Bruno Pereira Carreira

Proliferative Mechanisms Controlled by Nitric Oxide in Neural Stem Cells

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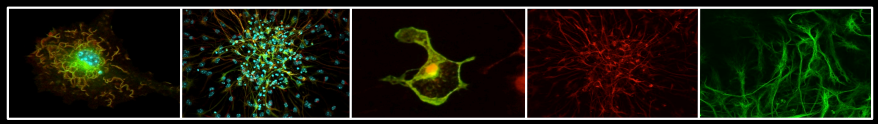

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Regulação dos mecanismos de proliferação de células estaminais neurais pelo óxido nítrico

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Bruno Pereira Carreira

Dissertação apresentada à Faculdade de Ciências e Tecnologia da Universidade de Coimbra, para prestação de provas de doutoramento em Biologia, na especialidade de Biologia Celular.

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FCT Fundação para a Ciência e a Tecnologia
MINISTÉRIO DA CIÊNCIA, TECNOLOGIA E ENSINO SUPERIOR

Aos meus Pais,
ao meu irmão
à Inês.

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Abbreviations

7-AAD	7-Amino-actinomycin
AD	Alzheimer's disease
a.f.u.	Arbitrary fluorescence units
AG1478	N-(3-chlorophenyl)-6,7-dimethoxy-4-quinazolinamine
Aβ	β-amyloid peptide
ANOVA	Analysis of variance
APP	Amyloid precursor protein
bFGF	Basic fibroblast growth factor
BBB	Blood-brain barrier
BCA	Bicinchoninic acid
BDNF	Brain-derived neurotrophic factor
BH4	Tetrahydrobiopteridin
BSA	Bovine serum albumin
BrdU	5-bromo-2'-deoxyuridine
8-Br-cGMP	8-bromoguanosine 3',5'-cyclic monophosphate
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
CLAP	Chemystatin, leupeptin, antiparin, pepstatin A (cocktail of protease inhibitors)
CNS	Central nervous system
CREB	cAMP responsive element-binding
CSF	Cerebrospinal fluid
COX-2	Cyclooxygenase-2
DCX	Doublecortin
DG	Dentate gyrus
DIV	Days *in vitro*
DTT	Dithiothreitol
eNOS	Endothelial nitric oxide synthase
ECF	Enhanced chemifluorescence

EdU	5-ethynyl-2'-deoxyuridine
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ES	Embryonic stem
ERK	Extracellular signal-regulated kinase
FAD	Flavin adenine dinucleotide
FeTMPyP	Fe(III)tetrakis(1-methyl-4pyridyl)porphyrin pentachlorideporphyrin pentachloride
FMN	Flavin mononucleotide
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDNF	Glial cell-line derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
HBSS	Hank's balanced salt solution
HD	Huntington's disease
HO-1	Heme-oxygenase-1
IGF-1	Insulin-like growth factor
IFN-γ	Interferon-gamma
IL	Interleukin
iNOS	Inducible nitric oxide synthase
iPS	Induced pluripotent stem
KA	Kainic acid
KT5823	1,2-Dihydro-2-[(2-methyl-4-pyridinyl)methyl]-1-oxo-8-(2-pyrimidinylmethoxy)-4-(3,4,5-trimethoxyphenyl)-2,7-naphthyridine-3-carboxylic acid methyl ester hydrochloride 2,3,9,10,11,12-hexahydro-10R-methoxy-2,9-dimethyl-1-oxo-9S,12R-epoxy-1H-diindolo[1,2,3-fg:3',2',1'-kl]pyrrolo[3,4-i][1,6]benzodiazocine-10-carboxylic acid, methyl ester
L-NAME	N^{ω}-nitro-L-arginine methyl ester
LPS	Lipopolysaccharide
LV	Lateral ventricle

MAPK	Mitogen-activated protein kinase
M-CSF	Macrophage-colony stimulating factor
MCAO	Middle cerebral artery occlusion
MnTBAP	Mn(III)tetrakis(4-benzoic acid)porphyrin chloride
NFκB	Nuclear factor κB
NeuN	Neuronal nuclei
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOC-18	DETA/NONOate, (Z)-1-[2-(2-Aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate
NOS	Nitric oxide synthase
NSAIDS	Nonsteroidal anti-inflammatory drugs
NSCs	Neural stem cells
3-NT	3-nitrotyrosine
OB	Olfactory bulb
O_2^-	Superoxide anion
ODQ	1*H*-[1,2,4]Oxadiozolo[4,3-a]quinoxalin-1-one
$ONOO^-$	Peroxynitrite
PBS	Phosphate buffered saline
PD	Parkinson's disease
PDE	Phosphodiesterase
PGE2	Prostaglandin E2
PMSF	Phenylmethylsulphonyl fluoride
PSA-NCAM	Polysialic acid-neural cell adhesion molecule
PKG	Protein kinase G
PVDF	Polyvinylidene difluoride
p90RSK	p90 ribosomal S6 kinase
RMS	Rostral migratory stream
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SDF-1α	Stromal cell-derived factor-1 alpha

SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SE	*Status epilepticus*
SEM	Standard error of the mean
sGC	Soluble guanylyl cyclase
SGZ	Subgranular zone
SVZ	Subventricular zone
TBI	Traumatic brain injury
TBS	Tris buffered saline
TBS-T	Tris buffered saline with Tween-20
T0156	1,2-Dihydro-2-[(2-methyl-4-pyridinyl)methyl]-1-oxo-8-(2-pyrimidinylmethoxy)-4-(3,4,5-trimethoxyphenyl)-2,7-naphthyridine-3-carboxylic acid methyl ester hydrochloride
TGF-α	Transforming growth factor alpha
TLE	Temporal lobe epilepsy
TLRs	Toll-like receptors
TNF-α	Tumor necrosis factor alpha
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling
U0126	1,4-Diamino-2,3-dicyano-1,4-bis(o-aminophenylmercapto)butadiene monoethanolate
VEGF	Vascular endothelial growth factor

Publications

Most of the work presented in this dissertation is published or are submitted for publication in peer-reviewed scientific journals, as follows:

1. Bruno P. Carreira, Maria Inês Morte, Ângela Inácio, Gabriel Costa, Joana Rosmaninho-Salgado, Fabienne Agasse, Anália Carmo, Patrícia Couceiro, Patrik Brundin, António F. Ambrósio, Caetana M. Carvalho, Inês M. Araújo. (2010) "Nitric oxide stimulates the proliferation of neural stem cells bypassing the epidermal growth factor receptor." *Stem Cells* 28(7): 1219-30.

2. Bruno P. Carreira, Maria Inês Morte, Ana Sofia Lourenço, Ângela Inácio, António F. Ambrósio, Caetana M. Carvalho, Inês M. Araújo. (2010) “Nitric oxide increases the proliferation of neural stem cells via the guanylyl-cyclase-cyclic GMP-protein kinase G pathway.” (under revision)

3. Bruno P. Carreira, Maria Inês Morte, António F. Ambrósio, Caetana M. Carvalho, Inês M. Araújo. (2011) “Nitric oxide from microglia origin impairs neural stem cell proliferation via nitration of the epidermal growth factor receptor.”(*submitted*)

Summary

Neural stem cells proliferate in the adult central nervous system (CNS) in two main regions, the subgranular zone (SGZ) of the dentate gyrus (DG) of the hippocampus and the subventricular zone (SVZ) of the lateral ventricles. The finding that neural stem cells are able to divide, migrate and differentiate into several cellular types raised a new hope for restorative neurology. Nitric oxide (NO), a pleiotropic signaling molecule in the CNS has been described to be able to modulate the proliferation of neural stem cells, but whether it acts as a pro- or anti-proliferative agent is still controversial. Some evidence suggests that NO is a physiological inhibitor of cell proliferation. However, under certain conditions, NO can act as a proliferative agent, favoring cell proliferation. Thus, targeting the NO system may be a powerful strategy to control cell proliferation/differentiation. However, the exact mechanisms by which NO regulates neuronal proliferation and differentiation are not yet clarified, and further investigation on this matter is needed.

Therefore, the main goal of this work was to study the mechanisms that are involved in the dual effect of NO in neural stem cell proliferation. Cultures of neural stem cells isolated from the SVZ of mice were exposed to a NO donor, in a range of concentrations comprehending both physiological and pathophysiological concentrations. We found that depending on the concentration, NO can have opposite effects on the proliferation of neural stem cells. Relatively low levels of NO, but already considered in the pathopysiological range, increased the proliferation of neural stem cells, while much higher levels of NO reduced proliferation. Very likely, different mechanisms are responsible for this dual effect of NO on neural stem cell proliferation.

The proliferative effect of NO in neural stem cells was further investigated, and the underlying mechanisms involved were shown to be dependent on the activation of the mitogen-activated protein kinase (MAPK) ERK1/2 pathway. We observed that NO rapidly bypasses the epidermal

growth factor (EGF) receptor (EGFR) and directly activates p21Ras, as early as 2 min after exposure to NOC-18, triggering cell proliferation via activation of the ERK/MAPK pathway. Moreover, the activation of the ERK/MAPK pathway was shown to be involved in the activation of transcription factors, particularly c-myc, p90RSK and Elk-1. Indeed, activation of p90RSK resulted in a decrease in the nuclear presence of the cyclin-dependent kinase inhibitor 1, $p27^{KIP1}$, which allows for cell cycle progression.

On the other hand, since the main intracellular receptor for NO is guanylyl cyclase, we investigated whether the proliferative effect of NO was mediated by cyclic GMP. We found that short-term exposure of neural stem cell cultures to NO (6 h) increased cell proliferation in a cGMP-independent manner by activating the ERK/MAPK signaling pathway, while long-term exposure to NO (24 h) activated independently the two signaling pathways, MAPK/ERK and the soluble guanylyl cyclase/cyclic GMP/protein kinase G.

Concerning the antiproliferative effect of NO, we found that the release of high concentrations of NO by NOC-18 caused the nitration of the EGF receptor, with intermediate formation of peroxynitrite, in SVZ-derived neural stem cells expressing EGFR. Concomitantly with increased nitration in tyrosine residues, NO caused a decrease in the phosphorylation status of the EGFR. Moreover, using a culture model of SVZ-derived stem cells mixed with microglia isolated from wild-type mice ($iNOS^{+/+}$), similar results were obtained. Thus the increased release of NO by activated $iNOS^{+/+}$ microglial cells, following treatment with LPS plus IFN-γ, caused nitration of EGFR, an irreversible post-translational modification of tyrosine residues, which parallels the decrease in proliferation of SVZ-derived neural stem cells treated with the inflammatory stimulation. In addition, cells expressing EGF receptor showed a strong labeling for 3-nitrotyrosine, indicative of protein nitration, following treatment with NOC-18. MnTBAP, a scavenger of superoxide, was able to prevent the nitration of the EGFR, as well as increased its phosphorylation status. Furthermore, either MnTBAP, or FeTMPyP, which promotes the

degradation of peroxynitrite, were able to rescue the proliferation of neural stem cells in $iNOS^{+/+}$mixed cultures following inflammation.

Finally, using an *in vivo* model of injury-induced neuroinflammation and neurogenesis, the kainic acid model of temporal lobe epilepsy, we showed that cell proliferation is prevented when the production of NO is abolished by deleting the iNOS gene, in $iNOS^{-/-}$ mice, which strongly suggests that NO promotes neural stem cell proliferation under certain pathophysiological conditions *in vivo.*

Overall, the results presented in this work clarify the mechanisms by which NO regulates the proliferation of neural stem cells. Thus, we show for the first time that supraphysiological levels of NO have a dual effect on neural stem cell proliferation, with different signaling mechanisms. Thus, the p21/ERK/MAPK signaling pathway appears to be involved in the rapid effect of NO in promoting cell cycle progression and early cell proliferation. For a longer exposure to NO, two independent pathways appear to be active: the p21/ERK/MAPK, and also the guanylyl cyclase/cGMP/PKG. Much higher levels of NO administered to SVZ-derived stem cell cultures or in SVZ-microglia mixed cultures, have an antiproliferative effect by decreasing the signaling through the EGFR due to nitration of tyrosine residues in this receptor.

Based on our work in the animal model and in cell cultures, our data suggests that NO from inflammatory origin is mostly proliferative. This study suggests that the modulation of the nitrergic system may be useful to harness the potential of endogenous neural stem cells for brain repair.

Resumo

A proliferação de células estaminais neurais é um processo que ocorre no sistema nervoso central (CNS) adulto em duas regiões em particular: na zona subgranular (SGZ) do girus dentado (DG) do hipocampo e na zona subventricular (SVZ) que delimita os ventrículos cerebrais laterais. A descoberta de algumas propriedades das células estaminais neurais, como a capacidade proliferativa e de diferenciação em vários tipos celulares, trouxeram novas perspectivas para a terapia neuro-restaurativa. Portanto, o conhecimento dos mecanismos de regulação destas células constitui um foco de interesse da comunidade científica. O monóxido de azoto, também designado de óxido nítrico (NO), é uma molécula gasosa que intervém na sinalização de ínumeros processos biológicos. Em particular no CNS, o NO tem sido descrito como capaz de modular a proliferação de células estaminais neurais, mas se actua como um agente pró- ou anti-mitótico ainda é controverso, sendo cada vez mais aceite que possa actuar em ambos os sentidos. Alguns autores sugerem que o NO é um inibidor fisiológico da proliferação celular. No entanto, em certas condições, o NO pode actuar como um agente pró-mitótico, favorecendo a proliferação celular.

Assim, a modulação do sistema nitrérgico poderá ser uma estratégia poderosa de controlo da proliferação/diferenciação celular. De facto, os mecanismos pelos quais o NO regula a proliferação e diferenciação neuronal ainda não estão esclarecidos, e uma investigação mais aprofundada sobre este assunto é necessária.

Neste estudo, o objectivo principal foi estudar os mecanismos de regulação da proliferação das células estaminais neuronais pelo NO. Procurou-se entender qual o efeito do NO na proliferação destas células, se proliferativo ou antiproliferativo, e decifrar os mecanismos responsáveis por esses efeitos. Para a execução do trabalho foram conduzidas experiências *in vitro* e *in vivo*. Nas experiências *in vitro*, culturas de células estaminais neurais isoladas da SVZ de murganhos foram expostas a diferentes concentrações de

um fármaco dador de NO, o NOC-18. Foram utilizadas concentrações de NOC-18 que permitiram uma libertação de NO compreendida entre valores fisiológicos e fisiopatológicas, como confirmado pela medição dos níveis de NO nos meios de cultura pela reacção de Griess. Dependendo da concentração, o NO pode ter efeitos opostos na proliferação das células estaminais neurais. Observámos que níveis relativamente baixos de NO, embora na gama fisiopatológica, aumentam a proliferação de células estaminais neurais em cultura, enquanto níveis mais elevados de NO induzem uma redução da proliferação celular. Muito provavelmente, diferentes mecanismos são responsáveis por este duplo efeito do NO na proliferação de células estaminais neurais.

Estudámos em seguida os mecanismos envolvidos no efeito proliferativo do NO e observámos que o aumento de proliferação induzido pelo NO é dependente da activação da via de sinalização das proteína-cinases activadas por mitogénios (MAPK). O NO entra na célula activando directamente a p21Ras, ultrapassando o receptor do EGF (EGFR). De facto, o NO activa a p21Ras após 2 minutos de incubação, sinalizando pela via das ERK/MAPK, envolvida na activação de factores de transcrição, como o Myc, Elk-1 e p90RSK, que regulam a progressão do ciclo celular. De facto, observámos que a activação da p90RSK resulta numa diminuição dos níveis nucleares da cinase dependente de ciclina 1, $p27^{KIP1}$, permitindo a progressão do ciclo celular e consequentemente a mitose.

Sendo a via de sinalização da guanilato ciclase um dos principais alvos intracelulares do NO, estudámos também o hipotético envolvimento desta via de sinalização na mediação do efeito proliferativo do NO. Curiosamente, a exposição de curta duração (6 horas) ao NO aumenta a proliferação celular de uma forma independente de cGMP, activando a via das p21/ERK/MAPK. Por outro lado, a exposição de longa duração (24 horas) ao NO parece activar ambas as vias de sinalização: a das p21/ERK/MAPK,

que parece estar activa em todas as fases analisadas, e a da proteína cinase G dependente de cGMP.

Quanto ao efeito antiproliferativo do NO, observou-se que a liberação de altas concentrações de NO pelo dador de NO, NOC-18, causou a nitração do receptor de EGF, com formação intermédia de peroxinitrito, em células estaminais neurais isoladas da SVZ que expressam o EGFR. Observámos que concomitantemente com o aumento da nitração em resíduos de tirosina, o NO causou uma diminuição no estado de fosforilação do EGFR. Além disso, utilizando um modelo de cultura de células estaminais neurais derivadas da SVZ em co-cultura com células da microglia isoladas de murganhos de genótipo selvagem para a iNOS ($iNOS^{+/+}$), foram obtidos resultados semelhantes. Ou seja, o aumento da libertação de NO pela células da microglia $iNOS^{+/+}$ activadas, após tratamento com LPS mais IFN-γ, relaciona-se com o aumento da nitração do EGFR, que é concomitante com a diminuição da proliferação das células SVZ. Além disso, as células que expressam o EGFR apresentam uma forte marcação para 3-nitrotirosina, um índice experimental para a nitração de proteínas, após o tratamento com NOC-18. O uso de um "scavenger" de peroxinitrito e superóxido, o MnTBAP, previne a nitração do EGFR, bem como aumenta o seu estado de fosforilação. Além disso, quer o MnTBAP ou o FeTMPyP, que promove a degradação de peroxinitrito, foram capazes de resgatar a proliferação de células estaminais neurais em co-cultura com células da microglia $iNOS^{+/+}$.

Usando um modelo *in vivo* de neuroinflamação e neurogénese, nomeadamente o modelo de epilepsia do lobo temporal induzida por ácido caínico, observou-se que o aumento da proliferação celular obtido após epilepsia é anulado quando a produção de NO é abolida por interrupção do gene da iNOS em murganhos $iNOS^{-/-}$. Estes resultados sugerem fortemente que o NO promove a proliferação de células estaminais neurais em algumas condições fisiopatológicas *in vivo*, pelo menos neste modelo de lesão cerebral.

Globalmente, os resultados apresentados neste trabalho esclarecem os mecanismos pelos quais o NO regula a proliferação de células estaminais neurais. Assim, mostramos que o NO, quando em níveis supra-fisiológicos, poderá ter um efeito duplo na proliferação celular, mediante a activação de diferentes mecanismos de sinalização. Deste modo, a via das p21/ERK/MAPK parece estar envolvida num efeito rápido do NO no sentido de promover a progressão do ciclo celular. Para uma exposição mais longa ao NO, duas vias independentes parecem estar activas: a via das p21/ERK/MAPK, que é a primeira a ser activada, e a via da guanilato ciclase-cGMP-PKG. Para níveis muito mais elevados o NO tem um efeito antiproliferativo diminuindo a sinalização do EGFR por intermédio de um mecanismo de modificação proteica pós-translacional, a nitração.

De acordo com resultados obtidos no modelo animal e de culturas mistas, o NO de origem inflamatória tem um efeito predominantemente proliferativo. Estes estudo sugere portanto que a modulação do sistema nitrérgico poderá ser útil para aproveitar o potencial das células estaminais neurais endógenas na terapia neuro-regenerativa.

Chapter 1

General Introduction

1.1 Neurogenesis

Neurogenesis, the process of generating new neurons from progenitor cells, was long considered to be limited to embryonic development in the mammalian central nervous system (CNS). This understanding originated from early works describing the development of adult brain of humans and other mammals (Koelliker, 1896; His, 1904; Ramon y Cajal, 1999). In fact, the brain was thought to be fixed after birth, with no changes at the cellular level, such as mitotic divisions, although some works reported the existence of mitotic cells in the adult brain of mammals (Allen, 1912; Brians, 1959). Joseph Altman had a major contribution with a series of papers showing evidence for adult neurogenesis in the adult brain of rat and cat, using autoradiography to track tritiated ([^{3}H])-thymidine incorporated by dividing cells (reviewed by Gross, 2000). However, these works were based in non-convincing methods to prove whether these newborn cells would integrate into neurons and be integrated in the CNS, and the significance of these results was not recognized. Other works were published showing evidence for adult neurogenesis in songbirds, by repeating Altman's experiments combined with electron microscopy, but again these reports were not considered relevant for the scientific community (Kaplan and Hinds, 1977; Nottebohm, 1985). In the nineteen nineties new techniques to detect cell proliferation were developed, namely the use of the thymidine analogue, 5-bromo-2'-deoxyruridine (BrdU), as a proliferation marker, instead of [^{3}H]-thymidine. Moreover, the development of specific antibodies against glial or neuronal markers allowed the distinction of neurons from glial cells. In fact, these new methods helped the detection of adult neurogenesis, which has been demonstrated to occur until senescence in mammalians, including humans (Eriksson *et al.*, 1998). Furthermore, the integration of newborn neurons into the neuronal network was confirmed by experiments testing synapse formation, long-term potentiation and expression of immediate early genes following stimulation of

hippocampus (van Praag *et al.*, 1999; Song *et al.*, 2002; Benninger *et al.*, 2003; Jessberger and Kempermann, 2003).

1.1.1 Neurogenesis in the adult mammalian brain

Neurogenesis occurs throughout life in two discrete regions of the adult mammalian brain: the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampus, both located in the telencephalon. The several stages of adult neurogenesis, including the proliferation of adult neural stem cells (NSCs) or progenitors, differentiation and fate determination of progenitor cells, survival, maturation and integration in the existing neuronal network of newborn neurons, are regulated by physiological and pathological inputs that reach the neurogenic niches, Particularly in rodents, olfactory discrimination and some forms of learning and memory require the integration of newborn neurons in the olfactory bulb (OB) and in the hippocampus, respectively, promoting the recruitment of neural stem cells from their niches to these areas.

The existence of neurogenesis in areas beyond the SVZ and SGZ of the adult mammalian brain have also been reported, namely in the neocortex (Gould *et al.*, 1999; Dayer *et al.*, 2005), striatum, amygdala (Bernier *et al.*, 2002), hypothalamus (Gould *et al.*, 2001; Xu *et al.*, 2005), mesencephalon (Zhao *et al.*, 2003), dorsal vagal complex (Bauer *et al.*, 2005) and spinal cord (Yamamoto *et al.*, 2001). However, these findings need further experimental support and more studies are needed (Rakic, 2002; Gould, 2007). Moreover, progenitor cells from several non-classical neurogenic regions of the mammalian brain, such as the optic nerve, hypothalamus, cortex, spinal cord and cerebellum, have been used in *in vitro* studies and differentiated into neurons and macroglial cells (Kirschenbaum *et al.*, 1994; Palmer *et al.*, 1999; Kondo and Raff, 2000; Laywell *et al.*, 2000; Nunes *et al.*, 2003; Markakis *et al.*, 2004; Lee *et al.*, 2005). The continuous regeneration of astrocytes and

oligodendrocytes in the mammalian brain appears to be due to these progenitor cells (Laywell *et al.*, 2000; Levine *et al.*, 2001; Dawson *et al.*, 2003).

Neurogenesis is also believed to occur in the nonmammalian brain (reviewed in Kaslin *et al.*, 2008) and has been extensively studied in songbirds (Nottebohm, 2004; Chapouton *et al.*, 2007).

1.1.1.1 Neurogenic niches in the adult brain

1.1.1.1.1 The subventricular zone

The subventricular zone is a thin cell layer located next to the ependyma of the telencephalic lateral walls of the lateral ventricles (Altman, 1969). There are four types of cells in the SVZ: ependymal cells, type B glial fibrillary acidic protein (GFAP)-positive progenitors, type C transit amplifying cells, and type A migrating neuroblasts. Type B GFAP-positive neural progenitors are also referred as SVZ astrocytes, which are relatively quiescent (Fig. 1.1) (Doetsch *et al.*, 1999; Alvarez-Buylla and Garcia-Verdugo, 2002). The identity of the resident adult neural stem cells that give rise to new neurons in the SVZ has been the subject of several studies and different cell types have been suggested, including astrocytes (Doetsch *et al.*, 1999), ependymal cells (Johansson *et al.*, 1999) and subependymal cells (Morshead *et al.*, 1994). However, the ependymal cells have been shown to be quiescent, not presenting properties of NSCs (Doetsch *et al.*, 1999; Capela and Temple, 2002). At present, the hypothesis that seems most accepted is that SVZ astrocytes are neural stem cells expressing GFAP, which are morphologically identical to astrocytes from other brain regions (Doetsch *et al.*, 1999; Garcia *et al.*, 2004). The SVZ is thought to provide a specific microenvironment, also referred to as the stem cell “niche”, characterized by the presence of several proteins involved in the maintenance of self-renewal and/or multipotency of neural stem cells (Alvarez-Buylla and Lim, 2004). Examples of these proteins

include Notch1 (Chojnacki *et al.*, 2003), sonic hedgehog (Machold *et al.*, 2003), basic fibroblast growth factor (bFGF) (Zheng *et al.*, 2004) and ciliary neurotrophic factor (Emsley and Hagg, 2003).

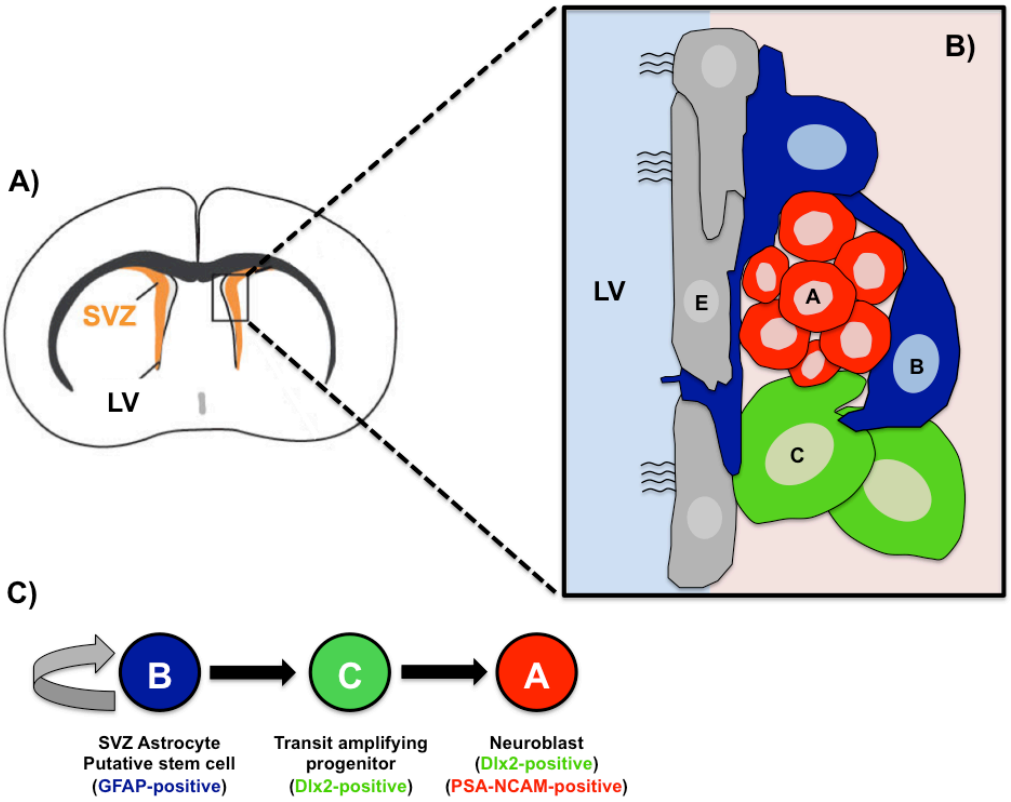


Figure 1.1. The neurogenic niche in the subventricular zone (SVZ). A) Cross-section of the adult mouse brain showing the subventricular zone (SVZ, orange), adjacent to the lateral ventricle (LV, light grey). **B)** Schematic illustration of the SVZ architecture and cell types. Multiciliated ependymal cells line the LV (E, gray). SVZ astrocytes or B cells (B, blue, GFAP-positive) act as neural stem cells and divide to give rise to rapidly dividing precursors, or C cells (C, green, Dlx2-positive). The C cells generate neuroblasts, or A cells (A, red, PSA-NCAM-positive) that migrate as chains through glial tunnels formed by SVZ slowly dividing astrocytes (B cells) into the olfactory bulb. The C cells can be found scattered in clusters along the network of chains. **C)** Specific cell markers appearing in each cell type. GFAP, glial fibrillary acidic-protein; Dlx2, homeobox protein Dlx2; PSA-NCAM, polysialylated-neural cell adhesion molecule (adapted from Alvarez-Buylla *et al.*, 2002).

In rodents and other mammals, type C transit amplifying cells are continuously generated from slow-dividing B stem cells, proliferate rapidly and originate type A migrating neuroblasts (Fig. 1.1 and 1.2). Signals like the Wnt-β-Catenin pathway regulate the proliferation and progression of type C transit amplifying cells, increasing the proliferation of these cells in the SVZ (Adachi *et al.*, 2007).

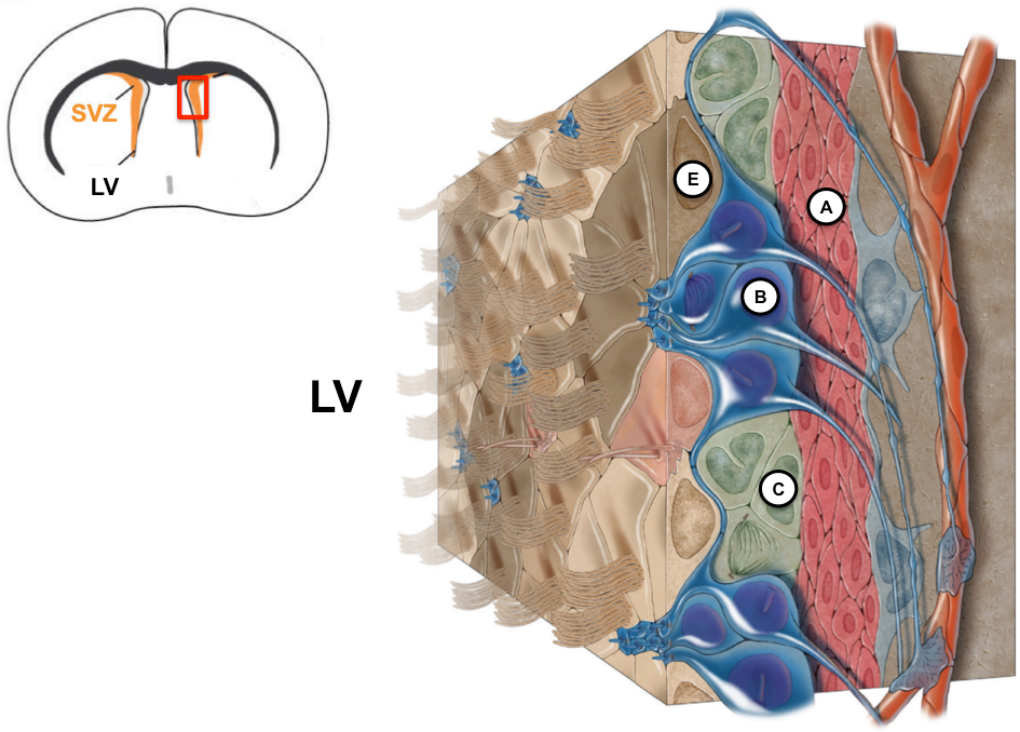


Figure 1.2. 3-Dimentional representation of the architecture of the SVZ. B cells (B, blue) present an apical ending at the ventrical surface and a long basal process that terminates on blood vessels. A cells (A, red) and C cells (C, green) and multiciliated ependymal cells (E, brown). LV – lateral ventrical (adapted from Mirzadeh *et al.*, 2008).

Unlike neural stem cells and type C cells, neuroblasts are already committed to the neuronal lineage. Few days after their formation, neuroblasts migrate tangentially, up to a distance of 5 mm in the rodent, from the SVZ to the olfactory bulb. This stream of tangentially and rostrally migrating

neuroblasts is referred to as the rostral migratory stream (RMS). In addition, a small number of precursors of oligodendrocytes is also produced in the SVZ, which migrate and are incorporated in the *corpus callosum* (Menn *et al.*, 2006). Upon reaching the OB, neuroblasts migrate radially from the RMS to the granule cell layer and glomerular layer and differentiate into inhibitory GABAergic granule interneurons and periglomerular dopaminergic interneurons, respectively. It has been suggested that the destination of each neuroblasts in the OB is already determined before it leaves the SVZ, however the mechanisms underlying this event remain to be elucidated (Merkle *et al.*, 2007). It is believed that these newly formed interneurons modulate the activity of glutamatergic neurons, mitral cells and tufted cells, thereby intervening in the olfactory system plasticity (reviewed by Lledo and Saghatelyan, 2005).

The migrating neuroblasts are usually bipolar, presenting extended processes that allow contact to adjacent cells and form "chains" in which migrating cells can slide on each other (Lledo and Saghatelyan, 2005). These chains of migrating neuroblasts are unsheathed by astrocytes, also referred to as glial tubes. Although the function of astrocytes in the migration of neuroblasts is not clear, astrocytes may be involved in the modulation of GABA levels, which are involved in the control of the speed of neuroblast migration. Several studies described the involvement of different molecules in the migratory behavior of neuroblasts and showed that rostral migration occurs in parallel with the directional flow of cerebrospinal fluid (CSF) in the lateral ventricle (Sawamoto *et al.*, 2006).

The CSF flow creates a concentration gradient of diffusible effectors secreted by the choroid plexus. Thus, the septum provides chemorepellent signals that might contain the axon guidance molecules Slit1 and Slit 2, which help guiding the rostral migration of SVZ neuroblasts. Modification of cytoskeleton also occurs, an event where the cyclin-dependent kinase 5 plays a crucial role in chain formation, and also in the speed and direction of the

neuroblasts migration through the RMS (Hirota *et al.*, 2007). Moreover, migration through the RMS and in the OB is regulated by cell-cell and cell-extracellular matrix interactions. Particularly, the EphB2-ephrin-B2 and neuregulin-ErbB4 pathways are involved in the proper organization of the RMS, while the polysialic acid-neural cell adhesion molecule (PSA-NCAM) protein, β1-integrin, proteoglicans and laminins are important for the regulation of the migration of SVZ neuroblasts. In the OB, the radial migration of neuroblasts is dependent on the extracellular matrix protein tenascin-R and the glycoprotein reelin (Lledo and Saghatelyan, 2005). Neuroblasts in the RMS are also attracted to the OB by several other molecules like netrin-1, prokineticin-2, glial cell-line derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF) (reviewed by Kaneko and Sawamoto, 2009).

Of all the cells formed in the SVZ, only a small number matures and integrates the olfactory system after the migration process. The remaining cells appear to degenerate in a process apparently dependent on caspases (Biebl *et al.*, 2005). The newly formed neurons in the OB go through different stages of development (Petreanu and Alvarez-Buylla, 2002; Lledo *et al.*, 2006), where GABAergic receptors develop before glutamatergic receptors or of dendritic spines formation (Lledo and Saghatelyan, 2005). About half of new neurons are eliminated after 6 weeks, although how this process is regulated remains unclear (Mizrahi *et al.*, 2006; Ninkovic *et al.*, 2007; Imayoshi *et al.*, 2008). However, the survival of new neurons is known to be dependent on external stimuli. In fact, for instance, odor deprivation reduces the complexity of dendritic arborization, suggesting that odorant cues and stimulation are important for survival of the newborn neurons (Petreanu and Alvarez-Buylla, 2002; Saghatelyan *et al.*, 2005; Yamaguchi and Mori, 2005).

The SVZ in the human brain is morphologically and functionally different from the SVZ of other mammals, since it is coated with a layer of astrocytes, that is not found in other mammals, except for humans and non-

human primates. Although there is proliferation in the SVZ of humans there is little evidence for the presence of RMS neuroblasts to the OB in humans (Sanai *et al.*, 2004; Quinones-Hinojosa *et al.*, 2006), but proliferation and neurogenesis were already detected in the olfactory bulb (Bedard and Parent, 2004).

1.1.1.1.2 The subgranular zone of the dentate gyrus

In the hippocampus, a proliferating population of multipotent precursors is found in the innermost subgranular cell layer of the SGZ of the dentate gyrus (Altman and Das, 1965). According to morphology and expression of specific molecular markers two types of neural progenitor cells could be identified in the SGZ: type 1 and type 2 (Fig. 1.2). Type 1 hippocampal progenitors have a radial process that spans the entire granule cell layer and branch out to the inner molecular layer. These progenitor cells express nestin, GFAP, Sry-related HMG box transcription factor and Sox-2 (Fukuda *et al.*, 2003; Garcia *et al.*, 2004; Suh *et al.*, 2007). Although expressing the astrocytic marker GFAP, these cells present morphological and functional aspects different from astrocytes. Type 2 hippocampal progenitors can arise from type 1 progenitor cells, but do not express GFAP. More interestingly, type 2 hippocampal progenitors expressing Sox-2 can differentiate into neurons or astrocytes (Suh *et al.*, 2007). In fact, the presence of the transcription factor Sox-2 is crucial for the maintenance of "stemness" of these adult stem cells, as well as neural stem cells from the SVZ, and also embryonic stem cells (ES) (reviewed in Jaenisch and Young, 2008).

In the SGZ, type 1 and 2 cells are in close contact with a dense layer of granule cells including differentiated neurons and newly formed neurons. The newly formed cells in the SGZ migrate a short distance as neuroblasts, being integrated in the granular cell layer of dentate gyrus as granule neurons (Fig. 1.3) (Eriksson *et al.*, 1998; Kempermann *et al.*, 2004; Seri *et al.*, 2004). Most of the newly formed SGZ cells die, but neurons that survive the first two

weeks are more likely to become mature and integrate a functional hippocampal neural circuitry (Kempermann *et al.*, 2003). In adult mice, newly generated granule neurons of the hippocampus undergo a continuous process of maturation that can take weeks, presenting electrophysiological activity after one month (van Praag *et al.*, 2002). Astrocytes, oligodendrocytes and other types of neurons can be identified in the granular zone microenvironment, where astrocytes seem to play an important role in promoting the differentiation of hippocampal progenitor cells, as well as the integration of new neurons, an event mostly mediated by Wnt signaling (Song *et al.*, 2002; Lie *et al.*, 2005).

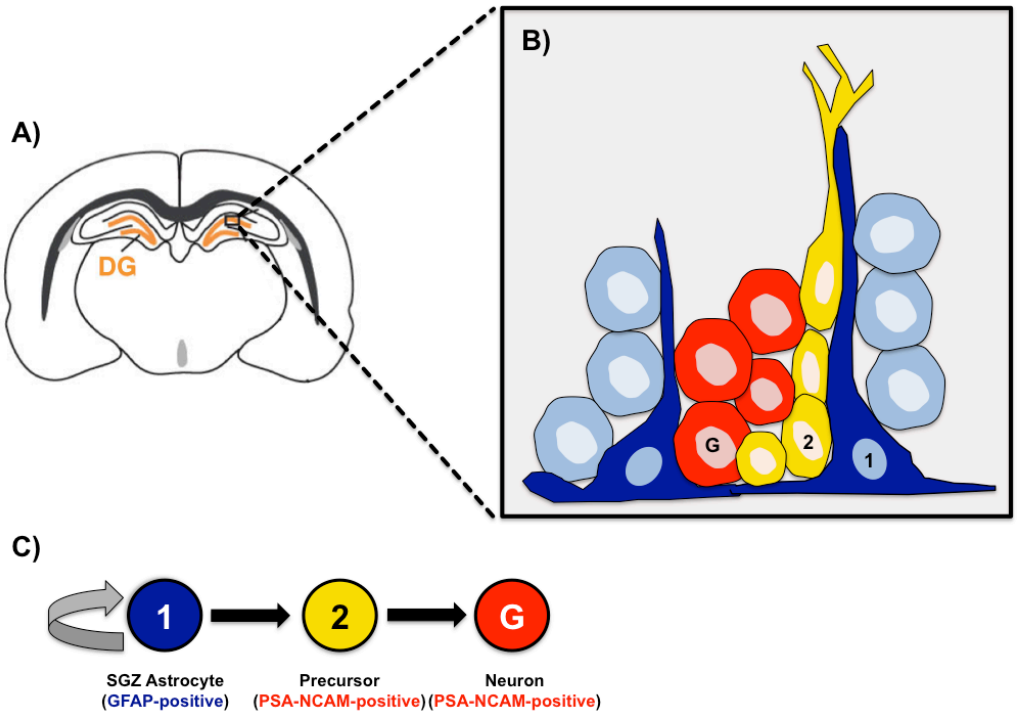


Figure 1.3. The neurogenic niche in the subgranular zone of the hippocampus. **A)** Cross-section of the adult mouse brain showing the hippocampus. **B)** Schematic illustration of the subgranular layer (SGL) and granule cell layer (GCL) architecture and cell types. SGL astrocytes or Type 1 cells (1, blue, GFAP-positive) divide to give rise to intermediate precursor cells, or type 2 progenitor cells (2, yellow), that divide and mature into new granule neurons (G, red). **C)** Specific cell markers appearing in each cell type. GFAP, glial fibrillary acidic-protein; PSA-NCAM, polysialylated-neural cell adhesion molecule (adapted from Alvarez-Buylla *et al.*, 2002).

1.1.1.2 Neural stem cells and neural progenitors

The term adult neural stem cells is given to all cells in the adult mammalian nervous system that have the capacity for self-renewal and differentiation into different types of nerve cells, including neurons, astrocytes and oligodendrocytes (Gage, 2000). The characteristics of stem cells were first described by Hall and Watts in 1989 (Hall and Watt, 1989), stating the conditions under which a cell was to be considered a stem cell:

a) Self-renewal capacity, where a pool of stem cells is maintained throughout the life of the organism by symmetrical mitotic divisions resulting in daughter cells with similar proliferative capacity;
b) Ability to generate progenitors, through asymmetric mitotic divisions, with limited proliferative capacity and committed to differentiation.

Various *in vitro* studies using neurospheres assays and adherent monolayer cultures were performed to demonstrate the existence of NSCs in the adult brain, as well as to show the capacity for self-renewal and the multipotent properties of these cells (reviewed by Taupin and Gage, 2002). However, these criteria should be demonstrated over an extended period of time, more than 5 passages in culture, and should coincide with a significant increase in progeny when compared with the number of cells in the initial population (Reynolds and Rietze, 2005). In 1992, Reynolds and Weiss made the first isolation and characterization *in vitro* of a population of stem cells isolated from the adult brain of mice (Reynolds and Weiss, 1992). In this study, a population of undifferentiated cells that expressed nestin was isolated from the striatal region, containing the SVZ. Nestin is an intermediate filament that has been characterized as a marker for stem cells during central nervous system development, and so considered as a marker for adult stem cells and adult neural progenitor cells (Frederiksen and McKay, 1988; Reynolds and Weiss, 1992). These cells were grown in a serum-free medium, supplemented

with epidermal growth factor (EGF), and formed floating aggregates, hence designated as neurospheres. Neurospheres are aggregates of stem cells and progenitor cells with proliferative capacity. These cells were multipotent and had the ability to differentiate into neurons, astrocytes and oligodendrocytes. Other studies were performed where astrocytes and oligodendrocytes were also obtained *in vitro* from rodent and also from human cultures of hipoocampal and SVZ cells, among others (Levison and Goldman, 1997; Luskin *et al.*, 1997; Palmer *et al.*, 2001; Sanai *et al.*, 2004).

In 1995, Gage and collaborators isolated and characterized *in vitro* a population of cells with identical properties to those isolated by Reynolds and Weiss, but now from the adult rat hippocampus (Gage *et al.*, 1995). Isolated cells were grown as monolayers, in culture medium supplemented bFGF. Other studies characterized this population of cells as containing self-renewing and multipotent NSCs (Gritti *et al.*, 1996; Palmer *et al.*, 1997). Since these studies, self-renewing, multipotent stem cells and neural progenitors have been isolated from the adult CNS of other species, including humans, particularly from the SVZ and the hippocampus (Taupin and Gage, 2002).

Although NSCs can be isolated from different areas of the adult CNS, adult neurogenesis has only been consistently found *in vivo* in the SVZ and in the SGZ (reviewed in Ma *et al.*, 2009). The SVZ and SGZ are hypothesized to have a microenvironment, known as neurogenic niche, with specific factors that promote differentiation and integration of newborn neurons (reviewed by Suh *et al.*, 2009).

1.1.2 Role of adult neural stem cells

Adult neural stem cells present in the SVZ of the lateral ventricles and in the SGZ of the DG are able to form new neurons (Gage, 2000). Although adult neurogenesis has been intensively studied over the past twenty years, only since 2002 it has been established that newly formed neurons in the adult brain are functional (Carlen *et al.*, 2002) and integrate into the pre-existing

neuronal network participating in specific physiological functions of the tissue. The neurogenic event is a tightly regulated process, even though its physiological functions have not been fully elucidated. Certain conditions are known to increase adult neurogenesis, like pregnancy (Shingo *et al.*, 2003), for instance, or damage to the brain (Arvidsson *et al.*, 2002; Zhao *et al.*, 2003).

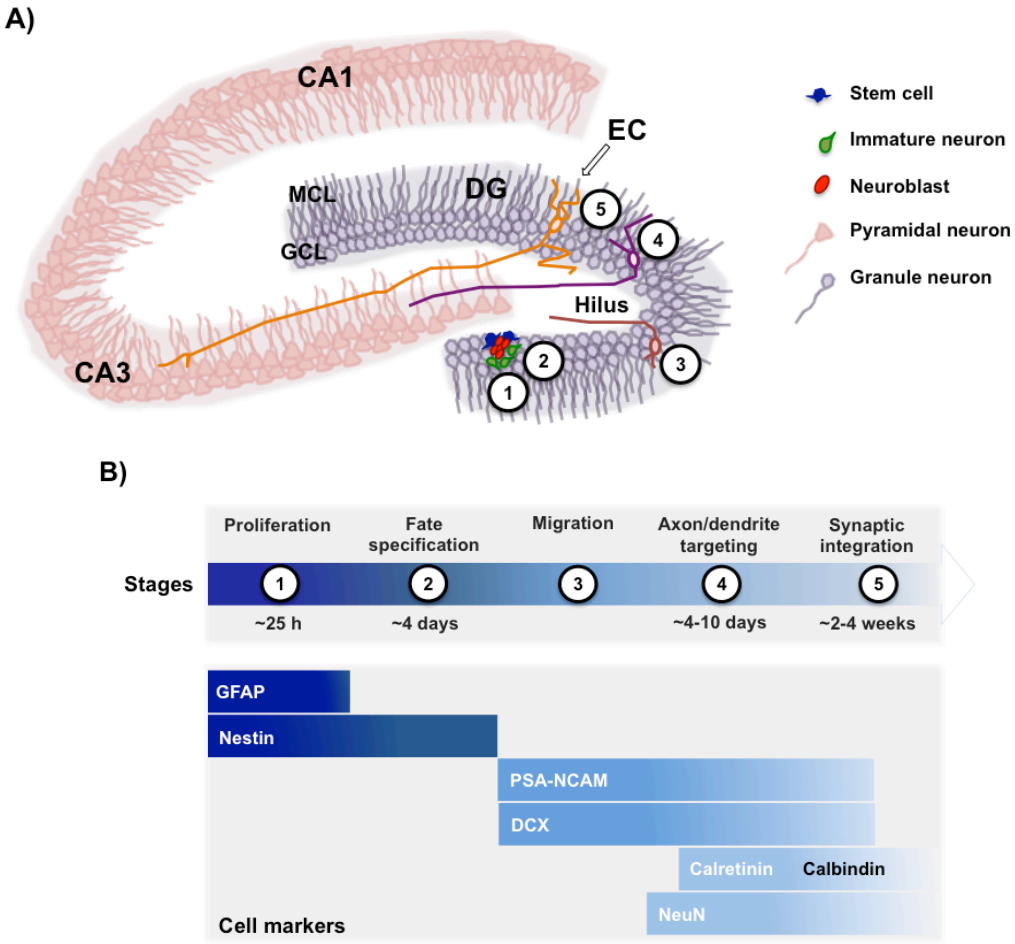


Figure 1.4. Adult neurogenesis in the dentate gyrus of the hippocampus. A) Adult neurogenesis in the dentate gyrus of the hippocampus undergoes five stages: **Stage 1 – Proliferation.** Stem cells (blue) located within the subgranular zone (SGZ) in the dentate gyrus present short processes that extent tangentially along the border of the granule cell layer and hillus, and radial processes that project through the granule cell layer. SGZ stem cells give rise to transient amplifying cells (red). **Stage 2 – Differentiation.** Transient amplifying cells differentiate into immature neurons

(green). **Stage 3 – Migration.** Immature neurons migrate into the granule cell layer (brown). **Stage 4 – Axon/Dendritic targeting.** Immature neurons (purple) project their axons towards the CA3 pyramidal cell layer, and their dendrites in the opposite direction into the molecular cell layer. **Stage 5 – synaptic integration.** New granule neurons (orange) receive and integrate input signals from the entorhinal cortex and send output signals to the CA3 and hillus regions. EC – entorhinal cortex; DG, dentate gyrus region; MCL, molecular cell layer; GCL, granular cell layer**. B)** The specific properties of each stage are summarized bellow. GFAP, glial fibrillary acidic-protein; DCX, doublecortin; PSA-NCAM, polysialylated-neural cell adhesion molecule (adapted from Ming and Song, 2005).

Newly generated neurons arising from the hippocampus mature and acquire electrophysiological properties similar to the neighboring neurons (Fig. 1.4), establish new synaptic connections and participate in functions such as learning and memory (Carlen *et al.*, 2002; van Praag *et al.*, 2002). Other studies suggest that neurogenesis plays an important role in mechanisms of specific memory acquisition (Rochefort *et al.*, 2002). Accordingly, the pharmacological suppression of hippocampal neurogenesis significantly reduces learning capacity in rats (Shors *et al.*, 2001).

Newborn neurons emerging from the SVZ migrate through the RMS and integrate into the neuronal network of the olfactory bulb (Fig. 1.5), establish functional synaptic connections and develop electrophysiological properties of mature neurons (Carlen *et al.*, 2002; Petreanu and Alvarez-Buylla, 2002; Belluzzi *et al.*, 2003). Furthermore, neurogenesis in the OB improves odor memory and discrimination, an important mechanism for offspring recognition by mice, after pregnancy, for instance (Gheusi *et al.*, 2000; Rochefort *et al.*, 2002; Shingo *et al.*, 2003).

Moreover, several models of injury in the rodent brain have been used to show that proliferation of stem cells is greatly increased in the SVZ and DG, following injury, which might be related to a repair attempt from the lesioned brain (Lowenstein and Parent, 1999).

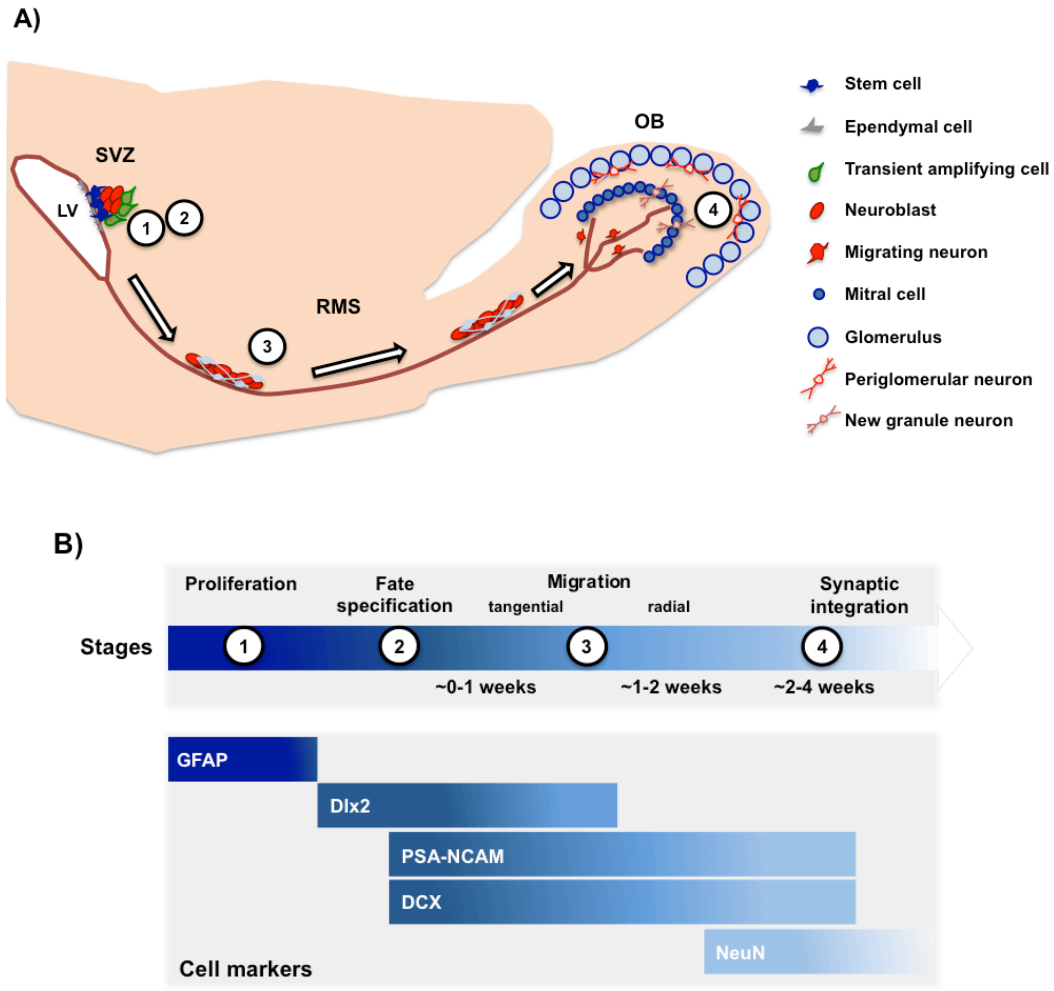


Figure 1.5. Neurogenesis in the olfactory system. A) Newly generated cells from the SVZ migrate through the rostral migratory stream (RMS) and integrate into the neuronal network of the olfactory bulb as interneurons (OB). **Stage 1 – Proliferation**. Stem cells (blue) in the SVZ give rise to transient amplifying cells (green). **Stage 2 - Fate specification**. Transient amplifying cells differentiate into neuroblasts (red). Ependymal cells (gray) of the lateral ventricle are essential to inhibit gliogenesis, favouring neuronal fate specification. **Stage 3 – Migration**. Neuroblasts migrate through the RMS into the OB. These immature neurons migrate as chains and are ensheathed by astrocytes. **Stage 4 - Synaptic integration**. New neurons that reach the OB, migrate radially into outer cell layers and differentiate into granule or periglomerular neurons. Lateral ventricle, LV; Olfactory bulb, OB; Rostral migratory stream, RMS; Subventricular zone, SVZ. **B)** Some specific properties of each stage are summarized bellow. GFAP, glial fibrillary acidic-protein; DCX, doublecortin; NeuN, Neuronal nuclei; PSA-NCAM, polysialylated-neural cell adhesion molecule (adapted from Ming and Song, 2005).

1.1.3 Regulation of adult neurogenesis

Adult neurogenesis is implicated in many forms of plasticity in the CNS, and understanding the fundamental mechanisms that control this process are of great relevance. Most of the studies about the regulation of adult neurogenesis focused on the understanding of cell cycle progression or in the expression of different markers during cell differentiation. The neurogenic process consists of three main steps: a) precursor cell proliferation, b) migration, and c) differentiation, integration and survival. Despite numerous limitations, in the last decade numerous factors that affect adult neurogenesis have been identified. However, the precise mechanisms that control neuronal fate in the adult nervous system remain largely unknown. Both intrinsic and extrinsic factors can interfere with the process of neurogenesis.

1.1.3.1 Hormones

Hormones in the adult mammalian brain regulate neurogenesis (reviewed by Abrous *et al.*, 2005). Estrogens have a proliferative effect on progenitor cells of DG, increasing their survival (Tanapat *et al.*, 1999). On the other hand, estrogen does not appear to affect neurogenesis in the SVZ of adult rats, although these cells express specific receptors for estrogen (Brannvall *et al.*, 2002; Isgor and Watson, 2005). Other hormones such as prolactin and thyroid hormones appear to increase neurogenesis in the SVZ of rodents (Giardino *et al.*, 2000; Shingo *et al.*, 2003), or in both the DG and SVZ, such as polyamines (Malaterre *et al.*, 2004). On the other hand, stress hormones such as corticosteroids, particularly glucocorticoids, decrease neurogenesis in the DG of young rats and primates (Gould *et al.*, 1998; Kippin *et al.*, 2004).

1.1.3.2 Trophic factors

Several trophic factors have been described as having a mitotic action on neurogenic regions of the adult brain. Thus, growth factors such as EGF and bFGF are potent agents for the maintenance and growth of adult NSCs *in vitro*. *In vivo*, both factors are proliferative in the SVZ, although only BFGF increases the number of newborn neurons in the olfactory bulb (Kuhn *et al.*, 1997). In 2002, Doetsch and colleagues found that EGF inhibits the differentiation of type C transit amplifying cells in neuroblasts (Doetsch *et al.*, 2002). Other studies have reinforced the idea that EGF is present in the adult SVZ, particularly after the re-expression of ErbB2, a receptor for EGF, and subsequent induction of radial glia morphology in GFAP-positive cells in the SVZ of young adult mice (Ghashghaei *et al.*, 2007). Although bFGF does not appear to increase the proliferation in the SGZ in young mice (Jin *et al.*, 2003), the knockdown of its receptor FGFR-1 in CNS decreases neurogenesis in this region (Zhao *et al.*, 2007). In 2009, Sun and collaborators showed that the intravenous administration of basic fibroblast growth factor increased proliferation both in the SVZ and DG, following traumatic brain injury (Sun *et al.*, 2009).

The transforming growth factor alpha (TGF-α) increases neurogenesis both in the SVZ and the DG, as demonstrated by studies in animal models (Craig *et al.*, 1996; Tropepe *et al.*, 1997; Battista *et al.*, 2006). The insulin-like growth factor 1 (IGF-1) also known as somatomedin C or mechano growth factor is a growth-promoting peptide hormone produced in the CNS by neurons and glial cells that exhibits neurotrophic properties in the adult mammalian brain (Niblock *et al.*, 2000; Anderson *et al.*, 2002). The IGF-1 increases cell proliferation in the SGZ as demonstrated in *in vivo* and *in vitro* (Aberg *et al.*, 2000; Trejo *et al.*, 2001; Aberg *et al.*, 2003; Perez-Martin *et al.*, 2003).

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family of growth factors, which prevents neurons from dying

during development (Hempstead, 2006; Reichardt, 2006). In the brain, BDNF is active in the cortex, hippocampus and basal forebrain, areas linked to memory and learning (Yamada and Nabeshima, 2003; Binder and Scharfman, 2004; Bekinschtein *et al.*, 2008). BDNF was shown to increase cell proliferation in the granule cell layer of the OB and in the DG of rodents (Zigova *et al.*, 1998; Benraiss *et al.*, 2001; Lee *et al.*, 2002). On the other hand, BDNF is able to to influence the survival and/or differentiation of newly born neurons, thus playing an important role in the maintenance of basal neurogenesis (Abrous *et al.*, 2005).

Vascular endothelial growth factor (VEGF) is a hypoxia-induced protein that stimulates growth of new blood vessels, restoring oxygen supply to tissues (Ferrara and Gerber, 2001). VEGF belongs to a subfamily of the platelet-derived growth factor family. This angiogenic protein exhibits neurotrophic and neuroprotective properties (Meirer *et al.*, 2001). VEGF stimulates cell proliferation both in the SVZ and the SGZ of rodents, suggesting that VEGF may be involved in the crosstalk between angiogenesis and neurogenesis (Jin *et al.*, 2002; Hansen *et al.*, 2008).

1.1.3.3 Neurotransmitters and neuromodulators

Neurogenesis in the adult brain can be modulated by neurotransmitters. Several studies evaluated the effect of neurotransmitters in the neurogenic process. Serotonin appears to be important for proliferation and maintenance of PSA-NCAM positive neurons in the SVZ and SGZ (Brezun and Daszuta, 1999). Depending on the location, glutamate may have a dual effect on neurogenesis, acting as an antiproliferative agent in the hippocampus, decreasing the formation of PSA-NCAM neurons (Nacher *et al.*, 2001), or, conversely, increasing the proliferation of SVZ cells *in vitro* (Brazel *et al.*, 2005). Similarly, noradrenaline also has different roles depending on their location. Depletion of noradrenaline decreases proliferation in the DG, but not in the SVZ of rat (Kulkarni *et al.*, 2002). Moreover, dopamine

stimulates the proliferation of SVZ cells *in vitro* (Coronas *et al.*, 2004; Van Kampen *et al.*, 2004), while the pharmacological elimination of dopaminergic nigrostrial projections decreases proliferation of neuronal precursors in the SVZ (Baker *et al.*, 2004; Hoglinger *et al.*, 2004).

Nitric oxide (NO) is a gaseous free radical synthesized in many cell types from L-arginine, a reaction catalyzed by NO synthase (NOS). NO is an intercellular messenger with multiple functions within the cardiovascular system, immune system and nervous system, where it can act as a non-canonical neurotransmitter (Alderton *et al.*, 2001). The effect of NO on neurogenesis appears to be dependent on the concentration achieved locally. Different studies reported a dual role of NO on cell proliferation, acting as a proliferative or antiproliferative agent in the adult brain. While the antiproliferative effect of NO is dependent on the inhibition of cyclin-dependent kinases and transcription factors by the p53 and Rb protein respectively (reviewed by Gibbs, 2003), the proliferative effect of NO is mediated by increased levels of cGMP in the SVZ and in the dentate gyrus of the hippocampus of the adult rodent brain (Zhang *et al.*, 2001; Zhang *et al.*, 2002). It appears that in physiological conditions, NO tonically inhibits neural stem cell proliferation in the brain (Packer *et al.*, 2003; Moreno-Lopez *et al.*, 2004; Matarredona *et al.*, 2005; Torroglosa *et al.*, 2007), while in pathophysiological conditions it exerts a proliferative effect on the dividing population of neuronal precursors (reviewed in Whitney *et al.*, 2009). However, other studies reported that supraphysiological concentrations of NO inhibit neural stem cell proliferation and promote astrogliogenesis in the SVZ (Covacu *et al.*, 2006). Moreover, it was found that exogenous administration of NO to adult rats significantly increased cell proliferation and migration in the SVZ and DG (Zhang *et al.*, 2001). Particularly, precursors that express neuronal NO synthase (nNOS) were identified in the regions of final differentiation in the SVZ of adult mice (Moreno-Lopez *et al.*, 2000).

The exact mechanisms by which NO regulates neuronal proliferation and differentiation are not yet clarified, and further investigation on this matter is needed. NO will be discussed in more detail in section 1.3 of the General Introduction, and the mechanisms underlying the role of NO on NSCs proliferation will be addressed in the present thesis.

1.1.3.4 Glial cells

Glial cells can regulate adult neurogenesis, particularly astrocytes which are important sensors of changes in the extracellular microenvironment (Alvarez-Buylla *et al.*, 2002). Astrocytes could be involved in the regulation of neurogenesis by releasing local signals (Song *et al.*, 2002), such as neurosteroids, cytokines, growth factors, glutamate, among others (reviewed by Abrous *et al.*, 2005). Moreover, the fact that some proliferating cells in the DG express a receptor for S-100, a small acidic calcium binding neurotrophic protein released by astrocytes, has reinforced the putative role of astrocytes in the regulation of adult neurogenesis (Abrous *et al.*, 2005).

1.1.3.5 Survival and fate selection

The last step of the formation of new neurons is their survival following differentiation and integration. Different molecules regulate neural stem cell survival and death pathways in the adult and embryonic brain. In fact, the same molecules may have different functions according to the developmental stage, and the differences between microenvironments are mostly reflected by the extrinsic regulators of the metabolism of neural stem cells.

The subgranular zone of the DG is one of the niches in the adult brain where new neurons are formed. A great percentage of these new neurons die after birth (Cameron *et al.*, 1993; Eriksson *et al.*, 1998; Rakic, 2002), but this event may be slowed or even prevented by environmental enrichment, particularly by performing cognitive tasks. However, the mechanisms

underlying the survival of newborn neurons remain unclear. Many authors have suggested that the significant decline in the number of newborn neurons after they are generated may be due to programmed cell death, confirmed by the fact that these cells are positive for terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). Survival may thus be due to the prevention of programmed cell death or rather to a generalized increase in hippocampal neurogenesis (Kempermann *et al.*, 1998; Gould *et al.*, 1999). The events that trigger cell death in newborn neurons are still under investigation. One possibility is that failure to correctly integrate or making the proper connections to receive synaptic inputs from the existing circuits may select for elimination cells that are not properly integrated with the surrounding tissue.

Newly generated neurons are also originated in the rostral migratory stream. As happens with newborn neurons in the dentate gyrus, TUNEL-positive cells are observed along the rostral migratory stream (Brunjes and Armstrong, 1996; Fiske and Brunjes, 2001), the SVZ, and the OB (Biebl *et al.*, 2000; Moreno-Lopez *et al.*, 2004). Sensory input failure for these cells may result in apoptosis, which is confirmed by an increased number of TUNEL-positive cells in the granule cell layer of the OB. However, whether cell death occurs solely in post-mitotic OB neurons or whether neural precursor cells are also subjected to cell death in the adult is not clear.

1.1.4 Neurogenesis and brain injury

It has been observed that injury and pathological conditions affect adult neurogenesis, having an impact in neurogenic regions but also in normally non-neurogenic areas (Ming and Song, 2005). The insult to the brain may be acute, like ischemic brain stroke, traumatic brain injury or prolonged seizures, or a slow-progressing neurodegenerative disease. Neurogenesis decreases with normal aging and is impaired in several neurodegenerative disorders, such as Huntington's disease (Lazic *et al.*, 2004; Gil *et al.*, 2005) or

Alzheimer's disease (Tatebayashi *et al.*, 2003). All these conditions are accompanied by an inflammatory response in the brain.

Many brain lesions lead to increased proliferation in the SVZ and the SGZ, causing migration of neural precursors to the lesioned areas. Specific types of injury were hypothesized to increase proliferation of neural progenitors and induce migration and differentiation of new neurons to regions where adult neurogenesis is extremely limited or non-existent (Magavi *et al.*, 2000; Magavi and Macklis, 2001). The factors that attract neural progenitors to the lesioned areas are still under investigation. Whether these newborn neurons are functionally integrated and survive in the existing neuronal circuitry remains a question of hot debate.

1.1.4.1 Acute brain lesions

1.1.4.1.1 Ischemia

Ischemic brain insults have been demonstrated to stimulate progenitor cell proliferation in the SGZ and the SVZ of adult rodents (Kokaia and Lindvall, 2003; Parent, 2003). Ischemic brain stroke have been used to show the migration of neuronal precursors from the SVZ to the damaged striatum, with subsequent differentiation (Arvidsson *et al.*, 2002). However, most of the newly formed neurons died prematurely within two to five weeks following stroke, which suggests that the local microenvironment, although essential for releasing of signals attracting immature neurons and further differentiation, is not adequate for long-term survival.

Multiple models of ischemia have also demonstrated an increase in neurogenesis. In models of transient global ischemia increased proliferation was demonstrated in the DG (Liu *et al.*, 1998; Takagi *et al.*, 1999; Kee *et al.*, 2001; Yagita *et al.*, 2001; Iwai *et al.*, 2002; Choi *et al.*, 2003; Bingham *et al.*, 2005; Darsalia *et al.*, 2005) and in the SVZ (Zhang *et al.*, 2001; Arvidsson *et al.*, 2002; Tang *et al.*, 2009) by an increase in the number of BrdU-positive

cells. In the ischemic brain, newly formed cells from the SGZ migrate to the granule cell layer of the DG (Liu *et al.*, 1998; Kee *et al.*, 2001; Yagita *et al.*, 2001; Iwai *et al.*, 2002; Bingham *et al.*, 2005), while cells from the SVZ migrate to the olfactory bulb, cortex (Zhang *et al.*, 2001) and striatum (Arvidsson *et al.*, 2002; Darsalia *et al.*, 2005). The majority of proliferating cells differentiated into neurons, as evaluated by the colocalization of BrdU with mature neuronal markers (Kee *et al.*, 2001; Yagita *et al.*, 2001; Arvidsson *et al.*, 2002; Iwai *et al.*, 2002; Bingham *et al.*, 2005). It is hypothesized that VEGF plays an important role in the increased neurogenesis following ischemia, by stimulating proliferation, migration and survival of neurons (Hansen *et al.*, 2008).

Overall, it has been demonstrated that following ischemia newborn neurons can integrate the CNS network and improve cognitive function. However, further studies are needed to proof a causal relationship between increased neurogenesis and improved recovery following ischemia (reviewed by Ekdahl *et al.*, 2009). Moreover, although neurogenesis has been shown to increase following ischemia, only a fraction of the newly generated neurons survive long-term (Arvidsson *et al.*, 2002).

1.1.4.1.2 Traumatic brain injury

Traumatic brain injury (TBI), a condition in which sudden trauma and secondary injury cause brain damage, increases cell proliferation in the dentate gyrus, in the SVZ as well as in the cortex (Dash *et al.*, 2001; Lu *et al.*, 2003; Rice *et al.*, 2003). Following TBI, progenitor cells from the SVZ and SGZ become activated. In facto, newly generated neurons survived 3 to 4 weeks post-TBI in the granule cell layer of the DG as demonstrated by co-labeling with BrdU or [^{3}H]-thymidine and calbindin, a mature neuronal marker of this region (Gould and Tanapat, 1997; Dash *et al.*, 2001; Rice *et al.*, 2003). Moreover, it was observed that exogenous administration of NO significantly increased proliferation, survival, migration and differentiation of neural

progenitors in the rat DG, as well other brain areas, and significantly improved neurological functional outcome following TBI (Lu *et al.*, 2003). However, it is still unclear whether the increased proliferation of progenitor cells results in stable neurogenesis, with long-term survival of the newly generated neurons post-injury (Richardson *et al.*, 2007).

1.1.4.1.3 Epilepsy

Temporal lobe epilepsy (TLE) is the most common type of epilepsy in adults. Spontaneous and recurrent seizures originating in the temporal lobe promote extensive neuronal loss in CA1, CA3 and the hilus of the DG of the hippocampus. Seizures, like other acute insults to the CNS such as ischemic stroke or traumatic brain injury, are followed by local inflammation and increased proliferation of neural stem cells and neuronal precursors in the main neurogenic areas. Studies using adult rodent models of acute seizures or limbic epileptogenesis demonstrated an increased neurogenesis in the SGZ and SVZ, following injury (Parent *et al.*, 1997; Gray and Sundstrom, 1998; Parent *et al.*, 2002). *Status epilepticus* (SE) in rat models is followed by an increase in neurogenesis in the DG (Palmer *et al.*, 1997; Bonde *et al.*, 2006). The majority of newborn cells generated in the SGZ of the DG migrate into the granule cell layer and differentiate into granule neurons, projecting axons to the CA3 region and dendrites to the molecular layer. However, some of the immature neurons and newly generated cells in the SGZ mislocate the hilus, develop the electrophysiological characteristics of dentate granule neurons and fire abnormal bursts in synchrony with the CA3 pyramidal neurons (Scharfman *et al.*, 2000). These abnormal neuronal circuits are thought to contribute to spontaneous and recurrent epileptic seizures, thus suggesting that aberrant neurogenesis following SE is involved in the development of epilepsy (Walter *et al.*, 2007). In the SVZ, proliferation is significantly increased following SE, and the newly formed neuroblasts rapidly migrate to the olfactory bulb, but some neuroblasts also migrate into other injured

forebrain regions (Parent *et al.*, 2002), which could suggests an attempt of endogenous recovery following SE.

1.1.4.2 Neurodegenerative disorders

Neurodegenerative disorders are characterized by slow and progressive neuronal death. In the absence of treatment for these disorders, the development of therapeutic strategies that can compensate the loss of neurons is of great interest, particularly the possibility of replacing the lost cells and restoring brain function. Changes in adult neurogenesis have been observed in some neurological disorders, like Alzheimer's, Parkinson's and Huntington's disease.

1.1.4.2.1 Huntington's disease

Huntington's disease (HD) is a neurodegenerative disease characterized by neuronal loss in the caudate-putamen. In humans, postmortem analysis has revealed that cell proliferation is increased in the subependymal layer of the caudate nucleus in HD (Curtis *et al.*, 2003). The newly generated cells express glial or neuronal markers. Curtis and collaborators also demonstrated that increased cell proliferation in the SVZ correlated with the severity of HD (Curtis *et al.*, 2003). Increased neurogenesis was also demonstrated in rodent models of HD (Tattersfield *et al.*, 2004; Vazey *et al.*, 2006; Lorincz and Zawistowski, 2009). Batista and colleagues also reported a progressive increase of cell proliferation in the SVZ of R6/2 transgenic mice. Interestingly, this study also reported deficits of cell migration between the SVZ to the olfactory bulb in R6/2 mice, due to redirection of new neurons to the striatum of R6/2 mice (Batista *et al.*, 2006). Taken together, these data indicate that attempts at neuronal regeneration may occur in the diseased adult brain, although these endogenous efforts do not address the

continued neurodegeneration of the affected brain areas, thus not being minimally effective.

However, other studies reported that cell proliferation and neurogenesis in the hippocampus is decreased in HD transgenic mouse models, either R6/1 or R6/2 mice (Gil *et al.*, 2004; Lazic *et al.*, 2004; Gil *et al.*, 2005; Phillips *et al.*, 2005; Lazic *et al.*, 2006; Phillips *et al.*, 2006). Interestingly, although the relationship between the decreased hippocampal neurogenesis and the progression of HD is not fully understand, van Dellen and colleagues reported that increased hippocampal neurogenesis delays progression of HD in the model mice (van Dellen *et al.*, 2000).

Further studies are needed to fully understand how neurogenesis is affected in HD patients or HD models, and whether cell therapies could be beneficial to ameliorating this disorder.

1.1.4.2.2 Alzheimer's disease

Alzheimer's disease (AD) is a chronic progressive disorder characterized by a widespread neuronal death in several brain areas, which ultimately leads to dementia and death. The AD brain is characterized by the presence of senile plaques containing β-amyloid peptide (Aβ), derived from amyloid precursor protein (APP), and neurofibrillary tangles, containing hyperphosphorylated microtubule-associated protein tau. Accumulation of Aβ causes neuronal loss and atrophy in the hippocampus and SVZ, decreases proliferation and differentiation of neural stem cells and progenitors, and promoting apoptosis (Donovan *et al.*, 2006; Ziabreva *et al.*, 2006).

Moreover, evidence suggesting enhanced neurogenesis has been found in patients with AD and in AD rodent models (Jin *et al.*, 2004; Jin *et al.*, 2004; Yu *et al.*, 2009). In mouse models, cell proliferation was stimulated in the DG at early stages of AD, however the newly generated neurons did not fully mature (Li *et al.*, 2008) or did not survive for long (Chen *et al.*, 2008; Gan

et al., 2008). Moreover, AD-specific proteins have been associated with AD-associated neurogenesis (Lopez-Toledano and Shelanski, 2007; Gan *et al.*, 2008; Rohe *et al.*, 2008). In fact, the complexity of the pathophysiological features of AD is not fully clarified, however it is hypothesized that deficits in neurogenesis contribute to the AD pathogenesis (reviewed by Kaneko and Sawamoto, 2009).

1.1.4.2.3 Parkinson's disease

Parkinson's disease (PD) is a motor disorder characterized by rigidity, resting tremors and bradykinesia. In PD patients, dopaminergic neurons in the substantia nigra *pars compacta* degenerate, causing loss of motor coordination in muscle movements. Increased neurogenesis in the substantia nigra has been a matter of debate in animal models of PD. Zhao and collaborators reported neurogenesis to be increased in the substancia nigra *pars compacta* in a mouse model of PD (Zhao *et al.*, 2003). However, in 2004, contradictory observations were published. Using similar methodological techniques in the same animal model of PD, Frielingsdorf and collaborators found no evidence of new dopaminergic neurons in the substantia nigra. Furthermore, this group also reported no evidence of neural stem cell migration from the cerebroventricular system into the substancia nigra (Frielingsdorf *et al.*, 2004). Shan and colleagues also demonstrated that NPCs successfully migrate to the substancia nigra, and differentiate into dopaminergic neurons (Shan *et al.*, 2006). However, other studies in patients and animal models suggest chronic inflammation during PD to enhance proliferation of NPCs from the SVZ and dentate gyrus and differentiation into neurons, but the inflammatory niche is not supportive of survival and incorporation of newborn neurons (Huisman *et al.*, 2004; Winner *et al.*, 2006; Jackson-Lewis and Przedborski, 2007; Peng *et al.*, 2008).

1.1.5 Brain repair and stem cell based therapies

Repair of damage tissues and organs is essential for the survival of organisms. The repair properties of any tissue or organ are linked to its intrinsic ability for cell replacement of dead cells and the correct integration of the newborn cells that, ideally, should restore the original structure. Therefore, repair of tissues with high cell turnover and low needs for reconstruction, such as skin or bone, is more efficient. The CNS, on the other hand, has weak capabilities for both endogenous cell replacement and pattern repair. Some strategies have been studied over the past years attempting brain repair, particularly therapies using stem cells attempting the enhancement of neurogenesis. Next, some of the most relevant therapeutic strategies for brain repair will be discussed.

1.1.5.1 Cell transplantation

Transplantation of neural precursors or stem cells is one of the promising methods being studied for the reconstruction of neuronal circuits. However, not all cells are good candidates to be used. The cells to be used for grafting must be phenotypically plastic, which means that they need to be able to differentiate into appropriate neurons or glial cells and most importantly, they must be able to proliferate *ex vivo* following stimulation with mitogens. Some authors have shown that grafted cells should be in the correct developmental stage to fully respond to the instructive niche of implantation. However, several studies have shown immature cells to fail integration in the hippocampus (Wang *et al.*, 1998; Sheen *et al.*, 1999). In fact, cells need to respond to the neurogenic signaling from the lesioned tissue, which means that cells should be used in a developmental state concomitant with the appropriate receptor competence to sense the external microenvironment.

In rodent models, SVZ-derived neural stem cells are an interesting starting material for intracerebral transplantation. These cells fulfill the major

criteria mentioned above (plasticity, multipotency, ability to proliferate *ex vivo*), and are good candidates for cell-replacement strategies. SVZ cells have been used in experimental models of Parkinson's disease, and successfully differentiated into mature neurons and integrated the neuronal network, thus contributing to improved motor performance (Zigova *et al.*, 1998; Richardson *et al.*, 2005). SVZ cells were also used in Huntington's disease with improved motor performance (Vazey *et al.*, 2006). In an animal model of multiple sclerosis, Cayre and collaborators observed that SVZ cells grafted into the subcortical white matter differentiated into oligodendrocytes and induced remyelination (Cayre *et al.*, 2006). In other study using this model, SVZ cells were injected in the lateral ventricles repopulating and differentiating into functional oligodendrocytes (Pluchino *et al.*, 2005). However, one of the limitations of a successful grafting is the absence of the required neuronal factors that allow differentiation of grafted cells in the site of injury. For instance, *ex vivo* pre-treatment with factors to correctly differentiate the cells in order to increase the graft efficiency is a desirable option (Zhang *et al.*, 2003), but may not be sufficient in determining the fate of the grafted immature cell. Moreover, the microenvironment of the injured brain plays a very important role, having instructive cues adequate to allow neuronal differentiation and its manipulation also seems to be necessary to improve efficiency and survival of the graft (Cao *et al.*, 2002).

In humans, the perspective of using adult neural stem cells is appealing for autologous transplantation, overcoming the need to find a matching donor, or the administration of immunosuppressive drugs (Galvin and Jones, 2006). However, isolation of sufficient stem cells from patients would involve invasive surgery, and the destruction of healthy brain structures, which limits the clinical application of this strategy (Taupin, 2006). The use of other cell types, such as embryonic stem (ES) cells or induced pluripotent stem (iPS) cells, is being investigated for cell transplantation strategies for the

treatment of several neurodegenerative disorders, such Alzheimer's disease, Parkinson's disease (Lindvall and Kokaia, 2010).

Although these strategies may be a promising approach for the treatment of neurodegenerative disorders, further studies need to be conducted so that they could be considered as a viable option for clinical therapy. Moreover, cell replacement could also be achieved by inducing endogenous stem cells in the adult CNS to differentiate into new neurons and glial cells, a less invasive strategy when compared to cell transplantation.

1.1.5.2 Stimulation of endogenous neurogenesis

In situ stimulation of endogenous adult neural stem cells and modulation of injury-induced neurogenesis is presently being considered as a potential therapeutic approach for neuronal repair in neurodegenerative disorders, as opposed to the more invasive approach of transplantation of exogenous stem cells (Picard-Riera *et al.*, 2004). Although grafting strategies may be an efficient approach for the treatment of neurodegenerative disorders affecting local neuronal populations such as in Huntington or Parkinson's diseases, in multifocal diseases affecting multiple regions of the brain stimulation of endogenous neurogenesis seems to be more advantageous. Since most brain disorders that could benefit from enhanced neurogenesis are accompanied by neuroinflammation, understanding how the inflammatory response affects neurogenesis is fundamental to better design therapeutic strategies for safe and efficient upregulation of endogenous neurogenesis.

Multiple studies have been conducted in order to increase neurogenesis in the SVZ. Injection of EGF and bFGF in the lateral ventricles of rodents increases proliferation and neurogenesis in this area (Craig *et al.*, 1996; Kuhn *et al.*, 1997). Moreover, administration of BDNF also increases cell proliferation and migration into the OB, but also to other brain regions, such as the striatum, the thalamus and the hypothalamus (Zigova *et al.*, 1998; Pencea *et al.*, 2001). Neurogenesis could also be increased by inhibiting bone

morphogenetic protein (BMP) actions, for instance with Noggin (Lim *et al.*, 2000). It has been demonstrated that intraventricular injection of adenoviruses encoding Noggin and BDNF increase the formation of new spiny neurons from progenitor cells in the adult striatal ventricular zone (Chmielnicki *et al.*, 2004).

Some drugs used in the clinics to treat different pathologies could also increase cell proliferation. Neuroleptics like olanzepine, (used for the treatment of schyzophrenia or bipolar disease) increase proliferation in the SVZ (Green *et al.*, 2006). Another example is the administration of nitric oxide donors or 5-phosphodiesterase inhibitors, such as sildenafil, which increase the neurogenesis in the OB and the DG of rats by a mechanism involving the intracellular increase of cGMP levels (Zhang *et al.*, 2002; Lu *et al.*, 2003; Zhang *et al.*, 2003; Zhang *et al.*, 2006). The development of strategies to increase endogenous proliferation and further migration of newly generated cells towards the damaged area is needed. For that purpose, the use of chemoattractive factors, like stromal cell derived-factor 1-alpha (SDF-1α), is being used together with proneurogenic factors like VEGF (Zhang *et al.*, 2003; Imitola *et al.*, 2004; Sun *et al.*, 2004; Sun *et al.*, 2006).

1.1.5.3 Other strategies

1.1.5.3.1 Neuroprotection

Neuroprotection concerns the mechanisms and strategies used to protect against neuronal loss or degeneration in the CNS. Neuronal loss is a common feature following an acute brain injury or as a result of chronic neurodegenerative diseases. The main goal of neuroprotection is to limit neuronal dysfunction and neuronal death, thus maintaining the integrity of cellular interactions in the brain. Several strategies are being investigated and some products can potentially be used for neuroprotection, such as free radical scavengers, antiexcitotoxic agents, apoptosis inhibitors, neurotrophic agents, ion channel modulators, metal ion chelators and gene therapy (Polazzi

and Monti, 2010). Neuroprotective strategies are not only useful to limit the extent of the disease-induced lesion or limiting neuronal loss, but may also enhance the survival of newborn cells.

1.1.5.3.2 Anti-inflammatory approaches

Inflammation is frequently associated with brain injury, neurodegenerative diseases and radiation treatment for brain tumors. Although inflammation is detrimental for adult neurogenesis (reviewed by Whitney *et al.*, 2009), it was demonstrated that neurogenesis can be restored by anti-inflammatory treatments (Ekdahl *et al.*, 2003; Monje *et al.*, 2003; Simard and Rivest, 2004). In fact, acute inflammation is important for the protection of the CNS against pathogens or insults and is also involved in the clearance of damaged or dead cells. An acute inflammatory response also enhances neurogenesis. However, chronic inflammatory may hinder neurogenesis. Although described by different authors that chronic inflammation may stimulate one or more processes of neurogenesis, such as proliferation, migration or differentiation, the problem remains in the reduced long-term survival of newly formed neurons (Whitney *et al.*, 2009).

In light of these facts, a full understanding of how the inflammatory response affects neurogenesis is fundamental to the development of therapeutic strategies that can induce neurogenesis from endogenous NPCs. The use of anti-inflammatory drugs that selectively block the anti-neurogenic effect of inflammatory mediators such as tumour necrosis factor alpha (TNF-α), interleukin (IL)-6 and IL-18, without preventing the neurogenic role of inflammation have been addressed as an approach to increase neurogenesis (reviewed by Ajmone-Cat *et al.*, 2008). Epidemiological evidence that chronic use of nonsteroidal anti-inflammatory drugs (NSAIDS) is associated with a decreased risk of developing neurodegenerative diseases such as AD or PD has been reported (McGeer and McGeer, 1995; Lim *et al.*, 2000; Chen *et al.*,

2003). In the case of acute lesions, the administration of the NSAID indomethacin following stroke increased the number of neuroblasts in the striatum of rats (Hoehn *et al.*, 2005), while minocycline was reported to increase the number of newborn neurons in the DG after occlusion of the middle cerebral artery in rats (Liu *et al.*, 2007).

This is an area with great interest where further research is needed. Although anti-inflammatory drugs may be useful for preventing the detrimental effects of inflammation on neurogenesis, it has been suggested that its use as adjuncts to other therapeutic agents, particularly drugs that aim to restore neuronal loss, could to be more advantageous mainly due to the multiple agents underlying the etiology of neurodegenerative disorders. Neuroinflammation will be discussed in detail in the following section.

1.2 Neuroinflammation

Inflammation is a complex biological response to harmful stimuli, such as stress, injury or infection (Nencini *et al.*, 2003; Schmidt *et al.*, 2005). The neuroinflammatory response attempts the protection of the affected organism by removing the injurious stimuli, removing dead and damaged cells initiating the healing process.

1.2.1 Inflammation following injury in the central nervous system

In the past, the CNS was considered immune-privileged because of its protection by the blood-brain barrier (BBB), which selectively allows certain inflammatory effectors to enter and exit. Presently, it is well established that immune surveillance does take place in the CNS, because of the selective permeability of its barrier to T cells, macrophages and dendritic cells (Hickey, 1999). Following damage or exposure to pathogens, an inflammatory response takes place, involving two types of immune cells: CNS resident microglial cells and astrocytes, and infiltrating lymphocytes, monocytes and macrophages of the hematopoietic system (Stoll and Jander, 1999; Streit *et al.*, 1999). Activated immune cells release a plethora of regulatory substances, like complement molecules, cytokines such as interferon gamma (IFN-γ), TNF-α, IL-1β, IL-18 and IL-6, chemokines such as SDF-1α and monocyte chemoattractant protein-1 (MCP-1), glutamate, reactive oxygen species and reactive nitrogen species like nitric oxide (Whitney *et al.*, 2009). These inflammatory mediators are responsible for the recruitment of resident microglia and stimulation of astrogliosis but also for the disruption of the BBB and recruitment of monocytes and lymphocytes from the hematopoietic system into the site of inflammation (Hickey, 1999; Lossinsky and Shivers, 2004; Taupin, 2008). Astrogliosis occurs following injury to the CNS (Latov *et al.*, 1979; Miyake *et al.*, 1988), and this event is believed to be necessary for

containing the inflammatory response, repairing the BBB and reducing further neuronal death (Bush *et al.*, 1999; Lossinsky and Shivers, 2004).

The activation of inflammatory-recruited cells leads to the release of inflammatory factors that creates a positive feedback loop of inflammatory activation, resulting ultimately in neuronal loss and/or neuronal damage. Inflammatory factors could have both beneficial and harmful effects on the cellular environment. Although CNS inflammation should be taken as a protective process, its injurious properties have also to be considered (Wyss-Coray and Mucke, 2002). In most organs, inflammation leads to collateral injury, which is normally reversible due to the inherent regenerative capacity of those tissues. However, collateral injury in the CNS is mediated by inflammatory factors that are neurotoxic themselves or responsible for the increased migration of inflammatory cells into the lesioned areas, which propagates the detrimental inflammatory status leading to neuronal loss and atrophy of the affected areas. To minimize this reciprocating cycle, an uncontrolled immune response is avoided by increasing the threshold needed to initiate the inflammatory process. In fact, the immune response in the brain is different from that in peripheral tissues, particularly the initiation and sensitivity to inflammation. Thus, a neuroinflammatory response requires higher levels of antigen or damage to occur compared to the levels in peripheral tissues (Matyszak, 1998; Perry, 1998).

The severity of neuroinflammation varies from mild acute to uncontrolled chronic inflammation, resulting in different activation states of inflammatory cell types and different biological outcomes (Stoll *et al.*, 2002). Neuroinflammation is now believed to be involved in the mechanisms leading to various CNS diseases, also affecting neurogenesis (Das and Basu, 2008).

1.2.2 Microglia: the immune resident cells of the brain

Microglia are glial cells found in the central nervous system structures, brain and spinal cord. Unlike astrocytes, oligodendrocytes and ependymal

cells, microglial cells derive from the mesodermal germ layer and were identified by del Rio Hortega (1919). In the adult CNS, microglia are numerous and are distributed throughout the parenchyma. In the healthy, brain, microglia are usually referred to as “resting microglia”, whose function has been clarified by different studies (Davalos *et al.*, 2005; Nimmerjahn *et al.*, 2005; Davalos *et al.*, 2008). Microglia in this resting state are in a constant surveillance activity of their immediate surrounding. Due to this constant probing activity of microglial cells, Hanish and Kettenmann suggested a new designation for this resting state – “surveying microglia” (Hanisch and Kettenmann, 2007). In fact, resting microglia present a typical morphology, with long slender processes extending from a small-elongated soma. Microglial processes and arborizations are highly dynamic and mobile, being randomly formed *de novo* or retracted (Nimmerjahn *et al.*, 2005). Such a dynamic and fine organization enables the stationary microglial cells to screen different brain regions without disturbing the neuronal structure. The active scanning by microglia rapidly changes to a targeted movement into a site of injury, and this response is apparently dependent on the activation of purinergic receptors and may involve astrocytes (Gehrmann, 1996; Haynes *et al.*, 2006). Moreover, microglia is distributed throughout the CNS with variable density in different brain regions (Lawson *et al.*, 1990). This ramified morphology occurs only in vivo, and seems to be relatively absent in isolated microglia in cell cultures.

Microglial can transition from an active probing state towards a more reactive state in response to a pathological event, a process characterized by morphological and functional changes. The microglial cells have the ability to adapt their activation status according to the pathological process occurring in the brain, thus exhibiting functional plasticity (Fig. 1.6).

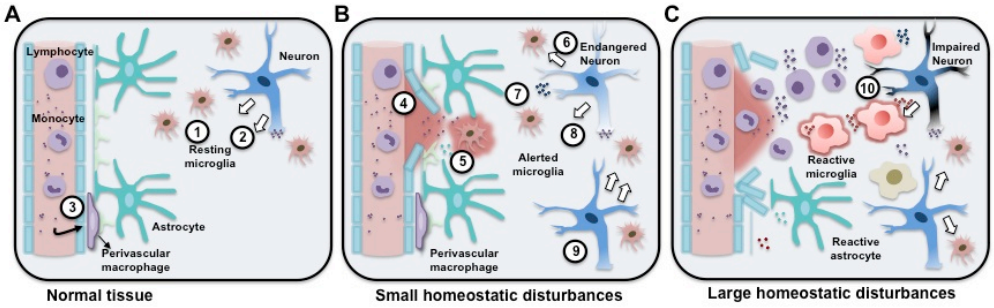


Figure1.6. Activity states of microglial cells. A) Microglial cells in normal tissue (1). Microglia in the resting state are in fact actively surveying their environment. (2) Microglia have receptors for different molecules which allows the detection of signals from disturbances in homeostasis. Neurons may release signaling molecules which keep microglial cells in this surveillance mode. (3) Circulating monocytes can differentiate into perivascular macrophages or into parenchymal microglial cells. **B) Small homeostatic disturbances** – focal and transient microglial activation. (4) Vascular or tissue damage is detected by microglial cells that rapidly respond changing its activity profile. (5) Astrocytes support the microglial response by releasing, for example, purinoreceptor ligands. (6) Disruption of normal cross-talk between neurons and microglia, allow endangered neurons to call microglial cells. (7) Alerted microglial cells can produce neurotrophic factors to support endangered neurons. (8) Endangered neurons can also release signaling molecules that are not usually released or release signaling molecules in critical concentrations, indicating functional disturbances. (9) Microglial cells may be able to restore normal homeostasis thus limiting further damage. **C) Large homeostatic disturbances** – strong insults to the CNS. (10) Microglial cells may adapt a more reactive behavior with drastic changes in phenotype. Excessive acute, chronic or maladaptative responses of microglial cells may be detrimental to neurons and glial cells (adapted from Hanisch and Kettenmann, 2007).

During the activation process, cell morphology changes from a ramified to a hyperramified and finally to an amoeboid morphology, which facilitates cell migration through the neuronal parenchyma (Raivich, 2005; Hanisch and Kettenmann, 2007). Reactive microglia have the capacity to rapidly upregulate a large number of receptor types, like cytokine receptors, toll-like receptors or cell adhesion molecules (see Table 1.1 for a complete description). On the other hand, these cells also release a plethora of inflammatory agents, like cytokines, chemokines, proteases or free radicals (see Table 1.2 for a full listing). The secreted products have been shown to act as pro- or anti-inflammatory agents, contributing to beneficial or detrimental outcomes in the

CNS. In fact, several of the products that have been reported to play an antimicrobial role have also been implicated in neurodegenerative disorders (reviewed by Graeber and Streit, 2010). Moreover, there is evidence that the microglial neurotoxic profile is due to both the loss of the beneficial functions and/or a shift of cellular function to a pro-inflammatory state, by releasing cytotoxic substances, including NO or superoxide (O_2^-), and the pro-inflammatory factors IL-1β and TNF-α, whose primary function is the destruction of invading pathogens (Block and Hong, 2005; Ransohoff and Perry, 2009; Graeber and Streit, 2010).

Table 1.1 Microglial cell membrane receptors (adapted from Block and Hong, 2005).

Cell Adhesion molecules
Immunoglobulins (Ig) superfamily
Major histocompatibility (MHC) class I and II glycoproteins
CD4 receptors
Ig Fc receptors (FcγRI, RII, RIII)
Intercellular adhesion molecule 1 (ICAM-1)
Integrins
Leucocyte function-associated antigen-1 (LFA-1, CD11a/CD18: CR1)
Mac-1 (CD11b/CD18; CR3)
p150, p95 (CD11c/CD18; CR4)
Complement receptors: C1q, C5a
Cytokine/chemokines receptors
Interferon (IFN)-α, IFN-β, IFN-γ
Interleukin (IL)-1, IL-6, IL-10, IL-12, IL-16, IL, 23
Tumor necrosis factor (TNF)-α
Macrophage-colony stimulating factor (M-CSF), Granulocyte-macrophage (GM)-CSF
CCR, CXCR, CX3CR
Opioid receptors (μ, κ)
Cannabinoid receptors (CB_1, CB_2)
Toll-like receptors
CD14 receptors
Mannose receptors
Purinogenic receptors
Benzodiazepine receptors (mitochondrial membrane)

Under certain pathological conditions, the resident microglial population of the adult CNS parenchyma can be supplemented with bone marrow-derived cells (Priller *et al.*, 2001; Bechmann *et al.*, 2005; Massengale *et al.*, 2005; Wirenfeldt *et al.*, 2007; Soulet and Rivest, 2008). Microglial-like cells migrating into the adult CNS from the peripheral blood and joining to the resident reactive microglia has been demonstrated in various models of acute injury (Priller *et al.*, 2001; Wirenfeldt *et al.*, 2007; Clausen *et al.*, 2008; Lambertsen *et al.*, 2009), as well as in chronic inflammation (Simard *et al.*, 2006; Remington *et al.*, 2007).

Table 1.2 Secretory products of microglial cells (adapted from Block and Hong, 2005).

Complement factors: C1, C3, C4
Amyloid precursor protein (APP)
Quinolinic acid, glutamate
Proteases: elastase, plasminogen
Matrix metalloproteinases: MMP-2, MMP-3, MMP-9
Growth factors: nerve growth factor, fibroblast growth factor
Cathepsins B and L
Eicosanoids: PGD_2, leukotriene C_4
Free radicals: superoxide, nitric oxide
Chemokines
CC: CCL2/MCP-1, CCL3/MIP-1α, CCL4/M1P-1β, CCL5/RANTES
CXC: CXCL8/IL-8, CXCL9/MIG, CXCL10/IP-10, CXCL12/SDF-1α
CX3C: CX3CL1/fractaline
Cytokines
IL-1α, IL-1β, IL-6, IL-10, IL-12, IL-16, IL-23
Transforming growth factor (TGF)-β, TNF-α

1.2.2.1 Acute microglia activation

In the brain, the innate immune response is predominantly characterized by an activation of microglia, which act as the resident macrophages of the CNS. Activated microglial cells engage in different actions, scavenging the

damaged or dead neurons, as well as initiating a local inflammatory reaction. Many molecules and conditions that indicate a threat to the CNS can trigger the transition from the resting (or surveying) state to active, alert and reactive microglia. Microglia recognize a wide range of signals from homeostatic or pathophysiological surveillance. High levels of factors that are not usually present or that occur in an abnormal format, such as protein aggregates, or others, such as microbial structures, are sensed by microglia receptors that present matching specificities. Activation of these receptors triggers signaling pathways that cause microglia to respond to the detected insult. Receptors such as the family of pattern recognition Toll-like receptors (TLRs), detect and distinguish between fungal, bacterial and viral structures (reviewed by Hanisch and Kettenmann, 2007). Activation of microglia can result in different phenotypes, which means functional diversity. When challenged by acute bacterial invasion, phagocytosis occurs together with the release of inflammatory mediators. On other hand, microglia release anti-inflammatory factors when removing apoptotic cells or cellular debris.

1.2.2.2 Chronic microglia activation

Chronic neuroinflammation persists for a long period following an initial injury or insult to the CNS. It is characterized by a long-standing microglial activation and subsequent sustained release of inflammatory mediators that leads to increase oxidative and nitrosative stress. These effects work to perpetuate the inflammatory response, activating additional microglial cells, promoting their differentiation, thus resulting in a self-perpetuating release of inflammatory factors (Frank-Cannon *et al.*, 2009).

The transition from acute to chronic activation of microglia can be exemplified by the alteration in cytokine production, with a progressive reduction in the production of IL-1β, IL-1α, TNF-α, IL-6 and free radicals, such as nitric oxide. In contrast, IL-10 and prostaglandin E2 (PGE2), that continue

being produced and released during chronic inflammation, are potent suppressors of the inflammatory function of microglia (Ekdahl *et al.*, 2009).

Chronic inflammation is often associated to be detrimental and damaging to the nervous tissue, however it could also play a protective role, as in the case of acute neuroinflammation. Whether inflammation is beneficial or detrimental to the brain may be dependent on the duration of the inflammatory response and also on the kind of microglial activation. Activated microglia change into a chronic profile following injury, maintaining an acute phenotype or changing into another activation state, whose effects could be neuroprotective or maladaptative (Cacci *et al.*, 2008).

1.2.3 Inflammatory neurodegeneration

Neurodegeneration is characterized by the slow, progressive dysfunction and loss of neurons in CNS. Immune activation within the CNS is a classical feature of ischemia, infections, trauma and neurodegenerative diseases (Fig. 1.7). Often it could contribute to collateral injury, which has been implicated in neuronal loss and atrophy in different brain regions. The susceptibility of neurons to cell death mediated by the innate inflammatory response (Boje and Arora, 1992; Chao *et al.*, 1992) and the failure of self-repair of the brain (Bjorklund and Lindvall, 2000), combined with the inhibition of axonal growth and limited repopulation by neuronal precursor cells are pointed as the main causes for the neurodegenerative event that follows inflammation (Goldberg and Barres, 2000; Fournier and Strittmatter, 2002). However, not all immune responses in the CNS are detrimental, and in many cases they actually aid repair and regeneration. Particularly, microglial cells seem to play an important role in facilitating the reorganization of neuronal circuits and triggering repair. It has been shown that insufficient clearance of cell debris by microglia, prevalent in several neurodegenerative diseases and declining with aging is associated with an adequate regenerative response (Neumann *et al.*, 2009).

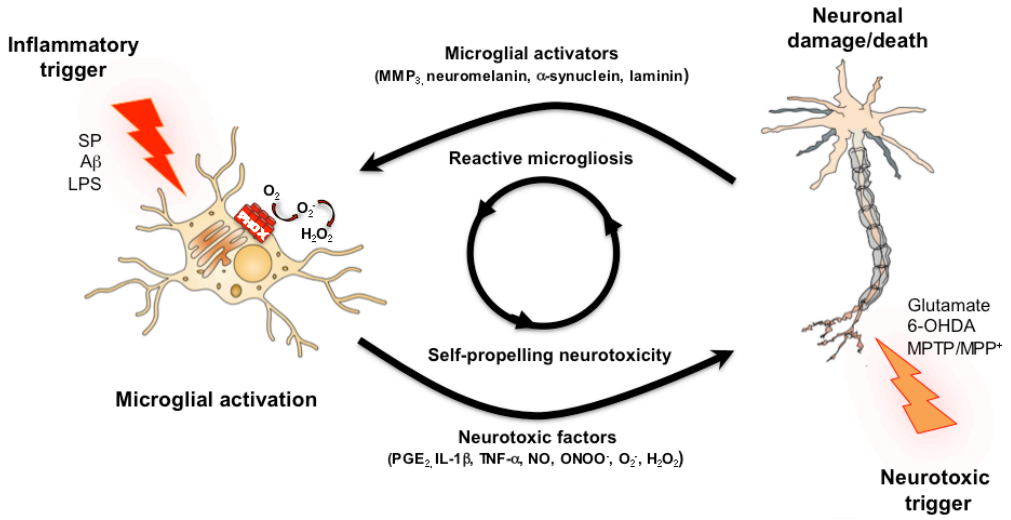


Figure 1.7. Reactive microgliosis leads to chronic neuronal damage. Microglial activation both with pro-inflammatory stimuli, such as lipopolysaccharide (LPS), and direct neuronal damage result in the release of neurotoxic factors, such as interleukin-1beta (IL-1β), nitric oxide (NO), tumor necrosis-alpha (TNF-α), peroxynitrite ($ONOO^-$), superoxide (O_2^-) and hydrogen peroxide (H_2O_2). Following damage with pro-inflammatory factors or direct neurotoxic triggers, neurons release microglial activators, such as MMP3, neuromelanin or α-synuclein, which activate microglial cells, thus perpetuating the cycle. This self-perpetuating cycle of microglia overactivation in response to neuronal damage, which results in progressive neurotoxicity, is known as reactive microgliosis. Reactive microgliosis has been suggested to underly the progressive neuronal damage in many neurodegenerative disorders Aβ, amyloid-β; MMP3, matrix metalloproteinase 3; MPP^+, 1-methyl-4-phenylpyridinium ion; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; 6-OHDA, 6-hydroxydopamine; PGE_2, prostaglandin E_2; PHOX, NADPH oxidase; SP, substance P (adapted from Block *et al.*, 2007).

Thus, microglia seem to play dual roles in neurodegeneration, both as a detrimental or beneficial agent (Ekdahl *et al.*, 2009). Immune activation is also important to limit viral infections and removal of necrotic cells following ischemia. Like microglia, T cells can also help recovery during neurodegenerative diseases, although the mechanisms remain unclear. The relationship between inflammation and neurodegeneration is being studied in models of CNS diseases such as Alzheimer's and Parkinson's disease, and suggest neuroinflammation to be a crucial process, if not the cause of CNS injury seen in these diseases (Whitney *et al.*, 2009; Lehnardt, 2010). However,

these studies also revealed complex neuroimmune interactions, both at cellular and molecular levels, demonstrating that immune cells secrete both neurotoxic and neuroprotective molecules (Amor *et al.*, 2010). Despite different triggering events, a common feature for the neurodegenerative event seems to be the chronic activation of microglia.

1.2.4 Brain inflammation and neurogenesis

Inflammation in the CNS is a complex process with different outcomes in neurogenesis. Besides the differences between mild acute and uncontrolled chronic inflammation, the shift from pro-neurogenic to anti-neurogenic inflammation seem to be dependent on the mechanism by which microglia, macrophages and/or astrocytes are activated, as well as on the duration of inflammation. Inflammation and microglia activation was initially thought to inhibit adult neurogenesis, however recent evidence indicates that microglia under certain circumstances can be beneficial to the neurogenic process.

Ekdahl and colleagues showed that lipopolysaccharide (LPS)-induced microglial activation impairs hippocampal neurogenesis in rats (Ekdahl *et al.*, 2003), apparently through the increased production of TNF-α, as also reported later by Liu (Liu *et al.*, 2005). Further support for the detrimental effect of LPS-activated microglia has been provided by Cacci and collaborators, showing that acute activation of microglia with LPS reduces neural stem cells survival and neuronal differentiation (Cacci *et al.*, 2008). The mechanism by which microglia exerts these adverse effects is through the release of the pro-inflammatory mediators IL-1β, IL-6, IFN-γ and TNF-α and reactive oxygen and nitrogen species, which seem to play an essential role in suppressing neurogenesis (Ben-Hur *et al.*, 2003; Cacci *et al.*, 2005; Iosif *et al.*, 2006; Koo and Duman, 2008). Moreover, suppression of activated microglia with an antibiotic, such as minocycline was shown to increase neurogenesis in the hippocampus, thus suggesting that the degree of impaired neurogenesis correlate with the number of activated microglial cells (Ekdahl *et al.*, 2003).

Neurogenesis was also restored following treatment with indomethacin, a non-steroidal anti-inflammatory drug, after irradiation-induced inflammation (Monje *et al.*, 2003) or focal cerebral ischemia (Hoehn *et al.*, 2005). Other studies report an increased survival of the newly generated neuroblasts in the striatum following stroke (Hoehn *et al.*, 2005), or in the dentate gyrus following middle cerebral artery occlusion (MCAO) (Liu *et al.*, 2007), when microglia activation is inhibited indomethacin or by minocycline, respectively.

However, in 2006, Butovsky and collaborators reported microglia to play a dual role of on neurogenesis, suggesting that microglia activated by inflammation inhibited neurogenesis, but also that microglial cells, when activated by IL-4 or low levels of IFN-γ, associated with T-helper cells, induced neurogenesis and oligodendrogenesis (Butovsky *et al.*, 2006). Moreover, a persistent production of neurons from adult neural stem cells has been observed, even after inhibition of acute microglia activation, during recovery after stroke (Kokaia *et al.*, 2006; Thored *et al.*, 2006). Furthermore, long-term survival of newborn neurons was observed following SE, concomitant with chronic microglial activation (Bonde *et al.*, 2006). In vitro studies have also showed an important role for microglia in directing the replacement of damaged or lost cells (Aarum *et al.*, 2003; Morgan *et al.*, 2004; Walton *et al.*, 2006; Nakanishi *et al.*, 2007). More recently, Jakubs and colleagues showed that microglia activation by LPS and inflammation enhances the integration of newborn neurons into the adult rat hippocampus (Jakubs *et al.*, 2008). These studies suggest a neuroprotective role of microglia for newborn cells.

More recently, Thored and colleagues have shown that long-term accumulation of activated microglia, although with a downregulated inflammatory profile, is concomitant with persistent neurogenesis in the adult SVZ after stroke (Thored *et al.*, 2009). These authors also suggested that IGF-1 plays an important role in the pro-neurogenic role of long-term accumulation of microglia, since it could promote proliferation and differentiation of neural progenitors, which is in agreement with previous studies (Kalluri *et al.*, 2007;

Zhu *et al.*, 2008). Other inflammatory mediators have also been implicated in the enhanced proliferation and migration of new neurons following brain damage, such as the SDF-1α and its receptor CXCR4 (Imitola *et al.*, 2004; Thored *et al.*, 2006). Moreover, the release of trophic factors like GNDF and BDNF are involved in the removal of synapses of damaged neurons, while the expression of the glutamate uptake protein GLT-1 by microglial cells during chronic microglial activation prevents glutamate-mediated neurotoxicity (Ekdahl *et al.*, 2009).

In summary, these studies suggest that although microglia has a detrimental action in early stages of the inflammatory response that follows acute insults, it could be converted into a protective state during chronic activation. However, future studies need to be conducted in order to assess the interaction between inflammation and neurogenesis, and more importantly, how newborn cells integrate the neuronal network. Different microglial phenotypes and morphologies occur during inflammation, thus the genetic and proteomic characterization will be of great interest to understand more accurately this complex crosstalk.

Astrocytes constitute the majority of glial cells in the CNS, and play an important role in neuroinflammation, as mentioned previously. Astrocytes, known collectively as astroglia, are roughly star-like and have broad end-feet on their processes. Considered in the past to be the packing material in the brain holding neurons in place, nowadays astrocytes are known to provide structural support for neurons, but also for playing important regulatory functions, including maintenance of extracellular ion balance, signaling to neurons through Ca^{2+}-dependent release of glutamate and repair and scarring process of the brain and spinal cord following injury (Svendsen, 2002). In inflammation, activated astrocytes release inflammatory factors, growth factors and regulate extracellular levels of excitatory amino acids, such as glutamate, which could induce neurogenesis from adult neural stem cells (Song *et al.*, 2002). Astrocytes are also involved in regulating the production of neural

synapses. However, like microglia, astrocytes have also been implicated in chronic inflammation that underlies neurodegeneration (Blasko *et al.*, 2004).

1.2.5 Inflammatory mediators

1.2.5.1 Cytokines

Cytokines are a group of small chemical messengers secreted by glial cells in the CNS and by numerous cells of the immune system. Cytokines can be classified as proteins, peptides or glycoproteins. Each cytokine has a cell-surface receptor, whose activation and subsequent intracellular signaling may involve upregulation and/or downregulation of several genes and their transcription factors that could lead to increased production of other cytokines, increased expression of receptors or, instead, the suppression of their own effect. The fact that cytokines trigger the release of other cytokines makes them important in chronic inflammatory processes.

The role of pro-inflammatory cytokines released by activated microglial cells, has been studied in the brain. Cytokines are described to have a differential effect on neurogenesis (Monje *et al.*, 2003). IL-6 and TNF-α decreased neurogenesis *in vitro* (Monje *et al.*, 2003), while IFN-γ has a detrimental effect on survival and proliferation of neural stem/progenitor cells (Ben-Hur *et al.*, 2003). Particularly IL-6 has been implicated in the mechanism underlying the negative effect of activated microglia on hippocampal neurogenesis (Monje *et al.*, 2003). IL-6 is also released by astrocytes, decreasing the number of newly formed of neurons (Vallieres *et al.*, 2002).

Other pro-inflammatory cytokines play an important role in modulating neurogenesis, such as TNF-α, that is produced in immune responses by activated astrocytes and microlial cells, as well as in some neurodegenerative diseases (Whitney *et al.*, 2009). TNF-α exerts its biological functions via interaction with TNF-α receptors (TNFR), TNF-R1 or TNF-R2 (Cacci *et al.*, 2005). Depending on the type of TNFR activated, TNF plays different effects

on neurogenesis (Iosif *et al.*, 2006). Thus, TNF-R1 acts as a suppressor of neural progenitor cells proliferation in the adult hippocampus, both in intact and in pathological brain. More recently, it has been shown that TNF-R1 is a negative regulator of stroke-induced SVZ progenitor proliferation (Iosif *et al.*, 2008). Contrarily, TNF-R2 enhances proliferation and survival of newly generated striatal and hippocampal neurons (Heldmann *et al.*, 2005).

Other studies reported TNF-α to be a positive regulator of neurogenesis both *in vivo* (Wu *et al.*, 2000) and *in vitro* (Widera *et al.*, 2006), an effect mediated by activation of nuclear factor κB (NF-κB) and further increased cyclin D1 expression. Cyclin D1 is necessary for cell cycle progression by promoting passage through the G1/S restriction point, thus promoting cell proliferation (Widera *et al.*, 2006). The proliferative effect of TNF-α has also be suggested to be a consequence of the TNF-α–mediated up-regulation of neurotrophins and growth factors such as nerve growth factor (NGF) and bFGF (Das and Basu, 2008). Besides regulating cell proliferation, TNF-α exerts a detrimental effect on differentiation and neuronal survival (Liu *et al.*, 2005). Other pro-inflammatory cytokines, such as IL-1β and IL-18, have been studied with respect to neurogenesis, however the exact role in the neurogenic process needs further research (Whitney *et al.*, 2009).

1.2.5.2 Complement system

The complement system is a part of the innate immune system with an important role in protection against infectious agents via inflammation, opsonization and cytolysis. Although complement proteins derived from serum/blood-brain barrier breakdown can contribute to injury or disease, infiltrating immune cells may represent an important local source of complement after injury. Astrocytes, microglia and neurons produce the complement proteins, but the role in normal and in ischemic CNS remains unclear (Thomas *et al.*, 2000; D'Ambrosio *et al.*, 2001). The complement

cascade is involved in triggering cell death and recruiting cells of the immune system to sites of inflammation, however, it might also have important neuroprotective roles that are only now coming to light. Recent evidence suggests that targeted activation of complement might be a potential approach for treatment of stroke and other acute neurodegenerative diseases (Yanamadala and Friedlander, 2010).

C3a and C5a are two complement molecules, both potent chemoattractants, expressed mainly in hippocampus and cortex in the normal brain (Davoust *et al.*, 1999; O'Barr *et al.*, 2001). It has been reported that neural progenitors and immature neurons express the receptors for complement fractions C3a and C5a. Mice lacking C3a or C3a receptor present reduced basal neurogenesis and impaired neurogenesis in the SVZ following ischemia, thus suggesting a beneficial involvement of these complement molecules in neurogenesis (Rahpeymai *et al.*, 2006).

More recent studies have implicated the complement system in diseases of the CNS, such Alzheimer's disease and other neurodegenerative conditions, such as spinal cord injuries (Yanamadala and Friedlander, 2010). Altogether, these reports open another aspect of modulation of neurogenesis by the inflammatory process, and more studies need to be done.

1.2.5.3 Cyclooxygenase-2

Cyclooxygenase-2, also referred as COX-2, is an inducible enzyme produced by macrophages and activated microglial cells following inflammation. The proliferative effect of COX-2 has been reported after inhibition or knockout of COX-2, which decreased progenitor cells proliferation in the SGZ of adult mice following transient forebrain ischemia (Sasaki *et al.*, 2003; Das and Basu, 2008). It has been hypothesized that COX-2 may affect neurogenesis through the production of prostaglandin E_2 (Uchida *et al.*, 2002), which may act directly via PGE2 receptor subtype EP3 receptors expressed in the granule cell layer of the dentate gyrus (Nakamura *et al.*, 2000) or indirectly

through bFGF (Sabbieti *et al.*, 1999). Moreover, administration of inhibitors of cyclooxygenase such as acetylsalicylic acid and indomethacin was also reported to reduce ischemia-induced proliferation in adult gerbils or rats (Kumihashi *et al.*, 2001; Sasaki *et al.*, 2003).

1.2.5.4 Nitric oxide

Nitric oxide, a short-lived diffusible gas, is an important cellular messenger involved in many physiological and pathological processes. NO is biosynthesized from L-arginine, oxygen and NADPH by various nitric oxide synthase (NOS) enzymes. NO is constitutively produced in neurons via neuronal NOS (nNOS) and has important roles in neuronal differentiation, survival, neurodegeneration and synaptic plasticity (Holscher, 1997). NO is also generated via inducible NOS (iNOS), expressed following an insult to the CNS such as ischemic neuronal death, thus acting as an inflammatory mediator (Estrada and DeFelipe, 1998). The role of NO as a modulator of neurogenesis is still unclear, and matter of strong debate. Controversial findings are found in the literature which ultimately illustrate that NO has influence in the neurogenic process both by inhibiting or stimulating neurogenesis depending on the source of NO. Thus, NO produced by the nNOS isoform has been demonstrated to have an antiproliferative effect both *in vitro* and *in vivo* (Packer *et al.*, 2003; Moreno-Lopez *et al.*, 2004; Zhu *et al.*, 2006; Torroglosa *et al.*, 2007). In the areas surrounding the SVZ, immature neurons express nNOS. The selective inhibition of nNOS with 7-nitroindazole (7-NI) greatly increased cell proliferation in the subventricular zone, RMS and olfactory bulb (Moreno-Lopez *et al.*, 2004). Furthermore, proliferation was increased in the mouse DG by nNOS inhibition (Zhu *et al.*, 2006) and in an nNOS knockout mouse model (Zhu *et al.*, 2006; Fritzen *et al.*, 2007). On the contrary, NO synthesized by endothelial NOS (eNOS) in the SVZ and iNOS in the dentate gyrus following focal ischemia, stimulates neurogenesis (Zhu *et al.*, 2003; Reif *et al.*, 2004; Cardenas *et al.*, 2005). Moreover, increased

immunoreactivity against iNOS following transient ischemia was shown to correlate with a decrease of nNOS in the hippocampus, which is concomitant with an increased neurogenesis (Luo *et al.*, 2007; Corsani *et al.*, 2008).

Altogether these findings illustrate that NO is a modulator of neurogenesis in diverse ways, and the different NO synthases are important players in this effect on neurogenesis (Estrada and Murillo-Carretero, 2005). NO effects on neurogenesis are dependent on the developmental period and source of NO. Apparently, under physiological conditions NO acts as a negative regulator of cell proliferation while in inflammatory conditions a decrease in nNOS and increase in iNOS may act as a mechanism to enhance neurogenesis. However more studies need to be conducted to determine the signaling mechanisms underlying the dual role of NO on neurogenesis. NO will next be discussed in detail in section 1.3, and the mechanisms underlying the role of NO on NSCs proliferation will be addressed in the present thesis.

1.3 Nitric oxide

1.3.1 Nitric oxide in the nervous system

Nitric oxide is a gaseous signaling molecule synthesized by the nitric oxide synthase (NOS) family of enzymes present in most cells in the body. NO is implicated in a wide range of physiological processes, being involved in several functions including blood-vessel tone, immune response and neurotransmission, but it can also be an important player in pathophysiological processes. Different members of the NOS family control different functions of NO. The discovery of NO in the nervous system was a breakthrough in the concept of neural communication. Indeed, the existence of nitrergic nerves dependent on the release of NO for transmission mechanisms emphasizes the uniqueness of this mediator (Moncada *et al.*, 1997). Unlike canonical neurotransmitters, NO is synthesized on demand, diffusing from nerve terminals and it is not stored in vesicles nor released by exocytosis.

In the peripheral nervous system, NO controls the relaxation of smooth-muscle cells, regulating the muscle tone of the gastrointestinal tract and urogenital tract, such as smooth-muscle relaxation in the *corpora cavernosa* allowing penile erection (Curro and Preziosi, 1998; Takahashi, 2003). In the CNS, NO is also associated with cognitive function, having an important role in synaptic plasticity important to the control of biological functions including body temperature, sleep-wake cycle, appetite and modulation of hormone release (reviewed by Calabrese *et al.*, 2007). NO can also act as a neuromodulator, particularly due to its diffusion distance of 40 ± 300 μm in radius (Garthwaite and Boulton, 1995), thus acting not only in cells that release NO, but acting on neighboring cells as an autocrine and/or paracrine messenger. Another distinctive feature from classical neurotransmitters is that, unlike them, NO ends its action after reacting with a substrate and not by enzymatic degradation or re-uptake. In addition, the key mechanism to regulate the activity of NO is the control of its synthesis.

Garthwaite and colleagues showed for the first time that in the CNS NO behaves as an intracellular messenger by increasing cyclic GMP levels, after the activation of glutamate receptors (Garthwaite *et al.*, 1988).

Physiologically, NO interacts with several intracellular targets activating different signaling pathways with a stimulatory or inhibitory response (reviewed by Guix *et al.*, 2005). However, NO could be toxic to cells, in a mechanism dependent on the formation of reactive nitrogen species (RNS) (Pacher *et al.*, 2007). Oxidative stress and nitrosative stress, a consequence of high levels of reactive oxygen species (ROS) and RNS, have been implicated in the pathogenesis of several neurodegenerative disorders (Moncada and Bolanos, 2006; Sultana *et al.*, 2006; Sultana *et al.*, 2007).

1.3.1.1 Nitric oxide synthases

The NOS family of enzymes is responsible for the synthesis of NO. Three different enzyme isoforms have been identified in mammalian cells: neuronal NOS (nNOS, type I), endothelial NOS (eNOS, type III) and inducible NOS (iNOS, type II) (Dawson and Snyder, 1994; Bredt, 1999; Guix *et al.*, 2005). Neuronal and endothelial NOS are constitutively expressed in specific tissues and require calcium-calmodulin complexes for their activation, while iNOS is calcium-independent and its regulation depends on *de novo* synthesis (reviewed by Guix *et al.*, 2005).

NO is synthesized by NOS, in a reaction that catalyzes the conversion of L-arginine, and oxygen into NO and L-citrulline (Palmer *et al.*, 1988; Moncada *et al.*, 1991). All three NOS isoforms need NADPH and co-factors for catalytic activity, specifically flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), iron protoporphyrin (heme), and tetrahydrobiopterin (BH4). FAD, FMN and heme are involved in the redox reactions leading to the synthesis of NO (Fig. 1.8). Heme and BH4 comprise the scaffold that maintains the substrate channel (Gorren and Mayer, 1998).

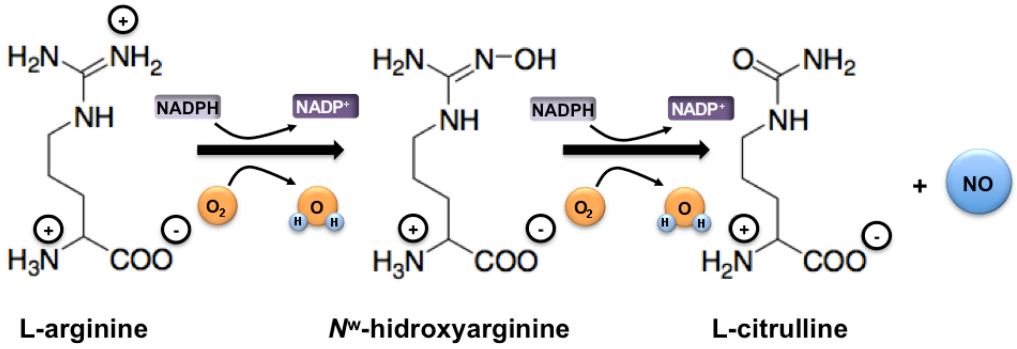


Figure 1.8. Synthesis of nitric oxide by nitric oxide synthase (NOS). L-arginine is oxidized to N^{w}-hidroxyarginine in the presence of NADPH and O_2. N^{w}-hidroxyarginine is re-oxidized to L-citrulline producing NO. NO easily diffuses within the cell or across the cell membrane, and is involved in multiple biological processes. All the NOS isoforms have four prosthetic groups that are required for the production of NO: flavin adenine dinucleotide (FAD), flavin adenine mononucleotide (FMN), tetrahydrobiopterin (BH4) and iron protoporphyrin IX (heme). The electron flow in the NO synthase reaction is: NADPH > FAD > FMN > heme > O_2.

The most common isoform in the brain is nNOS, and is present in different areas, such as the cerebral cortex, the thalamus, the hypothalamus, the striatum, the amydgala, the olfactory bulb, the cerebellum and the hippocampus, particularly in the CA1 region and in the DG (Bredt *et al.*, 1991; Vincent and Kimura, 1992; Dawson and Snyder, 1994; Rodrigo *et al.*, 1994). Although nNOS is present mainly in neurons, it has been found in astrocytes and cerebral blood vessels as well. The nNOS gene is present in chromosome 12, and four major nNOS isoforms have been described, the α (160 kDa), β (136 kDa) γ (125 kDa) and μ (165 kDa), isoforms that differ in size. nNOS can be regulated at the post-translational level via phosphorylation by protein kinase (PK) A, calmodulin-dependent kinases and PKC. Expression of nNOS is under the control of sex hormones, which increase nNOS in human neurons (Lee *et al.*, 2003).

Human eNOS is a 135 KDa protein with 1294 amino acids codified by a gene located in chromosome 7, present in the endothelium (Marsden *et al.*, 1993). In the brain, eNOS is expressed in cerebral endothelial cells, regulating cerebral blood flow, in granulle cells of the dentate gyrus and in pyramidal neurons of CA1, CA2 and CA3 hippocampal regions (reviewed by Calabrese

et al., 2007). eNOS has also been identified in other cell types including human and rat astrocytes (Colasanti *et al.*, 1998; Iwase *et al.*, 2000).

iNOS, whose gene is located in chromosome 17 (Lowenstein *et al.*, 1992), is expressed mainly in macrophages, astrocytes and microglial cells following inflammatory or immunological stimulation (Bredt, 1999; Calabrese *et al.*, 2006). iNOS is also expressed by neurons (Heneka and Feinstein, 2001). While the reversible binding of calcium to calmodulin regulates nNOS and eNOS, iNOS is regulated at the transcriptional level, not depending on calcium for activation. The fact that calmodulin is already bound to iNOS explains its calcium-independency (Guix *et al.*, 2005).

Although the main producing pathway of NO is dependent on the activation of NOS, NO can be synthesized by other mechanisms: the xanthine oxidase pathway, reduction of nitrates in acid and reducing conditions or by H_2O_2 and L-arginine by a non-enzymatic mechanism. Nevertheless, the synthesizing pathway responsible for the messenger role of NO is its enzymatic synthesis.

1.3.1.2 Cell survival and neuroprotection

NO is an important neuroprotective agent in the CNS, acting by multiple mechanisms to afford neuroprotection. NO may promote neuroprotection by S-nitrosylation, enhancing neuronal survival, particularly against excitotoxicity due to prolonged stimulation of NMDA receptors. Moreover, NO can also confer cytoprotection through the inhibition of caspase activity also by S-nitrosylation of the catalytic cysteine residues in caspases, reducing the activity of these proteases (Tenneti *et al.*, 1997; Liu and Stamler, 1999; Mannick *et al.*, 2001). The induction of heme oxygenase-1 (HO-1) is an early event in cellular response against oxidative stress and NO has been shown to induce HO-1 in the hippocampus (Kitamura *et al.*, 1998). Moreover, the effect of NO in the kinase Akt and the transcription factor cAMP responsive element-binding (CREB) has been shown to be involved in the

survival pathway of cerebellar granule cells, in a mechanism mediated by the sGC-cGMP-PKG pathway (Contestabile and Ciani, 2004; Riccio *et al.*, 2006).

1.3.1.3 Neurodegenerative diseases

Strong evidence has been reported in the literature to support a role of NO in the pathogenesis of neurodegenerative disorders, including autoimmune and chronic neurodegenerative diseases. This role of NO seems to be dependent on the concentration reached locally in the tissue. When NO is produced in excess, NO shifts from a physiological to a neurotoxic agent. NO overproduction can be due to nNOS activation following persistent glutamate excitatory input and/or to iNOS expression. Likewise, the excessive release of both glutamate and NO, coupled with oxidative stress and mitochondrial dysfunction, are involved in a number of neurodegenerative diseases. NO from glial origin has been reported as an important factor contributing for the vulnerability of neurons. Thus, iNOS expression and further increase in NO levels due to microglial activation causes neuronal death both *in vivo* and *in vitro* in rodents (Lee *et al.*, 2003; Cunningham *et al.*, 2005). Some authors suggested this neurotoxic effect to be a consequence of inhibition of the respiration, leading to hypoxia, and excitotoxicity (reviewed by Pacher *et al.*, 2007), hypothesizing the involvement of $ONOO^-$ in the mechanisms of neuronal death, due to excessive NO release by glial cells (Mander *et al.*, 2005). Protein nitration inhibits tyrosine phosphorylation which affects the signaling of many pathways involved in the control of cell survival, proliferation, or programmed cell death. Although NO has been implicated in acute injury events, particularly due to the massive release of NO from inflammatory response, its as also been associated to more slowly and progressive disorders can be sporadic or by genetic inheritance. Parkinson's disease, Alzheimer's disease, Huntington's disease, multiple sclerosis and amyotrophic lateral sclerosis are all neurodegenerative disorders in which NO has been suggested to be involved, since all of them show evidence of

oxidative and nitrosative stress (Guix *et al.*, 2005; Calabrese *et al.*, 2009). Furthermore, the presence of 3-nitrotyrosine, a marker for protein nitration, has been reported in several neurodegenerative diseases linked to oxidative stress such as AD or PD (Good *et al.*, 1996; Good *et al.*, 1998). Understanding the involvement of NO in the ethiology of these disorders highlights the potential beneficial role of selective NOS inhibitors. However, the best therapeutical approach would be the prevention of peroxynitrite formation with antioxidants (Calabrese *et al.*, 2007).

1.3.2 Nitric oxide signaling

1.3.2.1 Classical pathway

1.3.2.1.1 sGC-cGMP-PKG pathway

The main cellular signaling pathway stimulated by NO is the activation of soluble guanylate cyclase (sGC), subsequent production of cyclic guanosine-3',5'-monophosfate (cGMP) and further activation of protein kinases that regulate various physiological (Arnold *et al.*, 1977). Neurons synthesize cGMP in response to NO by activation of sGC, a heterodimeric heme-containing enzyme. NO reacts with the heme group of the sGC, which undergoes a conformational change, converting GTP into the second messenger cGMP (Fig. 1.9). Some studies suggest that NO can also downregulate sGC activity, particularly in some neuroinflammatory conditions (Sardon *et al.*, 2004).

cGMP-dependent kinases (PKG), which are serine/treonine kinases, are activated by cGMP and are involved in several physiological phenomena including long-term potentiation in the hippocampus and long-term depression in the cerebellum (Jurado *et al.*, 2005; Schlossmann and Hofmann, 2005). PKG type I is expressed as two isoforms: PKG Ia, which is activated at low cGMP concentrations, and is present in the cerebellar Purkinje cells and

smooth muscle cells; and PKG Ib, which requires higher concentrations of cGMP for activation, and is more abundant in the hippocampus and olfactory bulb. PKG II is a membrane-bound protein, in contrast to the cytosolic type I, which has been observed in the brain, intestine and kidney (Schlossmann and Hofmann, 2005).

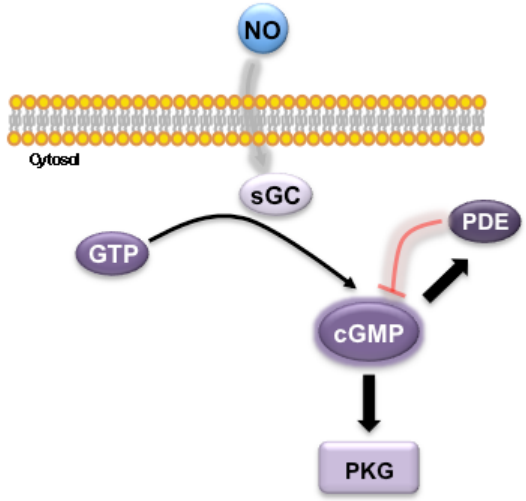


Figure 1.9. Nitric oxide signaling: guanylate cyclase activation. Nitric oxide reacts with the heme group of the soluble guanylyl cyclase (sGC), a cytosolic enzyme that catalyses the transformation of guanosine-5'-triphosphate (GTP) into 3,5-cyclic guanosine monophosphate (cGMP). cGMP is a second messenger with several downstream effectors, but the most important is protein kinase G (PKG). cGMP modulates the activity of certain phosphodiesterases of cyclic nucleotides (PDE), that catalyze the hydrolysis of cGMP, thus avoiding excessive accumulation of this molecule.

cGMP also modulates the activity of phosphodiesterares of cyclic nucleotides (PDE). The use of selective PDE inhibitors have been proven to be useful in clinic, particularly the inhibitors of PDE type 5, such as sildenafil citrate, sold as viagra, or tadalafil and vardenafil which are drugs used to treat erectile dysfuncion and pulmonary arterial hypertension (Krumenacker *et al.*, 2004; Burnett, 2006; Hemnes and Champion, 2006).

1.3.2.2 Post-translational modifications

1.3.2.2.1 S-nitrosylation

Nitric oxide can have different effects, depending on the local concentration and molecular environment. S-nitrosylation is a post-translational modification, and is a reversible regulatory mechanism on several proteins due to a direct interaction of NO with the sulphur from thiols groups in specific aminoacids in proteins, like cysteins, forming nitrosothiol adducts (Fig. 1.10) (Hanafy *et al.*, 2001).

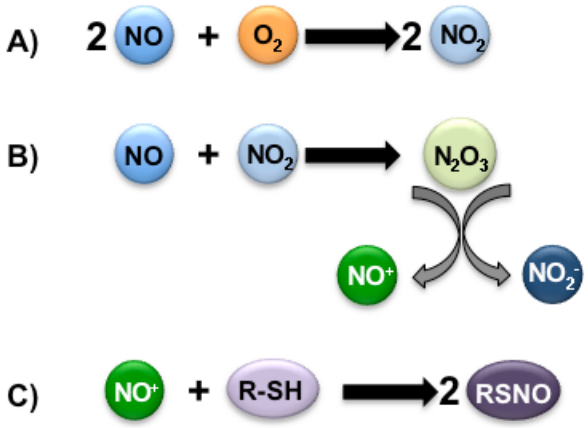


Figure 1.10. S-Nitrosylation. S-nitrosylation is a ubiquitous modification of cysteine thiol by nitric oxide (NO). S-nitrosylated proteins form when cysteine thiol reacts with NO in the presence of an electron acceptor (O_2). **A)** At high concentrations, NO may react with oxygen producing nitrogen dioxide (NO_2), which is a strong oxidant. **B)** Once sufficient levels of NO_2 are attained, NO is oxidized by NO_2 to form dinitrogen trioxide (N_2O_3). N_2O_3 can be partially dissociated into nitrite (NO_2^-) and nitrosium ion (NO^+). **C)** The nitrosium ion is responsible for nytrosylation of electrophilic compounds, such as thiols. NO^+ reacts with the sulfur atom of cysteine thiols (R-SH), forming S-nitrothiols (RSNO) (adapted from Hanafy *et al.*, 2001).

S-nitrosylation is one of the most important cellular mechanisms of NO for switching and regulating protein function (Hess *et al.*, 2005; Lipton, 1999). While nitrosylation of proteins, such as metalloproteinases and proteasome

components may lead to neurodegenerative disorders (Gu *et al.*, 2002; Halliwell, 2002), the nitrosylation of other proteins results in neuroprotection (Foster *et al.*, 2009). S-nitrosylation, as any physiologically relevant signal transduction mechanism, alters function, presents substrate selectivity, and is reversible (Hanafy *et al.*, 2001). S-nitrosylation of p21Ras is a good example signal transduction by NO, as detailed next.

1.3.2.2.1.1 p21Ras-MAPK pathway

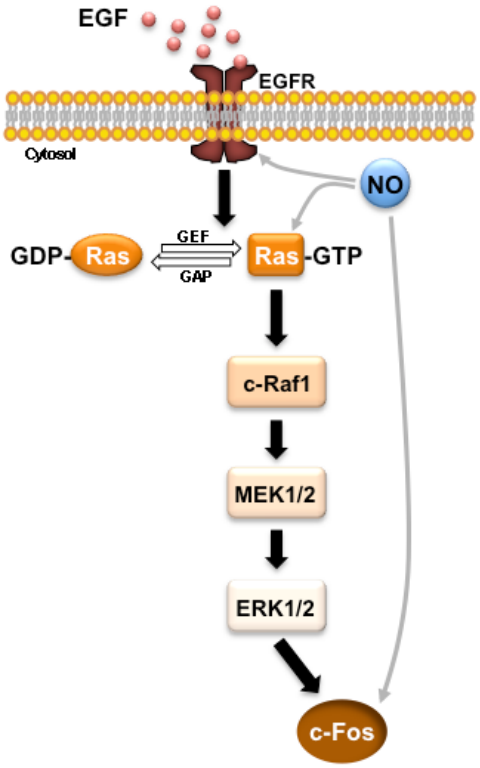


Figure 1.11. Nitric oxide signaling: the p21Ras-MAPK pathway. EGF-mediated dimerization and trans-(auto)phosphorylation of EGFR favours p21Ras activation (Ras-GTP), due to the exchange of GDP for GTP catalysed by a guanine nucleotide exchange factor (GEF). Folowing p21Ras activation a sequential phosphorylation cascade of kinases including Raf-family kinases, ERK/MAPK kinases (MEK1⁄2 and ERK1⁄2) occurs, leading to the activation of different transcription factors. The GTPase activity of Ras, enhanced by a GTPase-activating protein (GAP), transforms back active Ras (Ras-GTP) in its inactive form (Ras-GDP). NO targets the EGFR-p21Ras-MAPK proliferative pathway at different points including: S-nitrosylation of EGFR,

inhibiting its intrinsic tyrosine kinase activity; S-nitrosylation of p21Ras, favouring its activation by enhancing nucleotide exchange and inhibition of the promoter activity of the transcription factor c-fos.

NO is able to S-nitrosylate the p21 monomeric GTPase, p21Ras. Ras has 5 cysteine residues, but only cysteine 118 (Cys118) is nitrosylated with functional relevance (Lander *et al.*, 1995; Lander *et al.*, 1996; Lander *et al.*, 1997). Nitrosylation of p21Ras enhances its guanine nucleotide exchange and the subsequent recruitment of downstream effectors such as the mitogen activated kinase (MAPK) pathway (Fig. 1.11), playing a key role in proliferation, differentiation and apoptosis by modulation of cyclin-dependent kinases and their inhibitors (Guix *et al.*, 2005). Furthermore, S-nitrosylation of p21Ras also recruits the phosphatidylinositol-3 kinase (PI3-K) pathway (reviewed in Villalobo, 2006).

1.3.2.2.2 Nitration

Nitration has been proposed as an irreversible post-translational modification with important biological effects (Ischiropoulos, 2003). NO quickly reacts with superoxide anion (O_2^-), which leads to the formation of peroxynitrite ($ONOO^-$) (Reiter *et al.*, 2000). Furthermore, peroxynitrite can be synthesized after persistent inhibition of mitochondrial respiratory chain activity by NO (Moncada and Bolanos, 2006). Peroxynitrite is as strong oxidant, which can oxidize thiol residues to sulfenic and sulfonic acids and nitrate peptides and proteins at the phenyl side chain of tyrosine residues (Fig. 1.12). Tyrosine nitrated by peroxynitrite forms 3-nitrotyrosine that may impair some cellular functions, since tyrosine residues are important for phosphorylation signaling, for instance. Nitration of tyrosine residues interferes with the normal activity of proteins by inducing conformational changes leading ultimately to a loss of function (Hanafy *et al.*, 2001). Examples of this post-translational modification include heme oxygenases, histone deacetylase 2 or actin (Guix *et al.*, 2005).

Copper/zinc-superoxide dismutase (Cu/Zn-SOD or SOD1) deficiency facilitates peroxynitrite formation from NO since neurons are unable to eliminate O_2^- efficiently (Moncada and Bolanos, 2006). In some cases, nitration of tyrosine resides results in a gain of function, particularly in the case of protein kinase C, glutathione S-transferase, JNK and poly-ADP-ribose synthetase (for review see Guix *et al.*, 2005). Tyrosine-nitrated proteins have been detected in tissue samples from various inflammatory or degenerative diseases, which suggest that protein modification by peroxynitrite may be involved in the pathogenesis of several neurological diseases (Ischiropoulos and Beckman, 2003; Moncada and Bolanos, 2006).

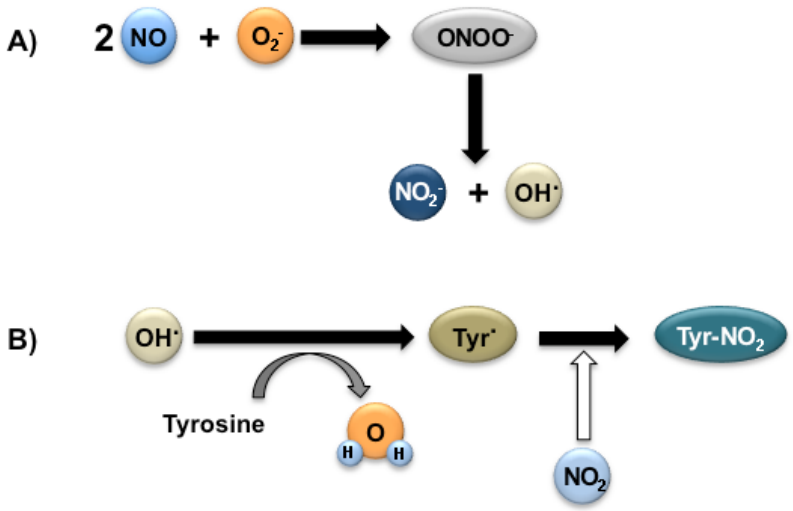


Figure 1.12. Protein nitration. Protein nitration consists in the addition of a nitro group (NO_2) to proteins, mainly with 3-position tyrosine residues (Tyr), with formation of 3-nitrotyrosine. **A)** Nitric oxide (NO) can react with superoxide anions (O_2^-) to form peroxynitrite ($ONOO^-$), which is a strong oxidant agent. The $ONOO^-$ mediated nitration depends on its intermediate products, such us nitrogen dioxide (NO_2) and hydroxyl radical ($OH^\cdot$). NO can be produced by inducible nitric oxide synthase (iNOS) under inflammatory conditions or by neuronal nitric oxide synthase (nNOS), as in the case of excitotoxicity. **B)** Tyrosine is oxidized to a tyrosine radical ($Tyr^\cdot$) by the hydroxyl radical. Next, the tyrosine radical binds to NO_2, forming nitrotyrosine ($Tyr\text{-}NO_2$) (adapted from Hanafy *et al.*, 2001).

1.3.3 Nitric oxide and adult neurogenesis

Nitric oxide was described to have a dual role on the regulation of adult neurogenesis. NO synthesized from nNOS appears to decrease neurogenesis or to act as an antiproliferative agent (Packer *et al.*, 2003; Moreno-Lopez *et al.*, 2004), whereas NO from iNOS and eNOS origin seems to stimulate neurogenesis (Zhu *et al.*, 2003; Reif *et al.*, 2004).

Indeed, some studies suggested NO to be antiproliferative under physiological conditions (Packer *et al.*, 2003; Moreno-Lopez *et al.*, 2004; Matarredona *et al.*, 2005; Covacu *et al.*, 2006; Torroglosa *et al.*, 2007; Murillo-Carretero *et al.*, 2009), while others reported NO to be proliferative, enhancing neurogenesis in pathological conditions (Zhang *et al.*, 2001; Zhu *et al.*, 2003). Next, the effect of NO in cell proliferation and neurogenesis will be discussed.

1.3.3.1 Cell proliferation and physiological neurogenesis

NO is an antiproliferative agent for a wide variety of cell types, including neuronal precursors (Packer *et al.*, 2003). Several mechanisms have been proposed to explain how NO exerts its anti-mitotic effect. In physiological conditions NO was shown to inhibit cell proliferation (Peunova *et al.*, 2001), in a mechanism independent of cGMP (Ignarro *et al.*, 2002; Murillo-Carretero *et al.*, 2002). NO can also exert its antiproliferative action via the p21Ras signaling pathway (Gonzalez-Zulueta *et al.*, 2000). An increase in the activity of PKA has also been described to mediate the antiproliferative effect of NO, being involved in increased expression of p53, p21Ras and heme oxigenase (Guix *et al.*, 2005). Transcription factors are common intracellular targets that mediate the antiproliferative effect of NO. Vossen and Erard suggested that NO downregulates the activity of transcription factors, and in particular nuclear receptors, via S-nitrosylation of cysteine residues as a possible mechanism to decrease cell proliferation (Vossen and Erard, 2002).

Moreover, the anti-proliferative effect of NO has been associated with the inhibition of cyclin A and the activation of the cyclin-dependent kinase inhibitor $p21^{Cip1/Waf1}$ by p42/p44 mitogen-activated-protein kinase (Ishida *et al.*, 1997; Bauer *et al.*, 2001). Poluha and collaborators, reported NO-mediated neuronal differentiation to use similar mechanisms to those described by Ishida and collaborators (Poluha *et al.*, 1997). Decreased cell proliferation and subsequent cell cycle arrest are phenomena closely linked to cell differentiation. Thus, the anti-mitotic effect of NO, which allows the action of differentiating factors, strongly suggests a role of NO in the maturation process that occurs during developmental and adult neurogenesis.

Indeed, NO is a regulator of neurogenesis in the CNS. The transient expression of nNOS during the development of the CNS suggest that NO participates in embryonic neurogenesis (Bredt and Snyder, 1994; Roskams *et al.*, 1994; Santacana *et al.*, 1998). In fact, NO may regulate cell proliferation during brain development, playing an important role in axonal projection patterning (Peunova and Enikolopov, 1995; Kuzin *et al.*, 1996). Endogenous NO appears to be a negative regulator of neurogenesis in the SVZ and DG where it usually reduces neuronal precursor proliferation, as demonstrated in nNOS knockout mice or by the treatment with inhibitors of NOS (Packer *et al.*, 2003; Matarredona *et al.*, 2004; Moreno-Lopez *et al.*, 2004). The decreased expression of the oncogene N-myc has been suggested as a possible mechanism for the antineurogenic effect of physiological NO (Ciani *et al.*, 2004).

Two major evidences from experimental studies suggest NO as an important modulator of cell proliferation and cell differentiation. First, the fact that several tumoral cell lines express different isoforms of NOS when exposed to differentiating factors, and second, the fact that inhibiting NOS activity prevents or delays cell differentiation. Altogether, these evidences suggest NO to be a pivotal player in the switch from immature proliferative neural stem cells to differentiating cells.

1.3.3.2 Cell proliferation and pathophysiological neurogenesis

NO from inflammatory origin has been reported as an agent promoting adult neurogenesis in the damaged ischemic brain (Zhu *et al.*, 2003; Sun *et al.*, 2005). Moreover, NO-releasing drugs can enhance recovery after brain injury, partly by increasing neurogenesis in the dentate gyrus and subventricular zone (Zhang *et al.*, 2001; Lu *et al.*, 2003; Chen *et al.*, 2004; Keynes and Garthwaite, 2004), following ischemic stroke (Zhang *et al.*, 2001) and traumatic brain injury (Lu *et al.*, 2003). It was suggested that nNOS-derived NO and iNOS-derived NO play opposite roles in regulating neurogenesis following cerebral ischemia (Luo *et al.*, 2007). Indeed, Luo and collaborators demonstrated that reduced nNOS is involved in ischemia-induced hippocampal neurogenesis by up-regulating iNOS expression, in a mechanism dependent on cAMP responsive element-binding protein phosphorylation (Luo *et al.*, 2007).

As seen, NO can have concentration-dependent effects, depending on the local concentration and surrounding molecular environment. At physiological concentrations NO is described to be a mediator in antiproliferative signaling on several tumoral cell lines, as well as on stem/progenitor cell cultures, favoring cell differentiation (Packer *et al.*, 2003; Matarredona *et al.*, 2004; Moreno-Lopez *et al.*, 2004), while in pathophysiological concentrations NO can affect neural stem cell proliferation (Covacu *et al.*, 2006; Torroglosa *et al.*, 2007; Murillo-Carretero *et al.*, 2009). However, the molecular mechanisms responsible for this dual effect of NO are not fully clarified.

1.4 Objectives

Nitric oxide is a pleiotropic signalling molecule with several distinct functions in the central nervous system including regulation of proliferation of stem cells, but contradictory observations are found in the literature. Some studies suggest that NO has an antiproliferative action, favoring astrogliogenesis (Covacu *et al.*, 2006; Torroglosa *et al.*, 2007), while others point out that NO can increase cell proliferation (Zhang *et al.*, 2001; Lu *et al.*, 2003; Zhu *et al.*, 2003; Chen *et al.*, 2004). Moreover, NO was shown to modulate neurogenesis in the adult CNS. Depending on the insult and on its source, NO can act either as an anti-proliferative agent (Packer *et al.*, 2003; Matarredona *et al.*, 2004), or stimulate neuronal precursor proliferation and differentiation (Zhang *et al.*, 2001; Zhu *et al.*, 2003). It appears that in physiological conditions, NO tonically inhibits cell proliferation in the brain, while in pathophysiological conditions, such as brain injury, it exerts a proliferative effect on the dividing population of neuronal precursors. However, the exact mechanisms by which NO regulates neuronal proliferation and differentiation are not yet clarified, and further investigation on this matter is needed. Since neuroinflammation is detrimental for adult neurogenesis, it would be of great interest to elucidate the role of inflammatory NO, produced by the transcriptionally regulated enzyme inducible nitric oxide synthase (iNOS), on the ongoing neurogenesis in these conditions.

Within this scenario, we propose to identify the mechanisms that are involved in the role of NO in neural stem cell proliferation. Therefore, the main goal of this work was to study the regulation of proliferation of neural stem cell by NO.

The aims of the specific chapters are presented as follows:

In Chapter 2, we investigated the effect of different concentrations of NO from an exogenous donor on the proliferation of derived neural stem cells isolated from the subventricular zone, at concentrations ranging from

physiological to pathophysiological levels. For *in vivo* studies, we used a model of injury-induced neuroinflammation and neurogenesis, the kainic acid (KA) model of temporal lobe epilepsy to evaluate cell proliferation in the SGZ of the dentate gyrus of the hippocampus, both in wild-type and in iNOS-deficient mice. Particularly the involvement of the EGRF-p21Ras-MAPK pathway was studied as an eventual target for NO-mediated proliferative events.

In Chapter 3, the involvement of the sGC-cGMP-PKG pathway in the proliferative effect of NO described in Chapter 2 was studied in an *in vitro* model of SVZ-derived neural stem cells. We investigated the involvement of the guanylyl cyclase and cGMP-dependent signaling in both early and late proliferation of neural stem cells.

In Chapter 4, we used both SVZ-derived neural stem cells cultures alone or in a mixed culture system with microglia isolated from wild-type or iNOS-knockout mice to investigated the antiproliferative effect of high concentrations of NO on neural stem cell proliferation and explored what the underlying mechanisms for the antiproliferative effect. We further studied whether the antiproliferative effect of high concentration of NO released either by an exogenous NO donor or by active microglial cells was mediated by peroxynitrite formation, and subsequent nitration of the EGF receptor, thus leading to decreased proliferative signaling.

Chapter 2

Nitric oxide stimulates the proliferation of neural stem cells bypassing the epidermal growth factor receptor

Part of the work presented in this chapter was published in
Stem Cells, **28**, 1219–1230 (2010)

1.5 Summary

Nitric oxide (NO) was described to inhibit the proliferation of neural stem cells. Some evidence suggests that NO, under certain conditions, can also promote cell proliferation, although the mechanisms responsible for a potential proliferative effect of NO in neural stem cells have remained unaddressed. In this work, we investigated and characterized the proliferative effect of NO in cell cultures obtained from the mouse subventricular zone. We found that the NO donor NOC-18 (10 μM) increased cell proliferation, whereas higher concentrations (100 μM) inhibited cell proliferation. Increased cell proliferation was detected rapidly following exposure to NO, and was prevented by blocking the mitogen-activated kinase (MAPK) pathway, without activating the EGF receptor. Downstream of the EGF receptor, NO activated p21Ras and the MAPK pathway, resulting in a decrease in the nuclear presence of the cyclin-dependent kinase inhibitor 1, $p27^{KIP1}$, allowing for cell cycle progression. Furthermore, in a mouse model that shows increased proliferation of neural stem cells in the hippocampus following seizure injury, we observed that the absence of inducible NO synthase ($iNOS^{-/-}$ mice) prevented the increase in cell proliferation observed following seizures in wild-type mice, showing that NO from iNOS origin is important for increased cell proliferation following a brain insult. Overall, we show that NO is able to stimulate the proliferation of neural stem cells bypassing the EGF receptor and promoting cell division. Moreover, under pathophysiological conditions *in vivo*, NO from iNOS origin also promotes proliferation in the hippocampus.

1.6 Introduction

Neural stem cells proliferate throughout life in two main regions of the adult central nervous system, the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus of the hippocampus. The newborn cells can differentiate into neurons or glia (Eriksson *et al.*, 1998; Gage, 2000; Alvarez-Buylla *et al.*, 2002). The proliferation of neural stem cells and neurogenesis in the SVZ or SGZ are often increased following a brain insult, such as ischemic stroke (Arvidsson *et al.*, 2002) or seizures (Parent *et al.*, 1997), which might be part of mechanisms supporting self-repair (Lowenstein and Parent, 1999; Curtis *et al.*, 2007). Thus, the identification of potential therapeutic targets for modulation of endogenous neurogenesis is of great interest.

Several recent studies have suggested that nitric oxide (NO) inhibits proliferation of neural stem cells under physiological conditions (Packer *et al.*, 2003; Moreno-Lopez *et al.*, 2004; Matarredona *et al.*, 2005). In isolated neural stem cells from the SVZ, supraphysiological concentrations of NO inhibit neural stem cell proliferation and promote differentiation of precursors into astrocytes (Covacu *et al.*, 2006; Torroglosa *et al.*, 2007). Torroglosa and colleagues have suggested that NO modulates the tyrosine kinase activity of the epidermal growth factor receptor (EGFR) (Torroglosa *et al.*, 2007), by a mechanism involving nitrosylation of specific cysteine residues in the EGFR (Murillo-Carretero *et al.*, 2009). On the other hand, NO was found to increase neurogenesis following ischemic brain damage (Zhu *et al.*, 2003). Moreover, treatment with a NO donor following middle cerebral artery occlusion increases cell proliferation, neurogenesis and functional recovery (Zhang *et al.*, 2001). Apparently, following a brain insult, the effect of NO on neural stem cells is proliferative, rather than antiproliferative, and enhances neurogenesis. However, there are no studies exploring the signaling pathways involved in the proliferative effect of NO following brain injury.

In this chapter, we investigated how pathophysiological concentrations of NO affect proliferation of neural stem cells and the underlying mechanisms involved. We show that NO bypasses the EGFR and directly activates p21Ras, triggering cell proliferation via activation of the mitogen-activated protein kinase (MAPK) ERK1/2. This kinase, in turn, modulates transcriptional and cell cycle regulators. Finally, using an *in vivo* model of brain insult, we show that cell proliferation is prevented when the production of NO is abolished by deleting the iNOS gene. This strongly suggests, contrary to earlier beliefs, that NO promotes neural stem cell proliferation under certain pathophysiological conditions *in vivo.*

1.7 Materials and Methods

1.7.1 Materials

Dulbecco's Modified Eagle's Medium:F-12 nutrient mixture, (D-MEM/F-12, with GlutaMAX^{TM}-I), B27 supplement, trypsin-EDTA solution (0.05% trypsin, 1 mM EDTA in HBSS), antibiotics (10,000 units/ml of penicillin, 10 mg/ml streptomycin), and trypsin (1:250) were purchased from GIBCO BRL, Life Technologies (Invitrogen, Paisley, UK). Deoxyribonuclease 1 (DNase-1), BrdU, PMSF, dithiothreitol, chymostatin, leupeptin, antiparin, pepstatin A, trypan blue, L-NAME, U0126, alkaline phosphatase-linked anti-rabbit secondary antibody and mouse anti-α-tubulin primary antibody were purchased from Sigma Chemical (St Louis, MO, USA). AG 1478 hydrochloride was obtained from Tocris Bioscience (Bristol, UK). EGF and bFGF were from Invitrogen (Paisley, UK). M-CSF was purchased from Peprotech (London, UK) and NOC-18 from Alexis Biochemicals (San Diego, CA, USA). BSA and MnTBAP were obtained from Calbiochem (San Diego, CA, USA). Hoechst 33342, anti-mouse IgG labeled with Alexa Fluor 594 or 488, and anti-rabbit IgG labeled with Alexa Fluor 594 or 488 secondary antibodies were purchased from Molecular Probes (Invitrogen, Paisley, UK). Griess Reagent System was

obtained from Promega (Madison, WI, USA). PVDF membranes and the Enhanced Chemifluorescence (ECF) reagent were obtained from Amersham Pharmacia Biotech (Buckinghamshire, UK). Other reagents used in immunoblotting experiments were purchased from BioRad (Hercules, CA, USA).

1.7.2 Animals

C57BL/6J mice or B6.129P2-*Nos2*^{tm1Lau}/J were obtained from Charles River (Barcelona, Spain) or The Jackson Laboratory (Bar Harbor, ME, USA), respectively, and kept in our animal facilities with food and water *ad libitum* in a 12h 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.

1.7.3 Subventricular zone cell cultures

Neural stem cell cultures were obtained from the SVZ of postnatal day 0-3 C57BL/6J mice. The SVZ was dissected as previously described (Agasse *et al.*, 2008), and the tissue was digested in 0.025% trypsin/0.265 mM EDTA, for 20 min at 37ºC, and mechanically dissociated. Single cells were ressuspended in fresh D-MEM/F-12 with GlutaMAX^{TM}-I, supplemented with 1% B27, 1% antibiotic (10,000 units/ml of penicillin, 10 mg/ml streptomycin), 10 ng/ml EGF and 10 ng/ml basic fibroblast growth factor (bFGF), and plated on uncoated Petri dishes at a density of 3,000 cells per cm^2. The SVZ stem cells were grown as floating aggregates in a 95% air-5% CO_2 humidified atmosphere at 37ºC, during 7 days. Next, the cells were collected and plated for 5 days on poly-L-lysine-coated plates, in the same medium as above, without added growth factors.

1.7.4 Experimental treatments in neural stem cell cultures

SVZ-derived neural stem cells were exposed to different concentrations of the NO donor DETA-NONOate/NOC-18 (1, 10 and 100 μM) for different periods of time, as indicated in the figure legends and in the text. The MEK1/2 inhibitor, U0126 (1 μM) or the EGFR inhibitor, AG 1478 hydrochloride (200 nM), were added 30 min before NOC-18 and kept throughout the incubation period.

1.7.5 Detection of cell proliferation and cell death by microscopy analysis

To analyze proliferation of neural stem cells, 10 μM 5-bromo-2'-deoxyuridine (BrdU) was added to the cultures 16 hours prior to fixation. BrdU is a thymidine analogue that is incorporated during DNA synthesis (Selden *et al.*, 1993). Nuclei that incorporated BrdU in this time-window were detected by immunofluorescence, as follows. Following 20 min fixation with 4% paraformaldehyde/4% sucrose, the cells were permeabilized with 1% Triton X-100 for 5 min, and DNA was denaturated by treatment with 1 M HCl for 30 min, at 37ºC. Non-specific binding was blocked with 3% bovine serum albumin (BSA) in 0.2% Tween-20 in phosphate-buffered saline (PBS) (PBS-T) for 1 h. BrdU-positive cells were labeled with a rat anti-BrdU antibody (1:50; Immunologicals Direct, Oxford, UK) for 90 min, at room temperature. The cells were then incubated with a secondary antibody goat anti-rat IgG conjugated with Alexa Fluor 594 (1:200), for 1 h at room temperature. Nuclei were stained with Hoechst 33342 (1 μg/ml) for 3 min.

Dead cells were detected by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay, which detects 3'-OH free ends in DNA fragments occurring in cells undergoing apoptosis. A commercial kit from Roche (In Situ Cell Death Detection kit, Fluorescein; Roche Applied Science, Mannheim, Germany) was used, and the standard protocol provided by the supplier was followed. Briefly, after BrdU staining, the cells were

incubated with the TUNEL reaction mixture in a humidified chamber for 1 h, at 37 ºC, in the dark. Coverslips were mounted on glass slides using DAKO fluorescence mounting medium (Dako Cytomation, Glostrup, Denmark), the cells were visualized in a fluorescence microscope (Axioskop 2 Plus, Zeiss, Jena, Germany) and images were acquired with the Axiovision software 4.7.

The number of BrdU-positive nuclei and/or apoptotic cells (TUNEL-positive cells) was counted in each coverslip, and the data are expressed as percentage of the total number of nuclei, counterstained with Hoechst 33342. A minimum of 4 independent experiments (from neural stem cell cultures prepared from different animals) was analyzed for each condition. In each coverslip, the cells of 7-10 randomly selected fields were counted, which represents approximately 900-1,200 cells per coverslip. Colocalization of BrdU and TUNEL was analyzed by laser scanning confocal microscopy, in at least 50 cells from each coverslip in a Leica DM IRE3 microscope (Leica Confocal Software Version 2.77, Leica, Wetzland, Germany). Double-labeled cells were analysed by orthogonal reconstruction of sections scanned at 1 µm-thickness.

1.7.6 Detection of cell proliferation and cell cycle analysis by flow cytometry

SVZ cell proliferation was also assessed by incorporation of 5-ethynyl-2'-deoxyuridine (EdU) and detected by flow cytometry. EdU is incorporated into DNA of dividing cells during S phase (Buck *et al.*, 2008; Cappella *et al.*, 2008; Chehrehasa *et al.*, 2009). SVZ cells were incubated with NOC-18 10µM for 24 h or 100 µM for 48 h, and EdU incorporation was used to assess cell proliferation, using a commercially available kit from Invitrogen (Click-iT® EdU Alexa Fluor® 488 Flow Cytometry Assay Kit). EdU was added to the SVZ cultures 16h before fixation. Fixation was performed with 70% ethanol overnight. Detection of EdU incorporation was based on click chemistry, a copper-catalysed reaction between an azide (conjugated to a fluorophore) and an alkyne (EdU). Fixed cells were incubated for 30 min with Alexa Fluor® 488

azide and copper sulfate. Next the cells were incubated with RNase and the nuclear dye 7-actinomycin D (7-AAD) for 30 min. Cells were analyzed for EdU incorporation and cell cycle on a FACScalibur using the Cellquest software (Becton Dickinson, San Jose, CA, USA). Fifty thousand events were acquired per experiment. Each condition was performed in duplicate, in at least 3 independent experiments. The flow cytometer was calibrated with fluorescent standard microbeads (CaliBRITE Beads; BD Biosciences, San Jose, CA, USA) for accurate instrument setting.

1.7.7 Immunocytochemistry

Fixed cultures (as described in section 2.3.5) were incubated with primary antibodies for 90 min, at room temperature, or overnight, at 4ºC. After rinsing with PBS, the cells were incubated with the appropriate secondary antibodies for 1 h (1:200, anti-mouse or anti-rabbit IgGs conjugated with Alexa Fluor 488 or 594), at room temperature. Nuclei were labeled with Hoechst 33342 (1 µg/ml) for 3 min. Images were acquired in a laser scanning microscope LSM 510 META (Zeiss, Jena, Germany). The primary antibodies and the dilutions used were as follows: mouse anti-Sox-2, 1:100 (R&D Systems, Minneapolis, MN, USA); rabbit anti-nestin, 1:100 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); rabbit anti-GFAP, 1:400 (Dako Cytomation, Glostrup, Denmark); mouse anti-nestin, 1:500 (BD Transduction, San Jose, CA, USA); mouse anti-Ki-67, 1:50 (Novocastra Laboratories, Newcastle, UK); rabbit anti-phospho-ERK1/2, 1:50 (Cell Signaling, Danvers, MA, USA); mouse anti-EGFR (Chemicon, Temecula, CA, USA), rabbit anti-EGFR (Cell Signaling), 1:50; rabbit anti-phospho-Tyr1173-EGFR, 1:200 (Cell Signaling); rabbit anti-$p27^{KIP1}$, 1:50 (Cell Signaling).

1.7.8 Nitric oxide production evaluation

NO release by NOC-18 was assessed by measuring the concentration of nitrites in the culture medium, using a commercial kit from Promega (Griess

Reagent System). The standard protocol provided by the supplier was followed. The concentration of nitrite for each sample was calculated from a standard curve performed using a sodium nitrite solution and the data were expressed in μM.

1.7.9 Ras GTPase activation assay

To detect the presence of activated Ras in cell lysates, a commercial kit was used (Millipore Iberica S.A.U., Madrid, Spain) and the standard protocol provided by the supplier was followed. Briefly, SVZ cultures were exposed to NOC-18 for different time periods, and lysates were prepared and analysed for the presence of GTP-bound p21Ras.

1.7.10 Western blot analysis

Cells were lysed in 50 mM Tris-HCl, 10 mM EGTA, 1% Triton X-100 and 2 mM $MgCl_2$, supplemented with 100 μM phenylmethylsufonyl fluoride (PMSF), 1 mM dithiothreitol, 1 μg/ml chymostatin, 1 μg/ml leupeptin, 1 μg/ml antiparin, 5 μg/ml pepstatin A, 1 mM sodium orthovanadate, 50 mM NaF, pH 7.4 at 4ºC. Protein concentration was determined by the bicinchoninic acid (BCA) method (BCA™ Protein Assay kit, Pierce, Rockford, IL, USA). The samples were used for Western blot analysis after adding 6x concentrated sample buffer (0.5 M Tris, 30% glycerol, 10% sodium dodecyl sulfate (SDS), 0.6 M dithiothreitol, 0.012% bromophenol blue) and heating, for 5 min, at 95ºC.

Equal amounts of protein were separated by electrophoresis on SDS-polyacrylamide gels, and transferred electrophoretically to polyvinylidene difluoride (PVDF) membranes. These were then blocked for 1 h at room temperature, in Tris-buffered saline (137 mM NaCl, 20 mM Tris-HCl, pH 7.6) containing 0.1% Tween-20 (TBS-T) and 3% BSA. Incubations with primary antibodies (rabbit anti-phospho-Tyr845/1068/1148/1173-EGFR, rabbit anti-phospho-ERK1/2; mouse anti-ERK1/2; rabbit anti-phospho-c-Raf (Ser338);

rabbit anti-phospho-p90RSK (Ser380); rabbit anti-phospho-Elk-1 (Ser383); rabbit anti-phospho-c-Myc (Thr58/Ser62); rabbit anti-phospho-CREB (Ser133); rabbit anti-$p27^{KIP1}$, all diluted 1:1,000; Cell Signaling, Danvers, MA, USA) in TBS-T 1% BSA were performed overnight, at 4ºC. Next, the membranes were incubated for 1 h at room temperature with alkaline phosphatase-linked secondary antibodies (anti-rabbit or anti-mouse IgG, 1:20,000) in TBS-T 1% BSA. After extensive washing in TBS-T 0.5% BSA, immunoreactive bands were visualized in the VersaDoc 3000 imaging system (BioRad, Hercules, CA, USA), following incubation of the membrane with ECF reagent for 5 min. Protein loading controls were performed using antibodies against either the total protein in study (e.g. total ERK1/2), or against α-tubulin (1:10,000).

1.7.11 Administration of kainic acid to mice

Adult male wild-type C57BL/6J mice or iNOS-deficient mice (B6.129P2-*Nos2*^{tm1Lau}/J) with 8-10 weeks of age were used. The weight of the animals varied between 20-25 g. Kainic acid (KA; Ocean Produce, Canada) was dissolved in a sterile saline solution (0.9% NaCl in water) and injected subcutaneously (25 mg/kg). All animals that received KA developed grade 5 seizures or higher (1972's Racine´s six-point scale modified for mice (Schauwecker and Steward, 1997)). In animals injected with saline solution alone, no seizures were observed. The mice were maintained for 1 to 5 days after the first generalized seizure. At least three animals survived in each experimental group. The genotype of each mice was confirmed by PCR using the primers recommended by The Jackson Laboratory for B6.129P2-*Nos2*^{tm1Lau}/J mice (5'- ACATGCAGAATGAGTACCGG-3', 5'-TCAACATCTCCTGGTGGAAC-3', and 5'-AATATGCGAAGTGGACCTCG-3'; TIB Biomol, Berlin, Germany). DNA was obtained from tail clips and purified with the QIAamp DNA mini kit (Qiagen Iberia, S.L., Madrid, Spain), and the PCR was run using the FastStart PCR Master mix from Roche Applied Science (Barcelona, Spain).

1.7.12 Immunohistochemistry

Cell proliferation in the subgranular zone of the hippocampus was assessed by BrdU incorporation. BrdU was delivered by intraperitoneal injection in four doses of 50 mg/kg, each 2 hours apart, resulting in a total of 200 mg/kg, up to 12 h before sacrificing the mice. The mice were perfused transcardially with 0.9% NaCl followed by 4% paraformaldehyde in PBS (pH 7.4), following deep anesthesia with sodium thiopental (0.5 g/kg; B. Braun Melsungen AG, Melsungen, Germany). The brains were removed and further kept overnight in 4% paraformaldehyde, and then dehydrated in 20% sucrose/0.1 M PBS for 24 h, at 4ºC. Coronal sections were cut and separated in eight series throughout the brain on a cryostat at a thickness of 30 µm. Free-floating brain sections were processed for detection by immunohistochemistry of BrdU incorporation in the SGZ, as detailed next.

Brain sections were treated with 1 M HCl for 30 min at 65ºC, for DNA denaturation, and then blocked for 1 h with 5% normal goat or donkey serum in 0.25% Triton X-100 in PBS. Slices were then incubated with the primary antibodies, mouse anti-BrdU (1:50; DAKO, Glostrup, Denmark) or rat anti-BrdU (1:50, AbD Serotec, Oxford, UK), mouse anti-Ki-67 (1:50, Novocastra Laboratories, Newcastle, UK), rabbit anti-GFAP (1:2000; DAKO), rat anti-CD45 (1:100; Abcam, Cambridge, UK), and goat anti-doublecortin (1:400; Santa Cruz Biotechnology, Santa Cruz, CA) overnight, at 4ºC. After rinsing with 0.25% Triton X-100 in PBS, the sections were incubated with goat or donkey anti-mouse or anti-rabbit IgG conjugated with Alexa Fluor 594 or Alexa Fluor 488 (1:200) for 2 h. Nuclei were stained with Hoechst 33342 (5 μg/ml) or with DRAQ5 (5 μM) for 10 min. The sections were mounted in 2% gelatin-coated slides with DAKO fluorescence mounting medium. Images were acquired in a laser scanning microscope LSM 510 META (Zeiss, Jena, Germany), and cell counting or quantification of immunoreactive area (using ImageJ, version 1.42o, NIH) was performed in the 5 mid sections of the hippocampus, of at least 3-4 animals, for the correspondent time points.

1.7.13 Data analysis

Data are expressed as means ± SEM. Statistical significance was determined by using two-tailed t tests, one-way or two-way analysis of variance (ANOVA), as appropriate. Differences were considered significant when $p < 0.05$.

1.8 Results

1.8.1 Characterization of SVZ primary cultures

Neural stem cells were isolated from the SVZ and cultured as floating aggregates (neurospheres), as originally described by Reynolds and Weiss (Reynolds and Weiss, 1992). For experiments, the cells were plated on poly-L-lysine-coated coverslips for 5 d, and characterized at this stage. The cells were stained against Sox-2, a transcription factor essential to maintain self-renewal of undifferentiated stem cells, and nestin, a neural precursor cell marker. The percentage of double-labeled cells was approximately 70%, suggesting that the majority of cells remained undifferentiated after plating (Fig. 2.1A-C). The number of Sox-2/nestin positive cells is similar to the number of cells positive for both nestin and glial fibrillary acidic protein (GFAP) (Fig. 2.1D-F), both markers expressed in type B cells of the SVZ (Doetsch *et al.*, 1997). Moreover, GFAP cells were mostly Sox-2 positive (Fig. 2.1G-I). In fact, the percentage of Sox-2/nestin positive cells is similar to values obtained in other studies in which the inhibitory effect of NO on the proliferation of neural stem cells isolated from the SVZ was reported (Covacu *et al.*, 2006; Torroglosa *et al.*, 2007).

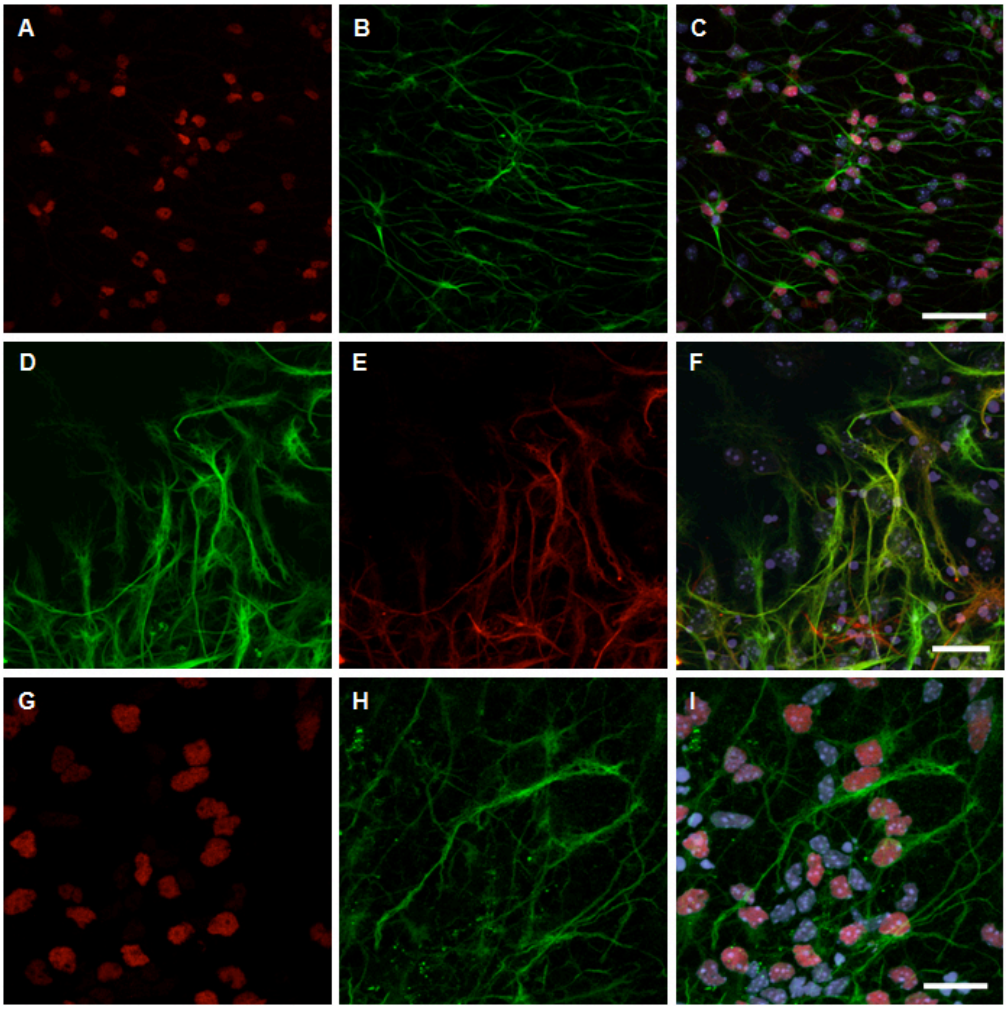


Figure 2.1. Characterization of SVZ stem cell cultures. The micrographs show laser scanning confocal images of SVZ cells labeled against Sox-2 (**A**, **G**, red), nestin (**B**, green; **E**, red) and GFAP (**D**, **H**, green). Merged images are shown in **C**, **F** and **I**. Nuclei were labeled with Hoechst 33342 (blue). Scale bars: 45 µm in **A**, **B** and **C**, 25 µm in **D**, **E** and **F**, and 20 µm in **G**, **H**, and **I**.

1.8.2 NO has a dual effect on the proliferation of neural stem cells

The NO donor NOC-18 was previously used to investigate the effect of NO on the proliferation of SVZ cultures (Covacu *et al.*, 2006; Torroglosa *et al.*, 2007). However, in a recent study, only concentrations above 30 µM were analysed (Torroglosa *et al.*, 2007), and another work, using lower

concentrations (starting at 10 μM), reported that NOC-18 had no positive effect on the proliferation of neural stem cells (Covacu *et al.*, 2006). In these reports, the effect of NO (in higher concentrations) in SVZ cells was found to be antiproliferative. Moreover, these studies were always performed in the presence of EGF, which could mask any potential proliferative effect of NO in these cells. Thus, to investigate whether NO could affect the proliferation of neural stem cells, we first evaluated the incorporation of BrdU by SVZ cell cultures, kept without EGF after plating, following treatment with a range of concentrations of NOC-18 for 24 or 48 h.. Exposure to 10 μM NOC-18 for 24 h increased BrdU incorporation (13.3 ± 0.4%, $p<0.001$), as compared to untreated cultures (8.8 ± 0.7%) (Figs. 2.2A and C), while 100 μM had no effect at 24 h. On the other hand, exposure for 48 h to 100 μM NOC-18, resulted in a significant decrease in proliferation (5.4 ± 0.3%, $p<0.05$), compared to control cultures (9.0 ± 0.8%) (Figs. 2.2B and C), which is in agreement with previous studies (Covacu *et al.*, 2006; Torroglosa *et al.*, 2007). 10 μM NOC-18 had no effect on proliferation after 48 h of treatment. The lowest concentration of NOC-18 used (1 μM) had no significant effect on cell proliferation at any time.

The effect of NO on the proliferation of neural stem cells was also studied by assessing the number of Ki-67-expressing cells. Ki-67 is present during G1, S, G2, and mitosis, but absent from non-dividing cells (G0), being considered a good marker to evaluate proliferation. We observed similar results to those obtained with BrdU incorporation: 10 μM NOC-18 for 24 h significantly increased the number of Ki-67 positive cells (10.5 ± 0.9%, $p<0.05$), when compared to untreated cultures (6.3 ± 0.2%) (Fig. 2.2D), while 100 μM NOC-18 for 48 h significantly decreased the number of Ki-67 positive cells (3.0 ± 0.1%, $p<0.05$) compared to untreated cultures (6.5 ± 0.6%) (Fig. 2.2E).

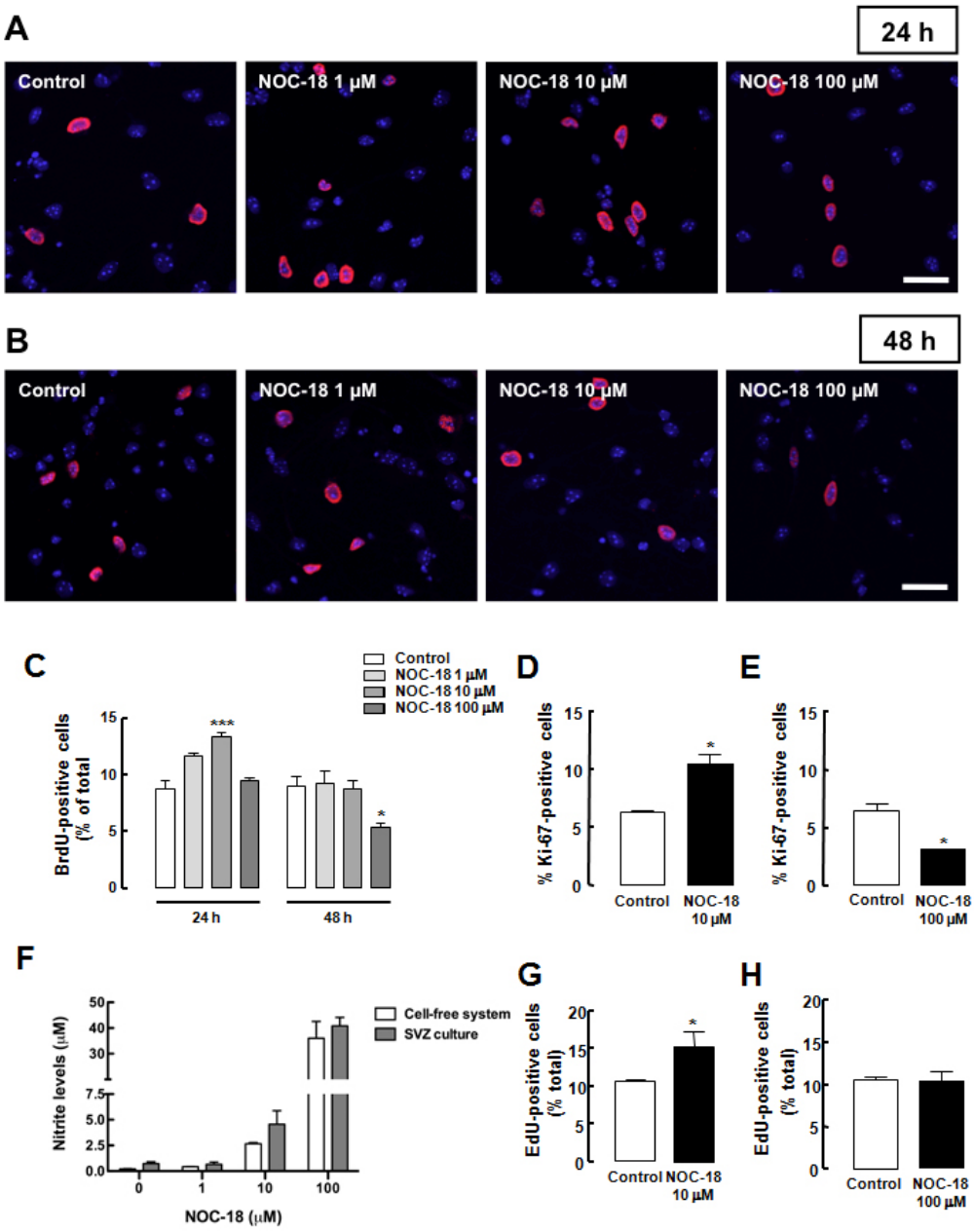


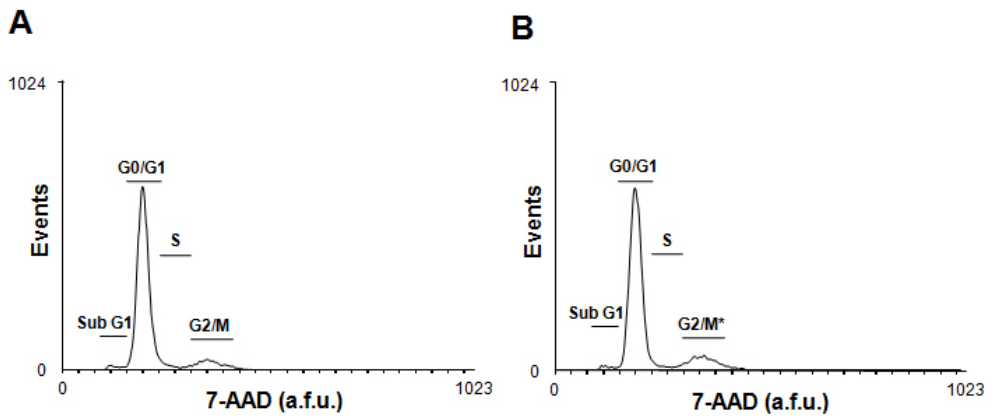
Figure 2.2. Effect of exposure to NO on the proliferation of neural stem cells. (**A, B**) Effect of the NO donor NOC-18 on the number BrdU-positive cells in SVZ cultures. Representative images of BrdU-positive cells (red) in neural stem cell cultures after exposure to increasing concentrations of NOC-18 for 24 h (**A**) and 48 h (**B**) are shown. Nuclei are labeled with Hoechst 33342 (blue). Scale bar: 30 µm. (**C**) The graph depicts the percentage of BrdU-positive cells in SVZ neural stem cells cultures after treatment with increasing NOC-18 concentrations for 24 and 48 h. Data are expressed as means ± SEM of at least 4 independent experiments. One-way ANOVA

(Dunnett's post-test). *p<0.05 and ***p<0.001, NOC-18 significantly different from control. The percentage of Ki-67-positive cells following treatment with NOC-18 (10 μM) for 24 h (**D**) or 100 μM NOC-18 for 48 h (**E**) is shown. Nitrite levels were measured in the culture medium of SVZ cultures or in culture medium alone, after 24h (**F**), as an indication of the amount of NO in the medium. Cell proliferation following treatment with NOC-18 (10 μM) for 24 h (**G**) or 100 μM NOC-18 for 48 h (**H**) was also assessed by incorporation of EdU and assessed by flow cytometry. Data are expressed as means ± SEM of at least 3 independent experiments. Two-tailed t-test, *p<0.05, NOC-18 significantly different from control.

The release of NO by NOC-18 was also evaluated, at 24 h (Fig. 2.2F). Starting at 10 μM, exposure to NOC-18 significantly increased the amount of NO, as assessed by measuring the levels of nitrites in the culture medium. Release of NO by 1 μM NOC-18 was bellow the detection limit of the Griess assay. Similar amounts of nitrites were detected in the culture medium, either in a cell-free system or in culture medium removed from SVZ cultures (two-factor ANOVA, $p>0.05$).

The proliferative effect of NO was also evaluated by a non-manual counting method, using detection of EdU incorporation by flow cytometry, as described in the 2.3.6 section. We observed that 24 h after addition of NOC-18 (10 μM) to SVZ cultures, incorporation of EdU significantly increased to 15.9 ± 2.0% ($p<0.05$) as compared to the EdU incorporation in control cultures (Fig. 2.2G). Furthermore, cell cycle analysis showed that NOC-18 (10 μM) induced a significant increase in the percentage of cells in G2/M (Fig. 2.3; 9.1 ± 0.4%, $p>0.05$), as compared to the control cultures (7.86 ± 0.3%). However, we failed to observe a decrease in cell proliferation induced by exposure to the higher concentration of NOC-18 (100 μM) for 48 h.

Since NO may induce apoptosis in neural cells (Canals *et al.*, 2001), cell death was evaluated following treatment with NOC-18, to test whether the effect of NO on proliferation could be masked by any potential cytotoxicity of NO. We found that the concentrations of NOC-18 used did not affect cell survival significantly, compared to untreated cultures (Table 2.1).



Sub G1	G0/G1	S	G2/M
6.04 ± 0.4 %	77.76 ± 0.5 %	2.62 ± 0.4 %	7.86 ± 0.3 %

Sub G1	G0/G1	S	G2/M
6.80 ± 1.0 %	78.79 ± 1.3 %	2.99 ± 0.3 %	9.10 ± 0.4 %*

Figure 2.3. NOC-18 increases the number of cells in G2/M phase. SVZ cell cycle analysis was assessed by flow cytometry following treatment with NOC-18. NOC-18 (10 μM, 24 h treatment) induced a significant increase in the percentage of cells in G2/M (**B**) as compared to control cultures (**A**). No differences were found for G0/G1 or S phases comparing both conditions. Moreover, NOC-18 had no toxic effect (sub G1). Data are expressed as means ± SEM of at least 3 independent experiments. Two-tailed t-test, *p<0.05, NOC-18 significantly different from control. a.f.u. – arbitrary fluorescence units.

Furthermore, besides incorporation during DNA synthesis, BrdU can also be incorporated in cells undergoing DNA repair. Co-localization of BrdU and TUNEL following exposure to NOC-18 (10 μM) for 24 h was not increased, comparing to untreated cells (Table 2.2). Moreover, 100 μM NOC-18 (48 h) did not change cell viability nor increased cell death.

We also investigated the participation of endogenous NO on the cell proliferation of SVZ cell cultures. The cultures were treated with L-NAME (500 μM), an inhibitor of NO production by the constitutive NO synthases. L-NAME had no effect in the proliferation of neural stem cells, as evaluated by BrdU incorporation (data not shown).

Table 2.1. Cell viability in neural stem cell cultures following exposure to NOC-18.

	Treatment	% live cells	% dead cells
24 h	Control	71.7 ± 1.2%	28.8 ± 1.0%
	1 μM NOC-18	73.2 ± 2.3% (n.s.)	29.0 ± 1.6% (n.s.)
	10 μM NOC-18	71.7 ± 2.9% (n.s.)	31.5 ± 0.5% (n.s.)
	100 μM NOC-18	75.5 ± 2.9% (n.s.)	25.2 ± 1.3% (n.s.)
48 h	Control	80.0 ± 2.0%	17.7 ± 0.7%
	1 μM NOC-18	77.2 ± 1.5% (n.s.)	20.5 ± 1.6% (n.s.)
	10 μM NOC-18	78.6 ± 2.1% (n.s.)	17.9 ± 1.7% (n.s.)
	100 μM NOC-18	77.5 ± 2.9% (n.s.)	19.8 ± 3.4% (n.s.)

Cell viability was assessed by analysis of nuclear morphology. Cells were considered dead when nuclei were condensed/fragmented and brightly stained with Hoechst 33342, and cells presenting a regular nuclear morphology and a light nuclear stain with bright nucleoli were considered live cells. Data are expressed as means ± SEM of at least 3 independent experiments. n.s. (non-significant) $p>0.05$, not different from the control, one-way ANOVA, Dunnett's post-test.

Table 2.2. Colocalization of BrdU-positive and TUNEL-positive cells in neural stem cell cultures following exposure to NOC-18.

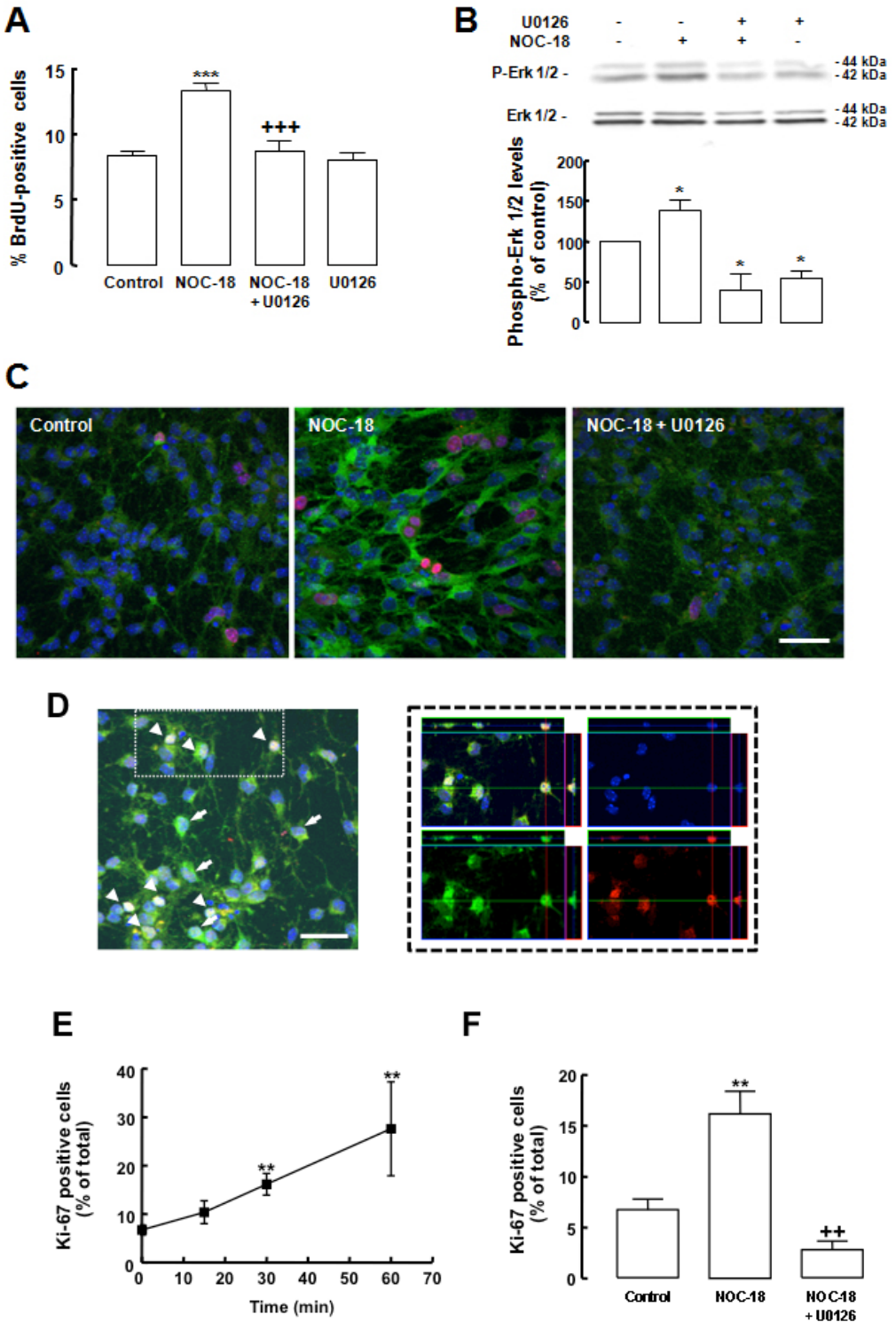
	Treatment	% $TUNEL^+$	% $BrdU^+$	% $BrdU^+/TUNEL^+$
24 h	Control	20.0 ± 1.4%	8.8 ± 0.7%	6.4 ± 1.8%
	1 μM NOC-18	22.4 ± 0.3% (n.s.)	11.7 ± 0.3%	4.6 ± 1.9% (n.s.)
	10 μM NOC-18	20.1 ± 2.7% (n.s.)	13.3 ± 0.4%***	4.8 ± 2.4% (n.s.)
	100 μM NOC-18	21.0 ± 2.2% (n.s.)	9.5 ± 0.2%	4.3 ± 3.4% (n.s.)
48 h	Control	20.6 ± 1.0%	9.0 ± 0.8%	3.6 ± 1.9%
	1 μM NOC-18	21.7 ± 1.6% (n.s.)	9.2 ± 1.1%	2.5 ± 1.7% (n.s.)
	10 μM NOC-18	21.2 ± 3.3% (n.s.)	8.8 ± 0.8%	4.9 ± 1.9% (n.s.)
	100 μM NOC-18	20.0 ± 0.5% (n.s.)	5.4 ± 0.3%*	3.9 ± 1.7% (n.s.)

Data are expressed as means ± SEM of at least 4 independent experiments. One-way ANOVA *$p<0.05$ and ***$p<0.001$, significantly different from control; n.s. (non-significant) $p>0.05$; (Dunnett's post-test).

1.8.3 The proliferative effect of NO is mediated by the activation of the ERK1/2 signaling pathway

We next evaluated the signaling pathway underlying the proliferative effect of NO on neural stem cells, using 10 μM NOC-18 as a stimulus. The antiproliferative effect of NOC-18 (100 μM) will be addressed in chapter 4. The main proliferative pathway in neural stem cells is triggered by activation of the epidermal growth factor receptor (EGFR) and activation of the signaling cascade of MAPK, with subsequent activation of transcription factors, modification of proteins involved in cell cycle progression and transcription of immediate early response genes (Shi *et al.*, 2008). Several kinases are part of the MAPK family, and the ERK1/2 kinases are the elements usually linked to cell proliferation (Meloche and Pouyssegur, 2007). Within this scenario, the involvement of the ERK1/2 pathway in the proliferative effect of NO was investigated.

Figure 2.4. NO increases cell proliferation via the ERK/MAPK signaling pathway (*opposite page*). **A**) Percentage of BrdU-positive cells in SVZ neural stem cell cultures following treatment with 10 μM NOC-18, for 24 h, with or without 1 μM U0126, a selective MEK1 and MEK2 inhibitor. Data are expressed as means ± SEM of at least 4 independent experiments. One-way ANOVA (Bonferroni's post-test). ***p<0.001, significantly different from control. $^{+++}$p<0.001, significantly different from NOC-18. **B**) Western blot analysis of phospho-ERK1/2 levels in lysates of neural stem cells. Exposure to 10 μM NOC-18 for 15 min enhanced ERK1/2 phosphorylation, which was blocked completely by U0126. Data are expressed as means ± SEM of at least 4 independent experiments. One-way ANOVA (Bonferroni's post-test). *p<0.05, significantly different from control. **C**) NOC-18 increased the immunoreactivity against phospho-ERK1/2 (green) in neural stem cells, following 30 min exposure. Nuclei are labeled with Hoechst 33342 (blue) and dividing cells are labeled with the mitotic marker Ki-67 (red). The images are representative of 3 independent experiments. Scale bar: 20 μm. **D**) Phospho-ERK1/2 colocalization with nuclear Ki-67 in proliferating cells upon treatment with NOC-18. Exposure to 10 μM NOC-18 for 30 min induced translocation of phospho-ERK1/2 (green) to the nuclei (blue), colocalizing with nuclear Ki-67 (red) in proliferating cells (arrowheads, left panel); the orthogonal reconstruction of the selected area is shown on the right panel. Cytosolic phospho-ERK1/2 is present in non-dividing cells (Ki-67-negative cells) (arrows). Nuclei are labeled with Hoechst 33342 (blue). Scale bar: 20 μm. **E**) Time-dependent increase in the percentage of Ki-67-positive cells after exposure to NOC-18 (10 μM). Data are expressed as means ± SEM of at least 3 independent experiments. One-way ANOVA (Dunnett's post-test). **p<0.01, significantly different from control. **F)** U0126 blocked the early increase in the percentage of Ki-67-positive cells following exposure to 10 μM NOC-18 for 30 min. Data are expressed as means ± SEM of at least 3 independent experiments. One-way ANOVA (Bonferroni's post-test). **p<0.01, significantly different from control. $^{++}$p<0.01, significantly different from NOC-18.


A
% BrdU-positive cells
15
10
5
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+++
Control
NOC-18
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44 kDa
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44 kDa
42 kDa
Phospho-Erk 1/2 levels (% of control)
200
150
100
50
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*
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C
Control
NOC-18
NOC-18 + U0126
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Ki-67 positive cells (% of total)
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20
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0
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Ki-67 positive cells (% of total)
20
15
10
5
0
**
++
Control
NOC-18
NOC-18 + U0126

The proliferative effect of NO was abolished by U0126, a selective inhibitor of MEK1/2, the kinase immediately upstream of ERK1/2 (Murphy and Blenis, 2006). U0126 prevented the increase in BrdU incorporation triggered by NOC-18 (10 μM), keeping cell proliferation similar to basal levels (8.7 ± 0.9%; $p<0.001$), when compared to NOC-18 alone (13.4 ± 0.6%; $p<0.001$), (Fig. 2.4A). MEK1/2 activates ERK1/2 (also referred to as p44/p42) by phosphorylation of Thr202/Tyr204 and Thr185/Tyr187 residues. Treatment with 10 μM NOC-18 rapidly increased ERK1/2 phosphorylation within 15 min of exposure (139.3 ± 13.8%, as compared to basal levels, $p<0.05$; Fig. 2.4B), and U0126 prevented ERK1/2 phosphorylation induced by NO ($p<0.05$). This effect was evident both by Western blot analysis (Fig. 2.4B) and by immunostaining, where a strong labeling of phospho-ERK1/2 was observed 30 min following exposure to NO (Fig. 2.4C). Moreover, NOC-18 caused translocation of phospho-ERK from the cytosol to the nucleus, as depicted in Fig. 2.4D, concomitantly with increased expression of Ki-67, meaning that these cells have started to divide. Within the first hour of exposure to NOC-18, NO significantly increased the number of Ki-67-expressing cells (Fig. 2.4E), and this proliferative effect detected after 30 min exposure to NOC-18 was blocked by U0126 (Fig. 2.4F).

After determining the involvement of the ERK1/2 pathway in the proliferative effect of NO, we investigated the entry point of NO in this signaling pathway. Upstream of ERK1/2, NO could be a) activating the EGFR directly, causing the receptor to transactivate and achieve phosphorylation levels high enough to trigger the downstream signaling of the ERK1/2 cascade; or b) activating directly p21Ras, which has a cysteine residue susceptible of being S-nitrosylated by NO (Lander *et al.*, 1995; Lander *et al.*, 1997), thus causing a conformational shift that allows the exchange of GDP for GTP and activation of p21Ras. Activation of the EGFR was evaluated by Western blot analysis of the phosphorylation of specific tyrosine residues (tyrosines 845, 1068, 1148 and 1173). Particularly, phosphorylation of tyrosine 1173 (Tyr1173-EGFR) is linked to increased downstream signaling towards ERK1/2, allowing for docking of the SHC scaffolding protein, which provides the link between EGFR and the Ras protein (Batzer *et al.*, 1994; Okabayashi *et al.*, 1994).

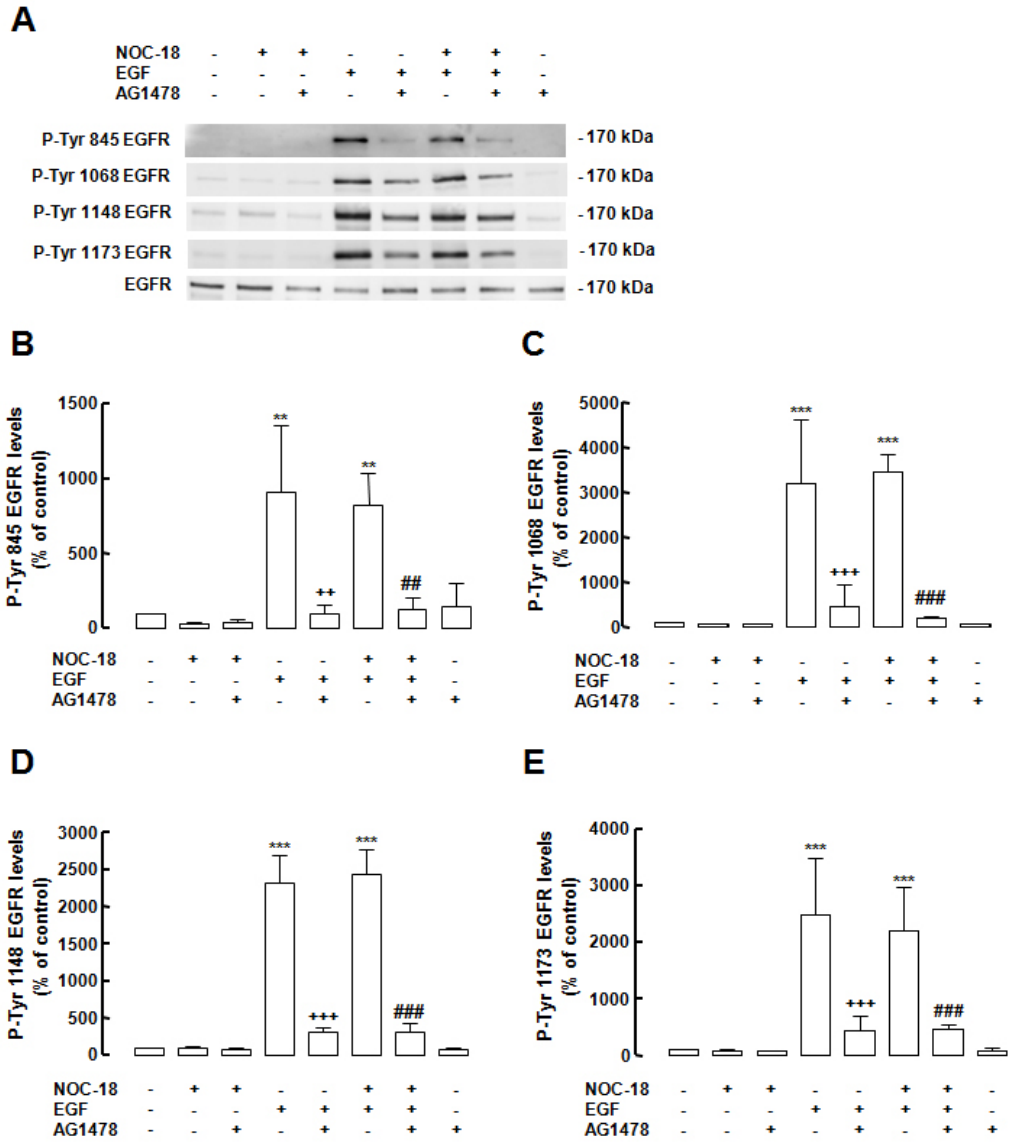


Figure 2.5. The proliferative effect of NO is independent from the activation of the EGF receptor. SVZ cells were treated with NOC-18 (10 µM) or EGF (20 ng/ml) for 5 min, in the presence or absence of AG1478 (200 ng/ml). EGFR activation was assessed using phospho-specific antibodies against tyrosine residues 845 (**B**), 1068 (**C**), 1148 (**D**) or 1173 (**E**). Representative images are shown in **A**. A loading control was performed by reprobing against EGFR. Data are expressed as means ± SEM of at least 4 independent experiments. One-way ANOVA (Bonferroni's post-test). ***p<0.001 and **p<0.01, significantly different from control; $^{+++}$p<0.001 and ++p<0.01, significantly different from EGF alone; $^{###}$p<0.001 and $^{##}$p<0.01, significantly different from NOC-18+EGF.

We observed that treatment with NO, up to 5 min, did not increase phosphorylation of EGFR in the analysed tyrosine residues (Fig. 2.5). Cells treated with EGF (20 ng/ml) for 5 min were used as a positive control of EGFR phosphorylation, showing a strong increase in the receptor phosphorylation, which was abolished by pretreatment with an inhibitor of EGFR, AG1478 (200 nM) (Fig. 2.5). Furthermore, EGF treatment increased the immunoreactivity of Tyr1173-EGFR, in EGFR-expressing cells (Fig. 2.6), in an AG1478-sensitive manner, while NOC-18 produced no effect on tyrosine 1173 phosphorylation.

Moreover, blocking EGFR with AG1478 did not prevent the increase in BrdU incorporation stimulated by NOC-18 (Fig. 2.7A). EGF alone increased cell proliferation, and this effect was abolished by AG1478 ($p<0.001$). Interestingly, when the proliferative effect of EGF was blocked in the presence of AG1478, exposure to NOC-18 still increased proliferation (NOC-18+EGF+AG1478; $p<0.001$), which confirms that the signaling of NO-induced proliferation is not dependent on the activation of the EGF receptor.

Since NO did not activate EGFR directly, we next evaluated the activation of p21Ras. We observed that NO significantly increased Ras activity at 2 min after exposure to NOC-18 (Fig. 2.7B). Furthermore, downstream of p21Ras, the phosphorylation of c-Raf increased 5 min after exposure to NOC-18 (142.9 ± 5.6%, as compared to basal levels, $p<0.001$), and this effect was not prevented by pretreatement with AG 1478 (132.8 ± 4.5%, compared to basal levels, Fig. 2.7C). These results suggest that NO bypasses the EGF receptor and activates directly p21Ras, which in turn increases the phosphorylation of c-Raf.

We next evaluated the effect of NO on the activation of several downstream targets of the ERK1/2 pathway, such as the p90 ribosomal S6 kinase (p90RSK) and the transcription factors c-Myc, Elk-1, and CREB, 1 h after the exposure to NOC-18. Treatment with NOC-18 increased the phosphorylation levels of p90 RSK (167.9 ± 17.0%, $p<0.05$, two-tailed t test), c-Myc (148.7 ± 8.3%, $p<0.01$, two-tailed t test) and Elk-1 (158.9 ± 11.6%, $p<0.01$, two-tailed t test), as compared to untreated control cultures (Fig. 2.7D), while the levels of CREB were unchanged by exposure to NO ($p>0.05$, two-tailed t test).

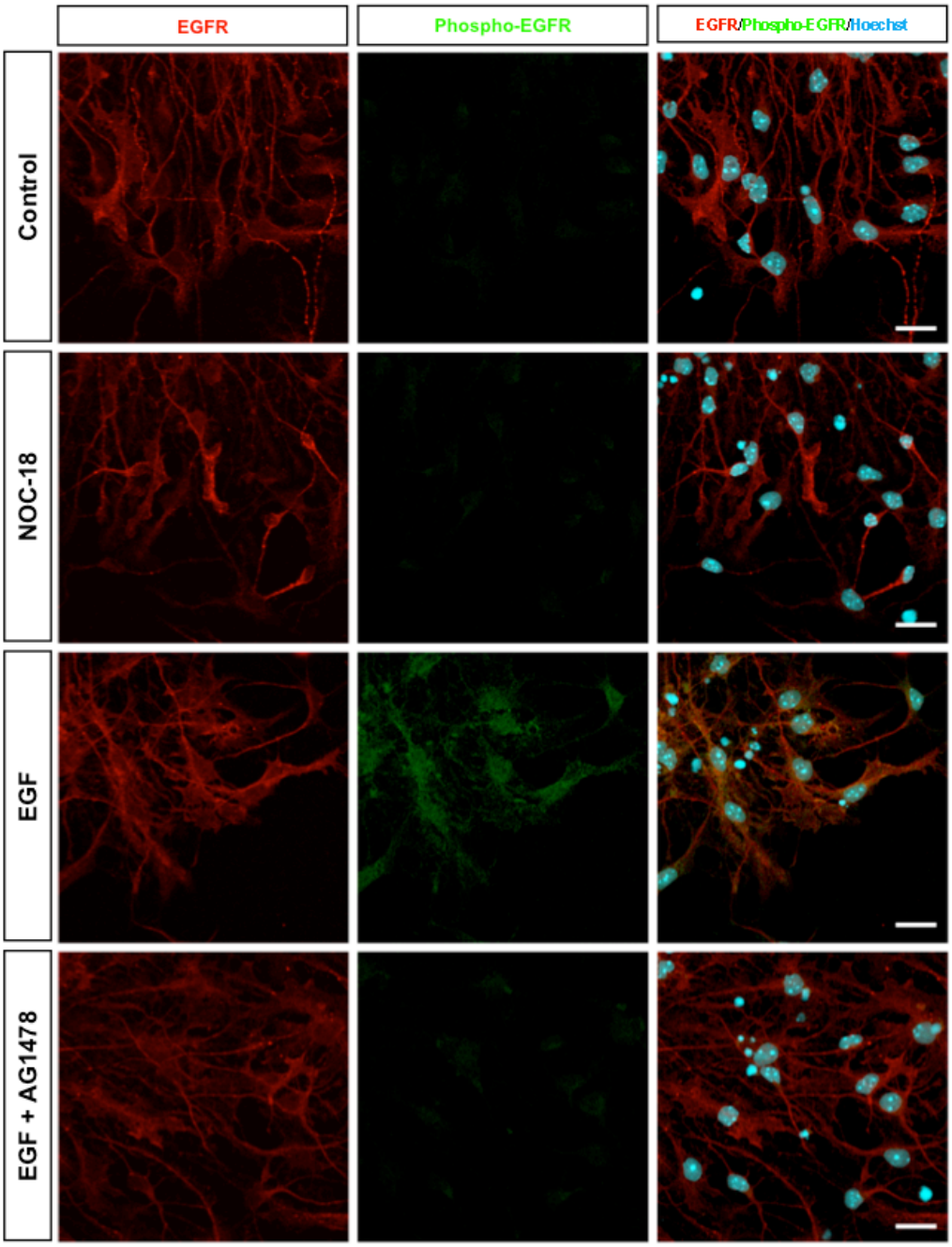


Figure 2.6. The proliferative effect of NO is independent of the activation of the EGF receptor. Exposure to NOC-18 (10 μM, 5 min) did not increase the phosphorylation of EGFR at Tyrosine 1173 (green), in EGFR-expressing cells (red). Nuclei are labelled with Hoechst 33342 (blue). The activation of the EGFR was induced by EGF (20 ng/ml), and could be prevented by pretreatment with AG 1478 (200 nM), an inhibitor of EGFR. Scale bar: 20 μm.

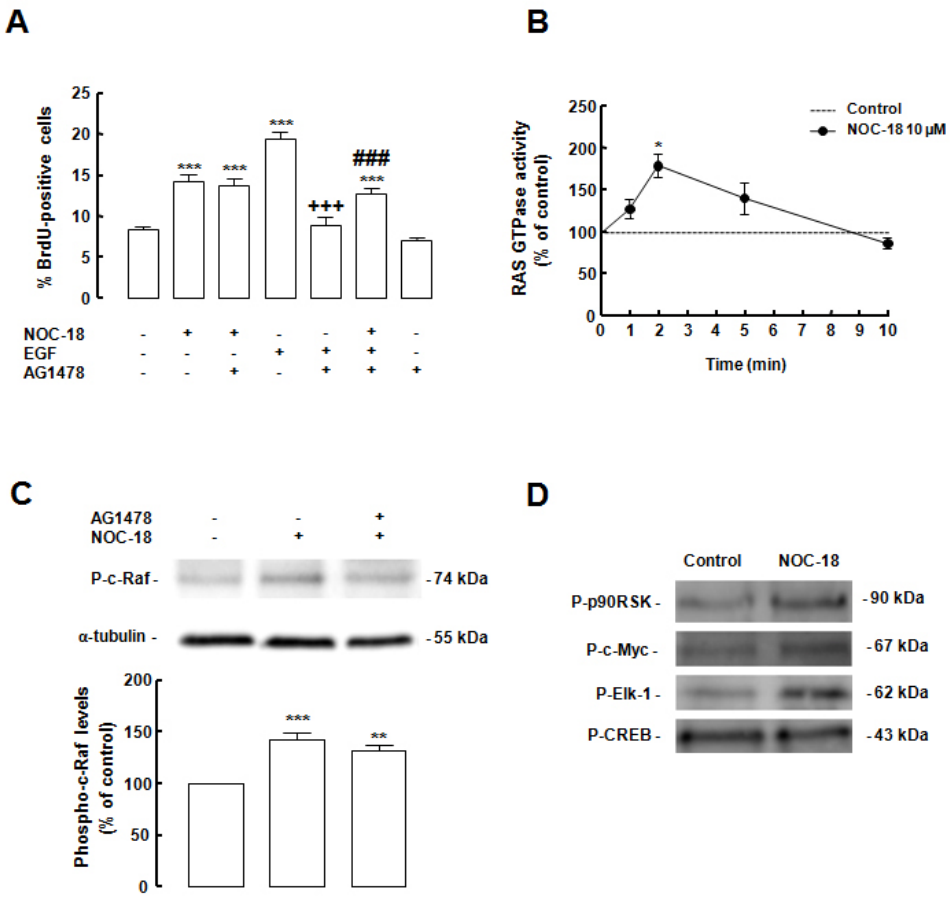


Figure 2.7. p21Ras is the entry point of NO in the ERK/MAPK signaling pathway. A) Effect of blocking the EGF receptor on the cell proliferation induced by exposure to NOC-18 (10 µM) for 24 h, as evaluated by BrdU incorporation. Data are expressed as means ± SEM of at least 3 independent experiments; one-way ANOVA, with Bonferroni's post-test. ***p<0.001, significantly different from control; $^{+++}$p<0.001, significantly different from EGF alone; $^{###}$p<0.001, significantly different from EGF+AG1478. **B)** Effect of NO on p21Ras GTPase activity. Data are expressed as means ± SEM of at least 4 independent experiments. One-way ANOVA (Dunnett's post-test). *p<0.05, significantly different from control. **C)** Western blot analysis of phospho-c-Raf levels in lysates of neural stem cell cultures treated with NOC-18 for 5 min. Exposure to 10 µM NOC-18 enhanced phosphorylation of c-Raf, which was not blocked by pretreatment with the EGFR inhibitor AG 1478 (200 nM). A loading control was performed by reprobing against α-tubulin. Data are expressed as means ± SEM of at least 4 independent experiments. One-way ANOVA (Bonferroni's post-test). ***p<0.001 and **p<0.01, significantly different from control. **D)** Western blot analysis of the phosphorylation levels of p90 RSK and of the transcription factors Elk-1, c-Myc and CREB, in lysates of neural stem cell cultures treated with NOC-18 for 1 h.

One of the targets of p90RSK is the cyclin-dependent kinase inhibitor $p27^{KIP1}$ (Fujita *et al.*, 2003). This inhibitor stalls cell division by inhibitory binding to cyclin/cyclin-dependent kinase complexes. In Fig. 2.4E we showed that NO increases the number of Ki-67-positive cells 1 h after exposure to NOC-18, i.e. dividing cells that lack $p27^{KIP1}$ in the nucleus (Fig. 2.8A). Non-dividing cells presented $p27^{KIP1}$ either in the nucleus or in the cytosol (Fig. 2.8A). Following exposure to NOC-18 (10 μM) for 1h, the number of cells presenting $p27^{KIP1}$ in the nucleus was significantly decreased ($p<0.001$), while the number of cells presenting $p27^{KIP1}$ in the cytosol or $p27^{KIP1}$-negative was significantly increased after exposure to NO ($p<0.01$ and $p<0.05$, respectively) (two-way ANOVA: treatment: $p<0.272$, $F= 1.325$, $df=1$; $p27^{KIP1}$ localization: $p<0.0001$, $F= 55.51$, $df=2$; treatment x $p27^{KIP1}$ localization (interaction): $p<0.0001$, $F= 29.12$, $df=2$) (Fig. 2.8B). Overall, the levels of $p27^{KIP1}$ were lower in lysates of SVZ cultures treated with NOC-18 for 1h ($p<0.01$, Fig. 2.8C). Moreover, blockade of the EGF receptor with AG1478 did not prevent the decrease in $p27^{KIP1}$ levels induced by exposure to NOC-18 ($p>0.05$), whereas U0126 abolished this effect ($p<0.001$, Fig. 2.8C).

1.8.4 Abolishment of cell proliferation in $iNOS^{-/-}$ mice following seizures

Finally, we investigated whether the effect of NO in the mouse brain, in pathological conditions, is proliferative or antiproliferative. Increased cell proliferation in the SGZ of the dentate gyrus of the hippocampus is a hallmark of epileptic seizures in rodents (Parent, 2007), and occurs simultaneously with increased neuroinflammation, which comprehends activation of microglial cells and expression of iNOS (De Simoni *et al.*, 2000). Microglial cells are, in such conditions, able to produce large amounts of NO (Murphy *et al.*, 1993). We used the kainic acid (KA) model of *status epilepticus* to investigate the role of NO in cell proliferation *in vivo*. Wild-type (WT) or $iNOS^{-/-}$ mice were treated with either saline or KA, as described in section 2.3.11.

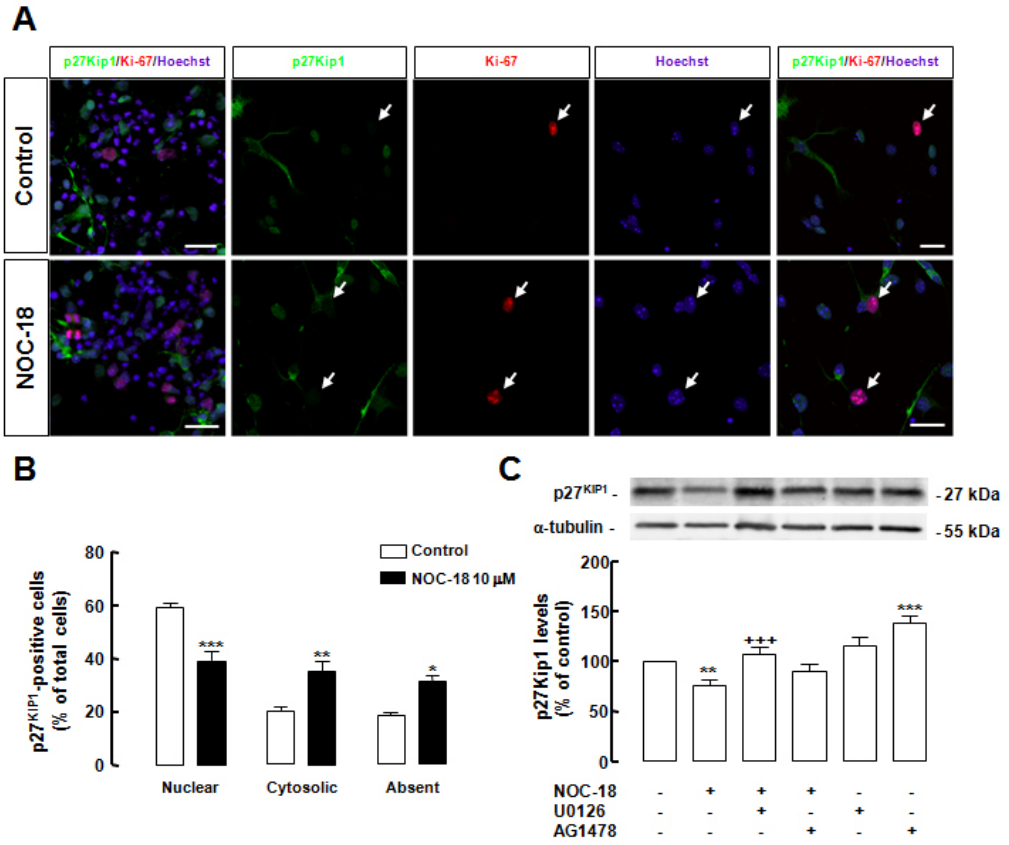


Figure 2.8. Effect of NO on the levels of the cyclin-dependent kinase inhibitor p27^{KIP1}. Neural stem cells treated with NOC-18 have more dividing cells and are negative for p27^{KIP1} in the nucleus **(A)**. Following exposure to NOC-18, Ki-67-positive cells (red), i.e. proliferating cells, lack p27^{KIP1} in the nucleus (arrows, lower panel). Non-dividing cells (negative for Ki-67) present p27^{KIP1} either in the nucleus or in the cytosol (green). Nuclei are labeled with Hoechst 33342 (blue). Scale bar: 20 µm. **B)** Percentage of cells presenting p27 ^{KIP1} in the nucleus, in the cytosol or cells where p27^{KIP1} is absent. Data are expressed as means ± SEM of at least 3 independent experiments. Two-way ANOVA (Bonferroni's post-test). ***p<0.001, **p<0.01 and *p<0.05, significantly different from control. **C)** Levels of p27^{KIP1}, as evaluated by Western blot analysis, following exposure for 1 h to 10 µM NOC-18, in the presence or absence of U0126 (1 µM) or AG1478 (200 nM). Data are expressed as means ± SEM of at least 4 independent experiments. One-way ANOVA (Bonferroni's post-test). **p<0.01 and ***p<0.001, significantly different from control; $^{+++}$p<0.001, significantly different from NOC-18.

BrdU incorporation was greatly increased following SE in the SGZ of wild-type C57BL/6J mice, up to 5 d after seizures following treatment with KA

(Fig. 2.9B; two-way ANOVA: treatment: $p<0.0001$, $F=118.7$, $df=3$; time: $p<0.0001$, $F=65.3$, $df=3$; treatment x time (interaction): $p<0.0001$, $F=26.3$, $df=9$). There was a significant increase in newly born cells as early as 48 h after SE in the wild-type KA-treated group ($p<0.05$), and a steady rise in cell proliferation up to day 5. At this time, the number of BrdU-positive cells in the SGZ of wild-type mice treated with KA was 4-fold higher (82.7 ± 5.9 cells/section, $p<0.001$) than that of saline-treated wild-type mice. In saline-treated wild-type mice, the number of proliferating cells in the granular cell layer of the dentate gyrus remained constant throughout the time points evaluated (average 22 cells/section). In $iNOS^{-/-}$ mice, the number of BrdU-positive cells in the dentate gyrus following treatment with KA was similar to that found in saline-treated $iNOS^{-/-}$ or wild-type mice ($p>0.05$), for all time points. These data suggest that abolishing NO of iNOS origin prevents the proliferation of neural stem cells *in vivo,* following the seizure insult.

We next evaluated whether the involvement of NO was specific to the proliferation of neuronal precursors (following seizures) or also involved in the proliferation of astrocytes or microglia cells. Doublecortin (DCX) was used as a marker of neuronal commitment, and GFAP was used to evaluate astrogliosis. We found that seizures increased DCX immunoreactivity (Fig. 2.9A and 2.9C) in wild-type mice, but not in $iNOS^{-/-}$ mice. On the other hand, GFAP immunoreactivity increased with KA treatment, and was elevated either in WT or $iNOS^{-/-}$ mice (Fig. 2.9A and 2.9D). Moreover, Ki-67-positive cells did not colocalize with GFAP or CD-45, a microglial marker (Fig. 2.10).

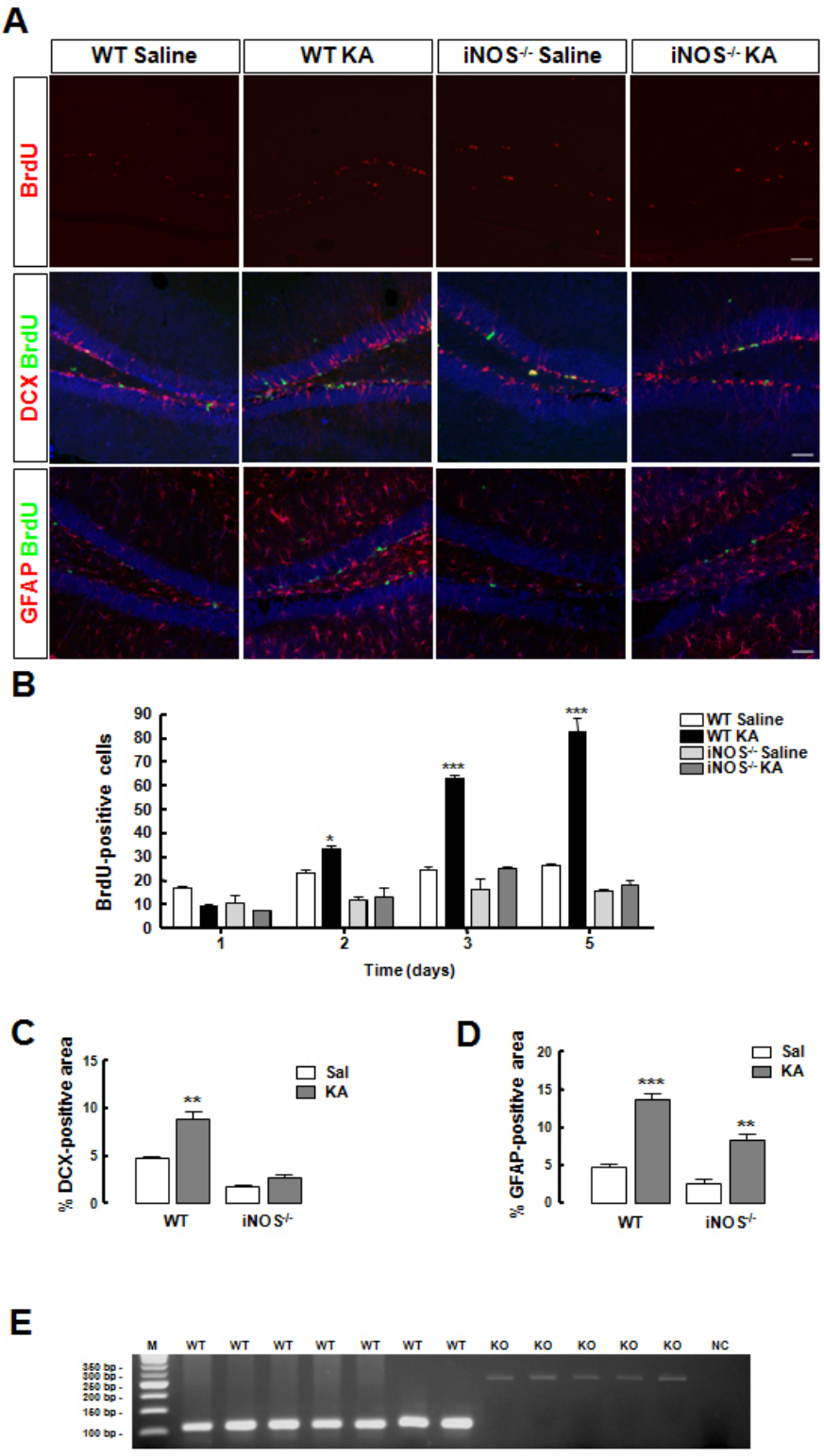

A
WT Saline
WT KA
iNOS-/- Saline
iNOS-/- KA
BrdU
DCX BrdU
GFAP BrdU
B
BrdU-positive cells
Time (days)
WT Saline
WT KA
iNOS-/- Saline
iNOS-/- KA
C
% DCX-positive area
Sal
KA
WT
iNOS-/-
D
% GFAP-positive area
Sal
KA
WT
iNOS-/-
E
M WT WT WT WT WT WT WT KO KO KO KO KO NC
350 bp
300 bp
250 bp
200 bp
150 bp
100 bp

Figure 2.9. Increased cell proliferation in the SGZ following seizures is abolished in iNOS$^{-/-}$ mice (*opposite page*). Evaluation of cell proliferation in the SGZ of wild-type (WT) *vs* iNOS$^{-/-}$ mice (KO) was assessed by BrdU incorporation, at several time points after *status epilepticus* induction **(B)**. Representative images are shown 5 d after treatment with KA **(A)**. The levels of doublecortin **(C**, red**)** and of GFAP **(D**, red**)** were also analysed. Data are expressed as means ± SEM. Two-way ANOVA (Bonferroni's post-test), *p<0.05, **p<0.01 and ***p<0.001, significantly different from control. Scale bar: 50 µm. **E)** Representative image from a 3% agarose gel electrophoresis of secondary PCR products. The genotype of the animals used in this study was confirmed by electrophoresis of PCR-amplified DNA of WT (108 bp) and iNOS-deficient mice (275 bp). M, 50-bp DNA ladder; WT, wild-type; KO, iNOS KO; NC, negative control in the PCR (water).

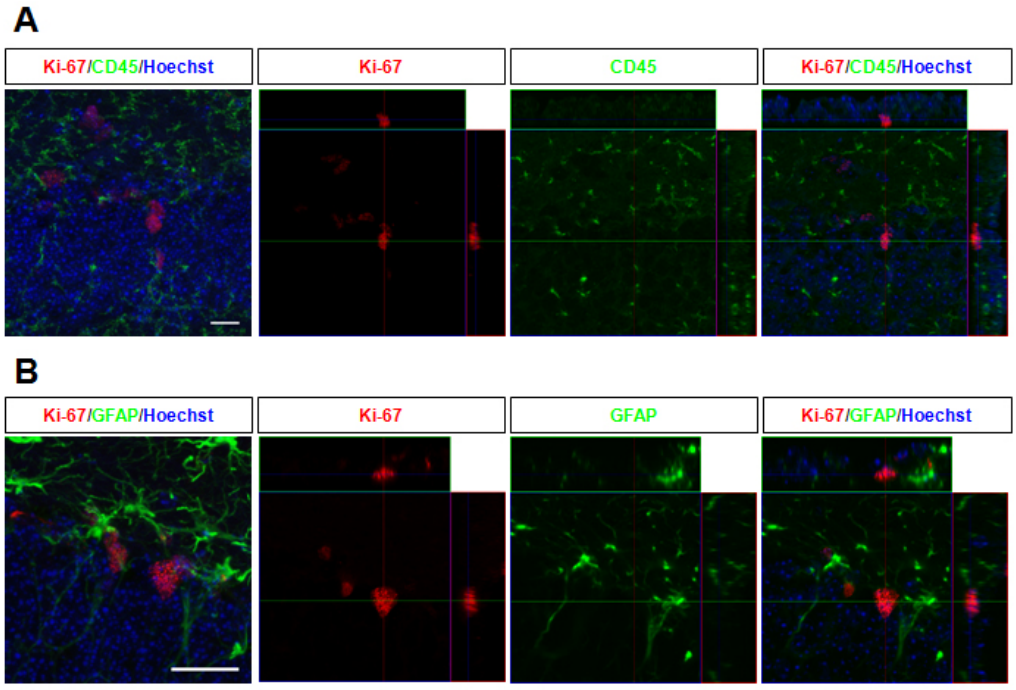


Figure 2.10. No evidence of colocalization of Ki-67 in CD45 or GFAP-positive cells in the dentate gyrus following seizures. Dividing cells (Ki-67-positive, in red) did not colocalize with either CD45 (**A**) or with GFAP (**B**). Representative images are shown 5 d after treatment with KA. Scale bar: 20 µm.

1.9 Discussion

In this chapter, we show that NO can have opposite effects on the proliferation of neural stem cells. NO either promotes or inhibits neural stem

cell proliferation, depending on the concentration and duration of exposure. In particular, we show that the proliferative effect of NO is due to the activation of p21Ras, bypassing the activation of the EGF receptor. As a result, activation of p90 RSK, Elk-1 and c-Myc increases and the levels of the cyclin-dependent kinase inhibitor $p27^{KIP1}$ decrease. Additionally, in an *in vivo* model of brain injury associated with increased cell proliferation, we show that NO from iNOS origin promotes cell proliferation.

1.9.1 NO induces the proliferation of neural stem cells bypassing the EGF receptor

Neural stem cells are able to self-renew and proliferate in response to EGF (Reynolds and Weiss, 1996; Doetsch *et al.*, 2002). Our results strongly suggest that NO bypasses the EGF receptor, using its signaling pathway, without requiring activation of the EGF receptor to exert its proliferative effect. Our results strongly suggest that NO signals via the MAPK pathway, but its target is downstream of the EGF receptor itself. To the best of our knowledge, our study is the first showing that NO induces proliferation of neural stem cells by directly activating the EGFR-ERK1/2 signaling pathway without activation of the EGF receptor. NO stimulated the downstream signaling of the EGF receptor pathway, rapidly activating p21Ras and increasing the phosphorylation of c-Raf. p21Ras is likely to be the entry point of NO in the signaling cascade, since this was the first detectable element of the ERK1/2 pathway that we found to be activated following exposure to NO. In vitro assays using recombinant p21Ras have shown that NO activates p21Ras, via S-nitrosylation of a critical cysteine residue (Cys118), inducing a conformational change and causing the release of GDP and binding of GTP, thus activating p21Ras (Lander *et al.*, 1995; Lander *et al.*, 1997). In our study, the activation of p21Ras was fast, peaking at 2 min after exposure to NOC-18, suggesting a direct effect of NO on p21Ras, without intermediate signaling. Downstream of p21Ras, the ERK1/2 kinases were activated following

exposure to NO. Blocking MEK1/2 with U0126 completely abolished ERK1/2 phosphorylation induced by NO and prevented the proliferative effect of NO, demonstrating that NO triggers proliferation upstream of ERK1/2.

Following activation of the ERK1/2 pathway, the proliferative effect of NO is fast and robust. We found that the number of dividing cells (Ki-67-positive) rapidly increased within the first hour of exposure to NO, which is accompanied by phosphorylation of several effectors of the ERK1/2 pathway, namely, p90RSK, Elk-1 and c-Myc. Phosphorylation of the transcriptional regulators Elk-1 and c-Myc strongly suggests that NO increases cell proliferation by activating the transcription of immediate early genes. More interestingly, NO strongly induced the phosphorylation of p90RSK, which can be activated following the binding of active ERK1/2 (Gavin and Nebreda, 1999). p90RSK plays an important role in the regulation of transcription factors and other regulators, translocating to the nucleus where it can phosphorylate, among others, c-Fos and the cyclic AMP response element-binding protein (CREB) (Chen *et al.*, 1993; Xing *et al.*, 1996). We did not observe increased phosphorylation of either CREB or c-Fos (data not shown). While in the nucleus, another important substrate of p90RSK is the cyclin-dependent kinase inhibitor 1, $p27^{KIP1}$. $p27^{KIP1}$ can be phosphorylated by p90RSK and then translocated to the cytosol, where it is ubiquitinated and degraded by the proteasome (Vlach *et al.*, 1997; Fujita *et al.*, 2003). $p27^{KIP1}$ prevents progression from G1 to S phase by complexing with cyclins and cyclin-dependent kinases, namely with cyclin E-CDK2 and cyclin D-CDK4 (Polyak *et al.*, 1994; Toyoshima and Hunter, 1994), and its nuclear export and translocation to the cytosol allow for progression into S phase. We detected a significant decrease in the levels of $p27^{KIP1}$ in lysates of cultures exposed to NOC-18 for 1 h, and this effect might explain the proliferative effect of NO. Moreover, we observed translocation of $p27^{KIP1}$ to the cytosol following treatment with NO, shown by a decrease in the number of cells presenting nuclear $p27^{KIP1}$, while the number of cells presenting cytosolic $p27^{KIP1}$ or

absence of this inhibitor was increased. This suggests that NO triggers cell-cycle reentry by promoting the translocation of $p27^{KIP1}$ from the nucleus to the cytosol, which is in agreement with the observation of an increased number of cells in G2/M. $p27^{KIP1}$ has been found to be a key regulator of the cell division of transit-amplifying progenitors from the SVZ (Doetsch *et al.*, 2002). Another study found that higher concentrations of NO, which decrease the proliferation of neural stem cells, are correlated with the nuclear presence of $p27^{KIP1}$ in stem cells of the mouse subventricular zone (Torroglosa *et al.*, 2007). Those results and ours suggest that $p27^{KIP1}$ is a likely mediator of the proliferative effect of NO.

Since NO can be cytotoxic (Boje and Arora, 1992; Dawson *et al.*, 1993; Dawson *et al.*, 1994; Bal-Price and Brown, 2001), there is the possibility that increased BrdU uptake is the result of DNA repair, following an insult caused by NO. On the other hand, if cell death occurs due to NO-mediated toxicity, it will result in fewer BrdU-labeled cells, which can be misinterpreted as decreased cell proliferation. Nevertheless, our results obtained by monitoring BrdU incorporation were paralleled by those obtained with proliferative markers (Ki-67), which argues against these possible interpretations. Moreover, we did not observe any significant changes in nuclear morphology or TUNEL staining. Therefore, taken together our data strongly suggest that the changes in BrdU incorporation following exposure to NO are not due to changes in cell viability, but in fact caused by changes in cell proliferation.

1.9.2 Nitric oxide as a proliferative versus antiproliferative agent

We used NOC-18 in a range of concentrations mimicking pathophysiological conditions, particularly neuroinflammation. At 10 μM, NOC-18 releases NO in a similar manner to lipopolysaccharide-treated microglial cells, whereas 100 μM NOC-18 generated 10-fold higher levels of NO (data not shown). The lower concentration of NOC-18 caused neural stem cells to proliferate, whereas the higher concentration inhibited cellular proliferation.

Varying the concentration of NO by approximately a factor of 10 profoundly affected its effect on the proliferation of neural stem cells. The higher concentration of NOC-18 (100 μM) used in other studies as an equivalent to NO released during neuroinflammation, in fact appears to be a concentration artificially much higher than what is attained during brain inflammation.

To evaluate the effect of NO on cell proliferation after injury *in vivo*, we examined the rodent model of *status epilepticus* in wild-type mice and mice that lack iNOS. *Status epilepticus* triggers a strong increase in hippocampal inflammation and greatly boosts proliferation of neural precursor cells in the SGZ of the dentate gyrus of rodents (Parent *et al.*, 1997; Gray and Sundstrom, 1998; Parent, 2007). We observed that proliferation was greatly increased in the dentate gyrus of wild-type mice after seizures. In contrast, mice lacking iNOS did not display an increase in cell proliferation in the dentate gyrus following seizures. These data strongly suggest that seizures stimulate cell proliferation by a mechanism dependent on NO produced by iNOS. An earlier report by Zhu and colleagues described that iNOS expression was necessary for increased cell proliferation in the dentate gyrus of mice subjected to focal cerebral ischemia, although the mechanism was not addressed in their study (Zhu *et al.*, 2003). Their work and ours support the fact that NO produced during a brain insult associated with an inflammatory reaction will favor cell proliferation. Studies that have used high concentrations of NO donors in models of neural stem cell proliferation have focused on the antiproliferative effects of NO (Covacu *et al.*, 2006; Torroglosa *et al.*, 2007), and have probably examined situations that are less relevant to those occurring *in vivo*.

In restorative neurology, both transplantation of exogenous stem cells and promotion of endogenous neurogenesis have been proposed as strategies to repair the damaged brain. Excessive proliferation of neural precursors or pluripotent stem cells associated with the formation of tumors is a major concern in the clinical application of both these strategies (Li *et al.*,

2008). Targeting the NO system may be a powerful strategy to control cell proliferation. According to our results this might moderately increase proliferation of neural stem cells. On the other hand, NO donors might also be used therapeutically, to enhance endogenous neurogenesis. Overall, this work sheds new light on the effects of NO on the proliferation of neural stem cells, and may help in steering research efforts towards modulating the nitrergic system to regulate proliferation of stem cells.

Chapter 3

Nitric oxide increases the proliferation of neural stem cells via the guanylyl cyclase - cyclic GMP - protein kinase G pathway

The work presented in this chapter is submitted for publication in a peer-reviewed international journal.

1.10 Summary

The aim of the work presented in chapter 3 was to test whether cGMP and the cGMP-dependent kinase (PKG) are involved in the proliferative effect triggered by NO described in chapter 2. For this purpose, cultures of neural stem cells isolated from the subventricular zone (SVZ) of C57BL/6J mice were used. Long-term exposure to the NO donor (24 h), NOC-18, increased cell proliferation, in a cyclic GMP-dependent manner, as determined by the inhibitory effect of a guanylate cyclase inhibitor, ODQ. Similarly to NOC-18, 8-Br-cGMP, a cGMP analogue, also increased cell proliferation following 24 h exposure. Interestingly, shorter exposures to NO (6 h) increased cell proliferation in a cGMP-independent manner, via the ERK/MAP kinase pathway, while 8-Br-cGMP had no effect on cell proliferation. Furthermore, the selective phosphodiesterase 5 (PDE5) inhibitor, T0156, enhanced the proliferative effect of NOC-18. On the other hand, the selective inhibitor of PKG, KT 5823, prevented the proliferative effect induced by NO. In conclusion, NO stimulates SVZ-derived stem cell proliferation, and this effect is mediated through the sGC/cGMP/PKG pathway at later stages, while initially the ERK/MAPK pathway is more relevant for the early proliferative effect of NO.

1.11 Introduction

We showed in Chapter 2 that the NO donor NOC-18 can stimulate neural stem cell proliferation, when used in low doses (1-10 μM). Nevertheless, the mechanisms underlying the proliferative effect of NO were not fully clarified.

The main biological target of NO is the heme-containing enzyme guanylyl cyclase. Guanylyl cyclase catalyzes the conversion of GTP to cyclic GMP (cGMP), which can then act on further downstream targets, such as the cGMP-dependent kinase (PKG) and cGMP-gated channels. NO and cGMP have been described as important effectors in several cellular processes, such as survival, differentiation, growth, axon guidance, proliferation or migration, through the activation of different downstream signalling cascades (Gomez-Pinedo *et al.,* 2010; Tegenge *et al.*, 2010; Madhusoodanan and Murad, 2007; Tegenge and Bicker, 2009). cGMP and cGMP-sparing agents such as sildenafil or tadalafil have been shown to positively affect neurogenesis (Zhang *et al.*, 2002; Wang *et al.*, 2005; Zhang *et al.*, 2006; Gomez-Pinedo *et al.*, 2010). It remains to be established whether cGMP and PKG are responsible for the proliferative effect of NO when applied to neural stem cells. Within this scenario, we investigated the role of the guanylyl cyclase-cGMP-PKG pathway in the proliferative effect of NO. We observed that cGMP analogues mimick the effect of NO in increasing cell proliferation. Moreover, blocking guanylyl cyclase prevented the proliferative effect of NO, thus pointing to a beneficial effect of NO-cGMP signalling pathway in enhancing neural stem cell proliferation.

1.12 Materials and Methods

1.12.1 Materials

Dulbecco's Modified Eagle's Medium:F-12 nutrient mixture, (D-MEM/F-12, with GlutaMAX^{TM}-I), B27 supplement, trypsin-EDTA solution (0.05%

trypsin, 1 mM EDTA in HBSS) and antibiotic (10,000 units/ml of penicillin, 10 mg/ml streptomycin) were purchased from GIBCO BRL, Life Technologies, Scotland. 5-bromo-2'-deoxyuridine (BrdU), 8-bromoguanosine 3',5'-cyclic monophosphate (8-Br-cGMP), phenylmethylsufonyl fluoride, dithiothreitol, orthovanadate, chymostatin, leuptin, antiparin, pepstatin A, trypan blue and N^{ω}-nitro-L-arginine methyl ester (L-NAME) and 1,4-Diamino-2,3-dicyano-1,4-bis(o-aminophenylmercapto)butadiene monoethanolate (U0126) were purchased from Sigma Chemical (St Louis, MO, USA). KT5823 was purchased from Alomone Labs (Jerusalem, Israel). 1*H*-[1,2,4]Oxadiozolo[4,3-a]quinoxalin-1-one (ODQ) and the phosphodiesterase 5 inhibitor (T0156) were obtained from Tocris Bioscience (Bristol, UK). Epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) and Click-iT® EdU Alexa Fluor® 488 Flow Cytometry Assay Kit were purchased from Invitrogen (Paisley, UK). Mouse anti-Sox-2 was purchased from R&D Systems (Minneapolis, MN, USA) and rabbit anti-nestin from Santa Cruz Biotechnology (Santa Cruz, CA, USA). DETA-NONOate (NOC-18) was obtained from Alexis Biochemicals (San Diego, CA, USA). DAKO fluorescent mounting medium was purchased from DakoCytomation (Glostrup, Denmark). Rat anti-mouse BrdU was obtained from Oxford Biotechnology. Hoechst 33342 dye, anti-rat IgG conjugated with Alexa Fluor 594, anti-mouse IgG conjugated with Alexa Fluor 594 and anti-rabbit IgG conjugated with Alexa Fluor 488 secondary antibodies were purchased from Molecular Probes (Leiden, The Nederlands). Polyvinylidene difluoride (PVDF) membranes, enhanced chemifluorescence (ECF) reagent, alkaline phosphatase-linked anti-rabbit and anti-mouse secondary antibodies were obtained from Amersham Pharmacia Biotech (Buckinghamshire, UK). Monoclonal mouse anti-p44/42 MAPK (ERK1/2) and rabbit anti-phospho-ERK1/2 antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). Other reagents used in immunoblotting experiments were purchased from BioRad.

1.12.2 Animals

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

1.12.3 Subventricular zone cell cultures

Neural stem cell cultures were obtained from the SVZ of postnatal day 0-3 C57BL/6J mice, as previously described in Chapter 2 (Carreira *et al.*, 2010). The SVZ stem cells were grown as floating neurospheres in a 95% air-5% CO_2 humidified atmosphere at 37ºC.

Seven days following plating the primary neurospheres were harvested, centrifuged and dissociated as single cells. Cells were then replated as above and allowed to grow as secondary neurospheres. Neurospheres with 2-4 passages were collected and plated for 5 days on 16-mm diameter glass coverslips, for immunocytochemistry assays, or on 12-well plates, coated with poly-L-lysine, in serum-free medium, without growth factors, for preparation of lysates or flow cytometry assays.

1.12.4 Experimental treatments of SVZ cell cultures

SVZ-derived neural stem cells were exposed to the NO donor NOC-18 (10 μM) or to the cGMP analogue 8-Br-cGMP (20 μM) for different periods of time, as indicated in detail in the figure legends and in the text. The soluble guanylyl cyclase inhibitor ODQ (50 μM), the protein kinase G (PKG) inhibitor KT5823 (1 μM), the phosphodiesterase 5 inhibitor T0156 (1 μM) and the MEK1/2 inhibitor U0126 (1 μM) were added 30 min before NOC-18 or 8-Br-cGMP and kept throughout the incubation period.

1.12.5 Detection of BrdU incorporation

Cell proliferation was assessed by the incorporation of the thymidine analogue, BrdU. BrdU (10 μM) was added to the cultures 16 h before fixation (Alvaro *et al.*, 2008; Carreira *et al.*, 2010). Nuclei that incorporated BrdU in this time-window were detected by immunofluorescence, as follows. Following 20 min fixation with 4% paraformaldehyde/4% sucrose in PBS, the cells were permeabilized with 1% Triton X-100 for 5 min, and DNA was denaturated by treatment with 1 M HCl for 30 min, at 37ºC. Non-specific binding was blocked with 3% BSA in PBS-T for 1 h, and then BrdU-positive cells were labeled with a rat anti-BrdU antibody (1:50) for 90 min, at room temperature. The cells were then incubated with a secondary antibody goat anti-rat IgG conjugated with Alexa Fluor 594 (1:200), for 1 h, at room temperature. Nuclei were stained with Hoechst 33342 (1 μg/ml) for 3 min. Coverslips were mounted on glass slides using DAKO fluorescence mounting medium Dako Cytomation. The cells were visualized in a fluorescence microscope (Axioskop 2 Plus, Zeiss, Jena, Germany) and images were acquired with the Axiovision software 4.7. The number of BrdU-positive nuclei was counted in 7-10 randomly selected fields for each coverslip (in a total of approximately 900-1,200 cells per coverslip), and the data were expressed as percentage of the total number of living cells. A minimum of 3 independent experiments was analyzed for each condition.

1.12.6 Detection of cell proliferation and cell cycle analysis by flow cytometry

Cell proliferation was also assessed by incorporation of EdU and detected by flow cytometry, using the Click-iT® EdU Alexa Fluor® 488 Flow Cytometry Assay Kit, as described in section 2.3.6 at Chapter 2.

1.12.7 Western blot analysis

Cells were lysed in 50 mM Tris-HCl, 10 mM EGTA, 1% Triton X-100 and 2 mM $MgCl_2$, supplemented with 100 µM PMSF, 1 mM dithiothreitol, 1 µg/ml chymostatin, 1 µg/ml leupeptin, 1 µg/ml antiparin, 5 µg/ml pepstatin A, 1 mM sodium orthovanadate, 50 mM NaF, pH 7.4 at 4ºC. Protein concentration was determined by the BCA method, and the samples were used for Western blot analysis, after adding 6x concentrated sample buffer (0.5 M Tris, 30% glycerol, 10% SDS, 0.6 M dithiothreitol, 0.012% bromophenol blue) and heating, for 5 min, at 95ºC.

Equal amounts of protein were separated by electrophoresis on SDS-polyacrylamide gels, and transferred electrophoretically to PVDF membranes. These were then blocked for 1 h at room temperature in Tris-buffered saline (137 mM NaCl, 20 mM Tris-HCl, pH 7.6) containing 0.1% Tween-20 (TBS-T) and 3% BSA. Incubations with primary antibodies (rabbit anti-phospho-ERK1/2 or mouse anti-ERK1/2 (1:1,000); Cell Signaling, Danvers, MA, USA) in TBS-T 1% BSA were performed overnight, at 4ºC. Next, the membranes were incubated for 1 h at room temperature with alkaline phosphatase-linked secondary antibodies (anti-rabbit or anti-mouse IgG, 1:20,000) in TBS-T 1% BSA. After extensive washing in TBS-T 0.5% BSA, immunoreactive bands were visualized at the VersaDoc 3000 imaging system (BioRad, Hercules, CA, USA), following incubation of the membrane with ECF reagent for 5 min. The results are expressed as the percentage of control of phospho ERK/total ERK ratio.

1.12.8 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), as appropriate, followed by *post hoc* Bonferroni's or Dunnet's tests, as indicated in the figure legends and in the text. Differences were considered significant when $p < 0.05$.

1.13 Results

1.13.1 NO increases cell proliferation via the guanylyl cyclase-cyclic GMP pathway

To investigate the involvement of cGMP in the proliferative effect of NO, we evaluated the incorporation of thymidine analogues (EdU or BrdU) by SVZ cell cultures following treatment with a NO donor (NOC-18). We have shown in chapter 2 that treatment with the NOC-18 in the range of 1-10 μM increases proliferation of SVZ cells, but whether cGMP is involved in NO-induced cell proliferation has not been addressed. We first investigated the involvement of cGMP in the proliferative effect of NO at 6h; and later at 24h of treatment with NOC-18 (10 μM). Exposure to NOC-18 for 6 h increased the incorporation of EdU to 146.4 ± 6.4% of the control ($p<0.05$) (Fig. 3.1A). At 24 h, NOC-18 treatment further increased EdU incorporation to 165.3 ± 10.2% of the control ($p<0.001$) (Fig. 3.1B). In control conditions (untreated cells), the number of EdU-positive cells (percentage of total living cells) was 2.4 ± 0.7% at 6h and 2.2 ± 0.9% at 24h.

The involvement of cGMP in the proliferative effect of NOC-18 was evaluated using the guanylyl cyclase inhibitor ODQ. Treatment with ODQ prevented the NO-induced incorporation of EdU at 24h ($p<0.05$), when comparing to SVZ cells treated with NOC-18 alone (Fig. 3.1B), suggesting that cGMP mediates the effect of NO on cell proliferation. However, the proliferative effect of NOC-18 at 6h of treatment was not affected by ODQ ($p>0.05$; Fig. 3.1A), suggesting that mechanisms other than guanylyl cyclase and cGMP are responsible for the proliferative effect of NO at 6h of incubation with NOC-18. Thus, we then investigated whether the mitogen-activated kinase ERK1/2 was involved in the latter effect. Inhibition of ERK1/2 activation by U0126 indeed prevented the increase in EdU-positive cells stimulated by NOC-18, at both 6h ($p<0.05$) and 24h ($p<0.001$) (Fig. 3.1C and 3.1D, respectively).

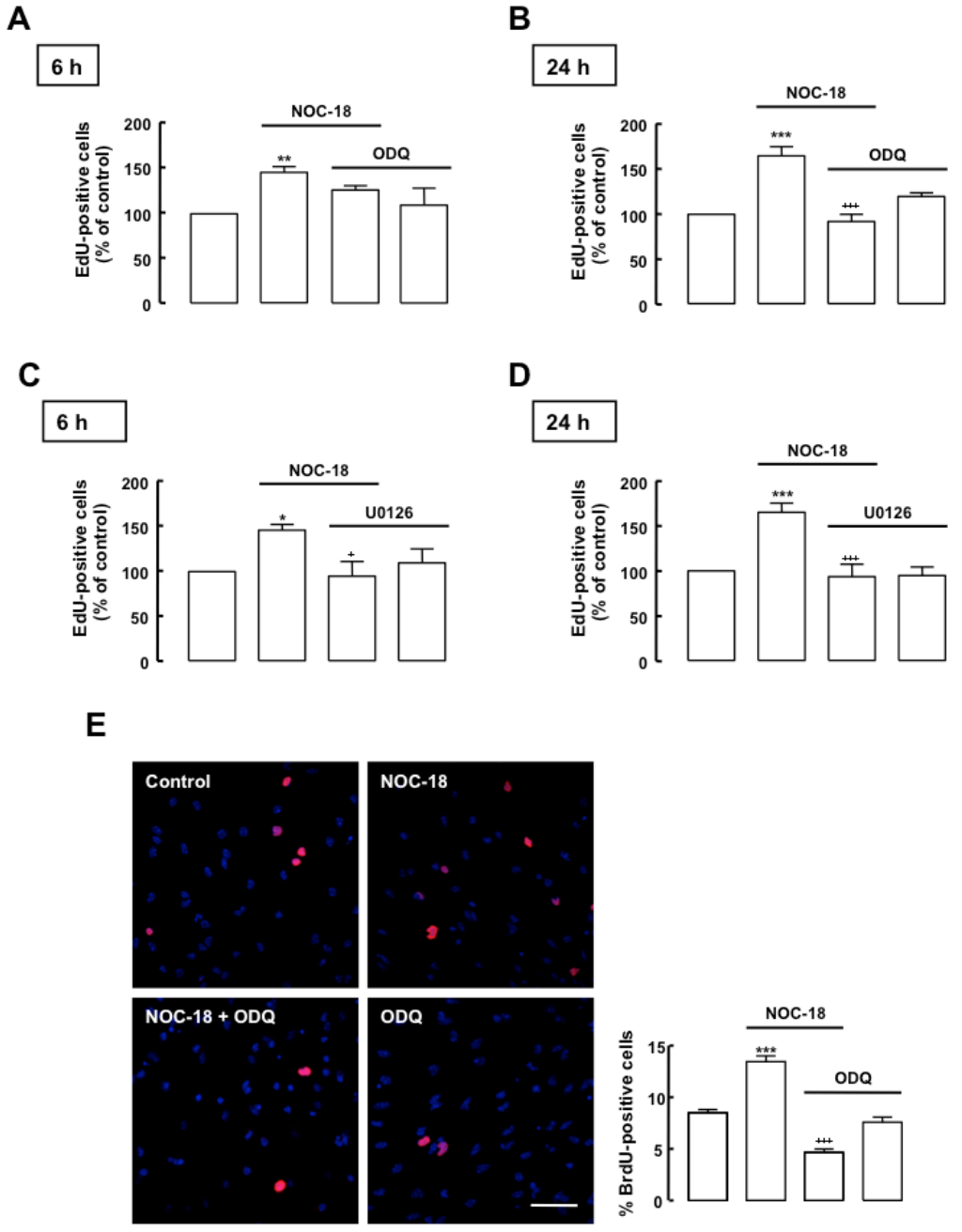


Figure 3.1. NO increases cell proliferation via the guanylyl cyclase-cGMP pathway for longer (24 h) but not for shorter (6 h) periods of cell exposure to NO. Cell proliferation following treatment with NOC-18 (10 μM), in the absence or presence of 50 μM ODQ, for 6 h (**A**) or 24h (**B**), evaluated by incorporation of EdU and assessed by flow cytometry. Data are expressed as means ± SEM of at least 4 independent experiments. One-way ANOVA (Bonferroni's post-test). **p<0.01 or

***p<0.001, significantly different from control; $^{+++}$p<0.001, significantly different from NOC-18. EdU incorporation in neural stem cells following exposure to NOC-18 (10 μM) in the absence or presence of 1 μM U0126, a selective MEK1 and MEK2 inhibitor, for 6 h (**C**) or 24 h (**D**), as assessed by flow cytometry. Data are expressed as means ± SEM of at least 4 independent experiments. One-way ANOVA (Bonferroni's post-test). *p<0.05 or ***p<0.001, significantly different from control; $^{+}$p<0.05 or $^{+++}$p<0.001, significantly different from NOC-18. **E**) BrdU incorporation in neural stem cells following exposure to NOC-18 (10 μM) for 24 h, in the absence or presence of a guanylyl cyclase inhibitor, ODQ (50 μM). ODQ completely blocks the increase in the number of BrdU-positive cells (red, representative images in the left panel). Nuclei are labeled by Hoechst 33342 (blue). Scale bar: 20 μm. Data are expressed as means ± SEM of at least 4 independent experiments (right panel). One-way ANOVA (Bonferroni's post-test). ***p<0.001, significantly different from control; $^{+++}$p<0.001, significantly different from 10 μM NOC-18.

Since ODQ only prevented EdU-incorporation 24h following treatment with NOC-18 (analysed by flow cytometry), we further confirmed this observation by evaluating the incorporation of BrdU by immunocytochemistry and microscopy analysis. Treatment with NOC-18 alone for 24h increased the number of BrdU-positive cells from 8.5 ± 0.3% of total cells (control) to 13.4 ± 0.5% (p<0.001), and ODQ also blocked this effect significantly (4.6 ± 0.3%, p<0.001), when compared to NOC-18 (Fig. 3.1E). Since NO may induce apoptosis in neural stem cells (Canals *et al.*, 2001), cell death was evaluated. Flow cytometry analysis of nuclei stained with 7-AAD, as described in the section 3.3.6, showed that the drugs used in this study did not affect cell survival significantly, compared to untreated cultures (Table 3.1).

Table 3.1 – Cell viability in neural stem cell cultures following exposure to NOC-18 with or without U0126 or ODQ.

	Treatment	% live cells	% dead cells
6 h	Control	90.9 ± 1.4%	9.1 ± 1.4%
	10 μM NOC-18	90.0 ± 1.7% (n.s.)	10.0 ± 1.7% (n.s.)
	10 μM NOC-18 + 1 μM U0126	90.2 ± 1.9% (n.s.)	9.8 ± 1.9% (n.s.)
	1 μM U0126	92.6 ± 1.2% (n.s.)	7.4 ± 1.2% (n.s.)
	10 μM NOC-18 + 50 μM ODQ	90.6 ± 1.7% (n.s.)	9.4 ± 1.7% (n.s.)
	50 μM ODQ	90.0 ± 1.6% (n.s.)	9.5 ± 1.8% (n.s.)
24 h	Control	90.6 ± 1.2%	9.4 ± 1.2%
	10 μM NOC-18	91.0 ± 1.4% (n.s.)	9.0 ± 1.4% (n.s.)
	10 μM NOC-18 + 1 μM U0126	90.1 ± 1.4% (n.s.)	9.9 ± 1.4% (n.s.)
	1 μM U0126	89.8 ± 1.8% (n.s.)	9.5 ± 1.6% (n.s.)
	10 μM NOC-18 + 50 μM ODQ	91.4 ± 1.1% (n.s.)	8.6 ± 1.1% (n.s.)
	50 μM ODQ	90.5 ± 2.0% (n.s.)	9.5 ± 2.0% (n.s.)

Cell viability was assessed by analysis of cell cycle distribution using the nuclear dye 7-Amino-actinomycin D (7-AAD), detected by flow cytometry. Data are expressed as means ± SEM of at least 3 independent experiments. n.s. (non-significant) $p>0.05$, not different from the control, one-way ANOVA (Dunnett's post-test).

1.13.2 The cGMP analogue 8-Br-cGMP mimics the proliferative effect of NOC-18

Since cGMP appears to mediate the proliferative effect of NO at 24h, we assessed the proliferative effect of a cGMP analogue, 8-Br-cGMP (20 μM), by flow cytometry. We observed a significant increase in EdU incorporation to 157.9 ± 14.6% ($p<0.01$) following 24 h of treatment (Fig. 3.2B), but not at 6 h (Fig. 3.2A), compared to control cultures. We further confirmed the 24 h observations by evaluating the incorporation of BrdU by immunocytochemistry and microscopy analysis. Treatment with 8-Br-cGMP for 24h significantly increased the number of BrdU-positive cells to 12.4 ± 0.5% ($p<0.01$) when comparing to control (4.6 ± 0.3%) (Fig. 3.2C).

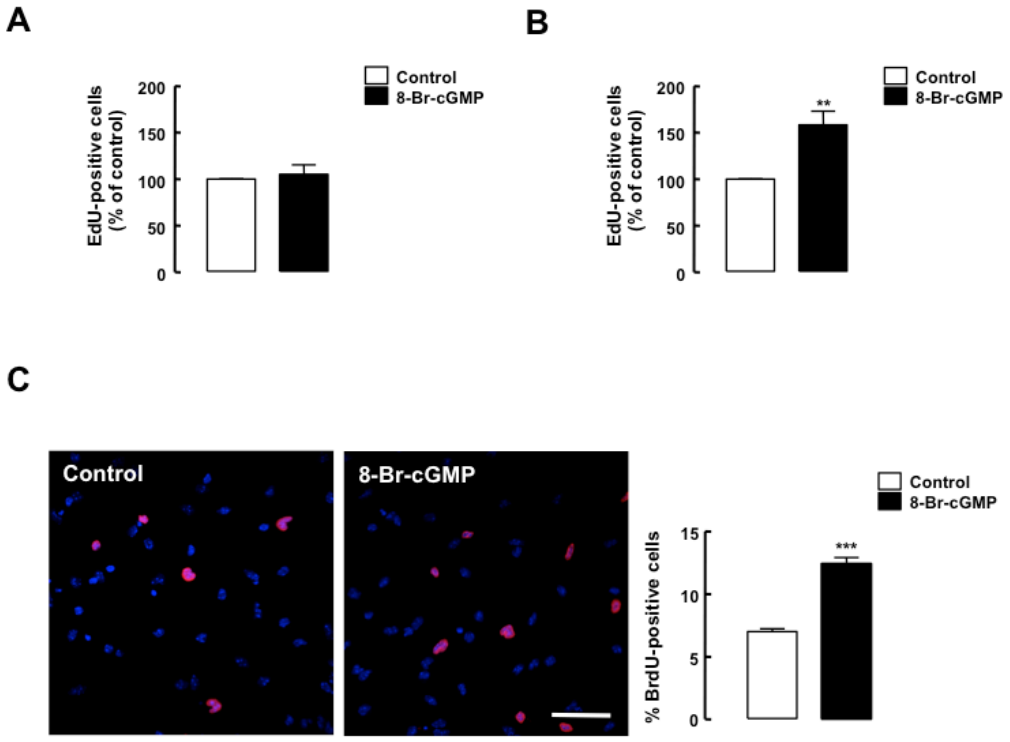


Figure 3.2. Effect of the cGMP analogue, 8-Br-cGMP, on cell proliferation in SVZ neurosphere cultures. A) EdU incorporation in neural stem cells following exposure to 20 μM 8-Br-cGMP as assessed by flow cytometry. 8-Br-cGMP for 6 h had no effect on cell proliferation. $p>0.05$, two tailed t-test. **B**) Longer exposure (24 h) to 8-Br-cGMP had a proliferative effect by increasing the incorporation of EdU. Two-tailed t-test. $^{**}p<0.01$, significantly different from control. Data are expressed as means ± SEM of at least 4 independent experiments. **C**) 8-Br-cGMP (20 μM) mimics the proliferative effect of NO, as determined by BrdU incorporation following 24 h treatment. Representative images of BrdU-positive cells (red) in neural stem cell cultures after exposure to 20 μM 8-Br-cGMP, for 24 h, are shown in the left panel. Nuclei are labeled with Hoechst 33342 (blue). Scale bar: 20 μm. The data in the graph represent the percentage of BrdU-positive cells and are expressed as means ± SEM of at least 3 independent experiments. Two-tailed t-test. $^{***}p<0.01$, significantly different from control.

1.13.3 NO-induced activation of the guanylyl cyclase pathway is independent of ERK/MAPK pathway activation

To identify the intracellular pathways that mediate the proliferative effect of NO, we investigated whether the guanylyl cyclase pathway is involved in the activation of the ERK/MAPK pathway in chapter 2. We showed

that NOC-18 alone stimulates proliferation of SVZ cultures by activating ERK1/2. To evaluate how fast the phosphorylation of ERK1/2 occurs following exposure to NOC-18, we analyzed the phospho-ERK1/2:total ERK1/2 immunoreactivity ratio at several time points after the stimulus (at 5, 15, 30 and 60 min). ERK1/2 phosphorylation increase to 138.1 ± 8.4% of the control 15 min after treatment with 10 μM NOC-18 ($p<0.001$) (Fig. 3.3A). At 30 min, the levels of phospho-ERK1/2 were similar to basal levels, suggesting that NO-induced phosphorylation of ERK1/2 is a transient event.

The activation of ERK by NO is an event independent of guanylyl cyclase. ODQ did not prevent the increase in the phosphorylation of ERK 1/2 triggered by NOC-18 (164.3 ± 6.5% of the control), suggesting that ODQ alone had no significant effect on ERK 1/2 phosphorylation (Fig. 3.3B).

Since cGMP can activate the cGMP-dependent protein kinase (PKG), the involvement of PKG in the activation of ERK1/2 was evaluated, following treatment with NOC-18. We observed that PKG does not appear to be involved in the early activation of ERK1/2 by NO, since KT5823 did not prevent the phosphorylation of ERK1/2 stimulated by exposure to NOC-18 for 15 min (Fig. 3.3C).

1.13.4 The cGMP analogue 8-Br-cGMP activates the ERK/MAPK pathway via PKG

We also analyzed the phospho-ERK1/2:total ERK1/2 immunoreactivity ratio at 5, 15, 30, 60 and 120 min following treatment with 8-Br-cGMP. There was a steady increase in the levels of phosphorylated ERK1/2 up to 2 h after treatment. At this time point, 20 μM 8-Br-cGMP induced a two-fold increase in phospho-ERK1/2:total ERK1/2 immunoreactivity ratio (221.1 ± 12.3 %), as compared to untreated cultures (Fig 3.4A). Inhibition of PKG with KT 5823 prevented ERK1/2 phosphorylation following exposure to 8-Br-cGMP (Fig. 3.4B).

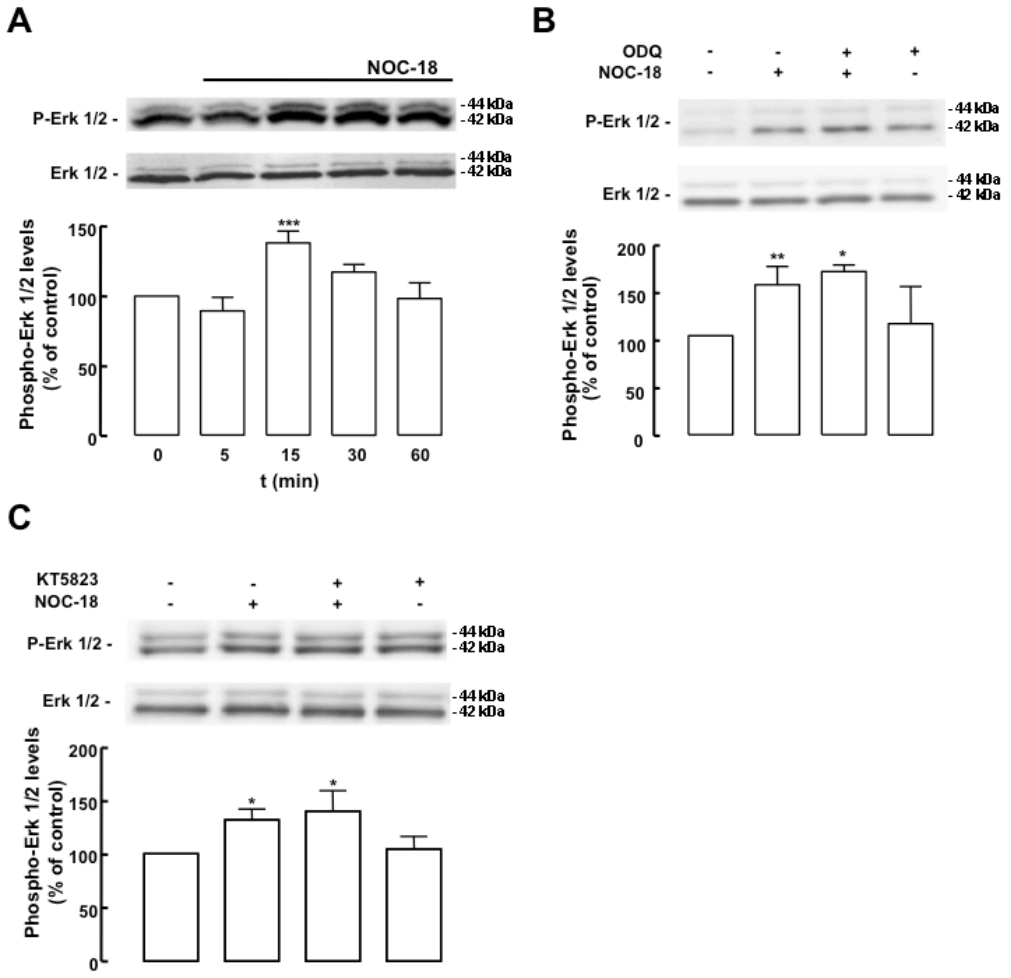


Figure 3.3. NO activates the ERK/MAPK pathway in a cGMP independent manner. A) Time course analysis of the phosphorylation of ERK1/2 following exposure to NOC-18 (10 μM). NOC-18 enhanced ERK1/2 phosphorylation as early as 15 min following treatment. Data are expressed as means ± SEM of at least 4 independent experiments. One-way ANOVA (Bonferroni's post-test). ***p<0.001, significantly different from Control. **B)** Western blot analysis of the involvement of guanylyl cyclase in the phosphorylation of ERK1/2, in lysates of neural stem cell cultures treated with NOC-18, for 15 min. No effect of ODQ (50 μM) on ERK1/2 phosphorylation was observed. Data are expressed as means ± SEM of at least 3 independent experiments. One-way ANOVA (Bonferroni's post-test). **p<0.01 and *p<0.05, significantly different from control. **C)** No effect of the PKG inhibitor (KT5823; 1 μM) on the phosphorylation of ERK1/2 stimulated by exposure to NOC-18 (10 μM), for 15 min. One-way ANOVA (Bonferroni's post-test). *p<0.05, significantly different from control.

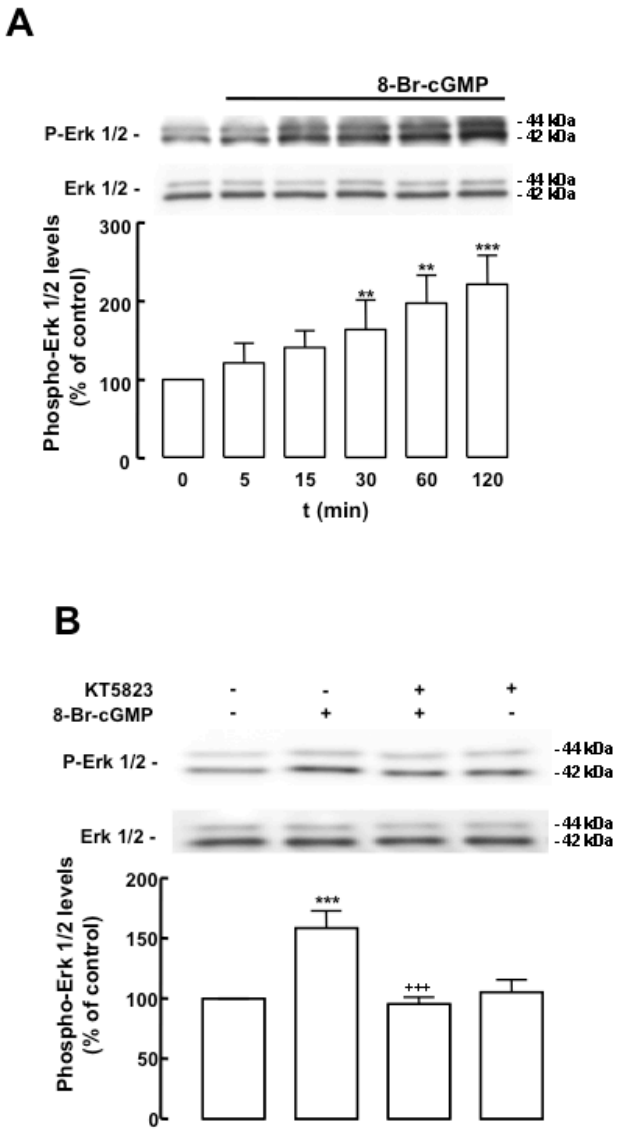


Figure 3.4. The cGMP analogue 8-Br-cGMP increases the phosphorylation of ERK1/2. A) Time course analysis of the phosphorylation of ERK1/2 upon treatment with 20 μM 8-Br-cGMP in lysates of neural stem cell cultures. Following exposure to 8-Br-cGMP, there is a time-dependent increase in the phosphorylation of ERK1/2, up to 120 min. Data are expressed as means ± SEM of at least 4 independent experiments. One-way ANOVA (Bonferroni's post-test). ***p<0.001 and **p<0.01, significantly different from control. **B)** KT5823 prevents the phosphorylation of ERK1/2 stimulated by treatment with 8-Br-cGMP for 2h One-way ANOVA (Bonferroni's post-test). ***p<0.001, significantly different from control. $^{+++}$p<0.001, significantly different from 8-Br-cGMP.

1.13.5 cGMP and PKG contribute to late but not to early proliferation induced by NO

Next, the involvement of cGMP and PKG in the proliferative effect of NO was studied by flow cytometry by evaluating the incorporation of EdU by SVZ cell cultures, following treatment with NOC-18 or 8-Br-cGMP. Blockade of PKG by KT5823 had no effect on early proliferation (6h) (Fig. 3.5A) but significantly prevented the EdU incorporation induced by NOC-18 treatment at 24 h (97.5 ± 15.2 %, $p<0.01$), as compared to cultures treated with NOC-18 alone (157.3 ± 12.4%) (Fig. 3.5B). Concerning the exposure of SVZ cells to the cGMP analogue, the inhibition of PKG prevented the proliferation induced by 8-Br-cGMP both for 6 h (102.918 ± 2.0 %, $p<0.05$) (Fig. 3.5C) and 24 h (83.8 ± 8.1 %, $p<0.05$) (Fig. 3.6D) when comparing to 8-Br-cGMP alone (123.757 ± 6.1 %, for 6 h and 162.7 ± 20.1 %, for 24 h). Flow cytometry analysis of nuclei stained with 7-AAD, as described, showed that the drugs used in this study did not affect cell survival significantly, compared to untreated cultures (Table 3.2).

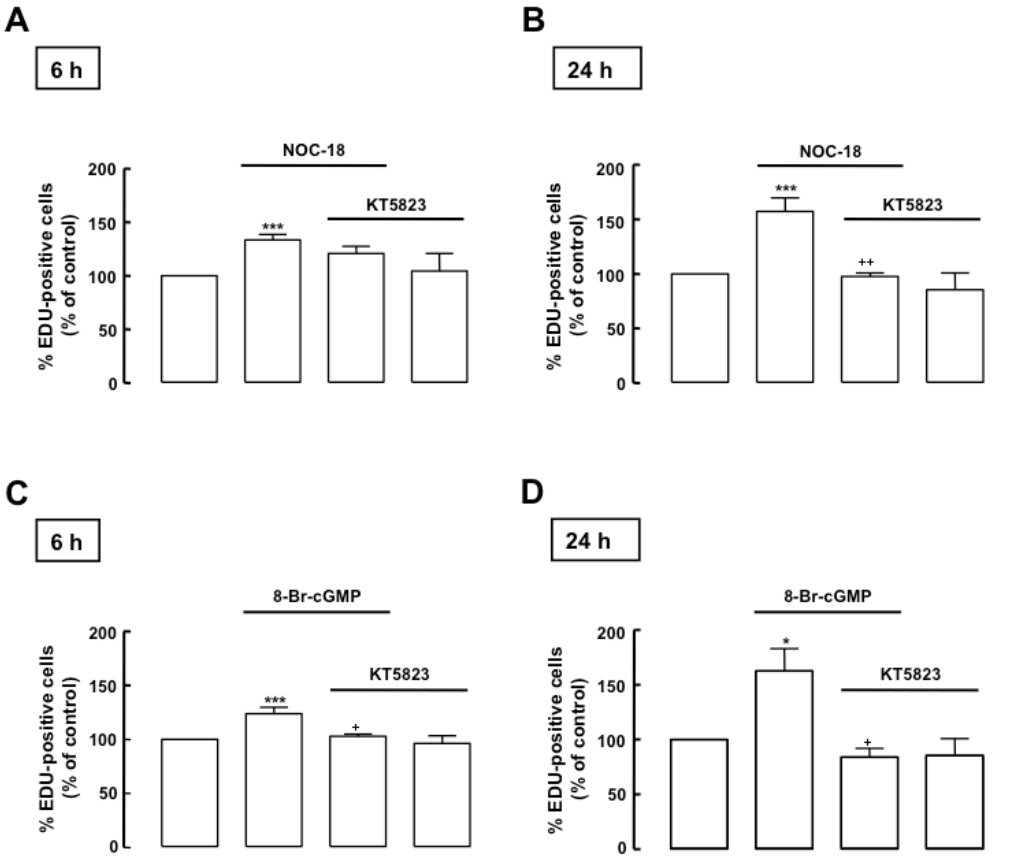


Figure 3.5. Involvement of the cGMP/PKG signalling pathway in the proliferation of neural stem cells. Cell proliferation following treatment with NOC-18 (10 μM), in the absence or presence of 1 μM KT5823, a selective PKG inhibitor, for 6 h (**A**) or 24 h **(B)**, evaluated by incorporation of EdU and assessed by flow cytometry. Data are expressed as means ± SEM of at least 4 independent experiments. One-way ANOVA (Bonferroni's post-test). ***p<0.001, significantly different from control; $^{++}$p<0.001, significantly different from NOC-18. EdU incorporation in neural stem cells following exposure to 8-Br-cGMP (20 μM) in the absence or presence of 1 μM KT5823, for 6 h (**C**) or 24 h **(D)**, as assessed by flow cytometry. Data are expressed as means ± SEM of at least 4 independent experiments. One-way ANOVA (Bonferroni's post-test). *p<0.05 or ***p<0.001, significantly different from control; $^{+}$p<0.05, significantly different from 8-Br-cGMP.

Table 3.2 – Cell viability in neural stem cell cultures following exposure to NOC-18 or 8-Br-cGMP with or without KT5823.

	Treatment	% live cells	% dead cells
6 h	Control	89.7 ± 1.1%	10.3 ± 1.0%
	10 μM NOC-18	91.0 ± 1.9% (n.s.)	9.0 ± 1.1% (n.s.)
	10 μM NOC-18 + 1 μM KT5823	89.2 ± 1.1% (n.s.)	10.8 ± 1.2% (n.s.)
	20 μM 8-Br-cGMP	93.0 ± 1.2% (n.s.)	7.0 ± 2.2% (n.s.)
	20 μM 8-Br-cGMP + 1 μM KT5823	90.2 ± 1.2% (n.s.)	9.8 ± 2.0% (n.s.)
	1 μM KT5823	92.1 ± 1.3% (n.s.)	7.9 ± 1.6% (n.s.)
24 h	Control	87.9 ± 1.9%	12.1 ± 1.9%
	10 μM NOC-18	91.0 ± 2.1% (n.s.)	9.0 ± 0.9% (n.s.)
	10 μM NOC-18 + 1 μM KT5823	92.1 ± 2.3% (n.s.)	7.9 ± 1.0% (n.s.)
	20 μM 8-Br-cGMP	89.9 ± 0.8% (n.s.)	10.1 ± 1.3% (n.s.)
	20 μM 8-Br-cGMP + 1 μM KT5823	90.0 ± 1.2% (n.s.)	10.0 ± 1.4% (n.s.)
	1 μM KT5823	93.5 ± 1.1% (n.s.)	6.5 ± 0.8% (n.s.)

Cell viability was assessed by analysis of cell cycle distribution using the nuclear dye 7-Amino-actinomycin D (7-AAD), detected by flow cytometry. Data are expressed as means ± SEM of at least 3 independent experiments. n.s. (non-significant) $p>0.05$, not different from the control, one-way ANOVA (Dunnett's post-test).

To further demonstrate the involvement of cGMP in the late proliferative effect of NO, SVZ cultures were treated with NOC-18 and a blocker of cGMP degradation and BrdU incorporation was assessed at 24 h. A selective phosphodiesterase 5 (PDE5) inhibitor, T0156, was used in these experiments. T0156 potentiated the increase in cell proliferation triggered by NOC-18 to 17.4 ± 1.9 % of BrdU-positive cells ($p<0.001$; Fig. 3.6), when compared to treatment with NOC-18 alone (12.7 ± 0.3 % of BrdU-positive cells). Proliferation in control cultures was 8.5 ± 0.3 %.

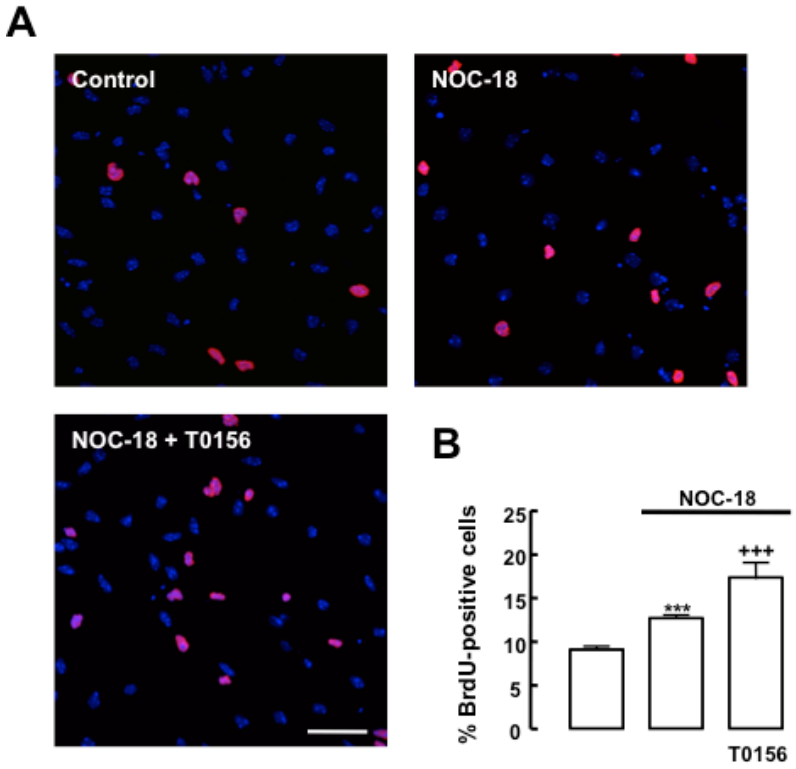


Figure 3.6. Blockade of cGMP degradation enhances the proliferative effect of NOC-18. A) Representative images of BrdU-positive cells (red) in neural stem cells following exposure to NOC-18 (10 μM) for 24h, in the presence or absence of a phosphodiesterase 5 inhibitor, T0156. Nuclei are labeled by Hoechst 33342 (blue). The images are representative of 3 independent experiments. Scale bar: 20 μm. **B)** Blockade of cGMP degradation by T0156 (1 μM) increased the proliferative effect of NO, assessed by evaluating the number of BrdU-positive cells. Data are expressed as means ± SEM of at least 4 independent experiments. One-way ANOVA (Bonferroni's post-test). ***p<0.001, significantly different from control; $^{+++}$p<0.001, significantly different from NOC-18.

1.14 Discussion

In this work, we show that cGMP and PKG are involved in the late proliferative effect triggered by nitric oxide (NO). The inhibition of guanylyl cyclase or PKG abolishes cell proliferation induced by NO, while blocking the degradation of cGMP further enhances the proliferative effect of NO. Although cGMP and PKG were not involved in the early activation of ERK1/2, they were

mandatory for cell proliferation following treatment with the NO-donor NOC-18 for 24 h. Moreover, the cGMP analogue 8-Br-cGMP had a similar effect to treatment with NOC-18 on cell proliferation.

We demonstrated that the late proliferative effect of NO is mediated by cGMP since the sGC inhibitor, ODQ, blocked NO-induced cell proliferation, as observed by the decreased incorporation of BrdU or EdU. Moreover, the blockade of cGMP degradation with a selective PDE5 inhibitor, T0156, potentiated the proliferative effect of NO. The sGC-cGMP pathway is the main effector pathway of NO biological effects as a second messenger. NO-mediated elevation of the intracellular levels of cGMP has been reported to directly regulate the activity of downstream effectors such as protein kinase G (PKG) (Fiscus *et al.*, 1983; Fiscus *et al.*, 1984; Forstermann *et al.*, 1990; Fiscus, 2002). More recently, it was reported that elevation of cGMP levels by PDE5 inhibition promoted cGMP/PKG activation, enhancing mesenchymal stem cell proliferation (Haider *et al.*, 2010). Other studies correlate elevation of cGMP levels to the enhancement of neurogenesis (Wang *et al.*, 2005; Zhang *et al.*, 2006; Zhang *et al.*, 2006).

Our results show that NO-induced activation of ERK1/2 is fast, occurring in a cGMP-independent manner, since ODQ did not prevent ERK1/2 phosphorylation. On the other hand, 8-Br-cGMP increased ERK1/2 phosphorylation at a slower rate than NOC-18. Previously, we demonstrated that phosphorylation of ERK1/2 is essential for the proliferative effect of NO, either at the early stages of cell proliferation following exposure to NO (after 30 min), or for later endpoints (24 h) (see Chapter 2). For short-term exposures (6 h), our results strongly suggest that the proliferative effect of NO is dependent on the activation of the ERK1/2 pathway and cGMP-independent, since in cultures treated with NOC-18 for 6 h, ODQ had no effect on NO-induced cell proliferation. Interestingly, long-term exposure to NO (24 h) increased cell proliferation in a cGMP-dependent manner.

To further demonstrate the involvement of cGMP in cell proliferation we assessed EdU incorporation following treatment with 8-Br-cGMP. On the other hand, in cultures treated with 8-Br-cGMP, the PKG inhibitor blocked cell proliferation at 24 h. Blockade of PKG inhibited ERK1/2 activation as well, thus suggesting that cGMP can cause ERK1/2 activation via PKG. Although 8-Br-cGMP results in increased ERK1/2 phosphorylation, activation of ERK1/2 by NO did not require cGMP or PKG activation. In cultures treated with NOC-18, the inhibitor of PKG inhibited cell proliferation at 24 h, but not the early activation of ERK1/2. Moreover, inhibition of PKG had no effect on early proliferation induced by NO.

Altogether, our results show that NO can activate two independent pathways, depending on the period of exposure of the cells, which induces the increase in neural stem cell proliferation: the ERK/MAP kinase and GC/cGMP/PKG pathways. Although there is no evidence of crosstalk between these two pathways for the early effect of NO, this possibility can not be excluded for NO-induced cell proliferation at later stages. While the early proliferation of neural stem cells triggered by NO is independent of cGMP and PKG, the complete blockade of the proliferative effect of NO at later stages by inhibition of either sCG, PKG or MEK1/2 suggests a crosstalk between the two pathways.

PKG is a serine/threonine kinase that is activated upon binding of cGMP, and it has been implicated in the regulation of gene expression, as reviewed by Madhusoodanan and Murad (Madhusoodanan and Murad, 2007). According to some authors cGMP is involved in the NO-mediated arrest of cell proliferation, in which PKG activation mediates the indirect inhibition of Raf-1 and subsequent decreased signaling by the MAPK pathway (Yu *et al.*, 1997; Guo *et al.*, 1998; Costa and Assreuy, 2005). These events have a negative effect on cell proliferation (Villalobo, 2006). Additionally, some studies suggest that the cGMP/PKG pathway is involved in the activation of the MAPK pathway, particularly ERK1/2 (Zaragoza *et al.*, 2002; Ota *et al.*,

2008), although we did not observe a crosstalk between the cGMP/PKG and ERK1/2 activation for early proliferation. While cGMP and PKG are undoubtedly involved and essential for the proliferative effect of NO in neural stem cells, the ERK pathway is not its target, and the precise mechanisms remain to be addressed.

Chapter 4

Nitric oxide from microglial origin impairs neural stem cell proliferation via nitration of the epidermal growth factor receptor

The work presented in this chapter is submitted for publication in a peer-reviewed international journal.

1.15 Summary

In Chapter 2, we found that the NO donor NOC-18 (100 μM), for 48 h, inhibited cell proliferation of SVZ-derived neural stem cells. We now investigated the mechanisms responsible for the antiproliferative effect of NO. We observed that NOC-18 caused the nitration of the EGF receptor in SVZ-derived neural stem cells expressing this receptor, which is concomitant with a decreased phosphorylation status of the EGF receptor. Using MnTBAP, a scavenger of peroxynitrite, nitration was prevented and cell proliferation rescued in SVZ-derived stem cell cultures. Moreover, using a culture system of SVZ-derived stem cells mixed with microglia isolated from wild-type mice ($iNOS^{+/+}$) or knockout mice ($iNOS^{-/-}$), we show that the increased release of NO by activated $iNOS^{+/+}$ microglial cells, following treatment with LPS plus IFN-γ, enhanced nitration of the EGF receptor, which is concomitant with decreased proliferation of SVZ-derived neural stem cells. Preventing peroxynitrite formation, by MnTBAP or FeTMyP, cell proliferation is restored to basal levels in $iNOS^{+/+}$ mixed cell cultures.

Overall, we show for the first time that NO has an antiproliferative effect in neural stem cell mediated by peroxynitrite formation, which causes nitration of the EGF receptor, leading to decrease in its phosphorylation status, thus preventing regular proliferation signaling.

1.16 Introduction

Brain inflammation was shown to be detrimental to neurogenesis (Ekdahl *et al.*, 2003; Monje *et al.*, 2003). However, the inflammatory factors involved in the negative effects of inflammation on the formation of new brain cells are not totally identified.

Activation of microglia is a hallmark of the neuroinflammatory process (Hanisch and Kettenmann, 2007). Microglial cells are highly dynamic sensors that continually scan the surrounding tissue (Davalos *et al.*, 2005; Nimmerjahn *et al.*, 2005). Upon an injury, or change in the environment, microglia promptly respond with morphological and biochemical changes, producing and releasing a plethora of signaling molecules (reviewed by Hanisch and Kettenmann, 2007). Inflammation has different effects on neurogenesis and under some circumstances, particularly following tissue damage it inhibits the neurogenic process (Ekdahl *et al.*, 2003; Kempermann and Neumann, 2003; Monje *et al.*, 2003; Ekdahl *et al.*, 2009). Upon neurotoxic, traumatic and inflammatory damage in the mammalian brain, nitric oxide (NO) is formed in high amounts, following the expression of the inducible nitric oxide synthase (iNOS) (Chao *et al.*, 1992; Galea *et al.*, 1992; Nathan and Xie, 1994; Murphy, 2000). NO can nitrosylate cysteine residues or nitrate tyrosine residues, typically leading to alterations in protein function (reviewed by Hanafy *et al.*, 2001). Reactive nitrogen and oxygen species are important factors in microglial-mediated inflammation (Rock *et al.*, 2004).

In isolated neural stem cells from the SVZ, supraphysiological concentrations of NO inhibit neural stem cell proliferation and promote differentiation of precursors into astrocytes (Covacu *et al.*, 2006; Torroglosa *et al.*, 2007). Torroglosa and colleagues have suggested that NO modulates the tyrosine kinase activity of epidermal growth factor (EGF) receptor (EGFR) (Torroglosa *et al.*, 2007), although the molecular mechanisms behind this effect remain unclear.

In the present study, we investigated how elevated concentrations of NO affect proliferation of neural stem cells, and what the underlying mechanisms are. To better understand whether NO mediates the detrimental effects of inflammation on neural stem cell proliferation, we used a culture system of SVZ-derived stem cells mixed with microglia isolated from wild-type mice ($iNOS^{+/+}$) or knockout mice ($iNOS^{-/-}$) for iNOS. We show that levels of NO similar to those produced by the brain's immune system have an antiproliferative effect mediated by peroxynitrite formation, which causes nitration of the EGF receptor, thus preventing regular signaling.

1.17 Materials and Methods

1.17.1 Materials

Dulbecco's Modified Eagle's Medium:F-12 nutrient mixture, (D-MEM/F-12, with GlutaMAX^{TM}-I), B27 supplement, trypsin-EDTA solution (0.05% trypsin, 1 mM EDTA in HBSS), gentamicin, antibiotic (10,000 units/ml of penicillin, 10 mg/ml streptomycin) and trypsin (1:250) were purchased from GIBCO BRL, Life Technologies, Scotland. Deoxyribonuclease 1 (DNase-1), 5-bromo-2'-deoxyuridine (BrdU), phenylmethylsufonyl fluoride, dithiothreitol, orthovanadate, chymostatin, leuptin, antiparin, pepstatin A, trypan blue, lipopolysaccharide (LPS) and alkaline phosphatase-linked anti-rabbit secondary antibody were purchased from Sigma Chemical (St Louis, MO, USA). Epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) and Click-iT® 5-ethynyl-2'-deoxyuridine (EdU) Alexa Fluor® 647 HCS Assay were purchased from Invitrogen (Paisley, UK). Macrophage colony stimulating factor (M-CSF) and interferon-gamma (IFN-γ) were purchased from Peprotech (London, UK) and DETA-NONOate (NOC-18) from Alexis Biochemicals (San Diego, CA, USA). BSA and MnTBAP were obtained from Calbiochem (San Diego, CA, USA). FeTMPyP was purchased from Cayman Chemical (Tallinn, Estonia). Rabbit anti-GFAP and DAKO fluorescent mounting medium were obtained from DakoCytomation (Glostrup, Denmark). Rat anti-mouse BrdU

was obtained from Oxford Biotechnology and rat anti-mouse-CD11b from Serotec (Oxford, UK). Mouse anti-nestin, rabbit anti-iNOS and mouse anti-GAPDH were purchased from BD Transduction (San Jose, CA, USA). Mouse anti-3-nitrotyrosine was purchased from Upstate Biotechnology (Charlottesville, VA, USA) and rabbit anti-EGFR from Cell Signaling (Danvers, MA, USA). Rabbit anti-nestin was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and mouse anti-Sox-2 from R&D Systems (Minneapolis, MN, USA); Hoechst 33342, anti-mouse IgG conjugated with Alexa Fluor 594 or 488, and anti-rabbit IgG conjugated with Alexa Fluor 633, 594 or 488 secondary antibodies were purchased from Molecular Probes (Invitrogen, Paisley, UK). Griess Reagent System was obtained from Promega (Madison, WI, USA). Polyvinylidene difluoride (PVDF) membranes, enhanced chemifluorescence (ECF) reagent and alkaline phosphatase-linked anti-rabbit and anti-mouse secondary antibodies were obtained from Amersham Pharmacia Biotech (Buckinghamshire, UK). Other reagents used in immunoblotting experiments were purchased from BioRad (Hercules, CA, USA).

1.17.2 Animals

C57BL/6J (iNOS$^{+/+}$) mice or B6.129P2-*Nos2*^{tm1Lau}/J (iNOS$^{-/-}$) were obtained from Charles River (Barcelona, Spain) or The Jackson Laboratory (Bar Harbor, ME, USA), respectively, and kept with food and water *ad libitum* in a 12h dark:light cycle. All experiments were performed in accordance with NIH and European guidelines (86/609/EEC) for the care and use of laboratory animals.

1.17.3 Primary microglial cell cultures

Primary mixed glial cultures were prepared from the brains of 0 to 3-day-old C57BL6 (iNOS$^{+/+}$) or B6.129P2-*Nos2*^{tm1Lau}/J (iNOS$^{-/-}$) mice according to the method of Giulian and Baker (Giulian and Baker, 1986). Briefly, the brains were removed from the skull, following decapitation, and placed in

dissection medium composed of Ca^{2+}- and Mg^{2+}-free 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, 1 mM sodium pyruvate, 10 mM HEPES, pH 7.4), supplemented with 0.25% gentamicin. The enveloping meninges and the cerebellum were discarded and the cortex tissue was mechanically dissociated and digested with trypsin (0.1%) and DNase 1 (0.001%) in Ca^{2+}- and Mg^{2+}- free HBSS for 20 min, at 37ºC. Cells were seeded in 75 cm^2 flasks coated with poly-L-lysine, at a density of 0.2 X 10^6 cells/cm^2 and cultured in D-MEM/F-12 with GlutaMAX™-I supplemented with 10% FBS, 0.25% gentamicin and 0.25 ng/ml M-CSF, at 37ºC and 95% air-5% CO_2 in a humidified incubator. Culture medium was changed every 3 - 4 days and confluency was achieved after 10 – 14 DIV. Microglia was detached from the mixed glial cultures 3-10 days after reaching confluency, by shaking at 200 r.p.m. for 2 h, and collected from the supernatant by centrifugation at 1500 r.p.m., for 5 min. Cells were then seeded for 3 days onto 16-mm diameter glass coverslips, for immunocytochemistry assays, or on 12-well plates, for preparation of lysates, both coated with poly-L-lysine, in serum-free medium, without M-CSF. Next, cultures were treated with an acute inflammatory stimulus (except the controls): 100 ng/ml LPS plus 0.5 ng/ml IFN-γ, for 24 h (Saura *et al.*, 2003).

1.17.4 Subventricular zone cell cultures

Neural stem cell cultures were obtained from the SVZ of postnatal day 0-3 wild-type or transgenic C57Bl6 mice expressing enhanced green fluorescent protein (eGFP) under the control of the actin promoter, as described in Chapter 2 (Carreira *et al.*, 2010). The SVZ-derived neural stem cells were allowed to develop as primary neurospheres in a 95% air-5% CO_2 humidified atmosphere at 37ºC, during 7 days. Next, neurospheres were collected and plated for 5 days on 16-mm diameter glass coverslips, for immunocytochemistry assays, or on 12-well plates, coated with poly-L-lysine, in the same medium as above, without growth factors, for preparation of lysates.

1.17.5 Mixed cell cultures

GFP-positive SVZ neurospheres were collected and plated together with microglial cell cultures (iNOS$^{+/+}$ or iNOS$^{-/-}$) on 16-mm diameter glass coverslips, for immunocytochemistry assays, or on 12-well plates, for preparation of lysates, both coated with poly-L-lysine, and kept in fresh D-MEM/F-12 with GlutaMAX^{TM}-I medium, supplemented with 1% B27, 0.25% gentamicin, 10 ng/ml EGF and 10 ng/ml bFGF, at 37ºC and 95% air-5% CO_2 in a humidified incubator, for 3 days. GFP-positive SVZ neurospheres were also seeded alone, for 3 days, on 16-mm diameter coverslips or on 12-well plates, coated with poly-L-lysine, and cultured in the same medium as above, for control experiments as indicated in the figure legends and in the text.

Cultures were treated with 100 ng/ml LPS plus 0.5 ng/ml IFN-γ, for 24 h. Control cultures were left untreated. The cell-permeable superoxide dismutase mimetic and peroxynitrite scavenger MnTBAP (100 μM) (Szabo *et al.*, 1996) or the peroxynitrite decomposition catalyst FeTMPyP (50 μM) (Misko *et al.*, 1998), when used, were added 30 min before LPS plus IFN-γ and kept throughout the incubation period.

1.17.6 Experimental treatments in SVZ-derived neural stem cell cultures

SVZ-derived neural stem cells were exposed to the NO donor DETA-NONOate/NOC-18 (100 μM) for 48 h, or to peroxynitrite ($ONOO^-$, 5 mM) for 10 min. MnTBAP (100 μM) or FeTMPyP (50 μM), were added 30 min before NOC-18 and kept throughout the incubation period.

1.17.7 Detection of BrdU incorporation in SVZ cell cultures

To analyze proliferation of SVZ-derived neural stem cells, 10 μM BrdU was added to the cultures 16 hours prior to fixation (Carreira *et al.*, 2010; Milenkovic *et al.*, 2004; Alvaro *et al.*, 2008). Nuclei that incorporated BrdU in this time-window were detected by immunofluorescence, as detailed next.

Following 20 min fixation with 4% paraformaldehyde/4% sucrose in phosphate-buffered saline (PBS, 0.1 M), the cells were permeabilized with 1% Triton X-100 for 5 min, and DNA was denaturated by treatment with 1 M HCl for 30 min, at 37ºC. Non-specific binding was blocked with 3% BSA in 0.2% Tween-20 in PBS (PBS-T) for 1 h, and then BrdU-positive cells were labeled with a rat anti-BrdU antibody (1:50) for 90 min, at room temperature. The cells were then incubated with a secondary antibody goat anti-rat IgG conjugated with Alexa Fluor 594 (1:200), for 1 h, at room temperature. Nuclei were stained with Hoechst 33342 (1 µg/ml) for 5 min. Coverslips were mounted on glass slides using DAKO fluorescence mounting medium DakoCytomation (Glostrup, Denmark). The images were acquired in a laser scanning microscope LSM 510 META (Zeiss, Jena, Germany) or in a fluorescence microscope (Axioskop 2 Plus, Zeiss, Jena, Germany. The number of BrdU-positive nuclei was counted in 8-10 randomly selected fields for each coverslip (in a total of approximately 900-1,200 cells per coverslip), and the data were expressed as percentage of the total number of living cells. A minimum of 3 independent experiments, from neural stem cell cultures prepared from different animals, were analyzed for each condition.

1.17.8 Detection of EdU incorporation

Neural stem cell proliferation was also assessed by incorporation of the EdU. EdU (10 µM) was added to the cultures 4 h prior to fixation. Nuclei that incorporated EdU were detected by immunofluorescence, as follows. Following 20 min fixation with 4% paraformaldehyde/4% sucrose in PBS (0.1 M), the cells were washed with 3% BSA/PBS and then permeabilized with 0.5% Triton X-100 / PBS for 15 min, at room temperature. The cells were then incubated with the Click-iT reaction cocktail [1x Click-iT Reaction Buffer 87.5% (v/v), $CuSO_4$ 2% (v/v), fluorescent azide (Alexa Fluor 647) 0.05% (v/v), and 1x Reaction Buffer Additive 10% (v/v)], protected from light. Cells were then washed twice in 3% BSA/PBS and an immunocytochemistry was performed

as detailed next. The number of EdU-positive nuclei was counted in 8-10 randomly selected fields for each coverslip (in a total of approximately 900-1,200 cells per coverslip), and the data were expressed as percentage of the total number of living cells. A minimum of 3 independent experiments was analyzed for each condition.

1.17.9 Immunocytochemistry

Following fixation and permeabilization, nonspecific binding was blocked with 3% BSA. Cells were incubated with the primary antibodies for 90 min, at room temperature. After rinsing with PBS, the cells were incubated with the appropriate secondary antibodies for 1 h (1:200, anti-mouse, anti-rabbit or anti-rat IgGs conjugated with Alexa Fluor 488, 594 or 633), at room temperature. All antibodies were prepared in blocking solution. Nuclei were labeled with Hoechst 33342 (1 μg/ml) for 5 min, after incubation with the secondary antibodies. Coverslips were mounted on glass slides, the cells were visualized using a fluorescence microscope (Axioskop 2 Plus, Zeiss, Jena, Germany) and the images were acquired with the Axiovision software (release 4.7) or in a laser scanning microscope LSM 510 META (Zeiss, Jena, Germany). The primary antibodies and the concentrations used were: mouse anti-Sox-2, 1:100; rabbit anti-nestin, 1:100; rabbit anti-GFAP, 1:400; mouse anti-nestin, 1:500; rat anti-mouse-CD11b, 1:200; rabbit anti-iNOS, 1:200; mouse anti-3-nitrotyrosine, 1:100 or rabbit anti-EGFR, 1:50.

1.17.10 Evaluation of nitric oxide production

NO production was assessed by measuring the concentration of nitrites in the culture medium (Green *et al.*, 1982), in primary microglia cultures, SVZ-derived neural stem cell cultures or mixed cell cultures, as indicated in the figure legends and in the text. A commercial kit from Promega (Griess Reagent System) was used, and the standard protocol provided by the supplier was followed. The concentration of nitrite for each sample was

calculated from a standard curve performed using a sodium nitrite solution and data were expressed in μM.

1.17.11 Western blot analysis

Cells were lysed in 50 mM Tris-HCl, 10 mM EGTA, 1% Triton X-100 and 2 mM $MgCl_2$, supplemented with 100 μM phenylmethylsufonyl fluoride, 1 mM dithiothreitol, 1 μg/ml chymostatin, 1 μg/ml leupeptin, 1 μg/ml antiparin, 5 μg/ml pepstatin A, 1 mM sodium orthovanadate, 50 mM NaF, pH 7.4 at 4ºC. Protein concentration was determined by the BCA method, and the samples were used for Western blot analysis, after adding 6x concentrated sample buffer (0.5 M Tris, 30% glycerol, 10% SDS, 0.6 M dithiothreitol, 0.012% bromophenol blue) and heating, for 5 min, at 95ºC. Equal amounts of protein were separated by electrophoresis on SDS-polyacrilamide gels, and transferred electrophoretically to PVDF membranes. These were then blocked for 1 h at room temperature, in Tris-buffered saline (137 mM NaCl, 20 mM Tris-HCl, pH 7.6) containing 0.1% Tween-20 (TBS-T) and 3% BSA. Incubations with primary antibodies (anti-iNOS or anti-GAPDH, 1:500) in TBS-T 1% BSA were performed overnight, at 4ºC. Next, the membranes were incubated for 1 h at room temperature with alkaline phosphatase-linked secondary antibodies (anti-rabbit or anti-mouse IgG, 1:20,000; respectively) in TBS-T 1% BSA. After extensive washing in TBS-T 0.5% BSA, immunoreactive bands were visualized in the VersaDoc 3000 imaging system (BioRad, Hercules, CA, USA), following incubation of the membrane with ECF reagent for 5 min.

1.17.12 Immunoprecipitation

Following the various experimental treatments as detailed in figure legends, the cultures were lysed in 20 mM Tris-HCl, 100 mM NaCl, 2 mM EDTA, 2 mM EGTA, supplemented with 100 μM PMSF, 1 mM dithiothreitol, 1

µg/ml chymostatin, 1 µg/ml leupeptin, 1 µg/ml antiparin, 5 µg/ml pepstatin A, 1 mM sodium orthovanadate, 50 mM NaF, pH 7.0, at 4ºC. Protein concentration was determined by the BCA method, and the samples were used for immunoprecipitation of nitrated proteins, using an antibody against 3-nitrotyrosine conjugated with agarose beads (#389549, Cayman Europe, Tallinn, Estonia), or immunoprecipitated against the EGF receptor, using an antibody against the EGF receptor (Cell Signaling, Danvers, MA, USA) conjugated with protein A sepharose beads (GE Healthcare Europe GmbH, Munich, Germany). Briefly, equal amounts of sample (250 µg of protein) were incubated with the antibody overnight at 4ºC, and then with the beads for 2 h at room temperature. Following rinsing, the supernatant was discarded and the beads were suspended in 2x concentrated sample buffer, boiled for 5 min, and centrifuged using Spin-X centrifuge tube filters (0.45 µm cellulose acetate; Corning Inc., Lowell, MA, USA), to separate the beads from the immunoprecipitates. Equal volumes of immunoprecipitate were loaded onto SDS-PAGE gels, and Western blotted as described above against the EGF receptor (#2232, Cell Signaling, Danvers, MA, USA; or #04-290, Millipore Iberica S.A.U., Madrid, Spain), in the case of the nitrated protein immunoprecipitates, or against phospho-tyrosine (P-Tyr 100, Cell Signaling, Danvers, MA, USA), in the case of the EGF receptor immunoprecipitates.

1.17.13 Statistical analysis

Data are expressed as means ± SEM. Statistical significance was determined by using two-tailed t tests, one-factor or two-factor analysis of variance (ANOVA) as appropriate, followed by *post hoc* Bonferroni's or Dunnet's tests, as indicated in the figure legends and in the text. Differences were considered significant when $p < 0.05$.

1.18 Results

1.18.1 Characterization of cell cultures

1.18.1.1 Primary microglial cell cultures

Microglial cells were isolated from mixed glial cultures by shaking, as detailed in the section 4.3.3. Cells were seeded on poly-L-lysine-coated coverslips for 3 d and characterized at this stage. The percentage of CD11b, a microglial cell marker, and glial fibrillary acidic protein (GFAP) positive cells was assessed by immunostaining. Approximately 90% of the cells were positive for CD11b, thus suggesting that the cultures were highly pure for microglial cells. No co-localization for CD11b/GFAP was observed.

1.18.1.2 SVZ-derived neural stem cells

Neural stem cells were isolated from the SVZ and cultured as floating aggregates (neurospheres). Cells were plated on poly-L-lysine-coated coverslips for 3 or 5 d, and characterized at these stages. Staining against the transcription factor Sox-2, and nestin, a neural precursor cell marker, was performed. The percentage of double-labeled cells was approximately 70%, which suggests that the majority of cells remained undifferentiated after plating as determined previously (Carreira *et al.*, 2010). The number of nestin/GFAP positive cells, both markers expressed in type B cells of the SVZ, was similar to the number of Sox-2/nestin positive cells. Moreover, GFAP-positive cells were mostly Sox-2 positive (Carreira *et al.*, 2010).

1.18.1.3 Mixed cell cultures

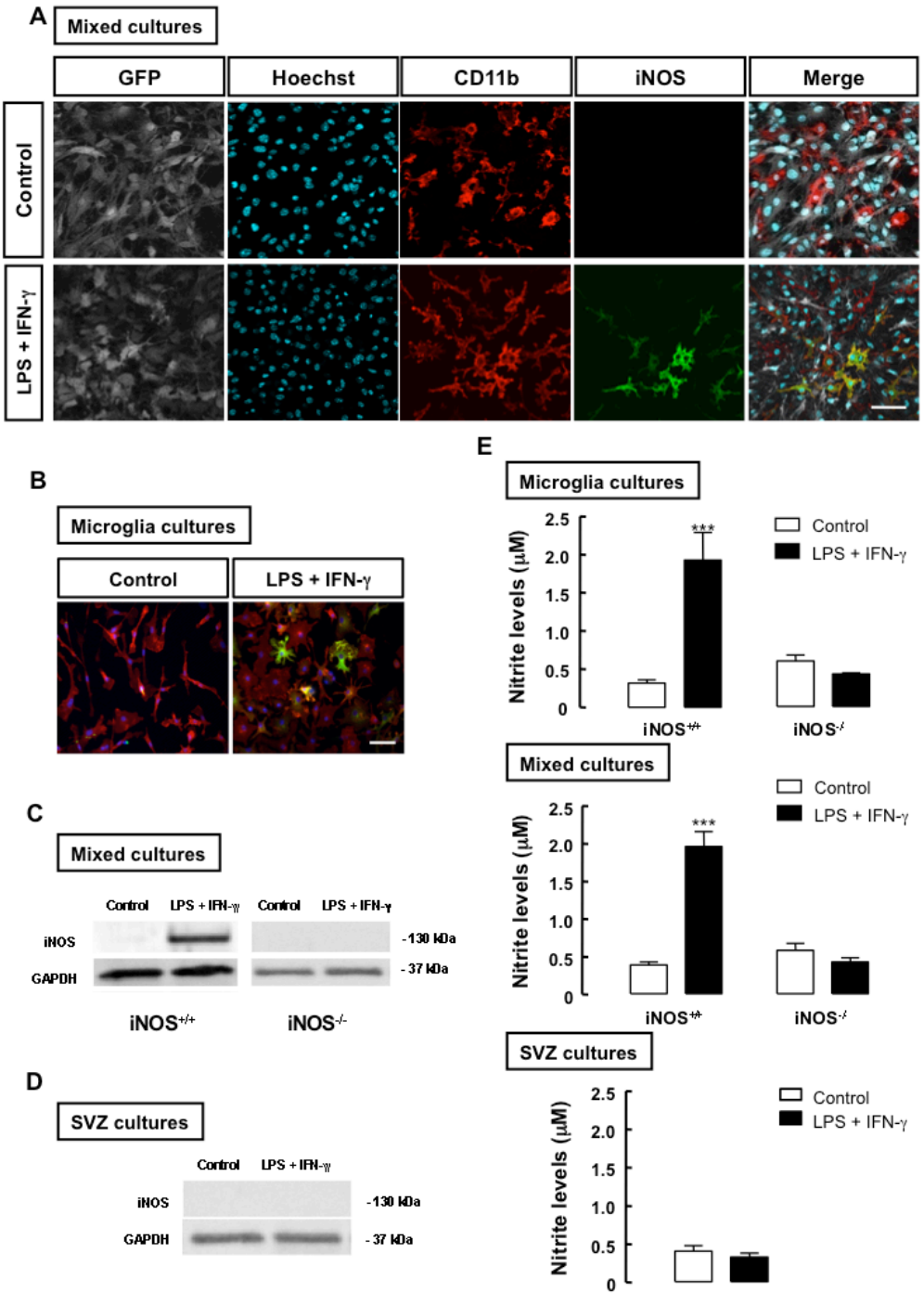
To investigate how NO from microglial origin could affect the proliferation of SVZ-derived neural stem cells, mixed cultures were performed as described in previous sections. Using LPS plus IFN-γ for 24h as an inflammatory stimulus, cultures were characterized by evaluating microglial cells morphology, iNOS expression and NO production. We observed the

presence of iNOS in wild-type ($iNOS^{+/+}$) microglial cells cultured together with GFP-positive SVZ cells ($iNOS^{+/+}$ mixed cultures), following treatment with LPS plus IFN-γ**.** This effect was clear both by immunostaining (Fig. 4.1A) and by Western blot analysis (Figure 4.1C). In cultures of $iNOS^{-/-}$ microglial cells with GFP-positive SVZ cells ($iNOS^{-/-}$ mixed cultures), we did not observe an increase in iNOS levels after the inflammatory stimulus, as assessed by Western blotting (Fig. 4.1C).

In $iNOS^{+/+}$ microglial cell cultures (Fig. 4.1B), exposure to LPS plus IFN-γ for 24h induced an increased immunoreactivity against iNOS, but not in $iNOS^{-/-}$ microglial cell cultures (data not shown). Concomitantly, following treatment with LPS plus IFN-γ, both $iNOS^{+/+}$ and $iNOS^{-/-}$ microglial cells exhibited an activated morphology with ovaloid cytoplasm, marked cellular hypertrophy and retraction of processes.

To determine whether SVZ-derived neural stem cells contributed to the increase in iNOS levels observed in mixed cell cultures (Fig. 4.1C), we also evaluated the presence of iNOS in SVZ-derived neural stem cell lysates, and observed a total absence of iNOS expression following treatment with LPS plus IFN-γ in these cultures (Fig. 4.1D).

Figure 4.1. Characterization of cell cultures (*opposite page*). A) Presence of iNOS-positive cells (green) in mixed cultures of wild-type ($iNOS^{+/+}$) microglia (CD11b-positive, red) together with GFP-positive SVZ cells (white), following treatment with LPS + IFN-γ for 24 h, as compared to controls (upper panels). Nuclei were labeled with Hoechst 33342 (blue). Representative images of immunocytochemistry are shown. Scale bar: 20 μm. **B)** Exposure to LPS + IFN-γ for 24 h induced expression of iNOS (green) in $iNOS^{+/+}$ microglia (CD11b-positive red). Nuclei were labeled with Hoechst 33342 (blue). The images are representative of 3 independent experiments. Scale bar: 50 μm. **C)** Detection of iNOS in mixed cultures of SVZ cells with $iNOS^{+/+}$ microglia, but not with $iNOS^{-/-}$ microglia, after treatment with LPS + IFN-γ for 24 h, as assessed by Western blotting. GAPDH was used as a loading control. The images are representative of 3 independent experiments. **D)** Absence of iNOS in SVZ cells cultured alone upon exposure to LPS + IFN-γ for 24 h. GAPDH was used as a loading control. The images are representative of 3 independent experiments. **E)** Production of NO, as measured by nitrite levels in the culture media, following treatment with LPS + IFN-γ, for 24 h, in microglia cells when cultured alone (microglia cultures) or together with SVZ cells (mixed cultures), or in SVZ cells cultured alone (SVZ cultures). Data are expressed as means ± SEM of at least 4 independent experiments. Two-way ANOVA (microglia cultures and mixed cultures); ***$p<0.001$, significantly different from control. Two-tailed t test (SVZ cultures); $p>0.05$.


A
Mixed cultures
GFP
Hoechst
CD11b
iNOS
Merge
Control
LPS + IFN-γ
B
Microglia cultures
Control
LPS + IFN-γ
C
Mixed cultures
Control
LPS + IFN-γ
Control
LPS + IFN-γ
iNOS
GAPDH
-130 kDa
-37 kDa
iNOS+/+
iNOS-/-
D
SVZ cultures
Control
LPS + IFN-γ
iNOS
GAPDH
-130 kDa
-37 kDa
E
Microglia cultures
Nitrite levels (μM)
2.5
2.0
1.5
1.0
0.5
0
***
Control
LPS + IFN-γ
iNOS+/+
iNOS-/-
Mixed cultures
Nitrite levels (μM)
2.5
2.0
1.5
1.0
0.5
0
***
Control
LPS + IFN-γ
iNOS+/+
iNOS-/-
SVZ cultures
Nitrite levels (μM)
2.5
2.0
1.5
1.0
0.5
0
Control
LPS + IFN-γ

In order to estimate the amount of NO produced by activated microglia in culture, we challenged microglial cells with LPS plus IFN-γ for 24 h, and assessed NO production by measuring nitrite levels in the culture media. Nitrite levels were higher in LPS plus IFN-γ-treated $iNOS^{+/+}$ microglial cell cultures (1.95 ± 0.3 μM, $p<0.001$), than in untreated cultures (0.32 ± 0.1 μM), corresponding to a 6-fold increase in NO production above control levels. Furthermore, this increased NO production was also observed in $iNOS^{+/+}$ mixed cultures (1.96 ± 0.2 μM, $p<0.001$) following treatment with LPS plus IFN-γ, as compared to untreated mixed cultures (0.39 ± 0.1 μM). In SVZ-derived neural stem cell cultures, in $iNOS^{-/-}$ mixed cultures and in $iNOS^{-/-}$ microglial cell cultures, treatment with LPS plus IFN-γ for 24 h did not significantly alter NO levels, as compared to untreated cultures (Fig. 4.1E).

1.18.2 NO from microglial origin has an antiproliferative effect on SVZ-derived neural stem cells

To investigate whether NO released by microglial cells could affect the proliferation of SVZ-derived neural stem cells, we evaluated the incorporation of EdU in cultures of SVZ-derived neural stem cells cultured with $iNOS^{+/+}$ or $iNOS^{-/-}$ microglia cells (from now on designated $iNOS^{+/+}$ or $iNOS^{-/-}$ mixed cultures), following treatment with LPS plus IFN-γ for 24 h. In $iNOS^{+/+}$ mixed cultures, we observed that 24 h following exposure to LPS plus IFN-γ EdU incorporation significantly decreased to 7.0 ± 1.09 % ($p<0.001$), as compared to control cultures (17.3 ± 0.88 %), but the same stimulus had no effect either in EdU incorporation in $iNOS^{-/-}$ mixed cultures (Fig. 4.2B) or in SVZ cultures alone (Fig. 4.2A). In addition, when MnTBAP or FeTMPyP are present during the inflammatory stimulus, cell proliferation is rescued to 15.8 ± 0.4 % or 14.7 ± 0.8 %, respectively, in $iNOS^{+/+}$ mixed cultures (Fig. 4.2B). MnTABP or FeTMPyP had no effect on the proliferation of $iNOS^{-/-}$ mixed cultures treated with LPS plus IFN-γ (Fig. 4.2B).

Moreover, we observed that SVZ-derived neural stem cells, but not microglia, incorporated EdU, as illustrated by the EdU immunostaining in the

GFP-positive SVZ cells and not in microglial cells, meaning that these are the dividing cells in mixed cultures (Fig. 4.2C). Furthermore, in Fig. 4.2C there is a decrease in the number of EdU positive cells in SVZ-iNOS$^{+/+}$ following incubation with LPS plus IFN-γ, in agreement with the data in Fig. 2A. We confirmed that LPS plus IFN-γ for 24 h did not change cell viability in iNOS$^{+/+}$ and iNOS$^{-/-}$ mixed cultures or in SVZ-cell cultures (data not shown).

1.18.3 High levels of NO induce nitration of the EGF receptor and decrease its phosphorylation status

We next investigated the possible NO-mediated nitration of the EGF receptor, which may affect the phosphorylation and further activation of the EGFR and downstream signaling, as a possible mechanism for the antiproliferative effect of NO. Nitration of tyrosine residues of the EGFR was evaluated in SVZ-derived neural stem cell cultures, but also in iNOS$^{+/+}$ and iNOS$^{-/-}$ mixed cultures.

SVZ-derived neural stem cells cultures were exposed to an NO donor, NOC-18 (100 μM) for 48 h. Immunoprecipitates of nitrated proteins were strongly labeled against EGFR after 48 h of treatment with 100 μM NOC-18. Moreover, this effect was similar to that observed in immunoprecipitates of nitrated proteins from SVZ cultures treated for 10 min with peroxynitrite, showing a strong nitration of the EGFR (Fig. 4.3A-B).

Concomitantly with increased nitration in tyrosine residues, NO caused a decrease in the phosphorylation status of the EGFR (Fig. 4.3C). In addition, cells expressing the EGFR showed a strong labeling for 3-nitrotyrosine, an experimental index for protein nitration (Radi *et al,* 2001), following treatment with NOC-18 (Fig. 4.3F). MnTBAP, a scavenger of peroxynitrite and superoxide, was able to prevent the nitration of the EGFR, as well as increased its phosphorylation status (Fig. 4.3B-C).

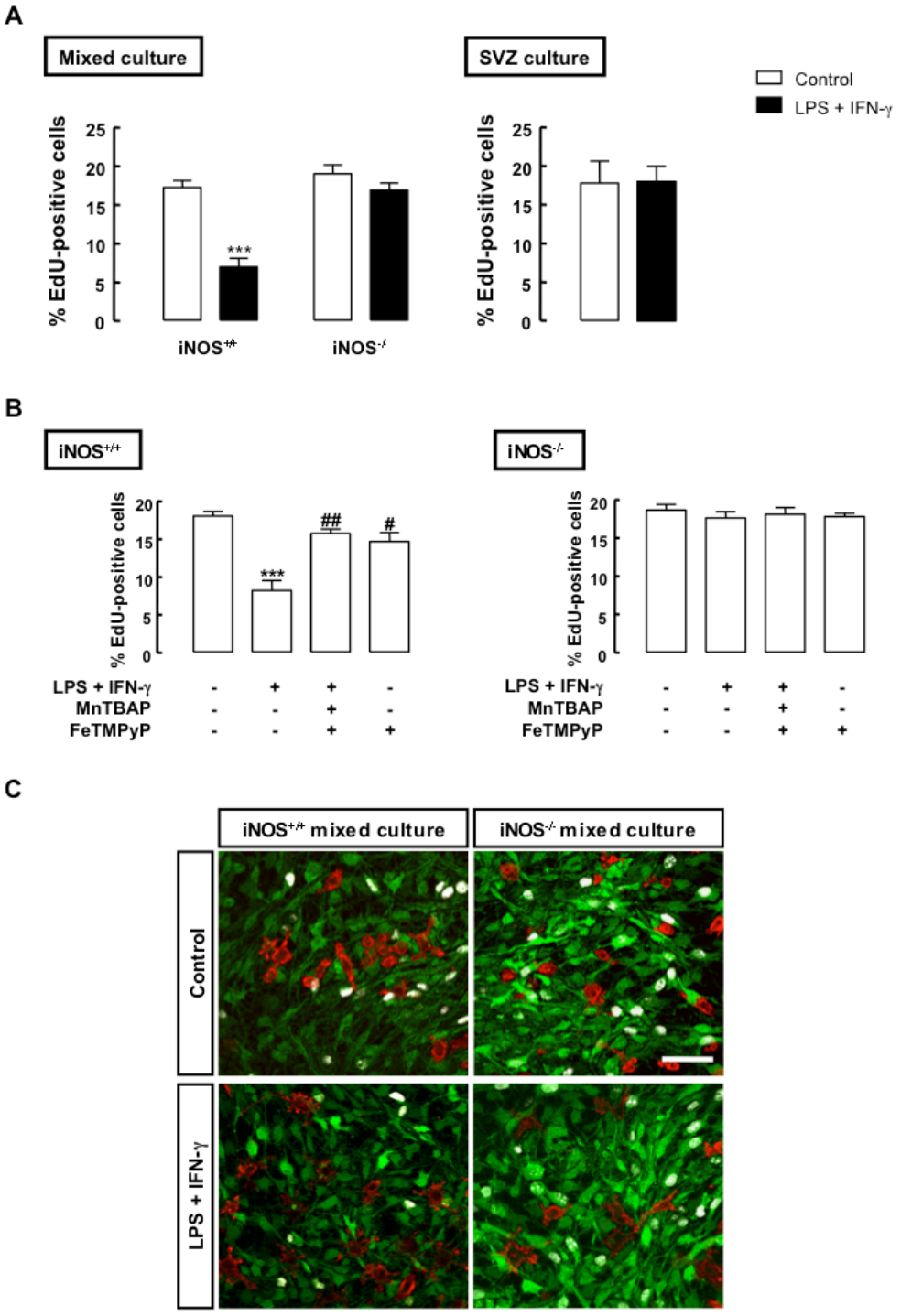


Figure 4.2. NO from microglial origin impairs the proliferation of SVZ cells. A) Incorporation of EdU in mixed or SVZ cultures following treatment with LPS + IFN-γ for

24 h. The data represent the percentage of EdU-positive cells and are expressed as means ± SEM of at least 3 independent experiments. Two-way ANOVA (mixed culture); ***p<0.001, significantly different from control. Two-tailed t test (SVZ culture); p>0.05. **B)** Incorporation of EdU in iNOS$^{+/+}$ or iNOS$^{-/-}$ mixed cultures upon treatment with LPS + IFN-γ for 24 h, with or without MnTBAP (100 μM) or FeTMPyP (50 μM). The data represent the percentage of EdU-positive cells and are expressed as means ± SEM of at least 3 independent experiments. One-way ANOVA (Bonferroni's post-test); ***p<0.001, significantly different from control; $^{\#\#}$p<0.01 or $^{\#}$p<0.05, significantly different from LPS + IFN-γ. **C)** Representative images of the incorporation of EdU (white) in GFP-positive SVZ cells (green) cultured together with iNOS$^{+/+}$ or iNOS$^{-/-}$ microglia (CD11b-positive, red), following exposure to LPS+IFN-γ for 24h. The images are representative of 3 independent experiments. Scale bar: 20 μm.

Next, we investigated whether scavenging superoxide and preventing the formation of peroxynitrite could rescue the antiproliferative effect of NOC-18 (100 μM) at 48 h. NOC-18 significantly decreased BrdU incorporation to 4.9 ± 0.2 % (p<0.001) as compared to BrdU incorporation in control cultures (7.7 ± 0.2 %), and MnTBAP rescued cell proliferation (7.9 ± 0.66 %), when present during the treatment with NOC-18 for 48 h (Fig. 4.3D).

Furthermore, in iNOS$^{+/+}$ mixed cultures, following treatment with LPS plus IFN-γ for 24 h, we observed an increase in the nitration of the EGF receptor in immunoprecipitates of nitrated proteins (127.4 ± 5.4 %, p<0.01) as compared to untreated cultures. Moreover, MnTBAP and FeTMPYP were able to prevent the nitration of EGFR in these cultures, after incubation with LPS plus IFN-γ, reducing EGFR nitration to 68.2 ± 7.2 % (p<0.001) or 84.9 ± 2.4 % (p<0.001) of the control, respectively (Fig. 4.3E). On other hand, this effect was not observed in iNOS$^{-/-}$ mixed cultures, where treatment with LPS plus IFN-γ did not increase the nitration of EGFR (Fig. 4.3 E).

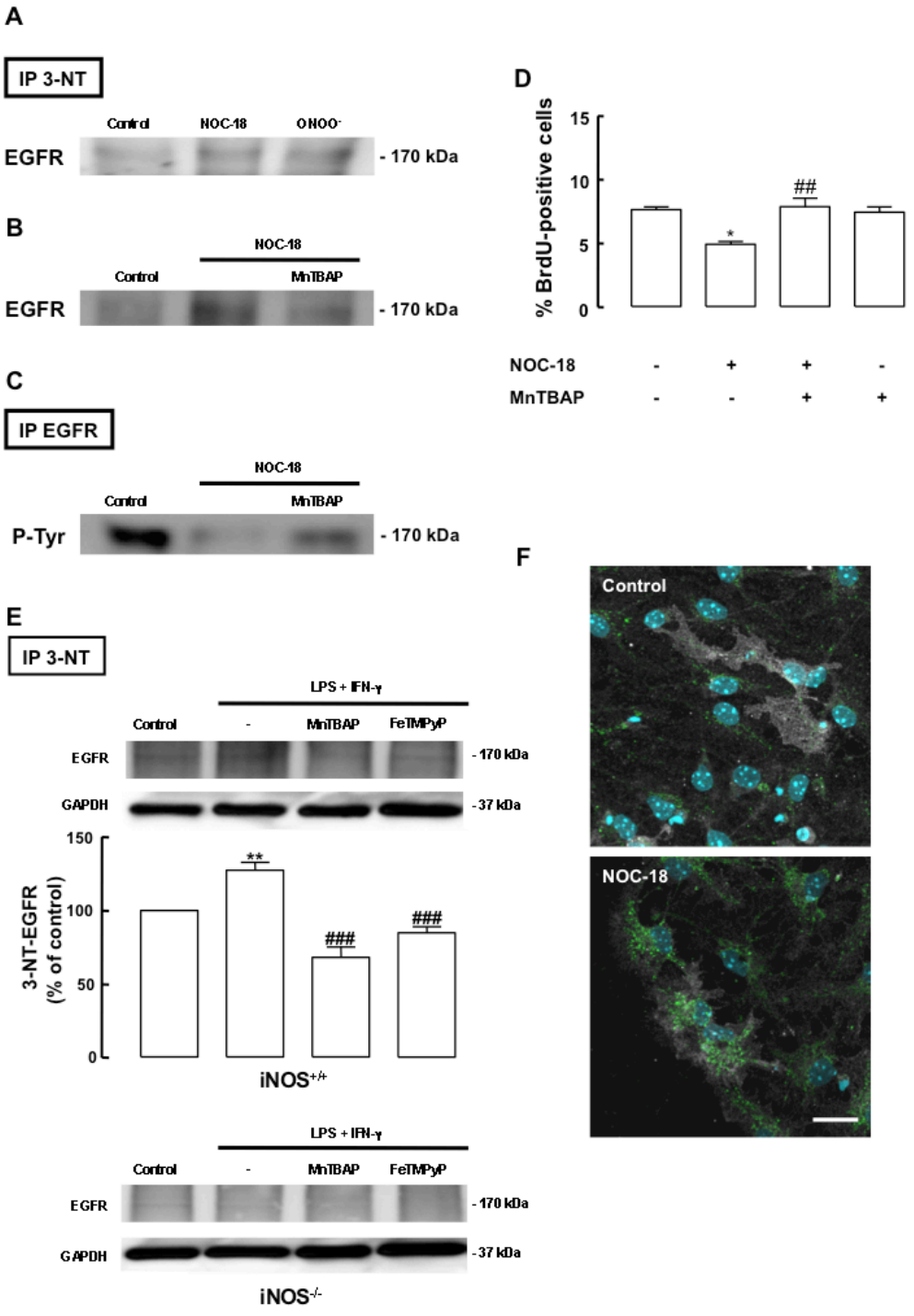


Figure 4.3. High levels of NO cause nitration of the EGF receptor, decreasing its phosphorylation status and SVZ cell proliferation. **A)** Nitration of EGFR upon exposure to NOC-18 (100 μM, 48 h) or $ONOO^-$ (5mM, 10 min), assessed by

immunoblotting against EGFR, after immunoprecipitation of the nitrated proteins (IP-3NT) in SVZ lysates. **B)** Superoxide dismutase (SOD) mimetic MnTBAP (100 μM), prevented the nitration of the EGFR following NOC-18 (100 μM) treatment. **C)** High levels of NOC-18 (100 μM) decreased the phosphorylation of tyrosine residues (P-Tyr) in immunoprecipitates of EGFR (IP-EGFR) in SVZ lysates. MnTBAP partially prevented the loss of tyrosine phosphorylation in the EGFR. **D)** MnTBAP (100 μM) prevented the antiproliferative effect of NOC-18 (100 μM) in SVZ cell cultures, as determined by BrdU incorporation. The data represent the percentage of BrdU-positive cells and are expressed as means ± SEM of at least 3 independent experiments. One-way ANOVA (Bonferroni's post-test). *$p<0.05$, significantly different from Control. $^{\#\#}p<0.01$, significantly different from NOC-18. **E)** Nitration of the EGFR in SVZ cells cultured in the presence of $iNOS^{+/+}$ microglia, but not with $iNOS^{-/-}$ microglia, following stimulation with LPS + IFN-γ for 24 h. MnTBAP (100 μM) and FeTMPyP (50 μM) prevented the nitration of the EGFR in SVZ cells cultured in the presence of $iNOS^{+/+}$ microglia, following exposure to LPS+IFN-γ for 24 h. Nitration was assessed by immunoblotting against the EGFR, after immunoprecipitation of the nitrated proteins (IP-3NT). A loading control was performed by reprobing against GAPDH. One-way ANOVA (Bonferroni's post-test). ***$p<0.001$, significantly different from Control. $^{\#\#\#}p<0.001$, significantly different from LPS+IFN-γ . **F**) Representative experiment showing that high concentrations of NOC-18 (100 μM) caused nitration of tyrosine residues in cells that express the EGF receptor (gray) after 48 h, as observed by immunostaining of 3-nitrotyrosine (green). Nuclei were labeled with Hoechst 33342 (blue). Scale bar: 10 μm.

1.19 Discussion

In this work, we show for the first time that NO has an antiproliferative effect in neural stem cell proliferation due to nitration of the EGF receptor, with intermediate formation of peroxynitrite, which causes the receptor to decrease its phosphorylation status. We also show for the first time that NO mediates the antiproliferative effect of inflammation in mixed cultures of neural stem cells and microglia challenged with LPS and IFN-γ. This effect is also mediated by peroxynitrite and involves nitration of the EGF receptor.

To study the effect of NO in SVZ-derived neural stem cells, primary cell cultures were exposed to the NO donor DETA-NONO:ate (NOC-18) in a concentration mimicking pathophysiological conditions, particularly neuroinflammation, when high concentrations of NO can be found locally in the brain. For this purpose, SVZ-derived neural stem cell cultures were incubated with 100 μM NOC-18 for 48 h, since it is a long-acting NO donor

with a half-life of approximately 22 h (Keefer *et al.*, 1996). At this concentration (100 μM), NOC-18 released NO to the same extent that we observed in LPS plus IFN-γ-treated microglial cells, thus mimicking what may be found in neuroinflammatory conditions.

Here we hypothesized that the antiproliferative effect of NO might be mediated by peroxynitrite, since high levels of NO can inhibit mitochondrial respiration and elicit superoxide production (Beltran *et al.*, 2002; Moncada and Bolanos, 2006).

In several pathophysiological conditions associated with the inflammatory process, activated inflammatory cells generate large amounts of reactive oxygen species such as superoxide, hydrogen peroxide and the hydroxyl radical, concomitantly with increased iNOS expression and production of large amounts of NO. Moreover, NO can induce the production of superoxide by mitochondria (Beltran *et al.*, 2002). In biological systems, NO and superoxide readily react to form peroxynitrite, which is an extremely reactive molecule (Beckman and Crow, 1993; Ischiropoulos and al-Mehdi, 1995; Pryor and Squadrito, 1995; Radi *et al.*, 2001). Nitration is an irreversible chemical modification that results from the reaction of peroxynitrite with tyrosine residues in proteins (Hanafy *et al.*, 2001), seriously affecting tyrosine phosphorylation/dephosphorylation signaling cascades (reviewed in Monteiro *et al.*, 2008). We observed that exposure to a high concentration of NO caused the nitration of the EGF receptor in SVZ-derived neural stem cells. Indeed, SVZ-derived neural stem cells expressing EGFR showed a strong labeling for 3-nitrotyrosine, a specific marker of protein nitration, following exposure to 100 μM NOC-18 for 48 h, pointing to the intermediate formation of peroxynitrite. In fact, we demonstrated here that treatment of SVZ neural stem cell cultures with peroxynitrite caused nitration of the EGF receptor. Our data also indicates that concomitantly with increased nitration in tyrosine residues of EGFR, NO decreases the phosphorylation status of this receptor, possibly inhibiting the tyrosine kinase activity of EGFR. The EGF receptor has over 15

tyrosine residues that can be phosphorylated and contribute to its activation. Since nitration of tyrosine residues appears to prevent their possibility of phosphorylation, which is paramount for activation of the EGFR and downstream signaling, a scavenger of peroxynitrite and superoxide, MnTBAP, was used. MnTBAP was able to partially prevent nitration of the EGF receptor in SVZ-derived neural stem cell cultures, as well as rescued cell proliferation to basal levels, suggesting that the antiproliferative effect of NO is mostly due to the formation of peroxynitrite and its reaction with tyrosine residues on the EGF receptor.

In this work, we also studied the effect of NO from inflammatory origin in the proliferation of SVZ-derived neural stem cell. We established mixed cultures of $iNOS^{+/+}$ or $iNOS^{-/-}$ microglia cells and SVZ-derived neural stem cells, and challenged them with LPS and IFN-γ during 24 h. We show that NO increased release by stimulated $iNOS^{+/+}$ microglia due to treatment with LPS plus IFN-γ, is concomitant with increased nitration of the EGFR, which correlates with a decreased proliferation of SVZ-derived neural stem cells. In fact, NO seems to be a key player for this effect, since its absence, and consequently peroxynitrite absence, prevented the nitration of the EGFR. In $iNOS^{-/-}$ mixed cultures, although there was activation of microglial cells following acute treatment with LPS plus IFN-γ, analysed by morphologic changes, no differences in cell proliferation were found in $iNOS^{-/-}$ mixed cultures following treatment with LPS plus IFN-γ, when compared to untreated cultures. Several studies showed that pharmacological blockade of inflammation elicited by injection of LPS (Monje *et al.*, 2003), or experimentally induced seizures (Ekdahl *et al.*, 2003), can restore hippocampal neurogenesis. However, some proinflammatory mediators, such as interleukin-6 (IL-6), released by activated microglia, seem to be important contributors to the inhibition of SGZ neurogenesis (Vallieres *et al.*, 2002; Monje *et al.*, 2003). On the other hand, microglia can also release trophic factors (Batchelor *et al.*, 1999), like brain-derived neurotrophic factor (BDNF), that have been reported to promote neurogenesis (Benraiss *et al.*, 2001).

Thus, activated microglia may have a beneficial or detrimental role, depending on the stimulus and local and temporal environmental changes during CNS lesion.

Although NO levels achieved after incubation with NOC-18 are very high when compared to those measured in $iNOS^{+/+}$ mixed cultures, it should be noted that in mixed cultures the cells are in close contact. Therefore, it is very likely that in $iNOS^{+/+}$ mixed cultures, NO levels achieved locally are quite high, as evidenced by the increased nitration of the EGF receptor following acute stimulation of microglial cells with LPS plus IFN-γ. Moreover, we show that scavenging peroxynitrite formation, by MnTBAP or FeTMyP treatment (Misko *et al.*, 1998; Salvemini *et al.*, 1998), basal cell proliferation is restored and nitration of the EGF receptor is prevented in $iNOS^{+/+}$ mixed cell cultures, which supports our hypothesis that the antiproliferative effect of NO is due to the formation of peroxynitrite and subsequent nitration of EGFR receptor (Fig. 4.4).

Some studies have reported that NO can have an antiproliferative effect by affecting the phosphorylation of the EGF receptor in fibroblasts (Estrada *et al.*, 1997), neuroblastoma cells (Murillo-Carretero *et al.*, 2002), and neural stem cells (Torroglosa *et al.*, 2007), but how such an effect is mediated was not demonstrated before. In Caco-2 cells (cell line obtained from human colon carcinoma) peroxynitrite was shown to cause nitration of EGFR (Uc *et al.*, 2003). To our knowledge, this is the first study showing that nitration of the EGF receptor is antiproliferative in neural stem cells. Several other players of the EGFR signaling cascade are also likely to be susceptible to peroxynitrite-mediated nitration, since they also have tyrosine residues that participate in EGFR signaling by phosphorylation/dephosphorylation and may, thus, be nitrated and inactivated, such as ERK1/2 or Raf-1. Although we did not address these other players in this work, it cannot be excluded that other participants in proliferation signaling, other than the EGF receptor, can be affected by NO and peroxynitrite-mediated nitration.

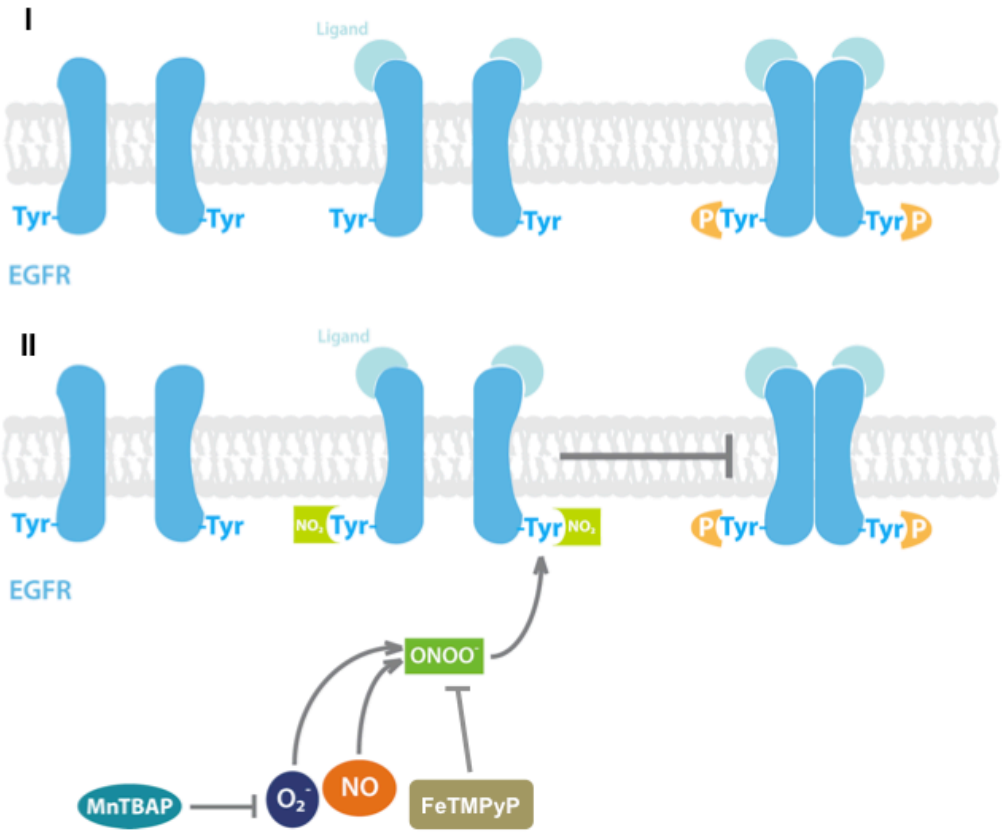


Figure 4.4. Illustration of tyrosine phosphorylation and signaling mediated by the EGF receptor (EGFR) and effects of peroxynitrite formation/removal. I – EGFR tyrosine kinase activity is linked to the signaling of proliferative events. Upon a stimulus, tyrosines of the C-terminal domain undergo phosphorylation. After oligomerization of the two domains, other tyrosine residues are phosphorylated and trigger intracellular signaling. **II** – Peroxynitrite ($ONOO^-$) nitrates the tyrosine residues of the EGFR, preventing its phosphorylation and further signaling, e.g., in inflammatory conditions. Prevention of tyrosine residues nitration with MnTBAP or FeTMPyP restores cell proliferation.

Moreover, the antiproliferative effect of NO in acute inflammatory conditions may be related to the enhancement of cell differentiation. Some publications reported that NO from inflammatory origin to be involved in astrogliogenesis (Covacu *et al.*, 2006), neurogenesis in the SVZ (Walton *et al.*, 2006), or oligodendrogenesis (Kempermann and Neumann, 2003; Butovsky *et al.*, 2006).

Overall, our findings highlight that control of the nitrergic system may be an important target in cell transplantation techniques, particularly in the case of grafting of neural stem cells in lesioned areas. Published evidence demonstrates that surviving grafts are influenced by the innate immune response, particularly due to acute microglia activation, which significantly impacts the survival and fate of both endogenous and transplanted neural progenitor cells (Monje *et al.*, 2002; Ekdahl *et al.*, 2003; Monje *et al.*, 2003; Ormerod *et al.*, 2008). Here we show that it is possible to prevent the antiproliferative effects of microglia during an acute inflammatory response, by preventing the formation of peroxynitrite.

Overall, our work sheds new light on the mechanisms of the effects of NO on the proliferation of neural stem cells, and may help in steering research efforts towards understanding how modulation of the nitrergic system can be used to regulate proliferation of stem cells in a regenerative context.

Chapter 5

General discussion

1.20 General discussion

Neurogenesis is not limited to embryonic development as previously thought, and occurs throughout the entire adult life of mammals, including humans. New neurons are continuously added to neural circuits and originate at two principal brain regions: the subventricular zone of the lateral ventricles, which generates olfactory bulb neurons, and the subgranular zone of the dentate gyrus of the hippocampus. Both regions harbor neural stem cells that can be isolated and cultured *in vitro* in the presence of growth factors, such as basic fibroblast growth factor, epidermal growth factor, or both. Indeed, cells can be expanded in these culture conditions, undergo several passages and keep an undifferentiated status and self-renewal. Moreover, the absence of growth factors results in the differentiation of cells into neurons, astrocytes or oligodendrocytes (reviewed in Suh *et al.*, 2009).

These singularities of neurogenesis have been studied exhaustively over the past years, and despite the great progress that has been achieved, the knowledge of the multiple aspects controlling the proliferation, differentiation or survival of NSCs are far from being known or understood. It was shown that neurogenesis decreases with aging and is impaired in several neurodegenerative disorders. Whether the insult is acute, such as ischemic brain stroke, traumatic brain injury or epileptic seizures, or is a slow-progressing disease like Alzheimer's disease, Huntington's disease or Parkinson's disease, all these conditions are accompanied by an inflammatory response in the brain (Amor *et al.*, 2010). Furthermore, it was shown that blockade of inflammation restores adult neurogenesis (Monje *et al.*, 2003). When an inflammatory brain response appears following an injury, activation of the brain immune cells takes place, particularly microglial cells. Microglial cells are known as an hallmark of the inflammatory process and are present throughout the brain in a "resting state", or more accurately "surveillance state", in physiological conditions (Hanisch and Kettenmann, 2007). In inflammatory conditions, microglial cells become "activated", and among a

pletora of morphological and immunological alterations, they are able to express the inducible nitric oxide synthase, producing high levels of nitric oxide (NO).

NO is a multifaceted gaseous signalling molecule with several distinct functions in the central nervous system (reviewed in Moncada and Bolanos, 2006). This Janus-faced molecule is simultaneously involved in neuroprotection and in neurotoxicity, and is also involved in neuroinflammatory mechanisms (Liu *et al.*, 2002; Calabrese *et al.*, 2007). NO was shown to modulate neurogenesis in the adult CNS (Contestabile and Ciani, 2004). Depending on the insult and on its source, NO can act as an anti-proliferative agent (Packer *et al.*, 2003; Moreno-Lopez *et al.*, 2004; Matarredona *et al.*, 2005), or stimulate neuronal precursor proliferation and differentiation (Zhu *et al.*, 2003). It appears that in physiological conditions, NO tonically inhibits cell proliferation in the brain, while in pathophysiological conditions it exerts a proliferative effect on the dividing population of neuronal precursors. Moreover, the physiological effect of NO is mostly mediated by nNOS, which is constitutively expressed, while pathophysiological levels of NO are attained following expression of iNOS. The physiological concentrations of NO in the normal brain have been estimated to range from low nM to 100 nM (Shibuki, 1990).

However, the exact mechanisms by which NO regulates neuronal proliferation and differentiation are not yet clarified, and further investigation on this matter is needed. Since neuroinflammation is detrimental for adult neurogenesis, it would be of great interest to elucidate the role of inflammatory NO on the ongoing neurogenesis in these conditions. Therefore, in the present work we investigated the mechanisms by which nitric oxide regulates the proliferation of neural stem cells.

In this study, we show that NO can have a dual effect on proliferation of neural stem cells, either promoting or inhibiting neural stem cell proliferation. Using the NO-donor, NOC-18, in a range of concentrations

mimicking NO release in physiological or pathophysiological events occurring in the brain, we observed that low concentrations of NO, but already in the pathological range, have a proliferative effect in cultured neural stem cells isolated from the SVZ. Moreover, the highest concentration of NOC-18 used in this work (100 μM, for 48 h) releases a massive amount of NO, and inhibits the proliferation of SVZ-derived neural stem cells.

In Chapters 2 and 3 we studied the intracellular targets of NO that underly the proliferative effect of NO, in particular, the possible involvement of the EGFR/p21Ras/ERK/MAPK and sGC/cGMP/PKG signaling pathways.

In Chapter 2, we showed an increased activity of p21Ras as soon as 2 min after treatment with 10 μM NOC-18, which is not dependent on the EGF receptor activation. Downstream of p21Ras, there is an increased activation of c-Raf and ERK1/2 following exposure to NO, which is also independent on EGFR signaling. We demonstrated that EGFR inhibition with the selective inhibitor AG1478 did not prevent the activation of c-Raf and ERK1/2 following treatment with NOC-18, demonstrating that NO triggers its proliferative effect in a EGFR independent fashion. In addition, we found that several transcription factor regulators, such as Elk-1, c-Myc and p90RSK, are activated by ERK1/2, following exposure to NO, suggesting that the NO proliferative effect is mediated by activating the transcription of immediate early genes. Particularly, p90RSK was studied due to its important role in the regulation of transcription factors, but also in the regulation of the cyclin-dependent kinase inhibitor, $p27^{KIP1}$ (Fujita *et al.*, 2003). $p27^{KIP1}$ can be phosphorylated by p90RSK and then translocated from the nucleus into the cytosol where it is ubiquitinated, thus allowing cell cycle progression (Fujita *et al.*, 2003). We found that concomitantly with the increased activity of p90RSK there is a decreased nuclear presence of $p27^{KIP1}$, contrasting with increased cytosolic $p27^{KIP1}$, suggesting that translocation of $p27^{KIP1}$ from the nucleus into the cytosol is occurring. This event has been described as essential for cell cycle progression, and has been found to be a key regulator of the cell division

of transit-amplifying progenitors from the SVZ (Doetsch *et al.*, 2002). Other studies have shown that higher concentrations of NO caused a decrease in cell proliferation of SVZ stem cells, and this event correlates with the nuclear presence of p^{27KIP1} (Torroglosa *et al.*, 2007). Moreover, we observed an increased Ki-67 immunoreactivity in cells that loose nuclear $p27^{KIP1}$. These findings are strong evidence of the cell cycle progression and further mitotic cell division following exposure to 10 μM NOC-18. Furthermore, we also have shown that there is an increased immunoreactivity against Ki-67 within the first hour of treatment with NOC-18, an event dependent on the activation of ERK1/2.

The p21Ras/ERK/MAPK pathway is rapidly activated by NO, bypassing the EGF receptor, following a short-term exposure to 10 μM NOC-18. However, as shown by flow cytometry experiments, the involvement of the ERK/MAPK signaling pathway in the proliferative effect of NO is also true for a long-term exposure to NOC-18 (24 h). Thus, in Chapter 3 we investigated other pathways that could be involved in the proliferative effect of NO, and, how they work or modulate the ERK/MAPK signaling pathway. We thus studied the possible involvement of the sGC/cGMP/PKG pathway.

We showed that activation of ERK1/2, which occurs rapidly, is cGMP-independent, following short-term exposure to NOC-18. However, we cannot exclude the possibility that cGMP may activate the ERK/MAPK pathway. In fact, the cGMP analog, 8-Br-cGMP, can activate the ERK/MAPK pathway, as shown by Western blotting experiments. Therefore, it should be considered that a gradual and long-term accumulation of cGMP could activate this pathway, thus keeping an increased cell proliferation rate upon exposure to NO for 24. It also should be noted that the NO donor used (NOC-18) is a long-acting NO-donor with an half-life of approximately 22 h (Keefer *et al.*, 1996). In addition, we showed by flow cytometry that both inhibition of sGC or PKG with ODQ or KT5823, respectively, did not prevent the activation of ERK1/2 following short-term (6 h) exposure to NO. Altogether these data reinforce the

hypothesis that short exposure to NO rapidly activates the ERK pathway in a mechanism independent of cGMP.

In contrast, long-term exposure (24h) to NO increased cell proliferation via ERK/MAPK activation in a cGMP-dependent manner. In fact, the data presented in Chapter 3 suggest that a crosstalk between both pathways may exist, and probably cGMP may act to keep ERK/MAPK active during the exposure to NO. This hypothesis is supported by the fact that upon inhibition of guanylyl cyclase or PKG, proliferation induced by NO is completely abolished, and cell proliferation returns to basal levels. If these pathways were signaling for cell cycle progression independently, would expect a partial decrease, but not a complete blockade, of the proliferative effect of NO following inhibition of sCG, PKG or MEK1/2.

Moreover, blockade of the degradation of endogenous cGMP, with the selective PDE5 inhibitor T0156, further enhanced the proliferative effect of NO. The same effect on cell proliferation was observed when the cGMP analog 8-Br-cGMP was used. Recently, it was reported that elevation of cGMP levels by PDE5 inhibition promoted cGMP/PKG activation, enhancing mesenchymal stem cell proliferation (Haider *et al.*, 2010). Other works also correlate elevation of cGMP levels to the enhancement of neurogenesis (Wang *et al.*, 2005; Zhang *et al.*, 2006; Zhang *et al.*, 2006).

Altogether, our results support the notion that the proliferative effect of NO is biphasic. NO can activate two independent pathways, depending on the period of exposure, that act to increase neural stem cell proliferation: the ERK/MAPK and the sGC/cGMP/PKG pathways, with no evidence of crosstalk between them in the early effect of NO, but for the long-term effects the pathways seem to interact. Some authors suggest cGMP involvement in NO-mediated arrest of cell cycle progression, with intermediate activation of PKG and indirect inhibition of Raf-1, and subsequent decreased signaling by the MAPK pathway (Yu *et al.*, 1997; Guo *et al.*, 1998; Costa and Assreuy, 2005). Other studies suggest the cGMP/PKG pathway to be involved in the activation

of the MAPK pathway, particularly ERK1/2 (Zaragoza *et al.*, 2002; Ota *et al.*, 2008).

We also investigated the effect of NO on cell proliferation after injury *in vivo*, using a rodent model of *status epilepticus* in wild-type mice and in mice that lack iNOS (iNOS$^{-/-}$). In Chapter 2, we show evidence that cell proliferation following seizures was greatly increased in the SGZ of wild-type animals but not in iNOS$^{-/-}$ mice. Abolishment of NO production in iNOS$^{-/-}$ mice prevents cell proliferation following seizures *in vivo*. Thus, these results strongly suggest NO is a key modulator of cell proliferation in the brain, following an insult. Moreover, we also investigated the phenotype of proliferating cells and observed increased immunoreactivity for DCX in WT mice, but not in iNOS$^{-/-}$ animals, suggesting that NO from inflammatory origin induces neuronal commitment of neural stem cells in the DG. We also observed increased astrogliosis and microgliosis in KA-treated WT mice, but not in iNOS$^{-/-}$ mice, suggesting that these events are independent of NO. Other authors have already shown that *status epilepticus* triggers proliferation of neural precursors in the SGZ of rodents (Parent *et al.*, 1997; Gray and Sundstrom, 1998; Parent, 2007). In addition, Zhu and colleagues described iNOS as necessary for increased neurogenesis in the DG of mice subjected to other brain insults, such as focal cerebral ischemia (Zhu *et al.*, 2003). These studies and ours support the hypothesis that NO from inflammatory origin favors cell proliferation in the lesioned brain. Other studies have been conducted in which high concentrations of NO-donors were used and anti-proliferative effects of NO were reported in cultured cells (Covacu *et al.*, 2006; Torroglosa *et al.*, 2007), but this probably is not representative of what happens *in vivo*.

In Chapter 4, we studied the antiproliferative effect of high concentrations of NO. Nitric oxide, when present in high concentrations can disrupt the mitochondrial respiratory chain, with subsequent formation of ROS, particularly O_2^-, that is highly reactive with NO, forming peroxynitrite, which is involved in nitration of proteins (Ledo *et al.*, 2004; Monteiro *et al.*, 2008). In

Caco-2 cells, peroxynitrite was shown to cause nitration of the EGFR, with subsequent inhibition of cell proliferation (Uc *et al.*, 2003). We investigated the existence of nitrated proteins in our cell culture models and we observed an increased nitration of the EGF receptor, with a concomitant decrease in cell proliferation, both in neural stem cell cultures treated with NOC-18 (100 μM, 48 h), and in cultures of neural stem cells mixed with microglia wild-type for iNOS activated with LPS plus IFN-γ. Scavenging peroxynitrite or superoxide with MnTBAP or FeMTPyP, reversed the antiproliferative effect of NO or inflammation, and cell proliferation was rescued to basal levels. These results strongly suggest a role of NO in this process, which is further supported by the fact that in mixed cultures of neural stem cells with non-expressing iNOS microglial cells. We did not observe changes neither in the nitration status of the EGFR nor in the proliferation of neural stem cells. Thus, among the mediators of the inflammatory response, NO can be considered as an agent of extreme importance in controlling cell proliferation.

Other proinflammatory agents, such as IL-6, released by activated microglial cells, have been reported to inhibit neurogenesis (Vallieres *et al.*, 2002). Other studies showed that pharmacological blockade of inflammation can restore neurogenesis (Ekdahl *et al.*, 2003; Monje *et al.*, 2003). Taking into consideration the data obtained and described in Chapter 4, it is plausible that the high concentrations of NO reached in the cultures, either in mixed cultures following microglial activation, or in neural stem cell cultures upon incubation with NOC-18, could be achieved locally in the brain, although in the seizure model the effect of NO was to induce proliferation in the subgranular zone.

1.20.1 Future directions

This work contributes for the understanding of the mechanisms that underly the role of NO in adult neural stem cell proliferation, although some aspects remain to be clarified. In fact, it will be interesting to see whether the increased proliferation induced by NO will lead to increased differentiation in

neurons. *In vivo*, we reported an increased proliferation of neuronal cells due to NO from inflammatory origin. It will be of interest to examine the survival of the newborn neurons, as well as their functional integration in the neuronal network. Electrophysiology studies may help clarifying these issues, as well as behavioral studies.

Stimulation of endogenous adult neural stem cells and modulation of injury-induced neurogenesis is presently being considered as a potential therapeutic approach for neuronal repair in neurodegenerative disorders, as opposed to the more invasive approach of transplantation of exogenous stem cells. Understanding how the inflammatory response affects neurogenesis is fundamental to better design therapeutic strategies for safe and efficient regulation of endogenous neurogenesis.

Therefore, understanding how the inflammatory agents modulate cell proliferation and/or differentiation of NSCs is of great usefulness, if their action could be correctly targeted and controlled, for instance with selective drugs for the agent of interest. NO has been drawing the attention of pharmaceutical companies in the latest years. Indeed, several non-steroidal anti-inflammatory NO-releasing drugs (NO-NSAID) are currently under investigation and were shown to be beneficial as neuroprotectants in models of several neurodegenerative conditions accompanied by inflammation (Keeble and Moore, 2002; Napoli and Ignarro, 2003). As an alternative to conventional NSAIDs with significant side effects (mainly in the gastrointestinal tract), pharmacologically improved and therapeutically enhanced NO releasing non-steroidal anti-inflammatory drugs with fewer side effects are being developed (Koc and Kucukguzel, 2009).

Chapter 6

Conclusions

1.21 Conclusions

The work presented in this thesis allowed us to draw the following main conclusions:

- Nitric oxide can have opposite effects on the proliferation of neural stem cells, either promoting or inhibiting neural stem cell proliferation, depending on the concentration and duration of exposure.
- The proliferative effect of NO is biphasic (Fig. 6.1). NO can activate two independent pathways, depending on the period of exposure: the ERK/MAPK and the sGC/cGMP/PKG pathways.
- cGMP and PKG are involved in the late proliferative effect triggered by NO. Although cGMP and PKG were not involved in the early activation of ERK1/2, they were mandatory for cell proliferation following long-term exposure to NO.
- NO rapidly activates the ERK/MAPK pathway, and the proliferative effect observed is due to the activation of p21Ras, bypassing the activation of the EGF receptor. As a result, activation of p90RSK, Elk-1 and c-Myc increases, and the nuclear levels of the cyclin-dependent kinase inhibitor $p27^{KIP1}$ decrease, which allows cell division. This pathway is active for all the stages analyzed – short-term (6 h) and long-term exposure (24 h) to NO.
- *In vivo*, nitric oxide from iNOS origin promotes cell proliferation and neuronal commitment, following an inflammatory response.
- High levels of NO, exogenously added or from microglial origin, have an antiproliferative effect in neural stem cells, mediated by nitration of the EGF receptor, which is concomitant with decreased phosphorylation of this receptor, thus preventing regular signaling.

In conclusion, we show for the first time the mechanisms underlying the dual effect of nitric oxide in the proliferation of adult neural stem cells:

I. a proliferative effect mediated by the early activation of ERK/MAPK pathway and by and later by the activation of sGC/cGMP/PKG pathway;

II. an antiproliferative effect, mediated by peroxynitrite formation, which causes nitration of the EGF receptor thus preventing regular signaling.

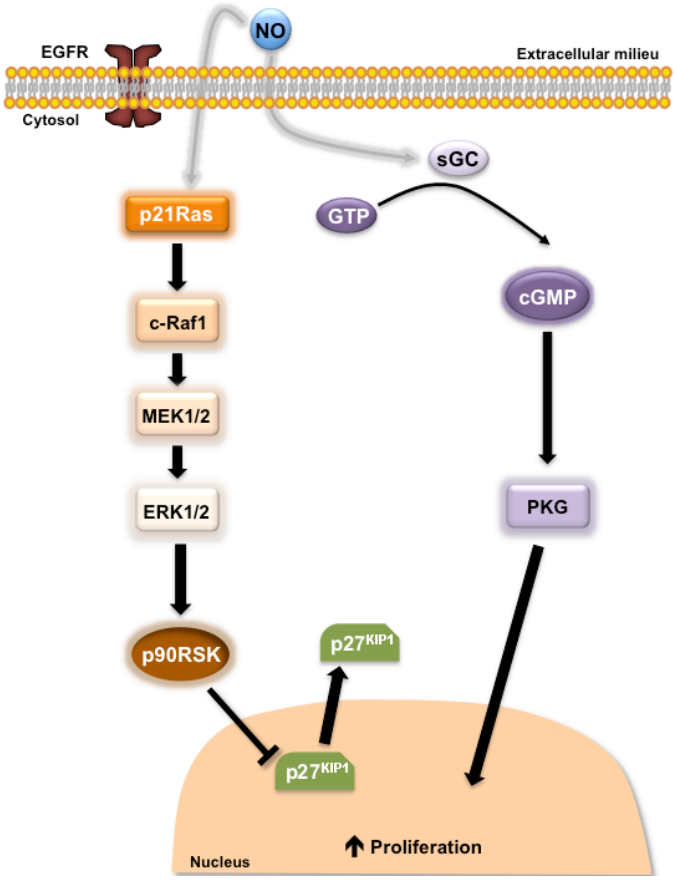


Figure 6.1. The proliferative effect of NO is biphasic. NO rapidly activates p21Ras, bypassing the EGF receptor, resulting in the activation of ERK1/2. Upon translocation to the nucleus, ERK1/2 phosphorylates kinases like p90RSK, which in turn triggers a decrease in the levels of p27Kip1. Upon phosphorylation, p27Kip1 is translocated to the cytosol. Loss of p27Kip1 results in increased cell proliferation. cGMP and PKG are also involved in the proliferative effect induced by long-term exposure to NO.

Chapter 7

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1.22 References

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