercules, CA, USA). All the antibodies used are described in Table **IV**.

Table IV. Primary and secondary antibodies used in Western blot (WB) and immunocytochemistry (IC).

Antibody	Host	Dilution	Application	Origin
Anti-Sox-2	Mouse	1:200	IC	R&D Systems (Minneapolis, MN, USA)
Anti-β-III-tubulin	Mouse	1:500	IC	Covance (Emersonville, CA, USA)
Anti-GFAP	Mouse	1:400	IC	Invitrogen (Paisley, UK)
Anti-nestin	Mouse	1:500	IC	BD biosciences (Franklin Lakes, NJ, USA)
Anti-musashi-1	Rabbit	1:250	IC	Abcam (Cambridge, UK)
Anti-guanylyl cyclase	Rabbit	1:500	WB	Abcam (Cambridge, UK)
Anti-nNOS	Mouse	1:500	WB	BD biosciences (Franklin Lakes, NJ, USA)
Anti-p27^{Kip1}	Rabbit	1:1,000	WB	Cell Signaling (Danvers, MA, USA)
Anti-phospho-ERK1/2	Rabbit	1:1,000	WB	Cell Signaling (Danvers, MA, USA)
Anti-ERK 1/2	Rabbit	1:1,000	WB	Cell Signaling (Danvers, MA, USA)
Anti-α-tubulin	Mouse	1:10,000	WB	Sigma Chemical (St Louis, MO, USA)
Anti-mouse IgG labeled with Alexa Fluor 594	Goat	1:200	IC	Invitrogen (Paisley, UK)
Anti-rabbit IgG labeled with Alexa Fluor 488	Goat	1:200	IC	Invitrogen (Paisley, UK)
Alkaline phosphatase-conjugated anti-rabbit	Mouse	1:20,000	WB	GE Healthcare Life Sciences (Buckinghamshire, UK)
Alkaline phosphatase-conjugated anti-mouse	Mouse	1:20,000	WB	GE Healthcare Life Sciences (Buckinghamshire, UK)

2.2. Methods

2.2.1. Animals

C57BL/6J mice were obtained from Charles River (Barcelona, Spain) and kept in our animal facilities with food and water *ad libitum* in a 12 hours dark:light cycle. All experiments were performed in accordance with institutional and European guidelines (86/609/EEC) for the care and use of laboratory animals.

2.2.2. Subventricular zone cell cultures

Neural stem cell cultures were obtained from the SVZ of 0-3 day C57BL/6J mice as described previously (Agasse *et al.* 2008). The brains were removed from the skull, following decapitation, and placed in a plate dish containing Hank's balanced salt solution (HBSS, 137 mM NaCl, 5.36 mM KCl, 0.44 mM KH_2PO_4, 0.34 mM $Na_2PO_4.2H_2O$, 4.16 mM $NaHCO_3$, 5 mM glucose, supplemented with 0.001 % phenol red, 1 mM sodium pyruvate, and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, HEPES, pH 7.4) supplemented with 0.24 % gentamicin. The enveloping meninges were removed and the brains were sectioned in 1 mm thickness coronal slices with a mouse brain matrix, from which the SVZ was excised. The sections were kept in 0.24 % gentamicin/HBSS and the SVZ was isolated from each section. The fragments of SVZ in 0.24 % gentamicin/HBSS were digested in 0.025 % trypsin /0.265 mM EDTA, for 15-20 minutes at 37 ºC, washed with 0.24 % gentamicin/HBSS and then mechanically dissociated by gentle dissociation with a pipette tip. The cells were resuspended in a 37 ºC D-MEM/F-12 with 2 mM GlutaMAX^{TM}-I (L-Ala-L-Gln), supplemented with 1 % B27, 1 % antibiotic (10,000 units/ml of penicillin, 10 mg/ml streptomycin), 10 ng/ml EGF and 5 ng/ml FGF, and plated on uncoated flasks with filter cap at a density of 100,000 cells/ml. Cell viability was evaluated by 0.1 % Trypan blue exclusion assay. The SVZ stem cells were grown as floating aggregates in a 95 % air/5

% CO_2 humidified atmosphere at 37 ºC, during approximately 7 days. Then, the primary neurospheres were harvested, centrifuged and mechanically dissociated as single cells. Cells were replated as above and allowed to grow as secondary neurospheres. 6-7 days later, the floating neurospheres were collected and plated for 2-3 days on poly-L-lisine-coated 16-mm diameter glass coverslips, for immunocytochemistry assays, or on 12-well plates for preparation of lysates or flow cytometry assays, in the same medium as above. Then, the medium was exchanged by a similar medium but without growth factors (EGF and FGF) and cells were kept in this medium for 24 h before the experiments.

2.2.3. Experimental treatments

SVZ cells were left 24 h without growth factors before applying the stimuli. For cell proliferation analysis, a 6-hour and 24-hour treatment was applied, except for YC-1 experiments, in which cell proliferation was only analyzed following 24 h of treatment. To analyze the phosphorylation of ERK 1/2 and the levels of $p27^{Kip1}$, SVZ neural stem cells were treated for 2 h, or as indicated in the figure legends. In both experiments, the stimuli applied were as follows: the PDE inhibitors, T0156 1 µM, sildenafil 1 µM and zaprinast 10 µM, and the sGC activator, YC-1 20 µM, were applied alone or together with the MEK 1/2 inhibitor, U0126 1 µM, the PKG inhibitor, KT5823 1 µM, and the sGC inhibitor, ODQ 50 µM. U0126, KT5823 and ODQ were applied 30 min before the treatment with the PDE5 inhibitors and YC-1.

For the experiments of pulldown of ubiquitilated $p27^{Kip1}$, cells were treated with 10 µM NOC-18 for 1 h and with T0156 (1 µM), sildenafil (1 µM) or zaprinast (10 µM) for 2 h.

For the determination of cGMP levels, cells were treated with T0156 (1 µM), sildenafil (1 µM) or zaprinast (10 µM) for 6 h.

All the experiments were performed together with the respective controls (untreated cells).

2.2.4. Immunocytochemistry

The culture medium was removed and the SVZ cells were washed with phosphate-buffered saline 0.01 M (PBS, 7.8 mM $Na_2HPO_4.2H_2O$, 2.7 mM $NaH_2PO_4.H_2O$, 154 mM NaCl, pH 7.2) and fixed with 4 % paraformaldehyde/4 % sucrose in PBS 0.01 M, for 20 min, and washed again with PBS 0.01 M. Fixed cells were permeabilized with Triton X-100 (1 % Triton in PBS 0.01 M), for 5 min, and the non-specific binding was blocked with albumin solution (3 % BSA, and 0.2 % Tween-20 in PBS 0.01 M) at room temperature, for 1 h. The incubation with primary antibodies in 3% albumin solution was performed overnight, at 4ºC. The primary antibodies and the dilutions used were as follows: mouse anti-Sox-2, 1:200; mouse anti-β-III-tubulin, 1:500; mouse anti-GFAP, 1:400; mouse anti-nestin, 1:500 and rabbit anti-musashi-1, 1:250. After rinsing with PBS 0.01 M, the cells were exposed to the appropriate secondary antibodies, anti-mouse or anti-rabbit IgGs conjugated with Alexa Fluor 488 or 594, 1:200, in 3 % albumin solution, for 90 min, at room temperature. Nuclei were stained with Hoechst 33342 (2 µg/ml) for 10 min. Cells were washed with PBS 0.01 M and mounted in uncoated glass slides with DAKO fluorescence mounting medium. Images were acquired in a laser scanning microscope LSM 510 META (Zeiss, Jena, Germany). Results of labeled cells are expressed in percentage of total of live cells of 3 independent experiments.

2.2.5. Analysis of cell proliferation by flow cytometry

SVZ neural stem cells proliferation was assessed by incorporation of ethynyl-2'-deoxyuridine (EdU) using the Click-iT® EdU Alexa Fluor® 488 HCS Assay kit. For the

6 h stimuli, 10 µM EdU (available in the kit) was added to the SVZ cultures at the same time of the treatment and for the 24 h treatment, EdU was added 4 h before cell fixation (20 h after stimuli). For fixation, cells were washed with sterile 0.01 M PBS and then sterile StemPro® Accutase® cell dissociation reagent was added to cells for 20 min, at 37 ºC. Cells were detached from the plate by gently pipetting and harvested into flow tubes. Following centrifugation at 180 x g for 20 min, the supernatant was discarded and cells were resuspended in 70 % ethanol overnight, at 4 ºC, as described previously (Carreira *et al.* 2010). Ethanol acts as a permeability and fixation agent, simultaneously. Cells can be stored in 70 % ethanol and used for flow cytometry for a maximum of 4 days.

Detection of EdU incorporation was based on click chemistry, a copper catalyzed covalent reaction between an azide (conjugated with the Alexa Fluor 488 fluorophore) and an alkyne (EdU). Fixed cells were pelleted by centrifugation at 218 x g for 20 min and the supernatant was discarded. A rinse with PBS 0.01 M was performed, followed by centrifugation at 218 x g for 15 min and the supernatant was discarded again. The reaction cocktail (Alexa Fluor® 488 azide, copper sulfate, 1x Click-iT reaction buffer and 1 x reaction buffer additive, available from the kit) was added and cells incubated for 30 minutes with the azide conjugate and copper sulphate, at room temperature, protected from light. After the reaction, the cells were pelleted at 218 x g for 15 min. The supernatant was removed and a new rinse with PBS 0.01 M was performed (218 x g, 15 min). The supernatant was discarded and PBS 0.01 M was added. Cells were incubated with ribonuclease A and the nuclear dye 7-actinomycin D (7-AAD), also available in the kit, for 30 min, at room temperature, protected from light. Ribonuclease A is used to ensure that 7-AAD only binds to DNA. The EdU incorporation was detected on a BD FACScalibur^{TM} Flow Cytometer, using the Cellquest Pro software, version 0.3.efab (Becton Dickinson, San Jose, CA, USA). Thirty thousand events were acquired per each experiment in the region of interest

(including apoptosis, G0/G1, S and G2/M). A minimum of 4 independent experiments was analyzed for each condition. Data were analyzed using the WinMDI2.9 software and are presented as means ± SEM of the number of non-apoptotic cells that incorporated EdU (% of control).

2.2.6. Preparation of cytosolic lysates

The treated cells were washed with PBS 0.01 M and scraped and lysed in 50 mM Tris-HCl, 0.15 M NaCl, 1 mM EDTA, 1 % Igepal and 10 % glycerol, supplemented with 200 µM PMSF, 1 µg/ml CLAP (chymostatin, leupeptin, antiparin, pepstatin A), 1 mM sodium orthovanadate, 1 µM dithiothreitol and 5 mM NaF, pH 7.5, 4ºC. Three freezing/thawing cycles followed by five 5-second sonication cycles were applied and the lysate was clarified by centrifugation at 14,000 x g, 4ºC. The cytosolic fraction was collected and the pellet (nuclear fraction) was discarded. Protein concentration was determined by the BCA method, according to manufacturer's instructions. 6x concentrated sample buffer was added and samples were denatured at 95ºC for 5 min. Samples were analyzed by Western blot.

2.2.7. Western blot analysis

Samples destined for Western blot analysis were electrophoresed in sodium dodecyl sulfate (SDS)-polyacrylamide gels using MiniPROTEAN® 3 systems (Bio-Rad Laboratories). Resolving gels were composed by 12 % bis-acrylamide, 25% Tris-HCl 1.5 M pH 8.0, 0.1 % SDS, 0.05 % TEMED and 0.05 % ammonium persulfate, in milliQ water, for all western blot experiments except for the identification of nNOS and sGC that resolving gels contained 8 % bis-acrylamide. Stacking gels were composed by 4 % bis-acrylamide, 25 % Tris-HCl 0.5 M pH 6.8, 0.1 % SDS, 0.05 % TEMED and 0.05 % ammonium persulfate, in milliQ water. Equal amounts of protein were applied on each

lane of the SDS-polyacrylamide gels submerged in a running buffer (25 mM Tris, 25 mM bicine and 0.1 % SDS, in milliQ water). Proteins were separated by electrophoresis, firstly at 60 V for 10 min and then at 120 V until proper bands separation were reached. A molecular ladder was used to control molecular weight separation. The polyvinylidene difluoride membranes were activated, first in 100 % methanol (2.5 to 5 min), followed by water and finally 15 to 30 min in electrotransference buffer (CAPS 10 mM, methanol 10 %, pH 11.0). Proteins were electrophoretically transferred to the activated membranes submerged in electrotransference buffer at 750 mA, for 90 min, at 4ºC, using the Trans-Blot Cell apparatus (Bio-Rad Laboratories). Membranes were blocked by 1 h incubation, at room temperature, with 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 or, for phosphorylated proteins, 3 % BSA. Incubations with the primary antibodies (rabbit phospho-ERK 1/2, rabbit anti-$p27^{kip1}$, 1:1,000; mouse anti-nNOS, rabbit anti-sGC, 1:500) in TBS-T containing 1 % blocking solution were performed overnight, at 4ºC. After rinsing with TBS-T (20 min, with 2 quick washes before and after), incubation with the appropriated alkaline phosphatase-linked secondary antibodies (anti-rabbit or anti-mouse, 1:20,000 in TBS-T containing 1 % blocking solution) was performed at room temperature, during 1 h. After extensive washing in TBS-T (for 1 h, changing into new TBS-T every 20 min), followed by the incubation of the membranes with the enhanced chemifluorescence reagent for the maximum of 5 min, immunoreactive bands were visualized in the VersaDoc 3000 imaging system (Bio-Rad, Hercules, CA, USA). Data were analyzed with the Quantity One software version 4.6.9 (Bio-Rad Laboratories). Protein control loadings were either performed after membranes reactivation (5-10 s in 100 % methanol and 20 min in TBS-T) using primary antibodies against rabbit ERK 1/2 (1:1,000) or mouse α-tubulin (1:10,000). The protocol used was the same as explained above.

2.2.8. Affinity binding precipitation of ubiquitinated $p27^{Kip1}$

SVZ cells were treated as explained above for 2 h, at 37ºC. Cells were briefly washed with PBS 0.01 M and cytosolic lysates were then prepared by adding 500 µl of lysis buffer (50 mM Tris-HCl, 0.15 M NaCl, 1 mM EDTA, 1 % Igepal and 10 % glycerol, supplemented with 200 µM PMSF, 1 µg/ml CLAP, 1 mM sodium orthovanadate, 5 mM NaF and 10 µM PR-619, pH 7.5, 4ºC) and scraping on ice. Freezing/thaw cycles followed by sonication cycles were applied and the lysate was clarified by centrifugation at 14,000 x g, 4ºC. The supernatant was collected and protein concentration was determined by the BCA method using the BCA™ Protein Assay kit, following the manufacturer's instructions. An *input* of 50 µg of protein was removed from each sample, for control of the cellular levels of $p27^{Kip1}$.

Agarose-TUBEs are Tandem Ubiquitin Binding Entities moieties that are coupled to agarose beads and are used for the identification and characterization of ubiquitinated proteins by Western blotting or downstream proteomic studies.

Agarose-TUBEs were equilibrated by inverting the vial several times to ensure a homogeneous solution and the necessary volume was collected and washed three times with TBS-T (20 mM Tris-HCl, 0.15 M NaCl, 0.1 % Tween-20, pH 8.0) followed by centrifugation at 1,000 x g at 4ºC. 600 µg of protein of each lysate were added to different tubes with the equilibrated slurry of agarose beads and incubated for 4 h at 4ºC, with agitation. The beads were collected by centrifugation at 1,500 x g and the supernatant was saved as the *unbound* fraction. Both, *input* and *unbound* fraction, were treated with 6 x concentrated sample buffer (0.5 M Tris-HCl/0.4 % SDS pH 6.8, 30 % glycerol, 10 % sodium dodecyl sulfate, 0.6 M dithiothreitol, 0.012 % bromophenol blue) and heated at 95ºC for 5 min. The agarose beads were resuspended in 50 µl of 2 x concentrated sample buffer followed by heating at 95ºC for 5 min. The *bound* fraction was separated from the beads by centrifugation at 13,000 x g. The ubiquitinated proteins were analyzed by Western blot in parallel with *inputs* and *unbound* fractions to

evaluate the levels of $p27^{Kip1}$. For the *inputs* 20 µg of protein were applied in the gel and 50 µg of protein in the case of the *unbound* fraction. As to the *bound* fraction, half of the final volume was used, i.e, 25 µl. The protocol used for Western blot was the same as explained above. The primary antibody used was the rabbit anti-$p27^{kip1}$, 1:1,000 and protein control loadings were performed using a primary antibody against mouse α-tubulin (1:10,000).

2.2.9. Determination of cGMP levels

The cGMP levels in cultured SVZ stem cells were determined after exposure to drugs for 6 h, using a cGMP Enzymeimmunoassay Biotrak System. This experimental assay is based on competition between unlabelled cGMP from cell lysates and a fixed quantity of peroxidase-labelled cGMP, for a limited number of binding sites on a cGMP specific antibody coated on a 96-well plate. Extraction and measurement was performed according to manufacturer's instructions. Briefly, cells were lysed with lysis buffer 1, available from the kit, which hydrolyses cell membranes to release intracellular cGMP. Cell lysis was facilitated by shaking during 10 min, followed by scraping. The concentrated samples were resuspended in assay buffer and acetylated in order to increase the sensitivity of the assay. Cell lysates were then immediately used for the enzyme immunoassay protocol, as previously described (Araujo *et al.* 2003). Optical density was read at 450 nm on a plate reader. All the experiments were carried out in duplicate. The results are expressed as fentomoles per million of cells.

2.2.10. Data analysis

Data are expressed as means ± SEM. Statistical significance was determined by using two-tailed t tests or one-factor analysis of variance (ANOVA) followed by Bonferroni's or Dunnett's post-tests, as appropriate and indicated in the figure legends

and in the text. Differences were considered significant when $p<0.05$. The software used was GraphPad Prism 5.0 (GraphPad Software, La Jola, CA, USA).

Chapter 3 Results

3.1. Characterization of SVZ cell cultures

3.1.1. NSC are predominant in the SVZ

Neural stem cells isolated from the subventricular zone and cultured as floating aggregates were plated on poly-L-lysine-coated coverslips for 4 days and were characterized at this stage. The cells were stained against Sox-2, a transcription factor essential to maintain self-renewal of undifferentiated stem cells and musashi-1, an evolutionary conserved RNA binding protein with maximum expression in proliferating multipotent neural precursor cells, decreasing during neuronal differentiation. The percentage of Sox-2-positive (80.2 % ± 3.2 %) and musashi-1-positive cells (83.5 % ± 4.3 %) was approximately 80 %, suggesting that the majority of cells remained undifferentiated after plating. The percentage of double-labeled cells was approximately 100 %, showing that both, Sox-2 and musashi-1, are good markers of undifferentiated cells (Fig. **5A**-**5C**). SVZ cells were also stained against the neural precursor marker, nestin, the glial fibrillary acidic protein (GFAP), a marker for astrocytes that also labels neural stem cells, and the neuron-specific marker, β-III-tubulin (Tuj1). Only 0.5 % of cells were positive for β-III-tubulin (0.5 % ± 0.1 %; Fig. **5J**-**5L**), suggesting that very few cells were differentiated into neurons at this stage. Moreover, approximately 20 % of live cells were GFAP positive (21.5 % ± 3.7 %; Fig. **5G**-**5I**) and almost 50 % expressed nestin (46.9 % ± 2.0 %; Fig. **5D**-**5F**). Furthermore, previous studies by our group showed that about 70 % of cells colocalize for nestin and GFAP (Carreira *et al.* 2010).

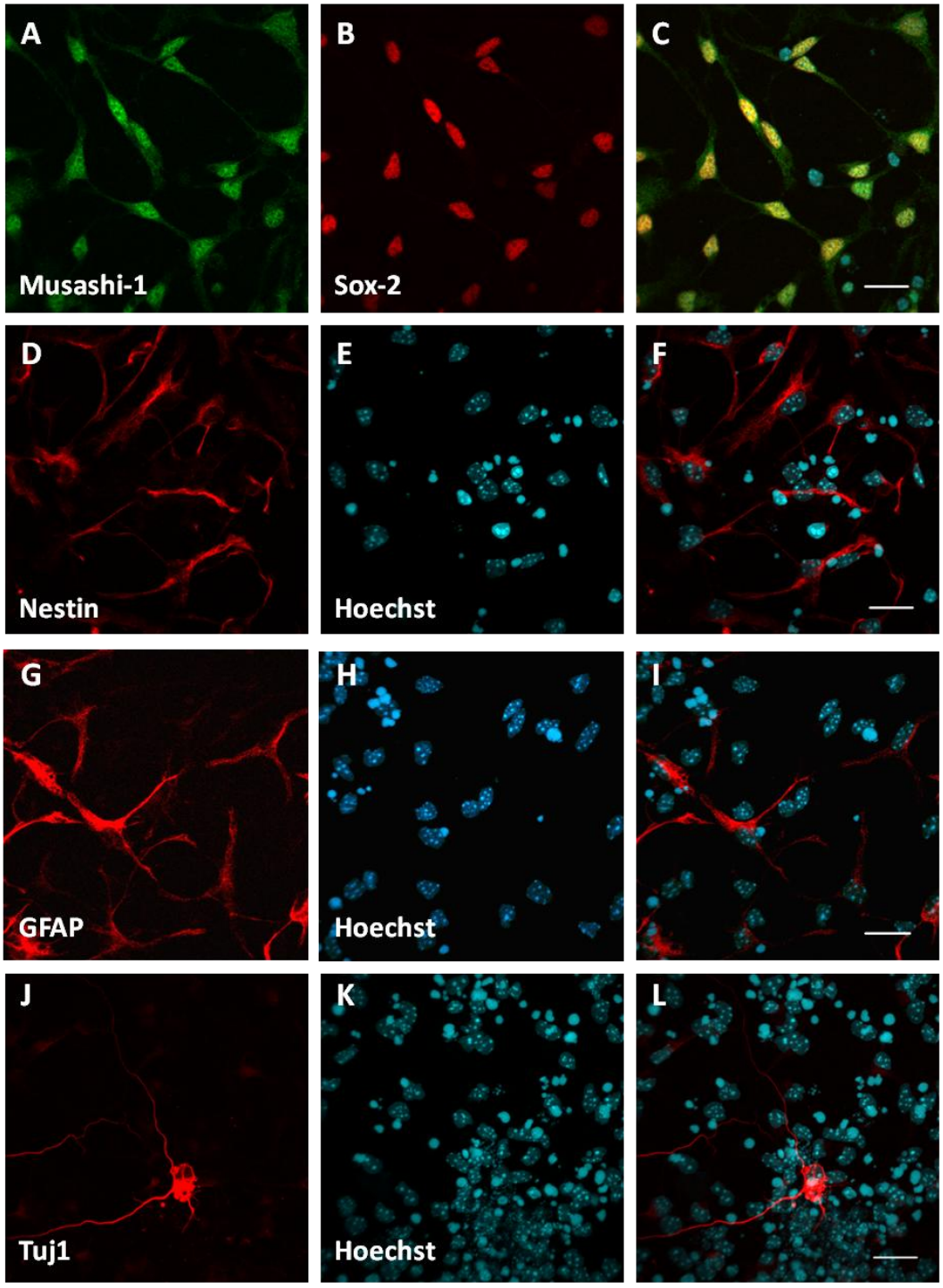

Figure 5. SVZ cultures are enriched in markers of NSC. The micrographs show laser scanning confocal images of SVZ cells labeled against musashi-1 (**A**, green), Sox-2 (**B**, red), nestin (**D**, red), GFAP (**G**, red) and Tuj1 (**J**, red). Nuclei were labeled with Hoechst 33342 (**E**, **H** and **J**, blue). Merged images are shown in **C**, **F**, **I** and **L**. Scale bars: 20 µM.

3.1.2. Presence of NO• and cGMP-producing enzymes

In order to confirm that sGC and nNOS were present in the SVZ cultures, we evaluated the presence of these proteins by Western blot. In fact, sGC was present in SVZ cells (Fig. **6A**). Likewise, the presence of the neuronal isoform of the nitric oxide synthase (nNOS) was evaluated. In agreement with sGC, nNOS was also present in the SVZ cultures (Fig. **6B**), meaning that endogenous NO• is produced in these cells. Furthermore, previous work by our group showed that these cells do not express iNOS or eNOS (B.Carreira, unpublished observations).

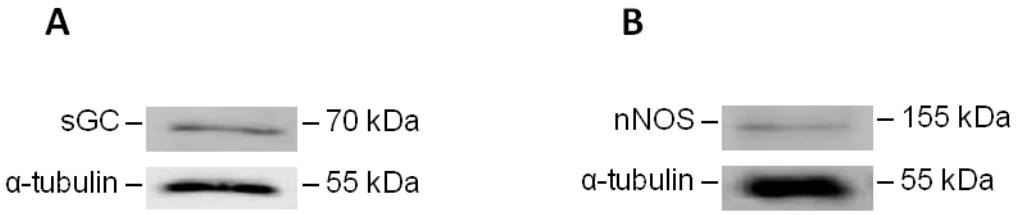

Figure 6. sGC and nNOS are present in the SVZ stem cell cultures. cGMP (**A**) and NO•-producing enzymes (**B**), respectively, can be detected in SVZ cultures by Western blot, using 40 µg of protein.

3.2. Modulation of SVZ cell proliferation by PDE inhibitors

3.2.1. PDE inhibitors stimulate neural stem cell proliferation

To initiate the studies with the three PDE5 inhibitors selected, different concentrations were tested for sildenafil and zaprinast. For the more selective inhibitor for PDE 5, T0156, the concentration used was the more used in the literature and used by our group in previous studies, 1 µM. A treatment of 6 h was performed in the presence of the PDE5 inhibitors. EdU incorporation was assessed in a flow cytometer.

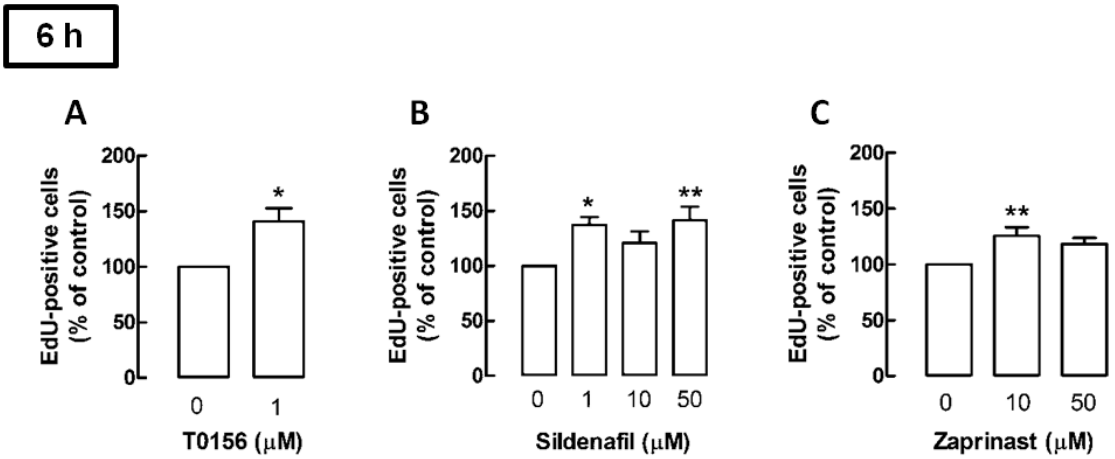

Figure 7. T0156, sildenafil and zaprinast stimulate the proliferation of SVZ neural stem cells following a 6 h exposure. Cells were treated for 6 h with 1 μM T0156 (**A**), 1 μM, 10 μM and 50 μM sildenafil (**B**) or 10 μM and 50 μM zaprinast (**C**). The incorporation of EdU was assessed by flow cytometry. Data are expressed as means ± SEM of at least five independent experiments. Two-tailed t test, *p<0.05, significantly different from control, and one-way ANOVA (Dunnett's post-test), *p<0.05 and **p<0.01, significantly different from control.

All the PDE5 inhibitors showed an increase in the proliferation of SVZ cells. Treatment with T0156 (1 μM) significantly increased the incorporation of EdU (140.7 % ± 11.9 %, p<0.05; Fig. **7A**), when compared to untreated cells. In the case of sildenafil, the three concentrations tested increased EdU incorporation (1 μM, 137.4 % ± 7.0 %, p<0.05; 10 μM, 120.8 % ± 10.4 %, p>0.05; 50 μM, 141.3 % ± 12.6 %, p<0.01; Fig. **7B**) as well as the two concentrations tested for zaprinast (10 μM, 125.6 % ± 7.8 %, p<0.05; 50 μM, 118.1 % ± 5.6 %, p>0.05; Fig. **7C**), in comparison to untreated cells. In agreement with this, 1 μM sildenafil and 10 μM zaprinast were selected for all the subsequent experiments.

In order to investigate whether this effect was maintained in time, the selected concentrations were tested for a longer period of exposure. SVZ cells were treated for

24 h with the chosen concentrations of the PDE5 inhibitors and EdU incorporation was evaluated by flow cytometry. The data are shown in Fig. **8**.

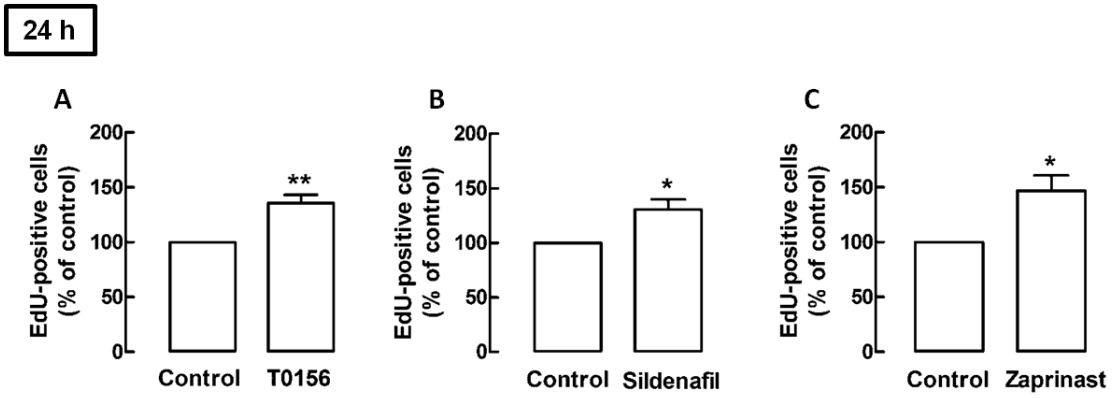

Figure 8. PDE5 inhibitors enhance SVZ cell proliferation following a treatment of 24 h. Cells were treated for 24 h with 1 µM T0156 (**A**) 1 µM sildenafil (**B**) or 10 µM zaprinast (**C**). The incorporation of EdU was assessed by flow cytometry. Data are expressed as means ± SEM of at least five independent experiments. Two-tailed t test, *p<0.05 and **p<0.01, significantly different from control.

In agreement with the results for 6 h of treatment, following 24 h of treatment, T0156 (1 µM; 135.6 % ± 7.3 %, p<0.01; Fig. **8A**), sildenafil (1 µM; 130.6 % ± 9.4 %, p<0.05; Fig. **8B**) and zaprinast (10 µM; 146.5 % ± 13.9 %, p<0.05; Fig. **8C**) also increased SVZ cell proliferation, in comparison to untreated cells.

3.2.2. Treatment with PDE5 inhibitors increases cGMP levels

In order to confirm that the stimulation of cell proliferation was due to an increase in the levels of cGMP by inhibition of PDE5, cGMP levels were measured using an immunoenzymatic commercial kit. SVZ cells were treated with T0156, sildenafil and zaprinast, according to the concentrations selected above.

Table V. PDE5 inhibitors increase the levels of cGMP in SVZ cells.

Treatment	cGMP levels (fmol/10^6 cells)
Control	7.4 ± 0.9
T0156	29.7 ± 4.2*****
Sildenafil	28.8 ± 5.1*****
Zaprinast	33.1 ± 6.1******

Cells were treated for 6 h with 1 µM T0156, 1 µM sildenafil or 10 µM zaprinast. Data are expressed as means ± SEM of two independent experiments. One-way ANOVA (Dunnett´s post-test), *p<0.05 and **p<0.01, significantly different from control.

In fact, cGMP levels were significantly increased in cultures exposed to T0156 (29.7 ± 4.2 fmol/10^6 cells, p<0.05), sildenafil (28.8 ± 5.1 fmol/10^6 cells, p<0.05) or zaprinast (33.1 ± 6.1 fmol/10^6 cells, p<0.01) in comparison to the values of cGMP levels in untreated cells (7.4 ± 0.9 fmol/10^6 cells). The data are shown in Table **V**.

3.2.3. Involvement of the MAPK and sGC pathways on SVZ cell proliferation

3.2.3.1. Inhibition of the MAPK pathway abolishes the proliferative effect of the PDE5 inhibitors

With the purpose of evaluating the involvement of the MAPK/ERK 1/2 pathway on the proliferation stimulated by the PDE5 inhibitors, we treated SVZ cultures with U0126, the inhibitor of MEK 1/2, the kinase immediately upstream to ERK 1/2. In the presence of U0126, together with the PDE5 inhibitors, the percentage of EdU incorporation decreased in comparison to what happened when T0156, sildenafil or zaprinast were administered alone.

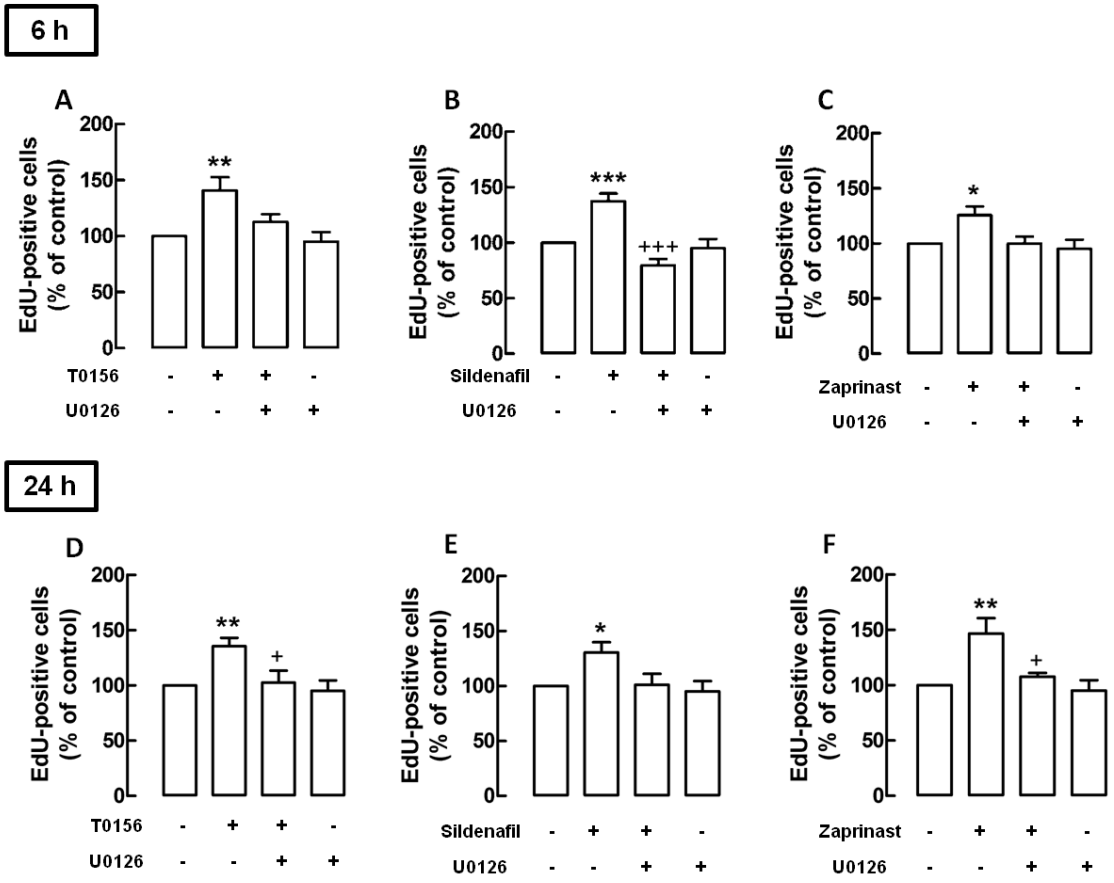

Figure 9. Exposure to the MEK 1/2 inhibitor, U0126, prevents proliferation of SVZ cells stimulated by PDE5 inhibitors. Cell proliferation following treatment with 1 µM U0126 and 1 µM T0156 (**A**, **D**), 1 µM sildenafil (**B**, **E**) or 10 µM zaprinast (**C**, **F**) was assessed by incorporation of EdU and evaluated by flow cytometry following 6 h and 24 h of treatment. Data are expressed as means ± SEM of at least four independent experiments. One-way ANOVA (Bonferroni´s post-test), *p<0.05, **p<0.01 and ***p<0.001, significantly different from control and +p<0.05, and +++p<0.001, significantly different from the PDE inhibitor.

U0126 prevented the increase in EdU incorporation triggered by sildenafil (6 h, 79.5 % ± 5.6 %, p<0.001; Fig. **9B**) in comparison to sildenafil alone (6 h, 137.4 % ± 7.0 %, p<0.001). Also, there was a non-statistically significant trend for the inhibition of the proliferative effect of T0156 (6 h, 112.7 % ± 6.8 %, p>0.05; Fig. **9A**) and zaprinast (6 h,

100.1 % ± 6.0, p>0.05; Fig.**9C**) by U0126, keeping cell proliferation similar to basal levels, in comparison to T0156 (6 h, 140.7 % ± 11.9 %, p<0.01) and zaprinast (6 h, 125.6 % ± 7.8 %, p<0.05) alone, respectively.

Similar results were obtained following 24 h of treatment. In the presence of U0126, the increase in the incorporation of EdU by T0156 (24 h, 102.5 % ± 10.7 %, p<0.05; Fig. **9D**) and zaprinast (24 h, 107.6 % ± 3.4 %, p<0.05; Fig. **9F**) was prevented, comparatively to T0156 (24 h, 135.6 % ± 7.3 %, p<0.01) and zaprinast (24 h, 146.5 % ± 13.9 %, p<0.01) alone, respectively. Moreover, U0126 appeared to prevent the increase in EdU incorporation by sildenafil (24 h, 101.2 % ± 9.9 %, p>0.05; Fig. **9E**) in comparison to sildenafil alone (24 h, 130.6 % ± 9.4 %, p<0.05), despite the lack of statistical significance.

3.2.3.2. sGC inhibition affects the proliferative effect of PDE5 inhibitors

To analyze the activation of the sGC pathway on cell proliferation stimulated by PDE5 inhibition, SVZ cells were exposed to the sGC inhibitor, ODQ. Together with the PDE5 inhibitors, ODQ appeared to have an inhibitory role on cell proliferation, mainly upon a 6 h treatment.

Following 6 h of treatment, ODQ prevented SVZ cell proliferation stimulated by T0156 (6 h, 105.5 % ± 7.9 %, p<0.05; Fig. **10A**), in comparison to T0156 alone (6 h, 140.7 % ± 11.9 %, p<0.01). Moreover, although not statistically significant, the treatment with ODQ appeared to inhibit the proliferative effect of sildenafil (6 h, 108.9 % ± 15.2 %, p>0.05; Fig. **10B**) and zaprinast (6 h, 113.4 % ± 9.7 %, p>0.05; Fig. **10C**) in comparison to sildenafil (6 h, 137.4 % ± 7.0 %, p<0.05) and zaprinast (6 h, 125.6 % ± 7.8 %, p<0.05) alone, respectively.

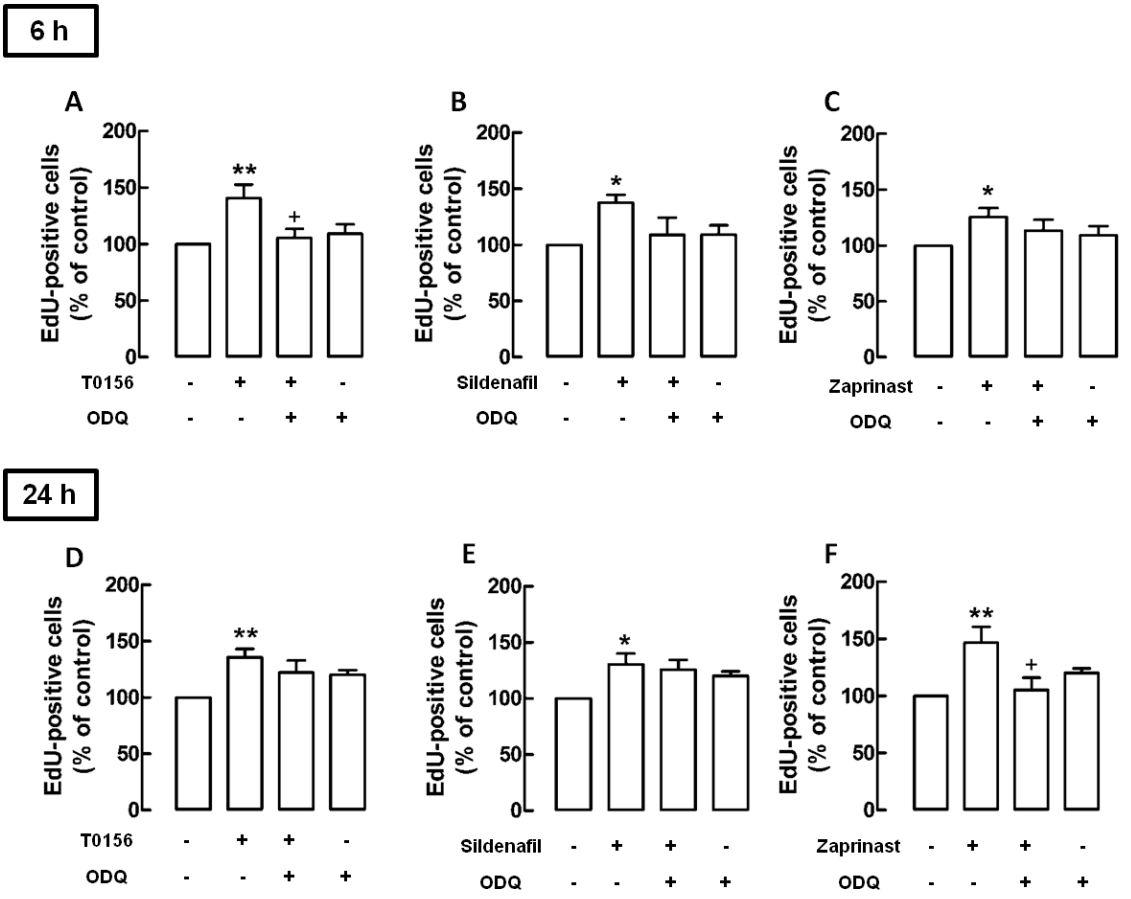

Figure 10. sGC inhibition by ODQ appears to abolish the proliferation of SVZ cells stimulated by PDEs inhibitors following 6 h of treatment. Cell proliferation following treatment with 50 µM ODQ and 1 µM T0156 (**A**, **D**), 1 µM sildenafil (**B**, **E**) or 10 µM zaprinast (**C**, **F**) was assessed by incorporation of EdU and evaluated by flow cytometry, following 6 h and 24 h treatment. Data are expressed as means ± SEM of at least four independent experiments. One-way ANOVA (Bonferroni´s post-test), *p<0.05 and **p<0.01, significantly different from control and +p<0.05, significantly different from the PDE inhibitor.

Following 24 h of treatment, ODQ appeared to be ineffective in preventing the increase of EdU-positive cells by T0156 (24 h; 121.9 % ± 10.9 %, p>0.05; Fig. **10D**) and sildenafil (24 h; 125.7 % ± 8.7 %, p>0.05, Fig. **10E**) in comparison to T0156 (24 h, 135.6 % ± 7.3 %, p<0.01) and sildenafil (24 h, 130.6 % ± 9.4 %, p>0.05) alone,

respectively. However, following the same period of treatment, the proliferative effect of zaprinast was prevented in the presence of ODQ (24 h, 105.1 % ± 10.1 %, $p<0.05$, Fig. **10F**) when compared to zaprinast alone (24 h, 146.5 % ± 13.9 %, $p<0.01$).

3.2.3.3. PKG inhibition abolishes the proliferative effect of PDE5 inhibitors

We next studied the effect of the inhibition of PKG, using the PKG inhibitor KT5823, on the proliferation triggered by PDE5 inhibition. PKG is activated by cGMP, inducing cell proliferation. Therefore, following 6 h of treatment with KT5823, the increase in the incorporation of EdU by T0156 (6 h, 102.6 % ± 7.1 %, $p<0.05$; Fig. **11A**), sildenafil (6 h, 101.1 % ± 9.0 %, $p<0.01$; Fig. **11B**) and zaprinast (6 h, 99.5 % ± 7.4 %, $p<0.05$; Fig. **11C**) was prevented, being cell proliferation similar to basal levels, comparatively to T0156 (6 h, 140.7 % ± 11.9 %, $p<0.01$), sildenafil (6 h, 137.4 % ± 7.0 %, $p<0.001$) and zaprinast (6 h, 125.6 % ± 7.8 %, $p<0.05$) alone, respectively.

Similarly, following 24 h of treatment with KT5823, the increase in cell proliferation induced by T0156 (24 h, 112.1 % ± 8.3 %, $p>0.05$; Fig. **11D**) and sildenafil (24 h, 107.6 % ± 6.0 %, $p>0.05$; Fig. **11E**) is decreased, in comparison to T0156 (24 h, 135.6 % ± 7.3 %, $p<0.05$) and sildenafil (24 h, 130.6 % ± 9.4 %, $p<0.05$) alone, respectively, even though the lack of statistical significance. Likewise, KT5823 prevented the increase of EdU incorporation triggered by zaprinast (24 h, 108.1 % ± 4.9 %, $p<0.05$; Fig. **11F**) when compared to zaprinast alone (24 h, 146.5 % ± 13.9 %, $p<0.05$).

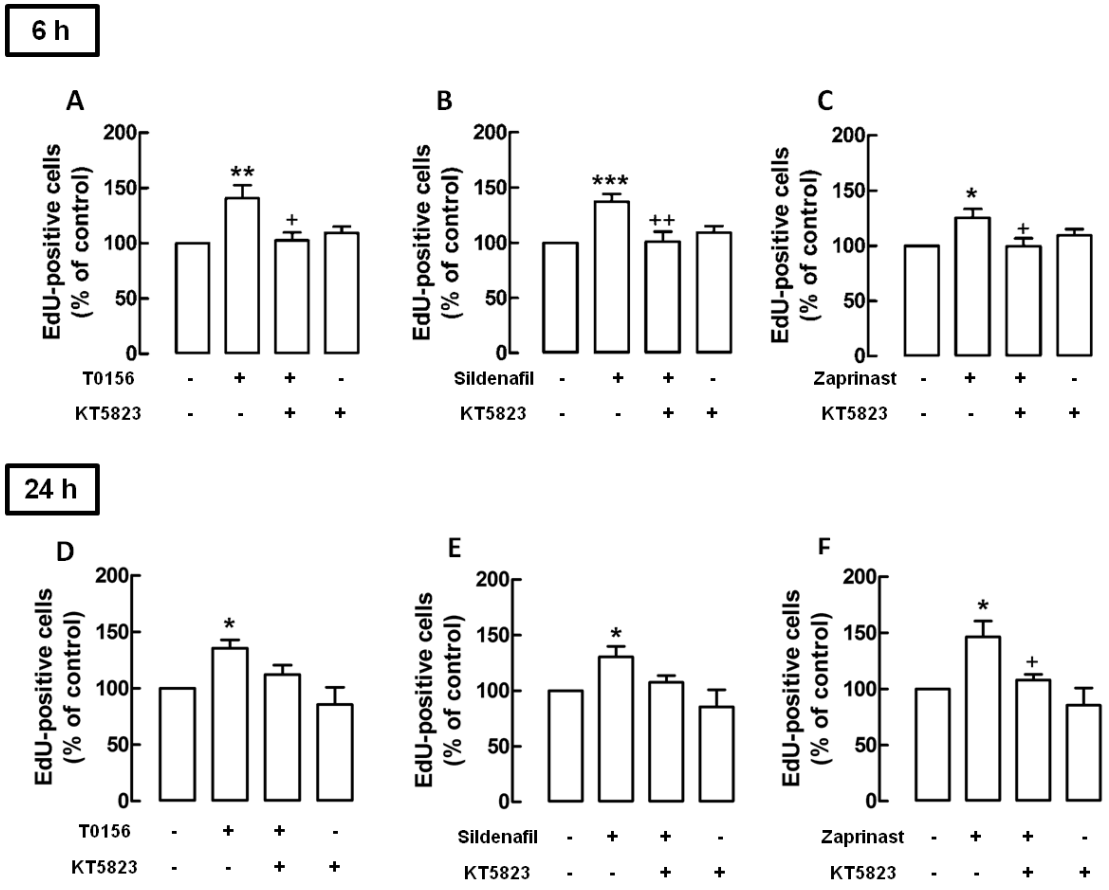

Figure 11. Inhibition of PKG, by KT5823, inhibits proliferation of SVZ cells stimulated by the PDE5 inhibitors. Cell proliferation following treatment with 1 µM KT5823 and 1 µM T0156 (**A**, **D**), 1 µM sildenafil (**B**, **E**) or 10 µM zaprinast (**C**, **F**) was assessed by incorporation of EdU and evaluated by flow cytometry, following 6 h and 24 h of treatment. Data are expressed as means ± SEM of at least four independent experiments. One-way ANOVA (Bonferroni´s post-test), *p<0.05, **p<0.01 and ***p<0.001, significantly different from control and +p<0.05 and ++p<0.01 significantly different from the PDE5 inhibitor alone.

3.2.4. Direct activation of sGC stimulates SVZ cell proliferation

In order to test the effects of the direct activation of sGC, SVZ cells were treated with 20 µM YC-1, a $NO^{\bullet}$-independent activator of sGC. Cell proliferation was assessed by flow cytometry following a treatment of 24 h with YC-1. Exposure to YC-1 increased

the incorporation of EdU by NSC (137.1 % ± 6.8 %, p<0.01; Fig. **12**), when compared to untreated cells. Treatment with the same concentration of YC-1 for 6 h did not alter the percentage of EdU-positive cells (data not shown).

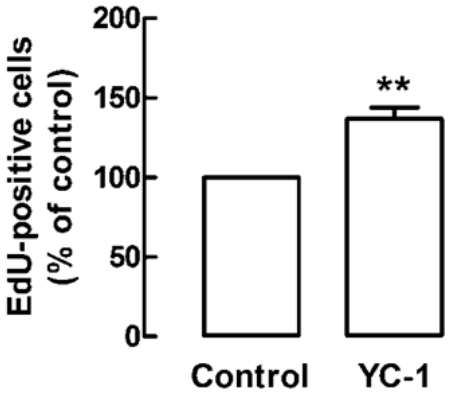

Figure 12. Direct activation of sGC by YC-1 stimulates SVZ cell proliferation. Cell proliferation following treatment with YC-1 (20 µM) for 24 h was assessed by the incorporation of EdU and analyzed by flow cytometry. Data are expressed as means ± SEM of at least four independent experiments. Two-tailed t test, **p<0.01, significantly different from control.

3.3. Activation of proliferative signaling pathways by inhibition of PDE5

3.3.1. PDE5 inhibition induces ERK 1/2 phosphorylation and decreases $p27^{Kip1}$ levels

Exposure of SVZ cell cultures to $NO^{\bullet}$ triggers proliferation by activation of ERK 1/2 signaling, which can be monitored by evaluating the phosphorylation of ERK 1/2 and the concomitant decrease in the levels of cyclin-dependent kinase inhibitor $p27^{Kip1}$ (Carreira *et al.* 2010). In order to choose the best time to study the effect of PDE5 inhibitors or of the activator of sGC (YC-1) on the levels of phospho-ERK 1/2 and

p27^{Kip1}, we treated the SVZ cultures with these drugs for different periods of time. Cells were treated with T0156 (1 µM; Fig. **13A**), sildenafil (1 µM; Fig. **13B**), zaprinast (10 µM; Fig. **13C**), or YC-1 (20 µM; Fig. **13D**) for 30 min, 1 h or 2 h. Treatment with T0156, zaprinast and YC-1 increased ERK 1/2 phosphorylation following 2 h of treatment. Furthermore, the cellular levels of p27^{Kip1} appear to be decreased, mainly following a 2 h treatment with the more selective inhibitor for PDE5 (T0156). According to these results, we selected a treatment of 2 h for the following experiments.

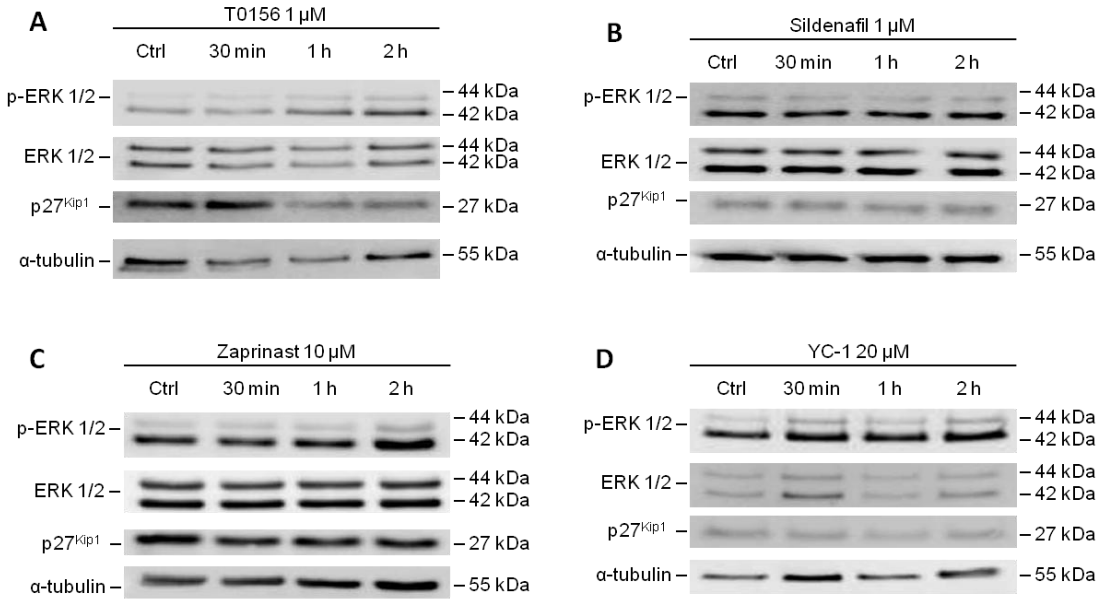

Figure 13. PDE5 inhibitors increase ERK 1/2 phosphorylation and decrease the levels of p27^{Kip1} following 2 h of treatment. SVZ stem cells were treated with 1 µM T0156 (**A**), 1 µM sildenafil (**B**), 1 µM zaprinast (**C**) and 20 µM YC-1 (**D**) for different times: 30 min, 1h and 2h.

3.3.2. Signaling through MAPK pathway: evaluation of ERK 1/2 phosphorylation

3.3.2.1. Treatment with PDE5 inhibitors increases ERK 1/2 phosphorylation

With the purpose of evaluating whether the increase in cell proliferation observed following the administration of PDE5 inhibitors was due to ERK 1/2 pathway activation, we analyzed the phosphorylation of ERK 1/2 by Western blot, following a treatment of 2 h with the PDE5 inhibitors.

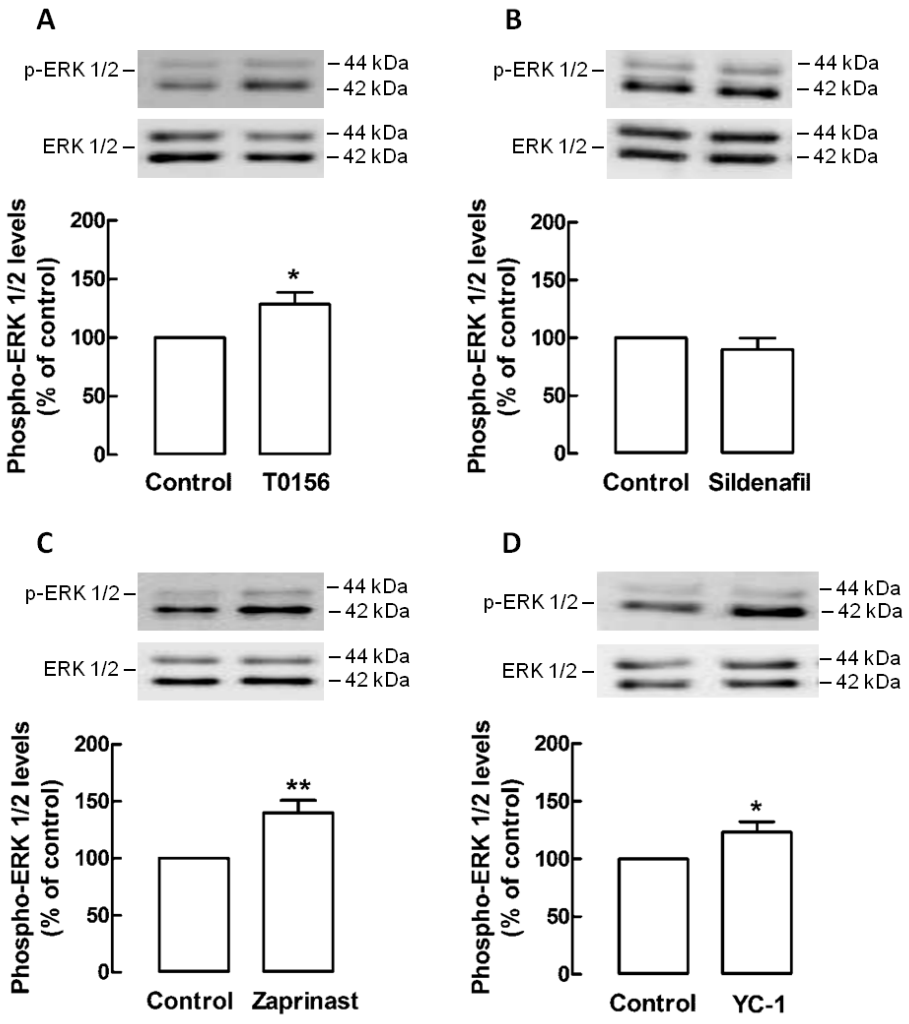

Figure 14. T0156, zaprinast and YC-1, but not sildenafil, increase ERK 1/2 phosphorylation following 2 h of treatment. Phospho-ERK 1/2 levels

following treatment with 1 µM T0156 (**A**), 1 µM sildenafil (**B**), 10 µM zaprinast (**C**) and 20 µM YC-1 (**D**) for 2 h were assessed by Western blot. Data are expressed as means ± SEM of at least 6 independent experiments. Two-tailed t test, *p<0.05 and **p<0.01, significantly different from control.

In fact, T0156 (1 µM; 128.5 % ± 10.0 %, p<0.05; Fig. **14A**) and zaprinast (10 µM; 140.0 % ± 11.0 %, p<0.01; Fig. **14C**) significantly increased ERK 1/2 phosphorylation in comparison to control. However, treatment with sildenafil (1 µM; 89.9 % ± 9.8 %, p>0.05; Fig. **14B**) did not increase the levels of phospho-ERK 1/2 in relation to basal levels. Furthermore, treatment with YC-1 increased ERK 1/2 phosphorylation (123.3 % ± 8.9 %, p<0.05; Fig. **14D**), in comparison to untreated cells.

3.3.2.2. Inhibition of PKG prevents the increase in the phospho-ERK 1/2 levels

In order to evaluate whether the inhibition of PKG was able to antagonize the effect of the PDE inhibitors and the sGC activator, samples obtained following 2 h of treatment were analyzed by Western blot.

Treatment with KT5823 appeared to suppress the increase of phospho-ERK 1/2 levels by T0156 (107.2 % ± 6.2 %, p>0.05; Fig. **15A**) and zaprinast (110.5 % ± 7.9 %, p>0.05; Fig. **15C**) in comparison to T0156 (128.5 % ± 10.0 %, p<0.05) and zaprinast (140.0 % ± 11.0 %, p<0.05) alone, respectively. On the contrary, treatment with KT5823 did not appear to decrease the levels of phospho-ERK 1/2 in the samples treated with sildenafil (120.2 % ± 11.0 %, p>0.05; Fig. **15B**) when compared to sildenafil alone (89.9 % ± 9.8 %, p>0.05).

Moreover, inhibition of PKG prevented the increase of phospho-ERK 1/2 levels by exposure to YC-1 (85.6 % ± 10.4 %, p<0.05; Fig. **15D**) in comparison to YC-1 alone (123.3 % ± 8.9 %, p>0.05).

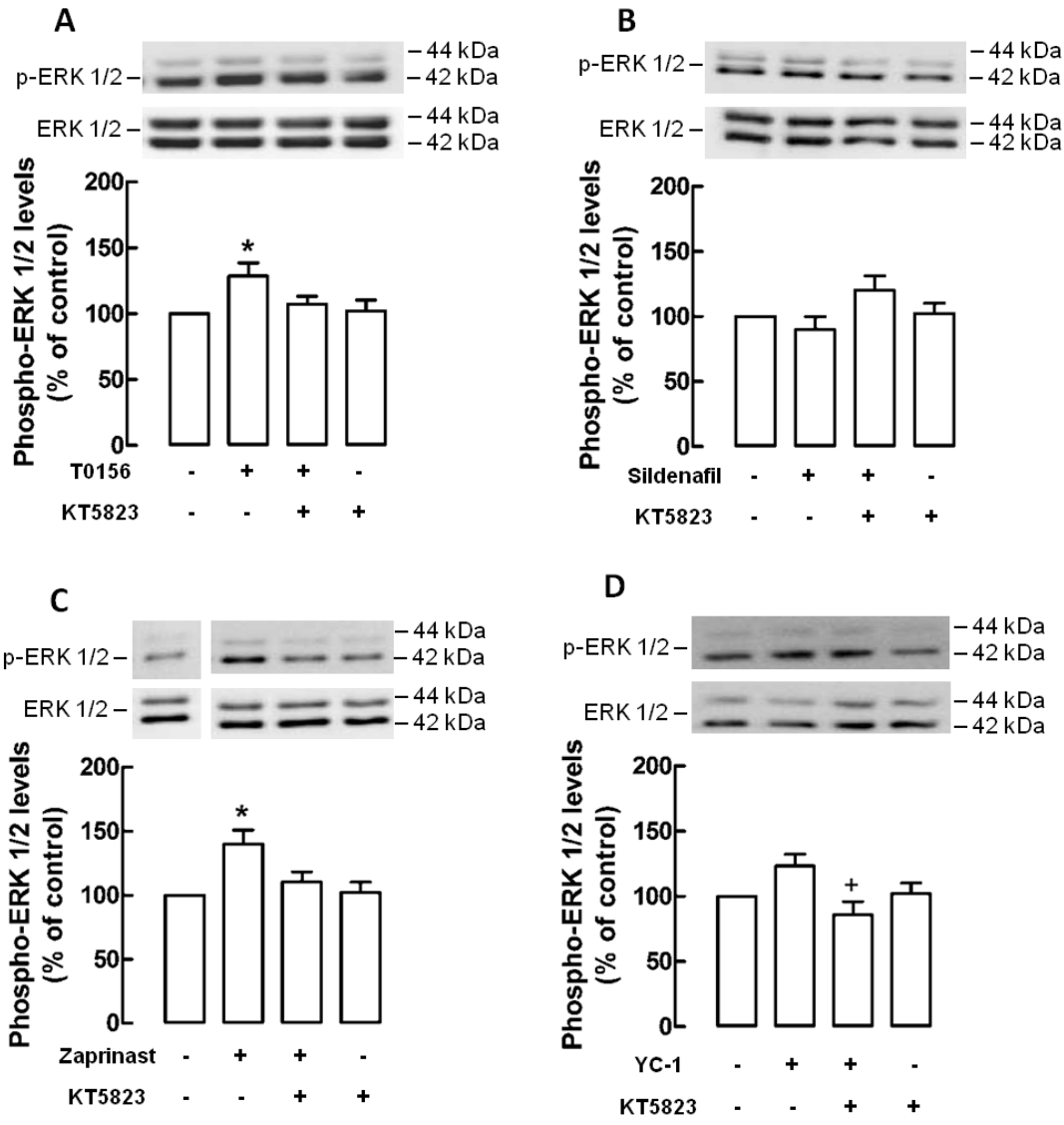

Figure 15. Inhibition of PKG, by KT5823, prevents the increase of ERK 1/2 phosphorylation. Levels of phospho-ERK 1/2 following treatment with 1 µM KT5823 and 1 µM T0156 (**A**), 1 µM sildenafil (**B**), 10 µM zaprinast (**C**) and 20 µM YC-1 (**D**) for 2 h were assessed by Western blot. Data are expressed as means ± SEM of at least 6 independent experiments. One-way ANOVA (Bonferroni´s post-test), *p<0.05, significantly different from control and +p<0.05, significantly different from YC-1.

3.3.3. Evaluation of $p27^{Kip1}$ levels

3.3.3.1. $p27^{Kip1}$ levels are decreased following treatment with T0156 and YC-1

In order to evaluate whether the $p27^{Kip1}$ levels were altered following 2 h of treatment, the protein levels were evaluated by Western blot for lysates of cells treated with T0156, sildenafil, zaprinast and YC-1 for 2 h.

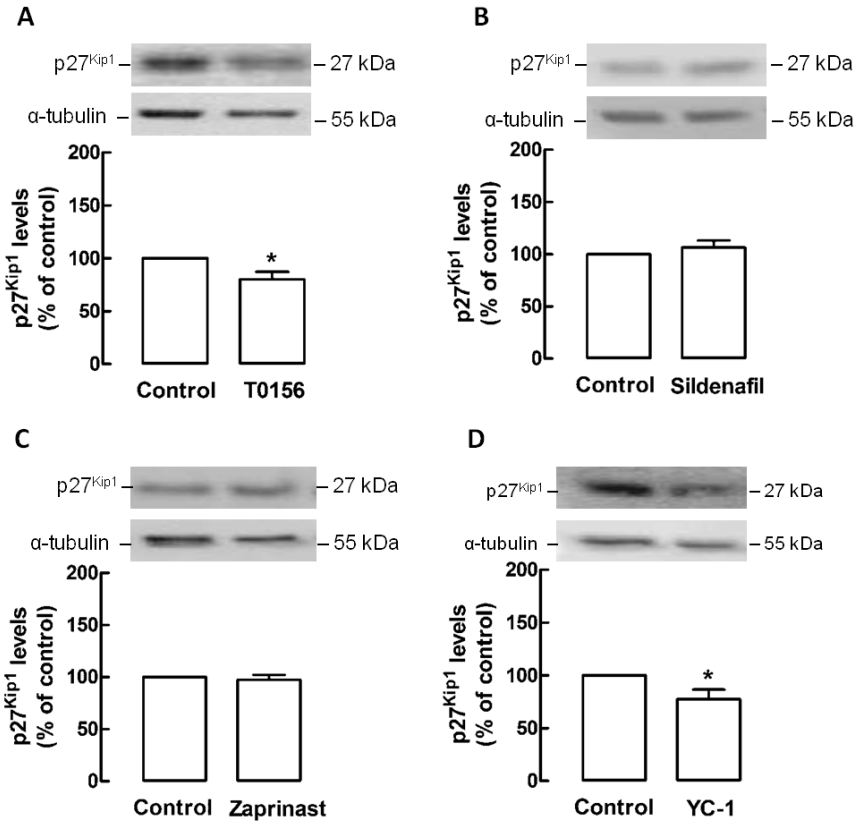

Figure 16. Treatment with T0156 and YC-1 decreases $p27^{Kip1}$ levels. $p27^{Kip1}$ levels following treatment 1 µM T0156 (**A**), 1 µM sildenafil (**B**) 10 µM zaprinast (**C**) and 20 µM YC-1 (**D**) for 2 h were assessed by Western blot. Data are expressed as means ± SEM of at least 6 independent experiments. Two-tailed t test, *p<0.05, significantly different from control.

Following treatment with T0156 (80.0 % ± 7.2 %, p<0.05; Fig. **16A**), $p27^{Kip1}$ levels significantly decreased in comparison to the levels in untreated cells. On the

contrary, treatment with sildenafil (106.4 % ± 6.7 %, p>0.05; Fig. **16B**) and zaprinast (97.4 % ± 4.8 %, p>0.05; Fig. **16C**) did not change the levels of $p27^{Kip1}$ in comparison to control. Furthermore, activating sGC with YC-1 significantly decreased $p27^{Kip1}$ levels (77.5 % ± 9.0 %, p<0.05, Fig. **16C**), in comparison to untreated cells.

3.3.3.2. Inhibition of MAPK pathway prevents the decrease of $p27^{Kip1}$ levels

As seen above, $p27^{Kip1}$ levels are decreased in the presence T0156 and the sGC activator, YC-1. In order to test whether this effect can be reversed by inhibiting the MAPK/ERK 1/2 pathway, treatment of the cells for 2 h with U0126 was performed.

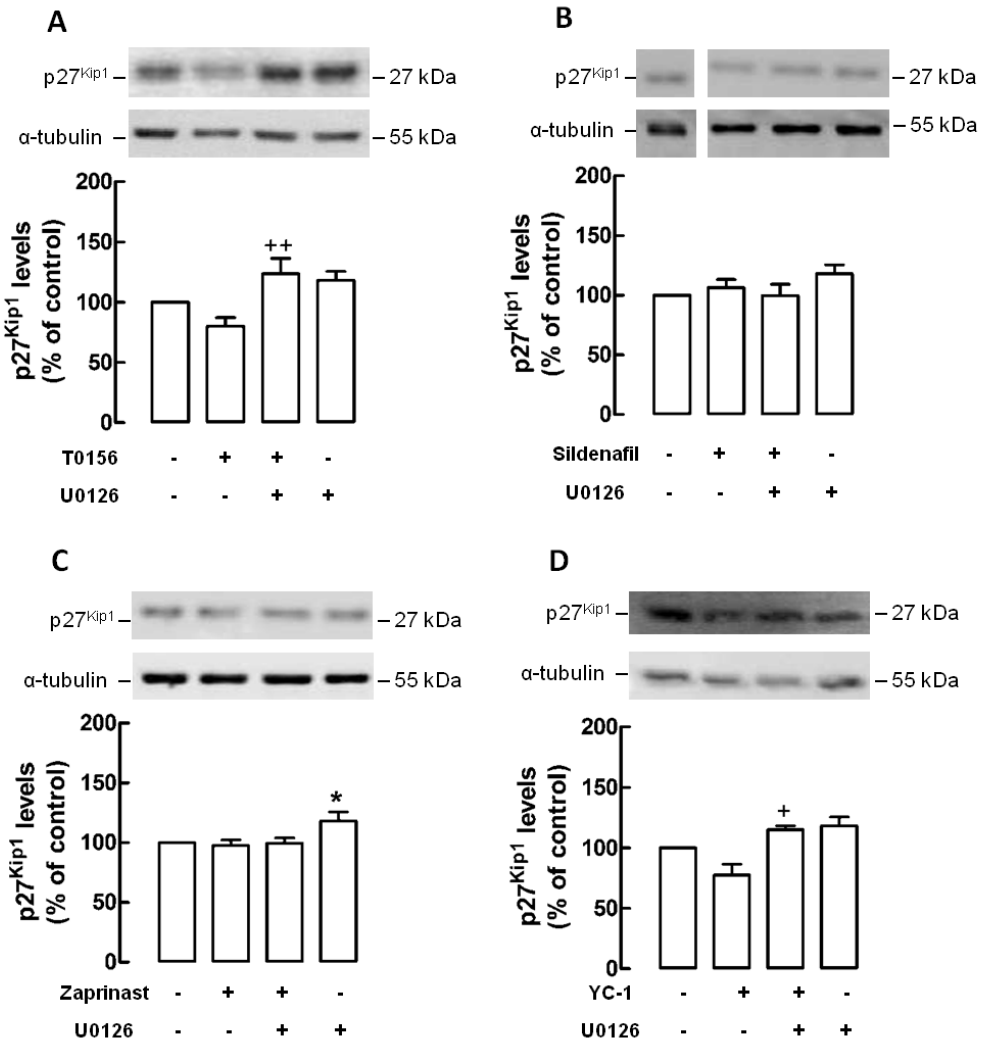

Figure 17. Inhibition of MEK 1/2, by U0126, blocks the decrease of p27^{Kip1} levels mediated by T0156 and sGC. p27^{Kip1} levels following treatment with 1 µM U0126 and 1 µM T0156 (**A**), 1 µM sildenafil (**B)**, 10 µM zaprinast (**C**) or 20 µM YC-1 (**D**) for 2 h were assessed by Western blot. Data are expressed as means ± SEM of at least 6 independent experiments. One-way ANOVA (Bonferroni´s post-test), *p<0.05, significantly different from control, +p<0.01, significantly different from YC-1 and ++p<0.01, significantly different from T0156.

The analysis of the Western blots revealed that U0126 alone increased p27^{Kip1} for levels superior to the baseline (118.0 % ± 7.5 %). Furthermore, the levels of p27^{Kip1} were significantly increased following treatment with U0126 and T0156 (123.5 % ± 13.0 %, p<0.01; Fig. **17A**) and YC-1 (114.9 % ± 3.4 %, p<0.05; Fig. **17D**), comparatively to T0156 (80.0 % ± 7.2 %, p>0.05) and YC-1 (77.5 % ± 9.0 %, p>0.05) alone, respectively.

On the other hand, U0126 did not change the levels of p27^{Kip1} in the samples treated with sildenafil (99.6 % ± 9.6 %, p>0.05; Fig. **17B**) neither zaprinast (99.3 % ± 4.6 %, p>0.05; Fig. **17C**) when compared to sildenafil (106.4 % ± 6.7 %, p>0.05) and zaprinast (97.4 % ± 4.8 %, p>0.05) alone, respectively.

3.3.3.3. PKG inhibition prevents the decrease in p27^{Kip1} levels by YC-1

We next evaluated whether the change in the levels of p27^{Kip1} could be reversed by inhibiting the sGC pathway at the PKG level, following a treatment of 2 h.

Inhibition of PKG did not appear to affect the levels of p27^{Kip1} following treatment with T0156 (84.1 % ± 10.2 %, p>0.05; Fig. **18A**), sildenafil (96.3 % ± 7.8 %, p>0.05; Fig. **18B**) and zaprinast (105.1 % ± 10.0 %, p>0.05; Fig. **18C**), in comparison to T0156 (80.0 % ± 7.2 %, p<0.05), sildenafil (106.4 % ± 6.7 %, p>0.05) and zaprinast (97.4 % ± 4.8 %, p>0.05) alone, respectively. Furthermore, treatment with KT5823 and

YC-1 raises p27^{Kip1} to levels close to basal (104.5 % ± 11.3 %, p>0.05; Fig. **18D**) when compared to YC-1 alone (77.5 % ± 9.0 %, p>0.05).

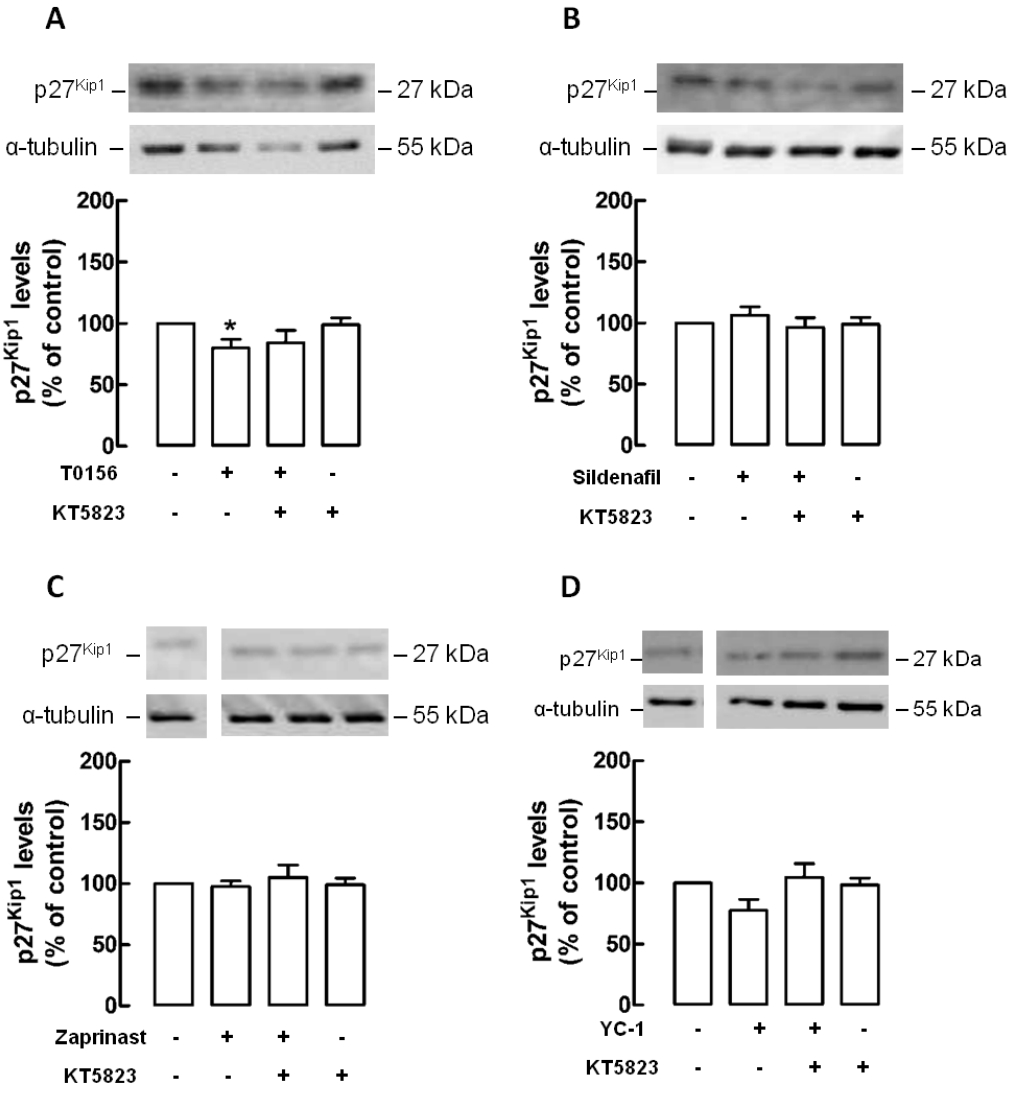

Figure 18. KT5823, inhibitor of PKG, prevents the decrease of p27^{Kip1} levels by YC-1. p27^{Kip1} levels following treatment with 1 µM KT5823 and 1 µM T0156 (**A**), 1 µM sildenafil (**B**), 10 µM zaprinast (**C**) and) or 20 µM YC-1 (**D**) for 2 h were assessed by Western blot. Data are expressed as means ± SEM of at least 6 independent experiments. One-way ANOVA (Bonferroni´s post-test), *p <0.05, significantly different from control.

3.3.4. Phospho-ERK 1/2 and p27^{Kip1} levels were not altered following treatment with sildenafil for longer treatments

As the treatment with sildenafil 1 µM for 2 h did not change the levels of phospho-ERK 1/2 and p27^{Kip1} contrarily to what was observed for T0156 and zaprinast, we decided to test a longer time of exposure. Thus, SVZ cells were treated for 6 h with sildenafil and cell lysates were analysed by Western blot. Accordingly to what was observed for 2 h of treatment, a longer treatment with sildenafil did not show any remarkable differences in the levels of ERK 1/2 phosphorylation (106.8 % ± 8.5 %, p>0.05; Fig. **19A**) neither p27^{Kip1} (91.7 % ± 8.9 %, p>0.05; Fig. **19B**) comparatively to control.

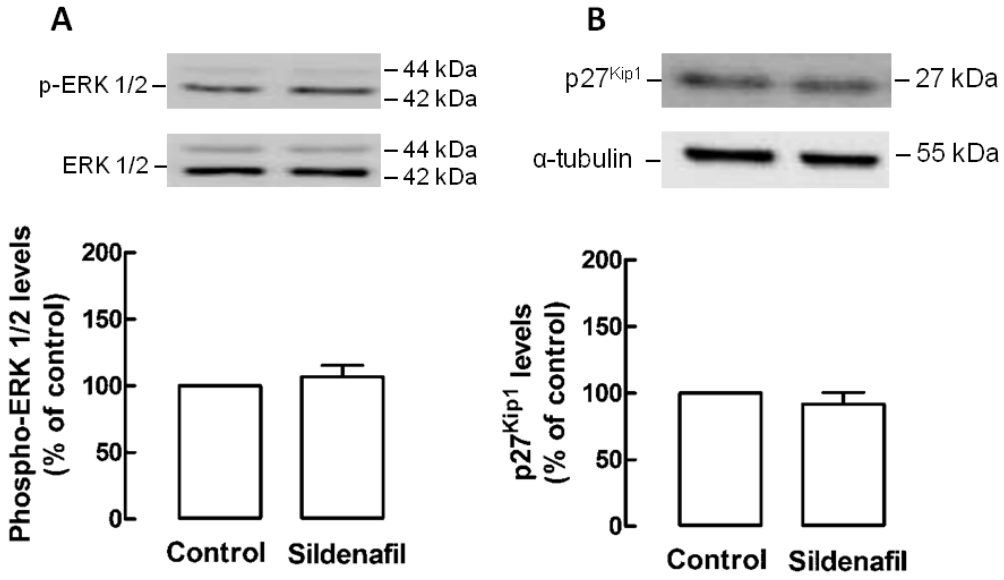

Figure 19. Sildenafil does not change the basal levels of phospho-ERK and p27^{Kip1} following 6 h of treatment. Phospho-ERK 1/2 (**A**) and p27^{Kip1} levels (**B**) following treatment with 1 µM sildenafil for 6 h were assessed by Western blot. Data are expressed as means ± SEM of at least 3 independent experiments. Two-tailed t test.

3.3.5. Ubiquitination of $p27^{Kip1}$ is not changed by PDE5 inhibition

As reported in a previous work from our group, treatment with a $NO^{\bullet}$ donor, 10 µM NOC-18, for 1 h, decreased the levels of $p27^{Kip1}$ in SVZ cells (Carreira *et al.* 2010). Here we tested whether $p27^{Kip1}$ in the SVZ cultures was being tagged with ubiquitin for subsequent degradation in the proteosome. In fact, $p27^{Kip1}$ levels were lower when cells were treated for 1 h with NOC-18 (*Inputs*; 89.0 %) and the levels of ubiquitinated $p27^{Kip1}$ were higher in the presence of NOC-18, as can be seen by an increase in the $p27^{Kip1}$ levels in the *bound* fraction (153.6 %) and a decrease in the *unbound* fraction (91.0 %), in comparison to untreated cells (Fig. **20A**).

A similar experiment was performed for the PDE inhibitors selected. In order to assess whether the results observed above for the levels of $p27^{Kip1}$ were due to protein tagging with ubiquitin for subsequent degradation, similarly to what happens in the presence of NOC-18, a 2 h treatment was applied. We chose this time of treatment as it was the time used in the previous experiments to evaluate the intracellular levels of $p27^{Kip1}$. In fact, the treatment with T0156 substantially decreased $p27^{Kip1}$ levels (*Inputs*; 49.5 %), but the levels of $p27^{Kip1}$ in the *bound* fraction were decreased (46.7 %) and increased in the *unbound* fraction (143.9 %), comparatively to the control (Fig. **20B**).

As for zaprinast and sildenafil, neither of them seemed to alter $p27^{Kip1}$ levels significantly (Fig. **20C**). However, with sildenafil (*Inputs*; 103.8 %), the percentage of $p27^{Kip1}$ bound to ubiquitin substantially decreases (*bound* fraction; *53.5* %) and the levels of unbound $p27^{Kip1}$ remained unaltered (*unbound* fraction; 100.5 %). In the presence of zaprinast (*Inputs*; 88.1 %), the amount of $p27^{Kip1}$ that bind to ubiquitin is similar to the basal (*bound* fraction; 108.0 %) and $p27^{Kip1}$ levels in the *unbound* fraction are slightly increased (139.0 %).

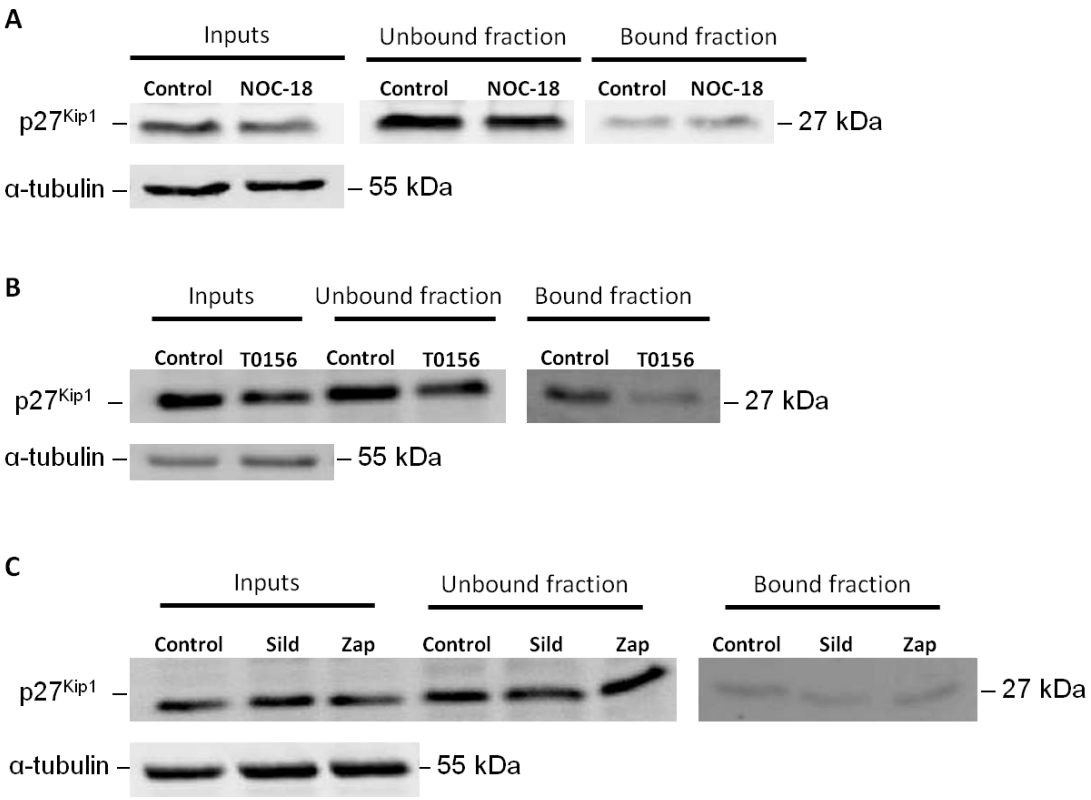

Figure 20. Exposure to NOC-18 and T0156 decreases the intracellular levels of p27^{Kip1} and the levels of ubiquitinated p27^{Kip1} increase following a NOC-18 stimulus. SVZ cells were treated with 10 µM NOC-18 for 1 h or with 1 µM T0156, 1 µM sildenafil, or 10 µM zaprinast for 2 h. *Inputs* refer to the cytosolic levels of p27^{Kip1} and the *unbound* fraction to the p27^{Kip1} that did not bound to agarose beads, the non-ubiquitinated p27^{Kip1}. The *bound* fraction represents the fraction of p27^{Kip1} that is ubiquitinated. Image representative of one experiment. Abbreviations: Sild, sildenafil; Zap, zaprinast.

Chapter 4 Discussion

In this study, we show that the inhibition of the PDE5 stimulates the proliferation of neural stem cells isolated from the SVZ to the same extent following 6 or 24 h of treatment. The three PDE inhibitors used in this work were able to increase proliferation. T0156, the more potent and selective inhibitor of PDE5, was particularly efficient in increasing SVZ cell proliferation. On the one hand, it increases ERK 1/2 activation and, on the other hand, it decreases the levels of the cyclin-dependent kinase inhibitor, $p27^{Kip1}$, responsible for the control of cell cycle progression.

4.1. T0156 stimulates proliferation of SVZ cells

Our analysis of cell proliferation shows a significant increase in the proliferation of NSC following 6 and 24 h of treatment with T0156. The inhibition of the production of cGMP by sGC, with ODQ, blocked this proliferative effect following 6 h of treatment but not upon 24 h. This effect may be due to the action of ODQ in other compensatory mechanisms at that time. Also in the sGC pathway, our observations in the prevention of cell proliferation by inhibition of PKG show that this pathway is effectively activated by T0156. However, inhibiting MEK 1/2, the proliferative effect of T0156 is suppressed at both the times tested, suggesting the involvement of the MAPK pathway on SVZ cell proliferation.

A previous study by our group showed that a cGMP analogue, 8-Br-cGMP, had a similar effect to this of T0156 in increasing SVZ cell proliferation, an effect that was also blocked by U0126 (Carreira *et al.* 2011). Here we show that the direct activation of sGC and thus, increasing cGMP production, has a similar effect in stimulating cell proliferation.

By the evaluation of phospho-ERK 1/2 levels, we show that T0156 and also YC-1 increase ERK 1/2 phosphorylation. These results are in agreement with the previous

observations of 8-Br-cGMP increasing phospho-ERK 1/2 levels (Carreira *et al.* 2011). Moreover, ERK 1/2 phosphorylation is prevented by the administration of the PKG inhibitor, KT5823, suggesting that PKG contributes to ERK 1/2 activation.

$p27^{Kip1}$ is a cyclin-dependent kinase inhibitor that prevents progression from G1 to S-phase, acting as a potent tumor suppressor protein. As previously reported by our group, $p27^{Kip1}$ levels are decreased in the presence of high levels of $NO^{\bullet}$ (Carreira *et al.* 2010). In agreement with this, here we show that inhibiting PDE5 with T0156 also decreases $p27^{Kip1}$ levels and hence, stimulates cell proliferation. Furthermore, exposure to U0126 alone increases $p27^{Kip1}$ to levels above the baseline and this effect is maintained even in the presence of T0156. On the other hand, the inhibition of PKG does not appear to restore basal levels of $p27^{Kip1}$.

Once in the cytosol, $p27^{Kip1}$ is ubiquitinated and, subsequently degraded by the proteosome (Fujita *et al.* 2003, Vlach *et al.* 1997), which allows for cell cycle progression. It had already been shown by our group that the treatment with 10 µM NOC-18 for 1 h significantly decreased the levels of $p27^{Kip1}$ in the SVZ cells (Carreira *et al.* 2010). In fact, we show that this $NO^{\bullet}$-induced decrease of $p27^{Kip1}$ levels is justified by its binding to ubiquitin, which tags $p27^{Kip1}$ for proteosomal degradation. Contrary to these observations, the decrease in the levels of $p27^{Kip1}$ in lysates treated for 2 h with T0156 does not seem to be justified by $p27^{Kip1}$ binding to ubiquitin. However, this could be explained by the time of detection of the levels of $p27^{Kip1}$. In fact, 2 h might be a very long time to test the ubiquitination of this protein and, at that time, $p27^{Kip1}$ may have already been degraded by the proteosome. Therefore, shorter times may be tested, such as 1 h of treatment as it was used for NOC-18. Within this scenario, $p27^{Kip1}$ may possibly be a mediator of the proliferative effect of $NO^{\bullet}$ and T0156.

Together, these results point out for the existence of a crosstalk between sGC and ERK 1/2 pathways. As the proliferative effect is abolished by inhibition of MEK 1/2,

in the MAPK pathway the crosstalk should occur at the level of MEK 1/2 or in an element upstream of it, namely, c-Raf1 or p21Ras.

4.2. Sildenafil stimulates cell proliferation but does not activate ERK 1/2 pathway

As shown in chapter 3, sildenafil significantly increases SVZ cell proliferation following 6 and 24 h of treatment. Similarly to what was observed to T0156, sildenafil proliferative effect was abolished following administration of ODQ, for 6 h, and KT5823 for 6 and 24 h treatment, showing the activation of sGC pathway. On the other hand, U0126 also prevents this effect, with both periods of treatment, suggesting the involvement of MAPK pathway in the enhancement of proliferation by sildenafil.

Despite the increase of EdU incorporation in cells treated with sildenafil and the blockade of this effect by inhibiting MAPK pathway, sildenafil did not increase ERK 1/2 phosphorylation in any of the times tested in this work. This effect goes against what we have observed for the other inhibitors. However, a similar lack of effect of sildenafil on ERK 1/2 phosphorylation had already been described by Wang and colleagues, in 2005. According to that study, sildenafil (300 nM) did not increase phosphorylation of ERK 1/2 following 1 h of treatment (Wang *et al.* 2005). Furthermore, these data suggest that in the SVZ, the increase in the levels of cGMP via the inhibition of PDE5 activity enhance neurogenesis through the PI3-K/Akt pathway, by Akt phosphorylation (Wang *et al.* 2005). However, there are some reports referring to an increase in the phosphorylation of ERK 1/2 and glycogen synthase kinase-3β, an element of the PI3-K/Akt pathway, in the induction of cardioprotection (Das *et al.* 2008) and the increase of ERK 1/2 phosphorylation in cultured endothelial cells (Pyriochou *et al.* 2007), following exposure to sildenafil.

Although this apparent lack of involvement of the ERK 1/2 pathway, we still observed that SVZ cell proliferation stimulated by sildenafil was suppressed by the

inhibition of MEK 1/2. In fact, MEK 1/2 can be indirectly activated by other factors, such as protein kinase A (Ambrosini *et al.* 2000) and protein kinase C (Kawakami *et al.* 2003), which are activated by cAMP. Therefore, sildenafil may possibly be acting by inhibition of a cAMP-hydrolyzing PDE that could indirectly activate MEK 1/2 and thereby increase cell proliferation. Within this scenario, sildenafil may also promote the activation of other pathways, namely the Akt/PI3-K pathway (Wang *et al.* 2005). ERK 1/2 can also be inhibited by other factors involved in other pathways. Thus, further studies on the sildenafil signaling pathways are needed in order to understand the mechanism of enhancement of endogenous neurogenesis, a mechanism that goes beyond the increasing levels of cGMP via the sGC pathway.

Moreover, and contrarily to T0156, in the presence of sildenafil the levels of $p27^{Kip1}$ are unaltered for both times tested in this work, a fact that may be related to the non-activation of ERK 1/2.

4.3. Zaprinast enhances SVZ cell proliferation

In agreement with the data obtained with the other PDE5 inhibitors tested, our results show that zaprinast effectively stimulates proliferation in the SVZ following 6 and 24 h of treatment. A prevention of this effect is observed inhibiting either sGC pathway, with ODQ or KT5823, or MAPK pathway, with U0126, showing that these pathways are both involved in the proliferative effect of zaprinast.

By the evaluation of phospho-ERK 1/2 levels, we show that zaprinast increases ERK 1/2 phosphorylation, an effect that is prevented by the administration of the PKG inhibitor, KT5823.

All these results are in agreement with the observations for T0156, supporting our hypothesis of the existence of a crosstalk between the two pathways. However, contrarily to T0156, the levels of $p27^{Kip1}$ do not decrease following treatment with

zaprinast, which could possibly be due to differences in their mechanisms of action. This point will be discussed in more detail in the next section.

4.4. T0156, sildenafil and zaprinast: Different mechanisms of action?

As introduced in Chapter 1, T0156, zaprinast and sildenafil are three inhibitors with different selectivity and potency for PDE5. Thus, depending on their concentration, they can inhibit other PDE, being one of the more commonly inhibited, the PDE6. PDE6 hydrolyzes cGMP as well as cAMP and is structurally similar to PDE5 (Lugnier 2006). PDE6 inhibition is reported to be responsible for some of the side effects of sildenafil, such as the impaired vision caused by the increase in cGMP levels in the retina (Laties *et al.* 2006). Therefore the interaction with other PDE and, consequently, the activation of other pathways could be pointed out as possible reasons for the different effects observed in this study for the three inhibitors. Also, it would be interesting to investigate which PDE are present in the SVZ cell cultures in order to understand the possible pathways involved.

Nevertheless, T0156, sildenafil and zaprinast have distinct chemical structures (Fig. **4**). Despite their action on PDE, we cannot exclude the hypothesis of these inhibitors act in other pathways, other than the cGMP pathway. In fact, inside the PDE family, these inhibitors can be selective to just one or two isoforms, but they might be able to directly act in other types of enzymes, which may then affect different signaling pathways.

Further studies on the activity and signaling pathways of these PDE5 inhibitors are needed, to clarify their mechanisms of action and, thus, to assess the best targets to enhance neurogenesis.

Chapter 5 Conclusions

5.1. Conclusion

The first aim of this work was to clarify the effect of PDE5 inhibitors on the proliferation of SVZ stem cell cultures. Our data indicate that cell proliferation is enhanced by the inhibition of PDE5 upon 6 and 24 h of treatment.

As a second goal, we proposed to investigate which pathways are activated by the PDE5 inhibitors. Our results point out for a dual activation: on the one hand, the sGC pathway and, on the other hand, the MAPK/ERK 1/2 pathway. Therefore, this study suggests that the effects of the PDE5 inhibitors on NSC proliferation are mediated by both cGMP-dependent and independent mechanisms. Although we suggested the existence of a crosstalk between both pathways, the element at which this interaction occurs is still unknown.

Therapies involving the specific inhibition of PDE5 are being evaluated for the treatment of several diseases. In regard to enhancing endogenous neurogenesis by this strategy, our study helps in better understanding the initial events that promote proliferation of SVZ cells by PDE5 inhibition. Our observations that cell proliferation is stimulated upon treatment with PDE5 inhibitors open many doors to the study of the subjacent mechanisms as well as their effect in the remaining neurogenesis steps.

5.2. Future perspectives

In our study, we suggested that NSC proliferation stimulated by PDE5 inhibition is due to a crosstalk between two or more pathways. Therefore, it would be of interest the study of the elements that mediate this response as well as clarifying whether there are other pathways involved besides the two studied here. For a more complete study of the effect of PDE5 inhibitors on neurogenesis, a similar study to this could be performed in cells from other neurogenic niches, such as the dentate gyrus, in order to assess whether PDE5 inhibition also stimulates cell proliferation in cells from other regions.

Furthermore, it would be useful to confirm that new neurons are effectively formed from the cells that proliferated. Following this, electrophysiological studies should be performed to understand whether the newly formed neurons are able to integrate the pre-existing neuronal network. This will provide us useful information about the functionality of those neurons.

Moreover, *in vivo* studies are needed in order to study the effect of the PDE5 inhibitor administration into a more complex system. The use of an *in vivo* model of brain injury, such as brain ischemia, will provide additional data about the effect of these compounds on the recovery of brain lesions. The proliferation and neuronal differentiation should be analyzed in these models.

Overall, this work may help to identify PDE5 as an interesting therapeutic target to enhance the formation of new brain cells.

Chapter 6 References

Agasse F, Bernardino L, Kristiansen H, Christiansen SH, Ferreira R, Silva B, Grade S, Woldbye DP and Malva JO (2008) Neuropeptide Y promotes neurogenesis in murine subventricular zone. *Stem Cells,* **26,** 1636-1645.

Altman J (1969) Autoradiographic and histological studies of postnatal neurogenesis. IV. Cell proliferation and migration in the anterior forebrain, with special reference to persisting neurogenesis in the olfactory bulb. *J Comp Neurol.,* **137,** 433-457.

Altman J and Das GD (1965) Autoradiographic and Historical Evidence of Postnatal Hippocampal Neurogenesis in Rats. *J. Comp. Neurol.,* **124,** 319-335.

Ambrosini A, Tininini S, Barassi A, Racagni G, Sturani E and Zippel R (2000) cAMP cascade leads to Ras activation in cortical neurons. *Brain Res Mol Brain Res,* **75,** 54-60.

Araujo IM, Ambrosio AF, Leal EC, Santos PF, Carvalho AP and Carvalho CM (2003) Neuronal nitric oxide synthase proteolysis limits the involvement of nitric oxide in kainate-induced neurotoxicity in hippocampal neurons. *J Neurochem,* **85,** 791-800.

Ballard SA, Gingell CJ, Tang K, Turner LA, Price ME and Naylor AM (1998) Effects of sildenafil on the relaxation of human corpus cavernosum tissue in vitro and on the activities of cyclic nucleotide phosphodiesterase isozymes. *J Urol,* **159,** 2164-2171.

Baratti CM and Boccia MM (1999) Effects of sildenafil on long-term retention of an inhibitory avoidance response in mice. *Behav Pharmacol,* **10,** 731-737.

Bender AT and Beavo JA (2004) Specific localized expression of cGMP PDEs in Purkinje neurons and macrophages. *Neurochem Int,* **45,** 853-857.

Bender AT and Beavo JA (2006) Cyclic nucleotide phosphodiesterases: molecular regulation to clinical use. *Pharmacol Rev,* **58,** 488-520.

Bernier PJ, Bedard A, Vinet J, Levesque M and Parent A (2002) Newly generated neurons in the amygdala and adjoining cortex of adult primates. *Proc Natl Acad Sci U S A,* **99,** 11464-11469.

Bischoff E (2004) Potency, selectivity, and consequences of nonselectivity of PDE inhibition. *Int J Impot Res,* **16 Suppl 1,** S11-14.

Blaiss CA, Yu TS, Zhang G, Chen J, Dimchev G, Parada LF, Powell CM and Kernie SG (2011) Temporally specified genetic ablation of neurogenesis impairs cognitive recovery after traumatic brain injury. *J Neurosci,* **31,** 4906-4916.

Boswell-Smith V, Spina D and Page CP (2006) Phosphodiesterase inhibitors. *Br J Pharmacol,* **147 Suppl 1,** S252-257.

Bredesen DE, Rao RV and Mehlen P (2006) Cell death in the nervous system. *Nature,* **443,** 796-802.

Brown J, Cooper-Kuhn CM, Kempermann G, Praag HV, Winkler J, Gage FH and Kuhn HG (2003) Enriched environment and physical activity stimulate hippocampal but not olfactory bulb neurogenesis. *European Journal of Neuroscience,* **17,** 2042-2046.

Cameron HA and Gould E (1994) Adult neurogenesis is regulated by adrenal steroids in the dentate gyrus. *Neuroscience,* **61,** 203-209.

Carlén M, Cassidy RM, Brismar H, Smith GA, Enquist LW and Frisén J (2002) Functional Integration of Adult-Born Neurons. *Current Biology,* **12,** 606-608.

Carreira BP, Morte MI, Inacio A et al. (2010) Nitric oxide stimulates the proliferation of neural stem cells bypassing the epidermal growth factor receptor. *Stem Cells,* **28,** 1219-1230.

Carreira BP, Morte MI, Lourenço AS, Santos AI, Inácio A, Ambrósio AF, Carvalho CM and Araújo IM (2011) Differential contribution of the guanylyl cyclase-cyclic GMP-protein kinase G pathway to the proliferation of neural stem cells stimulated by nitric oxide *(submitted)*.

Chalimoniuk M and Strosznajder JB (1998) Aging modulates nitric oxide synthesis and cGMP levels in hippocampus and cerebellum. Effects of amyloid beta peptide. *Mol Chem Neuropathol,* **35,** 77-95.

Chen J, Magavi SS and Macklis JD (2004) Neurogenesis of corticospinal motor neurons extending spinal projections in adult mice. *Proc Natl Acad Sci U S A,* **101,** 16357-16362.

Cheung WY (1970) Cyclic 3',5'-nucleotide phosphodiesterase : Demonstration of an activator. *Biochemical and Biophysical Research Communications,* **38,** 533-538.

Conover JC, Doetsch F, Garcia-Verdugo JM, Gale NW, Yancopoulos GD and Alvarez-Buylla A (2000) Disruption of Eph/ephrin signaling affects migration and proliferation in the adult subventricular zone. *Nat Neurosci,* **3,** 1091-1097.

Coquil JF, Brunelle G and Guedon J (1985) Occurrence of the methylisobutylxanthine-stimulated cyclic GMP binding protein in various rat tissues. *Biochem Biophys Res Commun,* **127,** 226-231.

Corbin JD (2004) Mechanisms of action of PDE5 inhibition in erectile dysfunction. *Int J Impot Res,* **16 Suppl 1,** S4-7.

Cserep C, Szonyi A, Veres JM, Nemeth B, Szabadits E, de Vente J, Hajos N, Freund TF and Nyiri G (2011) Nitric Oxide Signaling Modulates Synaptic Transmission during Early Postnatal Development. *Cereb Cortex*.

Curtis MA, Kam M, Nannmark U et al. (2007) Human Neuroblasts Migrate to the Olfactory Bulb via a Lateral Ventricular Extension. *Science,* **315,** 1243-1249.

Das A, Xi L and Kukreja RC (2008) Protein kinase G-dependent cardioprotective mechanism of phosphodiesterase-5 inhibition involves phosphorylation of ERK and GSK3beta. *J Biol Chem,* **283,** 29572-29585.

Devan BD, Bowker JL, Duffy KB, Bharati IS, Jimenez M, Sierra-Mercado D, Jr., Nelson CM, Spangler EL and Ingram DK (2006) Phosphodiesterase inhibition by

sildenafil citrate attenuates a maze learning impairment in rats induced by nitric oxide synthase inhibition. *Psychopharmacology (Berl),* **183,** 439-445.

Devan BD, Sierra-Mercado D, Jr., Jimenez M, Bowker JL, Duffy KB, Spangler EL and Ingram DK (2004) Phosphodiesterase inhibition by sildenafil citrate attenuates the learning impairment induced by blockade of cholinergic muscarinic receptors in rats. *Pharmacol Biochem Behav,* **79,** 691-699.

Dong XX, Wang Y and Qin ZH (2009) Molecular mechanisms of excitotoxicity and their relevance to pathogenesis of neurodegenerative diseases. *Acta Pharmacol Sin,* **30,** 379-387.

Duman RS, Malberg J and Nakagawa S (2001) Regulation of Adult Neurogenesis by Psychotropic Drugs and Stress. *Journal of Pharmacology and Experimental Therapeutics,* **299,** 401-407.

Emsley JG, Mitchell BD, Kempermann G and Macklis JD (2005) Adult neurogenesis and repair of the adult CNS with neural progenitors, precursors, and stem cells. *Progress in Neurobiology,* **75,** 321-341.

Enwere E, Shingo T, Gregg C, Fujikawa H, Ohta S and Weiss S (2004) Aging Results in Reduced Epidermal Growth Factor Receptor Signaling, Diminished Olfactory Neurogenesis, and Deficits in Fine Olfactory Discrimination. *J. Neurosci.,* **24,** 8354-8365.

Erceg S, Monfort P, Hernandez-Viadel M, Rodrigo R, Montoliu C and Felipo V (2005) Oral administration of sildenafil restores learning ability in rats with hyperammonemia and with portacaval shunts. *Hepatology,* **41,** 299-306.

Eriksson PS, Perfilieva E, Bjork-Eriksson T, Alborn A-M, Nordborg C, Peterson DA and Gage FH (1998) Neurogenesis in the adult human hippocampus. *Nat Med,* **4,** 1313-1317.

Essayan DM (2001) Cyclic nucleotide phosphodiesterases. *J Allergy Clin Immunol,* **108,** 671-680.

Forman MS, Trojanowski JQ and Lee VM-Y (2004) Neurodegenerative diseases: a decade of discoveries paves the way for therapeutic breakthroughs. *Nature Medicine,* **10,** 1055 - 1063.

Fujita N, Sato S and Tsuruo T (2003) Phosphorylation of p27Kip1 at threonine 198 by p90 ribosomal protein S6 kinases promotes its binding to 14-3-3 and cytoplasmic localization. *J Biol Chem,* **278,** 49254-49260.

Garthwaite J, Charles SL and Chess-Williams R (1988) Endothelium-derived relaxing factor release on activation of NMDA receptors suggests role as intercellular messenger in the brain. *Nature,* **336,** 385-388.

Gould E, Beylin A, Tanapat P, Reeves A and Shors TJ (1999) Learning enhances adult neurogenesis in the hippocampal formation. *Nat Neurosci,* **2,** 260-265.

Herczenik E and Gebbink MFBG (2008) Molecular and cellular aspects of protein misfolding and disease. *FASEB J.,* **22,** 2115-2133.

Hermann A, Maisel M, Wegner F, Liebau S, Kim DW, Gerlach M, Schwarz J, Kim KS and Storch A (2006) Multipotent neural stem cells from the adult tegmentum with dopaminergic potential develop essential properties of functional neurons. *Stem Cells,* **24,** 949-964.

Ignarro LJ (1991) Signal transduction mechanisms involving nitric oxide. *Biochem Pharmacol,* **41,** 485-490.

Im SH, Yu JH, Park ES, Lee JE, Kim HO, Park KI, Kim GW, Park CI and Cho SR (2010) Induction of striatal neurogenesis enhances functional recovery in an adult animal model of neonatal hypoxic-ischemic brain injury. *Neuroscience,* **169,** 259-268.

Jellinger K (2003) General aspects of neurodegeneration. *J Neural Transm Suppl,* **65,** 101-144.

Jellinger KA (2009) Recent advances in our understanding of neurodegeneration. *Journal of Neural Transmission,* **116,** 1111-1162.

Jin K, Sun Y, Xie L, Batteur S, Mao XO, Smelick C, Logvinova A and Greenberg DA (2003) Neurogenesis and aging: FGF-2 and HB-EGF restore neurogenesis in hippocampus and subventricular zone of aged mice. *Aging Cell,* **2,** 175-183.

Kahn RA, Weinberger J, Brannan T, Prikhojan A and Reich DL (1995) Nitric oxide modulates dopamine release during global temporary cerebral ischemia. *Anesth Analg,* **80,** 1116-1121.

Kawakami Y, Kitaura J, Yao L et al. (2003) A Ras activation pathway dependent on Syk phosphorylation of protein kinase C. *Proc Natl Acad Sci U S A,* **100,** 9470-9475.

Kee NJ, Preston E and Wojtowicz JM (2001) Enhanced neurogenesis after transient global ischemia in the dentate gyrus of the rat. *Experimental Brain Research,* **136,** 313-320.

Kempermann G, Kuhn HG and Gage FH (1997) More hippocampal neurons in adult mice living in an enriched environment. *Nature,* **386,** 493-495.

Kerwin JF, Jr., Lancaster JR, Jr. and Feldman PL (1995) Nitric oxide: a new paradigm for second messengers. *J Med Chem,* **38,** 4343-4362.

Kotera J, Fujishige K, Imai Y, Kawai E, Michibata H, Akatsuka H, Yanaka N and Omori K (1999) Genomic origin and transcriptional regulation of two variants of cGMP-binding cGMP-specific phosphodiesterases. *Eur J Biochem,* **262,** 866-873.

Kuhn H, Dickinson-Anson H and Gage F (1996) Neurogenesis in the dentate gyrus of the adult rat: age-related decrease of neuronal progenitor proliferation. *J. Neurosci.,* **16,** 2027-2033.

Laties A and Sharlip I (2006) Ocular safety in patients using sildenafil citrate therapy for erectile dysfunction. *J Sex Med,* **3,** 12-27.

Lau A and Tymianski M (2010) Glutamate receptors, neurotoxicity and neurodegeneration. *Pflugers Arch,* **460,** 525-542.

Lewis GD and Semigran MJ (2004) Type 5 phosphodiesterase inhibition in heart failure and pulmonary hypertension. *Curr Heart Fail Rep,* **1,** 183-189.

Lewis PD (1968) A quantitative study of cell proliferation in the subependymal layer of the adult rat brain. *Experimental Neurology,* **20,** 203-207.

Lie DC, Song H, Colamarino SA, Ming G-l and Gage FH (2004) Neurogenesis in the Adult Brain: New Strategies for Central Nervous System Diseases. *Annual Review of Pharmacology and Toxicology,* **44,** 399-421.

Lin CS, Lau A, Tu R and Lue TF (2000) Expression of three isoforms of cGMP-binding cGMP-specific phosphodiesterase (PDE5) in human penile cavernosum. *Biochem Biophys Res Commun,* **268,** 628-635.

Lincoln TM, Hall CL, Park CR and Corbin JD (1976) Guanosine 3':5'-cyclic monophosphate binding proteins in rat tissues. *Proc Natl Acad Sci U S A,* **73,** 2559-2563.

Lossinsky AS and Shivers RR (2004) Structural pathways for macromolecular and cellular transport across the blood-brain barrier during inflammatory conditions. Review. *Histol Histopathol,* **19,** 535-564.

Loughney K, Hill TR, Florio VA et al. (1998) Isolation and characterization of cDNAs encoding PDE5A, a human cGMP-binding, cGMP-specific 3',5'-cyclic nucleotide phosphodiesterase. *Gene,* **216,** 139-147.

Lugnier C (2006) Cyclic nucleotide phosphodiesterase (PDE) superfamily: a new target for the development of specific therapeutic agents. *Pharmacol Ther,* **109,** 366-398.

Magavi SS, Leavitt BR and Macklis JD (2000) Induction of neurogenesis in the neocortex of adult mice. *Nature,* **405,** 951-955.

Matarredona ER, Murillo-Carretero M, Moreno-López B and Estrada C (2004) Nitric oxide synthesis inhibition increases proliferation of neural precursors isolated from the postnatal mouse subventricular zone. *Brain Research,* **995,** 274-284.

Matarredona ER, Murillo-Carretero M, Moreno-López B and Estrada C (2005) Role of nitric oxide in subventricular zone neurogenesis. *Brain Research Reviews,* **49,** 355-366.

Ming G-l and Song H (2005) Adult Neurogenesis in the Mammalian Central Nervous System. *Annual Review of Neuroscience,* **28,** 223-250.

Mochida H, Takagi M, Inoue H et al. (2002) Enzymological and pharmacological profile of T-0156, a potent and selective phosphodiesterase type 5 inhibitor. *Eur J Pharmacol,* **456,** 91-98.

Moncada S, Palmer RM and Higgs EA (1989) Biosynthesis of nitric oxide from L-arginine. A pathway for the regulation of cell function and communication. *Biochem Pharmacol,* **38,** 1709-1715.

Moreno-Lopez B, Romero-Grimaldi C, Noval JA, Murillo-Carretero M, Matarredona ER and Estrada C (2004) Nitric Oxide Is a Physiological Inhibitor of Neurogenesis in the Adult Mouse Subventricular Zone and Olfactory Bulb. *J. Neurosci.,* **24,** 85-95.

Murad F (1994a) The nitric oxide-cyclic GMP signal transduction system for intracellular and intercellular communication. *Recent Prog Horm Res,* **49,** 239-248.

Murad F (1994b) Regulation of cytosolic guanylyl cyclase by nitric oxide: the NO-cyclic GMP signal transduction system. *Adv Pharmacol.,* **26,** 19-33.

Nakatomi H, Kuriu T, Okabe S, Yamamoto S-i, Hatano O, Kawahara N, Tamura A, Kirino T and Nakafuku M (2002) Regeneration of Hippocampal Pyramidal Neurons after Ischemic Brain Injury by Recruitment of Endogenous Neural Progenitors. *Cell,* **110,** 429-441.

Nilsson M, Perfilieva E, Johansson U, Orwar O and Eriksson PS (1999) Enriched environment increases neurogenesis in the adult rat dentate gyrus and improves spatial memory. *Journal of Neurobiology,* **39,** 569-578.

Parent JM (2003) Injury-Induced Neurogenesis in the Adult Mammalian Brain. *Neuroscientist,* **9,** 261-272.

Parent JM, Vexler ZS, Gong C, Derugin N and Ferriero DM (2002) Rat forebrain neurogenesis and striatal neuron replacement after focal stroke. *Ann Neurol,* **52,** 802-813.

Parent JM, Yu TW, Leibowitz RT, Geschwind DH, Sloviter RS and Lowenstein DH (1997) Dentate Granule Cell Neurogenesis Is Increased by Seizures and Contributes to Aberrant Network Reorganization in the Adult Rat Hippocampus. *J. Neurosci.,* **17,** 3727-3738.

Patel MD and Katz SD (2005) Phosphodiesterase 5 inhibition in chronic heart failure and pulmonary hypertension. *Am J Cardiol,* **96,** 47M-51M.

Pifarre P, Prado J, Baltrons MA, Giralt M, Gabarro P, Feinstein DL, Hidalgo J and Garcia A (2011) Sildenafil (Viagra) ameliorates clinical symptoms and neuropathology in a mouse model of multiple sclerosis. *Acta Neuropathol,* **121,** 499-508.

Prickaerts J, de Vente J, Honig W, Steinbusch HW and Blokland A (2002a) cGMP, but not cAMP, in rat hippocampus is involved in early stages of object memory consolidation. *Eur J Pharmacol,* **436,** 83-87.

Prickaerts J, Sik A, van der Staay FJ, de Vente J and Blokland A (2005) Dissociable effects of acetylcholinesterase inhibitors and phosphodiesterase type 5 inhibitors on object recognition memory: acquisition versus consolidation. *Psychopharmacology (Berl),* **177,** 381-390.

Prickaerts J, Sik A, van Staveren WC, Koopmans G, Steinbusch HW, van der Staay FJ, de Vente J and Blokland A (2004) Phosphodiesterase type 5 inhibition improves early memory consolidation of object information. *Neurochem Int,* **45,** 915-928.

Prickaerts J, van Staveren WC, Sik A, Markerink-van Ittersum M, Niewohner U, van der Staay FJ, Blokland A and de Vente J (2002b) Effects of two selective phosphodiesterase type 5 inhibitors, sildenafil and vardenafil, on object recognition memory and hippocampal cyclic GMP levels in the rat. *Neuroscience,* **113,** 351-361.

Privat A and Leblond C (1972) The subependymal layer and neighboring region in the brain of the young rat. *J Comp Neurol.,* **146,** 277-302.

Pyriochou A, Zhou Z, Koika V, Petrou C, Cordopatis P, Sessa WC and Papapetropoulos A (2007) The phosphodiesterase 5 inhibitor sildenafil stimulates angiogenesis through a protein kinase G/MAPK pathway. *J Cell Physiol,* **211,** 197-204.

Reif A, Schmitt A, Fritzen S et al. (2004) Differential effect of endothelial nitric oxide synthase (NOS-III) on the regulation of adult neurogenesis and behaviour. *Eur J Neurosci,* **20,** 885-895.

Rosen RC and Kostis JB (2003) Overview of phosphodiesterase 5 inhibition in erectile dysfunction. *Am J Cardiol,* **92,** 9M-18M.

Rutten K, Vente JD, Sik A, Ittersum MM, Prickaerts J and Blokland A (2005) The selective PDE5 inhibitor, sildenafil, improves object memory in Swiss mice and increases cGMP levels in hippocampal slices. *Behav Brain Res,* **164,** 11-16.

Sabayan B, Zamiri N and Farshchizarabi S (2010) Phoshphodiesterase-5 inhibitors: novel weapons against Alzheimer's disease? *Int J Neurosci,* **120,** 746-751.

Selkoe DJ (2004) Cell biology of protein misfolding: The examples of Alzheimer's and Parkinson's diseases. *Nat Cell Biol,* **6,** 1054-1061.

Shimizu-Albergine M, Rybalkin SD, Rybalkina IG, Feil R, Wolfsgruber W, Hofmann F and Beavo JA (2003) Individual cerebellar Purkinje cells express different cGMP phosphodiesterases (PDEs): in vivo phosphorylation of cGMP-specific

PDE (PDE5) as an indicator of cGMP-dependent protein kinase (PKG) activation. *J Neurosci,* **23,** 6452-6459.

Singh N and Parle M (2003) Sildenafil improves acquisition and retention of memory in mice. *Indian J Physiol Pharmacol,* **47,** 318-324.

Skovronsky DM, Lee VM-Y and Trojanowski JQ (2006) Neurodegenerative Diseases: New Concepts of Pathogenesis and Their Therapeutic Implications. *Annual Review of Pathology: Mechanisms of Disease,* **1,** 151-170.

St-John WM (1998) Neurogenesis of patterns of automatic ventilatory activity. *Prog Neurobiol,* **56,** 97-117.

Steiner MK, Preston IR, Klinger JR and Hill NS (2005) Pulmonary hypertension: inhaled nitric oxide, sildenafil and natriuretic peptides. *Curr Opin Pharmacol,* **5,** 245-250.

Takimoto E, Champion HC, Li M et al. (2005) Chronic inhibition of cyclic GMP phosphodiesterase 5A prevents and reverses cardiac hypertrophy. *Nat Med,* **11,** 214-222.

Taupin P (2008) Adult neurogenesis, neuroinflammation and therapeutic potential of adult neural stem cells. *Int J Med Sci.,* **5,** 127-132.

Tegenge MA, Stern M and Bicker G (2009) Nitric oxide and cyclic nucleotide signal transduction modulates synaptic vesicle turnover in human model neurons. *J Neurochem,* **111,** 1434-1446.

Torroglosa A, Murillo-Carretero M, Romero-Grimaldi C, Matarredona ER, Campos-Caro A and Estrada C (2007) Nitric Oxide Decreases Subventricular Zone Stem Cell Proliferation by Inhibition of Epidermal Growth Factor Receptor and Phosphoinositide-3-Kinase/Akt Pathway. *Stem Cells,* **25,** 88-97.

Turko IV, Ballard SA, Francis SH and Corbin JD (1999) Inhibition of cyclic GMP-binding cyclic GMP-specific phosphodiesterase (Type 5) by sildenafil and related compounds. *Mol Pharmacol,* **56,** 124-130.

Uthayathas S, Karuppagounder SS, Thrash BM, Parameshwaran K, Suppiramaniam V and Dhanasekaran M (2007) Versatile effects of sildenafil: recent pharmacological applications. *Pharmacol Rep,* **59,** 150-163.

van Praag H, Christie BR, Sejnowski TJ and Gage FH (1999a) Running enhances neurogenesis, learning, and long-term potentiation in mice. *Proceedings of the National Academy of Sciences of the United States of America,* **96,** 13427-13431.

van Praag H, Kempermann G and Gage FH (1999b) Running increases cell proliferation and neurogenesis in the adult mouse dentate gyrus. *Nature Neuroscience,* **2,** 266 - 270.

van Staveren WC, Glick J, Markerink-van Ittersum M, Shimizu M, Beavo JA, Steinbusch HW and de Vente J (2002) Cloning and localization of the cGMP-specific phosphodiesterase type 9 in the rat brain. *J Neurocytol,* **31,** 729-741.

Vlach J, Hennecke S and Amati B (1997) Phosphorylation-dependent degradation of the cyclin-dependent kinase inhibitor p27. *EMBO J,* **16,** 5334-5344.

Wallis RM, Corbin JD, Francis SH and Ellis P (1999) Tissue distribution of phosphodiesterase families and the effects of sildenafil on tissue cyclic nucleotides, platelet function, and the contractile responses of trabeculae carneae and aortic rings in vitro. *Am J Cardiol,* **83,** 3C-12C.

Wang L, Gang Zhang Z, Lan Zhang R and Chopp M (2005) Activation of the PI3-K/Akt pathway mediates cGMP enhanced-neurogenesis in the adult progenitor cells derived from the subventricular zone. *J Cereb Blood Flow Metab,* **25,** 1150-1158.

Whitney NP, Eidem TM, Peng H, Huang Y and Zheng JC (2009) Inflammation mediates varying effects in neurogenesis: relevance to the pathogenesis of brain injury and neurodegenerative disorders. *Journal of Neurochemistry,* **108,** 1343-1359.

Woulfe JM (2007) Abnormalities of the nucleus and nuclear inclusions in neurodegenerative disease: a work in progress. *Neuropathology and Applied Neurobiology,* **33,** 2-42.

Yamamoto S, Nagao M, Sugimori M et al. (2001a) Transcription factor expression and Notch-dependent regulation of neural progenitors in the adult rat spinal cord. *J Neurosci,* **21,** 9814-9823.

Yamamoto S, Yamamoto N, Kitamura T, Nakamura K and Nakafuku M (2001b) Proliferation of parenchymal neural progenitors in response to injury in the adult rat spinal cord. *Exp Neurol,* **172,** 115-127.

Yanamadala V and Friedlander RM (2010) Complement in neuroprotection and neurodegeneration. *Trends in Molecular Medicine,* **16,** 69-76.

Zhang F, White JG and Iadecola C (1994) Nitric oxide donors increase blood flow and reduce brain damage in focal ischemia: evidence that nitric oxide is beneficial in the early stages of cerebral ischemia. *J Cereb Blood Flow Metab,* **14,** 217-226.

Zhang L, Zhang RL, Wang Y, Zhang C, Zhang ZG, Meng H and Chopp M (2005) Functional recovery in aged and young rats after embolic stroke: treatment with a phosphodiesterase type 5 inhibitor. *Stroke,* **36,** 847-852.

Zhang L, Zhang Z, Zhang RL, Cui Y, LaPointe MC, Silver B and Chopp M (2006a) Tadalafil, a long-acting type 5 phosphodiesterase isoenzyme inhibitor, improves neurological functional recovery in a rat model of embolic stroke. *Brain Res,* **1118,** 192-198.

Zhang R, Wang L, Zhang L, Chen J, Zhu Z, Zhang Z and Chopp M (2003) Nitric oxide enhances angiogenesis via the synthesis of vascular endothelial growth factor and cGMP after stroke in the rat. *Circ Res,* **92,** 308-313.

Zhang R, Wang Y, Zhang L, Zhang Z, Tsang W, Lu M and Chopp M (2002) Sildenafil (Viagra) induces neurogenesis and promotes functional recovery after stroke in rats. *Stroke,* **33,** 2675-2680.

Zhang RL, Zhang Z, Zhang L, Wang Y, Zhang C and Chopp M (2006b) Delayed treatment with sildenafil enhances neurogenesis and improves functional recovery in aged rats after focal cerebral ischemia. *J Neurosci Res,* **83,** 1213-1219.

Zhao M, Momma S, Delfani K et al. (2003) Evidence for neurogenesis in the adult mammalian substantia nigra. *Proc Natl Acad Sci U S A,* **100,** 7925-7930.