UNIVERSIDADE DO ALGARVE
DEPARTAMENTO DE CIÊNCIAS BIOMÉDICAS E MEDICINA

IDENTIFICATION OF S-NITROSYLATED PROTEINS IN INJURY-INDUCED NEUROGENESIS

Ana Sofia Maximiano Leitão Ribeiro Lourenço

Tese para obtenção do Grau de Doutor em Ciências Biomédicas

Trabalho efectuado sob a orientação da

Professora Doutora Inês Maria Pombinho de Araújo

2018

IDENTIFICATION OF S-NITROSYLATED PROTEINS IN INJURY-INDUCED NEUROGENESIS

Declaração de autoria de trabalho

Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

Agradecimentos

A realização deste trabalho só foi possível devido à ajuda e colaboração de várias pessoas, às quais não posso deixar de agradecer.

Em primeiro lugar, à minha orientadora, a Professora Doutora Inês Araújo, pela oportunidade concedida e por todos os ensinamentos e conhecimentos partilhados ao longo destes anos.

Ao Centro de Neurociências e Biologia Celular (CNC), Centro de Biomedicina Regenerativa (CBMR) e ao Programa Doutoral em Ciências Biomédicas pela oportunidade, e à Fundação para a Ciência e a Tecnologia pelo financiamento.

À Professora Doutora Caetana Carvalho e Professor Doutor Eduardo Melo pelo acompanhamento e sugestões a este trabalho.

Ao Professor Doutor Paulo Martel pela disponibilidade e colaboração na análise e representação da estrutura tridimensional da PEBP-1.

Ao Professor Doutor Fred Gage por ter cedido as células estaminais de hipocampo.

A todos os colegas de laboratório ao longo deste tempo. Bruno e Inês por tudo o que me ensinaram no laboratório. Ana e Vanessa, por todos os momentos que passámos e tudo o que aprendemos e partilhámos quando embarcámos juntas nesta aventura. Ana, obrigada por confiares que eu poderia dar continuação ao teu trabalho. Vanessa, obrigada por estares presente e me ajudares nos momentos difíceis a manter a calma. À Daniela, Dorinda e Sónia que também participaram neste projecto. Sónia, obrigada pela companhia e os bons momentos, e também pelos conhecimentos partilhados. À Joana pela boa disposição e companheirismo.

A todos os outros colegas que de algum modo me ajudaram, nem que fosse com uma palavra amiga; vocês são muitos, mas sabem quem são.

A todos os técnicos do CNC e CBMR que de algum modo contribuíram. Pela possibilidade de utilização das unidades de cultura celular (obrigada Ana Luísa), microscopia (obrigada Cláudia) e biotério.

Aos meus amigos e à minha família, que sempre acreditaram em mim e me apoiaram e a quem devo tudo.

Abstract

In the adult mammalian brain, new neurons can be generated (adult neurogenesis) due to the presence of neural stem cells (NSC). Adult neurogenesis can be affected by several factors and is particularly altered in pathological conditions, which can increase NSC proliferation, migration and differentiation into neurons. However, neurogenesis after brain injury is not efficient due to low survival of new neurons. Brain insults trigger neuroinflammation with activation of microglia, which release several molecules, such as nitric oxide (NO). NO from inflammatory origin enhances adult neurogenesis through ERK/MAPK pathway signaling, but the exact mechanism is unknown. NO can directly modify protein function through S-nitrosylation, the addition of S-nitrosothiol to a cysteine thiol group. In this work, we aimed to validate new putative targets of S-nitrosylation by NO in NSC that are involved in the ERK/MAPK pathway, which were recently identified by our group. Here, we describe the S-nitrosylation of these new targets in conditions of increased post-injury neurogenesis mediated by NO and study in more detail phosphatidylethanolamine binding protein 1 (PEBP-1). PEBP-1 inhibits the ERK/MAPK pathway by binding to c-Raf and is the most promising target for neurogenesis. We show that S-nitrosylation of cysteine 133 of PEBP-1 is necessary for ERK phosphorylation induced by NO in NSC, which can be a mechanism involved in the release of PEBP-1 inhibitory function in the ERK/MAPK pathway. Moreover, in a model of post-injury neurogenesis we show that 14-3-3, 14-3-3 ε, hnRNP K and PEBP-1 are transiently S-nitrosylated following seizures, according to their role in the ERK/MAPK pathway, preceding the onset of proliferation of NSC. Overall, our data shows that these proteins may be important for regulation of post-injury neurogenesis and suggests that they could be good candidates for regulation, in order to enhance NSC proliferation and neuronal replacement efficiency following brain injury.

Keywords: Adult neurogenesis, nitric oxide, S-nitrosylation, neural stem cells, brain injury, hippocampus

Resumo

No cérebro adulto dos mamíferos, são produzidos novos neurónios (neurogénese) devido à presença de células estaminais neurais (NSC). A neurogénese pode ser afectada por diversos factores e está particularmente alterada em condições patológicas, o que pode aumentar a proliferação de NSC, migração e diferenciação em neurónios. No entanto, a neurogénese após lesão cerebral não é eficiente devido à baixa sobrevivência dos novos neurónios. Diversas lesões cerebrais desencadeiam neuroinflamação com activação da microglia, que liberta diversas moléculas, tal como o óxido nítrico (NO). O NO de origem inflamatória melhora a neurogénese adulta através de sinalização pela via da ERK/MAPK, mas o mecanismo exacto é desconhecido. O NO pode modificar directamente a função de proteínas através de S-nitrosilação, pela adição de S-nitrosotiol ao grupo tiol de uma cisteína. Neste trabalho, tivemos como objectivo validar novos possíveis alvos de S-nitrosilação pelo NO envolvidos na via da ERK/MAPK em NSC, e que foram recentemente identificados pelo nosso grupo. Aqui, descrevemos a S-nitrosilação destes novos alvos em condições de neurogénese pós-lesão aumentada pelo NO e estudamos em mais detalhe a proteína de ligação a fosfatidiletanolamina 1 (PEBP-1). A PEBP-1 inibe a via da ERK/MAPK ao ligar-se à c-Raf e é o alvo mais promissor para a neurogénese. Mostramos que a S-nitrosilação da cisteína 133 da PEBP-1 é necessária para a fosforilação da ERK induzida pelo NO em NSC, o que pode ser um mecanismo envolvido em ultrapassar a função inibitória da PEBP-1 na via da ERK/MAPK. Além disso, mostramos que num modelo de neurogénese pós-lesão as proteínas 14-3-3, 14-3-3 ε, hnRNP K e PEBP-1 estão S-nitrosiladas de forma transiente após convulsões, de acordo com a sua função na via da ERK/MAPK, e precedendo o início da proliferação de NSC. No geral, este trabalho mostra que estas proteínas poderão ser importantes para a regulação da neurogénese pós-lesão e sugere que possam ser boas candidatas para serem reguladas, de forma a melhorar a proliferação de NSC e a eficiência da substituição neuronal após lesão cerebral.

Palavras chave: Neurogénese, óxido nítrico, S-nitrosilação, células estaminais neurais, lesão cerebral, hipocampo

Table of contents

Abbreviations

ANOVA - analysis of variance
BDNF - brain-derived neurotrophic factor
bFGF - basic fibroblast growth factor
BSA - bovine serum albumin
cAMP - cyclic adenosine monophosphate
cGMP - cyclic guanosine monophosphate
CREB - cAMP response element-binding
CysNO - S-nitrosocysteine
DEA/NO - diethylamine NONOate
DG - dentate gyrus
D-MEM/F-12 - Dulbecco's modified eagle medium: nutrient mixture F-12
DTT - dithiothreitol
EDTA - ethylenediaminetetraacetic acid
EdU - 5-ethynyl-2'-deoxyuridine
EF - elongation factor
EGF - epidermal growth factor
EGFR - epidermal growth factor receptor
eNOS - endothelial nitric oxide synthase
ERK - extracellular signal-regulated kinase
GRK2 - G protein-coupled receptor kinase 2
HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hnRNP K - heterogeneous nuclear ribonucleoprotein K
IGF-1 - insulin-like growth factor 1
iNOS - inducible nitric oxide synthase
KA - kainic acid
KO - Knockout
MAPK - mitogen-activated protein kinase
MEK - mitogen-activated protein kinase kinase
NF-κB - Nuclear factor kappa-light-chain-enhancer of activated B cells
nNOS - neuronal nitric oxide synthase
NOC-18 - DETA NONOate
NOS - nitric oxide synthase
NSC - neural stem cells

OB - olfactory bulb

PBS - phosphate-buffered saline

PCNA - proliferating cell antigen

PEBP-1 - phosphatidylethanolamine-binding protein 1

P-ERK - phospho-ERK

PenStrep - Penicillin/Streptomycin

PKC - protein kinase C

PKG - cGMP-dependent protein kinases

PTM - post-translational modification

PVDF - polyvinylidene difluoride

RMS - rostral migratory stream

RT - room temperature

SDS - sodium dodecyl sulfate

Ser - serine

sGC - soluble guanylyl cyclase

SGZ - subgranular zone

SVZ - subventricular zone

TBI - traumatic brain injury

TBS-T - Tris-buffered saline with tween 20

VEGF - vascular endothelial growth factor

Wnt - Wingless-type

WT - wild type

Chapter 1. General introduction

Chapter 1

Chapter 1

1.1. Adult neurogenesis

The formation of new neurons (neurogenesis) in the brain was thought for a long time to only occur during embryogenesis. Ramón y Cajal in 1894 described for the first time the complex structure of brain neuronal networks and speculated that they were fixed and irreplaceable. This led to the idea that, contrary to other tissues, there was no way to replace neurons that are lost in the adult brain due to injury or aging. However, in 1965, Altman and Das observed proliferation of cells in rat hippocampus (Altman & Das 1965), contradicting the previous established paradigm. Since then, adult neurogenesis has been an intense subject of investigation and, nowadays, it is well established that there is formation of new neurons during the adult life of mammals, both in physiological and pathological conditions.

Neurogenesis in the adult occurs to maintain brain structure and plasticity, and can increase in response to harmful stimuli. It is present in different animal species, ranging from invertebrates (Tanaka & Reddien 2011, Koizumi & Bode 1991, Fernandez-Hernandez *et al.* 2013) to non-mammalian (Chapouton *et al.* 2007, Alunni & Bally-Cuif 2016) and mammalian (Brus *et al.* 2013) vertebrates. With the discovery of adult neurogenesis in humans (Eriksson *et al.* 1998) emerged the possibility of brain regeneration taking advantage of neural stem cells (NSC) for transplantation or enhancement of endogenous neurogenesis, in situations detrimental to normal brain functioning. Neurogenesis is a complex process, comprising proliferation, migration, differentiation and survival of the newly formed neurons, with functional integration in the existing neuronal circuits. Despite its importance, neurogenesis in mammals is restricted to certain areas of the adult brain, due to the presence of pools of NSC in specific regions. In the subventricular zone (SVZ) and in the hippocampus, new neurons are generated during adulthood. Neurogenesis in the SVZ is important for replacement of neurons of the olfactory bulb (OB), which is crucial for odor discrimination and processing of sensory information. Unlike several species, in humans there are few new OB neurons originating from neurogenesis in the SVZ, and OB neurogenesis seems to have little relevance (Bergmann *et al.* 2012). Interestingly, humans present more new neurons in the adult striatum than other mammals, but it is still elusive if these new neurons originate in the SVZ (Ernst *et al.* 2014). Hippocampal neurogenesis is critical for learning, memory and cognition and is conserved across mammals, including in humans (Gould *et al.* 1999, Spalding *et al.* 2013).

1.1.1. Neurogenic niches

In the adult mammalian brain, the SVZ that lines the lateral walls of the lateral ventricles (Fig. 1.1A) and the subgranular zone (SGZ) of the dentate gyrus (DG) of the hippocampus (Fig. 1.1D) are the main regions that contain NSC. These cells have the ability of self-renewal, slowly proliferating by either symmetric or asymmetric divisions (Morrison & Kimble 2006), and are multipotent, having the ability to originate neurons, astrocytes and oligodendrocytes (Lois & Alvarez-Buylla 1993).

In the SVZ, ependymal cells line the ventricles and separate the SVZ from the cerebrospinal fluid (Fig. 1.1B). NSC can contact the cerebrospinal fluid extending an apical process between rosettes of ciliated ependymal cells (Lim & Alvarez-Buylla 2016). Blood vessels also provide the necessary nutritional support to NSC, as well as access to regulatory and signaling molecules (Mercier *et al.* 2002). NSC (B cells) give rise to transient amplifying progenitors (C cells), rapidly proliferative cells with limited self-renewal capacity, which in turn originate neuroblasts (A cells, immature neurons) (Doetsch *et al.* 1997). Neuroblasts migrate long distances in chains along the rostral migratory stream (RMS) towards the OB (Lois & Alvarez-Buylla 1994, Lois *et al.* 1996), where they differentiate into granular and periglomerular neurons and integrate in the existing neuronal circuits as inhibitory interneurons (Alvarez-Buylla & Garcia-Verdugo 2002). Additional paths of migration have been described in mouse brain, with neuroblasts derived from the SVZ migrating towards forebrain regions such as cortex, striatum, and nucleus accumbens (Inta *et al.* 2008). In humans, neurogenesis in the OB is limited and the presence of a RMS is controversial (Curtis *et al.* 2007, Sanai *et al.* 2011), but neuroblasts derived from the SVZ may migrate to striatum (Ernst et al. 2014).

The SGZ (Fig. 1.1E), located between the hilus and the granule cell layer of the hippocampus, is also a vascularized region (Palmer *et al.* 2000). There, radial glia-like cells (type-1 cells) proliferate and originate transient amplifying progenitors (type-2a and 2b cells) (Kempermann *et al.* 2004), which proliferate rapidly and give rise to neuroblasts (type-3 cells) (Doetsch 2003). Neuroblasts migrate short distances towards the granule cell layer of the DG and differentiate into functional granular neurons that extend their axons into the CA3 region (van Praag *et al.* 2002).

In the neurogenic niches, NSC are maintained in a stem state due to the presence of several factors and interaction with different cells. NSC transplanted to different regions of the brain differentiate into neurons or glia depending on the transplanted site, and regardless of the origin of NSC (Shihabuddin *et al.* 2000, Seidenfaden *et al.* 2006). Therefore, environmental cues are necessary for the maintenance of NSC properties and fate commitment. The different types of cells present in the niches can be distinguished and identified according to specific cell markers (Fig. 1.1C,F). NSC are

from a glial lineage (Kriegstein & Alvarez-Buylla 2009) and therefore express glial fibrillary acidic protein, which is absent in neural progenitor cells that in turn express sex determining region Y-box 2 (Sox2) and nestin (both also present in NSC) (Filippov *et al.* 2003, Zhang & Jiao 2015). Upon commitment towards a neuronal fate, neuroblasts express doublecortin, but not nestin (Kronenberg *et al.* 2003). Fully mature neurons can be identified by expression of Neuronal nuclei (NeuN) and β-III-tubulin. Neurogenesis and its regulation can be studied using cultures of NSC. They can be isolated and cultured *in vitro* both as an adherent monolayer or as floating aggregates (neurospheres), being maintained as multipotent by epidermal growth factor (EGF) and/or basic fibroblast growth factor (bFGF) (Weiss *et al.* 1996, Kuhn *et al.* 1997).

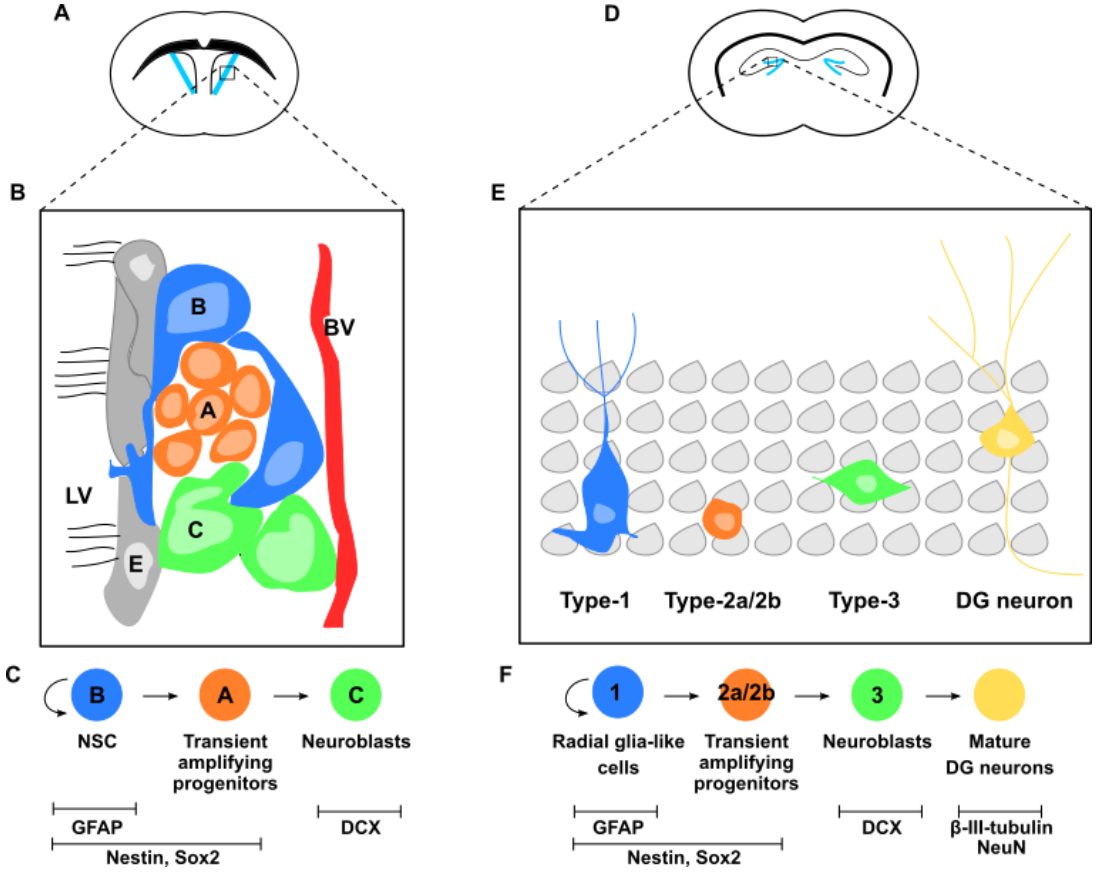


Figure 1.1 - Neurogenic niches in the adult mammalian brain. Representation of the neurogenic niches of the SVZ **(A-C)** and the SGZ of the DG **(D-F)**. The SVZ lines the lateral wall of the lateral ventricles **(A),** where NSC (B cells) contact the cerebrospinal fluid through ciliated ependymal cells (E) and give rise to highly proliferative transient amplifying progenitors (A cells), which in turn originate neuroblasts (C cells) **(B).** In the SGZ of the DG of the hippocampus **(D)**, radial glia-like cells (type-1) give rise to transient amplifying progenitors (type-2a and 2b), which originate neuroblasts (type-3) that migrate and differentiate into DG neurons **(E).** The different types of cells can be distinguished by expression of different markers **(C,F).** LV, lateral ventricle; BV, blood vessel; GFAP, glial fibrillary acidic protein; Sox2, sex determining region Y-box 2; DCX, doublecortin; NeuN, Neuronal nuclei.

1.1.2. Regulation of neurogenesis

Neurogenesis is a highly-regulated process, being affected by several components of the neurogenic niches, such as intrinsic factors of NSC, immune cells, extracellular matrix components, vascular system and signaling pathways (Christie & Turnley 2012). EGF promotes NSC proliferation in the SVZ and glial differentiation in both the SVZ and SGZ (Kuhn et al. 1997). bFGF increases NSC proliferation and neuronal differentiation in the SVZ (Kuhn et al. 1997), and is necessary for neurogenesis in the SGZ (Kang & Hebert 2015). Insulin-like growth factor 1 (IGF-1) promotes migration of neuroblasts to the OB and neuronal differentiation (McCurdy *et al.* 2005, Hurtado-Chong *et al.* 2009) and, in the SGZ, it enhances NSC proliferation and increases cell survival (Aberg *et al.* 2000, Lichtenwalner *et al.* 2006). Notch signaling is important for the self-renewal capacity and maintenance of NSC pool in the SVZ (Imayoshi *et al.* 2010). In the SGZ, Notch is necessary for neurogenesis, affecting proliferation, differentiation and survival of new neurons (Breunig *et al.* 2007). Sonic hedgehog signaling is necessary for maintenance of NSC pool in both the SVZ and SGZ (Machold *et al.* 2003) and migration of neuroblasts in the SVZ (Balordi & Fishell 2007). Canonical Wingless-type (Wnt) signaling in the SVZ increases NSC proliferation (Adachi *et al.* 2007) and post-injury neurogenesis (Shruster *et al.* 2012), while in the SGZ it is important for maintenance of NSC pool (Wexler *et al.* 2009) and for increase in neurogenesis (Lie *et al.* 2005). Non-canonical Wnt signaling influences maturation of new neurons in the OB (Pino *et al.* 2011) and DG (Varela-Nallar *et al.* 2010). Bone morphogenetic protein signaling in the SVZ decreases neurogenesis by increasing glial differentiation, which is prevented by Noggin (Lim *et al.* 2000). Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling is necessary for differentiation of new hippocampal neurons (Rolls *et al.* 2007).

Factors outside the niches also regulate neurogenesis. Hormonal differences between males and females affect neurogenesis. In female rats, estrogen during proestrous increases proliferation of NSC and enhances neurogenesis in the hippocampus (Tanapat *et al.* 1999). Pregnancy hormones also increase proliferation of NSC, in mouse and rat SVZ (Shingo *et al.* 2003, Furuta & Bridges 2005). Testosterone affects neurogenesis, so male castration of certain mouse strains leads to increase in proliferating cells in the SVZ (Tatar *et al.* 2013).

Physical exercise, particularly running, enhances hippocampal neurogenesis (van Praag *et al.* 1999b, Naylor *et al.* 2008, Bednarczyk *et al.* 2011) and improves cognition (van Praag *et al.* 1999a). This effect is mediated by increase in brain-derived neurotrophic factor (BDNF) (Farmer *et al.* 2004) and presence of serotonin (Klempin *et al.* 2013). Exercise induces angiogenesis (Black *et al.* 1990, Swain *et al.* 2003), releasing factors

that also increase neurogenesis, such as vascular endothelial growth factor (VEGF) (Jin *et al.* 2002).

Environmental enrichment is the improvement in housing conditions of laboratory animals, to increase cognitive stimulation (Baumans & Van Loo 2013). Some of the strategies used are bigger cages, new objects and social interaction by contacting with other animals. Environmental enrichment leads to increased survival of mice and rat hippocampal newborn granular neurons (Kempermann *et al.* 1997, Kempermann *et al.* 2002, Segovia *et al.* 2006). Animals with more neurogenesis also display better performance in cognitive tests that evaluate spatial memory, highlighting the association between the amount of new neurons and ability to learn (Sisti *et al.* 2007). This cognitive stimulation in adulthood helps prevent a decline in neurogenesis with aging, by maintaining the pool of NSC (reviewed in Kempermann 2008). Environmental enrichment also increases the number of neuronal branches and dendritic spines (Rampon *et al.* 2000), which improves synaptic activity. These effects are mediated by an increase in transcription factors, such as cyclic adenosine monophosphate (cAMP) response element-binding (CREB) (Williams *et al.* 2001), in proteins involved in synaptogenesis (Frick & Fernandez 2003, Nithianantharajah *et al.* 2004) and in neurotrophic factors, such as BDNF (Zhang *et al.* 2016). Running wheels are usually a part of environmental enrichment, so some studies evaluated the contribution of exercise for the effect in neurogenesis observed with environmental enrichment, concluding that increase in BDNF and proliferation of hippocampal NSC results mainly from physical exercise (Kobilo *et al.* 2011, Bechara & Kelly 2013). Therefore, physical exercise is responsible for increasing the proliferation of NSC, and environmental enrichment is responsible for increasing the survival of new neurons (reviewed in Olson *et al.* 2006). Environmental enrichment is able to reduce stress in mice (Olsson *et al.* 1994, Sztainberg *et al.* 2010). Chronic stress impairs hippocampal neurogenesis, due to increase in glucocorticoids that inhibit the proliferation of NSC and/or differentiation into neurons (Ridder *et al.* 2005, Kronenberg *et al.* 2009, Hodes *et al.* 2012, Anacker *et al.* 2013). High levels of glucocorticoids also lead to decreased levels of BDNF (Smith *et al.* 1995, Gourley *et al.* 2009). Astrocytes are important for neurogenesis (Song *et al.* 2002, Sultan *et al.* 2015) and may have a major role in modulating neurogenesis in conditions of stress (reviewed in Luarte *et al.* 2017).

Aging impairs all steps of neurogenesis. In aged brains, NSC enter a more quiescent state, which leads to a decrease in proliferating NSC both in the SVZ and SGZ (Bouab *et al.* 2011, Lugert *et al.* 2010). Fate specification is also altered with aging, resulting in increased glial differentiation and decreased neuronal differentiation (Encinas *et al.* 2011). The environment of neurogenic niches is a main contributor to the differences

observed in neurogenesis of young and aged mice (Villeda *et al.* 2011, Katsimpardi *et al.* 2014). Alterations in Wnt signaling (Piccin *et al.* 2014), and in the factors IGF-1, bFGF, VEGF (Shetty *et al.* 2005) and EGF (Enwere *et al.* 2004) have been described to play a role in decreasing neurogenesis in the aged brain. In humans, neurogenesis in the SVZ declines earlier (Sanai et al. 2011) than hippocampal neurogenesis (Spalding et al. 2013).

1.1.3. Neurogenesis in pathological conditions

Adult neurogenesis is affected in pathological conditions, such as stroke, traumatic brain injury (TBI), epilepsy and neurodegenerative diseases. In animal models of both transient global brain ischemia and focal brain ischemia, neurogenesis is increased in the SVZ (Tonchev *et al.* 2005, Jin *et al.* 2001) and SGZ (Kee *et al.* 2001, Arvidsson *et al.* 2001). Proliferation of NSC is increased following ischemic stroke (Takagi *et al.* 1999), and cultured NSC derived from ischemic brain show more proliferative capacity and differentiation into neurons (Deierborg *et al.* 2010). Moreover, neuroblasts derived from proliferation of NSC in the SVZ switch their migration route from the RMS towards the site of lesion in the striatum (Arvidsson *et al.* 2002, Zhang *et al.* 2004). There is also increased angiogenesis in the SVZ (Zhang *et al.* 2014) and in the dorsomedial striatum near the SVZ, so the new blood vessels provide a scaffold for migration of new neuroblasts (Thored *et al.* 2007), like in the RMS in physiological conditions. Factors of the neurogenic niches, such as bFGF (Lin *et al.* 1997) and IGF-1 (Yan *et al.* 2006) increase in the ischemic cortex following stroke, and their absence prevents enhancement of neurogenesis (Yoshimura *et al.* 2001, Yan et al. 2006). Despite the enhancement of neurogenesis, in ischemic stroke conditions the new neurons have low long-term survival (Arvidsson et al. 2002, Thored *et al.* 2006), and aberrant morphologies that lead to maladaptive integration in the existing circuits and memory impairment (Niv *et al.* 2012, Woitke *et al.* 2017).

In animal models of TBI, there is increase in NSC proliferation in the SVZ and SGZ (Chirumamilla *et al.* 2002, Gao *et al.* 2009). Moreover, there is enhancement of neuronal differentiation in some models (Dash *et al.* 2001, Rice *et al.* 2003, Villasana *et al.* 2014), whereas in others there is only increase in glial differentiation (Bye *et al.* 2011, Gao & Chen 2013), which can be modulated by age (Sun *et al.* 2005). VEGF mediates increased hippocampal neurogenesis following TBI, by promoting survival of the new granule neurons (Lee & Agoston 2010). In the SVZ, Eph receptor B3, which is necessary for negative regulation of cell proliferation and survival by EphrinB3 (Ricard *et al.* 2006), is transiently reduced following TBI and allows proliferation and survival of NSC (Theus

et al. 2010). Other factors involved in the increase in neurogenesis and survival of new neurons following TBI are bFGF (Sun *et al.* 2009b), EGF (Sun *et al.* 2010), IGF-1 (Carlson *et al.* 2014) and BDNF (Gao & Chen 2009).

Epilepsy is characterized by spontaneous recurrent seizures, which can disrupt adult neurogenesis. Several days following seizures, NSC proliferation is increased both in the SVZ (Parent *et al.* 2002) and SGZ (Gray & Sundstrom 1998), returning to physiological levels after a few weeks (Parent *et al.* 1997, Bonde *et al.* 2006). Most newborn cells differentiate into granule neurons (Jessberger *et al.* 2007). Notch (Sibbe *et al.* 2012), Sonic hedgehog (Banerjee *et al.* 2005) and Wnt (Jang *et al.* 2013) signaling pathways are involved in increasing neurogenesis after seizures. Expression of factors such as BDNF (Isackson *et al.* 1991) and VEGF (Newton *et al.* 2003) are altered after seizures and may also affect proliferation of NSC. Many hippocampal granule neurons born after seizures present abnormal morphologies, presenting hilar basal dendrites (Ribak *et al.* 2000, Dashtipour *et al.* 2003, Shapiro *et al.* 2005) and mossy fiber sprouting (Kron *et al.* 2010). Changes in the glial scaffold may be involved in this process (Shapiro et al. 2005). Nevertheless, functional integration occurs, with establishment of recurrent excitatory networks, of the new abnormal hippocampal granule neurons (Jessberger et al. 2007), and seems to be accelerated by seizures (Overstreet-Wadiche *et al.* 2006). Migration is also altered after seizures. In the SVZ, some neuroblasts leave the RMS towards non-olfactory forebrain regions, where they are not able to survive (Parent et al. 2002). In the DG, some granule cells migrate ectopically to the hilus and the border hilus/CA3 and integrate the existing circuits (Parent et al. 1997). However, this integration is abnormal and the new ectopic granule neurons burst in synchrony with CA3 pyramidal cells (Scharfman *et al.* 2000). Loss of reelin signaling (Gong *et al.* 2007) and excessive mechanistic target of rapamycin (mTOR) signaling (Pun *et al.* 2012) may be involved in this abnormal migratory behavior. Despite the initial increase in neurogenesis, the potential for neurogenesis is decreased several weeks following seizures (Hattiangady *et al.* 2004, Kralic *et al.* 2005), due to a shift to symmetrical divisions, differentiation into reactive astrocytes and depletion of NSC pool (Sierra *et al.* 2015).

In neurodegenerative diseases such as Alzheimer's disease, Huntington's disease and Parkinson's disease neurogenesis is also altered. Studies with animal models of Alzheimer's disease show contradictory effects on neurogenesis, due to a large variability of factors between studied models. A single mutation of amyloid precursor protein transgene results in decreased neurogenesis (Donovan *et al.* 2006), while double and triple mutations lead to increased proliferation and survival of new neurons (Haughey *et al.* 2002, Mirochnic *et al.* 2009). A triple transgenic mouse model, containing

mutation of amyloid precursor protein, presenilin1 and tau genes, also results in impaired neurogenesis, associated with presence of β-amyloid plaques (Rodriguez *et al.* 2008). Imbalance between hippocampal GABAergic and glutamatergic neurotransmission caused by β-amyloid leads to decrease in neurogenesis (Sun *et al.* 2009a); and apolipoprotein E4, a risk factor for developing Alzheimer's disease, also has a detrimental effect in neurogenesis (Li *et al.* 2009). In animal models of Huntington's disease, NSC proliferation in the SGZ is decreased (Lazic *et al.* 2004, Gil *et al.* 2005), and there are less neuroblasts and immature neurons (Fedele *et al.* 2011). Quiescence of NSC pool is increased, CREB signaling is decreased and transforming growth factor-β signaling seems to be involved in modulating neurogenesis (Kandasamy *et al.* 2010). In the SVZ, proliferation of NSC is maintained, but there are less new OB neurons (Phillips *et al.* 2005, Kohl *et al.* 2010). Local environment seems to be critical for survival of new neurons in the striatum, with contribution of BDNF and Noggin (Cho *et al.* 2007). In animal models of Parkinson's disease, neurogenesis is decreased in the SVZ and DG due to presence of α-synuclein. NSC proliferation is impaired in some models (Crews *et al.* 2008, Kohl *et al.* 2012), while in others there is decrease in survival and integration of new neurons (Winner *et al.* 2004, Nuber *et al.* 2008, Winner *et al.* 2012). Alterations in CREB (Winner et al. 2012), BDNF and glial cell-derived neurotrophic factor (Kohl et al. 2012) may contribute to the impairment of neurogenesis observed in Parkinson's disease.

1.1.3.1. Neuroinflammation

A common factor in brain pathological conditions is neuroinflammation. Inflammation in the brain is characterized by activation of microglia, the main immune cells of the brain, release of inflammatory cytokines and disruption of the blood brain barrier, further exacerbating the inflammatory response (Russo *et al.* 2011). In physiological conditions, microglia present a ramified morphology and are continuously monitoring the central nervous system environment (Nimmerjahn *et al.* 2005). Upon harmful stimuli, microglia become activated and release high amounts of free radicals and cytokines, which can be either pro or anti-inflammatory. When activated by lipopolysaccharide, microglia release reactive oxygen and nitrogen species, such as nitric oxide (NO), and pro-inflammatory cytokines, such as interleukin (IL)-1β, IL-6 and tumor necrosis factor-α, in order to protect the organism from foreign pathogens. However, this pro-inflammatory activation also affects healthy neurons. Another way of microglial activation involves supporting tissue repair and angiogenesis, by releasing anti-inflammatory cytokines, such as IL-4, IL-10 and transforming growth factor-β. The balance between these two

types of activation is critical for the outcome of the inflammatory process and differently affects neurogenesis (reviewed in Ekdahl 2012). Acute neuroinflammation appears to be detrimental to neurogenesis. Inflammation caused by activation of microglia with lipopolysaccharide impairs neurogenesis in rat DG both in normal and injured brain due to pro-inflammatory cytokines, such as IL-6 (Ekdahl *et al.* 2003, Monje *et al.* 2003). This effect results from decreased survival of new neurons, and can be prevented using minocycline, which suppresses microglia activation, or indomethacin, a non-steroidal inflammatory drug (Ekdahl et al. 2003, Monje et al. 2003). Survival of new neurons in the OB is also impaired by microglial activation, and can be rescued using minocycline (Lazarini *et al.* 2012). In a model of stroke, inflammation alters proliferation of NSC from the SVZ and survival of new neurons, which can be prevented by treatment with indomethacin (Hoehn *et al.* 2005). On the other hand, activation of microglia by low levels of interferon-γ stimulates differentiation of NSC into neurons (Butovsky *et al.* 2006). Inflammation can also be important for functional integration of new neurons in the hippocampus (Jakubs *et al.* 2008).

The presence of neuroinflammation in pathological conditions that increase neurogenesis may also decrease the survival of new neurons and lead to low efficiency of neuronal replacement. It is necessary to understand how cells in different stages of neuronal formation/differentiation are affected by inflammatory factors. Strategies to overcome this problem are needed, in order to enhance endogenous neurogenesis for brain repair. Enhancing the proliferation of NSC, the amount of potential new neurons that can survive and functionally integrate in the neuronal circuits increases. Of the molecules released by microglia during inflammation, NO has emerged as an important regulator of neurogenesis, so its role during this process deserves further investigation.

1.2. Nitric oxide

1.2.1. Biology of NO

NO is a free radical that is synthesized by the enzyme nitric oxide synthase (NOS) from the conversion of L-arginine into L-citrulline (Fig. 1.2). There are three isoforms of NOS. Neuronal NOS (nNOS), mainly present in neurons, and endothelial NOS (eNOS), mainly present in endothelial cells, are constitutively expressed and are regulated by calcium levels and binding of calmodulin (Alderton *et al.* 2001). In the brain, inducible NOS (iNOS) expression in microglia and astrocytes is triggered by insults to the central nervous system and its regulation is independent of calcium-calmodulin, being active once expressed (Alderton et al. 2001). nNOS and eNOS release low levels of NO (picomolar to nanomolar range), while iNOS releases higher amounts of NO (micromolar

range). Levels of NO released and the kinetics of NO synthesis and consumption *in vivo* and *in vitro* are critical to the outcome of the biological response induced by NO (Hall & Garthwaite 2009).

The use of NO donors, carriers of NO that release it over time, is a common strategy to induce NO release in *in vitro* studies. In this work, three NO donors were used to release NO. Diazeniumdiolates (NONOates) spontaneously decompose in solution, at physiological temperature and pH (Morley & Keefer 1993). There are several NONOates available, with different half-lives. Diethylamine NONOate (DEA/NO) has a short half-life of 2 min at 37°C, while the half-life of DETA NONOate (NOC-18) is 20 h at 37°C (Fitzhugh & Keefer 2000). S-nitrosothiols, such as S-nitrosocysteine (CysNO), contain a single chemical bond between a thiol group (R-SH) and the NO moiety (Zhang & Hogg 2005). They release NO under certain biological conditions, or directly transfer the nitroso group (R-NO) to other thiol groups (transnitrosylation) (Singh *et al.* 1996). In aqueous solutions, CysNO has a half-life shorter than 2 min, at 37°C (Mathews & Kerr 1993).

NO is a gaseous molecule with a short half-life (0.64 s in rat cortex) and is highly diffusible (diffusion coefficient in rat cortex is 2.2×10^{-5} cm^2/s) (Santos *et al.* 2011), being able to rapidly reach several biological targets at certain limited range. Due to its high reactivity, NO lacks a specific molecular target and interacts with several molecules. So, it is important for many different processes in the organism, such as vasodilation and smooth muscle relaxation, synaptic plasticity, inflammation and apoptosis, as well as neurogenesis. The effects of NO are produced through different signaling mechanisms, as described next.

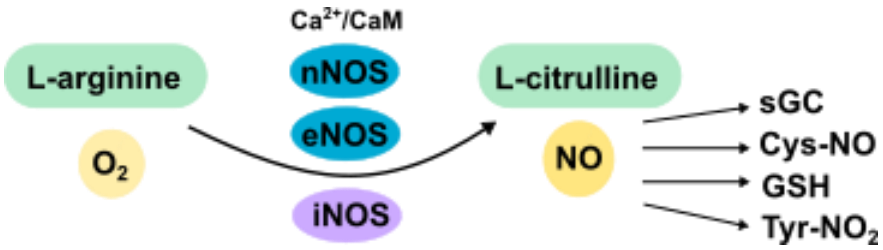


Figure 1.2 - NO is synthesized by NOS enzymes and acts in several pathways. NO is synthesized from L-arginine and oxygen by calcium/calmodulin (CaM)-dependent (nNOS and eNOS) and calcium-independent (iNOS) isoforms of NOS. It is important for several cellular processes and acts by different signaling mechanisms.

1.2.2. NO signaling

1.2.2.1. Classical NO signaling

Guanylyl cyclases are enzymes that synthesize cyclic guanosine monophosphate (cGMP) and exist in two forms: membrane-bound and soluble. Membrane-bound guanylyl cyclases are plasma membrane receptors that form homodimers and have an extracellular ligand binding domain, a short transmembrane region, and an intracellular region with the catalytic domain (Potter 2011). Soluble guanylyl cyclase (sGC) is homologous to the catalytic domains of the membrane-bound forms of guanylyl cyclase and is a receptor of NO (Fig. 1.3). sGC is a heterodimeric enzyme composed by two homologous subunits: an α subunit (α1 or α2) and a β subunit (β1) (Friebe & Koesling 2009). The α2 subunit interacts with many proteins and allows the α2β1 isoform localization to the plasma membrane, while the β1 subunit, which contains a ferrous (Fe(II)) heme, is essential for the dimerization and catalytic activity of sGC. At nanomolar concentrations, NO binds to sGC and promotes the rupture of the His–Fe(II) bond within the heme, leading to a conformational change in the His ligand (Russwurm & Koesling 2004, Rodriguez-Juarez *et al.* 2007). This increases the conversion of guanosine triphosphate into cGMP by sGC. Submicromolar concentrations of cGMP activate cGMP-dependent protein kinases (PKGs), homodimeric serine-threonine kinases. This interaction leads to a change in the conformation of PKGs and releases autoinhibitory contacts. Phosphodiesterases, particularly 5, 6 and 9, stop sGC/cGMP/PKG signaling by degrading cGMP (Lugnier 2006).

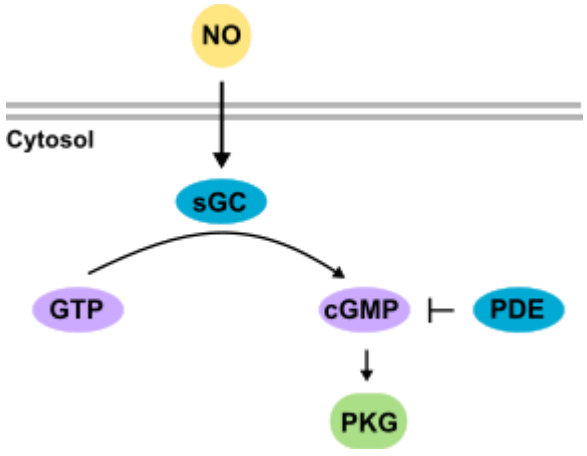


Figure 1.3 - Classical NO signaling. NO binds to the heme group of sGC, which produces cGMP from conversion of guanosine triphosphate. cGMP activates PKG, and can be degraded by phosphodiesterases (PDE).

1.2.2.2. Non-classical NO signaling

NO can produce its effects in a way that is independent of cGMP and that does not involve binding to metal centers, by inducing protein post-translational modifications (PTM), such as S-nitrosylation, S-glutathionylation or tyrosine nitration (Fig. 1.4).

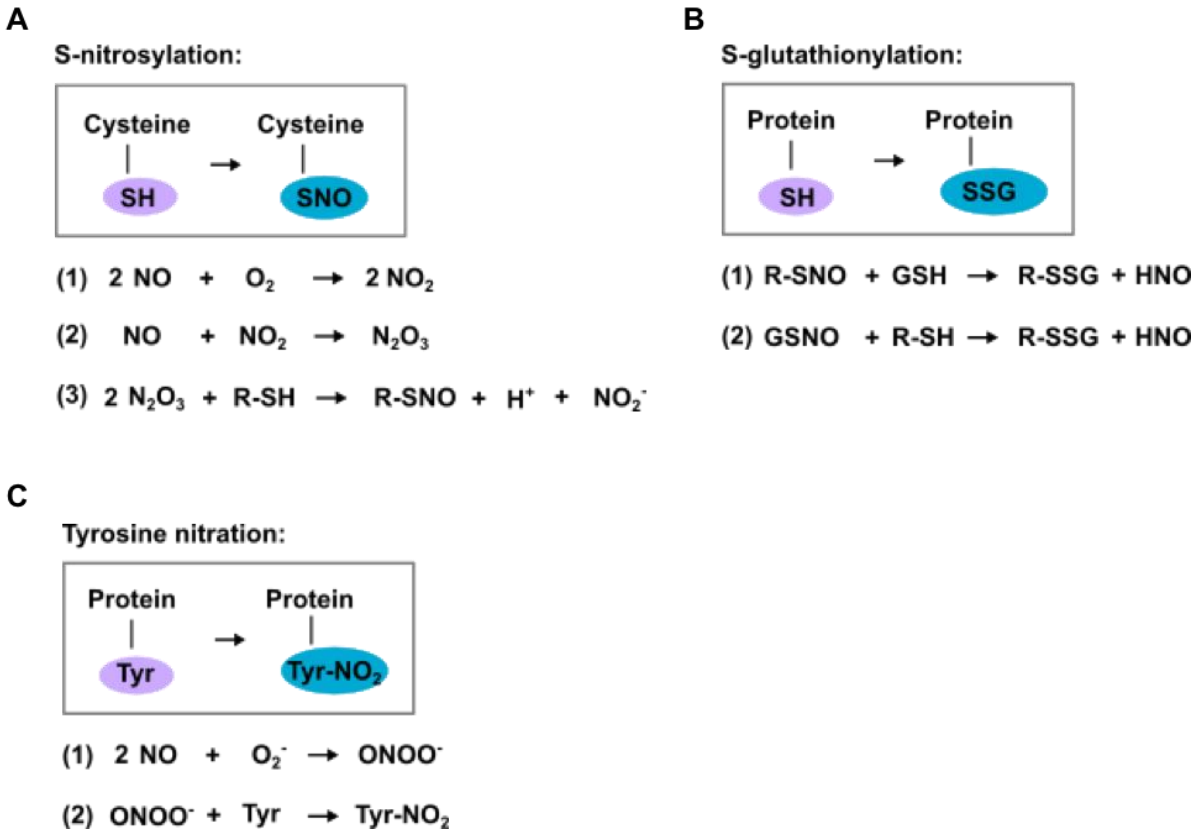


Figure 1.4 - Non-classical NO signaling. NO can directly modify protein function by inducing PTM, such as S-nitrosylation **(A)**, S-glutathionylation **(B)** and tyrosine nitration **(C)**. **(A, S-nitrosylation)** NO, in the potent oxidant form N_2O_3 **(1, 2)**, can react with free thiol groups (R-SH) of cysteines leading to the formation of a S-nitrosothiol (R-SNO) **(3)**. **(B, S-glutathionylation)** Glutathione (GSH) can react with S-nitrosylated proteins and lead to the formation of a mixed disulfide bond with those cysteines (SSG) **(1)**. Alternatively, NO can react with glutathione, originating S-nitrosoglutathione (GSNO) that can react with free thiols, and lead to formation of the mixed disulfide bond **(2)**. **(C, tyrosine nitration)** When in high amounts, NO reacts with superoxide anion and originates peroxynitrite ($ONOO^-$) **(1)**, which can react with protein 3-position tyrosine residues and form 3-nitrotyrosine (Tyr-NO_2) **(2)**.

1.2.2.2.1. S-nitrosylation

S-nitrosylation, also called S-nitrosation (Martinez-Ruiz & Lamas 2004), is a reversible PTM in which a nitroso group (R-NO) is covalently attached to a cysteine thiol (R-SH), forming a S-nitrosothiol (R-SNO) (Martinez-Ruiz *et al.* 2011). Formation and breakage of S-nitrosylation does not rely completely on enzymes because the specificity of this

reaction does not depend on the recognition of a target by an enzyme. Instead, S-nitrosylation is dependent on the reactivity between a nitrosylating agent and a target. There are three crucial regulatory factors for increasing the specificity and selectivity of S-nitrosylation for particular proteins and avoid broad reactivity: subcellular compartmentalization, site specificity and denitrosylation specificity (Derakhshan *et al.* 2007). Subcellular compartmentalization ensures that higher concentrations of the nitrosylating agent around selected cysteine residues lead to increased specificity. S-nitrosylation is considered a short-range signaling mechanism (Martinez-Ruiz *et al.* 2013). Since it is not able to directly react with cysteine thiols (unless in a thiyl radical form), high concentrations of NO are needed to induce formation of reactive nitrosylating species. S-nitrosothiols can be formed by different mechanisms (Guikema *et al.* 2005), such as the reaction of NO and O_2 that results in N_2O_3, a very potent nitrosylating species. Site specificity involves the reactivity of individual cysteine residues in certain protein microenvironments, being more susceptible to being S-nitrosylated. Accordingly, in physiological conditions only certain residues are modified, while very high concentrations of nitrosylating agents may be able to modify slower-reacting cysteines. Levels of cellular S-nitrosothiols are usually low, which suggests that denitrosylation is a highly active mechanism. The rate of denitrosylation is another critical factor for S-nitrosylation specificity (Benhar *et al.* 2009). Of the different mechanisms of denitrosylation, two are similar to the pathways used to reduce thiols with other oxidative PTM. On the one hand, reaction of S-nitrosothiols with glutathione leads to either protein glutathionylation or transnitrosylation. On the other hand, thioredoxin can directly act on protein S-nitrosothiols (Lillig & Holmgren 2007).

1.2.2.2.2. S-glutathionylation

S-glutathionylation is the addition of glutathione to a protein by formation of a mixed disulfide bridge with a cysteine residue (Klatt & Lamas 2000, Mieyal *et al.* 2008). S-glutathionylation is a stable mechanism of redox signaling controlled by several enzymatic mechanisms, and is not dependent on NO, but can be induced by NO and reactive nitrogen species. So, protein S-glutathionylation can be induced by peroxynitrite (Okamoto *et al.* 2001, Adachi *et al.* 2004). Moreover, it can result from the reaction of a free thiol and a S-nitrosothiol, so that glutathione can react with a cysteine of a S-nitrosylated protein, or S-nitrosoglutathione can be formed and react with the cysteine thiol. S-glutathionylation has been suggested as being a mechanism to protect the cell against thiol oxidation to sulfinic or sulfonic acid (Klatt & Lamas 2000).

1.2.2.2.3. Tyrosine nitration

Tyrosine nitration consists of the addition of a nitro group (-NO_2) to the phenolic ring of tyrosine residues, resulting in the formation of a 3-nitrotyrosine residue (Martinez-Ruiz et al. 2011). It is an irreversible modification, thermodynamically stable under physiological conditions. Peroxynitrite, which results from the reaction of NO with superoxide, is one of the main nitrating agents, though other reactive species have been described (Radi 2004, Souza *et al.* 2008). There is selectivity for increased nitration at individual proteins (Souza *et al.* 1999, Ischiropoulos 2003), but the factors that regulate it are still being investigated (Abello *et al.* 2009).

1.2.3. NO and neurogenesis

NO has been established as a regulator of adult neurogenesis. Several studies show that physiological levels of NO, released by nNOS, decrease neurogenesis both in the SVZ and DG. Abolishment of NO release, in *nNOS* knockout mice and by pharmacological inhibition of rat NOS, increases NSC proliferation in the SVZ and in the DG (Packer *et al.* 2003). Selective inhibition of nNOS increases NSC proliferation in the SVZ and causes a delay in neuronal differentiation (Moreno-Lopez *et al.* 2004). The anti-proliferative effect of NO released by nNOS is also observed in cultures of NSC derived from the SVZ (Matarredona *et al.* 2004). This effect is mediated by inhibition of epidermal growth factor receptor (EGFR) and phosphoinositide-3-kinase/Akt pathway (Torroglosa *et al.* 2007), through S-nitrosylation of EGFR (Murillo-Carretero *et al.* 2009). In the DG of *nNOS* knockout mice or after selective inhibition of nNOS, there is increased NSC proliferation and survival of newborn cells (Zhu *et al.* 2006, Fritzen *et al.* 2007).

On the other hand, NO released by iNOS during inflammation seems to promote neurogenesis. In a model of stroke, there is an increase in the proliferation of NSC in rat DG, which is prevented by inhibition of iNOS, and in *iNOS* knockout mice (Zhu *et al.* 2003). Our group has been extensively studying the role of NO from inflammatory origin in neurogenesis. In SVZ-derived NSC treated with concentrations of a NO donor (NOC-18) within the pathological range (1, 10 and 100 μM), NO has a dual role in the proliferation of NSC, dependent on its concentration and time of exposure (Carreira *et al.* 2010). Exposure to high levels of NO for a long period of time decreases the proliferation of NSC. In mixed cultures of SVZ-derived NSC and microglia, NO released by microglia also decreases NSC proliferation, which is prevented abolishing NO release by using *iNOS*$^{-/-}$ microglia (Carreira *et al.* 2014). This anti-proliferative effect of NO from inflammatory origin results from inhibition of the extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) pathway due to nitration of EGFR by

peroxynitrite, which prevents EGFR phosphorylation and its consequent activation (Carreira et al. 2014). Exposure of SVZ-derived NSC to low levels of NO (10 μM) for a short period of time increases NSC proliferation in a biphasic manner (Carreira et al. 2010, Carreira *et al.* 2013). In an initial stage, the proliferative effect of NO is mediated by activation of the ERK/MAPK pathway, while at a later stage the effect is mediated by activation of the sGC/cGMP/PKG pathway (Carreira et al. 2010, Carreira et al. 2013). In a mouse model of excitotoxic brain lesion, NO from inflammatory origin increases the proliferation of NSC in the DG, but impairs the survival of new neurons (Carreira et al. 2010, Carreira *et al.* 2015). Our group recently identified S-nitrosylation of p21Ras in cysteine 118 as the mechanism by which NO activates the ERK/MAPK pathway and increases NSC proliferation, both in cultures of NSC from the SVZ and in the model of excitotoxic brain lesion (Santos *et al.* 2017). This ultimately leads to translocation of cyclin-dependent kinase inhibitor $p27^{KIP1}$ from the nucleus to the cytosol and allows the progression of cell cycle (Carreira et al. 2010) (Fig. 1.5).

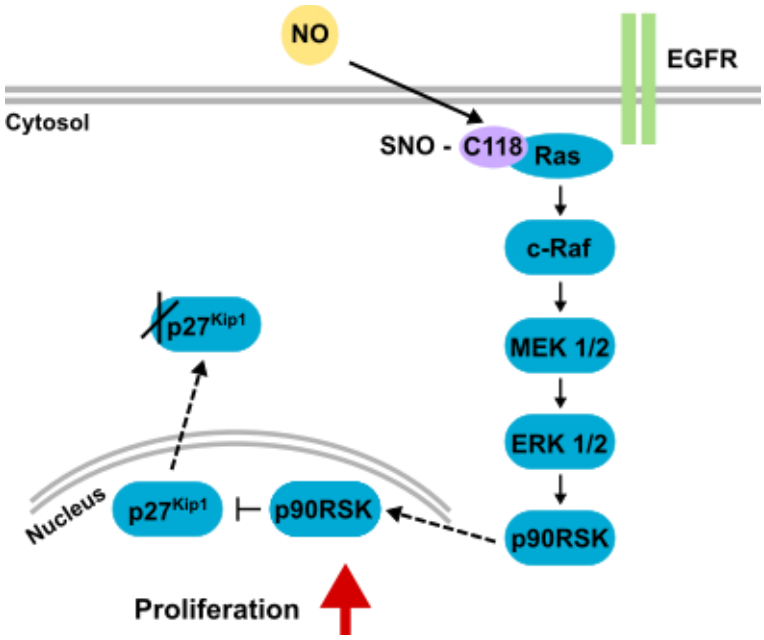


Figure 1.5 - NO increases NSC proliferation through ERK/MAPK pathway signaling. NO bypasses the EGFR and S-nitrosylates cysteine 118 of p21Ras, which activates the downstream signaling cascade of protein kinases, ultimately leading to translocation of $p27^{KIP1}$ to cytosol and its degradation, allowing cell proliferation to occur.

1.2.3.1. Identification of new putative targets of S-nitrosylation by NO in NSC

The ERK/MAPK pathway is crucial for the proliferative effect of NO, which can regulate protein function by S-nitrosylation. Involvement of S-nitrosylation of some proteins in neurogenesis has been described (reviewed in Santos *et al.* 2015). S-nitrosylation of

myocyte enhancer factor 2 transcription factors acts as a redox switch to inhibit both neurogenesis and neuronal survival (Okamoto *et al.* 2014). On the contrary, S-nitrosylation of histone deacetylase 2 in embryonic cortical neurons regulates dendritic growth and branching, due to chromatin remodeling and activation of CREB-dependent genes involved in neuronal development, promoted by neurotrophic factors (Nott *et al.* 2008). Moreover, S-nitrosylation of histone deacetylase 2 also regulates neuronal radial migration during cortical development (Nott *et al.* 2013).

Due to the low efficiency of post-injury neurogenesis, the identification of new proteins that could be targets of S-nitrosylation by NO and increase neurogenesis is of great interest. Therefore, our group identified S-nitrosylated proteins in NSC, in the presence of NO (Ana I. Santos, Ana S. Lourenço and Inês Araújo, submitted). SVZ-derived NSC were treated with a S-nitrosothiol (CysNO), and proteins were separated by bidimensional electrophoresis. Using a technique that allows fluorescent labeling of oxidized cysteines (fluorescence switch), there were several spots that appeared on the gel of NSC treated with CysNO, but not on the control (non-treated NSC). Those spots corresponded to oxidized proteins, which were then collected and analyzed by mass spectrometry. Several proteins were identified and, after researching their different biological functions, the involvement of some in the ERK/MAPK pathway regulation and cell proliferation was evident (Fig. 1.6). The ERK/MAPK pathway is involved in several cellular processes such as cell proliferation, differentiation, survival and apoptosis (Kolch 2005). Through binding of extracellular molecules, such as growth factors and hormones, to cell surface receptors, signals are transmitted by different effectors, which ultimately alters gene expression and affects cellular behavior. Receptor tyrosine kinases span the cell membrane and have an extracellular ligand binding domain and an intracellular tyrosine kinase domain, existing as inactive monomers. Ligand binding induces dimerization and receptor activation by autophosphorylation. This leads to activation of the GTPase p21Ras that binds and activates Raf kinase. Raf phosphorylates and activates mitogen-activated protein kinase kinase (MEK), which in turn phosphorylates and activates ERK. ERK can either interact with other effectors that can translocate to the nucleus, or translocate to the nucleus itself. In NSC, ERK/MAPK signaling is involved in cell proliferation mainly through binding of EGF and bFGF to their receptors (Sutterlin *et al.* 2013).

S-nitrosylation of those proteins of interest was assessed, and some were shown to be S-nitrosylated: 14-3-3 (particularly 14-3-3 ε), proliferating cell antigen (PCNA), heterogeneous nuclear ribonucleoprotein K (hnRNP K), elongation factor (EF)-1β and phosphatidylethanolamine-binding protein 1 (PEBP-1).

14-3-3 proteins are a family of 7 isoforms of adaptor proteins that regulate cell signaling pathways and are highly expressed in the brain (Cornell & Toyo-Oka 2017). During postnatal formation of the rat brain, 14-3-3 proteins are differentially expressed and have a role in neuronal development and synaptogenesis (Umahara *et al.* 2011). In the adult, they are necessary for hippocampal long-term potentiation and associative learning and memory (Qiao *et al.* 2014). 14-3-3 can activate the ERK/MAPK pathway, which is involved in the proliferative effect of NO in NSC (Carreira et al. 2010), by interacting with c-Raf (Chang & Rubin 1997, Roberts *et al.* 1997). The isoform 14-3-3 ε is highly expressed in the human hippocampus and is upregulated in temporal lobe epilepsy (Schindler *et al.* 2006). Moreover, 14-3-3 ε has an important role in neurogenesis and neuronal migration (Toyo-oka *et al.* 2014), and has been described to be S-nitrosylated in mesangial cells (Kuncewicz *et al.* 2003).

PCNA is necessary for DNA replication and is, therefore, essential for cell proliferation. Its regulation by PTM is essential for inducing a different set of cellular functions during DNA replication (Choe & Moldovan 2017). In response to EGFR signaling, which is involved in NSC proliferation (Gritti *et al.* 1995), PCNA is phosphorylated and protected from degradation (Wang *et al.* 2006). PCNA can be S-nitrosylated in cysteine 81 (Lam *et al.* 2010), which blocks its interaction with caspase-9 and promotes apoptosis in a human neuroblastoma cell line (Yin *et al.* 2015).

hnRNP K belongs to a family of proteins that bind RNA and are involved in nucleic acid metabolism (Dreyfuss *et al.* 1993). hnRNP K is essential for axonogenesis in Xenopus (Liu & Szaro 2011) and its inactivation, coupled with activation of the neuronal Hu-p21 pathway, may be essential to the switch from proliferation to differentiation in NSC (Yano *et al.* 2005). The activity of hnRNP K is regulated by several PTM (reviewed in Lu & Gao 2017), but S-nitrosylation is still yet to be described.

EF-1α and EF-1β are part of the EF 1 complex, which is responsible for translation elongation. EF-1α delivers aa-tRNAs to the A site of the ribosome (Carvalho *et al.* 1984) and EF-1β replaces EF-1α-bound GDP for GTP (Pittman *et al.* 2006). EF-1β and its regulation are essential for the function of EF-1α. Although a role in neurogenesis is not described, EF-1α can interact with and regulate Akt (Pecorari *et al.* 2009, Khwanraj *et al.* 2016), a pathway involved in the proliferation of NSC (Peltier *et al.* 2007, Le Belle *et al.* 2011). EF-1β can be regulated by phosphorylation (Venema *et al.* 1991), but its S-nitrosylation has not been previously described.

PEBP-1 regulates the ERK/MAPK pathway, preventing MEK phosphorylation by binding to c-Raf (Yeung *et al.* 1999), and its overexpression increases neuronal differentiation, both in a human neuroblastoma cell line (Hellmann *et al.* 2010) and in adult rat

hippocampal progenitor cells (Sagisaka *et al.* 2010). PEBP-1 is regulated by phosphorylation (Corbit *et al.* 2003), but its S-nitrosylation has not been described. Its role in neurogenesis is therefore interesting to study in more detail, so we focused more our study in this protein.

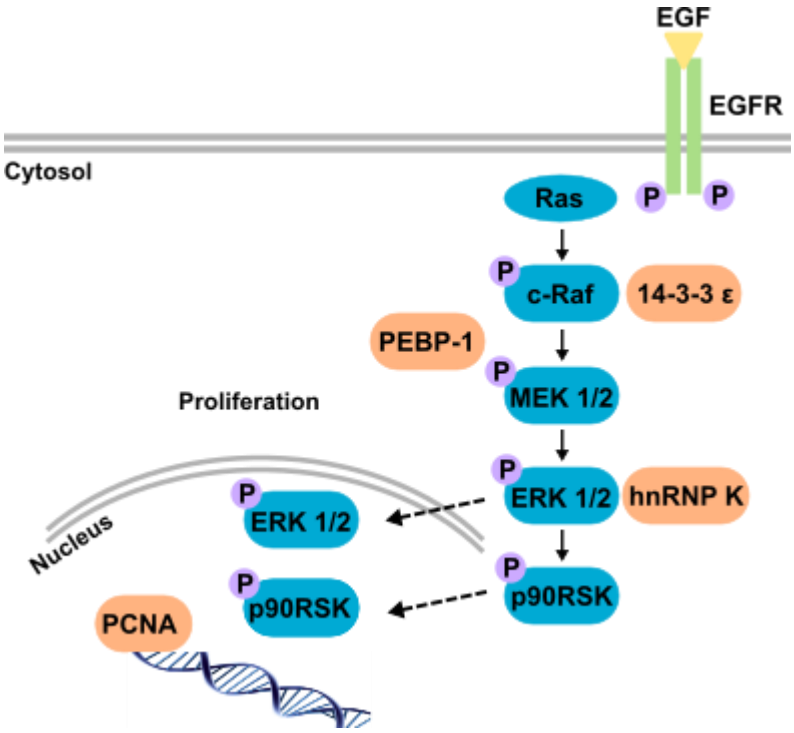


Figure 1.6 - ERK/MAPK pathway signaling through EGFR. Upon ligand binding (EGF), its receptor (EGFR) dimerizes and becomes activated by autophosphorylation. p21Ras is activated by adaptor proteins (not represented) and the downstream protein kinases are sequentially activated by phosphorylation, namely c-Raf, MEK and ERK. ERK can be translocated to the nucleus or interact with other proteins that are then translocated. In the nucleus, gene expression can be altered and lead to cell proliferation. 14-3-3 ε, PCNA, hnRNP K and PEBP-1 are involved in different steps of this signaling pathway.

1.2.3.1.1. Phosphatidylethanolamine-binding protein 1 (PEBP-1)

PEBP-1 belongs to a family of proteins characterized by having a conserved region that was a binding site for the phospholipid O-phosphatidylethanolamine (Serre *et al.* 1998). It is a highly conserved family of proteins, existing in organisms that range from bacteria to mammals. *PEBP-1* gene is composed of 4 exons localized in mouse chromosome 5, and human and rat chromosome 12. Knockout mice for *PEBP-1* revealed that *PEBP-1* is mainly expressed in testis and in the brain, mostly in structures of the limbic system, including the hippocampus (Theroux *et al.* 2007). These animals are viable but develop olfactory deficits. The protein has 187 aminoacids, of which in mouse 3 are cysteines (13, 133 and 168). Human and rat PEBP-1 have 2 cysteines (133 and 168). Cleavage

of the N-terminal of PEBP-1 originates the hippocampal cholinergic neurostimulating peptide, which corresponds to PEBP-1 aminoacids 2-12, and increases production of choline acetyltransferase in the hippocampus (Ojika *et al.* 2000).

PEBP-1 is involved in the regulation of different signaling pathways (Fig. 1.7). It is also called raf kinase inhibitory protein (RKIP) because it regulates the ERK/MAPK pathway by binding to c-Raf and MEK, preventing their association and phosphorylation of MEK, which results in inhibition of the signaling pathway (Yeung et al. 1999, Yeung *et al.* 2000). Phosphorylation of PEBP-1 serine 153 by protein kinase C (PKC) releases the association between PEBP-1 and c-Raf (Corbit et al. 2003) and triggers dimerization of PEBP-1, increasing its specificity for binding and inhibiting G protein-coupled receptor kinase 2 (GRK2) ability to phosphorylate G protein-coupled receptors (Lorenz *et al.* 2003, Deiss *et al.* 2012). PEBP-1 also regulates the NFκB pathway by interacting with several kinases, acting as a scaffold protein, and both its overexpression and downregulation impair the degradation of inhibitory κB upon stimulation by IL-1β (Yeung *et al.* 2001, Tang *et al.* 2010). Glycogen synthase kinase 3 β signaling can be directly activated by PEBP-1, through prevention of its inhibition by phosphorylation (Al-Mulla *et al.* 2011).

In the brain, PEBP-1 has different functions, such as mediating PKC-dependent MAPK activation during cerebellar long-term depression (Yamamoto *et al.* 2012). Several studies show that PEBP-1 is important for neurogenesis. Hippocampal progenitor cells secrete PEBP-1 to culture medium (Dahl *et al.* 2003). Moreover, PEBP-1 is involved in directing differentiation of hippocampal progenitor cells and neuroblastoma cells towards a neuronal lineage (Sagisaka et al. 2010, Hellmann et al. 2010) through enhanced crosstalk of PKC and ERK/MAPK and enhancement of G protein-coupled receptor signaling (Hellmann et al. 2010). In neuroblastoma cells, long-term ethanol exposure impairs neuronal differentiation, which is accompanied by downregulation of PEBP-1 and PKC, and by decreased activation of the ERK/MAPK pathway by BDNF (Hellmann *et al.* 2009). In an ischemic model, PEBP-1 regulates differentiation towards a neuronal lineage and decreases differentiation into astrocytes, in the hippocampus (Toyoda *et al.* 2012).

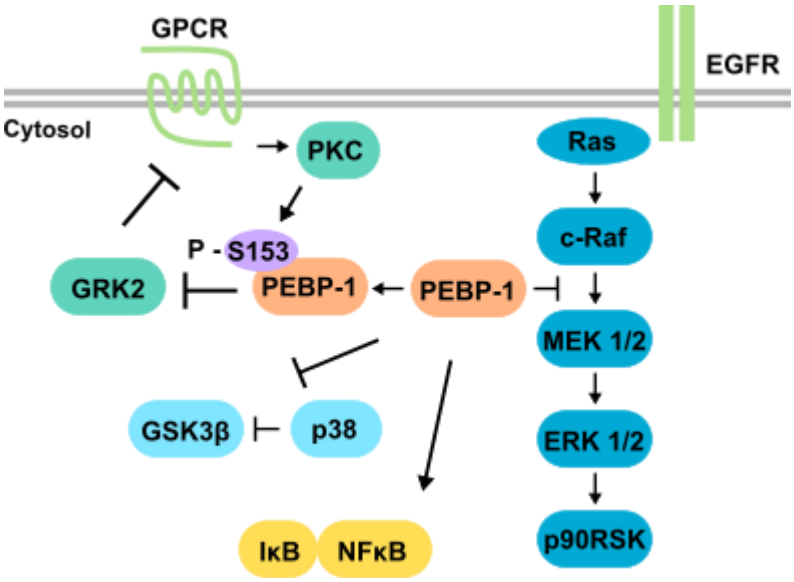


Figure 1.7 - PEBP-1 regulates several signaling pathways. PEBP-1 inhibits ERK/MAPK pathway signaling by binding to c-Raf and MEK, and by preventing phosphorylation of MEK by c-Raf. Upon activation through G protein-coupled receptor (GPCR), PKC phosphorylates serine 153 of PEBP-1. This induces the release of PEBP-1 inhibitory effect in the ERK/MAPK pathway, and prevents the inhibitory effect of GRK2 in GPCR signaling. PEBP-1 can also regulate NFκB signaling by preventing degradation of inhibitory κB, and can activate glycogen synthase kinase 3 β (GSK3β) by preventing its inhibitory phosphorylation.

1.3. Objectives

Post-injury neurogenesis is not an efficient process, which impairs the potential of brain regeneration. NO is an important regulator of adult neurogenesis, being able to enhance the proliferation of NSC in conditions of neuroinflammation. One of the ways by which NO can produce its effects is by inducing S-nitrosylation of proteins by reacting with thiol groups of cysteine residues. A mechanism of activation of the ERK/MAPK pathway through S-nitrosylation by NO and enhancement of proliferation of NSC has been described by our group (Santos et al. 2017). Moreover, our group also recently identified a group of proteins that are S-nitrosylated by NO in NSC (Ana I. Santos, unpublished results). Some of these proteins are involved in cell proliferation by interacting with the ERK/MAPK pathway. PEBP-1, one of the identified targets, is a regulator of cell proliferation by means of c-Raf inhibition, being a promising target to study in detail. In this work, we propose to investigate the role of PEBP-1 S-nitrosylation by NO in neurogenesis, particularly in the proliferation of NSC. In addition, we also propose to investigate the biological role of the other newly identified targets of S-nitrosylation of NO in post-injury neurogenesis.

The methods used in this work are described in detail in chapter 2.

In chapter 3.1, the role of S-nitrosylation of PEBP-1 by NO in proliferation was evaluated in SVZ-derived NSC using normal or NO-insensitive forms of PEBP-1, lacking specific cysteines. The contribution of each cysteine for S-nitrosylation of PEBP-1, and its effect on ERK/MAPK signaling and cell proliferation was evaluated, after treatment with the S-nitrosothiol CysNO or the NO donor NOC-18. Moreover, S-nitrosylation of PEBP-1 in post-injury neurogenesis was evaluated using a mouse model of excitotoxic brain lesion associated with increased hippocampal neurogenesis mediated by NO (Carreira et al. 2015). S-nitrosylation of PEBP-1 was assessed in the DG of wild type mice and mice lacking iNOS, and also in the stem cells of the DG, using cultured hippocampal stem cells treated with CysNO.

In chapter 3.2, the role of S-nitrosylation of the other identified proteins by NO in post-injury neurogenesis was evaluated using the mouse model described above. S-nitrosylation of 14-3-3, hnRNP K, PCNA and EF-1β was assessed in mouse DG, and in hippocampal stem cells treated with CysNO.

Chapter 4 includes an integrated discussion of the results presented in this work, and chapter 5 presents the conclusions.

Chapter 1

Chapter 2. Methods and materials

Chapter 2

2.1. Methods

2.1.1. Animals

C57Bl6 male mice were purchased from Charles River (Barcelona, Spain), and B6.129P2- *Nos*^{2tm1Lau}/J (knockout for *iNOS, iNOS* KO) male mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). The animals were kept in the animal facilities of Center for Neuroscience and Cell Biology (Coimbra) and Center for Biomedical Research (University of Algarve, Faro) with food and water *ad libitum* in a 12-hour dark:light cycle. All experiments were performed in accordance with institutional and European guidelines (2010/63/EU and DL 113/2013) for the care and use of laboratory animals, under the project license 0421/000/000/2013.

2.1.2. Genotyping

The genotype of B6.129P2-Nos2^{tm1Lau}/J mice was confirmed by polymerase chain reaction, using the primers recommended by The Jackson Laboratory (Table 2.1) and NZYTaq 2x Green Master Mix. DNA was obtained from the tip of the tail, after purification with NZY Tissue gDNA Isolation kit. After denaturation at 94°C for 5 min, 35 cycles of 94°C, 55°C and 72°C for 1 min each were performed, followed by a final extension at 72°C for 10 min.

Table 2.1 - Primer sequences for genotyping.

	DNA sequence (5'-3')
iNOS	ACA TGC AGA ATG AGT ACC GG
	TCA ACA TCT CCT GGT GGA AC
	AAT ATG CGA AGT GGA CCT CG

The amplicons were run in a gel electrophoresis (2% agarose with GreenSafe Premium), and were detected using ChemiDoc XRS+ (BioRad Laboratories Inc., Hercules, CA, USA), resulting in a product of 108 base pairs for the wild type allele, and a product of 270 base pairs for the recombinant allele (Fig. 2.1).

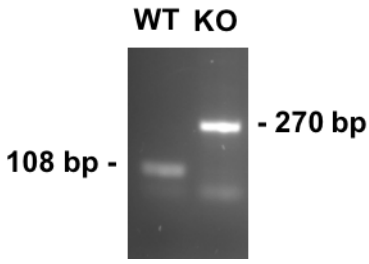


Figure 2.1 - Genotyping profile of WT and *iNOS* KO mice. Representative image of PCR products of wild type (WT) and *iNOS* KO mice genotyping.

2.1.3. SVZ-derived NSC cultures

NSC were isolated from the SVZ of P0-3 C57Bl6 mice as previously described (Morte *et al.* 2013). Briefly, mice were decapitated, the brain and meninges were removed, and slices of 1 mm were cut coronally. Tissue lining the lateral walls of the lateral ventricles was dissected and enzymatically digested in 0.025% Trypsin/0.265 mM ethylenediaminetetraacetic acid (EDTA) in Hanks' Balanced Salt Solution, 15 min at 37°C. Single cells were counted using 0.1% trypan blue exclusion assay and plated in uncoated T-25 flasks at a density of 100,000 cells/ml. NSC were allowed to grow as floating aggregates (neurospheres) in proliferation medium (Dulbecco's modified eagle medium: nutrient mixture F-12 (D-MEM/F-12) with 2 mM GlutaMAX™-I (L-Ala-L-Gln) supplemented with 1% B27, 1% antibiotic (PenStrep, 10,000 units/ml of penicillin, 10 mg/ml streptomycin), 5 ng/ml bFGF and 10 ng/ml EGF, in 95% air/5% CO_2 humidified atmosphere at 37°C. Approximately 5 days later, NSC were mechanically dissociated and resuspended in fresh proliferation medium (passage). After at least 2 passages, NSC were plated on 0.1 mg/ml poly-L-lysine-coated multiwells or coverslips, and maintained with proliferation medium until the desired confluency was achieved. Each culture was used until reaching a maximum of 12 passages.

2.1.4. Hippocampal stem cell cultures

Hippocampal stem cells were kindly provided by Dr. Fred H. Gage (Salk institute, USA). These cells were isolated from the hippocampus of adult Fischer 344 rats, as described by Gage *et al.* (1995), and their capacity for self-renewal and multipotency was characterized by Palmer *et al.* (1997). For maintenance of the cell line, hippocampal stem cells were cultured adherent on plates coated with polyornithine (10 µg/ml) and laminin (10 µg/ml), in D-MEM/F-12 with 2 mM GlutaMAX™-I (L-Ala-L-Gln) supplemented with 1% PenStrep, 1% N2 and 20 ng/ml bFGF, and in 95% air/5% CO_2 humidified atmosphere at 37°C. Culture medium was replaced every 3-4 days and, when confluent,

cells were detached using 0.25% Trypsin/EDTA for 1 min at 37°C. After centrifugation at 1,000 rpm for 5 min, cells were resuspended in fresh culture medium and allowed to grow.

2.1.5. Mutagenesis of PEBP-1

To overexpress PEBP-1, a commercially available plasmid of pCMV-SPORT6 backbone with mouse *PEBP-1* cDNA was purchased (Thermo Scientific, Waltham, MA, USA). To replace cysteine for serine, the nucleotide cytosine was changed to guanine. For each of the 3 cysteines of PEBP-1, two complementary oligonucleotides, containing the necessary mutation, were designed using QuikChange Primer Design Program (Agilent Technologies, Santa Clara, CA, USA), synthesized and purified (Table 2.2). QuickChange II XL Site-Directed Mutagenesis Kit was used to induce the mutations, following the manufacturer's instructions. Briefly, polymerase chain reaction amplification was performed with an initial denaturation of 95°C for 1 min, followed by 18 cycles of 95°C for 50 s, 60°C for 50 s and 68°C for 5 min, followed by an overall extension at 68°C for 7 min. The amplicons were then digested with *Dpn* I for 1 h at 37°C. Bacterial transformation was performed by inducing a heat-pulse in a 42°C water bath for 30 s, followed by a 2 min-incubation on ice. Bacteria were left to grow at 37°C with shaking at 230 rpm, for 1 h. After selection on agar plates with 100 μg/ml ampicillin, 2 colonies were picked, and plasmid DNA was purified with NZYMiniprep and sequenced by Stab Vida (Caparica, Portugal). One clone of each mutant was chosen and expanded, after which DNA was purified with Endotoxin-free Plasmid DNA Purification and spectrophotometrically quantified at 260/280 nm with NanoDrop 2000C (Thermo Scientific, Waltham, MA, USA).

Table 2.2 - Primer sequences for PEBP-1 mutagenesis.

	DNA sequence (5'-3')
C13S	CCG GGC CCT TGT CCT TGC AGG AGG T
	ACC TCC TGC AAG GAC AAG GGC CCG G
C133S	AGC CGC TGA GCT CCG ACG AGC CC
	GGG CTC GTC GGA GCT CAG CGG CT
C168S	GGC GGG CAC GTC CTA CCA AGC CG
	CGG CTT GGT AGG ACG TGC CCG CC

2.1.6. PEBP-1 overexpression

NSC were transfected with the PEBP-1 constructs using Lipofectamine LTX following the manufacturer's instructions. Briefly, NSC were plated until reaching 70% of confluency, and culture medium was replaced with proliferation medium without antibiotic (PenStrep) in the day of transfection. DNA (1.5 μg) and Plus Reagent (2.5 μl) were added to Opti-MEM I reduced serum medium, and after 10 min Lipofectamine LTX (2 μl) was added. Complexes were formed during 30 min, after which they were added to cells drop-by-drop and left for 30-48 h. Culture medium was replaced by medium without growth factors (EGF and bFGF) 24 h before cell treatment. In cell signaling analysis and proliferation experiments, NSC overexpressing PEBP-1 were identified by immunocytochemistry.

2.1.7. Synthesis of S-nitrosocysteine

CysNO synthesis was prepared as previously described (Jourd'heuil *et al.* 2000). 1 volume of L-Cysteine (200 mM in 1 M HCl) was mixed with 1 volume of $NaNO_2$ (200 mM) and kept in the dark. After 30 min at room temperature (RT), 2 volumes of K_2HPO_4 buffer (1 M, pH=7.4) were added. From that point on, the solution was kept on ice until storage at -80°C. Concentration was calculated using the extinction coefficient of 900 M^{-1} cm^{-1} at an absorbance of 338 nm (DeMaster *et al.* 1995), measured in NanoDrop 2000C (Thermo Scientific, Waltham, MA, USA).

2.1.8. Experimental treatments

Cell treatment was performed 24 h after changing the culture medium for medium without growth factors. To evaluate S-nitrosylation of PEBP-1, NSC were exposed to a S-nitrosothiol (CysNO, 100 μM) for 1, 2 and 15 min, and a NO donor (NOC-18, 10 μM) for 2.5, 5, 7.5 and 10 min. To evaluate protein oxidation, NSC were treated with CysNO (100 μM) and DEA/NO (100 μM and 1 mM) for 15 min, and NOC-18 (10 μM) for 1 h. In hippocampal stem cells, S-nitrosylation of proteins was evaluated after treatment with CysNO at different concentrations (10, 50 and 100 μM) for 15 min. To analyze cell signaling, NSC were treated with CysNO (10 μM) for 5 min and EGF (20 ng/ml) for 20 min. To evaluate cell proliferation, NSC were treated with NOC-18 (10 μM) for 1 h.

2.1.9. Evaluation of cell proliferation by incorporation of EdU

To evaluate cell proliferation, 5-ethynyl-2'-deoxyuridine (EdU, 10 μM), a thymidine analogue that incorporates in DNA in the S phase of cell division, was added to cultures

for 1 h. NSC were rinsed in 0.01 M phosphate-buffered saline (PBS, 7.8 mM $Na_2HPO_4.2H_2O$, 2.7 mM $NaH_2PO_4.H_2O$, 154 mM NaCl, pH 7.2) and fixed for 20 min with 4% paraformaldehyde/4% sucrose in PBS. EdU was detected by click chemistry reaction, a copper-mediated covalent coupling of the ethynyl group of EdU with a fluorescent dye-conjugated azide, using Click-iT EdU Alexa Fluor 488 HCS Assay Kit, following the manufacturer's instructions. Nuclei were labeled with Hoechst 33342 (1 μg/ml) for 5 min. Cells that incorporated EdU were detected by fluorescence microscopy (Axioimager Z2 APOTOME, Zeiss, Gena, Germany). The whole coverslip of each condition was analyzed for EdU-positive transfected cells. Ten representative images were randomly acquired for each coverslip, averaging around 500 analyzed cells per condition. The number of non-transfected cells that incorporated EdU of the total non-apoptotic cells was counted using ImageJ (version 1.47v, National Institutes of Health, Bethesda, MD, USA).

2.1.10. Immunocytochemistry

Overexpression of PEBP-1 and levels of phosphorylated ERK (P-ERK) were analyzed by immunocytochemistry. NSC were rinsed in PBS and fixed with 4% paraformaldehyde/4% sucrose in PBS for 20 min. After being rinsed in PBS, cells were permeabilized with 0.5% Triton X-100 for 15 min and blocked in 3% bovine serum albumin (BSA)/0.2% Tween 20 for 1 h. Primary antibodies (Table 2.3) were incubated overnight at 4°C and, after rinsing with PBS, secondary antibodies (Table 2.4) were incubated for 1 h 30 at RT. Nuclei were labeled with Hoechst 33342 (1 μg/ml, 5 min). Coverslips were mounted with DAKO fluorescence mounting medium on slides. At least 5 representative images were obtained using Axioimager Z2 APOTOME or LSM 710 confocal (Zeiss, Jena, Germany). Fluorescence of P-ERK was analyzed using ImageJ (version 1.47v, National Institutes of Health, Bethesda, MD, USA) to measure the mean gray value of the green channel, in transfected cells, as previously described (Santos et al. 2017).

Table 2.3 - Primary antibodies used in immunocytochemistry

Antibody	Host	Dilution	Company	Reference
PEBP-1	Goat	1:500	Abcam	ab2634
P-ERK	Rabbit	1:50	Cell Signaling	4370

Table 2.4 - Secondary antibodies used in immunocytochemistry

Antibody	Host	Dilution	Company	Reference
Anti-goat Alexa Fluor 594	Donkey	1:200	Invitrogen	A11058
Anti-rabbit Alexa Fluor 488	Donkey	1:200	Invitrogen	A21206

2.1.11. Western Blot analysis

To analyze PEBP-1 levels by Western Blot, cytosolic protein lysates were prepared after treatment, as previously described (Carreira et al. 2010). NSC were rinsed with cold PBS, and scrapped with cold lysis buffer (100 mM Tris-HCl, 10 mM ethylene glycol tetraacetic acid, 1% Triton X-100 and 2 mM $MgCl_2$) supplemented with protease and phosphatase inhibitors, and dithiothreitol (DTT, 1 mM). Samples were sonicated 6 times for 2 s with pauses of 4 s, and centrifuged at 15,800 g for 10 min at 4°C. Supernatant was collected and protein quantification was performed with BCA Protein Assay following the manufacturer's instructions.

Protein samples were denatured for 5 min at 95°C after addition of 6x concentrated Sample buffer (0.5 M Tris-HCl/0.4 % sodium dodecyl sulfate (SDS) pH 6.8, 30 % glycerol, 10 % SDS, 0.6 M DTT, 0.012 % bromophenol blue), and separated by electrophoresis in a 12% acrylamide gel. Proteins were then transferred to a polyvinylidene difluoride (PVDF) membrane using Trans-Blot® Turbo™ Transfer System (BioRad Laboratories Inc., Hercules, CA, USA) for 10 min. Membranes were blocked with 3% BSA in Tris-buffered saline with tween 20 (TBS-T) for 1 h at RT. PEBP-1 primary antibody (Table 2.5) was incubated overnight at 4°C. After washing with TBS-T, anti-goat HRP conjugate (Table 2.6) was incubated 1 h at RT. NZY Supreme ECL HRP Substrate was added to the membranes during 5 min and chemiluminescence was detected using ChemiDoc XRS+ (BioRad Laboratories Inc., Hercules, CA, USA). The images were acquired and analyzed with ImageLab 5.0 (BioRad Laboratories Inc., Hercules, CA, USA).

2.1.12. Preparation of cell protein lysates for fluorescence switch and biotin switch assays

After rinsing with cold 0.9% NaCl pH=7.4, cells were scrapped in cold TENT (50 mM Tris pH 7.2, 1 mM EDTA, 0.1 mM neocuproine, 1% Triton X-100) supplemented with protease inhibitors and 50 mM N-ethylmaleimide. Membrane proteins were removed with a

centrifugation of 10,760 g for 10 min at 4°C, and 2% SDS was added to the cytosolic samples. Free thiols (SH) were blocked with N-ethylmaleimide (50 mM) for 30 min at 37°C, and protein was quantified using BCA Protein Assay, following the manufacturer's instructions. The protocol was performed protected from light.

2.1.13. Fluorescence switch assay

To evaluate protein oxidation (Fig. 2.2A), 50 μg of protein were used and processed as previously described (Izquierdo-Alvarez *et al.* 2012). Protein was precipitated in 3 volumes of acetone for 10 min at -20°C and centrifuged at 10,760 g for 5 min at 4°C. The supernatant was discarded and the pellet resuspended in 2.5 mM DTT. After reduction for 10 min at RT, protein was precipitated as before, and resuspendend in TENS (50 mM Tris pH 7.2, 1 mM EDTA, 0.1 mM neocuproine, 1% SDS) with 40 μM BODIPY FL maleimide. Binding to free thiols (SH) was performed for 1 h at RT, stopped with addition of 2.5 mM DTT, and followed by acetone precipitation. To evaluate protein S-nitrosylation (Fig. 2.2B), 100 μg of protein were used. After protein precipitation, the pellet was resuspended in TENS with 100 mM sodium ascorbate and 40 μM BODIPY FL maleimide. Binding to free thiols (SH) was performed for 1 h at RT, stopped with addition of 4 mM N-ethylmaleimide, and followed by acetone precipitation. To analyze by SDS-PAGE, samples were resuspended in 2x non-reducing Laemmli buffer (100 mM Tris pH 7.6, 8 mM EDTA, 2% SDS, 20% glycerol, 0.04% Bromophenol blue) and run in a 10% acrylamide gel. Fluorescence was detected in ChemiDoc XRS+ (BioRad Laboratories Inc., Hercules, CA, USA), and images acquired using ImageLab 5.0 (BioRad Laboratories Inc., Hercules, CA, USA). Until this point, the protocol was performed in the dark. To stain the total protein, gel was fixed in 50% methanol/7% acetic acid for 15 min, and stained with 0.2% Coomassie Blue (45% methanol/10% acetic acid) for 1 h. After washing in 10% methanol/7% acetic acid for 30 min, gel was washed in water, and images were acquired in ChemiDoc XRS+ (BioRad Laboratories Inc., Hercules, CA, USA), using ImageLab 5.0 (BioRad Laboratories Inc., Hercules, CA, USA).

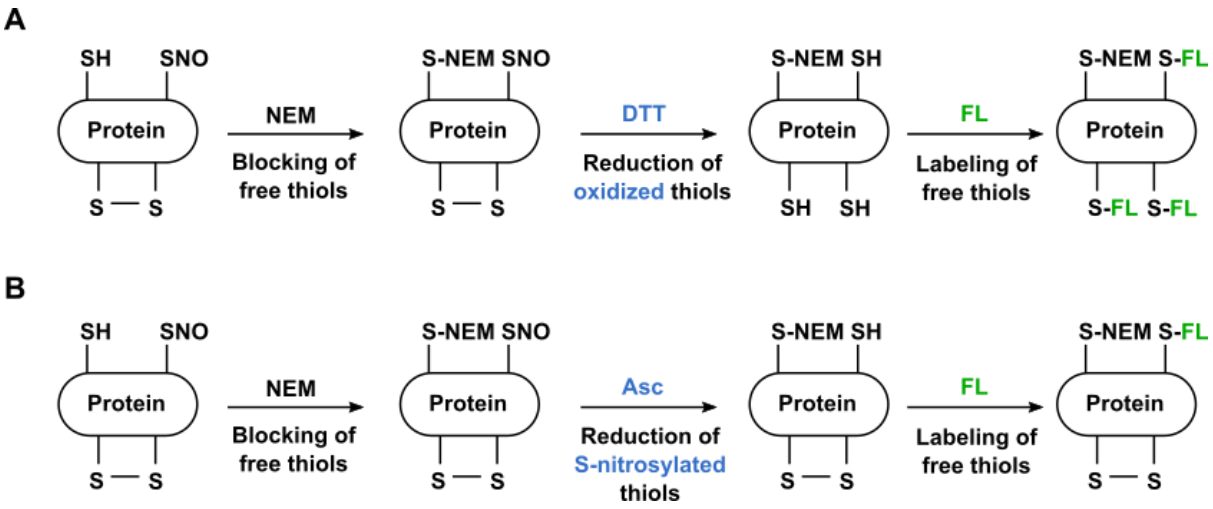


Figure 2.2 - Scheme of the fluorescence switch assay. Protein free thiol groups are blocked with N-ethylmaleimide (NEM), followed by reduction of oxidized groups by DTT **(A)** or S-nitrosylated groups by ascorbate (asc) **(B)** to free thiols. BODIPY FL maleimide is then able to bind to the free thiols.

2.1.14. Mouse model of kainic acid-induced seizures

To validate the targets of S-nitrosylation, a model of seizures epilepsy caused by kainic acid (KA) was used as a mouse model of post-injury neurogenesis. KA is an agonist of AMPA/kainate class of glutamate receptors. Injection of KA induces seizures and excitotoxicity, which results in neuronal degeneration in the CA1 and CA3 regions of the hippocampus (Schauwecker & Steward 1997). This model is characterized by increased neurogenesis in the DG after seizures (Parent 2007), which can be prevented in the absence of NO release by iNOS (Carreira et al. 2010).

C57Bl6 (wild type, WT) and B6.129P2- *Nos*^{2tm1Lau}/J (knockout for *iNOS*, *iNOS* KO) male mice with 12 weeks were subcutaneously injected with KA solution (25 mg/kg) or with saline solution (0.9% NaCl, control, SAL). Rating of seizures was performed according to the modified Racine scale as previously described (Schauwecker & Steward 1997): stage I - immobility, stage II - tail extension/forelimb/rigid posture, stage III - repetitive movements/ head bobbing, stage IV - rearing and falling, stage V - continuous rearing and falling, stage VI - tonic-clonic seizures. Only mice that reached epileptic seizures of stage V or VI were used in the experiment. After injection (1, 2, 3 and 5 days), mice were sacrificed by cervical dislocation, decapitated, the brain was removed and the DG dissected from the hippocampus.

2.1.15. Preparation of tissue protein lysates for biotin switch assay

To evaluate protein S-nitrosylation, proteins were extracted from the dissected mouse DG. Until binding of biotin, the protocol was performed in the dark, as previously described (Santos et al. 2017). Tissue was lysed by manually homogenizing with a tissue grinder, in cold TENT (50 mM Tris pH 7.2, 1 mM EDTA, 0.1 mM neocuproine, 0.5% Triton X-100) supplemented with protease inhibitors and 50 mM N-ethylmaleimide. Samples were sonicated 4 times for 2 s with 6 s pauses, and 2% SDS was added. Free thiols (SH) were blocked with N-ethylmaleimide (50 mM) during 30 min at 37°C, and protein was quantified using BCA Protein Assay, following the manufacturer's instructions.

2.1.16. Biotin switch assay

Biotin switch assay allows the identification of protein oxidation or S-nitrosylation, depending on the reducing agent used: DTT for oxidation (Fig. 2.3A) and ascorbate for S-nitrosylation (Fig. 2.3B). Detection of oxidation requires less amount of protein and is more sensitive, thus being easier to detect.

Evaluation of oxidation was performed using 250 μg of protein, and of S-nitrosylation was performed using 500 μg for cell cultures, and 1000 μg for DG lysates, as previously described (Santos et al. 2017). Until binding of biotin, the protocol was performed in the dark. Protein was precipitated with acetone at -20°C for 10 min and centrifuged at 10,760 g for 10 min. Pelleted protein was resuspended in TENS (50 mM Tris pH 7.2, 1 mM EDTA, 0.1 mM neocuproine, 1% SDS). For detection of oxidation, 2.5 mM DTT was added and incubated for 10 min at RT. After acetone precipitation, samples were resuspended in TENS with 1 mM Biotin-HPDP for 1 h at RT. DTT reduced oxidized thiols (SOx), including disulfide bonds, to free thiols (SH), leaving them available to bind Biotin-HPDP. For detection of S-nitrosylation, 100 mM sodium ascorbate and 1 mM Biotin-HPDP were added to TENS, and incubated for 1 h at RT. Ascorbate reduced S-nitrosylated thiols (SNO) to free thiols (SH), leaving them available to bind Biotin-HPDP. Protein was again precipitated with acetone at -20°C for 10 min, centrifuged at 10,760 g for 10 min, and resuspended in HENS (250 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.7, 1 mM EDTA, 0.1 mM neocuproine, 1% SDS) and Jaffrey's (20 mM HEPES pH 7.7, 100 mM NaCl, 1 mM EDTA, 0.5% Triton X-100). After centrifugation at 12,020 g for 2 min, a portion of the supernatant was saved (total protein). NeutrAvidin Plus UltraLink resin was equilibrated by addition of Equilibration buffer (20 mM HEPES pH 7.7, 100 mM NaCl, 1 mM EDTA) and centrifugation at 1,790 g for 1 min, 3 times. The remaining protein supernant was added

to the resin and left for 2 h in agitation, so that proteins bound to biotin (initially oxidized or S-nitrosylated) were retained in the resin, due to the high-affinity binding of biotin to neutravidin. The supernatant containing the proteins not bound to the resin was saved (non-retained protein). Samples were washed 3 times with Washing buffer (20 mM HEPES pH 7.7, 600 mM NaCl, 1 mM EDTA, 0.5% Triton X-100), 1 time with Equilibration buffer, and centrifuged at 1,790 g for 1 min each time. Proteins were released from the resin with Elution buffer (Equilibration buffer with 100 mM β-mercaptoethanol) for 20 min at 37°C, by disruption of biotin-protein binding due to β-mercaptoethanol. After centrifugation at 12,020 g for 1 min, the supernatant was saved (eluted protein) and concentrated using Vacuum Concentrator 5301 (Eppendorf, Hamburg, Germany) at 30°C in vacuum, in order to fit the well of the immunoblot gel.

To evaluate the outcome of the protocol by analyzing the presence or absence of biotin in total protein and non-retained protein, respectively, 4x non-reducing Laemmli buffer (200 mM Tris pH 7.6, 8 mM EDTA, 4% SDS, 40% glycerol, 0.08% Bromophenol blue) was added to a portion of these samples. Proteins were separated by electrophoresis in a 10% acrylamide gel, and transferred to a PVDF membrane using Trans-Blot® TurboTM Transfer System (BioRad Laboratories Inc., Hercules, CA, USA) for 10 min. Membranes were blocked in 3% BSA in TBS-T for 1 h at RT, and incubated with Streptavidin, Peroxidase conjugate (1:2,000) for 30 min at RT. After washing with TBS-T, NZY Supreme ECL HRP Substrate was added to the membranes for 5 min. Chemiluminescence was detected using ChemiDoc XRS+ (BioRad Laboratories Inc., Hercules, CA, USA), and the images were acquired with ImageLab 5.0 (BioRad Laboratories Inc., Hercules, CA, USA).

To analyze oxidation and S-nitrosylation, total protein and eluted protein samples were denatured for 5 min at 95°C after addition of reducing 4x Laemmli buffer with 20% β-mercaptoethanol, and separated by electrophoresis in a 12% acrylamide gel. Proteins were then transferred to a PVDF membrane using Trans-Blot® Turbo™ Transfer System (BioRad Laboratories Inc., Hercules, CA, USA) for 10 min. Membranes were blocked with 3% BSA or 5% milk in TBS-T for 1 h at RT. Primary antibodies were incubated overnight at 4°C (Table 2.5), except for hnRNP K that was incubated 48 h at 4°C. After washing with TBS-T, secondary antibodies (Table 2.6) were incubated 1 h at RT. NZY Supreme ECL HRP Substrate was added to the membranes for 5 min and chemiluminescence was detected using ChemiDoc XRS+ (BioRad Laboratories Inc., Hercules, CA, USA). The images were acquired and analyzed with ImageLab 5.0 (BioRad Laboratories Inc., Hercules, CA, USA). Levels of oxidized/S-nitrosylated protein were normalized with levels of total protein.

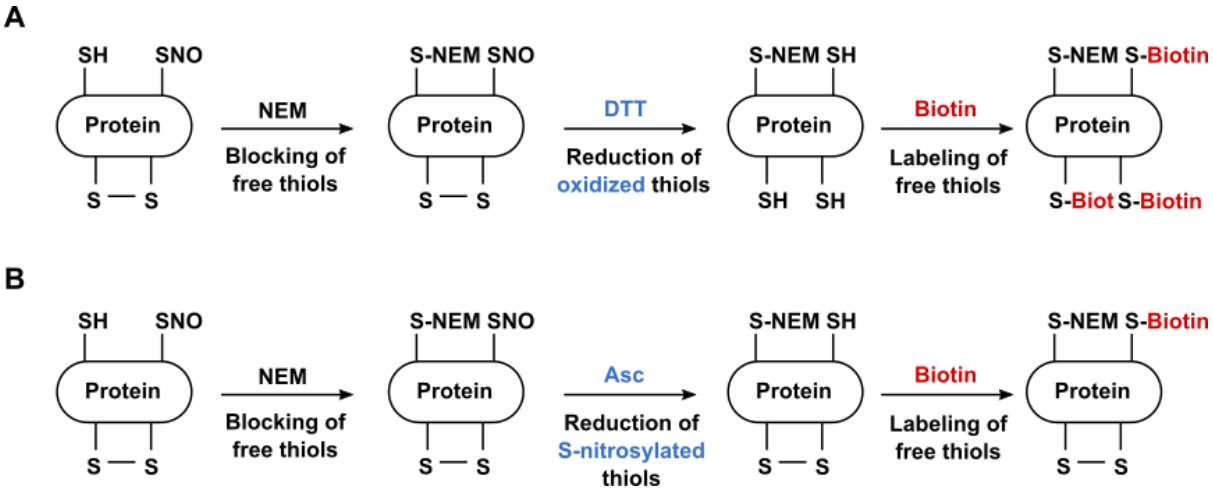


Figure 2.3 - Scheme of the biotin switch assay. Protein free thiol groups are blocked with N-ethylmaleimide (NEM), followed by reduction of oxidized groups by DTT **(A)** or S-nitrosylated groups by ascorbate (asc) **(B)** to free thiols. Biotin is then able to bind to the free thiols.

Table 2.5 - Primary antibodies used in Western Blot

Antibody	Host	Dilution	Company	Reference
hnRNP K	Rabbit	1:1,000	Cell Signaling	4675S
PCNA	Mouse	1:500	Santa Cruz Biotechnology	sc-56
14-3-3	Rabbit	1:1,000	Cell Signaling	8312S
14-3-3 ε	Rabbit	1:1,000	Cell Signaling	9769S
EF-1β	Goat	1:500	Santa Cruz Biotechnology	sc-82870
PEBP-1	Goat	1:10,000	Abcam	ab2634

Table 2.6 - Secondary antibodies used in Western Blot

Antibody	Host	Dilution	Company	Reference
Anti-goat HRP conjugate	Donkey	1:2,000	Life Technologies	A15999
Anti-rabbit HRP conjugate	Goat	1:2,000	Cell Signaling	7074
Anti-mouse HRP conjugate	Donkey	1:2,000	Cell Signaling	7076S

2.1.17. Statistical analysis

Statistical analysis was performed using GraphPad Prism 6.0 with One-way analysis of variance (ANOVA) followed by Dunnett's or Bonferroni's post-hoc tests, Two-way ANOVA and Two-tailed Student's t-test, as indicated in the figure legends. $p<0.05$ was considered statistically significant.

2.2. Materials

Trypsin-EDTA, DMEM-F12 GlutaMAX, B27, N2, PenStrep, Opti-MEM I reduced serum medium, Lipofectamine LTX, laminin, Click-iT EdU Alexa Fluor 488 HCS Assay Kit, Hoechst 33342, BODIPY FL maleimide, BCA Protein Assay, Biotin-HPDP and NeutrAvidin Plus UltraLink resin were purchased from Thermo Fisher Scientific (Waltham, MA, USA). bFGF and EGF were acquired from PeproTech (London, UK). Trypan blue, poly-L-lysine, polyornithine, HBSS, N-ethylmaleimide, neocuproine, sodium ascorbate, Tween 20, coomassie blue, methanol, HEPES, HBSS, MgCl2, EGTA, EDTA, glycerol, acetone, Ampicillin, diethylamine NONOate (DEA/NO) and β-mercaptoethanol were purchased from Sigma Aldrich (St Louis, MO, USA). Primers, NZYMiniprep, NZY Supreme ECL HRP Substrate, NZY Tissue gDNA Isolation kit, NZYTaq 2x Green Master Mix, GreenSafe Premium, DTT, acrylamide, TEMED, ammonium persulfate, Tris and BSA were acquired from NZYTech (Lisboa, Portugal). 10% SDS, SDS, polyvinylidene difluoride (PVDF) membranes and 12% precast gels were from Bio Rad Laboratories Inc. (Hercules, CA, USA) and low-fat dry milk from Nestlé (Vevey, Switzerland). QuickChange II XL Site-Directed Mutagenesis Kit and DAKO fluorescence mounting medium were acquired from Agilent Tecnologies (Santa Clara, CA, USA). cOmplete miniprotease inhibitor cocktail and PhosSTOP phosphatase inhibitor cocktail were purchased from Roche Applied Science (Penzberg, Germany). DETA NONOate (NOC-18) was acquired from Enzo Life Sciences Inc. (Farmingdale, NY, USA), Streptavidin, Peroxidase conjugate from Calbiochem (San Diego, CA, USA), and Endotoxin-free Plasmid DNA Purification from Macherey-Nagel (Duren, Germany). Kainic acid was from Ocean Produce International (Shelburne, Canada) and Tocris Bioscience (Bristol, UK). The origin of primary and secondary antibodies used in immunocytochemistry and Western Blot are listed in tables 2.3, 2.4, 2.5 and 2.6, respectively.

Chapter 2

Chapter 3. Results

Chapter 3

Chapter 3

3.1. PEBP-1 as a target of S-nitrosylation

3.1.1. S-nitrosylation of PEBP-1 in SVZ-derived NSC

3.1.1.1. PEBP-1 is S-nitrosylated by NO in NSC

To investigate if PEBP-1 was S-nitrosylated by NO in NSC, we first evaluated PEBP-1 oxidation, which is easier to detect, more sensitive and requires less amount of protein than analysis of S-nitrosylation. NSC were treated for 15 min with CysNO (100 μM) and oxidation was assessed by DTT-reducing biotin switch assay (Fig. 3.1A). We observed that treatment with CysNO increased the oxidation of PEBP-1, comparing with control (no treatment).

Next, after NSC were treated for 15 min with CysNO (100 μM), S-nitrosylation was evaluated by ascorbate-reducing biotin switch assay (Fig. 3.1B). We observed an increase in S-nitrosylation of PEBP-1 in treated cells, compared to control.

Using shorter exposure periods, NSC were treated with CysNO (100 μM, 1 and 2 min) and S-nitrosylation of PEBP-1 was evaluated by ascorbate-reducing biotin switch assay (Fig. 3.1C). We observed that exposure to CysNO during 1 min (3517.3 ± 1017.3%, $p<0.05$) and 2 min (3905.9 ± 1254.7%, $p<0.05$) significantly increased S-nitrosylation of PEBP-1 comparing with control.

Using a NO donor at a concentration that stimulates proliferation of NSC (NOC-18, 10 μM; (Carreira et al. 2010)), NSC were treated for different times (2.5, 5, 7.5 and 10 min) with NOC-18 to investigate the S-nitrosylation of PEBP-1 by NO over time, assessed by ascorbate-reducing biotin switch assay (Fig. 3.1D). We observed that exposure to NOC-18 for 2.5 min (197.3 ± 45.4%), 5 min (158.5 ± 19.2%) and 7.5 min (174.3 ± 56.9%) showed a tendency to increase S-nitrosylation of PEBP-1 comparing with control. Exposure to NOC-18 for 10 min significantly increased S-nitrosylation of PEBP-1 (314.1 ± 62.5%, $p<0.05$) comparing with control.

It should be noted that the effect on S-nitrosylation of the NO donor NOC-18 is much more modest than the effect of CysNO, while nonetheless it is detectable and significant.

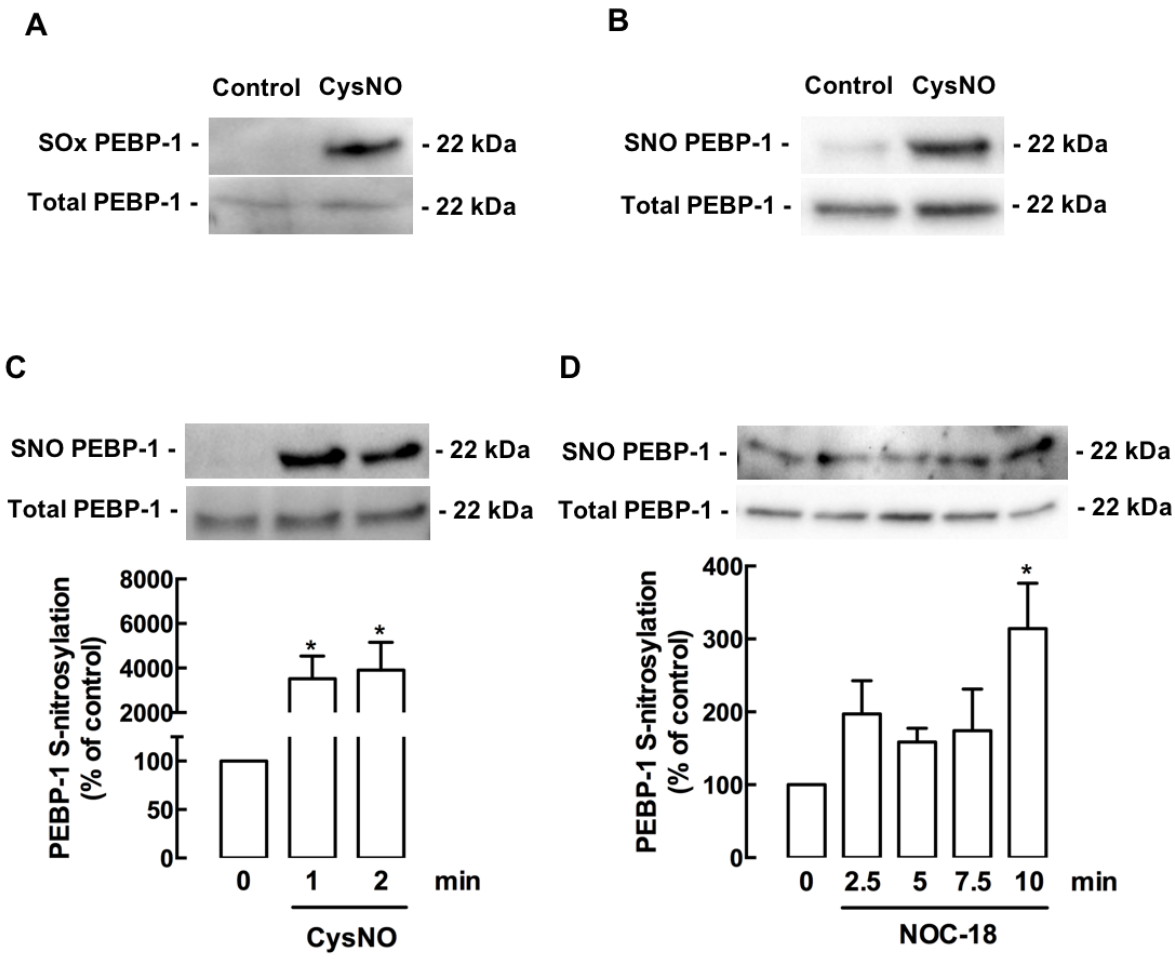


Figure 3.1 - Treatment of NSC with CysNO and NOC-18 induces S-nitrosylation of PEBP-1. SVZ-derived NSC were exposed to CysNO 100 µM for 15 **(A, B)**, 1 and 2 min **(C)**, and to NOC-18 10 µM for 2.5, 5, 7.5 and 10 min **(D)**. Oxidation **(A)** and S-nitrosylation **(B-D)** of PEBP-1 were evaluated by DTT-reducing **(A)** and ascorbate-reducing biotin switch assays **(B-D)**. Data from 6-8 **(C)** and 3-4 **(D)** independent experiments are presented as means ± SEM (% of control, 0, no treatment). One-way ANOVA (Dunnett's post-hoc test), * $p<0.05$ significantly different from control **(C, D)**. SOx PEBP-1, oxidized protein; SNO PEBP-1, S-nitrosylated protein.

3.1.1.2. Mutagenesis of PEBP-1

To investigate the role of PEBP-1 S-nitrosylation by NO in neurogenesis, we overexpressed PEBP-1 and NO-insensitive forms of PEBP-1 in NSC. Since mouse PEBP-1 has 3 cysteines, its tridimensional structure was analyzed in order to identify which of the cysteines would be more exposed and therefore would be more prone to react with NO. C13 (Fig. 3.2A) and C133 (Fig. 3.2B) are located at the surface of the protein, while C168 (Fig. 3.2C) is at the center of PEBP-1 tridimensional structure and less accessible; so it was speculated that mutation of C13 and C133 would produce NO-insensitive PEBP-1, but not mutation of C168. Mutagenesis was performed switching a cysteine for serine, which is also a polar aminoacid but has a hydroxyl group instead of

a thiol group and therefore cannot be S-nitrosylated. We produced constructs with a single mutation of each cysteine (C13S, C133S and C168S), and a double mutant of the most exposed cysteines (C13S/C133S).

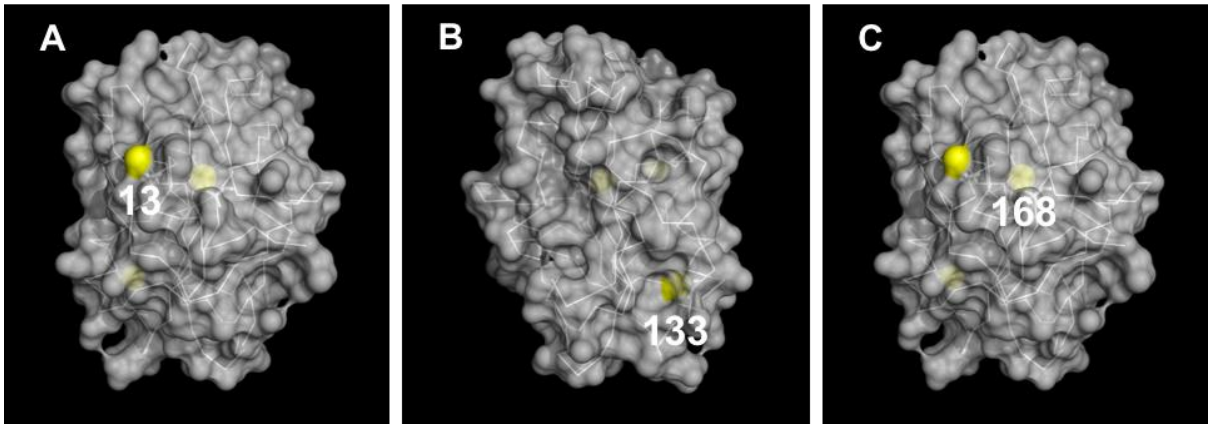


Figure 3.2 - Localization of cysteine amino acids in mouse PEBP-1 tridimensional structure. Schematic representation of mouse PEBP-1 was obtained by similarity with rat PEBP-1 crystal structure (Protein Data Bank), using SWISS-MODEL (Institute of Bioinformatics at the Biozentrum, University of Basel, Switzerland). Cysteines 13 **(A)**, 133 **(B)** and 168 **(C)** are highlighted in yellow. In collaboration with Paulo Martel.

3.1.1.2.1. Construct C13S

C13 was changed to a serine (Ser) by mutating the guanine nucleotide of that amino acid to a cytosine. Efficacy of this procedure was confirmed after sequencing PEBP-1 C13S (C13S, Fig. 3.3A) and comparing it to the sequence of PEBP-1 WT (PEBP-1, Fig. 3.3B). We observed that C13 had, in fact, been mutated to Ser and that no other mutations were introduced in the coding sequence of the gene.

(A) C13S coding sequence

ATG GCC GCC GAC ATC AGC CAG TGG GCC GGG CCC TTG **TCC** TTG CAG GAG GTG GAC GAG CCG CCC CAG CAC GCC CTG CGG GTC GAC TAC GCC GGG GTG ACG GTG GAC GAG CTG GGC AAA GTG CTA ACG CCC ACC CAG GTT ATG AAC AGG CCC AGC AGC ATT TCA TGG GAC GGC CTT GAT CCT GGG AAA CTC TAC ACC CTG GTC CTC ACA GAC CCC GAT GCT CCC AGC AGG AAG GAT CCC AAA TTC AGG GAG TGG CAC CAC TTC CTG GTG GTC AAC ATG AAG GGT AAT GAC ATT AGC AGT GGC ACT GTC CTC TCA GAT TAT GTG GGC TCC GGG CCT CCC AGT GGC ACA GGT CTC CAC CGC TAT GTC TGG CTG GTG TAC GAG CAG GAA CAG CCG CTG AGC **TGC** GAC GAG CCC ATT CTC AGC AAC AAG TCT GGA GAC AAT CGC GGC AAG TTC AAG GTG GAG ACC TTC CGC AAG AAG TAT AAC CTG GGA GCC CCG GTG GCG GGC ACG **TGC** TAC CAA GCC GAG TGG GAT GAC TAT GTG CCC AAG CTG TAC GAG CAG CTG TCA GGG AAG TAG

Ser13; C133; C168

(B) PEBP-1 coding sequence

ATG GCC GCC GAC ATC AGC CAG TGG GCC GGG CCC TTG **TGC** TTG CAG GAG GTG GAC GAG CCG CCC CAG CAC GCC CTG CGG GTC GAC TAC GCC GGG GTG ACG GTG GAC GAG CTG GGC AAA GTG CTA ACG CCC ACC CAG GTT ATG AAC AGG CCC AGC AGC ATT TCA TGG GAC GGC CTT GAT CCT GGG AAA CTC TAC ACC CTG GTC CTC ACA GAC CCC GAT GCT CCC AGC AGG AAG GAT CCC AAA TTC AGG GAG TGG CAC CAC TTC CTG GTG GTC AAC ATG AAG GGT AAT GAC ATT AGC AGT GGC ACT GTC CTC TCA GAT TAT GTG GGC TCC GGG CCT CCC AGT GGC ACA GGT CTC CAC CGC TAT GTC TGG CTG GTG TAC GAG CAG GAA CAG CCG CTG AGC **TGC** GAC GAG CCC ATT CTC AGC AAC AAG TCT GGA GAC AAT CGC GGC AAG TTC AAG GTG GAG ACC TTC CGC AAG AAG TAT AAC CTG GGA GCC CCG GTG GCG GGC ACG **TGC** TAC CAA GCC GAG TGG GAT GAC TAT GTG CCC AAG CTG TAC GAG CAG CTG TCA GGG AAG TAG

C13; C133; C168

Figure 3.3 - Coding sequences of C13S (A) and PEBP-1 (B). After mutation of guanine nucleotide number 59 to a cytosine, sequencing of C13S was compared with the sequence of PEBP-1. Ser13: serine 13; C13: cysteine 13; C133: cysteine 133; C168: cysteine 168.

3.1.1.2.2. Construct C133S

C133 was changed to Ser by mutating the guanine nucleotide of that amino acid to a cytosine. Efficacy of this procedure was confirmed after sequencing PEBP-1 C133S (C133S, Fig. 3.4A) and comparing it to the sequence of PEBP-1 WT (PEBP-1, Fig. 3.4B). We observed that C133 had, in fact, been mutated to Ser and that no other mutations were introduced in the coding sequence of the gene.

(A) C133S coding sequence

ATG GCC GCC GAC ATC AGC CAG TGG GCC GGG CCC TTG **TGC** TTG CAG GAG GTG GAC GAG CCG CCC CAG CAC GCC CTG CGG GTC GAC TAC GCC GGG GTG ACG GTG GAC GAG CTG GGC AAA GTG CTA ACG CCC ACC CAG GTT ATG AAC AGG CCC AGC AGC ATT TCA TGG GAC GGC CTT GAT CCT GGG AAA CTC TAC ACC CTG GTC CTC ACA GAC CCC GAT GCT CCC AGC AGG AAG GAT CCC AAA TTC AGG GAG TGG CAC CAC TTC CTG GTG GTC AAC ATG AAG GGT AAT GAC ATT AGC AGT GGC ACT GTC CTC TCA GAT TAT GTG GGC TCC GGG CCT CCC AGT GGC ACA GGT CTC CAC CGC TAT GTC TGG CTG GTG TAC GAG CAG GAA CAG CCG CTG AGC **TCC** GAC GAG CCC ATT CTC AGC AAC AAG TCT GGA GAC AAT CGC GGC AAG TTC AAG GTG GAG ACC TTC CGC AAG AAG TAT AAC CTG GGA GCC CCG GTG GCG GGC ACG **TGC** TAC CAA GCC GAG TGG GAT GAC TAT GTG CCC AAG CTG TAC GAG CAG CTG TCA GGG AAG TAG

C13; Ser133; C168

(B) PEBP-1 coding sequence

ATG GCC GCC GAC ATC AGC CAG TGG GCC GGG CCC TTG **TGC** TTG CAG GAG GTG GAC GAG CCG CCC CAG CAC GCC CTG CGG GTC GAC TAC GCC GGG GTG ACG GTG GAC GAG CTG GGC AAA GTG CTA ACG CCC ACC CAG GTT ATG AAC AGG CCC AGC AGC ATT TCA TGG GAC GGC CTT GAT CCT GGG AAA CTC TAC ACC CTG GTC CTC ACA GAC CCC GAT GCT CCC AGC AGG AAG GAT CCC AAA TTC AGG GAG TGG CAC CAC TTC CTG GTG GTC AAC ATG AAG GGT AAT GAC ATT AGC AGT GGC ACT GTC CTC TCA GAT TAT GTG GGC TCC GGG CCT CCC AGT GGC ACA GGT CTC CAC CGC TAT GTC TGG CTG GTG TAC GAG CAG GAA CAG CCG CTG AGC **TGC** GAC GAG CCC ATT CTC AGC AAC AAG TCT GGA GAC AAT CGC GGC AAG TTC AAG GTG GAG ACC TTC CGC AAG AAG TAT AAC CTG GGA GCC CCG GTG GCG GGC ACG **TGC** TAC CAA GCC GAG TGG GAT GAC TAT GTG CCC AAG CTG TAC GAG CAG CTG TCA GGG AAG TAG

C13; C133; C168

Figure 3.4 - Coding sequences of C133S (A) and PEBP-1(B). After mutation of guanine nucleotide number 419 to a cytosine, sequencing of C133S was compared with the sequence of PEBP-1. Ser133: serine 133; C13: cysteine 13; C133: cysteine 133; C168: cysteine 168.

3.1.1.2.3. Construct C13S/C133S

C13 and C133 were changed to Ser by mutating the guanine nucleotide of those amino acids to cytosine. Efficacy of this procedure was confirmed after sequencing PEBP-1 C13S/C133S (C31S/C133S, Fig. 3.5A) and comparing it to the sequence of PEBP-1 WT (PEBP-1, Fig. 3.5B). We observed that both C13 and C133 had, in fact, been mutated to Ser and that no other mutations were introduced in the coding sequence of the gene.

(A) C13S/C133S coding sequence

ATG GCC GCC GAC ATC AGC CAG TGG GCC GGG CCC TTG **TCC** TTG CAG GAG GTG GAC GAG CCG CCC CAG CAC GCC CTG CGG GTC GAC TAC GCC GGG GTG ACG GTG GAC GAG CTG GGC AAA GTG CTA ACG CCC ACC CAG GTT ATG AAC AGG CCC AGC AGC ATT TCA TGG GAC GGC CTT GAT CCT GGG AAA CTC TAC ACC CTG GTC CTC ACA GAC CCC GAT GCT CCC AGC AGG AAG GAT CCC AAA TTC AGG GAG TGG CAC CAC TTC CTG GTG GTC AAC ATG AAG GGT AAT GAC ATT AGC AGT GGC ACT GTC CTC TCA GAT TAT GTG GGC TCC GGG CCT CCC AGT GGC ACA GGT CTC CAC CGC TAT GTC TGG CTG GTG TAC GAG CAG GAA CAG CCG CTG AGC **TCC** GAC GAG CCC ATT CTC AGC AAC AAG TCT GGA GAC AAT CGC GGC AAG TTC AAG GTG GAG ACC TTC CGC AAG AAG TAT AAC CTG GGA GCC CCG GTG GCG GGC ACG **TGC** TAC CAA GCC GAG TGG GAT GAC TAT GTG CCC AAG CTG TAC GAG CAG CTG TCA GGG AAG TAG

Ser13; Ser133; C168

(B) PEBP-1 coding sequence

ATG GCC GCC GAC ATC AGC CAG TGG GCC GGG CCC TTG **TGC** TTG CAG GAG GTG GAC GAG CCG CCC CAG CAC GCC CTG CGG GTC GAC TAC GCC GGG GTG ACG GTG GAC GAG CTG GGC AAA GTG CTA ACG CCC ACC CAG GTT ATG AAC AGG CCC AGC AGC ATT TCA TGG GAC GGC CTT GAT CCT GGG AAA CTC TAC ACC CTG GTC CTC ACA GAC CCC GAT GCT CCC AGC AGG AAG GAT CCC AAA TTC AGG GAG TGG CAC CAC TTC CTG GTG GTC AAC ATG AAG GGT AAT GAC ATT AGC AGT GGC ACT GTC CTC TCA GAT TAT GTG GGC TCC GGG CCT CCC AGT GGC ACA GGT CTC CAC CGC TAT GTC TGG CTG GTG TAC GAG CAG GAA CAG CCG CTG AGC **TGC** GAC GAG CCC ATT CTC AGC AAC AAG TCT GGA GAC AAT CGC GGC AAG TTC AAG GTG GAG ACC TTC CGC AAG AAG TAT AAC CTG GGA GCC CCG GTG GCG GGC ACG **TGC** TAC CAA GCC GAG TGG GAT GAC TAT GTG CCC AAG CTG TAC GAG CAG CTG TCA GGG AAG TAG

C13; C133; C168

Figure 3.5 - Coding sequences of C13S/C133S (A) and PEBP-1 (B). After mutation of guanine nucleotides number 59 and 419 to cytosines, sequencing of C13S/C133S was compared with the sequence of PEBP-1. Ser13: serine 13; Ser133: serine 133; C13: cysteine 13; C133: cysteine 133; C168: cysteine 168.

3.1.1.2.4. Construct C168S

C168 was changed to Ser by mutating the guanine nucleotide of that aminoacid to a cytosine. Efficacy of this procedure was confirmed after sequencing PEBP-1 C168S (C168S, Fig. 3.6A) and comparing it to the sequence of PEBP-1 WT (PEBP-1, Fig. 3.6B). We observed that C168 had, in fact, been mutated to Ser and that no other mutations were introduced in the coding sequence of the gene.

(A) C168S coding sequence

ATG GCC GCC GAC ATC AGC CAG TGG GCC GGG CCC TTG **TGC** TTG CAG GAG GTG GAC GAG CCG CCC CAG CAC GCC CTG CGG GTC GAC TAC GCC GGG GTG ACG GTG GAC GAG CTG GGC AAA GTG CTA ACG CCC ACC CAG GTT ATG AAC AGG CCC AGC AGC ATT TCA TGG GAC GGC CTT GAT CCT GGG AAA CTC TAC ACC CTG GTC CTC ACA GAC CCC GAT GCT CCC AGC AGG AAG GAT CCC AAA TTC AGG GAG TGG CAC CAC TTC CTG GTG GTC AAC ATG AAG GGT AAT GAC ATT AGC AGT GGC ACT GTC CTC TCA GAT TAT GTG GGC TCC GGG CCT CCC AGT GGC ACA GGT CTC CAC CGC TAT GTC TGG CTG GTG TAC GAG CAG GAA CAG CCG CTG AGC **TGC** GAC GAG CCC ATT CTC AGC AAC AAG TCT GGA GAC AAT CGC GGC AAG TTC AAG GTG GAG ACC TTC CGC AAG AAG TAT AAC CTG GGA GCC CCG GTG GCG GGC ACG **TCC** TAC CAA GCC GAG TGG GAT GAC TAT GTG CCC AAG CTG TAC GAG CAG CTG TCA GGG AAG TAG

C13; C133; Ser168

(B) PEBP-1 coding sequence

ATG GCC GCC GAC ATC AGC CAG TGG GCC GGG CCC TTG **TGC** TTG CAG GAG GTG GAC GAG CCG CCC CAG CAC GCC CTG CGG GTC GAC TAC GCC GGG GTG ACG GTG GAC GAG CTG GGC AAA GTG CTA ACG CCC ACC CAG GTT ATG AAC AGG CCC AGC AGC ATT TCA TGG GAC GGC CTT GAT CCT GGG AAA CTC TAC ACC CTG GTC CTC ACA GAC CCC GAT GCT CCC AGC AGG AAG GAT CCC AAA TTC AGG GAG TGG CAC CAC TTC CTG GTG GTC AAC ATG AAG GGT AAT GAC ATT AGC AGT GGC ACT GTC CTC TCA GAT TAT GTG GGC TCC GGG CCT CCC AGT GGC ACA GGT CTC CAC CGC TAT GTC TGG CTG GTG TAC GAG CAG GAA CAG CCG CTG AGC **TGC** GAC GAG CCC ATT CTC AGC AAC AAG TCT GGA GAC AAT CGC GGC AAG TTC AAG GTG GAG ACC TTC CGC AAG AAG TAT AAC CTG GGA GCC CCG GTG GCG GGC ACG **TGC** TAC CAA GCC GAG TGG GAT GAC TAT GTG CCC AAG CTG TAC GAG CAG CTG TCA GGG AAG TAG

C13; C133; C168

Figure 3.6 - Coding sequences of C168S (A) and PEBP-1 (B). After mutation of guanine nucleotide number 524 to a cytosine, sequencing of C168S was compared with the sequence of PEBP-1. Ser168: serine 168; C13: cysteine 13; C133: cysteine 133; C168: cysteine 168.

3.1.1.3. Overexpression of PEBP-1 in NSC

Overexpression of normal and mutant PEBP-1 in NSC was assessed by immunocytochemistry and detected by fluorescence microscopy. The antibody used for detection of PEBP-1 was shown to detect PEBP-1 in the cytoplasm of NSC (Fig. 3.7).

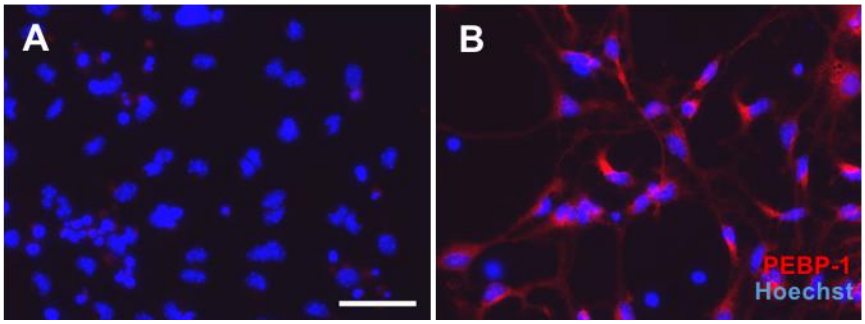


Figure 3.7 - PEBP-1 is present in the cytoplasm of NSC. Presence of PEBP-1 in SVZ-derived NSC was assessed by immunocytochemistry. Specificity of antibody binding was evaluated without anti-PEBP-1 antibody **(A)**. Representative image of PEBP-1 in SVZ-derived NSC **(B)**. PEBP-1, red; nuclei labeled with Hoechst 33342, blue. Scale bar: 40 µm.

To assess the outcome of transfection, NSC were transfected with the constructs of normal (Fig. 3.8B) and mutant PEBP-1 (Fig. 3.8C-F), and overexpression was evaluated by immunocytochemistry. Signal was considered positive for overexpression when the signal of endogenous PEBP-1 was reduced to background levels by the strongly labeled transfected cells. We observed that transfection of NSC with the PEBP-1 constructs induced overexpression resulting in high levels of this protein (Fig. 3.8B-F). Overexpression of normal PEBP-1 was also assessed by Western blot, which confirmed the increase in PEBP-1 levels (Fig. 3.8A).

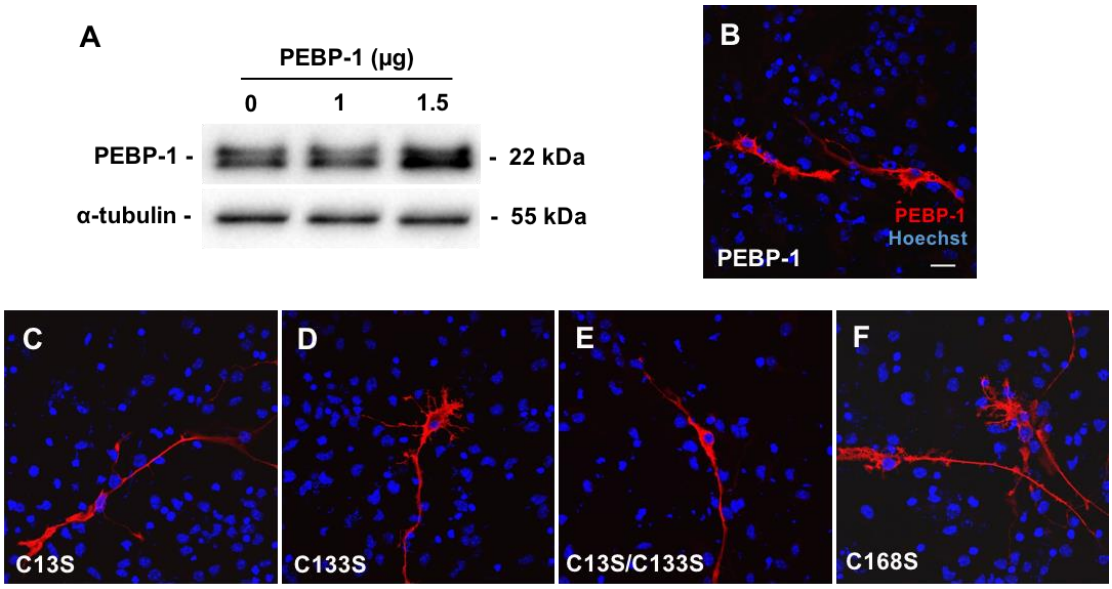


Figure 3.8 - Transfection of NSC with the different constructs induces PEBP-1 overexpression. SVZ-derived NSC were transfected with PEBP-1 **(A, B)**, C13S **(C)**, C133S **(D)**, C13S/C133S **(E)** and C168S **(F)** constructs. Overexpression of PEBP-1 was evaluated by Western blot using α-tubulin as a loading control **(A)** and by immunocytochemistry **(B-F)**. PEBP-1, red; nuclei labeled with Hoechst 33342, blue. Scale bar: 40 µm.

3.1.1.4. S-nitrosylation of PEBP-1 mutants

To assess if the observed S-nitrosylation of PEBP-1 was due to reaction of NO with PEBP-1 cysteines, we evaluated S-nitrosylation of this protein in the absence of each cysteine, by ascorbate-reducing biotin switch assay. NSC were transfected with normal and mutant PEBP-1 constructs and treated with 10 µM NOC-18 for 10 min, the time point at which we previously observed highest S-nitrosylation levels of PEBP-1. We observed that treatment of transfected NSC with NOC-18 did not change the levels of S-nitrosylation of PEBP-1 comparing with non-treated cells (Fig. 3.9).

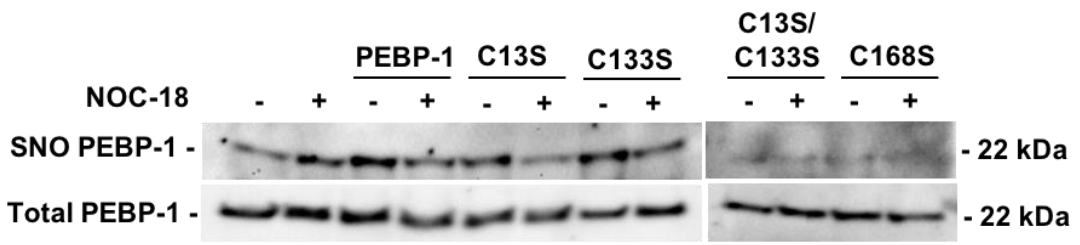


Figure 3.9 - Treatment of NSC with NOC-18 maintains S-nitrosylation of PEBP-1 after transfection with the different constructs. SVZ-derived NSC were transfected with PEBP-1, C13S, C133S, C13S/C133S and C168S constructs and treated with 10 µM NOC-18 for 10 min. S-nitrosylation of PEBP-1 was evaluated by ascorbate-reducing biotin switch assay. Representative image of 3 independent experiments. SNO PEBP-1, S-nitrosylated protein.

Due to the lack of effect of treatment of NOC-18, we compared the potential to induce S-nitrosylation of proteins of CysNO, NOC-18 and another NO donor with a shorter half-life (DEA/NO) using fluorescence switch assay. However, detection of S-nitrosylation (Fig. 3.10A, ascorbate) using this technique required higher amount of protein and was more difficult to achieve, when comparing with oxidation (Fig. 3.10A, DTT). Since S-nitrosylation is an oxidative reaction, we compared the effect of the compounds in protein oxidation. NSC were treated with 100 μM CysNO for 15 min, 10 μM NOC-18 for 1 h, and 100 μM and 1 mM DEA/NO for 15 min. We observed that treatment with both NO donors did not change protein oxidation, comparing with control, and that CysNO induced oxidation of proteins (Fig. 3.10B).

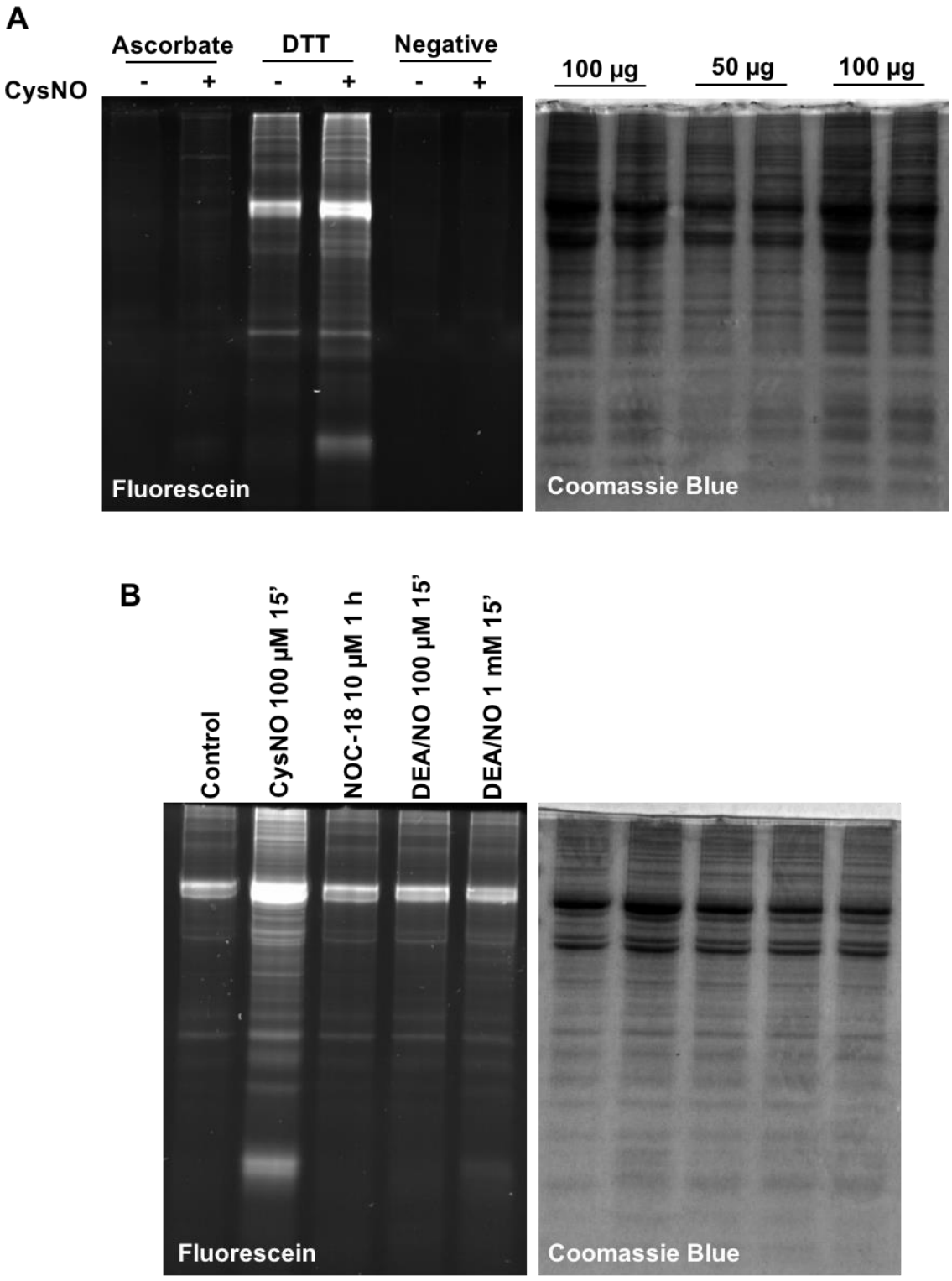


Figure 3.10 - Treatment of NSC with CysNO, but not NOC-18 or DEA/NO, induces strong oxidation of proteins. SVZ-derived NSC were treated with 100 μM CysNO for 15 min **(A, B)**, 100 μM and 1 mM DEA/NO for 15 min, and 10 μM NOC-18 for 1 h **(B)**. Oxidation was assessed by DTT-reducing fluorescence switch **(A, B)** and S-nitrosylation was assessed by ascorbate-reducing fluorescence switch **(A)**. A negative control without addition of DTT and ascorbate was performed. Fluorescein, S-nitrosylated or oxidized protein; Coomassie Blue, total protein.

Having established that treatment with CysNO was the best option to be able to detect protein oxidation/S-nitrosylation, S-nitrosylation of PEBP-1 was assessed in transfected NSC treated with 100 μM CysNO for 15 min. We observed that treatment of transfected NSC with CysNO induced S-nitrosylation of PEBP-1, regardless of cysteine mutation (Fig. 3.11).

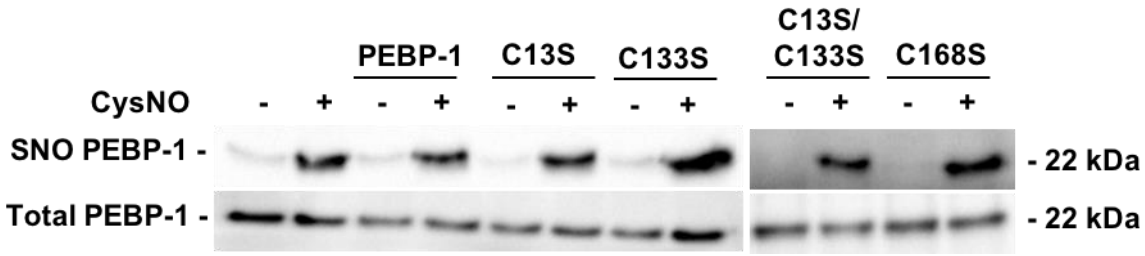


Figure 3.11 - Treatment of NSC with CysNO induces S-nitrosylation of PEBP-1 after transfection with the different constructs. SVZ-derived NSC were transfected with PEBP-1, C13S, C133S, C13S/C133S and C168S constructs and treated with 100 μM CysNO for 15 min. S-nitrosylation of PEBP-1 was evaluated by ascorbate-reducing biotin switch assay. Representative image of 2-4 independent experiments. SNO PEBP-1, S-nitrosylated protein.

3.1.1.5. PEBP-1 cysteine 133 is necessary for NO-dependent activation of ERK

If NO-mediated S-nitrosylation of PEBP-1 affects neurogenesis stimulated by NO, the next step would be to investigate whether S-nitrosylation of PEBP-1 can affect the activation of the ERK/MAPK pathway, which was described to occur shortly after NO treatment in NSC (Carreira et al, 2010), in cells expressing either PEBP-1 or the various cysteine mutants. ERK phosphorylation was assessed by immunocytochemistry. To evaluate the optimal conditions for ERK phosphorylation induced by NO, NSC were treated with 10 μM CysNO for 5 min and 10 μM NOC-18 for 20 min. As a positive control NSC were treated with 20 ng/ml EGF for 20 min. Untreated cells were considered the control. We observed that treatment with CysNO (Fig. 3.12B) and NOC-18 (Fig. 3.12C) increased ERK phosphorylation comparing with control (Fig. 3.12A). The highest increase in P-ERK levels was achieved in cells treated with the positive control EGF (Fig. 3.12D).

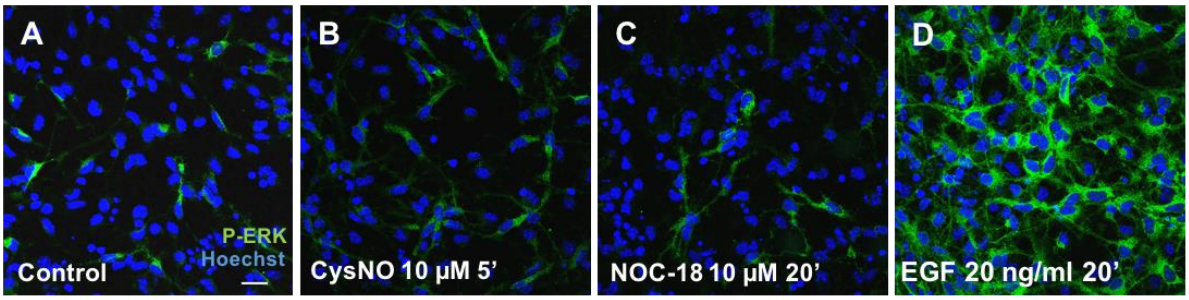
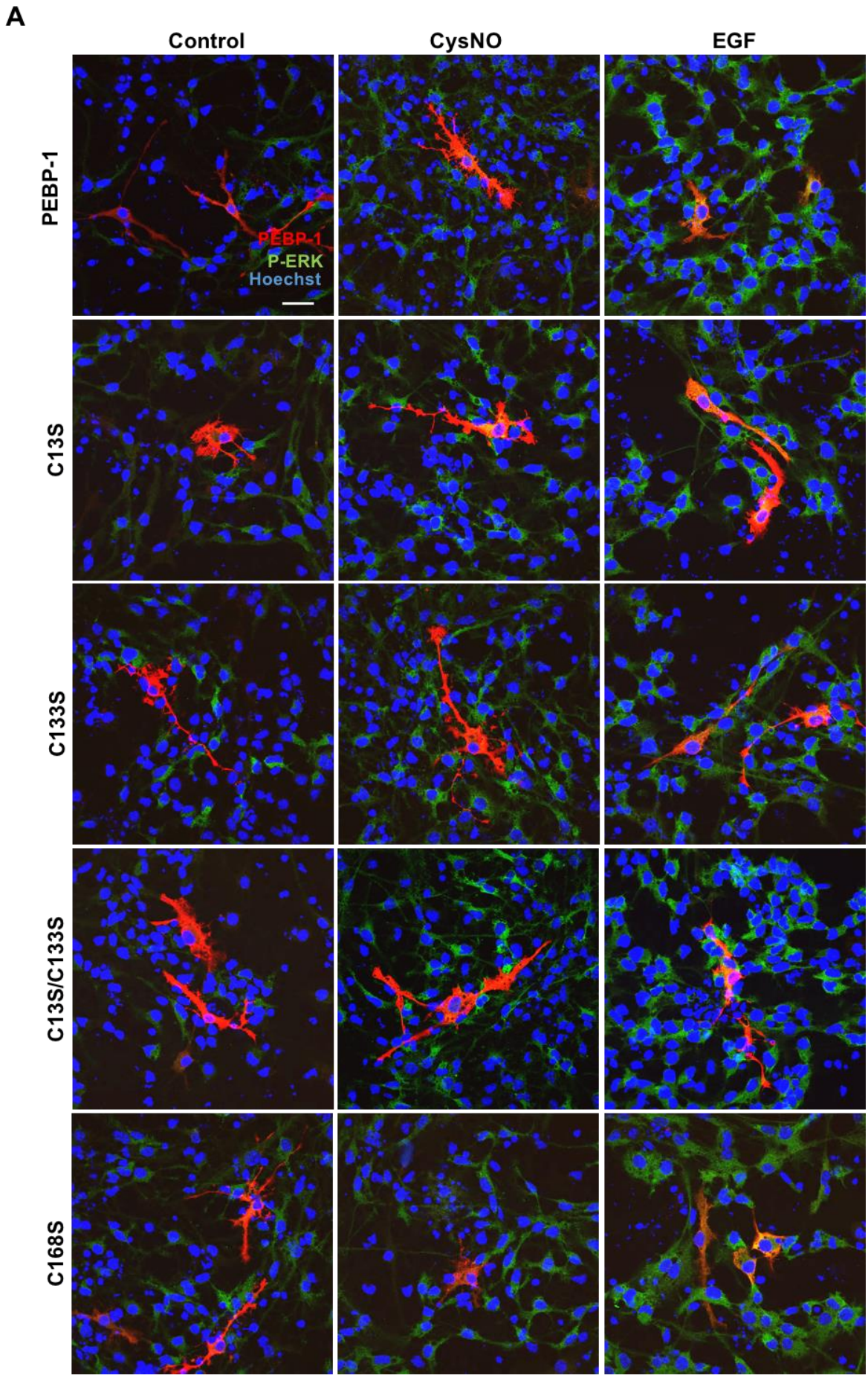


Figure 3.12 - Treatment of NSC with CysNO and NOC-18 increases ERK phosphorylation. SVZ-derived NSC were treated with 10 μM CysNO for 5 min **(B)** and 10 μM NOC-18 for 20 min **(C)**. Cells without treatment **(A)** were considered the control, and cells treated with 20 ng/ml EGF for 20 min were used as a positive control **(D)**. ERK phosphorylation was detected by immunocytochemistry. P-ERK, green; nuclei labeled with Hoechst 33342, blue. Scale bar: 40 μm.

To assess the role of PEBP-1 S-nitrosylation in ERK phosphorylation caused by NO, NSC were transfected with normal and mutant PEBP-1 constructs and treated with 10 μM CysNO for 5 min (Fig. 3.13A). Cells treated with 20 ng/ml EGF were used as a positive control, and cells with no treatment were considered the control (Fig. 3.13A). Fluorescence of P-ERK was measured in PEBP-1-overexpressing cells (Fig. 3.13B, 3.13C). Treatment of NSC transfected with PEBP-1 with CysNO significantly increased P-ERK levels (11.4 ± 1.1%, $p<0.05$, Fig. 3.13B), comparing with control (7.4 ± 1.3%). NSC transfected with C13S (10.6 ± 0.9%) and C168S (10.1 ± 1.2%) treated with CysNO showed a tendency to have increased P-ERK levels, comparing with control (C13S, 8.1 ± 0.9%; C168S, 8.1 ± 1.2%). Transfection of NSC with C133S (8.9 ± 1.4%) and C13S/C133S (7.8 ± 0.7%) did not increase P-ERK levels after treatment with CysNO, comparing with control (C133S, 7.6 ± 0.4%; C13S/C133S, 7.4 ± 1.3%). Treatment with EGF significantly increased P-ERK levels in transfected NSC, regardless of the construction (PEBP-1, 18.6 ± 1.4% $p<0.001$; C13S, 16.6 ± 2.1% $p<0.01$; C133S, 16.7 ± 1.7% $p<0.001$; C13S/C133S, 19.7 ± 1.7% $p<0.001$; C168S, 18.9 ± 1.5% $p<0.001$, Fig. 3.13C), comparing with the respective controls.


A
Control
CysNO
EGF
PEBP-1
C13S
C133S
C13S/C133S
C168S
PEBP-1
P-ERK
Hoechst

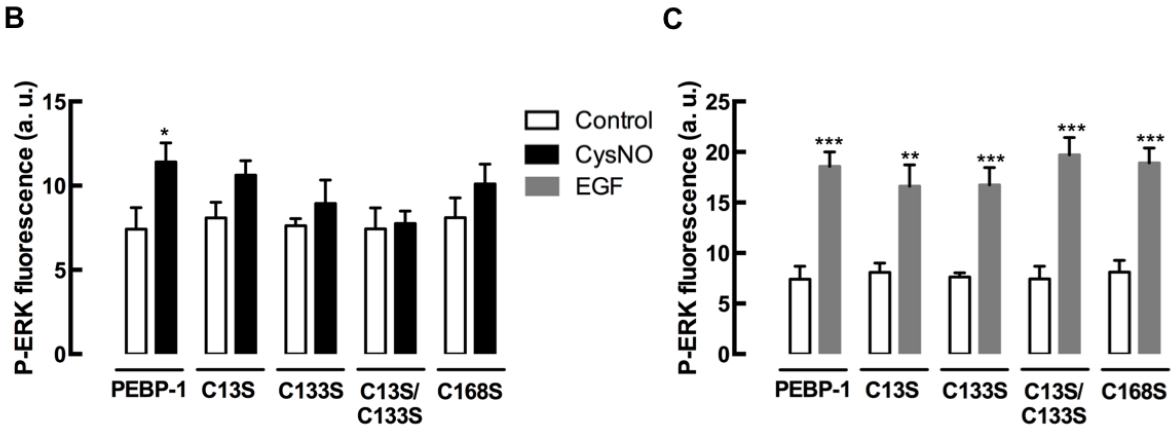
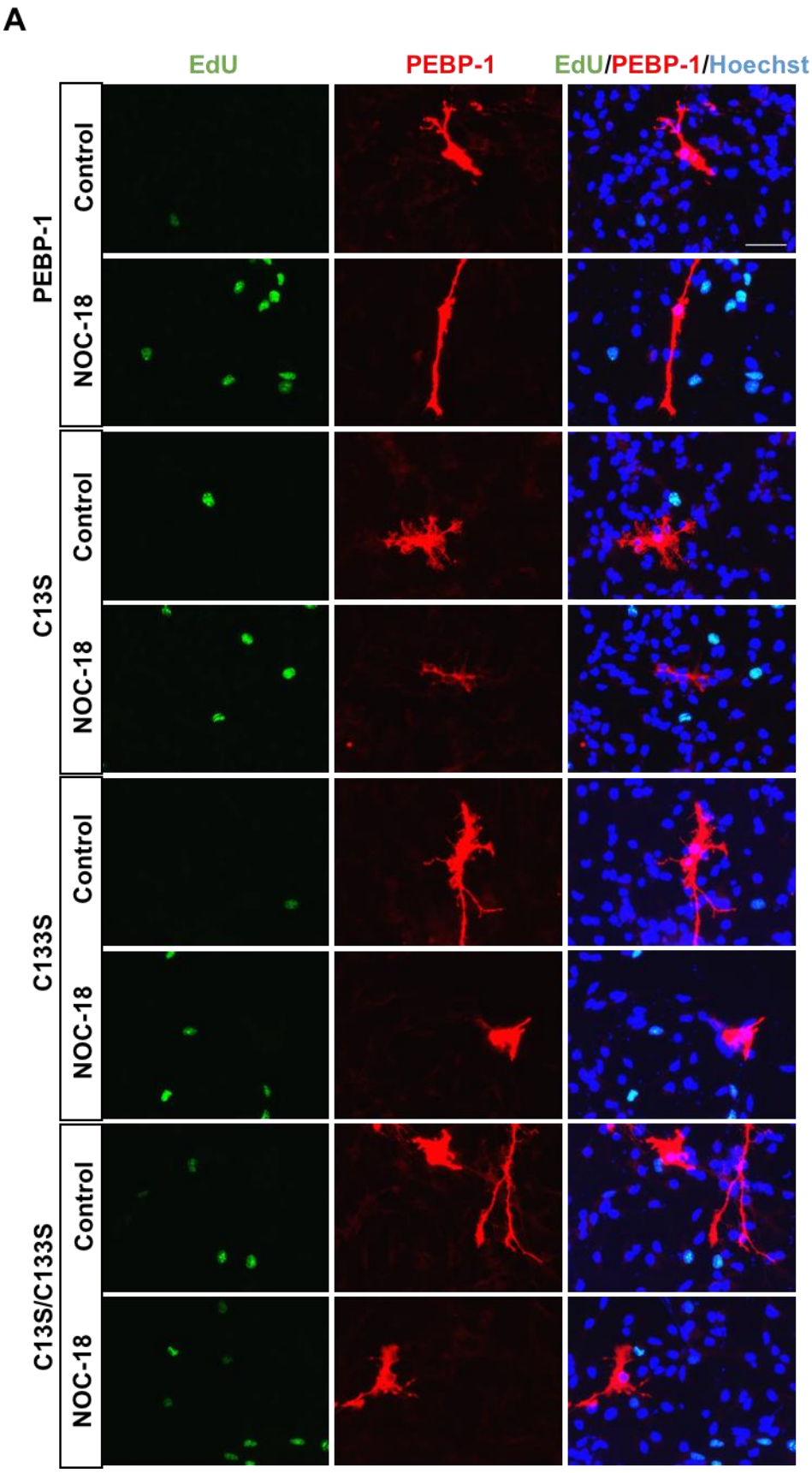


Figure 3.13 - Transfection of NSC with C13S/C133S construct prevents the increase in ERK phosphorylation induced by CysNO. SVZ-derived NSC were transfected with PEBP-1, C13S, C133S, C13S/C133S and C168S constructs, and treated with 10 µM CysNO for 5 min. Cells without treatment were considered the control, and cells treated with 20 ng/ml EGF for 20 min were used as a positive control. ERK phosphorylation and overexpression of PEBP-1 were detected by immunocytochemistry. Representative images are shown **(A)**. PEBP-1, red; P-ERK, green; nuclei labeled with Hoechst 33342, blue. Scale bar: 40 µm. Data from 4-5 independent experiments are presented as means ± SEM of arbitrary units of P-ERK fluorescence **(B, C)**. Two-tailed Student's t-test, * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ significantly different from control.

3.1.1.6. PEBP-1 overexpression inhibits NSC proliferation

To evaluate the effect of S-nitrosylation of PEBP-1 in cell proliferation, NSC were transfected with normal and mutant PEBP-1 constructs, treated with 10 µM NOC-18 for 1 h, and the incorporation of EdU was analyzed (3.14A). Cells with no treatment were considered the control. Treatment with NOC-18 significantly increased the number of EdU-positive cells in non-transfected cells, comparing with control, in PEBP-1 (control 1.6 ± 0.3%, NOC-18 5.3 ± 1.1%, $p<0.05$), C13S (control 2.2 ± 0.3%, NOC-18 5.1 ± 0.2%, $p<0.01$), C133S (control 1.7 ± 0.4%, NOC-18 4.7 ± 0.7%, $p<0.05$) and C13S/C133S (control 2.3 ± 0.3%, NOC-18 5.5 ± 0.5%, $p<0.05$) transfection conditions (Fig. 3.14B). We also observed that non-treated transfected cells were EdU-negative, and that this effect was not prevented with treatment with NOC-18 (Fig. 3.14A).


A
EdU
PEBP-1
EdU/PEBP-1/Hoechst
PEBP-1
Control
NOC-18
C13S
Control
NOC-18
C133S
Control
NOC-18
C13S/C133S
Control
NOC-18

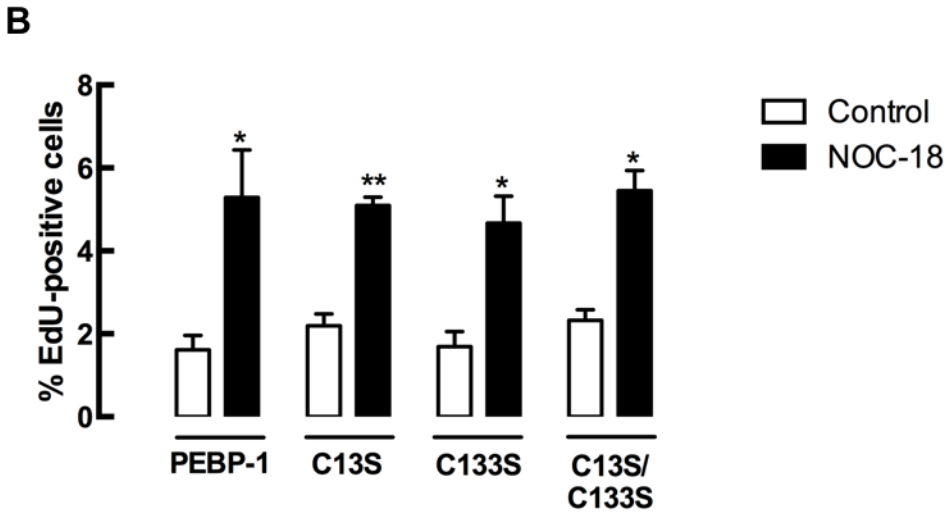


Figure 3.14 - Overexpression of normal and mutant PEBP-1 inhibits proliferation of NSC, which is not prevented by treatment with NOC-18. SVZ-derived NSC were transfected with PEBP-1, C13S, C133S and C13S/C133S constructs, and treated with 10 µM NOC-18 for 1 h. Cells without treatment were considered the control. Proliferation was assessed by incorporation of EdU (10 µM, 1 h), analyzed by fluorescence microscopy. Representative images are shown (A). EdU, green; PEBP-1, red; nuclei labeled with Hoechst 33342, blue. Scale bar: 40 µm. Data from 2-3 independent experiments are presented as means ± SEM of the number of positive cells for EdU of the total non-apoptotic cells (%) (B). Two-tailed Student's t-test, * $p<0.05$ and ** $p<0.01$ significantly different from control.

3.1.2. S-nitrosylation of PEBP-1 in hippocampus after seizures

3.1.2.1. PEBP-1 is S-nitrosylated by NO in the DG after seizures

To study the role of PEBP-1 S-nitrosylation in post-injury neurogenesis, we used an excitotoxic lesion mouse model that is characterized by an increase in neurogenesis in the DG after induction of seizures with KA (Parent 2007). In this model, NO is essential for the increased neurogenic response observed as a reaction to seizures induced by KA (Carreira et al. 2010). WT mice were injected with KA (25 mg/kg, s.c.) or SAL (control) and sacrificed 1, 2, 3 and 5 days later for dissection of the DG. S-nitrosylation of PEBP-1 was evaluated by ascorbate-reducing biotin switch assay (Fig. 3.15A). We observed that, 1 day after KA injection, S-nitrosylation of PEBP-1 significantly increased (173.9 ± 16.4%, $p<0.01$) comparing with control (SAL). S-nitrosylation of PEBP-1 was unchanged 2 (86.3 ± 11.8%), 3 (123.2 ± 31.4%) and 5 days (63.2 ± 10.8%) after seizure onset, comparing with control (SAL).

To assess the role of NO in the increase of PEBP-1 S-nitrosylation 1 day after SE, *iNOS* KO^- mice were injected with KA or SAL, sacrificed 1 day later and DG was dissected. PEBP-1 S-nitrosylation was evaluated by ascorbate-reducing biotin switch assay (Fig.

3.15B). We observed that S-nitrosylation of PEBP-1 significantly decreased 1 day after seizure onset (58.3 ± 8.9%, p<0.01) comparing with control (SAL).

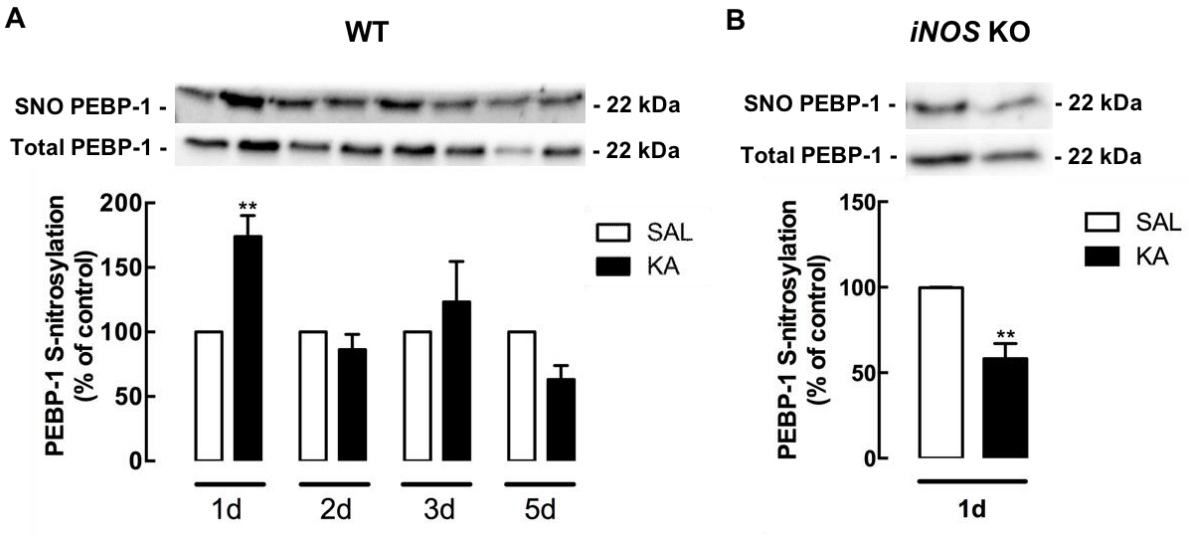


Figure 3.15 - PEBP-1 S-nitrosylation is transiently induced by NO after seizures, in the DG. Wild type **(A)** and *iNOS* KO **(B)** C57Bl6 mice were subcutaneously injected with a sterile solution of kainic acid (KA, 25 mg/kg) in 0.9% NaCl, achieving epileptic seizures. Control animals were injected the vehicle saline solution (SAL). The animals were sacrificed 1, 2, 3 and 5 days after the injection, and protein lysates of the DG were used to analyze S-nitrosylation of PEBP-1 by ascorbate-reducing biotin switch assay. Data from 5-6 animals are presented as means ± SEM and (% of control, SAL). Two-way ANOVA followed by Bonferroni's post-hoc test **(A)** and Student's t-test **(B),** ** p<0.01 significantly different from saline. SNO PEBP-1, S-nitrosylated protein; SAL, saline; KA, kainic acid; d, days.

To investigate S-nitrosylation of PEBP-1 in the stem cells of the DG, cultured hippocampal stem cells were treated with different concentrations of CysNO (10, 50 and 100 μM) for 15 min, and S-nitrosylation of PEBP-1 was analyzed by ascorbate-reducing biotin switch (Fig. 3.16). We observed that S-nitrosylation of PEBP-1 was unchanged after treatment with 10 μM CysNO (98.4 ± 5.4%), comparing with control. Treatment with 50 μM CysNO showed a tendency to increase S-nitrosylation of PEBP-1 (204.3 ± 73.7%) and treatment with 100 μM CysNO significantly increased PEBP-1 S-nitrosylation (339.9 ± 100.0%, p<0.05), comparing with control.

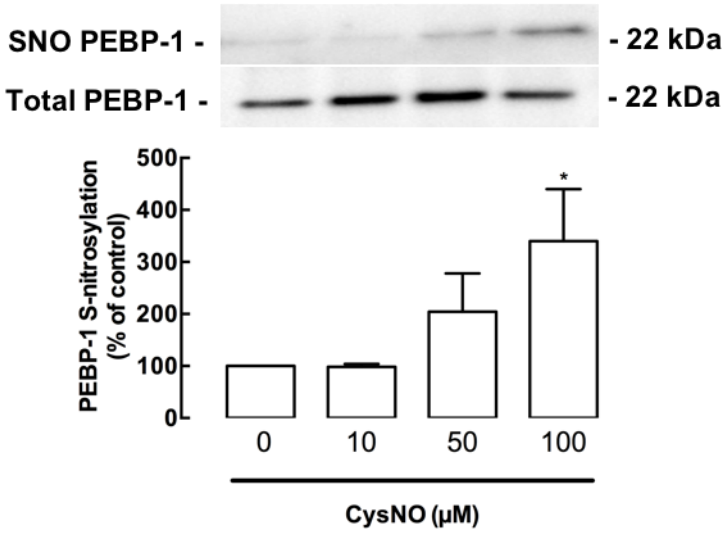


Figure 3.16 - Treatment of hippocampal stem cells with CysNO induces PEBP-1 S-nitrosylation in a dose-dependent manner. Hippocampal stem cells were treated with 10 µM, 50 µM and 100 µM CysNO for 15 min. S-nitrosylation of PEBP-1 was assessed by ascorbate-reducing biotin switch assay. Data from 4 independent experiments are presented as means ± SEM (% of control, 0, no treatment). One-Way ANOVA followed by Dunnett's post-hoc test, * $p<0.05$ significantly different from control. SNO PEBP-1, S-nitrosylated protein.

Chapter 3

3.2. S-nitrosylation targets of NO after brain injury

3.2.1. hnRNP K, 14-3-3 and 14-3-3 ε are S-nitrosylated after KA-induced seizures

Previous work by our group identified several proteins that are S-nitrosylated by NO in SVZ-derived NSC (Ana I. Santos, unpublished results). We aimed to assess if these proteins were S-nitrosylated in a model of hippocampal post-injury neurogenesis mediated by NO. To validate the new putative S-nitrosylation targets of NO, we used the same seizure model as described above for PEBP-1. Mice were injected with KA (25 mg/kg) or SAL (control) and sacrificed 1, 2, 3 and 5 days later for dissection of the DG. S-nitrosylation of proteins was evaluated by ascorbate-reducing biotin switch assay.

Of the analyzed proteins, we observed S-nitrosylation of hnRNP K, 14-3-3 and 14-3-3 ε, but not of PCNA or EF-1β (Fig. 3.17A). hnRNP K S-nitrosylation significantly increased 2 days after seizure onset (195.5 ± 45.0%, $p<0.05$, Fig. 3.17B), comparing with control. 1 day (139.8 ± 30.7%), 3 days (94.4 ± 29.0%) and 5 days (86.0 ± 16.9%) after KA treatment, S-nitrosylation of hnRNP K was similar to control. We first evaluated S-nitrosylation of all 14-3-3 proteins isoforms, which significantly increased 1 day after KA treatment (149.9 ± 34.8%, $p<0.05$, Fig. 3.17C), and was similar to control 2 days (112.2 ± 22.9%), 3 days (88.5 ± 14.3%) and 5 days (71.9 ± 15.4%) after seizure onset. S-nitrosylation of 14-3-3 ε, the most interesting isoform regarding neurogenesis, showed a tendency to increase 1 day (147.8 ± 37.2%, Fig. 3.17D) and 2 days (141.6 ± 28.4%) after seizure onset, and was maintained 3 days (75.7 ± 18.4%) and 5 days (69.7 ± 18.5%) after seizure onset, comparing with control.

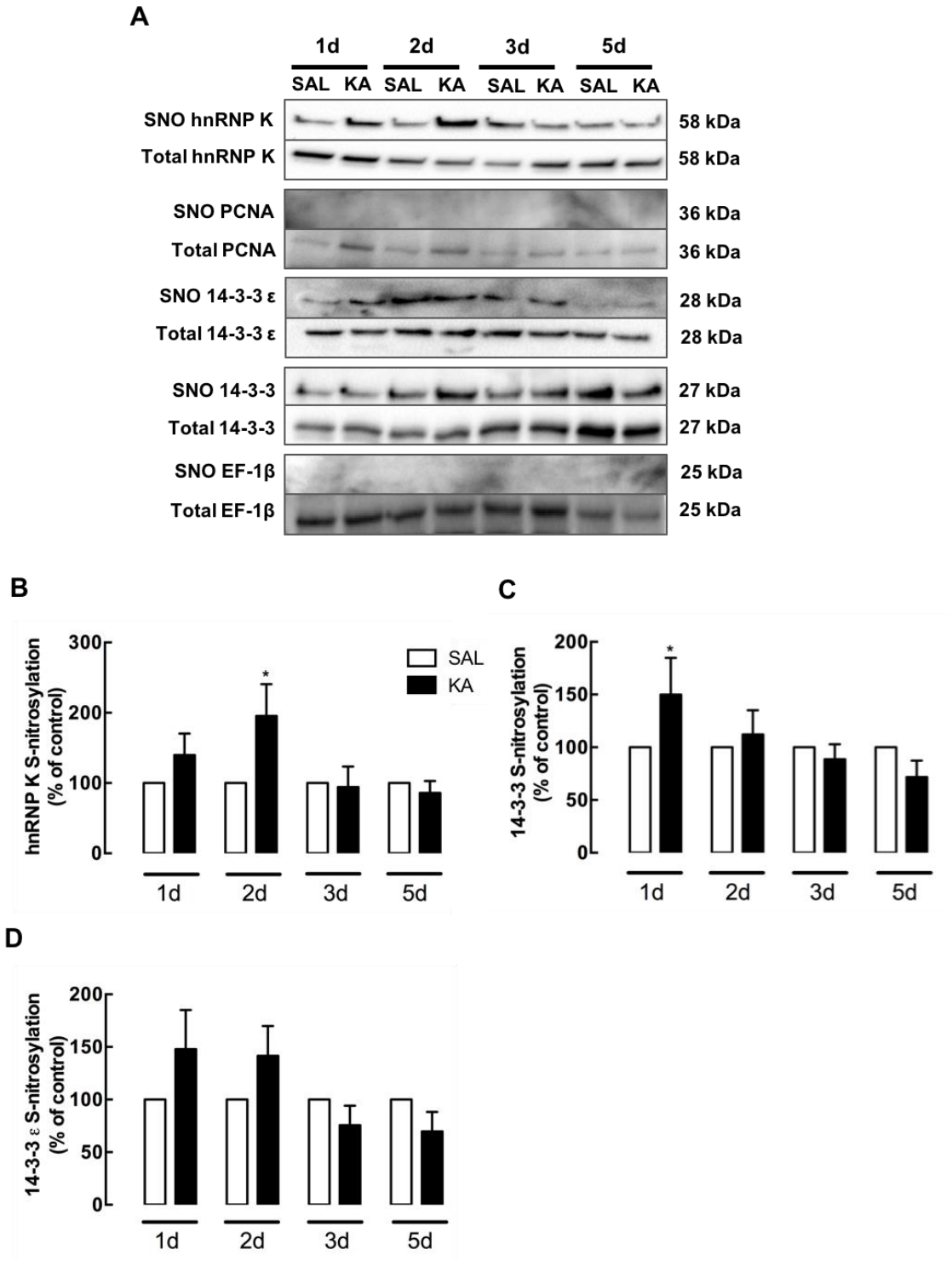


Figure 3.17 - Induction of epileptic seizures in mice leads to S-nitrosylation of proteins in the DG. Wild type C57Bl6 mice were subcutaneously injected with a sterile solution of kainic acid (KA, 25 mg/kg) in 0.9% NaCl, achieving epileptic seizures. Control animals were injected the vehicle saline solution (SAL). The animals were sacrificed 1, 2, 3 and 5 days after the injection, and protein lysates of the DG were used to analyze S-nitrosylation of hnRNP K, PCNA, 14-3-3 proteins and EF-1β by ascorbate-reducing biotin switch assay **(A)**. Data from at least 3 animals are presented as means ± SEM and (% of control, SAL) **(B-D)**. Two-way ANOVA followed by Bonferroni's post-hoc test**,** * $p<0.05$ significantly different from saline. SNO protein, S-nitrosylated protein; SAL, saline; KA, kainic acid; d, days.

3.2.2. hnRNP K, PCNA, 14-3-3 ε and EF-1β are S-nitrosylated in hippocampal stem cells

To investigate S-nitrosylation of the identified proteins in stem cells of the DG, we treated hippocampal stem cells with different concentrations of CysNO (10, 50 and 100 μM) for 15 min and analyzed S-nitrosylation of hnRNP K, PCNA, 14-3-3 ε and EF-1β by ascorbate-reducing biotin switch (Fig. 3.18A). We observed that hnRNP K S-nitrosylation showed a tendency to increase after treatment with 10 (243.2 ± 77.2%, Fig. 3.18B) and 50 μM CysNO (294.0 ± 97.1%), and significantly increased after treatment with 100 μM CysNO (487.9 ± 112.65%, $p<0.01$), comparing with control. Treatment with 100 μM CysNO significantly increased PCNA S-nitrosylation (260.7 ± 85.9%, $p<0.05$, Fig. 3.18C), while with treatment with 10 (134.7 ± 25.8%) and 50 μM CysNO (156.2 ± 31.8%) S-nitrosylation was similar to control. S-nitrosylation of 14-3-3 ε showed a tendency to increase after treatment with 10 (241.5 ± 44.1%, Fig. 3.18D) and 50 μM CysNO (256.7 ± 21.0%), and significantly increased after treatment with 100 μM CysNO (682.1 ± 209.3%, $p<0.05$), comparing with control. S-nitrosylation of EF-1β was maintained after treatment with 10 μM CysNO (186.3 ± 32.5%, Fig. 3.18E), and significantly increased after treatment with 50 (256.1 ± 34.6%, $p<0.01$) and 100 μM CysNO (234.2 ± 34.4%, $p<0.05$), comparing with control.

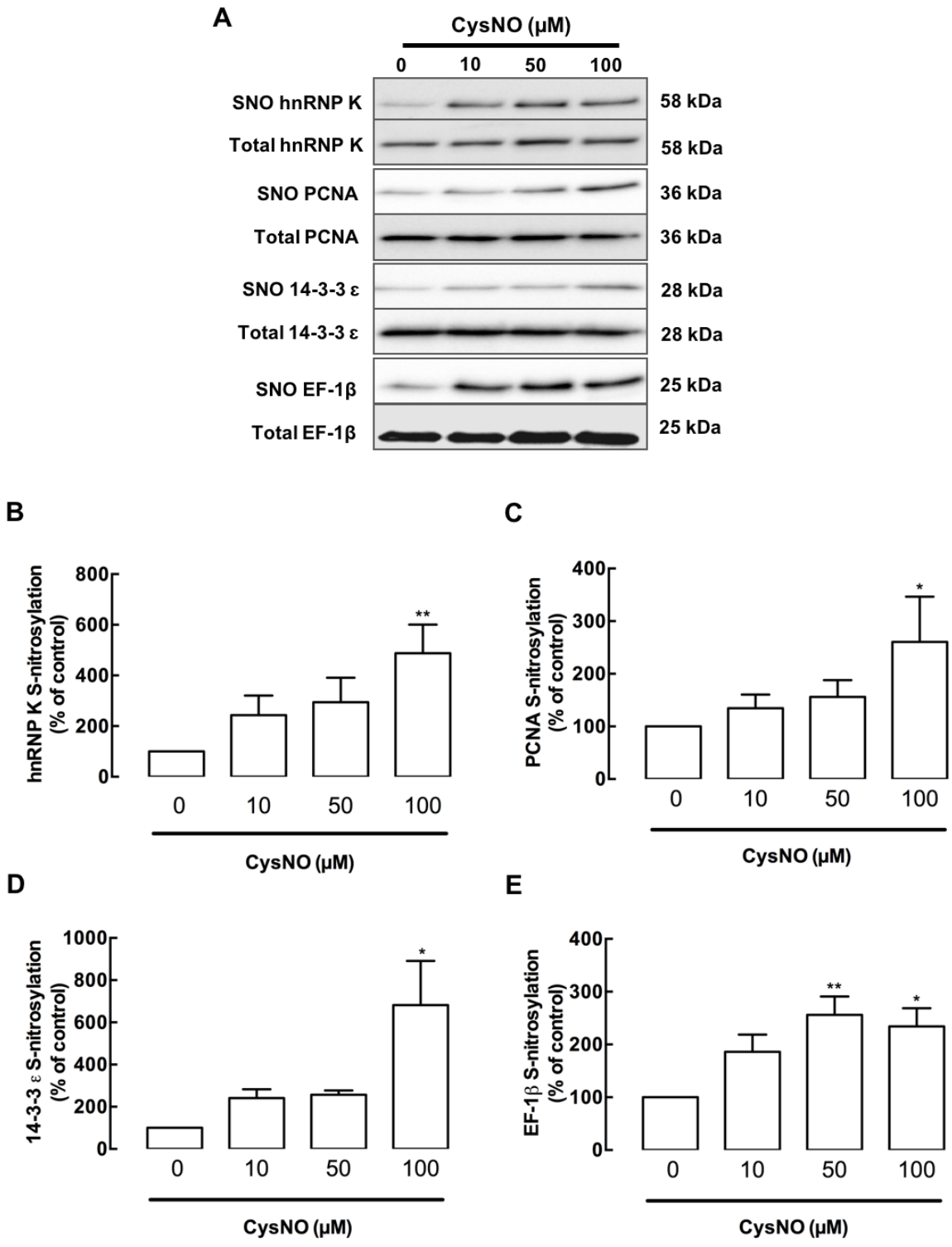


Figure 3.18 - Treatment of hippocampal stem cells with CysNO induces S-nitrosylation of proteins in a dose-dependent manner. Hippocampal stem cells were treated with 10 μM, 50 μM and 100 μM CysNO for 15 min. S-nitrosylation of hnRNP K, PCNA, 14-3-3 ε and EF-1β was assessed by ascorbate-reducing biotin switch assay **(A)**. Data from at least 3 independent experiments are presented as means ± SEM (% of control, 0, no treatment) **(B-E)**. One-Way ANOVA followed by Dunnett's post-hoc test, * $p<0.05$ and ** $p<0.01$ significantly different from control. SNO PEBP-1, S-nitrosylated protein.

Chapter 3

Chapter 4. Discussion

Chapter 4

Chapter 4

NO is a signaling molecule with several functions in the organism. Through S-nitrosylation, NO can modify the function of proteins relevant for many processes. As an important regulator of adult endogenous neurogenesis, NO is able to increase NSC proliferation after brain injury (Carreira et al. 2010). However, this repair process is not efficient (Carreira et al. 2015). The proliferative effect of NO is mediated by activation of the ERK/MAPK pathway, bypassing the EGFR, and directly activating p21Ras by S-nitrosylation (Carreira et al. 2010, Carreira et al. 2013, Santos et al. 2017, Carreira *et al.* 2012). The identification of proteins that could contribute to improve post-injury neurogenesis outcome is therefore of particular importance.

Our group identified recently several proteins as new targets of S-nitrosylation by NO (Ana I. Santos, unpublished results). PEBP-1, one of the identified targets, is an important regulator of the ERK/MAPK pathway, being essential in the regulation of cell proliferation and differentiation. The presence of this protein in rat hippocampal stem cells is associated with more differentiation towards a neuronal lineage (Sagisaka et al. 2010), making it a very interesting target to study regarding neurogenesis. It is particularly important to understand the regulation of PEBP-1 activity, in order to be possible to modulate it to increase neurogenesis. With this work, we aimed to investigate the role of S-nitrosylation of the new targets in neurogenesis, and study in more detail the role of S-nitrosylation of PEBP-1 in NSC proliferation. Using normal PEBP-1 and mutant forms of PEBP-1 insensitive to NO, we evaluated S-nitrosylation of PEBP-1 in NSC and its effect on ERK/MAPK signaling and proliferation. Moreover, we assessed the biological relevance of S-nitrosylation of PEBP-1 in a model of increased post-injury neurogenesis and in hippocampal stem cells. S-nitrosylation of other identified proteins that are related to ERK/MAPK pathway was also evaluated in the model of post-injury neurogenesis and in hippocampal stem cells.

Our results show that PEBP-1 is oxidized by CysNO, which is in accordance with our first results obtained upon the identification of oxidized proteins by NO, using a different labeling technique (Ana I. Santos, unpublished results). Moreover, we show that CysNO specifically induces S-nitrosylation of PEBP-1, and that this reaction is fast (1-2 min). Given the fact that CysNO is a S-nitrosothiol compound, it makes sense that the transfer of the S-nitrosothiol group from CysNO to PEBP-1 would be a quick process. Since our main interest was to study the S-nitrosylation of PEBP-1 under proliferative conditions, we used a NO donor, at a concentration previously established as being proliferative, and different exposure times (Carreira et al. 2010, Carreira et al. 2013, Santos et al. 2017). Our results show that S-nitrosylation of PEBP-1 increases after exposure to NO during 10 min. The detection of S-nitrosylation of PEBP-1 by NO after treatment with 10 μM NOC-18 was difficult to achieve, and the levels of S-nitrosylation induced by 100 μM

CysNO were much higher than those induced by NOC-18. In transfected cells, we were not able to detect an increase in S-nitrosylation of PEBP-1 after treatment with NOC-18. So, we evaluated which could be a better compound to induce S-nitrosylation, using a fluorescence switch assay. This technique allows the labeling of oxidized or S-nitrosylated proteins present in a protein lysate, depending on the reducing agent used. Detection of S-nitrosylation by fluorescence switch was difficult, due to the high specificity and low sensitivity of the assay. Since S-nitrosylation is an oxidation process, a compound that does not induce oxidation of proteins will also not induce S-nitrosylation. Therefore, we evaluated oxidation instead of S-nitrosylation. We used the S-nitrosothiol CysNO at the concentration that had previously induced S-nitrosylation of PEBP-1, and NOC-18 at the known proliferative concentration, but longer exposure time. Additionally, since S-nitrosylation is a fast reversible reaction, we used DEA/NO, a NO donor with a short half-life, at high concentrations. We were only able to detect protein oxidation induced by CysNO. The different response to treatment to CysNO and NO donors can occur because CysNO and NO released from NO donors are metabolized differently (Hickok *et al.* 2012). In order to produce nitrosothiols and induce transnitrosylation, CysNO does not need to release NO, while NO released from NO donors requires an oxidation reaction. Moreover, CysNO generates higher amounts of nitrosothiols than NO, which could differently affect the biological effect of both compounds.

Therefore, we performed the experiments of S-nitrosylation detection and cell signaling analysis using CysNO. Cell proliferation analysis was done using the NO donor, to be more representative of the biological response of NO. Treatment of transfected cells with CysNO induced PEBP-1 S-nitrosylation, regardless of the mutation present in the protein. This can be explained by the low transfection efficiency of the constructs in NSC. The observed signal may be from the endogenous PEBP-1 present, which has all 3 cysteines available to react with NO. NSC are very difficult cells to transfect so, since we could easily detect the presence of endogenous PEBP-1, we can assume that its levels were high enough to interfere with the exogenous modified PEBP-1. One way to analyze S-nitrosylation only in PEBP-1-overexpressing cells could be to add a tag to our constructs. That would allow us to separate transfected cells from non-transfected cells by immunoprecipitation and evaluate S-nitrosylation only in that fraction of protein. Alternatively, this experiment could be done using viral vectors or nucleofection, which can be more efficient than cationic lipid mediated transfections (Rappa *et al.* 2004, Cesnulevicius *et al.* 2006).

Since cell transfection efficiency was not enough to allow the detection of substantial differences by Western blot, immunocytochemistry was used for cell signaling and

proliferation experiments, as we were able to analyze only the transfected cells. Treatment of NSC with 10 μM CysNO for 5 min increases P-ERK levels detected by Western Blot (Ana I Santos, unpublished results), so we tested if we could detect this effect by immunocytochemistry. CysNO and NOC-18 increased P-ERK levels of NSC. We used EGF, the EGFR agonist and an activator of the ERK/MAPK pathway as the positive control in the experiments of ERK phosphorylation, since its effect in activation of this pathway should be independent of the effect of NO. That way we could assure that possible differences in ERK phosphorylation between mutant forms of PEBP-1 were not due to a secondary effect caused by the mutations that affect protein function in such a way that it affects by itself the signaling pathway. Treatment with EGF induced ERK phosphorylation in PEBP-1-overexpressing NSC regardless of cysteine mutation, which shows that PEBP-1 mutants are functional and respond to EGF stimulation as normal PEBP-1. In NSC overexpressing normal PEBP-1, P-ERK levels were significantly increased by treatment with CysNO. However, in NSC transfected with C13S/C133S construct this effect was abolished, and also at a lower extent in NSC transfected with C133S. This may indicate that C133S is important for S-nitrosylation of PEBP-1 and that this reaction lowers its inhibitory effect in the ERK/MAPK pathway activation. PEBP-1 binds to and inhibits c-Raf (Yeung et al. 1999) so, in order to allow ERK/MAPK activation and thus cell proliferation, its inhibitory effect on c-Raf must be released. The fact that prevention of ERK phosphorylation is more evident in the double mutant indicates that both C13 and C133 may be potential sites for S-nitrosylation of NO, but with C133 being the most important and preferential site for reacting with NO. In the absence of C133, C13 S-nitrosylation may be able to compensate at some extent, while in the opposite scenario C133 S-nitrosylation appears to be enough to increase ERK phosphorylation. In hippocampal stem cells, we observed S-nitrosylation of PEBP-1 induced by treatment with CysNO, dependent on its concentration. C13 is only present in mouse PEBP-1, being replaced by a serine in human and rat forms of PEBP-1. So, it makes sense that C133 can be more relevant than C13, with its biological role being present across different species.

Regarding cell proliferation, overexpression of all forms of PEBP-1 inhibited proliferation of NSC, and that was not prevented with treatment with the proliferative concentration of NOC-18. P-ERK levels could be increased by CysNO, but treatment with NOC-18 was not able to induce proliferation of cells overexpressing PEBP-1. It is possible that the strong inhibitory effect caused by overexpression of PEBP-1 prevented the activation of ERK/MAPK pathway by NO to a threshold that could result in cell proliferation. Alternatively, NSC could have turned towards a more differentiated state upon overexpression of PEBP-1. Overexpression of PEBP-1 in neuroblastoma cells has been

shown to be able to promote neuronal differentiation, even in the absence of factors that induce differentiation of these cells (Hellmann et al. 2010). Differentiation is usually irreversible so, even in the presence of a proliferative agent, proliferation would not occur, regardless of the increase of P-ERK levels. One mechanism by which PEBP-1 is regulated is phosphorylation. PEBP-1 binds to c-Raf (Yeung et al. 2000) and, after being phosphorylated by PKC at Ser153 (Corbit et al. 2003), it binds to GRK2 (Lorenz et al. 2003), allowing the activation of ERK/MAPK pathway. So, besides S-nitrosylation, PEBP-1 may need to be also phosphorylated in order to stop its inhibitory effect and allow cell proliferation to occur, although this was not addressed in this work. A three-state allosteric model for regulation of PEBP-1 has been proposed (Skinner & Rosner 2014). This model states that there is an intermediary state of PEBP-1 with which PKC interacts, rather than directly interacting with PEBP-1 bound to c-Raf. This is based on the fact that Ser153 is in a stable helix and to interact with a kinase it was expected to be part of an unfolded helix. Moreover, the authors hypothesize that the stability of this intermediary state of PEBP-1 is important for PEBP-1 regulation and that it could be affected by PTM. S-nitrosylation could be a mechanism by which this regulation of PEBP-1 occurs, facilitating phosphorylation by PKC.

The biological relevance of S-nitrosylation of PEBP-1 was evaluated using a mouse model of excitotoxic brain lesion that has increased proliferation in the DG following seizures (Parent 2007). PEBP-1 was S-nitrosylated 1 day after epileptic seizures. Given that in mice lacking *iNOS* there was no induction of S-nitrosylation of PEBP-1 at this time point, we can assume that NO from inflammatory origin (produced by iNOS in microglia) is responsible for PEBP-1 S-nitrosylation. The reaction of S-nitrosylation is reversible, and the NO-dependent proliferation in this model occurs 3-5 days after seizures (Carreira et al. 2015). If S-nitrosylation of PEBP-1 is important for interaction of PKC with this protein, this could explain how PEBP-1 releases its inhibitory function in the ERK/MAPK pathway and allows the increase in proliferation, which is observed later than PEBP-1 S-nitrosylation. p21Ras, which is upstream of c-Raf, is S-nitrosylated 2 days after seizures in this model (Santos et al. 2017). So, upon being produced by iNOS due to the inflammation after KA-induced excitotoxicity, NO could react preferentially with C133 of PEBP-1, thereby facilitating PEBP-1 phosphorylation by PKC and binding to GRK2. Without PEBP-1 bound to c-Raf, S-nitrosylation of p21Ras could initiate the signaling cascade of ERK/MAPK and ultimately induce cell proliferation.

To validate other S-nitrosylation targets we have previously identified in our group, we used the same mouse model of excitotoxic brain lesion described above. Transient S-nitrosylation of hnRNP K, 14-3-3 and 14-3-3 ε was detected in this model. S-nitrosylation was present 1 day (14-3-3 and 14-3-3 ε) or 2 days (hnRNP K) after the induction of

epileptic seizures. This occurs before the onset of proliferation of NSC in this model, which is observed 3 days after seizures and is dependent on NO released from iNOS (Carreira et al. 2015). Therefore, it is possible that, through regulation by NO from inflammatory origin, these proteins can contribute for the process of neurogenesis in this model, especially in proliferation. The fact that S-nitrosylation was observed at different times after seizures may be related to the different functions of these proteins. 14-3-3 proteins seem to have an important role in excitotoxicity. In rat hippocampus, 4 h following KA-induced excitotoxicity, the levels of total 14-3-3 are reduced (Schindler *et al.* 2004). This decrease is isoform-selective and dependent on localization, with 14-3-3 γ and 14-3-3 δ levels being decreased in microsome-enriched fractions (Schindler et al. 2006). A reduction in 14-3-3 protein levels has also been found in rat frontal cortex 24 h after KA treatment, with 14-3-3 γ, 14-3-3 ε, 14-3-3 η and 14-3-3 τ levels being decreased. This effect is accompanied by an increase in ERK phosphorylation levels (Smani *et al.* 2017), which is necessary for NSC proliferation. The decrease in 14-3-3 isoforms levels in certain cellular compartments after KA-induced excitotoxicity may be due to degradation by proteolysis (Henshall *et al.* 2002). However, it is probable that in others, such as the cytoplasm, regulation of 14-3-3 isoforms may occur through PTM, namely S-nitrosylation. As discussed above, PEBP-1 is S-nitrosylated 1 day after seizure onset in this mouse model. Both 14-3-3 proteins and PEBP-1 regulate the ERK/MAPK pathway and are rapidly S-nitrosylated following seizures. In a previous work, our group observed that p21Ras is S-nitrosylated 2 days after seizures (Santos et al. 2017), which suggests that, before acting directly on components of the ERK/MAPK pathway, NO reacts with regulators of this signaling pathway.

hnRNP K is a substrate of ERK1/2, being accumulated in the cytoplasm upon phosphorylation by this kinase (Habelhah *et al.* 2001). Thus, it is not surprising that it is S-nitrosylated in a later time point than regulators of the ERK/MAPK pathway. In summary, it is possible that NO first S-nitrosylates PEBP-1 and 14-3-3 proteins, releasing the inhibitory effect of PEBP-1 and promoting the activating effect of 14-3-3 in c-Raf, followed by S-nitrosylation of members of the ERK/MAPK pathway (namely p21Ras) and of downstream proteins, such as hnRNP K (Fig. 4.1).

Although S-nitrosylation of PCNA and EF-1β was not detected in this seizure model, their regulation by S-nitrosylation in other systems is not excluded. To evaluate S-nitrosylation of the identified targets in DG stem cells, we treated hippocampal stem cells with different concentrations of CysNO. S-nitrosylation of all the identified proteins was detected in a dose-dependent manner, with higher levels as the concentration of CysNO increased. The range of CysNO concentrations used in this study included positive and negative effects on cell proliferation. Treatment with the lowest concentrations of CysNO (10 and

50 μM) for 1 h enhances the proliferation of SVZ-derived NSC, while treatment with the highest concentration of CysNO (100 μM) inhibits this process (Ana I. Santos and Inês Araújo, data not shown). Interestingly, the proteins that were S-nitrosylated in the *in vivo* model showed a tendency to be S-nitrosylated by CysNO at the proliferative concentrations. Moreover, the proteins whose S-nitrosylation was not detectable in the *in vivo* model showed lower levels of S-nitrosylation, even at the highest CysNO concentration used, despite being S-nitrosylated by CysNO in SVZ-derived NSC (Ana I. Santos, Ana S. Lourenço and Inês Araújo, submitted). This further indicates a possible physiological relevance of the identified proteins for hippocampal neurogenesis.

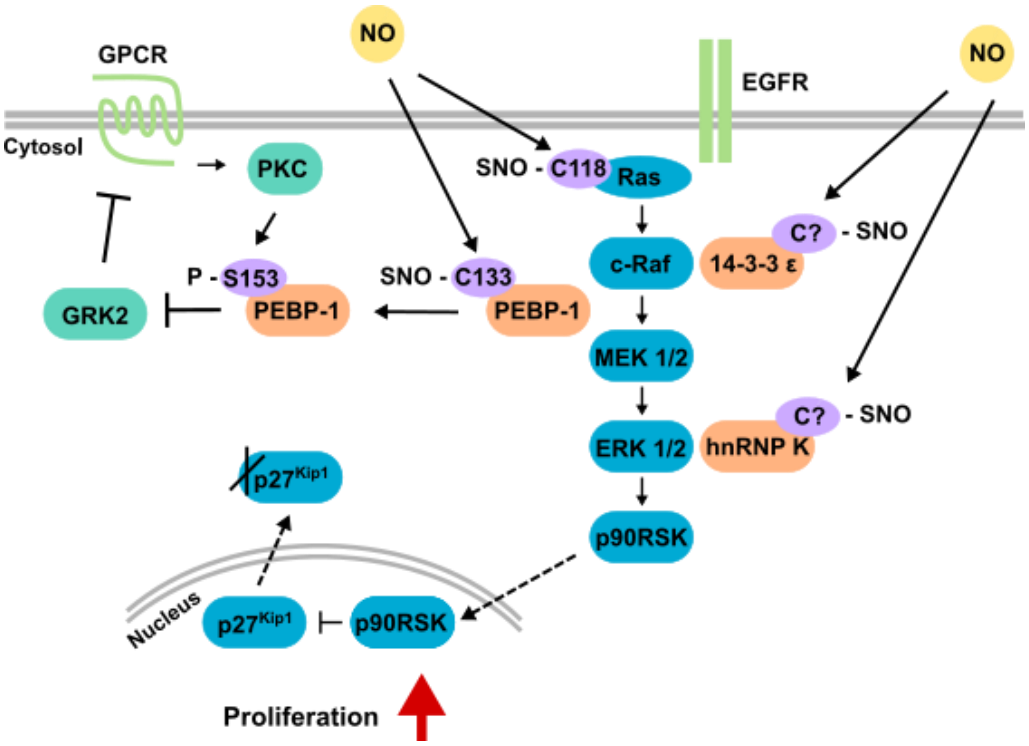


Figure 4.1 - Proposed model for S-nitrosylation of proteins by NO during post-injury neurogenesis. NO S-nitrosylates cysteine 133 of PEBP-1, facilitating phosphorylation of serine 153 by PKC and releasing the inhibitory effect of PEBP-1 in c-Raf, and also S-nitrosylates 14-3-3 ε, promoting the activating effect of 14-3-3 ε in c-Raf. This is followed by S-nitrosylation of members of the ERK/MAPK pathway, such as p21Ras, and of downstream proteins, such as hnRNP K, allowing cell proliferation.

Chapter 5. Conclusions

Chapter 5

Chapter 5

The discovery of neural stem cells and replacement of lost neurons in the adult brain of mammals allowed the possibility for repair of damage after a brain lesion by regenerative medicine. The potential of endogenous neurogenesis for therapeutic use has been subject of investigation in the last years. NO has emerged as a regulator of neurogenesis, particularly in cell proliferation. A hallmark of inflammation is the activation of iNOS and release of high amounts of NO. By reacting with thiol groups of cysteine residues, NO regulates targets of S-nitrosylation relevant in post-injury hippocampal neurogenesis.

We studied in more detail PEBP-1, one of the promising targets known to be involved in regulation of cell proliferation. S-nitrosylation of C133 seems to the function of several proteins by S-nitrosylation, resulting in their activation or inhibition. The identification and validation of S-nitrosylation targets of NO that could improve neurogenesis in conditions of brain lesion seems a crucial step for obtaining regenerative therapies through modulation of protein function. Our goal was to validate new be necessary for activation of the ERK/MAPK pathway and may be a mechanism by which it could function as a way of releasing the inhibitory effect of PEBP-1, probably facilitating its phosphorylation by PKC.

Moreover, our work allowed the validation of S-nitrosylation of 14-3-3, 14-3-3 ε, hnRNP K and PEBP-1 in conditions of brain lesion. Overall, some of the studied proteins were shown to be S-nitrosylated following epileptic seizures, according to their cellular functions: first the regulators of ERK/MAPK pathway, then a downstream protein. This effect was observed before the onset of proliferation of NSC, which suggests that these proteins could have biological importance for the enhancement of hippocampal neurogenesis following brain lesion. The roles of the new identified targets of NO in neurogenesis deserve further investigation. Using mutant proteins with cysteines replaced with other aminoacids, or by direct genome editing with CRISPR-Cas9, the functions of these S-nitrosylation targets can be investigated on several processes, including proliferation of NSC. It would be interesting to evaluate S-nitrosylation of PEBP-1 using mutant animals after genome editing of PEBP-1 cysteines, in both WT and *iNOS* KO mice, allowing the identification of the preferential S-nitrosylation sites of PEBP-1 in a context of brain lesion. Moreover, the study of the role of S-nitrosylation of PEBP-1 in the ability of PKC to phosphorylate this protein would also be of interest.

More studies can now be developed in order to further understand the role of S-nitrosylation of these proteins in conditions of neuroinflammation, to advance the knowledge about the role of NO in neurogenesis and its potential application to increase proliferation of NSC and enhance neuronal replacement.

Chapter 5

Chapter 6. References

Chapter 6

Abello, N., Kerstjens, H. A., Postma, D. S. and Bischoff, R. (2009) Protein tyrosine nitration: selectivity, physicochemical and biological consequences, denitration, and proteomics methods for the identification of tyrosine-nitrated proteins. *J Proteome Res,* **8,** 3222-3238.

Aberg, M. A., Aberg, N. D., Hedbacker, H., Oscarsson, J. and Eriksson, P. S. (2000) Peripheral infusion of IGF-I selectively induces neurogenesis in the adult rat hippocampus. *J Neurosci,* **20,** 2896-2903.

Adachi, K., Mirzadeh, Z., Sakaguchi, M., Yamashita, T., Nikolcheva, T., Gotoh, Y., Peltz, G., Gong, L., Kawase, T., Alvarez-Buylla, A., Okano, H. and Sawamoto, K. (2007) Beta-catenin signaling promotes proliferation of progenitor cells in the adult mouse subventricular zone. *Stem Cells,* **25,** 2827-2836.

Adachi, T., Weisbrod, R. M., Pimentel, D. R., Ying, J., Sharov, V. S., Schoneich, C. and Cohen, R. A. (2004) S-Glutathiolation by peroxynitrite activates SERCA during arterial relaxation by nitric oxide. *Nat Med,* **10,** 1200-1207.

Al-Mulla, F., Bitar, M. S., Al-Maghrebi, M., Behbehani, A. I., Al-Ali, W., Rath, O., Doyle, B., Tan, K. Y., Pitt, A. and Kolch, W. (2011) Raf kinase inhibitor protein RKIP enhances signaling by glycogen synthase kinase-3beta. *Cancer Res,* **71,** 1334-1343.

Alderton, W. K., Cooper, C. E. and Knowles, R. G. (2001) Nitric oxide synthases: structure, function and inhibition. *Biochem J,* **357,** 593-615.

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

Alunni, A. and Bally-Cuif, L. (2016) A comparative view of regenerative neurogenesis in vertebrates. *Development,* **143,** 741-753.

Alvarez-Buylla, A. and Garcia-Verdugo, J. M. (2002) Neurogenesis in adult subventricular zone. *J Neurosci,* **22,** 629-634.

Anacker, C., Cattaneo, A., Musaelyan, K., Zunszain, P. A., Horowitz, M., Molteni, R., Luoni, A., Calabrese, F., Tansey, K., Gennarelli, M., Thuret, S., Price, J., Uher, R., Riva, M. A. and Pariante, C. M. (2013) Role for the kinase SGK1 in stress, depression, and glucocorticoid effects on hippocampal neurogenesis. *Proc Natl Acad Sci U S A,* **110,** 8708-8713.

Arvidsson, A., Collin, T., Kirik, D., Kokaia, Z. and Lindvall, O. (2002) Neuronal replacement from endogenous precursors in the adult brain after stroke. *Nat Med,* **8,** 963-970.

Arvidsson, A., Kokaia, Z. and Lindvall, O. (2001) N-methyl-D-aspartate receptor-mediated increase of neurogenesis in adult rat dentate gyrus following stroke. *Eur J Neurosci,* **14,** 10-18.

Balordi, F. and Fishell, G. (2007) Hedgehog signaling in the subventricular zone is required for both the maintenance of stem cells and the migration of newborn neurons. *J Neurosci,* **27,** 5936-5947.

Banerjee, S. B., Rajendran, R., Dias, B. G., Ladiwala, U., Tole, S. and Vaidya, V. A. (2005) Recruitment of the Sonic hedgehog signalling cascade in electroconvulsive seizure-mediated regulation of adult rat hippocampal neurogenesis. *Eur J Neurosci,* **22,** 1570-1580.

Baumans, V. and Van Loo, P. L. (2013) How to improve housing conditions of laboratory animals: the possibilities of environmental refinement. *Vet J,* **195,** 24-32.

Bechara, R. G. and Kelly, A. M. (2013) Exercise improves object recognition memory and induces BDNF expression and cell proliferation in cognitively enriched rats. *Behav Brain Res,* **245,** 96-100.

Bednarczyk, M. R., Hacker, L. C., Fortin-Nunez, S., Aumont, A., Bergeron, R. and Fernandes, K. J. (2011) Distinct stages of adult hippocampal neurogenesis are regulated by running and the running environment. *Hippocampus,* **21,** 1334-1347.

Benhar, M., Forrester, M. T. and Stamler, J. S. (2009) Protein denitrosylation: enzymatic mechanisms and cellular functions. *Nat Rev Mol Cell Biol,* **10,** 721-732.

Chapter 6

Bergmann, O., Liebl, J., Bernard, S., Alkass, K., Yeung, M. S., Steier, P., Kutschera, W., Johnson, L., Landen, M., Druid, H., Spalding, K. L. and Frisen, J. (2012) The age of olfactory bulb neurons in humans. *Neuron,* **74,** 634-639.

Black, J. E., Isaacs, K. R., Anderson, B. J., Alcantara, A. A. and Greenough, W. T. (1990) Learning causes synaptogenesis, whereas motor activity causes angiogenesis, in cerebellar cortex of adult rats. *Proc Natl Acad Sci U S A,* **87,** 5568-5572.

Bonde, S., Ekdahl, C. T. and Lindvall, O. (2006) Long-term neuronal replacement in adult rat hippocampus after status epilepticus despite chronic inflammation. *Eur J Neurosci,* **23,** 965-974.

Bouab, M., Paliouras, G. N., Aumont, A., Forest-Berard, K. and Fernandes, K. J. (2011) Aging of the subventricular zone neural stem cell niche: evidence for quiescence-associated changes between early and mid-adulthood. *Neuroscience,* **173,** 135-149.

Breunig, J. J., Silbereis, J., Vaccarino, F. M., Sestan, N. and Rakic, P. (2007) Notch regulates cell fate and dendrite morphology of newborn neurons in the postnatal dentate gyrus. *Proc Natl Acad Sci U S A,* **104,** 20558-20563.

Brus, M., Keller, M. and Levy, F. (2013) Temporal features of adult neurogenesis: differences and similarities across mammalian species. *Front Neurosci,* **7,** 135.

Butovsky, O., Ziv, Y., Schwartz, A., Landa, G., Talpalar, A. E., Pluchino, S., Martino, G. and Schwartz, M. (2006) Microglia activated by IL-4 or IFN-gamma differentially induce neurogenesis and oligodendrogenesis from adult stem/progenitor cells. *Mol Cell Neurosci,* **31,** 149-160.

Bye, N., Carron, S., Han, X., Agyapomaa, D., Ng, S. Y., Yan, E., Rosenfeld, J. V. and Morganti-Kossmann, M. C. (2011) Neurogenesis and glial proliferation are stimulated following diffuse traumatic brain injury in adult rats. *J Neurosci Res,* **89,** 986-1000.

Carlson, S. W., Madathil, S. K., Sama, D. M., Gao, X., Chen, J. and Saatman, K. E. (2014) Conditional overexpression of insulin-like growth factor-1 enhances hippocampal neurogenesis and restores immature neuron dendritic processes after traumatic brain injury. *J Neuropathol Exp Neurol,* **73,** 734-746.

Carreira, B. P., Carvalho, C. M. and Araujo, I. M. (2012) Regulation of injury-induced neurogenesis by nitric oxide. *Stem Cells Int,* **2012,** 895659.

Carreira, B. P., Morte, M. I., Inacio, A., Costa, G., Rosmaninho-Salgado, J., Agasse, F., Carmo, A., Couceiro, P., Brundin, P., Ambrosio, A. F., Carvalho, C. M. and Araujo, I. M. (2010) Nitric oxide stimulates the proliferation of neural stem cells bypassing the epidermal growth factor receptor. *Stem Cells,* **28,** 1219-1230.

Carreira, B. P., Morte, M. I., Lourenco, A. S., Santos, A. I., Inacio, A., Ambrosio, A. F., Carvalho, C. M. and Araujo, I. M. (2013) Differential contribution of the guanylyl cyclase-cyclic GMP-protein kinase G pathway to the proliferation of neural stem cells stimulated by nitric oxide. *Neurosignals,* **21,** 1-13.

Carreira, B. P., Morte, M. I., Santos, A. I., Lourenco, A. S., Ambrosio, A. F., Carvalho, C. M. and Araujo, I. M. (2014) Nitric oxide from inflammatory origin impairs neural stem cell proliferation by inhibiting epidermal growth factor receptor signaling. *Front Cell Neurosci,* **8,** 343.

Carreira, B. P., Santos, D. F., Santos, A. I., Carvalho, C. M. and Araujo, I. M. (2015) Nitric Oxide Regulates Neurogenesis in the Hippocampus following Seizures. *Oxid Med Cell Longev,* **2015,** 451512.

Carvalho, M. D., Carvalho, J. F. and Merrick, W. C. (1984) Biological characterization of various forms of elongation factor 1 from rabbit reticulocytes. *Arch Biochem Biophys,* **234,** 603-611.

Cesnulevicius, K., Timmer, M., Wesemann, M., Thomas, T., Barkhausen, T. and Grothe, C. (2006) Nucleofection is the most efficient nonviral transfection method for neuronal stem cells derived from ventral mesencephali with no changes in cell composition or dopaminergic fate. *Stem Cells,* **24,** 2776-2791.

Chang, H. C. and Rubin, G. M. (1997) 14-3-3 epsilon positively regulates Ras-mediated signaling in Drosophila. *Genes Dev,* **11,** 1132-1139.

Chapouton, P., Jagasia, R. and Bally-Cuif, L. (2007) Adult neurogenesis in non-mammalian vertebrates. *Bioessays,* **29,** 745-757.

Chirumamilla, S., Sun, D., Bullock, M. R. and Colello, R. J. (2002) Traumatic brain injury induced cell proliferation in the adult mammalian central nervous system. *J Neurotrauma,* **19,** 693-703.

Cho, S. R., Benraiss, A., Chmielnicki, E., Samdani, A., Economides, A. and Goldman, S. A. (2007) Induction of neostriatal neurogenesis slows disease progression in a transgenic murine model of Huntington disease. *J Clin Invest,* **117,** 2889-2902.

Choe, K. N. and Moldovan, G. L. (2017) Forging Ahead through Darkness: PCNA, Still the Principal Conductor at the Replication Fork. *Mol Cell,* **65,** 380-392.

Christie, K. J. and Turnley, A. M. (2012) Regulation of endogenous neural stem/progenitor cells for neural repair-factors that promote neurogenesis and gliogenesis in the normal and damaged brain. *Front Cell Neurosci,* **6,** 70.

Corbit, K. C., Trakul, N., Eves, E. M., Diaz, B., Marshall, M. and Rosner, M. R. (2003) Activation of Raf-1 signaling by protein kinase C through a mechanism involving Raf kinase inhibitory protein. *J Biol Chem,* **278,** 13061-13068.

Cornell, B. and Toyo-Oka, K. (2017) 14-3-3 Proteins in Brain Development: Neurogenesis, Neuronal Migration and Neuromorphogenesis. *Front Mol Neurosci,* **10,** 318.

Crews, L., Mizuno, H., Desplats, P., Rockenstein, E., Adame, A., Patrick, C., Winner, B., Winkler, J. and Masliah, E. (2008) Alpha-synuclein alters Notch-1 expression and neurogenesis in mouse embryonic stem cells and in the hippocampus of transgenic mice. *J Neurosci,* **28,** 4250-4260.

Curtis, M. A., Kam, M., Nannmark, U., Anderson, M. F., Axell, M. Z., Wikkelso, C., Holtas, S., van Roon-Mom, W. M., Bjork-Eriksson, T., Nordborg, C., Frisen, J., Dragunow, M., Faull, R. L. and Eriksson, P. S. (2007) Human neuroblasts migrate to the olfactory bulb via a lateral ventricular extension. *Science,* **315,** 1243-1249.

Dahl, A., Eriksson, P. S., Persson, A. I., Karlsson, G., Davidsson, P., Ekman, R. and Westman-Brinkmalm, A. (2003) Proteome analysis of conditioned medium from cultured adult hippocampal progenitors. *Rapid Commun Mass Spectrom,* **17,** 2195-2202.

Dash, P. K., Mach, S. A. and Moore, A. N. (2001) Enhanced neurogenesis in the rodent hippocampus following traumatic brain injury. *J Neurosci Res,* **63,** 313-319.

Dashtipour, K., Wong, A. M., Obenaus, A., Spigelman, I. and Ribak, C. E. (2003) Temporal profile of hilar basal dendrite formation on dentate granule cells after status epilepticus. *Epilepsy Res,* **54,** 141-151.

Deierborg, T., Roybon, L., Inacio, A. R., Pesic, J. and Brundin, P. (2010) Brain injury activates microglia that induce neural stem cell proliferation ex vivo and promote differentiation of neurosphere-derived cells into neurons and oligodendrocytes. *Neuroscience,* **171,** 1386-1396.

Deiss, K., Kisker, C., Lohse, M. J. and Lorenz, K. (2012) Raf kinase inhibitor protein (RKIP) dimer formation controls its target switch from Raf1 to G protein-coupled receptor kinase (GRK) 2. *J Biol Chem,* **287,** 23407-23417.

DeMaster, E. G., Quast, B. J., Redfern, B. and Nagasawa, H. T. (1995) Reaction of nitric oxide with the free sulfhydryl group of human serum albumin yields a sulfenic acid and nitrous oxide. *Biochemistry,* **34,** 11494-11499.

Derakhshan, B., Hao, G. and Gross, S. S. (2007) Balancing reactivity against selectivity: the evolution of protein S-nitrosylation as an effector of cell signaling by nitric oxide. *Cardiovasc Res,* **75,** 210-219.

Doetsch, F. (2003) A niche for adult neural stem cells. *Curr Opin Genet Dev,* **13,** 543-550.

Doetsch, F., Garcia-Verdugo, J. M. and Alvarez-Buylla, A. (1997) Cellular composition and three-dimensional organization of the subventricular germinal zone in the adult mammalian brain. *J Neurosci,* **17,** 5046-5061.

Donovan, M. H., Yazdani, U., Norris, R. D., Games, D., German, D. C. and Eisch, A. J. (2006) Decreased adult hippocampal neurogenesis in the PDAPP mouse model of Alzheimer's disease. *J Comp Neurol,* **495,** 70-83.

Dreyfuss, G., Matunis, M. J., Pinol-Roma, S. and Burd, C. G. (1993) hnRNP proteins and the biogenesis of mRNA. *Annu Rev Biochem,* **62,** 289-321.

Ekdahl, C. T. (2012) Microglial activation - tuning and pruning adult neurogenesis. *Front Pharmacol,* **3,** 41.

Ekdahl, C. T., Claasen, J. H., Bonde, S., Kokaia, Z. and Lindvall, O. (2003) Inflammation is detrimental for neurogenesis in adult brain. *Proc Natl Acad Sci U S A,* **100,** 13632-13637.

Encinas, J. M., Michurina, T. V., Peunova, N., Park, J. H., Tordo, J., Peterson, D. A., Fishell, G., Koulakov, A. and Enikolopov, G. (2011) Division-coupled astrocytic differentiation and age-related depletion of neural stem cells in the adult hippocampus. *Cell Stem Cell,* **8,** 566-579.

Enwere, E., Shingo, T., Gregg, C., Fujikawa, H., Ohta, S. and Weiss, S. (2004) Aging results in reduced epidermal growth factor receptor signaling, diminished olfactory neurogenesis, and deficits in fine olfactory discrimination. *J Neurosci,* **24,** 8354-8365.

Eriksson, P. S., Perfilieva, E., Bjork-Eriksson, T., Alborn, A. M., Nordborg, C., Peterson, D. A. and Gage, F. H. (1998) Neurogenesis in the adult human hippocampus. *Nat Med,* **4,** 1313-1317.

Ernst, A., Alkass, K., Bernard, S., Salehpour, M., Perl, S., Tisdale, J., Possnert, G., Druid, H. and Frisen, J. (2014) Neurogenesis in the striatum of the adult human brain. *Cell,* **156,** 1072-1083.

Farmer, J., Zhao, X., van Praag, H., Wodtke, K., Gage, F. H. and Christie, B. R. (2004) Effects of voluntary exercise on synaptic plasticity and gene expression in the dentate gyrus of adult male Sprague-Dawley rats in vivo. *Neuroscience,* **124,** 71-79.

Fedele, V., Roybon, L., Nordstrom, U., Li, J. Y. and Brundin, P. (2011) Neurogenesis in the R6/2 mouse model of Huntington's disease is impaired at the level of NeuroD1. *Neuroscience,* **173,** 76-81.

Fernandez-Hernandez, I., Rhiner, C. and Moreno, E. (2013) Adult neurogenesis in Drosophila. *Cell Rep,* **3,** 1857-1865.

Filippov, V., Kronenberg, G., Pivneva, T., Reuter, K., Steiner, B., Wang, L. P., Yamaguchi, M., Kettenmann, H. and Kempermann, G. (2003) Subpopulation of nestin-expressing progenitor cells in the adult murine hippocampus shows electrophysiological and morphological characteristics of astrocytes. *Mol Cell Neurosci,* **23,** 373-382.

Fitzhugh, A. L. and Keefer, L. K. (2000) Diazeniumdiolates: pro- and antioxidant applications of the "NONOates". *Free Radic Biol Med,* **28,** 1463-1469.

Frick, K. M. and Fernandez, S. M. (2003) Enrichment enhances spatial memory and increases synaptophysin levels in aged female mice. *Neurobiol Aging,* **24,** 615-626.

Friebe, A. and Koesling, D. (2009) The function of NO-sensitive guanylyl cyclase: what we can learn from genetic mouse models. *Nitric Oxide,* **21,** 149-156.

Fritzen, S., Schmitt, A., Koth, K., Sommer, C., Lesch, K. P. and Reif, A. (2007) Neuronal nitric oxide synthase (NOS-I) knockout increases the survival rate of neural cells in the hippocampus independently of BDNF. *Mol Cell Neurosci,* **35,** 261-271.

Furuta, M. and Bridges, R. S. (2005) Gestation-induced cell proliferation in the rat brain. *Brain Res Dev Brain Res,* **156,** 61-66.

Gage, F. H., Coates, P. W., Palmer, T. D., Kuhn, H. G., Fisher, L. J., Suhonen, J. O., Peterson, D. A., Suhr, S. T. and Ray, J. (1995) Survival and differentiation of

adult neuronal progenitor cells transplanted to the adult brain. *Proc Natl Acad Sci U S A,* **92,** 11879-11883.

Gao, X. and Chen, J. (2009) Conditional knockout of brain-derived neurotrophic factor in the hippocampus increases death of adult-born immature neurons following traumatic brain injury. *J Neurotrauma,* **26,** 1325-1335.

Gao, X. and Chen, J. (2013) Moderate traumatic brain injury promotes neural precursor proliferation without increasing neurogenesis in the adult hippocampus. *Exp Neurol,* **239,** 38-48.

Gao, X., Enikolopov, G. and Chen, J. (2009) Moderate traumatic brain injury promotes proliferation of quiescent neural progenitors in the adult hippocampus. *Exp Neurol,* **219,** 516-523.

Gil, J. M., Mohapel, P., Araujo, I. M., Popovic, N., Li, J. Y., Brundin, P. and Petersen, A. (2005) Reduced hippocampal neurogenesis in R6/2 transgenic Huntington's disease mice. *Neurobiol Dis,* **20,** 744-751.

Gong, C., Wang, T. W., Huang, H. S. and Parent, J. M. (2007) Reelin regulates neuronal progenitor migration in intact and epileptic hippocampus. *J Neurosci,* **27,** 1803-1811.

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

Gourley, S. L., Kedves, A. T., Olausson, P. and Taylor, J. R. (2009) A history of corticosterone exposure regulates fear extinction and cortical NR2B, GluR2/3, and BDNF. *Neuropsychopharmacology,* **34,** 707-716.

Gray, W. P. and Sundstrom, L. E. (1998) Kainic acid increases the proliferation of granule cell progenitors in the dentate gyrus of the adult rat. *Brain Res,* **790,** 52-59.

Gritti, A., Cova, L., Parati, E. A., Galli, R. and Vescovi, A. L. (1995) Basic fibroblast growth factor supports the proliferation of epidermal growth factor-generated neuronal precursor cells of the adult mouse CNS. *Neurosci Lett,* **185,** 151-154.

Guikema, B., Lu, Q. and Jourd'heuil, D. (2005) Chemical considerations and biological selectivity of protein nitrosation: implications for NO-mediated signal transduction. *Antioxid Redox Signal,* **7,** 593-606.

Habelhah, H., Shah, K., Huang, L., Ostareck-Lederer, A., Burlingame, A. L., Shokat, K. M., Hentze, M. W. and Ronai, Z. (2001) ERK phosphorylation drives cytoplasmic accumulation of hnRNP-K and inhibition of mRNA translation. *Nat Cell Biol,* **3,** 325-330.

Hall, C. N. and Garthwaite, J. (2009) What is the real physiological NO concentration in vivo? *Nitric Oxide,* **21,** 92-103.

Hattiangady, B., Rao, M. S. and Shetty, A. K. (2004) Chronic temporal lobe epilepsy is associated with severely declined dentate neurogenesis in the adult hippocampus. *Neurobiol Dis,* **17,** 473-490.

Haughey, N. J., Nath, A., Chan, S. L., Borchard, A. C., Rao, M. S. and Mattson, M. P. (2002) Disruption of neurogenesis by amyloid beta-peptide, and perturbed neural progenitor cell homeostasis, in models of Alzheimer's disease. *J Neurochem,* **83,** 1509-1524.

Hellmann, J., Rommelspacher, H., Muhlbauer, E. and Wernicke, C. (2010) Raf kinase inhibitor protein enhances neuronal differentiation in human SH-SY5Y cells. *Dev Neurosci,* **32,** 33-46.

Hellmann, J., Rommelspacher, H. and Wernicke, C. (2009) Long-term ethanol exposure impairs neuronal differentiation of human neuroblastoma cells involving neurotrophin-mediated intracellular signaling and in particular protein kinase C. *Alcohol Clin Exp Res,* **33,** 538-550.

Henshall, D. C., Araki, T., Schindler, C. K., Lan, J. Q., Tiekoter, K. L., Taki, W. and Simon, R. P. (2002) Activation of Bcl-2-associated death protein and counter-response of Akt within cell populations during seizure-induced neuronal death. *J Neurosci,* **22,** 8458-8465.

Hickok, J. R., Vasudevan, D., Thatcher, G. R. and Thomas, D. D. (2012) Is S-nitrosocysteine a true surrogate for nitric oxide? *Antioxid Redox Signal,* **17,** 962-968.

Hodes, G. E., Brookshire, B. R., Hill-Smith, T. E., Teegarden, S. L., Berton, O. and Lucki, I. (2012) Strain differences in the effects of chronic corticosterone exposure in the hippocampus. *Neuroscience,* **222,** 269-280.

Hoehn, B. D., Palmer, T. D. and Steinberg, G. K. (2005) Neurogenesis in rats after focal cerebral ischemia is enhanced by indomethacin. *Stroke,* **36,** 2718-2724.

Hurtado-Chong, A., Yusta-Boyo, M. J., Vergano-Vera, E., Bulfone, A., de Pablo, F. and Vicario-Abejon, C. (2009) IGF-I promotes neuronal migration and positioning in the olfactory bulb and the exit of neuroblasts from the subventricular zone. *Eur J Neurosci,* **30,** 742-755.

Imayoshi, I., Sakamoto, M., Yamaguchi, M., Mori, K. and Kageyama, R. (2010) Essential roles of Notch signaling in maintenance of neural stem cells in developing and adult brains. *J Neurosci,* **30,** 3489-3498.

Inta, D., Alfonso, J., von Engelhardt, J., Kreuzberg, M. M., Meyer, A. H., van Hooft, J. A. and Monyer, H. (2008) Neurogenesis and widespread forebrain migration of distinct GABAergic neurons from the postnatal subventricular zone. *Proc Natl Acad Sci U S A,* **105,** 20994-20999.

Isackson, P. J., Huntsman, M. M., Murray, K. D. and Gall, C. M. (1991) BDNF mRNA expression is increased in adult rat forebrain after limbic seizures: temporal patterns of induction distinct from NGF. *Neuron,* **6,** 937-948.

Ischiropoulos, H. (2003) Biological selectivity and functional aspects of protein tyrosine nitration. *Biochem Biophys Res Commun,* **305,** 776-783.

Izquierdo-Alvarez, A., Ramos, E., Villanueva, J., Hernansanz-Agustin, P., Fernandez-Rodriguez, R., Tello, D., Carrascal, M. and Martinez-Ruiz, A. (2012) Differential redox proteomics allows identification of proteins reversibly oxidized at cysteine residues in endothelial cells in response to acute hypoxia. *J Proteomics,* **75,** 5449-5462.

Jakubs, K., Bonde, S., Iosif, R. E., Ekdahl, C. T., Kokaia, Z., Kokaia, M. and Lindvall, O. (2008) Inflammation regulates functional integration of neurons born in adult brain. *J Neurosci,* **28,** 12477-12488.

Jang, M. H., Bonaguidi, M. A., Kitabatake, Y., Sun, J., Song, J., Kang, E., Jun, H., Zhong, C., Su, Y., Guo, J. U., Wang, M. X., Sailor, K. A., Kim, J. Y., Gao, Y., Christian, K. M., Ming, G. L. and Song, H. (2013) Secreted frizzled-related protein 3 regulates activity-dependent adult hippocampal neurogenesis. *Cell Stem Cell,* **12,** 215-223.

Jessberger, S., Zhao, C., Toni, N., Clemenson, G. D., Jr., Li, Y. and Gage, F. H. (2007) Seizure-associated, aberrant neurogenesis in adult rats characterized with retrovirus-mediated cell labeling. *J Neurosci,* **27,** 9400-9407.

Jin, K., Minami, M., Lan, J. Q., Mao, X. O., Batteur, S., Simon, R. P. and Greenberg, D. A. (2001) Neurogenesis in dentate subgranular zone and rostral subventricular zone after focal cerebral ischemia in the rat. *Proc Natl Acad Sci U S A,* **98,** 4710-4715.

Jin, K., Zhu, Y., Sun, Y., Mao, X. O., Xie, L. and Greenberg, D. A. (2002) Vascular endothelial growth factor (VEGF) stimulates neurogenesis in vitro and in vivo. *Proc Natl Acad Sci U S A,* **99,** 11946-11950.

Jourd'heuil, D., Gray, L. and Grisham, M. B. (2000) S-nitrosothiol formation in blood of lipopolysaccharide-treated rats. *Biochem Biophys Res Commun,* **273,** 22-26.

Kandasamy, M., Couillard-Despres, S., Raber, K. A., Stephan, M., Lehner, B., Winner, B., Kohl, Z., Rivera, F. J., Nguyen, H. P., Riess, O., Bogdahn, U., Winkler, J., von Horsten, S. and Aigner, L. (2010) Stem cell quiescence in the hippocampal neurogenic niche is associated with elevated transforming growth factor-beta signaling in an animal model of Huntington disease. *J Neuropathol Exp Neurol,* **69,** 717-728.

Kang, W. and Hebert, J. M. (2015) FGF Signaling Is Necessary for Neurogenesis in Young Mice and Sufficient to Reverse Its Decline in Old Mice. *J Neurosci,* **35,** 10217-10223.

Katsimpardi, L., Litterman, N. K., Schein, P. A., Miller, C. M., Loffredo, F. S., Wojtkiewicz, G. R., Chen, J. W., Lee, R. T., Wagers, A. J. and Rubin, L. L. (2014) Vascular and neurogenic rejuvenation of the aging mouse brain by young systemic factors. *Science,* **344,** 630-634.

Kee, N. J., Preston, E. and Wojtowicz, J. M. (2001) Enhanced neurogenesis after transient global ischemia in the dentate gyrus of the rat. *Exp Brain Res,* **136,** 313-320.

Kempermann, G. (2008) The neurogenic reserve hypothesis: what is adult hippocampal neurogenesis good for? *Trends Neurosci,* **31,** 163-169.

Kempermann, G., Gast, D. and Gage, F. H. (2002) Neuroplasticity in old age: sustained fivefold induction of hippocampal neurogenesis by long-term environmental enrichment. *Ann Neurol,* **52,** 135-143.

Kempermann, G., Jessberger, S., Steiner, B. and Kronenberg, G. (2004) Milestones of neuronal development in the adult hippocampus. *Trends Neurosci,* **27,** 447-452.

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

Khwanraj, K., Madlah, S., Grataitong, K. and Dharmasaroja, P. (2016) Comparative mRNA Expression of eEF1A Isoforms and a PI3K/Akt/mTOR Pathway in a Cellular Model of Parkinson's Disease. *Parkinsons Dis,* **2016,** 8716016.

Klatt, P. and Lamas, S. (2000) Regulation of protein function by S-glutathiolation in response to oxidative and nitrosative stress. *Eur J Biochem,* **267,** 4928-4944.

Klempin, F., Beis, D., Mosienko, V., Kempermann, G., Bader, M. and Alenina, N. (2013) Serotonin is required for exercise-induced adult hippocampal neurogenesis. *J Neurosci,* **33,** 8270-8275.

Kobilo, T., Liu, Q. R., Gandhi, K., Mughal, M., Shaham, Y. and van Praag, H. (2011) Running is the neurogenic and neurotrophic stimulus in environmental enrichment. *Learn Mem,* **18,** 605-609.

Kohl, Z., Regensburger, M., Aigner, R., Kandasamy, M., Winner, B., Aigner, L. and Winkler, J. (2010) Impaired adult olfactory bulb neurogenesis in the R6/2 mouse model of Huntington's disease. *BMC Neurosci,* **11,** 114.

Kohl, Z., Winner, B., Ubhi, K., Rockenstein, E., Mante, M., Munch, M., Barlow, C., Carter, T., Masliah, E. and Winkler, J. (2012) Fluoxetine rescues impaired hippocampal neurogenesis in a transgenic A53T synuclein mouse model. *Eur J Neurosci,* **35,** 10-19.

Koizumi, O. and Bode, H. R. (1991) Plasticity in the nervous system of adult hydra. III. Conversion of neurons to expression of a vasopressin-like immunoreactivity depends on axial location. *J Neurosci,* **11,** 2011-2020.

Kolch, W. (2005) Coordinating ERK/MAPK signalling through scaffolds and inhibitors. *Nat Rev Mol Cell Biol,* **6,** 827-837.

Kralic, J. E., Ledergerber, D. A. and Fritschy, J. M. (2005) Disruption of the neurogenic potential of the dentate gyrus in a mouse model of temporal lobe epilepsy with focal seizures. *Eur J Neurosci,* **22,** 1916-1927.

Kriegstein, A. and Alvarez-Buylla, A. (2009) The glial nature of embryonic and adult neural stem cells. *Annu Rev Neurosci,* **32,** 149-184.

Kron, M. M., Zhang, H. and Parent, J. M. (2010) The developmental stage of dentate granule cells dictates their contribution to seizure-induced plasticity. *J Neurosci,* **30,** 2051-2059.

Kronenberg, G., Kirste, I., Inta, D., Chourbaji, S., Heuser, I., Endres, M. and Gass, P. (2009) Reduced hippocampal neurogenesis in the GR(+/-) genetic mouse model of depression. *Eur Arch Psychiatry Clin Neurosci,* **259,** 499-504.

Kronenberg, G., Reuter, K., Steiner, B., Brandt, M. D., Jessberger, S., Yamaguchi, M. and Kempermann, G. (2003) Subpopulations of proliferating cells of the adult

hippocampus respond differently to physiologic neurogenic stimuli. *J Comp Neurol,* **467,** 455-463.

Kuhn, H. G., Winkler, J., Kempermann, G., Thal, L. J. and Gage, F. H. (1997) Epidermal growth factor and fibroblast growth factor-2 have different effects on neural progenitors in the adult rat brain. *J Neurosci,* **17,** 5820-5829.

Kuncewicz, T., Sheta, E. A., Goldknopf, I. L. and Kone, B. C. (2003) Proteomic analysis of S-nitrosylated proteins in mesangial cells. *Mol Cell Proteomics,* **2,** 156-163.

Lam, Y. W., Yuan, Y., Isaac, J., Babu, C. V., Meller, J. and Ho, S. M. (2010) Comprehensive identification and modified-site mapping of S-nitrosylated targets in prostate epithelial cells. *PLoS One,* **5,** e9075.

Lazarini, F., Gabellec, M. M., Torquet, N. and Lledo, P. M. (2012) Early activation of microglia triggers long-lasting impairment of adult neurogenesis in the olfactory bulb. *J Neurosci,* **32,** 3652-3664.

Lazic, S. E., Grote, H., Armstrong, R. J., Blakemore, C., Hannan, A. J., van Dellen, A. and Barker, R. A. (2004) Decreased hippocampal cell proliferation in R6/1 Huntington's mice. *Neuroreport,* **15,** 811-813.

Le Belle, J. E., Orozco, N. M., Paucar, A. A., Saxe, J. P., Mottahedeh, J., Pyle, A. D., Wu, H. and Kornblum, H. I. (2011) Proliferative neural stem cells have high endogenous ROS levels that regulate self-renewal and neurogenesis in a PI3K/Akt-dependant manner. *Cell Stem Cell,* **8,** 59-71.

Lee, C. and Agoston, D. V. (2010) Vascular endothelial growth factor is involved in mediating increased de novo hippocampal neurogenesis in response to traumatic brain injury. *J Neurotrauma,* **27,** 541-553.

Li, G., Bien-Ly, N., Andrews-Zwilling, Y., Xu, Q., Bernardo, A., Ring, K., Halabisky, B., Deng, C., Mahley, R. W. and Huang, Y. (2009) GABAergic interneuron dysfunction impairs hippocampal neurogenesis in adult apolipoprotein E4 knockin mice. *Cell Stem Cell,* **5,** 634-645.

Lichtenwalner, R. J., Forbes, M. E., Sonntag, W. E. and Riddle, D. R. (2006) Adult-onset deficiency in growth hormone and insulin-like growth factor-I decreases survival of dentate granule neurons: insights into the regulation of adult hippocampal neurogenesis. *J Neurosci Res,* **83,** 199-210.

Lie, D. C., Colamarino, S. A., Song, H. J., Desire, L., Mira, H., Consiglio, A., Lein, E. S., Jessberger, S., Lansford, H., Dearie, A. R. and Gage, F. H. (2005) Wnt signalling regulates adult hippocampal neurogenesis. *Nature,* **437,** 1370-1375.

Lillig, C. H. and Holmgren, A. (2007) Thioredoxin and related molecules--from biology to health and disease. *Antioxid Redox Signal,* **9,** 25-47.

Lim, D. A. and Alvarez-Buylla, A. (2016) The Adult Ventricular-Subventricular Zone (V-SVZ) and Olfactory Bulb (OB) Neurogenesis. *Cold Spring Harb Perspect Biol,* **8**.

Lim, D. A., Tramontin, A. D., Trevejo, J. M., Herrera, D. G., Garcia-Verdugo, J. M. and Alvarez-Buylla, A. (2000) Noggin antagonizes BMP signaling to create a niche for adult neurogenesis. *Neuron,* **28,** 713-726.

Lin, T. N., Te, J., Lee, M., Sun, G. Y. and Hsu, C. Y. (1997) Induction of basic fibroblast growth factor (bFGF) expression following focal cerebral ischemia. *Brain Res Mol Brain Res,* **49,** 255-265.

Liu, Y. and Szaro, B. G. (2011) hnRNP K post-transcriptionally co-regulates multiple cytoskeletal genes needed for axonogenesis. *Development,* **138,** 3079-3090.

Lois, C. and Alvarez-Buylla, A. (1993) Proliferating subventricular zone cells in the adult mammalian forebrain can differentiate into neurons and glia. *Proc Natl Acad Sci U S A,* **90,** 2074-2077.

Lois, C. and Alvarez-Buylla, A. (1994) Long-distance neuronal migration in the adult mammalian brain. *Science,* **264,** 1145-1148.

Lois, C., Garcia-Verdugo, J. M. and Alvarez-Buylla, A. (1996) Chain migration of neuronal precursors. *Science,* **271,** 978-981.

Lorenz, K., Lohse, M. J. and Quitterer, U. (2003) Protein kinase C switches the Raf kinase inhibitor from Raf-1 to GRK-2. *Nature,* **426,** 574-579.

Lu, J. and Gao, F. H. (2017) The Molecular Mechanisms and the Role of hnRNP K Protein Post- Translational Modification in DNA Damage Repair. *Curr Med Chem,* **24,** 614-621.

Luarte, A., Cisternas, P., Caviedes, A., Batiz, L. F., Lafourcade, C., Wyneken, U. and Henzi, R. (2017) Astrocytes at the Hub of the Stress Response: Potential Modulation of Neurogenesis by miRNAs in Astrocyte-Derived Exosomes. *Stem Cells Int,* **2017,** 1719050.

Lugert, S., Basak, O., Knuckles, P., Haussler, U., Fabel, K., Gotz, M., Haas, C. A., Kempermann, G., Taylor, V. and Giachino, C. (2010) Quiescent and active hippocampal neural stem cells with distinct morphologies respond selectively to physiological and pathological stimuli and aging. *Cell Stem Cell,* **6,** 445-456.

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

Machold, R., Hayashi, S., Rutlin, M., Muzumdar, M. D., Nery, S., Corbin, J. G., Gritli-Linde, A., Dellovade, T., Porter, J. A., Rubin, L. L., Dudek, H., McMahon, A. P. and Fishell, G. (2003) Sonic hedgehog is required for progenitor cell maintenance in telencephalic stem cell niches. *Neuron,* **39,** 937-950.

Martinez-Ruiz, A., Araujo, I. M., Izquierdo-Alvarez, A., Hernansanz-Agustin, P., Lamas, S. and Serrador, J. M. (2013) Specificity in S-nitrosylation: a short-range mechanism for NO signaling? *Antioxid Redox Signal,* **19,** 1220-1235.

Martinez-Ruiz, A., Cadenas, S. and Lamas, S. (2011) Nitric oxide signaling: classical, less classical, and nonclassical mechanisms. *Free Radic Biol Med,* **51,** 17-29.

Martinez-Ruiz, A. and Lamas, S. (2004) S-nitrosylation: a potential new paradigm in signal transduction. *Cardiovasc Res,* **62,** 43-52.

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

Mathews, W. R. and Kerr, S. W. (1993) Biological activity of S-nitrosothiols: the role of nitric oxide. *J Pharmacol Exp Ther,* **267,** 1529-1537.

McCurdy, R. D., Feron, F., McGrath, J. J. and Mackay-Sim, A. (2005) Regulation of adult olfactory neurogenesis by insulin-like growth factor-I. *Eur J Neurosci,* **22,** 1581-1588.

Mercier, F., Kitasako, J. T. and Hatton, G. I. (2002) Anatomy of the brain neurogenic zones revisited: fractones and the fibroblast/macrophage network. *J Comp Neurol,* **451,** 170-188.

Mieyal, J. J., Gallogly, M. M., Qanungo, S., Sabens, E. A. and Shelton, M. D. (2008) Molecular mechanisms and clinical implications of reversible protein S-glutathionylation. *Antioxid Redox Signal,* **10,** 1941-1988.

Mirochnic, S., Wolf, S., Staufenbiel, M. and Kempermann, G. (2009) Age effects on the regulation of adult hippocampal neurogenesis by physical activity and environmental enrichment in the APP23 mouse model of Alzheimer disease. *Hippocampus,* **19,** 1008-1018.

Monje, M. L., Toda, H. and Palmer, T. D. (2003) Inflammatory blockade restores adult hippocampal neurogenesis. *Science,* **302,** 1760-1765.

Moreno-Lopez, B., Romero-Grimaldi, C., Noval, J. A., Murillo-Carretero, M., Matarredona, E. R. and Estrada, C. (2004) Nitric oxide is a physiological inhibitor of neurogenesis in the adult mouse subventricular zone and olfactory bulb. *J Neurosci,* **24,** 85-95.

Morley, D. and Keefer, L. K. (1993) Nitric oxide/nucleophile complexes: a unique class of nitric oxide-based vasodilators. *J Cardiovasc Pharmacol,* **22 Suppl 7,** S3-9.

Morrison, S. J. and Kimble, J. (2006) Asymmetric and symmetric stem-cell divisions in development and cancer. *Nature,* **441,** 1068-1074.

Morte, M. I., Carreira, B. P., Machado, V., Carmo, A., Nunes-Correia, I., Carvalho, C. M. and Araujo, I. M. (2013) Evaluation of proliferation of neural stem cells in vitro and in vivo. *Curr Protoc Stem Cell Biol,* **Chapter 2,** Unit 2D 14.

Murillo-Carretero, M., Torroglosa, A., Castro, C., Villalobo, A. and Estrada, C. (2009) S-Nitrosylation of the epidermal growth factor receptor: a regulatory mechanism of receptor tyrosine kinase activity. *Free Radic Biol Med,* **46,** 471-479.

Naylor, A. S., Bull, C., Nilsson, M. K., Zhu, C., Bjork-Eriksson, T., Eriksson, P. S., Blomgren, K. and Kuhn, H. G. (2008) Voluntary running rescues adult hippocampal neurogenesis after irradiation of the young mouse brain. *Proc Natl Acad Sci U S A,* **105,** 14632-14637.

Newton, S. S., Collier, E. F., Hunsberger, J., Adams, D., Terwilliger, R., Selvanayagam, E. and Duman, R. S. (2003) Gene profile of electroconvulsive seizures: induction of neurotrophic and angiogenic factors. *J Neurosci,* **23,** 10841-10851.

Nimmerjahn, A., Kirchhoff, F. and Helmchen, F. (2005) Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. *Science,* **308,** 1314-1318.

Nithianantharajah, J., Levis, H. and Murphy, M. (2004) Environmental enrichment results in cortical and subcortical changes in levels of synaptophysin and PSD-95 proteins. *Neurobiol Learn Mem,* **81,** 200-210.

Niv, F., Keiner, S., Krishna, Witte, O. W., Lie, D. C. and Redecker, C. (2012) Aberrant neurogenesis after stroke: a retroviral cell labeling study. *Stroke,* **43,** 2468-2475.

Nott, A., Nitarska, J., Veenvliet, J. V., Schacke, S., Derijck, A. A., Sirko, P., Muchardt, C., Pasterkamp, R. J., Smidt, M. P. and Riccio, A. (2013) S-nitrosylation of HDAC2 regulates the expression of the chromatin-remodeling factor Brm during radial neuron migration. *Proc Natl Acad Sci U S A,* **110,** 3113-3118.

Nott, A., Watson, P. M., Robinson, J. D., Crepaldi, L. and Riccio, A. (2008) S-Nitrosylation of histone deacetylase 2 induces chromatin remodelling in neurons. *Nature,* **455,** 411-415.

Nuber, S., Petrasch-Parwez, E., Winner, B., Winkler, J., von Horsten, S., Schmidt, T., Boy, J., Kuhn, M., Nguyen, H. P., Teismann, P., Schulz, J. B., Neumann, M., Pichler, B. J., Reischl, G., Holzmann, C., Schmitt, I., Bornemann, A., Kuhn, W., Zimmermann, F., Servadio, A. and Riess, O. (2008) Neurodegeneration and motor dysfunction in a conditional model of Parkinson's disease. *J Neurosci,* **28,** 2471-2484.

Ojika, K., Mitake, S., Tohdoh, N., Appel, S. H., Otsuka, Y., Katada, E. and Matsukawa, N. (2000) Hippocampal cholinergic neurostimulating peptides (HCNP). *Prog Neurobiol,* **60,** 37-83.

Okamoto, S., Nakamura, T., Cieplak, P., Chan, S. F., Kalashnikova, E., Liao, L., Saleem, S., Han, X., Clemente, A., Nutter, A., Sances, S., Brechtel, C., Haus, D., Haun, F., Sanz-Blasco, S., Huang, X., Li, H., Zaremba, J. D., Cui, J., Gu, Z., Nikzad, R., Harrop, A., McKercher, S. R., Godzik, A., Yates, J. R., 3rd and Lipton, S. A. (2014) S-nitrosylation-mediated redox transcriptional switch modulates neurogenesis and neuronal cell death. *Cell Rep,* **8,** 217-228.

Okamoto, T., Akaike, T., Sawa, T., Miyamoto, Y., van der Vliet, A. and Maeda, H. (2001) Activation of matrix metalloproteinases by peroxynitrite-induced protein S-glutathiolation via disulfide S-oxide formation. *J Biol Chem,* **276,** 29596-29602.

Olson, A. K., Eadie, B. D., Ernst, C. and Christie, B. R. (2006) Environmental enrichment and voluntary exercise massively increase neurogenesis in the adult hippocampus via dissociable pathways. *Hippocampus,* **16,** 250-260.

Olsson, T., Mohammed, A. H., Donaldson, L. F., Henriksson, B. G. and Seckl, J. R. (1994) Glucocorticoid receptor and NGFI-A gene expression are induced in the hippocampus after environmental enrichment in adult rats. *Brain Res Mol Brain Res,* **23,** 349-353.

Overstreet-Wadiche, L. S., Bromberg, D. A., Bensen, A. L. and Westbrook, G. L. (2006) Seizures accelerate functional integration of adult-generated granule cells. *J Neurosci,* **26,** 4095-4103.

Packer, M. A., Stasiv, Y., Benraiss, A., Chmielnicki, E., Grinberg, A., Westphal, H., Goldman, S. A. and Enikolopov, G. (2003) Nitric oxide negatively regulates mammalian adult neurogenesis. *Proc Natl Acad Sci U S A,* **100,** 9566-9571.

Palmer, T. D., Takahashi, J. and Gage, F. H. (1997) The adult rat hippocampus contains primordial neural stem cells. *Mol Cell Neurosci,* **8,** 389-404.

Palmer, T. D., Willhoite, A. R. and Gage, F. H. (2000) Vascular niche for adult hippocampal neurogenesis. *J Comp Neurol,* **425,** 479-494.

Parent, J. M. (2007) Adult neurogenesis in the intact and epileptic dentate gyrus. *Prog Brain Res,* **163,** 529-540.

Parent, J. M., Valentin, V. V. and Lowenstein, D. H. (2002) Prolonged seizures increase proliferating neuroblasts in the adult rat subventricular zone-olfactory bulb pathway. *J Neurosci,* **22,** 3174-3188.

Parent, J. M., Yu, T. W., Leibowitz, R. T., Geschwind, D. H., Sloviter, R. S. and Lowenstein, D. H. (1997) Dentate granule cell neurogenesis is increased by seizures and contributes to aberrant network reorganization in the adult rat hippocampus. *J Neurosci,* **17,** 3727-3738.

Pecorari, L., Marin, O., Silvestri, C., Candini, O., Rossi, E., Guerzoni, C., Cattelani, S., Mariani, S. A., Corradini, F., Ferrari-Amorotti, G., Cortesi, L., Bussolari, R., Raschella, G., Federico, M. R. and Calabretta, B. (2009) Elongation Factor 1 alpha interacts with phospho-Akt in breast cancer cells and regulates their proliferation, survival and motility. *Mol Cancer,* **8,** 58.

Peltier, J., O'Neill, A. and Schaffer, D. V. (2007) PI3K/Akt and CREB regulate adult neural hippocampal progenitor proliferation and differentiation. *Dev Neurobiol,* **67,** 1348-1361.

Phillips, W., Morton, A. J. and Barker, R. A. (2005) Abnormalities of neurogenesis in the R6/2 mouse model of Huntington's disease are attributable to the in vivo microenvironment. *J Neurosci,* **25,** 11564-11576.

Piccin, D., Tufford, A. and Morshead, C. M. (2014) Neural stem and progenitor cells in the aged subependyma are activated by the young niche. *Neurobiol Aging,* **35,** 1669-1679.

Pino, D., Choe, Y. and Pleasure, S. J. (2011) Wnt5a controls neurite development in olfactory bulb interneurons. *ASN Neuro,* **3,** e00059.

Pittman, Y. R., Valente, L., Jeppesen, M. G., Andersen, G. R., Patel, S. and Kinzy, T. G. (2006) Mg2+ and a key lysine modulate exchange activity of eukaryotic translation elongation factor 1B alpha. *J Biol Chem,* **281,** 19457-19468.

Potter, L. R. (2011) Guanylyl cyclase structure, function and regulation. *Cell Signal,* **23,** 1921-1926.

Pun, R. Y., Rolle, I. J., Lasarge, C. L., Hosford, B. E., Rosen, J. M., Uhl, J. D., Schmeltzer, S. N., Faulkner, C., Bronson, S. L., Murphy, B. L., Richards, D. A., Holland, K. D. and Danzer, S. C. (2012) Excessive activation of mTOR in postnatally generated granule cells is sufficient to cause epilepsy. *Neuron,* **75,** 1022-1034.

Qiao, H., Foote, M., Graham, K., Wu, Y. and Zhou, Y. (2014) 14-3-3 proteins are required for hippocampal long-term potentiation and associative learning and memory. *J Neurosci,* **34,** 4801-4808.

Radi, R. (2004) Nitric oxide, oxidants, and protein tyrosine nitration. *Proc Natl Acad Sci U S A,* **101,** 4003-4008.

Rampon, C., Jiang, C. H., Dong, H., Tang, Y. P., Lockhart, D. J., Schultz, P. G., Tsien, J. Z. and Hu, Y. (2000) Effects of environmental enrichment on gene expression in the brain. *Proc Natl Acad Sci U S A,* **97,** 12880-12884.

Rappa, G., Kunke, D., Holter, J., Diep, D. B., Meyer, J., Baum, C., Fodstad, O., Krauss, S. and Lorico, A. (2004) Efficient expansion and gene transduction of mouse neural stem/progenitor cells on recombinant fibronectin. *Neuroscience,* **124,** 823-830.

Ribak, C. E., Tran, P. H., Spigelman, I., Okazaki, M. M. and Nadler, J. V. (2000) Status epilepticus-induced hilar basal dendrites on rodent granule cells contribute to recurrent excitatory circuitry. *J Comp Neurol,* **428,** 240-253.

Ricard, J., Salinas, J., Garcia, L. and Liebl, D. J. (2006) EphrinB3 regulates cell proliferation and survival in adult neurogenesis. *Mol Cell Neurosci,* **31,** 713-722.

Rice, A. C., Khaldi, A., Harvey, H. B., Salman, N. J., White, F., Fillmore, H. and Bullock, M. R. (2003) Proliferation and neuronal differentiation of mitotically active cells following traumatic brain injury. *Exp Neurol,* **183,** 406-417.

Ridder, S., Chourbaji, S., Hellweg, R., Urani, A., Zacher, C., Schmid, W., Zink, M., Hortnagl, H., Flor, H., Henn, F. A., Schutz, G. and Gass, P. (2005) Mice with genetically altered glucocorticoid receptor expression show altered sensitivity for stress-induced depressive reactions. *J Neurosci,* **25,** 6243-6250.

Roberts, R. L., Mosch, H. U. and Fink, G. R. (1997) 14-3-3 proteins are essential for RAS/MAPK cascade signaling during pseudohyphal development in S. cerevisiae. *Cell,* **89,** 1055-1065.

Rodriguez, J. J., Jones, V. C., Tabuchi, M., Allan, S. M., Knight, E. M., LaFerla, F. M., Oddo, S. and Verkhratsky, A. (2008) Impaired adult neurogenesis in the dentate gyrus of a triple transgenic mouse model of Alzheimer's disease. *PLoS One,* **3,** e2935.

Rodriguez-Juarez, F., Aguirre, E. and Cadenas, S. (2007) Relative sensitivity of soluble guanylate cyclase and mitochondrial respiration to endogenous nitric oxide at physiological oxygen concentration. *Biochem J,* **405,** 223-231.

Rolls, A., Shechter, R., London, A., Ziv, Y., Ronen, A., Levy, R. and Schwartz, M. (2007) Toll-like receptors modulate adult hippocampal neurogenesis. *Nat Cell Biol,* **9,** 1081-1088.

Russo, I., Barlati, S. and Bosetti, F. (2011) Effects of neuroinflammation on the regenerative capacity of brain stem cells. *J Neurochem,* **116,** 947-956.

Russwurm, M. and Koesling, D. (2004) NO activation of guanylyl cyclase. *EMBO J,* **23,** 4443-4450.

Sagisaka, T., Matsukawa, N., Toyoda, T., Uematsu, N., Kanamori, T., Wake, H., Borlongan, C. V. and Ojika, K. (2010) Directed neural lineage differentiation of adult hippocampal progenitor cells via modulation of hippocampal cholinergic neurostimulating peptide precursor expression. *Brain Res,* **1327,** 107-117.

Sanai, N., Nguyen, T., Ihrie, R. A., Mirzadeh, Z., Tsai, H. H., Wong, M., Gupta, N., Berger, M. S., Huang, E., Garcia-Verdugo, J. M., Rowitch, D. H. and Alvarez-Buylla, A. (2011) Corridors of migrating neurons in the human brain and their decline during infancy. *Nature,* **478,** 382-386.

Santos, A. I., Carreira, B. P., Izquierdo-Alvarez, A., Ramos, E., Lourenco, A. S., Santos, D. F., Morte, M. I., Ribeiro, L. F., Marreiros, A., Sanchez-Lopez, N., Marina, A., Carvalho, C. M., Martinez-Ruiz, A. and Araujo, I. M. (2017) S-Nitrosylation of Ras Mediates Nitric Oxide-Dependent Post-Injury Neurogenesis in a Seizure Model. *Antioxid Redox Signal*.

Santos, A. I., Martinez-Ruiz, A. and Araujo, I. M. (2015) S-nitrosation and neuronal plasticity. *Br J Pharmacol,* **172,** 1468-1478.

Santos, R. M., Lourenco, C. F., Gerhardt, G. A., Cadenas, E., Laranjinha, J. and Barbosa, R. M. (2011) Evidence for a pathway that facilitates nitric oxide diffusion in the brain. *Neurochem Int,* **59,** 90-96.

Santos, A. I., Lourenço, A. S., Simão, S., Silva, D., Santos, D., Onofre, A. P., Pereira, A. C., Izquierdo-Álvarez, A., Ramos, E., Araújo, I. M., Martínez-Ruiz, A. Nitric oxide-induced regulation of neurogenesis: Identification of new targets of S-nitrosylation in NSC (submitted).

Scharfman, H. E., Goodman, J. H. and Sollas, A. L. (2000) Granule-like neurons at the hilar/CA3 border after status epilepticus and their synchrony with area CA3 pyramidal cells: functional implications of seizure-induced neurogenesis. *J Neurosci,* **20,** 6144-6158.

Schauwecker, P. E. and Steward, O. (1997) Genetic determinants of susceptibility to excitotoxic cell death: implications for gene targeting approaches. *Proc Natl Acad Sci U S A,* **94,** 4103-4108.

Schindler, C. K., Heverin, M. and Henshall, D. C. (2006) Isoform- and subcellular fraction-specific differences in hippocampal 14-3-3 levels following experimentally evoked seizures and in human temporal lobe epilepsy. *J Neurochem,* **99,** 561-569.

Schindler, C. K., Shinoda, S., Simon, R. P. and Henshall, D. C. (2004) Subcellular distribution of Bcl-2 family proteins and 14-3-3 within the hippocampus during seizure-induced neuronal death in the rat. *Neurosci Lett,* **356,** 163-166.

Segovia, G., Yague, A. G., Garcia-Verdugo, J. M. and Mora, F. (2006) Environmental enrichment promotes neurogenesis and changes the extracellular concentrations of glutamate and GABA in the hippocampus of aged rats. *Brain Res Bull,* **70,** 8-14.

Seidenfaden, R., Desoeuvre, A., Bosio, A., Virard, I. and Cremer, H. (2006) Glial conversion of SVZ-derived committed neuronal precursors after ectopic grafting into the adult brain. *Mol Cell Neurosci,* **32,** 187-198.

Serre, L., Vallee, B., Bureaud, N., Schoentgen, F. and Zelwer, C. (1998) Crystal structure of the phosphatidylethanolamine-binding protein from bovine brain: a novel structural class of phospholipid-binding proteins. *Structure,* **6,** 1255-1265.

Shapiro, L. A., Korn, M. J. and Ribak, C. E. (2005) Newly generated dentate granule cells from epileptic rats exhibit elongated hilar basal dendrites that align along GFAP-immunolabeled processes. *Neuroscience,* **136,** 823-831.

Shetty, A. K., Hattiangady, B. and Shetty, G. A. (2005) Stem/progenitor cell proliferation factors FGF-2, IGF-1, and VEGF exhibit early decline during the course of aging in the hippocampus: role of astrocytes. *Glia,* **51,** 173-186.

Shihabuddin, L. S., Horner, P. J., Ray, J. and Gage, F. H. (2000) Adult spinal cord stem cells generate neurons after transplantation in the adult dentate gyrus. *J Neurosci,* **20,** 8727-8735.

Shingo, T., Gregg, C., Enwere, E., Fujikawa, H., Hassam, R., Geary, C., Cross, J. C. and Weiss, S. (2003) Pregnancy-stimulated neurogenesis in the adult female forebrain mediated by prolactin. *Science,* **299,** 117-120.

Shruster, A., Ben-Zur, T., Melamed, E. and Offen, D. (2012) Wnt signaling enhances neurogenesis and improves neurological function after focal ischemic injury. *PLoS One,* **7,** e40843.

Sibbe, M., Haussler, U., Dieni, S., Althof, D., Haas, C. A. and Frotscher, M. (2012) Experimental epilepsy affects Notch1 signalling and the stem cell pool in the dentate gyrus. *Eur J Neurosci,* **36,** 3643-3652.

Sierra, A., Martin-Suarez, S., Valcarcel-Martin, R., Pascual-Brazo, J., Aelvoet, S. A., Abiega, O., Deudero, J. J., Brewster, A. L., Bernales, I., Anderson, A. E., Baekelandt, V., Maletic-Savatic, M. and Encinas, J. M. (2015) Neuronal hyperactivity accelerates depletion of neural stem cells and impairs hippocampal neurogenesis. *Cell Stem Cell,* **16,** 488-503.

Singh, R. J., Hogg, N., Joseph, J. and Kalyanaraman, B. (1996) Mechanism of nitric oxide release from S-nitrosothiols. *J Biol Chem,* **271,** 18596-18603.

Sisti, H. M., Glass, A. L. and Shors, T. J. (2007) Neurogenesis and the spacing effect: learning over time enhances memory and the survival of new neurons. *Learn Mem,* **14,** 368-375.

Skinner, J. J. and Rosner, M. R. (2014) RKIP structure drives its function: a three-state model for regulation of RKIP. *Crit Rev Oncog,* **19,** 483-488.

Smani, D., Sarkar, S., Raymick, J., Kanungo, J., Paule, M. G. and Gu, Q. (2017) Downregulation of 14-3-3 Proteins in a Kainic Acid-Induced Neurotoxicity Model. *Mol Neurobiol*.

Smith, M. A., Makino, S., Kvetnansky, R. and Post, R. M. (1995) Stress and glucocorticoids affect the expression of brain-derived neurotrophic factor and neurotrophin-3 mRNAs in the hippocampus. *J Neurosci,* **15,** 1768-1777.

Song, H., Stevens, C. F. and Gage, F. H. (2002) Astroglia induce neurogenesis from adult neural stem cells. *Nature,* **417,** 39-44.

Souza, J. M., Daikhin, E., Yudkoff, M., Raman, C. S. and Ischiropoulos, H. (1999) Factors determining the selectivity of protein tyrosine nitration. *Arch Biochem Biophys,* **371,** 169-178.

Souza, J. M., Peluffo, G. and Radi, R. (2008) Protein tyrosine nitration--functional alteration or just a biomarker? *Free Radic Biol Med,* **45,** 357-366.

Spalding, K. L., Bergmann, O., Alkass, K., Bernard, S., Salehpour, M., Huttner, H. B., Bostrom, E., Westerlund, I., Vial, C., Buchholz, B. A., Possnert, G., Mash, D. C., Druid, H. and Frisen, J. (2013) Dynamics of hippocampal neurogenesis in adult humans. *Cell,* **153,** 1219-1227.

Sultan, S., Li, L., Moss, J., Petrelli, F., Casse, F., Gebara, E., Lopatar, J., Pfrieger, F. W., Bezzi, P., Bischofberger, J. and Toni, N. (2015) Synaptic Integration of Adult-Born Hippocampal Neurons Is Locally Controlled by Astrocytes. *Neuron,* **88,** 957-972.

Sun, B., Halabisky, B., Zhou, Y., Palop, J. J., Yu, G., Mucke, L. and Gan, L. (2009a) Imbalance between GABAergic and Glutamatergic Transmission Impairs Adult Neurogenesis in an Animal Model of Alzheimer's Disease. *Cell Stem Cell,* **5,** 624-633.

Sun, D., Bullock, M. R., Altememi, N., Zhou, Z., Hagood, S., Rolfe, A., McGinn, M. J., Hamm, R. and Colello, R. J. (2010) The effect of epidermal growth factor in the injured brain after trauma in rats. *J Neurotrauma,* **27,** 923-938.

Sun, D., Bullock, M. R., McGinn, M. J., Zhou, Z., Altememi, N., Hagood, S., Hamm, R. and Colello, R. J. (2009b) Basic fibroblast growth factor-enhanced neurogenesis contributes to cognitive recovery in rats following traumatic brain injury. *Exp Neurol,* **216,** 56-65.

Sun, D., Colello, R. J., Daugherty, W. P., Kwon, T. H., McGinn, M. J., Harvey, H. B. and Bullock, M. R. (2005) Cell proliferation and neuronal differentiation in the dentate gyrus in juvenile and adult rats following traumatic brain injury. *J Neurotrauma,* **22,** 95-105.

Sutterlin, P., Williams, E. J., Chambers, D., Saraf, K., von Schack, D., Reisenberg, M., Doherty, P. and Williams, G. (2013) The molecular basis of the cooperation between EGF, FGF and eCB receptors in the regulation of neural stem cell function. *Mol Cell Neurosci,* **52,** 20-30.

Swain, R. A., Harris, A. B., Wiener, E. C., Dutka, M. V., Morris, H. D., Theien, B. E., Konda, S., Engberg, K., Lauterbur, P. C. and Greenough, W. T. (2003) Prolonged exercise induces angiogenesis and increases cerebral blood volume in primary motor cortex of the rat. *Neuroscience,* **117,** 1037-1046.

Sztainberg, Y., Kuperman, Y., Tsoory, M., Lebow, M. and Chen, A. (2010) The anxiolytic effect of environmental enrichment is mediated via amygdalar CRF receptor type 1. *Mol Psychiatry,* **15,** 905-917.

Takagi, Y., Nozaki, K., Takahashi, J., Yodoi, J., Ishikawa, M. and Hashimoto, N. (1999) Proliferation of neuronal precursor cells in the dentate gyrus is accelerated after transient forebrain ischemia in mice. *Brain Res,* **831,** 283-287.

Tanaka, E. M. and Reddien, P. W. (2011) The cellular basis for animal regeneration. *Dev Cell,* **21,** 172-185.

Tanapat, P., Hastings, N. B., Reeves, A. J. and Gould, E. (1999) Estrogen stimulates a transient increase in the number of new neurons in the dentate gyrus of the adult female rat. *J Neurosci,* **19,** 5792-5801.

Tang, H., Park, S., Sun, S. C., Trumbly, R., Ren, G., Tsung, E. and Yeung, K. C. (2010) RKIP inhibits NF-kappaB in cancer cells by regulating upstream signaling components of the IkappaB kinase complex. *FEBS Lett,* **584,** 662-668.

Tatar, C., Bessert, D., Tse, H. and Skoff, R. P. (2013) Determinants of central nervous system adult neurogenesis are sex, hormones, mouse strain, age, and brain region. *Glia,* **61,** 192-209.

Theroux, S., Pereira, M., Casten, K. S., Burwell, R. D., Yeung, K. C., Sedivy, J. M. and Klysik, J. (2007) Raf kinase inhibitory protein knockout mice: expression in the brain and olfaction deficit. *Brain Res Bull,* **71,** 559-567.

Theus, M. H., Ricard, J., Bethea, J. R. and Liebl, D. J. (2010) EphB3 limits the expansion of neural progenitor cells in the subventricular zone by regulating p53 during homeostasis and following traumatic brain injury. *Stem Cells,* **28,** 1231-1242.

Thored, P., Arvidsson, A., Cacci, E., Ahlenius, H., Kallur, T., Darsalia, V., Ekdahl, C. T., Kokaia, Z. and Lindvall, O. (2006) Persistent production of neurons from adult brain stem cells during recovery after stroke. *Stem Cells,* **24,** 739-747.

Thored, P., Wood, J., Arvidsson, A., Cammenga, J., Kokaia, Z. and Lindvall, O. (2007) Long-term neuroblast migration along blood vessels in an area with transient angiogenesis and increased vascularization after stroke. *Stroke,* **38,** 3032-3039.

Tonchev, A. B., Yamashima, T., Sawamoto, K. and Okano, H. (2005) Enhanced proliferation of progenitor cells in the subventricular zone and limited neuronal production in the striatum and neocortex of adult macaque monkeys after global cerebral ischemia. *J Neurosci Res,* **81,** 776-788.

Torroglosa, A., Murillo-Carretero, M., Romero-Grimaldi, C., Matarredona, E. R., Campos-Caro, A. and Estrada, C. (2007) Nitric oxide decreases subventricular zone stem cell proliferation by inhibition of epidermal growth factor receptor and phosphoinositide-3-kinase/Akt pathway. *Stem Cells,* **25,** 88-97.

Toyo-oka, K., Wachi, T., Hunt, R. F., Baraban, S. C., Taya, S., Ramshaw, H., Kaibuchi, K., Schwarz, Q. P., Lopez, A. F. and Wynshaw-Boris, A. (2014) 14-3-3epsilon and zeta regulate neurogenesis and differentiation of neuronal progenitor cells in the developing brain. *J Neurosci,* **34,** 12168-12181.

Toyoda, T., Matsukawa, N., Sagisaka, T., Uematsu, N., Kanamori, T., Kato, D., Mizuno, M., Wake, H., Hida, H., Borlongan, C. V. and Ojika, K. (2012) Suppression of astrocyte lineage in adult hippocampal progenitor cells expressing hippocampal cholinergic neurostimulating Peptide precursor in an in vivo ischemic model. *Cell Transplant,* **21,** 2159-2169.

Umahara, T., Uchihara, T., Nakamura, A. and Iwamoto, T. (2011) Differential expression of 14-3-3 protein isoforms in developing rat hippocampus, cortex, rostral migratory stream, olfactory bulb, and white matter. *Brain Res,* **1410,** 1-11.

van Praag, H., Christie, B. R., Sejnowski, T. J. and Gage, F. H. (1999a) Running enhances neurogenesis, learning, and long-term potentiation in mice. *Proc Natl Acad Sci U S A,* **96,** 13427-13431.

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

van Praag, H., Schinder, A. F., Christie, B. R., Toni, N., Palmer, T. D. and Gage, F. H. (2002) Functional neurogenesis in the adult hippocampus. *Nature,* **415,** 1030-1034.

Varela-Nallar, L., Alfaro, I. E., Serrano, F. G., Parodi, J. and Inestrosa, N. C. (2010) Wingless-type family member 5A (Wnt-5a) stimulates synaptic differentiation and function of glutamatergic synapses. *Proc Natl Acad Sci U S A,* **107,** 21164-21169.

Venema, R. C., Peters, H. I. and Traugh, J. A. (1991) Phosphorylation of valyl-tRNA synthetase and elongation factor 1 in response to phorbol esters is associated with stimulation of both activities. *J Biol Chem,* **266,** 11993-11998.

Villasana, L. E., Westbrook, G. L. and Schnell, E. (2014) Neurologic impairment following closed head injury predicts post-traumatic neurogenesis. *Exp Neurol,* **261,** 156-162.

Villeda, S. A., Luo, J., Mosher, K. I., Zou, B., Britschgi, M., Bieri, G., Stan, T. M., Fainberg, N., Ding, Z., Eggel, A., Lucin, K. M., Czirr, E., Park, J. S., Couillard-Despres, S.,

Aigner, L., Li, G., Peskind, E. R., Kaye, J. A., Quinn, J. F., Galasko, D. R., Xie, X. S., Rando, T. A. and Wyss-Coray, T. (2011) The ageing systemic milieu negatively regulates neurogenesis and cognitive function. *Nature,* **477,** 90-94.

Wang, S. C., Nakajima, Y., Yu, Y. L., Xia, W., Chen, C. T., Yang, C. C., McIntush, E. W., Li, L. Y., Hawke, D. H., Kobayashi, R. and Hung, M. C. (2006) Tyrosine phosphorylation controls PCNA function through protein stability. *Nat Cell Biol,* **8,** 1359-1368.

Weiss, S., Dunne, C., Hewson, J., Wohl, C., Wheatley, M., Peterson, A. C. and Reynolds, B. A. (1996) Multipotent CNS stem cells are present in the adult mammalian spinal cord and ventricular neuroaxis. *J Neurosci,* **16,** 7599-7609.

Wexler, E. M., Paucer, A., Kornblum, H. I., Palmer, T. D. and Geschwind, D. H. (2009) Endogenous Wnt signaling maintains neural progenitor cell potency. *Stem Cells,* **27,** 1130-1141.

Williams, B. M., Luo, Y., Ward, C., Redd, K., Gibson, R., Kuczaj, S. A. and McCoy, J. G. (2001) Environmental enrichment: effects on spatial memory and hippocampal CREB immunoreactivity. *Physiol Behav,* **73,** 649-658.

Winner, B., Lie, D. C., Rockenstein, E., Aigner, R., Aigner, L., Masliah, E., Kuhn, H. G. and Winkler, J. (2004) Human wild-type alpha-synuclein impairs neurogenesis. *J Neuropathol Exp Neurol,* **63,** 1155-1166.

Winner, B., Regensburger, M., Schreglmann, S., Boyer, L., Prots, I., Rockenstein, E., Mante, M., Zhao, C., Winkler, J., Masliah, E. and Gage, F. H. (2012) Role of alpha-synuclein in adult neurogenesis and neuronal maturation in the dentate gyrus. *J Neurosci,* **32,** 16906-16916.

Woitke, F., Ceanga, M., Rudolph, M., Niv, F., Witte, O. W., Redecker, C., Kunze, A. and Keiner, S. (2017) Adult hippocampal neurogenesis poststroke: More new granule cells but aberrant morphology and impaired spatial memory. *PLoS One,* **12,** e0183463.

Yamamoto, Y., Lee, D., Kim, Y., Lee, B., Seo, C., Kawasaki, H., Kuroda, S. and Tanaka-Yamamoto, K. (2012) Raf kinase inhibitory protein is required for cerebellar long-term synaptic depression by mediating PKC-dependent MAPK activation. *J Neurosci,* **32,** 14254-14264.

Yan, Y. P., Sailor, K. A., Vemuganti, R. and Dempsey, R. J. (2006) Insulin-like growth factor-1 is an endogenous mediator of focal ischemia-induced neural progenitor proliferation. *Eur J Neurosci,* **24,** 45-54.

Yano, M., Okano, H. J. and Okano, H. (2005) Involvement of Hu and heterogeneous nuclear ribonucleoprotein K in neuronal differentiation through p21 mRNA post-transcriptional regulation. *J Biol Chem,* **280,** 12690-12699.

Yeung, K., Janosch, P., McFerran, B., Rose, D. W., Mischak, H., Sedivy, J. M. and Kolch, W. (2000) Mechanism of suppression of the Raf/MEK/extracellular signal-regulated kinase pathway by the raf kinase inhibitor protein. *Mol Cell Biol,* **20,** 3079-3085.

Yeung, K., Seitz, T., Li, S., Janosch, P., McFerran, B., Kaiser, C., Fee, F., Katsanakis, K. D., Rose, D. W., Mischak, H., Sedivy, J. M. and Kolch, W. (1999) Suppression of Raf-1 kinase activity and MAP kinase signalling by RKIP. *Nature,* **401,** 173-177.

Yeung, K. C., Rose, D. W., Dhillon, A. S., Yaros, D., Gustafsson, M., Chatterjee, D., McFerran, B., Wyche, J., Kolch, W. and Sedivy, J. M. (2001) Raf kinase inhibitor protein interacts with NF-kappaB-inducing kinase and TAK1 and inhibits NF-kappaB activation. *Mol Cell Biol,* **21,** 7207-7217.

Yin, L., Xie, Y., Yin, S., Lv, X., Zhang, J., Gu, Z., Sun, H. and Liu, S. (2015) The S-nitrosylation status of PCNA localized in cytosol impacts the apoptotic pathway in a Parkinson's disease paradigm. *PLoS One,* **10,** e0117546.

Yoshimura, S., Takagi, Y., Harada, J., Teramoto, T., Thomas, S. S., Waeber, C., Bakowska, J. C., Breakefield, X. O. and Moskowitz, M. A. (2001) FGF-2

regulation of neurogenesis in adult hippocampus after brain injury. *Proc Natl Acad Sci U S A,* **98,** 5874-5879.

Zhang, J. and Jiao, J. (2015) Molecular Biomarkers for Embryonic and Adult Neural Stem Cell and Neurogenesis. *Biomed Res Int,* **2015,** 727542.

Zhang, R., Zhang, Z., Wang, L., Wang, Y., Gousev, A., Zhang, L., Ho, K. L., Morshead, C. and Chopp, M. (2004) Activated neural stem cells contribute to stroke-induced neurogenesis and neuroblast migration toward the infarct boundary in adult rats. *J Cereb Blood Flow Metab,* **24,** 441-448.

Zhang, R. L., Chopp, M., Roberts, C., Liu, X., Wei, M., Nejad-Davarani, S. P., Wang, X. and Zhang, Z. G. (2014) Stroke increases neural stem cells and angiogenesis in the neurogenic niche of the adult mouse. *PLoS One,* **9,** e113972.

Zhang, X. Q., Mu, J. W., Wang, H. B., Jolkkonen, J., Liu, T. T., Xiao, T., Zhao, M., Zhang, C. D. and Zhao, C. S. (2016) Increased protein expression levels of pCREB, BDNF and SDF-1/CXCR4 in the hippocampus may be associated with enhanced neurogenesis induced by environmental enrichment. *Mol Med Rep,* **14,** 2231-2237.

Zhang, Y. and Hogg, N. (2005) S-Nitrosothiols: cellular formation and transport. *Free Radic Biol Med,* **38,** 831-838.

Zhu, D. Y., Liu, S. H., Sun, H. S. and Lu, Y. M. (2003) Expression of inducible nitric oxide synthase after focal cerebral ischemia stimulates neurogenesis in the adult rodent dentate gyrus. *J Neurosci,* **23,** 223-229.

Zhu, X. J., Hua, Y., Jiang, J., Zhou, Q. G., Luo, C. X., Han, X., Lu, Y. M. and Zhu, D. Y. (2006) Neuronal nitric oxide synthase-derived nitric oxide inhibits neurogenesis in the adult dentate gyrus by down-regulating cyclic AMP response element binding protein phosphorylation. *Neuroscience,* **141,** 827-836.