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UNIVERSIDADE DO ALGARVE
DEPARTAMENTO DE CIÊNCIAS BIOMÉDICAS E MEDICINA

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Departamento de Ciências Biomédicas e Medicina

Differential regulation of hippocampal neurogenesis by nitric oxide following seizures

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Daniela Filipa Jorge da Cruz Santos

Dissertação de Mestrado

Mestrado em Ciências Biomédicas

Orientação científica da Professora Doutora Inês Araújo

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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

Hoje sei que a vida é feita de todos os sentimentos. Quase como uma receita: temos os ingredientes (os que nascem connosco e os que nos são ensinados), e depois misturamos tudo. Nem sempre na ordem certa, mas no fim temos sempre qualquer coisa com a qual podemos aprender a melhorar.

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"(…) no dia em que nasceste, o Mundo sorriu e tu choraste. Vive a tua vida de forma a que, no dia em que morras, tu sorrias e o mundo chore."

Table of Contents

Abbreviations

ANOVA analysis of variance
bFGF basic fibroblast growth factor
BBB blood-brain barrier
BMP bone morphogenetic proteins
BrdU 5-bromo-2'-deoxyuridine
CldU 5-cloro-2'**-**deoxyuridine
CNS central nervous system
CREB cyclic response element binding protein
DCX doublecortin
DG dentate gyrus
ECM extracellular matrix
EdU 5-ethynyl-2'-deoxyuridine
EGF epidermal growth factor
eNOS endothelial nitric oxide synthase
GABA γ-aminobutyric acid
GFAP glial fibrillary acidic protein
IdU 5-iodo-2'-deoxyuridine
IGZ inner granular zone
iNOS inducible nitric oxide synthase
iNOS$^{+/+}$ inducible nitric oxide synthase wildtype (mice)
iNOS$^{-/-}$ inducible nitric oxide synthase knockout (mice)
KA kainic acid
NeuN neuronal nuclei
NGS normal goat serum
NO nitric oxide
nNOS neuronal nitric oxide synthase
NSC neural stem cell
OB olfactory bulb
OGZ outer granular zone
PCNA proliferating cell nuclear antigen
PBS phosphate-buffered saline
PFA paraformaldehyde
RMS rostral migratory stream
SE *status epilepticus*
SEM standard error of the mean
SGZ subgranular zone
SVZ subventricular zone
TLE temporal lobe epilepsy
TUNEL terminal deoxynucleotidyl transferase dUTP nick end labeling
VEGF vascular endothelial growth factor

Abstract

The fact that the adult brain is able to produce new neurons or glial cells from neural stem cells (NSC) became one of the most interesting and challenging fields of research in neuroscience. Endogenous adult neurogenesis occurs in two main regions of the brain: the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) in the dentate gyrus. Brain injury may be accompanied by increased neurogenesis, although neuroinflammation promotes the activation of microglial cells that can be detrimental to the neurogenic process. Nitric oxide (NO) is one of the factors released by microglia that can be proneurogenic. The mechanism by which NO promotes the proliferation of NSCs has been intensively studied. However, little is known about the role of NO in migration, survival and differentiation of the newborn cells. The aim of this work was to investigate the role of NO from inflammatory origin in proliferation, migration, differentiation and survival of NSCs from the dentate gyrus in a mouse model of *status epilepticus*. We also assessed neuroinflammation in the same injury model. Our work showed that NO increased proliferation of the early-born cells after seizures, but is detrimental for their survival. NO also increased migration of neuroblasts. Moreover, NO was important to maintain long-term neuroinflammation. Taken together, these results show that NO may be a good target to promote proliferation and migration of NSCs following seizures, but compromises survival of early-born cells.

Key words: neural stem cells, adult neurogenesis, hippocampus, nitric oxide, *status epilepticus*

Resumo

A descoberta de que o cérebro adulto é capaz de produzir novas células nervosas ou da glia a partir de células estaminais neurais tornou-se uma das mais interessantes e desafiantes áreas da neurociência. A neurogénese adulta endógena ocorre em duas principais regiões do cérebro de mamíferos, ou nichos neurogénicos: a zona subventricular nos ventrículos laterais e a zona subgranular no giro dentado do hipocampo.

Apesar ter sido proposto que a neurogénese ocorre como uma tentativa do cérebro em reparar zonas lesionadas por um insulto, nestas condições, a neuroinflamação que resulta do evento promove a activação das células da microglia. As células da microglia são consideradas as células imunitárias do sistema nervoso central e ao serem activadas libertam factores inflamatórios que podem prejudicar o processo neurogénico.

O monóxido de azoto, ou óxido nítrico (do inglês *nitric oxide*, NO), é um dos factores libertados pela microglia activada e que apresenta um efeito dual na neurogénese, promovendo-a ou inibindo-a, dependendo da concentração e tempo de exposição. Esta molécula pleiotrópica resulta da actividade enzimática de uma das três isoformas da sintase do óxido nítrico: neuronal, endotelial ou indutível. O mecanismo pelo qual o NO participa na proliferação das células estaminais neurais tem sido intensivamente estudado por diversos grupos em todo o Mundo. Sabe-se que em condições fisiológicas o NO participa como um factor anti-neurogénico, mas, em condições que antecedem um insulto cerebral, o NO promove a proliferação de células estaminais em ambos os principais nichos neurogénicos. No entanto, pouco se sabe sobre a função do NO na migração, sobrevivência e diferenciação das novas células.

O objectivo deste trabalho foi investigar o papel do NO de origem inflamatória na proliferação, migração, diferenciação e sobrevivência de novas células formadas no giro dentado na sequência de um insulto cerebral. Foi também investigado o envolvimento do NO na resposta neuroinflamatória no mesmo modelo. Neste trabalho foi usado um modelo de murganho de *status epilepticus* induzido por administração intraperitoneal de ácido caínico, uma vez que é um modelo *in vivo* de lesão cerebral capaz de mimetizar os eventos neurogénicos pós-lesão de uma forma muito robusta, nomeadamente ao nível do aumento da proliferação de células estaminais, migração e diferenciação de novas células.

Os nossos resultados mostram que o tratamento com ácido kaínico aumentou o número de células em proliferação até 14 dias após lesão. Na ausência da enzima iNOS o tratamento com ácido kaínico diminuiu a proliferação de novas células até 14 dias após lesão, excepto no 7º dia, sugerindo uma regulação bifásica da proliferação pelo NO. Assim, é possível definir um período em que a proliferação é regulada por uma mecanismo dependente de NO (até 5 dias após lesão) e outro em que o NO parece não estar envolvido (7 dias após lesão). A migração de neuroblastos aumenta após lesão e é dependente de NO 14 dias após lesão. A distribuição das novas células ao longo do giro dentado formadas 3 dias após lesão foi alterada após crises epilépticas, mas o mecanismo pelo qual é regulado é independente de NO. Nas condições analisadas, o número de novos neurónios que resultam de células formadas 3 dias após lesão diminui, sugerindo que o NO é importante para a sobrevivência dos novos neurónios. A diferenciação das novas células em astrócitos não foi alterada após lesão. Por fim, a astrogliose está aumentada 28 dias após lesão.

O nosso trabalho mostra que o NO de origem inflamatória está envolvido na proliferação e sobrevivência dos novos neurónios formados numa fase inicial da proliferação após crises epilépticas. Os novos neurónios formados após lesão por *status epilepticus* parecem sobreviver melhor quando são formados numa fase mais tardia do processo proliferativo (7 dias após lesão), uma vez que o tratamento com ácido caínico não promoveu qualquer alteração no número de novos neurónios formados durante esta janela temporal. Além disso, o NO mostra ser importante para a migração de neuroblastos, uma vez que se verifica um aumento da imunoreactividade destas células após lesão. Verificou-se também que a neuroinflamação está presente 28 dias após lesão, o que sugere que o NO é importante para a manutenção da activação de astrócitos a longo prazo, indicando uma persistência de inflamação no hipocampo.

Assim, concluímos que o NO de origem inflamatória participa em diferentes fases da neurogénese no hipocampo, abrindo a possibilidade de explorar abordagens terapêuticas baseadas nos efeitos do NO em situações de lesão cerebral.

Palavras-chave: células estaminais neurais, neurogénese adulta, hipocampo, monóxido de azoto, *status epilepticus*

Chapter 1

Introduction

1. Introduction

Neurodegenerative diseases are receiving increasing attention and effort as their prevalence increases in an aging population. Replacing damaged neurons or lesioned areas by grafting new cells has been an active area of research. The discovery that the brain is able to produce new cells throughout adult life has further encouraged biomedical research in order to understand how this process is regulated and how to use it for the benefit of patients suffering from neurological disorders or brain lesions. The transplantation of neural stem cells (NSC) or endogenous manipulation of adult neurogenesis have been proposed as therapeutic strategies for the treatment of neurological disorders and the damaged brain. Thus it is important to understand the neurogenic process and how newborn cells survive in a context of brain injury.

1.1 Neurogenesis and neural stem cells

Neurogenesis is a complex multistep process by which new nerve cells are produced from neural stem cells (NSCs) and functional integrated into the central nervous system (CNS). For over 100 years it was believed that the production of new neurons was confined to embryonic development (Ramón y Cajal, 1928), stopping just before puberty and brain plasticity occurred as a result of structural changes involving synapses of pre-existing neurons (Alvarez-Buylla and Kirn, 1997). This concept was accepted and supported by the principal figures at the time, and became a central dogma in neuroscience. The term “neural stem cell” became popular in the last 20 years but the terminology is not yet fully defined, once the criterion is not common worldwide. NSCs are self-renewing and multipotent progenitor cells with the ability to differentiate into neural lineages including neurons and glia cells (Götz and Huttner, 2005). NSCs were first identified in the second half of the 19th century, with histological observations made by Wilhelm His of diving cells in the embryonic human brain (His, 1904). These germinal cells were different from the similar cells in other organs and stopped dividing after neuronal commitment. In the first half of the 20th century, the lack of detection methods for cell division and differentiation made the occasional references to postnatal neurogenesis in mammals to be ignored. In 1944, the neurosurgeon Globus and the neuropathologist Kuhlenbeck analyzed *post mortem* brain tissue and described bipotencial mother cells that differentiated into unipolar or bipolar neuroblasts (reviewed in Curtis et al., 2011). In the mid twentieth century, Joseph Altman and his colleagues labeled dividing cells with [^{3}H]-thymidine and revealed a constitutive production of new neurons in the adult hippocampus (Altman

and Das, 1965) and adult olfactory bulb (Altman, 1969). Twenty years later, the combination of [^{3}H]-thymidine labeling and electron microscopy showed mitosis in the subventricular zone (SVZ) of adult macaque monkeys (Kaplan, 1983), and later in the 1990's, adult neurogenesis was demonstrated in songbirds (Alvarez-Buylla and Kirn, 1997), validating the pioneering work of Altman. Since then, adult neurogenesis has been identified in many different animal species, such as crustaceans (Beltz and Sandeman, 2003; Schmidt, 2007), reptiles (Font et al., 2001), insects (Scotto-Lomassese et al., 2003; Cayre et al., 2007), fish (reviewed in Kizil et al., 2011) and mammals, including humans (Eriksson et al., 1998; Gage et al., 1995).

1.1.1 Endogenous adult neurogenesis

In most brain regions, the generation of new nerve cells is indeed restricted to the embryonic development period. In the adult central nervous system (CNS), endogenous neurogenesis occurs due to the existence of NSC in two specific brain regions, called neurogenic niches: the subventricular zone (SVZ) in the lateral walls of the lateral ventricles (Doetsch et al., 1997; Doetsch and Scharff, 2001; Curtis et al., 2007) (Fig. 1.1 A) and the subgranular zone (SGZ) of the dentate gyrus of the hippocampus (Eriksson et al., 1998; Limke and Rao, 2002) (Fig. 1.1 B). There is also some evidence of adult neurogenesis in mammalian brain outside this regions, namely in neocortex (Gould et al., 1999; Dayer et al., 2005), amygdala (Bernier et al., 2002), hypothalamus (Gould et al., 2001; Xu et al., 2005; Lee and Blackshaw, 2012) and spinal cord (Yamamoto et al., 2001), to mention a few.

The neurogenic niche is a microenvironmental system characterized by the presence of NSCs, supporting glial cells, vascular supply and local signals that regulate self-renewal capacity, activation and fate determination of NSCs (Alvarez-Buylla and Lim, 2004; Morrison and Spradling, 2008). The architecture and molecular signaling in the niche promote cell-cell and cell-extracellular matrix interactions, important to maintain stem cells location and characteristics (Conover and Notti, 2008; Suh et al., 2009). The interaction between stem cells and their somatic cells neighbors, as well as the extracellular matrix that surrounds the cells and confers structural organization, are very important to maintain NSCs within their niche and their stem cells characteristics. Astrocytes function as sensors of the neurogenic niche environment, contributing to detect alterations in neuronal and precursor number and translate signals from the blood vessels and other cells (Lim and Alvarez-Buylla, 1999). Despite the similarities between both neurogenic niches, namely the somatic cell signaling and presence of an

extracellular matrix and membrane basement to cell anchoring, there are structural differences between the SVZ and SGZ.

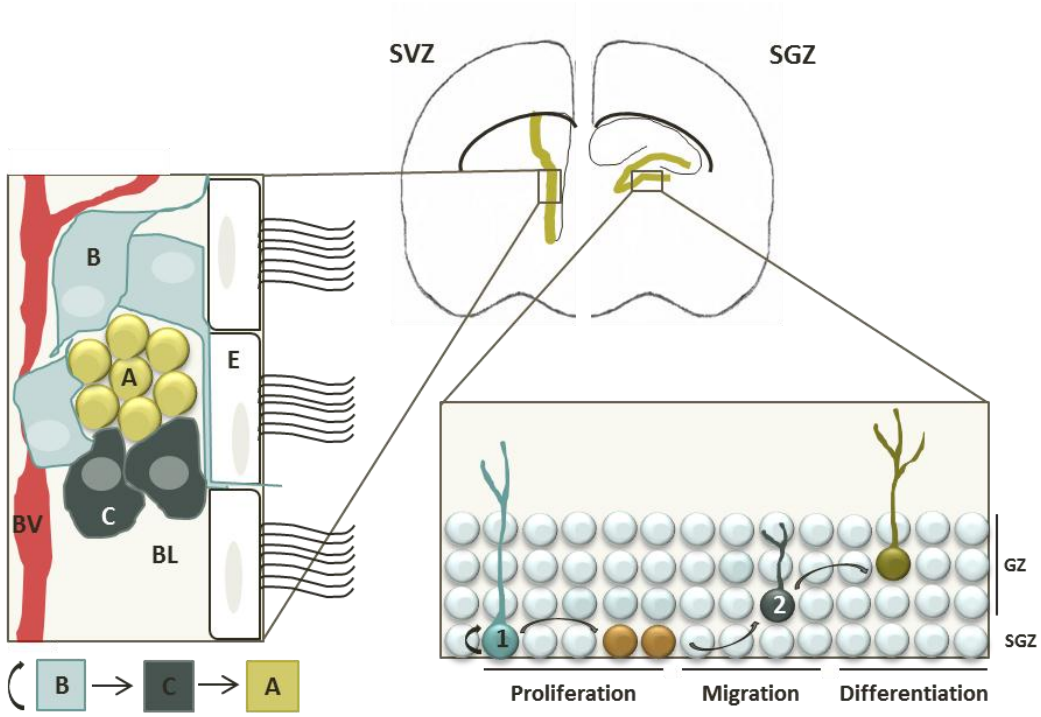

Figure 1.1 – Neurogenic niches and cell types. SVZ pannel, Coronal schematic section of adult mouse brain showing the location of the subventricular zone (SVZ) of lateral ventricles (green). SVZ is composed of a single layer of multi-ciliated ependymal cells **(E, white).** SVZ astrocytes, or type B cell **(B, light blue)**, are self-renewal primary precursors, and give rise to focal clusters of rapidly dividing transit amplifying cells, or type C cell **(C, brown)**. Neuroblasts, or type A cells **(A, light green)**, derived from type C cells, migrate throughout rostral migratory stream to the olfactory bulb, where they become local interneurons. Blood vessels **(BV)** and basal lamina **(BL)** are in close proximity to the SVZ. **SGZ pannel,** Coronal schematic section of adult mouse brain showing the location of the subgranular zone (SGZ) of dentate gyrus of hippocampus. NSCs **(blue)** from the SGZ with self-renewal capacity proliferate **(brown)** and migrate **(dark blue)** towards the granular zone (GZ) of the DG of hippocampus. After neuronal commitment and differentiation **(dark green)**, cells extend axonal projections to CA3 region of hippocampus.

The SVZ is a multicellular layer extending along the lateral wall of the lateral ventricles, considered the largest pool of NSCs of adult brain (Carleton et al., 2003). SVZ is composed by four main cell types: neuroblasts (type A cells), SVZ astrocytes (type B

cells), immature precursors (type C cells) and a single layer of multi-ciliated ependymal cells that separates the SVZ from the lateral ventricle (Fig. 1.1 A). Ependymal cells are very important in maintenance of the undifferentiated state of NSCs by producing noggin, an antagonist of bone morphogenetic proteins (BMP) signaling, and therefore preventing the glial differentiation of SVZ cells induced by BMPs. Under normal physiological conditions, the newborn cells spread along the rostral migratory stream (RMS) to the olfactory bulb, where they migrate radially and differentiate into functional granule and periglomerular neurons (Carleton et al., 2002; Doetsch, 2003), with functional synapses and electrophysiological activity (Carlen et al., 2002; Petreanu and Alvarez-Buylla, 2002; Belluzzi et al., 2003). Among these cells is also an oligodendrocyte precursor cell, known as O2A cell, making the SVZ a site of oligodendrocyte generation (Nait-Oumesmar *et al.*, 1999). Neurogenesis in the adult olfactory bulb has been identified in mammals, including humans (Curtis et al., 2007), with important function in odor memory and discrimination (Gheusi et al., 2000; Rochefort et al., 2002).

The SGZ of the dentate gyrus is described as a thin germinal layer, corresponding to the space between the beginning of granular layer of dentate gyrus and the hilus (Fig. 1.1 B) (Scharfman and Gray, 2007). Hippocampal neurogenesis (Fig 1.2) occurs locally and involves three sequential main steps: 1) proliferation of NSCs, 2) migration and 3) differentiation. The first step is a mitotic phase where astrocyte-like cells (type 1 cells) asymmetrically divide for about 7 to 8 days into immature precursors (neuroblast or type 2 cells) (Seri et al., 2004). During this period, type 1 cells, similarly to type B cells in SVZ, have a unique radial process and ramified structure at its end. Their morphology and conserved expression of glial fibrillary acidic protein (GFAP), Nestin and *Sox2* is consistent with radial glial cells that give rise to the first neurons during embryonic development (Suh et al., 2009). Type 2 cells, or neuroblasts, are nonradial cells similar to type C cells in SVZ. Both types of cells are very important in maintenance of neurogenesis, since its ablation results in the cessation of production of newborn cells in both neurogenic niches (Suh et al., 2009). After fate determination, the cells that survive start to migrate through granular zone of the dentate gyrus and during this 2 week period, cells start to extend axons and express doublecortin (DCX) and PSA-NCAM, a neural cell adhesion molecule. These cells give rise to functional granular neurons that extend axonal projection into the mossy fiber pathway to integrate into pre-existing circuits in CA3 pyramidal cells of hippocampus (Stanfield and Trice, 1988; Markakis and Gage, 1999) with identical electrophysiological properties to the other granule cells (van Praag et al., 2002). The last main step is differentiation of

the newborn cells into mature neurons or glial cells, which starts with integration of the new cells into the pre-existent network, where they mature, and ends with a crucial event of long-term survival. This last step is the longest, lasting up to 6 weeks. Mature neurons can be assessed at this stage with staining against neuronal nuclei (NeuN) cell marker.

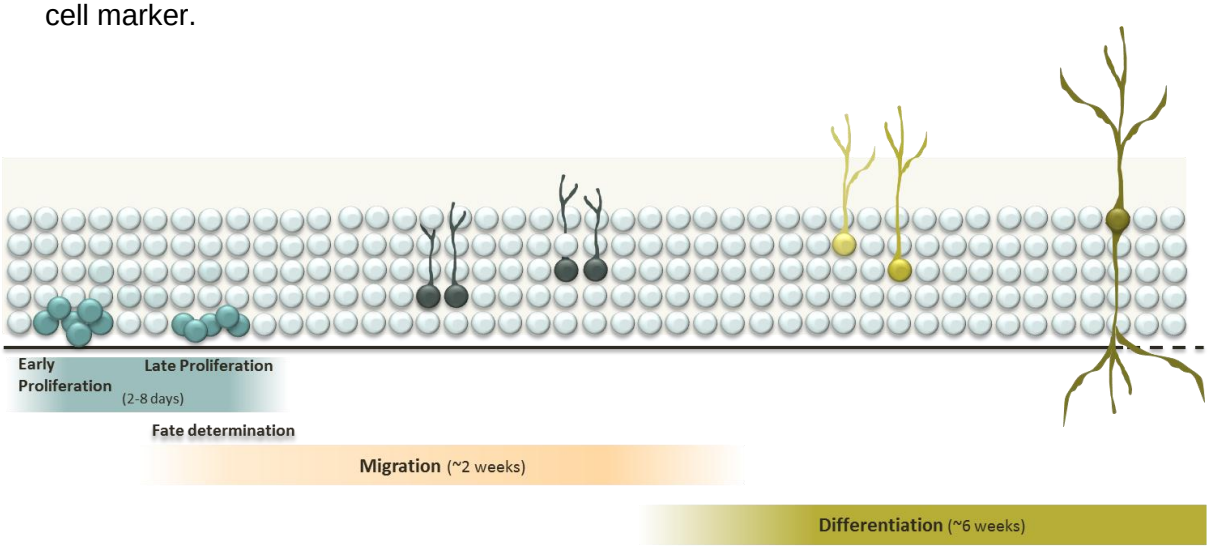

Figure 1.2 – Adult neurogenesis in the dentate gyrus. NSCs proliferate in the first 7 to 8 days before fate determination. Only cells that survive migrate in the next 2 weeks. The neurogenic process continues to differentiation of cells into mature neurons after integration of newborn cells into the pre-existent network, for about 6 weeks. Last, but very important, mature cells have to survive in a long-term period.

Each step is regulated by intrinsic and extrinsic factors that will be described in section 1.2.1 of this chapter.

Hippocampal neurogenesis has a great impact in learning and memory (Shors et al., 2006; Drapeau et al., 2008). Ablation of hippocampal neurogenesis through irradiation, antimitotic agents or inducible genetic methods, has been used as a tool to assess the importance of formation of new granule cells in learning and memory functions. Gould and his colleagues showed that rats trained in a hippocampal dependent-task leads to an increase in the number of adult granule cells (Gould et al., 1999). Moreover, this type of experiments showed that hippocampal adult neurogenesis is required for long-term retention of spatial memory (Dupret et al., 2008; Jessberg et al., 2009), contextual fear memory (Ko et al., 2009) and impaired short-term memory by neurogenesis ablation (reviewed in Marín-Burgin and Schinder, 2012). Despite the extensive investigation in hippocampal neurogenesis and its function in adult brain, there are

some important questions that remain without a consensual answer. For example, understanding specific tasks that depend on the dentate gyrus function and, in particular, how newborn neurons in the adult brain can be functional integrated in both physiological and pathological conditions.

Although the new nerve cells are able to functionally integrate into the pre-existent network (Carlen et al., 2002), there are some important differences between newly generated neurons and more mature ones. Recent studies have demonstrated that adult young granule cells have a lower threshold for induction of long-term potentiation and long-term depression (Mongiat et al., 2009), show hyperexcitability and form synaptic connections before full maturation and exhibit different membrane properties than the existent cells (Schmidt-Hieber et al., 2004). A more deep investigation on these features may be important to understand synaptic plasticity in the hippocampus.

1.2 Regulation of adult neurogenesis

1.2.1 Physiological neurogenesis

Adult neurogenesis is very important for brain plasticity. Understand how this process is regulated and the interaction between newborn cells and the intervening factors may contribute to design and improve therapeutic approaches to neurological disorders. The neurogenic process is influenced by several different factors (Table 1.1), being the SVZ mostly regulated by internal signals and the SGZ by extrinsic factors (reviewed in Suh et al., 2009).

In SVZ and SGZ, the pool of stem cells is maintained by the canonical Wnt/β-catenin signaling pathway (Wexler et al., 2009). Self-renewal and multipotency of NSCs are regulated by expression of *Sox2* (Graham et al., 2003; Suh et al., 2007), since downregulation of *Sox2* is associated with differentiation of NSCs in embryonic, postnatal and adult neurogenesis (reviewed in Suh et al., 2009).

Proliferation is affected by a great range of factors, from hormones to neurotransmitters. Prolactin and thyroid hormones promote proliferation in the SVZ (Giardino et al., 2000; Shingo et al., 2003) and co-expression of *Sox2* and *Pax6* are also important pro-neurogenic factors (Favaro et al., 2009; Ehm et al., 2010). Among the neurotransmitters envolved in proliferation of NSCs are serotonin, γ-aminobutyric acid (GABA) and glutamate. Serotonin seems to be important in cell proliferation and maintenance of expression of PSA-NCAM in both SVZ and SGZ (Brezun and Daszuta, 1999). GABA controls the levels of proliferation of $GFAP^+$ NSCs of the SVZ by

activation of $GABA_A$ receptors in these cells, acting as a negative feedback signal (Liu et al., 2005). Activation of NMDARs, a type of receptor of glutamate, dramatically decreased proliferation in SGZ (Cameron et al., 1995). Growth factors, such epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF), are both related with an increase in proliferation in the SVZ (Doetsch et al., 2002). In fact, NSCs produce the EGF and bFGF receptors (Doetsch et al., 2002; Frinchi et al., 2008), which reinforces their importance in proliferation of newborn cells. Cells isolated from the neurogenic regions can be cultured with EGF, bFGF, or both, supplemented medium. Previous studies in our group reported that initial proliferation of NSCs following exposure to nitric oxide is mediated by the ERK/MAPK pathway and at later stages by the GC/cGMP/PKG pathway (Carreira et al., 2013).

Both niches are in close proximity with the vascular system, sharing some transcription and growth factors, as vascular endothelial growth factor (VEGF). In the adult SGZ, overexpression of VEGF induces hippocampal neurogenesis and angiogenesis (Jin et al., 2002), suggesting a cross-talk between both systems.

Neuroblast migration in the SVZ can be affected by disruption of eph/ephrins signaling (Conover et al., 2000). Survival of the newborn cells is greatly influenced by *Prox1* (Lavado et al., 2010) and *NeuroD* (Kuwabara et al., 2009), and particularly increased in the SGZ by estrogen (Tanapat et al., 1999). However, corticosteroids have the opposite effect, decreasing neurogenesis in SGZ of young rats and primates (Gould et al., 1998; Kippin et al., 2004). Exposure to thyroid hormone T_3, showed to increase the number of DCX-positive cells and speed up the neuronal maturation of hippocampal progenitors (Kapoor et al., 2012).

Finally, the newborn cells are challenged to integrate and mature into the pre-existent neuronal network. Astrocytes from hippocampus and SVZ have an active role in the maturation of neurons in their niches (Lim and Alvarez-Buylla, 1999), mediated by *wnt3* signaling (Lie et al., 2005). In the SGZ the maturation and integration process can also be modulated by CREB signaling (Magill et al., 2010). Differentiation of NSCs in the SGZ are influenced by the transcription factors *Neurog2* and *Tbr2* to become glutamatergic neurons in the hippocampus (Ozen et al., 2007) and overexpression of *Ascl1* leads to differentiation into oligodendrocytes in SVZ (Kim et al., 2007, 2011a; Jessberger et al., 2008). Differentiation of type C cells into neuroblasts is promoted by GABAergic signals (Tozuka et al., 2005; Ge et al., 2006) but inhibited by EGF (Doetsch et al., 2002).

Hippocampal neurogenesis has been studied in several different models and it is now known that can be influenced by different factors. More than one step of the neurogenic process can be affected by aging (Kuhn et al., 1996; Rothman et al., 2009), environmental stimulation (Kempermann et al., 1998; Scotto-Lomassese et al., 2000), intensive exercise, genetic background and stress (Gould et al., 1997; reviewed in Schoenfeld and Gould, 2013).

Table 1.1 – Physiological regulation of adult neurogenesis.

Signal	Effect	Reference
Hormones		
Prolactin	Promotes proliferation of NSCs in the SVZ	Shingo et al., 2003
Thyroid hormones	Increase DCX^+ cells and maturation of neuronal progenitors	Giardino et al., 2000
Estrogen	Increases survival of newborn cells in SGZ	Tanapat et l., 1999
Corticosteroids	Decrease neurogenesis in SGZ	Gould et al., 1998; Kippin et al., 2004
Growth Factors		
EGF	Increases proliferation in SVZ	Doetsch et al., 2002
bFGF	Increases proliferation in SVZ	Doetsch et al., 2002
BDNF	Increases proliferation in SVZ and SGZ	Benraiss et al., 2001; Scharfman et al., 2005
VEGF	Induces hippocampal neurogenesis and angiogenesis	Jin et al., 2002;
Transcription factors		
Sox2	Maintenance of self-renewal and multipotency of NSCs	Graham et al., 2003; Suh et al., 2007
Prox1	Increases survival of newborn cells	Kuwabara et al., 2009
Pax6	Promotes neuronal differentiation in the SVZ	Kohwi et al., 2005
NeuroD	Increases survival of newborn cells	Lavado et al., 2010
Neurog2	Differentiation of SGZ newborn cells into glutamatergic neurons	Ozen et al., 2007
Tbr2	Differentiation of SGZ newborn cells into glutamatergic neurons	Ozen et al., 2007
Ascl1	Differentiation of SVZ newborn cells into oligodendrocytes	Kim et al., 2007, 2011a Jessberger et al., 2008
Neurotransmitters		
Serotonin	Cell proliferation; Maintenance of PSA-NCAm expression	Brezun and Daszuta, 1999
γ-aminobutyric acid (GABA)	Controls levels of proliferation of $GFAP^+$ cells in SVZ; Differentiation of type C cells into neuroblasts	Liu et al., 2005 Ge et al., 2006; Tozuka et al., 2005
Glutamate	Decreased proliferation in SGZ	Cameron et al., 1995

Glial cells		
Astrocytes	Maturation of neurons	Lim and Alvarez-Buylla, 1999
Intrinsic signal pathways		
Wnt/β-catenin	Maintains pool of stem cells	Wexler et al., 2009
CREB	Maturation and integration in SGZ	Magill et al., 2010
Shh	Required for neuroblast migration na SVZ	Balordi and Fishell, 2007
BMP	Decreases neurogenesis; promotes neuroblast survival	Lim et al., 2000
ERK/MAPK	Promotes initial proliferation of NSCs following NO exposure	Carreira et al., 2013
GC/cGMP/PKG	Promotes late proliferation of NSCs following NO exposure	Carreira et al., 2013

1.2.2 Neurogenesis in pathological conditions

1.2.2.1 Neurogenic response to lesion

Neurogenesis is highly influenced by pathological conditions, such as neurodegenerative disorders, ischemia, stroke, trauma and seizures. In these conditions there is a pro-neurogenic response from the damaged and surrounding cells that can be part of some of the spontaneous recovery (reviewed in Lowenstein and Parent, 1999). However, inadequate cell differentiation or excessive amount of new neurons could disturb existing neural circuits and contribute to impairment of the functional recovery. Extrinsic factors are released and have different effects depending on the type of disorder affecting the brain.

Expression of growth factors such as EGF and bFGF are increased following ischemia and traumatic brain injury. *In vivo* experiments in an injured mice model showed that infusion of EGF into the lateral ventricles increased proliferation of NSCs in the SVZ and induced their migration (Gonzalez-Perez et al., 2009) and in rat model of ischemia EGF alone promotes regeneration of injured area (Kolb et al., 2007). The combination of EGF with bFGF has already been successful in increasing proliferation in SVZ, hippocampus and hypothalamus (Oya et al., 2008) and repopulation of the damaged CA1 hippocampal neurons (Nakatomi et al., 2002). Expression of VEGF is also related with increased proliferation and migration in SVZ (Wittko et al., 2009; Calvo et al., 2011).

Neurodegenerative diseases are chronic inflammatory diseases characterized by slow and progressive neuronal death. Cell proliferation is altered in both neurogenic niches.

Patients and mouse models of Alzheimer's disease demonstrated high levels of expression of early neuronal differentiation markers (Jin et al., 2004a, b) and cell proliferation was induced in the dentate gyrus at early stages of the disease (Chen et al., 2008; Gan et al., 2008). Also, in a rat model for Huntington's disease, the cell proliferation in the SGZ increased due to the increase in *Sox2*-positive stem cells and a decrease in CREB signaling (Kandasamy et al., 2010). Chronic inflammation in Parkinson's disease seems to be related with enhancement of proliferation and differentiation of neuronal precursors to neurons (Shan et al., 2006). Overall, chronic brain injury promotes cell proliferation and increases neurogenesis as demonstrated by rodent models of Huntington's disease (Curtis et al., 2003), Alzheimer's disease (Yu et al., 2009) and Parkinson's disease (Zhao et al., 2003).

Acute brain disorders, such as ischemia, stroke, traumatic brain injury and epilepsy, are known to have a stimulating effect of proliferation of NSCs. Ischemic brain insults stimulate progenitor cell proliferation in both niches (Jin et al., 2001; reviewed in Kokaia and Lindvall, 2003; Parent, 2003), as already seen in animal models of transient global ischemia by an increase in BrdU-positive cells (Liu and Huang, 1998; Kee et al., 2001; Zhang *et al.*, 2001; Arvidsson et al., 2002; Choi *et al.*, 2003; Bingham et al., 2005; Tang et al., 2009). Although most of the proliferating cells differentiated into neurons (Kee et al., 2001; Arvidsson et al., 2002; Bingham et al., 2005), the long-term survival of the newborn neurons are not very successful (Arvidsson et al., 2002).

Seizures are also pro-neurogenic lesions, stimulating proliferation of NSCs and long-term survival of newly neurons (Bonde et al., 2006). Increased neurogenesis has been reported in animal models of *status epilepticus* (SE), both in SVZ (Parent et al., 2002) and in SGZ (Parent et al., 1997; Sankar et al., 2000). Many of the neurons that are born in the dentate gyrus after seizures migrate correctly (into the granule cell layer) (Parent et al., 1997) and are functionally integrated into the hippocampal circuitry (Scharfman et al., 2000). However, studies showed that some cells migrate into the hilus (Parent et al., 1997; Scharfman et al., 2000), which may contribute to abnormal integration of new neurons into the CA3 regions of the hippocampus (Scharfman et al., 2000). Studies with animal model for seizures showed that proliferative progenitor response to seizures occurs independently of cell death (Smith, 2005). In this work we used the kainic acid model of *status epilepticus* as a model to study injury-induced neurogenesis.

1.2.2.2 Neuronal death, neuroinflammation and neurogenesis

Whenever a brain insult takes place, whether is acute, such as traumatic brain injury, stroke or epileptic seizures, or chronic like Alzheimer's disease, Huntington's disease, or Parkinson's disease, multiple mechanisms are triggered, namely excitotoxicity events, free radical damage and inflammation (Amor et al., 2010; Xiong et al., 2010).

Neuroinflammation is characterized by the disruption of blood-brain barrier and recruitment of hematopoietic immune cells and activation of CNS resident microglial cells as a response of the brain to infections, diseases and injuries (Nencini et al., 2003; Schmmidt et al., 2005). This complex biological process attempts to protect the brain from harmful stimuli by removing dead and damaged cells and start the healing process. However, several works have already demonstrated the importance of neuroinflammation and its severity in the pathophysiology of neurological diseases, since this inflammatory process creates a positive feedback loop of inflammatory activation that leads to the progressive death of neuronal cells, called neurodegeneration (Das and Basu, 2008; Whitney et al., 2009; Goldberg and Barres, 2000).

The relationship between neuroinflammation and neurogenesis it is not clear yet. However, studies to date shows a dependence on the mechanism(s) by which immune cells from the CNS and macrophages are activated and interact and the type of inflammatory factors released (Ekdahl et al., 2009). In this conditions, release of cytokines, interleukins, chemokines and reactive oxygen species or reactive nitrogen species (Whitney et al., 2009), among others, promotes the recruitment of microglial cells and disruption of the BBB. These factors have influence in different stages of neurogenesis (Monje et al., 2003; Ekdahl et al., 2003), affecting proliferation, migration and differentiation of the newborn cells following a brain lesion (Jakubs et al., 2008). Recently, it has been showed that synaptic connectivity of the new neurons may be influenced by the inflammatory environment (Hennberger et al., 2005). IFN-γ, for example, promotes neuronal differentiation (Wong et al., 2004) and the chemokine SDF-1α induces the migration and promotes survival of neural precursors (Krathwohl and Kaiser, 2004a). Reactive oxygen and nitrogen species are also very important in regulation of the neurogenic response following a brain lesion (Rock et al., 2004). One of the most studied is nitric oxide (NO), and its function on adult neurogenesis will be developed next (section 1.3).

Astrocytes are the main glial cells in the CNS, with structural and several regulatory functions (Svendsen, 2002). Activation of astrocytes during neuroinflammation,

promotes the release of inflammatory and growth factors, such as neurotrophins and glutamate, that regulate the inflammatory response of the brain (Song et al., 2002). Studies in animal models of depression have reported the importance of the brain-derived neurotrophin factor (BDNF) and their contribution to regulation of adult neurogenesis by the BDNF-TrkB pathway.

1.3 Nitric oxide and neurogenesis

Nitric oxide (NO) is a free radical gaseous molecule product of the oxidation of L-arginine to L-citrulline, a biological process catalyzed by nitric oxide synthase (NOS). There are three known isoforms of NOS, which are products of different genes and therefore have different localization, regulation, biochemical and pharmacological properties, and inhibitor sensitivity. NOS isoforms are: neuronal NOS (nNOS), located at human chromosome 12 (Kisgimoto et al., 1992; Xu et al., 1993) and predominantly expressed in neuronal tissue; endothelial NOS (eNOS), located at chromosome 7 (Xu et al., 1994) with expression in vascular endothelial cells; and inducible NOS (iNOS), located in either side of chromosome 17 (Xu et al., 1994). nNOS and eNOS are considered constitutively expressed and calcium-dependent isoforms, while iNOS expression is calcium-independent and inducible by cytokines or bacterial components (Bredt and Snyder, 1994; Geller and Billar, 1998; Alderto et al., 2001).

NO is a very interesting molecule due to its short half-life, signaling properties (Whitney et al., 2009) and diffusivity that can go up to 100 µm from the point of synthesis (Lancaster, 1997). It is involved in many physiological mechanisms, but it is mostly known by its role in regulation of vascular system and inflammatory responses. In the CNS, NO can participate in sensory motor function (Moreno-López et al., 1996) and control of cerebral blood flow (Estrada et al., 1993).

The role of NO as a modulator of neurogenesis is still unclear. However, great progress has been done in terms of identifying the effect of NO in proliferation, differentiation and survival of NSCs and the signal pathways involved. Under physiological conditions, NO seems to inhibit proliferation of NSCs (Cheng et al., 2003; Packer et al., 2003; Moreno-Lopez et al., 2004; Matarredona et al., 2005; reviewed in Calabrese et al., 2007), but promoting formation on newborn cells under pathological situations (Zhu et al., 2003; reviewed in Kokaia and Lindvall, 2003 and Whitney et al., 2009; Carreira et al., 2010). The neurogenic response mediated by NO depends on the pathophysiological state of the tissue, source of NO and time of exposure (Carreira et

al., 2010). NO from nNOS modulates synaptic activity and plasticity (Moreno-Lopez et al., 1996), and neuronal differentiation and survival (reviewed in Holscher, 1997). There are significant evidences of the role of NO in regulation of hippocampal neurogenesis. Also, NO has been shown as a modulator of the expression of GFAP in astrocytes (Covacu et al., 2006) and their morphological changes in inflammatory conditions (Borán and García, 2007). However, the exact mechanisms by which NO triggered this effects is still unknown. Additional investigation on the biological targets by which NO regulates neuronal proliferation and its function on differentiation and survival of NSCs is still needed.

In physiological conditions, microglial cells have a typical morphology and dynamic and are in a "resting" state, functioning as sensors of the homeostatic environmental (Davalos et al., 2005, 2008). In a neuroinflammatory context, microglial cells are active and express iNOS, which continuously produces high amounts of NO (Murphy et al., 1993; Murphy, 2000). In these conditions, NO from nNOS seems to have a great impact on regulation of NSCs function in SVZ and SGZ (Packer et al., 2003; Moreno-López et al., 2004), since nitrogenic neurons expressing nNOS are in close proximity with both niches through vascular system. In fact, chronic nNOS inhibition enhances neurogenesis in SVZ, RMS an OB, but not in SGZ, in adult mice (Moreno-López et al., 2004). NO expression from iNOS in SGZ has a positive effect in the ischemic-induced neurogenesis in mice (Zhu et al., 2003), correlated with the activation of NMDA receptors (Arvidsson et al., 2001). Covacu and colleagues showed that NO from iNOS is also involved in astrogliogenesis of NSCs by activation of the JAK/STAT-1 signal transduction pathway (Covacu et al., 2006). Long-term survival of newborn neurons is impaired after *SE* despite chronic inflammation and activation of microglia (Bonde et al., 2006). Recently it has been demonstrated that the early proliferative effect of NO in SVZ-derived NSCs bypasses activation of EGF receptor (Carreira et al., 2010), and later proliferation involves activation of the cGMP/PKG signaling pathway (Carreira et al., 2013). These findings show that the effect of NO in neurodegeneration seems to be greatly influenced by its source and concentration on the tissues, since overexpression of NO showed to be neurotoxic as a consequence of inhibition of the respiratory chain enzymes (reviewed in Carreira et al., 2012).

The relationship between NO and the proliferation of NSCs following brain injury is a relatively well characterized process. However, less is known about the role of NO on survival of the newborn cells in an injury context.

1.3.1 Neurogenic targets in nitrergic pathways

NO has the ability to interact with several intracellular targets in order to trigger a stimulatory or inhibitory signal pathway. Improvement of NO-based therapies implies to increase the knowledge about their endogenous targets, in order to minimize the side effects of NO in the brain. The regulation of biological effects of NO can be mediated by its main target, the guanylyl cyclase, which catalyzes the conversion of GTP to cGMP (Bredt and Snyder, 1989). cGMP can activate several downstream molecules, such as PKG, phosphodiasterases and ion channels (Paupardin-Tritsch et al., 1986), and therefore modulates the activity of several different cellular substrates. Together, NO and cGMP can activate downstream signaling cascades involved in enhancement of proliferation of NSCs (Carreira et al., 2012), survival, differentiation (Gomez-Pinedo et al., 2010), growth and axon guidance or migration (Tegenge et al., 2011), among other processes.

NO can also interact with both, the constitutive and the inducible isoforms, of ciclooxygenase (Mollace et al., 2005). By activation of this enzyme, NO combined with NO-releasing compounds (NO-NSAID) is able to decrease inflammatory response as a result of blocking the synthesis of prostaglandins. However, side effets are described in the gastrointestinal tract, cardiovascular system and kidneys from chronic use of NSAID (reviewed in Scheiman and Fendrick., 2007; Harirforoosh et al., 2009).

The involvement of NO in promotion of cell survival and neuroprotective functions may be due to its interaction with CREB (Riccio et al., 2006) and Akt kinase pathways (reviwed in Contestabile et al., 2004). Targeting these signal pathways will be also a possibility to adress nitrergic therapeutic approaches.

1.4. How to study adult neurogenesis in the mouse brain

Detection of neurogenesis and cell proliferation can be achieved by different techniques. The most commons involves the use of analogs of nucleotides that are incorporated during DNA replication in mitosis. 5-bromo-2'-deoxyuridine (BrdU), 5-ethynyl-2'-deoxyuridine (EdU), 5-iodo-2'-deoxyuridine (IdU) and 5-chloro-2'-deoxyuridine (CldU) (Leuner et al., 2009) are examples of synthetic thymidine analogues that incorporate dividing cells during S-phase of DNA synthesis, and therefore allow evaluation of different stages of neurogenesis, depending on the time between their administration and cell fixation. BrdU can be used both *in* vitro and *in* vivo and then detected by immunohistochemistry, microplate assay or flow cytometry.

BrdU detection causes no visible toxicity (Dolbeare, 1995) and allows double- and triple-labeling with cell markers for mature neurons, such as MAP-2, TUJ1 and NeuN, for identification of new neurons (Kuhn et al., 1996). However, BrdU methodology requires aggressive acid treatment for DNA denaturation before detection, which can lead to the loss of binding sites for DNA dyes or antibodies and damage of the tissue (Zeng et al., 2010). BrdU and EdU can be used simultaneously, allowing investigation of proliferation at different time points (Morte et al., 2013). Despite the advantages of using BrdU method in neurogenesis detection, the amount of BrdU is diluted each time cells divide leading to an underestimation of the real number of dividing cells (Karpowicz et al., 2005). The use of endogenously-produced cell cycle markers, such as proliferating cell nuclear antigen (PCNA), Ki67, phospho-histone H3, and minichromosome marker-2, may overcome the problems of BrdU method but does not allow to determine the fate of the cells since are rapidly degraded as the cell cycle progresses.

Retroviral labeling can also be used to assess mitotic cells *in* vivo, by complete integration of green fluorescent protein, or other live reporters, into dividing cells (van Praag et al., 2002; Carleton et al., 2003; Magavi et al., 2005). This approach allows direct visualization and morphological analysis of an entire cell population. However, it requires stereotaxic injection onto specific brain regions and does not exceed the efficiency of labeling with BrdU.

The most recent method to study dynamic of neurogenesis and the age of neurons is the measurement of radioactive ^{14}C content. ^{14}C is a stable component in DNA tissues and thus can be detected and measured in *post mortem* tissue by using a radiomass spectrometer (Spalding et al., 2005; 2013). Atmospheric ^{14}C is reflected in animals at all times through uptake of CO_2 metabolized by autotrophic organisms (Harkness, 1972; Libby et al., 1964; Spalding et al., 2005b). When cells divide, the correspondent concentration of atmospheric ^{14}C at the time is integrated into the synthesized genomic DNA creating a date mark (Spalding et al., 2005a). This methodology is particularly useful to study the kinetics of a cell population and dynamics.

Animal models of brain injury are also commonly used to study neurogenic process *in vivo*. One example is the rat model of temporal lobe epilepsy. Epilepsy is defined as a medical condition characterized by the transient and periodic occurrence of epileptic seizures. As a result, glutamatergic synapses are excessively activated leading to brain excitotoxicity and cell death. The hippocampus is very affected by these events, since it is very rich in glutamatergic synapses, and hippocampal damage can be mimicked in

rodent models by systemic injection of kainic acid (KA), an agonist of AMPA/KA receptors, or tetanus toxin (Jiruska et al, 2013), with pro-convulsive properties. After induction of *SE*, cell proliferation is increased in the hippocampus (Parent et al., 1997; Parent, 2007; Gray and Sundstrom, 1998; Jiruska et al., 2013) as well as neuroinflammation, leading to activation of microglial cells and expression of iNOS (De Simoni et al., 2000).

1.5. Therapeutic approaches for brain repair

Enhancement of endogenous neurogenesis

One of the approaches considered for adult NSC-based therapy in the CNS is the stimulation of endogenous NSCs and enhancement of adult neurogenesis. Local cell proliferation has already been demonstrated near the lesion site (Parent et al., 2002), but the lack or insufficiency of the signals that are able to maintain the process makes it impossible to have a successful response. The self-repair capacity is thus limited to the lesion extension and environmental signals, and requires other regenerative strategies.

One of the major challenges in implementing this therapeutic approach is to find a way to increase the migration distance of the cells from the neurogenic niche to lesion sites, especially in a large size brain, such is the human brain (reviewed in Marr et al., 2010). On the other hand, recruitment of endogenous stem cells does not raise ethical issues regarding the origin and immunogenicity of the cells. Nevertheless, it is important to expand the knowledge about signals and factors that stimulate stem cells to proliferate and regulate their migration, integration and differentiation in both neurogenic and non-neurogenic regions of the brain. Especially in the aged brain, the expression of pro-neurogenic factors is decreased and therefore is important to investigate the combination of factors needed and their benefit as therapeutic approach in neurodegenerative disorders.

Transplantation therapies

Transplantation of exogenous stem cells has also been considered as a potential therapy for neuronal repair in neurodegenerative diseases. However, is a much more invasive approach than stimulation of endogenous neurogenesis and so there is still some reluctance in applying this strategy. Exogenous stem cells may be isolated from embryonic or tissue stem cells and expanded *in vitro*. Grafting adult-derived NSCs into

non neurogenic sites results in the absence of neuronal lineage commitment (Suhonen et al., 1996; Sheen et al., 1999; Shihabuddin et al., 2000) but can stimulate the local production of new neurons in aged neurogenic regions (Park et al., 2010). In the adult brain, stem cells must be grafted to a neurogenic region in order to differentiate.

Direct cell reprogramming in the brain

The first studies that demonstrated that it is possible to directly reprogram cells in the brain were performed in astrocytes. Several other studies have been performed from then on and in 2010 Werning and colleagues introduced the BAM cocktail, a mix of 3 transduction genes – *Ascl1, Brn2,* and *Mytl1* (BAM) – that was sufficient to directly reprogram primary postnatal mouse fibroblasts into induced neurons (Vierbuchen et al., 2010). Recent studies have showed that adult differentiated astrocytes can be reprogrammed into proliferative neuroblasts in the adult mouse brain by the single transcription of *Sox2* (Niu et al., 2013).

In any case, the possibility of formation of teratomas due to excessive cell proliferation is a major concern (Li et al., 2008) in the application of any of the strategies. Therapeutic approaches for brain repair also will have to take into account the effects of factors released in an inflammatory context on differentiation and survival of the newborn/transplanted cells, and use accurate *in vitro* and *in vivo* models that allows investigation of neurogenesis.

1.6. Objective

Despite the intensive investigation on the effect of NO on the proliferation of NSCs, there is a lack of information about the role of NO in migration, differentiation and survival on newborn cells following brain injury.

The aim of this work was to investigate the role of NO from iNOS in the regulation of hippocampal neurogenesis after a brain insult. In order to do that we analyzed proliferation of NSCs, migration of the newborn cells, differentiation and survival using a mouse model of *status epilepticus*.

Chapter 2

Material and Methods

2.1 Materials

Normal goat serum (NGS), parafolmaldehyde (PFA), Triton X-100 and 5-bromo-2'-deoxyuridine (BrDU) were purchased from Sigma-Aldrich (St. Louis, Mo., USA). Mouse anti-NeuN and Mouse anti-GFAP were purchased from Millipore (Billerica, MA). Rat anti-BrdU was purchased from AbD Serotec (Oxford, UK). Alexa Fluor® 488 Goat Anti-Rat IgG (H+L) and Alexa Fluor® 594 Goat Anti-Mouse IgG (H+L) were purchased from Invitrogen (Paisley, UK). DAKO fluorescence mounting medium was from dakoCytomation (Glostrup, Denmark). Kainic acid (KA) was from Ocean Produce (Canada). Sodium thiopental was from B. Braun Melsungen (Germany, DE). Hoechst 33342 dye was from Molecular Probes (Leiden, The Netherlands). Doublecortin (C-18) (DCX) was obtained from Santa Cruz Biotechonology (Dallas, Texas, USA).

2.2 Animals

2-month old C57BL/6J ($iNOS^{+/+}$ and iNOS wildtype) and B6.129P2-$Nos2^{tm\ 1\ Lau}$/J ($iNOS^{-/-}$ and iNOS knockout) male mice, were obtained from Charles River (Barcelona). $iNOS^{-/-}$ mice are highly susceptible to tumors as well as bacterial and viral pathogens, resistant to sepsis-induced hypotension (Mashimo and Goyal, 1999), and show a decrease in neuronal injury after stroke and in diet-induced atherosclerosis (Liu and Huang, 2008). The weight of the animal varied between 18 and 26 g. The animals were kept in animal facilities with food and water *ad libitum* in a 12 hours dark:light cycle. All experiments were performed in accordance with institutional and European guidelines (86/609/EEC) for the care and use of laboratory animals.

2.3 Administration of kainic acid in mice

Kainic acid was dissolved in a sterile saline solution (0.9% NaCl in water) and injected subcutaneously (25 mg/kg). All animals that received KA developed grade five seizures or higher according to 1972s Racine's six-point scale modified for mice (Schauwecker and Steward, 1997). In animal injected with saline solution alone, no seizures were observed and animals were used as controls. At least 3 animals survived in each experimental group, except for $iNOS^{-/-}$ mice treated with BrdU 24 h after seizures.

2.4 5-bromo-2'-deoxyuridine incorporation

To assess proliferation of NSCs, all animals were treated with KA or saline solution and treated with BrdU (intraperitoneal (i.p.) injection, 4 doses, 50 mg/kg) each 2 hours apart, with a total of 200 mg/kg, up to 12 hours before sacrificing at different time points (Fig 2.1). In order to analyze distribution of NSCs along dentate gyrus, animals were treated with BrdU (i.p. injections, four doses, 50 mg/kg) every 12 hours, three and seven days after KA or saline administration. Three weeks later mice were sacrificed (Fig 2.2). In both experiments mice were transcardially perfused with 0.9% NaCl followed by 4% PFA in 0.01 M phosphate buffer saline (PBS, 7.8 mM $Na_2HPO_4.2H_2O$, 2.7 mM $NaH_2PO_4.H_2O$, 154 mM NaCl, pH 7.2), after deep anesthesia with sodium pentobarbital. Brains were removed and kept overnight in 4% PFA, and then dehydrated in 20% sucrose/0.2 M phosphate buffer (PB, 48 mM $NaH_2PO_4.H_2O$, 152 mM $Na_2HPO_4.2H_2O$, pH 7.2), at 4ºC. Coronal sections from the hippocampal region were cryosectioned (30 µm thick, in 8-series) and stored in an antifreeze solution (0.05 M PB, 30 % ethylene glycol, 30 % glycerol), at 4ºC.

2.5 Immunohistochemistry

Free-floating coronal hippocampal sections were processed for immunohistochemistry against DCX or BrdU and NeuN or BrdU and GFAP. Brain sections were treated with 1M HCl for 20 min at 65ºC, for DNA denaturation, and then blocked for 1 h with 5% NGS in 0.25% Triton X-100 in 0.01 M PBS. Slices were then incubated with the primary antibodies, goat anti-DCX (1:400) or rat anti-BrdU (1:50) and mouse anti-NeuN (1:200) or mouse anti-GFAP (1:7000), 48 h at 4ºC. After rinsing once with 0.25% Triton X-100 in PBS and twice with 2% NGS in 0.25% Triton X-100 in 0.01 M PBS, the sections were incubated with the correspondent secondary antibodies (1:200), in 2% block solution, for 2 h in the dark, at room temperature. For nuclear staining, brain sections were incubated with 2 µg/ml Hoechst 33342 solution prepared with 0.01 M PBS, for 10 minutes. After rinsing once with 0.25% Triton X-100 in 0.01 M PBS and twice with 0.01 M PBS, the sections were kept in 0.01 M PBS solution, at 4ºC, until setting in 2% gelatin-coated slides with DAKO fluorescence mounting medium.

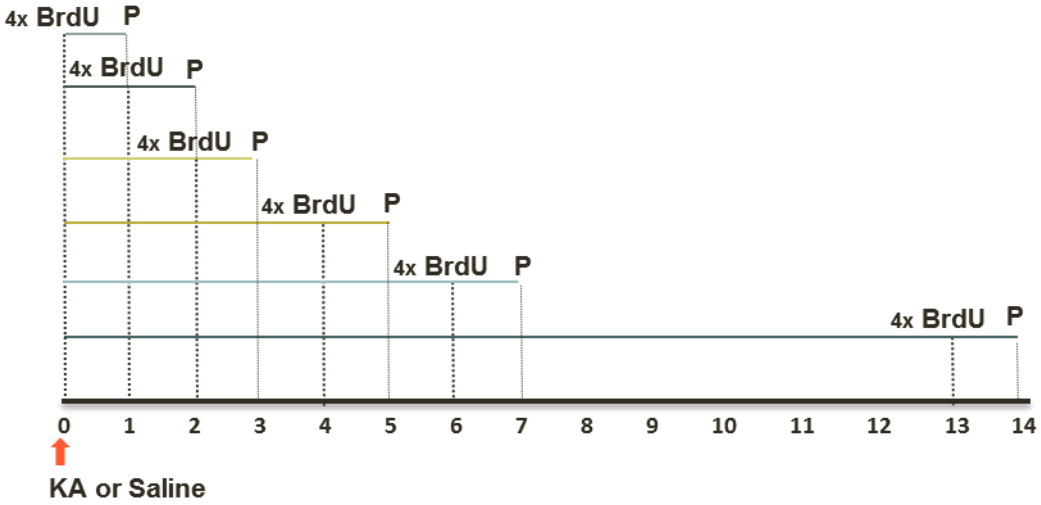

Figure 2.1 – Experimental protocol for assessment of proliferation of NSCs in the dentate gyrus. Intraperitoneal injections (i.p.) of BrdU (4 doses, 50 mg/kg) were administrated every 2 hours, up to 12 hours before perfusion **(P)**.

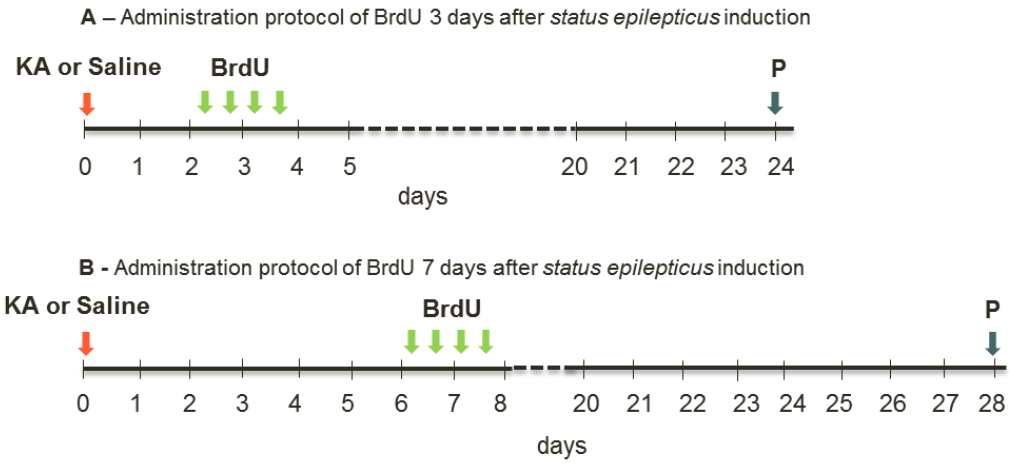

Figure 2.2 – Experimental protocol for assessment of differentiation of NSCs in the dentate gyrus. A, Administration protocol of BrdU 3 days after *status epilepticus*. **B,** Administration protocol of BrdU 7 days after *status epilepticus*. Intraperitonial injections (i.p.) of BrdU (4 doses, 50 mg/kg) were administrated every 12 hours. Perfusions **(P)** were performed 3 weeks after BrdU treatment, following anesthesia.

2.6 DCX- and GFAP-immunoreactivity

DCX and GFAP immunoreactive areas were analyzed using ImageJ software. Snap images were acquired in a Zeiss Axioimager (Zeiss, Jena, Germany, http://www.zeiss.com) with a 20x objective. The threshold value was set for each staining and the percentage of dark background area was measured, excluding more anterior and posterior dentate gyri.

2.7 Analysis of co-localization of BrdU with NeuN or GFAP

$BrdU^+$ cells in dentate gyrus for each animal were counted using epifluorescence microscope (20x objective). Images (0.73 µm z-stacks) from 50 $BrdU^+$ cells of each brain were acquired in a laser scanning microscope LSM 510 META or LSM 710 (Zeiss, Jena, Germany, http://www.zeiss.com) with Argon/2 (488 nm) and DPSS 561-10 (561 nm) lasers (63x oil-immersion objective). Orthogonal projections in y axis were performed and counted the number of $BrdU^+/NeuN^+$ or $BrdU^+/GFAP^+$ cells. The percentage of colocalized cells were achieved by dividing the total number of $BrdU^+/NeuN^+$ or $BrdU^+/GFAP^+$ cells by 50 $BrdU^+$ cells.

2.8 Statistical analyses

The data are presented as means ± SEM. Statistical significance was determined using a two-way analysis of variance (ANOVA, Bonferroni's post-test) in GraphPad Prism 5. Differences were considered significant when $p<0.05$.

Chapter 3

Results

3.1. NO is involved in proliferation of NSCs and migration of neuroblasts after seizures

3.1.1 Proliferation of neural stem cells in the hippocampus following seizures comprises a NO-dependent and NO-independent phase

To investigate the role of NO in cell proliferation, we used an *in vivo* KA model of *status epilepticus*. iNOS$^{+/+}$ or iNOS$^{-/-}$ mice were treated with either saline or KA, as described in *Materials and Methods*. All animals that received KA developed grade five seizures or higher according to the Racine's six-point scale modified for mice (Schauwecker and Steward, 1997). In animals injected with saline solution alone, no seizures were observed. Proliferation of newborn cells was evaluated by the incorporation of BrdU, a thymidine analogue. The number of BrdU-positive cells in the dentate gyrus was assessed by immunohistochemistry.

In iNOS$^{+/+}$ mice, treatment with KA increased significantly the incorporation of BrdU in the SGZ from 3 days after treatments up to 14 days, when compared to saline-treated mice (Fig 3.1 B, two-way ANOVA; treatment: 31.95, F=151.5, df=3; time: 29.71, F=84.57, df=5; treatment x time [interaction]: 33.70, F=31.97, df=15). The number of BrdU^{+} cells in iNOS$^{+/+}$ mice treated with saline was 16.83 ± 0.92 cells/section at 24 hours, 23.13 ± 1.31 cells/section at 2 days, 24.46 ± 1.78 cells/section at 3 days, 26.32 ± 1.13 cells/section at 5 days, 21.60 ± 0.49 cells/section at 7 days, and 21.71 ± 1.77 cells/section at 14 days. The number of BrdU^{+} cells in iNOS$^{+/+}$ mice treated with KA was 9.39 ± 1.04 cells/section at 24 hours ($p>0.05$), 33.23 ± 1.86 cells/section at 2 days ($p>0.05$), 63.36 ± 1.36 cells/section at 3 days after *SE* ($p<0.001$), 82.70 ± 5.87 cells/section at 5 days after *SE* ($p<0.001$), 82.46 ± 3.44 cells/section for 7 days after *SE* ($p<0.001$), and 37.48 ± 1.20 cells/section for 14 days after *SE* ($p<0.01$). In iNOS$^{+/+}$ mice treated with saline solution, the number of BrdU^{+} cells did not change significantly during the analyzed period of time ($p>0.05$ for all time points).

In iNOS$^{-/-}$ mice, BrdU incorporation was unchanged with KA treatment up to 5 days after seizures (7.73 ± 1.43 cells/section at 1 day after *SE* ($p>0.05$), 13.35 ± 3.87 cells/section for 2 days after *SE* ($p>0.05$), 25.64 ± 0.53 cells/section for 3 days after *SE* ($p>0.05$), and 18.36 ± 1.99 cells/section for 5 days after *SE* ($p>0.05$)). Interestingly, the number of BrdU^{+} cells in dentate gyrus was significantly increased 7 days after *SE* (87.08 ± 8.40 cells/section, $p<0.001$), compared with saline-treated iNOS$^{-/-}$ mice (20.08 ± 0.61 cells/section). Finally, the incorporation of BrdU returned to basal levels 14 days after *SE* with KA treatment (27.33 ± 2.17 cells/section, $p>0.05$). In saline-treated iNOS$^{-/-}$

mice, the incorporation of BrdU was similar for all time points (11.31 ± 2.86 cells/section at 1 day, 12.10 ± 1.33 cells/section at 2 days, 16.71 ± 4.06 cells/section at 3 days, 15.58 ± 0.96 cells/section at 5 days, 20.08 ± 0.61 cells/section at 7 days, and 17.15 ± 1.39 cells/section at 14 days, p>0.05).

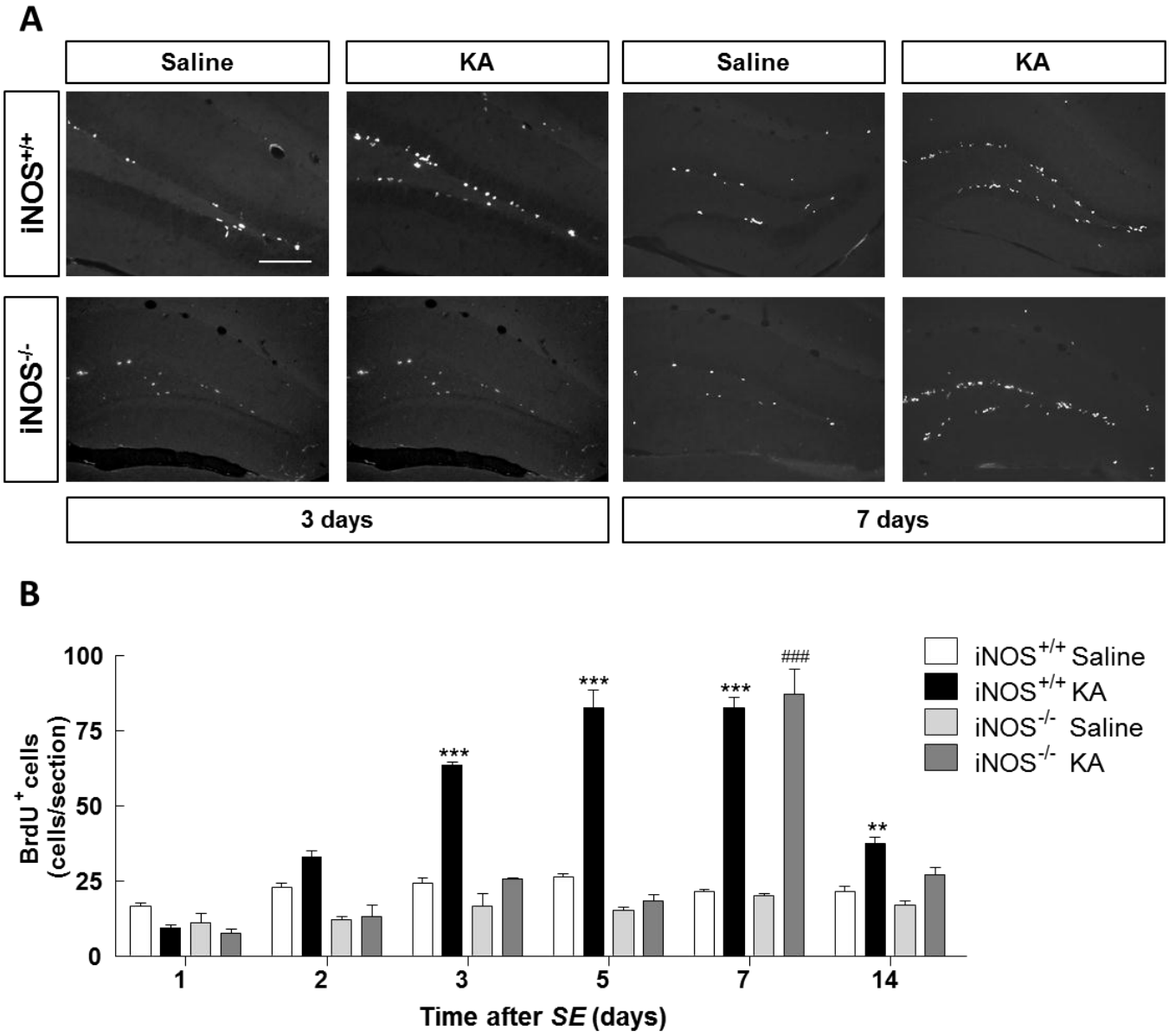

Figure 3.1 – NO increases NSCs proliferation following treatment with KA.
A, Representative images of $BrdU^+$ cells (white), 3 and 7 days after KA or saline treatment in $iNOS^{+/+}$ and $iNOS^{-/-}$ mice. **B,** Number of $BrdU^+$ cells at different time points (1, 2, 3, 5, 7 and 14 days) after seizures. Data are expressed as means ± SEM. Two-way ANOVA (Bonferroni's post-test), N=2 to 5, $^{**}p<0.01$ and $^{***}p<0.001$ are different from $iNOS^{+/+}$ Saline; $^{###}p<0.001$ is different from $iNOS^{-/-}$ Saline. Scale bar: 50 µm.

3.1.2. Migration of neuroblasts following seizures is dependent of NO

To investigate the role of NO on the migration of neuronal precursors, $iNOS^{+/+}$ or $iNOS^{-/-}$ mice were treated with either saline or KA, as described in *Materials and Methods*. The migration of new neuroblasts occurs around 2 weeks in the neurogenic process. According to this fact, we choose to analyze this event at 7 days and 14 days after treatments. DCX was used as a marker of neuroblast migration (Nacher et al., 2003) and DCX-immunoreactive area was assessed by immunohistochemistry (Carreira *et al*., 2010).

The DCX-immunoreactive area was increased in $iNOS^{+/+}$ mice, 14 days after seizures compared to saline-treated mice, but not in KA-treated $iNOS^{-/-}$ compared to saline-treated mice (Fig 3.2 A). At 7 days after seizures, the percentage of DCX-immunorective area tends to increase with KA treatment in both $iNOS^{+/+}$ (169.01 ± 33.50 % of control, $p>0.05$) and $iNOS^{-/-}$ (145.64 ± 32.75 % of control, $p>0.05$), although this increase is not significant compared to saline-treated mice (100.00 ± 14.14 % of control in $iNOS^{+/+}$ mice and 100.00 ± 10.99 % of control in $iNOS^{-/-}$ mice) (Fig 3.2 B, two-way ANOVA; genotype: 0.79, $F=0.1382$, $df=1$, $p>0.05$; treatment: 18.90, $F=3.326$, $df=1$, $p>0.05$; genotype x treatment [interaction]: 0.78, $F=0.1381$, $df=1$, $p>0.05$).

At 14 days after seizures the DCX-positive area duplicated in $iNOS^{+/+}$ mice (209.32 ± 4.07 % of control, $p<0.05$), when compared to saline-treated mice of the same genotype (100.00 ± 24.75 % of control). In $iNOS^{-/-}$ mice, treatment with KA did not change the DCX-immunoreactive area (87.57 ± 5.48 % of control, $p>0.05$), when compared with the saline-treated mice (100.00 ± 39.13). (Fig 3.2 C, two-way ANOVA; genotype: 23.01, $F=6.420$, $df=1$, $p<0.05$; treatment: 14.57, $F=4.065$, $df=1$, $p>0.05$; Genotype x treatment [interaction]: 23.01, $F=6.420$, $df=1$, $p<0.05$)

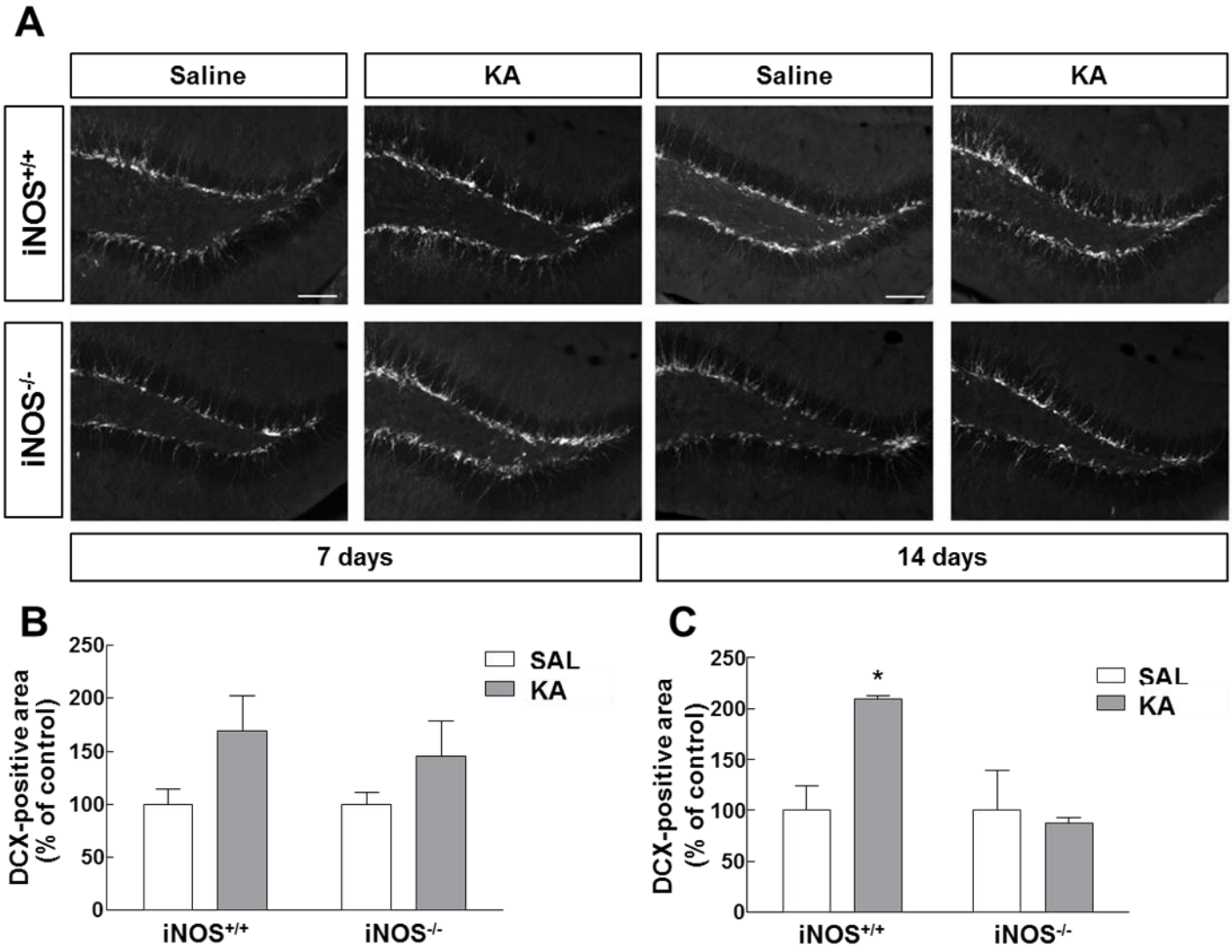

Figure 3.2 – DCX immunoreactivity is dependent of No 14 days after seizures.

A Representative images of DCX (white) immunoreactivity in the dentate gyrus, 7 and 14 days after KA or saline treatment in iNOS$^{+/+}$ and iNOS$^{-/-}$ mice. **B,** DCX-immunoreactive area 7 days after *SE*. **C,** DCX-immunoreactive area 14 days after *SE*. Data are expressed as means ± SEM. Two-way ANOVA (Bonferroni's post-test), N=3 to 6, $^{*}p < 0.05$ is different from iNOS$^{-/-}$ saline. Scale bar: 100 µm.

3.2. Distribution of newborn cells in the dentate gyrus is modified by NO after seizures

3.2.1. Distribution of newborn cells formed 3 days after seizures in the dentate gyrus is independent of NO, 21 days after treatment with BrdU.

We next investigate the role of NO in the distribution of newborn cells along the dentate gyrus when proliferation is NO-dependent. $iNOS^{+/+}$ or $iNOS^{-/-}$ mice were treated with either saline or KA and BrdU was injected in all animals 3 days later. The distribution of the new cells formed at 3 days after seizures were assessed in the SGZ, inner granular zone (IGZ) and outer granular zone (OGZ) of the dentate gyrus, 21 days after BrdU treatment.

$BrdU^+$ cells increased with KA treatment in $iNOS^{+/+}$ and $iNOS^{-/-}$ mice (Fig 3.3 A). The total number of $BrdU^+$ cells significantly increased with KA treatment in both $iNOS^{+/+}$ and $iNOS^{-/-}$ mice (Fig 3.3 B, two-way ANOVA; genotype: 5.68, F=2.334, df=1, $p>0.05$; treatment: 49.79, F=20.47, df=1, $p<0.001$; genotype x treatment [interaction]: 0.76, F=0.3113, df=1, $p>0.05$). For $iNOS^{+/+}$ mice, treatment with KA duplicated $BrdU^+$ cells (29.24 ± 2.91 $BrdU^+$ cells/section, $p<0.05$) compared to saline-treated mice (12.59 ± 1.66 $BrdU^+$ cells/section). For $iNOS^{-/-}$ mice, KA treatment also doubled the number of $BrdU^+$ cells (37.99 ± 7.75 $BrdU^+$ cells/section, $p<0.01$) compared to saline-treatment (16.66 ± 2.89 $BrdU^+$ cells/section. In both genotypes, treatment with saline did not change the number of $BrdU^+$ cells (12.59 ± 1.66 $BrdU^+$ cells/section for $iNOS^{+/+}$ mice, and 16.66 ± 2.89 $BrdU^+$ cells/section for $iNOS^{-/-}$ mice).

In $iNOS^{+/+}$ mice, KA-treatment significantly increased $BrdU^+$ cells in SGZ (16.68 ± 1.56 $BrdU^+$ cells/section, $p<0.01$) comparatively to saline-treated mice (9.49 ± 1.18 $BrdU^+$ cells/section) (Fig 3.3 C, two-way ANOVA; treatment: 25.52, F=14.81, df=3, $p<0.001$; regions: 41.85, F=36.43, df=2, $p<0.001$; treatment x regions [interaction]: 1.62, F=0.4690, df=6, $p>0.05$). For these mice, $BrdU^+$ cells also increased with KA treatment in IGZ (9.35 ± 1.97 $BrdU^+$ cells/section, $p<0.01$), when compared with saline-treated mice (2.48 ± 0.47 $BrdU^+$ cells/section). $BrdU^+$ cells did not change significantly in OGZ with KA treatment (3.21 ± 0.53 BrdU-positive cells/section, $p>0.05$) compared to saline-treated mice (0.62 ± 0.08 BrdU-postive cells/section).

Similarly, in $iNOS^{-/-}$ mice (Fig 3.3 C), $BrdU^+$ cells significantly increased after seizures in SGZ (17.98 ± 2.82 $BrdU^+$ cells/section, $p<0.05$) and IGZ (12.37 ± 3.78 $BrdU^+$ cells/section, $p<0.05$), compared with saline-treated $iNOS^{-/-}$ mice. Treatment with KA

did not change the number of BrdU$^+$ cell in OGZ (7.64 ± 2.19 BrdU$^+$ cells/section, p>0.05) compared to saline treatment (1.17 ± 0.26 BrdU$^+$ cells/section).

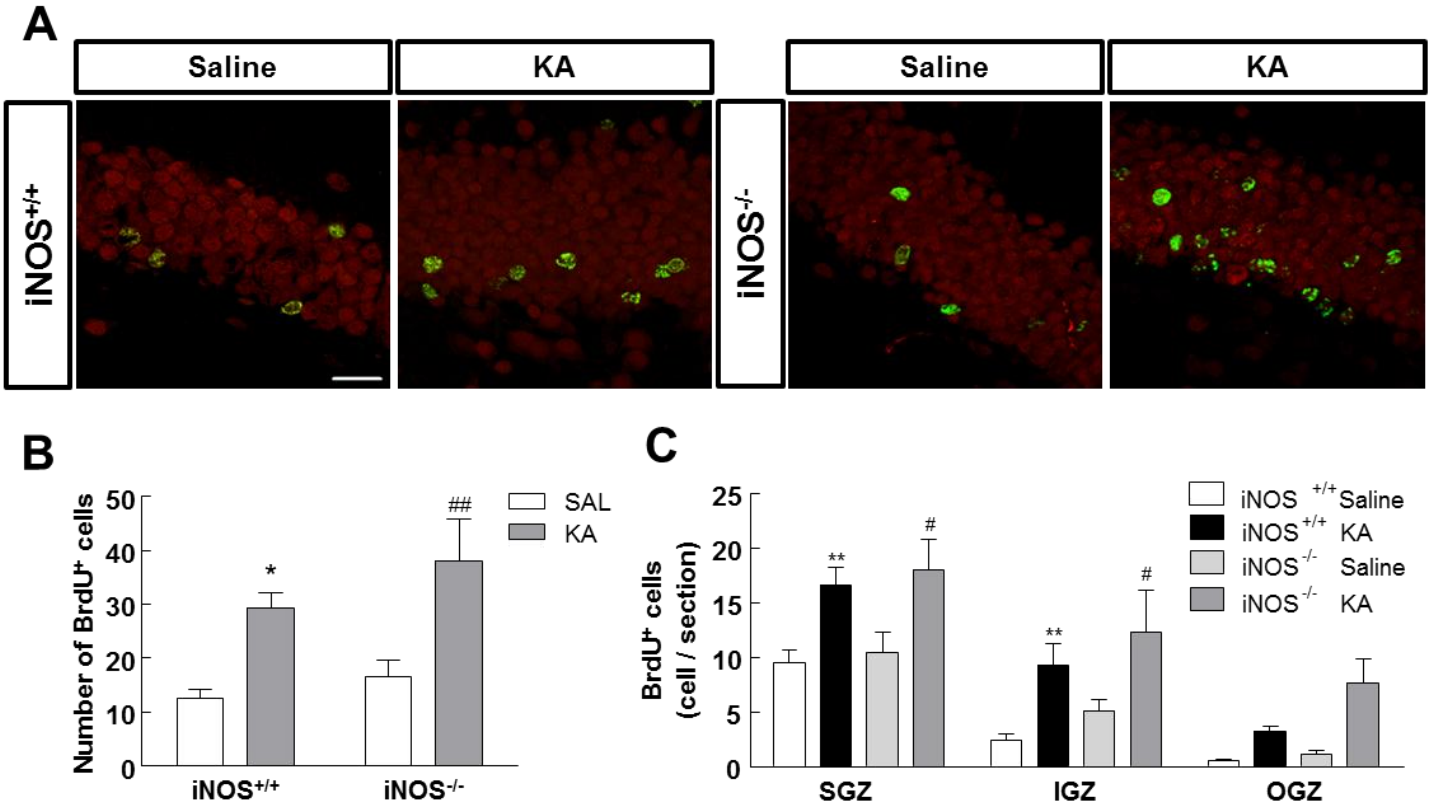

Figure 3.3 – The number of BrdU$^+$ cells in the dentate gyrus increase following seizures, 21 days after BrdU treatment, by a NO-independent mechanism.

A, Representative images of BrdU (green) and NeuN (red) positive cells in the dentate gyrus, 3 days after treatment with KA or saline in iNOS$^{+/+}$ and iNOS$^{-/-}$ mice . **B,** Number of BrdU$^+$ cells in iNOS$^{+/+}$ and iNOS$^{-/-}$ mice. Data are expressed as means ± SEM. Two-way ANOVA (Bonferroni's post-test), N=5 to 7, $^*p<0.05$ is different from iNOS$^{+/+}$ saline; $^{\#\#}p < 0.01$ is different from iNOS$^{-/-}$ saline. **C,** BrdU$^+$ cells in the 3 regions of the dentate gyrus. Data are expressed as means ± SEM. Two-way ANOVA (Bonferroni's post-test), N=5 to 7, $^{**}p<0.01$ is different from iNOS$^{+/+}$ saline; $^{\#}p<0.05$ is different from iNOS$^{-/-}$ saline.
Scale bar: 20 µm.

3.2.2. Abolishment of NO does not affect distribution of newborn cells formed 7 days after seizures in the dentate gyrus, 21 days after treatment with BrdU

Taking into account our initial results about proliferation, we next investigated the role of NO in the distribution of newborn cells along the dentate gyrus when proliferation is NO-independent, for example, cells that are formed 7 days after seizures. $iNOS^{+/+}$ or $iNOS^{-/-}$ mice were treated with either saline or KA, BrdU was injected in all animals 7 days later and perfusions performed 21 days after BrdU treatment.

Cells formed 7 days after seizures, treatment with KA did not change significantly the number of $BrdU^+$ cells along the dentate gyrus for neither genotype (Fig 3.4 A). The number of $BrdU^+$ cells did not change with genotype (Fig 3.4 B, two-way ANOVA, treatment: 10.61, F=2.180, df=1, p>0.05; genotype: 15.95, F=3.278, df=1, p>0.05; treatment x genotype [interaction]: 0.45, F=0.09155, df=1, p>0.05). In $iNOS^{+/+}$ mice, the number of $BrdU^+$ cell in KA treated-mice was 19.46 ± 5.26 $BrdU^+$ cells/section (p>0.05) and in saline-treated mice is 10.63 ± 0.89 $BrdU^+$ cells/section. In $iNOS^{-/-}$ mice, the difference between saline- (21.15 ± 4.10 $BrdU^+$ cells/section) and KA-treated mice (26.99 ± 6.70 $BrdU^+$ cells/section, p>0.05) was similar.

In $iNOS^{+/+}$ mice, the number of $BrdU^+$ cells in KA-treated mice was 10.49 ± 2.18 $BrdU^+$ cells/section in SGZ (p>0.05), 6.47 ± 2.29 $BrdU^+$ cells/section in IGZ (p>0.05), and 2.52 ± 0.93 $BrdU^+$ cells/section in OGZ (p>0.05), and for saline-treated mice was 8.00 ± 0.43 $BrdU^+$ cells/section in SGZ, 2.25 ± 0.54 $BrdU^+$ cells/section in IGZ, and 0.38 ± 0.07 $BrdU^+$ cells/section in OGZ (Fig 3.4 C, two-way ANOVA; treatment: 11.25, F=4.592, df=3, p<0.01; regions: 48.07, F=29.44, df=2, p<0.001; treatment x regions [interaction]: 3.95, F=0.8072, df=6, p>0.05).

In $iNOS^{-/-}$ mice, the number of $BrdU^+$ cells in mice treated with KA was 13.93 ± 2.76 $BrdU^+$ cells/section in SGZ (p>0.05), 9.80 ± 2.40 $BrdU^+$ cells/section in IGZ (p>0.05) and 3.25 ± 1.68 $BrdU^+$ cells/section in OGZ (p>0.05) (Fig 3.4 C). Saline treated mice show 14.48 ± 2.64 $BrdU^+$ cells/section in SGZ, 5.29 ± 1.04 $BrdU^+$ cells/section in IGZ and 1.38 ± 0.47 $BrdU^+$ cells/section in OGZ.

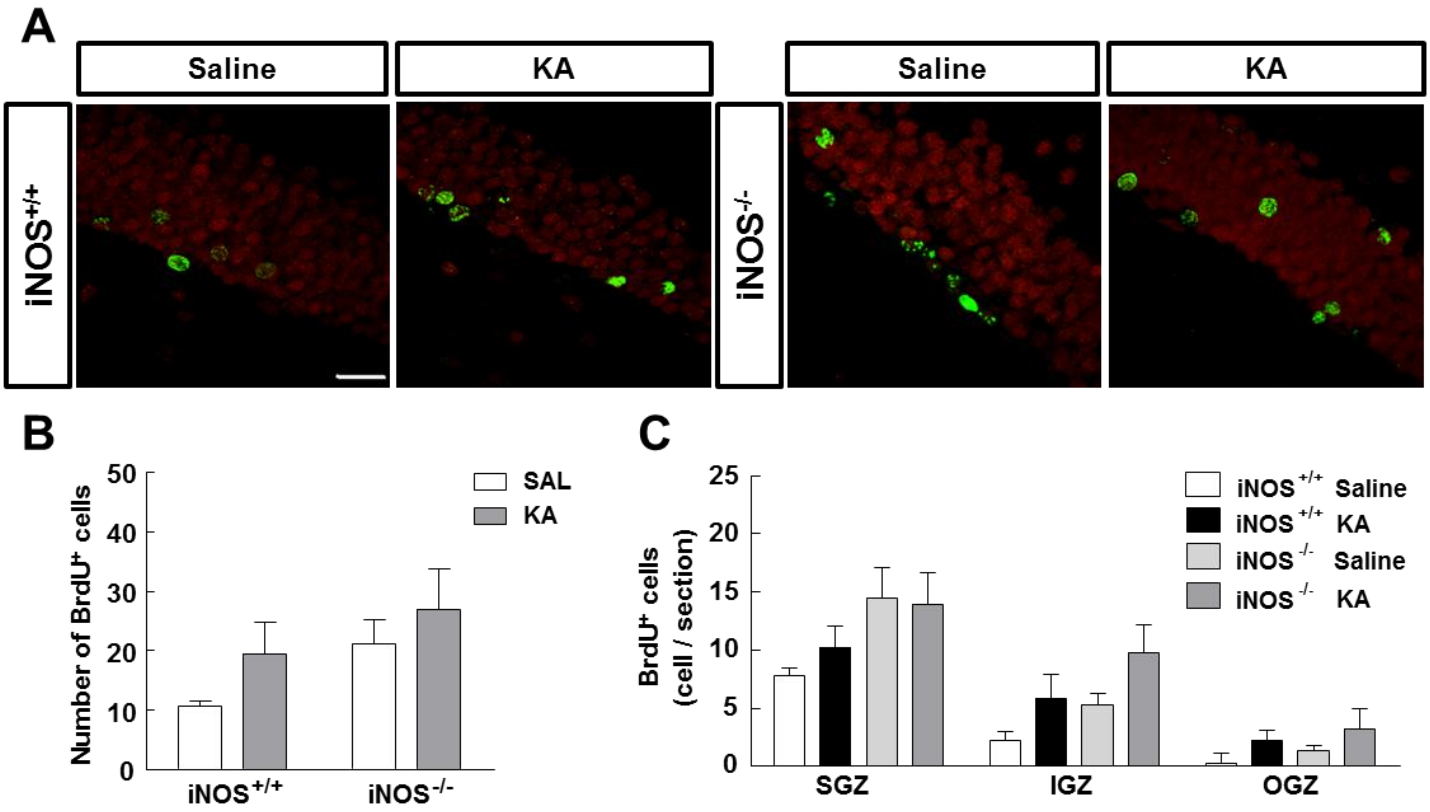

Figure 3.4 – The number of BrdU$^+$ cells in the dentate gyrus born 7 days after *SE* is not affected by NO following seizures, 21 days after treatment with BrdU.

A, Representative images of BrdU (green) and NeuN (red) positive cells in the dentate gyrus, 7 days after treatment with KA or saline in iNOS$^{+/+}$ and iNOS$^{-/-}$ mice. **B,** Number of BrdU$^+$ cells in iNOS$^{+/+}$ and iNOS$^{-/-}$ mice. Data are expressed as means ± SEM. Two-way ANOVA (Bonferroni's post-test), N=4 to 6. **C,** BrdU$^+$ cells in the three regions of the dentate gyrus. Data are expressed as means ± SEM. Two-way ANOVA (Bonferroni's post-test), N=4 to 6. Scale bar: 20 µm.

3.3. NO has different effects in neuronal and astrocytic differentiation

3.3.1. Neuronal differentiation of newborn cells formed 3 days after *SE* is decreased by NO.

To investigate the survival of newborn cells 3 and 7 days after *SE*, co-localization of $BrdU^+/NeuN^+$-cells were assessed by immunohistochemistry. NeuN is neuronal marker for mature neurons and co-localization with BrdU allows the investigation of new neurons formed at the time point of treatment with BrdU. Images of 50 $BrdU^+$-cells of each animal were acquired by laser scanning microscopy and orthogonal projections in y axis were performed for each image (Fig 3.5 A).

At 21 days after treatment with BrdU, the percentage of new neurons born 3 days after *SE*, decreased in $iNOS^{+/+}$ mice treated with KA (53.50 ± 7.04 % of $BrdU^+/NeuN^+$-cells, $p<0.05$), compared to saline-treated mice (72.29 ± 3.48 % of $BrdU^+/NeuN^+$-cells), but not in $iNOS^{-/-}$ mice (43.50 ± 6.95 % of $BrdU^+/NeuN^+$-cells for saline and 43.20 ± 5.68 % of $BrdU^+/NeuN^+$-cells for KA-treated mice, $p>0.05$) (Fig 3.5 B, two-way ANOVA: genotype: 7.79, F=2.682, df=1, $p>0.05$; treatment: 32.65, F=11.25, df=1, $p<0.01$; genotype x treatment [interaction]: 7.30, F=2.516, df=1, $p>0.05$).

For neurons born 7 days after *SE*, the number of new neurons in $iNOS^{+/+}$ mice was very similar between treatments, with 55.6 ± 7.22 % of $BrdU^+/NeuN^+$-cells ($p>0.05$) in KA-treated mice and 62.00 ± 5.03 % of $BrdU^+/NeuN^+$-cells for saline-treated mice. In $iNOS^{-/-}$ mice, treatment with KA (69.50 ± 5.85 % of $BrdU^+/NeuN^+$-cells) also did not change the number of new neurons, compared to saline treated mice (58.80 ± 8.31 % of $BrdU^+/NeuN^+$-cells) (Fig 3.5 C, two-way ANOVA; genotype: 3.15, F=0.5348, df=1, $p>0.05$; treatment: 0.53, F=0.09010, df=1, $p>0.05$; genotype x treatment [interaction]: 8.07, F=1.372, df=1, $p<0.05$).

A detailed analysis of the effect of treatment with KA in newborn cells 3 and 7 days after seizures, in both genotypes (Fig 3.5 D, two-way ANOVA; genotype: 0.40, F=0.09596, df=1, $p>0.05$; time: 17.89, F=4.320, df=1, $p>0.05$; genotype x time [interaction]: 11.31, F=2.732, df=1, $p>0.05$), shows that the number of new neurons, born 3 days after seizures, in $iNOS^{-/-}$ mice was 43.20 ± 5.68 % of $BrdU^+/NeuN^+$-cells ($p>0.05$) and in $iNOS^{+/+}$ mice was 52.67 ± 7.51 % of $BrdU^+/NeuN^+$-cells ($p>0.05$). The number of neurons born 7 days after seizures in $iNOS^{-/-}$ mice was 69.50 ± 5.85 % of $BrdU^+/NeuN^+$-cells, ($p>0.05$) and in $iNOS^{+/+}$ mice was 55.67 ± 7.22 % of $BrdU^+/NeuN^+$-cells.

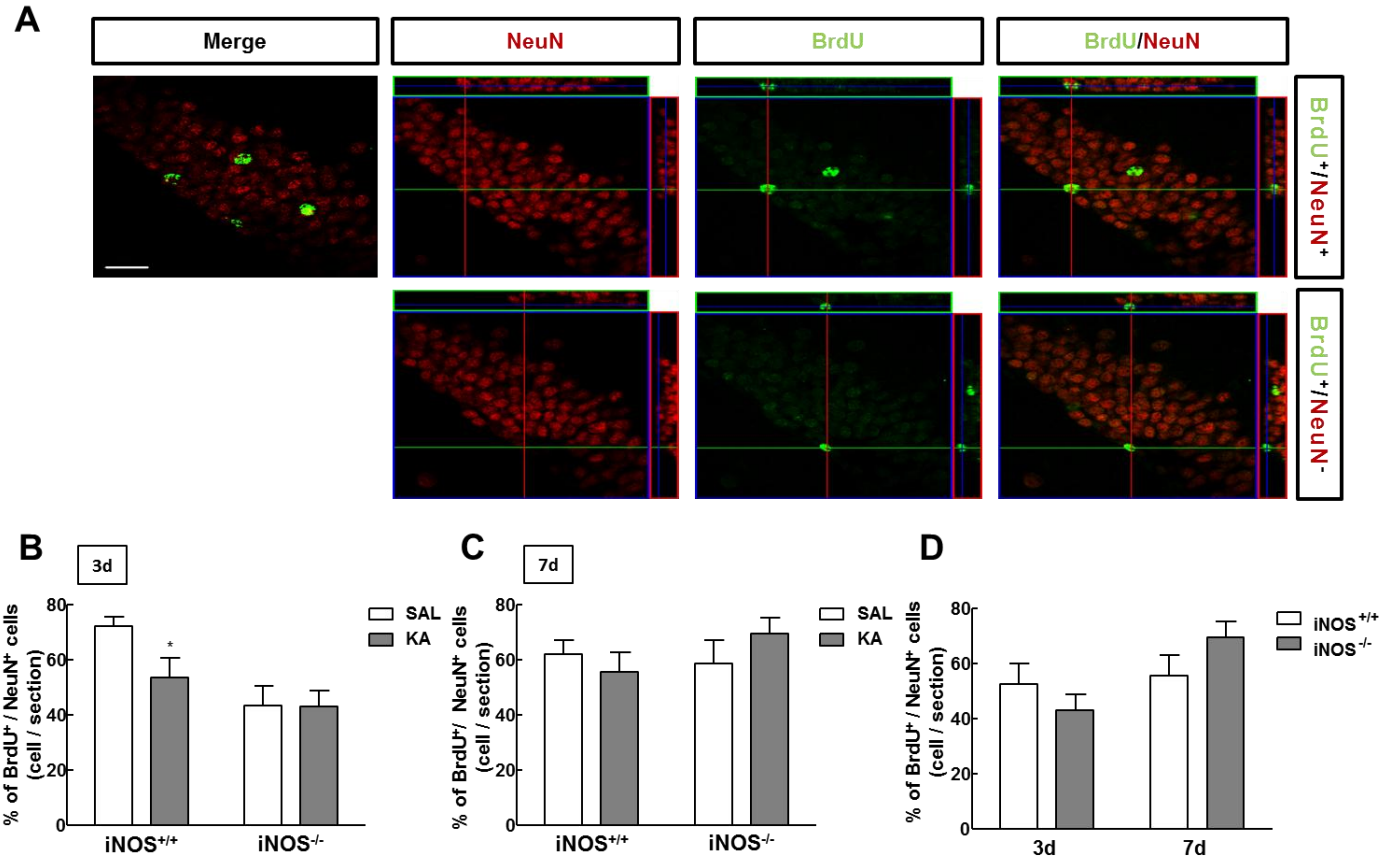

Figure 3.5 – NO decreases the number of newborn neurons born 3 days after *SE* in iNOS$^{+/+}$ mice.

A, Orthogonal projections of representative images of BrdU^{+}/NeuN^{+} and BrdU^{+}/NeuN^{-}-cells. **B,** Percentage of BrdU^{+}/NeuN^{+-}-cells 3 days after *SE*, assessed by immunohistochemistry. Data are expressed as means ± SEM. Two-way ANOVA (Bonferroni's post-test), N=4 to 7, $^{*}p<0.05$ is different from iNOS$^{+/+}$ saline. **C,** Percentage of BrdU^{+}/NeuN^{+}-cells 7 days after *SE*. Data are expressed as means ± SEM. Two-way ANOVA (Bonferroni's post-test), N=4 to 6. **D,** Percentage of BrdU^{+}/NeuN^{+}-cells in KA-treated iNOS$^{+/+}$ and iNOS$^{-/-}$ mice, 3 and 7 days after *SE*. Data are expressed as means ± SEM. Two-way ANOVA (Bonferroni's post-test), N=4 to 7, $p>0.05$. Scale bar: 20µm.

3.3.2. Astrogliogenesis is not affected by abolishment of NO after seizures

Following the previous results, we were interested in understanding whether the proliferating cells could be differentiating into astrocytes. In order to analyze that, we assessed $GFAP^+$ cells formed at 3 and 7 days after seizures by immunohistochemistry 21 days after treatment with BrdU. GFAP is a protein expressed by astrocytes, and co-localization with BrdU allows the identification of newborn astrocytes at the time point of treatment with BrdU. Images of 50 $BrdU^+$ cells of each animal were acquired in laser scanning microscope and orthogonal projections in y axis were performed for each image (Fig 3.6 A).

For cells born 3 days after *SE* in $iNOS^{+/+}$ mice, seizures did not change the number of new astrocytes (3.50 ± 0.34 % of $BrdU^+/GFAP^+$-cells, $p>0.05$), compared with saline-treated mice (2.71 ± 0.61 % of $BrdU^+/GFAP^+$-cells). In $iNOS^{-/-}$ mice, the number of $BrdU^+/GFAP^+$-cells in KA-treated mice was 6.00 ± 1.22 % of $BrdU^+/GFAP^+$-cells ($p>0.05$) and 5.25 ± 1.11 % of $BrdU^+/GFAP^+$-cells in mice treated with saline (Fig 3.6 B, two-way ANOVA, genotype: 36.46, $F=10.30$, $df=1$, $p<0.01$; treatment: 3.39, $F=0.9584$, $df=1$, $p>0.05$; genotype x treatment [interaction]: 0.00, $F=0.0005183$, $df=1$, $p>0.05$).

For cell born 7 days after *SE*, seizures did not change the number of $BrdU^+/GFAP^+$-cells in both genotypes (3.60 ± 0.51 % of $BrdU^+/GFAP^+$-cells in $iNOS^{+/+}$ mice and 2.75 ± 1.11 % of $BrdU^+/GFAP^+$ cells in $iNOS^{-/-}$ mice, $p>0.05$). The percentage of $BrdU^+/GFAP^+$-cells in saline-treated mice was very similar between $iNOS^{+/+}$ (2.00 ± 0.71 % of $BrdU^+/GFAP^+$-cells) and $iNOS^{-/-}$ mice (1.20 ± 0.37 % of $BrdU^+/GFAP^+$-cells) (Fig 3.6 C, two-way ANOVA; genotype: 7.07, $F=1.473$, $df=1$, $p>0.05$; treatment: 25.76, $F=5.369$, $df=1$, $p<0.05$; genotype x treatment [interaction]: 0.01, $F=0.001353$, $df=1$, $p>0.05$).

Analyzing the KA-treated mice alone (Fig 3.6 D, two-way ANOVA, genotype: 4.55, $F=1.137$, $df=1$, $p>0.05$; time: 16.60, $F=4.143$, $df=1$, $p>0.05$; genotype x time [interaction]: 18.77, $F=4.686$, $df=1$, $p<0.05$), the percentage of $BrdU^+/GFAP^+$-cells, for newborn cells 3 days after *SE*, in $iNOS^{-/-}$ mice was 6.00 ± 1.22 % of $BrdU^+/GFAP^+$-cells ($p>0.05$) and 3.50 ± 0.34 % of $BrdU^+/GFAP^+$-cells in $iNOS^{+/+}$ mice. Also, the percentage of $BrdU^+/GFAP^+$-cells, for newborn cells 7 days after seizures, was not affected by treatment with KA in both genotypes (3.60 ± 0.51 % of $BrdU^+/GFAP^+$-cells in $iNOS^{+/+}$ mice and 2.75 ± 1.11 % of $BrdU^+/GFAP^+$-cells in $iNOS^{-/-}$ mice, $p>0.05$).

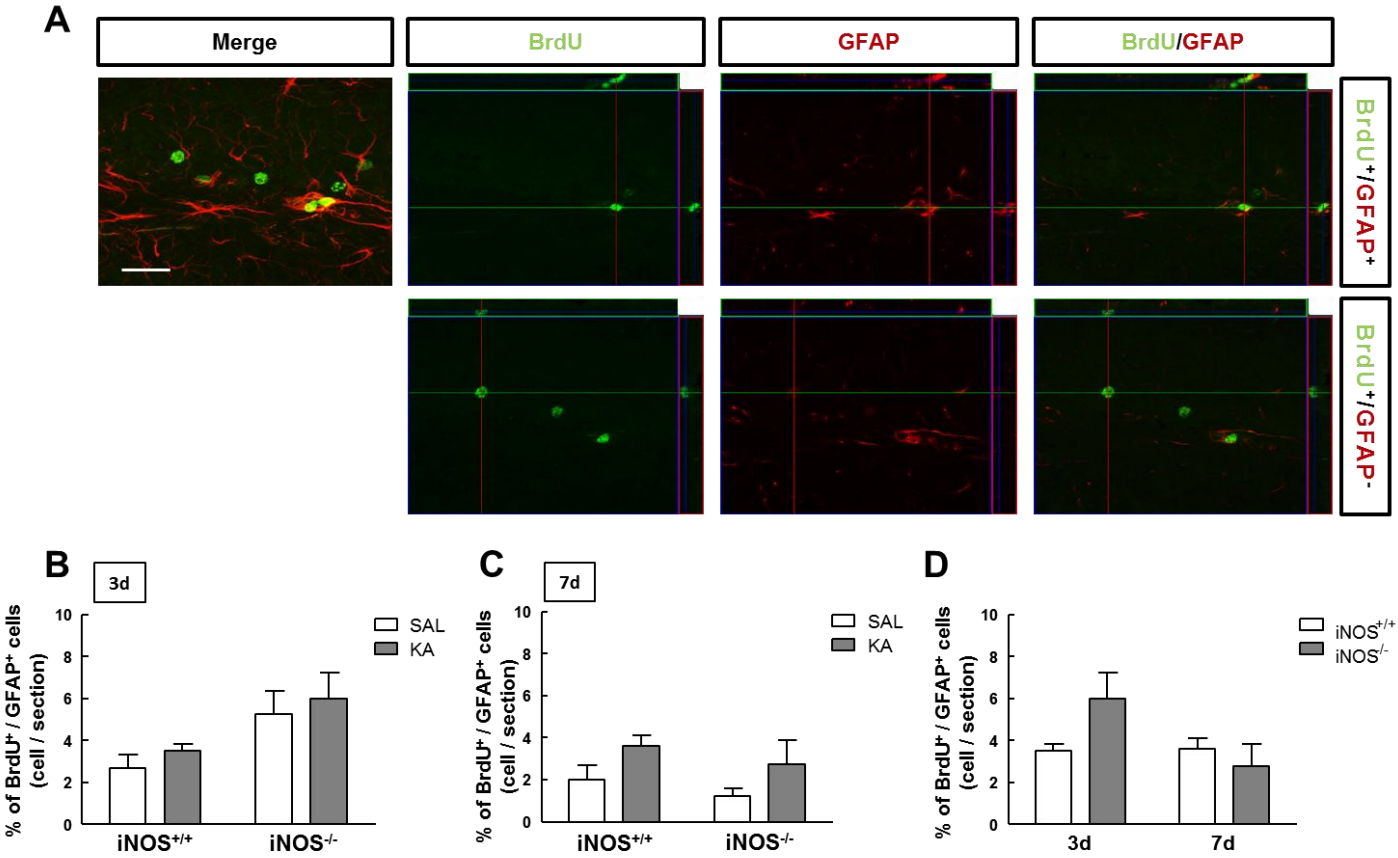

Figure 3.6 – Differentiation of newborn cells formed 3 and 7 days after *SE* into astrocytes is not affected by NO.

A, Orthogonal projections of representative images of BrdU$^+$/GFAP$^+$-cells and BrdU$^+$/GFAP-cells. **B,** Percentage of BrdU$^+$/GFAP$^+$-cells born 3 days after *SE*. Data are expressed as means ± SEM. Two-way ANOVA (Bonferroni's post-test), N=4 to 7, p>0.05. **C,** Percentage of BrdU$^+$/GFAP$^+$-cells born 7 days after *SE*. Data are expressed as means ± SEM. Two-way ANOVA (Bonferroni's post-test), N=4 to 5, p>0.05. **D,** Percentage of BrdU$^+$/GFAP$^+$-cells in KA-treated iNOS$^{+/+}$ and iNOS$^{-/-}$ mice, 3 and 7 days after *SE*. Data are expressed as means ± SEM. Two-way ANOVA (Bonferroni's post-test), N=4 to 7, p>0.05. Scale bar: 20µm.

3.4 NO is important for astrogliosis in iNOS$^{+/+}$ mice 28 days after treatment

We next evaluated the possibility of the involvement of NO in neuroinflammation, 28 days after seizures. GFAP immunoreactivity was assessed by immunohistochemistry and because it is proportional to the intensity of GFAP staining, it can be used as a measure for astrogliosis. (Fig 3.7 A).

In iNOS$^{+/+}$ mice, KA treatment increased GFAP immunoreactivity (170.45 ± 15.74 % of control, $p<0.05$) when compared with saline-treated mice (100.00 ± 23.87 % of control) 28 days after treatment. In iNOS$^{-/-}$ mice treatment with KA does not change GFAP immunoreactivity (124.76 ± 24.76 % of control, $p>0.05$) compared with mice treated with saline solution (100.00 ± 9.81 % of control) (Fig 3.7 B, two-way ANOVA, genotype: 28.27, $F=6.721$, $df=1$, $p<0.05$; treatment: 6.41, $F=1.527$, $df=1$, $p>0.05$; genotype x treatment [interaction]: 6.42, $F=1.527$, $df=1$, $p<0.05$).

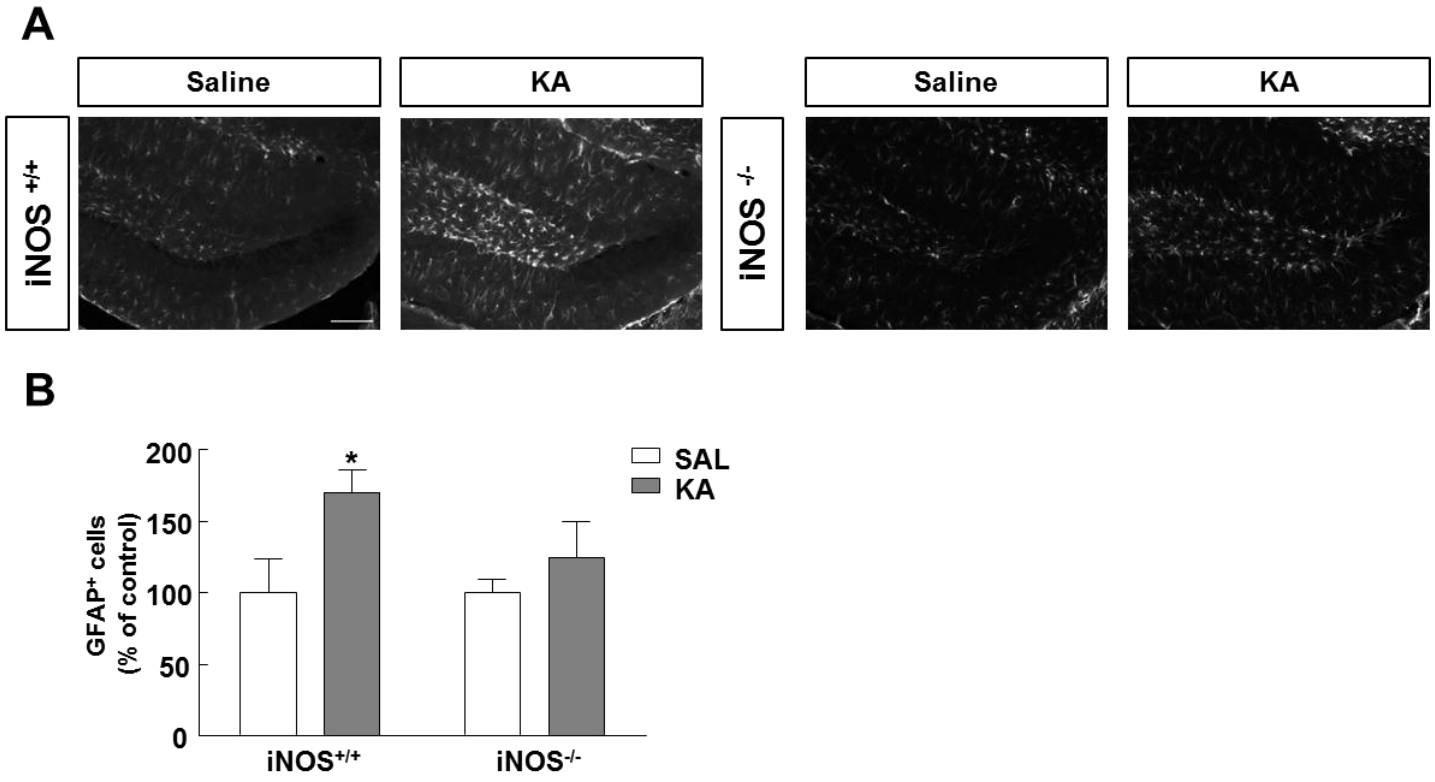

Figure 3.7 – Astrogliosis is affect by abolishment of NO, 28 days after seizures. A, Representative images of GFAP (white) immunoreactivity 28 days after KA or saline treatment in iNOS$^{+/+}$ and iNOS$^{-/-}$ mice. **B,** GFAP immunoreactivity 28 days after *SE*. Data are expressed as means ± SEM. Two-way ANOVA (Bonferroni's post-test), N=4 to 5, $^{*}p<0.05$ is significantly different from iNOS$^{+/+}$ saline.. Scale bar: 100 µm.

Chapter 4

Discussion

During the present work our main goal was to study the hippocampal neurogenesis and the involvement of NO from inflammatory origin in the different stages of the neurogenic process after seizures. In this work we show that NO is involved in proliferation, migration and survival of the newborn cells after seizures, as shown in Fig 4.1. We also investigated the involvement of NO from iNOS in astrogliogenesis and neuroinflammation following seizures.

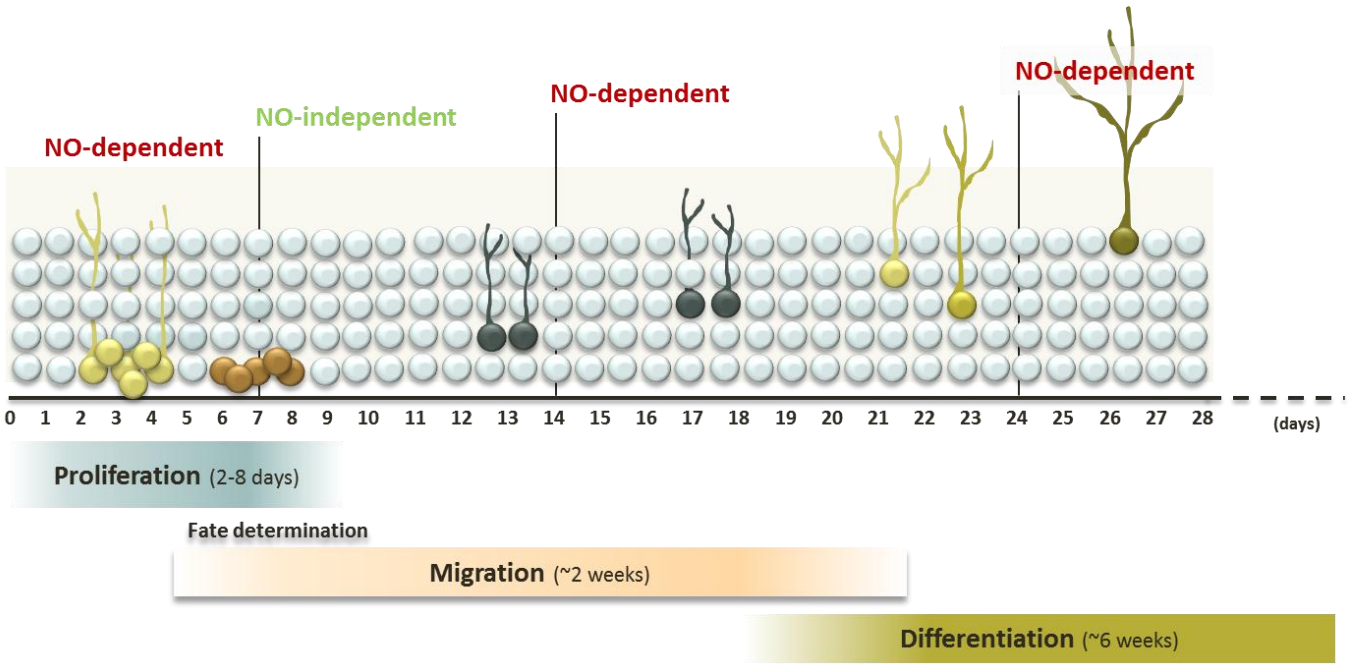

Figure 4.1 – Involvement of nitric oxide in regulation of hippocampal neurogenesis. Nitric oxide is involved in different stages of the hippocampal neurogenic process, controlling early proliferation, migration of the newborn cells and the number of new neurons generated after seizures. Early proliferation of NSCs, migration of new neuroblasts and survival of early born cells are regulated by a NO-dependent mechanism, while late proliferation of NSCs seems to be regulated by a NO-independent mechanism.

4.1. NO is involved in early proliferation of NSCs after seizures

Adult neurogenesis starts with proliferation of NSCs in the neurogenic niches. Particularly in the hippocampus, proliferation of NSCs occurs in the SGZ of the dentate gyrus. In order to study the involvement of NO in proliferation of newborn cells after a brain injury, we used a *status epilepticus* mouse model and counted the number of $BrdU^+$ cells at different time points, as described in *Chapter 2 - Material and Methods*.

Here we show that proliferation of NSCs is highly increased up to 14 days after seizures, with a peak at 5 days after treatment with KA, compared to saline-treated iNOS$^{+/+}$ mice. These results are in line with previous findings that rodent model of *SE* trigger neuroinflammation and stimulate proliferation of newborn cells in the SGZ of the dentate gyrus (Parent et al., 1997, 2007; Gray and Sundstrom, 1998). Cell proliferation is also increased in different acute injured-animal models, such as stroke (Parent et al., 2003; Zhu et al., 2003) and traumatic brain injury (Dash et al., 2001; Rice et al., 2003).

Production of inflammatory factors from microglia, such as NO, has already been reported as essential for proliferation of neuronal progenitors cells in the hippocampus (Yoneyama et al., 2010), functioning as second messengers in intracellular signal pathways. Our group previously described the mechanism by which NO triggers the initial proliferation in SVZ cells *in vitro* (Carreira et al., 2010, 2012). In these studies, we reported that NO is able to bypass the EGF receptor and directly activate upstream components of ERK 1/2/MAPK signaling pathway, resulting in increased cell proliferation of NSCs in early stages (Carreira et al., 2010). Moreover, late proliferation depends on the activation of cGMP and PKG, suggesting a biphasic mechanism of proliferation trigged by NO (Carreira et al., 2013).

The removal of NO showed that proliferation of NSCs can be differentially affected by NO in two distinct time periods. Proliferation of NSCs is not affected by abolishment of NO up to 5 days and at 14 days after treatment with KA, suggesting a NO-dependent regulation of the early proliferation. At 7 days after seizures, the number of BrdU^{+} cells in mice lacking NO treated with KA also increased when compared to saline-treated mice of the same genotype. At this time point, proliferation seems to be regulated by a NO-independent mechanism.

One of the signal pathways that may play an important function at this time point is the neurotrophin signaling mediated by BDNF/TrkB. Previous studies showed that BDNF increases the number and survival of newborn neurons in the SVZ and olfactory bulb (Kirschenbaum and Goldman, 1995; Zigova et al., 1998; Bath et al., 2008) and the dentate gyrus (Lee et al., 2002). Also the NO-cGMP pathway is an important mediator of the proliferative effects of neuropeptide Y in the hippocampus (Howell et al., 2005; Agasse et al., 2008; Cheung et al., 2012).

Since NO can be cytotoxic (Boje and Arora, 1992; Bal-Price and Brown, 2001), it may influence BrdU uptake, increasing BrdU incorporation as a result of DNA repair or decreasing as a result of neurodegeneration following a brain insult, so this should be evaluated. Our group has analyzed that nuclear morphology of NSCs at these times

was not affected, which indicates that these cells were not compromised. DNA repair as a result of DNA fragmentation may be detected by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining in cells that incorporated BrdU. Colocalization of BrdU and TUNEL was assessed in this model and *in vitro* after exposure to NOC-18 (10µM), a NO donor, for 24 hours, and it was not increased in either condition (data not shown). Together, these findings suggest that the changes in BrdU incorporation are due to cell proliferation and not by DNA repair.

iNOS$^{-/-}$ mice show a decrease in neuronal injury after stroke (Liu and Huang, 2008) leading to a possible decrease in the recruitment of pro-neurogenic factors, such as NO, released by microglial cells in the neuroinflammatory environment. However, *SE* model and stroke model are different from each other and this phenotypic characteristic may not be present in the rodent epilepsy models.

4.2. Involvement of NO in migration and distribution of newborn cells in the dentate gyrus following seizures

4.2.1. NO increased migration of neuroblasts after seizures

Neuronal migration is a key point in the neurogenic process. We next investigated the role of NO in migration of neuroblasts in the dentate gyrus following *SE*.

Our results showed that DCX-immunoreactivity area does not change 7 days after seizures, although it tend to increase. At 14 days after *SE*, DCX-immunoreactivity area increased after seizures, by a NO-dependent mechanism.

Previous studies have reported the important role of NO in regulation of fate of the newborn cells in the dentate gyrus. Recently, it has been shown that the number of DCX^{+} neuroblasts significantly increased following treatment with L-NAME, a NOS inhibitor, and KA together (Cosgrave et al., 2010). Moreover, in the some studies, inhibition of NOS alone increased the number of BrdU^{+} newborn cells in the hilus, which suggest a role of NO in their correct migration into the granular zone of the dentate gyrus. Having this into account, our results showed that NO from an inflammatory origin it is not involved in migration of the neuroblasts, at least, at 2 weeks after seizures.

4.2.2. Distribution of early-born cells in the dentate gyrus is affected following seizures by a NO-independent mechanism

Our results show that the number of newborn cells after seizures, formed early (3 days) after seizure, was increased in SGZ and IGZ. Also at this time, the number of newborn cells increased in the SGZ and IGZ in mice lacking iNOS after seizures, suggesting that NO does not contribute to the way the cells distribute throughout the dentate gyrus.

At 7 days after seizures, the distribution along the different regions of the dentate gyrus was not affected by treatment with KA.

According to our study of the proliferation of the NSCs after seizures, at 7 days after KA treatment the proliferation of newborn cells is regulated by a NO-independent mechanism. Therefore, the fact that NO did not change the distribution of the new cells along the dentate gyrus suggest that NO is not involved in how cells are distributed along the dentate gyrus after seizures.

4.3. Differentiation and survival of newborn cells are limited by NO

4.3.1. NO limits survival of the cells that proliferate early (3 days) but not later (7 days) after seizures

The last stage of neurogenesis is differentiation. Survival of newborn cells may limit the final number of mature neurons. During this 6 week period, newborn cells undergo integration into the pre-existent network, maturation in neurons or glial cells and, last but very important, long-term survival. Neuronal differentiation can be assessed from 3 to 4 weeks after neurogenesis starts by analysis of cellular markers, cell morphology or gene expression. In this study we used NeuN as a mature neuron marker, and counted the percentage of $BrdU^+$ cells colocalized with $NeuN^+$ cells.

We show that the number of early-born cells (3 days after *SE*) that survive up to 21 days after treatment with BrdU decreased (Fig 4.2), compared to late-born cells (7 days after *SE*) (Fig 4.3). These results suggest the survival of newborn cells after seizures is regulated by a NO-dependent mechanism, similar to proliferation of NSCs in these conditions.

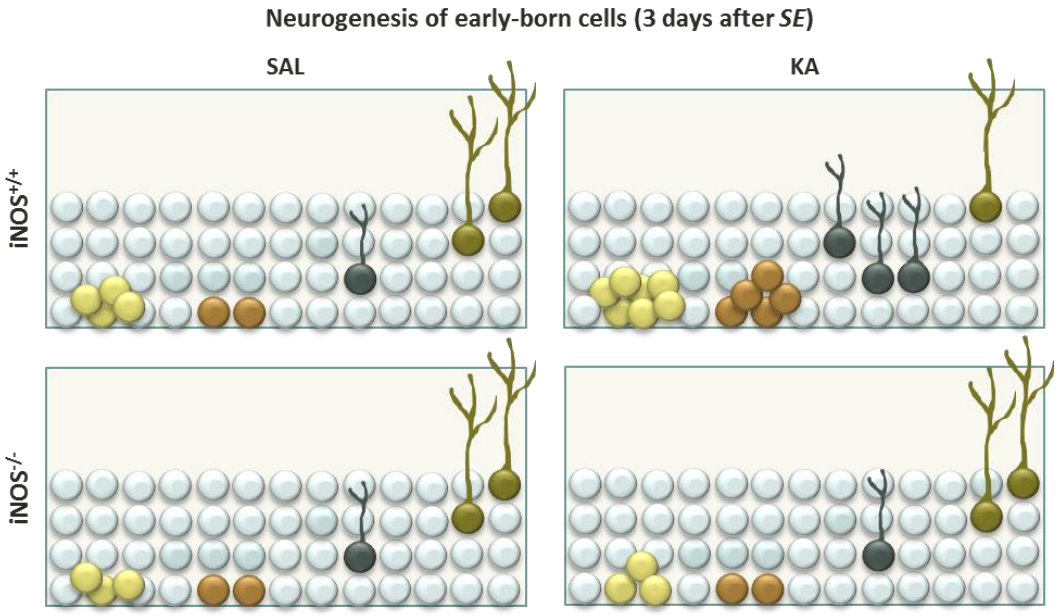

Figure 4.2 – Schematic representation of hippocampal neurogenesis, in iNOS$^{+/+}$ and iNOS$^{-/-}$ mice 3 days after treatment with saline or KA solution. iNOS$^{+/+}$ mice treated with KA show an increased proliferation of NSCs 3 days after seizures. At this time point, the number of newborn neurons decreased compared with iNOS$^{+/+}$ treated with saline solution. iNOS$^{-/-}$ mice.

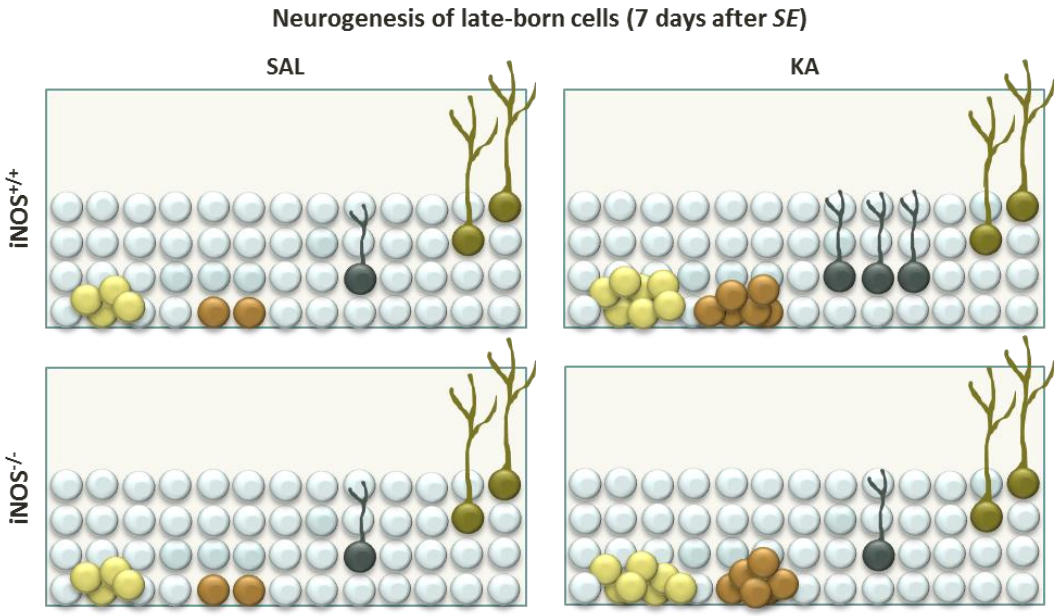

Figure 4.3 – Schematic representation of hippocampal neurogenesis, in iNOS$^{+/+}$ and iNOS$^{-/-}$ mice treated either saline or KA solution. Proliferation of NSCs is increased in both iNOS$^{+/+}$ or iNOS$^{-/-}$ mice 7 days after treatment with KA, but formation of new neurons is not affected.

These results suggest that cells that proliferate in a NO-independent phase become neurons that survive better than cells that proliferate up to 5 days after the onset of seizures. Supraphysiological levels of NO are toxic to neurons and neural apoptosis was evident after administration of a NO donor in a febril seizure rat model (Chen et al., 2008). NO is also been proposed as an inhibitor of cell-cycle progression in many cell types, through activation of p53 or Rb signaling pathways (Ishida et al., 1997; Nakaya et al., 2000). This relationship of NO and programmed cell death might have influence in survival rate of the newborn cells.

4.3.2. Astrogliogenesis is not altered by NO following seizures

Astrogliogenesis was also analyzed for cells formed during early or late proliferative periods after seizures. GFAP is an astrocyte marker and therefore, colocalization of BrdU/GFAP allows the investigation of newborn astrocytes at the time point of treatment with BrdU.

Our results showed that differentiation into astrocytes of new cells born 3 and 7 days after seizures, assessed 21 days after treatment with BrdU, is not affected.

In vitro studies reported that exposure to pathological levels of NO (0.1 mM for 24 hours) promotes astroglial fate determination in neural stem cells over neuronal commitment or selectively depletes early neuronal progenitor cells (Covacu et al., 2006). In this particular model, astrogliogeneses seems to be positively regulated by exposure to NO. Here we show that exposure to NO from iNOS is not involved in astroglial differentiation from neural stem cells after a brain injury, which did not change *per se* the number of new born $GFAP^+$ cells.

4.4 Astrogliosis by NO following seizures

Astrogliosis is defined as an abnormal increase in the number of reactive astrocytes. Astrocyte activation is a cellular response to injury or disorders in the CNS and may influence neuronal survival, as a component of the neuroinflammatory response to lesion.

Here we showed that GFAP-immunoreactive area was increased 28 days after seizures, in a NO-dependent manner, suggesting that neuroinflammation is still present at this time.

Previously, our group studied neuroinflammation 5 days after seizures, and showed an increase in the number of reactive astrocytes either in $iNOS^{+/+}$ or $iNOS^{-/-}$ treated with KA (Carreira et al., 2010). Here we show that activation of astrocytes is maintained up to 28 days after seizures. However, the astrogliosis is not observed in the mice lacking iNOS at this time point, suggesting that late astrogliosis is NO-dependent.

4.5 Regulation of physiological *versus* patophysiological neurogenesis by NO.

The role of NO in regulation of neurogenesis is still unclear. Overall, NO seems to negatively regulate neurogenesis in physiological conditions, while in patophysiological situations it shows proneurogenic action (Tabela 4.1).

Several studies reported decrease in proliferation of NSCs (Packer et al., 2003; Torroglosa et al., 2007; Moreno-López et al., 2004) and survival of the new born cells (Covacu et al., 2006). NO can also modulate differentiation of new precursors by increasing neuronal (Cheng et al., 2003; Moreno-López et al., 2004) or astrocytic differentiation (Covacu et al., 2006).

After a brain insult, NO has been reported as pro-neurogenic factor, since increase in proliferation of NSCs is reported in most of the injury-induced models (Zhu et al., 2003; Hua et al., 2008). Although differentiation is positively regulated by NO following brain insults (Moreno-López et al., 2004, the survival of the new born cells seems to be decreased by NO (Ciani et al., 2006).

Our results, together with previous findings, suggest that not only proliferation of NSCs are regulated by NO-dependent mechanisms following a lesion, but also migration and survival of the new born cells are somehow regulated by the presence of NO following seizures. The fact that NO is important to maintain neuroinflammation up to 28 days

after seizures may have influence in survival of new born cells, and contribute to the failure in new neurons efficiently surviving in such conditions.

Table 4.1 – Regulation of adult neurogenesis by NO (physiological *versus* pathological conditions).

	Effect	References
Physiological conditions		
Proliferation	Decrease	Pacher et al., 2003; Torroglosa et al., 2007 Moreno-López et al., 2004
Differentiation	Increase neurogenesis and astrogliogenesis	Cheng et al., 2003; Covacu et al., 2006; Moreno-López et al., 2004
Survival	Decrease	Covacu et al., 2006
Patophysiological conditions		
Proliferation	Increase	Zhu et al., 2003; Hua et al., 2008; Kokaia and Lindvall, 2003
Differentiation	Increase	Moreno-López et al., 2004
Survival	Decrease	Ciani et al., 2006

Chapter 5

Conclusions

With this work we aimed to understand the involvement of NO produced from iNOS in the hippocampal neurogenesis in a *status epilepticus* mouse model. Our results showed that:

- Production of NO in an inflammatory context increased proliferation of the early-born NSCs following a brain insult.

- Migration of neuroblasts was increased following seizures by a NO-dependent mechanism.

- Distribution of newborn cells along the dentate gyrus was modified by seizures, but NO was not involved in this phenomenon.

- Survival of the new neurons formed at an early stage of the proliferation (3 days after seizures) is decreased by NO.

- Astrogliogenesis was not affected by seizures.

- NO showed to be important in maintenance of neuroinflammation up to 28 days after seizures.

Altogether, these findings helped us to understand the involvement of NO produced by iNOS in different stages of adult neurogenesis following injury and open the possibility to explore new NO-based therapeutic approaches to brain repair after an insult. However, the mechanisms by which NO can act as proliferative factor, and more importantly, how it modulates the survival, differentiation and integration of the newborn cells into the existent synapses is still unclear. There are still many unanswered questions about how NO can modulate neurogenesis in order to improve the proposed therapeutic approaches for brain repair, and more studies are still needed, using both *in vitro* and *in vivo* models.

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