Universidade do Minho
Escola de Ciências da Saúde

Ana Luísa Mendanha Falcão

Novel perspectives on the subependymal zone complexity and modulation

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UMinho | 2012

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Tese de Doutoramento em Ciências da Saúde

Trabalho efetuado sob a orientação do
Prof. Doutor João Carlos Sousa

Trabalho efetuado sob a co-orientação da
Professora Doutora Joana Almeida Palha

Outubro de 2012

DECLARAÇÃO RELATIVA ÀS CONDIÇÕES DE REPRODUÇÃO

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Número do Bilhete de Identidade: 12545777

Título dissertação:

Novel perspectives on the subependymal zone complexity and modulation.

Orientadores:

Doutor João Carlos Sousa e Doutora Joana Almeida Palha

Ano de conclusão: 2012

Designação Ramo de Conhecimento do Doutoramento:

Ciências da Saúde – Ciências da Saúde

Universidade do Minho, 10 de Setembro de 2012

Assinatura: _____

...para que nós voemos na atmosfera serão precisas as forças concertadas do sol, do âmbar, dos ímanes e das vontades, mas as vontades são, de tudo, o mais importante, sem elas não nos deixaria subir a terra...

José Saramago in *Memorial do Convento*

Aos meus Pais e irmão

Acknowledgments

I would like to name all the wills that walked along and pushed, in one way or another, this PhD forward.

To begin, because they are the first reason that drove me to science, to my Karolinska Ola's lab mates. In particular to crazy **Therese**, doce **Shirin**, **Ana Teixeira** and sporty **Anna Cascante**. You taught me how fun and cheerful a lab can be! To **Gonçalo Castelo-Branco**, my first direct supervisor and inspiration, for all the knowledge and motivation you have granted me, and above all, for believing and encouraging me to proceed in research.

Luísa and **Lina,** my dearest friends. Together we shared so many times our sorrows and our glories; you keep reminding me that distance is no barrier for true friendship. Thank you for the papers you have downloaded for me and Lu your password is magic and helped a lot!!

Lília and **Liliana**. I appreciate your long and sincere friendship; our relaxing weekends still remind me how simple happiness is. Paradoxically, your names and addresses together, are a complex paradigm to my brain. Lília, you are the definition of enthusiasm in person, you have taught me that frogs and trees are actually quite interesting☺.

Sofia, Diogo and **Isa & Tânia.** For the long lasting conversations about everything and nothing. You are what my neuroscientist colleagues call "environmental enrichment to my life cage". Sofi and Diogo good luck for your PhD studies! I am sure you will make the CEHUM proud!

Joaninha Fraga and **Irene.** You have given much of you to me… that was why it was so difficult to watch you leave… I won't forget our "maluqueiras", friday night dinners and mostly the help and support. Friends like you, it's to keep forever.

Jovica, Magdalena, Julia, Elsa and all the **people in Goetz's lab** for the kind reception and all the help and input you gave me in my four months journey in Munich. **Osborn & lab members, Isabel** and **Leila** for being so friendly, for the barbecues and walk tours that cheered my days.

Joana Barbosa, as if not enough your friendship you have offered me your home! It was so good to have an old childhood friend waiting for me in Munich…you made me feel home! Who knew our scientific goals would cross?! I miss our unidirectional gossiping…it kept me updated and made me laugh more than the situation required! I also miss hearing you say that my cooking was delicious ☺ it's always good to know that I gave something in return!!

To Fundação para Ciência e Tecnologia for the financial support through a PhD grant (SFRH / BD / 44485 / 2008).

All the members of the **ECS**. Prof **Cecilia Leão,** the smile and gentleness shown to everyone makes us feel at home.

Nerds and **technicians** from **ICVS**, with special regards to:

Goretti, for all the support and nice conversations. **Luis and Miguel** for always being available to help even when you couldn't! **Susana** for always taking care (of what I forgot) and for being always ready to help despite the moody expression☺. **Magda, Carlos, Celine**. **Manuela,** for the endless patience you've shown me when I knocked on your door n times…

Pedro Leão Lion, **Mário** sempre alerta, **Ana** Joni, **Sara & Andreia, Hugo** SEAT fellow, **Filipa** ahahah, **Eduardo, Ana Raquel, Adriana** Psi, **Anabela, Armando, Patrícia M.** and **Tó.** The first researchers at ICVS with whom I've chattered nonsense and science too.

Morgates (aka Morgado), **Vitinho** (aka Vitor Hugo) buddies of science&fun, great times in DC&PD&NY (vitinho, you still own me some pictures!!). **Olibiéra** (aka J.Oliveira) for the advices, **Castra** (aka Andreia) for the companionship and for sharing some cookies.

Susanas M. and C., Silvi, Nuno, Miguel and **Fabinho.** For the lab cheerfulness and for being such great persons! **Diana, Luís J., Patrícia** and **Antonio P.** my neurogenic mates, **Dinis,** for solving my computer issues and for making my friend happy! To you all: I wish all the best for your PhD studies!

Ana Rita (your crazy theories made me laugh), **Paula** my course&PhD partner, for treating me bad and wishing me well! **A. Melo** for some good advices!

Nadine, for being so kind and for reviewing my English, **Margarida**, **Roqueira,** my immune partners, **ZéMiguel** (for teaching science + some curiosities of the time of your teens) and **J. Cerqueira,** for receiving me so well in your office, for the enthusiastic input and ideas and for being always in a good mood.

Luísa P. for the support, scientific input and coffees accompanied with nice neurogenic and non-neurogenic discussions.

Bessa, Monica. For the fruitful neurogenic discussions, for your friendship and for always crossing fingers for me and letting me know I can count on you!

Sandro my partner in crime (i.e. volley&sardine&camping), for the support and friendship. **Ashley**, much more than a colleague, a friend. For the many times you have gladly helped me, for borrowing me your spoon for my yogurt, and for the coffees and great talks about science-related and non-related stuff. **Catarina** or Châta, thanks for calling me "mini-chefinha" (I think is cute), and for sometimes listening to what I tell you ☺. Your help was precious for this thesis, but most important than that, I won a friend for life. (P.S: I will be waiting for your visit...)

Nuno Sousa and Joana Palha. You are inspiring as scientists and as persons. Thanks for showing us that there are no limits for knowledge. Nuno, for the brainstorming and Joana, for the guidance, for always being ready to discuss and for being so freely accessible. You have taught me that everything is possible as long as we fight for it. Thank you!

Fernanda Marques

João Sousa

Whatever I write it won't be enough.

Fernanda, thank you for the endless times you have helped me, even when I didn't ask you were always there to help! Your honesty, altruism and sense of justice make you a unique person and a special friend. Also, for the daily scientific and personal support (even when I was in Munich), it meant a lot to me every time you said: "vai correr tudo bem"! (friend4ever).

João, this thesis was only possible because you believed in it and always have backed me up. I

am thankful to have supervisors that were encouraging me all the time. With you I have grown not only as a scientist but also as a person. More than a mentor for science, you were a source of general knowledge. A supervisor that cares about his students and provides not only scientific guidance but also good music and good books is a friend. THANK YOU my friend!

E porque sem eles, até a mais lógica das ciências não faria sentido para mim...

Para a minha família,

Se os mencionasse todos teria de escrever mais duas ou três páginas, por isso vou abreviar: para os meus **primos todos** e afins **(&Cia)**, nomeadamente ao **Nuninho (&Cia)**, por aquele abraço, à **Belinha (&Cia)**, pelas imensas sextas-feiras que passamos juntos e que em muito contribuem para a minha felicidade e à **Sara F. (&Cia)** pelos jantares, conversas e apoio incondicional!

Para um grande pedaço de mim: a minha **mãe**, **pai** e **irmão**. Por existirem e darem cor, norte e felicidade ao meu mundo!

Por isto e por tudo... se eu não for uma pessoa de sorte, então o mundo gira ao contrário!

Abstract

In the mammalian brain adult neurogenesis occurs in two restricted sites: the subependymal zone (SEZ) and the subgranular zone of the hippocampal dentate gyrus. The SEZ comprises neural stem and progenitor cells that lie adjacent to the ependyma layer of the lateral ventricles. SEZ born neuroblasts migrate anteriorly in the rostral migratory stream towards the olfactory bulbs where they differentiate and integrate into neuronal circuitries. Because cells in the SEZ niche sense alterations in brain homeostasis and are able to alter their proliferative, migratory and differentiation profiles in response to injury, it is of particular interest to completely understand this dynamics both in physiological and pathological conditions. In this context, the present thesis addresses three main aspects in regard to SEZ niche complexity and modulation.

First, in regard to SEZ heterogeneity, we performed a topographic analysis of the rat SEZ niche along the anterior-posterior and dorsal-ventral axes. We found that the SEZ cell proliferation decreases along the anterior-posterior axis and varies considerably according to the position in the dorsal-ventral axis. Furthermore, these differences were associated with relevant gradients in the neuroblasts population and in the neural stem cell (NSC) population throughout the dorsal-ventral axis.

Next, we performed the same analysis on the proliferative and progenitor population profile in the mouse adult neurogenic niche, and found relevant species-specific differences between rat and mouse models, two closely related species. The proliferation gradients and the neuroblasts distribution observed in rat were absent in mice.

Finally, in regard to SEZ modulation/modulators in disease, we report the impact of a peripheral inflammatory stimulus, triggered by lipopolysaccharide (LPS), on the SEZ, choroid plexus (CP) and cerebrospinal fluid (CSF). The CP is the structure of the brain that produces and secretes most of the CSF that is in direct contact with the NSCs of the SEZ. In response to an acute peripheral inflammatory stimulus CP gene expression of modulators of the SEZ is altered and this is partially reflected on the CSF composition. The same inflammatory stimulus triggered a rapid and transient increase on SEZ cell proliferation. The peak of CP response to the inflammatory trigger was at 6h and 12h upon LPS administration and the induction of SEZ cell proliferation occurred specifically after 12h of LPS stimulus.

In summary, the data presented here reveals relevant topographical specificities of the rat and mouse SEZ and highlights species-specific differences. Moreover, it gives further insights on the SEZ response to acute peripheral inflammatory stimulus and pinpoints relevant CP synthetized molecules that when secreted towards the CSF can modulate the SEZ dynamics.

Resumo

No cérebro adulto dos mamíferos existem dois locais onde ocorre neurogénese: a zona subependimal (SEZ) e a zona subgranular que constitui a circunvolução denteada do hipocampo. A SEZ compreende células estaminais neurais e células progenitoras que se encontram adjacentes à camada do epêndima dos ventrículos laterais. Os progenitores de neurónios (também designados por neuroblastos) provenientes da SEZ migram anteriormente na via rostral de migração para os bolbos olfativos onde se diferenciam e integram nos circuitos neuronais.

Como as células da SEZ percecionam alterações na homeostasia do cérebro e são capazes de alterar os seus perfis de proliferação, migração e diferenciação, em resposta a uma lesão, torna-se particularmente interessante entender completamente esta dinâmica, tanto em condições fisiológicas como patológicas. Neste contexto, esta tese aborda três aspetos principais em relação à complexidade e modulação da SEZ.

Em primeiro lugar, no que diz respeito à heterogeneidade da SEZ, foi realizada uma análise topográfica deste nicho neurogénico no rato ao longo dos eixos anterior-posterior e dorsal-ventral. Os resultados obtidos demonstraram que a proliferação das células da SEZ diminui ao longo do eixo anterior-posterior e varia consideravelmente de acordo com a posição no eixo dorsal-ventral. Para além disso, estas diferenças estão associadas a uma distribuição diferencial na população dos neuroblastos e na população das células estaminais neurais (NSCs) ao longo do eixo dorsal-ventral.

Em seguida, foi realizada a mesma análise na SEZ do murganho adulto. A análise destes resultados revelou diferenças relevantes entre estas duas espécies filogeneticamente muito próximas. A proliferação e a distribuição diferencial das NSCs e dos neuroblastos observada em ratos não ocorrem em murganhos.

Finalmente, no que diz respeito à modulação da SEZ em resposta a insultos ao cérebro, foi estudado o impacto de um estímulo inflamatório periférico, desencadeada por um lipopolissacarídeo (LPS), componente da parede das bactérias gram negativas, sobre a SEZ, o plexus coroideus (CP) e o líquido cefalorraquidiano (CSF). O CP é a estrutura do cérebro que produz e segrega a maioria do CSF que por sua vez está em contacto direto com as NSCs da SEZ. Em resposta a um estímulo periférico inflamatório, a expressão genética no CP de moduladores da SEZ é alterada, sendo esta alteração parcialmente refletida na composição

proteica do CSF. O mesmo estímulo inflamatório desencadeou um aumento rápido e transitório na proliferação celular na SEZ. O pico da resposta do CP ao estímulo inflamatório desencadeado pelo LPS foi às 6h e 12h após a administração de LPS e a indução da proliferação celular na SEZ ocorreu especificamente após 12h de estímulo com LPS.

Em resumo, os resultados apresentados nesta tese revelam especificidades topográficas da SEZ no rato e no murganho, destacando ainda diferenças específicas entre estas duas espécies. Além disso, revela também a resposta da SEZ a um estímulo periférico inflamatório agudo e realça algumas moléculas sintetizadas no CP que quando segregadas para o CSF podem modular as células da SEZ.

Contents

List of abbreviations

AD	-	Alzheimer disease
BBB	-	Blood-brain barrier
BCSFB	-	Blood-cerebrospinal fluid barrier
CNS	-	Central nervous system
CP	-	Choroid plexus
CPEC	-	Choroid plexus epithelial cells
CSF	-	Cerebrospinal fluid
DCX	-	Doublecortin
EGF	-	Epidermal growth factor
EGFR	-	Epidermal growth factor receptor
FGF2/bFGF	-	Fibroblast growth factor 2/basic fibroblast growth factor
GABA	-	γ-aminobutyric acid
GFAP	-	Glial fibrillary acidic protein
IL	-	Interleukin
LPS	-	Lipopolysaccharide
MS	-	Multiple sclerosis
NSC/NSCs	-	Neural stem cell(s)
OB/OBs	-	Olfactory Bulb(s)
Prom 1	-	Prominin 1
PSA-NCAM	-	Polysialylated form of the neural cell adhesion molecule
RMS	-	Rostral migratory stream
SEZ	-	Subependymal zone
TAP	-	Transit amplifying progenitor
TJ	-	Tight junction

1. Introduction

1.1 Neurogenesis

Neurogenesis is the process of formation of neurons from neural stem and progenitor cells. It is mostly active during embryonic development, when the vast majority of neurons are formed from multipotent neural stem cells (NSC) (Gotz & Huttner 2005). However, neurogenesis also occurs during adult life in restricted areas of the brain. We will focus particularly in adult neurogenesis.

1.2 Adult neurogenesis

Adult neurogenesis is the formation of new functional neurons in the adult brain. The establishment of a novel mature, functional and totally integrated neuron in the adult central nervous system (CNS) requires four steps: i) the proliferation of the "mother cell", i.e., the NSC, that in turn gives rise to a pre-committed neuronal precursor/progenitor cell, ii) the migration of the progenitor cell towards the site where it will be integrated, iii) the differentiation of the precursor cell into a neuron and iv) the final integration into neural circuitries (Ming & Song 2005). The outcome of these processes is the formation of a functional neuron in the adult brain. When these same steps occur but instead of the formation of a neuronal precursor a glial precursor is generated, originating an astrocyte or oligodendrocyte, the process is designated adult gliogenesis (Aguirre & Gallo 2004). In the adult brain two very well described places for the birth of new neurons exist: the subependymal zone (SEZ), also known as adult subventricular zone (SVZ), and the subgranular zone of the dentate gyrus of the hippocampus (Figure 1). The first feeds the olfactory bulbs (OBs) whereas the second supplies new neurons for the hippocampal granular zone (Ming & Song 2011).

In addition to these two neurogenic areas, adult neurogenesis has also been described in the hypothalamus (Yuan & Arias-Carrion 2011), the amygdala (Fowler et al 2008) and the subcallosum zone (Seri et al 2006). Nevertheless, it remains debatable if in these brain areas new functional adult neurons are formed from NSCs (Gould 2007).

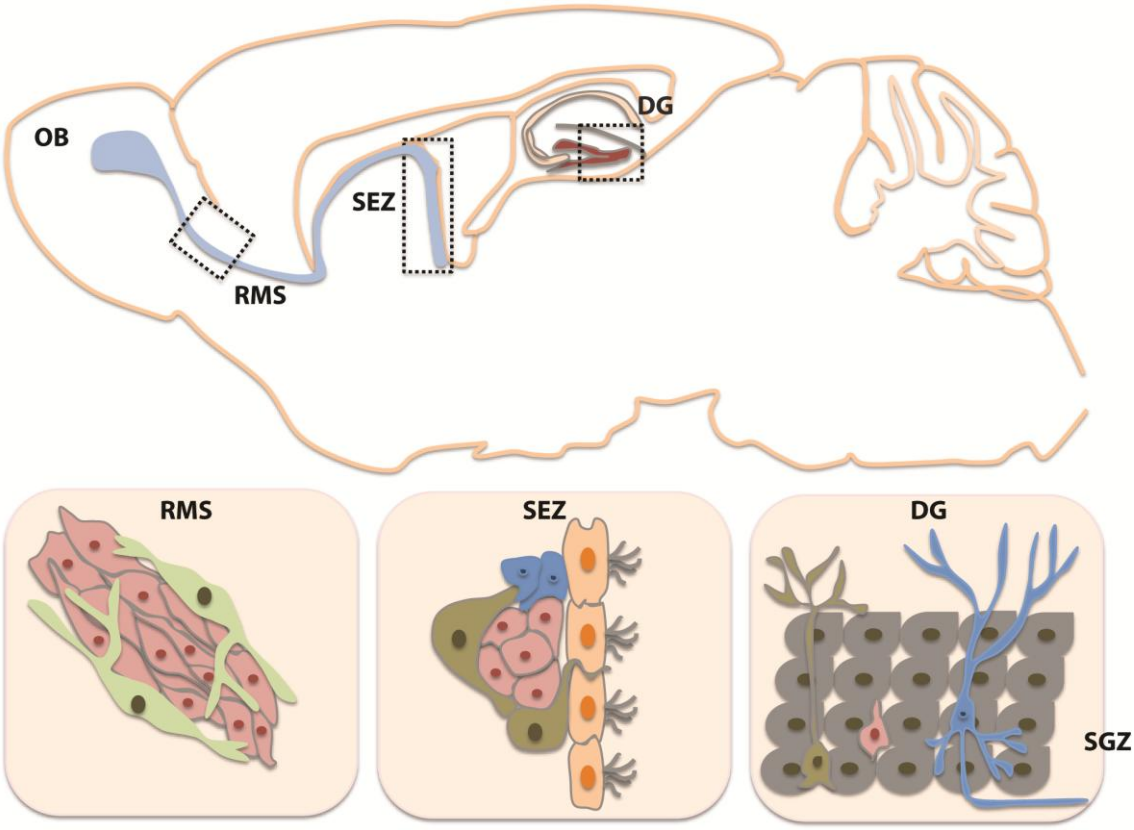

Figure 1: Neurogenic niches in the adult brain. Adult neurogenesis is confined to two major areas: the subependymal zone (SEZ) and the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG). Neurons originated in the SEZ and SGZ migrate to the olfactory bulbs (OB) and the dentate gyrus, respectively. Chains of neuroblasts migrate anteriorly through the rostral migratory stream (RMS) towards the OB. Both adult neurogenic niches are constituted by three different cell types.

1.2.1 Historical perspective

For a long time it was believed that the adult brain could not form new neurons. In 1913 the neuroscientist Ramón y Cajal published a study proclaiming that neurogenesis occurred exclusively in the developing prenatal brain (Ramon y Cajal 1913). This theory was for long accepted since it was believed that neurons do not divide and there was no evidence for the existence of a stem cell pool in the adult brain. Moreover, at the time, it was difficult to comprehend how new neurons could integrate in complex neuronal circuitries. Ezra Ellen, in 1912, made the first report of cell divisions in the lateral wall of the lateral brain ventricle (Allen 1912). However, the pioneer studies that began to overturn this dogma arose only in the late 50's and early 60's. The first evidence for adult neurogenesis was the observation of mitosis underneath the ependymal layer of the lateral ventricle in adult young mice (Messier et al 1958).

Shortly after, the existence of proliferation in the adult brain was confirmed and evidence was brought for the existence of mitosis in another brain area, the dentate gyrus (Smart and Leblond 1961). These findings were also described in rats (Bryans 1959) and cats (Altman 1963), not only in physiological conditions but also in response to adult brain injury (Altman 1962). Despite all these indications, the major turning point favouring adult neurogenesis was in 1965 when Joseph Altman provided histological evidence for the existence of adult neurogenesis in the hippocampus (Altman & Das 1965). Four years later, he further described the migration of cells from a subependymal layer of the lateral ventricles to the OBs where they integrate and differentiate into neurons (Altman 1969). This study was further confirmed through electron-microscopy by Michael Kaplan eight years later (Kaplan & Hinds 1977). The subsequent step that reinforced interest in adult neurogenesis emerged in the early 90's with the first *in vitro* evidence for the existence of adult NSCs in the brain (Lois & Alvarez-Buylla 1993, Reynolds & Weiss 1992). Cells isolated from the striatum (that encompassed the SEZ) had the capability to proliferate in response to the epidermal growth factor (EGF) and subsequently give rise to both neurons and glial cells (Reynolds & Weiss 1992). Henceforward several studies emerged relying on adult neurogenesis. Some of those confirmed previous reports by using new techniques such as retroviral tracing (Luskin 1993) and 5-bromo-2'-deoxyuridine (BrdU) labelling, a thymidine analog that incorporates into the DNA in the S phase of mitosis (Corotto et al 1993); others described in detail the rostral migratory stream (RMS), a migratory pathway of the SEZ born cells to the OBs (Doetsch & Alvarez-Buylla 1996, Lois & Alvarez-Buylla 1994, Lois et al 1996). The identity of the adult NSCs (Chiasson et al 1999, Doetsch et al 1999a, Johansson et al 1999b), the anatomy and functional organization of these cells in the subependymal layer (Doetsch et al 1997), the effect of stress (Cameron & Gould 1994) and environmental enrichment (Kempermann et al 1998, Kempermann et al 1997) on adult hippocampal neurogenesis were the major topics in the adult neurogenesis field in the 90's. In addition, an important discovery in the late 90's came from the work of Peter Eriksson that reported, for the first time, new neurons in the adult hippocampus of humans (Eriksson et al 1998).

The current challenges focus on the deep understanding of neurogenic niches heterogeneity and modulation, the exact molecular mechanisms regulating the formation and functional integration of new neurons. Of notice, in parallel with the studies in rodents many efforts have been done to characterize these NSCs niches in the adult human brain. Ultimately, the goal is to gain insights for the manipulation of these niches for regenerative purposes in humans.

1.2.2 The subependymal zone

The SEZ is the major site of adult neurogenesis in the adult brain. In rodents, it is commonly described to be constituted by a thin layer of cells and it is located below the ependyma that lines the lateral walls of the lateral ventricles (Alvarez-Buylla & Garcia-Verdugo 2002) (Figure 2).

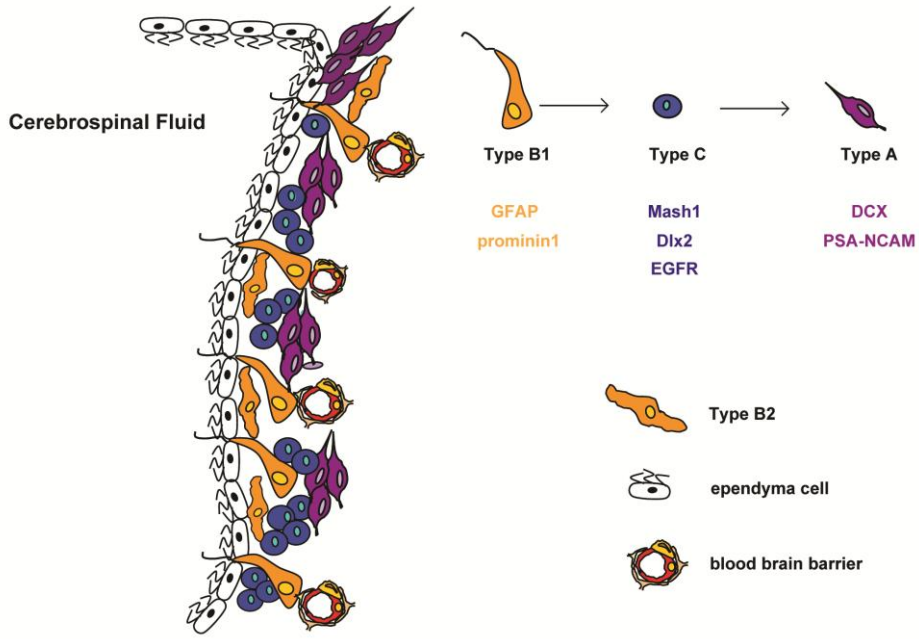

Figure 2: Morphological representation of the subependymal zone niche (SEZ). SEZ cells are located below the ependyma layer that lines the lateral ventricles filled with cerebrospinal fluid (CSF). This region comprise type B1 cells, the neural stem cells, that give rise to type C cells, also known as transit amplifying progenitors, that originate type A cells, also known as neuroblasts. Also in the SEZ are type B2 cells or niche astrocytes and blood vessels from the blood brain barrier (BBB). Type B1 cells are in contact both with the CSF, by projecting a cilium to the ventricle, and with the BBB.

Currently, the definition of the SEZ is broader, and it is not exclusively restricted to the lateral wall of the ventricles, but also to the dorsal and medial ventricular walls from where new neurons can also arise (Alvarez-Buylla et al 2008).

In the literature the SEZ is mostly referred as the SVZ. The reasoning for the designation of SEZ is due to its location underneath the ependyma and to distinguish it from the embryonic SVZ. In fact, given the recent findings that adult NSCs from this niche can also have their cell bodies in direct contact with the ventricle, both designations are not completely appropriate (Mirzadeh et al 2008).

For long, the origin of the SEZ was described to be the lateral ganglionic eminence, its embryonic counterpart (Kohwi et al 2005, Stenman et al 2003). More recently, the embryonic origin of the SEZ was determined by Cre-lox fate mapping of progenitors from the distinct parts of the embryonic ventricular zone until adulthood (Young et al 2007). Contrarily to what was anticipated, neural stem and progenitor cells originated not only from the lateral ganglionic eminence but also from the medial eminences and the cerebral cortex (Merkle et al 2007). Further insights on the maintenance, heterogeneity and other aspects of this niche are reviewed below.

1.2.2.1 Cellular composition and structural organization

We will next further discuss the cellular origin of the SEZ derived new neurons; two possibilities exist, either they are born from an adult NSC pool that generates neuronal progenitors, or directly from residual neuronal precursor cells. The definition of a NSC encloses two main properties, the capacity of self-renewal and multipotency (Gage 2000). These two features can be easily shown *in vitro*. By isolating cells from the adult striatum (including the SEZ), Reynolds et al were the first to show the formation of neurospheres (agglomerates of proliferating cells) in the presence of EGF and the subsequent differentiation into both neurons and glial cells, ascribing to the SEZ cells features of stemness (Reynolds et al 1992).

The search for the identity of the NSCs and their progeny *in vivo* emerged when Doetsch and colleagues introduced the glial fibrillary acidic protein (GFAP) expressing cells as the NSCs of the SEZ (Doetsch et al 1999a). These cells, also named type B cells originated transient amplifying progenitors (TAPs) or type C cells, that in turn gave rise to neuroblasts (neuronal precursors) or type A cells (Doetsch et al 1999a, Doetsch et al 1997, Doetsch et al 1999b) (Figure 2). On the other hand, it was proposed that the ependymal cells were in fact the neural stem cells of the SEZ, namely because ependymal cells proliferate rapidly and turned into neurons that reached the OBs (Johansson et al 1999a). To demonstrate that the type B cells were the "mother cells" that generated all other cell types, Doetsch et al (Doetsch et al 1999b) studied the effect of the administration of a cytostatic drug into the mouse brain ventricle and showed that the highly proliferative cells that were ablated from the SEZ niche at the end of the infusion were type A and C cells. Few type B cells were spared but these were enough to allow the progressive recovery of these mice several days after. These data indicate that type B cells are quiescent and slowly

dividing cells able to repopulate the neurogenic niche (Doetsch et al 1999a). Nevertheless, the question of whether these type B cells could be originated from ependymal cells remained open. At the present moment it is well established that the NSCs of the SEZ are the type B astrocytes (Doetsch 2003) and that ependymal cells might function as a reservoir of neurons and glia cells for the adult injured brain (Carlen et al 2009). The cellular types residing in the SEZ are next described in more detail.

Type E cells or ependymal cells form a monolayer that outlines the ventricular walls (Figure 2). Amongst other functions ependymal cells constitute a physical barrier to the direct and free exchange of molecules between the cerebrospinal fluid (CSF) and brain parenchyma (Bruni 1998, Bruni et al 1985). In the lateral wall two distinct ependymal cells have been described according to the number of cilia. The type E1 ependymal cells are multiciliated whereas the E2 ependymal cells display only two long cilia (Mirzadeh et al 2008). The E2 ependymal cells represent only 5% of the total ependymal cells in the lateral wall (Mirzadeh et al 2008). In physiological conditions these cells proliferate rarely (Altman 1963, Coskun et al 2008) or do not proliferate at all (Mirzadeh et al 2008, Spassky et al 2005). These cells are commonly marked with the S100β marker (Carlen et al 2009).

Type B cells are astrocytic cells and express the intermediate filament GFAP. In the SEZ two types of GFAP positive cells were distinguished according to ultrastructural differences. Type B2 astrocytes, or niche astrocytes, display a highly branched morphology and are frequently found in the interface of the SEZ and the striatum (Doetsch et al 1997). Type B1 astrocytes are radial-glia like cells that extend long processes both towards blood vessels settled deeper in the SEZ and to the ventricular space (Mirzadeh et al 2008) (Figure 2). The latter are recognized as the NSCs of the SEZ based on the following facts: i) they are structurally similar to their ancestor's radial glia progenitor cells that extend an apical primary single cilium towards the ventricle, ii) they increase their contacts with the ventricle when proliferation in the SEZ is stimulated, iii) they express both nestin and prominin1 (Prom1 or CD133), markers for NSCs, iv) they are mitotic, expressing Ki67 for instance, and give rise to neuroblasts and neurons. The type B1 astrocytic NSCs organize in pinwheel structures and the apical ending, i.e., the primary cilium, is in direct contact with the ventricular lumen and is surrounded by ependymal cells (Mirzadeh et al 2008).

Many attempts have been made to find specific cellular markers that could differentiate type B1 cells from type B2 and from ependymal cells. It is now consensual that at least some NSCs are $GFAP^+$ and $Prom1^+$ (Beckervordersandforth et al 2010). Moreover, activated NSCs also express

the epidermal growth factor receptor (EGFR) (Pastrana et al 2009). When the AraC, a cytostatic drug, is administrated into the ventricle, the activated NSCs ($GFAP^+EGFR^+$) and their progeny are eliminated. Only the $GFAP^+EGFR^-$ NSCs survive, then becoming active (hence $EGFR^+$) and start repopulating the SEZ niche (Pastrana et al 2009). Nevertheless some controversy still exists since it was suggested that Prom1 is solely expressed in ependymal cells (but not in all) and that Prom1 cells were able to form new neurons in the OB, indicating these cells as an additional source of more quiescent cells (Coskun et al 2008, Pfenninger et al 2007).

Combining all these recent findings, it remains uncertain the molecular identification of NSCs. Structural data indicates that B1 cells are the NSCs and GFAP and Prom1 seem to be the better markers to label these cells. Intriguingly, not all B1 cells pinwheels express Prom1 (approximately 29% express Prom1) (Mirzadeh et al 2008). Are they both NSCs? If so, what's the difference between them? Is EGFR expressed preferentially in any of these cells? Can Prom1 and/or GFAP expressing ependymal cell be another source of NSCs? These are some of the questions that remain unanswered concerning the identity of the NSCs.

Type C cells, or TAPs, are the progeny of the NSCs. These rapidly dividing cells are organized in clusters of immature precursors that express distal-less homeobox 2 (Dlx2), achaete-scute complex homolog 1 (Ascl1or Mash1) and EFGR (Ciccolini et al 2005, Ming & Song 2011) (Figure 2). A short pulse of BrdU mainly labels TAPs indicating these cells are the largest pool of proliferating cells in the SEZ. Infusions of EGF into the brain ventricles induce a massive increase in proliferation in the SEZ and the production of new astrocytes in the OB concomitant with a reduction in neurogenesis (Kuhn et al 1997), suggesting multipotency of TAPs triggered by EGF. Furthermore, these cells behave as multipotent NSCs *in vitro* in response to EGF, further reinforcing that TAPs can be reprogrammed when exposed to EGF (Doetsch et al 2002).

Type A cells or neuroblasts are born from type C cells and constitute the neuronal precursors. These cells travel a long distance from the SEZ to the OBs where they become fully mature neurons. Most type A cells express the polysialylated form of the neural cell adhesion molecule (PSA-NCAM) and doublecortin (DCX) (Figure 2), which are associated to their migratory properties (Ming & Song 2011). Although these molecules are expressed in neuronal precursors, it should be stated that it is also possible to find them in postmitotic neurons (Gascon et al 2007, Klempin et al 2011). Neuroblasts divide actively in the SEZ and also in the RMS. However, as neuroblasts approach the OBs the proliferation rates become much slower and the cell cycles are lengthened (Smith & Luskin 1998). Once in the OBs, A cells give rise to different types of

interneurons and are integrated in distinct layers of the OB. The process of migration and the heterogeneity of these cells will be further discussed below.

Tanycytes (Chojnacki et al 2009, Doetsch et al 1997), microglia (in response to injury) (Ekdahl et al 2009, Thored et al 2009) and endothelial cells of the blood vessels (Shen et al 2008, Tavazoie et al 2008) are also cellular components of the SEZ niche.

1.2.2.2 Topography and heterogeneity

The classical view of adult neurogenesis in the SEZ has restricted the existence of NSCs exclusively to the lateral wall of the lateral ventricles (Alvarez-Buylla & Garcia-Verdugo 2002). Recent findings suggest that news neurons are not only generated from the lateral wall but also from the dorsal (Brill et al 2009, Merkle et al 2007, Ventura & Goldman 2007) and medial walls (Kohwi et al 2007, Merkle et al 2007). In fact, evidence exists indicating that SEZ cells from any point in the entire lateral ventricle are able to generate new neurons (Merkle et al 2007).

The neurogenic potential is not confined to the lateral ventricles but also encompasses the RMS (Gritti et al 2002) and the OB core (rostral/anterior extension of the RMS) (Liu & Martin 2003). When isolated for *in vitro* clonal analysis, the RMS and the OB core cells were able to self-renew and originated both neurons and glia *in vitro*. Moreover, according to the progenitor's position in the RMS the outcome progeny was distinct. For instance, progenitors residing in more posterior parts of the RMS originated preferentially oligodendrocytes *in vitro* (Gritti et al 2002). Thus, NSCs capable of generating neuroblasts are present along the entire SEZ-RMS-OB path. Still, are these progenitors similar to each other albeit the distinct origin? The discrimination between SEZ cells and the subsequent characterization of these distinct populations is strictly necessary for understanding the neurogenic niche dynamics. As aforementioned, different stem/progenitor cell types were identified in the SEZ. Each one of these populations displayed common traits that include ultrastructural properties and molecular markers. As referred previously, it is currently generally accepted that there are three major neural stem and progenitor cell types and that NSCs give rise to a subset of TAPs that generate neuroblasts for the OB. However, it is becoming evident that even within these three defined populations lays a remarkable heterogeneity either due to inherited intrinsic or epigenetic factors (Alvarez-Buylla et al 2008) and/or an additional diversity in the surrounding microenvironment cues. Naturally, an accurate look on the topography of the SEZ discloses major anatomical differences. For instance, as the ventricle

extends from anterior to posterior levels (where it partially collapses forming the subcallosum zone also with neurogenic potential) the SEZ modifies in length and contact with the ventricle. Interestingly, it was described that the choroid plexus (CP) secrets slits and generates CSF gradients (Nguyen-Ba-Charvet et al 2004, Sawamoto et al 2006). Thus, it is conceivable that according to the position on the ventricular axis singular microenvironments are provided to the SEZ cells. These facts highlight the need to specify the precise locations where SEZ analysis is performed in all studies addressing SEZ dynamics.

Multiple studies evidenced that the NSC pool is highly heterogeneous both in the origin and in cellular fate (Alvarez-Buylla et al 2008, Merkle et al 2007, Young et al 2007). The fate of SEZ born cells is the OB where they become interneurons. Through GLAST::Cre-ERT2 fate mapping, it was demonstrated that a pool of NSCs that subsists in the adult dorsal wall of the lateral ventricles also gives rise to glutamatergic neurons to the OBs (Brill et al 2009). Furthermore, it was reported that SEZ NSCs also originate oligodendrocytes and oligodendrocyte precursors that migrate to the striatum, corpus callosum and fimbria fornix (Menn et al 2006, Nait-Oumesmar et al 1999, Picard-Riera et al 2002). Despite all the evidence referred before, the question of whether the NSCs from the SEZ are multipotent *in vivo* remains unanswered. For instance, is a single NSC able to generate both neurons and glia *in vivo*? Recent findings show that rather than a uniform pool of NSCs, heterogeneous and already pre-committed NSCs exist in specific locations of the SEZ (Merkle et al 2007). Several studies report that migrating neuroblasts per se display a substantial heterogeneity (Baker et al 2001, De Marchis et al 2004, Jankovski & Sotelo 1996) before reaching the OB. Furthermore, neuroblasts born either in ventral, dorsal, anterior or posterior regions are distinct, produce different neuronal types and are integrated in different layers of the OB. As an example, neuroblasts from dorsal regions tend to give rise to superficial granule cells, whereas ventral regions born neuroblasts yield mostly deep granule cells (Merkle et al 2007). Experiments with homotopically or heterotopically transplanted grafts from ventral or dorsal origins have uncovered the role of the niche microenvironment versus intrinsic properties of NSCs. Surprisingly, even after *in vitro* culture, heterotopic transplanted cells from dorsal or ventral origin gave rise to the same neuronal types as previously determined. This suggests that the NSCs are intrinsically pre-determined to become a certain type of neuron *in vivo* (Merkle et al 2007). The reason why different regionally placed NSCs give rise to distinct progeny has been speculated. In developmental systems like the forebrain telencephalon, the orchestration of individual neuronal types is associated with the expression of particular transcription factors in

the ventricular walls (Campbell 2003). Similarly, for the adult SEZ it is documented the expression of distinct transcription factors in both overlapping and non-overlapping regions of the SEZ. Some of these transcription factors were further correlated with the SEZ embryonic origin (Stenman et al 2003, Waclaw et al 2006, Young et al 2007). In fact, a regional specific pattern of transcription factors expression in the SEZ NSCs is associated both with their embryonic origin and adult neuronal fate (Young et al 2007). Generally, NSCs in the lateral ventricular wall ubiquitously express Dlx1, 2 and 5 and Mash1, while empty spiracles homeobox 1 (Emx1) expression is exclusive to the dorsal wall of the ventricle. Furthermore, the transcription factors NK2 homeobox 1 (Nkx2.1) and paired box gene 6 (Pax6) outline the ventral and dorsal regions of the lateral wall, respectively (Alvarez-Buylla et al 2008, Weinandy et al 2011). The current challenge is to understand how different combinations of transcription factors can orchestrate different neuronal types.

1.2.2.3 Migration, cellular fate and functional relevance of progenitor cells

The most well documented fate for the SEZ derived neuroblasts are the OBs. Neuronal precursors leave the SEZ, migrate anteriorly in the RMS, reach the OBs (Figure 1) and originate several types of inhibitory interneurons (Altman 1969, Doetsch & Alvarez-Buylla 1996, Lois & Alvarez-Buylla 1994, Luskin 1993). It is estimated that the time that a neuronal precursor takes to reach its final destination in rodents is at least 2 days (Lois & Alvarez-Buylla 1994). A time-lapse experiment performed in *in vitro* cultures of neuroblasts showed a chain migration of neuroblasts at a speed of 122 μm/h (Wichterle et al 1997). In fact, the RMS is organized as a complex tangential network of migrating neuroblasts chains (Doetsch & Alvarez-Buylla 1996). Wrapped by astrocytes in tubular–like structures, these chains of neuroblasts use each other as a support for the contiguous migration (Lois et al 1996). Because the neuroblasts chain migration towards the OBs occurs even in the absence of glial tubes *in vitro* and *in vivo* (namely in rabbits), (Luzzati et al 2003) the role of astrocytes in the migration is not entirely understood. The speed for cell migration is considerable reduced in RMS glial-enriched organotypic cultures to approximately 40 μm/h (Mejia-Gervacio et al 2011) when compared with *in vitro* cultures (speed of 122 μm/h) of chain neuroblasts (Wichterle et al 1997). Furthermore, it was suggested that through regulation of γ-aminobutyric acid (GABA) availability, astrocytic cells could modulate the migration of neuronal precursors (Bolteus & Bordey 2004). Importantly, the astrocytic tubes can

also support migration guidance. Indeed, in a transgenic mouse model with disrupted β1 integrin function, the astrocytic sheath structure is disturbed resulting in neuroblast migration out of the RMS. Accordingly, it was demonstrated that β1 integrin is essential for neuroblast's cell-to-cell contact and thus for migration (Belvindrah et al 2007). In addition, the neuroblasts markers PSA-NCAM and DCX were also described to play a role in migration (Chazal et al 2000, Ocbina et al 2006).

At end of the RMS, neuroblasts begin to migrate radially from the OB core to granule cell and periglomerular layers. Unlike the RMS tangential migration, radial migration does not require the presence of a glial scaffold but instead an interaction between the cells and the blood vessels. Specifically, migrating cells establish contact with the extracellular matrix and the astrocytic end feet in the blood vessels (Bovetti et al 2007) to reach the outer layers of the OBs.

The mechanism by which attractive and repulsive signals direct thousands of neuroblasts to travel anteriorly, with scarce cellular dispersion, remains unclear. It is well recognized that migratory instructive signals have a wide range of origins such as the septum, the CP through the CSF, the glial cells that form the RMS tubular structure and the OBs (Hu 1999, Liu & Rao 2003, Mason et al 2001, Wu et al 1999). Even though still debatable, the cooperation between chemorepulsive molecules ascending in most posterior regions and the chemoattractive signals sent by the OBs is thought to drive rostrally the neuroblasts stream. In particular, slit proteins are chemorepulsive molecules produced and secreted by the septum and by the CP in the CSF and repel neuroblasts (that express slit ligand Robo) towards the OBs (Nguyen-Ba-Charvet et al 2004, Sawamoto et al 2006). Furthermore, the CSF flow was also implicated in this process. Sawamoto and colleagues showed that the ependymal cilia beating mediated CSF flow generates a gradient in Slit2 and directs neuroblast migration. Transgenic mice exhibiting deficient cilia and consequently disturbed CSF flow display abnormal neuroblast migration (Sawamoto et al 2006).

The role of OBs in neuroblasts migration is controversial. Some studies show that when the OBs are removed neuroblasts migration still takes place leading to an accumulation of progenitors in the end of the RMS remnants (Kirschenbaum et al 1999). Others claim that OBs are required to attract and provide the proper migration of the new neurons (Liu & Rao 2003). Generally, these are the factors involved in neuronal migration: netrin-1 (Murase & Horwitz 2002), neuroregulins and Erb4 (Anton et al 2004, Perroteau et al 1999), brain derived neurotrophic factor (BDNF) (Chiaramello et al 2007), EGF (Aguirre et al 2005, Kim et al 2009) and Reelin (Hack et al 2002).

Neuronal precursors continue to divide in the RMS or exit cell cycle and begin to differentiate. Once in the OBs, new granular cells (deep, superficial and calretin positive) and periglomerular cells (calretin positive, calbidin positive and tyrosin hydroxylase positive) are formed (Lledo et al 2008). Most of these new neurons are granule cells integrated in the granule cell layer and are GABAergic. As referred previously, a small group of glutamatergic interneurons was also identified (Brill et al 2009) in the OBs.

Thousands of new cells reach the OBs daily (Kaplan et al 1985, Lois & Alvarez-Buylla 1994), although the volumetric size of the OBs doesn't change substantially (Biebl et al 2000). Thus, programmed cell death is absolutely required to maintain the total number of cells. In fact, many studies showed neuronal death occurs mainly at the level of the OBs (Petreanu & Alvarez-Buylla 2002). Although debatable, the reason why it takes place preferentially in the OBs, and not in the RMS or the SEZ, might be related to the decision process of what cells are necessary at a determined time in the OBs.

The functional relevance of the newly generated neurons in the OBs is a key aspect (Lazarini & Lledo 2011). Electrophysiological studies have been performed to compare the neuronal properties of these new neurons, namely synaptic plasticity, with the pre-existing neurons (Belluzzi et al 2003). Although new neurons seem completely integrated and functional, many gaps remain for the enlightenment of the final purpose for adult olfactory neurogenesis. A recent study showed that upon deletion of adult generated neurons by conditionally expression of the diphtheria toxin in nestin-positive cells, there was no pronounced behavioural difference in the olfactory mediated behaviours even upon six months of progenitor depletion (Imayoshi et al 2008). Conversely, other studies demonstrated that disturbing adult olfactory neurogenesis (in NCAM-deficient mice) leads to an impairment in odour discrimination (Gheusi et al 2000). On the other hand, a similar analysis performed in mice subjected to cranial irradiation of the SEZ, revealed that only the long-term olfactory memory was impaired (Lazarini et al 2009).

1.2.2.4 Intrinsic and extrinsic regulation

The SEZ adult neural stem and precursor cell population is not considerably altered throughout life. This steady-state level is due to two major facts: i) the tightly regulated processes that control NSC self-renewal encompassing asymmetric divisions that produce a new NSC and a progenitor cell and ii) the migration of the generated progenitors (neuroblasts) out of the niche. New

emerging insights support a negative feedback loop between the differentiated progeny and the NSCs that regulates the formation of new cells in the SEZ (Miller & Gauthier-Fisher 2009). On the basis of this theory is the fact that NSCs express receptors for several neurotransmitters, such as GABA (Bordey 2007), serotonin (Banasr et al 2004) and dopamine (Kim et al 2010), and are innervated by axons of mature neurons (Baker et al 2004).

The interplay between extrinsic and intrinsic factors determines the niche homeostasis. Intrinsic factors are set of signals produced by the progenitors that together with exterior microenvironment cues (extrinsic factors) instruct distinct neurogenic phases and ultimately the cellular fate.

As examples of intrinsic factors are the transcription factors expressed by space-restricted NSCs and all the intracellular receptors involved in the progenitor's proliferation and neuroblasts migration (referred above). Other intrinsic modulators of the SEZ are Mash1 in TAPs involved in the induction of neuronal specification (Fode et al 2000) and the neuroblasts markers PSA-NCAM and DCX implicated in progenitor migration (Gascon et al 2007, Ocbina et al 2006). Epigenetic regulation is widely recognized to play a fundamental role in stem cell multipotentiality, specification and differentiation (Hsieh & Gage 2004). However, only recently, studies relying on the epigenetic control of adult SEZ dynamics are beginning to emerge (Sun et al 2011). Epigenetic factors implicate relevant modifications to the genome without any alteration on its sequence; it comprises DNA methylation, histone modifications and non-coding RNAs as main mechanisms of action. As an example of epigenetic regulation, knockout mice for Bmi1, a protein part of a complex that methylate histones, exhibit post-natal depletion of NSCs (Molofsky et al 2003) while overexpression of Bmi1 in cultures or *in vivo* triggers NSCs expansion and self-renewal (Fasano et al 2009). The histone deacetylase 2 (HDAC2) inducible deletions in adult NSCs (through GLAST::CreERT2 mice) led to a deficit in formation of new neurons in the OBs and an accumulation of proliferating cells in the SEZ, suggesting that HDAC2 is needed for the neuronal lineage progression (Jawerka et al 2010). Another recent finding is related to the microRNA miR-124 that was proposed to be a neuronal fate determinant that makes the switch from NSC to TAP and neuroblasts. miR-124 is only expressed in NSCs progeny and when knocked-down *in vivo* induces the appearance of ectopic cells of glial nature in the OBs (Akerblom et al 2012, Cheng et al 2009).

Extrinsic factors for the SEZ regulation comprise several trophic and growth factors, neurotransmitters, morphogens, hormones and cytokines. Generally, the extrinsic factors

described to impact on adult SEZ neurogenesis are fibroblast growth factor 2 (FGF2 or bFGF) (Jin et al 2003), EGF (Kuhn et al 1997), transforming growth factor alpha (TGF-α) (Craig et al 1996), ciliary neurotrophic factor (CNTF) (Emsley & Hagg 2003), retinoic acid (Wang et al 2005), neurotrophins (Bath & Lee 2010), prolactin (Shingo et al 2003) and thyroid hormones (Fernandez et al 2004). Extracellular signalling molecules are of diverse origins, namely from ependymal cells, neural stem and precursor cells and neurons. The neurotransmitters aforementioned are good examples of extrinsic factors of neuronal origin. The neurotransmitter GABA is reported to inhibit NSCs proliferation and is produced by niche neuroblasts (Bordey 2007, Platel et al 2008, Young & Bordey 2009). This is suggested to be a negative feedback loop to control the formation of new cells in the SEZ. Contrarily to GABA, dopamine and serotonin stimulate NSCs proliferation (Banasr et al 2004, Coronas et al 2004).

The ependymal layer, adjacent to the SEZ, produces and secretes noggin, an antagonist of bone morphogenic proteins (BMP) signalling, that prevents differentiation and induces proliferation of NSCs (Li & LoTurco 2000, Lim et al 2000). In addition, together with endothelial cells of the blood-brain barrier (BBB), the ependymal layer is a source of pigment epithelium derived factor (PEDF) that induces NSC self-renewal and proliferation in *vitro* and *in vivo* (Ramirez-Castillejo et al 2006). In fact, the endothelial cells of the BBB are in close contact with the SEZ cells and are a source of molecules that promote NSC maintenance. For instance, an *in vitro* study showed that endothelial cells inhibit differentiation and stimulates self-renewal of NSCs by activating the transcription factor Hes1 (Shen et al 2004). Of notice, and much less explored, is the CP and the septum that also provide to the SEZ cells molecules that direct neuroblasts migration, as above-mentioned (Nguyen-Ba-Charvet et al 2004, Sawamoto et al 2006).

Additional players are involved in the SEZ dynamics such as microglia (Thored et al 2009) that are particularly relevant in response to brain injury or in diseases.

1.3 Barriers of the brain

The evolutionary brain complexity ascended, amongst other reasons, from the formation of barriers that physically separate the brain microenvironment milieu from the periphery. For an efficient neuronal processing the brain must be sheltered from the persistent variations in the periphery blood contents. In mammals, there are three structures separating the brain and the blood flow: the network of capillaries that constitute the BBB, the arachnoid membrane and the

epithelial cells of the CP forming the blood-cerebrospinal fluid barrier (BCSFB) (Abbott 2005). These barriers largely exceed the physical separation function; they play a dynamic role in the selective and active transport of nutrients and toxic agents into and out of the brain; they also constitute a primary line of defence against peripheral insults protecting the brain from external noxious events. In fact, the brain has for long been considered immune-privileged in part due to its barriers (Abbott 2005).

1.3.1 Blood-brain barrier

The BBB constitutes a physical barrier between the peripheral blood and the extracellular fluid of the brain parenchyma. Unlike vessels in the periphery, brain capillaries are constituted of endothelial cells interconnected by tight junctions (TJs) that restrain the passage of blood derived molecules and cells. Endothelial cells display extremely low pinocytic activity that further inhibits transcellular passage of molecules across the barrier (Ballabh et al 2004). The influx of molecules into the brain requires the existence of specific transporters and the only exceptions are small molecules such as O_2 and CO_2 that diffuse freely through their concentration gradients. The endothelial cells in capillaries forming the BBB are wrapped by a basal lamina, pericytes and astrocytic end-feets that all together constitute the neurovascular unit (Persidsky et al 2006).

1.3.2 Blood-cerebrospinal fluid barrier

The BCSFB is in the interface of the peripheral blood circulating in fenestrated capillaries and the CSF filling the brain ventricles. The physical interface is either the CP epithelial cells (CPEC), or the arachnoid membrane which surrounds the brain. Unlike the capillaries that form the BBB, CP capillaries are fenestrated and do not have TJs; the barrier properties are therefore ascribed to the CPEC (Abbott 2005), as will be next described in more detail.

1.3.2.1 Choroid plexus structure and function

The CP is a monolayer of epithelial cells connected by TJs that extend from the ependyma to the ventricles (the two laterals, the third and the fourth ventricles). The apical membrane of the CP

epithelium faces the CSF whereas the basolateral membrane lays over a stromal core that comprises different cell types (such as fibroblasts, dendritic cells and macrophages) (Emerich et al 2005) and is enriched in fenestrated capillaries that provide a blood flow four to ten times greater than the rest of the brain (Keep & Jones 1990) (Figure 3).

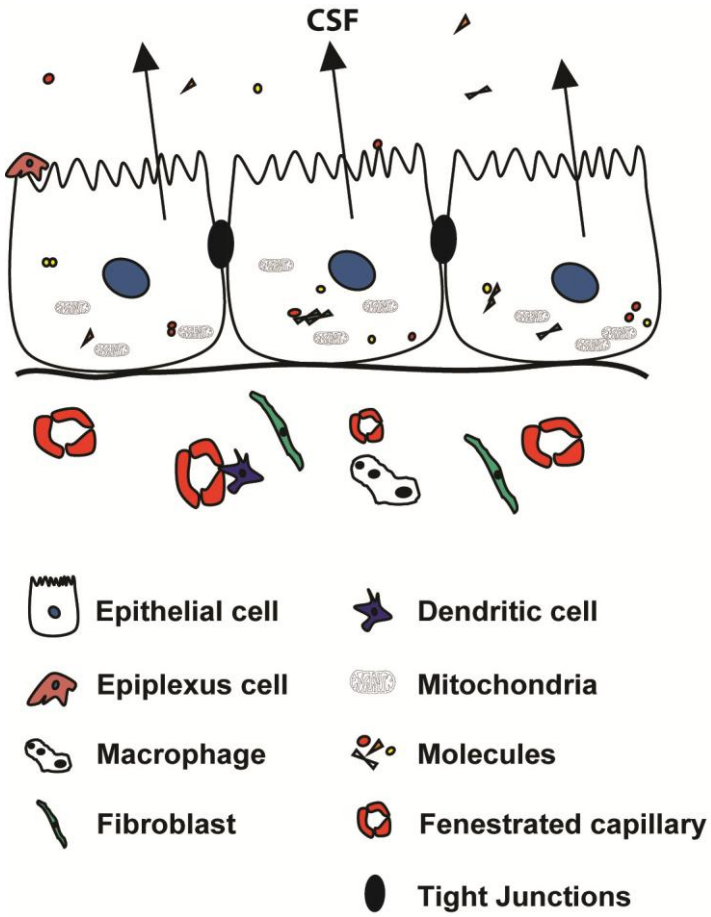

Figure 3: Schematic representation of the choroid plexus. The choroid plexus is constituted by a monolayer of epithelial cells bound together by tight junctions. The epithelial cells display two sides, the basolateral side where cells lie on a basal lamina and the apical side that faces the CSF. The choroid plexus epithelial cells are enriched in mitochondria which provide a metabolic capability to secrete many molecules towards the CSF. The basolateral side is a highly vascularized connective tissue that contains macrophages, dendritic cells, fibroblasts and fenestrated capillaries throughout the stroma. Epiplexus cells are attached to the villosities of the epithelial cells.

Epiplexus cells are also observed residing in the apical side of the epithelium (Figure 3). The basal lamina and the TJs constitute the typical hallmark of the CPs highly polarized epithelium. The presence of the TJs in the CPEC prevents the free paracellular diffusion of water-soluble molecules. The TJs are dynamic structures whose integrity can be altered under several circumstances, conditioning the barrier properties of the CPs. Generally the proteins that

constitute TJs from the CPs include occludin, claudins and junctional adhesion molecules (Vorbrodt & Dobrogowska 2003). The barrier properties of CPECs that are conferred by TJs can be assessed in primary *in vitro* cultures by evaluating transepithelial electric resistance (Strazielle & Preston 2003). CPECs *in vitro* cultures are important tools to study the barrier properties in the context of brain diseases, with lower values obtained for transepithelial electric resistance reflecting a leaky CPEC barrier, which compromises brain homeostasis.

The best described function for the CPs is the production and secretion of the CSF. In fact, CPECs are enriched in mitochondria that provide a metabolic work capacity for both secretory activity and maintenance of ionic gradients across the BCSFB (Cornford et al 1997). Moreover, they display a dense apical cover of microvilli that expand the surface area of contact with the brain ventricles (Figure 3). The major proteins constitutively expressed by the CP, and therefore enriched in the CSF, are transthyretin (Dickson et al 1985), lipocalin-type prostaglandin D2 synthase (Urade et al 1993) and transferrin (Zakin et al 2002). Furthermore, the CPEC are rich in transporters and in receptors both in the basolateral and in the apical sides and therefore mediate the transport of several particles in (e.g. vitamins, glucose, aminoacids) and out (e.g. amyloid β peptide) of the brain (Chodobski & Szmydynger-Chodobska 2001); and respond to various stimuli (e.g. lipopolysaccharide [LPS], interleukin 6 [IL-6] and interleukin [IL-1β]) (Marques et al 2009a, Marques et al 2007).

1.3.2.2 Cerebrospinal fluid source, function and composition

The CSF is a colourless fluid that fills the brain ventricles and the subarachnoid space. It circulates from the lateral ventricles via the interventricular foramina to the third ventricle, and via the cerebral aqueduct towards the fourth ventricle. The flow ends at the subarachnoid space and the spinal cord where it is finally reabsorbed. The CSF flow is generated both by the beating of ependymal cilia, hydrostatic pressure gradient and the pressure created by the expansion of the arteries in the CPs due to the heart beating (Johanson et al 2008).

At least 2/3 of the CSF is produced by the CPs (Speake et al 2001). The remaining comes from the interstitial fluid adjacent to the ventricular walls resulting from the metabolism of the brain parenchyma and from the BBB (Johanson et al 2008). The CSF is estimated to renew approximately three to four times per day in humans (Wright 1978) and up to eleven times per

day in young adult rats (Johanson et al 2008). CSF renewal is absolutely required for the metabolic homeostasis of the brain.

There are many functions attributed to the CSF. Firstly, the most observable one is the mechanical protection and the buoyancy that it confers to the brain. The subsequent functions are all related to the CSF components that by gaining proximal and distal contact to the brain parenchyma provide CP-born molecules a privileged access to a vast extension of the CNS (Agnati et al 1995). The CSF is a source of ions, lipids, hormones, glucose, and biologically active proteins and molecules (Chodobski & Szmydynger-Chodobska 2001). It also receives metabolites from the brain parenchyma for posterior removal from the brain through the CPs. Alterations in the CSF chemical and molecular composition leads to a dysfunctional brain, for instance, when the CSF renewal decreases, the rates of clearance of metabolic cellular wastes also decrease leading to their accumulation. Furthermore, disruption in the CSF flow, caused by dysfunctional cilium for instance, leads to hydrocephalus (Banizs et al 2005).

During development, the CSF plays a key role for the normal growth of the embryonic brain. Namely, it was observed that the hydrostatic pressure exerted by the CSF is essential for proper brain enlargement (Gato & Desmond 2009, Jelinek & Pexieder 1970, Pexieder & Jelinek 1970). The reduction of the intra-luminal pressure in chick embryos led to an impaired morphogenesis and a drastic decrease in neuroepithelial proliferation (Desmond & Jacobson 1977). In fact, embryonic neurogenesis depends on the combined actions of CSF pressure and CSF growth factors (Lehtinen & Walsh 2011). Several mitogens and growth factors present in embryonic CSF were described to play a role on developmental neurogenesis (Gato et al 2005). For instance, CSF bFGF regulates neuroepithelial cell proliferation and neurogenesis during early stages of development in chick embryos (Martin et al 2006). In the mouse developing cortex, CSF insulin growth factor 2 (IGF2) was shown to regulate growth and neuronal survival (Lehtinen et al 2011). Another example is retinoic acid signalling that contributes to cortical neural formation and to cerebellum development (Alonso et al 2011, Crandall et al 2011, Siegenthaler et al 2009, Zhang et al 2003). Although much is known on the role of the CSF in embryonic neurogenesis, knowledge on the role of CSF in adult neurogenesis is less studied. The CSF influence on adult neurogenesis will be exploited hereafter.

1.4 Subependymal zone regulation by the barriers of the brain

From development to adulthood, the continuous occurrence of NSCs in the lateral ventricles is only possible due to the existence of a particular niche that together with NSC intrinsic properties nurtures cell stemness. Curiously, SEZ type B1 cells generally accepted as adult NSCs are contacting both the CSF and the endothelial cells of the BBB, suggesting that endothelial cells of the BBB and epithelial cells of the CPs are natural sources of molecules of relevance for NSC maintenance, which we will refer to next.

1.4.1 Subependymal zone regulation mediated by the blood brain barrier

The anatomical basis of the interaction between vascular endothelial cells and SEZ cells was recently revealed by demonstrating that the proliferative progeny lie adjacent to the blood vessels (Shen et al 2008) and that the type B cells contact blood vessels at sites that lack either the astrocytic endfeet or the pericyte coverage (Tavazoie et al 2008). This unique property of the BBB enables vascular derived signals access to the SEZ niche (Ming & Song 2011). Previous work using SEZ rat explants and human vascular endothelial cells suggested that brain vascular cells support neuronal migration and maturation, but not mitogenesis, through the action of BDNF (Leventhal et al 1999). Other studies indicate that the endothelial cells secretome is also an important component for NSC expansion (Shen et al 2004). In fact, endothelial cells are a source of factors that influence neural stem and precursor cells, for instance vascular endothelial growth factor (VEGF) and bFGF (Biro et al 1994, Jin et al 2002). Of notice, the role of endothelial cells derived PEDF in supporting NSC survival and maintaining multipotency (Elahy et al 2012, Ramirez-Castillejo et al 2006).

1.4.2 Subependymal zone regulation mediated by the choroid plexus

Until recently, it was believe that adult neural stem and precursor cells of the lateral walls of the ventricles were physically separated from the CSF by a juxtaposed ependymal layer. Even though ependymal cells do not exhibit TJs, such as the CPECs, molecules present in the CSF would have to cross ependymal cells or diffuse through it to gain access to SEZ cells. Recently, a comprehensive view of the cytoarchitecture of the SEZ cells by using whole mounts of the lateral

walls (Mirzadeh et al 2010), brought to light the presence of single ciliated astrocytic cells, or type B1 cells, contacting directly the ventricle and hence the CSF. Type B1 cells are encircled by ependymal cells forming pinwheels like structures as described previously. These pinwheels were frequently found in the walls of the lateral ventricles (Mirzadeh et al 2008).

Similarly to brain embryogenesis where radial glial cells (from which type B cells are originated) receive, directly from the CSF, instructive cues to proliferate and expand (Gato & Desmond 2009, Lehtinen et al 2011) also type B1 cells from the SEZ neurogenic niche are likely capable of process signals derived from the CSF. Because the CPs are the main producers of the CSF, these findings highlight both the CSF and the CP potential role in adult neurogenesis, establishing a path from the CPs to the CSF and hence towards SEZ.

The proof of concept that CSF contents impact on the SEZ cell dynamics originally came from experiments with intracerebroventricular injections (Jin et al 2003, Kuhn et al 1997). Infusions of FGF2 and EGF into the CSF of the brain ventricles resulted in remarkable effects on the SEZ cell proliferation, migration and cellular fate (Kuhn et al 1997). Importantly, it was shown that Slit2, a chemorepellent molecule produced by the CPs and secreted into the CSF is required for neuroblasts migration towards the OBs (Sawamoto et al 2006).

While the significance of CP born molecules in neurogenesis was previously reported during brain development (Lehtinen & Walsh 2011, Zappaterra & Lehtinen 2012), during adulthood the role of the CP secretome is largely unknown. A growing number of publications illustrate the existence of numerous morphogens, mitogens and trophic factors in the CPs (Chodobski & Szmydynger-Chodobska 2001, Cuevas et al 1994, Timmusk et al 1995). Recently, the CP basal transcriptome was characterized and revealed many more biologically active molecules that could be of relevance for SEZ modulation (Marques et al 2011). Some of these proteins were previously shown to influence the NSC niche both *in vitro* and *in vivo*.

1.5 The subependymal zone in the context of brain diseases and insults

Because a plethora of extracellular signals of different origins command the fate and migration of SEZ born cells, any alteration to this finely tuned regulatory process is able to impact on the niche homeostasis. Indeed, this is observed in many non-physiological conditions as is next summarized for several brain injuries and neurodegenerative disorders.

Alzheimer's disease (AD) is characterized by a progressive cognitive decay and memory impairment and is associated with the formation of Aβ deposits, the amyloid plaques and

intracellular neurofibrillary tangles composed of tau proteins (Tam & Pasternak 2012). Intracerebroventricular injections of Aβ reduced the proliferation in the SEZ and mice models for AD display impaired neurogenesis (Haughey et al 2002). Interestingly, examinations on AD patients correlate deficits in olfactory capacities with the progression of the disease, another indication that SEZ-OB network is impaired in AD (Murphy et al 1990).

Huntington disease (HD), an autosomal dominant disease, is characterized by the expansion of a CAG triplet in the huntingtin protein (Zheng & Diamond 2012). Postmortem analysis in HD human patients revealed an expansion in the SEZ suggesting enhanced neurogenesis (Curtis et al 2003b, Curtis et al 2005). Accordingly, rat models of HD, where a neuronal striatal lesion is induced by quinolinic acid, display increased SEZ cell proliferation (Tattersfield et al 2004). Moreover, neuroblasts were shown to migrate towards the lesion site forming new immature neurons (Batista et al 2006, Tattersfield et al 2004).

Parkinson disease (PD) patients display severe motor deficits and progressive dopaminergic neuronal loss in the substancia nigra (Corti et al 2011). A reduction of proliferating cells in the SEZ is observed upon postmortem analysis of human brains from PD patients (Hoglinger et al 2004). These findings are in line with the previously described function of dopamine in promoting proliferation in the SEZ (Hoglinger et al 2004). In fact, in the 6-hydroxydopamine rat model of PD a decrease in proliferation is detected but also a redirection of neuroblasts towards the lesion site in the striatum is observed (Baker et al 2004, Winner et al 2008).

Multiple sclerosis (MS) is a demyelinating inflammatory disease (Nylander & Hafler 2012). MS postmortem brains examination demonstrated increased proliferation in the SEZ and PSA-NCAM positive cells homing to areas of lesion (Nait-Oumesmar et al 2007). Additional studies in mouse models of MS showed increased oligodendrogenesis (Jablonska et al 2010, Picard-Riera et al 2002, Pluchino et al 2008) and decreased neurogenesis, resulting in olfactory deficits measured at the molecular and behavioural levels (Tepavcevic et al 2011).

Stroke is an ischemic episode caused by an obstruction in the cerebral arteries and leads to brain cellular death (Hossmann 2006). In the medial cerebral artery occlusion (MCAO) mouse model of ischemic stroke, the proliferation in the SEZ is augmented and neuroblasts migrate radially to the lesion site where new mature neurons are formed (Arvidsson et al 2002, Yamashita et al 2006, Zhang et al 2004). In accordance, strong evidence exists in humans for the formation of new neurons following an ischemic incident (Jin et al 2006, Minger et al 2007).

Accumulating evidence consistently indicates that brain injuries and disorders, as the ones just described, share an inflammatory component (Minghetti 2005). The role of neuroinflammation in adult neurogenesis is still controversial (Carpentier & Palmer 2009, Gonzalez-Perez et al 2010, Taupin 2008). Each insult or disease displays a particular cascade of inflammatory events. For example, microglia and astroglia exhibit singular temporal and spatial cocktails of proinflammatory and anti-inflammatory molecules that can strongly impact on NSC proliferation, specification and ultimately on the cellular fate of SEZ born cells. Therefore, depending on the inflammatory milieu context inherent to a particular disease, neuroinflammation may either promote or prevent adult neurogenesis and gliogenesis.

An important step to regenerate tissue loss is the migration of progenitor cells to sites of injury. Further highlighting the importance of the inflammatory component to neurogenesis is the fact that neural stem and precursor cells express a wide-range of receptors for chemokines (Tran et al 2007) that when stimulated during neuroinflammation processes constitute crucial homing signals for progenitor cells (Belmadani et al 2006). At sites of injury, stromal-derived factor 1α (SDF1α or Cxcl12) expression is enhanced by endothelial and astrocytic cells, initiating a chemoattractive process of Cxcl12 receptor chemokine (C-X-C motif) receptor 4 (CXCR4) expressing NSCs. Additionally, this molecule also promoted NSC proliferation *in vitro* (Imitola et al 2004). This role was also reported in several diseases such as MS, brain trauma and brain tumours (Calderon et al 2006, Itoh et al 2009, van der Meulen et al 2009). An additional chemokine involved in neuronal progenitor's migration is the monocyte chemoattractant protein-1 (MCP1or Ccl2). Neural progenitor cells transplanted into non-ischemic areas migrate preferentially towards infarcted sites where microglia and astrocytes upregulate MCP1 expression levels (Liu et al 2007, Yan et al 2007). Furthermore, chains of neuroblasts expressing the MCP1 receptor chemokine (C-C motif) receptor 2 (CCR2) are found migrating towards areas where MCP1 was infused (Yan et al 2007).

Importantly, several cytokines have also been described as modulators of NSCs (Bauer et al 2007, Das & Basu 2008, Nagao et al 2007). Leukemia inhibitory factor (LIF) stimulates NSC proliferation and self-renewal (Bauer & Patterson 2006). Interestingly, its expression levels are induced in microglia and reactive astrocytes in ischemia and traumatic brain injury (Banner et al 1997, Suzuki et al 2000). Similarly, the inflammatory cytokine IL-6 was described to promote NSC self-renewal and to inhibit the formation of new neurons (Covey et al 2011, Vallieres et al 2002). Other cytokines also modulating adult neurogenesis are: IL-1β, interferon gamma (IFNγ)

and tumor necrosis factor alpha (TNF-α) (Das & Basu 2008, Iosif et al 2006, Monje et al 2003, Wong et al 2004). Nevertheless, there is still controversy on the function of this cytokines. As an example, TNF-α, a major proinflammatory cytokine was demonstrated to play both pro and anti-neurogenic effects. Upon infusion of neutralizing antibodies for TNF-α in animals models of stroke, the formation of new striatal neurons was suppressed, suggesting a pro-neurogenic action of TNF-α (Heldmann et al 2005). On the other hand, TNF-α was further suggested as a negative modulator of hippocampal neurogenesis (Cacci et al 2005, Iosif et al 2006) but through TNFR1 exclusively (Iosif et al 2006). Further analysis suggested that the concentration of TNF-α itself might determine the final outcome (Bernardino et al 2008).

Commonly, stimuli given by the administration of LPS, an endotoxin of the Gram-negative bacteria (Alexander & Rietschel 2001, Raetz & Whitfield 2002), has been used to mimic neuroinflammation because it rapidly triggers cellular expression of cytokines and chemokines through toll-like receptor 4 (TLR4). LPS actions are frequently associated with impaired adult hippocampal neurogenesis (Carpentier & Palmer 2009, Ekdahl et al 2003, Fujioka & Akema 2010, Monje et al 2003, Russo et al 2011). LPS brain infusions strongly reduce hippocampal neurogenesis, an effect triggered by microglial activation; inhibition of microglial activation restores hippocampal neurogenesis (Ekdahl et al 2003). Endorsing results showed that treatment with a nonsteroidal inflammatory drug after a single systemic injection of LPS restores hippocampal neurogenesis (Monje et al 2003). Also of interest is the subsequent functionality of the newly formed neurons upon LPS treatment. Recent reports claim that upon local injections of LPS in the hippocampal dentate gyrus, newly formed neurons display different synaptic plasticity properties (Jakubs et al 2008). Microglia and astrocytic responses to LPS driven inflammation are not unique. The effects of LPS in the CPs were recently unveiled by Marques et al (2007, 2009a, 2009b), which will next be referred.

1.6 The blood-cerebrospinal fluid barrier in the context of brain disorders and insults

Rising expectations for the development of therapies based on brain endogenous regenerative capacity firstly motivated by the discovery of adult NSCs in the brain were further encouraged by the observation of naturally-occurring NSCs response to brain insults and neurodegenerative disorders (Curtis et al 2003a, Curtis et al 2007, Sierra et al 2011). To enhance and improve

NSC-based therapies on cellular regeneration and/or replacement, the mechanisms underlying these processes must be uncovered.

In this sense, we have described so far the BCSFB as a crucial element for brain homeostasis maintenance; hence, the ability of the brain to adapt to an injury also relies on the CP-CSF response. There are several brain diseases and injuries associated to CP-CSF alterations. Even physiological aging in itself alters BCSFB permeability, CSF volume, composition and turnover (Pfefferbaum et al 1994, Preston 2001). These alterations are exacerbated in brain disorders such as AD. The accumulation of Aβ is thought to be the major hallmark in AD. Interestingly, low levels of Aβ-binding proteins, like transthyretin (TTR), are reduced in the CSF of AD patients indicating an inefficient clearance of this peptide from the CNS (Hansson et al 2009, Mesquita et al 2012, Serot et al 1997).

In physiological situations the CNS is free of peripheral immune cells, however, events that trigger neuroinflammatory processes can elicit leakage of the barriers and the entrance of blood cells into the brain. This is the case of the nervous system autoimmune diseases such as MS. It is reported that in the experimental autoimmune encephalomyelitis (EAE) mouse model of MS, inflammatory cells are recruited to the CNS and can be detected in the CSF. In accordance, the CP also displays major alterations, namely in the expression of the adhesion molecules ICAM1 and VCAM1 (Engelhardt et al 2001). Moreover, the overall gene expression profile of the CPs and the CSF composition is altered in EAE (Marques et al 2012). Together these data associate the CP-CSF nexus to the pathophysiology of MS. Another neuroinflammatory event that has been implicated with CPs and CSF disturbance is traumatic brain injury (TBI). Following TBI the BCSFB is also damaged, thus a subsequent increase in the traffic of leucocytes and blood derived molecules from the periphery to the brain occurs (Johanson et al 2011). This leads to the disruption of the CP-CSF functions including the brain support for nutrients and removal of metabolic waste products (Johanson et al 2011). However, the BCSFB also develops mechanisms to restore brain homeostasis. The CP intensifies the expression, synthesis and secretion of neurotrophins and growth factors that will reach the lesion sites and help on the regeneration/repair process (Johanson et al 2011). A similar strategy is acquired by the CP after a transient forebrain ischemia in adult rats (Johanson et al 2000).

Importantly, the CP-CSF not only participates in brain parenchyma derived inflammatory events but also respond to peripheral inflammatory triggers. In fact, the interplay between the immune

system and the brain is mediated by the BCSFB and the BBB. It was recently described the CP gene expression profile in response to an acute peripheral inflammation triggered by LPS (Marques et al 2009b). After a short LPS stimulus the CPs were able to robustly change their transcriptome, and these were also reflected at the CSF level (Marques et al 2007). Amongst the altered molecules were chemokines (such as Ccl2, Ccl10), cytokines (such as IL-6 and TNF-alpha) and adhesion molecules (Marques et al 2009a, Marques et al 2009b). Similarly, experiments conducted in an *in vitro* model for the BCSFB, helped to clarify the origin of the response observed *in vivo*, showing that the CP response is triggered by LPS directly and/or by blood-borne molecules. Accordingly, CPEC stimulated at the basolateral side by LPS or by serum derived from animals injected with LPS, display individual enhanced expression for cytokines and chemokines (Marques et al 2009b).

While LPS elicited inflammation was able to impact on hippocampal neurogenesis, the significance of this insult for the SEZ adult neurogenesis remains unknown.

1.7 Project aims

The purpose of this study was to give insights in emerging questions concerning SEZ complexity and modulation. More specifically we aimed at:

1. Characterize the topographical proliferation profiles and the distribution of neural stem and progenitor cells throughout the anterior-posterior and dorsal-ventral axes of the SEZ, both in rat and mouse;
2. Determine inter-species variations in the SEZ topographical profiles between rat and mouse;
3. Analyse the impact of an acute peripheral inflammatory stimulus on the SEZ niche dynamics;
4. Investigate the role of CP secreted molecules on the SEZ modulation upon an acute peripheral inflammatory stimulus.

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2. Topographical analysis of the subependymal zone neurogenic niche

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Topographical Analysis of the Subependymal Zone Neurogenic Niche

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Abstract

The emerging model for the adult subependymal zone (SEZ) cell population indicates that neuronal diversity is not generated from a uniform pool of stem cells but rather from diverse and spatially confined stem cell populations. Hence, when analysing SEZ proliferation, the topography along the anterior-posterior and dorsal-ventral axes must be taken into account. However, to date, no studies have assessed SEZ proliferation according to topographical specificities and, additionally, SEZ studies in animal models of neurological/psychiatric disorders often fail to clearly specify the SEZ coordinates. This may render difficult the comparison between studies and yield contradictory results. More so, by focusing in a single spatial dimension of the SEZ, relevant findings might pass unnoticed. In this study we characterized the neural stem cell/progenitor population and its proliferation rates throughout the rat SEZ anterior-posterior and dorsal-ventral axes. We found that SEZ proliferation decreases along the anterior-posterior axis and that proliferative rates vary considerably according to the position in the dorsal-ventral axis. These were associated with relevant gradients in the neuroblasts and in the neural stem cell populations throughout the dorsal-ventral axis. In addition, we observed spatially dependent differences in BrdU/Ki67 ratios that suggest a high variability in the proliferation rate and cell cycle length throughout the SEZ; in accordance, estimation of the cell cycle length of the neuroblasts revealed shorter cell cycles at the dorsolateral SEZ. These findings highlight the need to establish standardized procedures of SEZ analysis. Herein we propose an anatomical division of the SEZ that should be considered in future studies addressing proliferation in this neural stem cell niche.

Citation: Falcão AM, Palha JA, Ferreira AC, Marques F, Sousa N, et al. (2012) Topographical Analysis of the Subependymal Zone Neurogenic Niche. PLoS ONE 7(6): e38647. doi:10.1371/journal.pone.0038647

Editor: Domingos Henrique, Instituto de Medicina Molecular, Portugal

Received October 17, 2011; **Accepted** May 13, 2012; **Published** June 20, 2012

Funding: This work has received financial support from FEDER through the COMPETE program and FCT – Fundação para a Ciência e a Tecnologia under the projects PTDC/SAU-OSM/104475/2008 and PTDC/SAU-NEU/105180/2008. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

The subependymal zone (SEZ), generally described as a thin layer of proliferative cells lining the lateral wall of the lateral brain ventricles, is a major source of multipotent neural stem cells (NSCs) in the adult brain [1,2]. The fate of this pool of stem cells is to generate new neurons that migrate anteriorly along the rostral migratory stream (RMS) towards the olfactory bulb where they differentiate into different types of interneurons [3,4]. Additionally, it was shown that SEZ NSCs generate oligodendrocytes [5,6]. Alterations in the proliferative and migratory profile of the SEZ NSC population are extensively described for several animal models of neurological disorders, such as Alzheimer's and Parkinson's diseases, stroke and epilepsy [7]. Altogether, such studies have raised expectations for the development of endogenous regenerative therapies based on the manipulation of the SEZ neurogenic niche. However, to fully explore the regenerative potential of the SEZ stem cell niche, a better knowledge of how the niche is maintained and regulated, both in physiological and pathological conditions, is needed.

Recent studies demonstrated that, in mice, the SEZ stem cell niche is not topographically and functionally uniform; indeed, the SEZ niche is not restricted to the lateral walls of the ventricles, but rather extends to more dorsal portions of the ventricle walls [8] and to the RMS [9]. In accordance, several reports extend the analysis of the SEZ to the beginning of the RMS [10–13]. In addition, it is becoming increasingly evident that the SEZ NSC population is heterogeneous as supported by *in vitro* studies which show a large variation in the number of neurosphere forming cells extracted from serial brain slices along the anterior-posterior axis [14]. Furthermore, there is also evidence that the expression of transcription factors by NSCs varies according to their position along the ventricular neuraxis [15–17]. Interestingly, a correlation between the regionalization of type B cells and cell-fate specification has also been described [18]; for example, SEZ cells were found to generate not only GABAergic neurons, but also glutamatergic olfactory bulb interneurons specifically derived from the dorsal SEZ [8].

Taken together, the literature reflects the heterogeneity and complexity of the SEZ stem cell niche and anticipates the pitfalls that may occur when data obtained from specific regions in the anterior-posterior and dorsal-ventral axes are used for extrapolations to the entire SEZ. Also of consideration, the lack of consistency or specificity in topographical mapping may generate discrepancies between studies and mask relevant changes in

specific regions when the analysis is made as a whole [19]. Therefore, we thought of relevance to characterize the proliferation pattern of SEZ cells throughout the anterior-posterior and dorsal-ventral axes. Taking into consideration the profile encountered, we propose a standard division for the anterior-posterior SEZ and define the dorsal-ventral regions in the SEZ based on differences in cell proliferation and on anatomic parameters.

Results

Analysis of Cell Proliferation Rate Along the Anterior-posterior Axis

Analysis of the SEZ cell proliferation rate along the anterior-posterior axis, as defined in the material and methods section and in Figure 1, revealed that the anterior SEZ displays the highest number of Ki67 positive cells per mm^2 ($6.40\pm0.27\times10^3$) that comparatively decreases 48% and 52% at the intermediate and posterior SEZ divisions, respectively (Figure 2A). Similarly, analysis of proliferation with BrdU revealed that at the intermediate and posterior levels of the SEZ, BrdU incorporation was 45% and 34% lower than in the anterior division ($2.86\pm0.29\times10^3$ BrdU positive cells/mm^2) (Figure 2A). These results showed that the SEZ cell proliferation rate is higher in the anterior division than in the intermediate and posterior divisions and that the latter two display very similar proliferation patterns. The proliferation analysis was extended further posteriorly along the anterior posterior axis, into a division here designated post-posterior which is anatomically found at the same level of the hippocampus, the other major neurogenic niche of the adult brain. Post-posterior SEZ exhibited the lowest proliferation rates of the anterior-posterior axis with both Ki67 ($1.15\pm0.09\times10^3$ cells/mm^2) and BrdU ($0.77\pm0.12\times10^3$ cells/mm^2) markers (Figure 2A). The present data highlights the heterogeneity in cell proliferation rates in the SEZ along the anterior-posterior axis.

Analysis of Cell Proliferation Rates Along the Dorsal-ventral Axis

Along the dorsal-ventral axis, proliferation was assessed separately in every 150 μm length fragment, beginning at the top of the lateral wall (dorsally positioned) to the ventral tip. The proliferation rate, assessed by Ki67 and BrdU labelling, decreased in the lateral wall along the dorsal to ventral axis (Figure 1, lower panel). Interestingly, as for the anterior-posterior axis, there was a position in the dorsal-ventral axis where the proliferation rate decreased steeply (indicated by the arrow in the lower panel of Figure 1). These observations prompted for the division of the lateral wall of the SEZ in two different regions: dorsolateral and ventral. The dorsolateral SEZ comprises the dorsal part of the lateral wall and extends to the beginning of the ventral SEZ. At this point there is a directional switch of the lateral wall that starts elongating perpendicular to the dorsal SEZ. Thus, taking into account these observations, four distinct regions were considered to estimate the proliferation rates throughout the dorsal-ventral axis: RMS (specifically the beginning of the RMS), dorsal, dorsolateral and ventral (illustrated in Figure 1 middle and lower panels). To the best of our knowledge, this is the first study that separately estimates proliferation rates in different dorsal-ventral regions of the SEZ.

Examination of both Ki67 and BrdU positive cells along the SEZ dorsal-ventral axis revealed major differences in cell proliferation rates between the four defined regions (Figure 2B). The RMS displayed the highest values for Ki67-positive cells ($5.80\pm0.37\times10^3$ cells/mm^2), with this value decreasing 23% in the dorsolateral region ($4.45\pm0.27\times10^3$ cells/mm^2). In contrast, the dorsal SEZ presented a number of Ki67 positive cells/mm^2 of only approximately 10% comparatively to the RMS and the dorsolateral SEZ, the lowest proliferation densities of the four regions. The ventral SEZ also displayed low values for proliferation, 55% bellow the value displayed by the anatomically contiguous dorsolateral SEZ. Interestingly, the SEZ proliferation pattern estimated by BrdU incorporation did not completely mirror the data obtained for Ki67. The number of BrdU positive cells in the dorsolateral SEZ was significantly higher ($p < 0.01$) than in the RMS (Figure 2B).

Combined Analysis of Proliferation in the Anterior-posterior and Dorsal-ventral Axes

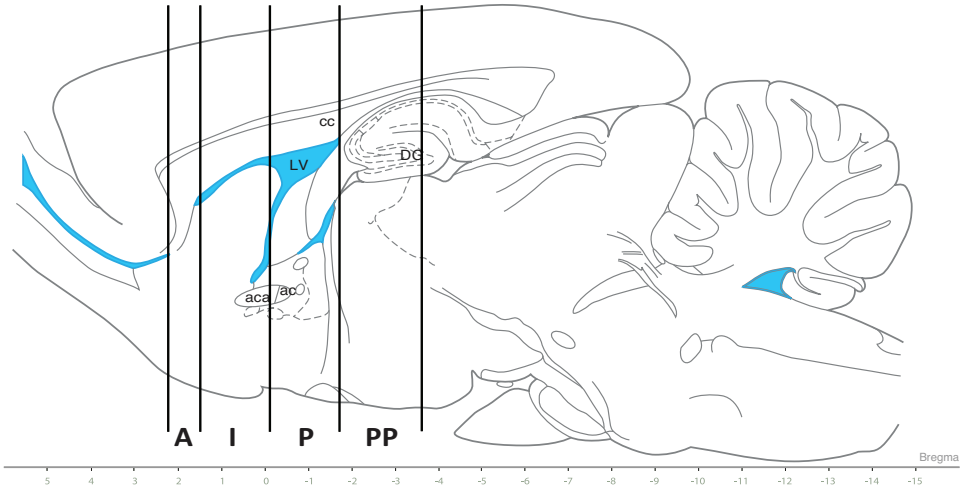
Since the proliferation rates vary along the anterior-posterior axis, as described above, the four different dorsal-ventral regions were further analysed separately in the anterior, intermediate and posterior divisions (Figure 3). According to the criteria used to define these four regions, only the RMS is identified in the anterior SEZ. The intermediate SEZ comprises all four regions and the posterior SEZ contains the dorsal, dorsolateral and ventral regions. While the proliferation rates of intermediate and posterior SEZ of dorsolateral and ventral regions remained constant, RMS proliferation, assessed both by Ki67 and BrdU, significantly decreased from the anterior to the intermediate divisions. In contrast, proliferation in the dorsal SEZ increased from the intermediate to the posterior division (Figure 3A and 3B).

Analysis of the Neuroblast and NSC Populations Along the SEZ Axes

The observed dissimilarities in the proliferative patterns given by the proliferation markers Ki67 and BrdU led us to discriminate which cell type population/populations could explain these findings. A 2 hours BrdU pulse labels mostly fast dividing cells, i.e., neuroblasts and transit amplifying progenitors (TAPs). In order to obtain a comprehensive view of the SEZ neuroblasts, a wholemount staining of the entire wall of the SEZ was performed. Figure 4A shows a pronounced distribution of the neuroblasts towards the dorsal part of the lateral wall equivalent to the dorsolateral SEZ. Furthermore, the estimation of the rates of neuroblasts (DCX positive cells) in the various regions showed similar rates from the anterior to the posterior SEZ (Figure 4B). Conversely, at the dorsal-ventral axis, the dorsolateral SEZ ($6.20\pm0.35\times10^3$ DCX positive cells/mm^2) displayed higher rates for neuroblasts when compared with the ventral SEZ ($2.28\pm0.27\times10^3$ DCX positive cells/mm^2) (Figure 4C). This finding is in line with the proliferative pattern referred above. Importantly, the analysis of proliferating neuroblasts (double DCX/BrdU positive cells, Figure 4F) provided a similar profile (Figure 4E). Furthermore, BrdU retaining cells double labelled with GFAP (an approach to label NSC) revealed a decreasing gradient from the dorsolateral SEZ to the ventral SEZ (Figure 4D).

Estimation of the BrdU/Ki67 Ratio throughout the SEZ Axes

To verify whether the oscillations in proliferation densities along the entire SEZ resulted from diverse mitotic rates, the ratio between BrdU and Ki67 throughout the SEZ was next determined. This ratio provides an estimation of cell cycle length since Ki67 labels all phases of the cell cycle (excluding G0), and BrdU is incorporated exclusively in the S phase [20]. It is important to note that the length of the S phase remains relatively constant whereas the G1 phase regulates cell cycle length [21]. A 2 hours BrdU pulse was given to avoid secondary cell divisions that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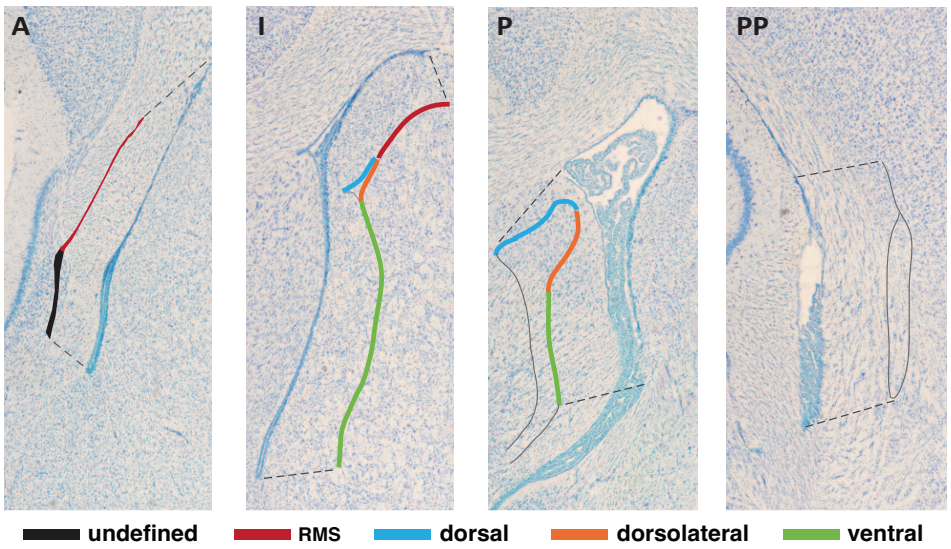
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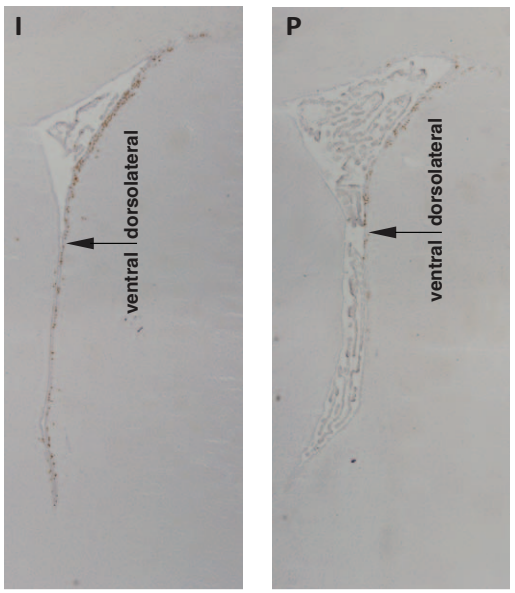
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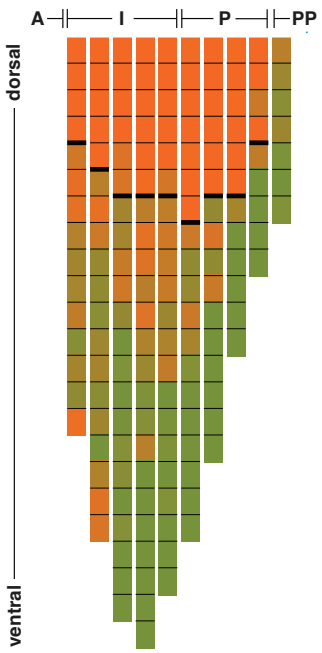
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Figure 1. Representation of the subependymal zone divisions defined at the anterior-posterior and dorsal-ventral axes. In the *upper panel* four anterior to posterior divisions are defined according to the SEZ anatomical heterogeneity along the neuraxis: anterior (A), intermediate (I), posterior (P) and post-posterior (PP). For the established divisions, regions are further defined in a dorsal to ventral SEZ orientation, as outlined in the colored traces (*middle panel*): rostral migratory stream (RMS; red trace), dorsal (blue trace), dorsolateral (orange trace), and ventral (green trace). In the anterior division of the SEZ, the area containing proliferating cells that cannot be defined as RMS is designated undefined (black trace). In the post-posterior division of the SEZ, few proliferating cells are found lining the ventricle wall and therefore no dorsal-ventral region is defined (ventricle walls outlined in grey). The topography of each region varies across the SEZ divisions (*middle panels*). Along the lateral wall of the brain ventricles proliferation decreases from the most dorsal portion to the ventral tip (*left lower panels*). Dorsolateral and ventral SEZ regions were defined, by subdividing the lateral wall of the ventricle in 150 μm-long contiguous fragments, and proliferating cells per area along the anterior to posterior axis were counted. The density of proliferating cells is graphically and spatially represented in the colored tiled map (*right lower panel*); the color scale ranges from orange to green, representing higher to lower density of proliferating cells, respectively. A pronounced decrease in the number of proliferating cells is observable at specific locations of the lateral wall defining the boundary between dorsolateral and ventral SEZ (represented by an arrow in the *left lower panels* and by a bold line in each column of the colored map). ac, anterior commissure; aca, anterior commissure, anterior part; cc, corpus callosum; DG, dentate gyrus; LV, lateral ventricle.
doi:10.1371/journal.pone.0038647.g001

would allow BrdU dilution; thus the BrdU/Ki67 ratio provides an estimation of the cell cycle length [20,22]. Interestingly, the posterior and post-posterior SEZ presented the highest BrdU/Ki67 ratio, when compared to anterior and intermediate SEZ (p?0.01)(Figure 5A). Considering the dorsal-ventral axis regionalization, again major differences were found in the BrdU/Ki67 ratio between the dorsolateral and the dorsal SEZ and RMS (40% and 45% decreased, respectively, when compared to the dorsolateral SEZ) (Figure 5B). Combined analysis of BrdU/Ki67 in the anterior-posterior and dorsal-ventral axes revealed similar results; however, the BrdU/Ki67 ratio at the ventral SEZ was lower than at the dorsolateral SEZ at intermediate levels (Figure 5C).

Estimation of the Neuroblasts Cell Cycle Length throughout the SEZ Axes

The cell cycle length estimated for the overall neuroblasts population (labeled by DCX) in the SEZ was of 26.9 (0.23) hours; this value was calculated from the parameters given by the graph of Figure 6A, (GF = 0.79, slope = 0.02957). The same analysis was performed to estimate neuroblasts cell cycle length along the anterior-posterior axis (anterior, intermediate and posterior SEZ) and dorsal-ventral axis (dorsolateral and ventral SEZ). Although no significant differences were found in the neuroblasts cell cycle length along the anterior-posterior axis [anterior, intermediate and posterior levels were 27.9 (0.28), 27.1 (0.27) and 26.6 (0.24) hours, respectively], we found a statistically significant difference between the dorsolateral and ventral SEZ [24.7 (0.31) and 28.1 (0.35) hours, respectively] at the intermediate level (Figure 6B). Dorsolateral and ventral SEZ displayed different kinetic profiles that ultimately lead to differences in the cell cycle lengths. A significant difference in the GF was observed between the dorsolateral SEZ and the ventral SEZ [0.79 (0.03) and 0.68 (0.03), respectively].

Analysis of Proliferating Cells Surrounding the SEZ

We were also interested in studying the number of cells proliferating in the vicinity of the SEZ; that is, within 100 μm apart from SEZ (Figure 7A), along the anterior-posterior axis. Data analysis indicates that the number of Ki67 proliferating cells in the SEZ vicinity decreased from anterior to posterior divisions (Figure 7B). These results were similar when analysed by BrdU labelling. When cells were labelled with BrdU (Figure 7C), the number of dividing cells in posterior SEZ (5±2) was decreased when compared either with the anterior or the intermediate SEZ (16±2 and 13±3, respectively; p?0.05); no differences were observed between anterior and intermediate SEZ.

Discussion

This study provides the first unbiased stereological analysis of the SEZ proliferative pattern throughout the anterior-posterior and the dorsal-ventral axes of the adult rat brain. For this purpose the SEZ was subdivided into anterior, intermediate, posterior and post-posterior divisions (in the anterior-posterior axis) and into RMS, dorsal, dorsolateral and ventral regions (in the dorsal-ventral axis). The analyses performed, taking into consideration these divisions, revealed substantial spatial variations on cell proliferation, cell population and cell-cycle length, which reinforce the need to establish clear topographical references - which we propose herein - for studies addressing cell population dynamics in the SEZ.

The SEZ cell population comprises three main types of cells: A, B and C. Type B cells, which are quiescent stem cells that give rise to type C cells (also known as transient-amplifying progenitors), the precursors of type A cells (neuroblasts) [23]. These last two cell types are mitotically active and comprise the majority of the SEZ cell population that is labelled by short-pulse BrdU and Ki67. Evaluation of proliferation by these markers revealed heterogeneity in cell proliferation rates in the SEZ along the dorsal-ventral and anterior-posterior axes position. Specifically, with respect to the dorsal-ventral axis, the dorsolateral SEZ displayed substantially higher proliferative rates than the ventral SEZ. In the anterior-posterior axis, the anterior SEZ exhibited the highest number of proliferating cells. Of notice, the most anterior part of the SEZ comprehends a large extension of the beginning of the RMS, classically recognized as the pathway for SEZ born neuroblasts migrating towards the olfactory bulbs [3]. The fact that neuronal precursors are converging anteriorly to this pathway prompted us to investigate the contribution of the population of neuroblasts to the increased rates of proliferation in the anterior SEZ. Neuroblasts are known to migrate in response to insult/modulation [24]. Surprisingly, no differences were found in the neuroblasts population, as assessed by the number of DCX positive cells per mm^2, at the anterior, intermediate and posterior SEZ. Conversely, at the dorsal-ventral axis the majority of the DCX positive cells were found at the dorsolateral SEZ, as observed in the DCX wholemount staining and estimated by the rates of DCX positive cells in the dorsolateral and ventral SEZ. Accordingly, the rates of proliferating neuroblasts were also reduced in ventral SEZ when compared to the dorsolateral SEZ, which is in agreement with the proliferative pattern observed herein.

As the rates of neuroblast progenitors are variable in the dorsal-ventral axis, we next asked if the stem cells from which they are derived were also differently distributed through this axis. For that purpose quiescent cells were labelled by a daily injection of BrdU over 2 weeks followed by 2 more weeks of chase to allow

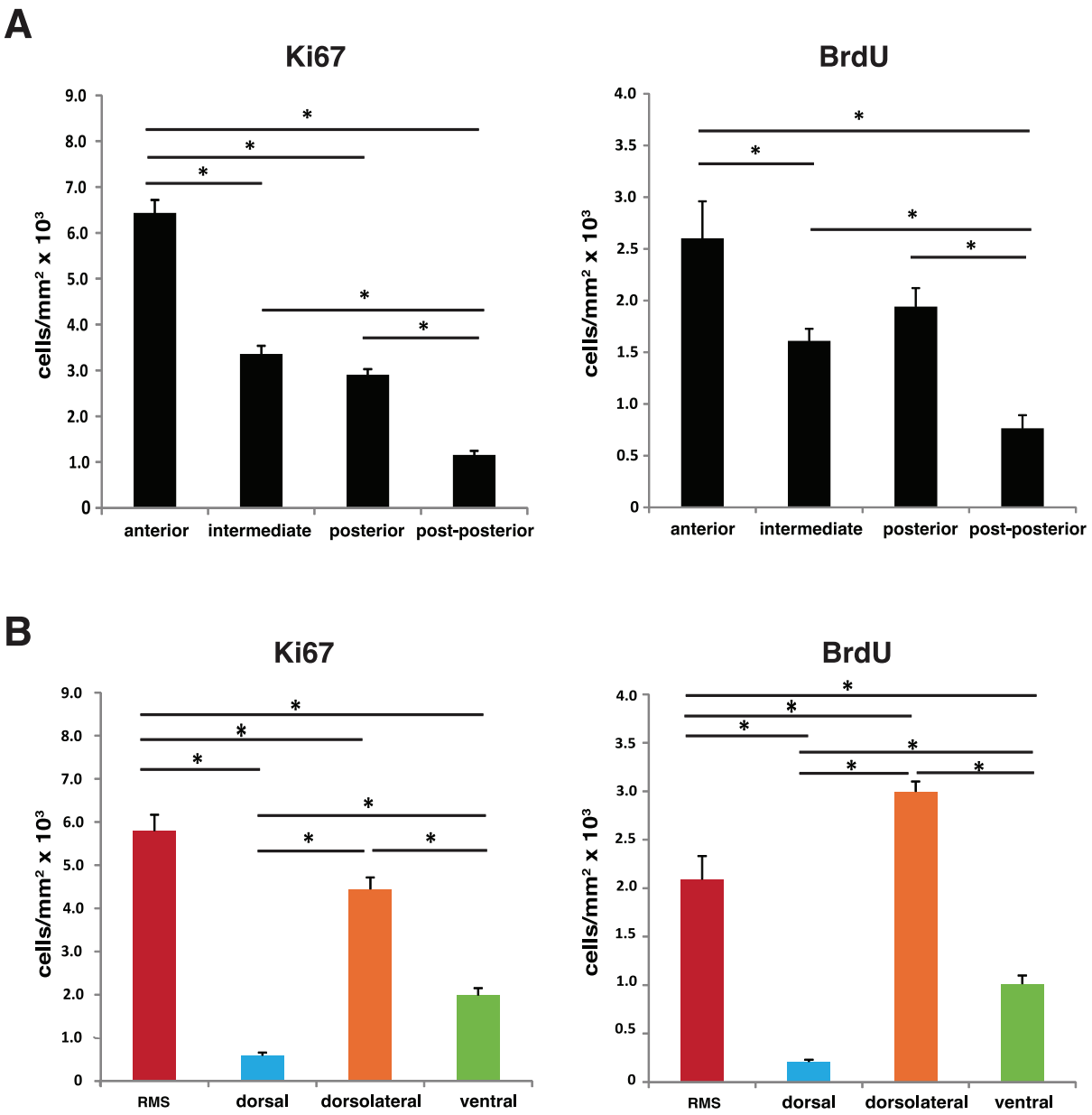

Figure 2. The subependymal zone cell proliferation pattern is dependent on the anterior-posterior and dorsal-ventral axes position. (A) SEZ total proliferation analysis throughout anterior-posterior divisions shows the highest number of Ki67 and BrdU positive cells in the anterior SEZ, decreasing along the intermediate, posterior and post-posterior levels. (B) Cell proliferation varies according to the SEZ dorsal-ventral axis position. Proliferation is expressed as number of Ki67 or BrdU positive cells per area (mm^2). The threshold value for statistical significance was set at 0.05 (* $p<0.05$).
doi:10.1371/journal.pone.0038647.g002

progenitor cells to leave the SEZ and/or dilute BrdU label. Because this method is not specific to label NSCs we further performed double staining for BrdU and GFAP, a consensual marker of NSCs [25,26]. While this approach may label astrocytes in the proliferating niche, it is unlikely that this is a major confounder since astrocytes are not described to proliferate significantly under physiological conditions [27]. Our results show a higher rate of NSCs at the dorsolateral SEZ. This finding suggests that the number of NSCs declines from dorsal to ventral regions, which is also indicative of fewer progenitors and, thus, less proliferation. Our results are in agreement with a study that described a higher frequency of pinwheels (another method to label type B stem cells) [28] at the most dorsal part of the lateral wall, which corresponds to the herein designated dorsolateral SEZ.

Interestingly, we observed highly divergent proliferation rates along the dorsal-ventral axis. Dorsal SEZ exhibited the lowest proliferation rate of all four regions. In contrast, the dorsolateral region of the SEZ displayed the highest proliferative rate and BrdU/Ki67 ratio when compared tothe RMS, dorsal, and ventral SEZ (at intermediate levels) suggesting faster cell cycles in this region. Accordingly, the cell cycle length for DCX positive cells of the dorsolateral SEZ was confirmed to be shorter than that of the ventral SEZ. Furthermore, the rate for proliferating neuroblasts (GF) at the ventral SEZ was considerably lower than at the

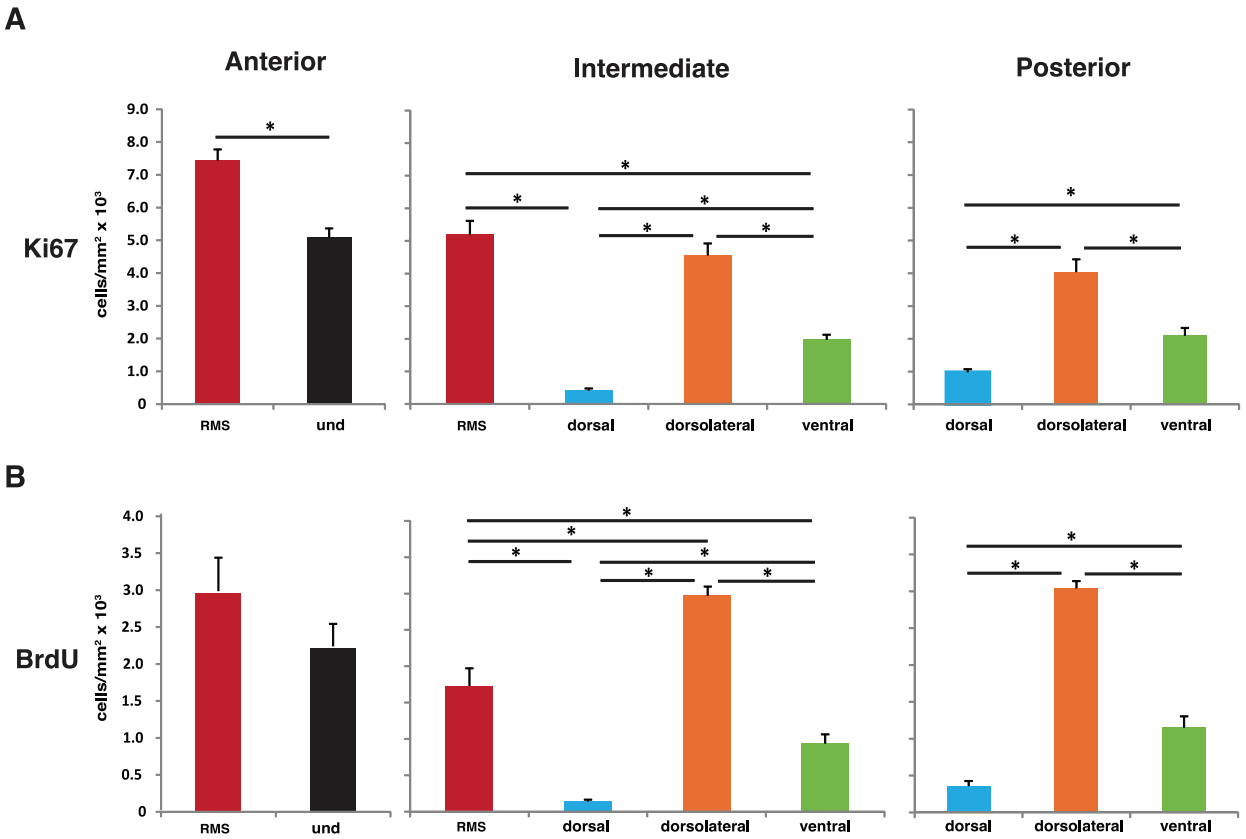

Figure 3. Combinatorial analysis of cell proliferation in the subependymal zone anterior-posterior and dorsal-ventral axes. The proliferation rate in the different dorsal-ventral regions was assessed at the anterior, intermediate and posterior levels either with Ki67 **(A)** or BrdU **(B)**. Proliferation pattern analysis in dorsal-ventral SEZ regions along the defined anterior to posterior axis revealed that proliferation in the RMS significantly decreased from the anterior to the intermediate division. Cell proliferation in the dorsal, dorsolateral and ventral regions was not significantly affected in the intermediate to posterior divisions transition. Proliferation is expressed as number of Ki67 and BrdU positive cells per area (mm^2). The threshold value for statistical significance was set at 0.05 (* $p<0.05$).
doi:10.1371/journal.pone.0038647.g003

dorsolateral SEZ. These results reinforce the dissimilarities between the neuroblasts populations at the lateral wall.

The mitotic rates were also determined for the anterior-posterior axis. Intriguingly, the BrdU/Ki67 ratio is augmented at the posterior and post-posterior SEZ, suggesting that the cell cycle length is shortened in the most posterior portions of the SEZ, even though the proliferation rate is inferior or equivalent to that in the anterior and in the intermediate SEZ, respectively. Also, the DCX positive cell cycle lengths were not statistically significant different at the anterior, intermediate and posterior SEZ. Most likely the TAPs are also contributing to the observed BrdU/Ki67 ratio**,** even thought there was a trend in the neuroblast population to shorten the cell cycle length at more posterior levels. Although differences in NSCs proliferation along the anterior RMS have been shown (stem cells derived from distal rostral extensions of the SEZ, i.e., near the olfactory bulbs proliferate significantly more slowly than caudally placed RMS cells) [9], the same has never been shown for the SEZ.

Notably, an *in vitro* study showed that the number of label-retaining cells (commonly used to identify putative stem cells in the adult brain) obtained from 400 µm thick slices declines in posterior regions [14]. Similarly, higher frequency of pinwheels is found at the more anterior levels of the SEZ [28]. All together these observations suggest that the increased rates of proliferation at anterior levels may result from an increase in the NSCs population.

To the best of our knowledge this is the first study reporting distinct gradients in cell proliferation along the dorsal-ventral axis of the rat SEZ; it is interesting to note that it recapitulates the domains containing different types of progenitors in the germinal zone [15]. Moreover, we have estimated for the first time the cell cycle length for the neuroblasts, which is approximately 27 hours. The cell cycle length for the entire SEZ population has been estimated to be approximately 19 hours [29,30]. Of interest, this discrepancy in time is certainly a consequence of the heterogeneity in the populations that constitute the SEZ [31], as highlighted here. In addition, it further suggests that the neuroblasts display longer cell cycles than TAPs. In fact, a short pulse BrdU labels approximately only 35% of DCX positive cells; the remaining 65% are other cellular types, mostly TAPs. Our data provides indication that the TAPs display the shorter cell cycle length of the SEZ population.

The novel methodological approach we propose here to characterize the SEZ cell population dynamics allowed a combined proliferation analysis along the anterior-posterior and dorsal-ventral axes. This approach highlighted the variations in proliferation along SEZ axes as well as the individual specificities of each dorsal-ventral region in the context of the overall SEZ proliferative rates at anterior-posterior divisions. For instance, the

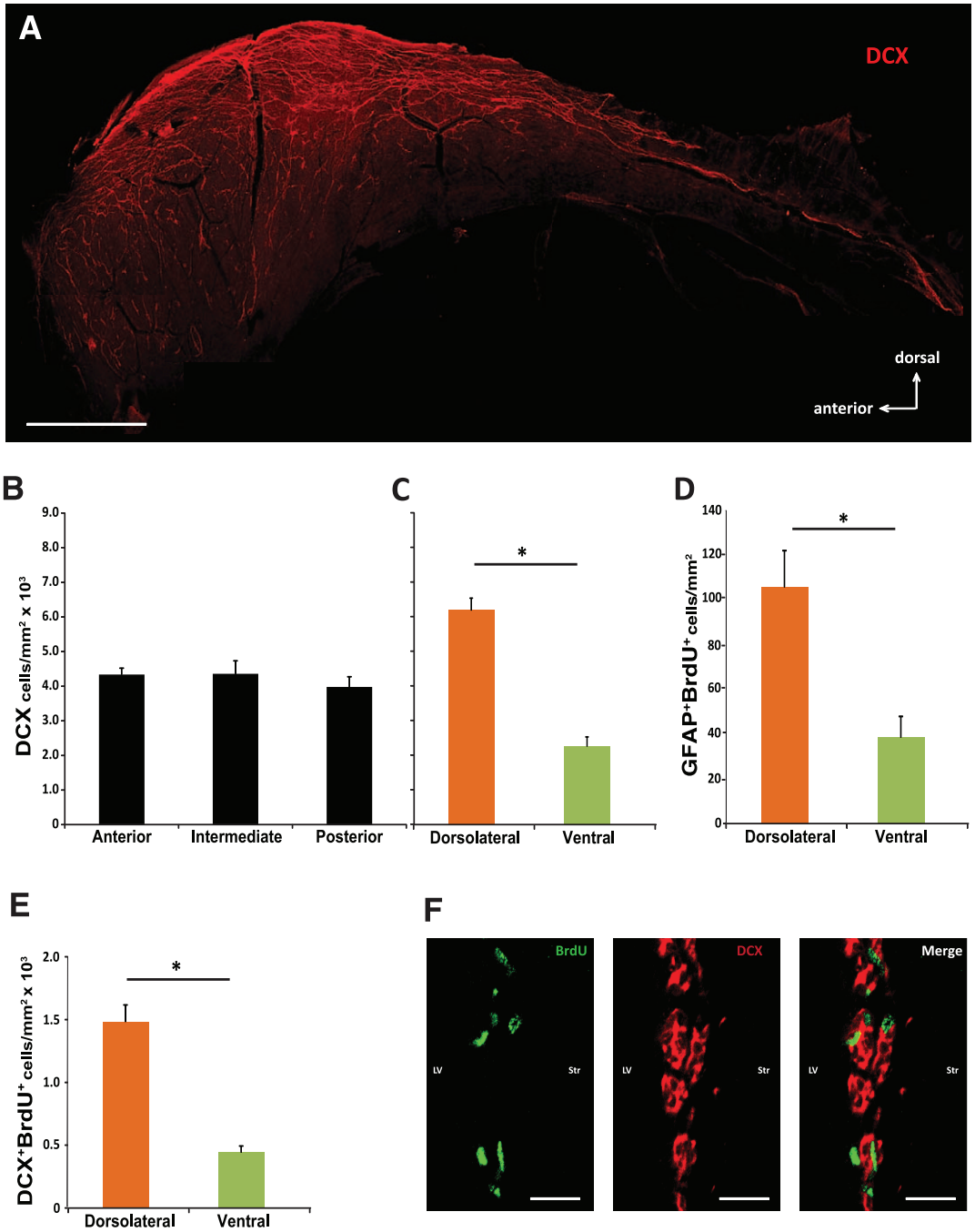

Figure 4. Neural stem and progenitor cells decrease along the subependymal zone dorsal-ventral axis. A DCX wholemount staining for the lateral wall is represented in **(A)** (Scale bar = 1 mm). DCX positive cell rates were estimated through the lateral wall for anterior, intermediate and posterior SEZ **(B)**, dorsolateral and ventral SEZ **(C)**. BrdU retaining cells were double stained with GFAP and assessed in the dorsolateral and ventral SEZ **(D)**. The same analysis was performed for proliferating neuroblasts (double BrdU/DCX positive cells) **(E)**. The images for the BrdU, DCX and BrdU/DCX staining are represented in **(F)** (Scale bar = 20 μm). LV, lateral ventricle; Str, striatum. All results are expressed as number of positive cells per area (in mm^2). The threshold value for statistical significance was set at 0.05 (* $p<0.05$).
doi:10.1371/journal.pone.0038647.g004

RMS proliferative pattern is not uniform along the SEZ, diminishing from the anterior to the posterior coordinates. On the other hand, dorsal SEZ rates of proliferating cells are higher in the posterior SEZ.

The present observations support the view that the SEZ stem cell niche is more than the initially thought thin layer of cells lining the anterior wall of the lateral brain ventricles. Besides this well-defined niche, the most ventral portion of the lateral wall [18], the RMS [9], the dorsal and the entire lateral wall of the lateral ventricles [8,18], display progenitor cells that ultimately generate new neurons. Most importantly, it confirms dissimilarities between adult NSCs along the anatomical axes [15,18,31,32]; as an example, it was demonstrated that different olfactory bulb interneurons are derived from specific locations in the SEZ [17].

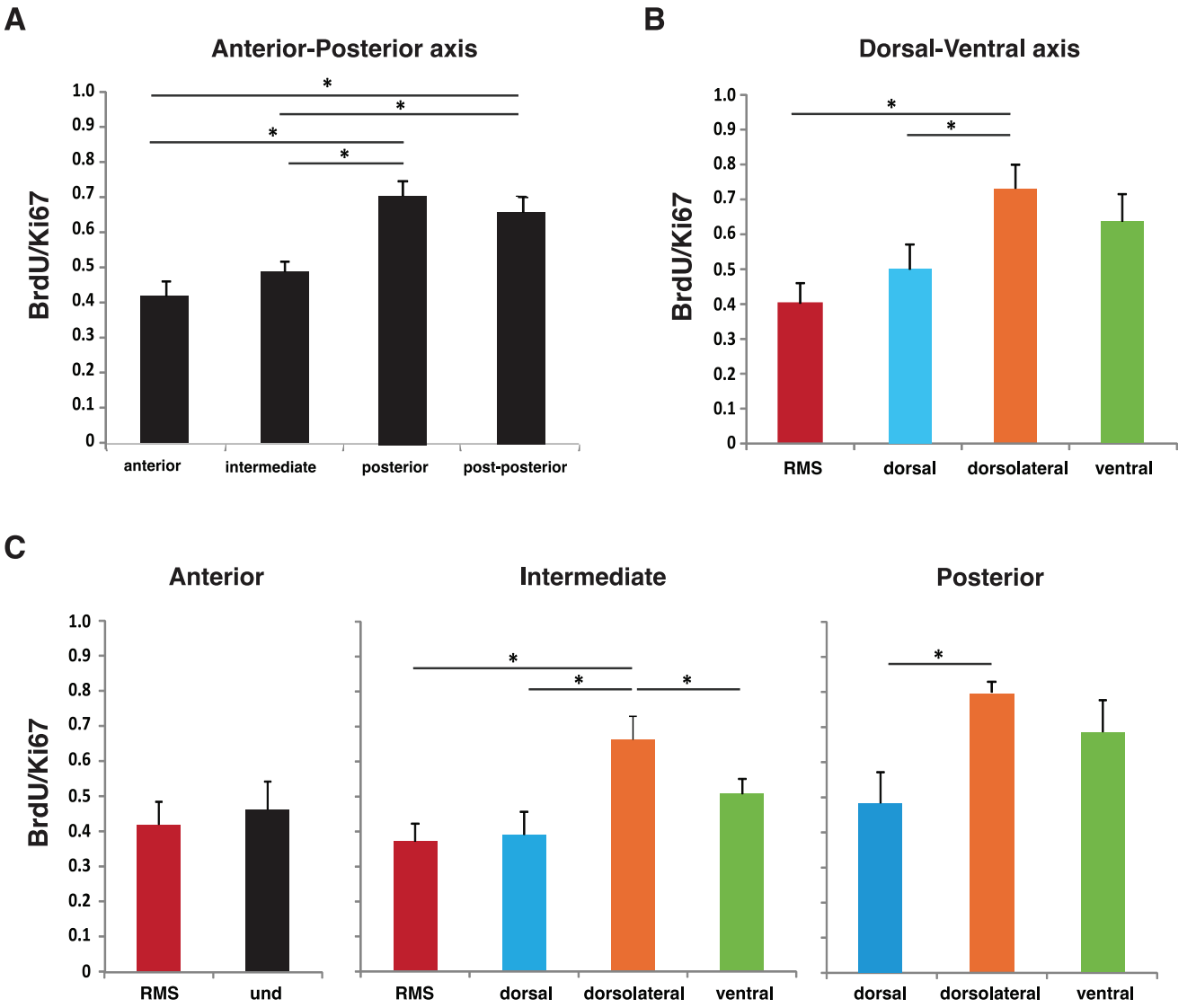

Figure 5. The BrdU/Ki67 ratio differs throughout the subpendymal zone. The SEZ total BrdU/Ki67 ratio is represented for the anterior-posterior **(A)** and dorsal-ventral axes **(B)**. For the different dorsal-ventral regions the BrdU/Ki67 ratios were assessed at the anterior, intermediate and posterior levels **(C)**. The threshold value for statistical significance was set at 0.05 (* $p<0.05$).
doi:10.1371/journal.pone.0038647.g005

As a consequence, we propose the existence of a spatial code of SEZ progenitors. This spatial code matches the regional proliferation pattern we found along the dorsal-ventral axis, supporting the concept that the spatial regionalization observed in the adult SEZ partially relates to its embryonic origin and to the distinct transcription factor expression profiles throughout the SEZ dorsal-ventral axis [15].

Adult NSCs scattered throughout the SEZ give rise to neuroblasts that converge into the RMS and migrate tangentially to the olfactory bulb [3]. However, numerous studies report the occurrence of non-tangential migration of SEZ born cells in non-physiological conditions [33]. We show here that, even in physiological conditions, there are cells proliferating in the vicinity of the SEZ that may derive from the SEZ niche. Our data demonstrate that the number of these proliferating cells under basal conditions increases towards the anterior SEZ in the same manner as SEZ proliferation. Although the fate of these proliferating cells remains to be elucidated, it is known that they increase in response to brain insults, as many SEZ derived neuronal progenitors leave the SEZ and migrate towards areas of damage [33,34]. Assuming that some of these proliferating cells are SEZ born, we here describe a standardized method to assess non-tangential migration that should be considered in studies comprising the migration of cells outside the SEZ, in both physiological and pathological conditions.

In conclusion, this study indicates that the prevalent analysis of lateral wall of the lateral brain ventricles [35–38] as a proxy of the entire SEZ is biased and lacks precision as it overshadows highly relevant SEZ region specific differences. As these regional differences might also translate functional implications, their knowledge is of relevance to the development of regenerative strategies conveying the usage of endogenous SEZ cells. Thus we propose herein a SEZ topographical division model (Figure 1) that takes into consideration regional differences along the SEZ axes that will be useful to normalize and compare the results on various experimental models that assess SEZ cell dynamics.

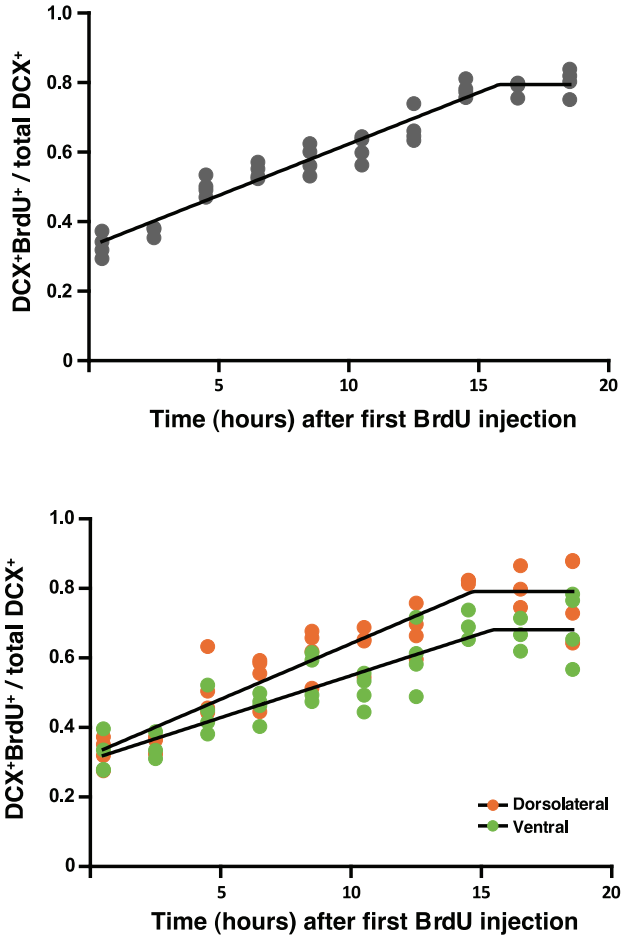

Figure 6. Estimation of the cell cycle length of the DCX positive cell population reveals differences between dorsolateral and ventral subependymal zone at intermediate levels. A cumulative BrdU labeling protocol was performed to determine cell cycle length for DCX positive cells. The time points for BrdU injections are plotted against the percentage of the total DCX population (DCX positive cells) that is proliferating (double DCX/BrdU positive cells) at each time point. When this percentage is constant (the graphic reaches a plateau) it is named Growth Fraction (GF). The parameters to calculate cell cycle length (Tc) are obtained from the following parameters: GF and slope of the first linear fragment. This procedure was performed for DCX positive cells from the entire SEZ (A) or from dorsolateral and ventral SEZ at intermediate levels separately (B).
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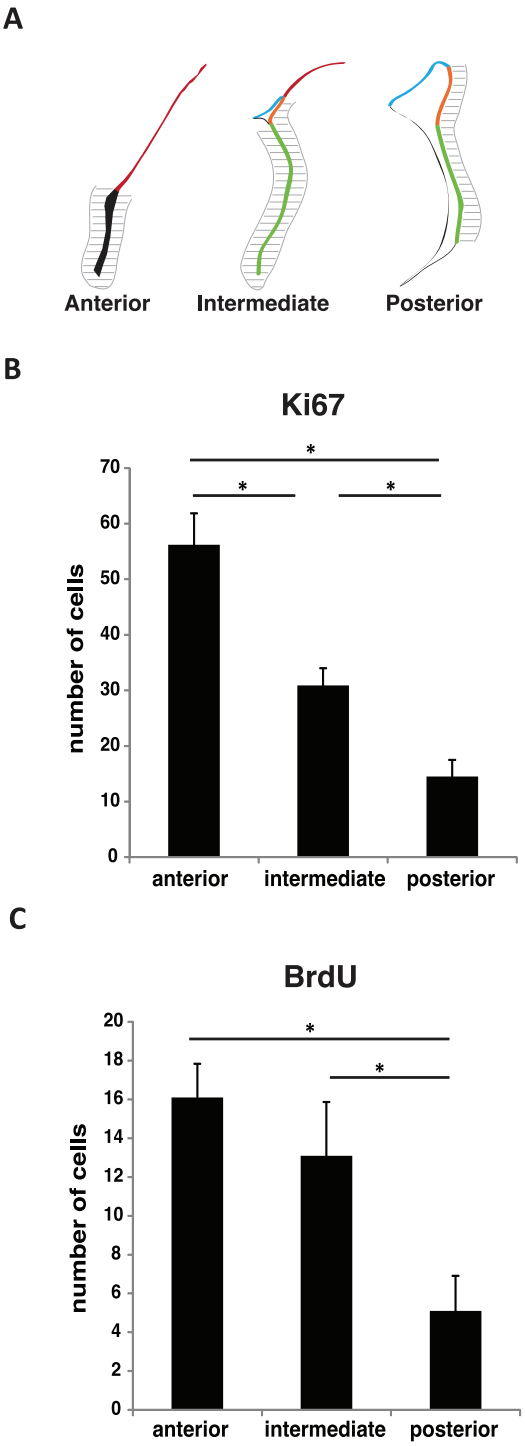

Figure 7. The number of cells proliferating in the vicinity of the subependymal zone decrease along the anterior-posterior axis. (A) An area within a distance of 100 µm apart from the SEZ was defined in the anterior, intermediate and posterior divisions. Ki67 (B) and BrdU (C) positive cells located in the area surrounding the SEZ, as illustrated in (A), were counted. Results are represented as number of Ki67 or BrdU positive cells per section. The threshold value for statistical significance was set at 0.05 (* $p<0.05$).
doi:10.1371/journal.pone.0038647.g007

Materials and Methods

Ethics Statement

This study was approved by the Portuguese national authority for animal experimentation, Direcção Geral de Veterinária (ID: DGV9457). Animals were kept and handled in accordance with the guidelines for the care and handling of laboratory animals in the Directive 2010/63/EU of the European Parliament and of the Council.

Animals

All experiments were conducted in 10-week-old male Wistar rats (Charles River, Barcelona, Spain). Animals were maintained in 12 hours light/dark cycles at 22 to 24°C and 55% humidity and fed with regular rodent's chow and tap water *ad libitum*. To reduce stress-induced changes in the hypothalamus–pituitary axis associ-

ated with the injection, all animals were daily handled for 1 week until the day of sacrifice.

Administration of 5-bromo-2′-deoxyuridine (BrdU) for Proliferation Assessment and for BrdU Label Retaining Cells Estimation

For the purpose of SEZ proliferation assessment 5 animals were administered with BrdU (50 mg/Kg) intraperitoneally (*ip*) and sacrificed 2 hours later. This protocol labels SEZ fast dividing cells.

To label a quiescent pool of cells at the SEZ a group of 4 animals were daily injected with BrdU (50 mg/Kg) *ip* for 2 weeks followed by another 2 weeks period of chase. The progeny of stem cells that exit the cell cycle and retain the BrdU labelling exit the SEZ during the chase period.

Cumulative BrdU Labelling for Cell Cycle Length Analysis

To estimate the cell cycle length of the SEZ neuroblasts population a protocol based on that previously established by Nowakowski et al [39] was performed. In accordance, three assumptions were made: 1) the proliferating population is part of a single asynchronous population 2) it is growing at a steady state and 3) there are not non-proliferating cells to consider. Based on these assumptions different groups of rats were progressively exposed to a series of BrdU injections. A total of 40 animals (n = 4 in each goup) were injected with BrdU (50 mg/Kg) *ip* at 2 hours intervals (up to a maximum of 10 time points), in a total period of 18 hours. The last BrdU injection was followed by a 0.5 hour delay before sacrifice, which allowed unlabelled proliferating cells to enter the S phase and incorporate BrdU. Thus, the first group, time point 0.5 hour, had a single BrdU injection, whereas the last group, time point 18.5 hours, received ten BrdU injections.

The interval between BrdU injections has to be shorter than the time of the S phase (Ts) to ensure that every cell that passes through the S phase incorporates BrdU at least once. This cumulative BrdU labeling will ultimately lead to saturation on the BrdU labeling of the proliferative population. At this stage every proliferating cell has incorporated BrdU and therefore a plateau is reached. At the end of the analysis a graph is obtained where the time points of BrdU injections are plotted against the percentage of the total population that is proliferating at each time point. When this percentage is constant (the plateau), it is named Growth Fraction (GF). A least squares approach was performed by using the segmental linear regression data fit. The parameters taken from the graph were used to calculate the cell cycle length (Tc). Tc was calculated from the equation slope = GF/Tc [39,40] where GF is the growth fraction and the slope corresponds to the slope of the first linear segment. This procedure was performed for each of the SEZ regions determined in this study.

Immunohistochemistry

Animals were anesthetized with sodium pentobarbital and transcardially perfused with cold saline for the stereological analysis of the SEZ and with 4% paraformaldehyde (PFA) in 0.01 M PBS for fluorescence immunohistochemistry. Brains were removed, embedded in O.C.T. compound and snap-frozen; serial coronal sections (20 μm) were cut in a cryostat and collected to slides for immunohistochemistry.

For the stereological analysis of the SEZ slides were post-fixed in 4% PFA in 0.01 M PBS for 30 min and antibodies against markers that evaluate cell proliferation were used: BrdU at a dilution of 1:50 (Mouse Anti-Bromodeoxyurine, Clone Bu20a, DAKO, Spain) and Ki67 (an endogenous marker of cell proliferation) at a dilution of 1:100 (Ki67 antigen, rabbit polyclonal antibody, Novocastra, UK). Primary antibodies were detected by the Ultravision Detection System (Lab Vision, Freemont, CA), and the reaction developed with 3,3'-diamino-benzidine substrate (Sigma Aldrich, St.Louis, MO, USA); sections were subsequently counterstained with hematoxylin.

Fluorescence immunohistochemistry was performed to label proliferating neuroblasts (double BrdU/DCX positive cells), neuroblasts (DCX positive cells) and neural stem cells (double BrdU/GFAP positive cells). The following antibodies against markers of SEZ populations were used: doublecortin (DCX) (rabbit polyclonal to doublecortin -neuroblast marker, Abcam, UK) at a dilution of 1:500 and glial fibrillary acidic protein (GFAP) (polyclonal rabbit anti-GFAP, DAKO, Spain) at a dilution of 1:100 together with BrdU (rat anti-BrdU, BU1/75 clone, Abcam) at a dilution of 1:100. Fluorescent secondary antibodies (Invitrogen, Carlsbad, CA, USA), anti-rabbit and anti-rat were used to detect the primary antibodies at a dilution of 1:1000. To label the nucleus, incubation with 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) at a dilution of 1:1000 was performed. Primary and secondary antibodies were diluted in PBS-0.5%Triton/10% FBS and incubated overnight at 4°C, for the primary antibody, and 2 hours at room temperature, for the secondary antibody.

Wholemount Staining

Wholemount staining for DCX was performed according to the technique described by Mirzadeh et at [41]. Briefly, the entire lateral wall, from rat brains perfused with cold saline, was dissected under a stereomicroscope and incubated in 4% PFA-0.5% Triton overnight at 4°C. Primary (anti-DCX 1:250, Abcam) and secondary (1:500, Invitrogen) antibodies were each incubated for 2 days at 4^{a}C.

Stereology

Estimation of cell density in the different regions of the SEZ was obtained using the Visiopharm Integrator system (VIS) software in an Olympus BX51 microscope (Olympus, Hamburg, Germany). Coronal sections for proliferation analysis comprised SEZ between bregma coordinates 2.28 mm and −3.60 mm [42].

Proliferation in the SEZ was assessed by Ki67, an endogenous marker expressed during all phases of mitosis, except for the resting phase G0 [43]; and by the exogenous marker BrdU, a thymidine analogue that is incorporated in the DNA during the S phase. The number of Ki67 and BrdU positive cells was counted and results expressed as Ki67 or BrdU positive cells per area (in mm^2). Every sixteenth section from the anterior SEZ, bregma 2.28 mm (at this level the initial section was randomly selected to certify unbiased sampling), until posterior SEZ, bregma −3.60 mm, was analysed. The use of the VIS Software allowed delimitation, at low magnification (40×), of the areas of interest in the SEZ and the counting of Ki67 or BrdU positive cells within the defined areas at high magnification (400×). The divisions of the SEZ in the anterior-posterior axis were defined between bregma coordinates 2.28 mm and −3.60 mm (Figure 1, upper panel). Table 1 summarizes the anatomical criteria used to define anterior, intermediate, posterior and post-posterior SEZ. The SEZ anterior division starts at the beginning of the genu of the corpus callosum where a very well defined ependymal layer is observed and finishes at the end of the genu of the corpus callosum (bregma 2.28 mm to 1.44 mm); intermediate SEZ begins with the end of the genu of the corpus callosum and extends up to the decussation of the anterior commissure (bregma 1.44 mm to 0.12 mm); the posterior division of the SEZ begins at the decussation of the anterior commissure and extends to the beginning of the hippocampus, bregma −1.72 mm; the post-

Table 1. Anterior-posterior axis landmarks of the SEZ divisions.

SEZ	Bregma coordinates (mm)	Anatomical references
Anterior	[2.28; 1.44[	From the beginning to the end of the genu of the corpus callosum
Intermediate	[1.44; −0.12[	From the end of the genu of the corpus callosum to the decussation of the anterior commissure
Posterior	[−0.12; −1.72[	From the decussation of the anterior commissure to the beginning of the hippocampus
Post Posterior	[−1.70; −3.60]	From the beginning of the hippocampus to the fusion of the dorsal and ventral parts of the lateral ventricle

Bregma coordinates are according to Paxinos & Watson (2004) [42].
doi:10.1371/journal.pone.0038647.t001

posterior division extends up to bregma −3.60 mm. From this position on, sparse proliferating cells were detected in the SEZ.

Dorsal-ventral axis regionalization was performed as follows (see also Figure 1, middle panel). The dorsal SEZ located in the upper part of the lateral ventricles and the beginning of the RMS at the dorsal corner of the lateral wall. For the ventricles' lateral wall of the SEZ the analysis was extended by evaluating for the presence of a gradient in the proliferation rate along the dorsal to ventral extension of this region. Specifically, the lateral wall of the SEZ was subdivided into contiguous 150 μm-long fragments at its length (coloured tiled map on the right of Figure 1 lower panel). The proliferation for each fragment was determined and plotted according to the dorsal-ventral axis position. This analysis comprised the intermediate and posterior SEZ and it further allowed for the division of the lateral wall into the ventral and the dorsolateral SEZ regions, illustrated in Figure 1 (middle and lower panels).

Confocal Imaging and Quantitative Analysis

To estimate the number of neuroblasts (DCX positive cells) and proliferating neuroblasts (double DCX/BrdU positive cells) from the cumulative BrdU labelling along the anterior-posterior and dorsal-ventral axes, 6 sections per animal (2 sections at anterior levels, 2 at intermediate levels and 2 at posterior levels) were analysed. For each section, pictures were taken for the entire lateral wall of the SEZ using a confocal microscope (FV1000; Olympus) and the total number of DCX positive cells and the number of double DCX/BrdU positive cells was counted. The percentage of proliferating neuroblasts was calculated using the ratio double DCX/BrdU positive cells/total DCX positive cells.

For the single pulse BrdU labelling, double DCX/BrdU positive cells through the lateral wall, i.e., at dorsolateral and ventral regions, were counted. The rate for proliferating neuroblasts was estimated by dividing the number of double positive cells for the corresponding area. The areas were determined using the Image J software.

To estimate the total number of DCX positive cells throughout the SEZ, the same procedure as described above was performed. The DCX rates were estimated by dividing the number of double positive cells by the corresponding area.

For the BrdU label retaining cells, the rates for double GFAP/BrdU positive cells throughout the lateral wall, i.e., at dorsolateral and ventral regions, were estimated as described above for the single pulse BrdU labelling (double DCX/BrdU positive cells). The number of double GFAP/BrdU positive cells was divided for the corresponding area.

Confocal images of wholemount preparations of the lateral wall were taken with a 10× objective.

Statistical Analysis

Data [presented as the mean (± SEM) or time (SE)] was analysed with GraphPad PRISM 5 software (GraphPad Software Inc., San Diego, CA). The analysis consisted of one-way analysis of variance (ANOVA) with Bonferroni multiple comparison post-test analysis for single-factor multiple group comparisons to determine differences between three or more groups or Student's t test for two-group comparisons. To compare Tc between different regions a Z statistic test [40] was used. The threshold value for statistical significance was set at $p<0.05$ and $Z>1.96$.

Acknowledgments

Ana Mendanha Falcão and Fernanda Marques are recipients of PhD and post-doctoral fellowships, respectively, from Fundação para a Ciência e Tecnologia.

Author Contributions

Conceived and designed the experiments: AMF JAP NS JCS. Performed the experiments: AMF ACF FM. Analyzed the data: AMF JAP NS JCS. Contributed reagents/materials/analysis tools: JAP NS JCS. Wrote the paper: AMF JAP NS JCS.

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3. The subependymal zone proliferative pattern and progenitor cell distribution in mice: species matters!

The subependymal zone proliferative pattern and progenitor cell distribution in mice: species matters!

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Abstract

The subependymal zone (SEZ), the major neurogenic niche of the adult brain, responds to brain insults and is modified in many neurodegenerative diseases, eliciting expectations for the development of new endogenous regenerative therapies that ultimately envisage human applications. Nevertheless, much remains to be done to fully understand the regulation and the potential of the SEZ cells both in physiological and non-physiological conditions. So far, the most widely used models to study adult neurogenesis in the SEZ are rodents. Interestingly, when comparing the SEZ niche in different species, differences are observed. To translate studies from animal models to humans for clinical applications, it is necessary to understand the SEZ species-specific variations. In the past few years progress was made in understanding the SEZ niche heterogeneity in rodents. Given our previous findings on the topographical mapping of adult rat SEZ proliferation, we now extend the study to the adult mouse brain. We found no differences in the proliferation in the anterior, intermediate and posterior SEZ and along the lateral wall. However, we report relevant differences between two close related species, rats and mice that should be taken into account when extrapolating data on the SEZ from mice to rats and vice-versa.

Key words

Subependymal zone, mice, species-specific, variability

Introduction

The occurrence of neural stem cells (NSCs) in discrete zones of the adult brain has been reported in several mammals (Barker et al 2011). Generally, NSCs are confined to two major brain areas: the subependymal zone (SEZ) lining the lateral wall of the lateral ventricles and the subgranular zone (SGZ) of the hippocampal dentate gyrus (Ming & Song 2005). The most well recognized fate for SEZ born progenitor cells is the olfactory bulbs (OB) where they integrate into circuitries mostly as GABAergic interneurons (Lois & Alvarez-Buylla 1994). Although the presence of NSCs in the lateral ventricles is transversal to all mammalian species, the formation of new functional adult olfactory neurons has only been unequivocally described in rodents (rat and mice) and in some primates (Lindsey & Tropepe 2006). Importantly, the amount of proliferation and new neurons integrated in the OBs is variable across species. When compared to rodents, primates display a substantial decrease both in the number of NSCs in the SEZ and in the olfactory neurogenesis (Azim et al 2012). Interestingly, a recent study relying on ^{14}C birth dating analysis of adult human olfactory neurons has demonstrated the limited, if any, existence of new olfactory neurons in adult human OBs (Bergmann et al 2012). Additionally, other studies demonstrated that adult neurogenesis in humans seems limited to the childhood (Sanai et al 2011). The major variation in NSCs occurrence and fate across species indicates its intrinsic species-specific heterogeneity and ultimately may reflect distinct functional adaptations. Regardless of species, SEZ alterations are observed in many pathophysiological conditions (Curtis et al 2007). As an example, following ischemic stroke enhanced proliferation is observed in the ipsilateral SEZ both in human and in rodents (Marti-Fabregas et al 2010). These observations highlight an additional role for NSCs of the adult mammalian SEZ as a reservoir of cells for regenerative purposes. Therefore, many efforts have been concentrated in understanding the potentiality and modulation of NSCs in the adult brain.

Due to a short life span, together with high reproduction rates and known genetic background, rodents are the most widely used models to study adult neurogenesis either in physiological conditions or in the context of disease. Generally, it is expected that adult neurogenesis follow the same rules between closely related species. For instance, rats and mice belong to the same sub-family and results obtained from one species are often extrapolated to the other. However, there is evidence for variability between NSCs from rat and mouse. Specifically, *in vitro* experiments have revealed that neurospheres isolated from the entire brain displayed different properties

between mice and rat (Ray & Gage 2006). Furthermore, hippocampal derived neurospheres from rat and mice exhibit significant differences in proliferative capacity and differentiation fate (Steffenhagen et al 2011). Strengthening the *in vitro* data, *in vivo* work demonstrated differences in adult hippocampal neurogenesis between rats and mice. Specifically, newly generated hippocampal neurons matured more rapidly and were more frequent in rat than in mice (Snyder et al 2009). Also of notice, radial glial tubes forming the rostral migratory stream (RMS), the path of neuroblasts migration from the SEZ to the OBs, are more complex in rats than in mice (Peretto et al 2005). Altogether, these findings raise questions about the differences across close related species in the intrinsic heterogeneity and potential of NSCs, as well as their extrinsic regulation.

Despite some reports about distinctive hippocampal neurogenesis in rat and mouse, data is missing on these potential differences in adult SEZ neurogenesis between these two species. We have recently described that the proliferative profile of SEZ cells throughout the lateral ventricles in rat varies in the anterior-posterior and dorsal-ventral axes and that the NSCs are heterogeneously distributed in the SEZ axes (Falcao et al 2012) (Chapter 2). In the present work, we characterized the proliferative pattern and NSCs distribution in mice.

Materials and Methods

Ethics statement

This study was approved by the Portuguese national authority for animal experimentation, Direção Geral de Veterinária (ID: DGV9457). Animals were kept and handled in accordance with the guidelines for the care and handling of laboratory animals in the Directive 2010/63/EU of the European Parliament and of the Council.

Animals

All experiments were conducted in 10-weeks-old male C57BL/6 mice (Charles River, Barcelona, Spain). Animals were maintained in 12 h light/dark cycles at 22 to 24°C and 55% humidity and fed with regular rodent's chow and tap water *ad libitum*. To reduce stress-induced changes in the hypothalamus–pituitary axis associated with 5-bromo-2'-deoxyuridine (BrdU) injections, all animals were daily handled for 1 week until the day of the experimental procedure.

Administration of BrdU for proliferation assessment and for BrdU label retaining cells estimation

For the purpose of SEZ proliferation assessment, five animals were administered with BrdU (a thymidine analogue that is incorporated in the DNA during the S phase; 50mg/Kg) intraperitoneally (*i.p.*) and sacrificed 2h later. This protocol labels SEZ fast dividing cells. To assess neuroblast proliferating cells an additional group of 5 animals was injected with BrdU and sacrificed 2h later.

To label a quiescent pool of cells at the SEZ, a group of four animals was daily *i.p.* injected with BrdU (50mg/Kg) for 2 weeks followed by another 2 weeks chase period. The neural stem cell progeny that exit the cell cycle and retain the BrdU labelling leaves the SEZ during the chase period.

Immunohistochemistry

Animals were anesthetized with ketamine hydrochloride (150mg/kg) plus medetomidine (0.3mg/kg), and transcardially perfused with either cold saline for the stereological analysis of the SEZ, or with 4% paraformaldehyde (PFA) in 0.01M PBS, a pre-fixation of the brain, for fluorescence immunohistochemistry. Brains were removed, embedded in O.C.T. compound and snap-frozen; serial coronal sections (20 μm) were cut in a cryostat and collected to slides for

immunohistochemistry.

For the stereological analysis of the SEZ, animals injected with a single pulse of BrdU were analysed by immunohistochemistry. Briefly, slides were post-fixed in 4% PFA in 0.01M PBS for 30 min and BrdU or Ki67 antibodies, to assess proliferation, were used at a dilution of 1:100 (rat Anti-Bromodeoxyurine, BU1/75 (ICR1) clone, Abcam, UK; Ki67 antigen, rabbit polyclonal antibody, Novocastra, UK). For BrdU staining, a pre-treatment of 30 min in HCl 2N was required. For Ki67 an additional antigen retrieval step (in 10 mM sodium citrate buffer at pH 6) was performed. Primary antibodies were detected with the Ultravision Detection System (Lab Vision, Freemont, CA, USA), and the reaction developed with 3,3'-diaminobenzidine substrate (Sigma, St.Louis, MO, USA); sections were subsequently counterstained with hematoxylin.

Fluorescence immunohistochemistry was performed to label proliferating neuroblasts [double BrdU/doublecortin (DCX) positive], neuroblasts (DCX positive cells) and neural stem cells [double BrdU/ glial fibrillary acidic protein (GFAP) positive]. Antibodies against the following markers of SEZ populations were used: DCX (rabbit polyclonal to doublecortin-neuroblast marker, Abcam) at a dilution of 1:500 and GFAP (polyclonal rabbit anti-GFAP, DAKO, Spain) at a dilution of 1:100, together with BrdU (Abcam) at a dilution of 1:100. Fluorescent secondary antibodies (Invitrogen, Carlsbad, CA, USA), anti-rabbit and anti-rat were used to detect the primary antibodies at a dilution of 1:1000. To label the nucleus, incubation with 4',6-diamidino-2-phenylindole (DAPI; Sigma) at a dilution of 1:1000 was performed. Primary and secondary antibodies were diluted in PBS-0.5%Triton/10% FBS and incubated overnight at 4°C, for the primary antibody, and 2h at room temperature for the secondary antibody.

Wholemount staining

Wholemount staining for DCX and Ki67 was performed as previously described (Mirzadeh et al 2010). Briefly, the entire lateral wall from rat and mouse brains perfused with cold saline was dissected under a stereomicroscope and incubated in 4% PFA-0.5% Triton overnight at 4°C. For Ki67 staining, wholemounts were incubated in 10 mM sodium citrate buffer (at pH 6) at 80°C for 30 min. Primary (anti-DCX 1:250 Abcam, anti-ki67 1:100 Novocastra) and secondary (1:500, Invitrogen) antibodies were each incubated for 2 days at 4°C.

Stereology

Estimation of cell density in the different regions of the SEZ was performed as described before (Falcao et al 2012). Coronal sections for proliferation analysis comprised SEZ between bregma coordinates 1.18mm and -1.94mm. The number of BrdU or Ki67 positive cells and the areas were estimated by means of the Visiopharm Integrator system (VIS) software in an Olympus BX51 microscope (Olympus, Hamburg, Germany). Results were expressed as BrdU or Ki67 positive cells per area (in mm^2). Every sixteenth section from the anterior SEZ, bregma 1.18mm, until posterior SEZ, bregma -1.94mm, was analysed. The use of the VIS Software allowed delimitation, at low magnification (40x), of the areas of interest in the SEZ and the counting of BrdU positive cells within the defined areas at high magnification (400x). The divisions of the SEZ in the anterior-posterior and dorsal-ventral axes were defined on the basis of established anatomical references (Table 1), as previously described (Falcao et al 2012). Briefly, at the anterior-posterior axis, the SEZ anterior division starts and finishes with the beginning and the end of the genu of the corpus callosum, respectively (bregma 1.18mm to 0.74mm); intermediate SEZ begins with the end of the genu of the corpus callosum and extends up to the decussation of the anterior commissure (bregma 0.74mm to 0.14mm); the posterior division of the SEZ begins at the decussation of the anterior commissure and extends to the beginning of the hippocampus, bregma -0.94mm; the post-posterior division extends up to bregma -1.94mm (Table 1).

Confocal imaging and quantitative analysis

To estimate the number of neuroblasts (DCX positive cells) and proliferating neuroblasts (double DCX/BrdU positive cells) along the anterior-posterior and dorsal-ventral axes, 6 sections per animal were analysed. For each section, images were taken for the entire lateral wall of the SEZ using a confocal microscope (FV1000; Olympus) and the total number of DCX positive cells and the number of double labelled DCX/BrdU positive cells was counted. The rate for neuroblasts and proliferating neuroblasts was estimated by dividing the number of positive and double positive labelled cells for the corresponding area. The areas were determined using the Image J software. The same analysis was performed to estimate rates of double GFAP/BrdU positive cells.

Confocal imaging of wholemount preparations of the lateral ventricle wall was performed to label neuroblasts and proliferating cells. Images taken from the lateral wall were then assembled to show the entire lateral wall staining.

Table 1: Anterior-posterior axis landmarks of the mice SEZ divisions. Bregma coordinates are according to Paxinos & Franklin (2001).

SEZ	Bregma coordinates (mm)	Anatomical references
Anterior	[1.18; 0.74[	From the beginning to the end of the genu of the corpus callosum
Intermediate	[0.74; -0.14[	From the end of genu of the corpus callosum to the decussation of anterior commissure
Posterior	[-0.14; -0.94[	From the decussation of anterior commissure to the beginning of the hippocampus
Post Posterior	[-0.94; -1.94]	From the beginning of the hippocampus to the fusion of the dorsal and ventral parts of the lateral ventricle

Statistical analysis

All the data (presented as the mean ± SEM) was analysed with GraphPad PRISM 5 software (GraphPad Software Inc., San Diego, CA). The analysis consisted of one-way analysis of variance (ANOVA) with Bonferroni multiple comparison test post-test analysis for single-factor multiple group comparisons to determine differences between three or more groups. Student's t test was performed for two-group comparisons. The threshold value for statistical significance was set at $p<0.05$.

Results

In the anterior-posterior axis, SEZ cell proliferation is lower in the post-posterior region

To assess proliferation along the anterior-posterior axis we have estimated the number of BrdU and Ki67 positive cells per area in four SEZ levels: anterior, intermediate, posterior and post-posterior (Figure 1). These regions were previously defined according to external anatomic cues for the rat SEZ (Falcao et al 2012), and the same criteria for the definition of SEZ regions were applied in this analysis for the mouse. Our results show no differences in cell proliferation between anterior, intermediate and posterior SEZ, $2.37\pm0.08 \times10^{3}$, $2.32\pm0.14 \times10^{3}$ and $2.37\pm0.24 \times10^{3}$ BrdU positive cells per mm^{2}, respectively (Figure 1A). However, at post-posterior levels, we found a reduction in proliferation to $1.51\pm0.18 \times10^{3}$ BrdU positive cells per mm^{2}. Similar results were achieved when the same analysis was performed using the endogenous proliferation marker Ki67 (Figure 1B).

In the dorsal-ventral axis, the dorsal SEZ displays the lowest cell proliferation

At the dorsal-ventral axis the SEZ was divided into four distinct parts: dorsal, RMS, dorsolateral and ventral SEZ. Dorsal SEZ is at the dorsal wall of the SEZ, RMS constitutes the beginning of the RMS, localized at the dorsal corner of the ventricular lateral wall, dorsolateral and ventral are outlined from the lateral wall (Falcao et al 2012). The proliferation at the RMS, dorsolateral and ventral SEZ was similar, $3.00\pm0.35 \times10^{3}$, $2.60\pm0.28 \times10^{3}$ and $2.51\pm0.14 \times10^{3}$ BrdU positive cells per mm^{2}, respectively (Figure 1C). However, the dorsal SEZ exhibited the lowest proliferation rates of the four regions, $0.36\pm0.08 \times10^{3}$ BrdU positive cells per mm^{2}, only 12-14% of the total proliferation found in the other regions (Figure 1C). The same analysis was performed using the Ki67 marker and the results were generally similar (Figure 1D).

The proliferation rates of the SEZ regions are similar along the anterior-posterior axis in the lateral wall but not in the dorsal wall

To further investigate if the proliferation rates estimated for the four SEZ regions were preserved along the anterior-posterior axis, we independently analysed cell proliferation within anterior, intermediate and posterior levels of the SEZ. The proliferation, assessed by BrdU staining, in the SEZ lateral wall did not change from intermediate to posterior positions (Figure 2). Also, no significant differences were observed on the beginning of the RMS from anterior to intermediate

levels. However, when analysing the anterior SEZ, the proliferation rates for RMS were higher than in undefined (und) region. Of notice, dorsal SEZ proliferation rate increases 4 times at posterior levels from 0.12±0.12 x10^3 to 0.58±0.06 x10^3 BrdU positive cells per mm^2 (Figure 2). A similar increase in cell proliferation in the dorsal SEZ at posterior levels was found with the Ki67 marker (Figure 2).

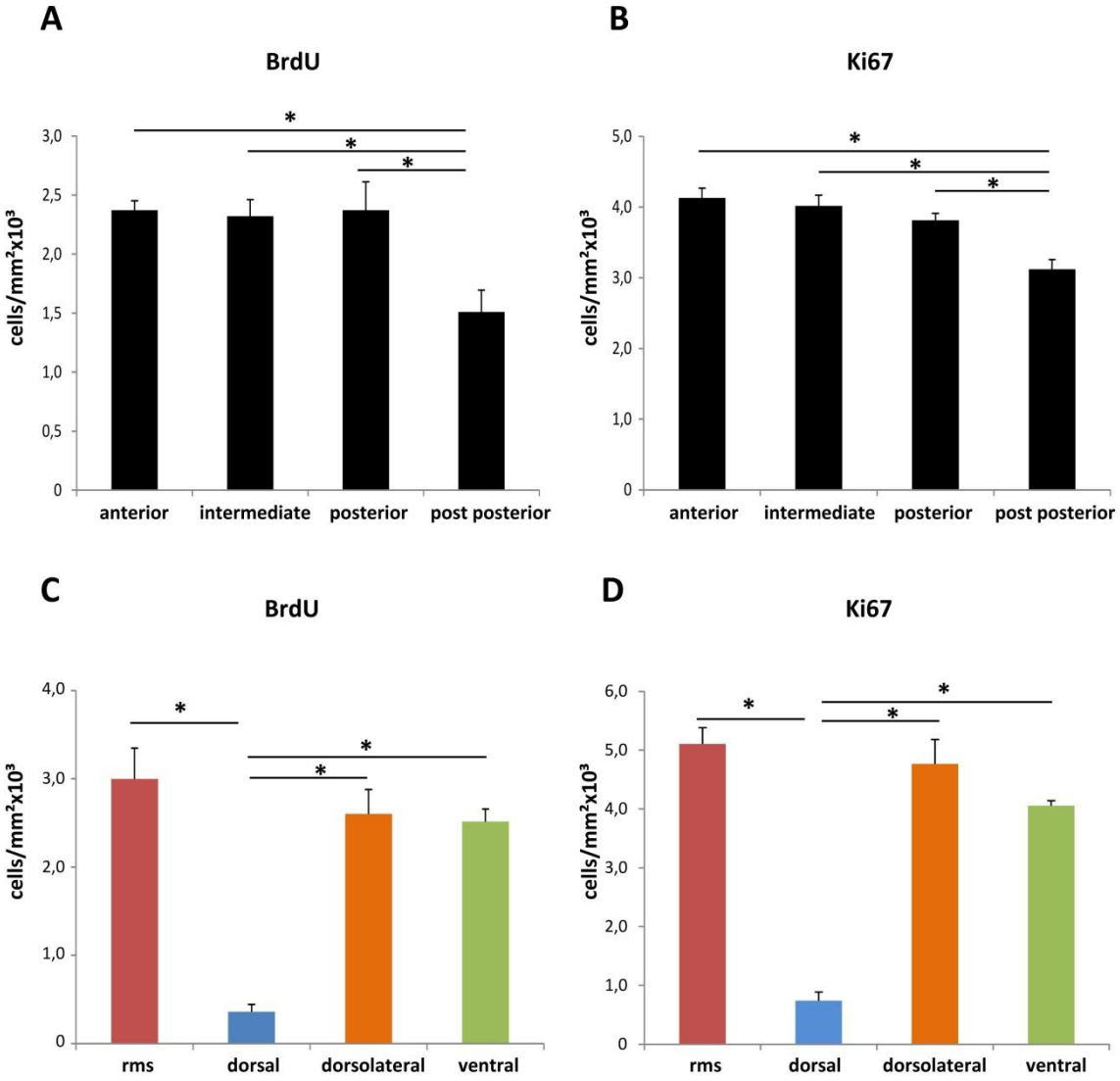

Figure 1 - Proliferative pattern of the mice subependymal zone (SEZ) cells throughout the anterior-posterior and dorsal-ventral axes. **A, B**. The SEZ proliferation, assessed by the number of BrdU and Ki67 positive cells, was reduced at the post-posterior SEZ in the anterior-posterior axis. **C, D.** At the dorsal-ventral axis, the dorsal SEZ displayed the lowest levels of proliferation. Proliferation was expressed as number of BrdU or Ki67 positive cells per area (mm^2). The threshold value for statistical significance was set at 0.05 (* $p<0.05$).

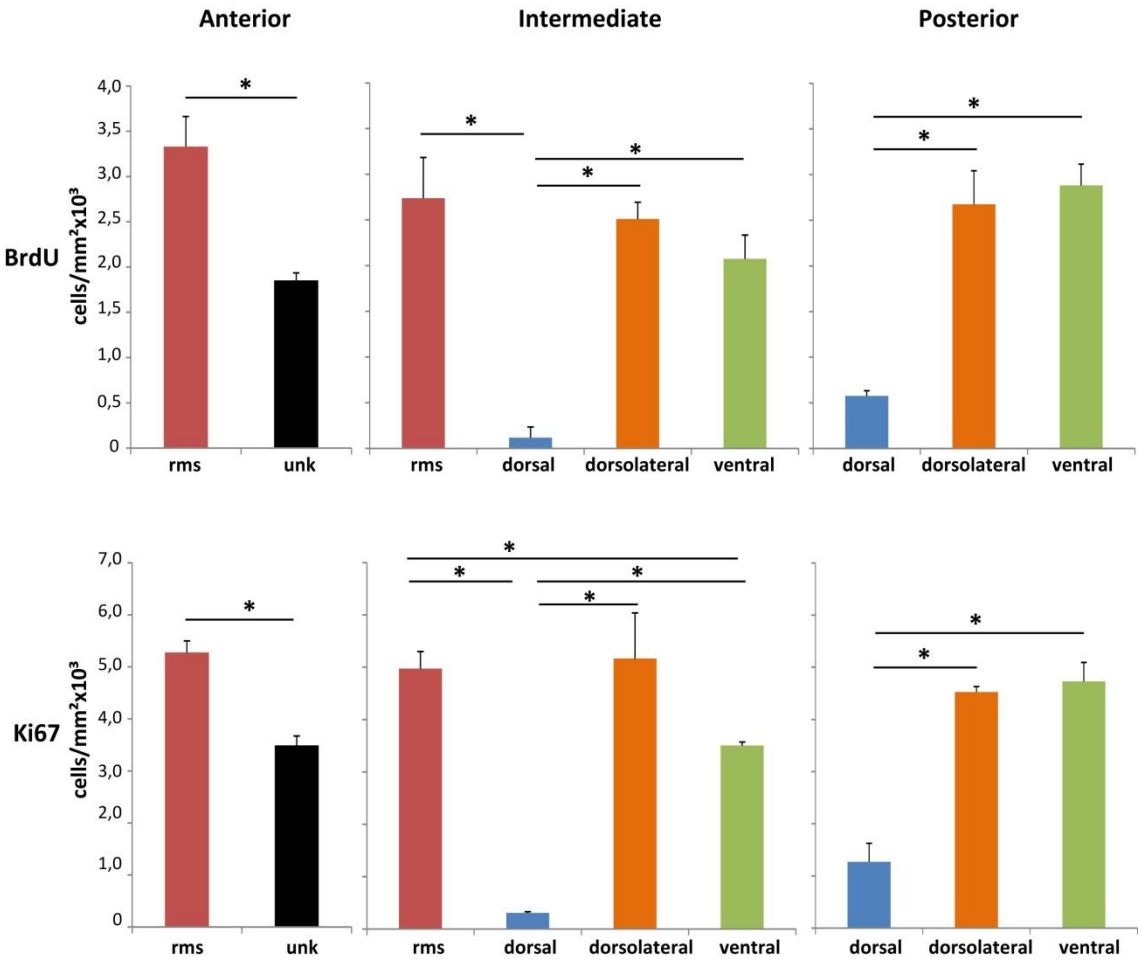

Figure 2 - Combined analysis of cell proliferation in the subependymal zone (SEZ) anterior-posterior and dorsal-ventral axes. Proliferation pattern analysis was assessed by the number of BrdU and Ki67 positive cells in dorsal-ventral SEZ regions along the anterior to posterior axis. The pattern of proliferation between different regions of the SEZ was similar along anterior-posterior axis. However, the dorsal SEZ rates of proliferation were increased at the posterior SEZ. Proliferation was expressed as number of BrdU and Ki67 positive cells per area (mm^2). The threshold value for statistical significance was set at 0.05 (* $p<0.05$).

Neural stem and precursor cells are homogeneously distributed in the lateral wall

Despite the observed relative homogeneity in proliferative patterns throughout the lateral SEZ wall, we analysed the neuroblast distribution in the lateral wall. No major differences were detected in the number of DCX positive cells and the double BrdU/DCX positive cells between dorsolateral and ventral SEZ at the intermediate levels (Figure 3). Furthermore, when assessing NSC rates by double staining of BrdU and GFAP, this population was homogeneously distributed within the lateral wall (Figure 3). Since GFAP is a consensual marker for NSCs and the population that constitutes label retaining cells (BrdU positive cells) are relatively quiescent cells, we identified the NSCs in the SEZ by labelling cells with GFAP and BrdU (from label retaining cells).

These results suggest that neural stem and progenitor cells are uniformly distributed in the mice SEZ.

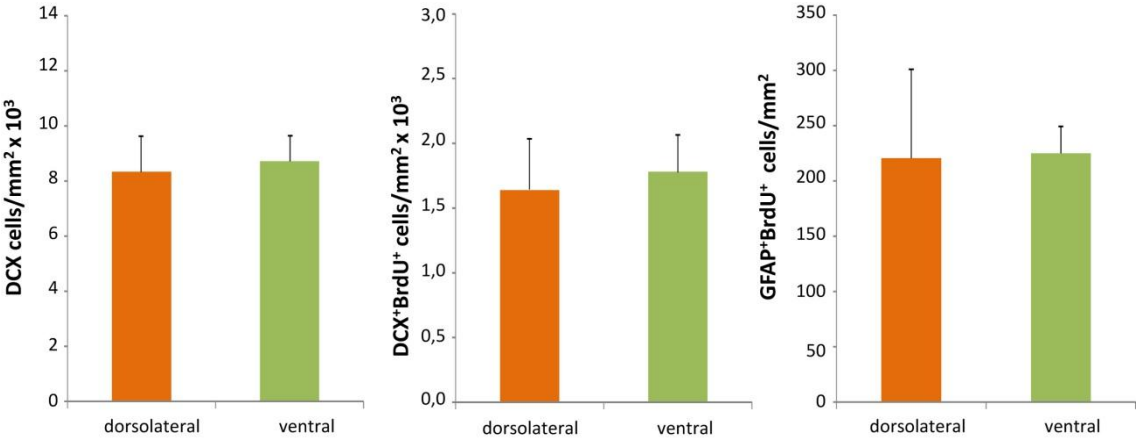

Figure 3 - The distribution of neural stem cells and neuroblasts was similar in the dorsal-ventral axis. DCX and double BrdU/DCX staining was used to assess neuroblasts and proliferating neuroblasts, respectively, at the dorsolateral and ventral SEZ. Neural stem cells were detected by double staining BrdU retaining cells with GFAP and assessed in the dorsolateral and ventral SEZ. All results are expressed as number of positive cells per area (in mm^2). The threshold value for statistical significance was set at 0.05 (* $p<0.05$).

The BrdU/Ki67 ratios are similar throughout the SEZ

We have estimated the BrdU/Ki67 ratio which provides an estimation of the cell cycle lengths between different dorsal-ventral regions and anterior-posterior divisions of the SEZ. These were constant throughout both axes suggesting that there are no major differences in the cell cycle length of the SEZ cells between the analysed regions (Figure 4).

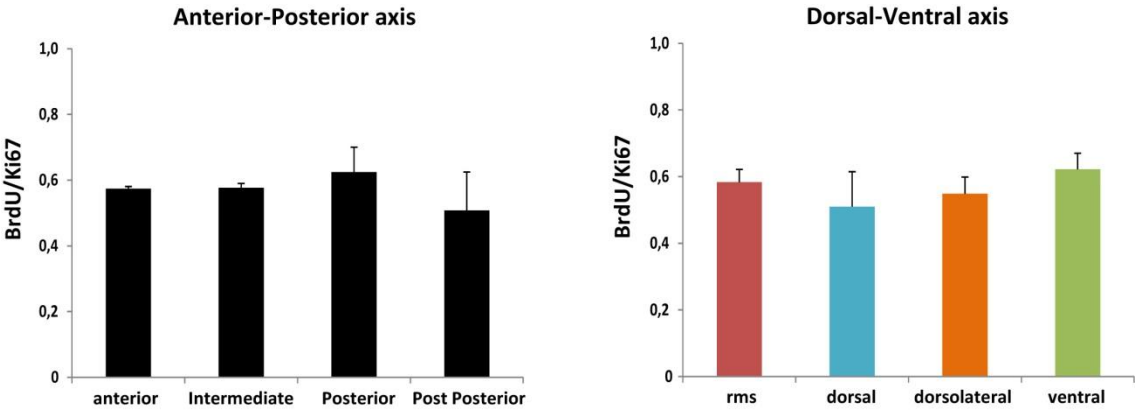

Figure 4 - The BrdU/Ki67 ratio throughout the subependymal zone was constant. The ratios BrdU/Ki67 were represented for the SEZ at anterior-posterior and dorsal-ventral axes. The threshold value for statistical significance was set at 0.05 (* $p<0.05$).

The number of cells in the vicinity of the SEZ diminishes from anterior to posterior divisions

The estimation of the number of cells proliferating less than 100μm apart from the SEZ revealed a remarkable reduction in the BrdU and Ki67 positive cells from anterior to posterior divisions. The anterior division presented higher numbers of BrdU and Ki67 positive cells ectopically placed, 9±1 and 27±3 respectively, when compared to intermediate and posterior divisions (Figure 5).

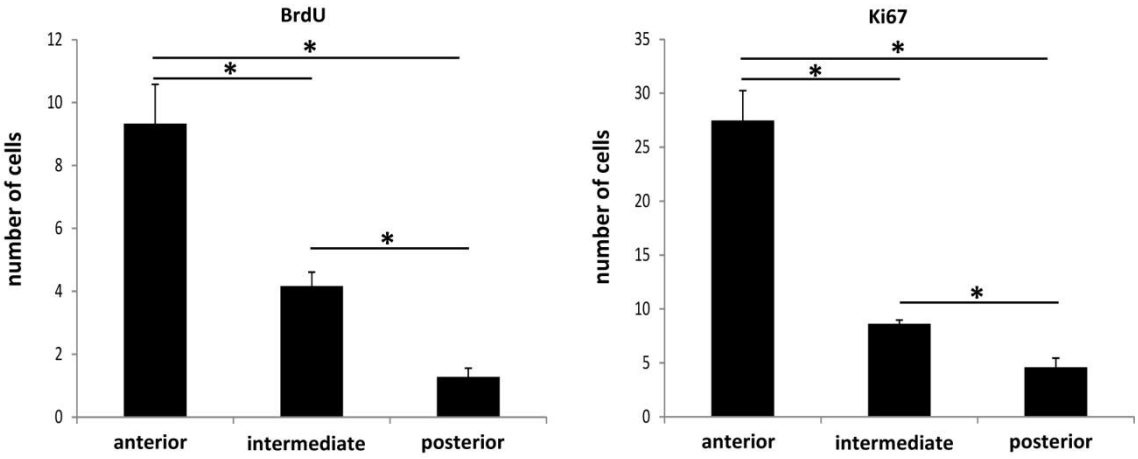

Figure 5 - The number of cells proliferating in the vicinity of the subependymal zone decreased along the anterior-posterior axis. The BrdU and Ki67 positive cells located within a distance of 100 μm apart from the SEZ were estimated. Results were represented as number of BrdU or Ki67positive cells per section. The threshold value for statistical significance was set at 0.05 (* $p<0.05$).

Discussion

By unravelling the topographical specificities in the proliferative rates in the rat SEZ, we have previously emphasized the relevance to take into account the location within the SEZ when studying the NSCs. Defining the exact coordinates in which analysis is performed is necessary to make appropriate and valid associations and comparisons across studies. In this sense, it is not surprising that similar studies on the SEZ dynamics give contradictory results, probably due to the use of different anatomic clues or specific SEZ regions. Because we found significant differences in the rat proliferation and neuroblast population throughout the SEZ axes, in this study we have performed a topographical analysis of the adult SEZ NSC niche in mice, using a similar approach to that described previously for the rat SEZ (Falcao et al 2012). The present study gives further insights about the similarities and differences between the SEZ of two close related species that must be taken into account for future analysis when extrapolating results obtained from mice to rat and vice-versa.

So far, studies performed in mice shed light to what is becoming increasingly evident: the adult NSC pool of the SEZ is heterogeneous regarding to its intrinsic properties and spatial locations (Alvarez-Buylla et al 2008). Although *in vitro* cultures of SEZ derived cells support the existence of multipotent NSCs by showing its self-renewal capacity and multipotentiality (Doetsch et al 2002), *in vivo* studies show that NSCs are already pre-committed to form certain types of interneurons at the OBs (Merkle et al 2007, Young et al 2007). Furthermore, NSCs from mice also express different transcription factors (TFs) along the dorsal-ventral axis, which in part overlap its embryonic counterpart's identity and TFs expression pattern (Alvarez-Buylla et al 2008, Weinandy et al 2011). In this sense, it was further suggested that combinations between TFs on the NSCs determine their cellular fate (Hack et al 2005).

Despite the reported cellular heterogeneity in the SEZ, and contrary to the described for rats, we found minor differences on mice proliferative patterns along the SEZ anterior-posterior and dorsal-ventral axes. By using two different proliferation markers, the acutely administrated exogenous marker BrdU, and the endogenous marker Ki67, we found a reduction in proliferation only at post-posterior levels of the SEZ. Noteworthy, most studies disregard the analysis of SEZ at post-posterior levels because it is generally assumed that new neurons at the OBs are born exclusively from the SEZ regions corresponding to the herein defined anterior, intermediate and posterior SEZ. Interestingly, a study performed by Merkle and colleagues (2007) revealed

additional sites of birth of new OB neurons (Merkle et al 2007). Amongst others, new neurons arise both from post-posterior levels of the SEZ and from the most anterior part of the RMS. Concerning the dorsal-ventral axis of the SEZ, we detected a sharp decline in proliferating cells at the dorsal SEZ. In fact, dorsal SEZ is largely omitted in studies in the SEZ and only recently its neurogenic potential was recognized (Brill et al 2009, Merkle et al 2007). Also, it was documented that the medial wall hosts NSCs that originate new OB neurons but only at most anterior levels (Merkle et al 2007), equivalent to the here defined anterior SEZ.

We have further analysed the BrdU/Ki67 ratios, an estimation of the cell cycle length, along the SEZ axes and found no alterations throughout both anterior-posterior and dorsal-ventral axes. This result is in accordance with the uniform proliferation rates between anterior, intermediate and posterior SEZ concerning the anterior-posterior axis and between dorsolateral and ventral SEZ in regards to the dorsal-ventral axis.

Recently, the NSC distribution profile throughout the mouse lateral wall was described by means of pinwheels structures analysis in wholemounts (Mirzadeh et al 2008). Interestingly, type B1 cell clusters (located in the middle of the pinwheels) were more numerous in anterior-ventral and posterior-dorsal positions. Type B1 cells are generally accepted as NSCs that give rise to all cellular populations at the SEZ (Doetsch et al 1999). These single cilium cells are identified by GFAP and gamma-tubulin labelling surrounded by beta-catenin from ependymal cells, forming a pinwheel-like structure. Altogether, data obtained from us and others (Mirzadeh et al 2008) suggests that the heterogeneous distribution of type B1 cells does not impact on the proliferative profile along the lateral wall.

It was suggested that the cells that comprise the pinwheel structure are heterogeneous namely on the quiescence/mitotic activity (Shook et al 2012). Therefore, the inclusion of a mitotic marker, such as PH3 or Ki67, in the estimations for the number of mitotic NSC in pinwheels could considerably change the outcome. For instances, the aged SEZ displays fewer type B1 cells but equal percentages for mitotic type B1 cells (Shook et al 2012). In the present work, we label NSCs by co-labelling GFAP with label retaining cells, given by BrdU staining. The analysis of NSCs using this approach revealed no statistically significant differences in the rates of NSCs between dorsolateral and ventral SEZ. Similarly, the distribution of neuroblasts in the lateral wall was also uniform, i.e., dorsolateral and ventral SEZ display similar rates of both neuroblasts and proliferating neuroblasts. Taken together, these results further support the data from the proliferation analysis. Although progenitors scattered from diverse parts of the lateral wall give

rise to different OB neuronal types, our data suggests that in what concerns the proliferation rate, neuroblasts are similar along the lateral wall. Likewise, there was no spatial-specific distribution of the general neuroblast population, as assessed by DCX staining.

We assessed the number of proliferating cells along the anterior-posterior axis and observed a significant decrease in the number of cells proliferating in the vicinity of the SEZ at intermediate and posterior levels. The migration of progenitor cells, namely neuroblast cells, out of SEZ towards sites of injury has been described in the context of brain damage (Zhang et al 2004). Although most of proliferating cells migrate tangentially, radial migration should not be excluded, at least in response to injury.

A combined analysis of rat and mouse proliferation patterns along the SEZ highlights the following inter-species discrepancies: i) the uppermost proliferation rate observed at the anterior SEZ in rats is absent in mice, ii) the remarkable decline in proliferation from dorsolateral to ventral SEZ present in the rat lateral wall does not occur in mice (Supplementary Figure 1), iii) the differential cell cycle lengths of rat SEZ cells, namely between dorsolateral and ventral progenitors, does not occur in mice, as given by similar BrdU/Ki67 ratios and iv) the differential spatial distribution of rat neuroblasts (Supplementary Figure 1) and NSCs along the lateral wall is homogeneous in mice. Nevertheless, some major similarities between the SEZ of both species were found, such as the proliferative decay at post-posterior levels, the limited proliferation at the dorsal SEZ, the augment of dorsal SEZ proliferation at posterior levels, and the decrease of proliferating cells surrounding the SEZ at intermediate and posterior levels.

Commonly, many conclusions on numerous SEZ related studies in the rat are tacitly assumed to hold true for mice, and vice-versa. The above outlined species-specific differences between these two most common laboratory rodent models points to the need to revise and consider SEZ from rats and mice as two distinct heterogeneous niches of neural stem and progenitor cells. Most importantly, it may ultimately reflect distinct functional differences both in health and disease. The existence of adult NSCs in the SEZ has been described in many mammalian species (Bedard et al 2002, Kornack & Rakic 2001, Sawamoto et al 2011, Seaberg & van der Kooy 2002). The closest to human the lesser extent is the formation of new neurons at the OBs. Thus, it is reasonable to infer that species highly dependent on olfaction, such as rodents, require an increased number of new OB neurons than species that do not depend as much on olfaction. In this sense, nonhuman primates display a less developed SEZ-RMS-OB nexus than rodents.

In summary, although species-specific differences on hippocampal neurogenesis were

demonstrated, both *in vivo* and *in vitro* (Snyder et al 2009, Steffenhagen et al 2011) this is the first report on the topic concerning the adult SEZ neurogenic niche.

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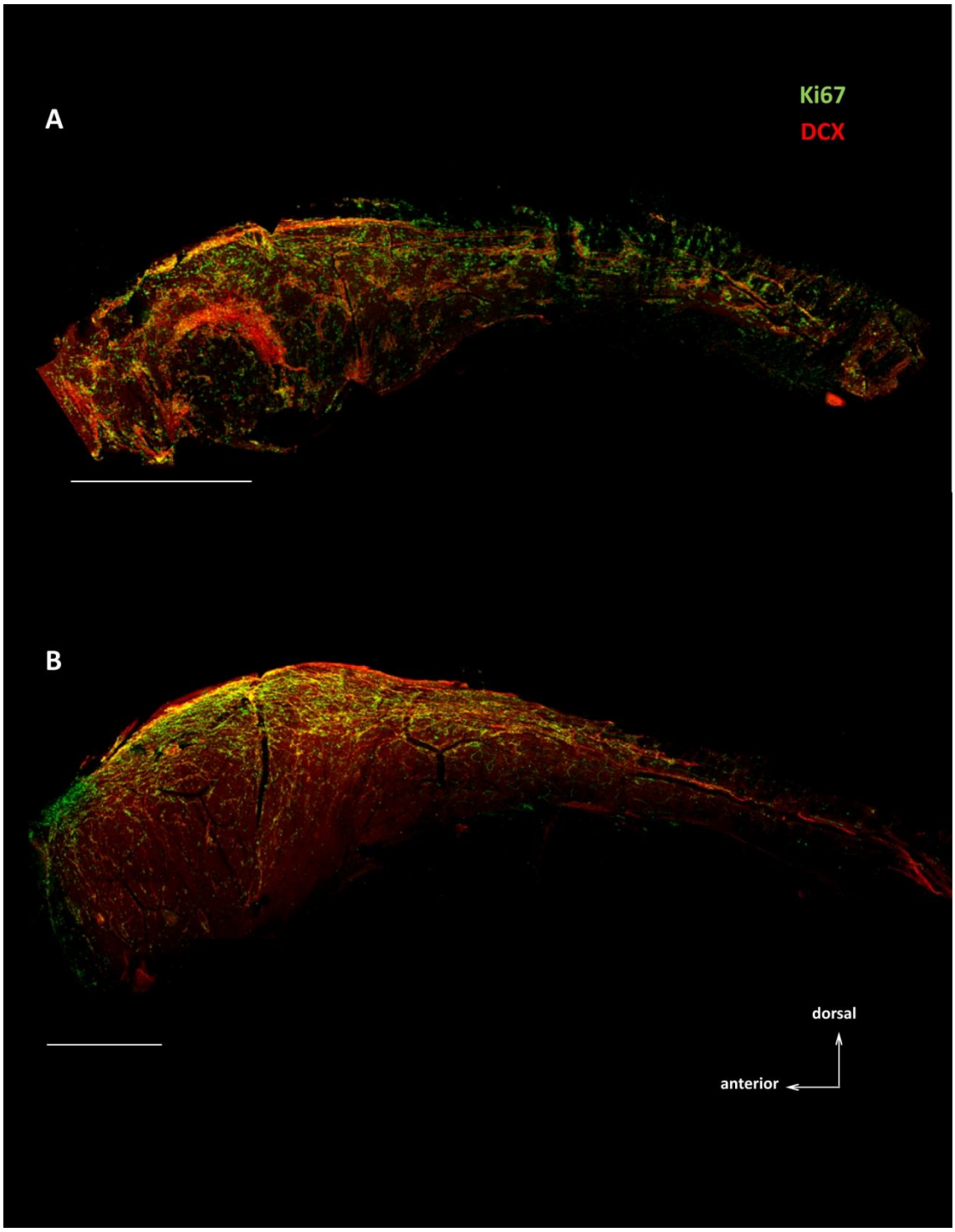

Supplementary figure 1 - En face views of proliferation and neuroblasts population in the lateral wall of rat and mice. Wholemount staining for proliferation, Ki67 (green) and neuroblasts, DCX (red) revealed a different distribution of cells in the mouse (A) and rat (B) lateral wall. Scale bar=1mm.

4. Acute peripheral inflammation impacts on subependymal zone, choroid plexus and cerebrospinal fluid

Acute peripheral inflammation impacts on subependymal zone, choroid plexus and cerebrospinal fluid

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Abstract

The subependymal zone (SEZ) is the major site of neurogenesis in the adult brain that originates new neurons for the olfactory bulbs (OBs). The interplay between intrinsic and extrinsic factors regulates the SEZ cell proliferation, fate and migration. The extrinsic cues that influence SEZ cells in a paracrine manner are of diverse origins: ependymal cells, endothelial cells of the blood-brain barrier, neurons, microglia and neural stem and precursor cells. Recently, we and others have suggested that the cerebrospinal fluid (CSF), whose proteins are mostly synthetized and secreted by the choroid plexus (CP), can also modulate the SEZ niche dynamics. Of notice, the CSF composition is significantly altered in response to inflammation. Interestingly, inflammatory conditions that considerably change the environment milieu of the SEZ niche have been shown to trigger alterations in the proliferative and migratory profiles of the SEZ cells. In this study, we challenged the peripheral immune system by administrating a single injection of lipopolysaccharide (LPS) and analysed the CP expression profile of various genes, the CSF composition and the SEZ cells. We observed an increase in proliferation of SEZ cells, namely in the neuroblasts population, upon 12h of LPS stimulus. Furthermore, we report a rapid and transient CP gene expression alteration of SEZ modulators, such as growth factors, that is also reflected in the CSF. Our data suggest that alterations in the CP and thus in the CSF composition triggered by LPS will likely impact on SEZ niche dynamics.

Key words

Subependymal zone, choroid plexus, cerebrospinal fluid, inflammation, neurogenesis

Introduction

In the past decades, the regenerative capabilities of the neurogenic areas of the adult brain, both in physiological and in pathological conditions, have been extensively studied (Kaneko & Sawamoto 2009, Vandenbosch et al 2009). The subependymal zone (SEZ), located in the lateral wall of the lateral ventricles, is the major source of new neurons in the adult brain (Alvarez-Buylla & Garcia-Verdugo 2002, Ming & Song 2011); SEZ born neuroblasts migrate anteriorly towards the olfactory bulbs (OBs) where they differentiate into interneurons (Whitman & Greer 2009). Moreover, stem cells of the SEZ are also capable of generating oligodendrocytes *in vivo* that in turn migrate to the corpus callosum and the striatum (Menn et al 2006).

In the context of regeneration, the SEZ neural stem cell (NSC) niche is of particular interest given the observed pronounced modifications upon brain injuries and in several disorders (Vandenbosch et al 2009). In fact, progenitors from the SEZ display altered proliferative and migratory profiles in a putative attempt to rescue the degenerating tissues (Curtis et al 2007). Understanding the exact mechanisms underlying such responses will certainly originate novel therapeutic targets for these conditions.

Of interest, many of these disorders and insults present an inflammatory component (Amor et al 2010), namely microglia activation and release chemokines and cytokines, which were shown to modulate adult neurogenesis in the hippocampal dentate gyrus (Das & Basu 2008, Ekdahl et al 2009).

A growing number of studies suggest a key role for the choroid plexus (CP) in brain inflammation (Marques et al 2009b, Marques et al 2009c) and in diseases such as multiple sclerosis (Marques et al 2012, Reboldi et al 2009). The CP is a structure of the brain where the majority of the cerebrospinal fluid (CSF) is produced (Speake et al 2001) and is part of the blood-CSF barrier. The presence of receptors for several immune mediators, in both the apical and in the basolateral CP membrane, and its secretory ability make it ideally positioned for transferring signals into and out of the brain, through alterations in the CSF composition. Importantly, the SEZ NSCs are in direct contact with the CSF and thus ideally positioned to be modulated by CP-born molecules (Falcao et al 2012a, Lehtinen & Walsh 2011). In fact, the access of CSF proteins to the SEZ was unequivocally demonstrated in studies with intracerebroventricular (ICV) injections of growth factors that resulted in the modification of the SEZ cell population dynamics (Jin et al 2003, Kuhn et al 1997).

Whether alterations in the CSF composition, triggered by inflammation, have any impact on the

SEZ cell population remains unexplored. Here we show that a single acute peripheral inflammatory stimulus is able to alter the CP expression of growth factors and other proteins involved in stem cells dynamics, which correlates with alterations in cell proliferation in the SEZ niche.

Materials and Methods

Ethics statement

This study was approved by the Portuguese national authority for animal experimentation, Direção Geral de Veterinária (ID: DGV9457). Animals were kept and handled in accordance with the guidelines for the care and handling of laboratory animals in the Directive 2010/63/EU of the European Parliament and of the Council.

Animals and experimental groups

All experiments were conducted in 10-weeks-old male Wistar rats (Charles River, Barcelona, Spain). Animals were maintained in 12h light/dark cycles at 22 to 24°C and 55% humidity and fed with regular rodent's chow and tap water *ad libitum*.

For the analysis of the SEZ cell population, four groups of animals (n=5 per group) were compared. Three groups received a single intraperitoneal (*i.p.*) injection of lipopolysaccharide (LPS; *Escherichia coli*, serotype O26:B6; Sigma, St Louis, USA) at a 5mg/Kg body weight dose for 6, 12 or 24h; a control group was injected with vehicle (0.9% NaCl) alone. Subsequent to each of these periods of time, all groups received a single injection of 5-bromo-2'-deoxyuridine (BrdU) at 50mg/kg, 2h before sacrifice (Figure 1A). To reduce stress-induced changes in the hypothalamus–pituitary axis associated with the *i.p.* injections, animals were daily handled for 1 week until the day of sacrifice.

For CP gene expression and CSF protein analysis, two additional groups were included (n=5 per group) for which LPS was administered 1 and 3h prior to sacrifice; the BrdU injection was not performed in these animals. The inclusion of these two additional groups intended to perform the kinetic profile of the changes in the CSF composition.

Immunohistochemistry

Animals were anesthetized with sodium pentobarbital and transcardially perfused with cold saline and brains were removed, embedded in O.C.T. compound and snap-frozen. Serial coronal sections (20 μm) were cut in a cryostat and collected to slides for immunohistochemistry. The antibodies used against markers that evaluate cell proliferation were BrdU at a dilution of 1:50 (Mouse Anti-Bromodeoxyurine, Clone Bu20a, DAKO, Barcelona, Spain) and Ki67 at a dilution of

1:100 (Ki67 antigen, rabbit polyclonal ab, Novocastra, UK). For BrdU staining a pre-treatment with HCl 2N for 30 min was required. Primary antibodies were detected by the Ultravision Detection System (Lab Vision, Freemont, CA, USA), and the reaction developed with 3,3'-diaminobenzidine substrate (Sigma); sections were subsequently lightly counterstained with hematoxylin. Fluorescence immunohistochemistry was performed to label proliferating neuroblasts [double BrdU/ doublecortin (DCX) positive] and neuroblasts (DCX positive cells). The following antibodies against markers of SEZ populations were used: DCX (rabbit polyclonal to doublecortin-neuroblast marker, Abcam) at a dilution of 1:500 together with BrdU (rat anti-BrdU, BU1/75 clone, Abcam) at a dilution of 1:100. Fluorescent anti-rabbit and anti-rat secondary antibodies (Invitrogen, Carlsbad, CA, USA) were used to detect the primary antibodies at a dilution of 1:1000. To label the nucleus, incubation with 4',6-diamidino-2-phenylindole (DAPI; Sigma) at a dilution of 1:1000 was performed. Primary and secondary antibodies were diluted in PBS-0.5%Triton/10% FBS and incubated overnight at 4°C for the primary antibody, and 2h at room temperature for the secondary antibody.

Stereology

Estimation of cell density in the different regions of the SEZ was obtained using the Visiopharm Integrator system (VIS) software in an Olympus BX51 microscope (Olympus, Hamburg, Germany). Coronal sections for proliferation analysis comprised SEZ between bregma coordinates 2.28mm and -1.72mm. Proliferation in the SEZ was assessed by Ki67, an endogenous marker expressed during all phases of mitosis, except for the resting phase G0; and by the exogenous marker BrdU, a thymidine analogue that is incorporated in the DNA during the S phase. The number of Ki67 and BrdU positive cells was counted and results expressed as Ki67 or BrdU positive cells per area (in mm^2). The analysis was performed as we previously described (Falcao et al 2012b). Briefly, every sixteenth section from the anterior SEZ, bregma 2.28mm until posterior SEZ, bregma -1.72mm, was analysed. The VIS Software was used to delimitate, at low magnification (40x), the areas of interest in the SEZ and to count the Ki67 or BrdU positive cells within the defined areas at high magnification (400x). We have analysed the different regions of the SEZ separately throughout the brain axis (Falcao et al 2012b).

CP and CSF collection

Animals were anesthetized with sodium pentobarbital (200mg/Kg) and transcardially perfused

with sterile RNAase free cold saline. Brains were removed and CP isolated under a stereomicroscope (SZX7; Olympus), frozen in dry ice, and stored at -80°C. CSF was collected from the cisterna magna, an aliquot was kept to confirm the absence of blood contamination (Huang et al 1995), and the remainder kept at -80°C for later determination of specific protein concentration.

RNA extraction, cDNA synthesis and qPCR analysis

Total RNA was isolated from the CP samples using Trizol reagent (Invitrogen). 500ng of total RNA was amplified using the Superscript RNA amplification system (Invitrogen), according to the manufacturer's instructions. Subsequently, 1µg of CP RNA was reverse transcribed using random primers of the superscript first-strand synthesis system for reverse transcription PCR (Invitrogen). Quantitative real-time PCR analysis was used to measure the expression levels of hypoxanthine guanine phosphoribosyl transferase (Hprt), basic fibroblast growth factor (bFgf), nerve growth factor (Ngf), monocyte chemoattractant protein 1 (Mcp1), insulin growth factor 1 (Igf1), pigment epithelium-derived factor (Pedf), amphiregulin (Areg), bone morphogenetic protein 7 (Bmp7), chemokine (C-X-C motif) ligand 12 (Cxcl12), netrin1, ciliary neurotrophic factor (Cntf), and slit homologue 2 (Slit2) mRNA transcripts. The reference gene, Hprt, was used as internal standard for normalization since it has been previously shown that these conditions (*i.p.* injection of LPS) do not interfere with the expression of Hprt (Marques et al 2007). The quantitative real-time PCR reactions were conducted using equal amounts of cDNA from each sample and were performed on a Bio-Rad CFX96™ using the QuantiTect SYBR Green RT-PCR reagent kit (Qiagen, Hamburg, Germany). The oligonucleotide primers for bFgf, Ngf, Mcp1, Igf1, Pedf, Areg, Bmp7, Cxcl12, Netrin1, Cntf and Slit2 (Table 1) were designed using the Primer3 software (Rozen & Skaletsky 2000), on the basis of the respective GenBank sequences.

ELISA

The quantitative determination of MCP1 and NGF in the CSF was performed by enzyme-linked immunosorbent assays (ELISA). Two microliters of CSF was used to detect the levels of these proteins at the different LPS time points and in the control group. MCP1 was detected following the manufacturer's instructions for ELISA kits, rat MCP-1 ELISA Set (BD Biosciences, Europe); and NGF by a direct enzyme-linked immunosorbent assay using rabbit anti-NGF antibody (ab66459, Abcam) at 1:600 dilution, followed by a biotinylated secondary anti-rabbit antibody at

a dilution of 1:500 (ab6720, Abcam) for detection with peroxidase-conjugated streptavidin (Thermoscientific, UK) (1:1000 dilution) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) (Sigma). The reaction was stopped using 0.1mol/L citric acid and read at an optical density of 405 nm. The standard curve was made with a NGF peptide (ab66458, Abcam).

Table 1: List of qPCR primers sequence, forward and reverse, and the respective product size.

Gene	Forward primer sequence (5' - 3')	Reverse primer sequence (5' - 3')	Product size (bp)
Hprt	gcagactttgctttccttgg	tccactttcgctgatgacac	208
bFgf	cacgtcaaactacagctcca	aggcgttcaaagaagaaaca	149
Ngf	ggacgcagctttctatcctg	ctccctctgggacattgcta	131
Igf1	ggcattgtggatgagtgttg	gtcttgggcatgtcagtgtg	128
Mcp1	tagcatccacgtgctgtctc	tgctgctggtgattctcttg	122
Pedf	atgaaggcgacgttaccaac	gttccacttgggtgagcttc	129
Areg	ctgctggtcttaggctcagg	accacaagtccaccagcact	110
Bmp7	gaaaacagcagcagtgacca	cagagggaaggcacactctc	144
Cxcl12	gctctgcatcagtgacggta	cagccttgcaacaatctgaa	145
Netrin1	cactgccactactgcaagga	ggtggtttgattgcaggtct	126
Cntf	ggacctctgtagccgttctatctg	ggtacaccatccactgagtcaagg	129
Slit2	tgtgtgtgcaaaccatcctt	acagtgatatggggcaggag	147

Statistical analysis

Data (presented as the mean ± SEM) was analysed with GraphPad PRISM 5 software (GraphPad Software Inc., San Diego, CA). The analysis consisted of a non-parametric Mann–Whitney test for two-group comparisons between LPS treated animals and controls. The threshold value for statistical significance was set at $p < 0.05$.

Results

12 h after LPS peripheral administration the neuroblast proliferation in the SEZ is increased

The peripheral inflammation triggered a rapid and transient 50% induction on SEZ cells BrdU incorporation 12h after the LPS stimulus (from 1.5 ± 0.08 $x10^3$ BrdU positive cells per mm^2 in saline injected animals to 2.24 ± 0.33 $x10^3$ BrdU positive cells per mm^2) (Figure 1B). This result was further confirmed by the endogenous marker of proliferation Ki67; in accordance with the results obtained for BrdU, the number of Ki67 positive cells in the SEZ was augmented in animals challenged for 12h with LPS (Figure 1B). This increase was observed in the various divisions of the SEZ (anterior, intermediate and posterior as well as in the RMS, ventral and dorsolateral) (Figure 1C). We have further estimated the proliferating neuroblasts, by double BrdU/DCX labeling, after 12h of LPS administration and found an increase in the double BrdU/DCX positive cells from 0.95 ± 0.11 $x10^3$ cells per mm^2 to 1.30 ± 0.10 $x10^3$ cells per mm^2 after the inflammatory trigger (Figure 1D). Taken together, these results show a time specific and transient enhancement of the SEZ cell proliferation in response to a peripheral stimulus.

The number of BrdU positive cells found in the vicinity of the SEZ is increased after the LPS challenge

The number of cells proliferating in the vicinity of the SEZ (that could be migrating radially) up to a maximal distance of 100μm were estimated. This analysis revealed an increase in the number of BrdU positive cells surrounding SEZ at the time point of 12h subsequent to LPS administration (Figure 1E). On the other hand, we did not find any difference in Ki67 positive cells migrating away from the ventricles, when comparing controls and LPS groups (Figure 1E).

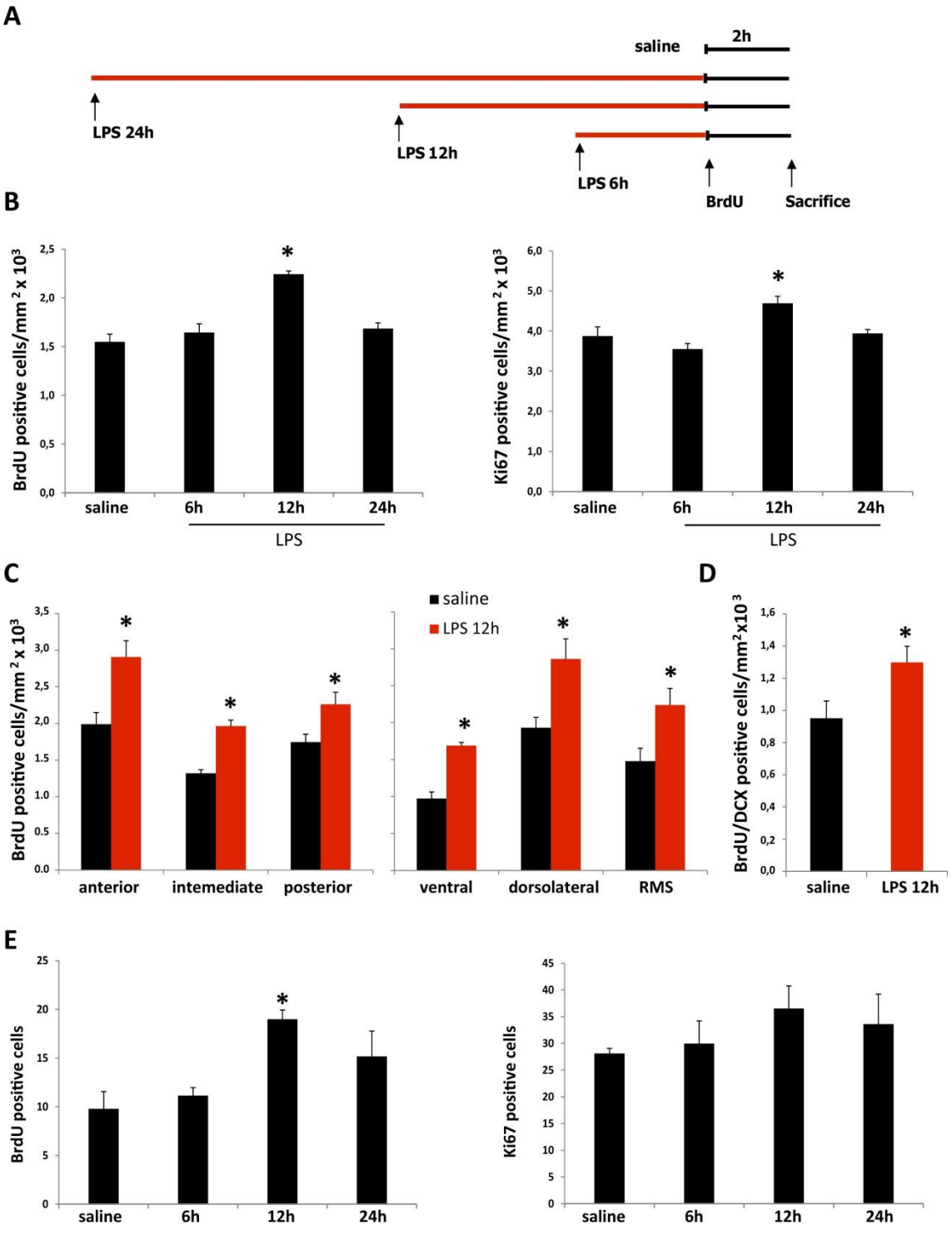

Figure 1: An acute peripheral inflammatory stimulus increases cell proliferation in the SEZ. **A.** Schematic representation of experimental design. Animals were administrated with a single LPS injection for different periods of time (6, 12 and 24h) or injected with saline. Subsequently, a single injection of BrdU was administrated to all groups and after 2h animals were sacrificed. **B.** Analysis of BrdU positive cells in the SEZ show a sharp increase in BrdU incorporation in the SEZ of 12h LPS. In agreement, the number of Ki67 positive cells also increased after 12h of LPS injection. **C.** The increased proliferation in SEZ cells upon 12h of LPS stimulus was constant throughout the different regions of the SEZ in both anterior-posterior and dorsal-ventral axes. **D.** The SEZ neuroblast population, double

BrdU/DCX cells, displayed increased proliferation in response to LPS challenge. **E.** The number of BrdU positive cells in the vicinity of SEZ upon 12h of LPS stimulus was increased, but the number of Ki67 labeled cells was not altered throughout time. The results are expressed as number of positive cells per SEZ area (mm^2). The threshold value for statistical significance was set at 0.05 (* $p < 0.05$). In all graphs, results are expressed as mean ± SEM and n=5.

CP gene expression profile is significantly altered during an acute peripheral inflammation

We performed CP gene expression profile analysis on specific proteins that are synthesized in the CP and simultaneously described to have an effect in the SEZ niche. We compared CP gene expression on those proteins between control animals and animals injected with LPS for 1, 3, 6, 12 and 24h. In response to inflammation, CP exhibited an upregulation in the expression of genes encoding growth factors such as bFgf and Ngf. The expression of bFgf displayed a 5 and 7-fold increase after 6 and 12h of LPS injection, respectively (Figure 2A). Similarly, Nfg genes displayed an increased expression from 1 to 12 h after the LPS challenge (Figure 2A). The gene expression of the chemokine Mcp1 was upregulated after 3, 6, 12 and 24h of LPS injection, peaking at 12h with a 95-fold increase relative to control (Figure 2A). On the contrary, Igf-1 was down-regulated at 6, 12 and 24h upon inflammatory stimulus (Figure 2A). We further analysed the expression levels of other genes and found that the mRNA levels of Areg, Netrin1, Cntf and Slit2 were upregulated, while the levels of Pedf, Bmp7 and Cxcl12 mRNA were reduced as depicted in Figure 2B. The time point where major differences were found on gene expression was at 12h after LPS administration; at this time point 8 out of the 11 genes analysed were altered.

The increased gene expression levels of Mcp1 is reflected in the CSF protein composition

We next evaluated if the CP gene expression changes upon an inflammatory stimulus were reflected at the protein level in the CSF for some of the modulators. The MCP1 protein was below the level of detection on saline injected animals but was detected after 3, 6, 12 and 24h after the LPS systemic challenge. In fact, a robust increase in MCP1 protein levels was observed at 6 and 12h to 363 ± 107 ng/mL and 648 ± 112 ng/mL, respectively (Figure 3). On the other hand, the levels of NGF were not altered in response to LPS despite the alterations previously found in the Ngf expression levels in the CP.

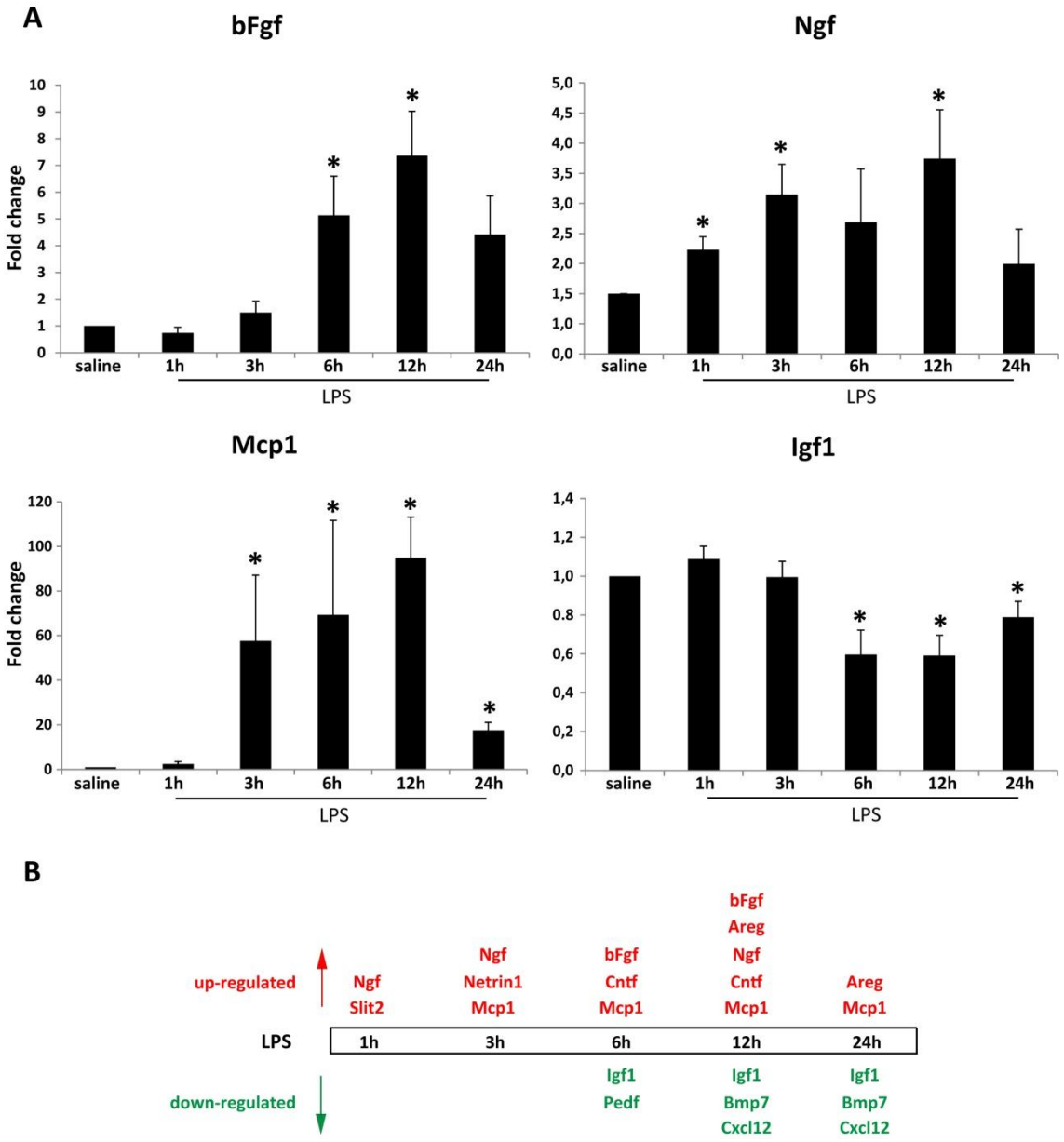

Figure 2: Modulators of SEZ population that are synthesized by the CP display altered gene expression in response to inflammation. **A.** The mRNA levels of the growth factors bFgf and Ngf were induced from 3h to 12h and 1h to 12h after LPS stimulus, respectively. Furthermore, the expression of the chemokine Mcp1 displayed the highest fold change increase upon 3, 6, 12 and 24h of LPS stimulus. Conversely, Igf-1 mRNA levels were reduced at 6, 12 and 24h after LPS injection. **B.** Schematic representation of the genes whose expression in the CP were altered in response to peripheral inflammation triggered by LPS at different time points: 1, 3, 6, 12 and 24h. Associated with each LPS time point are the genes found altered in CP, up (red) or down-regulated (green). Gene expression was normalized by the housekeeping gene Hprt and expressed as fold change relative to the saline injected control group. The threshold value for statistical significance was set at 0.05 (* $p < 0.05$). Results are expressed as mean ±SEM and n=5.

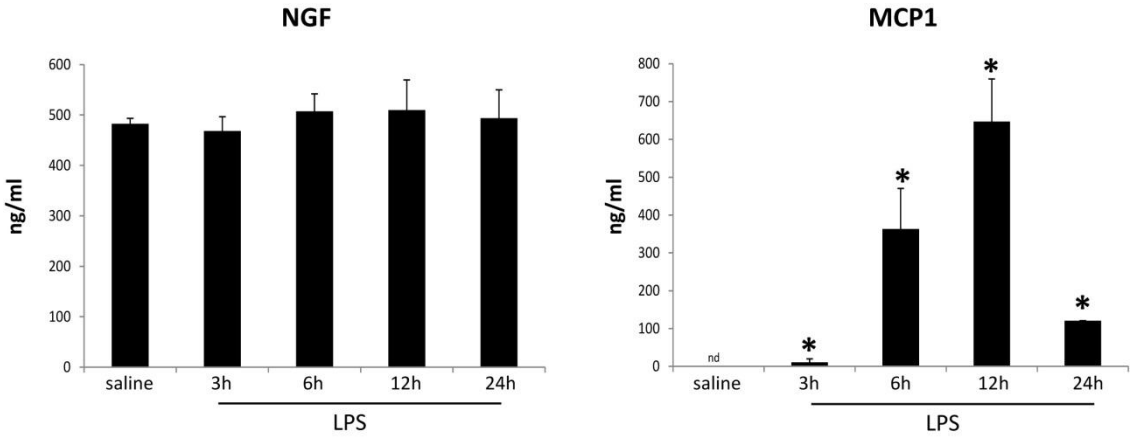

Figure 3: Determination of protein concentrations of MCP1 and NGF in the CSF after a peripheral inflammatory stimulus. The CSF levels of MCP1 were drastically increased upon 3h of LPS stimulus and remained high until 24h of LPS stimulus. These levels in the control group were below the detection limit. In contrast, the CSF levels of NGF were unaltered upon LPS peripheral stimulus. The threshold value for statistical significance was set at 0.05 (* $p < 0.05$). In all graphs, results are expressed as mean ± SEM and n=5.

Discussion

In the present work we show that a single acute peripheral inflammatory stimulus results in increased cell proliferation in the SEZ. By studying the CP expression profile of genes known to modulate the SEZ and their CSF concentration, the data collected in this study suggests that alterations in CP-born molecules, and thus in the CSF protein components, are likely to impact on the SEZ population dynamics.

It has been reported that the SEZ responds to several injuries and that is altered in neurodegenerative diseases, by either increasing or decreasing proliferation levels (Curtis et al 2007) and exhibiting abnormal progenitor migratory pathways towards sites of injury (Zhang et al 2004). Many, if not all of these insults to brain homeostasis share an inflammatory component (Minghetti 2005). The role of neuroinflammation on adult neurogenesis is complex. Although many molecules are commonly found as part of the inflammatory immune response in brain diseases, there is still high heterogeneity on the inflammatory response namely due to spatial-temporal specific secretion of the inflammatory mediators. In fact, the effect of the immune system on adult neurogenesis has been debated for long (Carpentier & Palmer 2009, Gonzalez-Perez et al 2010a). More specifically, the effects of LPS-triggered inflammation on adult neurogenesis have been mostly reported for the subgranular zone of the hippocampal dentate gyrus (Russo et al 2011b). Intracortical infusions of LPS for 28 days negatively regulate the formation of new neurons in the hippocampus, but no differences were detected in the proliferation rates (Ekdahl et al 2003). Conversely, acute intracerebroventricular injections of LPS resulted in a decrease in proliferation but also survival and differentiation of hippocampal progenitor cells (Russo et al 2011a). When administrated systemically, a single LPS injection triggers a reduction in hippocampal proliferation (Fujioka & Akema 2010) and in neurogenesis (Monje et al 2003). Many reports suggest that microglia activation and immune mediators secreted by these and other cells are accountable for this effect (Ekdahl et al 2009). Conversely to what was described for the hippocampus, we show, for the first time for the SEZ niche, that a single LPS injection is able to trigger a specific and transient increase in the SEZ cell proliferation pattern, namely in the neuroblast population. Because we have previously demonstrated that the proliferative profile was heterogeneous throughout the axes in the rat SEZ (Falcao et al 2012b), we further assessed proliferation in the various SEZ regions and observed a consistent increase in proliferation in all analysed SEZ areas. In addition to the alteration in the proliferation of the SEZ cells, we observed an increase in the number of BrdU positive cells in the vicinity of the SEZ.

Conversely, no differences were found in the number of Ki67 positive cells. Assuming that these cells are SEZ-derived, these might mean that an abnormal migration of SEZ cells is occurring and that the mismatch between BrdU and Ki67 suggests that cells are starting to exit the cell cycle to differentiate.

The source and the identity of the molecule(s) that could trigger the herein observed differences in the rate of proliferation of SEZ cells are diverse. On one hand, LPS might directly stimulate NSCs due to a leaky blood-brain barrier (BBB). In fact, *in vitro* and *in vivo* studies using rat SEZ derived NSCs show that toll-like receptors are expressed in the SEZ, namely the LPS receptor TLR4 (Covacu et al 2009, Rolls et al 2007). Nevertheless, a role for a direct effect of LPS in the SEZ is conflicting. Namely, TLR4 agonists, such as LPS, had no effect on proliferation and differentiation of SEZ derived NSCs (Covacu et al 2009); in contrast, another study shows that activation of TLR4 by LPS decreases the number of neurospheres formed (Rolls et al 2007). Of notice, TLR4 deficient mice displayed enhanced NSC proliferation both in the dentate gyrus of the hippocampus and in the SEZ (Rolls et al 2007). Another possible modulator of the SEZ response to LPS is microglia. Microglia are the immune cells of the central nervous system and are well described to play a role in response to neuroinflammation in the hippocampus, namely in response to LPS (Ekdahl et al 2009). These cells produce and secrete several cytokines and chemokines that influence NSC survival, proliferation, differentiation and migration (Gonzalez-Perez et al 2010b). Other candidate that could be modulating the SEZ in response to a peripheral insult is the blood brain barrier (BBB) itself. It has been reported that progenitor cells in the SEZ lie close to blood vessels, and moreover, blood vessels at the SEZ are modified in a way that allows the contact of NSCs in places that either lack the astrocytic endfeet or pericytes in the BBB (Shen et al 2008, Tavazoie et al 2008). The BBB response to LPS, in other brain areas, has also been described in several studies (Banks & Erickson 2010). When peripherally administrated, LPS might induce a leakage in the BBB and induce the production of several immune mediators such as interleukin 6 (IL-6) and interleukin 1beta (IL-1β) (Banks & Erickson 2010). In fact, these factors were also reported to be synthetized at the level of microglia in response to LPS (Loughlin & Woodroofe 1996). Furthermore, these interleukins have both been described to modulate NSC. In one hand, IL-6 was reported to stimulate self-renewal of NSCs and inhibit the formation of new neurons (Covey et al 2011, Vallieres et al 2002). On the other hand, a recent study shows that, when injected in the lateral ventricle, IL-1β decreased the proliferating cells through upregulation of VCAM1 that maintains NSCs by inhibiting lineage

progression (Kokovay et al 2012). Although LPS can induce barrier breakdown, it was suggested that LPS acts indirectly by stimulating cells constituting the barriers of the brain, rather than entering into the brain parenchyma, since radioactive iodinated LPS, injected peripherally, did not reach the brain parenchyma, even when repeatedly injected (Banks & Robinson 2010). Thus, the impact of systemic LPS on SEZ cells is likely indirect through stimulus of the BBB and/or blood-cerebrospinal fluid barrier (BCSFB), whose response will then impact on parenchymal cells, such as microglia, ependyma, and neural stem and progenitor cells.

Recently, the role of the BCSFB in response to inflammation has been extensively examined, namely upon an LPS acute inflammatory stimulus (Marques et al 2009a, Marques et al 2008, Marques et al 2009b, Marques et al 2007). The gene expression profile of CP cells is profoundly altered after an acute peripheral inflammatory stimulus triggered by LPS (Marques et al 2009b). Many of the altered expressed molecules are immune modulators, such as cytokines and chemokines, including IL-6 and IL1β. Furthermore, the CP gene expression alterations are reflected in the CSF content (Marques et al 2007). The immune molecular players found altered in response to LPS stimulus are similar between CP, BBB and microglia. More specifically, IL-1β, IL-6 and tumor necrosis factor alpha (TNF-α) are commonly found induced in LPS mediated immune responses. Importantly, these cytokines were reported to influence neural stem and progenitor cell proliferation and differentiation in distinct manners, as referred above. The fact that cytokines with different neurogenic actions are induced in several cellular types in response to LPS and can be simultaneously present in the SEZ, does not allow us, in the present study, to identify the precise origin of the molecules influencing the SEZ increased proliferation herein described. It is however likely that molecules such as bFgf, Ngf, Igf1, Pedgf, Areg, BmP7, Netrin1, Cntf, Slit2, Cxcl12 and Mcp1 whose transcription is altered in the CP upon the LPS challenge, which in some cases is reflected in the CSF composition, are relevant SEZ modulators. All of these molecules were described to play a role in proliferation and/or migration of neural stem and progenitor cells (Emsley & Hagg 2003, Falk & Frisen 2002, Gonzalez-Perez et al 2010a, Hurtado-Chong et al 2009, Imitola et al 2004, Lim et al 2000, Liu et al 2007, Murase & Horwitz 2002, Ramirez-Castillejo et al 2006, Sawamoto et al 2006, Yan et al 2007). For instances, the role of bFgf in stimulating proliferation of neural stem and progenitor cells has been well described both *in vitro* and *in vivo*, namely the intracerebroventricular injection of bFGF results in a massive induction of SEZ cell proliferation (Kuhn et al 1997). Of notice, NGF is generally described to play a role on neuronal growth and differentiation. In particular, it has

been described as up-regulated, namely in the CSF of EAE rats (Micera et al 1995) and in the CSF of multiple sclerosis patients (Laudiero et al 1992). Nevertheless, in this study although the mRNA levels of NGF were altered in the CP, this effect was not detected at the level of the CSF, which demonstrates that further mechanisms regulate the protein synthesis in the CP. Notably, on the other hand, the CSF levels for MCP1 correlated with the CP gene expression. The CSF should next be analysed for the above described SEZ modulators and the functional implications of their increase/decrease in the CSF should be addressed, for instance by intracerebroventricular injections.

In summary, in this study, we provide evidence, for the first time, that a single acute peripheral inflammatory stimulus can impact in a specific and transient manner in SEZ cell proliferation. Of interest, specific modulators of these altered dynamics might originate from the CP/CSF.

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5. General discussion and future perspectives

Since the first discovery of mitotic activity in the adult brain, the number of studies relying on adult neurogenesis has exponentially grown. The past four years comprised more than half (52%) of the total papers published on adult neurogenesis (based on PubMed search on “adult” AND “neurogenesis" OR "neural stem cells" from September 2008 up to September 2012 vs the total number of papers until September 2012). This drastic increase on research in adult neurogenesis reflects the enthusiasm of the neuroscientist community on the potential of adult neural stem cells (NSCs) for the development of new therapies for central nervous system (CNS) repair. Indeed, there are two possibilities to promote brain regeneration: cell transplantation therapies and activation/modulation of the endogenous brain regenerative capacity. Regarding the first, numerous studies report a successful therapeutic usage of cell transplantation in animal models for spinal cord injury (McDonald et al 1999), stroke (Ishibashi et al 2004, Kelly et al 2004), Parkinson's disease (Sawamoto et al 2001) and demyelinating disorders (Pluchino et al 2003). Nevertheless, the clinical success of cell transplantation therapies in human patients, for instances in Parkinson's disease, did not reach, thus far, the expectations (Freed et al 2001, Olanow et al 2003). Of notice, it was suggested that the adult mammalian brain, *per se*, displays regenerative capacity, however with low efficiency (Okano 2006, Okano & Sawamoto 2008). In this context, the subependymal zone (SEZ) niche has a central role as the major source of adult NSCs in the mammalian brain and given its response to brain damage. In fact, analysis performed both in animal models and in postmortem tissue from human patients clearly demonstrates that neural stem and precursor cells of the SEZ display abnormal proliferative and migratory profiles following brain injury, possibly as an attempt to rescue neuronal loss. As an example, patients who suffered an ischemic episode exhibit increased ipsilateral SEZ cell proliferation (Marti-Fabregas et al 2010). Moreover, proliferating neuroblasts are found in human ischemic-induced lesions, suggesting the existence of ectopic neurogenesis (Jin et al 2006, Macas et al 2006). The functional significance of these new cells remains unexplored, but certainly encourages further investigation on the brain endogenous regenerative capacity.

In order to enhance and modulate the endogenous regenerative capacity of adult NSCs towards effective brain regeneration, it is essential to comprehend how the process of neurogenesis in health and disease is intrinsically regulated.

In this sense, the present work gives further insights on the following themes:

i) The SEZ niche heterogeneity;

ii) The inter-species SEZ variability;

iii) The SEZ response to peripheral inflammation;

iv) The choroid plexus (CP) as a modulator of the SEZ niche.

These four topics and the contribution of the results herein presented to the general understanding of the SEZ neural stem cell niche dynamics will next be discussed in detail.

i) The SEZ niche heterogeneity

The definition of a NSC encompasses two main characteristics: self-renewal capacity and multipotency. Indeed, the existence of a NSC pool in the lateral wall of the lateral ventricles was first demonstrated *in vitro* by collecting cells of the lateral wall and testing them for self-renewal i.e., unlimited proliferative capacity and ability to generate progeny (through asymmetric divisions), while maintaining an undifferentiated state, and the multipotentiality, i.e., the ability to generate the three cellular types of the CNS: neurons, astrocytes and oligodendrocytes (Ming & Song 2005). In this sense, it was for long tacitly assumed that the SEZ comprised a uniform pool of NSCs that generated neuroblasts which would ultimately give rise to all types of interneurons of the OBs. Additionally, it was shown that the SEZ NSCs can also give rise to Olig2 positive progenitor cells that differentiated in oligodendrocytes in the corpus callosum and in demyelinating areas (in response to demyelinating insults) (Menn et al 2006). In the past five years, a new concept regarding SEZ NSCs heterogeneity has ascended based on *in vivo* studies which showed that neuronal diversity comes from diverse and spatially confined stem cell populations (Alvarez-Buylla et al 2008) instead of from a uniform pool of NSC, as previously thought. The fact that NSCs are already in a pre-committed stage, and even when heterotopically transplanted give rise to the same neuron type as intrinsically pre-determined, raises questions about the multipotentiality of NSCs *in vivo* (Merkle et al 2007). Moreover, whether the SEZ progenitor cells that generate oligodendrocytes in the corpus callosum are originated from a different population of pre-committed NSCs remains unknown. Because it is becoming evident that NSCs are heterogeneous throughout the anterior-posterior and dorsal-ventral axes, studies addressing SEZ cell dynamics should be carefully analysed and take into consideration these spatial differences. In fact, a brief examination of the literature reveals that many studies in the SEZ do not specify the coordinates in which the analysis was performed, which may render difficult the comparison between studies and yield contradictory results. Furthermore, the vast

majority of the studies do not perform an independent analysis of the different regions of the SEZ and do not take into account that the topography of the SEZ is highly variable throughout the brain axes. So far, no studies have independently addressed the proliferation and progenitor population in the regions herein described. In this sense, the anatomical divisions that we proposed herein, based on external landmarks, will help, *per se*, to standardize the analysis of the SEZ. Specifically, we analysed the SEZ cells proliferative profile in the rat anterior-posterior and dorsal-ventral axes and showed that:

- The SEZ cell proliferation decreases along the anterior-posterior axis and varies considerably according to the position in the dorsal-ventral axis;
- These differences are associated with relevant gradients in the NSC population and in the neuroblasts progenitor population throughout the dorsal-ventral axis;
- The neuroblasts display shorter cell cycles at the dorsolateral SEZ than in the ventral SEZ

These findings support the previously reported heterogeneity of the SEZ cells and further reinforce the need to independently analyse the different regions of the SEZ.

The fact that different locations in the SEZ anterior-posterior and dorsal-ventral axes display different rates of proliferation might be explained by the heterogeneous distributions of NSCs throughout the axes since there was a correlation in the NSC density and the proliferation rates between dorsolateral and ventral SEZ. Nonetheless, this raised the question of why are NSCs preferentially located in the dorsolateral SEZ? Regardless of NSCs and neuroblasts distribution throughout the axes, we also found differences on the cell cycle length between neuroblasts from dorsolateral and ventral SEZ that clearly show the presence of two distinct neuroblast populations. Whether similar differences on the cell distribution and cell cycle length also occur in the TAP cells remains undetermined, and should next be analysed.

Together with the literature, our findings raise more questions: the existence of different progenitor populations along the SEZ axes is exclusively determined intrinsically or is also triggered by spatially restricted niche signals? For instances, is there a uniform distribution of axon terminals (a source of neurotransmitters known to modulate SEZ cells) throughout the SEZ? Do cerebrospinal fluid (CSF) protein concentration gradients play a role in this signalling? And, most importantly, do cells in different anterior-posterior and dorsal-ventral regions display the same potential to respond to brain damage? The evidence collected so far urges for further investigation on the differences between both NSCs and neuroblasts isolated from distinct SEZ regions. Specifically, it would be interesting to investigate the gene expression profiles and the

epigenetic states of cells from spatial confined populations. The stereotaxic method developed by Merkle et al (2007) that allows the injection of viruses to label radial glia in a regionally specific manner in P0 mice would be of great value to further isolate SEZ populations from specific regions. The delivery of lentivirus to different populations of radial glia cells where the expression of a fluorescent protein is under the control of the GFAP or DCX promoter will only label cells with active expression of GFAP and DCX, and thus label NSCs and neuroblasts, respectively. These cells could then be isolated by FACS sorting for further study with gene expression analysis, epigenetics, and cell culture assays. Future studies should also address the response of spatially confined populations to different brain injuries, by using the same labelling strategy described above.

We performed the same aforementioned analysis for the rat SEZ in the mouse. By using the same external landmarks, we determined the patterns of proliferation and neuroblast population throughout the mice SEZ. Our main findings in the mouse SEZ topographical analysis are:

- At the anterior-posterior axis, the proliferation rates for anterior, intermediate and posterior SEZ are similar, but at the post-posterior SEZ the proliferation is reduced comparatively to the anterior, intermediate and posterior SEZ;
- At the dorsal-ventral axis, the dorsal SEZ displays the lowest proliferation rates of the dorsal-ventral regions, while proliferation rates for RMS, ventral and dorsolateral SEZ are similar;
- The distribution of neuroblasts, proliferating neuroblasts and NSCs is similar between the dorsolateral and the ventral SEZ.

Hence, the proliferation profile of the mouse SEZ, when comparing the dorsolateral and ventral SEZ, is in accordance with the neuroblast and NSCs distribution.

In contrast with our data, Mirzadeh et al (2008) have showed, also in mice, that the distribution and frequency of the type B1 cells, assumed as NSCs, was heterogeneous throughout the lateral ventricle. For instances, more NSCs were allocated at the dorsal aspect of the lateral wall (equivalent to the herein defined dorsolateral) when compared to more ventral parts (Mirzadeh et al 2008). As the proliferating progeny is originated from NSCs, it would be expected to find more progenitors in places with more NSCs. However, we found that the neuroblast progenitors and the proliferation profile are relatively uniform. Moreover, we assessed NSC rates by staining label retaining cells with GFAP, and found no difference between NSC frequency in the dorsolateral and ventral SEZ. Together with the data by Mirzadeh et al (2008), our findings suggest that NSCs assessed by pinwheels encompass different types of NSCs populations, active and quiescent,

and that the active population seems uniformly distributed in the lateral wall. This hypothesis can be tested by determining the frequency and distribution of the NSCs in pinwheels, including the mitotic marker Ki67.

We will next discuss the species-specific differences found in the rat and in the mouse.

ii) The species-specific SEZ variability

A major purpose of research in adult neurogenesis is to apply the discoveries generated in laboratory animal models to the development of trials and studies in humans. A good translational research project can impact on human health by enriching our understanding of human pathology that ultimately leads to the raise of new therapeutic targets for brain regeneration. In this sense, it is crucial to characterize and comprehend species-specific variations on the SEZ neurogenic niche.

In this study, we report relevant species-specific differences in the adult SEZ neurogenic niche between rat and mouse models, two closely related species and the most used laboratory animal models for the study of brain pathological conditions. Conversely to what was observed in the rat SEZ, no differences were detected between the proliferation at anterior, intermediate and posterior levels, and between dorsolateral and ventral areas, in the SEZ of the mouse. In agreement, no evidence was found for differences in the cell cycle length between SEZ cells placed in different regions in the mouse. These findings raise several questions; for instances, why does the ventral SEZ in rat display fewer progenitor cells than dorsolateral SEZ, when in mice these two are similar? What is the functional implication of this species-specific difference? Since cells expressing calbindin in the OBs are derived preferentially from ventral regions of the adult mice SEZ (Merkle et al 2007), does the rat OBs have fewer periglomerular calbindin positive interneurons when compared to mice OBs? The next step would be to compare in the rat and mouse the amount and identity of new neurons, particularly calbindin positive interneurons that integrate olfactory circuitries. Of relevance, differences between rat and mouse in the number, maturation and functional properties of new hippocampal neurons have been previously reported (Snyder et al 2009).

Also of interest is to compare the impact of the ablation of the SEZ-mediated neurogenesis in the rat and in the mouse, by means of SEZ irradiation (Lazarini et al 2009) or infusion of anti-mitotic drugs in the lateral ventricles (Doetsch et al 1999). Rats and mice would then be evaluated in terms of behaviour in olfaction dependent tasks, since this is the major functional role described

for adult SEZ born neurons.

Our *in vivo* findings further support previous *in vitro* studies that show species-specific differences between rat and mouse derived whole brain and hippocampal neurospheres (Ray & Gage 2006). Future studies should also address *in vitro* species-specific differences between mouse and rat SEZ-derived neurospheres regarding the cellular identity of the originated populations, proliferative capacity, and response to mitogens and other subtracts. This *in vitro* analysis would give us further insights on the *in vivo* function and potential of the NSCs.

In the adult brain, the presence of neural stem and progenitor cells in the SEZ was detected in all the mammalian species studied so far (Barker et al 2011). Nonetheless, the formation of new functional and mature neurons has only been well described in rodents and in nonhuman primates (Lindsey & Tropepe 2006). However, it is known that in nonhuman primates the formation of new neurons for the OBs is far less extended when compared to rodents. Importantly, two recent studies report that the formation of new neurons in the OBs of the human brain only occurs in childhood (Bergmann et al 2012, Sanai et al 2011). Moreover, no evidence was found for the existence of a neuroblast chain migration towards the OBs in adult humans (Sanai et al 2007). Despite the absence of new neurons in the OBs, NSCs could be successfully isolated from the adult human brain. Also of interest, it was recently described a robust migratory pathway to the pre-frontal cortex from the infant (4-6 months old) human SEZ (Sanai et al 2011). Similarly, studies performed in early postnatal mice revealed a massive migration of SEZ-born cells into numerous forebrain regions, including the cortex, striatum, and nucleus accumbens (Inta et al 2008). Importantly, rodent models of many neurodegenerative diseases and brain injuries display similar responses to damage by the SEZ (Curtis et al 2007, Sierra et al 2011).

Taken together, these findings demonstrate many common features of the SEZ dynamics between animal models and humans, both in health and in disease. They also suggest an evolutionary purpose of adult SEZ neurogenesis, since species depending on olfaction for daily basis tasks exhibit greater formation of new neurons at the OBs, when compared to species that do not depend on olfaction for survival, as it is the case of humans. The reason why rats and mice, both dependent on olfaction, display a different profile for SEZ proliferation and NSC and neuroblasts distribution remains unknown. Also unknown is whether there is a functional implication for the observed dissimilarities.

iii) The SEZ response to peripheral inflammation

A growing number of studies identified an alteration in the proliferative and migratory profiles of the SEZ cells in response to brain insults. Importantly, an inflammatory component is thought to be part of this response. The contribution of the inflammatory mediators to neurogenesis seems to depend on the context and/or on the modulator, since opposite effects have been reported. On one hand, inflammation has been shown to negatively influence adult hippocampal neurogenesis, a process that was restored upon treatment with anti-inflammatory drugs (Ekdahl et al 2003). Conversely, ischemia induced SEZ neurogenesis was suppressed upon administration of anti-inflammatory drugs, suggesting a positive role of inflammation on the SEZ neurogenesis (Kim et al 2009). The role of neuroinflammation, by itself, has been discussed mainly in the context of the formation of new hippocampal neurons, where it seems detrimental (Ekdahl et al 2003). However, the impact of inflammation on SEZ cells is still poorly investigated. In the present thesis, by studying the effect of an acute peripheral lipopolysaccharide (LPS) stimulus, we found that:

- The proliferation of SEZ cells, namely of neuroblasts, is acutely and transiently enhanced after the LPS stimulus;
- The number of cells proliferating in the vicinity of SEZ is enhanced 12h after the LPS stimulus.

Whether the increase in the neuroblast proliferation will originate additional new neurons remains undetermined. Of interest, more cells were found proliferating in the vicinity of the SEZ. The determination of the identity of these cells is crucial to understand if an ectopic migration is occurring in response to LPS. Also, further studies are needed to investigate if there are any changes in the identity and in the fate of the neurons formed during LPS stimulus. To address this question, BrdU should be administrated during the peak of the SEZ response, LPS 12h, followed, three weeks later, by assessment of new neurons at the OBs.

The mechanisms underlying the observed SEZ response to peripheral LPS remains unidentified. In fact, it is known that inflammatory stimulus elicit the production and secretion of numerous chemokines and cytokines from cells of the brain parenchyma, such as microglia, endothelial cells of the blood-brain barrier and epithelial cells of the CP. Moreover, these immune mediators have been described to influence neural stem and progenitor cells proliferation and migration, some of them in opposite ways. Therefore, it is difficult to discriminate the exact mechanisms by which inflammation is modulating the SEZ dynamics. We will focus our discussion on the putative contribution of the CP derived molecules next.

iv) The CP as a modulator of the SEZ niche

The CP produces and secretes the vast majority of the CSF which, in turn, directly contacts the NSCs of the SEZ. Interestingly, a recent study shows that adult SEZ-derived cells are able to form neurospheres when cultured in adult CSF, suggesting a role for adult CSF molecules on SEZ dynamics (Lehtinen et al 2011). In fact, it was demonstrated that CSF gradients of Slit2, produced by the CP, direct neuroblast migration towards the OBs (Sawamoto et al 2006).

It was previously described that in response to an acute LPS stimulus, the CP changes its transcriptome and secretome and thus the CSF composition (Marques et al 2007); the molecules induced in the CP and increased in the CSF comprised mainly immune mediators, such as interleukins (Marques et al 2009). Herein, we show that in response to peripheral inflammation, the CP is able to change the expression levels of factors known to modulate the SEZ. Specifically, our findings are:

- Gene expression of molecular modulators of the SEZ, such as bFgf, Igf2, Mcp1 and Ngf are altered upon an acute peripheral inflammatory stimulus;
- The alterations in the CP gene expression are reflected in the CSF protein content of these SEZ modulators.

Nevertheless, whether all the alterations detected in the CP gene expression of SEZ modulators reflects in CSF composition needs to be determined. Furthermore, this work would benefit from experiments with cultures of SEZ cells with physiological CSF and CSF collected from animals stimulated with LPS, thus allowing to further unravel the role of the CP-CSF nexus on the SEZ dynamics upon a peripheral inflammatory stimulus. In addition, intracerebroventricular injections of candidate molecules, such as MCP1, would help to determine their function upon an inflammatory challenge. On the other hand, the intracerebroventricular injections of neutralizing antibodies for candidate proteins at the same time as the LPS trigger could be performed to assess if the effect on the SEZ was suppressed. Alternatively, a lentivirus based approach (Regev et al 2010) could be used to overexpress or knockdown candidate molecules specifically in the CP, and hence in the CSF.

Our findings shed to light to the putative functions of the CP in modulating SEZ niche dynamics in response to brain insults. In fact, CP gene expression and CSF composition are altered in response to neurodegenerative diseases disorders (Emerich et al 2004, Marques et al 2012). Future studies on brain insults and neurodegenerative diseases should analyse CP-CSF nexus alterations and correlate with differences found in the SEZ niche.

In summary, the work presented herein gives novel perspectives on emerging subjects in the SEZ adult neurogenesis and raises further questions regarding the complexity of the SEZ cells and the SEZ niche modulation.

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6. Annexes

frontiers in
CELLULAR NEUROSCIENCE

REVIEW ARTICLE
published: 09 August 2012
doi: 10.3389/fncel.2012.00034

The path from the choroid plexus to the subventricular zone: go with the flow!

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In adult mammals, under physiological conditions, neurogenesis, the process of generating new functional neurons from precursor cells, occurs mainly in two brain areas: the subgranular zone in the dentate gyrus of the hippocampus, and the subventricular zone (SVZ) lining the walls of the brain lateral ventricles. Taking into account the location of the SVZ and the cytoarchitecture of this periventricular neural progenitor cell niche, namely the fact that the slow dividing primary progenitor cells (type B cells) of the SVZ extend an apical primary cilium toward the brain ventricular space which is filled with cerebrospinal fluid (CSF), it becomes likely that the composition of the CSF can modulate both self-renewal, proliferation and differentiation of SVZ neural stem cells. The major site of CSF synthesis is the choroid plexus (CP); quite surprisingly, however, it is still largely unknown the contribution of molecules specifically secreted by the adult CP as modulators of the SVZ adult neurogenesis. This is even more relevant in light of recent evidence showing the ability of the CP to adapt its transcriptome and secretome to various physiologic and pathologic stimuli. By giving particular emphasizes to growth factors and axonal guidance molecules we will illustrate how CP-born molecules might play an important role in the SVZ niche cell population dynamics.

Keywords: choroid plexus, cerebrospinal fluid, subventricular zone, growth factors

INTRODUCTION

The adult subventricular zone (SVZ) neural stem cell niche, also designated as subependymal zone to distinguish from the embryonic SVZ, is the major source of novel neurons in the adult brain (Whitman and Greer, 2009). The properties of this neural progenitor cells niche are being increasingly studied, in light of the potential usage of endogenous sources of regenerative cells in disorders of the central nervous system. The adult SVZ stem cell population is heterogeneous, in a region-specific manner, along the wall of the brain ventricles. This stem cells heterogeneity is a consequence of the pattern of transcription factors (intrinsic factors) they express (Alvarez-Buylla et al., 2008), and results in the generation of different types of novel neurons in the olfactory bulb (Lledo et al., 2008). In addition several extrinsic factors [other brain cells, blood vessels, and the cerebrospinal fluid (CSF)] in the vicinity of the SVZ also participate in the regulation of the SVZ niche and in fate determination of these progenitor cells. The CSF, whose composition is mainly determined by the choroid plexus (CP) secretome, is a major source of proteins and smaller molecules that signal the SVZ. Understanding the contribution of the CP in the interplay between extrinsic factors and intrinsic properties of the SVZ neural progenitor cells is not only of biological relevance, but also of interest in pathological conditions that may alter the CP transcriptome and/or secretome, and ultimately impact on the SVZ.

THE CP MORPHOLOGY AND FUNCTION

The CPs are thin membranes that float in the CSF filled lateral, third and fourth brain ventricles. The CP is mainly composed of a monolayer of epithelial cells derived from the ependymal cells that line the wall of the brain ventricles (**Figure 1**). Underneath this monolayer of epithelial cells lays a stroma perfused with highly permeable fenestrated blood vessels, fibroblasts, and immune cells such as dendritic cells and macrophages. The CP is a highly vascularized structure with a 10 times fold higher blood flow when compared to the brain parenchyma (Keep and Jones, 1990).

CP epithelial cells display a clearly polarized cellular morphology bearing: (1) an apical surface (facing the brain ventricles, and hence the CSF) composed of a large number of microvilli of variable length that extensively increases the contact area with the CSF; (2) a smother basolateral membrane (facing the CP connective tissue, hence the blood side); and (3) lateral membranes, the surface contact area between adjacent epithelial cells. At the most apical portion of the lateral membranes the existence of tight junctions limits the paracellular passage of blood derived cells and proteins (Vorbrodt and Dobrogowska, 2003). Tight junctions, together with the expression of several basolateral and apical transporters, make CP epithelial cells the effectors of the blood-CP-CSF barrier (Spector, 2010). The CP is responsible for the generation of at least two-thirds of the CSF volume via the secretion of water, ions, and macromolecules (Johanson et al., 2008). In fact, the CP epithelial cells display several transporters

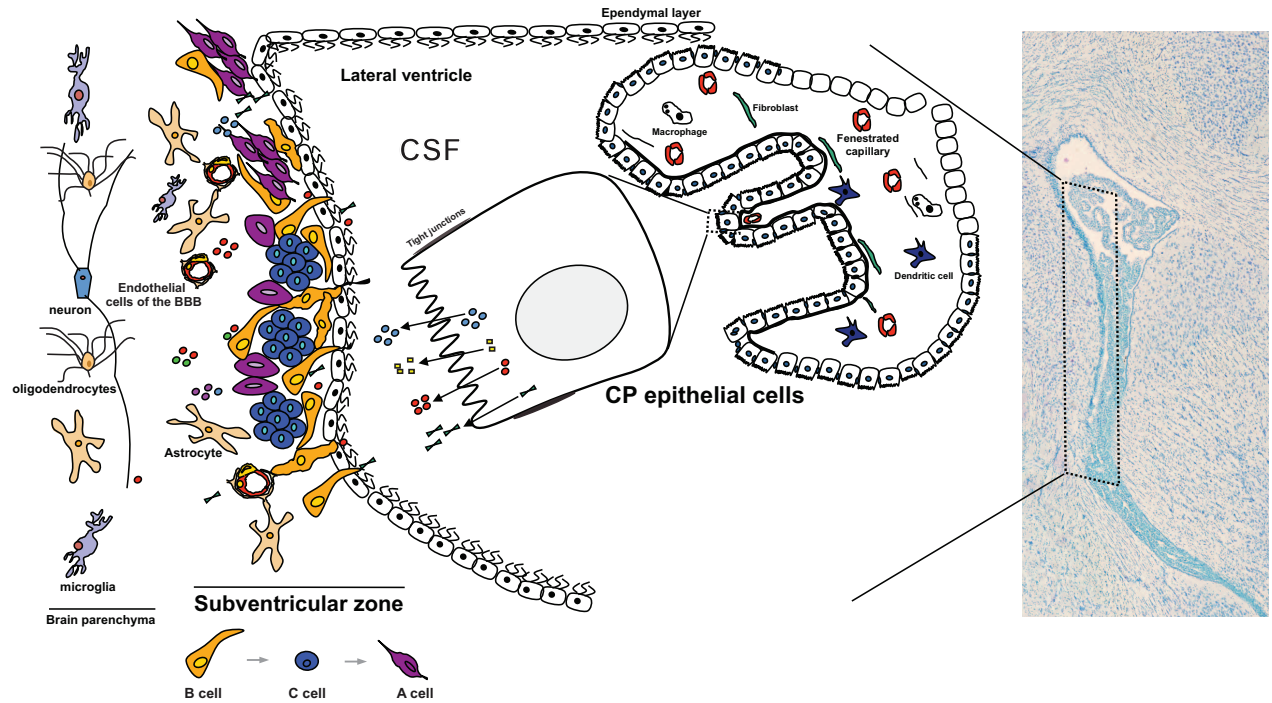

FIGURE 1 | The CP influences the CSF composition that baths the neural progenitor cells in the SVZ. Due to its highly secretory capacity and its particular location facing the lateral wall of the brain ventricles, and hence the SVZ, the proteins being secreted by the CP rapidly flow in the CSF and enter in contact with the SVZ. Proteins and other molecules that are secreted toward the CSF penetrate the interstitial space between the cells adjacent to the wall of the ventricles. Being composed of a thin cell layer, the SVZ is influenced by the paracrine effect of the CP. In particular, type B1 neural stem cells are in direct contact with the CSF by projecting a primary cilium toward the ventricle. These cells are considered the stem cells of the adult SVZ and give rise to type C cells (transit amplifying progenitors) that in turn originate the type A cells (neuroblasts). Also in the SVZ are the resident astrocytes, microglia and endothelial cells of the blood-brain barrier (BBB). Together with the ependymal cells and the CP-born molecules, these are the modulators of the adult SVZ cell niche. Cells of the brain parenchyma lay in the SVZ neighborhood.

for water molecules and ions, transporters for small peptides and polypeptides, and have the capacity to synthetize, and then secrete several proteins toward the CSF (Praetorius, 2007; Johanson et al., 2008). The necessary energy to feed this highly secretory capacity is provided by a high density of mitochondria (Redzic and Segal, 2004). In addition, the CP epithelial cells have receptors, both in the apical and basolateral sides, for molecules such as neurotransmitters, cytokines, bacterial toxins, amongst others; importantly, several of these receptors have been shown to signal downstream cascades that ultimately influence the CP transcriptome and secretome (Marques et al., 2009a, 2011; Johanson et al., 2011).

PROTEINS THAT ARE EXPRESSED BY THE CP ARE SECRETED TOWARD THE CSF

The high secretory capacity of the CP is reflected in the composition of the CSF (Chodobski and Szmydynger-Chodobska, 2001; Thouvenot et al., 2006) (**Figure 1**). Amongst the most abundant proteins in CSF are CP-secreted proteins, such as transthyretin (Sousa et al., 2007), transferrin and prostaglandin D2 synthase (Chodobski and Szmydynger-Chodobska, 2001). Reflecting the importance of the CP-CSF nexus in the normal brain functioning, these and other proteins have been independently explored as unique biomarkers of psychiatric and neurological disorders.

In the last decade, the continuous improvement of large screening proteomic techniques resulted in a more comprehensive characterization of the CSF protein content in several species and different ages (Parada et al., 2006; Zappaterra et al., 2007; Stoop et al., 2010), both in physiological and in neuropathological conditions such as Alzheimer's disease and depression (Ditzen et al., 2011; Menon et al., 2011; Ringman et al., 2012). However, changes in the CSF content may result not only from alteration in the CP, but also (or rather) be the consequence from an altered brain parenchyma metabolism under the pathological condition.

THE SVZ STEM CELL NICHE IS IN CLOSE CONTACT WITH THE CSF

The adult SVZ niche is located along the lateral walls of the lateral brain ventricles (**Figure 1**). It is composed of slow-dividing (type B) and fast-dividing (type C) stem cells, and neuroblasts (type A cells) (García-Verdugo et al., 1998). The slow-dividing stem cells are divided in two distinct types, B1 and B2 cells, based on cellular characteristics and positioning in the SVZ. Type B1 cells are located closer to the ventricular space, with cell bodies immediately below the layer of ependymal cells, and are in direct contact with the CSF by a unique short non-motile primary cilium that extends

toward the ventricles (Mirzadeh et al., 2008). In addition, type B1 cells are also in contact, by means of basal processes, with the extensive network of blood vessels that cross the periventricular space (Shen et al., 2008). By means of gap and adherens junctions, type B1 cells also contact each other and ependyma cells (Mirzadeh et al., 2008). Type B2 stem cells have astrocytic characteristics and are not in direct contact with the CSF, but rather located closer to the striatum. Type C cells, also known as transit-amplifying cells (TAPs), are the direct progeny of type B cells and are in close contact with their progenitors and with the vasculature. These fast-proliferating cells originate neuroblasts (type A cells) that migrate along the rostral migratory stream (RMS) anteriorly toward the olfactory bulbs where they differentiate mainly into GABAergic interneurons (Whitman and Greer, 2009). The astrocytic processes together with the blood vessels form a scaffold that directs these chains of migrating neuroblasts toward the olfactory bulbs (Whitman and Greer, 2009; Bozoyan et al., 2012). The SVZ also gives rise to oligodendrocyte progenitors, although in lower number when compared with neuroblasts (Menn et al., 2006).

The cells in the SVZ are influenced by several types of extrinsic factors namely growth factors, neurotransmitters and other effectors of signaling pathways. These extrinsic factors originate from blood vessels (Shen et al., 2008), the ependymal cell layer (Lim et al., 2000), the nervous projections toward the lateral walls of the brain ventricles (Lennington et al., 2011), and most importantly, from the CSF. In fact, the distinctive architecture of the adult SVZ neural stem cell niche makes the CSF an essential player that influences the dynamics of the SVZ cell niche. This CSF born molecules influence, by direct contact, type B1 cells via the primary cilium and ependyma cells that in turn interact with type B1 cells. Moreover they also diffuse into the lateral wall parenchyma and thus can exert an effect in type B2, type C and type A cells.

CP BORN MOLECULES MODULATE THE SVZ

The relevance of the CSF content during brain development has been extensively reported. For instance, CSF insulin-growth factor 2 (IGF2), is well described to promote growth and neuronal survival in the mouse developing cortex (Lehtinen et al., 2011). Another example is retinoic acid (Parada et al., 2008; Lehtinen et al., 2011); both meningeal- and CP-derived retinoic acid signaling were shown to contribute to cortical neuron formation and migration, and to cerebellum development (Zhang et al., 2003; Siegenthaler et al., 2009; Crandall et al., 2011).

While the relevance of CSF-derived molecules for neurogenesis during brain development has been evidenced, the potential of CSF-derived molecules to determine neural stem cells renewal, proliferation and migration in the postnatal neurogenic niches, namely the SVZ, has not received the same attention. Moreover, the specific contribution from CP secreted proteins has been seldom highlighted and only rarely demonstrated (Sawamoto et al., 2006). We will next refer to molecules that have been shown to influence the SVZ neural stem cells population dynamics, and that also are expressed/secreted by the adult CP.

INSULIN-LIKE GROWTH FACTORS

IGF2 is highly expressed, as shown by in situ hybridization, in CP epithelial cells not only during development (Lehtinen et al., 2011) but also in adulthood (Bondy et al., 1992). Under physiological conditions IGF2 was the second highest expressed gene found in a microarray study of the adult CP (Marques et al., 2011); the other member of the insulin growth factor family, insulin-like growth factor 1 (IGF1), is also expressed by adult CP epithelial cells, but only modestly. While the CP is not the only contributor to the presence of IGF1 and IGF2 in the CSF during development (other sites are the meninges and the endothelial cells of the brain blood vessels) (Lehtinen et al., 2011) it is certainly well positioned to rapidly influence the SVZ by paracrine effects. Both IGF1 and IGF2 proliferative actions are signaled via the insulin-like growth factor type 1 receptor (IGFR1) (Weber et al., 1992). IGF2 was found to be highly associated with the primary cilium of cortical progenitor cells that projects directly toward the CSF, indicating that IGF signaling occurs via IGF1R located in the primary cilia (Lehtinen et al., 2011). In fact, ablation of IGFR1 expression solely in neural precursor cells resulted in impaired cortical formation, namely microcephaly (Kappeler et al., 2008), similarly to what was found in IGF2-null mice (Lehtinen et al., 2011). Noticeably, IGFR1 is present both in the apical portion of the developing cortical ventricular zone surface, and in the adult SVZ (Yan et al., 2006), which highlights the importance of CP-CSF derived IGF signaling. In turn, IGF1's role in adult neurogenesis has been extensively demonstrated for the dentate gyrus since IGF1 infusion into the hippocampus increases proliferation and neurogenesis (Anderson et al., 2002). Similarly, when infused into the ventricles, IGF1 promotes cell proliferation and neurogenesis in the adult hypothalamus (Pérez-Martín et al., 2010). Furthermore, IGF1 seems to promote the exit of neuroblasts from the adult SVZ and their migration toward the olfactory bulb (Hurtado-Chong et al., 2009). This mismatch in the role of IGF1 in the hippocampus and adult SVZ niches results not only from the differences in the cellular distribution pattern of IGFR1 in both regions (Anderson et al., 2002), but also from the interaction of the IGF1 signaling cascade with other signaling factors such as BDNF and VEGF (Llorens-Martín et al., 2009). Noteworthy is the fact that the action of IGF1 in the two principal adult niches might also be conditioned by its binding to insulin growth factor binding proteins that might inhibit or potentiate the action of IGF1.

Of interest, the adult CP also expresses (Marques et al., 2011) and secretes (Thouvenot et al., 2006) several other insulin growth factor related proteins, such as insulin growth factor binding proteins, insulin growth factor receptors and insulin growth factor binding protein-like 1 (Igfbpl1). Of notice, the latter, is a protein found enriched in adult neural stem cells when compared with parenchymal astrocytes obtained by fluorescence-activated cell sorting (FACS) of SVZ cells (Beckervordersandforth et al., 2010). As for insulin-like growth factor binding protein 2 (Igfbp2), which is very highly expressed by the adult CP (Marques et al., 2011), its ability to support the survival and cycling of hematopoietic stem cells has been recently shown (Huynh et al., 2011). Whether IGFBP2 has a similar effect in the neural progenitor cell population in the adult SVZ is still unknown.

FIBROBLAST GROWTH FACTORS

Another important group of growth factors expressed by the adult CP (Marques et al., 2011) is the fibroblast growth factor (FGF) family and related proteins. Their involvement in several processes of brain development (neural stem cell induction, cell differentiation, brain regions patterning, and neuronal circuit assembly) has been extensively demonstrated (Guillemot and Zimmer, 2011). In fact, the embryonic brain has several sources of different FGF family members that contribute to the determination of brain regionalization; for example, the contribution of CSF-derived FGFs to embryonic brain development was shown for FGF2 since it promotes precursor proliferation in chick embryos (Martín et al., 2006). FGF8, potentially derived from the CP-CSF, has also been described to participate in the patterning of brain regions in the chick (Parada et al., 2005) and mouse embryos (Fukuchi-Shimogori and Grove, 2001).

As for the adult brain, FGF2 injected in the ventricles increased proliferation in the SVZ and neurogenesis in the olfactory bulb (Kuhn et al., 1997). Furthermore subcutaneously injected FGF2, in both early post-natal and in young rats, crossed the brain barriers and increased in the CSF while promoting proliferation in both the subgranular zone of the dentate gyrus and the SVZ (Wagner et al., 1999). The importance of FGF2 for neuronal proliferation is illustrated by the extensive use of FGF2 (also known as bFGF) as a mitogen in SVZ neurosphere assays (Pastrana et al., 2011).

While less is known for the role of other FGF family members in adult neurogenesis it is of notice that FGF family members, such as FGF3, FGF9 and FGF10, are expressed under basal physiological conditions in the adult CP (Marques et al., 2011). Interestingly, FGF10 participation in the maintenance of the neurogenic potential of the adult SVZ was already suggested (Hajihosseini et al., 2008). Thus, under particular conditions, alterations in the expression of FGFs and their secretion toward the CSF may impact in the SVZ population.

EPIDERMAL GROWTH FACTOR (EGF) AND TRANSFORMING GROWTH FACTOR ALPHA (TGFa)

In vitro, when exposed to epidermal growth factor (EGF), adult SVZ derived cells form neurospheres that display multipotent and self-renewal properties (Pastrana et al., 2011). Although expressed in a relative small number of type B1 cells, EGF receptor (EGFR) expressing cells that form neurospheres in vitro are derived mainly from transit amplifying C cells (Doetsch et al., 2002). *In vivo*, it was shown that high levels of EGF administered by intracerebroventricular infusion impacts on the SVZ by increasing proliferation and generating progeny that occupies the surrounding brain parenchyma, and also diverts SVZ cells from the neuronal lineage to the oligodendrocytic lineage (Doetsch et al., 2002; Gonzalez-Perez et al., 2009). While it is believed (Doetsch et al., 2002) that the probable source of EGFR signaling occurs via transforming growth factor alpha (TGFa), given its expression in the CP (Seroogy et al., 1993; Marques et al., 2011), we cannot exclude that this signaling pathway occurs through EGF, also expressed by the CP (Marques et al., 2011). The relevance of TGFa/EGF for EGFR in this context is illustrated by the observation that the decreased proliferation in the SVZ displayed by the TGFa knockout mice can be corrected by supplementation with EGF (Tropepe et al., 1997). Interestingly, TGFa signaling via EGFR was also shown to influence the migratory properties of cells in the RMS (Kim et al., 2009) and of oligodendrocyte precursors derived from SVZ cells (Gonzalez-Perez and Quiñones-Hinojosa, 2010). In addition, a role for TGFa/EGFR signaling in promoting migration of cells derived from the SVZ was highlighted by TGFa infusion to the dopamine-depleted striatum of rodent models of Parkinson's disease (Cooper and Isacson, 2004; de Chevigny et al., 2008). Despite the interesting potential of the CP as a source of TGFa/EGF for the modulation of the SVZ its specific physiological contribution has never been demonstrated, which certainly deserves additional research.

PLATELET DERIVED GROWTH FACTORS (PDGF)

PDGF signaling also occurs via the primary cilium and, interestingly, the PDGF signaling pathway modulates neural stem cells and affects lineage fate. For instance, *in vitro* experiments showed that PDGF increased neurosphere formation (Jackson et al., 2006). Whether SVZ GFAP-positive neural stem cells express the platelet derived growth factor receptor alpha polypeptide (PDGFRa) is disputable (Jackson et al., 2006; Chojnacki et al., 2011; Ihrie and Álvarez-Buylla, 2011), but infusion of PDGF into the ventricle is known to bolster proliferation in the SVZ (Jackson et al., 2006). The endogenous source of this ligand has not been determined (Ihrie and Álvarez-Buylla, 2011) but recently we found that the CP expresses several PDGFs mRNAs (Marques et al., 2011), with particular emphasis to PDGFa. Once again, the physiological significance of this expression and its influence over the SVZ neural stem cell niche remains to be established.

BONE MORPHOGENETIC PROTEINS (BMPs), SONIC HEDGEHOG (Shh) AND Wnt

During the formation of the central nervous system, FGF signaling action is frequently aligned with and/or counteracted by signaling from BMPs, Shh and Wnt pathways. The result of this interaction, impacting on cell proliferation and cell fate, is dependent of the highly dynamic spatiotemporal variation in the expression of the various effector proteins (Guillemot and Zimmer, 2011).

The role of BMP, Shh, and Wnt proteins derived from the CP-CSF during development has been shown. For instance, Shh expression in the hindbrain CP is high and CSF Shh was demonstrated to be essential for cerebellar development by promoting proliferation of granule precursors (Huang et al., 2009, 2010). BMPs, that together with Wnt and FGF proteins, participate in cortical development (Shimogori et al., 2004), display a very dynamic presence in the CSF in an age dependent manner (Lehtinen et al., 2011).

In the adult SVZ, all these proteins participate in the regulation of the SVZ niche. Adult SVZ type B and C cells express both BMP2 and BMP4 and their respective receptors, and SVZ ependymal cells alter the activity of BMPs (Lim et al., 2000; Peretto et al., 2004). The CP origin of these ligands should also be considered since it expresses BMP1, BMP2, BMP4, BMP6 and BMP7 under basal physiological conditions (Marques et al.,

2011). Noteworthy, the presence of some of these BMPs in the adult CSF was also recently confirmed (Lehtinen et al., 2011). Furthermore, growth differentiation factors 3 and 8 (Gdf3 and Gdf8), known to modulate BMP signaling, are also expressed by the adult CP (Marques et al., 2011) and are secreted toward the CSF (Lehtinen et al., 2011).

Shh and Wnts pathways are also active in the adult SVZ and were implicated in the formation, maintenance, proliferation and migration of adult neural stem cells. Shh is produced by ventral forebrain neurons that extend projections toward the SVZ, and persistent Shh signaling determines a specific neural progeny (Ihrie et al., 2011). Shh is also expressed by the CP (Marques et al., 2011) but its levels in the adult CSF have not been determined. As for Wnt, evidence exists that it may participate in the regulation of the adult SVZ population, namely enhancing Wnt signaling via beta-catenin in type C cells increases proliferation and results in a higher number of neurons in the olfactory bulb (Adachi et al., 2007). On the other hand, during embryonic development Wnt may rather play a role in maintaining the SVZ stem cell pool (Piccin and Morshead, 2011). Similarly to Shh and BMPs, several members (for instance Wnt5a, 5b, and 10b) of the Wnt family are expressed in the adult CP, and specifically secreted by CP epithelial cells, under basal conditions (Thouvenot et al., 2006; Marques et al., 2011).

One of the most interesting features of the Shh and Wnt signaling pathways is that they are modulated by the primary cilium (Louvi and Grove, 2011), making the cilia projected by type B1 cells toward the CSF a particularly well positioned route for CP derived "messages".

EPHRINS, SEMAPHORINS AND SLITS

Neuroblasts derived from the SVZ form a stream of moving cells that converge in the RMS; they are ensheated by a layer of astrocyte processes and use each other as guides in the migration process toward the olfactory bulb (Lledo et al., 2008). Several proteins from the group of classical axon guidance molecules were implicated not only in the regulation of the migration of neuroblasts, but also in the proliferation of type B cells. For instance, SVZ cells express the EphA and EphB receptors; the ligands for these receptors are the transmembrane molecules ephrins. The relevance of these molecules in the modulation of the SVZ was shown by the infusion of the EphB2 ligand in the lateral ventricles, which disrupted the migratory chain of neuroblasts and increased the proliferation of type B1 cells (Conover et al., 2000). Interestingly, a role for EphB2 signaling was suggested in the conversion of ependymal cells to astrocytes after lesion of the ventricular wall (Nomura et al., 2010). Also, it has recently been shown that ephrinB3-EphB3 signaling in the SVZ is transiently inhibited to allow the expansion and survival of neural progenitor cells upon traumatic brain injury (Theus et al., 2010). EphrinB3 is one of the ephrins expressed by the CP at very low levels under basal physiological conditions (Marques et al., 2011). While the fact that these molecules are attached to the membrane might diminish the relevance of their expression in the CP in the modulation of the SVZ neural progenitors, the data provided by infusion experiments (Conover et al., 2000) suggest that the cleavage and release of ephrins from the membrane of CP cells toward the CSF, under particular conditions, could influence the response of type B1 cells.

Semaphorins and slits are guidance molecules that, contrary to ephrins, are secreted factors, and hence exert their effects to a certain distance, in a paracrine fashion. Several semaphorin family members and slits 1, 2, and 3 are expressed (Marques et al., 2011) and secreted by the CP (Hu, 1999; Sawamoto et al., 2006). Semaphorin signaling is usually associated with endothelial cells (Tamagnone and Mazzone, 2011); semaphorin-3a and its respective receptor are highly expressed in the endothelial cells that are present along the RMS (Meléndez-Herrera et al., 2008), suggesting a role for semaphorin-3a in the migration of neuroblasts. Whether semaphorins specifically secreted by the CP influence the migration of neuroblasts toward the olfactory bulb has not been determined.

On the other hand, a role for slits specifically derived from the CP in the modulation of SVZ neural progenitors niche was clearly shown; for instance, slit 2 was shown to participate in the regulation of neuronal migration of neurons in the developing brain (Hu, 1999). Interestingly both the adult SVZ and the RMS express the receptors Robo2 and Robo3 through which slit1 and slit 2 exert their chemorepulsive activities (Nguyen-Ba-Charvet, 2004). Importantly, it was shown (Sawamoto et al., 2006) that ciliary beating from ependymal cells that line the wall of the brain ventricles contribute to the movement of the CSF, hence creating gradients of the chemorepelent slits secreted by the CP (Nguyen-Ba-Charvet, 2004; Marques et al., 2011) and thus contributing to the anterior migration of neuroblasts to the olfactory bulbs. Nevertheless, the role of slits in directing the migration of future olfactory bulb neurons goes behind the influence of the CP since type C and type A cells also express slit1 (Nguyen-Ba-Charvet, 2004), which seems to avoid the invasion of the RMS by astrocytic processes (Kaneko et al., 2010).

PATHOLOGICAL CONDITIONS CAN ALTER THE CP TRANSCRIPTOME/SECRETOME AND IMPACT THE SVZ

Being placed at the interface between the periphery and the central nervous system, the CP is particularly well positioned to sense alterations, and respond to, in both its basolateral side (the blood side) and its apical interface (the CNS side). On the other hand, any response the CP mounts to external stimuli will ultimately reflect in its secretome, and hence in the CSF that surrounds the brain parenchyma. In fact, since the CP epithelial cells are equipped with transporters for several proteins and metabolites, pathological damage to the CP itself will alter CSF composition and ventricular volume, as in the case of hydrocephalus (Johanson et al., 2011).

One relevant example of a peripheral event that impacts in the CP transcriptome is peripheral inflammation. When a single intraperitoneal injection of lipopolysaccharide, the membrane component of gram negative bacteria, was given, the CP displayed an acute and transient but profound change in its transcriptome that reflected in the CSF composition (Marques et al., 2007, 2009a). When a similar but repeated inflammatory stimulus was given, the result was still present but more

attenuated (Marques et al., 2009b). These alterations in the CP properties and secretome may impact, even if transiently, in the SVZ niche population. During development, it was recently reported that maternal peripheral inflammation alters fetal ventricular zone proliferation that reflects in cortical layers formation. The effect was shown to involve the barriers of the brain, including the blood-CP-CSF barrier (Stolp et al., 2011). While several reports refer to the impact of neuroinflammation in the adult neurogenesis niches, as in multiple sclerosis, Alzheimer's disease and brain injury (Tepavčević et al., 2011; Wood et al., 2011; L'Episcopo et al., 2012) (Hamilton and Holscher, 2012), the more subtle effects of inflammation-induced alterations in the CP *per se*, and its impact in the adult SVZ, are presently unknown.

A physiological situation where alterations in the CP properties, in the CSF content and in neurogenesis occur is aging. During aging, mainly at the later ages of life, the CP becomes progressively less efficient in protein synthesis and CSF secretion (Redzic et al., 2005). This malfunction in CSF secretion and abnormal removal of CSF toxic compounds, such as the Alzheimer's disease abeta peptide, might contribute to neuropathology (Preston, 2001; Carro et al., 2005). In fact, alterations in the CP-CSF nexus properties might contribute to the alterations observed in the SVZ in rodents during aging and in models and Alzheimer's disease (Jin et al., 2004; Sotthibundhu et al., 2009; Conover and Shook, 2011).

CONCLUDING REMARKS

Under basal normal physiological conditions the CP displays the ability to express several genes encoding for proteins known to promote proliferation, differentiation, and survival of neural progenitor cells. These proteins are secreted toward the CSF, which is as a route for the delivery of CP-born proteins/molecules to the SVZ. *Per se* this is physiologically relevant; yet under disruptive conditions that alter regular CP homeostatic balance, the CSF protein content will be modified and this will lately impact on the SVZ. Whether this culminates in disease developing outcomes (such as is the case of glioblastomas), or might function as potential rescue mechanisms for brain parenchyma lesions (such as in stroke, Parkinson's disease or multiple sclerosis), will be a key issue in adult neural stem cell research in the future. In fact, modulating the CP-CSF nexus in pathologies of the central nervous system could become an important aspect in the usage of endogenous/exogenous neural progenitor cells for stem cell based therapies in the brain.

ACKNOWLEDGMENTS

Ana M. Falcão and Ashley Novais are recipients of Ph.D. fellowships from the Fundação para a Ciência e Tecnologia (FCT, Portugal). Fernanda Marques is a recipient of postdoctoral fellowship from the Fundação para a Ciência e Tecnologia (FCT, Portugal). This work is supported by a grant from FCT (PTDC/SAU-OSM/104475/2008).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 30 April 2012; accepted: 24 July 2012; published online: 09 August 2012.
Citation: Falcão AM, Marques F, Novais A, Sousa N, Palha JA and Sousa JC (2012) The path from the choroid plexus to the subventricular zone: go with the flow! Front. Cell. Neurosci. ***6****:34. doi: 10.3389/fncel.2012.00034*