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Avaliação da resposta neurogénica à ativação do recetor de estradiol acoplado à proteína G (GPER)

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For you grandmas,
who will always be looking after me.

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Resumo

O estradiol pertence ao grupo das hormonas esteroides e desempenha um papel importante, sendo essencial não só na manutenção das funções normais do cérebro, mas também na proteção contra lesões neurais. São cada vez mais os estudos que evidenciam o efeito neuroprotetor do estradiol, nomeadamente através da ativação do recetor de estrogénio acoplado à proteína G (GPER). No entanto, não se sabe se esses efeitos neuroprotetores envolvem uma ação pró-neurogénica deste recetor. Assim, o objetivo deste estudo foi avaliar se a ativação do GPER é capaz de desencadear uma resposta neurogénica na região subventricular (SVZ). Foi confirmada a presença do GPER em culturas de células estaminais neurais (NSC) isoladas a partir da SVZ, assim como em fatias de SVZ obtidas de murganhos. A estimulação de culturas de NSC derivadas de SVZ com agonistas do GPER (G1) desencadeou um aumento significativo dos níveis de expressão do recetor bem como um aumento da diferenciação de NSC em neurónios e células da glia, avaliadas através do número de células positivas para $Ki67^+$/ *Neuronal nuclei* ($NeuN^+$) e $Ki67^+$/ *Glial fibrillary acidic protein* ($GFAP^+$), respetivamente. Pelo contrário, a ativação seletiva do GPER não aumentou o número de neuroblastos ($Ki67^+$/ Doublecortin (DCX^+)) ou células da glia ($Ki67^+/DCX^-$) proliferativos, diferindo do efeito mediado pelo estradiol, o qual aumentou significativamente a proliferação de células neurais. A injeção subcutânea de murganhos com G1 (190μg/Kg) e intraperitoneal com Bromodeoxiuridina (BrdU) mostrou que o G1, *per se*, aumenta a proliferação neural na SVZ de murganhos. Em conjunto, os nossos resultados sugerem que a ativação do GPER promove a neurogénese na SVZ, apresentando elevado potencial para ser utilizado como uma estratégia regeneradora.

Palavras-chave

Neurogénese, Células estaminais neurais, Zona subventricular, Estradiol, Recetor de estrogénio acoplado à proteína G

Resumo alargado

O cérebro contém, entre outras características, neuroplasticidade e é capaz de gerar novas células cerebrais ao longo da sua vida, tanto em condições normais como neurodegenerativas. A neurogénese é um processo biológico que requer a proliferação, migração, sobrevivência e diferenciação de células recém-geradas em células neurais maduras. Ao longo dos últimos anos tem vindo a crescer o número de estudos que atribuem às hormonas esteroides, nomeadamente ao estradiol (E_2), um papel importante quer na manutenção de funções cerebrais normais, quer na proteção do cérebro contra lesões neurais. No entanto, ainda não se sabe se o papel protetor mediado pelo recetor de estrogénio acoplado à proteína G (GPER) envolve uma ação pró-neurogénica. Desta forma, o principal objetivo deste estudo foi avaliar se a ativação do GPER é capaz de desencadear uma resposta neurogénica na região subventricular (SVZ). Para tal, foi confirmada a presença do GPER em culturas de células estaminais neurais (NSC) provenientes da SVZ, assim como na região que ladeia os ventrículos laterais, região onde se localiza a SVZ, em fatias de cérebro de murganhos. A ativação do GPER em culturas de NSC derivadas de SVZ com um agonista específico, G1, promoveu um aumento significativo da expressão do recetor bem como um aumento da diferenciação de NSC em neurónios e células da glia maduros, avaliadas através do número de células positivas para $Ki67^+$/ *Neuronal nuclei* ($NeuN^+$) e $Ki67^+$/ *Glial fibrillary acidic protein* ($GFAP^+$), respetivamente. Pelo contrário, a ativação do GPER não aumentou o número de neuroblastos ($Ki67^+$/ Doublecortin (DCX^+)) ou células da glia ($Ki67^+/DCX^-$) proliferativos, diferindo do efeito mediado pelo estradiol que aumentou significativamente a proliferação de células neurais. Procedeu-se ainda ao estudo da ativação do GPER num modelo *in vivo* e, para isso, murganhos adultos foram injetados subcutaneamente com G1 e intraperitonealmente com bromodeoxiuridina. Os resultados obtidos mostraram que o G1, *per se,* foi capaz de aumentar a proliferação neural na SVZ de murganhos. No entanto, relativamente à avaliação da diferenciação celular, para nenhum dos marcadores foram encontradas células positivas para BrdU na região da SVZ dos murganhos, sugerindo que as células proliferativas já tinham seguido o seu trajeto de migração e diferenciação em direção ao bulbo olfativo.

Em conjunto, os nossos resultados sugerem que a ativação do GPER promove a neurogénese na SVZ, apresentando elevado potencial para ser utilizado com estratégia terapêutica regeneradora.

Abstract

Estradiol is a steroid hormone that plays an important trophic and protective role in the adult brain, being essential not only to maintaining normal brain functions but also to protect the brain against neural injuries. There is an increasing number of studies that evidence the neuroprotective effect of estradiol, namely through the activation of the G protein-coupled estrogen receptor (GPER). However, it is not known if this protective effect involve a pro-neurogenic action of this receptor. Therefore, the aim of this study was to evaluate whether GPER activation triggers a neurogenic response in the subventricular zone (SVZ), a neurogenic niche. We confirmed the presence of GPER both in neural stem cell (NSC) cultures from SVZ and in the cells present in the borders of the lateral ventricles of mice brain, where SVZ is located. We analyse the effects evoked by GPER activation *in vitro*, using G1, the GPER specific agonist. GPER activation promoted a significant increase in the expression levels of the receptor as well as an increase of NSC differentiation into mature neurons and glial cells, assessed by the number of $Ki67^+$/Neuronal nuclei ($NeuN^+$) and $Ki67^+$/Glial fibrillary acidic protein ($GFAP^+$)- positive cells, respectively. Contrariwise, GPER activation did not increase neuroblast ($Ki67^+$/ Doublecortin (DCX^+)) or glial ($Ki67^+/DCX^-$) proliferation *in vitro*, in contrast estradiol significantly promoted neural cell proliferation. The *in vivo* analysis of GPER activation showed that G1 *per se* was able to increase neural proliferation in the mice SVZ. Taken together, the results showed that GPER activation effectively promote neurogenesis in the SVZ and has potential to be used as a neurogenic therapeutic agent.

Keywords

Neurogenesis, Neural stem cells, Subventricular zone, Estradiol, G protein-coupled estrogen receptor

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List of Abbreviations

AC	Adenylyl cyclase
BDNF	Brain-derived neurotrophic factor
BLBP	Brain lipid-binding protein
BMP	Bone morphogenic proteins
BMPRs	Bone morphogenic protein receptors
BrdU	Bromodeoxyuridine
BTC	Betacellulin
CREB	Cyclic-AMP-responsive-element binding protein
DCX	doublecortin
EGF	Epidermal growth factor
EGFRs	Epidermal growth factor receptors
ERK 1/2	Extracellular-signal-regulated kinase 1/2
ERs	Estrogen receptors
ERα	Estrogen receptor α
ERß	Estrogen receptor ß
E_2	17ß-estradiol
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
GC	Granule cells
GFAP	Glial fibrillary acidic protein
GLAST	Glutamate aspartate transporter
GPCR	G protein-coupled receptors
GPER	G protein-coupled estrogen receptor
GSK-3ß	Glycogen synthase kinase-3ß
HBSS	Hanks balanced salt solution
HIF-1α	Hypoxia-inducible factor 1α
i.p.	Intraperitoneal
LV	Lateral ventricle
MAPK	Mitogen-activated protein kinase
MEK 1/2	MAPK/ERK kinase 1/2
MMPs	Matrix metalloproteinases
MMS	Medial migratory stream
MPP^+	1-methyl-4-phenylpyridinium
NeuN	Neuronal nuclei
NO	Nitric oxide
NOS	Nitric oxide synthase
NT-3	Neurotrophin-3
OB	Olfactory bulb
PBS	Phosphate buffered-saline
PBS-T	Phosphate buffered-saline 0.1% Tween
PDL	Poli-D lysine
PFA	Paraformaldehyde
PG	Periglomerular neurons
PKA	Protein kinase A

PI3K	Phosphatidylinositol 3-kinase
PLC	Phospholipase C
Pro-HB-EGF	Proheparine-binding epidermal growth factor-like growth factor
RMS	Rostral migratory stream
RT	Room temperature
s.c.	subcutaneous
SERMs	Selective estrogen receptor modulators
SFM	Serum-free medium
SGZ	Subgranular zone
SphK	Sphingosine kinase
SVZ	Subventricular zone
TBS-T	Tris buffer saline solution 0.1% Tween
TEMED	Tetramethylethylenediamine
TGF-α	Transforming growth factor α
V	Ventricle
VEGF	Vascular endothelial growth factor
VZ	Ventricular zone

Work presented in this thesis has resulted in:

Poster presentation at the XII ANNUAL CICS-UBI SYMPOSIUM 2017:
Chendo C, Santos T, Bernardino L & Baltazar G, Evaluation of the neurogenic response evoked by activation of the G protein-coupled estradiol receptor. (7th July) CICS-UBI – Health Sciences Research Centre, University of Beira Interior, Covilhã, Portugal.

Chapter 1 – Introduction

1.1 Neurogenesis

The mammalian brain is plastic and continues to generate new neurons throughout adulthood under normal as well as degenerative conditions (1). Neurogenesis require the proliferation, migration, survival and differentiation of newly generated cells into neurons (2). The neurogenic niches are a major repository of neural stem cells (NSC). The production of new neuronal cells in the adult brain is typically limited to the subventricular zone (SVZ), which lines the lateral ventricles and generates cells (neuroblasts) that migrate along the rostral migratory stream (RMS) to the olfactory bulb (OB), where they differentiate into functional olfactory interneurons, and the subgranular zone (SGZ) of the hippocampus, associated to functions such as learning, memory and mood regulation (1–3).

Multipotent NSC are capable of producing multiple types of both neuron and glia and are located in the SVZ, while neural progenitor cells located in the SGZ divide more frequently and into limited cell subtypes, as they may only produce daughter cells of either a defined glial or neuronal lineage (4–6).

In addition to being responsible for cell production and replacement, under pathological conditions like traumatic brain injury and cerebral ischemia, enhanced NSC proliferation and differentiation was described. Therefore, newborn neurons and glia derived from neurogenic niches are able to redirect the normal migration pathway toward the sites of injury like cortex and striatum. Then, the newly generated cells can differentiated into the specific cell types lost after injury (7–13).

1.1.1 The formation of the SVZ

The primordial SVZ of a rodent embryo delaminates from the ventricular zone (VZ) at around day 10.5 of the mouse forebrain development (14).

At day 14 of the embryonic development, the walls of the lateral ventricles are lined by proliferating VZ cells while the SVZ is most prominent in primordia of basal ganglia and extends as a thin layer into the lateral neocortex. The location of the embryonic SVZ as well as its large size and widespread migratory capacity of their cells supports the idea that the embryonic SVZ supplies a large number of new cells for many regions during the forebrain development (14).

In the postnatal/adult brain, the walls of the lateral ventricles are lined by a thin layer of ependymal cells and adjacent to it remains a thin layer of proliferative SVZ (figure 1.1) (14,15).

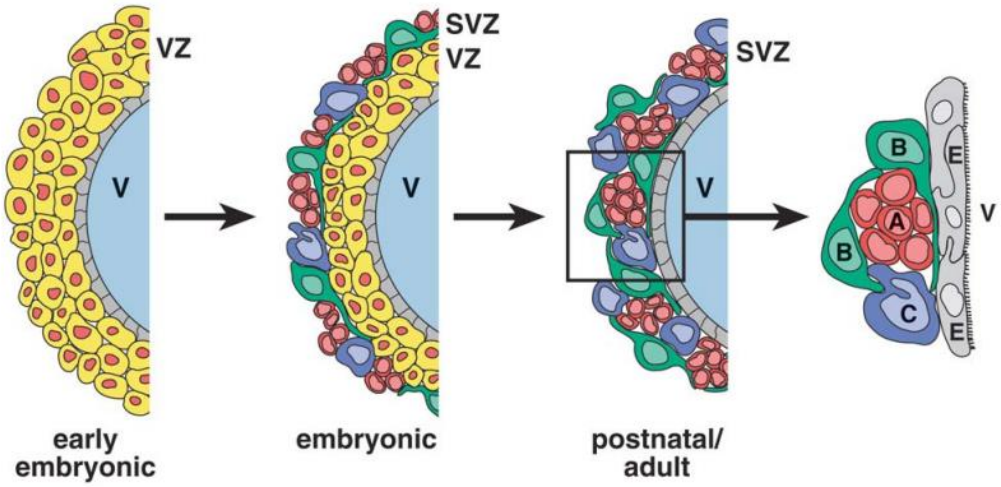

Figure 1.1- The proliferative ventricular zone and subventricular zone during development and into adulthood. The reduced SVZ in the adult is composed of migrating neuroblasts, astrocytes, transitory amplifying progenitor cells separated from the ventricle by a monolayer of ependymal cells. Abbreviations: V, ventricle; VZ, ventricular zone; SVZ, subventricular zone; A, neuroblasts; B, type B cells; C, transit-amplifying cells; E, ependymal cells. From (15).

1.1.2 Cellular architecture of the SVZ

The cells that serve as adult stem cells are called type B cells and express, among others, glial fibrillary acidic protein (GFAP), glutamate aspartate transporter (GLAST) and brain lipid-binding protein (BLBP). These cells are derived from the embryonic neural stem cells, the radial glia (16). The type B cells make direct contact with the ventricles lumen by extending a thin cellular process between ependymal cells (E cells). Activated B cells divide slowly and give rise to transit-amplifying precursors (type C cells), which then divide rapidly and generate neuroblasts or type A cells. These cells, that express migratory markers such as doublecortin (DCX) and PSA-NCAM, form elongated aggregates called chains and continue to divide while migrating to the OB through tunnels formed by the processes of type B cells, as seen in figure 1.2 (14–16). After reaching the core of the OB, they move radially into granular and periglomerular layers, where they differentiate into mature neurons (17,18).

The generation of large colonies of mature neurons requires the division of type B cells as described suggesting that type B cells act as stem cells in the SVZ region (19). When all migrating neuroblasts and type C cells are eliminated the cellular regeneration is maintained by B cells. Although neuroblasts have the ability to divide, cultures of purified A cells lose the capacity of self-renewing (20).

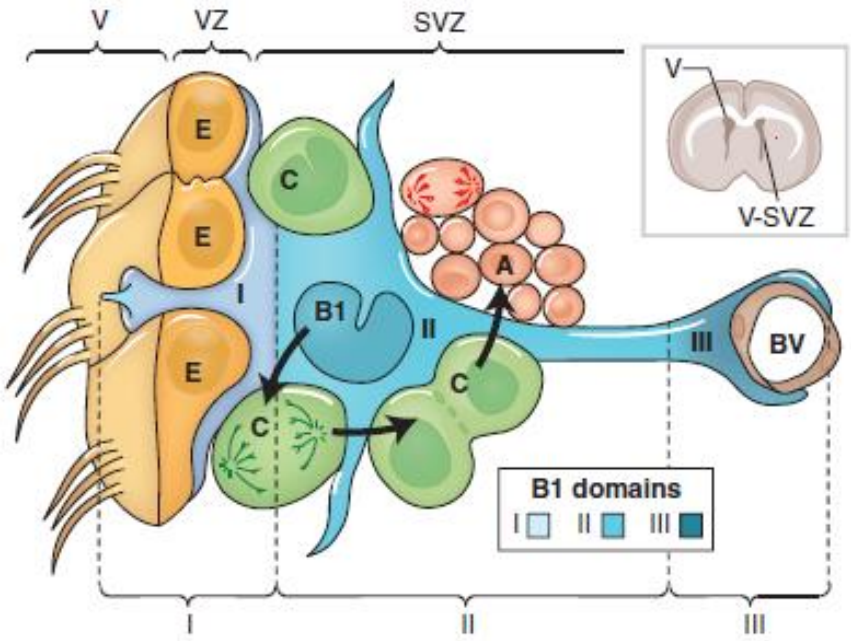

Figure 1.2- Cellular architecture of the SVZ. Type B cells, astrocytes that serve as the SVZ adult stem cells; Type C cells, give rise to type A cells (migratory neuroblasts); Ependymal cells (E), form an epithelial layer that separates the SVZ from the ventricle (V). The SVZ can be subdivided into three domains based on the structure and the spatial arrangement of the B cells: Domain I (apical) contains the type B cells apical processes and the body of ependymal cells; Domain II (intermediate) contains the cell body of the most B cells, which are in contact with the type C and A cells; Domain III (basal) contains the B cell's basal process with end-feet on blood vessels. From (17).

1.1.3 Rodent vs Human SVZ

With increasing demand for new therapeutic self-repair agents and treatment for neurodegenerative diseases, it is crucial to understand the differences in composition and structure of SVZ between rodents and humans.

The postnatal human SVZ contains a small number of proliferative and migrating cells and appears to be organised in a heterogenic thickness with a single cell row in the dorsal part and multiple cell rows in the ventral zone. Different from SVZ of rodents, the human neurogenic niche has a prominent gap layer (GAP) devoid of cells and populated by a dense network of interconnected processes from astrocytes and ependymal cells. Below this GAP, there is cellular "ribbon" of multipotent astrocytes with NSC properties, lining the human lateral ventricle (21,22). Besides a RMS that allow neuroblasts migration from SVZ to OB, the human brain has a medial migratory stream (MMS) that connects SVZ with the medial prefrontal cortex. It should be noticed that contrary to the rodent brain, in the human brain migration from SVZ to the OB ceases in the early postnatal stage (22,23).

Despite the differences, human SVZ cells express the same cell markers of rodent SVZ and present an organization with cells migrating in chains, suggesting similar neurogenic mechanisms between the two species (23,24).

1.1.4 Neurogenic modulators

It is known that neurogenesis in the SVZ is modulated by many factors capable of regulating cell proliferation and/or migration. One of these regulators is the microenvironment of the neurogenic niche, which is responsible for controlling stem cells self-renewal and progenitor cell differentiation through soluble and non-soluble signals from stem cells themselves, their progenitors and neighboring cells. These signals can be, among others, cytoplasmic factors, niche factors, transcriptional factors and epigenetic regulators (17,25). Some of these factors will be described in more detail below.

- ✓ Growth factors

One of the factors implicated in the regulation of neurogenesis is the brain-derived neurotrophic factor (BDNF). This regulator exists in two forms, the precursor proBDNF and mature form, BDNF. The majority of the studies points to mature BDNF as the principal regulator of the postnatal SVZ neurogenesis, regulating survival and differentiation of neural stem cells and neuroblasts (26,27). Intraventricular administration of BDNF in the lateral ventricles of adult rats, promotes an increase in the number of newborn neurons in the OB (28). Kirschenbaum & Goldman (1995) verified also that BDNF administration *in vitro* promotes an increase in the long-term survival of neuroblasts (29).

Others growth factors such as epidermal growth factor (EGF) and fibroblast growth factor (FGF) can stimulate the proliferation of SVZ cells *in vivo* and are the principal mitogens used to induce the formation of neurospheres with the characteristics of NSC *in vitro* (30–32). These growth factors can also control the cell-fate of the NSC as well as their differentiation (33). Zheng *et al.* (2004) and Tropepe *et al.* (1997) demonstrate that mice null for FGF or for EGF receptor ligand-transforming growth factor α (TGF-α), respectively, have significantly reduced neurogenesis on the SVZ region (34,35).

Vascular endothelial growth factor (VEGF) is also a known regulator of neurogenesis in the SVZ. Its intraventricular infusion enhances SVZ-proliferation and promotes migration (36).

Neurotrophin-3 (NT-3) is a growth factor secreted by endothelial cells. NT-3 signalling regulates the quiescence of the NSC population through rapid phosphorylation of endothelial nitric oxide synthase (NOS) that lead to the production of nitric oxide (NO), which in turn acts as a cytostatic factor (37).

Betacellulin (BTC) is a EGF-like growth factor also secreted by the endothelial cells. *In vivo* BTC infusion increase cell proliferation in SVZ as well as OB neurogenesis whereas BTC inhibition cause a decrease of the type B and A cell number (38).

- ✓ Bone morphogenic proteins (BMP) and Notch ligands

In addition to factors that promote cell differentiation, there are others capable of maintaining stem cells in an undifferentiated state. The ependyma-SVZ interaction plays an important role in this process. The BMP and their receptors (BMPRs) are expressed throughout the SVZ while Noggin, a BMP-binding protein that act as antagonist, is expressed by ependymal cells. Higher BMP levels or reduced competition of Noggin for BMP promote gliogenesis. On the other hand, when Noggin is widely expressed it blocks endogenous BMP signals and promote neurogenesis instead of gliogenesis (figure 1.3A) (15,39).

Besides the BMP-Noggin interactions, the Notch 1 receptor and its ligand are also an example of the ependyma-SVZ interaction. The binding of Notch 1 receptor in NSC to ligands expressed on neighbouring cells is thought to maintain neural stem cells in their undifferentiated state. This mechanism promotes self-renewal and symmetrical division rather than asymmetrical division and consequent differentiation, or promotes gliogenesis. On the contrary, absence of Notch signalling promotes neurogenesis (figure 1.3B) (39,40).

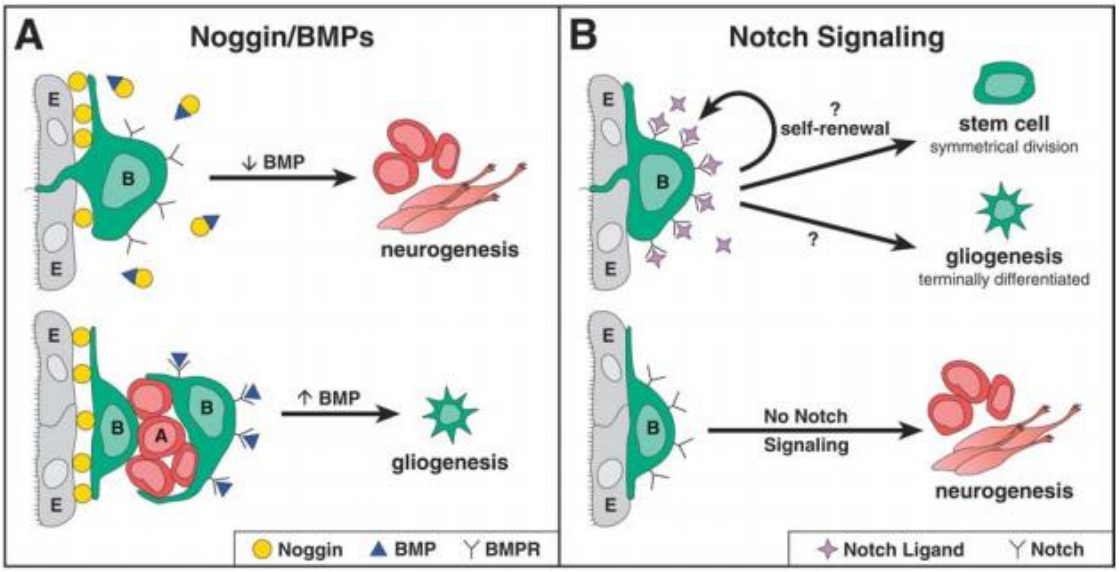

Figure 1.3- Signalling molecules and their actions within the SVZ. A, neuroblasts; B, type B cells; E, ependymal cells. Acting as antagonist, Noggin is thought to block BMP signalling through its receptor, BMPR, promoting neurogenesis instead of gliogenesis. Higher BMP levels or reduced competition of Noggin for BMP results in gliogenesis (A). Notch signalling by neural stem cells is thought either to promote self-renewal or result in gliogenesis. Absence of Notch signalling allows neurogenesis (B). From (15).

- ✓ Neurotransmitters

Neurotransmitters, such as serotonin (41), dopamine (42,43), acetylcholine (44,45), glutamate and GABA (46,47) are capable of modulating the migration, maturation, integration and survival of newly generated cells (39). For instance, serotonin acts through receptors in the SVZ, increasing cell proliferation (41).

The SVZ receives dopaminergic innervation from the midbrain and Hoglinger *et al.* (2004) showed that dopaminergic denervation leads to decreased SVZ-proliferation and OB neurogenesis whereas that administration of levodopa, a dopamine precursor, is able to restore neural cell proliferation (43).

Adult SVZ neuroblasts release GABA which inhibits the proliferation of precursors and enhances its migration (46). More importantly, the newborn cells have to be previously activated by GABAergic synaptic inputs from local interneurons and then by glutamatergic inputs for synaptic integration on the neural network (figure 1.4) (39). On the other hand, NMDA receptor signalling can regulate the survival of newborn neurons in the SVZ (47).

Nitrergic neurons can be found in the proximity of SVZ and express the neuron-specific form of NOS, responsible for NO production. NO is a free-radical signalling molecule reported to inhibit adult neurogenesis (48).

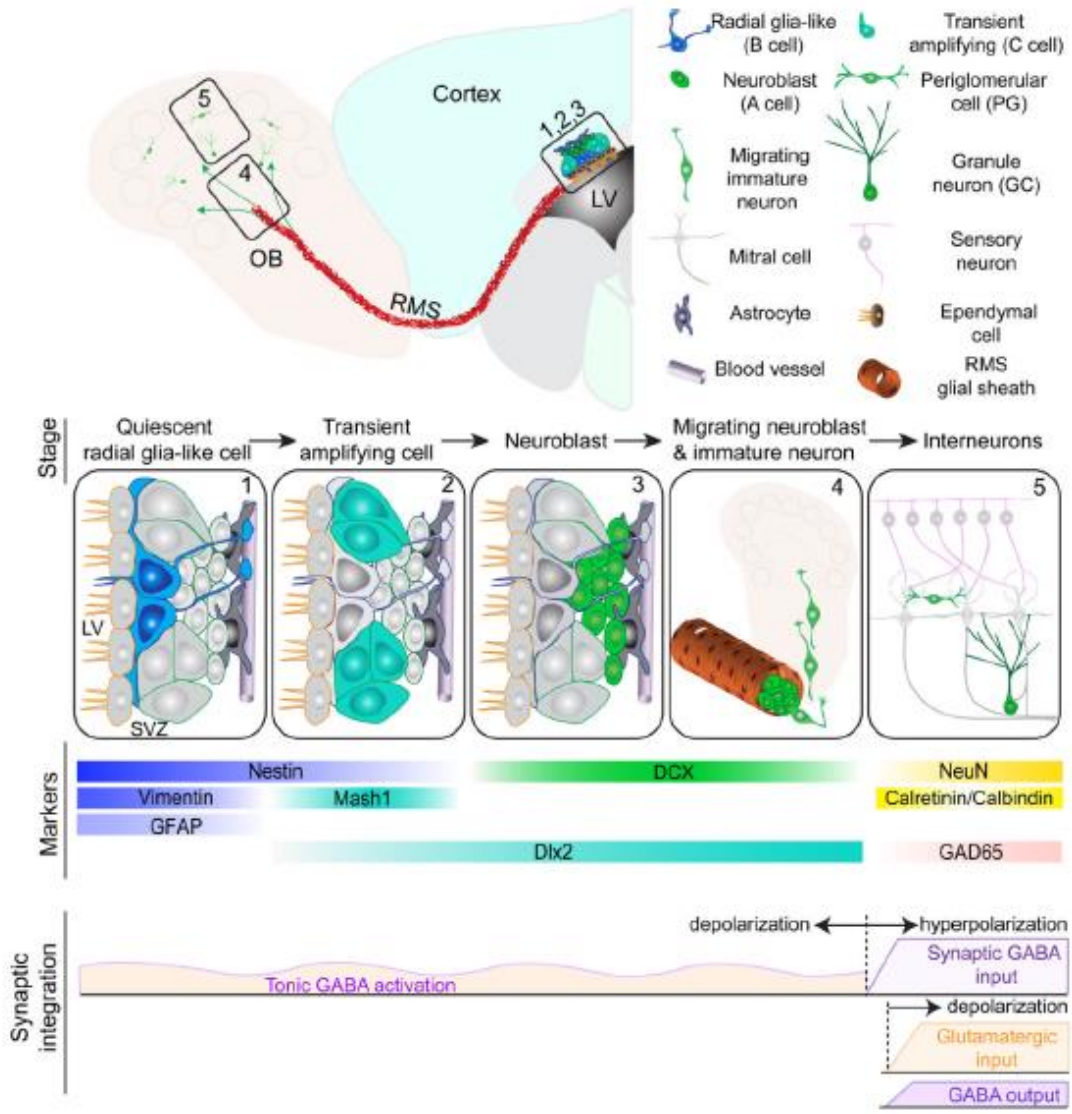

Figure 1.4- Overview of the adult neurogenesis in the SVZ and OB. Schematic representation of the development stages in adult SVZ neurogenesis: (1) activation of radial glia-like cells in the subventricular zone in the lateral ventricle (LV); (2) proliferation of transient amplifying cells; (3) generation of neuroblasts; (4) chain migration of neuroblasts within the rostral migratory stream (RMS) and radial migration of immature neurons in the olfactory bulb (OB); (5) Synaptic integration and maturation of granule cells (GC) and periglomerular neurons (PG) in the OB. Below, the expression of specific markers of each cellular development stage. Lastly, the sequential process of synaptic integration. Abbreviations: GFAP, glial fibrillary acidic protein; DCX, doublecortin; NeuN, neuronal nuclei. Adapted from (39).

- ✓ Wnt signalling

The Wnt proteins appear to control multiple functions in neural development, acting at distinct stages of the neurogenic lineage and playing role in stem cell maintenance, cell proliferation and differentiation and well as cell migration (17). Yu *et al.* (2006) demonstrated that Wnt-3a and Wnt-5 increase proliferation and differentiation of neural progenitor cells isolated from post-natal and adult mice brain (49). The overexpression of

Diversin, a component of the Wnt signalling pathways, enhance the proliferation of neuroblasts and increase the number of DCX-positive cells that reach the OB (50).

- ✓ Steroid hormones

Over the past years, many studies implicated steroids hormones such as testosterone and estradiol as potential regulators of the adult neurogenesis (51–53). In mammals, evidences suggest that in the reproductive cycle, when estrogen levels are higher, there is an increase in the cell proliferation in the dentate gyrus of the hippocampus. On the contrary, a reduction of the circulating estrogens results in a significant decrease in the proliferation of hippocampal precursors (54). In the next section, the role of estradiol as a neurogenic regulator will be discussed more deeply.

1.2 Estradiol: a brief sneek peek

Estradiol belongs to the steroid hormones group, being an estrogen, and exists in two optical isomers, 17 α-estradiol and 17 ß-estradiol (E_2), being 17 ß-estradiol the more potent and the physiological active form of the two estrogens (55). Estrogens can be synthesized peripherally in the reproductive organs and extragonadal sites and cross the blood-brain barrier to act in the brain, and can also be synthesised in the nervous system by neurons and astrocytes from cholesterol. Both cell types present all the enzymatic machinery necessary for the production of estradiol (figure 1.5) (56).

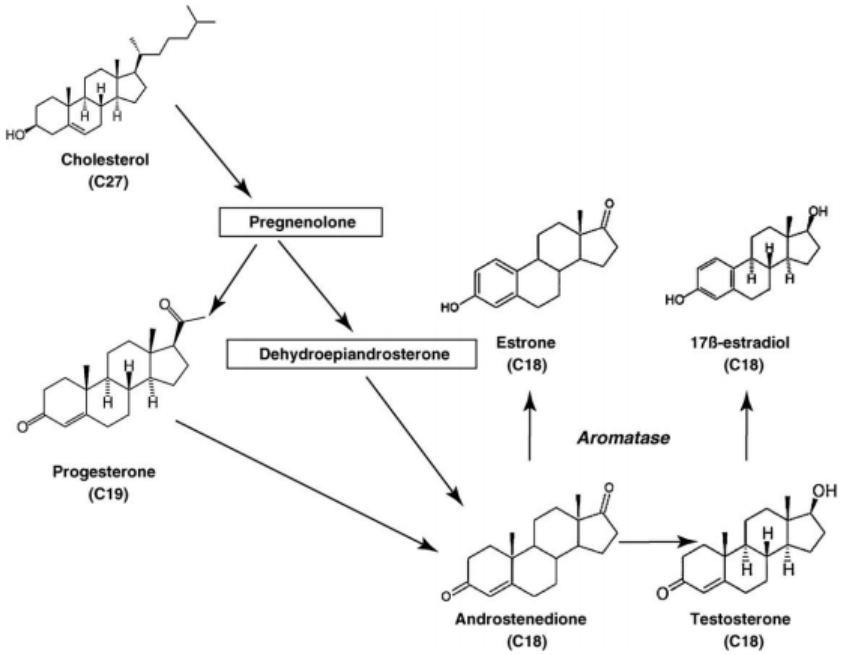

Figure 1.5- Estradiol metabolic pathway. Estrogens and other steroid hormones are derived from cholesterol, with pregnenolone formed from cholesterol through the activity of cytochrome P-450. Other early steps in estrogen biosynthesis are the conversion of pregnenolone to dehydroepiandrosterone, and the conversion of progesterone to androstenedione. 3ß-hydroxysteroid dehydrogenases catalyse several reactions in the androgen pathway, leading to production of

androstenedione and testosterone. The enzyme aromatase catalyses the conversion of C19 steroids to estrogens. From (57)

Several studies indicate that E_2 plays important roles in regulating the nervous system, acting directly on neurons and glial cells, preventing cell death, promoting neuronal survival, enhancing neurite outgrowth, stimulating synaptogenesis and regulating synthesis of neurotransmitters and their receptors (58–60). During embryonic and fetal brain development, estradiol exerts its actions by modulating neurogenesis, cell migration and differentiation (58). Figure 1.6 shows some of the possible pathways of estrogen-mediated neuroprotection.

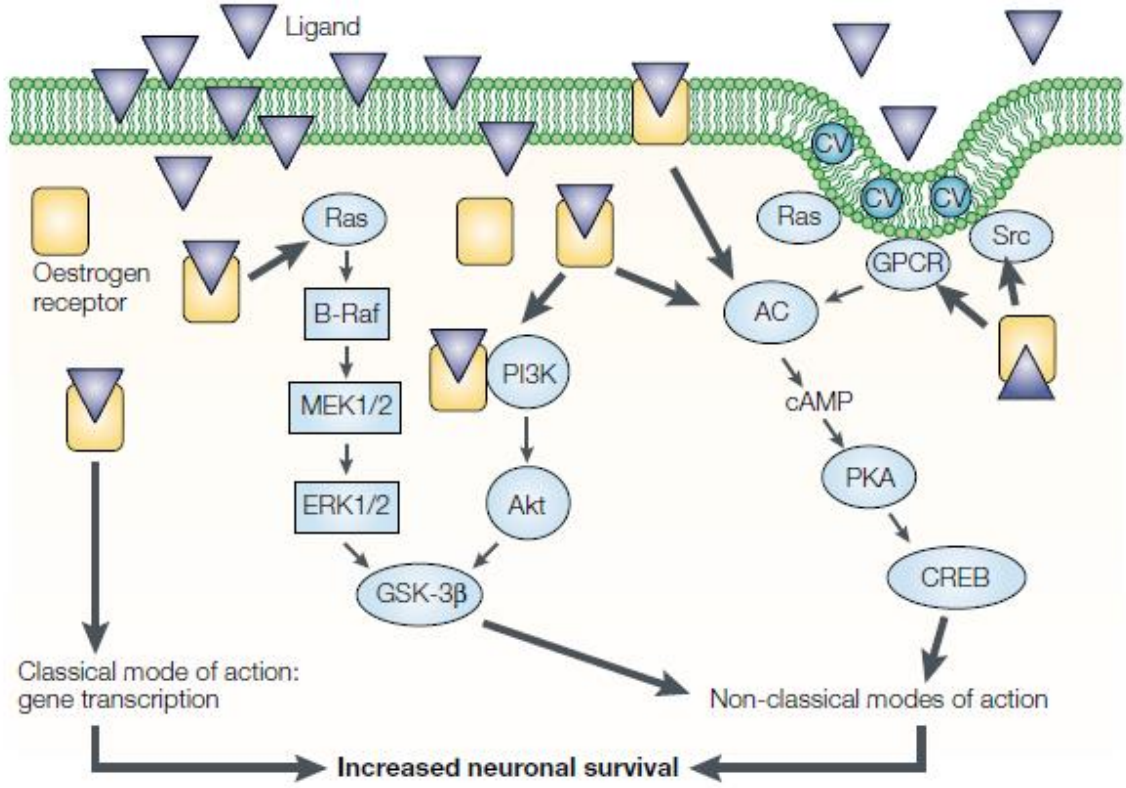

Figure 1.6- Pathways of estrogen-mediated neuroprotection. Through a classical genomic response, estrogens binds to the nuclear ERs and induce regulation of potentially neuroprotective genes. Estrogens can also interact directly with the mitogen-activated protein kinase (MAPK) signalling pathway through the activation of Ras, B-Raf and MAPK/ERK kinase 1/2 (MEK 1/2). Estrogen rapidly induces the phosphorylation of extracellular-signal-regulated kinase 1/2 (ERK 1/2). Likewise, ERs interact with the phosphatidylinositol 3-kinase (PI3K) signalling pathway, leading to the activation of its downstream effector Akt. Interestingly, a central downstream target of MAPK and Akt signalling is the enzyme glycogen synthase kinase-3ß (GSK-3ß)— an important modulator of nerve-cell survival. Moreover, estrogen rapidly induces the phosphorylation of cyclic -AMP-responsive-element binding protein (CREB) through adenylyl cyclase (AC) and protein kinase A (PKA). Various intracellular signalling pathways, including G protein-coupled receptors (GPCR) and Src, could be brought together locally with ERs. Crosstalk at intracellular signalling has also been proposed for putative membrane ERs that remain to be clearly identified. The non-classical mode of estrogen action includes an indirect genomic activity and structural actions. From (61).

Estrogens can act through genomic and non-genomic mechanisms, depending on the estrogen receptor activated. There are two forms of classic estrogen receptors (ERs), ERα or ERß,

which are typically located in the cell cytoplasm or nucleus (figure 1.7). The binding of estrogen to its receptors induces a cascade of events that starts with conformational changes in the receptor and culminates with gene transcription and consequent gene expression. This sequence of events that form the ER-mediated signalling includes receptor dimerization, interaction of receptor dimers with DNA and recruitment of co-activators and transcriptional factors that then form a complex protein-DNA assembly called the preinitiation complex of gene transcription. The ERs can form homo or heterodimers that translocate to the nucleus and bind to estrogen response elements (EREs) located in the promoter regions of target genes. Besides that, ERs can also regulate gene expression through other mechanisms like via protein-protein interactions with other transcription factors and their respective response elements (61,62).

Although classic estrogen receptors trigger signalling cascades that take hours to days to occur, some evidence suggests that classical nuclear receptors are required for some rapid estrogen-mediated responses in various cell types (63).

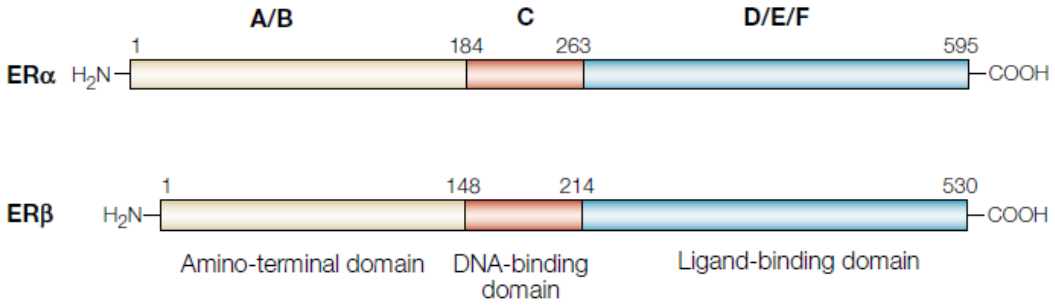

Figure 1.7- The nuclear estrogen receptors. Both receptors share the same structural domains (A-F). ERs can be divided into three parts: amino-terminal or A/B domain, DNA-binding or C domain, ligand-binding or E domain. The F domain is responsible for distinguishing estrogen agonists from estrogen antagonists. From (61).

In 2005 a third estrogen receptor was identified. Due to its structural characteristics of 7 transmembrane domains and coupling to G proteins this receptor was called G protein-coupled estrogen receptor (GPER). This receptor is responsible for E_2 rapid effects (64). This receptor will be described in more detail in the next section.

1.2.1 Estradiol as a potent neurogenic regulator

E_2 plays an important trophic as well as protective role in the adult brain being essential not only to the maintenance of normal brain functions but also to protecting the brain against neural injuries through different mechanism including the stimulation of neurogenesis. Physiological concentrations of E_2 are capable of supressing neuronal apoptosis and enhancing the production of adult-born neurons in the SVZ of ovariectomized mice after ischemic stroke as well as their differentiation and migration to the ischemic region, potentially replacing damaged cells. Furthermore, Suzuki and co-workers showed that by knocking out the classic

estrogen receptors, the ability of E2 to increase neurogenesis is lost (65). Additionally to E_2, other estrogen receptor modulators (SERMs), like tamoxifen or raloxifene has been studied as potential neurogenic modulators following focal cerebral ischemia. To note that this two SERMs have antagonistic action towards ERα and ERβ and act as agonists of GPER (66). Both intact and ovariectomized adult female rats treated with E_2 or raloxifene present increased neurogenesis in the ipsilateral SVZ following transient middle-cerebral artery occlusion. In contrast animals treated with tamoxifen did not presented increased neurogenesis (67). On the other hand, Farinetti *et al.* (2015) reported that E_2 and testosterone treatment increases proliferation in the SVZ of prepuberal castrated male rats. Additionally, they showed that such effect is restricted to males, suggesting that adult neurogenesis is differently modulated depending the genre (51). This is also supported by a study in which both gonadectomized male and female rats were treated repeatedly with E2. The results demonstrated a clear sex difference in the response to E2 of hippocampal neurogenesis and apoptosis, with adult females being more responsive to the effects of E2 than males (68). The neurogenic effect of E2 is not restricted to rodents. In fact, E_2 induced proliferation of human neuronal progenitor cells associated with DNA replication, elevated cell cycle protein expression and centrosome amplification, which was associated with augmentation of total cell number (69). In a study undertaken to evaluate the effects of E_2 on the proliferation and neural differentiation of human adipose stem cells during neurogenic differentiation, the results showed that E_2 treatment significantly increase the proliferation rate of differentiated cells, with a higher expression of precursor and mature neuronal markers (70).

1.2.2 G protein-coupled estrogen receptor: a possible target for brain repair

As mentioned in the previous section, a third estrogen receptor was discovered in the late ‘90s, when specific binding sites for estrogen at the outer surface of isolated rat endometrial cells was reported by Pietras & Szego (1997) (71). A few years later it was assigned to this receptor the role of mediator of estrogen rapid non-genomic actions (66). Thus, GPER (also called GPER1 or GPR30) was proposed to be the first identified intracellular transmembrane ER that contributes to normal estrogen physiology as well as pathophysiology (72). Being a GPCR, this receptor seems to mediate rapid actions of estrogens through second messenger signalling pathways. In this way, estrogen binding to the receptor may lead to activation of different intracellular signalling pathways such as kinase activation, cAMP production, calcium mobilization and transcriptional activation. Estrogen is freely cell permeable, gaining access to intracellular estrogen receptors. Its binding to GPER activates heterotrimeric G proteins, which in turn can activate multiple effectors, such as adenylyl cyclase and consequent cAMP production, Src, and sphingosine kinase (SphK) (figure 1.8). These two pathways appear to be involved in the activation of matrix metalloproteinases (MMPs), which cleave proheparin-binding-EGF-like growth factor (pro-HB-EGF), releasing free HB-EGF that can then transactivate epidermal growth factor receptors (EGFRs). EGFR activation leads to

multiple downstream events, including the activation of phospholipase C (PLC), mitogen-activated protein kinases (MAPKs), and phosphatidylinositol 3-kinases (PI3Ks). PLC activation leads to intracellular calcium mobilization through the actions of inositol triphosphate (IP3). The activation of MAPKs and PI3Ks results in the activation of numerous cytosolic pathways as well as the activation of nuclear proteins that regulate transcription factors. Thus, estrogen stimulation can give rise to the transcription of gene targets whose promoters do not contain steroid response elements (72–74). The cellular consequences can range from cell proliferation and survival to cell differentiation and migration.

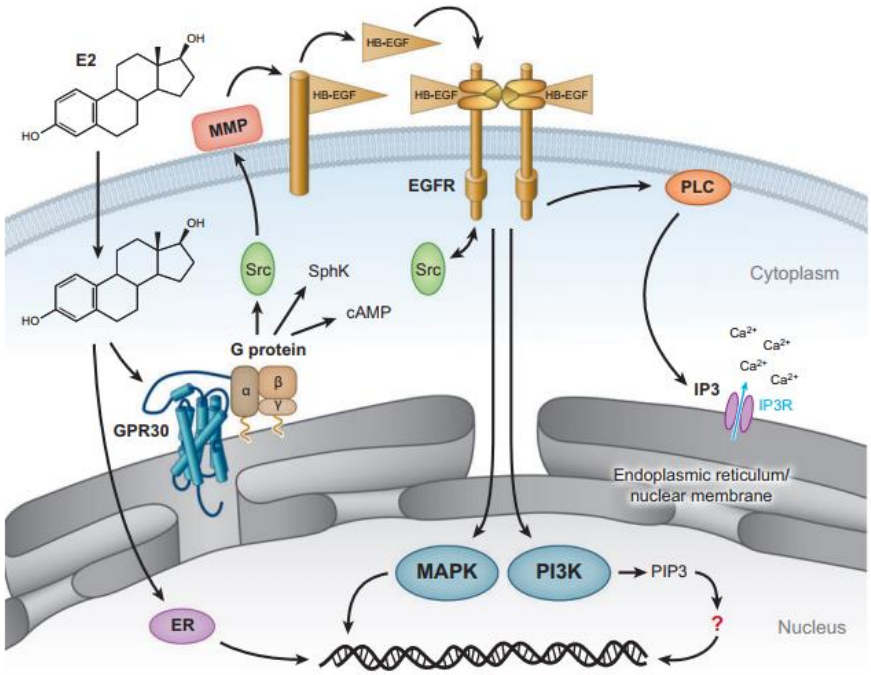

Figure 1.8- Pathways triggered by GPER activation. Estrogen binding to classical ER leads to direct transcriptional activation as well as signal transduction events. Upon agonist binding, GPER activates heterotrimeric G proteins, which in turn can activate multiple effectors, including kinase activation, cAMP production, calcium mobilization and transcriptional activation. The combined effects of these cytosolic signalling and nuclear transcription events often result in cell proliferation. Abbreviations: GPR30, G protein-coupled estrogen receptor; SphK, sphingosine kinase; MMPs, matrix metalloproteinases; EGFRs, Epidermal growth factor receptors; PLC, phospholipase C; MAPKs, mitogen-activated protein kinases; PI3K, phosphatidylinositol 3-kinase; HB-EGF, heparin-binding epidermal growth factor-like growth factor; IP3R, inositol triphosphate receptor. From (74).

Through the years, a growing number of molecules targeting GPER with agonistic or antagonistic activity have been emerging. Among others, GPER is activated by endogenous natural estrogens like E_2 and estrone as well as phytoestrogens such quercetin, genistein, epicatechin or resveratrol. Endocrine disruptors/xenoestrogens such as bisphenol A, atrazine and nonylphenol also activate GPER (75). Selective synthetic, non-steroidal, small molecules compounds such as G1 and ER antagonists/SERMs like tamoxifen and ICI182,780 were also shown to exert an agonist action in GPER (64,66). On the other hand, progesterone,

testosterone, cortisol and synthetic small molecule compounds G15 and G36 inhibits GPER activity (64,75). G1 was identified as a non-steroidal compound by Bologa *et al.* (2006) through a combination of virtual and biomolecular screening targeting GPER. It was obtained a binding affinity of the synthetic compound of 11 nM compared with the 6 nM of the estrogen (76).

GPER not only exerts direct effects upon ligand activation but its function involves the crosstalk with nuclear steroid receptors, including ERα and ERβ (figure 1.9) (75). For example, in the BG-1 ovarian cancer cells the expression of c-fos is both ERα and GPER-dependent. Down-regulation of ERα and GPER inhibited proliferation and c-fos transcription, indicating the use of the same signalling via. Moreover, both receptors promoted cell division, however the silencing of each individual inhibited it (77).

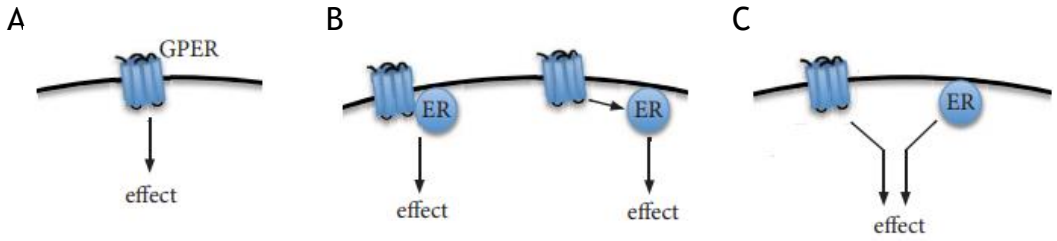

Figure 1.9- Possible crosstalk between nuclear estrogen receptors and G protein-coupled estrogen receptor. A- GPER acts autonomously; B- GPER and ER form a complex or GPER activates ER; C- GPER and ER activate parallel pathways that lead to the same effect. Abbreviations: GPER, G protein-coupled estrogen receptor; ER, estrogen receptor. Adapted from (78).

1.2.2.1 GPER localization in the brain

In the few studies undertaken to determine the distribution of GPER in the central nervous system, this receptor was identified, among others, in the hypothalamic-pituitary axis, autonomic nuclei of the brain stream, rostro-caudal extent of lateral globus pallidus, striatum, isocortex and hippocampal dentate gyrus hilus, where interestingly the ERα and ERβ expression is either low or absent. In the midbrain a high concentration was found in the substantia nigra (79–81).

The subcellular location of GPER remains controversial. Initially, it was described as being located on the plasmatic membrane, like the majority of GPCR (64). However, another study suggests that GPER is also located intracellularly, in particular in the endoplasmic reticulum and Golgi apparatus (82). The subcellular localization of GPER may depend on cell or tissue type (83).

1.2.2.2 GPER-mediated neuroprotection

Studies that link the E_2 signalling through GPER to neuroprotective effects are increasing. Lebesque *et al.* (2010) demonstrated that acute administration of non-classic ER agonists delayed the loss of hippocampal neurons in a global ischemia model (84). Studies conducted

by our group showed that GPER selective activation is able to modulate microglial responses and protect nigral dopaminergic degeneration and motor functions against injury induced by an inflammatory insult (85). In another study aimed at evaluating the contribution of GPER to estrogen-mediated dopaminergic neuroprotection against an insult induced by 1-methyl-4-phenylpyridinium (MPP^+), effective G1-mediated protection of dopaminergic neurons was demonstrated (86).

1.2.2.3 Role of GPER in neurogenesis

Duarte-Guterman *et al.* (2015) evaluated the role of GPER in hippocampal cell proliferation in adult female rats and the regulation of GPER expression, in the hippocampus. E2 induced a reduction in GPER expression in the granule cell layer but not in the CA1 and CA3 regions whereas treatment with GPER agonist decreased cell proliferation in adult ovariectomized rats, indicating a GPER-independent role of estradiol in hippocampal neurogenesis or alternatively an antagonistic effect of intracellular and membrane bound ER activation to maintain the levels of neurogenesis. GPER did not co-localized with progenitor cells in the subgranular zone of the dentate gyrus, indicating that the effects of GPER activation on neurogenesis may be indirect (87).

Chapter 2 – Aims

GPER appears to play a crucial role in estrogen-mediated neuroprotection. On the other hand, it is crucial to identify mechanisms that promote adult neurogenesis because such knowledge may allow the development of therapeutic strategies for neuronal replacement, fighting brain injuries like neurodegenerative diseases. In this sense, we aim to evaluate the effects of GPER activation on SVZ neurogenesis under physiological conditions. More specifically, the aims of this work are:

- To evaluate the effect of GPER activation in NSC cultures from SVZ and *in vivo* in cell proliferation and differentiation;

- To analyse the GPER expression in NSC cultures from SVZ and *in vivo*.

Chapter 3 – Materials and Methods

3.1 *In vitro* studies

3.1.1 SVZ cell cultures and experimental treatments

All experiments related to the use of experimental animal models were conducted in agreement with protocols approved by the national ethical requirements for animal research and the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purpose (Decreto-Lei n.º 113/2013¸ European Union Directive number 192 2010/63/EU).

For SVZ derived-neurospheres culture, a protocol adapted from Gil-Perotín *et al.* (2013) was used (88).

SVZ cultures were prepared from 1 to 3 day-old Wistar rats. Briefly, brains were removed and placed into Hanks Balanced Salt Solution (HBSS; 137mM NaCl, 5.36mM KCl, 4.16mM $NaHCO_3$, 0.44mM KH_2PO_4, 0.34mM $Na_2HPO_4.2H_2O$, 5mM glucose, 1mM sodium pyruvate, 10mM HEPES, pH 7.4) supplemented with 100U/mL penicillin and 100µg/mL streptomycin (all from Sigma). SVZ fragments were dissected from 450µm-thick coronal brain sections and digested in 0.5g/L trypsin and 0.2g/L EDTA diluted in phosphate buffered-saline (PBS; NaCl 140mM, KCl 2.7mM, KH2PO4 1.5mM and Na2HPO4 8.1mM, pH7.4), and digested by mechanical dissociation. The cell suspension was diluted in serum-free medium (SFM) composed of Dulbecco's modified Eagle medium [(DMEM)/F12+GlutaMAX^{TM}-1)] supplemented with 100U/mL penicillin, 100µg/mL streptomycin, 1% B27 supplement, 10ng/mL epidermal growth factor (EGF) (all from Life technologies). Single cells were then plated on uncoated Petri dishes (Corning Life Science, NY, USA) and the neurospheres were allowed to develop in an incubator with 5% CO_2 and 95% atmospheric air at 37°C. Five to eight days after plating, neurospheres were seeded onto poly-D-lysine (PDL; Sigma-Aldrich Co. LLC, St. Louise, MO, USA) coated 24-well plates for immunofluorescence assay or in PDL coated 12-well plates for western blot analysis, in SFM medium devoid of growth factors.

For GPER immunostaining and western blot, cells were exposed to E_2 or G1 (100nM, Calbiochem) for 24h. For proliferation assay, cells were incubated with the GPER antagonist G15 (100nM, Calbiochem) for 30 min before adding E_2 or G1 for further 48 hours. For cell differentiation studies the same protocol was used and the experience was finished 7 days after the beginning of the stimulation. A schematic representation of the *in vivo* experimental assays is shown (Fig. 3.1).

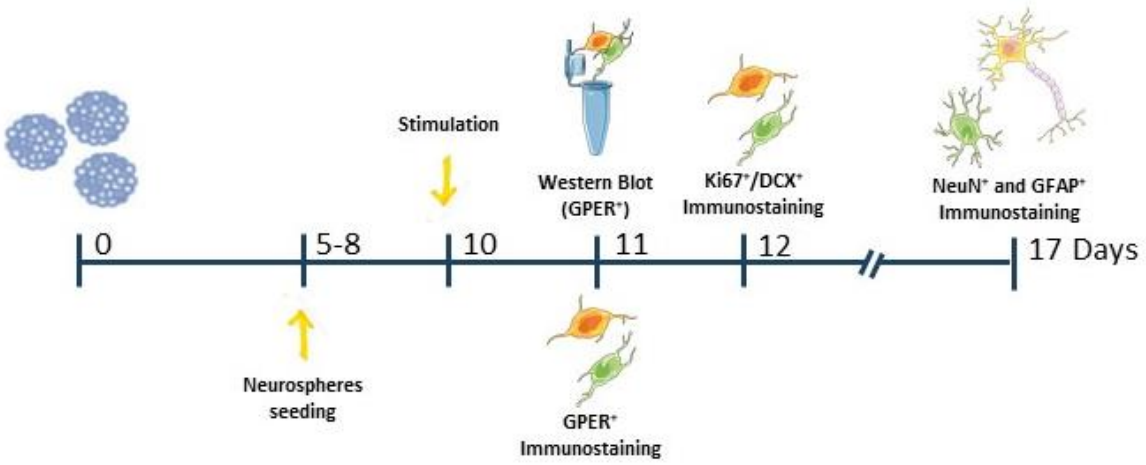

Figure 3.1- Schematic representation of the *in vitro* experimental assay. Abbreviations: GPER, G protein-coupled estrogen receptor; Ki67, cell proliferation marker; DCX, doublecortin; NeuN, neuronal nuclei; GFAP, glial fibrillary acid protein.

3.1.2 Immunocytochemistry

Cells were fixed with 10% formalin solution for 10 minutes and permeabilized in 1% Triton X-100 diluted in PBS for 5 minutes. After permeabilization, cells were incubated, for 2h at RT, in a blocking solution of PBS containing 0.1% Tween (PBS-T) and 20% fetal bovine serum (FBS). After washing with PBS-T, cells were incubated for 72 hours at 4ºC, with appropriate primary antibodies (Table 2.1) diluted in a solution of PBS-T and 1% FBS. After incubation with the primary antibodies, the cells were rinsed in PBS-T and incubated with the secondary antibodies (1:1000) in the PBS-T and 1% FBS, for 2 hours at RT, and after that were incubated with Hoechst 2µM (1:1000, Invitrogen) diluted in PBS solution containing 1% FBS, for 10 minutes at RT. Finally, the cells were rinsed in PBS-T and mounted in fluorescence mounting medium (DAKO; Glostrup, Denmark). Images were acquired using an inverted epifluorescence microscope (AxioObserver Z1, Zeiss), using a 63x lens.

Table 3.1 – Primary and secondary antibodies used in immunocytochemistry assays. (GPER, G protein-coupled estrogen receptor; DCX, doublecortin; GFAP, glial fibrillary acid protein; NeuN, neuronal nuclei).

PRIMARY ANTIBODY	DILUTION	COMPANY	SECUNDARY ANTIBODY	COMPANY
RABBIT ANTI-GPER	1:200	Santa Cruz Biotechnology	Goat anti-rabbit 546	Invitrogen
RABBIT ANTI-KI67	1:500	Abcam	Goat anti-rabbit 488	Invitrogen
GOAT ANTI-DCX	1:200	Santa Cruz Biotechnology	Donkey anti-goat 546	Life Technologies
RABBIT ANTI-NEUN	1:100	Merck Millipore	Goat anti-rabbit 546	Invitrogen
RABBIT ANTI-GFAP	1:2000	DAKO	Goat anti-rabbit 546	Invitrogen

3.1.3 Cell counting and immunofluorescence quantification

To analyse the immunolabelling, cells at the border of seeded neurospheres, where the cells formed a pseudo-monolayer, were counted. All experimental conditions were performed in triplicate in three independent cell cultures. Quantification of cells and of the fluorescence intensity was performed in twenty random fields *per* coverslip.

All analyses were performed using the software ImageJ (NIH Image, Bethesda, MD, USA).

3.1.4 Western blot

For preparation of protein extracts, cells were rinsed twice with PBS. Then, cells were lysed on ice with lysis buffer (25mM Tris, 2.5mM EDTA, 1% Triton X-100, 1mM DTT, 1mM phenylmethylsulfonyl fluoride, 25µg/mL leupeptin). The cell extracts were collected and the total amount of protein concentration was quantified using the Bradford protein assay. After the quantification, the protein samples were treated with a denaturation solution (62.5mM Tris-HCl, pH6.8, 2% SDS, 10% glycerol, 140mM ß-mercaptoethanol, 0.1% bromophenol blue) and boiled for 5 minutes at 100°C. Then, 20µg of total protein were loaded into each lane of

the stacking gel constituted by 4% acrylamide in 0.5M Tris-HCl, 10% sodium dodecyl sulfate (SDS), 0.05% ammonium persulfate (PSA) 10% and 0.1% tetramethylethylenediamine (TEMED). The proteins were separated by their molecular weight in the resolution gel (12% acrylamide in 1.5M Tris-HCl, 10% SDS, 0.05% PSA 10%, 0.05% TEMED) by SDS-PAGE electrophoresis at 160V until the front of the race reach the final of the gel, in a running buffer solution (25mM Tris, 190mM glycine pH8.3, 0.1% SDS) at RT. Subsequently, proteins were transferred to a polyvinylidene difluoride membrane (PVDF, GE Healthcare, Amersham, UK) through semi-dry transfer during 25 minutes at 1.0A, 25V, using transfer buffer (10mM CAPS, 10% methanol) at RT. After that, membranes were blocked with 5% non-fat milk (Páturage, France) in Tris buffer saline solution – Tween 20 (TBS-T; 20mM Tris, 137mM NaCl, 1M HCl 0.38%, 0.1% Tween 20) for 1h at RT. Membranes were then incubated overnight at 4°C with a rabbit anti-GPER antibody (1:800; 42kDa; Santa Cruz Biotechnology) and further incubated with the goat anti-rabbit antibody conjugated with horseradish peroxidase (1:5000 Santa Cruz Biotechnology) at RT for 1h. After incubation with the secondary antibody, the membranes were exposed to Luminata TM Crescendo Western HRP Substrate (Millipore) for 5 minutes. Protein bands were detected using the ChemiDoc™ MP Imaging System (Bio-Rad).

3.2 *In vivo* studies

3.2.1 Animals

All animal experimental procedures were performed in accordance with CICS-UBI regulations and national and European Community guidelines (86/609/EEC; 2010/63/EU). In this study were used 24 adult C57BL/6 mice (10-13 week-old) male. All animals were maintained in appropriate cages, under temperature controlled conditions (22±2°C) with a fixed 12h light/ dark cycle, with food and water freely available.

3.2.2 Experimental treatments

The animals were separated in two experimental protocols, one for cell proliferation analysis and another for cell differentiation evaluation. Each experimental protocol was constituted by two groups of six animals: the vehicle group and the G1 group.

Mice were subject to a subcutaneous injection (s.c.) of G1 (190µg/Kg, Calbiochem) (85) diluted in 0.9% NaCl containing 0.3% gelatin or vehicle (0.9% NaCl 0.3% gelatin) every 12 hours. To label dividing cells, bromodeoxyuridine (BrdU, Sigma) dissolved in a sterile solution of 0.9% NaCl was administrated by intraperitoneal injection (i.p.) twice a day (12h-12h) at two different concentrations depending the experimental protocol.

For cell proliferation analysis, 100mg BrdU /Kg were administrated for 2 days. The animals were sacrificed the day after the last BrdU injection and the brains were removed for further immunolabelling assays.

To evaluate the effect of G1 on cell differentiation in SVZ, BrdU was injected by i.p. injection at 50mg BrdU/Kg for 3 days and the animals were maintained for 21 days before being euthanized for immunohistochemistry analysis. Throughout the experiments, the animals did not show significant weight variations. A schematic representation of the *in vivo* experimental assays is shown below.

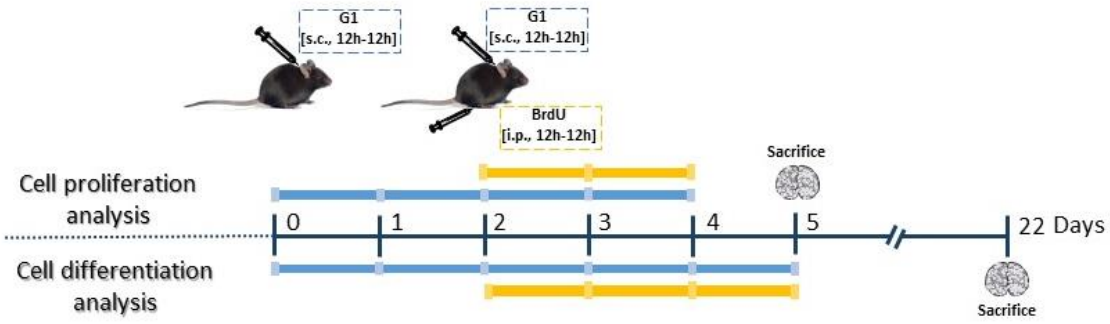

Figure 3.2- Schematic representation of the *in vivo* experimental assays. Abbreviations: BrdU, bromodeoxyuridine.

3.2.3 Immunohistochemistry

3.2.3.1 Preparation of the brain tissue

At the end of each *in vivo* experimental protocols and to unveil the neurogenic response evoked by activation of the GPER in the SVZ, mice were deeply anesthetized with a mixture of ketamine (90mg/Kg of mouse weight, Imalgene 1000, Merial, Lyon, France) and xylazine (10mg/Kg of mouse weight, Rompun 2%, Bayer, Leverkusen, Germany), and then perfused intracardially with a saline solution followed by 4% paraformaldehyde (PFA). The brains were removed and post-fixed in 4% PFA for 24h at 4°C, followed by immersion in a 30% sucrose solution (Fisher Scientific) in PBS at 4 °C, until the tissue got fully dehydrated and sunk. Brains were frozen in the vapour of liquid nitrogen and stored at -80°C until sectioning. Thereafter, the brains were sectioned in coronal sections of 40µm from the olfactory bulb towards midbrain using a cryostat (Leica CM30505, Leica Microsystems, Nussloch, Germany) at -20°C. The sections were collected sequentially in series of 6, spaced 240µm from each other, and were kept in a cryopreservation solution (30% glycerol, 30% ethylene glycol, 30% water and 10% phosphate buffer solution (0.2M)) at -20°C until used in the immunohistochemistry assays.

3.2.3.2 Immunostaining assay

For immunohistochemistry analysis, a protocol adapted from Wojtowicz & Kee (2006) (89) was used.

Firstly, tissue sections were washed three times, for 5 minutes each, with PBS to remove the cryopreservation solution. After that, to allow DNA denaturation, the slices were incubated with 2M HCl for 25 minutes at 37°C. Then, the tissue sections were rinsed with PBS and immersed in a blocking solution of PBS with 0.3% Triton X-100 and 2% FBS, for 2 hours at RT. After this period, slices were incubated with the following primary antibodies diluted in the blocking solution for 72 hours at 4ºC: mouse anti-BrdU (1:500, Santa Cruz Biotechnology), goat anti-DCX (1:1000, Santa Cruz Biotechnology) or rabbit anti-GFAP (1:2000, Invitrogen). Thereafter, sections were rinsed in PBS and then incubated with Hoechst (1:1000, Invitrogen) and the respective secondary antibodies: Alexa Fluor-488 donkey anti-mouse (1:1000; Life Technologies), Alexa Fluor-546 donkey anti-goat or anti-rabbit (1:1000, Life Technologies), diluted in a PBS solution with 0.3% Triton X-100, for 2 hours at RT. Completed the secondary body incubation time, the tissue sections were rinsed in PBS and mounted in fluorescence mounting medium (DAKO; Glostrup, Denmark) for further analysis. Photomicrographs were obtained with an AxioObserver LSM 710 confocal microscope (Carl Zeiss), using a 40x magnification.

3.2.4 Cell counting

To evaluate the cell proliferation induced by the GPER agonist G1 in the SVZ, $BrdU^+$ and $BrdU^+$/ DCX^+ or $BrdU^+$/ $GFAP^+$ cells of at least 4 animals were counted. The sections considered were constituted by 5 slices spaced by 240µm. Cells in the dorso-lateral region of the SVZ (figure 3.3) were counted from three different Z axis-positions *per* field (totalling approximately 90 fields *per* mouse).

The cell counts were performed using the software ImageJ (NIH Image, Bethesda, MD, USA).

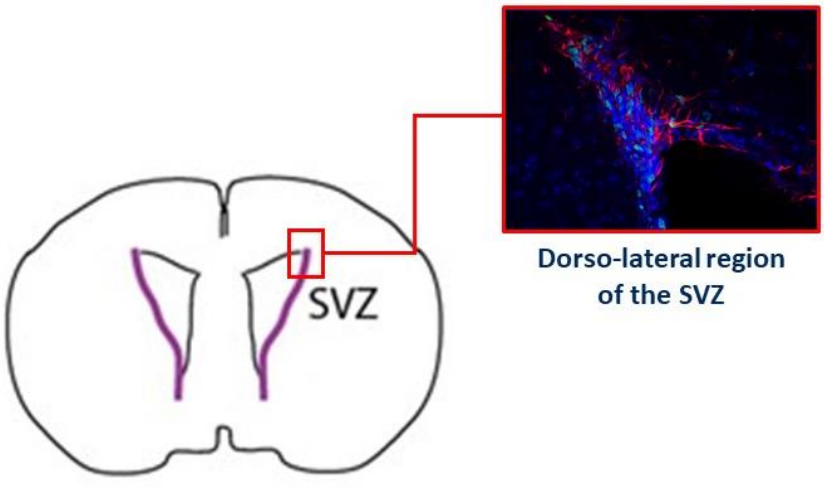

Figure 3.3- Schematic representation of the region where cells were counted in *in vivo* experiments.

3.3 Statistical analysis

Data are shown as the mean ± standard error of the mean (SEM), expressed as percentages of values obtained in control/vehicle conditions. Statistical analysis was performed using one-way ANOVA, followed by Bonferroni's Multiple Comparison Test or unpaired one-tailed Student's t test, with values of $P<0.05$ considered statistically significant. All statistical analysis was achieved using GraphPad Prism 5 Demo (GraphPad Software, San Diego, CA, USA).

Chapter 4 - Results

4.1 GPER is expressed by NSC cultures derived from the SVZ and by cells present in the border of the lateral ventricles

To better understand how the activation of the GPER modulates the neurogenic response in the SVZ, first was evaluated the expression of the receptor in this region. For that reason, immunostainings for GPER were performed in SVZ cells of mice brain and in NSC cultures from SVZ of rat. As shown in figure 4.1A and 4.1B, the GPER is expressed both in the cells of the lateral ventricles of mice brain and in rat NSC cultures from SVZ.

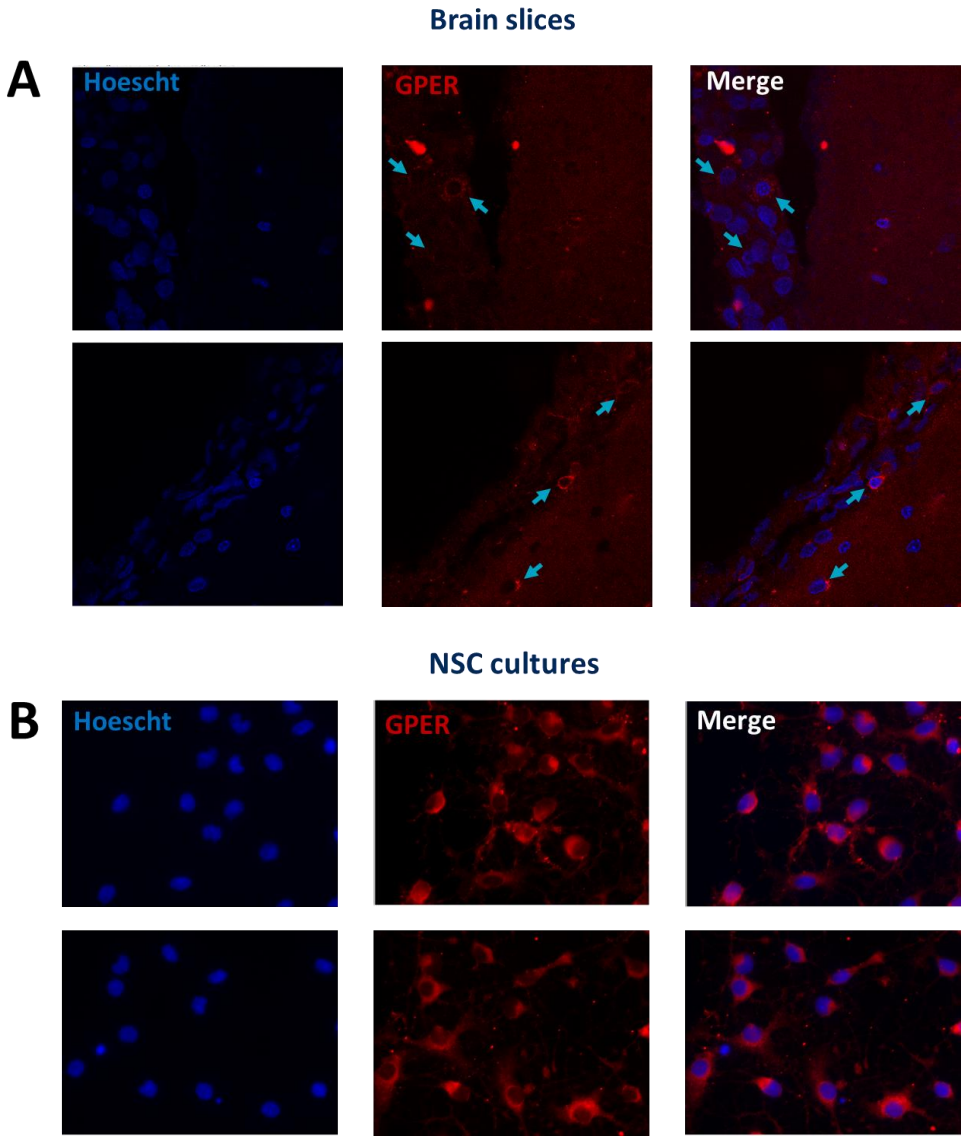

Figure 4.1- Expression of the GPER in *brain slices and* in NSC cultures. Representative images of immunostaining for GPER^{+} cells in SVZ cells of mice brain **(A)** and in NSC cultures from SVZ of rat **(B)**. Blue arrows highlight cells labeling for the GPER marker.

4.2 Exposure to G1 increases the expression of GPER by NSC

To investigate whether estrogen receptors modulate GPER expression, the intensity of GPER fluorescence in cells stimulated with the GPER agonists were analysed. GPER levels were significantly increased in NSC cultures treated with G1 (100nM) (figure 4.2A). On the other hand, when exposed to E_2 (100nM), a non-statistically significant increase was observed ($mean_{E2}$=107.0±1.5; $mean_{G1}$=116.7±3.6). In addition to changes in the levels of fluorescence intensity of the receptor, significant alterations were also observed in the cell morphology of cultures exposed to G1 (figure 4.2A).

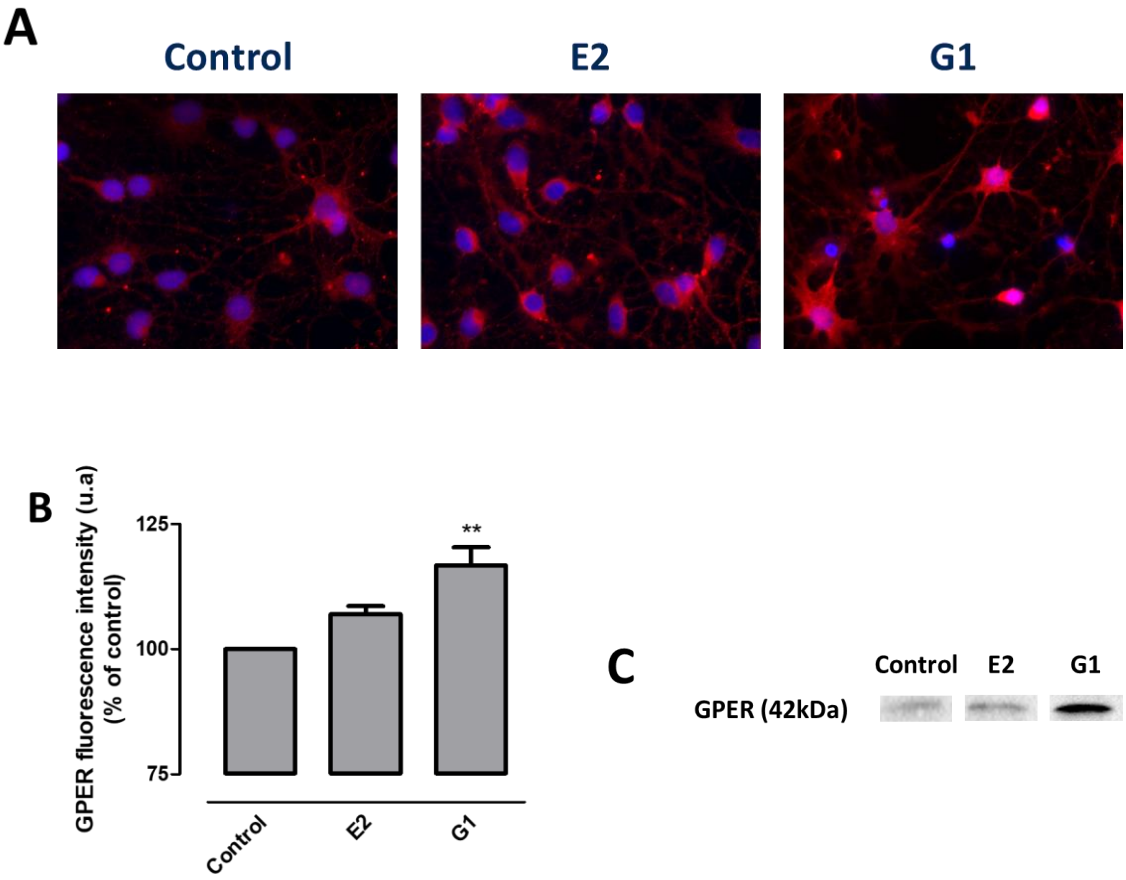

Figure 4.2- GPER expression levels in NSC cultures from SVZ. Representative images of immunostaining for GPER in NSC cultures from SVZ (A) Graph depicts the percentage of GPER fluorescence intensity of $GPER^+$ cells in NSC cultures from SVZ (B). Data are expressed as percentage of control ± SEM (n=3). **P<0.01 G1 vs control using one-way ANOVA, followed by the Bonferroni's multiple comparison test. Changes of GPER expression assessed by western blot (representative image) (C).

4.3 Estradiol, but not GPER agonists, promotes cell proliferation

Given that E_2 is involved in pathways that regulate cell proliferation and differentiation (51,65,90) we evaluate whether these effects are GPER-mediated. To analyse neural proliferation, Ki67, an endogenous protein expressed throughout the active phases of the cell cycle but absent in the quiescent cells (89), and widely used as a cell proliferation marker, was used.

The results showed that E_2 increased significantly the number of proliferative neuroblasts ($Ki67^+/DCX^+$; $mean_{E2}$=176.7±22.2; figure 4.3B) and exerts a similar proliferative effect on cells that are not directed to neuronal lineage, that is, proliferative cells ($Ki67^+$) not labelled for the marker of neuronal precursors and immature neurons, doublecortin (DCX). E_2 increases $Ki67^+/DCX^-$ cells by 105.4% ($mean_{E2}$=205.4±18.3; figure 4.3C).

Neither the proliferative neuroblast population ($mean_{G1}$=79.1±14.4; figure 4.3B) nor the non-neuronal precursors $Ki67^+/DCX^-$ ($mean_{G1}$=121.7±25.6; figure 4.3C) were affected by exposure to the GPER selective agonist, G1.

Surprisingly when E_2 was applied in the presence of the GPER antagonist, G15, the proliferative effect of E_2 was lost both in neuronal ($mean_{G15+E2}$=96.0±13.0; figure 4.3B) and non-neuronal ($mean_{G15+E2}$=112.0±9.0; figure 4.3C) cell population, suggesting that the effect of E_2 is dependent on GPER activation.

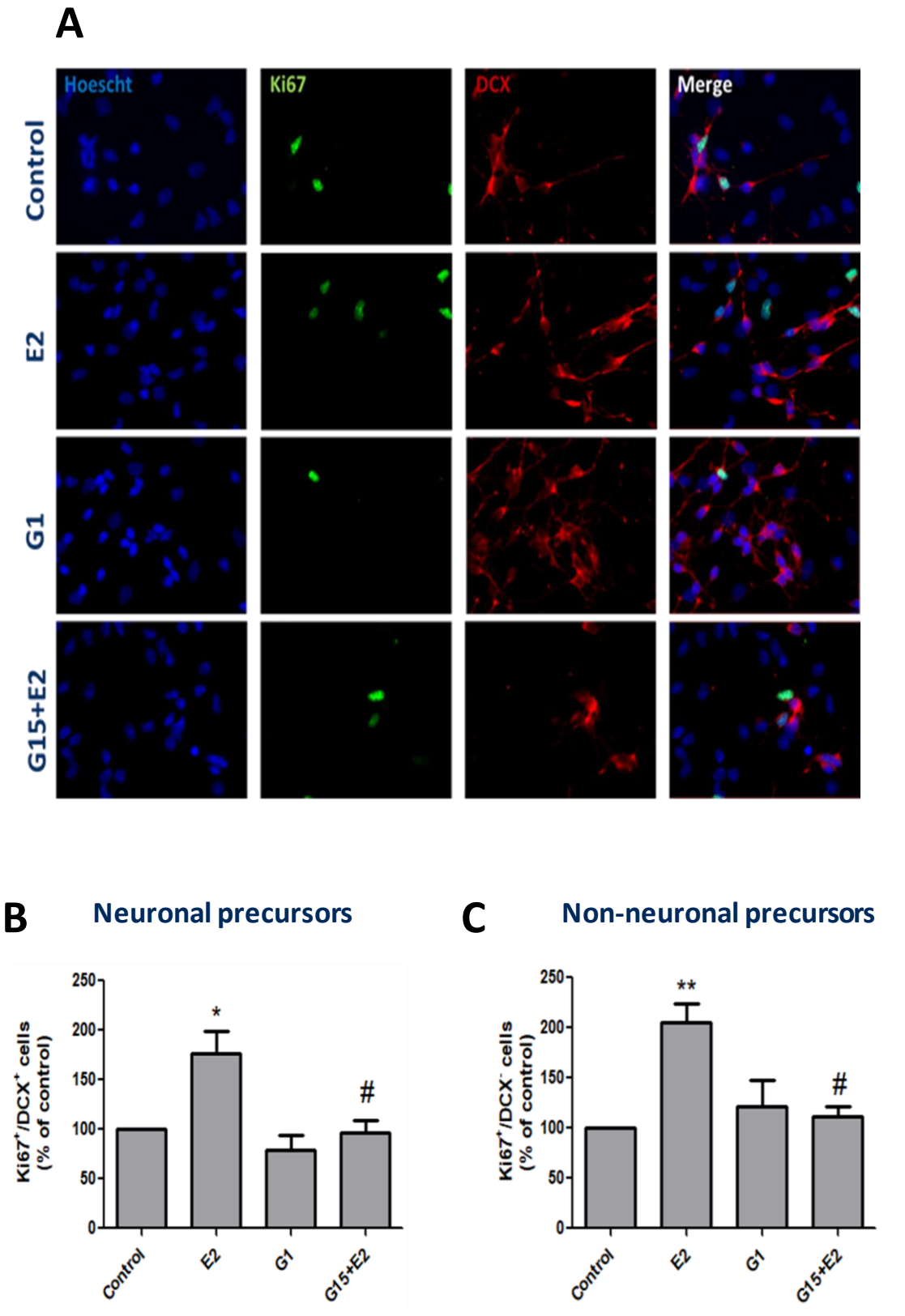

Figure 4.3- Cell proliferation in NSC cultures from SVZ. Representative images of immunostaining for Ki67/DCX in NSC cultures from SVZ (A). Graphs depict the percentage of $Ki67^{+}/DCX^{+}$ (B) and $Ki67^{+}/DCX^{-}$ (C) cells in NSC cultures from SVZ. Data are expressed as percentage of control ± SEM (n = 3). *P<0.05, **P<0.01, E_2 vs control; #P<0.05, E_2 vs G15+E_2 using one-way ANOVA, followed by the Bonferroni's multiple comparison test.

4.4 GPER selective activation increases neuronal and glial differentiation

In addition to evaluating how GPER affects cell proliferation in NSC cultures, we also analysed whether activation of this receptor interferes with the differentiation of these cells. E_2 induced a small, non-statistically significant, increase of $NeuN^+$ cells ($mean_{E2}$=132.2±23.1; figure 4.4B). On the other hand, treatment with G1 increased by 93% the number of $NeuN^+$ cells ($mean_{G1}$=193.2±20.8; figure 4.4B).

E_2 induced also a small, non-statistically significant, increase in the number of glial cells ($GFAP^+$ cells; $mean_{E2}$=133.0±14.5; figure 4.5B), whereas G1 promoted a robust increase of 114% in the number of $GFAP^+$ cells ($mean_{G1}$=213.9±30.2; figure 4.5B).

Treatment with GPER antagonist, G15, *per se* did not affected the number of either neurons ($mean_{G15+E2}$=96.6±7.2; figure 4.4B) or glial cells ($mean_{G15+E2}$=124.4±11.0; figure 4.5B).

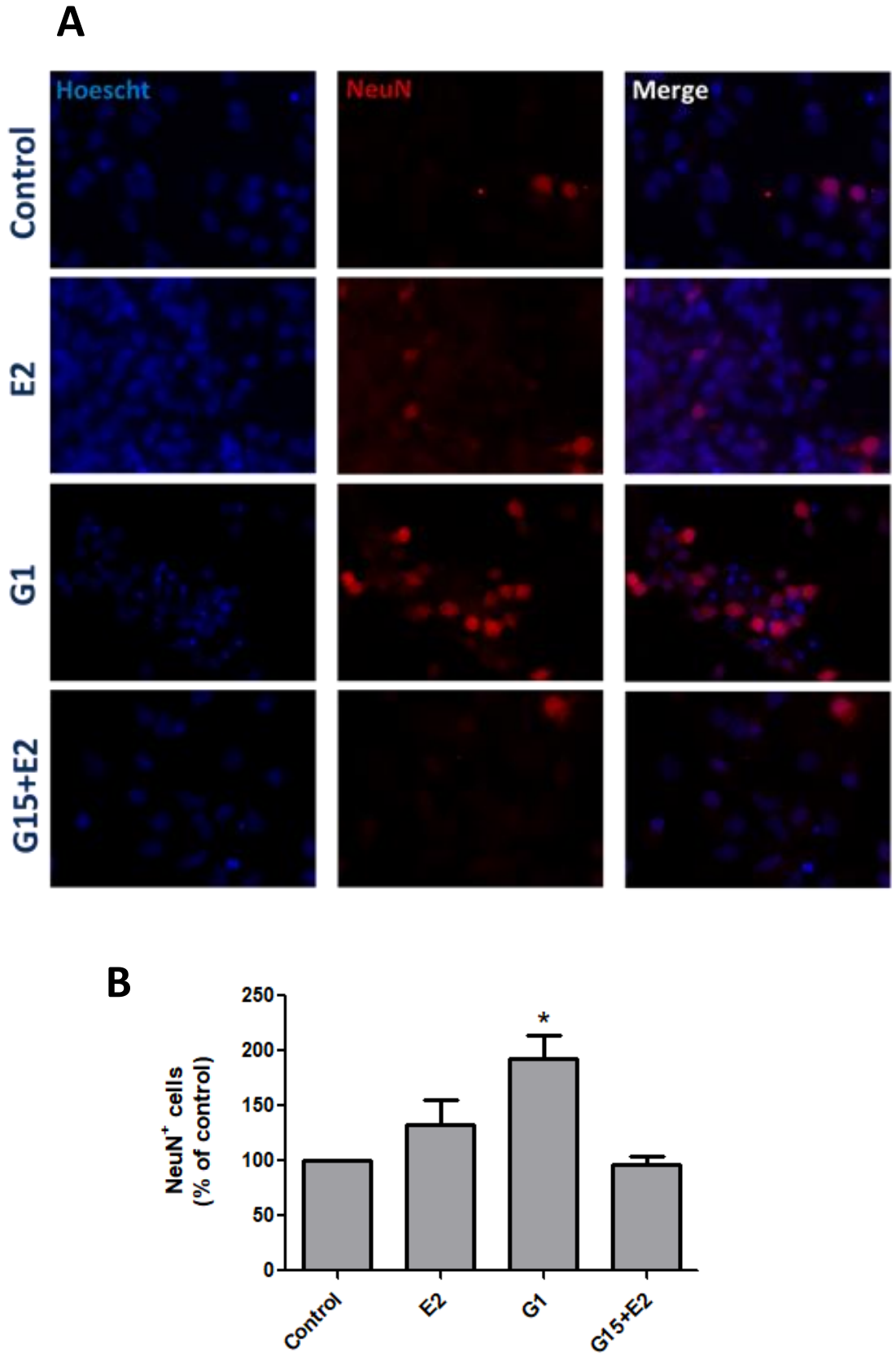

Figure 4.4- Neuronal differentiation of NSC cultures from SVZ. Representative images of immunostaining for NeuN in NSC cultures from SVZ (A). Graph depicts the percentage of NeuN$^+$ cells in NSC cultures from SVZ (B). Data are expressed as percentage of control ± SEM (n = 3). *P<0.05, **P<0.01 G1 vs control using one-way ANOVA, followed by the Bonferroni's multiple comparison test.

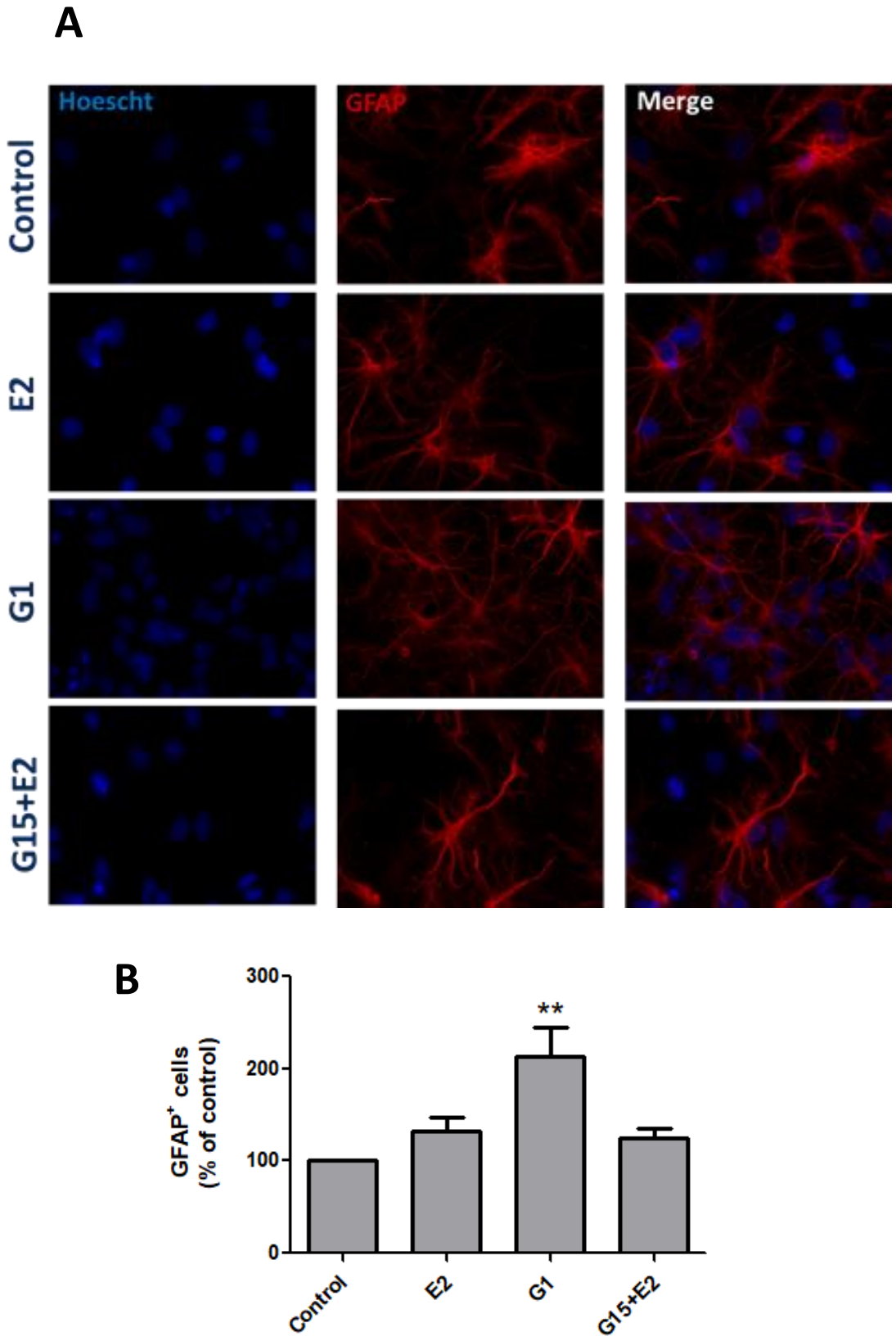

Figure 4.5- Glial differentiation of NSC cultures from SVZ. Representative images of immunostaining for GFAP in NSC cultures from SVZ (A). Graph depicts the percentage of GFAP$^+$ cells in NSC cultures from SVZ (B). Data are expressed as percentage of control ± SEM (n = 3). *P<0.05, **P<0.01 G1 vs control using one-way ANOVA, followed by the Bonferroni's multiple comparison test.

4.5 GPER activation is associated with cell proliferation in the SVZ of mice brain

We further investigate the effect of G1 on the proliferation of neuroblasts in the SVZ. For this purpose, a BrdU incorporation assay was implemented. This assay consists in the evaluation of the ability of dividing cells to incorporate BrdU instead of thymidine (89). Proliferative cells ($BrdU^+$ cells) marked as neuroblasts ($BrdU^+/DCX^+$ cells) were counted on the dorso-lateral region of the SVZ (figure 3.3 of the methods section).

As seen in figure 4.6B, treatment with G1 significantly increased the proliferative neuroblasts ($BrdU^+/DCX^+$ cells) in the SVZ, as compared with control animals ($mean_{vehicle}$=100.2±5.2, n=5; $mean_{G1}$=116.7±2.8, n=6).

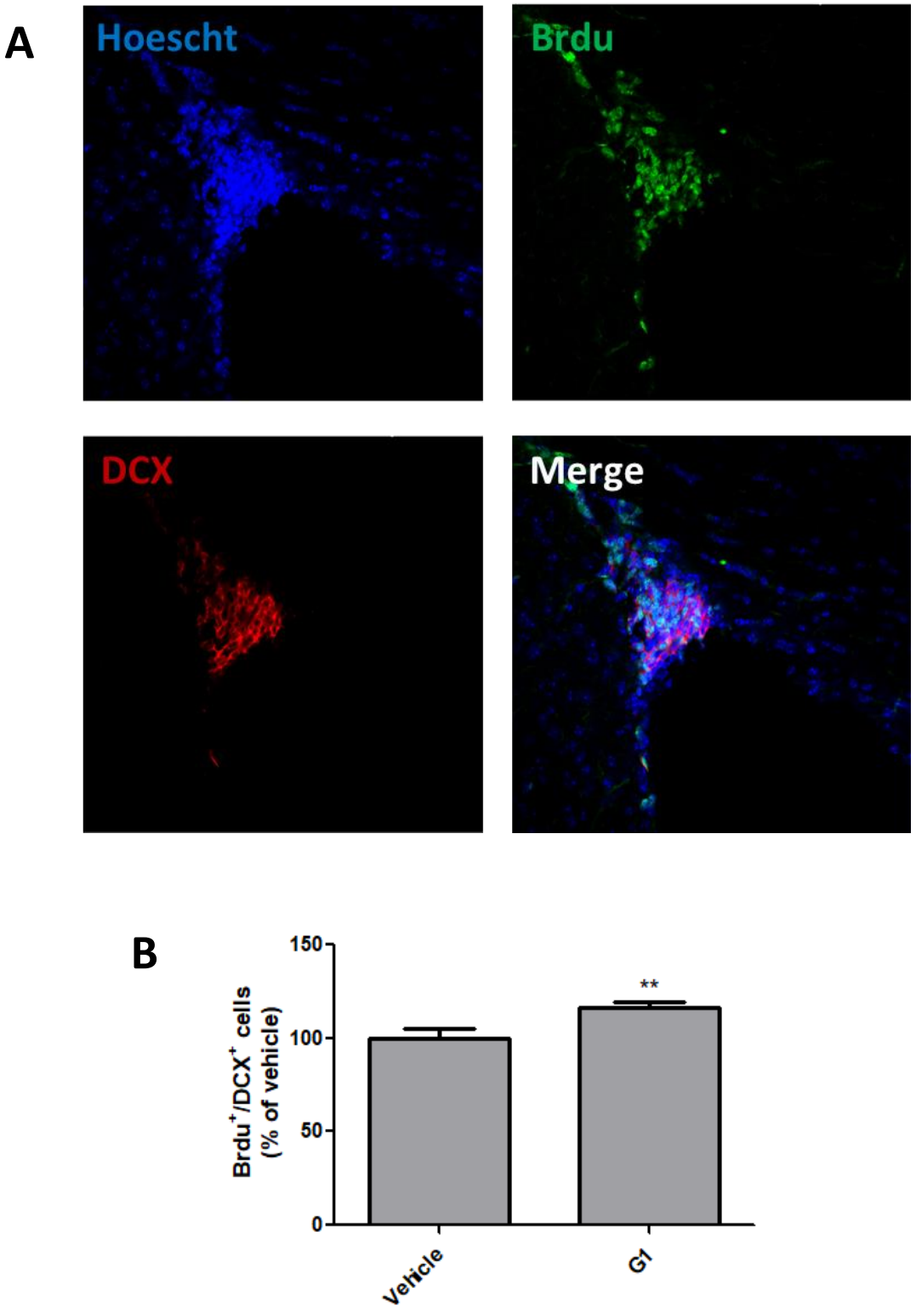

Figure 4.6- Neuronal proliferation of NSC in SVZ mice brain. Representative images of immunostaining for BrdU/DCX in NSC in SVZ mice brain (A). Graph depicts the percentage of $BrdU^+/DCX^+$ cells in NSC in SVZ mice brain (B). Data are expressed as percentage of control ± SEM (n =5-6). **P<0.01 G1 vs vehicle using one-tailed unpaired Student's t-test.

Activation of GPER was also effective in stimulating the proliferation of glial cells. G1 (100nM) treatment for 5 days increased the number of $BrdU^+/GFAP^+$ cells by 16.2% ($mean_{vehicle}$=100.0±2.9, n=4; $mean_{G1}$=116.2±6.2, n=5; figure 4.7B).

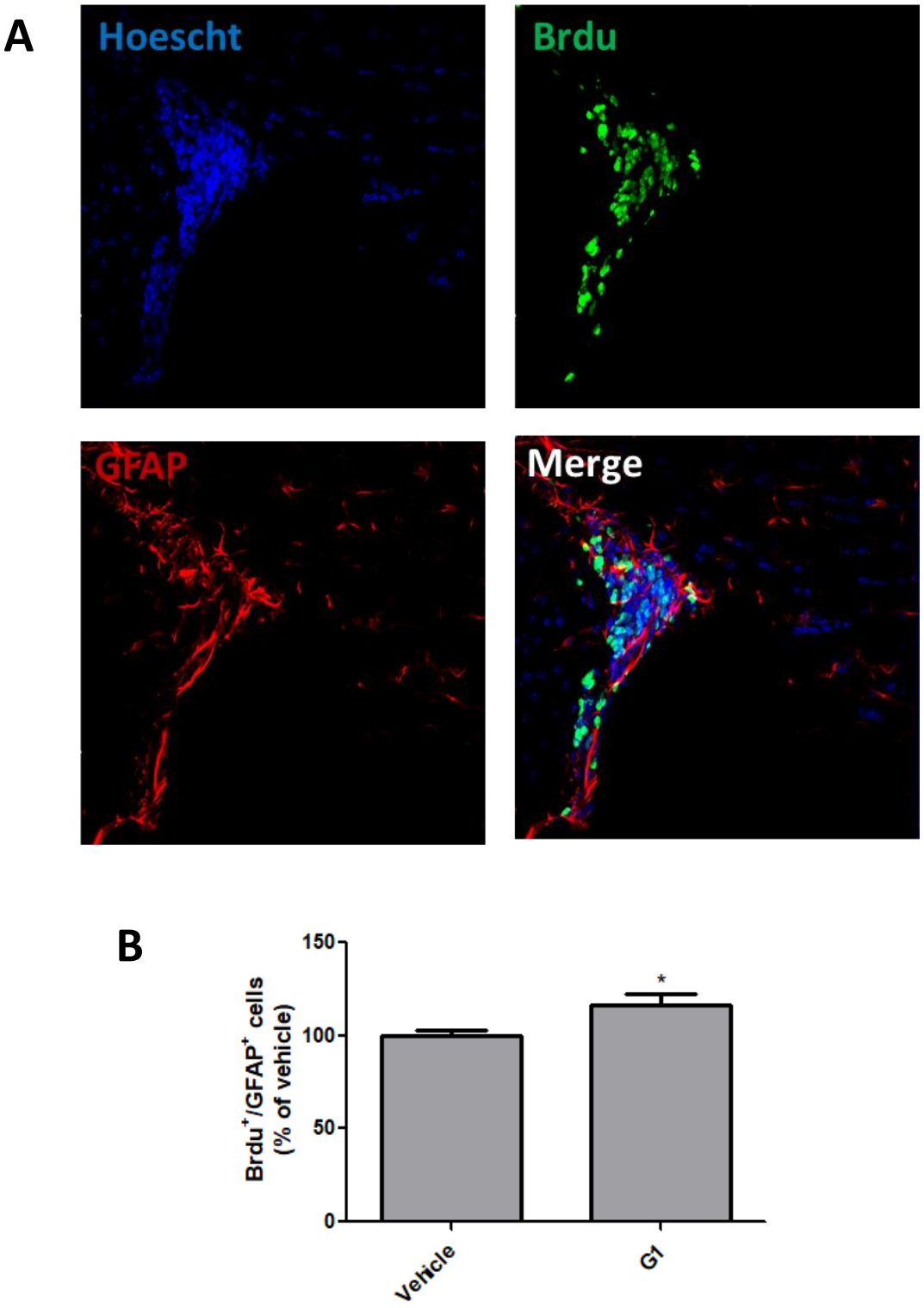

Figure 4.7- Glial proliferation of NSC in SVZ mice brain. Representative images of immunostaining for Brdu/GFAP in NSC in SVZ mice brain (A). Graph depicts the percentage of $Brdu^+/GFAP^+$ cells in NSC in SVZ mice brain (B). Data are expressed as percentage of control ± SEM (n = 4-5). *P<0.05 G1 vs vehicle using one-tailed unpaired Student's t-test.

4.6 Migration of proliferative cells from the SVZ of mice brain

Neurogenesis involves the proliferation, migration, survival and differentiation of newly generated cells into neurons (2). Taking this in account, we analysed the effects of GPER selective activation on long-term survival of newborn neuronal and glial cells in the SVZ niche, 17 days after treatment with G1 (see methodology in figure 3.2)

The results from the immunohistochemistry assays using double labeling for proliferative neurons ($BrdU^+/NeuN^+$; figure 4.8) and glia ($BrdU^+/GFAP^+$; figure 4.9), showed no BrdU labelling cells in the SVZ region. These results suggest that the recently generated cells had already initiated their migration path towards the OB. So, an immunohistochemistry using the same markers in the OB was performed. It was found BrdU labelling cells in the OB. However, the tissue of the animals of both conditions was not in the proper condition and, unfortunately, could not be used for cell quantifications.

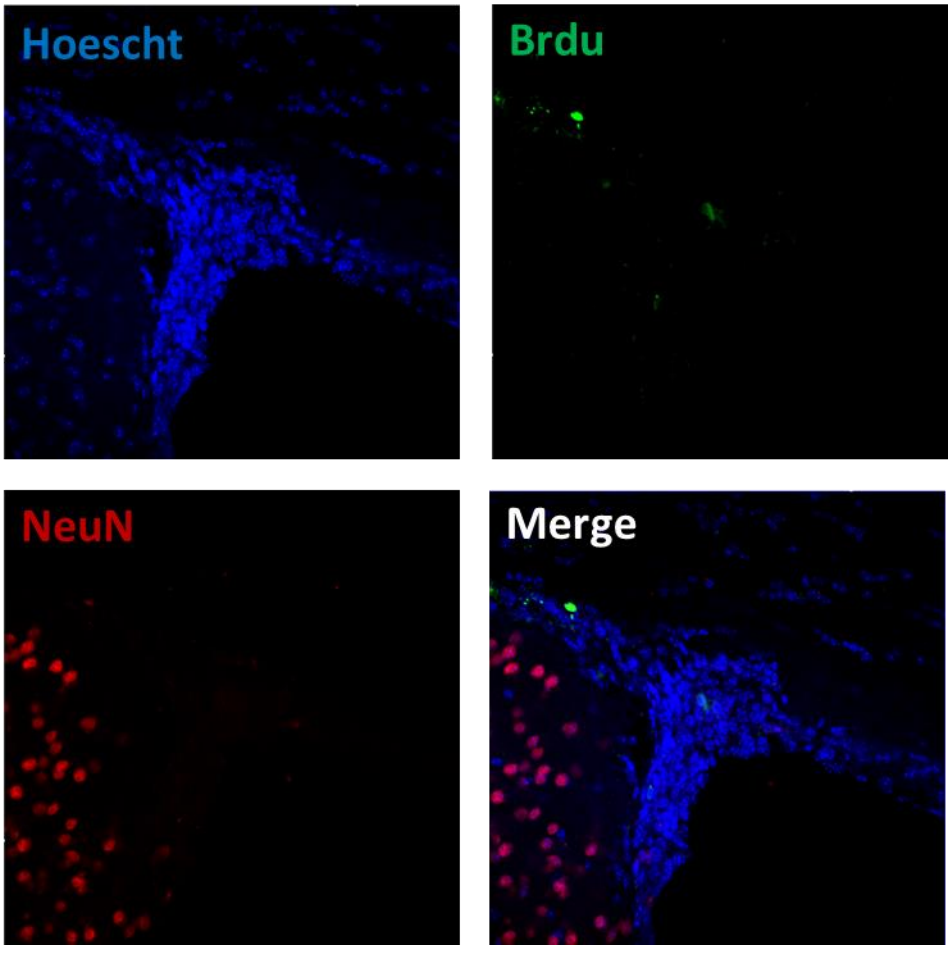

Figure 4.8- Migration of proliferative neuroblasts in SVZ mice brain. Representative images of immunostaining for Brdu/NeuN in NSC in SVZ mice brain.

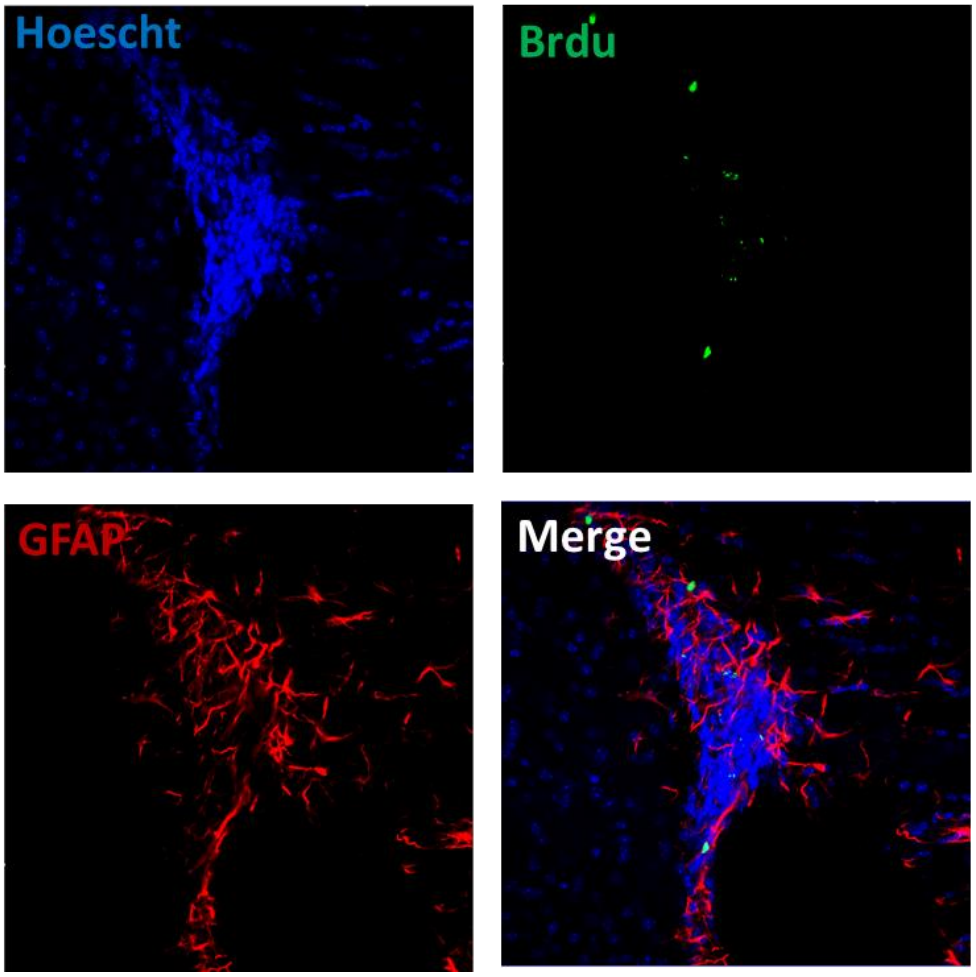

Figure 4.9- Migration of proliferative glia in SVZ mice brain. Representative images of immunostaining for Brdu/GFAP in NSC in SVZ mice brain.

Chapter 5 – Discussion

In our study, we demonstrated that the activation of the non-classic estrogen receptor, GPER, is able to modulate its levels of expression as well as proliferation and differentiation of newborn cells in the SVZ. Firstly, we analyse the GPER expression in NSC cultures from SVZ and *in vivo* by immunostaining and we showed that GPER is expressed either in NSC cultures or in brain slices from mice (figure 4.1). Additionally, we observed that the selective activation of the receptor increases the GPER expression while E_2 had no significant effect. These results contrast with data from a previous study, performed in the in the dentate gyrus of the hippocampus, that showed that GPER expression is downregulated by G1 and E_2 (87). However, the mentioned study analysed a different neurogenic niche and the GPER expression was assessed by double-label immunofluorescence for GPER and Ki67 while in in the present study GPER expression was assessed in all cells and was not restricted to the proliferative population.

In the rat dentate gyrus, steroid hormones can modulate the adult neurogenesis by differentially affecting cell proliferation and survival in both male and female. In female rats, different levels of cell proliferation are achieved in each phase of the estrous cycle, peaking during the proestrus, when estrogen levels are higher, and decreasing during estrus or in diestrus (54). Ovariectomy induces a decrease in cell proliferation which can be reversed by acute treatment with E_2 (91). On the other hand, in male rats, testosterone and dihydrotestosterone, but not E_2, enhance cell survival in the dentate gyrus (92). In addition to the GPER expression profile, we also evaluated whether GPER modulates SVZ neurogenesis. Our results agree with the findings of Farinetti *et al.* (2015) where they demonstrate that E_2 increase cell proliferation in the SVZ of adult male rats (51), as well as of data from Saravia *et al.* (2004) showing that E_2 restores cell proliferation in the SVZ of diabetic mice. Suzuki *et al.* (2007) suggested that the proliferative actions of E_2 are confined to neuronal precursors and do not influence gliosis (65). In our study, we observed a significant increase in the number of proliferating neuroblasts ($Ki67^+/DCX^+$) as well as neuroglia ($Ki67^+/DCX^-$) with E_2, contrary to the effect of the GPER selective agonist, G1. Okada *et al.* (2010) suggested that E_2 mediates its proliferative action through classic receptors since the stimulatory effect of E_2 on the proliferation of neural stem/progenitor cells is inhibited by treatment with ICI182,780 (90). Furthermore, knocking out both estrogen receptors α and ß has been shown to blocks the ability of E_2 to increase neurogenesis (65). However, others showed that estrogens rapidly increase cell proliferation possibly through a non-classic, non-genomic mechanism (93). It is known that GPER not only exerts direct effects upon ligand activation but its function involves the crosstalk with nuclear steroid receptors (75), which is in agreement with our results showing that treatment with the GPER antagonist, G15, resulted in a significant downregulation in the neural proliferation induced by E_2. Taken together, these results may

indicate that cell proliferation mediated by the nuclear receptor requires also the interaction with GPER. More studies are required to clarify how this regulation occurs.

Adult neurogenesis comprises several steps in which proliferative NSC differentiate into mature neural cell lines (94). Therefore, we also analysed the role of GPER in cell differentiation. The results obtained showed that E_2 tends to increase both neuronal and glial proliferation, assessed by the number of $NeuN^+$ and $GFAP^+$ cells respectively, while treatment with G1 significantly increase NSC differentiation into neurons and glial cells. When stimulated with non-classic estrogen receptor antagonist, the number of mature neurons and glia is similar to basal numbers. These data suggest that GPER is directly involved in signalling mechanisms that trigger the differentiation mediated by estrogens. These results are in accordance with a previous study showing that differentiation of oligodendroglia from NSC is stimulated via putative membrane-associated ER (93) and E_2 can promote mouse neural stem cell migration and differentiation into endothelial lineage in injured peripheral nerve (95). Furthermore E_2 can rapidly activate extracellular signal-regulated ERK 1/2 and interact with others neurotrophic factors such as BDNF, regulating its expression (90). Thus, activation of GPER may induce and enhance BDNF expression and consequent transcription, leading to differentiation of proliferative NSC into mature neurons and glial cells as well as increasing hypoxia-inducible factor 1α (HIF-1α) and VEGF expression (96).

It has been established that neuroblasts and newborn glia are able to migrate toward a site of injury such as cortex and striatum and then differentiate into the specific cell types lost after injury (7–13). Yamashita *et al.* (2006) demonstrated that part of the neuroblasts from SVZ differentiates into mature neurons and starts to express NeuN at the sites of injury, where they begin to be incorporated into the existing neuronal circuit by forming synapses with neighbouring cells (12). Indeed, Cheng *et al.* (2013) reported that E_2 treatment induced an increase of BrdU/DCX positive cells in the dentate gyrus of the hippocampus and the SVZ in ischemic female ovariectomized rats, , possibly through increase of HIF-1α and VEGF protein expression (96). Walters and co-authors demonstrated that intracerebral estradiol administration increases neurogenesis in the injured zebra fish brain (97). However, others showed that proliferation of progenitor cells in adult male rats was not influence by E_2 (68,92). Therefore, it is important to identify neurogenesis modulators and their mechanisms of action. In an attempt to obtain more information on this subject, we evaluate SVZ neurogenesis in adult mice at two different time points: i) 1 day after G1 treatment to assess the number of proliferative cells ($BrdU^+$ cells) as well as proliferative neuroblast population ($BrdU^+/DCX^+$ cells) and newborn glial cells ($BrdU^+/GFAP^+$), and ii) 3 weeks after G1 treatment to assess the number of newborn neurons ($Brdu^+/NeuN^+$ cells) and glia ($BrdU^+/GFAP^+$ cells). Our experience demonstrates that G1 *per se* increases in a significant way the number of proliferative neuroblasts. The same pattern was achieved with the newly-generated glia. These results are not in accordance with a study led by Duarte-Guterman *et al.* (2015) in

which treatment with GPER agonist decreased cell proliferation in adult ovariectomized rats, indicating an estradiol-independent role of GPER in hippocampal neurogenesis or alternatively an antagonistic effect of intracellular and membrane bound ER activation to maintain levels of neurogenesis (87). However, these results points to some ambiguities between studies evaluating adult neurogenesis such as differences in animal species and genre (68), neurogenic niche analysed, G1 doses administered and different timing of G1 and BrdU injections.

The existing data points to a role of E_2 in the turnover of OB newborn neurons and its survival in the accessory OB of adult female rats (98). Progesterone and E2, through activation of ERα induce also an increase of differentiated motor neurons derived from mouse embryonic stem cells (99). However, other studies point to decrease in the rate of newly generated cells in the OB of adult female mice (100) and rats (53). In humans, E_2 could promote differentiation and survival of dopaminergic neurons derived from human stem cells (101). To uncover the effects of GPER selective activation on the long-term survival of newborn neuronal and glial cells in the SVZ niche we assess the number of proliferative cells ($BrdU^+$ cells) as well as the number of cells double-labelled for $BrdU^+/NeuN^+$ and $BrdU^+/GFAP^+$. The data showed that BrdU labelled cells were absent from the SVZ region, which suggests that the proliferative cells had already followed their path of migration towards the olfactory bulb. In order to demonstrate this hypothesis, an immunohistochemistry using the same markers was performed in the olfactory bulb. Unfortunately, the tissue of the animals was not in proper conditions and could not be used for analysis of the long-term survival of newborn neuronal and glial cells. Hence, to obtain more conclusive results, we should further increase the number of animals used per group and evaluate the functional integration of new neurons and glia in the mice bulb olfactory.

Chapter 6 – Conclusions

Neurogenesis is a biological process responsible for the generation of neural stem cells throughout the lifespan of mammalian brain. Analysis of previous studies demonstrated that proliferation and survival of newborn cells can be modulated by steroid hormones such as estradiol. GPER was proposed to play a role in estrogen-mediated neuroprotective effect. However, it is not known whether this protective effect involves a pro-neurogenic action of the non-classic estrogen receptor. Hence, in this present study we pretended to evaluate if GPER activation triggers a neurogenic response in the subventricular zone. After confirming the presence of GPER both in NSC cultures from SVZ and in the cells of the neurogenic niche, we showed that stimulation with GPER agonist, G1, increases the expression levels of the receptor as well as the differentiation of NSC into mature neurons and glial cells. On contrary, GPER activation could not increase neuroblast as well as glial proliferation *in vitro*, diverge from estradiol that significantly increase cell proliferation. Interestingly, although this result suggests that GPER is not involved in the proliferation increase mediated by estradiol, the use of the GPER antagonist demonstrates that this receptor is somehow involved in this proliferative pathway. Additionally, G1 *per se* was able to increase neural proliferation in the mice SVZ *in vivo*. Taken together, our results showed that GPER activation effectively promote neurogenesis in the SVZ and has potential to be used as a regenerative strategy in neurodegenerative disorders.

Further experiments will be required to better understand the potential of GPER activation as a regenerative agent. Specifically, studies to identify the specific neurogenic pathways triggered by GPER activation as well as analysis of the functional integration of new neurons and glia in the olfactory bulb. Additional studies focused in the neurogenic response under pathological conditions would be interesting to perform.

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