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# Lineage-specific manipulation of subventricular zone germinal activity for neonatal cortical repair

Diane Angonin

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Université Claude Bernard Lyon 1

Ecole Doctorale  
Biologie Moléculaire Intégrative et Cellulaire

Spécialité de doctorat : Biologie  
Discipline : Neurosciences

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# Lineage-specific manipulation of subventricular zone germinal activity for neonatal cortical repair

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# ABSTRACTS

## Short abstract (French)

L'hypoxie périnatale chronique est un modèle murin de lésion cortical qui reproduit de nombreux déficits observés chez les grands prématurés. Elle entraîne une dégénérescence, une atrophie et un délai de maturation des oligodendrocytes (OL) et des neurones glutamatergiques (NG) du cortex cérébral causant des déficits cognitifs à long terme. Des études ont montré une régénération spontanée bien que partielle de ces types cellulaires, dont l'origine reste à déterminer.

Nos résultats mettent en évidence une réponse spontanée et dynamique du microdomaine dorsal de la zone sous-ventriculaire (dSVZ), une zone germinale dans laquelle des populations de progéniteurs d'OL et de NG persistent après la naissance, qui produit des neurones et des oligodendrocytes corticaux en réponse à l'hypoxie. Les cellules de la dSVZ gardent donc leur compétence à produire de NG corticaux après la naissance.

L'administration par voie intranasale d'un inhibiteur de Gsk3 $\beta$  nommé CHIR, identifié à partir d'une étude bio-informatique comme promouvant les lignages « dorsaux » de la SVZ juste après la période d'hypoxie, potentialise cette réponse spontanée. Nos résultats montrent que CHIR augmente la production de neurones corticaux à partir de la dSVZ encourage l'acquisition de marqueurs de sous types de neurones corticaux, mais reste insuffisant pour promouvoir leur survie. De plus, CHIR promeut la production d'OL corticaux, ainsi que leur maturation et leur survie. Enfin, CHIR entraîne une activation des cellules souches de la dSVZ qui persiste à long terme. Pour conclure, notre étude identifie la dSVZ comme une source malléable de cellules pour la réparation corticale suite à une lésion du cerveau immature.

Mots clés : hypoxie néonatale, régénération cellulaire, Wnt, pharmacogénomique, zone sous-ventriculaire, cellules souches neurales, oligodendrocytes, neurones corticaux

## Short abstract (english)

Perinatal hypoxia is a model of premature brain injuries that reproduces many of the deficits observed in very preterm babies. It leads to degeneration, atrophy and delayed maturation of oligodendrocytes (OLs) and cortical glutamatergic neurons, which often lead to long-term cognitive impairments. Previous studies demonstrated a spontaneous but partial regeneration of those cell types following hypoxia, but their origins remained a matter of debate.

Our results reveal that OL progenitors (OPCs) and glutamatergic neuron progenitors, which persist in the dorsal microdomain of the subventricular zone (dSVZ), are recruited toward the cortex following hypoxia. Permanent labelling of dSVZ neural stem cells by targeted electroporation revealed an increased migration of OLs as well as the induction of a de novo, although transient glutamatergic neurogenesis into the cortex of hypoxic mice. Hence, dSVZ cells participate to post-hypoxia cellular regeneration and thereby keep the competency to produce cortical neurons after birth. We next performed a bioinformatics analysis to identify small bioactive molecules to promote this regenerative attempt. We selected the small molecule CHIR, a GSK3 $\beta$  inhibitor, to use in the context of chronic hypoxia. After validating in vivo the positive effect of CHIR on dSVZ lineages in non-pathological conditions, CHIR was administered intranasally immediately after hypoxia, resulting in a promotion of short-term cortical neurogenesis and OL maturation as well as a long-term OL survival. Furthermore, CHIR promoted dSVZ stem cell activation more than one month after the treatment. Altogether, our study identifies the dSVZ as a malleable source for post-hypoxia cortical repair.

Key words: Hypoxia, cellular regeneration, Wnt, pharmacogenomic, subventricular zone, neural stem cells, oligodendrocytes, neurogenesis

## Long abstract (french)

C'est à partir d'observations cliniques qu'il a été mis en évidence que le cerveau immature présente un meilleur potentiel régénératif que le cerveau adulte. Cet « effet de la lésion précoce » ou « principe de Kennard » a guidé la recherche en médecine régénérative pendant plusieurs dizaines d'années et a permis de mettre en évidence une période de temps critique pour la réparation du système nerveux juste après la naissance. En effet, durant cette période, les circuits corticaux se font et se raffinent, mais surtout, une forte activité germinale persiste dans le cerveau, permettant une certaine réparation tissulaire et une réorganisation des circuits en place.

Dans le système nerveux central (SNC) postnatal comme dans celui de l'adulte, la zone sous-ventriculaire (SVZ) est la principale source de cellules souches neurales, générant en permanence de nouveaux neurones du bulbe olfactif ainsi que des oligodendrocytes, cellules myélinisantes du SNC. La SVZ est sous-divisée en différents microdomaines, contenant des cellules souches neurales (CSN) provenant de différentes régions de l'embryon et produisant des cellules de lignages cellulaires distincts. Dans notre laboratoire, nous sommes particulièrement intéressés par le microdomaine dorsal de la SVZ. En effet, ses cellules produisent de manière continue des progéniteurs d'oligodendrocytes et de neurones glutamatergiques, deux types cellulaires particulièrement sensibles dans le contexte de lésions périnatales. De nombreuses évidences indiquent que des facteurs intrinsèques ainsi que des facteurs provenant du microenvironnement participent à établir et maintenir cette hétérogénéité régionale. Ces facteurs extrinsèques représentent des cibles potentielles qui pourraient être exploitées pour manipuler des lignages spécifiques de la SVZ en conditions physiologiques, mais surtout en conditions pathologiques, par exemple après la perte ou dégénérescence de types cellulaires particuliers qui devraient être remplacés spécifiquement.

L'hypoxie périnatale est un modèle de lésion cortical qui reproduit de nombreux déficits observés chez les grands prématurés. Elle entraîne une dégénérescence, une atrophie des cellules du cortex cérébral qui entraîne des déficits cognitifs à long terme. Notamment, les oligodendrocytes présentent un délai de maturation et les neurones

glutamatergiques dégénèrent en partie. Des études ont montré une régénération spontanée bien que partielle de ces types cellulaires, dont l'origine reste à déterminer.

Nos résultats mettent en évidence une réponse spontanée et dynamique des cellules du microdomaine dorsal de la SVZ, des progéniteurs d'oligodendrocytes et de neurones glutamatergiques, qui sont affectées différemment par la période d'hypoxie mais sont toutes deux recrutées pour la réparation corticale. En effet, elles produisent des neurones glutamatergiques et des oligodendrocytes corticaux en réponse à l'hypoxie périnatale. Les cellules de la SVZ dorsale gardent donc leur compétence à produire des neurones glutamatergiques corticaux après la naissance.

Afin de promouvoir cette réponse endogène des cellules de la SVZ dorsale, nous avons décidé d'utiliser une approche pharmacogénomique. Une étude bioinformatique consistant à comparer les transcriptomes des cellules souches et progéniteurs des différents microdomaines de la SVZ, a permis l'identification de signatures transcriptionnelles correspondant aux différents lignages produits par chacun des microdomaines. Ces signatures transcriptionnelles ont été comparées aux changements d'expression induits par une liste de petites molécules afin d'identifier celles qui pourraient promouvoir les lignages dorsaux de la SVZ. Parmi les petites molécules ayant le potentiel de promouvoir les lignages dorsaux, nous avons choisi l'inhibiteur de Gsk3 $\beta$  de seconde génération AR-A014418, dont nous avons immédiatement comparé l'efficacité avec CHIR99021, un inhibiteur de Gsk3 $\beta$  de troisième génération. Les inhibiteurs de Gsk3 $\beta$  sont largement décrits et utilisés comme promouvant la voie Wnt/ $\beta$ -caténine. En condition physiologique, pendant la période postnatale et chez l'adulte, les deux petites molécules augmentent la production de progéniteurs d'oligodendrocytes et de neurones glutamatergiques dans la SVZ dorsale. CHIR99021 étant plus efficace *in vivo* qu'AR-A014418, nous l'avons sélectionné afin de tester sa capacité à promouvoir la régénération cellulaire dans le contexte de l'hypoxie périnatale. De manière intéressante, nos résultats montrent que l'administration intranasale de CHIR99021 juste après la période d'hypoxie potentialise la réponse spontanée de la SVZ dorsale à l'hypoxie. En effet, CHIR99021 augmente la production de neurones corticaux à partir de la dSVZ et semble promouvoir leur survie à long terme. De plus, CHIR99021 promeut la production d'oligodendrocytes corticaux, accélère leur maturation et augmente leur survie. Enfin, CHIR99021

entraîne une activation des cellules souches de la dSVZ qui persiste plus d'un mois après le traitement.

Nos résultats valident l'efficacité de notre étude pharmacogénomique qui permet d'identifier des petites molécules influençant le devenir des cellules souches de la SVZ. En particulier, les inhibiteurs de Gsk3 $\beta$  ont un fort pouvoir dorsalisant dans un contexte physiologique comme dans un contexte pathologique. De plus, nous avons montré une tentative spontanée de la SVZ dorsale à promouvoir une régénération corticale suite à une hypoxie périnatale. Nous montrons que l'activation de la voie Wnt/ $\beta$ -caténine par l'administration intranasale d'inhibiteurs de Gsk3 $\beta$  dans le contexte de l'hypoxie périnatale améliore la régénération cellulaire corticale spontanée et endogène suivant la lésion.

Ainsi, nous identifions la zone sous-ventriculaire dorsale comme une source malléable de cellules souches et de progéniteurs pour la réparation corticale suivant l'hypoxie périnatale.

## List of abbreviations

**AEP** : anterior entopeduncular  
**Akt (PKB)** : protein kinase B  
**Apc** : adenomatous polyposis coli  
**Ascl1 (Mash1)** : Achaete-scute homolog 1  
**BBB** : blood brain barrier  
**BDNF** : brain-derived neurotrophic factor  
**BGal** :  $\beta$ -galactosidase  
**Bmp** : bone marrow protein  
**BrdU** : bromodeoxyuridine  
**Cas3A** : active caspase3  
**CC1** : Apc antibody clone  
**Chat** : choline acetyltransferase  
**Ck1** : casein kinase 1  
**CldU** : chlorodeoxyuridine  
**CMAP** : connectivity map  
**CNS** : central nervous system  
**CSF** : cerebrospinal fluid  
**Ctip** : Chicken ovalbumin upstream promoter transcription factor-interacting protein  
**Cux** : cut-like homeobox 1  
**Dcx** : double cortin  
**DWMI** : diffuse white matter injury  
**EdU** : ethynyldeoxyuridine  
**EGF, EGFR** : epidermal growth factor, epidermal growth factor receptor  
**EPO, dEPO, IEPO** : electroporation, dorsal electroporation, lateral electroporation  
**Erk** : extracellular signal–regulated kinase  
**FDA** : food drug and administration  
**FezF** : Fez family zinc finger protein  
**FGF** : fibroblast growth factor  
**GCL** : glomerular cell layer

**GDNF** : glial cell derived neurotrophic factor  
**Gfap** : glial fibrillary acidic protein  
**GFP** : green fluorescent protein  
**GL** : granular cell layer  
**Gsk3** : glycogen synthase kinase 3 beta  
**HDAC** : histone deacetylases  
**HIF** : hypoxia-induced factor  
**IdU** : iododeoxyuridine  
**IGF** : insulin-like growth factor  
**IN** : intranasal  
**IPC** : intermediate progenitor cell  
**Lef** : lymphoid enhancer factor  
**LGE** : lateral ganglionic eminence  
**mAChR** : muscarinic acetylcholine receptor  
**Mbp** : myelin binding protein  
**Mcm2** : minichromosome maintenance complex component 2  
**MGE** : medial ganglionic eminence  
**NP** : neural progenitor  
**NeuN** : neuronal nuclei  
**NSC, aNSC, qNSC** : neural stem cell, active neural stem cell, quiescent neural stem cell  
**OB** : olfactory bulb  
**OL** : oligodendrocyte  
**Olig1/2** : oligodendrocyte transcription factor 1/2  
**PDGF** : platelet-derived growth factor  
**Pi3K** : phosphoinositide 3-kinase  
**PrV** : parvalbumin  
**qPCR** : quantitative polymerase chain reaction  
**S100 $\beta$**  : s100 calcium-binding protein B  
**Satb2** : Special AT-rich sequence-binding protein 2  
**SDS-PAGE** : sodium dodecyl sulfate polyacrylamide gel electrophoresis  
**RGC** : radial glia cells

**RMS** : rostral migratory stream

**RNA, siRNA** : ribonucleic acid, small interferent ribonucleic acid

**Scf $\beta$ -TrCP** : Skp1–cullin 1– f-box

**Shh** : sonic hedgehog

**Sox2 (SRY)** : Sex determining region Y-box 2

**SST** : somatostatin

**SVZ, ISVZ, dSVZ** : subventricular zone, dorsal subventricular zone, lateral subventricular zone

**TAPs** : transient amplifying progenitors

**Tbr2 (Eomes)** : T-brain gene-2

**Tcf** : T-cell factor

**Tgf** : transforming growth factor

**VZ** : ventricular zone

**VLBW** : very low birth weight

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## INTRODUCTION

### **A. The activity of the ventricular-subventricular zone persists at postnatal ages but declines over time.**

#### **1. A germinal activity persists in the ventricular-subventricular zone throughout life**

##### **a. History of postnatal neurogenesis discovery**

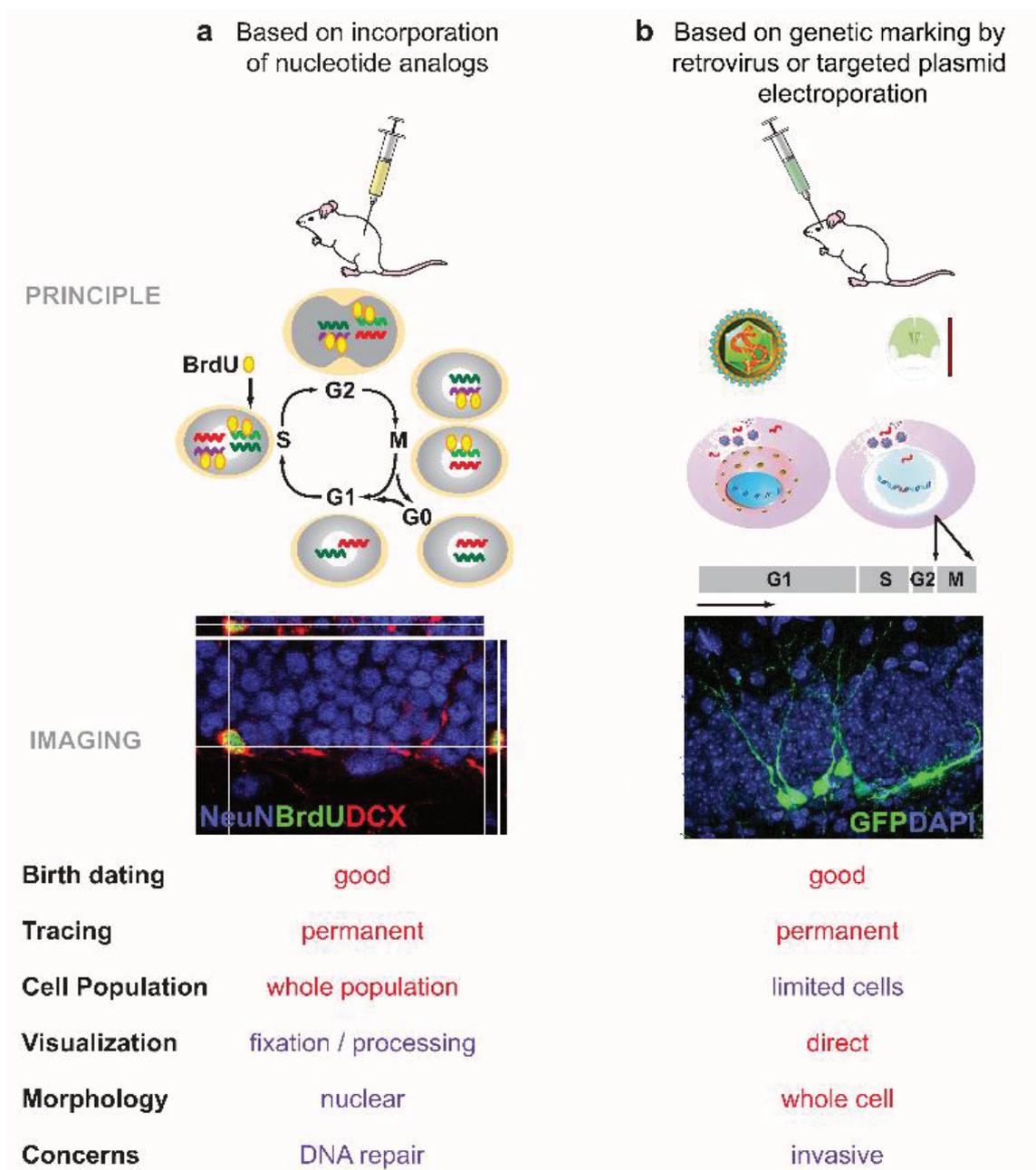
Neurons, first described in the XIXe century, are excitable cells collecting and transmitting electrical signals through chemical synapses. They assemble into networks and constitute the main components of the CNS. Santiago Ramon y Cajal is at the origin of a major step in the neuroscience field as he proved the neuron doctrine, meaning that the brain is made of cell units connected to each other, and not a single continuous network (like described in the reticular theory). However, he also concluded that neurons could be generated only before birth (Berciano et al., 2001).

The first evidences of an adult neurogenesis were provided by Altman and Das in 1960's who used a method to label postnatal proliferating cells in the S-phase of the cell cycle using thymidine-H3 in the rat brain. Thymidine-H3 is detectable by autoradiography and allows to trace the fate of dividing cells, which, based on ultrastructural criteria, appeared to become neuron rather than glial cells (SIDMAN et al., 1959). Using this approach, it was proposed that the production of neurons continues after birth in the hippocampus, the neocortex and the olfactory bulb (Altman, 1966, 1969; Altman and Das, 1965; ALTMAN and DAS, 1965). However, evidences were indirect and it took longer to the scientific community to accept the notion of postnatal neurogenesis which was refuting the old dogma of an exclusively embryonal birth of all neurons (Berciano et al., 2001; Ming and Song, 2011). Later, in the 70's,

this dogma was challenged by two other scientists. Fernando Nottebohm worked on the canary and showed that those birds have the capacity to seasonally grow new neurons when learning new songs. In addition of confirming that postnatal neurogenesis can be observed in some species, these observations also suggested a functional role for adult born neurons (Nottebohm et al., 1976). Kaplan and others provided further information showing adult born neuron integrate, survive long term in the hippocampus, and seemed to receive synaptic inputs and extend axonal projections in different areas of the rodent brain (Kaplan and Bell, 1983; Kaplan and Hinds, 1977; Stanfield and Trice, 1988). Eventually, neural stem cells (Nestin-expressing cells with the capacity to proliferate and differentiate into neuronal and glial cells) were isolated from the adult rodent brain (Reynolds and Weiss, 1992), and then from the human brain (Kukekov et al., 1999). These landmark studies, provided the last piece of evidence which convinced the researchers of the existence of an adult neurogenesis in the mammalian brain (Gross, 2000).

#### **b. Tools to study proliferating cells and their fate**

This conceptual evolution was made possible because of the emergence of new tools to study the fate of neural stem cells. Bromodeoxyuridine (BrdU) for instance, a synthetic thymidine analogue is another S-phase marker of the cell cycle like thymidine-H3 (Gratzner, 1982). Importantly, BrdU, in contrast to thymidine-H3, can be revealed by immunocytochemistry and can therefore be detected together with other cell-specific markers, such as neuronal markers, which allows to assess more directly the identity of the newborn cells. Adult neurogenesis using BrdU incorporation was widely shown to occur in the brain of various mammalian species, including humans, in the 90's (Eriksson et al., 1998). BrdU incorporation is still widely used to label and follow the fate of global populations of stem cells and progenitors (**Fig I1.1A**), as well as the EdU, IdU and CldU, other nucleotide analogues which allow to combine different proliferation markers (Yennek and Tajbakhsh, 2013). The major drawback of this approach is its lack of information on the morphology of the cells due to its exclusive nuclear location. Retroviral-based lineage tracing (Sanes et al., 1986) as well as targeted electroporation (Boutin et al., 2008; Fernández et al., 2011) can be used to circumvent this limitation (**Fig I1.1B**).

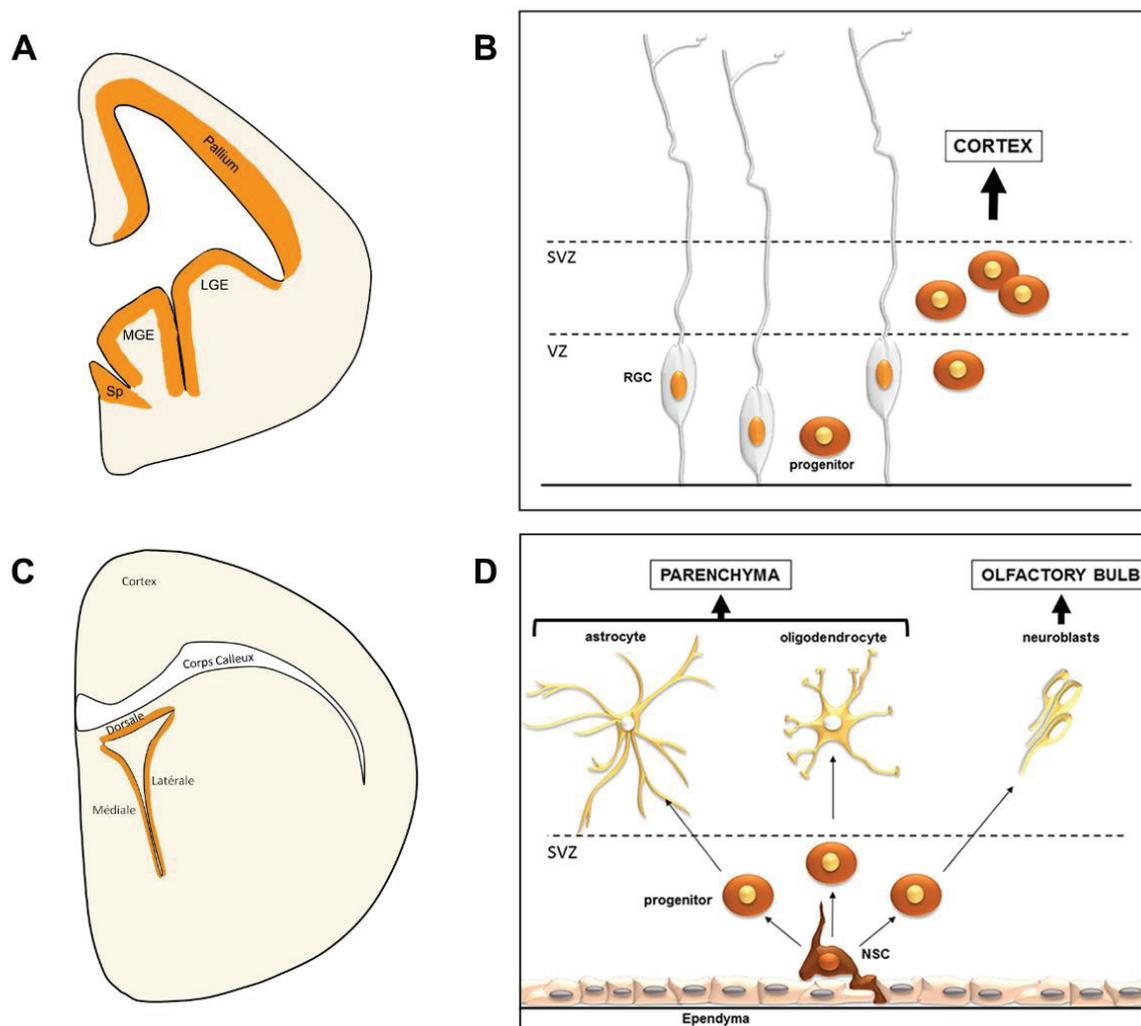


**Figure I1.1: Illustration summarizing different approaches to highlight and analyze adult neurogenesis in vivo.** (A) Approach based on marking proliferating cells (thereby immature) and their progeny by the incorporation of nucleotide analogs during DNA replication in the S-phase of the cell cycle. Immunostaining confirms the colocalization of bromodeoxyuridine (BrdU) and post-mitotic neuron-specific markers. (B) Approaches based on retroviral genetic marking or targeted plasmid electroporation. Retrovirus integrate the host genome specifically during the M phase of the cell cycle when cell membrane breaks down. Targeted electroporation requires oriented electric pulses after plasmid injection in the ventricular system. When performed in the first few days after birth, it allows the efficient electroporation of neural stem cells. Microphotography shows the expression of green fluorescent protein (GFP) in newborn cells two weeks after stereotaxic injection of the retrovirus into the hilus region of the adult mouse hippocampus. Similar cells are observed after electroporation of a GFP plasmid to the subventricular zone. Those approaches allows direct visualization of the morphology of newborn cells. They can also be used for genetic manipulation of neural stem cells and of their progeny. Adapted from (Ming and Song, 2005)

### c. Neurogenesis persists over time in the VZ-SVZ

At the embryonic stage, the ventricular zone (VZ) surrounding the lateral ventricle, together with the subventricular zone (SVZ) are the source of an intense neurogenesis producing the neurons of the different regions of the CNS (Rakić and Sidman, 1969), including the cortex (Anderson et al., 1997; de Carlos et al., 1996; Gao et al., 2014) (**Fig I1.2A,B**). Although postnatal neurogenesis activity was first described in the hippocampus, where it is associated with learning and memory (Gonçalves et al., 2016), evidences also showed a postnatal neurogenesis persisting in the postnatal SVZ. Indeed, while the VZ disappears rapidly around birth, the SVZ continues to produce neurons as well as glial cells, and this, throughout life (**Fig I1.2C,D**).

Even though the germinal activity of the VZ-SVZ at the embryonic stage has long been demonstrated, the proof of its ability to produce functional neurons at postnatal and adult stages is more recent. Since the demonstration of neural progenitor cells in the adult SVZ 25 years ago (Reynolds and Weiss, 1992), numerous studies focused on the competency of those progenitors to produce functional neurons and on the possibility to manipulate their fate.



**Figure I1.2: Both embryonic and adult VZ-SVZ contain neural stem cells and progenitors.** (A, C) Diagram representing a coronal view of an embryonic (A) and adult (C) brain section. The germinal region appears in orange. (B, D) Representation of the neural stem cells (NSCs) and progenitor populations as well as the location where they migrate while differentiating, in the embryonic and adult VZ-SVZ. In the embryonic pallial VZ-SVZ, NSCs are radial glia cells (RGCs), dividing through symmetric divisions to give rise to two daughter RGCs or through asymmetric divisions to give rise to one RGC and one progenitor. Progenitors migrate to the SVZ, proliferate and differentiate into cortical neurons. In the adult SVZ, NSCs produce proliferating progenitors. Progenitors give rise to parenchymal astrocytes and oligodendrocytes, or neuroblasts migrating toward the olfactory bulb. Abbreviations: Sp: septum ; MGE: medial ganglionic eminence ; LGE: lateral ganglionic eminence ; RGC: radial glia cell ; NSC: neural stem cell.

## 2. The VZ-SVZ undergo major cytoarchitectural changes around birth

### a. The embryonic pallial VZ-SVZ produces cerebral cortex neurons

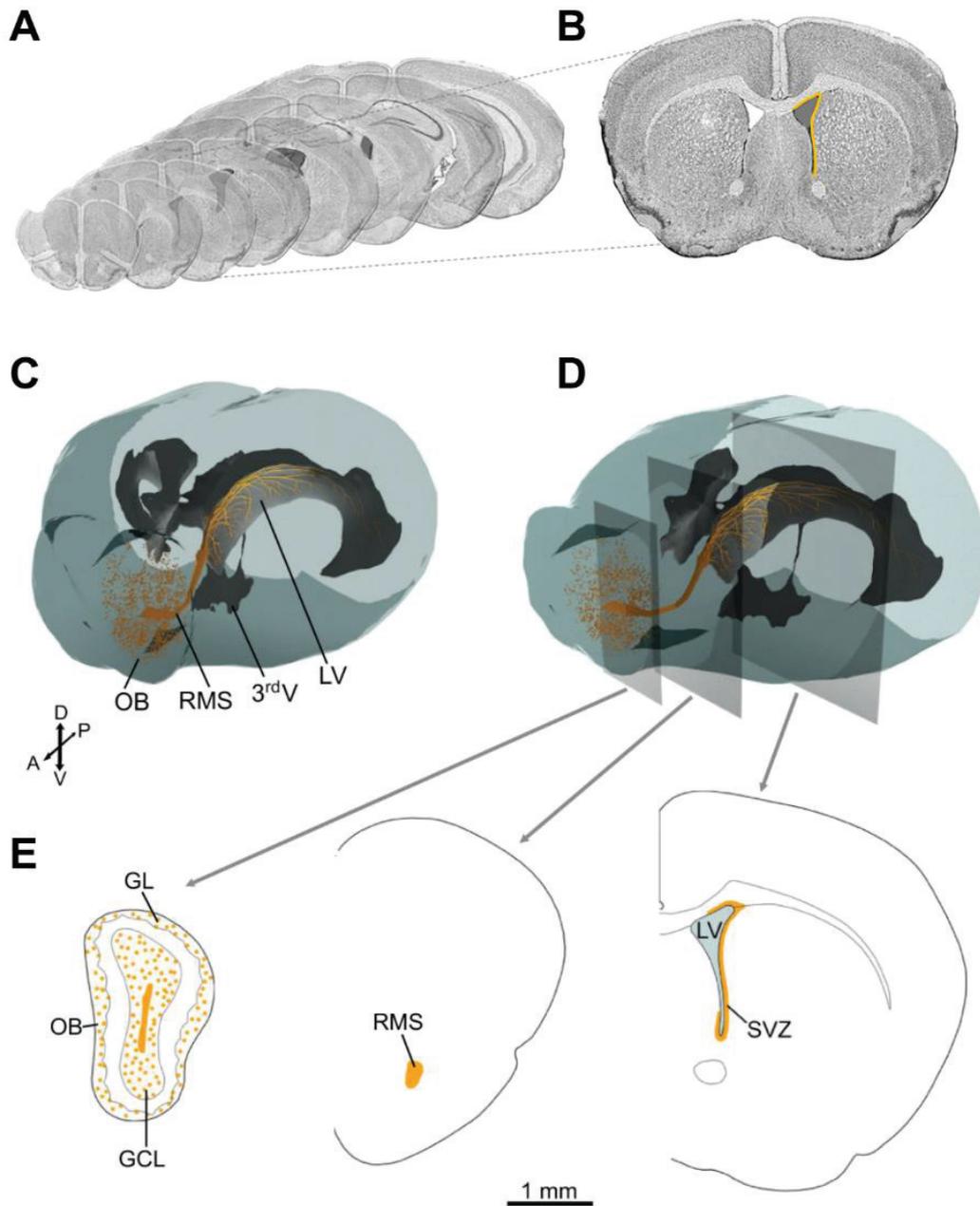
At the embryonic stage, the activity of the VZ-SVZ is intense and notably leads to the formation of the cerebral cortex. Radial glia cells (RGCs), derived from the original neuroepithelial cells, are located within the VZ, around the lateral ventricle. They have two thin processes extending from the ventricular surface to the outer surface of the cortex, with an apical-basal polarity. This particular morphology lead the RGCs to be first only considered for a long time as scaffold cells for the radial migration of the cortical neurons (Rakic, 1971). Their function of early precursor of neuronal and glial cells was only demonstrated later (Malatesta et al., 2000; Noctor et al., 2001). At the onset of neurogenesis around E10, RGCs proliferate through symmetric divisions to give rise to two daughter radial glia cells or differentiate through asymmetric division to give rise to a daughter radial glia cell and either a neuron or an intermediate progenitor cell (IPC) (Takahashi et al., 1996). At E12-E13, a new compartment appears, the subventricular zone (SVZ), composed of some randomly organized dividing cells located at the basal border of the VZ (Smart, 1973), where the IPC migrate from the VZ. There, they divide symmetrically to give rise to two neurons. As neurogenesis proceeds, the rate of asymmetric divisions increase progressively at the expense of symmetric divisions. Moreover, IPCs amplify the neurogenic pool prior to terminal neurogenic divisions. Thus, as neurogenesis continues the RGC pool remains largely stable while the IPC pool expands progressively until the end of cortical neurogenesis around E17 (Noctor et al., 2007, 2008).

The mammalian cerebral cortex is a highly organized structure underlying higher brain functions such as abstract thinking, learning and memory. In the mouse, cortical neurogenesis takes place mostly from E10 (10 days following fecundation) to E19 ; the majority of cortical neurons being produced between E12,5 and E17,5 (García-Moreno et al., 2007). Although cortical neurogenesis was long thought to be occurring exclusively before birth, a recent study challenges this view with evidences of a minor postnatal cortical neurogenesis originating in the meninges (Bifari et al., 2017). The cortex is composed of glutamatergic excitatory projection neurons (~80%) and GABAergic neurons (~20%) that assemble to form stereotyped cortical circuits (Bédard

et al., 2002; Kornack and Rakic, 2001a; Sawamoto et al., 2011). In addition, oligodendrocytes and astrocytes modulate the circuits activity with their pleiotropic roles (Müller and Best, 1989). The cortical cytoarchitecture is often defined based on its glutamatergic neuron components reflecting its layered organization (Toma and Hanashima, 2015). Cortical shape as well as cortical genesis and organization will be described with more details later in this manuscript.

### **b. The postnatal SVZ renews olfactory bulb neurons**

After birth, the SVZ remains capable of producing neurons and participate largely to the renewal of the olfactory bulb (OB) GABAergic neurons (Doetsch and Alvarez-Buylla, 1996; Kirschenbaum and Goldman, 1995) but also of a smaller proportion of the OB glutamatergic neurons (Brill et al., 2009). Indeed, postnatal SVZ progenitors produce neuroblasts that migrate tangentially, in chains, through the rostral migratory stream (RMS) formed of tube-like structures of astrocytes, from the SVZ to the OB. Eventually neuroblasts differentiate into local interneurons in the OB, dedicated to odor information processing, where they integrates into local circuits (Lim and Alvarez-Buylla, 2016; Nagayama et al., 2014) (**Fig I1.3**). In total, the postnatal SVZ participates to the renewal of the olfactory bulb neurons by producing about 30000 new neurons a day in young adult mice (Lois and Alvarez-Buylla, 1994). Interestingly, OB neurogenesis persists in non-human primates (Bédard et al., 2002; Kornack and Rakic, 2001b; Sawamoto et al., 2011) but the diversity of produced lineages is rapidly restricted after birth (Azim et al., 2013). In humans, OB neurogenesis declines during infancy (Sanai et al., 2011). However, a striatal interneuron genesis seems to occur postnatally and persists longer throughout life (Akhtar and Breunig, 2015; Ernst et al., 2014a).



**Figure 11.3: The VZ-SVZ produces olfactory neurons throughout life.** Schematic representation of the neurogenic process along the entire extent of the ventricular system of the neonatal/adult rodent forebrain. **(A)** Series of coronal sections of a postnatal brain with Nissl staining. **(B)** Representation of the VZ-SVZ (orange) on a coronal section of the forebrain. **(C, D)** 3D representation of V-SVZ neurogenesis. Neural stem cells (NSCs) are located in the walls of the lateral ventricle (LV) and generate an irregular network of migratory neuroblasts (orange) that converge into the rostral migratory stream (RMS). Upon arrival at the olfactory bulb (OB), the neuroblasts migrate radially to reach their final destination and mature into GABAergic or glutamatergic neurons: granular neurons in the granular cell layer GCL and periglomerular neurons in the glomerular layer (GL). **(E)** Sectioning planes through the OB, RMS and LV show the distribution of newly generated neuroblasts in these three forebrain compartments. 3<sup>rd</sup>V, third ventricle; SVZ, subventricular zone. Adapted from (Fiorelli et al., 2015).

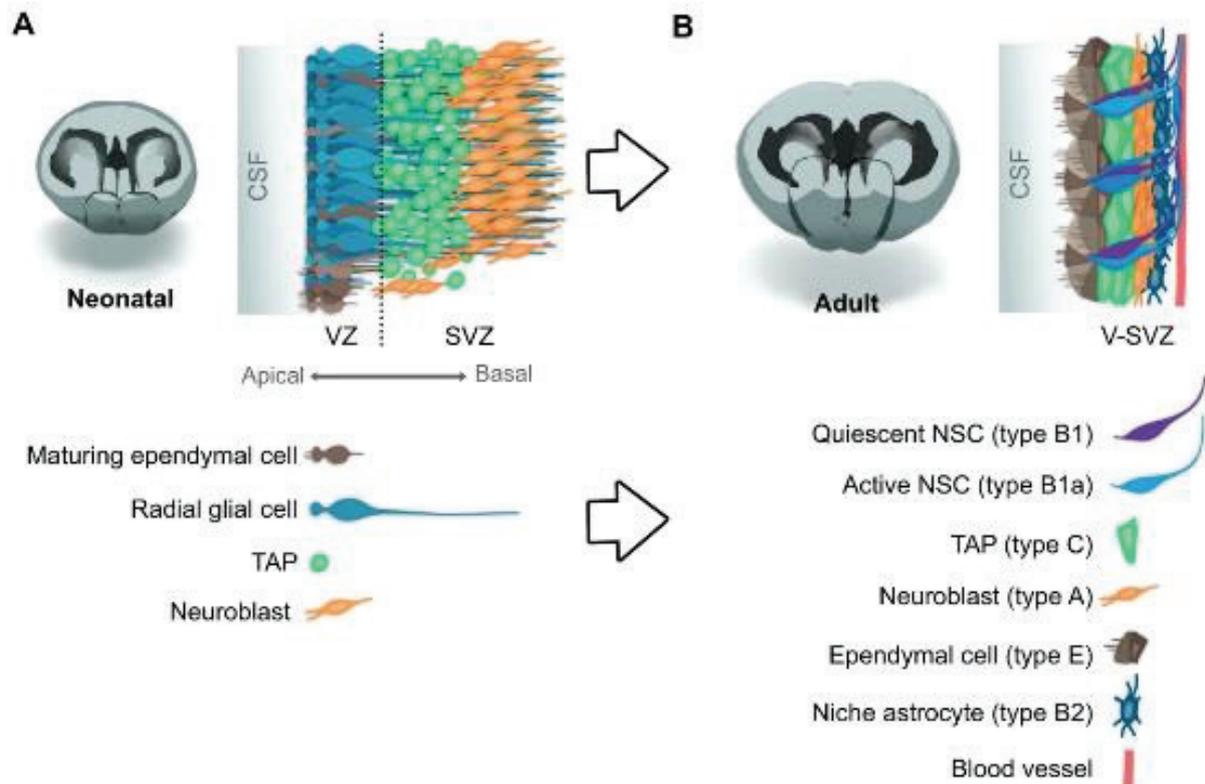
### c. Cytoarchitectural reorganization of the VZ-SVZ around birth

Around birth, the neurogenic niche undergoes a major cytoarchitectural reorganization, which correlates with a decreased neurogenic activity. Indeed, during the first two postnatal weeks, radial glial cells (RGCs) change morphology to adopt astrocytic characteristics, such as the expression of the cytoskeleton marker GFAP. In addition, their basal process retracts from the pial surface to contact blood vessels. Some RGCs will give rise to neural stem cells (NSCs) called type B NSCs. There is currently a debate regarding the identity of RGCs that give rise to postnatal NSCs. Indeed, the classic view implies that RGCs transform into NSCs around birth while recent studies suggest that this transition occurs earlier during embryonic development. Indeed, recent studies have described a small cohort of slow cycling progenitors segregate from other RGCs between E13,5 and E15,5 giving rise to the adult SVZ NSCs (Fuentealba et al., 2015; Furutachi et al., 2015a). Part of the RGCs also differentiate into ependymal cells or adopt a glial fate (Merkle et al., 2004; Tramontin et al., 2003). A thin epithelium-like layer appears gradually during the first postnatal weeks, forming an interface with the cerebrospinal fluid (CSF) contained in the ventricle and the neurogenic niche, and composed of multiciliated ependymal cells (also called type E cells) born earlier in time, before birth (Mirzadeh et al., 2008; Spassky et al., 2005). This first layer is surrounded by type B NSCs, which keep an apical cilium in contact with the CSF, allowing them to sense circulating cues (**Fig I1.4**). It is possible to visualize this organization with whole-mount preparation of lateral ventricle wall, revealing a pinwheel characteristic organization (Mirzadeh et al., 2008). There are two categories of Type B NSCs, the quiescent and the active NSCs (Li and Clevers, 2010). NSCs remain active for only a few weeks before getting depleted for a few week, generating transient activated progenitors (TAPs, or type C cells), which have indeed a high rate of proliferation.

In summary, even though the cytoarchitecture of the VZ-SVZ as well as the intensity of the germinal activity changes over time and more precisely around birth, pre-natal and post-natal VZ-SVZ keep containing the same categories of cells. 3-Dimensional representations of the VZ-SVZ niche allows a better comprehension of its structure as well as of the extrinsic elements from the microenvironment, which potentially influence the activity of the residing NSCs (**Fig I1.4**).

In this manuscript, I decided to call NSCs rather than progenitors the astrocyte-like cells of the postnatal SVZ expressing Sox2 marker (Ellis et al., 2005; Kriegstein and Alvarez-Buylla, 2009). As mentioned before, NSCs can be in a quiescent or active state. Interestingly, quiescent and active NSCs can interconvert *in vitro*, highlighting the role of the microenvironment in NSCs state. Moreover, active NSCs are heterogeneous and can be separated into two categories according to their proliferating rate (Codega et al., 2014a). In this manuscript, I will differentiate the quiescent NSCs from the activated and actively cycling NSCs according to their expression of the proliferating markers Ki67 and Mcm2. Quiescent NSCs indeed do not express those proliferative markers as they remain at the G0 cell cycle stage. Activated NSCs express Mcm2 in addition to Sox2 but not Ki67. Eventually, actively cycling NSCs express Sox2, Mcm2 and Ki67 (Maslov et al., 2004a). Indeed, several studies showed that it is possible to separate the active VZ-SVZ NSCs in two categories according to their proliferative rate, with the active NSCs cycling in 12 hours while actively cycling NSCs cycle in about 15 days (Doetsch et al., 1999; Morshead et al., 1994, 1998; Tropepe et al., 1997). Furthermore, they have the ability reconstitute spontaneously their pool of actively cycling NSCs when depleted by Ara-C, an anti-mitotic drug (Doetsch et al., 1999). The transient amplifying progenitors (TAPs) are the highly proliferating as well and already committed cells expressing lineage-specific markers such as Tbr2 for the glutamatergic neuronal lineage and Olig2 for the oligodendrocyte lineage and do not express Sox2. Migrating neuroblasts express the immature neuron marker Dcx (Azim et al., 2012a; Maslov et al., 2004a). Both embryonic and postnatal VZ-SVZ contain NSCs (called radial glia cells, RGCs, before birth), immature or mature ependymal cells, TAPs and neuroblasts. However, the differences in the cytoarchitecture changes their interactions with the microenvironment. For instance, postnatal SVZ neural stem cells create physical contacts with blood vessels and their contact with the ventricle containing the cerebrospinal fluid (CSF). Blood vessels capillaries located in the VZ-SVZ provide an incomplete blood-brain barrier (BBB) as they are not fully enwrapped by pericytes and astrocyte endfeet, which allows a better permeability to growth factors and small molecules (Sawada et al., 2014; Tavazoie et al., 2008). Furthermore, factors from the blood vessels have an age-dependant effect on the VZ-SVZ cell proliferation (Lehtinen et al., 2011). Of note, a population of choline acetyltransferase (ChAT+) neurons was recently described in the SVZ for having the ability to modulate neurogenic

proliferation, suggesting another level of control of SVZ activity (Paez-Gonzalez et al., 2014). In addition, a very recent study showed that proopiomelanocortin neurons from the hypothalamus could regulate specific NSC populations of the SVZ in response to physiological and environmental signals (Paul et al., 2017).

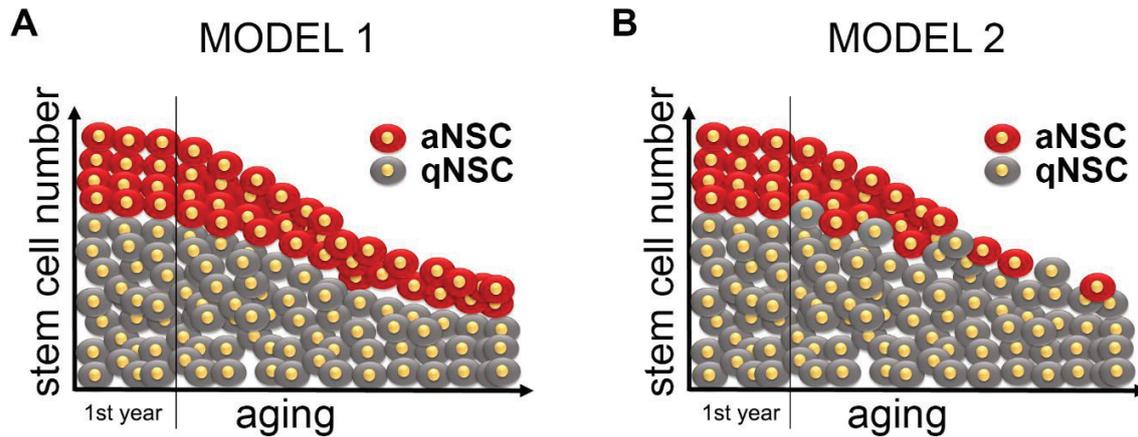


**Figure I1.4: Cellular organization of the postnatal and adult VZ-SVZ.** (A) In the first days after birth, the lateral wall is layered in a ventricular zone (VZ), which is composed of three layers of radial glial cells (RGCs), and a subventricular zone (SVZ), which is populated by proliferating transit amplifying progenitors (TAPs) and migrating neuroblasts. In the lateral wall, RGCs begin to retract their long basal processes following a ventrodorsal gradient. Some RGCs begin to differentiate into ciliated ependymal cells. (B) Following postnatal maturation, the V-SVZ becomes considerably thinner and clearly structured (from ~300  $\mu\text{m}$  at birth to <100  $\mu\text{m}$  in the adult mouse). Adult quiescent and actively proliferating NSCs contact the CSF with an apical process that is surrounded by ciliated ependymal cells arranged in stereotypical pinwheel structures, although the density and organization of these pinwheels vary throughout walls and rostrocaudal coordinates. TAPs and neuroblasts are also more tightly organized compared with the neonatal V-SVZ – respectively in clusters (green) and dorsally migrating chains (orange) in close proximity to the NSCs. On the parenchymal side of the V-SVZ, the NSC basal end-feet contact the blood vessels. Note that the pattern of the pinwheel structures is shown as being very regular for simplification purposes; it is actually more complex and irregular *in vivo*. (Fiorelli et al., 2015)

#### d. NCSs activity decreases with aging

Following the dramatic cellular changes that occurs during the first post-natal weeks, germinal activity will continue decreasing over time. With aging, the SVZ evolves with a stenosis of the lateral ventricle walls, leading to a deterioration of most of the ventral SVZ, and restricting the neurogenic region to the dorsolateral corner of the SVZ. The integrity of the ependymal border is maintained, even though it is thinner and contains a large number of astrocytes that are intermingled with ependymal cells (Conover and Shook, 2011; Luo et al., 2006). The total number of proliferative cells is reduced by 50 to 75%.

The pool of postnatal NSCs remain quite stable until middle-age (about one year) (Piccin et al., 2014) in the SVZ. However, NSCs lose progressively their proliferative capacities (Ahlenius et al., 2009) to become more and more quiescent (Bouab et al., 2011). It remains unclear how stem cell heterogeneity changes over time and the importance of extrinsic and intrinsic factors in this phenomenon. Two models were proposed to explain NSCs loss of activity. The first one postulates that the proportion of quiescent and active stem cells remain stable and the decreased activity is due to overall decrease in stem cell population, likely combined with a decreased proliferation rate at the cellular level. The second model postulates that active stem cell population disappears progressively to become more and more quiescent (**Fig 11.5**) (Chaker et al., 2016). The pool of progenitors, on an other hand dramatically decrease with aging (Balordi and Fishell, 2007; Giachino et al., 2014). In parallel, olfactory bulb (OB) neurogenesis is also impaired with aging with a number of new neurons that arrive in the OB decreasing over time (Enwere et al., 2004). Indeed, studies using label retention strategies such as BrdU demonstrated a decline of SVZ neurogenesis of about 50% between old (23 to 25 months old) and young mice (2 to 4 months old) (Tropepe et al., 1997), associated functional changes in odor discrimination.



**Figure 11.5: Aging-related changes in quiescent and active neural stem cell populations in the SVZ.** NSC number decreases over time, after the first year of life. Two nonexclusive models were proposed to explain this phenomenon. Diagrams represent the evolution of the number of neural stem cells (NSC) with aging as well as the proportion of active NSC (aNSC, grey) and quiescent NCSs (qNSC, red). **(A)** Model 1 suggests that NSC dynamics are altered throughout time probably due to a decreased proliferation rate. This can be due to multiple factors including changes in NSC recruitment and/or division rate. **(B)** Model 2 suggests a progressive shift of adult NSCs from the active to the quiescent state. Adapted from (Chaker et al., 2016).

### 3. The postnatal SVZ is regionalized according to the fate of the stem cells it contains

#### a. The postnatal SVZ is divided into three microdomains

Although the SVZ contains progenitor populations all along the ventricle, those populations are not homogeneous. For instance, a study carried out by Merkle, revealed that neural stem cells from the different part of the postnatal SVZ produce very specific and different subtypes of olfactory bulb neurons. In this study, SVZ neural stem cells from different areas were labelled permanently neonatally (P0) and the nature of the olfactory bulb neurons originating from the different populations of SVZ neural stem cells was assessed 4 weeks later (Merkle et al., 2007). Interestingly, the localization of the neural stem cells from which the neurons originate from does not

only determine the nature of the olfactory bulb neurons but also their pattern of dendritic arborization (Kelsch et al., 2007). To simplify, three microdomains can be defined within the postnatal SVZ: the lateral wall, the dorsal wall and the medial wall of the postnatal SVZ. The lateral microdomain of the SVZ is the most active in term of number of cells being produced. It mostly contains several kinds of GABAergic neuron progenitors and is biased to generate granular neurons (~90%) instead of periglomerular cells (~10%). In contrast, the medial microdomain of the SVZ, is thought to produce less cells, and is biased to produce periglomerular neurons (>85%). Eventually, the dorsal microdomain contains the most diverse populations of progenitors, including progenitors of glutamatergic and GABAergic neurons. Further, it produces a balanced amount of neurons that integrate into both the PGC and GC layers (Fiorelli et al., 2015) (**Fig I1.6D**). Moreover, the dorsal microdomain of the SVZ contains oligodendrocyte progenitors producing new oligodendrocytes throughout life.

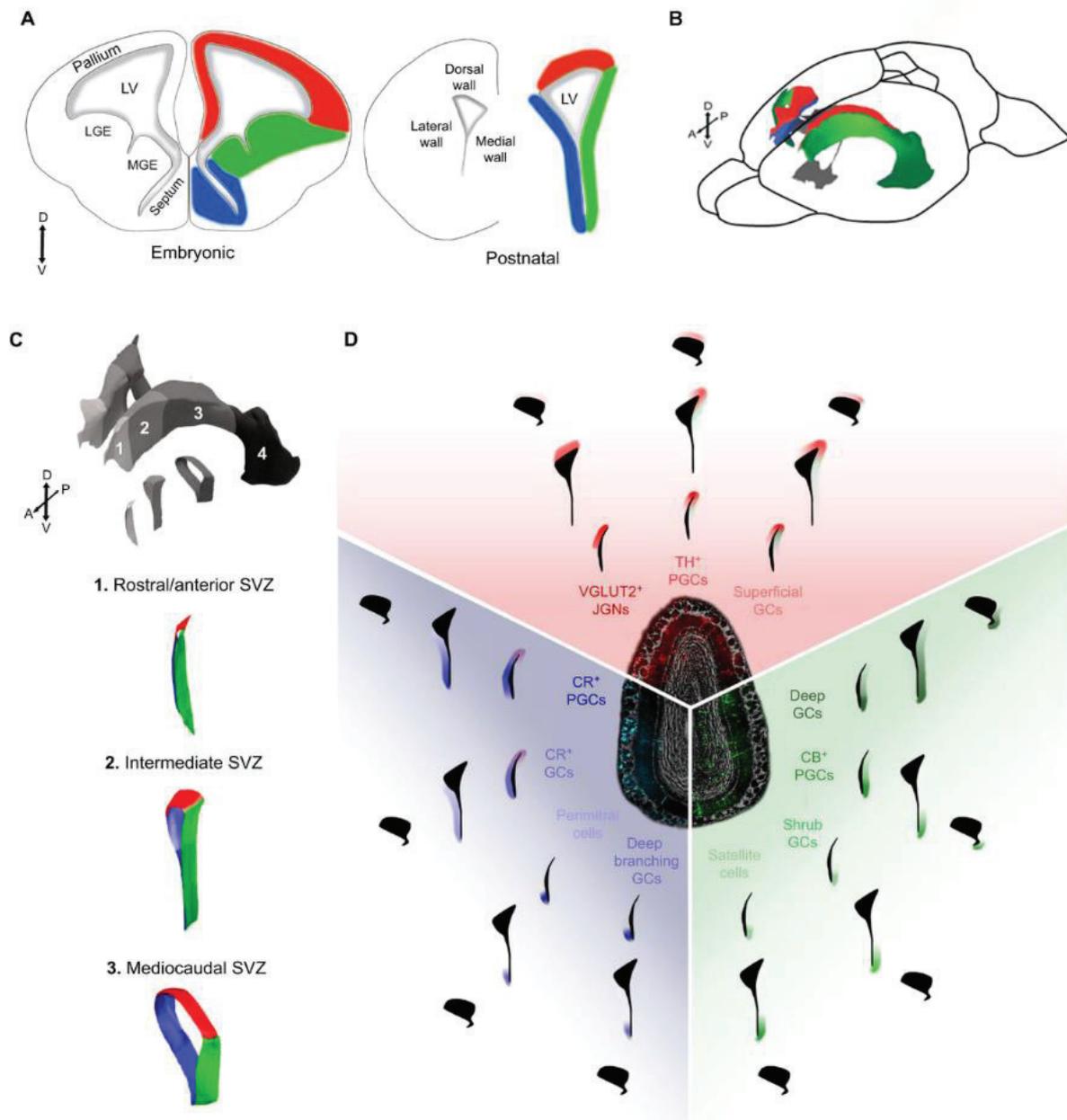
The heterogeneity of the SVZ is due to intrinsic properties of the cells it contains (Merkle et al., 2007). A study carried out by Young showed that cells located in the different microdomains of the SVZ originate from different parts of the embryo, using CRE-lox fate-mapping of pallial and subpallial embryonic cells (Merkle et al., 2007). The lateral microdomain of the VZ-SVZ originates from the lateral ganglionic eminence (LGE) and medial ganglionic eminence (MGE) while the medial microdomain of the VZ-SVZ originates from the septum. Eventually, the dorsal microdomain of the VZ-SVZ originates from the pallium (Kohwi et al., 2007; Ventura and Goldman, 2007; Willaime-Morawek and Van Der Kooy, 2008; Willaime-Morawek et al., 2006) (**Fig I1.6A-C**). Single cell analysis of SVZ NSCs highlighted another level of heterogeneity. Indeed, at the adult stage, individual NSCs appears to be already primed toward a neuronal or a glial fate (Llorens-Bobadilla et al., 2015a).

However, extrinsic factors play a key role in this heterogeneity. SVZ microdomains are actively maintained by factors contained in the microenvironment. Several signaling pathways were identified to be active in a regionalized manner of the VZ-SVZ. Indeed, microarray analysis showed Shh, Bmp2 and Tgfa are signaling specifically activated in the lateral microdomain of the SVZ while Wnt/ $\beta$ -catenin, Bmp4 and Igf1 are specifically activated in the dorsal microdomain of the SVZ, as revealed by the increased expression of the targets of those pathways (Azim et al., 2015, 2016). The

activity of those signaling pathways is tightly linked to the fate of SVZ NSCs and will be discussed later in the manuscript.

Altogether, these observations demonstrate a heterogeneity of SVZ neural stem cells between microdomains, regulated by intrinsic as well as extrinsic mechanisms. A better understanding of this heterogeneity is particularly interesting to trigger a lineage specific recruitment in the context of brain regeneration (Donega and Raineteau, 2017).

Of note, the particular 3-dimensional organization of the ventricular system and the orientation of the different walls (corresponding to each of the microdomains discussed above) led to the emergence of new tools to target and fate map SVZ stem cells and their progenies. For instance, in addition to the “classic” viral-mediated transgenesis technique (Braun et al., 2013; Zuccotti et al., 2014), to manipulate SVZ cells, it is now possible to target specifically those 3 regions with electroporation to investigate further the progeny of those different stem cell populations (de Chevigny et al., 2012; Fernández et al., 2011).



**Figure 11.6: The postnatal SVZ is divided into 3 different microdomains according to the fate of the stem cells they contain.** (A) The postnatal SVZ walls or microdomains (right) derive from their embryonic counterparts (left): the pallium, lateral and medial ganglionic eminences (LGE and MGE) and septum. Color-coding: red, pallium/dorsal wall; blue, septal/medial wall; green, ganglionic eminences/lateral wall. (B) Fate-mapping studies have shown that fate specification programs are conserved from development to adulthood in the NSCs of the different walls. Dorsal NSCs derive from pallial progenitors (red domain). Medial wall NSCs derive from ventral septal progenitors expressing (blue and cyan domains). Lateral wall NSCs, the most abundant population in the adult, derive from ventral subpallial progenitors (green and yellow domains). (C) A volumetric 3D reconstruction of the LVs is shown with the 3 microdomains of the postnatal SVZ. (D) The color-coding in A-C is conserved here to illustrate the SVZ microdomains of origin of specific OB neuron subtypes. The DAPI-counterstained OB in the center shows neurons in their final destination in specific OB layers following fate mapping of their region-specific NSC pools. Neurons are colored according to their origin: red, green and blue define neurons that have originated from NSCs of the dorsal, ventral and medial microdomains of the SVZ, respectively. Adapted from (Fiorelli et al., 2015)

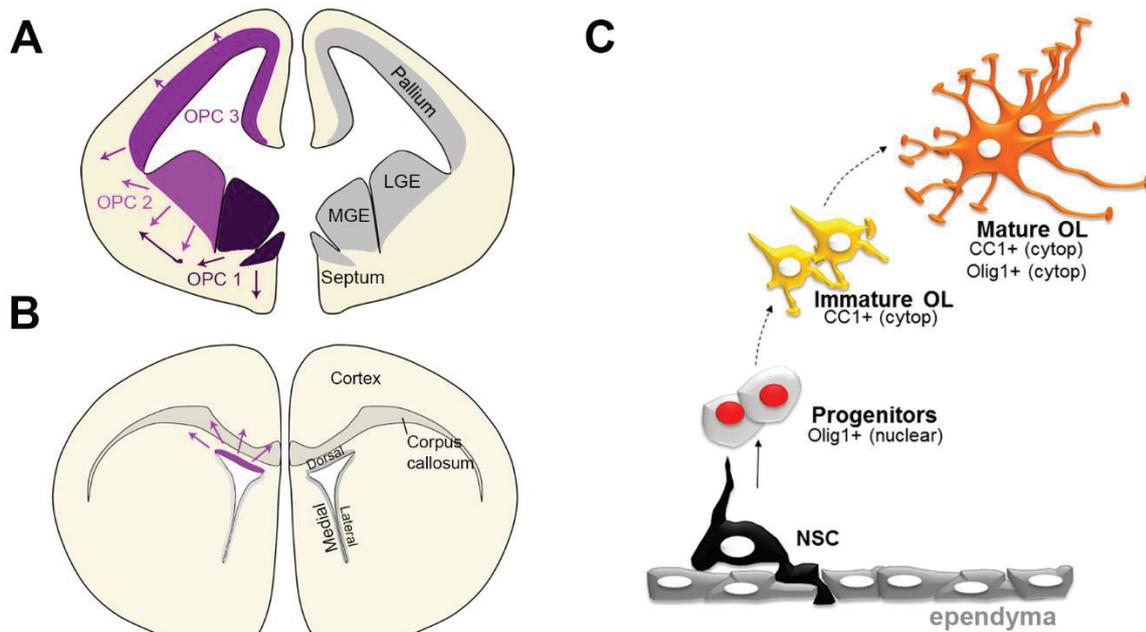
## **b. Different waves of oligodendrocytes are sequentially produced over time**

VZ-SVZ progenitors do not only produce neurons but also glial cells throughout time. Although gliogenesis includes both oligodendrogenesis and astrogenesis, in this manuscript I will exclusively focus on the former.

Most oligodendrocytes are produced during embryogenesis and early postnatal life before they migrate toward both white and grey matter (Qi et al., 2002; Thomas et al., 2000). The origin of oligodendrocytes has long been under debate (Olivier et al., 2001; Pringle and Richardson, 1993; Rowitch, 2004; Timsit et al., 1995; Warf et al., 1991). The classic view before the 1990's was favoring a unique origin, from the ventral telencephalon, mediated by the morphogen sonic hedgehog (Shh). However, ever since, this theory has been refuted. The adopted view now is that oligodendrocytes can be generated under different signaling pathways, in different parts of the embryo and at different time points. This reflects a heterogeneity of germinal activity in the VZ-SVZ and could suggest that there are different kinds of oligodendrocytes with different functions and properties. Indeed, it is well admitted now that there are 3 different waves of oligodendrogenesis, arising from distinct ventro-dorsal regions of the forebrain. The first one originates from the embryonic ventral telencephalon around E12,5 (Kessaris et al., 2001) from the medial ganglionic eminence MGE to the anterior entopeduncular (AEP) region and will give rise to a transitory wave of oligodendrocytes. This first wave will disappear from the cortex short after birth and gradually from the other parts of the brain to be almost eradicated by adulthood (Spassky et al., 1998; Woodruff et al., 2001). The reason of this oligodendrocyte renewal remains unclear. A second wave arises from the lateral and caudal ganglionic eminences and start at E15,5 and is followed by the last one, arising from the postnatal cortex. Interesting experiment showed that when one of those oligodendrocyte populations born during the 3 waves is ablated, a phenomenon of compensation occurs. Indeed, the neighboring oligodendrocytes expand to generate the normal number of oligodendrocytes in the mature brain, suggesting that the different populations of oligodendrocytes are functionally equivalent (El Waly et al., 2014). To summarize, oligodendrocytes are produced in a time specific and regionalized manner, and continue to be produced until adulthood (**Fig I1.7A,B**).

In order to be functional, oligodendrocyte progenitors need to go through a maturation process. Indeed, it takes several days for the progenitors to go through maturation process and reach the mature stage at which they are functional. Several intermediate stages have been highlighted to study the kinetic of oligodendrocyte maturation. The early and late progenitor stages both characterized by the expression of Olig1 marker in the nucleus. The immature stage (iOL) come after, characterized by the expression of CC1 (APC antibody clone) without cytoplasmic nor nuclear Olig1 expression. Eventually, mature OL express cytoplasmic Olig1, CC1 and MOG (Nakatani et al., 2013) (**Fig I1.7C**).

Several signaling are essential for an efficient OPC generation and proper oligodendrocyte maturation. For instance, PDGF signaling play a critical role in timing oligodendrocyte maturation by promoting early progenitor proliferation, preserving the pool of immature oligodendrocytes and preventing premature differentiation (Noble et al., 1988; Raff et al., 1988). Wnt/ $\beta$ -catenin signaling has been suggested to play a key role in oligodendrocyte progenitor proliferation and/or oligodendrocyte maturation as its manipulation influence greatly one or the other of those two phenomenon. However the results obtained by different groups remain conflicting and thereby the role of Wnt/ $\beta$ -catenin on oligodendrocyte production and maturation remains unclear (Dai et al., 2014). Indeed, while some studies show that an activation of Wnt/ $\beta$ -catenin signalling increase oligodendrocyte progenitor production (Fancy et al., 2009; Langseth et al., 2010; Shimizu et al., 2005) other studies suggest that its inhibition is crucial for proper oligodendrocyte maturation (Azim et al., 2014a; Dai et al., 2014; Tawk et al., 2011). Together, those data suggest that Wnt/ $\beta$ -catenin signalling is a key player for oligodendrogenesis process and regulates oligodendrocyte progenitor proliferation, differentiation and oligodendrocyte maturation in a stage specific manner.



**Figure 11.7: Oligodendrocyte production and maturation throughout time.** (A, B) Three sequential waves of OPCs are generated from different regions of the forebrain ventricular zone (VZ). The first wave, OPC 1 (dark purple) arises from the medial ganglionic eminence (MGE), and starts at E12.5. The second wave, OPC 2 (purple arrows) arise from the lateral and medial ganglionic eminences (LGE), starting at E15.5. The last wave, OPC 3, (light purple arrows) arises from the pallium and starts around birth (A). After birth, oligodendrocytes continue to be produced from the dorsal SVZ (B). (C) Maturation of oligodendrocytes (OLs). NSCs subsequently generate oligodendrocyte (OL) progenitors, immature OLs and eventually mature OLs.

Oligodendrocytes are a type of neuroglia, which in addition to fulfill a supportive role in the CNS produces myelin. Myelin defines a sheath of lipoprotein wrapped around the neuronal axons of both central and peripheral nervous system and play a critical role in the isolation of the axons and thereby the fast propagation of the action potential along neuron axons. Myelin started to be described at the 17<sup>th</sup> century, however it was long thought to be produced by neuronal axons and not by oligodendrocytes. It is only in 1962 that the definitive proof that oligodendrocytes secrete myelin in the CNS was established. Indeed, myelin is essential for protecting and insulating axons, and enhance their transmission of electrical impulse and several diseases are associated to myelin defects. Demyelination is a consequence of some neurodegenerative autoimmune disease including multiple sclerosis, inherited demyelinating diseases such as leukodystrophy but is also observed in premature brain injuries (Boullerne, 2016; Martin et al., 1981; Menn et al., 2006; Naruse et al., 2016; Ulrich and Lardi, 1978;

Weiss et al., 2004). Of note, oligodendrocytes are also important secretors of trophic factors such as glial cell line-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), or insulin-like growth factor-1 (IGF-1) which influence the behavior of nearby neurons and play a role in neuroprotection (Bankston et al., 2013).

To conclude this part, the two first week postnatal in mice constitute a window of intense postnatal SVZ activity and plasticity, with a high production of neural and glial cells supported by an intense activity of key signaling pathways. Indeed, SVZ activity decline drastically after this period.

## **B. Chronic hypoxia is a model of injury to study premature brain plasticity.**

### **1. Oxygen is a key environmental regulator of cell fate**

Oxygen is vital for nearly all form of life through its importance in energy homeostasis. It is also a key element of the cellular microenvironment, influencing cell behavior (Yun et al., 2002). Oxygen content is an important regulator of physiological and pathological cellular processes, during development but also for maintenance of normal tissue function in adult. Variations of oxygen content creates rapidly an oxidative stress with diverse consequences according to the tissue but in certain case leading to cell apoptosis (Kannan and Jain, 2000). Brain tissue is particularly sensitive to oxygen variations. Indeed, reduced oxygen level during brain development often leads to cognitive and behavioral deficits later in life (Basovich, 2010), which will be described later in this manuscript.

#### **a. Oxygen content influences neural stem cell behavior *in vitro***

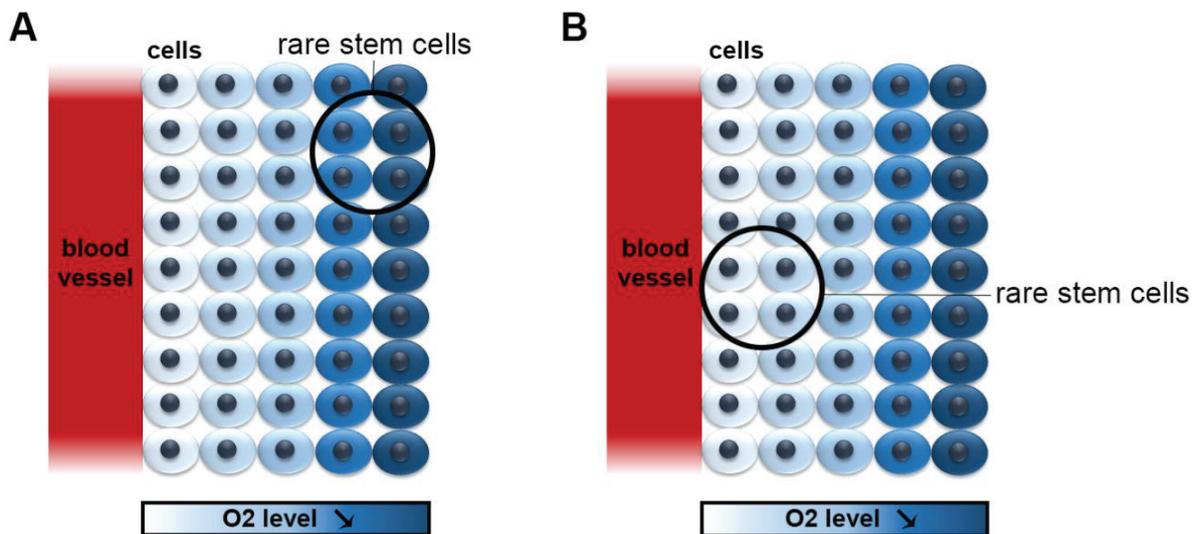
Several *in vitro* studies showed the importance of an appropriate oxygen content on brain development and stem cell fate. Already in the 80's, Morriss and New made a connection between oxygen level and mammalian embryogenesis, showing that low-oxygen content was essential for the development of the neural tube by ex utero mouse embryo in culture (Morriss and New, 1979). Later, several studies highlighted a role of oxygen in the balance between neural stem cell (NSC) maintenance and differentiation. For instance, a study carried out by Morrison showed that a decreased oxygen level resulted in the promotion of survival, proliferation, and differentiation of cultured rat neural crest stem cells (Morrison et al., 2000). Similar experiments were carried out later, showing a promotion of rat NSCs proliferation, and dopamine neuron

generation, as well as a reduced cell death in a lowered oxygen environment (Studer et al., 2000). Eventually, human central nervous system precursor cells appeared to have a higher proliferating rate under hypoxic conditions (Storch et al., 2001, 2003). However, the notion of “hypoxia” varies between these *in vitro* studies. Recently, a study carried out by Ortega reported that small changes in oxygen content induced profound changes on proliferation and differentiation of human radial glia cells (RGCs). Notably, a “physiological” hypoxia (3% of oxygen, corresponding to oxygen level measured within the neural stem cell niche) promoted RGC differentiation and neurogenesis while “severe” hypoxia (1% of oxygen) and “anoxia” (below 1% of oxygen), had a negative effect on RGC differentiation, preventing both neurogenesis and oligodendrogenesis. Glutamatergic neuron formation was particularly affected (Ortega et al., 2016). This last study highlighted the importance of taking into account the physiological hypoxia in brain tissue. Together, those results suggest that a mild hypoxia could promote NSC differentiation and neurogenesis while severe hypoxia might have an opposite effect (Zhu et al., 2005). Of note, *in vitro* studies allowed to unravel the molecular mechanisms responsible for the effects of hypoxia and notably to identify hypoxia-inducible factors (HIFs) as key regulators of hypoxic response in cells and tissues (Bruick, 2003; Semenza, 1999).

#### **b. Oxygen content regulates neural stem cell behavior *in vivo***

As mentioned before, *in vivo* studies are needed to fully understand the role of oxygen in physiological conditions. Indeed, oxygen level is heterogeneous in the different parts of the organism at both embryonic and adult stages, creating specific niches and influencing cell behavior (Maltepe and Simon, 1998; SIMON et al., 2002). In the germinal zones of the developing forebrain, the concentration of oxygen in the developing zone is much lower (from 1 to 5%) than in the atmosphere (about 21%), resulting in a phenomenon of “physiologic” hypoxia. This low oxygen level seems essential for promoting NSCs proliferation (Mohyeldin et al., 2010; Simon and Keith, 2008a), while pathological hypoxia (oxygen level below 1%) triggers NSCs quiescence and apoptosis (Ezashi et al., 2005; Santilli et al., 2010). Lowered oxygen level does not only play a role on NSCs behavior but could also be important for proper differentiation of RGCs into glutamatergic neurons during corticogenesis (Malik et al.)

as well as for the development of cortical connectivity (Kozberg et al., 2016). Interestingly, increasing oxygen content specifically in the neurogenic niche promoted the formation of a novel neurogenic layer basal to the subventricular zone, participating to corticogenesis (Wagenfuhr et al., 2015). Of note, a tight coupling exists between neural activity and blood flow which provides the oxygen to the brain. Stem cell populations do not always develop in low oxygen content environment apart from the neural blood vessels. For instance, the perivascular Slam+ stem cells develop in a high oxygen content environment (**Fig I2.1**) (Simon and Keith, 2008a). Altogether, those data show a strong influence of oxygen content of the microenvironment on NSCs proliferation and fate, particularly glutamatergic neuron lineage, and suggest a role in the establishment of cortical networks.



**Figure I2.1: Oxygen content of the microenvironment influences stem cell development.** Certain populations develop in a high oxygen content like the Slam+ perivascular stem cells (**A**) while other populations develop in a low oxygen content (**B**). Adapted from (Simon and Keith, 2008b).

## 2. Chronic hypoxia, a model for studying premature children neurological disorders

### **a. Perinatal hypoxia has various causes**

Perinatal hypoxia is a pathology resulting from deprivation of oxygen to a newborn infant lasting long enough during the birth process to cause physical harm. Perinatal Hypoxia may involve virtually every organ system of the body. However, the brain damages resulting from it are the most commonly studied, perhaps because the brain is the least likely to quickly or completely heal. Perinatal hypoxia may occur *in utero*, during labor and delivery, or in the immediate postnatal period, with different ranges of severity. There are numerous causes. Indeed, perinatal hypoxia may be due to a trauma (including placental abruption, cord compression, birth trauma) pharmacological compound (including transplacental anesthetic or narcotic administration), a deleterious environment (intrauterine pneumonia or maternal opiates), or developmental issues like congenital cardiac or pulmonary anomalies, and birth trauma (McGuire, 2007). Preterm infants and very low birth weight (VLBW) infants are particularly at risk. They defined by a weight under 1,500 g and a birth before 32 gestational weeks of age. Weight and gestational week number remain reliable markers of potential hypoxia induced brain lesions, even though, gestational age is a better predictor of mortality and morbidity than is birthweight. In France, about 10000 infants per year are born prematurely and in European countries, it is 1.1-1.6% of live births. The progress in quality of care and efficiency of therapies allowed to increase the survival of premature children, but raised new issues about their developmental outcomes (Larroque et al., 2008). Indeed, in some hospitals, babies born after only 22 gestational weeks are saved, leading to a very high risk of severe disabilities. For instance, cerebral palsy affects 18% of premature infants born at 26 gestational weeks, 9% of those born before 32 gestational weeks while only about 1% of the babies born at term (Abbott, 2015).

### **b. Broad neurological consequences of perinatal hypoxia**

The spectrum of consequences both VLBW and prematurity have on the development of the brain is broad. For instance, Converging data suggest that preterm birth perturbs the genetically determined program of corticogenesis in the developing brain. Indeed

preterm infants develop more cerebral palsy, mental retardation, and sensory impairments associated with cerebral gray and white matter abnormalities, ventriculomegaly and decreased cortical and basal ganglia volumes in the early years of development, and later long term cognitive difficulties, such as those involved with language and executive functions, and psychiatric illnesses including autistic spectrum and anxiety disorders (Anderson et al., 2003; Johnson et al., 2010). Premature infants also have a high risk to develop diffuse white matter injuries (DWMI) previously called periventricular leukomalacia (Back, 2006) and often associated with significant disruption of myelination (**Fig I2.2A,B**) (Kinney and Back, 1998). Unfortunately, there are currently only few prognostic indicators allowing to determine if the child will suffer from those neurological disorders by the adulthood. It is possible to improve those cognitive and neurological sequelae, with an adapted nutrition (Dutta et al., 2015) and on a longer term, with the use of extra resources such as special education services and occupational and physical therapy. However, many VLBW children won't show any significant sign of improvement by the adolescence, even with an adapted way of life (Luu et al., 2011; Ment et al., 2003). Neuroimaging studies suggest that preterm children establish aberrant pattern of connectivity after birth, maybe due to a prolonged period of plasticity, resulting in brain asymmetries and abnormal connectivity (Myers et al., 2010). Magnetic resonance imaging studies revealed the most common types of neural abnormalities associated with prematurity, a significant decrease in grey and white matter volume in premature brains, a compromised microstructural organization and integrity of white matter, and hippocampal abnormalities. These alterations last until adolescence and might cause the neurological issues observed in premature infants (Beauchamp et al., 2008; Brown et al., 2009; Volpe, 2003). However, the poor information on premature brain at the cellular level leave many questions unsolved and emphasized the need of an animal model for studying prematurity consequences on the brain.

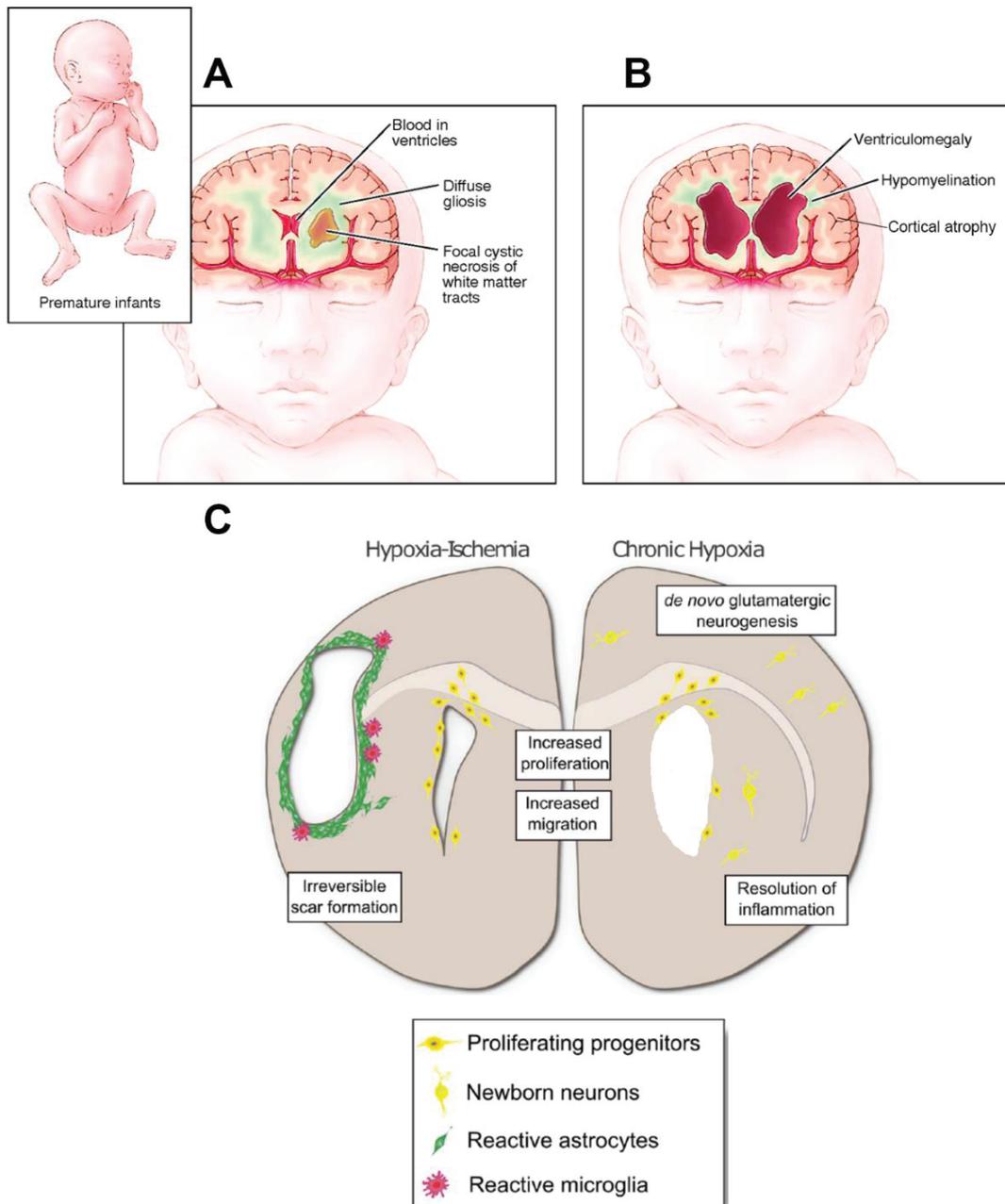
### **3. Chronic hypoxia is a model system to study premature brain repair and rewiring**

### a. Animal models of perinatal hypoxia

Over the past decades, several animal models have been developed to study premature brain injuries. First, a sheep model of global ischemia in preterm animals recapitulates quite faithfully the developmental events occurring in preterm humans. In this model, a global ischemia is induced at the end of the pregnancy, at a gestational point more or less equivalent to the third semester. Neurological consequences of this perinatal ischemia were a global decrease of the brain volume, a white matter injury and reactive astrogliosis and/or reactive microglia and macrophages (Back et al., 2012).

In mice, two models have been established to study the VLBW brain disruption. The hypoxia/ischemia (HI) model proposed by Vanucci in which mice are exposed to hypoxia after carotid artery occlusion. Even though the Vanucci reproduces several functional and behavioral abnormalities of the preterm infants (Cengiz et al., 2011; FAN et al., 2005), the model reproducing most of the aspects of premature infants is the chronic hypoxia model. In this model, mice are exposed to a hypoxic environment between P3 and P11 (Salmaso et al., 2014a; Scafidi et al., 2009). The chronic hypoxia model reproduces the cellular recovery observed in human with a number of cortical neurons, as well as volume and brain weight back to normal after 5 weeks. Unlike for the HI model, inducing a scar formation and gliosis, chronic hypoxia does not induce a severe inflammatory reaction (Salmaso et al., 2014b), thereby limiting additional loss of tissue and potential cyst formation (**Fig I2.2C**) (Hagberg et al., 2012). However, hypoxic mice still suffer from long-term behavioral sequelae such as impairment in learning tasks and discrimination, hyperactivity and increased anxiety, some of them appearing to be permanent (Chahboune et al., 2009; Dell'Anna et al., 1991; Nyakas et al., 1996; Weiss et al., 2004).

Taken together, these data show that the chronic hypoxia model reproduces both cellular and behavioral consequences of prematurity and is pertinent to study potential therapies.



**Figure I2.2: Common types of injuries associated with prematurity and corresponding mouse models.** (A) Key characteristic of premature brain with intraventricular hemorrhage. White matter is often impacted with a focal cystic necrosis and diffuse gliosis. (B) Sequelae of premature brain with hypomyelination resulting from failure of lesion repair, cortical atrophy and ventriculomegaly resulting in significant loss of brain parenchyma. (C) Premature brain injury models. The Hypoxia-ischemia model induces a thick scar formation composed of reactive astrocytes and microglia, contrary to the chronic hypoxia model. Both models promote subventricular zone progenitor proliferation and migration, whereas spontaneous *de novo* neurogenesis is only observed in the chronic hypoxia model. Adapted from (Donega and Raineteau, 2017; Silbereis et al., 2010)

## **b. Chronic hypoxia highlights a spontaneous but partial attempt for cellular regeneration**

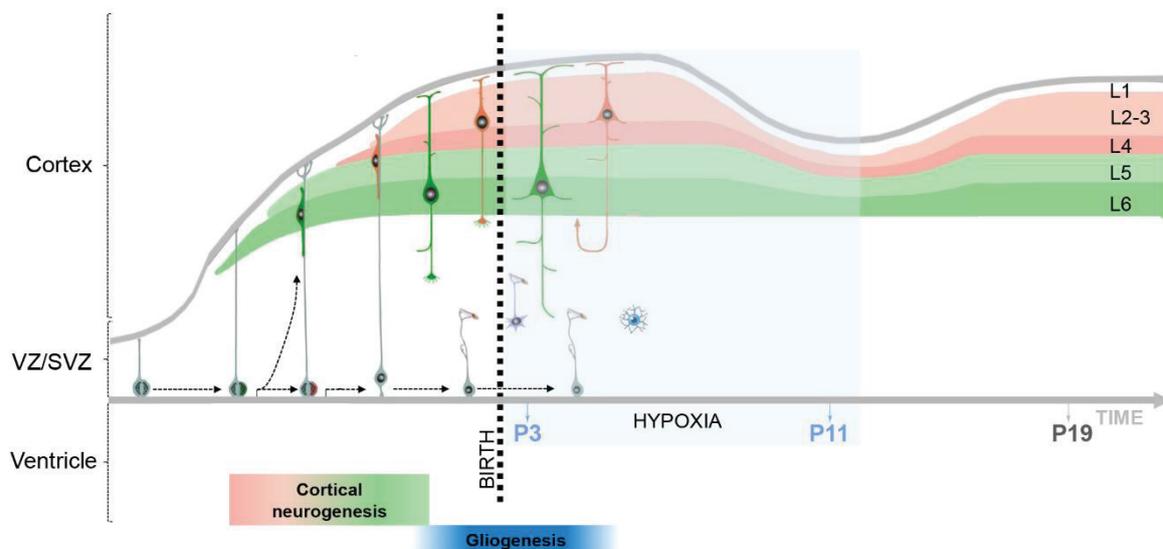
### **- Consequences on neural stem cells**

The chronic hypoxia model is a model of premature brain injury. In mice, the brain is still very immature during the two first weeks after birth, with an intense gliogenesis for instance. In this model, mice are subjected to a hypoxic environment containing 10% oxygen from P3 (3 days postnatal) to P11. The cortical volume of hypoxic mice gets back to normal within a few weeks following the end of the hypoxia period (Fagel et al., 2006). At the cellular level this could be explained by a delay in maturation of cortical cells or cell death followed by a reactivation of cortical neurogenesis and gliogenesis. Indeed, the amount of cortical neurons and particularly glutamatergic excitatory and GABAergic inhibitory neurons in the cortex recovers by the time the mice reach early adulthood. A study carried out by Fagel et al suggests that hypoxia affects astroglial cells properties allowing them to give rise to cortical neurons. Indeed, a subset of astroglial cells persists in the parenchyma including the neocortex, in addition to the two neurogenic niches, and exhibits “stem cell like properties” during the first postnatal week to be eventually completely extinguished by P15 (Laywell et al., 2000) in Normoxic brains. In hypoxic brains, however, this period seems to be extended (Bi et al., 2011a). Such a mechanism is likely to explain partly the post-hypoxia cortical neuron recuperation, however, other potential mechanisms remain to be investigated. For instance, a similar delay of the astroglial “stemness” was shown in the SVZ, suggesting that SVZ could be a source of postnatal cortical neurogenesis (Bi et al., 2011a; Salmaso et al., 2012). Chronic hypoxia also has consequences on the second postnatal neurogenic niche with a long-term shift from astroglial to neuronal fate of Gfap+ cells.

Of note, trophic factors such as Brain Derived Growth Factor (BDNF) and Fibroblast Growth Factor (FGF) seems to play a key role in the post hypoxia recovery as mice presenting a genetic deletion of FGF receptor present a much worse phenotype following hypoxia and are unable to recover a total neuron number (Fagel et al., 2006, 2009).

- Consequences on cortical neurons

In contrast to the excitatory neurons recovering in term of number by one month following the injury (**Fig 12.3**), GABAergic inhibitory neuron population remain chronically depleted. In addition, decreased expression of the mature interneuron markers parvalbumin (PrV) and somatostatin (SST) suggests a perturbed maturation of these interneuron subtypes and could be partly responsible for long term neurobehavioral impairment associated with chronic hypoxia (**Fig12.4A,B**). A study carried out by Fagel investigated the short term and long term consequences of hypoxia on cortical interneurons and highlighted the importance of the environment in post-hypoxia cellular recovery. Indeed, they showed that an enriched environment was able to reverse the defect in GABAergic neurons, in addition to a stimulation of the production of glutamatergic neurons in the dentate gyrus associated with improved performance in spatial memory (Fagel et al., 2006, 2009).



**Figure 12.3: Chronic hypoxia model and consequences on the cerebral cortex.** In physiological condition, cortical neurogenesis including glutamatergic neuron formation (green and red) happens at embryonic stages from neural stem cells located in the ventricular zone (VZ). In chronic hypoxia model, mice are subjected to a hypoxic environment containing 10% oxygen from 3 to 11 days after birth (P3 to P11). Cortical cell atrophy and death occurs in response to lack of oxygen, resulting in a decreased cortical volume. Interestingly, cortical volume gets back to normal by 8 days following the end of the hypoxic period, suggesting a reactivation of cortical neurogenesis and gliogenesis as well as a recovery of the atrophy.

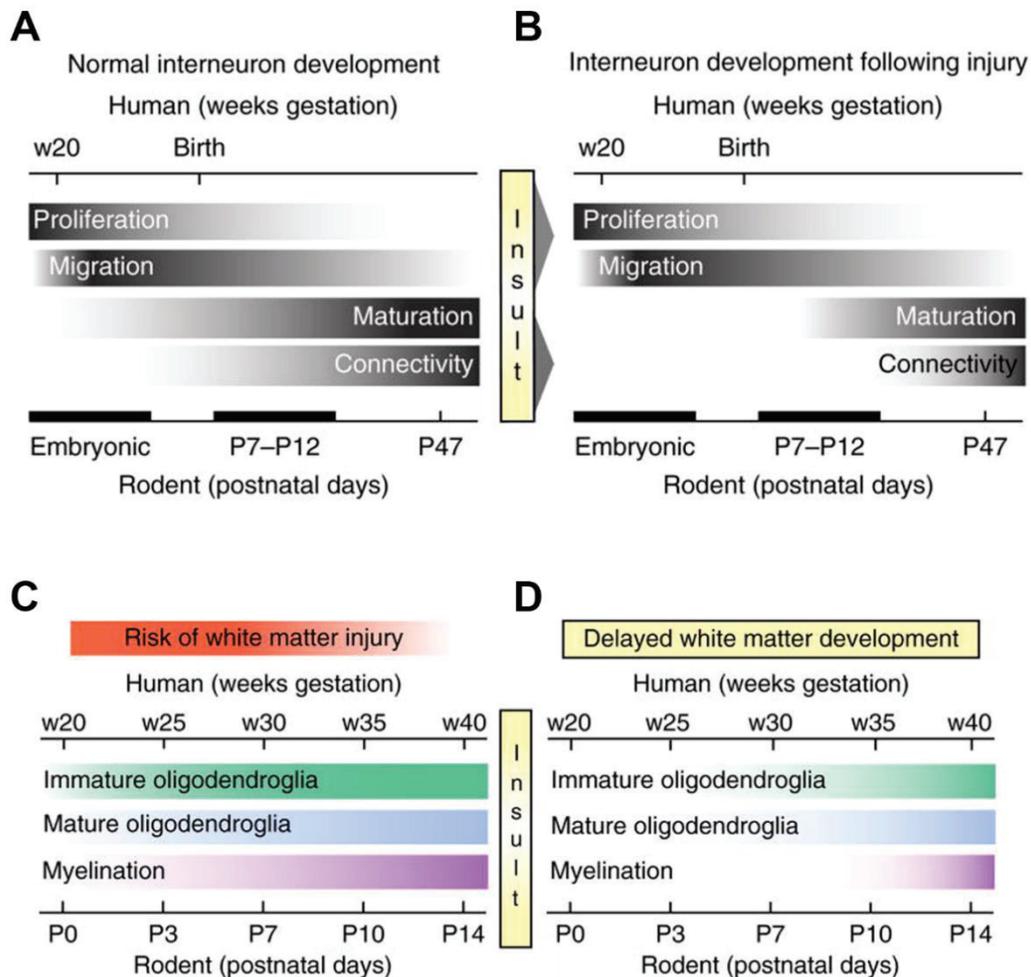
- Consequences on oligodendrocytes

The highest risk for developing DWMI occurs for premature infants born between 23 and 32 weeks of gestation. Indeed, it corresponds to the beginning of pre-myelinating oligodendrocytes production and early white matter myelination. Oligodendrogenesis occurs after the cortical neurogenesis period in rodents, mostly during the first and second postnatal weeks, corresponding to the last part of gestation in human. Indeed, newborn mouse brain is less mature than human brain at birth, which makes of chronic hypoxia a good model for studying brain damage of premature children. Of note, the period of hypoxia is a critical time for oligodendrocyte maturation, which is intense during the very first postnatal weeks in normoxic conditions (**Fig I2.4A,B**). Thus, chronic hypoxia decreasing the oxygen supply at this period has consequences on maturation of oligodendrocytes and thereby on axon myelination.

A study carried out by Jablonska allowed to identify two phases following the hypoxic period: first, an injury phase occurring during the few days directly following the end of hypoxia and a second phase, later, suggesting a cellular and molecular recovery. Of note, two similar phases were identified in developing infants following a premature birth (Back et al., 2001; Volpe, 2001). During the first phase, chronic hypoxia induces a significant hypomyelination due to oligodendrocyte apoptosis and delayed oligodendrocyte differentiation. In parallel, a regenerative attempt occurs with an increased proliferation of oligodendrocyte progenitors within both the white matter and the SVZ, suggesting an endogenous potential for cellular recovery following perinatal injury. Indeed, a “recovery phase” follows. By P47 (47 days after birth), myelination in white matter appeared to be increased in the hypoxic brains as well as the population of oligodendrocytes (**Fig I2.3C,D**). At the molecular level, a few proteins like Cdk2 and Cdk4 cyclins were identified as playing a critical role in the “recovery phase”. Indeed, Cdk2 regulates OPC proliferation in the white matter while Cdk4 regulates OPC proliferation in the SVZ (Jablonska et al., 2012).

The recovery phase following hypoxia and leading to a reconstitution of the pool of oligodendrocytes at least partly from the pool of SVZ oligodendrocyte progenitors is consistent with recent studies demonstrating the same time-course for post-hypoxia neurogenesis leading to a reconstitution of cortical neurons (Fagel et al., 2009). Moreover, it defines a developmental time window critical for myelination and

stimulation of oligodendrogenesis, which could be targeted with new therapies such as the use of small molecules.



**Figure 12.3: Chronic hypoxia causes a delay in maturation of both interneurons and oligodrocytes.** (A, B) Representation of the time-course of interneuron development in the human and mouse cortex in normal condition (A) and after exposure to hypoxic injury (B). (C, D) Representation of the time-course of oligodendrocyte maturation in the human and mouse cortex in normal condition (C) and after exposure to hypoxic injury (D). Adapted from (Salmaso et al., 2014a)

Cortical cell loss followed by cellular regeneration make of this model, in addition to a pertinent model for studying prematurity, an interesting model for studying postnatal reactivation of cortical neurogenesis and oligodendrogenesis. However, it is important

to note that this cellular recovery is only partial and is probably at the origin of the long-term cognitive impairment observed in hypoxic mice. Indeed, although a few studies already tried to unravel the mechanism underlying the regenerative attempt following hypoxia several questions remain under debate. For instance, **from where does the postnatal-born cortical neurons and oligodendrocytes originate? Does newborn cells induced by hypoxia specify, mature correctly, and benefit the system?**

## **C. Advances and remaining challenges to promote forebrain cellular repair**

Several studies have demonstrated a spontaneous attempt for cellular repair in various forebrain regions. Based on those observations, efforts have been made to experimentally promote this spontaneous attempt. Although these studies represent proofs of principle that cellular regeneration might be achieved in the postnatal forebrain, several challenges remain to realistically appreciate the feasibility of such an approach. Indeed, as highlighted in the first part of this introduction, the regionalization of the postnatal SVZ raises concerns regarding the competence of postnatal NSCs to produce specific neural cell types, including sub-types of neurons. Increasing knowledge of this regionalization and of the signals that guides it, may however offer new means to promote the generation of relevant neural cell types in regenerative contexts.

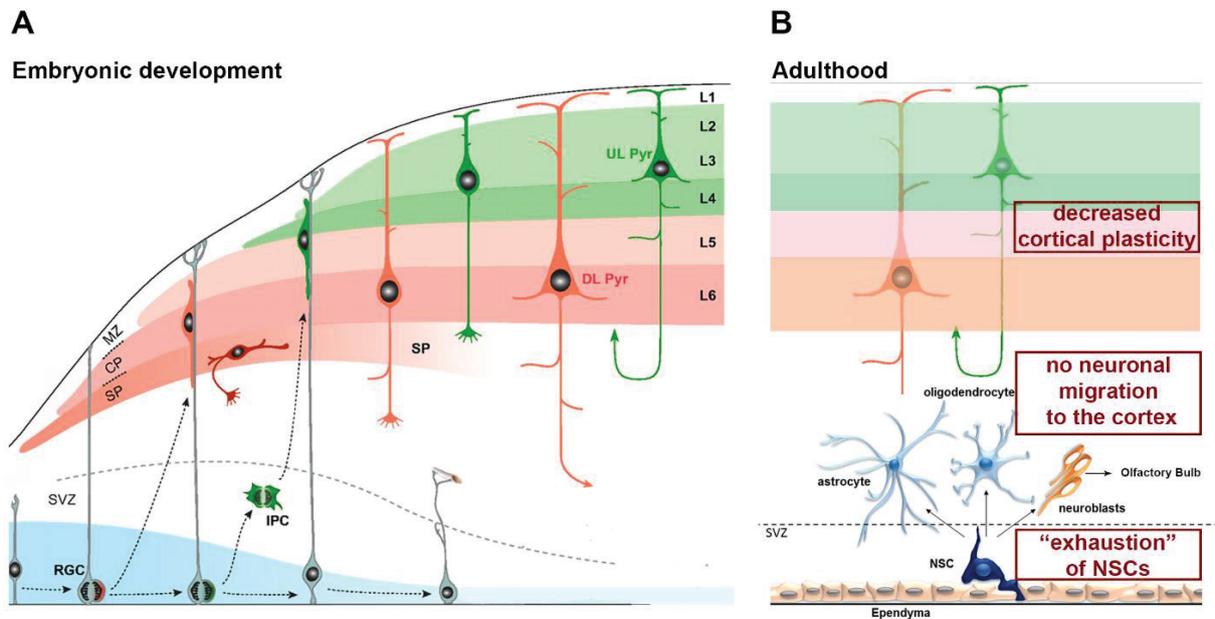
### **1. Barriers to postnatal cortical regeneration can be overcome early in development**

#### **a. Many obstacles to cortical neurogenesis after birth**

Corticogenesis is a tightly regulated phenomenon, imbricating spatial and temporal cues. Therefore cortical regeneration at adult stages raises many challenges. Indeed, projection cortical neurons reach their sometimes very distant target early in development and it seems very unlikely that long-distance connexions could be regenerated. Indeed, layer V neurons reaching the spinal cord or layer VI neurons reaching the thalamus for instance. Secondly, the population of radial glia cells disappears around birth and therefore the scaffold necessary for the neuron migration into the different cortical layers (Tramontin et al., 2003). Finally, the neurons being

postmitotic cells, they originate from progenitors and NSCs. Although a population of progenitors and NSC persists in the adult brain and throughout adulthood, notably in the VZ-SVZ, they seem to largely lose their capacity to produce cortical excitatory neurons (Sanai et al., 2011). In addition, cortical plasticity is decreased in adult, preventing or making difficult the generation of neurons from parenchymal glia, which can be observed earlier in time.

Several approaches has been used to achieve postnatal cortical repair (Breunig and Arellano, 2007; Urbán and Guillemot, 2014). First, the redevelopment of “embryonic-like” radial glia cells to be both a source of cortical neurons and a scaffold for their correct migration (Ghashghaei et al., 2007). Others tried to transplant neural stem cells and progenitors directly into the cortex or to boost endogenous neural stem cells to promote cortical repair. Those different approaches will be described in more details in the following paragraph. However, they raise many questions regarding to the competency of neural stem cells to produce cell types that are normally produced earlier in time. Moreover, the microenvironment less plastic, less permissive in the postnatal cortex could prevent maturation, specification and integration of the newborn cells (**Fig 13.1**).



**Figure 13.1: Barriers to cortical regeneration during adulthood. (A)** Cortical development during embryogenesis. Radial glia cells (RGC) produce cortical neurons (deep layer pyramidal neurons, DL Pyr ; upper layers pyramidal neurons, UP Pyr) or neuronal progenitor (IPC). **(B)** In the adult cortex, there are several barriers to regeneration. First, the neurogenic activity of SVZ neural stem cells (NSCs) decrease and is exclusively restricted to olfactory bulb neurogenesis. Newborn neurons do not migrate toward the cortex. This might be partly due to the lack of RGC which, in addition to their neuronal activity, are scaffold proteins for cortical neuron migration during embryonic corticogenesis. Finally, the cortex is not as plastic in adults as it is in embryo, thereby preventing the integration and survival of newborn neurons. Adapted from (Akhtar and Breunig, 2015)

**b. Kennard principle: postnatal period is more permissive than adulthood for tissue repair and circuit rewiring**

Many of the difficulties highlighted above can be overcome if the lesion happens early enough, during the window of time described in the first part of this introduction, which is the case in the context of chronic hypoxia.

The idea that the developing brain recovers better than the adult brain started to be evoked from the 20<sup>th</sup> century by the neuropsychologist Hans-Lukas Teuber from clinical observations (TEUBER and RUDEL, 1962), short after William James's work who first introduced the notion of brain plasticity in 1890 (Finger and Wolf, 1988; James and William, 1890). However, it was Margaret Kennard who showed the effect of age

on brain injury recovery thanks to her systematic work on monkey of different ages. From there was born the concept of “early lesion effect”, also called “Kennard principle”. Indeed, this principle describes the immature brain as more plastic than mature brain, with a better capacity to remap and reorganize and therefore, more resilient to injuries (KENNARD, 1942). Some studies highlight other factors influencing the resilience of immature brain such as the number of lesions, their frequency or their gravity. For instance, a mild perinatal injury have the potential to evolve into significant pathologies at longer term even though the lesion happens early in development. This is the case for premature infants who suffered from hypoxia, or for cerebral palsy (Bennet et al., 2012). However, the “Kennard principle” is widely accepted in the field (Bennet et al., 2013) and for decades, has guided regenerative research. Indeed, a critical window for brain repair and more particularly for neocortical neurogenesis was highlighted during the postnatal period. In the first part of this introduction we already discussed the intense activity of the SVZ during the two first weeks following birth. In the cortex, it corresponds to a period of circuit making and refining, suggesting that this period is permissive to both tissue regeneration and circuit re-writing.

## **2. Regenerative strategies for promoting brain repair**

Several approaches were already used to treat brain injuries. Neuroprotection aims at decreasing cell damage and death induced by the injury, mainly by influencing the cellular microenvironment. Neuroregeneration, on another hand, promotes brain plasticity and postnatal germinal activity. Ideally, regenerative strategies would combined both aspects. I will focus on two different regenerative approaches: the transplantation of exogenous neural stem cells, and the stimulation of endogenous neural stem cells.

### a. Exogenous NSCs to repair the injured forebrain

Cellular transplantation was one of the first strategy used for brain repair (**Fig I3.2**). Indeed, it allows to target both neuroprotective and neuroregenerative mechanisms. Diverse types of cells were used for transplantation following traumatic brain injuries (Longhi et al., 2005). Stem cells are however often used for many reasons. First, they are a great source of trophic factors, but they also can adapt to their environment and thereby evolve with the pathology. Eventually, according to the type of stem cell, they have a great capacity of differentiation and have the potential to replace the cells of the central nervous system that are lost following the injury (Kondziolka et al., 2002; Lladó et al., 2004; Lu et al., 2003; Mahmood et al., 2006; Ourednik et al., 2002; Pluchino and Martino, 2008; Rafuse et al., 2005; Sladek et al., 1988; Wong et al., 2005; Yan et al., 2004). The fate of the transplanted cells can be pre-determined in vitro or is dictated by the host environment (Yandava et al., 1999). Transplanting neural stem cells into the injured brain may promote the spontaneous regenerative attempt of the brain by increasing the neurogenic and gliogenic environment it created. Indeed, transplantation of neural stem cells after traumatic brain injury promote motor and cognitive recovery (Philips et al., 2001; Riess et al., 2002; Schouten et al., 2004). Furthermore, in the neonatal brain, intranasal administration of mesenchymal stem cells after hypoxia/ischemia lesion promotes cortical neurogenesis and decrease gliosis (Donega et al., 2014a). The source of transplanted cells is a key issue of regenerative medicine using stem cell transplants in a translational perspective. So far, transplantations of neural stem cells derived from embryo (Brüstle et al., 1997; Okabe et al., 1996; Reubinoff et al., 2001; Tropepe et al., 2001), from the skin (McKenzie et al., 2006), and from bone-marrow-derived stem cells (Deng et al., 2001; Kabos et al., 2002) were used in addition to fetal and adult more common sources, offering a certain diversity of cell sources. However, several issues come with cell transplants. Indeed, they have a limited survival and interaction with host tissue, and their effect on the central nervous system is highly variable. Furthermore, they might induce an immune response from the immune system (Jones et al., 2004). The beneficial effect of transplanted cells might be due to the factor they secrete. A study carried out by Kleindienst shows that it is possible to improve cognitive recovery following traumatic brain injury by infusing directly in the cerebrospinal fluid ventricle of the mice S100 $\beta$ , a

protein normally secreted by astrocytes (Kleindienst et al., 2005). Hence, another strategy to promote cortical repair is the use of small molecules. Indeed, a pharmacological approach potentially provides the trophic factors also produced by exogenous stem cells but allows also to promote the endogenous stem cells and progenitors that persist in postnatal tissue.

### **b. Activating endogenous NSCs to repair the injured forebrain**

- The brain does regenerate spontaneously in particular contexts

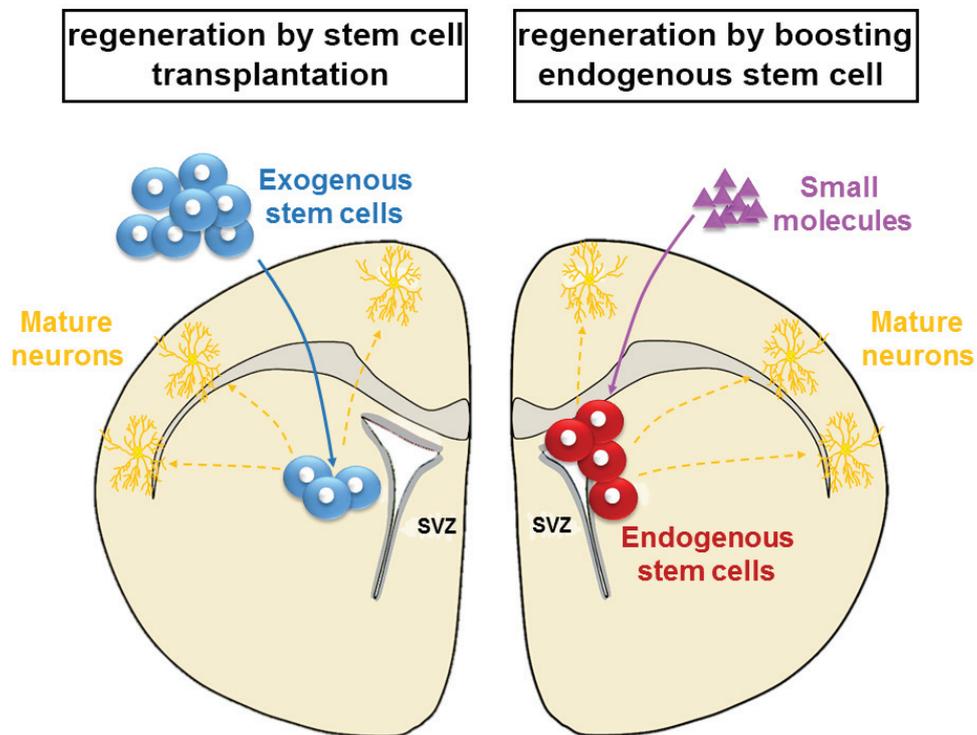
In the 80s, a study carried out by David and Aguayo demonstrated for the first time that neuronal axons from the central nervous system could regenerate, and highlighted the importance of an appropriate microenvironment. Indeed, the study showed that peripheral nerve grafts implanted in the optic nerve allowed a regeneration of axons within the peripheral nerve graft but not beyond or in the surrounding tissue (David and Aguayo, 1981). Moreover, after many adult brain or spinal cord lesions, a spontaneous neurogenesis was observed in the injured area. For instance, following traumatic brain injury in adults, an increased neurogenesis and angiogenesis was observed within the first months following injury (Dash et al., 2001; Kernie et al., 2001; Ramaswamy et al., 2005). Because of their key role in brain plasticity, the two germinal niches of the central nervous system were studied with great attention in the regenerative context as potential substrates for cell repair. In particular, the subventricular zone (SVZ), due to its proximity with the cortex and the fact that cortical neurons originate from the pallial prenatal VZ-SVZ, is a potential source for cortical cellular repair. Postnatal neurogenesis originating from the SVZ was described following a stroke, giving rise to mature neurons in the striatum, however, the presence of new neurons maturing correctly in the cortex following stroke remains less well established (Lindvall and Kokaia, 2015). In accordance with the Kennard effect, the brain seems to be more plastic and inclined to regenerate at younger ages. Indeed, after neonatal hypoxic/ischemic injury, a reactivation of neurogenesis as well as a migration of newborn toward the site of injury was observed in much larger proportions. Whether the newborn neurons or glial cells specify, mature and integrate correctly seems to be

largely dependent on the microenvironment, particularly the extrinsic factors driving specific signaling pathways. Altogether, those data show spontaneous regenerative attempts of the neonatal and adult brain following injury, which guided the development of several strategies to enhance post-injury regeneration (Hayashi et al., 2005; Shoichet et al., 2008).

- Endogenous stem cell activity can be boosted to promote brain repair

Over the past years several studies tried to target the pathological consequences following traumatic brain injuries as well as promotion of regeneration. Even though a few clinical trials such as the use of glutamate receptor antagonists, radical scavengers and steroids to prevent excitotoxicity failed to show consistent effect in human, pharmacological approaches remain of great interest in the context of brain regeneration (**Fig 13.2**) (Ikonomidou and Turski, 2002; Narayan et al., 2002; Willis et al., 2003). Importantly, the timing of the treatment and the dosage, as well as the route of administration are critical and need a strong optimization for being efficient (Shoichet et al., 2008). Notably, the low diffusion through the blood brain barrier represent a limitation to the approach (Pardridge, 2012). The administration of bioactive molecules showed promising results following several central nervous system injuries. For instance, intraventricular administration of epidermal growth factor (EGF) and erythropoietin (EPO) after a stroke promotes the recruitment of endogenous neural stem cells toward the site of injury, resulting in an improved functional recovery (Kolb et al., 2007). Administration of allopurinol, an inhibitors of xanthine oxidase, after hypoxia/ischemia had neuroprotective effect in rats (Palmer et al., 1993) and lamb (Cerio et al., 2013; Shadid et al., 1998). Eventually, an interesting study carried out by Scafidi showed that intranasal administration of heparin-binding EGF following chronic hypoxia decreased oligodendroglial death and promoted replacement of lost oligodendrocytes by enhancing oligodendrocyte production from progenitors and their maturation, resulting in a promotion of functional recovery. Furthermore, EGF administration had a positive effect on the behavioral deficits observed long-term after the injury (Scafidi et al., 2014). This last study illustrates the possibility to boost endogenous neural stem cells by manipulating the EGF signaling pathway. However, chronic hypoxia induce neuronal deficits in addition to glial deficits. The next step would

be to identify pathways and the corresponding drugs to induce specific fate in neural stem cell populations.



**Figure 13.2: Therapeutic strategies using exogenous and endogenous stem cells for neuronal repair.** On the left, exogenous stem cells are transplanted into a damaged brain to trigger neuronal repair. Exogenous stem cells are often neural stem cells derived from embryonic stem cells or induced pluripotent stem cells. On the right, endogenous stem cells from the subventricular zone (SVZ) are recruited using a pharmacological approach. Indeed, small molecules are administered to trigger SVZ neural stem cells recruitment toward neuronal repair. Similar approach can be applied for glial cell repair.

### 3. Remaining challenges for promoting cellular repair in the postnatal forebrain

Several questions remain to be answered regarding to the cortical regeneration following chronic hypoxia. First, the competency of the neural stem cells from the dorsal microdomain of the subventricular zone (dorsal SVZ) to produce appropriate

cortical neurons after birth in an early lesion context was never clearly established. During embryogenesis, cortical neuron subtypes production follows a strict temporal order. The first challenge to overcome is to understand the role of dorsal SVZ stem cells in post-hypoxia neuronal repair which will highlight their competency of dorsal SVZ stem cells to produce various subtypes of neurons, and notably the earliest produced in development. The second challenge will be to elaborate a pertinent approach to promote specific lineages of the SVZ to eventually promote cortical repair.

**a. 1<sup>st</sup> challenge : does postnatal dorsal subventricular zone stem cells keep their competency to produce cortical neurons ?**

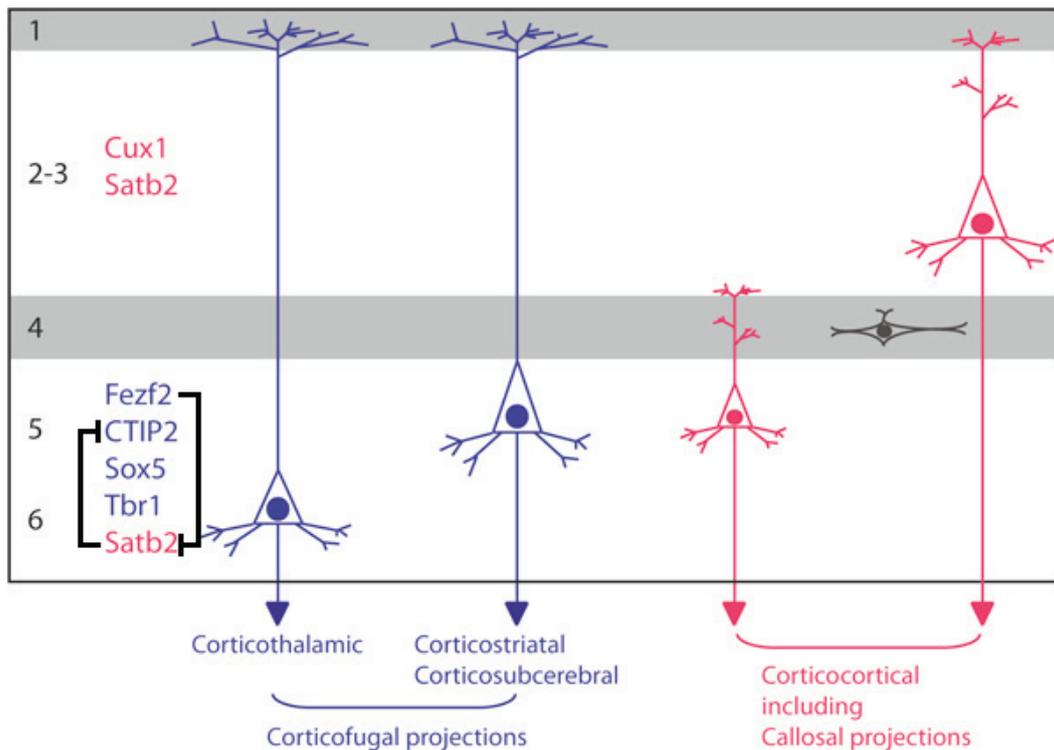
- Different neuronal subtypes are sequentially produced during corticogenesis

The mammalian cortex is a highly organized structure underlying higher brain functions such as abstract thinking, learning and memory. The formation of the cortex relies on the expansion of neural stem cells as well as radial glia cells, neural progenitors and the subsequent generation of post-mitotic neurons and happens exclusively at the embryonic period. The cortex structure is often described based on the identity of the glutamatergic neurons it contains which represent about 80% of the total cortical neuron population. Conversely, the GABAergic inhibitory neurons represent roughly the rest of the cortical neuron population, about 20% (Chu and Anderson, 2015). The cortex is indeed subdivided into 6 different layers, as Cajal and Golgi first described it. Neurons from the different layers differ in term of morphology as well as in term of marker they express and are generated at different times of the development. Indeed, successive waves of radial glia cells differentiate resulting in the generation of glutamatergic cortical neurons, from the deepest layer (layer 6) to the most superficial one (layer 1), in an inside-out manner and throughout the embryonic period. The neurons belonging to the different cortical layers differ in term of morphology, projections and marker they express. This specific time-course of layer specific generation of neurons raise many interrogations regarding to the competency of radial

glial cells over time and their ability to produce neurons from those different layers at other timepoints, for instance in a regenerative context.

The earliest neurons arising from the radial glia cells of the VZ reside in a structure known as the preplate emerging around E10,5 (10,5 days after fecundation). Maturation of the preplate into cortical plate starts about one day later and the production of pyramidal neurons is regulated following a strict sequence until E18. Techniques such as retrograde tracing and intracellular dye injections allowed a precise description of the pattern of connections of the cortical neurons. The first neurons to be produced are going to constitute the deepest layer, the layer 6. Most of those neurons are corticofugal cells forming feedback cells to the thalamic nuclei. Together with layer 5 neurons they form subcortical projections to reach, for instance, the spinal cord. Neurons located in the layers 1 and 5 extend axons on shorter distance, locally in the cortex. Layers 2 and 3 form cortico-cortical projection including callosal projections (Butt et al., 2005) (**Fig I3.3**).

Many transcription factors are characteristically expressed by both germinal cells and neurons from specific cortical layers (Gray et al., 2004; Lodato et al., 2015; Molyneaux et al., 2007). Neurons and progenitors from the preplate express *Nurr1* (Watakabe et al., 2007). While glutamatergic neuron progenitors express *Tbr2*, most of the glutamatergic neurons express *Tbr1* (Hevner et al., 2001). *Fezf2*, *Sox5*, and *Ctip2* (Molyneaux et al., 2007) are transcription factors required to specify these early-born cortical neurons from deep layer cortical neurons (layers 5 and 6). *Fezf2* plays its role in layer specification partly by repressing the chromatin remodeling protein *Satb2* (Chen et al., 2008). Indeed, *Satb2*, is involved in the specification of the superficial layer neurons by repressing the activity of *Ctip2* (Britanova et al., 2008; Sugitani et al., 2002). *Cux1/2* are expressed in upper cortical layer neurons (layers 2 to 4). Further, an in utero fate mapping study suggests that a subpopulation of *Cux2*<sup>+</sup> progenitors produce exclusively superficial cortical neurons (Kohwi and Doe, 2013).

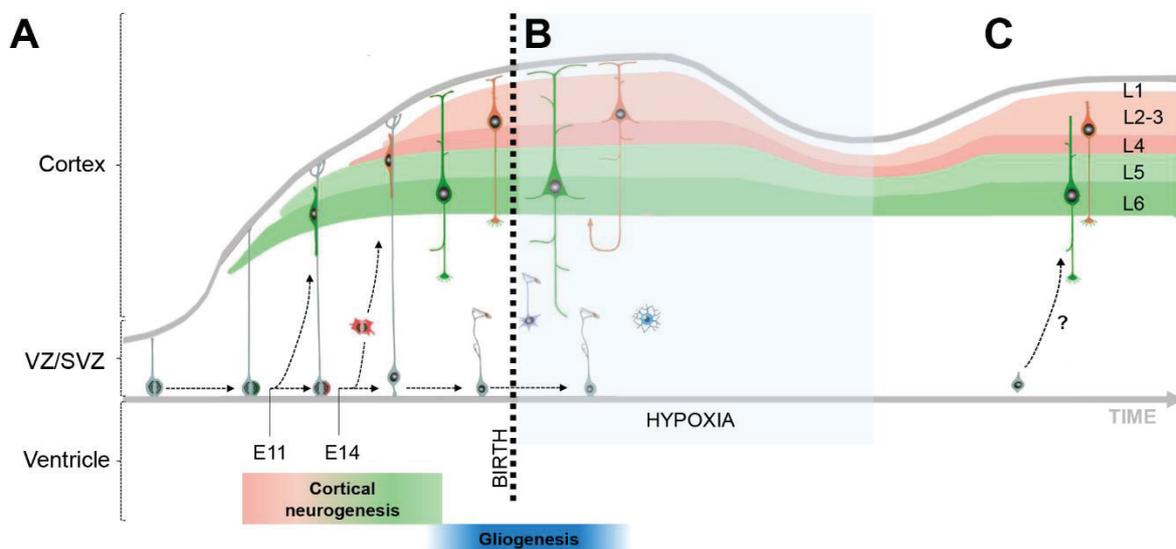


**Figure 13.3: Transcription factors involved in layer-specification of cortical neurons.** Diagram representing the laminar organization of the cortex in terms of gene expression and axonal projections. Deep layer cortical neurons express Fezf2, CTIP2, Sox5 involved in deep layer cortical neuron specification (blue transcription factors), which makes corticofugal projections including corticothalamic, corticostriatal and corticosubcerebral projections (blue neurons). Fezf2 represses Satb2 which represses CTIP2. Satb2 is expressed in both deep and upper cortical neurons. Eventually, Cux1 triggers upper layer cortical neurons which make corticocortical projections (red transcription factor and neurons). Adapted from (Gaspard and Vanderhaeghen, 2011)

Both ventricular zone (VZ) and subventricular zone (SVZ) stem cells and progenitors can generate cortical neurons before birth. Studies showed that VZ cells express mostly genes involved in deep layer cortical neurons while SVZ cells express mostly genes involved in upper layer cortical neurons such as Cux1. This suggests that VZ cells generate more likely the deep layer neurons while SVZ cells generate upper layers progenitors, which are the latest produced (Götz et al., 1998; Hack et al., 2005; Nieto et al., 2004; Tarabykin et al., 2001). The expression of Cux2 is already detected in the VZ, before the formation of the SVZ, suggesting that those early progenitors might be committed already to form the SVZ and to eventually produce the upper layer neurons. Of note, Satb2 and Fezf2 regulate genes involved in neurological disorders,

providing a link between corticogenesis defects and pathologies such as autism (Srinivasan et al., 2012).

Whether postnatal SVZ neural stem cells keep the competency to spontaneously produce cortical neurons that will specify and integrate correctly in the already established cortical layers after chronic hypoxia cortical injury remains to determine (**Fig I3.4**) and will be investigated in Chapter 1.



**Figure I3.4: Chronic hypoxia cortical model provides information on the competency of the postnatal SVZ stem cell competency.** (A) Sequential generation of the cortical layers in an inside-out manner from the VZ-SVZ neural stem cells during embryogenesis. This period of cortical neurogenesis is followed by a perinatal period of cortical gliogenesis (B). The perinatal period of hypoxia (10% of oxygen from 3 to 11 days after birth in the environment) leads to a cortical atrophy followed by a cellular regeneration attempt. (C) The correct specification of the newborn cortical neurons remains under debate. We propose that the postnatal SVZ as a source for post-hypoxia cortical repair. Adapted from (Kwan et al., 2012)

#### - Competency of VZ-SVZ neural stem cells

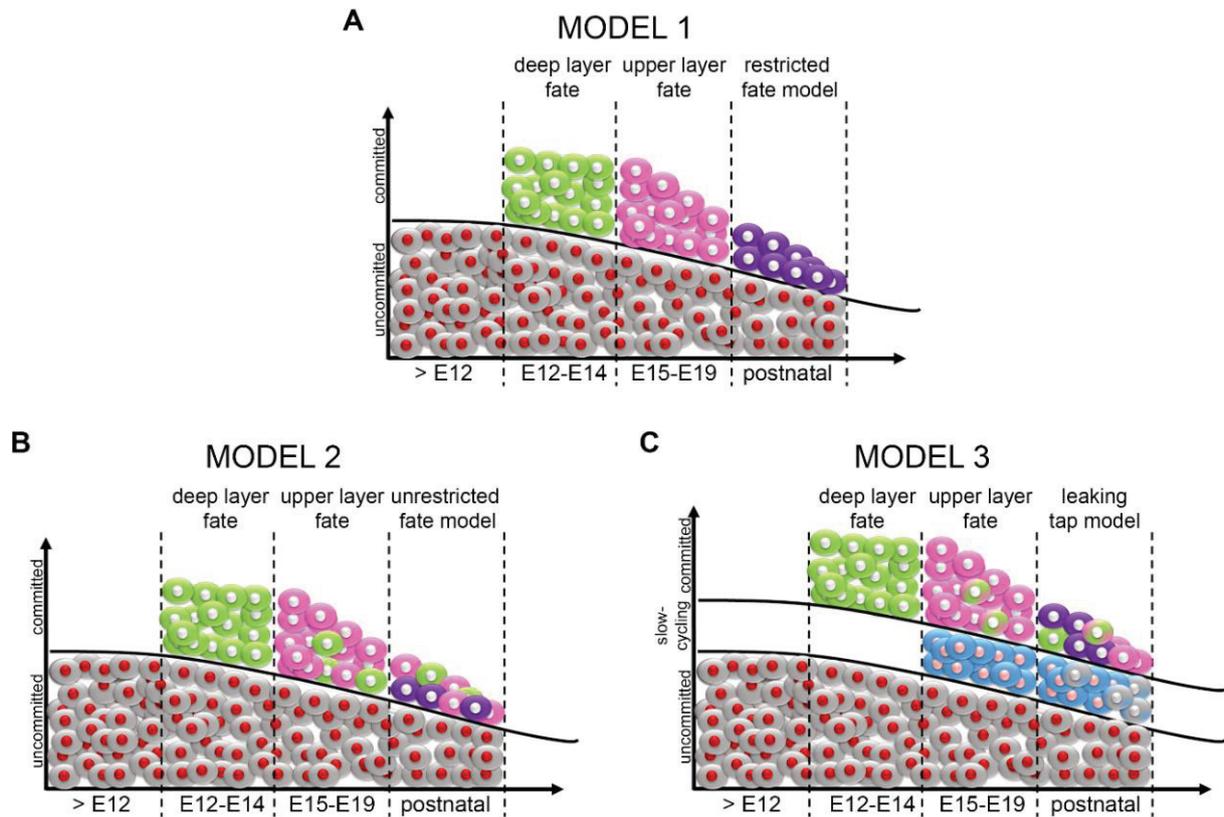
The NSCs from the postnatal SVZ have the ability to regenerate oligodendrocytes in the context of regeneration following an injury such as hypoxia. However, little is known about the capacity of those neural stem cells to regenerate cortical neurons, particularly the excitatory glutamatergic neurons produced embryonically in such a

sequential manner. Few studies show that RGC fate is influenced by the microenvironment. However, the role of a few proteins and growth factors in corticogenesis has been highlighted. Reelin, for instance, is a protein secreted by Cajal-Retzius neurons, a transient neuron population located in the marginal zone, which regulates migration and positioning of cortical neurons. Mutants for reelin are unable to produce the cortical layers in an inside-out manner and their cortical layers are arranged in the opposite manner (Polleux et al., 1998; Tarabykin et al., 2001). Furthermore, BDNF acts directly on the RGC fate through a modulation of their cell cycle (Fukumitsu et al., 2006) and endocannabinoids are involved in proliferation, pyramidal specification and axonal navigation (Diaz-Alonso et al., 2012). However, even if some extrinsic factors can influence or refine corticogenesis, this process remains largely due to intrinsic factors and original studies let little hope for the possibility of an endogenous cortical repair originating from the VZ-SVZ cells. Several studies of heterochronic grafts have shown a loss of competency over time. For instance the transplantation of late cycling embryonic progenitors into the early embryonic ferret SVZ showed that the late progenitors failed to produce deep layer neurons and were already committed to produce upper layer neurons (Frantz and McConnell, 1996). Moreover, a study carried out by Desai showed that transplantation of mid-stage progenitors into earlier stage embryonic mouse could only produce later stage cortical neurons but not the ones produced normally at the age of the receiving embryo (Desai and McConnell, 2000). However, it seems that a population of quiescent NSCs persist in the VZ-SVZ, which has the competency to produce cortical neurons long after birth, while the proliferating progenitors and active NSCs lost it overtime. Indeed, in both of those studies, only the proliferative progenitors were selected for the grafting. In contrast, a study carried out by show that SVZ explants containing not only cycling progenitors but also quiescent embryonically derived NSC from postnatal and 3 months old adult mice transplanted onto embryonic sections had the competency to produce pyramidal glutamatergic neurons (Fuentealba et al., 2015; Furutachi et al., 2015b; Sequerra et al., 2010).

- Three models for SVZ neural stem cells competency

Different models have been proposed to explain the competency of VZ-SVZ neural stem cells to produce different lineages overtime. The classic model propose that while

the uncommitted NSC pool decrease progressively, the committed NSC lose their competency to produce cortical neurons. First, they lose the competency to produce deep layer neurons, and eventually to produce upper layer neurons during embryogenesis. Eventually, after birth they lose completely their competency to produce deep layer neurons and might still be able to produce upper layer neurons. This is the restricted fate model (**Fig I3.5A**). in line with the recent demonstration of an embryonic origin of postnatal and adult NSCs, it is possible that embryonically derived NSCs keep the competency to produce both deep and cortical layer neurons, if recruited in the context of brain lesion for instance. Two alternative models were recently proposed by Donega and Raineteau (ref). The unrestricted model proposes that there is no restriction of fate for the committed cells: they keep their competency to produce all cortical neurons (**Fig I3.5B**). Eventually, the leaking tap model proposes that a pool of slow cycling/quiescent stem cells continues to produce progenitors through adulthood even though this production declines with age. Thus, the population of embryonically-derived neural stem cells maintain its potential to be “reactivated” in the context of injury (**Fig I3.5C**). If so, extrinsic factors such as morphogens are likely to be involved in this process (Donega and Raineteau, 2017).



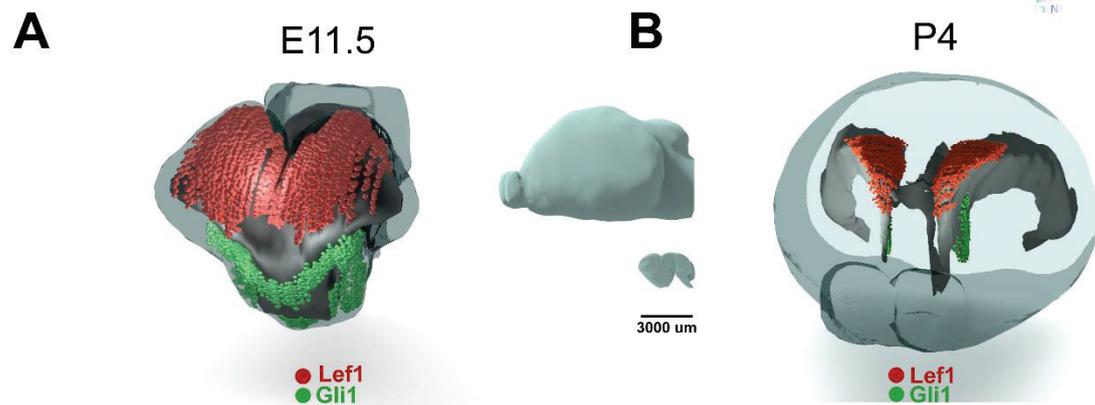
**Figure 13.5: Competency of neural stem cells in the embryonic and postnatal VZ-SVZ.** (A) The classic view (Model 1) proposes a restriction of neural stem cell fate over time. Neural stem cells would lose little by little their competency to produce cortical neurons. Postnatal neural stem cells would not be able to produce deep layer cortical neurons but only a few upper layer cortical neurons. (B) The second model (Model 2) proposes that neural stem cells committed to produce both deep and upper cortical layers persist in the postnatal VZ-SVZ and can therefore be recruited in the context of brain injury. (C) The last model (Model 3) proposes that a population of slow/cycling neural stem cells accumulates during development and could acquire either a deep or upper cortical layer fate or acquire another fate. Adapted from (Donega and Raineteau, 2017)

**b. 2nd challenge : elaborating a translational strategy to promote cortical repair in a lineage-specific manner**

Since the heterogeneity of the subventricular zone (SVZ) was established, many studies focused on signaling pathways that may act in specific regions of the postnatal SVZ.

- The Wnt/ $\beta$ -catenin and Sonic hedgehog signaling pathways control the regionalization of the subventricular zone

Specific signaling pathways regulate the different microdomains of the postnatal SVZ (Merkle et al., 2007). For instance, a study carried out by Ihrie shows that shutting down sonic hedgehog (Shh) signaling in the NSCs located in the lateral microdomain of the SVZ results in a decrease of olfactory bulb neurons produced. On another hand, the ectopic activation of the pathway in dorsal SVZ NSC resulted in a respecification of their progeny into laterally derived OB neurons, showing at the same time the permissivity of the dorsal SVZ NSC to Shh pathway. More importantly, this study highlighted the dependency of the NSCs from the lateral wall to Shh signaling, in accordance with Shh ventralizing role during development (Hoch et al., 2009; Ihrie et al., 2011). Another interesting study carried out previously in the group showed that it is possible to increase specifically the proliferation and the number of dorsal SVZ progenitors by activating Wnt pathway in the SVZ, and thereby showed that the lateral SVZ progenitors do not answer to Wnt/ $\beta$ -catenin signaling. In this study, different tools were used to activate Wnt signaling, most notably a pharmacological approach involving intraventricular infusion of a small molecule (Azim et al., 2014b). Moreover, Wnt/ $\beta$ -catenin signaling is active exclusively in the dorsal microdomain of the SVZ at postnatal time point (Azim et al., 2014b; Ortega et al., 2013), as in the pallial cells from which it originates (Hoch et al., 2009; Lee et al., 2000).. Altogether, those studies suggest that the environment largely influence NSC fate. However, it seems that all NSC do not have the same amenability to respecification by the environment, and the same permissivity to the different signaling pathways. Identifying key signaling pathways responsible for NSC commitment into different lineages allows not only to challenge the SVZ plasticity but could also allow to elaborate therapeutical approaches in the context of brain repair (Angonin et al., 2015).



**Figure I3.6: Wnt/ $\beta$ -catenin and Sonic hedgehog signaling contribute to the postnatal SVZ heterogeneity.** (A, B) Volumetric reconstructions of E11.5 and P4 mouse forebrains depicting the brain surface (blue, semi-transparent) and ventricular system (dark gray). Note that most of the brain volume is occupied by the ventricular system at E11.5. The embryonic expression of specific signaling pathway downstream target genes (red, Lef1 or BatGal for canonical Wnt signaling; green, Gli1 for Shh signaling) persists after birth into postnatal life. Reproduced from (Fiorelli et al., 2015).

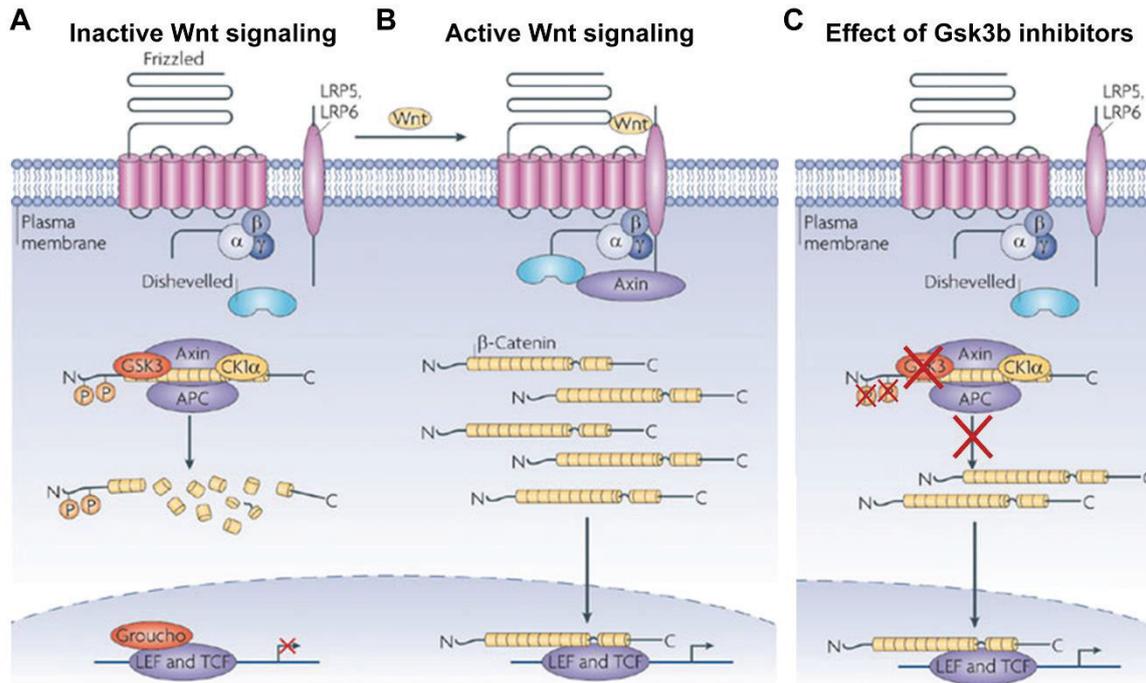
Because we are particularly interested in glutamatergic cortical neuron repair as well as oligodendrocyte repair, we decided to focus on Wnt/ $\beta$ -catenin signaling pathway. Indeed, Wnt/ $\beta$ -catenin signaling actively maintain the NSCs and progenitors contained in the dorsal microdomain of the SVZ which contains glutamatergic neuron progenitors and oligodendrocyte progenitors, two populations that suffer significantly from chronic hypoxia (Fagel et al., 2006).

#### - Wnt pathway discovery and signaling

Wnt/ $\beta$ -catenin signaling is a highly conserved cascade of signaling across species. The name of the protein initiating the signaling cascade, Wnt, was given in the 1990's after researchers discovered the homology between the protein Int1 studied in the mouse and the protein Wingless studied in the drosophila. Int1 was indeed of interest at this time as it was one of the first genes producing a developmental phenotype to be knocked out by homologous gene targeting in mice (Nusse and Varmus, 2012). Indeed, Wnt proteins are highly conserved in evolution and are active in every branch of the animal kingdom. It took more than 10 years to fully understand the functioning of the canonical Wnt pathway, mobilizing a lot of resources. However, the implication of Wnt signaling in development, stem cell regulation, but also in several diseases such

as cancer, or neurodegenerative diseases makes crucial to unravel the cellular and molecular mechanisms it underlies. Indeed, Wnt signaling is indeed involved at many timepoints of the development and in many different tissues. It is a pleiotropic signaling initiated by the extracellular, cysteine-rich, glycosylated protein Wnt. The Wnt proteins are a family of nineteen highly conserved secreted glycoproteins, triggering three signaling pathways: the Wnt/ $\beta$ -catenin signaling pathway, the Wnt/PCP (planar cell polarity) signaling pathway and the Wnt/ $\text{Ca}^{2+}$  signaling pathway. However, the molecular mechanisms underlying the two  $\beta$ -catenin independent pathways remain poorly defined. Here, we focus on the Wnt/ $\beta$ -catenin signaling pathway, also called the canonical Wnt pathway. In the absence of Wnt ligands, three cytoplasmic proteins, glycogen synthase kinase 3 (Gsk3 $\beta$ ), casein kinase 1 $\alpha$  (Ck1 $\alpha$ ), Axin and adenomatous polyposis coli (Apc), bind together to form a degradation complex. This protein complex promotes the phosphorylation of  $\beta$ -catenin on serine 33 and 37 and threonine 41 by Gsk3 (Yost et al., 1996), which leads to its ubiquitination by Skp1–cullin 1–f-box (Scf $\beta$ -TrCP) E3 ligase. Eventually the  $\beta$ -catenin is degraded by the 26S proteasome (Kimelman and Xu, 2006). When Wnt protein is secreted in the extracellular interspace, it can bind to its transmembrane Frizzled and the co-receptor low density lipoprotein receptor-related protein 5 (Lrp5) or Lrp6. Dishevelled cytoplasmic phosphoproteins are then activated, which inhibit the degradation complex, thereby blocking the phosphorylation of  $\beta$ -catenin by Gsk3 and its degradation.  $\beta$ -catenin, which contains an NLS sequence is then guided to the nucleus where it accumulates. There, it can exert its co-activator role binding to lymphoid enhancer-binding factor (Lef) and T cell factor (Tcf) proteins to modulate the expression of target genes. The good understanding of the Wnt/ $\beta$ -catenin pathway allowed the elaboration of transgenic tools to manipulate the signaling, using a transgenic or a pharmacologic approach.

In comparison to other growth factors, Wnt signaling has several unique properties, including a short range of action. Indeed, it predominantly mediates signaling locally, between neighboring cells. It also often acts as a gradient and its level of activity will determine which promotor it influences.



**Figure 13.7: Wnt/β-catenin signaling pathway and its manipulation by Gsk3β inhibitors.** (A) In the absence of Wnt, β-catenin is phosphorylated by a destruction complex composed of Gsk3, Axin and Ck1a, leading to its ubiquitination and eventually to its degradation by the proteasome. (B) In presence of Wnt, the destruction complex is inhibited and β-catenin stabilizes in the cytoplasm to eventually translocate into the nucleus where it exerts its transcription factor role together with Lef and Tcf. (C) Gsk3β inhibitors active Wnt signaling by inhibiting the destruction complex. Adapted from (Angers and Moon, 2009)

- Tools to study Wnt/β-catenin signaling

The emergence of numerous new tools, notably quantitative reporters such as TopFlash in addition to Wnt protein as reliable end points for signaling have simplified the study of Wnt signalling in vivo and in vitro, and attracted many new investigators. The generation of Wnt reporter mice, initially Top-gal animals from Elaine Fuchs (DasGupta and Fuchs, 1999), later of animals with transgenic markers driven by Axin promoters (van Amerongen, 2012; Lustig et al., 2002) and eventually, of the BatGal mouse line, in which expression of β-galactosidase is under the control of seven Tcf/Lef binding sites, provided yet more experimental opportunities (Maretto et al., 2003a). It is now possible to trace Wnt responding cells in any tissue of the mouse, examine the origin of these cells, and follow their fate in normal settings or after injury (van Amerongen, 2012; Barker and Clevers, 2006). These new experimental tools

have led to a rapidly growing list of Wnt signaling components, built on the core pathway.

Wnt signaling is a key player already at the early stages of development, particularly in the establishment of the rostral-caudal and medial-lateral tissue patterning, by spatially graded  $\beta$ -catenin activity. Canonical Wnt signaling has been mapped in the developing embryo and subsequently forebrain using several reporter models above which BAT-gal reporter mice, in which expression of  $\beta$ -galactosidase is under the control of seven Tcf/Lef binding sites, and thereby reflecting the activity of Wnt/ $\beta$ -catenin signaling (Maretto et al., 2003a). This approach, completes the informations provided by transcriptional analysis.

There are many ways to manipulate Wnt/ $\beta$ -catenin signaling. However, the most translational in the context of brain repair is the use of a pharmacological approach. Notably, the small molecules inhibiting Gsk3 $\beta$ , AR-A014418 and CHIR99021 are commonly used in our lab because of their efficiency to activate Wnt/ $\beta$ -catenin, when administrated intraventricularly, intraperitoneally but also intranasally.

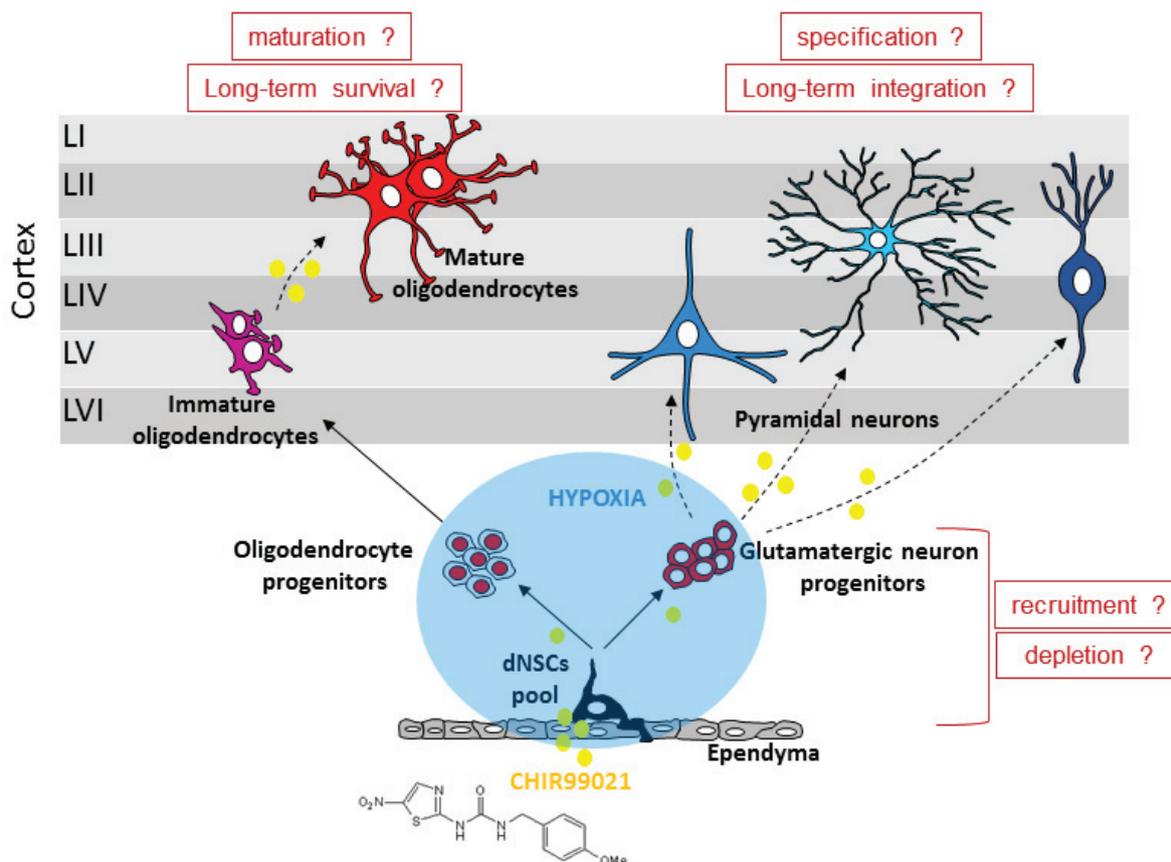
- Wnt/ $\beta$ -catenin signaling promotes brain repair

Several studies showed that new neurons generated in the subventricular zone (SVZ) have the ability to migrate toward sites of injury in the context of brain repair, particularly after stroke in adult (Arvidsson et al., 2002; Yamashita et al., 2006). Wnt/ $\beta$ -catenin signaling seems to play a key role in the recruitment of SVZ neural stem cells. Indeed, its activity is increased in the SVZ and support symmetric divisions of NSCs leading to the expansion of the NSC pool (Piccin and Morshead, 2011) in ischemic stroke model, characterized by striatal lesion. Moreover, an extrinsic decrease of Wnt/ $\beta$ -catenin signaling following the injury by intraventricular infusion of siRNA targeting  $\beta$ -catenin result in a decreased proliferation in the SVZ as well as a decreased number of newborn neurons in the striatum (Lei et al., 2008). In addition, a study carried out by Shruster and collaborators shows that increasing the expression of Wnt-3a in the SVZ or the striatum following focal ischemic injury with a lentiviral vector injection decrease the expansion of the injury at the neuronal level. Both injections in the SVZ and the striatum improved functional recovery (Shruster et al., 2012).

Proof of the involvement of Wnt/ $\beta$ -catenin in cortical repair following postnatal injury, in addition to the ability of Wnt/ $\beta$ -catenin to promote dorsal lineages of the SVZ, makes the manipulation Wnt/ $\beta$ -catenin activity of great interest in the context of chronic hypoxia.

## D. Key issues and objectives of the thesis

One of the major challenge of regenerative medicine is to trigger tissue repair by recruiting endogenous stem cells. However, the efficiency of this approach in vivo remains largely limited. First, the endogenous stem cells may have lost their competency to produce the damaged cells. Second, if/when they differentiate correctly, they need to migrate toward the site of injury. Eventually, the microenvironment at the lesion site has to be permissive enough to allow a correct the integration of the newborn cells. Chronic hypoxia is a model of mild cortical injury followed by a spontaneous cellular regeneration. Although a few studies focused on cortical oligodendrocyte and GABAergic neurons repair (Fagel et al., 2006; Jablonska et al., 2012), none focused on the source of glutamatergic neurons born after the hypoxic period. Postnatal subventricular zone stem cells represent a potential cellular source for cortical repair following injury. The most translational way to promote their participation to tissue repair is to use a pharmacological approach, i.e. small molecules, to increase their activity and trigger cellular recruitment and integration at the site of injury. Timely controlled modulation of signaling pathways is necessary to trigger neuronal specification and oligodendrocyte maturation. The selection of relevant small molecules will therefore depend on finding a tight balance between its ability to promote dorsal SVZ stem cell proliferation, trigger specific fate but also promote or at least not prevent cell maturation (**Fig I4.1**).



**Figure I4.1: Key issues and objectives of the thesis.** The goal of the thesis was to identify small molecules to manipulate specific lineages of the postnatal SVZ and promote cortical repair in the context of chronic hypoxia. The mechanisms involved in cortical repair investigated in the thesis are: neural stem cells and progenitor recruitment and depletion, correct specification and integration of the newborn glutamatergic neurons, and eventually, correct maturation and survival of newborn oligodendrocytes.

The main objective of my thesis was to explore the competency of postnatal neural stem cells to participate to forebrain cortical repair and design new means to encourage this cortical repair in a lineage specific manner. The project had three thematic approaches:

**Aim 1 :** To investigate the contribution of the dorsal SVZ to the spontaneous cellular repair observed following chronic neonatal hypoxia.

We first investigated if the dorsal microdomain of the postnatal subventricular zone (dorsal SVZ), which continues producing oligodendrocyte and glutamatergic neuron progenitors throughout life, was a source of cell for cortical repair following chronic

hypoxia. For this purpose, after validating the model by insuring that apoptosis indeed occurred in the cortex, we fate mapped specifically postnatal dorsal SVZ stem cells after the necessary time for cellular recovery after chronic hypoxia. We also investigated the nature of the cells originating from the dorsal SVZ that migrated in the cortex to conclude on the competency of postnatal dorsal SVZ neural stem cells to produce cortical neurons expressing layer specific markers.

**Aim 2** : To develop a pharmacogenomics approach for identifying small molecules for lineage specific manipulation of postnatal forebrain germinal activity

The second aim of our work consisted in identifying the most efficient small molecule to promote post-hypoxia cortical repair. For this purpose, we performed a pharmacogenomic analysis to highlight small molecules with the potential to trigger oligodendrocyte and glutamatergic neuron fates. This approach was validated by testing small molecules in the neonatal brain, in the adult brain and in the hypoxic brain, thereby showing the amenability of the dorsal SVZ stem cells to pharmacological treatment in different contexts. CHIR99021, a Gsk3 $\beta$  inhibitor, was eventually selected for its efficiency to induce dorsal SVZ cell proliferation, oligodendrocyte and glutamatergic neuron fates, but also for its efficiency using intranasal administration.

**Aim 3** : To test small molecules to promote tissue repair following chronic neonatal hypoxia.

The last aim of my thesis was to determine the effect of CHIR99021, on the progeny of dorsal SVZ stem cells in the context of chronic hypoxia. To do this, CHIR99021 was administrated intranasally following the hypoxic period in the chronic. The effect of the drug on oligodendrocyte maturation, neuronal specification and on the maintenance of the pool of stem cells in the niche were investigated at different timepoints.

## MATERIAL AND METHODS

Unless stated, all materials were purchased from Sigma-Aldrich. All procedures were in accordance and approvals of the UK Home Office Animals Scientific Procedures Act (1986), Ethics Committee of the Veterinary Department of the Canton of Zurich (Approval ID 182/2011). Experiments in France were performed in accordance with European requirements 2010/63/UE and have been approved by the Animal Care and Use Committee CELYNE (APAFIS#187 & 188). Animal procedures were executed in accordance with UK/Swiss/French law, with strict consideration given to the care and use of animals. All mice were bred over wildtype C57/BL6 background for several generations and positive animals for Mash1-EGFP selected at birth under UV light.

### a. Part performed by Kasum Azim

#### Bioinformatics

Whole genome transcriptome datasets of the isolated SVZ microdomains, and region-specific NSCs and TAPs are described in detail in a recent study that aimed at describing transcriptional regulators acting in SVZ regionalization [1]. Briefly, the rostral periventricular regions of postnatal mice of different ages (P4, P8 and P11) of the *Ascl1-EGFP<sup>Bac</sup>* transgenic reporter mouse line were carefully microdissected under a fluorescent binocular microscope in RNase free and sterile conditions. SVZ microdomains isolated were derived from brain coordinates +1 relative to the bregma to 0 relative to the bregma. The *Hes5-EGFP* reporter mouse line was used in combination with Prominin-1 immunodetection to isolate NSCs from microdissected dorsal and lateral microdomains by fluorescence activated cell sorting. Similarly, the *Ascl1-EGFP<sup>Bac</sup>* transgenic reporter mouse line was used to isolate the 25% brightest cells, i.e. corresponding to TAPs, from either microdomain. Half a litter of animals were used to pool for each replicate throughout. In the present study, these datasets (recently made publically available from NCBI Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo>) GEO Series accession number GSE60905), were

analyzed using previously applied bioinformatics methods, with only minor modifications (Azim et al., 2015). In brief, data were cured (background subtraction, normalization, and summarization) using robust multi-chip analysis (RMA) using the Partek Genomic Suite software package version 6.6 using stringent false discovery rate (FDR) with p-values where necessary in the analysis. All data's were normalized collectively with datasets from previous studies of isolated NSC, NPs and glia (i.e. GSE60905, GSE9566, GSE18765) for optimal parameters. Partek was used assemble affymetrix data to generate hierarchical clustering and gene lists. GO's were generated using the latest MGI mouse GO datasets via the Broad Institute (<http://www.broadinstitute.org/gsea/index.jsp>). The numbers of probe that were differentially expressed across the 10 samples analysed (dNSCs, dTAPs, INSCs, ITAPs, P4 dorsal SVZ, P4 lateral SVZ, P8 dorsal SVZ, P8 lateral SVZ, P11 dorsal SVZ and P11 lateral SVZ) represented a total of ~37K probesets within the 10% FDR range. Genego Metacore (<https://portal.genego.com/>) and GSEA (<http://www.broadinstitute.org/gsea/msigdb/index.jsp>) were used to filter and select for probes associated as secreted morphogens (tropic factors, growth factors, extracellular signaling molecules, mitogens and secreted inhibitors of signaling pathways). The numbers of morphogen from this filtered list that were significantly altered amounted to 530 probes, representing approximately 330 individual genes. Identification of spatially enriched signaling ligands regardless of sample type (**Fig 1A-B**) was done by comparing all dorsal versus all lateral samples. This gene list was uploaded onto Genego Metacore and Process Network option selected using the default parameters. Determination of the spatial expression profiles of secreted signaling factors in SVZ microdomains (**Fig 1C-H**) was performed by comparing datasets using appropriate fold changes and FDR cut-offs (Partek, 1.65 fold change and FDR < 5%). For all analysis, raw expression values are provided and Heatmaps are presented in the manuscript.

### **SPIED analysis**

For SPIED identification of small molecules, the “dorsal NSCs/TAPs”, “lateral NSCs/TAPs”, “postnatal NSCS/TAPs”, “oligodendroglial lineage” and of the “rejuvenating” transcriptional signatures were defined using Partek (1.8-fold change,

FDR<5%) as follows. Probes significant across multiple normalised datasets in the background (representing ~40K probes) were processed. For identification of “dorsalizing” small molecules, dNSCs/dTAPs datasets (positive range) were compared with probes significantly different in the background. This list was then further refined and compared with other postnatal datasets (negative range) using the advanced tab in list manager followed by criteria configuration in generating lists with merged expression profiles. Inversely, for identification of “ventralizing” small molecules, INSCs/ITAPs datasets (positive range) were compared as above against with probes significantly different in the background. This list was further refined and compared with other postnatal datasets from the same study (negative range) (Azim et al., 2015). For identification of “pro-oligodendrogenic” small molecules, publicly available datasets of forebrain derived OL lineage cells (positive range; GSE9566) were compared to dNSCs and dTAPs (negative range) from which they emerge. Finally for identification of “rejuvenating” small molecules, publically available datasets of adult NSCs (Beckervordersandforth et al., 2010) (positive range), were compared with postnatal dNSCs/INSCs (negative range) (Azim et al., 2015). These expression profiles, consisting of gene symbols of “enriched” genes were next uploaded onto SPIED (<http://www.spied.org.uk/cgi-bin/HGNC-SPIED3.1.cgi>) to interrogate the CMAP initiative in an unbiased way and identify small molecules predicted to promote the positive ranges of gene signatures using default parameters. The Broad connectivity map 2.0 (CMAP2.0) database consists of the transcriptional profiles corresponding to the effects of small molecules at various concentrations and treatment times using panels of human cell lines. The data is available for download in the form of ranked probe sets for each microarray sample on the [HG-U133A] Affymetrix Human Genome U133A Array platform.

Identified small molecules cellular targets were exhaustively characterized using publically available drug repositories ([www.DrugBank.ca/](http://www.DrugBank.ca/); [www.genome.jp/kegg/drug/](http://www.genome.jp/kegg/drug/); [www.insilico.charite.de/](http://www.insilico.charite.de/); [www.pharmgkb.org/](http://www.pharmgkb.org/); [www.stitch.embl.de/](http://www.stitch.embl.de/)). Small molecules protein targets identified were cross-checked in <http://www.genecards.org/> for classifying them under general GO terms. All analysis presented in **Fig 2**, are shown as a percentage.

## Small molecule Target Gene analysis

“Target genes” are defined as the genes from the queried expression profiles that are also induced by a given small molecule. Target genes for each analysis were generated as follows. To generate lists of genes perturbed by the small molecules, gene replicates were pooled and the relative expression levels calculated. Changes passing the student’s t-test p-value of  $\leq 0.05$  were processed and when there were multiple probes for a given gene, the probe with the biggest fold change was assigned to the gene. These were aligned for matching signatures with the transcriptional profiles corresponding to the small molecules repurposed in the CMAP using pattern-matching algorithms that enable identification of functional connections between drugs, genes and diseases through the transitory feature of common gene-expression changes (Lamb et al., 2006). The entire database is available for download (<http://www.broadinstitute.org/gsea/msigdb/index.jsp>) in the form of ranked probesets for each microarray sample on the [HG-U133A] Affymetrix Human Genome U133A Array platform.

For analysis of “target genes” of select small molecules, we first performed hierarchical clustering of their expression profile in the various cell types and lineage that compose the SVZ, using the following datasets: purified postnatal NSCs and TAPs (GSE60905 (Azim et al., 2015)), for purified glial cells (GSE9566 (Cahoy et al., 2008)) and adult NSCs/NPs/ependymal cells (GSE18765 (Beckervordersandforth et al., 2010)). Target genes were then classified by “Process network and pathway maps” GO categories. Briefly, target gene lists containing the contrasts and fold changes were analyzed via the web platform <http://www.broadinstitute.org/cmap/index.jsp> and functionally classified using Genego Metacore (<https://portal.genego.com/>) for Process Networks and Pathway Maps.

Last, target genes were studied further for obtaining the shortest path between genes associated with highly ranked process networks and pathway maps using standard Dijkstra’s shortest paths algorithm and applying default parameters (Azim et al., 2016; Hwang et al., 2011). Background RMA normalized data for all probesets relevant for the postnatal and adult SVZ derived from postnatal NSCs and TAPs ((GSE60905 (Azim et al., 2015)), purified glial cells ((GSE9566 (Cahoy et al., 2008))), and adult NSCs/NPs/ependymal cells ((GSE18765 (Beckervordersandforth et al., 2010))) were

uploaded onto Genego Metacore (raw data's provided with the manuscript). This allows obtaining, visualizing, aligning and clustering the most relevant target genes based on small molecule target genes with reference to basally expressed genes in the SVZ. Objects within this network were restricted to 70-80 in accordance with those highly ranked and most significant within the earlier GO analysis and signaling-to-transcriptional options were selected. Internal clusters (2-4) within the network module were arranged according to the highest ranked GO pathways within the analysis in the pathway selection menu. A full description of the definition of objects and nodes can be found here: <https://portal.genego.com/legends/MetaCoreQuickReferenceGuide.pdf>

### **SVZ microdissection, qPCR and Western Blot**

The SVZ microdomains were microdissected using previously published protocols (Azim et al., 2015). In brief, mouse pups were killed humanely by cervical dislocation. In sterile and RNase free conditions, brains were rapidly dissected free and placed in ice-cold postnatal-specific coronal brain matrix (Zivic Instruments, USA) to obtain tissue segments of 500  $\mu$ m thickness containing the rostral periventricular tissue as above for whole genome transcriptome analysis. For examination of LY-294002 induced genes by qPCR, pups were treated by intraventricular infusion (see below) at P9 and P10 and 180 mins following final infusion, tissue was microdissected. Five pups were used to pool for individual 'n' numbers and RNA was systematically amplified for all n numbers as previous (Azim et al., 2014b). For western blot (**S1 Fig A**), pups aged at P10 were treated with LY-294002 (see below) and 45 mins following injection, pups were systematically sacrificed by cervical dislocation and tissue microdissected and flash frozen in lysis buffer in liquid nitrogen for storage at  $-80^{\circ}\text{C}$  (Azim et al., 2014b). One litter of pups was pooled to yield 1 'n' number. For qPCR experiments, relative gene expression was determined using the  $2^{-\Delta\Delta\text{CT}}$  method versus the housekeeping gene GAPDH (Glyceraldehyde-3-phosphate dehydrogenase). See **S7 Table** for all of primers. List of primers used in the study. Primers were designed by Primer Express 1.5 software and synthesized by Eurofins (Ebersberg, Germany). Unstated primers in main text were custom designed and obtained from (Qiagen).

Protein was extracted with lysis buffer and standard procedures as previous (Azim et al., 2014a). 15 µg was loaded for SDS-PAGE gels and transferred to a PVDF membrane (GE Healthcare, Amersham). Blots were preincubated in a blocking solution of 5% BSA in 0.2% TBST (0.1 M Tris base, 0.1% Tween 20, pH 7.4) for 1 hr at RT, incubated with primary antibodies overnight at 4°C and after washing, with a horseradish peroxidase-conjugated anti-rabbit antibody (1:10,000-1:25,000; Pierce Biotechnology). Primary antibodies were all obtained from Cell Signaling and used in concentrations of 1:500 for phospho-forms and 1:2000 for total forms of protein. Protein bands were detected by adding SuperSignal West Pico Chemiluminescent Substrate (Pierce) by exposing the blot in a Stella detector (Raytest). Densitometry analysis was performed with NIH software and by normalizing the band intensities to total Akt or total Erk1/2 values. Intensity values for pAkt were combined and pAkt-473 shown only.

Gene expression and western blot data are presented as mean + standard deviation of the mean (SD) or standard error of the mean (SEM) respectively, and samples compared for significance using unpaired t-test (t-test) or (Prism v3.02 software; GraphPad).

**b. Part performed by Diane Angonin (except experiments involving LY-294002)**

### **In Vivo Procedures**

Animals were killed humanely by cervical dislocation and brains removed rapidly to ice cold fixative. Mouse pups of similar size were used throughout. Mice aged P8 were treated by intraventricular infusion into the LV daily for 3 days, and brains sampled at P11, overnight following the final injection. Mice were deeply anaesthetized under isoflurane and differing concentrations of LY-294002 (Sigma-Aldrich), dissolved in sterile DMSO, sterile filtered and co-administered with sterile saline delivered into the

cerebrospinal fluid (CSF) of the LV using a Hamilton syringe, at a point 2 mm from the midline along the Bregma, and to a depth of 2 mm. Sterile saline/DMSO vehicle were used as controls throughout this study. Concentration of small molecules infused into the CSF of the lateral ventricle were based on the known CSF availability of agents in accordance with applied in vitro as performed previously by the authors [57] and elsewhere for determination of CSF volume and turnover in the adult mouse [58]. For the analysis of the effect of GW8510, 4 intraventricular injections of 2 $\mu$ l were performed between P2 and P3 in OF1 mice following the same procedure. GW8510 was diluted to reach a concentration of 1,5mM in sterile PBS. The controls received a vehicle solution of sterile PBS with the corresponding volume of DMSO. Mice were terminated at P3. Methods applied for studying postnatal oligodendrogenesis are based on previous studies using C57/BL6 mice and transgenic mouse line in which fluorescent reporters DsRed are under control of the proteolipid protein (PLP) promoter (Azim et al., 2012b, 2014a). Methods applied for studying Wnt/ $\beta$ -catenin signaling are based on previous studies using BatGal mice expressing the b-galactosidase reporter under the control of TCF/LEF binding sites, thereby reflecting the activity of Wnt/ $\beta$ -catenin signaling (Maretto et al., 2003b).

Methods applied for testing GSK3 $\beta$  inhibitor in postnatal SVZ were performed using 4 intraperitoneal (IP) injections of AR-A014418 or CHIR99021 at different concentrations (0.5 and 5mM for AR-A014418 and 0.5 and 1.5 for CHIR99021) or vehicle (sterile PBS ; DMSO) from P4 to P5 before termination at P6.

Methods applied for studying rejuvenation of the adult SVZ were performed by infusing GSK3 $\beta$  inhibitors into the ventricular system of adult postnatal day 90 mice. Animals were anesthetized with a subcutaneous injection of Ketamin (60 mg/kg body weight), Xylazine (13 mg/kg body weight) and Acepromazine (1.5 mg/kg body weight) before being fixed in a stereotaxic apparatus. After exposure of the skull surface, a canula (Alzet, Brain infusion kit 3) was stably at the following coordinates (Bregma -0.5mm; lateral 1mm, depth: 2.5mm) for intraventricular infusion of the GSK3 $\beta$  inhibitors CHIR99021 and AR-A014418. Delivering of the small molecules was achieved over a period of 3 days using osmotic minipumps (1  $\mu$ l/h, model 1003D; Alzet Osmotic Pumps) into the CSF. Sham animals received all surgical steps, catheter implantation and pump insertion.

For studying SVZ NSCs recruitment following hypoxia and small molecule administration, dorsal SVZ NSCs were permanently labelled by dorsal electroporation (Boutin et al., 2008; Fernández et al., 2011) of a Cre plasmid (Cambridge, MA, www.addgene.org, plasmids 13775) in Cre-reporter mice (ROSA26-Flox-Stop-Flox YFP, Jackson Laboratories). A pCAGs-Cre plasmid under a chicken  $\beta$ -actin promoter was obtained from Addgene (Cambridge, MA, www.addgene.org, plasmids 13775). Mice aged P1 were electroporated, then placed in a hypoxic rearing chamber maintaining at 9.5-10,5% O<sub>2</sub> concentration by displacement with N<sub>2</sub> as described previously (Fagel et al., 2006). Hypoxia began at the postnatal day 3 (P3) for 8 days until P11. A separate group was maintained in a normal atmosphere (normoxic group). CHIR99021 was administered by intranasal administration as previously described [45]. Mucus was first permeabilised by the use of type IV hyaluronidase, then, 10  $\mu$ l of CHIR99021 (Sigma) were administrated 4 times (starting at the end of the hypoxic period, then every 12 hours), at the concentration of 1.5mM in sterile PBS (Vehicle was used as a control). Mice were terminated after 8 days after cessation of hypoxia at P19, or at P45. In order to study the activity of Wnt/ $\beta$ -catenin signaling, we used the BAT-GAL mouse line (Maretto et al., 2003a) which were subjected to hypoxia and/or intranasal administration of CHIR99021 following protocols already described. Mice were terminated at P11 before treatment or at P15.

## Immunohistochemistry

Standard immunofluorescence protocols were applied as previously described (Azim et al., 2014a). Mice were sacrificed by injection with an intraperitoneal overdose of pentobarbital (Eutha77 in Ringer's solution), followed by transcardial perfusion with Ringers solution 4% paraformaldehyde (PFA) dissolved in 0.1M phosphate buffered saline (PBS; pH 7.4). Following removal, brains were post-fixed in 4% PFA overnight at 4°C and cut in coronal sections at 30-50  $\mu$ m thickness for obtaining serial sections. Primary antibodies used were goat anti-Dcx goat (1:400 Santa Cruz); mouse anti-Olig1 (1:100, Abcam); mouse anti-Satb2 (1:200, Santa Cruz); rat anti-Ctip2 (1:500, abcam); rabbit anti-Cux1 (1:200, Santa Cruz); rabbit anti-Cas3A (1:1000, Millipore); anti-Mcm2 (1:400 Santa Cruz); chicken anti- $\beta$ -galactosidase (1:500, Abcam); Mouse anti-Ki67 (1:500, BD Pharmingen); rabbit anti-Ki67 (1:500 Thermoscientific); 1:300,

Millipore); rabbit anti-GFAP (1:300, DAKO); rabbit anti-Olig2 (1:400, Millipore); Chicken anti-GFP (1:1000; Abcam, AB13970); Mouse anti-NeuN (1:500, Millipore, MAB377); Rat anti-MBP (1:300, Millipore, AB40390); Rabbit anti-Tbr2 (1:500, Abcam), sheep anti-GSK3 $\beta$ -P (Ser9) (1:100, Santa Cruz). Appropriate secondary antibodies conjugated with Alexafluor 488, 568, 405 or 647 (1:400, Molecular Probes) were applied. Control experiments were performed using appropriate blocking peptides where available or otherwise by omission of the primary antibody. Fluorescent labelling of cells in S-phase by EdU (5-ethynyl-2'-deoxyuridine) detection was performed following manufacturers guidelines using Click-it EdU Alexa Fluor 555 imaging kit (Invitrogen). Tissues were mounted on poly-lysine coated glass slides with Vectashield mounting media (Vector Laboratories) and sealed with coverslips.

### **In vitro procedures**

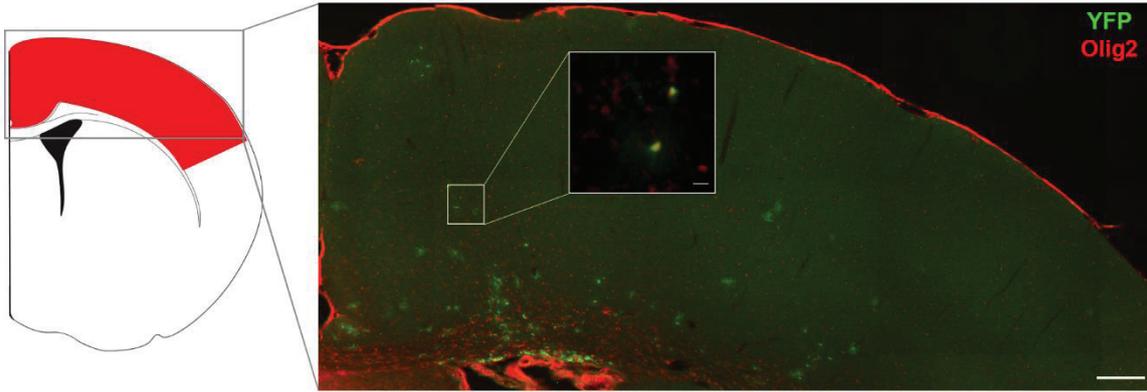
TNT229 cell line were cultured in DMEM (Gibco) with 10% fetal bovine serum (FBS, Life Technologies) and 1% Penicilline-Streptomycine. In order to test the effect of the Gsk3 $\beta$  inhibitors, cells were treated with CHIR99021, AR-A014418 or DMSO in a low-content FBS medium for 30 minutes. Cells were then fixated with PFA 4% 10 minutes at room temperature and eventually analyzed by immunohistochemistry.

### **Imaging and quantification procedures**

Imaging and analysis methods are described in detail in our previous methodological study (Azim et al., 2012a). All quantifications were performed using a homogenous sampling approach that has been optimized for 3-dimensional analysis of microdomains in the mouse SVZ [10], and provides an accurate quantification equivalent to exhaustive stereological methods from which it is adapted (Azim et al., 2012a). In brief, serial coronal sections were processed throughout the entire rostro-caudal extent of the SVZ (series of 6 sections for adult tissues and at least 3 for the postnatal SVZ). Quantification was performed on equivalent areas in each experimental group (Azim et al., 2014b). Images were captured using a Zeiss LSM

Meta 5.1, Zeiss LSM Meta 7.1 or Leica SPEII confocal microscope and processed with Zeiss LSM Image Examiner (V. 5.2.0.121) or LAS-AF software (V. 2.7), maintaining the acquisition parameters constant to allow comparison between samples. The number of cells expressing the markers Ki67, EDU, Olig2, Tbr2, Mcm2, Dcx, Ascl1, and GFAP were quantified with at least 3 fields of view per section on series of equally spaced sections of 40  $\mu\text{m}$  thickness encompassing the entire rostral lateral ventricle as previously described (Azim et al., 2012b, 2014b). The dorsal SVZ in both ages was defined based on DAPI counterstaining (Invitrogen). Quantifications were performed on confocal z-stacks of  $230 \mu\text{m}^2 \times 230 \mu\text{m}^2$  in the x-y-plane, and 30  $\mu\text{m}$  in the z-plane, with a volume of  $1.6 \times 10^6 \mu\text{m}^3$ . For the case of GFAP+ cells, i.e. NSCs, only cells directly adjacent to the ependymal layer were analyzed as previous for similar postnatal ages (Azim et al., 2014b). The myelin index, a means to measure postnatal myelination in the corpus callosum, was done in serial sections from PLP-DsRed mice (Azim et al., 2012b). the number of myelin sheaths crossing a diagonal transect was counted in each confocal z-section at 1, 5, 10, 15, 20, 25, and 30  $\mu\text{m}$  (captured using a 40 $\times$  objective), so that the myelin index (MI) represents the density of DsRed+ myelin sheaths within a volume of  $1.6 \times 10^6 \mu\text{m}^3$ .

Optical density of BGal staining in the Batgal mouse line was performed in the complete dorsal SVZ from epifluorescence microscope images, with Leica software. Values were normalized regarding to area and DAPI intensity using LAS-AF software. Quantifications of BGal+, Tbr2+, Ki67+, Mcm2+, and Olig2 at P11, P15, P19, P45 in the entire dSVZ were done from images taken with Leica SPEII confocal microscope, on 3 (for P11 and P15) or 4 sections (for P19, P45) per brain. Quantification of Dcx+ cells in the OB was performed using stereological analysis with Mercator software on 3 sections per brain. Estimation of the density of YFP+ cells originating from the dorsal electroporation of dsCRE plasmid in ROSA-YFP mice was performed by defining a volume of cortex reached by the electroporated cells (**Fig M1**). Statistical significances were tested using GraphPad Prism v302, for multiple variables, using or one-way analysis of variance (ANOVA) followed by Bonferroni's posthoc test, and for two variable using unpaired t-tests (referred to as t-test) where appropriate.



**Figure M1: Area of migration of dorsal subventricular zone cells progeny in the cortex.** On the left, drawing of a coronal section of a mouse brain. The red area represents the cortical area where YFP+ cells are located following the electroporation of a CRE plasmid in the dorsal subventricular zone of a ROSA-YFP mouse. On the right, an overview of a corresponding section shows the location of YFP+ cells in the cortex. Microphotograph showing two YFP+ cortical oligodendrocytes (YFP+/Olig2+). Scale bars: 100um in the overview; 20um in the microphotography.

# CHAPTER 1

## **Dorsal subventricular zone neural stem cells contribute to de novo cortical oligodendrogenesis and neurogenesis following chronic neonatal hypoxia.**

### **SUMMARY – Chapter 1**

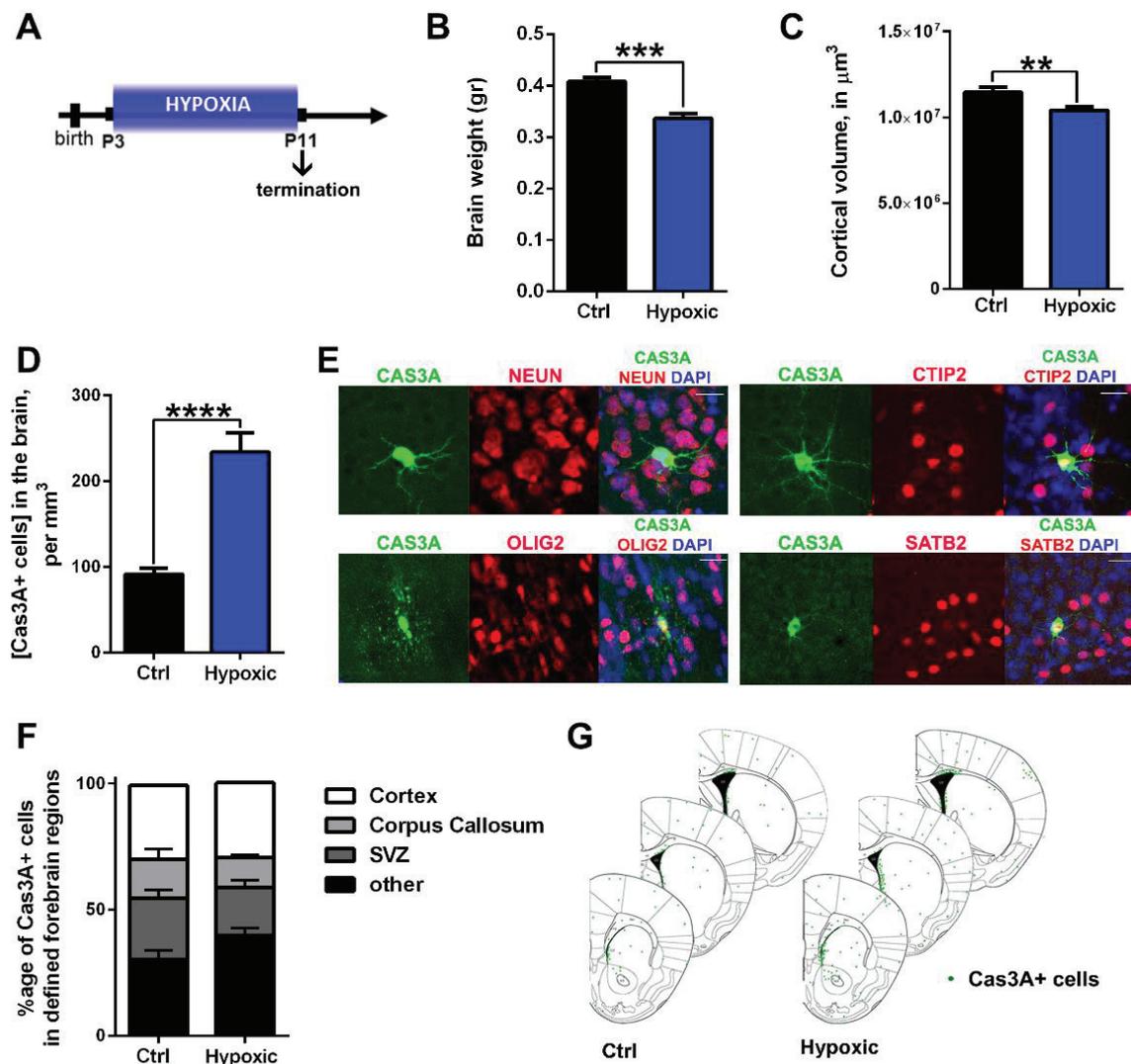
De novo cortical oligodendrogenesis and neurogenesis was described following neonatal hypoxia, but the source of these cells remains a matter of debate (Fagel et al., 2006). Here we aimed at investigating whether the neural stem cells (NSCs) and progenitors of the dorsal most regions of the subventricular zone (dSVZ) participate to this spontaneous regenerative attempt. In this first experimental chapter, we used an established model of chronic hypoxia (Scafidi et al., 2009), to characterize the immediate consequences of a neonatal period of hypoxia on cortical integrity. Our results confirmed that chronic neonatal hypoxia results in brain weight loss and decreased cortical volume, which were correlated to an increased apoptosis of both glial and neuronal cells. We next investigated the response of select SVZ progenitor population to the period of hypoxia. Our results highlighted a different response of progenitors of oligodendrocytes (Olig2+) and of glutamatergic neurons (Tbr2+). Indeed, while the first progenitor population increased following hypoxia, the second appeared to be halved by the end of the hypoxic period. These effects were however transient as the density of the two progenitor populations was back to normal by one week following the end of hypoxia. Interestingly, progenitors from both lineages increased their proliferation following hypoxia suggesting that they were recruited for cortical repair. Indeed, their fate mapping revealed their efficient migration towards the cortex where they generated new oligodendrocytes, and more remarkably new neurons.

Altogether, these data highlight a complex, lineage specific response of SVZ progenitors to neonatal hypoxia. Our results also demonstrated that progenitors of the dorsal SVZ contribute to de novo oligodendrogenesis and neurogenesis following neonatal hypoxia.

## RESULTS – Chapter 1

### a. Chronic hypoxia induces diffuse apoptosis of both glial and neuronal cells throughout the brain.

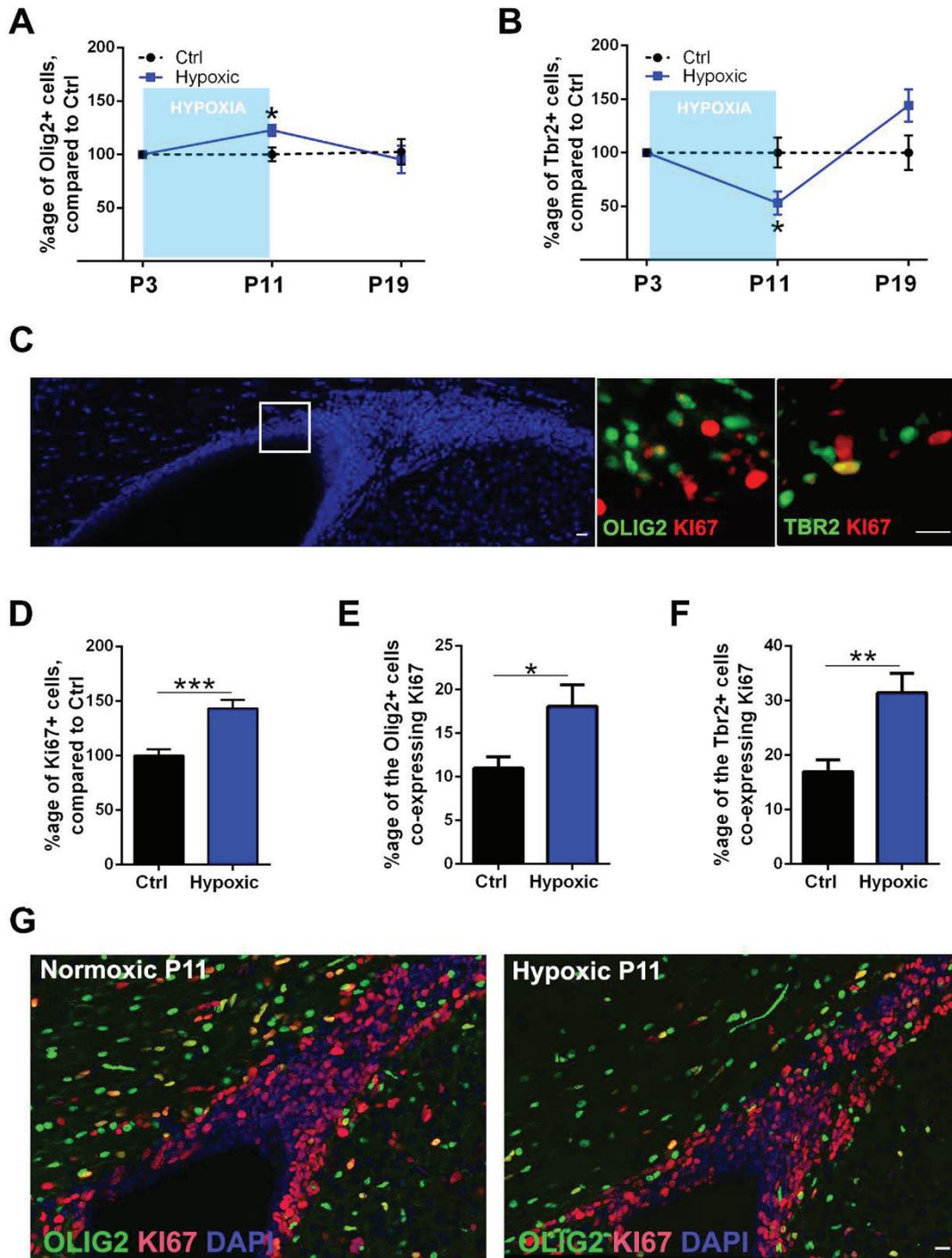
In order to study the contribution of the postnatal SVZ to tissue repair, we established a model of premature brain injury. We chose the model of chronic perinatal hypoxia, which has been described to lead to cell death without major inflammation or tissue disruption (Fagel et al., 2006; Ganat et al., 2002). Mice were exposed to a hypoxic environment containing 10% of O<sub>2</sub> from P3 to P11 (**Fig R1.1A**). To assess the consequences of the hypoxia period, brain weight as well as cortical volume were measured at P11. Brain weight was decreased by about 20% at the end of the hypoxic period (**Fig R1.1B**). Stereological estimates of the cortical volume confirmed this atrophy by revealing a significant loss of volume (**Fig R1.1C**). We next investigated if cell apoptosis could participate to this atrophy by quantifying the number of cells expressing the activated form of Caspase-3 (Cas3A) at P11. Quantification of Cas3A+ cells throughout the brain revealed a significant increase (~120%) of apoptosis in hypoxic (Hx) animals compared to the Normoxic (Ctrl) (**Fig R1.1D**). Analysis of their distribution failed to reveal a region specific enrichment, suggesting that apoptosis affects all cell types in all regions of the forebrain. Indeed, colocalisation of Cas3A with glial (Gfap and Olig2) and neuronal (NeuN) markers was observed as well as colocalisation of Cas3A with specific cortical glutamatergic markers Satb2 and Ctip2 (**Fig R1.1E**). Altogether, those results show that chronic hypoxia induces a diffuse cell death throughout the brain, including cortical glutamatergic neurons and oligodendrocytes.



**Figure R1.1: Chronic hypoxia induces increased diffused cell apoptosis in the forebrain including of cortical glutamatergic neurons and oligodendrocytes.** (A) Experimental design: OF1 mice were subjected to a hypoxic environment (10% O<sub>2</sub>) from P3 to P11 and terminated at the end of the hypoxic period. (B) Hypoxic (Hx) brains weight less than Normoxic brains (Ctrl). (C) Stereological analysis of cortical volume shows a significant decrease in Hx brains compared to Ctrl. (D) Stereological epifluorescence microscopy analysis revealed an increased density of Cas3A+ in Hx brains. (E) Cas3A+ cells co-express markers of oligodendrocytes (Olig2) and neurons (NeuN), as well as markers of subtypes of cortical glutamatergic neurons (Satb2, Ctip2). (F) Cas3A+ cell distribution remains unchanged following hypoxia, indicating that increased apoptosis occurs in all regions of the forebrain, including the cortex, corpus callosum and subventricular zone (SVZ). (G) Mapping of the Cas3A+ cells on 3 coronal sections of Ctrl and Hx brains. Abbreviations: P3/P11: respectively 3 and 11 days after birth ; Ctrl: control ; SVZ : subventricular zone. Scale bars: 20 microns in (E). P-values: \*\*< 0.01 ; \*\*\*<0.001 ; \*\*\*\*<0.0001.

**b. Dorsal SVZ progenitors of distinct lineages are differentially affected by the hypoxic period**

In the dorsal most regions of the postnatal SVZ, populations of oligodendrocyte (Olig2+) and glutamatergic neuron progenitors (Tbr2+) persist, constituting potential cellular sources for cortical repair. We investigated whether those progenitor populations were equally affected by the hypoxic period. At P11, the end of the hypoxic period, the population of Olig2+ progenitors is increased by about 20% compared to the normoxic (Ctrl) animals in the dorsal SVZ (**Fig R1.2A, C**). In contrast, the population of glutamatergic neurons progenitors is severely reduced by the period of hypoxia as revealed by a 50% decrease of the number of Tbr2+ cells (**Fig R1.2B, C**). Thus, progenitors of distinct lineages are differentially affected by the hypoxic period. Interestingly, these differences were not observed anymore at P19. At this later timepoint, while the number of Olig2+ progenitors returns to baseline, the number of Tbr2+ progenitors showed a rebound to slightly exceed those of normoxic animals (**Figure R1.2A, B**). This dynamic response of defined progenitor populations to hypoxia was paralleled by changes in proliferation, as reflected by the increase of Ki67+ cells observed in the dorsal SVZ following hypoxia (**Fig R1.2D**). At P11, both Olig2+ and Tbr2+ cells increased their proliferation, as revealed by the higher percentage of cells expressing the proliferative marker Ki67 (**Fig R1.2E, F, G**). Together, these results indicate that chronic hypoxia induced a dynamic, complex and lineage specific response of SVZ germinal activity.

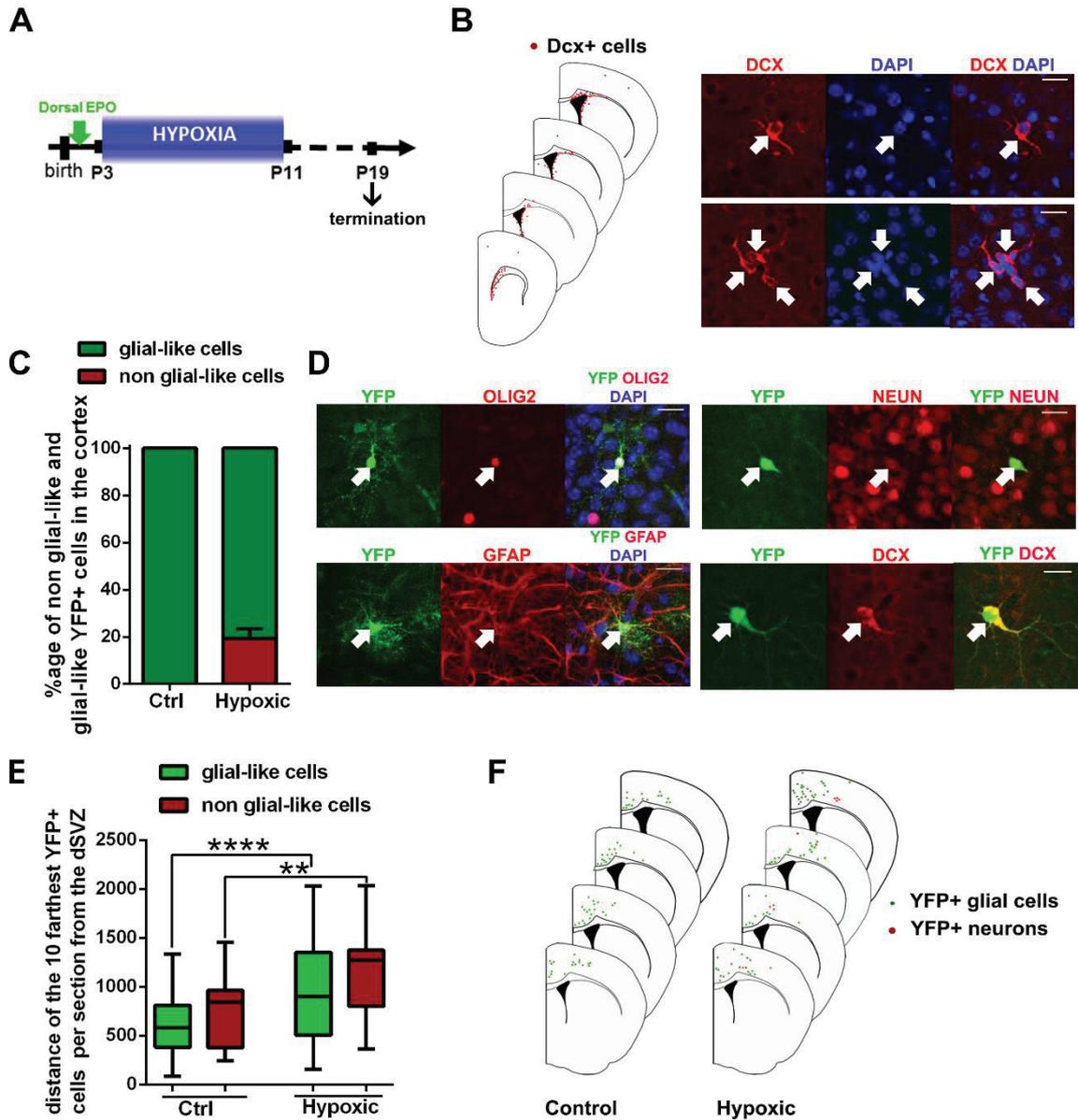


**Figure R1.2: Neonatal hypoxia differentially affects progenitors of distinct lineage in the dorsal SVZ (dSVZ).** (A-B) Quantifications of the changes of oligodendrocytes (olig2+) and glutamatergic neuron (Tbr2+) populations following hypoxia. Graphs show the changes in the two progenitors populations at the end of the hypoxic period (p11) and after 1 week of recovery (p19). (C) Overview of the dorsal SVZ with Dapi counterstaining. The box indicates the approximate location of inserts. Photomicrographs illustrate progenitors (Tbr2 and Olig2, green) and proliferation stainings (Ki67, red). (D) Quantification shows the changes in proliferation (Ki67+ cells) at the end of the hypoxic period. (E-F) Quantifications show the proportion of proliferative glutamatergic neuron progenitors (Ki67+/Tbr2+ within the Tbr2+ population) and proportion of proliferative oligodendrocyte progenitors (Ki67+/Olig2+ within the Olig2+ population). (G) Overview illustrates Olig2, Ki67 and Dapi staining in the dSVZ. Abbreviations: P3/P11/P19: respectively 3, 11 and 19 days after birth; Ctrl: control Scale bars: 20 microns in (C) and (G), p-values: \* < 0.05; \*\* < 0.01; \*\*\* < 0.001.

### c. The dorsal SVZ contributes to de novo cortical neurogenesis following chronic hypoxia

The dynamic response of dorsal SVZ (dSVZ) progenitors to hypoxia suggests their potential recruitment for cellular repair. Such recruitment was previously demonstrated for oligodendrocytes (Jablonska et al., 2012). Here we investigated if the dSVZ could also contribute to de novo neurogenesis following neonatal hypoxia. Supporting such de novo neurogenesis, immunodetection of the neuroblast marker Dcx revealed numerous migrating neuroblasts at P19 in the cortex of hypoxic animals, while they were almost absent in normoxic (Ctrl) animals (**Fig R1.3B**). In order to investigate whether the dorsal SVZ was the source of those newly generated neurons, a fate mapping experiment was performed. A CRE plasmid was electroporated in the dSVZ, 1 day after birth to permanently label dSVZ radial glial cells and their progeny by induction of the reporter fluorescent protein YFP. Cortex of hypoxic and normoxic mice were examined 8 days following the hypoxic period, at P19. At this timepoint, the YFP cytoplasmic expression allows to reliably distinguish the glial from the neuronal progeny (**Fig R1.3D**). Interestingly, a population of YFP+ cells with a neuronal morphology was identified specifically in the hypoxic brains (**Fig R1.3C, D**). The neuronal identity of those YFP+ cells was confirmed by immunodetection of Dcx and of the pan neuronal marker NeuN. On another hand, the “glial-like” YFP+ cells expressed either the oligodendroglial marker Olig2 or the astrocytic marker Gfap (**Fig R1.3D**). YFP+ neurons induced by hypoxia were not localized in specific areas or layers of the cortex but seemed to randomly distribute throughout the most dorsal cortical region (**Fig R1.3F**). Moreover, YFP+ cells from both glial and neuronal lineage migrate further from the dSVZ in the hypoxic condition, in the cortex and corpus callosum (**Fig 1.3E**). This suggests that chronic hypoxia influences cellular microenvironment to increase its permissiveness to cell migration. The contribution of the lateral microdomain of the SVZ to post-hypoxia cortical was investigated at P19, following lateral EPO at P1 and hypoxia. Immunostaining of the YFP+ cells revealed no YFP+ cells in the cortex (**Fig SR1**). This suggests that the contribution of the SVZ to post-hypoxia cortical repair might be exclusively originating from the dorsal microdomain of the SVZ.

Together, our results show that the postnatal dorsal SVZ progenitors are recruited after chronic hypoxia. In addition to the previously described de novo oligodendrogenesis observed following hypoxia (Jablonska et al., 2012) our results also confirmed that de novo cortical neurogenesis occurs and reveal that at least some of these neurons originate from the dorsal SVZ.



**Figure R1.3: Chronic hypoxia reactivates cortical neurogenesis.** (A) Experimental design: A CRE plasmid was electroporated in the dorsal microdomain of the SVZ of ROSA YFP mice (dorsal EPO) at P1 before the hypoxic period (10% O<sub>2</sub> from P3 to P11). Mice were terminated 8 days after the end of the hypoxic period, at P19. (B) Mapping of the Dcx+ cells in the cortex of hypoxic animals at P19. (C) Proportion of glial like and non-glial-like YFP+ cells in the cortex of ctrl and hypoxic animals at P19. (D) Representative pictures illustrating the expression of Olig2 or Gfap markers by glial-like YFP+ cells, and of the Neun and Dcx markers by neuronal-like YFP+ cells, confirming the glial vs neuronal nature of the two YFP+ cells subgroups. (E) Graph shows the repartition of the distance of migration of the 10 farthest YFP+ cells from the dorsal SVZ in both ctrl and hypoxic conditions. (F) Mapping of the YFP+ neuronal and glial cells in the cortex. Scale bars: 20 microns in B and D. Abbreviations: %age: percentage, EPO: electroporation, P3/P11/P19: respectively 3, 11 and 19 days after birth; Ctrl: control; P-values: \*\* < 0.01 ; \*\*\*\*<0.0001.

## DISCUSSION – Chapter 1

The activity of the subventricular zone (SVZ) decreases over time from embryonic stages to adulthood. However, the competency of the NSCs and progenitors it contains to participate to tissue repair remains under debate. Here we used a model of premature brain injury, the chronic hypoxia model to study the competency of specific populations of the SVZ to repair the cortex. Although a study carried out by Diaz showed that specific apoptosis of cortical glutamatergic neurons failed to induce an endogenous response triggering cellular repair (Diaz et al., 2013), other studies suggested a role of the SVZ following different cortical models of injury. For instance, a few studies suggested that cortical neurons originating from the SVZ could participate to cortical repair after stroke (Jiang et al., 2001; Jin et al., 2003; Zhang et al., 2006). Furthermore, a study carried out by Saha showed a recruitment of SVZ cells toward the injury site after motor cortex aspiration, differentiating into glia but also generating a few neurons (Saha et al., 2013). However, chronic hypoxia model is particularly interesting as the injury it implicates is mild and happens during a particular time window. Indeed, during the two first postnatal weeks a high proliferative activity remains in the SVZ. Furthermore, cortical oligodendrocyte production and maturation peaks during the very first postnatal weeks (Levine et al., 2001). After this period, progenitor populations decline in parallel of the germinal activity of the SVZ. The population of glutamatergic neuron progenitors declines particularly fast, and is almost extinguished by 2 months after birth (cf Chapter 2). These data suggest altogether a window of opportunity for production and maturation of SVZ derived neurons and oligodendrocytes. Hence, chronic hypoxia model provides interesting information on how changes in the microenvironment influence the fate of NSC as well as the maturation of both cell types.

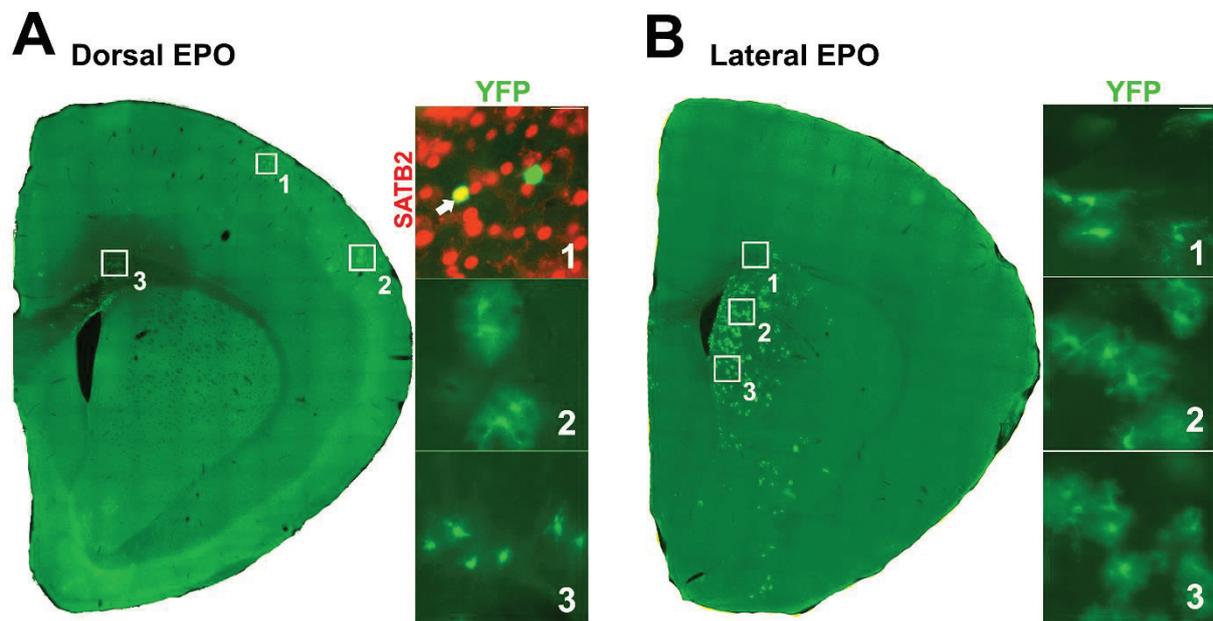
Our results confirmed that chronic hypoxia induces apoptosis in the brain resulting in a global loss of weight and decreased cortical volume, in accordance with other studies (Fagel and 2013, 2013; Fagel et al., 2006, 2009; Schwartz et al.). This decreased cortical volume may be explained by a decrease in both populations of GABAergic and glutamatergic neurons (Fagel et al., 2009), as well as of oligodendrocytes (Jablonska et al., 2012). The hypoxic period is followed by a regenerative attempt from the brain resulting in a recuperation in term of cell number by one week following the end of the

hypoxic period (Fagel et al., 2006). Reactivation of the “stem cell” properties of astrocyte-like (Gfap+) cells located in the cortical parenchyma was shown, with their ability to produce glutamatergic cortical neurons after hypoxia (Bi et al., 2011b). However, it is likely that the source of newborn neurons is not unique.

We have shown previously that a population of glutamatergic neuron progenitors as well as oligodendrocyte progenitors persist in a specific region of the SVZ, the dorsal microdomain of the SVZ (dSVZ). The role of the SVZ on post-hypoxia oligodendrocyte repair in the cortex was already suggested (Jablonska et al., 2012). However, the SVZ being a heterogeneous region, a better understanding of the origin within the SVZ of the oligodendrocytes involved in post-hypoxia cortical repair and the signaling pathways underlying this mechanism is important from a translational point of view. My results indeed showed a specific contribution of the dorsal most region of the SVZ to post-hypoxia oligodendrogenesis and neurogenesis.

In this chapter, we confirmed the effect of SVZ NSCs and progenitors in post-hypoxia cortical repair with an increased migration capacity of their progeny. Moreover, we showed that the dorsal microdomain of the SVZ was involved by producing neurons migrating toward the cortex, postnatally, following hypoxia. The lateral microdomain of the SVZ, which has the highest endogenous activity postnatally, did not seem to be involved in post-hypoxia cortical repair. This means that dorsal SVZ NSCs and progenitors keep their competency to produce cortical neurons postnatally. Whether the microenvironment is still permissive and allows a correct specification of those cortical neurons will be investigated in chapter 3.

## Supplementary data - Chapter 1



**Figure RS1.1 : Neural stem cells from the lateral microdomain of the subventricular zone does not participate to post-hypoxia cortical repair.** (A) Overview of a coronal section of hypoxic brain at P19 (19 days after birth). A CRE plasmid was electroporated in the dorsal microdomain of the subventricular zone (SVZ) of P1 Cre-reporter mice. Microphotographs show the morphology of YFP+ cells in the cortex corresponding to the boxed areas on the overview. Insert 1 shows YFP+ cortical neurons, one of which co-expresses *Satb2* (shown with a white arrow). Inserts 2 and 3 show YFP+ cells with glial morphologies. (B) Overview of a coronal section of hypoxic brain at P19, following a CRE electroporation in the lateral SVZ at P1. Microphotographs show the morphology of YFP+ cells in the cortex corresponding to the boxed areas on the overview. Inserts 1, 2 and 3 show YFP+ cells with typical glial morphologies. Scale bars: 20 microns in A and B. Abbreviation: EPO: electroporation.

## CHAPTER 2

### Pharmacogenomic identification of small molecules for lineage specific manipulation of subventricular zone germinal activity

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## SUMMARY – Chapter 2

The aim of this chapter is to identify small molecule(s), which have the ability to influence the fate of subventricular zone (SVZ) neural stem cells in a lineage specific manner.

First, a pharmacogenomic approach allowed to highlight fate-specific transcriptional signatures and to identify drug-like compounds with the capacity to induce those fate-specific transcriptional signatures. Transcriptional signatures of define SVZ microdomains or lineages at different postnatal timepoints were obtained from previously generated datasets. Indeed, the SVZ is divided into different microdomains notably according to the fate of the stem cells it contains (Chaker et al., 2016; Merkle et al., 2007). For instance, the dorsal microdomain of the SVZ is of particular interest, as it contains oligodendrocyte and glutamatergic neuron progenitors, two cell types particularly affected by neonatal hypoxia.

Two drugs that came out from this analysis were the pro-oligodendroglial LY-294002, and the dorsalizing and rejuvenating Gsk3 $\beta$  inhibitor AR-A014418. The effect of the different drugs were confirmed *in vivo* in different contexts. LY-294002 was confirmed to have pro-oligodendroglial effects following intraventricular infusion. AR-A014418 showed “dorsalizing” effects, as it induced both oligodendroglial and glutamatergic neuron lineages when administrated during the first week postnatal as well as in the adult brain. The effect of AR-A014418, a second generation Gsk3 $\beta$  inhibitor, was compared to the effect of the third generation Gsk3 $\beta$  inhibitor CHIR99021, which appeared to be more efficient. Eventually, intranasal administration of CHIR99021 following the hypoxic period in the context of chronic hypoxia promoted regenerative cortical neurogenesis and oligodendrogenesis.

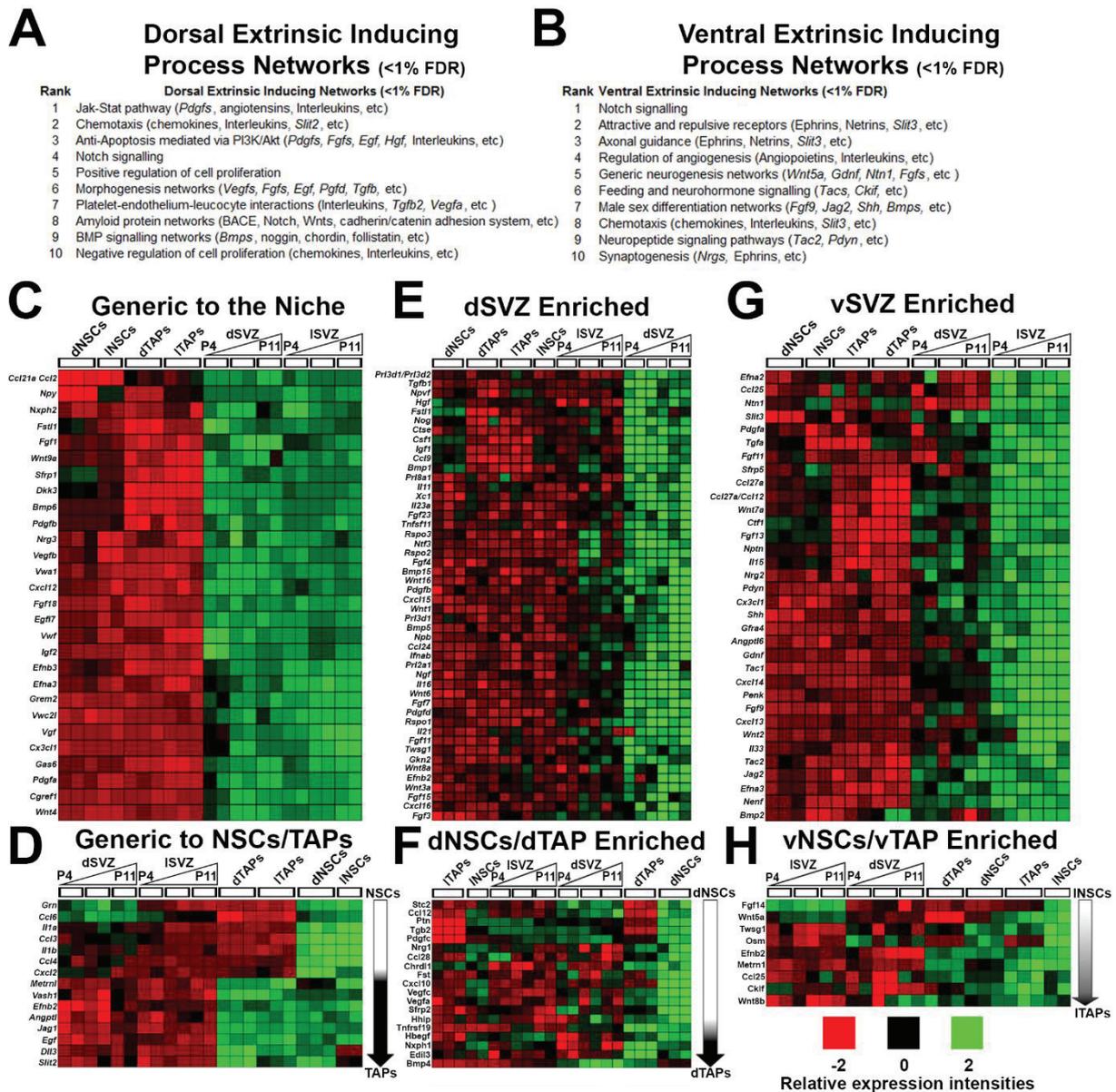
Altogether, those data validated the bioinformatics approach used to identify drug-like compounds for lineage specific manipulation of SVZ neural stem cells, in health as well as in regenerative contexts diseases.

## RESULTS – Chapter 2

### a. Identification of divergent signaling pathways in SVZ microdomains

The SVZ contains NSCs and their progeny, the transient amplifying progenitors (TAPs), which generate both NPs and OPs. The SVZ can be subdivided into discrete spatial microdomains (or niches) from which distinct neural lineages originate. While subtypes of GABAergic interneurons originate from all SVZ regions, the dorsal SVZ additionally gives rise to glutamatergic NPs and is the primary source of forebrain OPs (Reviewed in (Azim et al., 2016; Fiorelli et al., 2015; Weinandy et al., 2011)). To identify the molecular hallmarks that determine cell fate within these microdomains, we previously generated whole transcriptome datasets of NSCs, TAPs and their respective SVZ niches at postnatal day (P)4, P8 and P11 (Azim et al., 2015), which correspond to the postnatal period of greatest germinal activity and lineage diversity (Batista-Brito et al., 2008; Fiorelli et al., 2015). Here, we interrogated these datasets to identify signaling and metabolic processes that are unique to NSCs and TAPs and their respective SVZ niches. Transcripts enriched in dorsal vs. lateral datasets were compared using GeneGO Metacore for Process Networks and the function of individual genes were classified using <http://www.genecards.org>. The top 10 Metacore categories in each microdomain were ranked (**Fig R2.1A, B**), and only 2 categories overlapped, namely ‘Chemotaxis’ and ‘Notch signaling’, stressing the importance of these pathways within the neurogenic niche, as well as highlighting the existence of discrete signaling processes that are specific to the dorsal and lateral SVZ microdomains (**Fig R2.1A, B**). Among enriched transcripts generic to the SVZ niche, those coding for secreted signaling factors (grouped here for simplicity as ‘morphogens’) were prominent in both SVZ microdomains (**Fig R2.1C**); many of these were enriched in NSCs/TAPs (**Fig R2.1D**). Examination of the dorsal and lateral SVZ revealed a large number of genes differentially enriched within the two microdomains (**Fig R2.1E-H**). Notably, the dorsal SVZ contained the greatest number of genes that were uniquely expressed (**Fig R2.1E**), in line with the greater diversity of lineages originating from this microdomain. In particular, the Wnt ligands were specific to the dorsal SVZ, whilst Shh was specific to the lateral SVZ, in line with evidence that these signaling pathways have key roles in dorsalization and ventralization of the SVZ,

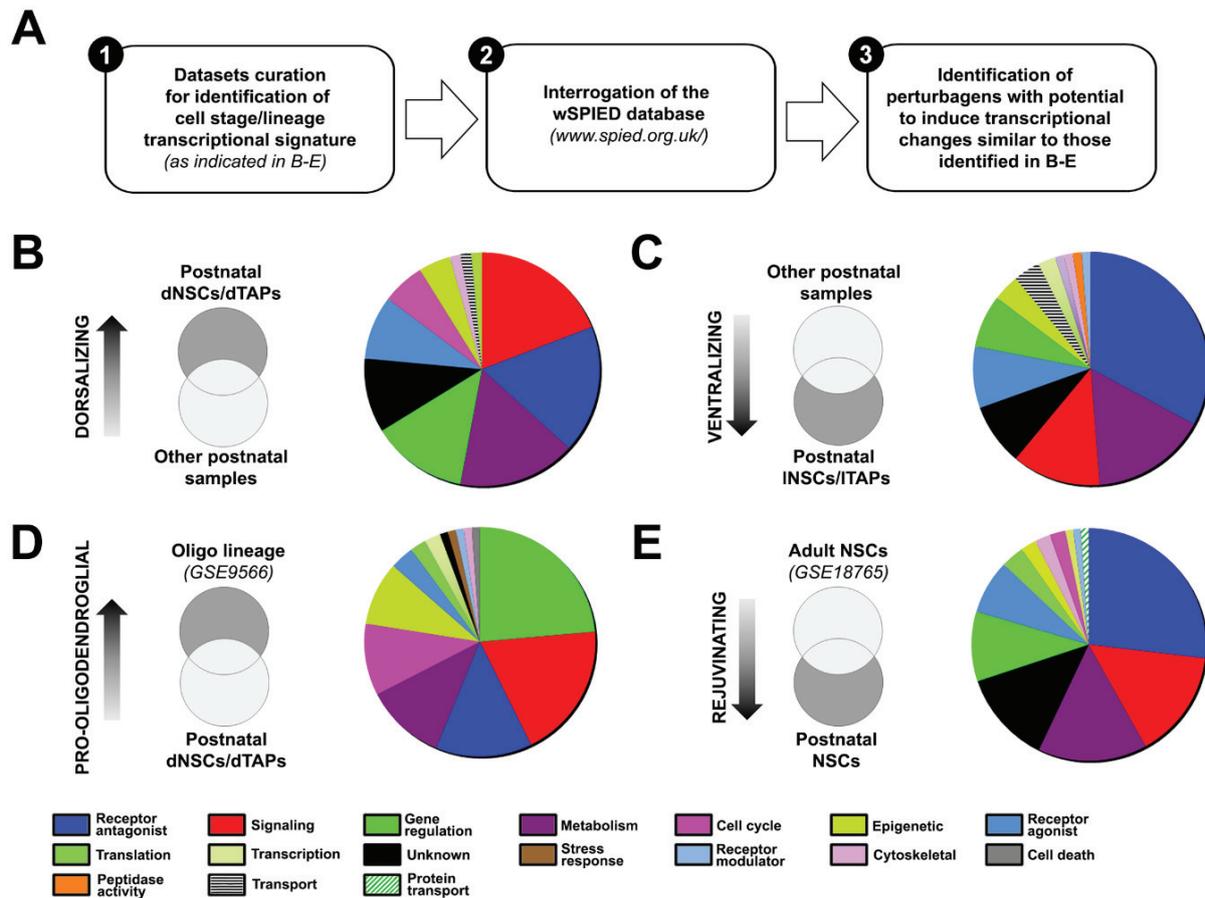
respectively (Azim et al., 2014a; Ihrle et al., 2011). In addition, several members of the TGF $\beta$ /Bmp family and their pathway inhibitor Noggin were enriched in the dorsal SVZ (**Fig R2.1E**), indicating they may have a specific role in driving dorsal SVZ fates. The lateral SVZ was enriched in the proneural determinants Bmp2 and Tgfa (Colak et al., 2008), as well as an abundance of chemokines and secreted molecules with undefined roles in neurogenesis (**Fig R2.1G, H**). A number of *Fgf* ligands were specific to the dorsal or lateral SVZ, indicative of functional divergence of FGF signaling within the microdomains (**Fig R2.1E, G**). Together, these results highlight major regionalization of signaling pathways within the SVZ, supporting the possibility they could be directly targeted to instruct lineage commitment of NSCs.



**Figure R2.1 : Spatial expression profiles of secreted signaling factors in SVZ microdomains.** (A-B) Significantly filtered genes in each region-derived samples were analysed on GeneGo Metacore for process networks and ranked numerically according to their FDR significance (1%<). NB: process networks categories were shortened to fit. (C-D) Heatmaps of genes generic and stable within microdomains across the varied time points are plotted, including those common in isolated NSCs/TAPs (D). (E-G) Heatmaps of genes enriched in region specific microdomains (generally stable temporal expression) versus the adjacent microdomain and NSCs/TAPs. The same was performed for region specific NSCs/TAPs (F, H). Note: the overlapping expression profiles for region specific NSCs with TAPs. dNSCs = dorsal NSCs; dTAPs = dorsal TAPs; dSVZ = dorsal SVZ; INSCs = lateral NSCs; ITAPs = lateral TAPs; ISVZ = lateral SVZ.

## **b. SPIED/CMAP identification of small molecules for manipulating SVZ regionalization and NSC fate**

We applied a novel pharmacogenomics approach to probe SVZ regionalization. To this end, a meta-analysis was performed to identify relationships between the transcriptional signatures of SVZ niches and/or lineages to those induced by exposure to small bioactive molecules in different contexts. This “connectivity map” approach allows the identification of small bioactive molecules capable of inducing transcriptional changes similar to those observed in the queried samples, and therefore to potentially manipulate cell fate in the SVZ (**Fig R2.2A**) (Lamb et al., 2006; Stegmaier et al., 2004; Williams, 2012). Small molecules were ranked according to the number of genes that are altered, referred to as ‘target genes’ (**See Table 1A-D**), and the protein targets of each small molecule were classified according to GO terms (**Fig R2.2B-E**) (see Materials and Methods for further details).



**Figure R2.2 : CMAP identification of perturbagens that promote region specific SVZ signatures, oligodendrogenesis and reactivation of adult NSCs.** (A) Schematic representation of the experimental flow. Microarray datasets were compared for obtaining expression signatures (detailed in Materials and Methods) and uploaded onto SPIED to interrogate the CMAP obtaining a list of perturbagens. These were further inspected for their known protein targets, categorized and presented in Figure as Pies (ordered clock wise following their ranking order). Note that small molecules related to distinct top ranked categories are observed in the different analysis. Genes enriched in: (B) dNSCs/dTAPs compared to other early postnatal datasets (i.e. dorsalization); (C) INSCs/ITAPs compared to other early postnatal datasets (i.e. ventralization); (D) OL lineage cells compared to dNSCs and dTAPs; (E) Postnatal NSCs compared to adult NSCs. dNSCs = dorsal NSCs; dTAPs = dorsal TAPs; INSCs = lateral NSCs; ITAPs = lateral TAPs.

**Table 1:** Top ranked small molecules identified from SPIED/CMAP analysis of small molecules that promote (A) dorsalization of the SVZ, (B) ventralization of the SVZ, (C) oligodendrogenesis, and (D) neurogenesis. Small molecules are ranked according to the largest numbers of 'target' or 'perturbed' genes. For the full list please refer to corresponding S1-S4 Tables.

<b>(A) DORSALIZATION</b>		<b>(B) VENTRALIZATION</b>	
<b>Small molecules</b>	<b>Number of target genes</b>	<b>Small molecules</b>	<b>Numbers of target genes</b>
<b>GW-8510</b>	1367	<b>verteporfin</b>	735
<b>ciclopirox</b>	475	<b>cephaeline</b>	598
<b>AR-A014418</b>	456	<b>terfenadine</b>	452
<b>oxprenolol</b>	366	<b>prenylamine</b>	420
<b>streptomycin</b>	357	<b>3-</b>	396
<b>cinchonine</b>	353	<b>abamectin</b>	361
<b>alfaxalone</b>	323	<b>rescinnamine</b>	354
<b>caffeic_acid</b>	323	<b>etanidazole</b>	336
<b>oxytetracycline</b>	323	<b>dicycloverine</b>	284
<b>azaperone</b>	301	<b>5707885</b>	263

<b>(C) OLIGODENDROGENESIS</b>		<b>(D) REJUVENATION</b>	
<b>Small molecules</b>	<b>Number of target genes</b>	<b>Small molecules</b>	<b>Numbers of target genes</b>
<b>trichostatin_A</b>	3292	<b>adiphenine</b>	1227
<b>tanespimycin</b>	2732	<b>helveticoside</b>	1157
<b>LY-294002</b>	<b>2296</b>	<b>thapsigargin</b>	1070
<b>vorinostat</b>	2247	<b>AR-A014418</b>	<b>998</b>
<b>GW-8510</b>	2093	<b>anisomycin</b>	989
<b>camptothecin</b>	1927	<b>lanatoside_C</b>	865
<b>phenoxybenzami</b>	1871	<b>viomycin</b>	776
<b>H-7</b>	1803	<b>monensin</b>	762
<b>sirolimus</b>	1783	<b>nadolol</b>	761
<b>irinotecan</b>	1752	<b>podophyllotoxin</b>	757

Small molecules that may drive NSC/TAP dorsalization or ventralization, were identified by comparing the transcriptome of dorsal and lateral NSCs/TAPs (**S1-S2 Tables**), as summarized in **Tables 1A and B**. Significantly, AR-A014418, which we have previously demonstrated dorsalizes the SVZ (Azim et al., 2014b), was identified in the top 10 dorsalizing perturbagens (**Table 1A**). Conversely, 3-nitropropionic acid ranked highly in the ventralizing screen (**Table 1B**) is an activator of GSK3 $\beta$  (Crespo-Biel et al., 2010), which we have shown represses SVZ dorsalization (Azim et al., 2014a). Drugs targeting the ventralizing Shh signaling pathway (Ihrie et al., 2011) were also identified, such as tolinaftate that has been described as inhibiting Shh signaling (Lipinski and Bushman, 2010). Altogether, these findings help validate our approach (see below). Among other small molecules, the most prominent category was ‘Receptor antagonists’ (**Fig R2.2B, C**), consistent with neurotransmitters being major regulators of neurogenesis in the SVZ (Berg et al., 2013). Notably, ventralizing perturbagens targeting muscarinic acetylcholine (mACh) receptors were highly ranked, e.g. terfenadine (**Table 1B**), suggesting mACh receptors are important determinants of interneuron specification in the lateral SVZ (Samarasinghe et al., 2014). The two other prominent categories of small molecules were associated with ‘Signaling’ and ‘Metabolism’ (**Fig R2.2B, C**), which included the most highly ranked dorsalizing perturbagen GW-8510, a potent Cdk inhibitor (**Table 1A**), and ciclopirox, an inhibitor of prolyl-4-hydroxylase that promotes Notch signaling and NSC activation (Ma et al., 2013; Zheng et al., 2008). The most highly ranked ventralizing perturbagen was verteporfin (**Table 1B**), which alters downstream transcriptional activity of the hippo pathway to regulate cell cycle and neuronal differentiation (Zhang et al., 2012).

Small molecules that may promote oligodendrogenesis were identified by comparing the transcriptome of dorsal NSCs/TAPs (i.e. the main postnatal forebrain source of OLs) with publically available transcriptional datasets of oligodendroglial lineage cells (Cahoy et al., 2008). The results are detailed in **S3 Table** and summarised in **Table 1C**. Most small molecules were related to ‘Gene regulation’ and ‘Signaling’ (**Fig R2.2D**). Many from the latter category, including two of the top 10 ranked pro-oligodendrogenesis drugs LY-294002 and sirolimus, are inhibitors of PI3K/Akt/mTor signaling (**Table 1C**), which acts downstream of several ligands enriched in the dorsal SVZ (**Fig R2.1E**). Notably, many of the small molecules associated with oligodendrogenesis were within “Epigenetic” and “Cell cycle” categories, which barely

featured in the other SPIED analyses (**Fig 2B-E**). The highest ranking amongst these were trichostatin-A and vorinostat (**Table 1C**), which are potent inhibitors of histone deacetylases (HDACs) with broad epigenetic activities, consistent with evidence that HDACs repression is required during NSC differentiation into OPs (Castelo-Branco et al., 2014; Wu et al., 2012). Similarly, the high ranking of the Cdk inhibitors GW-8510 and camptothecin (**Table 1C**) highlights the importance of inhibition of Cdks in regulating cell cycle progression and differentiation of OPs from NSCs, in support of previous studies (Caillava et al., 2011).

Small molecules that may rejuvenate the adult SVZ were identified by comparing the transcriptome of postnatal NSCs (Azim et al., 2015) and adult NSCs (Beckervordersandforth et al., 2010) (**Fig R2.2E; S4 Table**). The aim of this approach was to identify key changes underlying the decline in the activity and loss of competence of NSCs that occurs in adulthood, which is attributed to the combined upregulation of inhibitory and downregulation of positive cues (Hamilton et al., 2013), and is critical for the response of NSC to brain injury and degeneration (Curtis et al., 2007; Kim and Szele, 2008). In this manner, key small molecules were identified for 'rejuvenating' adult NSC (**Fig R2.2E; Table 1D**), approximately 20% of which overlapped with those required in dorsalizing or ventralizing the postnatal SVZ, and were categorised as 'Receptor antagonist', 'Signaling' and 'Metabolism'. Some interesting candidates included antagonists for  $\alpha/\beta$  adrenergic receptors, including the highly ranked nadolol (**Table 1D**), which have been described to promote NSC activation from quiescence in the dentate gyrus (Jhaveri et al., 2014), and enhance NP survival following exit from the SVZ (Bauer et al., 2003). A key signaling pertubagen was monensin, which impedes TGF $\beta$  processing (Mitchell et al., 2004), a major neurogenesis inhibitory factor during ageing (Pineda et al., 2013). Significantly, the GSK3 $\beta$  inhibitor AR-A014418 was one of the top ranking 'rejuvenating' small molecules (see below), which we have shown promotes the genesis of glutamatergic NPs in the postnatal SVZ via the canonical Wnt signaling pathway (Azim et al., 2014a, 2014b).

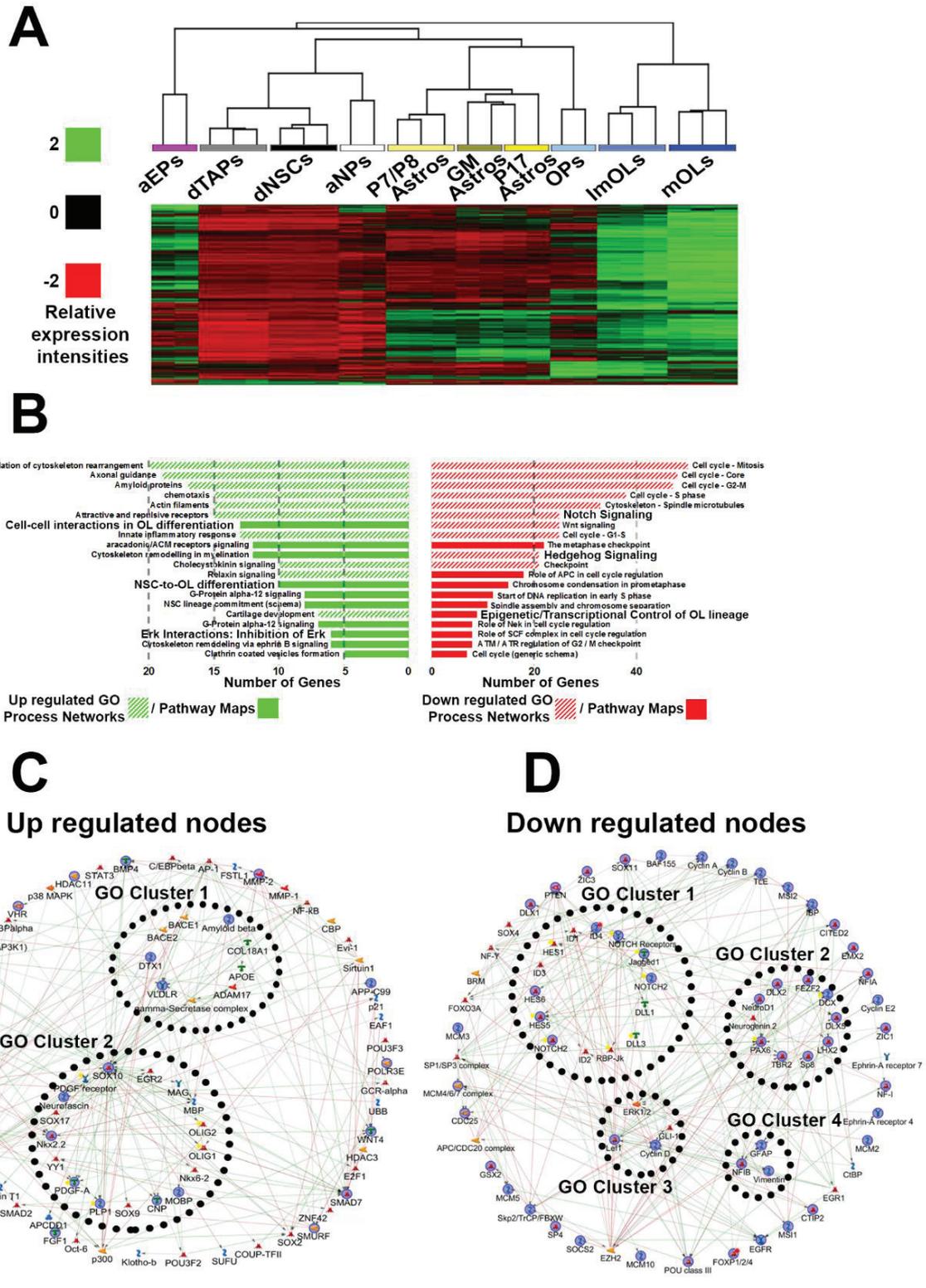
Based on these SPIED/CMAP analyses, LY-294002 and AR-A014418 were identified as promising agents that may specifically regulate oligodendrogenesis (**Table 1C**) or neurogenesis (**Table 1D**), respectively, and were selected for further analysis to

resolve signaling-to-transcriptional interactions by Genego Metacore network visualization and *in vivo* validation.

### **c. LY-294002 induces transcriptional changes that promote oligodendrogenesis**

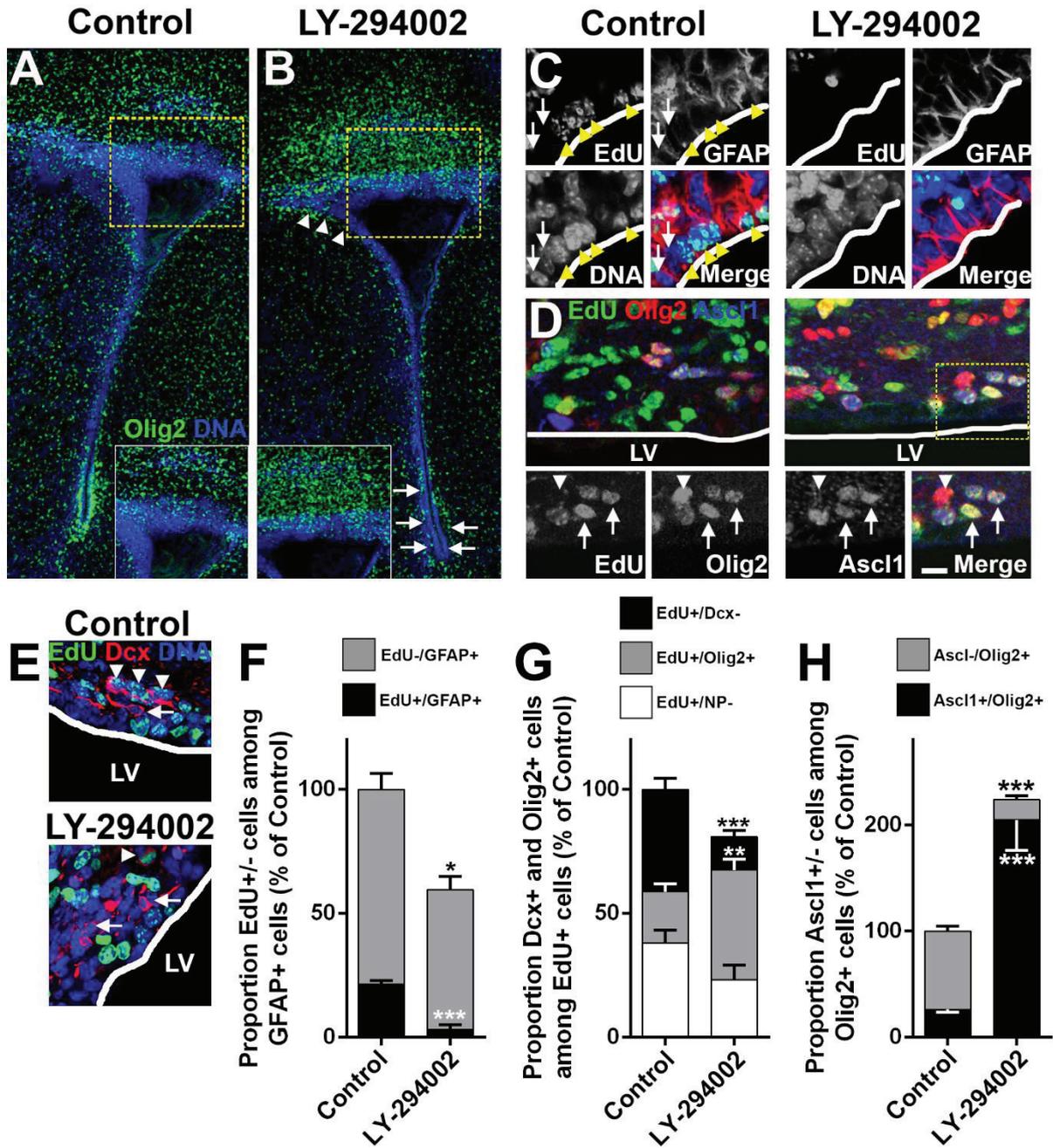
LY-294002 is a widely used and highly specific inhibitor of PI3K/Akt. Expression of the target genes of LY-294002 in SVZ cell types/lineages (**S5 Table**) was compared by hierarchical clustering, highlighting their association with late stage OLs compared to other cell lineages, including NSCs/TAPS of the dorsal SVZ (**Fig R2.3A**). Further target genes analysis provided additional information on the mode of action and predicted effects of LY-294002. Categorising target genes for GO Pathway Maps and Process Networks revealed up-regulation of genes associated with oligodendrogenesis and myelination and down-regulation of genes related to cell cycle behaviour and neurogenesis (**Fig R2.3B**). Finally, Genego Metacore network visualisation was applied to resolve signaling-to-transcriptional interactions (detailed in Materials and Methods). LY-294002 up-regulated transcriptional nodes were associated with oligodendrogenesis (**Fig R2.3C**), while down-regulated nodes included Notch signaling, proneuronal TFs and astroglial-related genes (**Fig R2.3D**). Altogether, LY-294002 appeared as a strong candidate for inducing specifically oligodendrogenesis in the postnatal SVZ. It was infused into the CSF of the lateral ventricle, commencing at P8 and the effects on the SVZ were determined at P11, by immunostaining (**Fig R2.4**), biochemical and qPCR analysis of its target genes (**Fig SR2.1A, B**), as described in our previous studies (Azim et al., 2014a, 2014b). Intraventricular infusion to achieve a CSF concentration of 3  $\mu$ M LY-294002 effectively inhibited Akt phosphorylation, the immediate target of PI3K, throughout the SVZ (**Fig RS2.1A**), and rapidly and specifically promoted oligodendrogenesis in the dorsal SVZ (**Fig R2.4A, B**). Quantification performed through the rostro-caudal axis of the lateral ventricle revealed a pronounced induction of the OL lineage marker Olig2, particularly in the most dorsal SVZ region (**Fig R2.4A, B**). NSCs were identified as GFAP immunopositive cells in direct contact with the lateral ventricle wall and their proliferative state was assessed using EdU (mice received a single i.p. injection of EdU

at P8). Compared to controls, GFAP immunoreactivity and the extent of GFAP+/EdU colocalisation were significantly reduced following LY-294002 infusion (**Fig R2.4C, F**), indicating a general loss of both proliferative and non-proliferative GFAP+ NSCs, consistent with evidence of their precocious differentiation into OPs. Immunolabelling for Dcx and Olig2 in combination with EdU to identify NPs and OPs, respectively, revealed different effects of LY-294002 on these two lineages. Notably, the numbers of Olig2+/EdU+ OPs were increased dramatically (**Fig R2.4D, G**) and a greater proportion of these cells expressed Ascl1 (**Fig R2.4D, H**), whereas Dcx+/EdU+ NPs were reduced, as was the overall number of Dcx+ cells in the dSVZ (**Fig R2.4E, G**). This indicates that LY-294002 promoted the early stages of oligodendrogenesis at the expense of neurogenesis. In addition, OL differentiation was also enhanced as revealed by a doubling of PLP-DsRed+ OLs and a subsequent 30% enhancement in myelination as revealed by myelin index measurements (**Fig RS2.1C**), by the end of the treatment.



**Figure R2.3 : CMAP pathway and network analysis for LY-294002 target genes.** Gene list generated to obtain the drug profiles for LY-294002 (**S5 Table**) was utilized to compile the CMAP “LY-294002 target-gene” list, and genes prospectively up regulation are presented as a heatmap in **A** showing enrichment in later OL lineage cells and downregulation in dorsal NSCs or TAPs. aEPs = adult ependymas; aNPs = adult NPs; GM = grey matter; astros = astrocytes; imOLs = immature OLs; mOLs = mature/myelinating OLs. (**B**) Prospectively up-regulated or down-regulated genes analysed by Genego Metacore for GO Pathway Maps and Process Networks,

and lists are ranked according to significance (FDR <2%)/numbers of genes present in each of the categories. **(C-D)** Short path network to visualize highly connected signaling-to-transcriptional nodes were performed for up and down regulated by LY-294002 target genes. Internal clusters were grouped by selecting the “link GO objects” in GeneGo Metacore. High lightened Blue objects are directly within data and remaining are within the background (or basal) data's.



**Figure R2.4 : LY-294002 promotes dorsal SVZ-oligodendrogenesis.** Pups were treated for 3 days with LY-294002 or saline/DMSO and examined for immunolabelling. (A-B) Periventricular sections show greater Olig2 immunostaining in LY-294002 in more dorsal periventricular regions compared to controls, illustrated in expanded insets. In the lateral SVZ, LY-294002 reduced Olig2 expression, as indicated by arrows (B). Arrowheads in B show reduced nuclei density in the dorsolateral horn of the SVZ where NPs migrate. Scale bar in A = 200  $\mu$ m. (C) arrowheads show a loss of EdU in GFAP+ cells facing directly the lateral wall in LY-294002, compared to controls. Arrows show examples GFAP+ cells that have not incorporated EdU that were increased following LY-294002. (D) Single plane confocal micrographs show greater EdU+Olig2 colocalization in LY-294002 and single panel captions of single planes illustrate most newly generated Olig2+ cells co-express EdU and Ascl1 (arrows) or have absent or lower levels of Ascl1 (arrowheads). Scale bar in D = 10  $\mu$ m in captions of D, 15  $\mu$ m in main panels of D, 15  $\mu$ m in C and 10  $\mu$ m in D. (E) Confocal micrographs illustrate fewer densities of Dcx+ cells in LY-294002 and loss of their proliferation (compare Dcx+ cells with

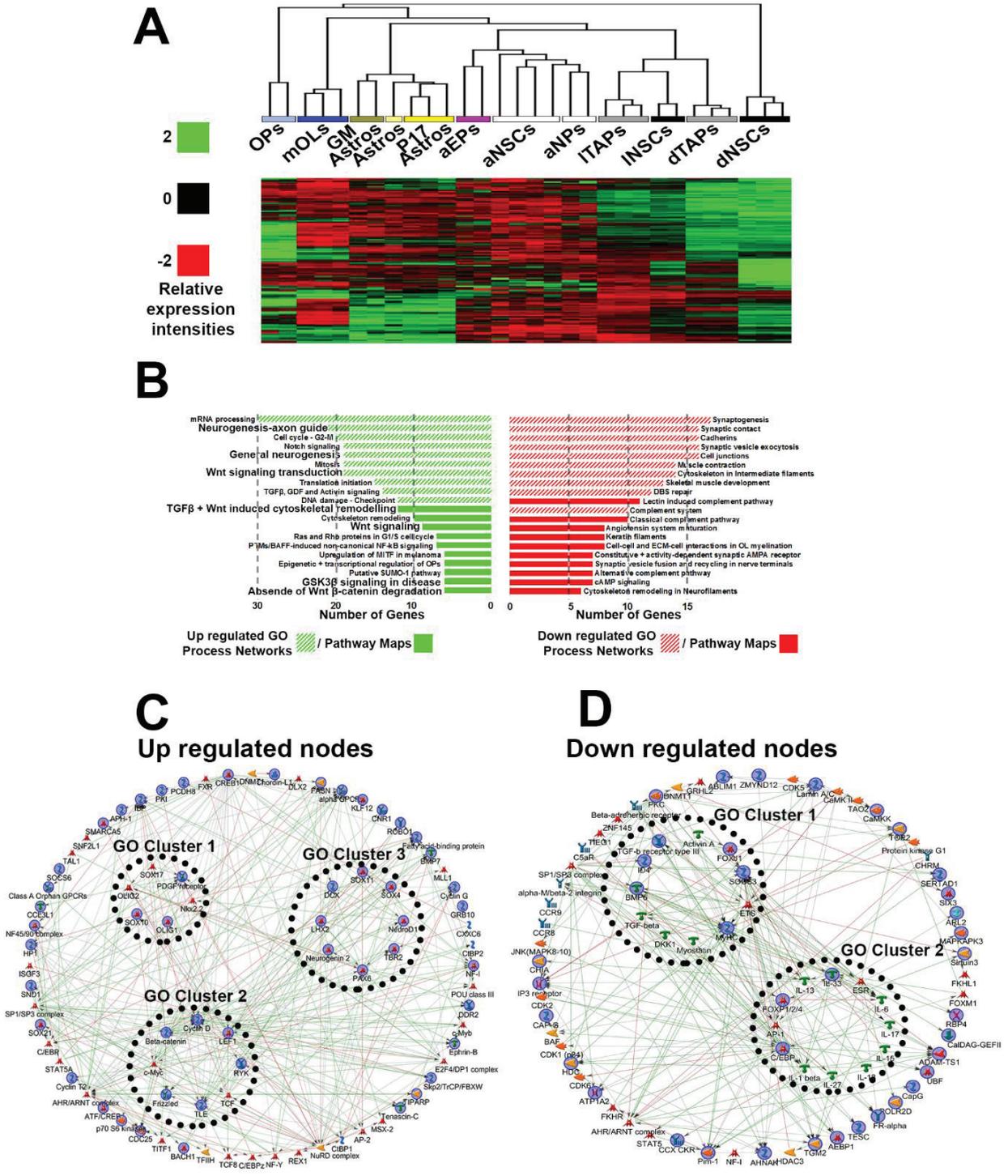
arrowheads to those marked by arrows). (F-H) Quantification of changes in GFAP+/EDU+ or EdU- cells directly facing the wall of the lateral ventricle (F). Quantification of changes in EdU+ cells expressing progenitor markers (Dcx, Olig2, or none of these markers (NP-)) (G). Quantification of changes in Olig2+/Ascl1+ or Ascl1- cells (H). Data are mean  $\pm$  SEM normalized to controls ((n=5 for control and LY-294004 for all quantifications); significance was tested using unpaired t-test throughout versus respective control; \*\*p<0.01; \*\*\*p<0.001.

Importantly, qPCR of the microdissected SVZ (**Fig RS2.1B**) confirmed LY-294002 acts via the target genes/nodes identified by the target gene analysis (**Fig R2.3**), and provided additional information on its modes of action. Analysis revealed *Fgf2* and *Igf1* were not increased (**Fig RS2.1B**), indicating they were not the mechanism of action of LY-294002. Conversely, LY-294002 significantly decreased Shh signaling, which promotes SVZ ventralization, together with Notch signaling (**Fig RS1B**), which stimulates NSCs self-renewal and is a major rate limiting determinant of OL differentiation (Basak et al., 2012; Wang et al., 1998). Overall, these analyses suggest that LY-294002-mediated PI3K/Akt inhibition promotes an environment permissive to oligodendrogenesis while inhibiting signaling pathways that promote neuronal cell fates.

#### **d. AR-A014418 induces transcriptional changes that promote rejuvenation of the adult SVZ**

AR-A014418 is a highly specific inhibitor of GSK3 $\beta$ , which the SPIED/CMAP analysis identified as having one of the highest number of target genes associated with rejuvenation (**Table 1D**), as well as being positively related to dorsalization of the SVZ (**Table 1A**) and negatively related to ventralization (**Table 1B**). Consistent with this, the target genes of AR-A014418 were enriched more prominently in dorsal NSCs/TAPs and OPCs, than other cell types (**Fig R2.5A**). Furthermore, pathway/network analysis of AR-A014418 target genes revealed an upregulation of multiple categories related to neurogenesis, oligodendrogenesis and Wnt signaling (**Fig R2.5B**), consistent with our recent evidence that AR-A014418 promotes generation of glutamatergic NPs and OPs from the postnatal dorsal SVZ via canonical Wnt signaling (Azim et al., 2014a, 2014b). Genego Metacore network visualisation identified up-regulated nodes mainly consisting of transcriptional regulators, notably

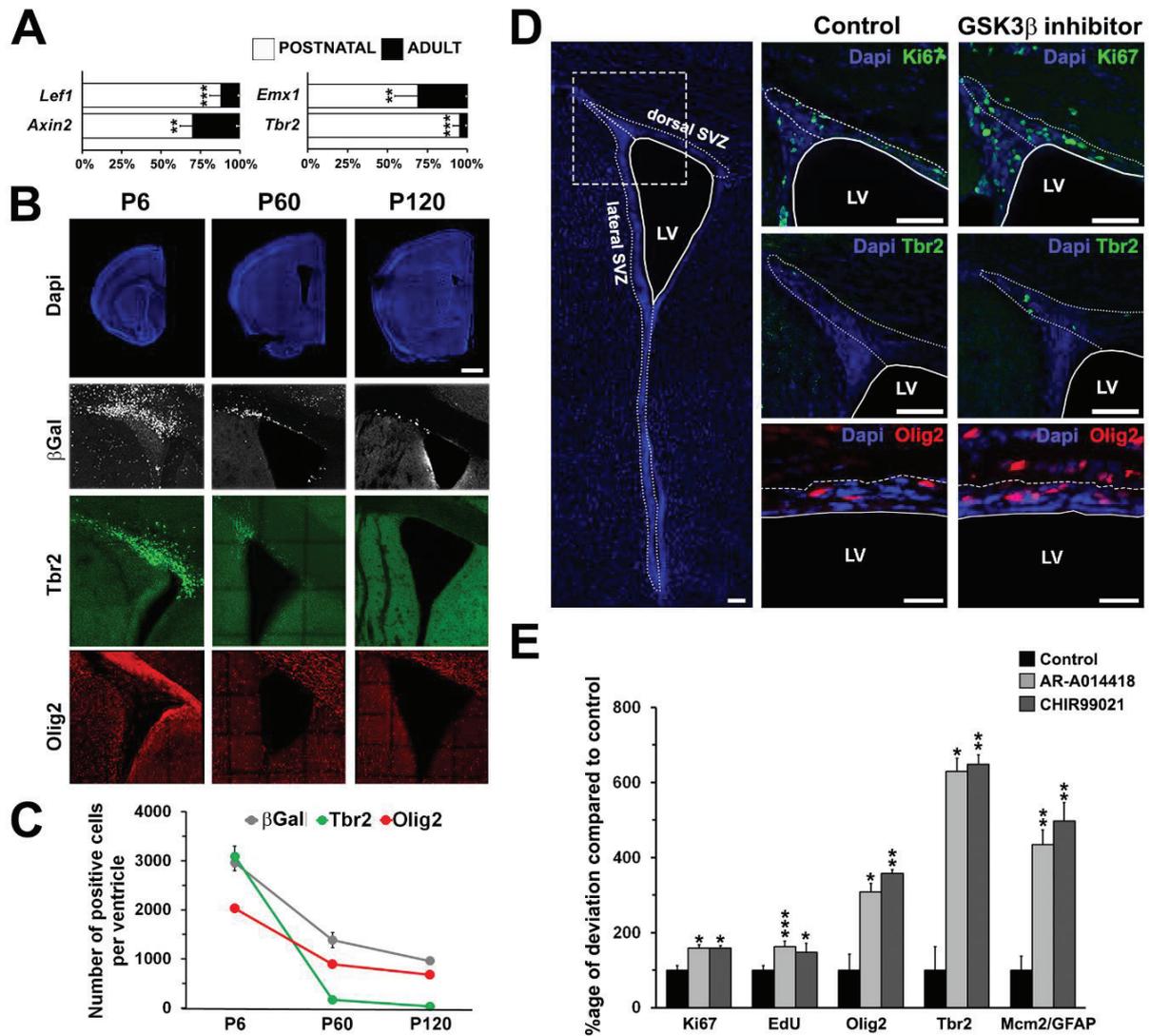
TFs associated with neurogenesis (**Fig R2.5C**; e.g. *Tbr2*, *NeuroD1*, *Pax6*) and oligodendrogenesis (**Fig R2.5C**; e.g. *Olig1/2*, *Sox10*). Down-regulated nodes included members of pro-inflammatory cytokines (**Fig R2.5D**), such as *IL-33* that likely inhibits neurogenesis (Gonzalez-Perez et al., 2010), as well as *Id4*, which is upregulated in adulthood and is a potent inhibitor of neurogenesis (Hirai et al., 2012). These analyses support AR-A014418 as a strong candidate for promoting lineages and signaling pathways that are characteristic of the early postnatal SVZ, whilst downregulating inhibitory factors associated with the decline in neurogenic capacity in the adult.



**Figure R2.5 : CMAP pathway and network analysis for ARA-014418 target genes.** Gene list generated to obtain the drug profiles for ARA-014418 (S6 Table) was utilized to compile the CMAP “ARA-014418” target-gene list, and genes prospectively up regulated are presented as a heatmap in **A** showing enrichment in earlier postnatal dorsal SVZ cells. aEPs = adult ependymas; aNPs = adult NPs; GM = grey matter; astros = astrocytes; mOLs = mature/myelinating OLs; aNSCs = adult NSCs; dNSCs = dorsal NSCs; dTAPs = dorsal TAPs; INSCs = lateral NSCs; ITAPs = lateral TAPs. **(B)** Prospectively up-regulated or down-regulated genes analysed by Genego Metacore for GO Pathway Maps and Process Networks, and lists are ranked according to significance (FDR <1%)/numbers of genes present in each of the

categories. **(C-D)** Short path network to visualize highly connected signaling-to-transcriptional nodes were performed for up and down regulated ARA-014418 target genes. Internal clusters were grouped by selecting the “link GO objects” in GeneGo Metacore. High lightened Blue objects are directly within data and remaining objects are within the background (or basal) data's.

An age-related decline in SVZ activity was confirmed by qPCR analysis of adult SVZ microdomains, which indicated a parallel decline in neurogenic potential and canonical Wnt/ $\beta$ -catenin signaling in the dorsal SVZ (**Fig RS2.2**). This was confirmed by an observed sharp decline in the expression of *Lef1* and *Axin2* and the dorsal SVZ\glutamatergic NP markers *Emx1* and *Tbr2* (**Fig R2.6A**). Analysis of BAT-gal mice (Maretto et al., 2003b) further demonstrated a decline in Wnt/ $\beta$ -catenin activity between P6 and P60, together with a decrease in the densities of Tbr2+ NPs and to a lesser extent Olig2+ OPs in the adult, and their apparent loss by P120 (**Fig R2.6B, C**). These analyses demonstrate the neurogenic capacity and lineage diversity of the dorsal SVZ declines in the adult brain (Hamilton et al., 2013; Shook et al., 2012) and, based on the SPIED/CMAP analysis, we predicted GSK3 $\beta$  inhibitors are strong candidates for rejuvenating the adult dorsal SVZ.



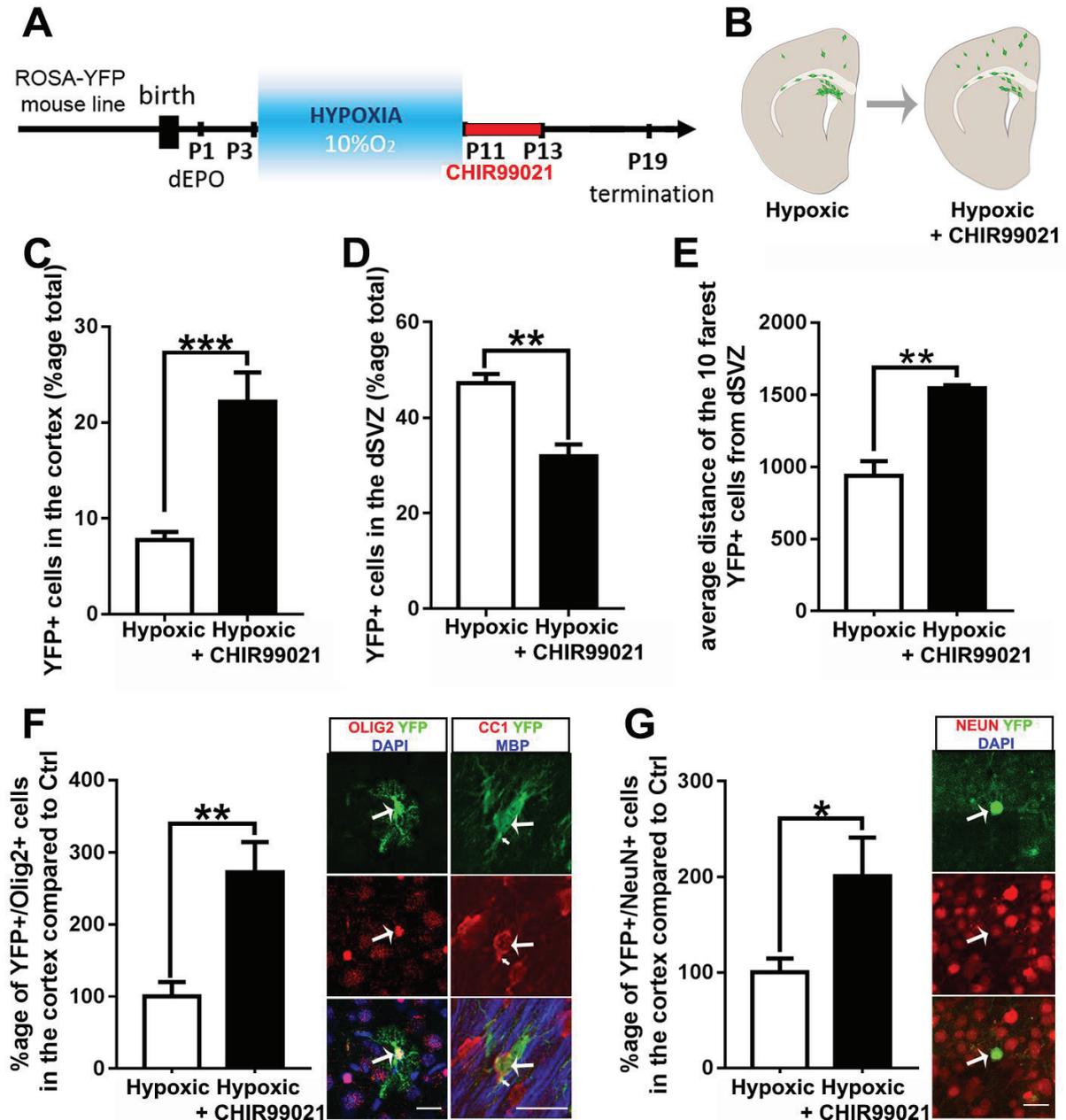
**Figure R2.6 : Pharmacological stimulation of Wnt/ $\beta$ -catenin signaling rescue OP and glutamatergic neuron progenitor numbers in the adult mouse.** (A) qPCR analysis reveals a pronounced decrease of Wnt targets genes *Lef1* and *Axin2* and pallial *Emx1* and *Tbr2* transcripts expression in the dorsal SVZ between P6 and P60 ( $n=3$  for P6 and P60). Results are expressed as percentage and normalized in comparison with *Gapdh* level of expression and compared using unpaired t test. (B) Representative coronal sections illustrating the pronounced and rapid decrease of Wnt canonical signaling in the  $\beta$ Gal reporter mouse ( $\beta$ GAL+), and the parallel decrease of glutamatergic neuron (*Tbr2*+ and OP (*Olig2*+)) in the SVZ of mouse brain at the age of 6 days (P6), 2 months (P60) and 4 months (P120) ( $n=3$  individual animals for each time point). (C) Quantification of the average number of  $\beta$ GAL+, *Tbr2*+ and *Olig2*+ cells in the dorsal wall of the SVZ in P6, P60, and P120 mice (3 animals per age). (D) Representative pictures of *Ki67*, *Tbr2* and *Olig2* expression in the adult (P90) SVZ before and after treatment with GSK3 $\beta$  inhibitors (AR-A014418, not shown, and CHIR99021, shown). (E) Percentage increase of proliferation (*Ki67*, EdU), OP (*Olig2*), glutamatergic neuron progenitor (*Tbr2*) and neural stem cell (*Mcm2*/GFAP) numbers following intraventricular infusion of AR-A014418 (3-10  $\mu$ M) and CHIR99021 (3-10  $\mu$ M). Values are normalized compared to the controls ( $n=5$  for each of control, AR-A014418 and CHIR99021). Error bars represent standard error of the mean (SEM). \*\*,  $p<0.01$ ; \*,  $p<0.05$ ; t test. Scale Bar = 1 mm (B) and 50  $\mu$ m (D).

The effects of the GSK3 $\beta$  inhibitors ARA-A014418 and second generation inhibitor CHIR99021 on dorsal lineages in the adult SVZ were examined directly *in vivo*, by infusion into the CSF of the lateral ventricle (**Fig R2.6D, E and RS2.3-5**). First, these agents were tested in postnatal mice, to confirm our previous findings that their infusion into the CSF effectively inhibits GSK3 $\beta$  activity and stimulates Wnt/ $\beta$ -catenin signaling in the dorsal SVZ (**Fig RS2.3**) [15], and promote the generation of glutamatergic NPs and OPs (**Fig RS2.4**). Next, we examined the effects of AR-A014418 or CHIR99021 in the adult (P90) dorsal SVZ; the agents were infusion into the lateral ventricle caudally, to ensure no damage to the SVZ by the injection procedure, whilst ensuring effective distribution of agents at a concentration of 3-10  $\mu$ M at the SVZ, which is rostral to the injection site (**Fig RS2.5**). Treatment with AR-A014418 and CHIR99021 dramatically stimulated the germinal activity of the adult dorsal SVZ, increasing proliferation as revealed by Ki67 immunolabelling and EdU incorporation, with profound effects on Mcm2/GFAP+ NSCs and Tbr2+ glutamatergic NPs, which were respectively increased 5-fold and 6-fold (**Fig R2.6D, E**). There was also an increase in oligodendrogenesis as evinced by a 3-fold increase in the number of Olig2+ cells (**Fig R2.6D? E**). Importantly, careful analysis revealed no marker co-expression (i.e. Olig2/Tbr2, and Dlx2/Tbr2, > 50 cells/brain in 5 animals), supporting appropriate and lineage specific progenitor specification. Thus, infusion of GSK3 $\beta$  inhibitors was able to rejuvenate the SVZ by promoting the re-emergence of lineages associated with early postnatal life, as predicted by the SPIED/CMAP analysis.

**e. Administration of small bioactive molecules that promotes SVZ germinal activity in a model of premature brain injury.**

The results reported above validate the SPIED/CMAP-based approach for lineage specific manipulation of SVZ germinal activity at various ages. Next, we explored the regenerative potential in a neuropathological context. We used a model of premature injury that lead to diffuse oligodendroglia and neuronal loss throughout the cortex (Fagel et al., 2006) to investigate the potential of small bioactive molecule to promote

the spontaneous cellular regeneration previously observed in this model (Bi et al., 2011b; Scafidi et al., 2014). We selected GSK3 $\beta$  inhibitors for their predicted capacity to induce dorsal lineages, i.e. oligodendrogenesis and neurogenesis (Azim et al., 2014b)[10] (**Fig R2.2**). We selected the second generation GSK3 $\beta$  inhibitors CHIR99021 over AR-A014418, due to its higher activity at lower concentration in inducing dorsal lineages (**Fig RS2.4C**). Dorsal electroporation of a Cre plasmid in Rosa-YFP Cre reporter mice allowed long term labelling and fate mapping of dorsal NSCs. Intranasal CHIR99021 delivery in hypoxic animals led to a significant decrease in the number of YFP+ cells in the SVZ, while their number concomitantly increased in the cortex (**Fig R2.7C, D**). This efficient cortical cellular recruitment was accompanied by a significant enhancement of distance of migration of YFP+ cells following CHIR99021 treatment (**Fig R2.7B, E**). Phenotypic characterization of the cells revealed significantly enhanced oligodendrogenesis (YFP+/Olig2+, **Fig R2.7F**), regeneration of new myelinating OLs (YFP+/CC1+/MBP+, **Fig R2.7F**), as well as increased neurogenesis (YFP+/NeuN+, **Fig R2.7G**) following hypoxia and CHIR99021 treatment. Expression of postmitotic marker (CC1 and NeuN) in OLs and neurons, respectively, support their successful differentiation following CHIR99021 treatment (**Fig R2.7G, H**). These results illustrate the capacity of small bioactive molecules identified in our bioinformatic approach to promote regeneration following forebrain injury.



**Figure R2.7 : CHIR99021 promotes dorsal SVZ derived cortical OL regeneration following chronic hypoxia.** (A) Schematic representation of the experimental workflow. A Cre plasmid was electroporated in the dorsal SVZ of ROSA-YFP mice 1 day after birth (P1) for permanent labeling of dorsal NSCs. Mice were placed in a hypoxic chamber containing 10% of O<sub>2</sub> from P3 to P11, then subjected to intranasal CHIR99021 administration from P11 to P13. Animals were sacrificed at P19 for analysis of recombined cell number, migration and differentiation. (B) Schematic representation of the results quantified in C-E. (C-D) CHIR99021 treatment following hypoxia leads to a decrease of YFP+ cells in the dSVZ (C), paralleled by a concomitant increase in the cortex (n=4 for hypoxic and n=4 for CHIR99021) (D). (E) The average distance of the 10 farthest YFP+ cells from the dorsal SVZ is increased following CHIR99021 treatment (n=4 for hypoxic and n=3 for CHIR99021). (F-G) CHIR99021 treatment promote de novo oligodendrogenesis (F, YFP+/Olig2+) and neurogenesis (G, YFP+/NeuN+; left confocal micrographs) following Hypoxia (n=7 for hypoxic and n=6 for CHIR99021). Right confocal

micrographs show expression of CC1 in YFP+ cells in the most superficial cortical layers support the successful differentiation of the newborn OLs (large arrow) that support myelin (small arrow shows colocalising YFP+/MBP+ myelinated fibers) in CHIR99021 treated animals. \*\*\* $p < 0.001$ ; \*\*,  $p < 0.01$ ; \*,  $p < 0.05$ ; t test used throughout. Scale bars = 20  $\mu\text{m}$  throughout.

## DISCUSSION – Chapter 2

Controlling the fate of NSCs would represent a key therapeutic strategy in neuroregenerative medicine. In the present study, we used a novel SPIED/CMAP strategy to identify small molecules that are predicted to regulate transcriptional changes associated with neurogenesis in the SVZ neurogenic niche. Importantly, we validate this approach by demonstrating that two of the identified small molecules, which target PI3K/Akt and GSK3 $\beta$ , were able to differentially direct the fate of NSCs *in vivo*, to promote oligodendrogenesis and neurogenesis, in the postnatal and adult SVZ. Moreover, we show that this approach can be used to promote regeneration in a clinically relevant mouse model of postnatal brain hypoxia, which demonstrates the power of the SPIED/CMAP-based approach for repurposing of clinically approved small molecules for promoting regeneration in neurodegenerative and demyelinating diseases.

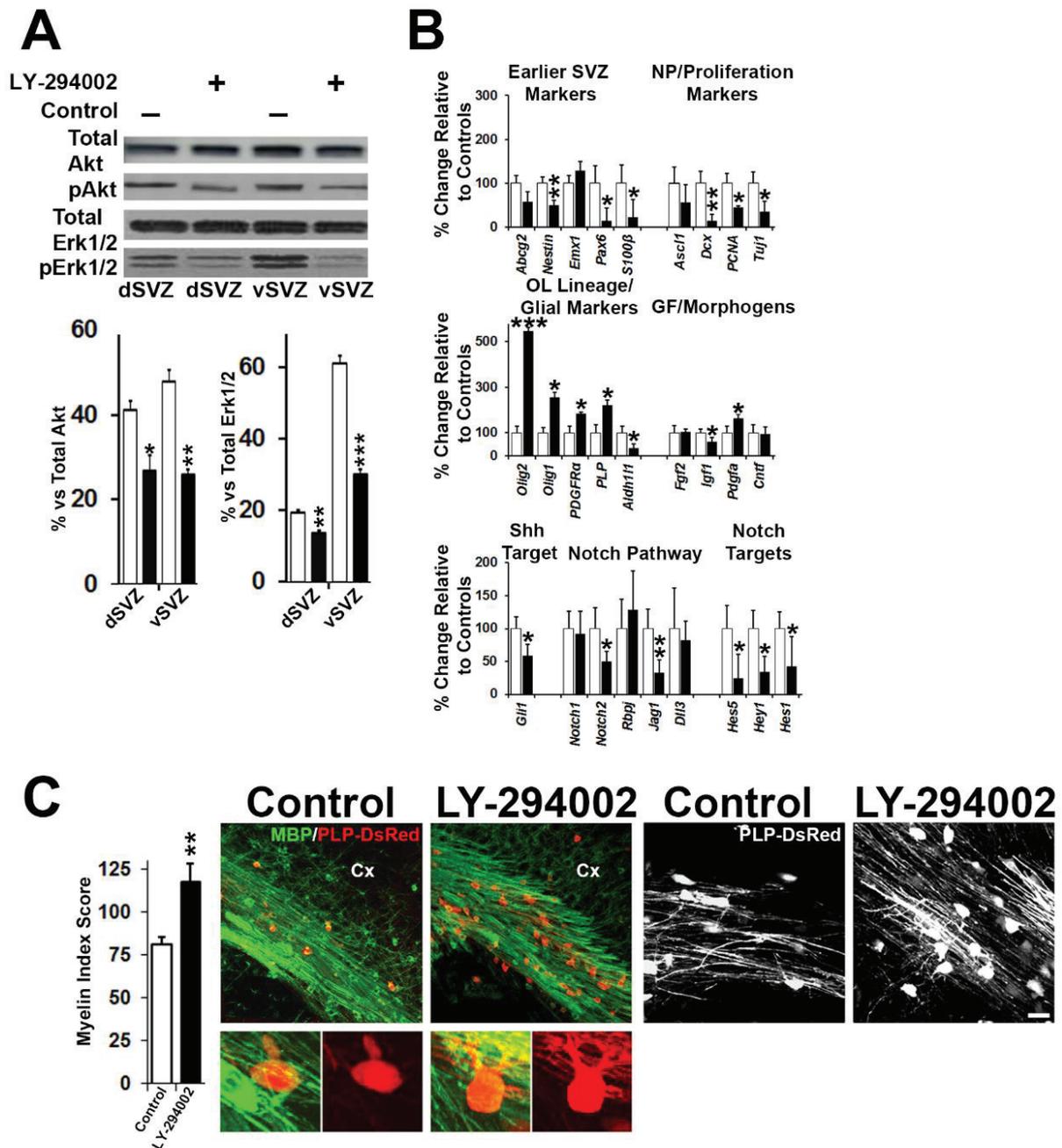
Transcriptome profiling revealed for the first time the marked heterogeneity of secreted ligands within the dorsal and lateral microdomains of the postnatal SVZ that are likely to contribute towards the regionalized fate of NSCs by regulating downstream transcriptional programs (Azim et al., 2014b, 2015; Ihrie et al., 2011). These regional differences suggested to us that specific signaling pathways could be probed as a means to manipulate SVZ regionalization, as well as the lineages associated with specific SVZ microdomains. To this end, we provide the first interrogation of the SVZ using the CMAP initiative, which has been used extensively in other systems (reviewed in (Lamb et al., 2006)). We identified small molecules that have the potential to manipulate region and/or lineage-specific gene expression signatures. Small molecules were further prioritized by additional analysis of their target genes. First, small molecules can be prioritized by the number of “target genes” (**Tables 1 and 2**). Additionally, “target genes” were examined further by GO term analysis, which enabled us to prioritize small molecules predicted to act on select lineages/signaling pathways and exclude those with undesirable side effects (e.g. cell death). Applying these selection criteria, we selected LY-294002 and AR-A014418 for validation *in vivo* and show they stimulate the genesis of specific lineages during development and in adulthood.

To target oligodendrogenesis, we selected the specific PI3K inhibitor, LY-294002 on the basis that our SPIED/CMAP analyses predicted an upregulation of signatures relevant to OL lineage cells. Network analysis revealed that dorsal-derived lineages are mediated by via PI3K/Akt signaling which were highly ranked in analysis performed Fig 1A, implying novel fate-specification roles. In addition, in vitro studies indicate an important role for PI3K/Akt signaling in OL development (Guardiola-Diaz et al., 2012; Vemuri and McMorris, 1996). Our findings demonstrate for the first time that intraventricular infusion of LY-294002 specifically targeted oligodendrogenesis in vivo in the dorsal SVZ, which is the primary source of forebrain OLs in the postnatal brain (Menn et al., 2006). Furthermore, a novel finding was that Notch2-signaling is a target of PI3K/Akt in vivo and may be a primary mechanism regulating fate decision of NSC along an oligodendroglial path (Basak et al., 2012; Wang et al., 1998), which was not predicted from previous genetic or in vitro studies. It is significant, therefore, that ciclopirox, an inhibitor of prolyl-4-hydroxylase that promotes Notch signaling and NSC activation (Ma et al., 2013; Zheng et al., 2008), was the second most highly ranked pertubagen predicted to promote oligodendrogenesis. Other factors identified by our analyses and worthy of further examination, inhibit HDACs, which is essential for generation of OPs (Castelo-Branco et al., 2014). Indeed, our analyses indicate the key to promoting oligodendrogenesis in the SVZ is to inhibit signaling pathways that promote neuronal cell fate and future strategies should examine the therapeutic potential of combinations of small molecules.

Gene expression profiling of the SVZ demonstrated a marked decline in NSC activity during adulthood, in support of other studies indicating an age-related loss of specific signaling cues that promote NSC proliferation and fate commitment (Luo et al., 2006; Maslov et al., 2004a; Shook et al., 2012). Most notable was the decline in canonical Wnt/ $\beta$ -catenin activity, which is consistent with an increase in Wnt inhibitors during adulthood (Zhu et al., 2014). It is significant, therefore, that our study demonstrates that small molecule inhibitors of GSK3 $\beta$  identified by the SPIED/CMAP analysis act as mimetics of Wnt/ $\beta$ -catenin and successfully reverse the decline in specific neural lineages in the adult SVZ. Significantly, we identified that the GSK3 $\beta$  inhibitors AR-A014418 and CHIR99021 have a profound effect on germinal activity in the dorsal SVZ of 3-month old mice. This was primarily illustrated by the pronounced effects on glutamatergic NPs, although OPs were also increased to a lesser extent.

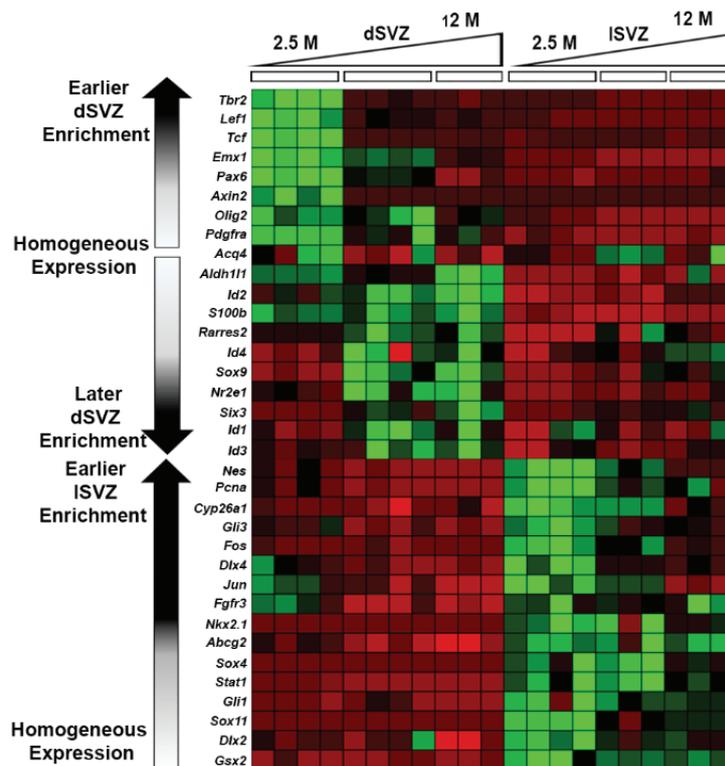
In summary, our analyses provide a comprehensive catalogue of small molecules that can be used to manipulate SVZ microdomain-specific lineages. The power of this approach is highlighted by our results demonstrating that two of these compounds, LY-294002 and AR-A014418, stimulate oligodendrogenesis and glutamatergic NPs generation in postnatal and adult contexts. Moreover, experiments performed in a model of postnatal brain injury reveal that this strategy can be applied to promote SVZ germinal activity in a regenerative context. Although further work is required to fully characterize the functional outcome of this repair, these encouraging results will stimulate further studies on repurposing other small molecules and determine their potential for lineage specific manipulation of SVZ germinal activity and regeneration in multiple neuropathologies. In this regard, it is noteworthy that identified small molecules, for example GSK3 $\beta$  inhibitors may be useful for reactivating Wnt-signalling which is down regulated during impaired cortical development leading to neuropsychiatric disorders in adulthood (Singh et al., 2010). Furthermore, our SPIED/CMAP analysis predicts that further specificity could be achieved by combinatorial approaches, most notably promoting adult neurogenesis using AR-A014418 combined with adiphénine, a nACh inhibitor that was the most highly ranked pro-rejuvenating and anti-oligodendrogenesis small molecules. Conversely, specific stimulation of oligodendrogenesis could be achieved by LY-294002 in combination with the HDAC inhibitor trichostatin-A, which was the most highly ranked pro-oligodendrogenesis and anti-neurogenic small molecule. Moreover, our analyses highlighted a number of small molecules that are predicted to have an “anti-dorsalizing” or “ventralizing” effect, hence would be specifically pro-neurogenic. This is of considerable translational interest, since the more ventral SVZ is more developed in humans (Bernier et al., 2000) and adult neurogenesis has recently been described in the striatum (Ernst et al., 2014b), which is more extended than in rodents (Inta et al., 2015). Moreover, the ventral SVZ gives rise to specific granule cells, such as those forming the Islands of Calleja (De Marchis et al., 2004), and our analyses identifies small molecules that may target the different factors that drive the differentiation of such specific neurons. In conclusion, our study establishes unequivocally the effectiveness of employing pharmacogenomic approaches for generating a robust framework that guides mechanistic experiments to manipulate neurogenic niches in the brain, which has considerable potential for identifying therapeutics for neurodegenerative and demyelinating diseases.

## Supplementary data – Chapter 2

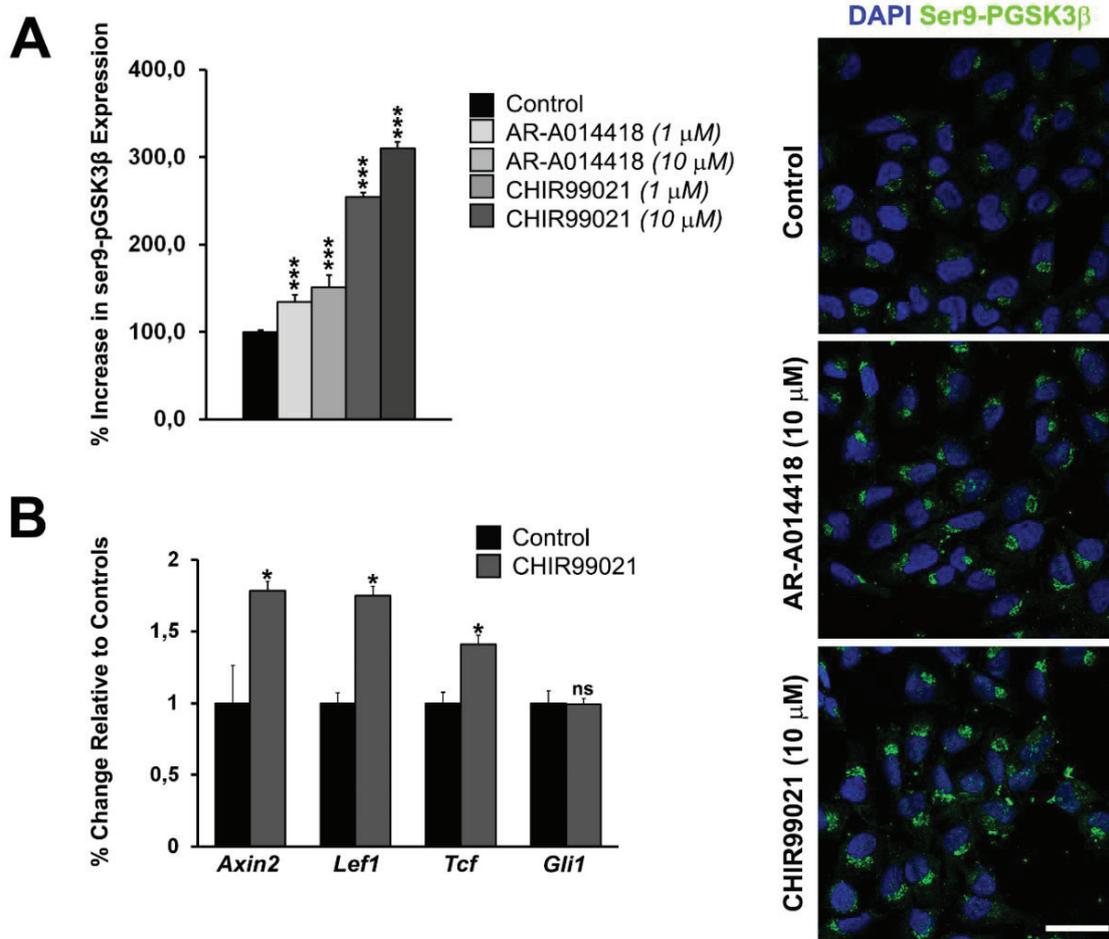


**Figure RS2.1 : LY-294004 inhibits PI3K/Akt signaling, modulates expression of transcripts, promotes specifically Olig2+ progenitors and augments myelination.** (A) P9 mice were treated with 0.06 mM LY-294002 and saline/DMSO as controls and SVZ microdomains were analyzed by western blot 45 mins after infusion. Representative immunoblots and mean densitometric values for protein levels ( $\pm$ SEM,  $n = 3$  for control and LY-294002), for total-Akt and pAkt and total Erk1/2 and pErk1/2. Significance was tested by t test. (B) qPCR was performed on microdissected dorsal SVZ 180 min following final infusion to detect cell specific transcripts in earlier NSC/NP lineages, glial lineages, secreted trophic factors, and signaling pathway components or target genes. Data's are expressed as the mean ( $\pm$  SD;  $n = 3$  for control and LY-294002) % change of relative expression values, GAPDH

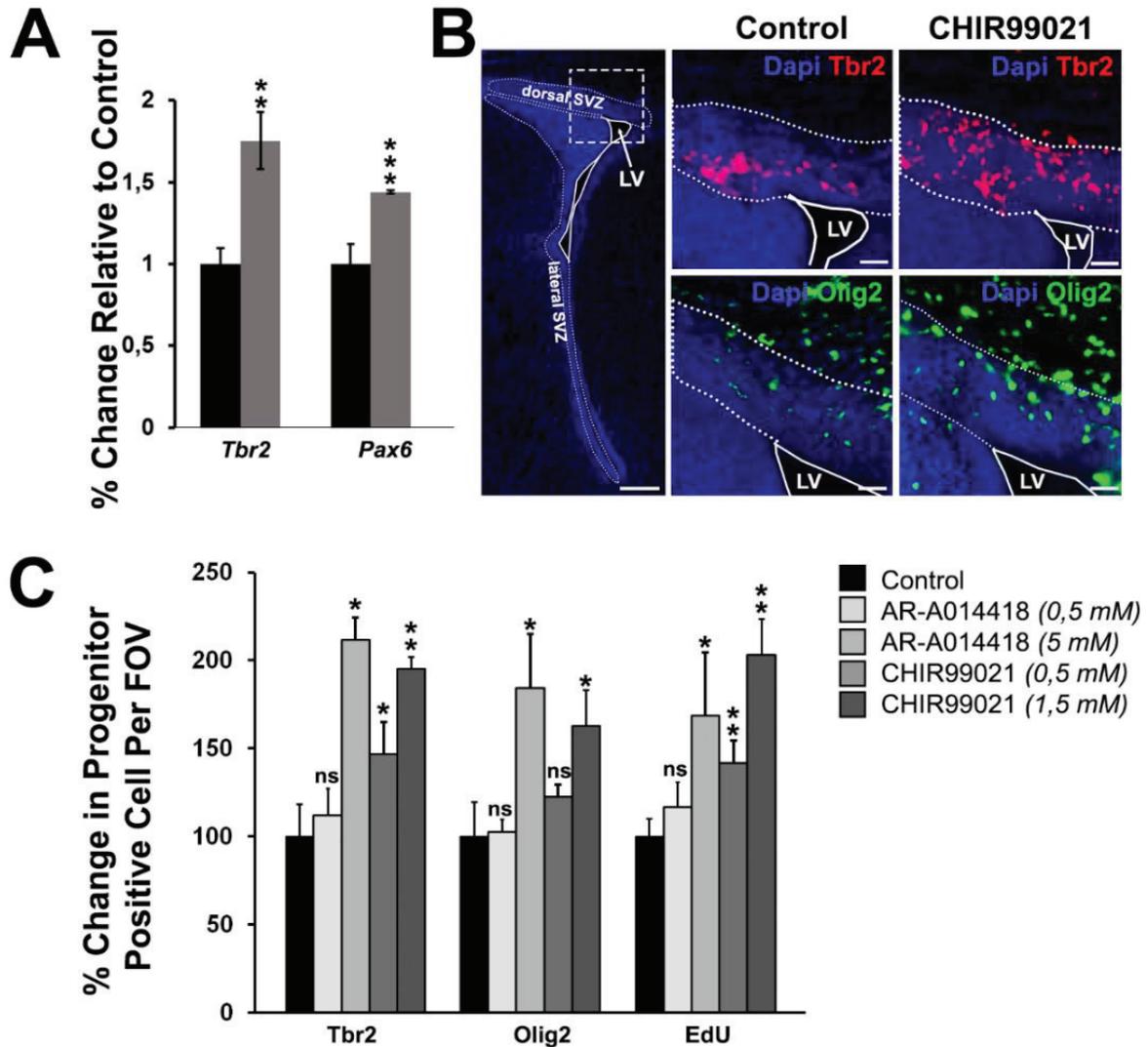
normalized. \* $p < 0.05$ ; \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ;  $t$  tests. White bars indicate controls and black bars for LY-294002. (C) P8 PLP-DsRed transgenic mice (for identifying OLs and myelin sheaths) were treated daily with 0.06 mM LY-294002 and saline/DMSO as controls for 3 days and sacrificed at P11 for immunolabelling with MBP for changes in myelination. Histogram of the myelin index in the corpus callosum; data are mean number of myelin sheaths  $\pm$  SEM ( $n = 4$  for control and LY-294002) in a constant volume and were tested for significance using unpaired  $t$  test (\*\*  $p < 0.01$ ). Confocal micrographs show enhanced MBP immunolabelling following LY-294002 and insets show LY-294002 induced PLP-DsRed+ OLs support more normal appearing myelin sheaths. Right panels show enhanced DsRed expression and greater densities in PLP-DsRed+ OLs. Images are flattened confocal z-stacks of thickness 15  $\mu$ m (left panels; captions are single z-sections), or 10  $\mu$ m thickness in right panels. Scale bar in right panels = 25  $\mu$ m; in left panels (5  $\mu$ m in captions) and 15  $\mu$ m in left single channel panels.



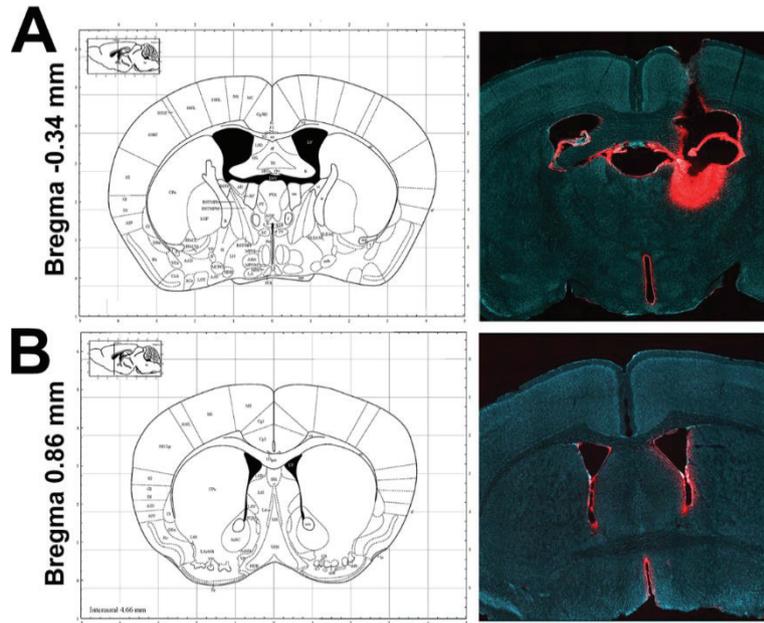
**Figure RS2.2 : Confirmation in the decline in SVZ activity, Wnt-signaling and dorsal-derived lineages.** The spatiotemporal gene expression changes in microdissected SVZ microdomains were examined by qPCR of selected genes and processed in Partek Genomics Suit 6.6 and presented as an intensity heatmap. Only transcripts that passed the criteria of  $p < 0.05$  ANOVA versus its adjacent (dorsal versus lateral) or temporal (2.5 months ( $n = 4$ ), 6 months ( $n = 3$ ) and 1 year ( $n = 3$ )) are presented.



**Figure RS2.3 : GSK3β inhibitors AR-A014418 and CHIR99021 successfully activate Wnt/β-catenin signaling *in vitro* and *in vivo*.** (A) GSK3β inhibitors (AR-A014418 and CHIR99021) activate the Wnt canonical pathway *in vitro*, as indicated by increased immunodetection of Ser9-GSK3β phosphorylation. Graph shows the quantification of optical density and  $n \geq 75$  cells analyzed per group. Images illustrate the experimental conditions. Scale Bar = 40 μm. (B) qPCR analysis of Wnt signaling target genes expression *Axin2*, *Lef1* and *Tcf* and the Shh signaling target gene *Gli1* in the dorsal SVZ following subcutaneous injections of CHIR99021 (500μM), as previously observed for AR-A014418 (Azim et al., 2014b). Error bars represent standard error mean (SEM). \*\*,  $p < .01$ ; \*,  $p < .05$ ; t test.



**Figure RS2.4 : Sub-cutaneous injections of AR-A014418 and CHIR99021 increase glutamatergic neuron and OL progenitor numbers in the early postnatal dorsal SVZ.** AR-A014418, CHIR99021 or a vehicle (CTR) was injected subcutaneously during 2 days before isolation of the brain. **(A)** qPCR analysis of the dorsal SVZ markers *Tbr2* and *Pax6* in the dorsal SVZ following subcutaneous injections of CHIR99021 (500 μM). **(B)** Representative picture of EDU and *Tbr2* stainings in the dorsal SVZ. Scale Bar = 100 μm (overview) and 40 μm (right panels). **(C)** Percentage increase of *Tbr2*<sup>+</sup>, *Olig2*<sup>+</sup> and Ki67<sup>+</sup> cells in the dorsal SVZ after AR-A014418 and CHIR99021 administration at different concentration. Values are normalized compared to the controls. Error bars represent standard error mean (SEM) and n = 7 for control and 3 n numbers for each GSK3β inhibitor group. \*\*, p<0.01; \*, p<0.05; t test.



**Figure S2.5 : Procedure for intraventricular infusion of GSK3 $\beta$  inhibitors in P90 mice. (A)** Infusion site (red arrow), on caudal coronal section from Mouse Paxinos Atlas. Here DAPI (in red) is infused to visualize the pattern of diffusion from the cerebrospinal fluid, on a coronal section stained with Nissl. **(B)** Note that the rostral regions of the lateral ventricles, where quantifications were performed remained intact.

## CHAPTER 3

### Intranasal administration of CHIR99021 promotes cellular repair following neonatal hypoxia

#### SUMMARY – Chapter 3

Previous chapters highlighted a spontaneous contribution of neural stem cells (NSCs) from the subventricular zone (SVZ) to participate to cortical cellular repair following perinatal hypoxia. They further demonstrated that SVZ germinal activity can be manipulated in a lineage specific manner. This chapter builds onto these observations. It aims at determining if activating Wnt/ $\beta$ -catenin signalling in the dorsal subventricular zone (dSVZ) following chronic hypoxia has beneficial effects on the (re)generation and maturation of cortical oligodendrocytes and glutamatergic neurons.

The pharmacogenomic approach, presented in Chapter 2, identified Gsk3 $\beta$  inhibitors (characterized notably as a Wnt/ $\beta$ -catenin signalling activators (Cohen and Goedert, 2004)) as small molecules with both “dorsalizing” and “pro-oligodendroglial” potentials. Further, we showed that administration of CHIR99021 promoted *de novo* cortical oligodendrogenesis and neurogenesis following neonatal hypoxia (Azim et al., 2017). Here, we investigated these effects in more details. We were in particular interested in identifying the subtypes of neurons being produced, as well as the long-term effects of such treatment onto the pools of NSCs and onto the maturation and survival of the newly-generated cells. To this end, we performed CHIR99021 intranasal administration following chronic hypoxia and sacrificed the animals at P19 and P45 in order to investigate the short and long term effects of this treatment onto SVZ NSCs as well as their oligodendroglial and neuronal progenies.

Our data confirmed that CHIR99021 promotes cellular repair in the forebrain following perinatal hypoxia. CHIR99021 administration promoted specification of dSVZ-derived but not their long-term survival. In contrast, CHIR99021 administration promoted both

the maturation of dSVZ-derived oligodendrocytes, as well as their long-term survival. These effects were paralleled by a long-term activation of dSVZ NSCs, which however did not result in their depletion by P45. Thus, short-term CHIR99021 treatment following chronic hypoxia showed both immediate and long-term effects onto cellular repair in the premature forebrain.

## RESULTS – Chapter 3

### a. Hypoxia and CHIR99021 promote Wnt/ $\beta$ -catenin pathway activity in the dorsal subventricular zone

In chapter 2, we identified CHIR99021, a small molecule activating Wnt/ $\beta$ -catenin signaling, for having the ability to promote dorsal lineage of the subventricular zone (SVZ), both oligodendrocyte and glutamatergic neuron progenitors, as well as for its rejuvenating effect in the adult SVZ. In a pathological context, we showed that CHIR99021 administration promoted endogenous cortical neurogenesis from the dorsal subventricular zone (dSVZ) following hypoxia. Wnt/ $\beta$ -catenin pathway plays indeed a crucial role in maintaining the dorsal identity of SVZ stem cells and progenitors and its activity decreases throughout life. Furthermore, intranasal administration of Wnt3a has a neuroprotective and regenerative effect on the brain after stroke (Zachory Wei et al.). We first investigated whether Wnt/ $\beta$ -catenin signalling was affected by the period of hypoxia in the dorsal SVZ and thereby if cortical regeneration following hypoxia could be partly due to this signalling pathway. To do this, we used the BatGal mice, expressing b-galactosidase under the control of a Wnt target promotor (Maretto et al., 2003b) subjected to chronic hypoxia and terminated at P11 (**Fig RS1A**). Interestingly, the optical density (OD) of b-galactosidase staining in the dorsal SVZ was increased in the hypoxic brains compared to the normoxic ones (Ctrl), suggesting a spontaneous activation of Wnt/ $\beta$ -catenin signalling after hypoxia (**Fig RS3.1B, D**). Moreover, the percentage of glutamatergic neuron progenitors (Tbr2+ cells) co-expressing b-galactosidase suggesting an increased activity of the pathway in the glutamatergic neuron lineage (**Fig RS3.1C, D**).

We next verified if CHIR99021 activated Wnt/ $\beta$ -catenin signalling in the dorsal SVZ, using the BatGal mice. First, we performed intra-peritoneal injections from P4 to P6 to analyse the b-galactosidase and Tbr2 expression at P6 in the dorsal SVZ. Both markers were upregulated (**Annexe**). Then, we performed intranasal administration of CHIR99021 from P11 to P13 and terminated the brains at P15 (**Fig RS3.1E**). Analysis revealed an increased optical density of b-galactosidase staining in the dorsal SVZ (**Fig RS1F**), as well as an increased percentage of glutamatergic neuron progenitors (Tbr2+ cells) co-expressing b-galactosidase (**Fig RS1G**). This shows that intranasal

administration of CHIR99021 does activate Wnt/ $\beta$ -catenin signalling in the dorsal SVZ, notably in the population of glutamatergic neuron progenitors.

Together, our results showed that chronic hypoxia promotes the activity of Wnt/ $\beta$ -catenin signalling in the dorsal SVZ, which could be involved in the recruitment of dorsal SVZ cells for cortical repair. Moreover, intranasal administration of CHIR99021 had a similar effect, increasing the activity of Wnt/ $\beta$ -catenin signalling in the dorsal SVZ. Administration of CHIR99021 after the hypoxic period could potentiate the effect of hypoxia on Wnt/ $\beta$ -catenin signalling.

#### **b. CHIR99021 administration promotes the early specification of hypoxia-induced cortical neurons but not their long term survival**

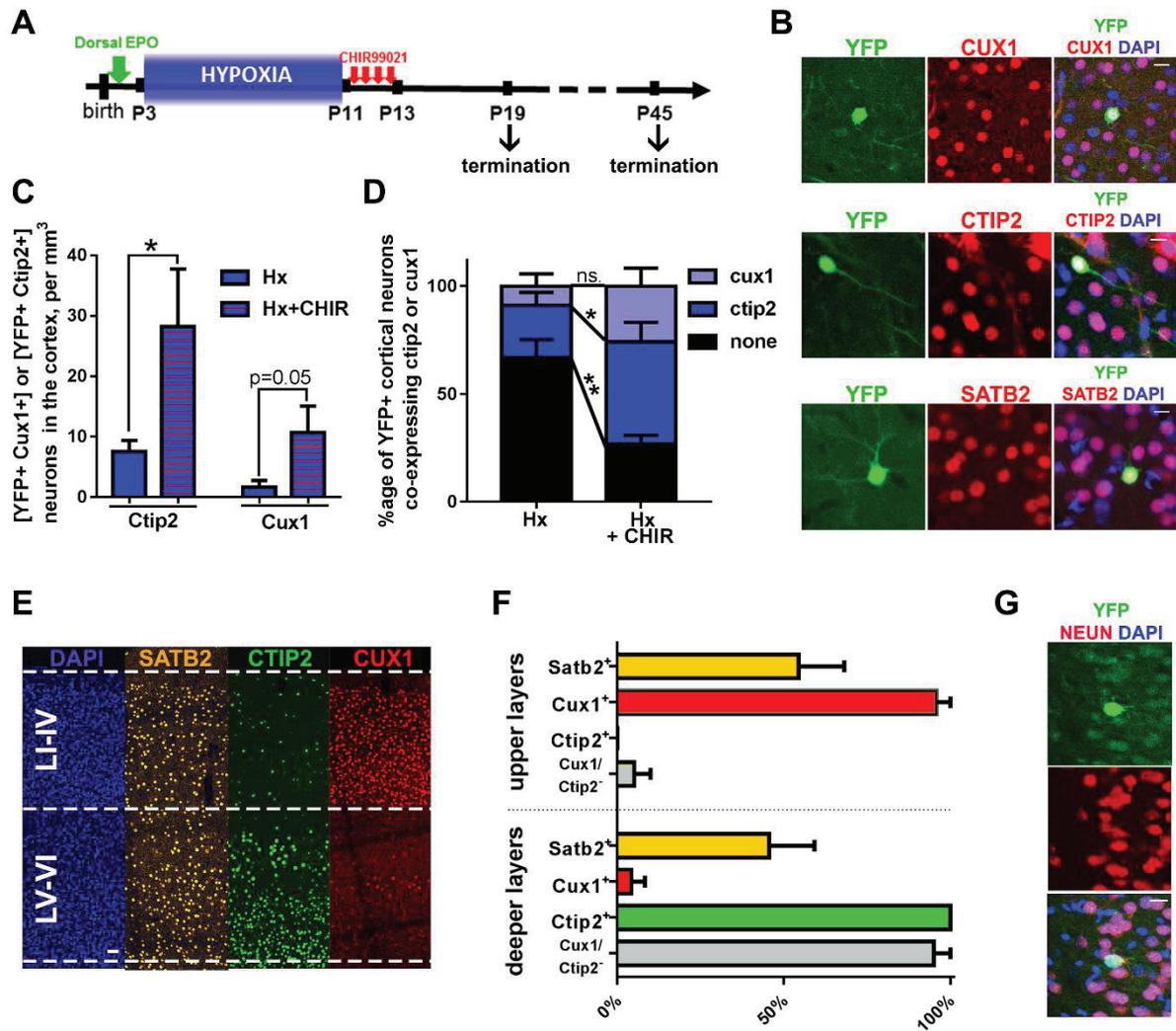
We first investigated the identity of postnatally born cortical neurons, using the same experimental design (**Fig R3.1A**). We selected several glutamatergic markers in order to investigate their expression by newborn cortical neurons at P19. We used *Satb2* that labels a population of neurons that can be observed throughout cortical layers (**Fig R3.1E**). In addition, we used *Cux1* and *Ctip2* as markers of upper (layers I-IV) and deeper (layers V-VI) cortical neurons, respectively. Interestingly, a significant fraction of YFP<sup>+</sup> neurons expressed one or the other of these markers. Thus, about 38% of the YFP<sup>+</sup> neurons expressed *Satb2* corresponding to a density of 25 per mm<sup>3</sup> in the area reached by the electroporation (**Fig RS3.2A, B, C**), 10% of the upper cortical layer *Cux1* and about 25% the deep layer cortical marker *Ctip2* (**Fig R3.1C**). Together, these results suggest that a large proportion of newborn neurons derived from the dorsal most SVZ regions are glutamatergic and rapidly acquire subtype-specific cortical neurons markers expression.

We next assessed the layer distribution of these positive neurons at P19. Interestingly, YFP<sup>+</sup> cortical were localized in the cortical layers corresponding to the marker they expressed. Indeed, more than 95% of YFP<sup>+</sup>/*Cux1*<sup>+</sup> neurons were located in upper cortical layers, where YFP<sup>+</sup>/*Ctip2*<sup>+</sup> neurons were consistently absent. Inversely, all YFP<sup>+</sup>/*Ctip2*<sup>+</sup> neurons were located in deeper cortical layers, where only 1

YFP+/Cux1+ neurons was observed. In agreement with the more widespread Satb2 expression, about 55% of the YFP+/Satb2+ cells were localized in the upper layers while about 45% were in the deeper layers (**Fig R3.1F**). Thus, newborn cortical neurons appear to rapidly specify to acquire layer specific markers in accordance with their final location.

We next investigated the effect of CHIR99021 intranasal administration onto newborn neurons specification. The increase number YFP+/NeuN observed following CHIR99021 administration (**see Fig7, Chapter 2**) appears to include an increase in the number of Cux1+ and more strikingly of Ctip2+ neurons being generated (**Fig R3.1C**). In parallel, the proportion of YFP+ neurons expressing one or the other of these layer specific cortical markers greatly increased from 35% to 75% following CHIR99021 treatment. Remarkably, the proportion of YFP+/Ctip2+ neurons showed the largest increase (25% to 60%, **Fig R3.1D**). The layer specific distribution of Cux1 and Ctip2+ neurons was preserved following CHIR99021 treatment.

In order to assess the long-term survival of newborn neurons, we repeated those analyses at P45. No YFP+ cortical neurons were observed in the hypoxic condition, indicating that the spontaneous cortical neurogenesis observed following neonatal hypoxia is mainly abortive. Further, only rare YFP+/NeuN+ cells were observed in the hypoxic brains following CHIR99021 administration (**Fig R3.1G**) and were all located in deep layers, suggesting that the faster acquisition of layer markers is largely insufficient to facilitate the integration and long-term survival of newborn neurons.



**Figure R3.1: CHIR99021 administration promotes acquisition of layer specific markers by newborn neurons following hypoxia.** (A) Experimental design: a CRE plasmid was electroporated in the dorsal microdomain of the SVZ of ROSA YFP mice (dorsal EPO) at P1 before to undergo a hypoxic environment (10% O<sub>2</sub>) from P3 to P11, followed by intranasal administration of CHIR99021 from P11 to P13. Mice were terminated at P19 or at P45. (B) Illustrations of neuron-like YFP<sup>+</sup> cells expressing the cortical neuron markers Cux1, CtIp2 and Satb2 at P19. (C-D) Density and proportion of neuron-like YFP<sup>+</sup> cells co-expressing Cux1 and CtIp2 following hypoxia (Hx) and CHIR99021 treatment (Hx+CHIR) at P19. (E) Representative pictures illustrating the pattern of expression of the cortical markers Satb2, CtIp2 and Cux1. Satb2 is localized in all cortical layers while CtIp2 is specific for deep and Cux1 specific from the upper cortical layers. (F) Percentage of YFP<sup>+</sup> neurons expressing Satb2, Cux1, CtIp2 or Cux1 and CtIp2 within the deeper or upper cortical layers at P19. (G) Microphotographs of a YFP<sup>+</sup> cortical neuron at P45 in Hx+CHIR conditions. Scale bars: 50 microns in E, 10 microns in B and G. Abbreviations: EPO: electroporation; Hx; hypoxic/Hx+CHIR: hypoxic treated with CHIR99021; P3/P11/P13/P19/P45 : respectively 3, 11, 13, 19 and 45 days after birth %age: percentage. LI-IV : cortical layers 1 to 4; LV-VI : cortical layers 5 and 6. P-values: ns > 0.05 ; \* < 0.05 ; \*\*<0.01.

**c. CHIR99021 promotes the maturation and long term survival of newborn cortical oligodendrocytes**

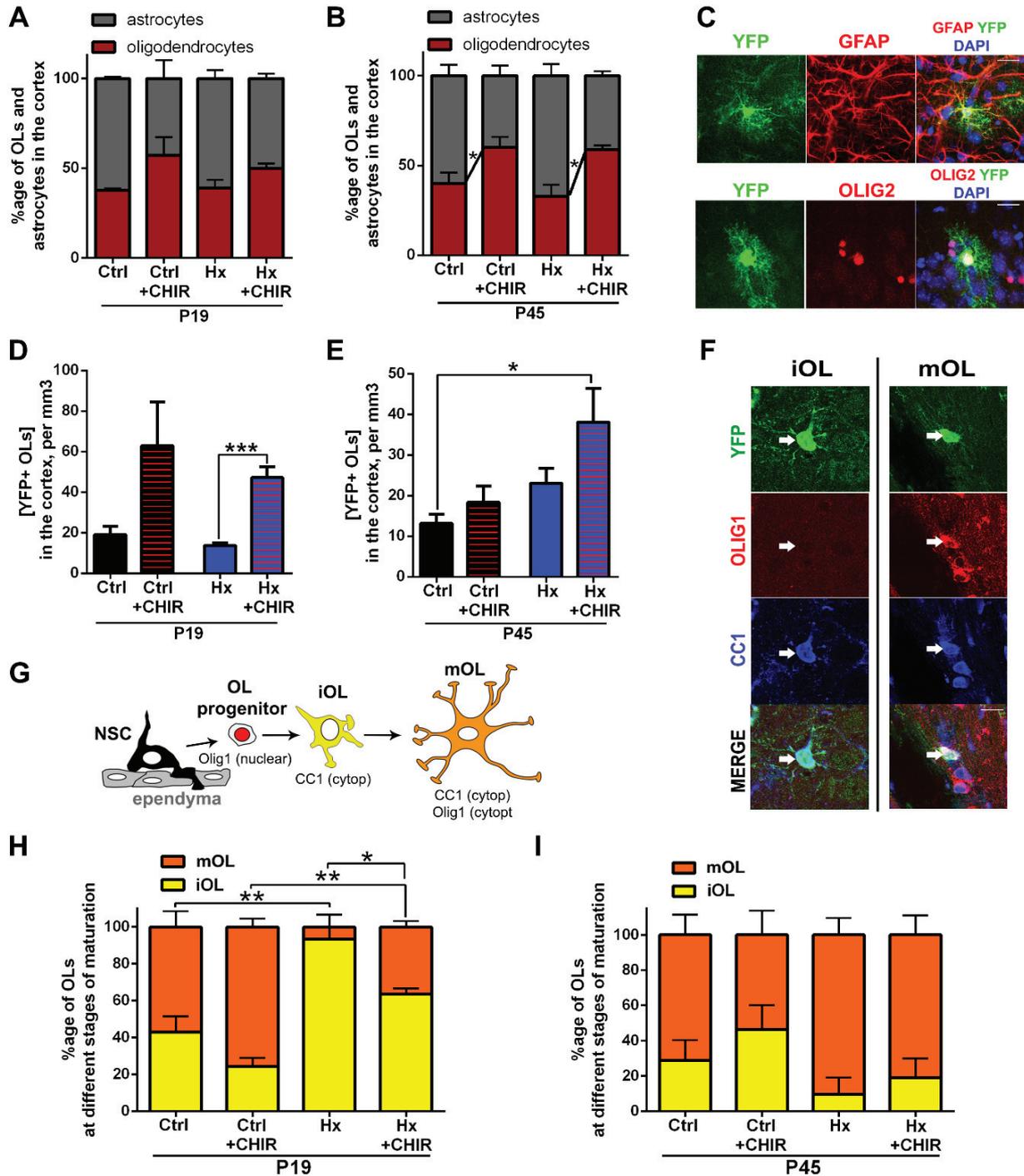
We next assessed in details the gliogenic response observed following hypoxia, as well as its modulation by the CHIR99021 treatment. To this end, cortical YFP+ glial cells were characterized by using the astrocytic marker Gfap and the oligodendroglial marker Olig2 (**Fig R3.2C**) at both P19 and P45 (**Fig R3.2A, B**).

For both timepoints, the balance between YFP+ engaging into an astrocytic fate (Gfap+, 40%) and oligodendrocytic fate (Olig2+, 60%) was not affected by hypoxia (compare Ctrl to Hx in **Fig R3.2A, B**). Interestingly, CHIR99021 treatment promoted oligodendrogenesis in both normoxic (Ctrl) and hypoxic (Hx) animals, as reflected by the increased proportion of YFP+ cells acquiring an oligodendroglial identity at P19 (**Fig R3.2A**). This tendency was confirmed at P45, by a significant increase in the proportion of YFP+ oligodendrocytes, at the expense of YFP+ astrocytes (**Fig R3.2A**). Quantification of the density of YFP+ oligodendrocytes at P19, confirmed the increased oligodendrogenesis following CHIR99021 administration, which appeared more consistent in hypoxic animals when compared to controls (**Fig R3.2D**). Analysis performed at P45, substantiated these observations by confirming the increased density of YFP+/Olig2+ cells within the cortex, following hypoxia and CHIR99021 treatment (**Fig R3.2E**).

We next assessed if this increased oligodendrogenesis was paralleled by an effect of CHIR99021 administration on the maturation of newborn oligodendrocytes. Indeed, Wnt/ $\beta$ -catenin signalling differentially controls distinct stages of oligodendrogenesis. Thus, activating the Wnt/ $\beta$ -catenin signalling pathway seems to promote progenitor proliferation and inhibit oligodendrocyte differentiation (Dai et al., 2014). In order to investigate the effects of hypoxia and/or CHIR99021 treatment onto oligodendrocyte maturation, we combined the immunodetection of two oligodendrocyte markers Olig1 and CC1. Previous studies have shown that OL progenitors express Olig1 in the nucleus, while maturing oligodendrocytes first express CC1 (i.e. immature oligodendrocytes, iOL), then co-express CC1 and Olig1 in their cytoplasm (i.e. mature oligodendrocytes, mOL; **Fig R3.2F, G**) (Dai et al., 2015; Nakatani et al., 2013). The proportion of YFP+ cortical iOL and mOL reveals a significant increase in the proportion of iOL which represent about 95% of the OL population in the hypoxic (Hx)

brains compared to about 40% in the controls (Ctrl), suggesting a delay in oligodendrocyte maturation due to hypoxia. CHIR99021 administration promoted the maturation of YFP+ cortical oligodendrocytes, increasing significantly the proportion of mOL from 5 to 30% and decreasing accordingly the proportion of iOL (**Fig R3.2H**). At P45, the ratios between the different stages of maturation are similar in all the conditions with a larger proportion of mOL (**Fig R3.2I**).

Altogether, those results highlighted an effect of hypoxia on oligodendrocyte maturation in the cortex by 8 days following the end of the hypoxic period, which can be reversed by CHIR99021 administration. In addition, those results showed that a larger number of cortical OLs is produced following CHIR99021 administration, which mature more rapidly.



**Figure R3.2: CHIR99021 promotes oligodendrocytes production and maturation following hypoxia.** (A-B) Graphs showing the proportion of astrocytes and OLs in the YFP+ glial population of the cortex at P19, in the normoxic (Ctrl) and hypoxic animals (Hx), with or without CHIR99021 treatment (Ctrl + CHIR; Hx + CHIR) at P19 (A) and P45 (B). (C) Representative pictures of YFP+ astrocytes (Gfap+) and oligodendrocytes (Olig2+). (D-E) Graphs showing the density of YFP+/Olig2+ cells in the cortex at P19 (D) and P45 (E). (F) Representative pictures of the markers used to characterize the distinct stages of OL maturation. (G) Diagram shows the different stages of maturation of oligodendrocytes: progenitor, iOL, mOL, characterized by the expression of a combination of CC1 and Olig1 markers. (H-I) Graph shows the proportion of YFP+ OLs at different stages of maturation in the

cortex at P19 (H) and P45 (I). Scale bars: 20 microns in C and F, Abbreviations: %age: percentage; OL: oligodendrocytes, iOL: immature oligodendrocytes, mOL: mature oligodendrocytes, P19/P45: respectively 19 and 45 days after birth; Ctrl: normoxic/Ctrl+CHIR: normoxic treated with CHIR99021/Hx; hypoxic/Hx+CHIR: hypoxic treated with CHIR99021. P-values: \* < 0.05 ; \*\*<0.01 ; \*\*\*<0.001.

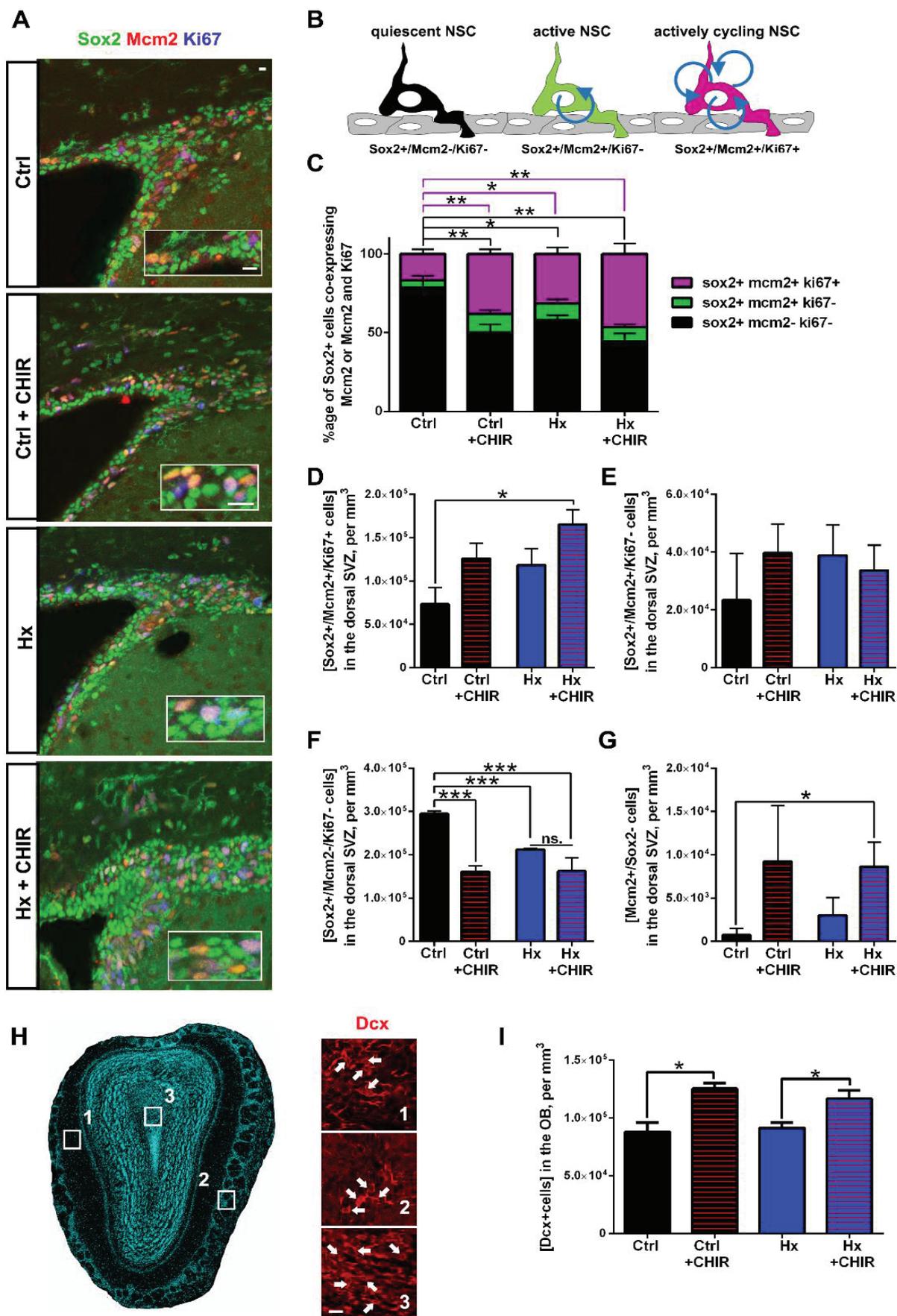
#### **d. Acute CHIR99021 treatment has long-term effects on dorsal subventricular zone germinal activity**

The results presented above revealed that a two-day treatment with CHIR99021 results in both short and long-term effects onto cellular regeneration following hypoxia. We wondered if such acute treatment could also have long-term effects on the reservoir of SVZ neural stem cells and therefore onto the germinal activity of this region.

Neural stem cells (NSCs) can be subdivided in several populations that reflect different stages of activation (ref). Here, we used expression of Sox2, Mcm2 and Ki67, to identify subpopulations of NSCs. Quiescent NSCs express the stem cell marker Sox2 but none of the proliferation markers Mcm2 and Ki67. Activated NSCs express Sox2 and Mcm2, while actively cycling NSCs also express Ki67 (**Fig R3.3A, B**). The proportion of NSCs subpopulations was quantified in the dorsal SVZ at P45, that is to say more than one month after hypoxia and/or CHIR99021 treatment. The analysis revealed a positive effect of hypoxia and/or CHIR99021 on the proportion of actively cycling NSCs. Consequently, hypoxia and/or CHIR99021 reduced the proportion of quiescent NSCs. Those results were confirmed by assessment of the density of each cell subpopulations in the dSVZ. Thus, a significant increase in the density of actively cycling NSCs was observed following hypoxia and CHIR99021 administration (**Fig R3.3D-F**). This was paralleled by an increased density of Mcm2+/Sox2- cells, which likely represent their immediate progeny (**Fig R3.3G**).

In order to investigate if the changes observed in the proportion of actively dividing NSCs resulted in changes of germinal activity, we assessed olfactory bulb (OB) neurogenesis in the different experimental groups. Quantification of OB neurogenesis was performed by estimating the density of immature (Dcx+) neurons within the OB of

P45 animals. Stereological quantifications revealed an increase OB neurogenesis in both normoxic (Ctrl) and hypoxic (Hx) animals following CHIR99021 administration. These results suggest that acute CHIR99021 administration shortly after birth have long term consequences on the activity of SVZ neural stem cells, even weeks later (**Fig R3.3H-I**).



**Figure R3.3: CHIR99021 treatment has long-term effects on the germinal activity of the dorsal SVZ.** (A) Representative picture of Sox2 Mcm2 and Ki67 staining in the dorsal subventricular zone (dSVZ) of normoxic (Ctrl) and hypoxic animals (Hx), with or without CHIR99021 treatment (Ctrl + CHIR and Hx + CHIR) at P45. (B) Diagram summarizing the distinct stages of SVZ neural stem cells (NSCs) activation: i.e. quiescent NSCs (Sox2+/Mcm2-/Ki67-, black), activated NSCs (Sox2+/Mcm2+/Ki67-, purple) and actively dividing NSCs (Sox2+/Mcm2+/Ki67+, green). (C) Graphs showing the proportion of quiescent, active and actively cycling NSCs in Ctrl, Ctrl + CHIR, Hx and Hx + CHIR p45 brains. (D-G). Graphs showing the density of quiescent NSCs (D), activated NSCs (E), actively cycling NSCs (F) and proliferating progenitor cells (G) within the dSVZ at p45 in all experimental groups. (H) Representative pictures of Dcx staining within the olfactory bulb (OB) of P45 animals. White arrows show Dcx+ cells in the different regions of the OB. (I) Graph show the density of Dcx+ cells in the OB of all experimental groups at p45. Scale bars: 10 microns in A and H, Abbreviations: NSC: neural stem cells ; SVZ: subventricular zone ; OB: olfactory bulb, Ctrl: normoxic/Ctrl+CHIR: normoxic treated with CHIR99021/Hx; hypoxic/Hx+CHIR: hypoxic treated with CHIR99021 ; P45: 45 days after birth. P-values: \* < 0.05 ; \*\*<0.01 ; \*\*\*<0.001.

## DISCUSSION – Chapter 3

Boosting endogenous stem cells to promote tissue regeneration following a lesion remains one of the main challenge in regenerative medicine. Using a pharmacological approach is a translational and pertinent way to promote recruitment of endogenous stem cells and/or progenitors of defined lineages (Azim et al., 2017). Here, we show that a short-term treatment with CHIR99021, immediately following neonatal hypoxia, induces both short and long-term effects onto neural stem cells (NSCs) activation and progenies.

We used a pharmacogenomic approach to identify a small molecule, CHIR99021, with the ability to promote germinal activity in a lineage-specific manner in the postnatal subventricular zone (Azim et al., 2017). CHIR99021 is a third generation Gsk3 $\beta$  inhibitor, which acts by enhancing Wnt/ $\beta$ -catenin signalling (Li et al., 2013; Wu and Pan, 2010). Our results, obtained in the BatGal reporter mice confirmed activation of this key signalling pathway following CHIR99021 intranasal delivery. Intranasal delivery, as a non-invasive and translational route in the context drug therapy, has increased in popularity over the past years. Indeed, it notably allows to bypass the blood brain barrier (BBB) (Fortuna et al., 2014; Hanson et al., 2008). Interestingly, chronic neonatal hypoxia similarly affected Wnt/ $\beta$ -catenin signalling, which might be causative to the spontaneous but partial cellular repair observed in this model ((Fagel et al., 2006), see also chapter 1). This is not surprising as a study carried out by Ortega showed that oligodendrocyte progenitors, which numbers are increased following hypoxia, produce canonical Wnt ligands (Ortega et al., 2016) . Of note, the glutamatergic neuron progenitor population seems particularly permissive to Wnt/ $\beta$ -catenin signalling as shown by the increased proportion of glutamatergic neurons in which Wnt/ $\beta$ -catenin signalling is active after both hypoxia and CHIR99021 administration.

In chapter 2, we showed that CHIR99021 promotes post-hypoxia cortical neurogenesis, as observed one week following the end of the hypoxic period. Although remarkable, a reactivation of neurogenesis following injury might however not always be beneficial for brain function. For instance, an increased neurogenesis occurs in the dentate gyrus following epilepsy which contributes to associated cognitive declines (Cho et al., 2015). Hence, in a regenerative context, the correct specification and

maturation of newborn neurons is particularly important to investigate. Our results demonstrated for the first time the expression of appropriate cortical layer marker by newborn neurons, in agreement with their final location. Thus, newborn neurons located in cortical layers I-IV expressed Cux1, while those in layers V-VI expressed Ctip2. Noticeably, CHIR99021 treatment promoted acquisition of those markers. The observation of newborn neurons expressing deep layer markers is particularly surprising. Indeed, deep layer neurons are produced during early embryogenesis and it is usually accepted that progenitors lose their capacity to produce them by E14.5. The re-emergence of deep layer neurons at postnatal timepoints questions the competency of the postnatal SVZ stem cells and progenitors and favours the non-restricted fate model and the leaking tap model rather than the restricted fate model (see **Fig 13.5**). Our experiments however suggest that the postnatal cortical environment is not permissive to integrate those late born neurons, which majority was lost by P45 (Akhtar and Breunig, 2015). While CHIR99021 intranasal administration promoted cortical de novo neurogenesis following hypoxia, as well as the proportion of neurons co-expressing specific cortical neuronal markers by one week following the end of the hypoxic period, it appeared to be insufficient to trigger successful cortical neuron integration. A similar observation was made in other brain regions or in different paradigms. Thus, while striatal neurogenesis can be observed following ischemia, new born neurons show only limited long-term survival (Kokaia et al., 2006). Similarly, reprogramming of NG2 cells into neurons following stab wound injury, failed to demonstrate their full integration (Heinrich et al., 2014). Combination approaches will be required to overcome this limited integration of new born neurons in the postnatal cortex. Both Bdnf and Noggin supply or a treatment with the histone deacetylase inhibitor, iANBs have been demonstrated to triggers the maturation and integration of neuroblasts into electrophysiologically mature and functional neurons, which integrate into the local neural network in adult (Niu et al., 2013). Combining CHIR99021 treatment with such approaches remains to be investigated.

Conflicting results have been reported on the role of Wnt/ $\beta$ -catenin pathway in oligodendrogenesis. Thus, while Wnt/ $\beta$ -catenin activity is required for oligodendrocyte progenitor proliferation (Azim et al., 2014b; Fancy et al., 2009; Langseth et al., 2010; Shimizu et al., 2005), it needs to be downregulated for proper oligodendrocyte

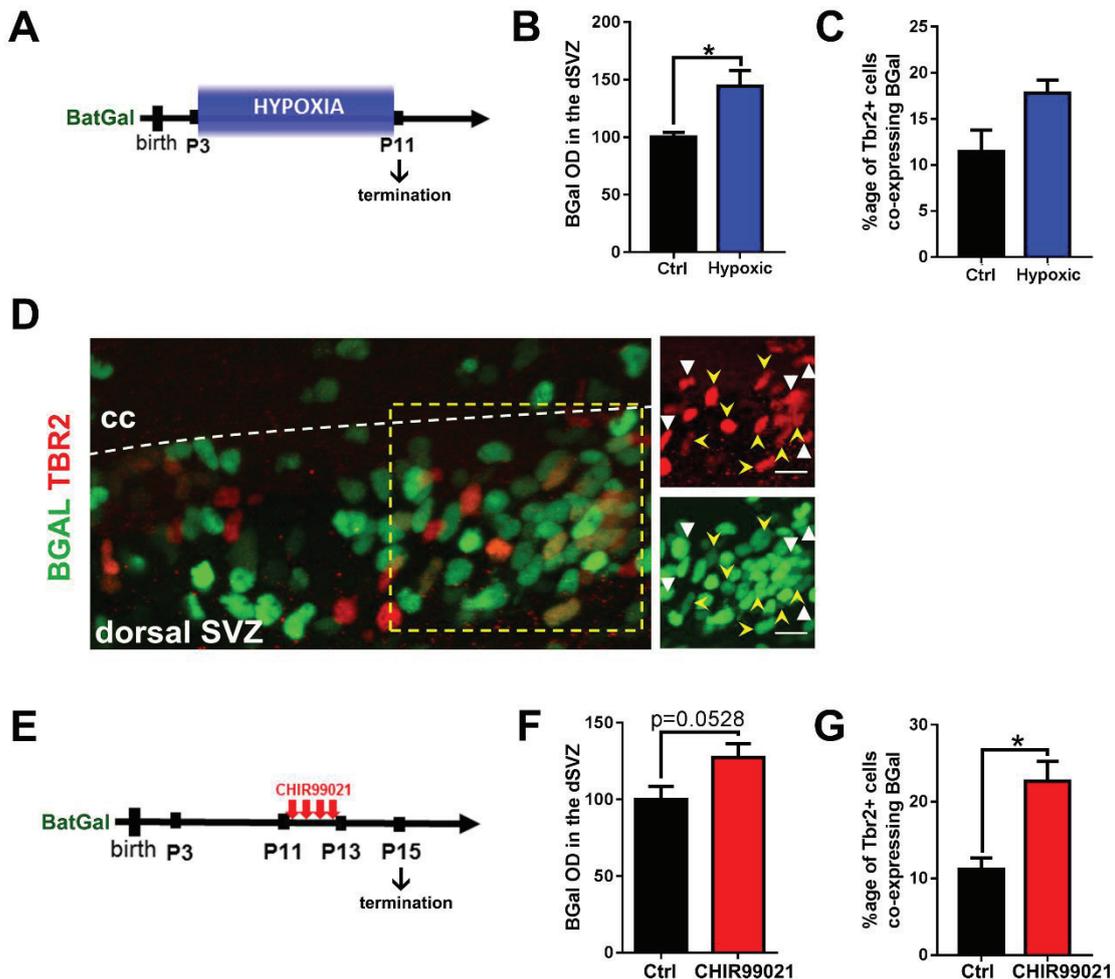
maturation (Azim et al., 2014a; Dai et al., 2014; Tawk et al., 2011). Our results contrast with this original findings by showing that a two-day CHIR99021 treatment following hypoxia promotes both oligodendrocytes, but also their maturation. Several experimental differences with previous studies may explain this discrepancy. First, the kinetics of the approach used to manipulate Wnt/ $\beta$ -catenin signalling needs to be taken into account. Indeed, previous studies used conditional Knock Out approaches, which are permanent and therefore very different from the pharmacological approach that we used in the present study. For instance, Dai and collaborators activated Wnt signalling with  $\beta$ -catenin  $\Delta$ Exon3 mice and inhibited it with  $\beta$ -catenin  $\Delta$ Exon2–6 mice (Dai et al., 2014). Pharmacological activation of Wnt/ $\beta$ -catenin signaling using CHIR99021 punctual administration following hypoxia may only affect oligodendrocyte progenitor proliferation and may not act long-enough to affect oligodendrocyte maturation. A larger pool of oligodendrocyte progenitors may increase the amount of maturing oligodendrocytes and therefore affect the ratio between immature and oligodendrocytes toward the mature stage. Moreover, because of diffusion properties, CHIR99021 may differentially impact subventricular zone cells from parenchymal cells. Thus, when oligodendrocyte progenitors start to migrate away from the subventricular zone, the activation of Wnt/ $\beta$ -catenin signaling may be interrupted (Pardridge, 2016). Finally, numerous regulatory loops in the Wnt canonical pathway. Indeed, Wnt signaling enhance the expression of Wnt signaling inhibitors Dkk1 (Chamorro et al., 2005) and Nkd1 (Angonin and Van Raay, 2013; Larraguibel et al., 2015) as well as of the Wnt signaling activators LEF/TCF genes (Arce et al., 2006). It is important to note that Dkk1 and Nkd1 act upstream from Gsk3 $\beta$  (CHIR99021 target), while LEF/TCF act downstream of Gsk3 $\beta$  (Clevers and Nusse, 2012). This may lead in the activation of a positive loop in which CHIR99021 would trigger the activation of Wnt/ $\beta$ -catenin signaling rather than its inhibition and therefore may explain the long-term effect of CHIR99021 observed on the activity of neural stem cells. Indeed, progressive loss of activity of hematopoietic stem cells with aging is associated with a decreased activity of Wnt/ $\beta$ -catenin signaling (Florian et al., 2013). This could be investigated with the BatGal mouse line (Maretto et al., 2003b). Whether CHIR99021 benefits the system by restoring the amount of myelin in the corpus callosum (significantly decreased in hypoxic brains until at least P30, (Scafidi et al., 2014)) remains to be investigated as well. This could be done by MBP or MOG immunostaining or western blotting, or by using diffusor tensor images (DTI) (Scafidi et al., 2014). Moreover, as several

behavioural deficits observed in hypoxic mice are due to defects of myelin, it would be interesting to perform behavioural tests such as the inclined beam-walking task (Scafidi et al., 2014).

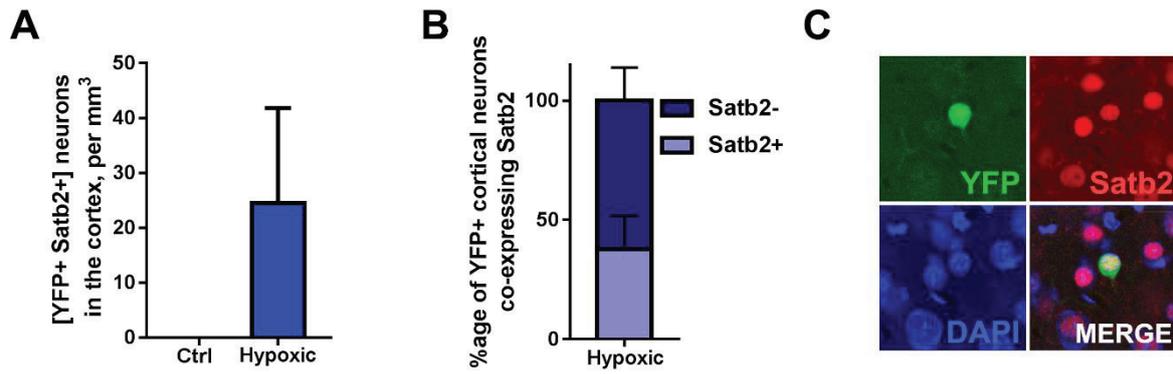
One of the main concern when using approaches aimed at activating NSCs is their possible long-term depletion, which might have long-term consequences on tissue homeostasis (Kinder et al., 2010). It is possible to separate the pool of SVZ stem cells in three groups according to their expression of select proliferative markers. Quiescent stem cells constitute a reservoir of non-cycling cells, which have the potential to be activated under certain conditions (Codega et al., 2014b). These quiescent NSCs may be primed, before cycling as recently demonstrated in single cell RNA-sequencing experiments (Llorens-Bobadilla et al., 2015b). Once activated, they produce glial and neuronal cells with different kinetics (Maslov et al., 2004a; Morshead et al., 1998) to eventually become exhausted several months later (Calzolari et al., 2015). Here we used expression of Sox 2 and of two proliferative markers, Mcm2 and Ki67 to identify and quantify these different cell populations. While actively proliferating cells express Ki67, Mcm2 is also expressed in cycle competent cells (Maslov et al., 2004b), as supported by our observation of a subpopulation of Sox2<sup>+</sup> cells expressing Mcm2 but not Ki67. Using these markers, we present evidences that neonatal hypoxia leads to a partial depletion of the pool of quiescent stem cells by P45. Importantly, CHIR99021 administration does however not result in an additional depletion. In agreement with these observations, we found that the pool actively cycling neural stem cells was increased more than one month after CHIR99021 administration in the hypoxic brain. This was paralleled by an increased olfactory bulb neurogenesis at P45. Thus, short-term treatment following hypoxia may have long-term effects on the balance of quiescent vs. activated stem cells, which will need to be investigated in more details. This could be achieved by flow cytometry analysis of quiescent vs activated NSCs as recently performed by several groups (Codega et al., 2014b; Daynac et al., 2015). Alternatively, one could perform behavioral tests to see whether the enhanced olfactory bulb neurogenesis has functional consequences (Valley et al., 2009).

Altogether, we show here promising effects of CHIR99021 to boost post-hypoxia cortical regeneration on the oligodendrocyte lineage. Additional approach could be used to improve its effect on cortical glutamatergic neuron repair. However, CHIR99021 might have long-term detrimental effects on brain tissue homeostasis by depleting the quiescent pool of SVZ stem cells and promoting their activity, which remain to be explored in more details.

## Supplementary data – Chapter 3



**Figure RS3.1 : Both chronic hypoxia and intranasal administration of CHIR99021 activate Wnt/ $\beta$ -catenin signaling in the dorsal subventricular zone.** (A) Experimental design for B-C graphs. BatGal mice, which express BGal under the control of a Wnt/ $\beta$ -catenin target promoter, were subjected to hypoxia from P3 to P11 and terminated at P11. (B) Optical density (OD) of BGal staining in the dorsal subventricular zone (dSVZ) in Normoxic (Ctrl) and Hypoxic mice. (C) Percentage of the Tbr2+ cell population co-expressing BGal in the dSVZ of Ctrl and Hypoxic mice. (D) Representative picture of BGal and Tbr2 stainings in the dSVZ and the above corpus callosum (cc). Microphotographies show the Tbr2+/BGal- cells (white arrows) and the Tbr2+/BGal+ cells (yellow arrows). (E) Experimental design for F-G graphs. CHIR99021 was administered intranasally four times between P11 and P13 in BatGal mice. Mice were terminated at P15. (F) Optical density (OD) of BGal staining in the dSVZ in control (Ctrl) and mice treated with CHIR99021 (CHIR99021). (G) Percentage of the Tbr2+ cell population co-expressing BGal in the dSVZ of Ctrl and mice treated with CHIR99021. Scale bar: 20 microns in D. Abbreviations: BGal: b-galactosidase ; OD: optical density ; P3/P11/P13/P15: respectively 3, 11, 13 and 15 days after birth ; Ctrl : control ; dSVZ: dorsal subventricular zone ; cc: corpus callosum. P-values: \* <0.05.

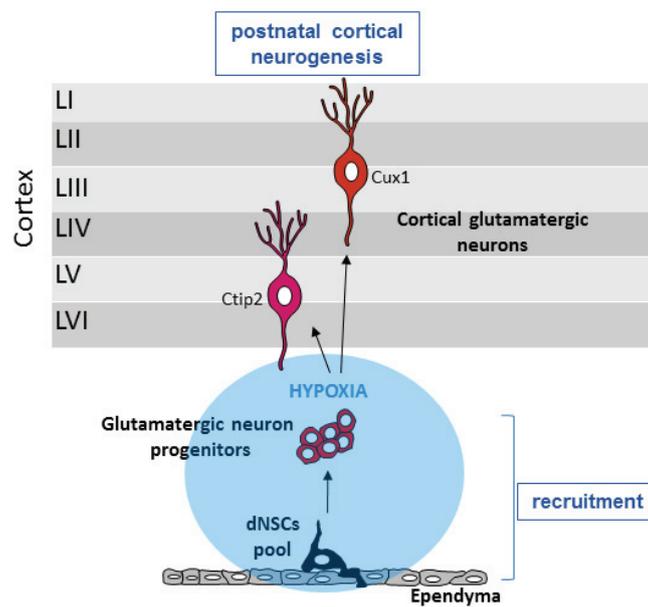


**Figure RS3.2: Hypoxia induces the postnatal production of cortical Satb2+ neurons originating from the dorsal subventricular zone. (A-B)** Density of neuron-like YFP+ cells co-expressing Satb2 in control (Ctrl) and Hypoxic brains at P19, following dorsal electroporation of a CRE plasmid in ROSA-YFP mice, 1 day after birth (A). Percentage of YFP+ cortical neurons co-expressing Satb2 in hypoxic brains (B). **(C)** Microphotographs representing a YFP+/Satb2+ cortical neuron. Abbreviations: %age: percentage; Ctrl: control. Scale bar: 20 microns in C.

## GENERAL CONCLUSION & PERSPECTIVES

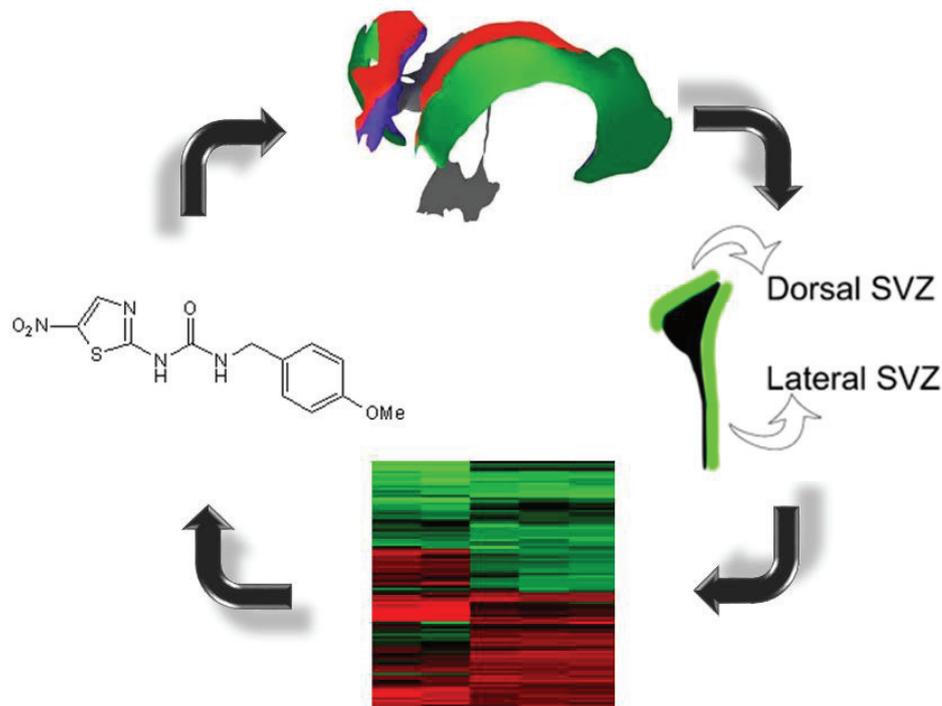
### General Conclusion

In this manuscript, we first showed that the neural stem cells and progenitors from the dorsal microdomain of the subventricular zone (dSVZ) kept the competency to produce cortical glutamatergic neurons in the young brain, in a pathological context (**Fig C1**).



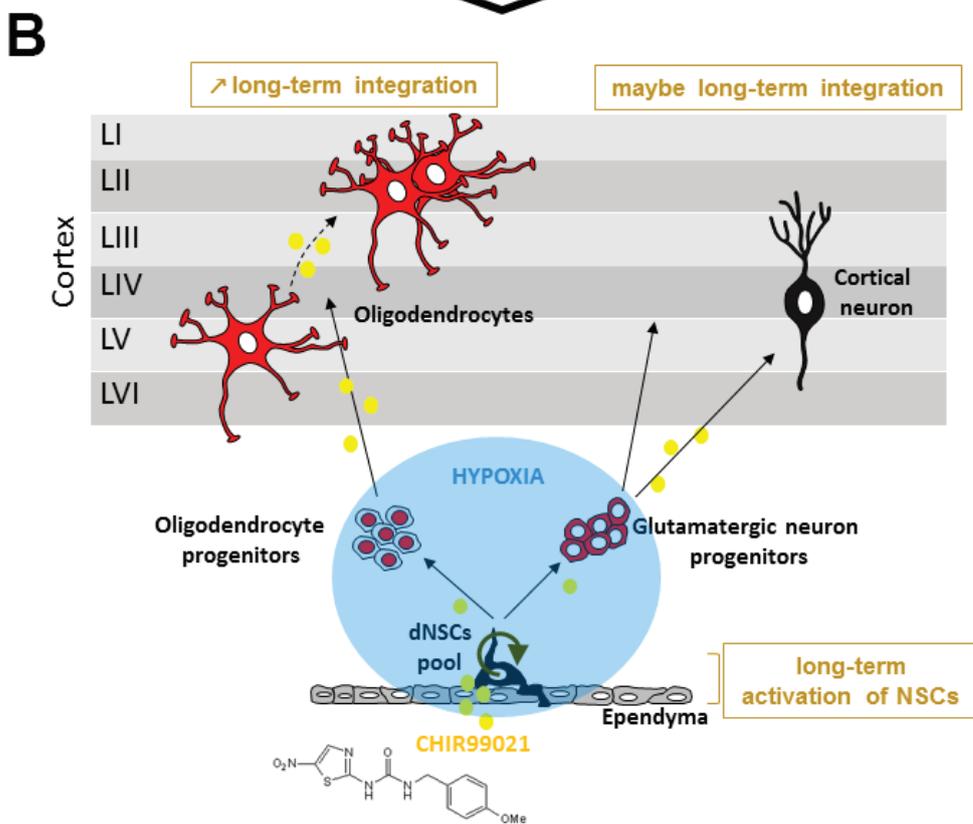
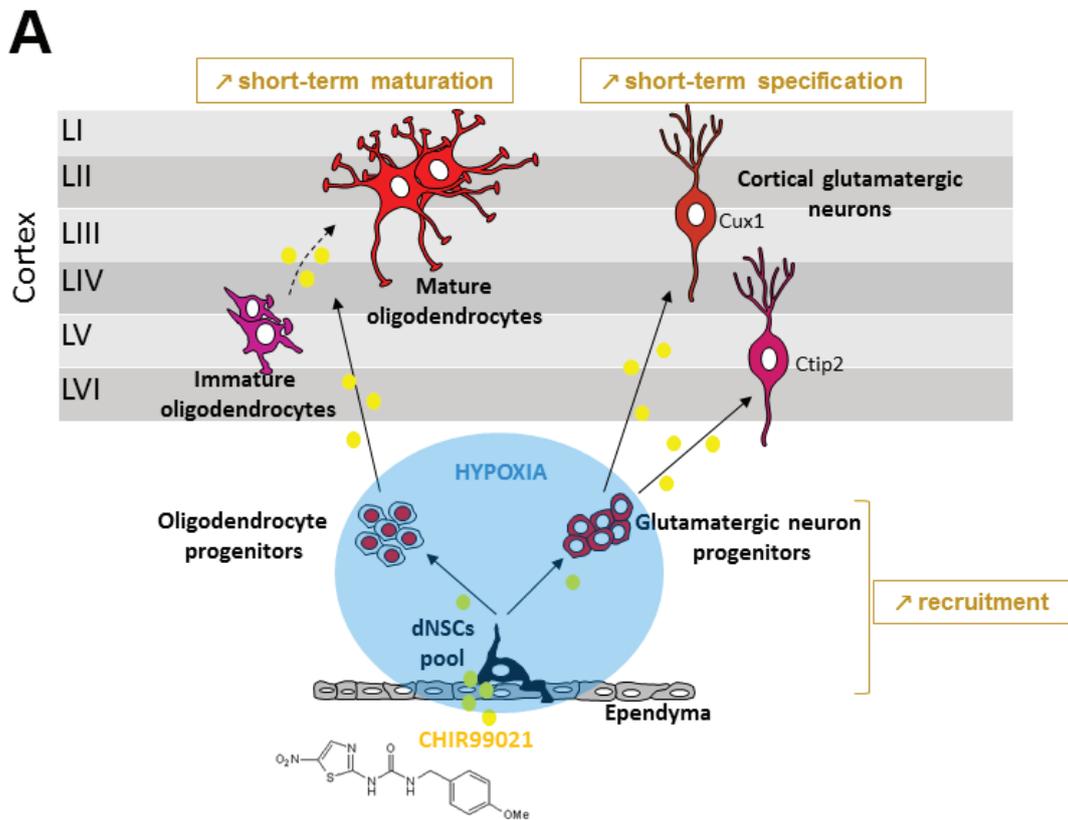
**Figure C1: Chronic hypoxia induces a reactivation of cortical glutamatergic neurogenesis.** Neural stem cells and glutamatergic neuron progenitors persist in the dorsal microdomain of the subventricular zone after birth. In physiological condition, they produce olfactory bulb neurons in addition to glial cells. In the context of chronic hypoxia, they are recruited, to produce cortical glutamatergic neurons expressing notably Ctip2 and Cux1. Abbreviation: dNSCs: dorsal neural stem cells.

We then used a bioinformatics approach allowing to identify drug-like compounds with the capacity to influence the cellular fate of subventricular zone cells, and validated the approach *in vivo* (**Fig C2**).



**Figure C2: Strategy used to identify small molecules for lineage specific manipulation of SVZ germinal activity.** The SVZ is divided into different microdomains according to the cell lineages of the stem cells they contain. Lineage associated transcriptional signatures were obtained from the comparison of dorsal and lateral microdomain stem cells and progenitors transcriptome. They were compared to the transcriptional signatures associated with the effect of small molecules. Small molecules inducing similar transcriptional changes than the one associated with specific stem cell fates were tested in vivo and their consequences on SVZ stem cells and progeny investigated.

Notably, we identified Gsk3 $\beta$  inhibitors as small molecule class promoting the dorsal fates of the SVZ, oligodendrocyte and glutamatergic neuron lineages. Finally, we showed that intranasal administration of CHIR99021, a Gsk3 $\beta$  inhibitor, following chronic hypoxia induces both short-term and long-term effects onto neural stem cells activation and progenies. Noticeably, while CHIR99021 failed to trigger significant long-term integration of cortical neurons, it promoted both cortical oligodendrocyte short-term maturation and long-term integration originating from the dSVZ (**Fig C3**).



**Figure C3: CHIR99021 intranasal administration affects both short-term and long-term dorsal subventricular zone (SVZ) neural stem cell activation and progenies in the context of chronic hypoxia. (A)** Short-term effects of CHIR99021 on dorsal SVZ cells and progenies following hypoxia. CHIR99021 promotes short-term dorsal SVZ spontaneous cell recruitment toward the cortex. Cortical oligodendrocyte mature faster and more cortical glutamatergic neurons are produced. **(B)** Long-term effects of CHIR99021 on dorsal SVZ cells and progenies following hypo. CHIR99021 promotes long-term activation of dorsal SVZ stem cells. Cortical oligodendrocytes integration is increased while cortical neurons integration might be possible.

To conclude, we identified the dorsal microdomain of the SVZ as an amenable source of endogenous stem cells for cortical repair following chronic hypoxia. The following paragraphs highlight several perspectives to this project.

## Perspectives

### **1. Better understanding of the mechanisms of action and functional consequences of CHIR99021 administration following hypoxia**

#### **a. Functional and behavior tests to highlight the potential of CHIR99021 in a translational perspective**

Regenerative medicine is based on either bringing into the injured tissue exogenous stem cells with the capacity to differentiate into a specific cell type or to stimulate endogenous stem cells to promote tissue repair. Here we proposed a strategy based on the second approach. We stimulated endogenous stem cells from the dorsal subventricular zone (dSVZ), during a postnatal window of time characterized by a high plasticity and permissivity. The consequence of the stimulation was studied in detail at the cellular level, on the cortical dSVZ derived oligodendrocytes, glutamatergic neurons as well as on the dSVZ NSCs. However, in order to use this approach in a translational context, some crucial information are still lacking at the tissue level and at the functional level. Indeed, while we showed that CHIR99021 administration promotes oligodendrocyte generation and maturation, it would be interesting to gain deeper knowledge in their capacity to myelinate axons in the cortex, but also in the corpus callosum, also largely affected by the hypoxic period, both in rodent and human (Jablonska et al., 2012; Pujol et al., 2004). To do this, analysis of myelin staining in these area of interest would be pertinent, as well as electronic microscopy analysis. In addition, ex vivo diffusion tensor image (DTI) analysis would allow a functional analysis of myelination in the corpus callosum for instance (Mori and Zhang, 2006). Finally, even though the chronic hypoxia lesion is mild, some precise behavioral defects persist several months after the injury such as a decreased efficiency with the beam walking task test (Scafidi et al., 2014). Whether CHIR99021 treatment improves long-term behavioral deficits remains to be investigated and would allow to conclude on a beneficial rather than detrimental effect of CHIR99021 on post-hypoxia spontaneous regeneration.

**b. Manipulating cortical microenvironment to allow glutamatergic neurons to specify and integrate correctly.**

Glutamatergic neurons originating from the dorsal SVZ failed to integrate into the cortex following chronic hypoxia, although they appeared to migrate correctly to the injury site and to express specific glutamatergic cortical markers. Indeed, those cortical markers triggered neuronal correct specification at embryonic timepoints during physiological corticogenesis. Even though the results obtained with CHIR99021 were not sufficient to conclude on a promotion of neuronal cortical repair, CHIR99021 triggered the generation of cortical neurons originating from the dorsal SVZ and promoted their specification as well as oligodendrocyte maturation in the cortex. This suggests that CHIR99021 might influence cortical microenvironment shortly after administration. Inflammation is often an obstacle to regeneration and many regenerative approaches tend to reduce inflammation to promote cellular repair (Donega et al., 2014b). Interestingly, our preliminary data revealed a decrease in Iba1+ cell density following CHIR99021 administration in the hypoxic cortex shortly after intranasal administration, suggesting a decreased diffuse inflammation, in accordance with the results obtained in the ischemic cortex following Gsk3 $\beta$  inhibitor administration (Yilmaz and Granger, 2008; Zhou et al., 2011) (**Fig SP1**). However, contrary to hypoxia/ischemia which leads to an acute inflammation, detrimental for cellular regeneration (Liu and McCullough, 2013), chronic hypoxia diffuse inflammation appears to be more permissive to efficient tissue repair (Bi et al., 2011a; Fagel et al., 2006, 2009). The role of diffuse inflammation in cellular regeneration following chronic hypoxia as well as the pertinence of using an approach which influences this phenomenon to promote cortical repair remains to be investigated further. It would be in particular of interest to investigate in more details the type1 and type 2 responses which seems to represent the imbalance between the activity of microglia and astrocytes (Siegel and Zalcman, 2009) following both hypoxia and CHIR99021. Information on how the cortical microenvironment changes after birth and following chronic hypoxia would be interesting to improve our approach. This could be done by combining different drugs at different timings for instance.

## **2. Investigate the molecular mechanisms underlying the predicted effects of the drugs**

### **a. Gsk3 $\beta$ inhibitors do not only affect Wnt/ $\beta$ -catenin signaling**

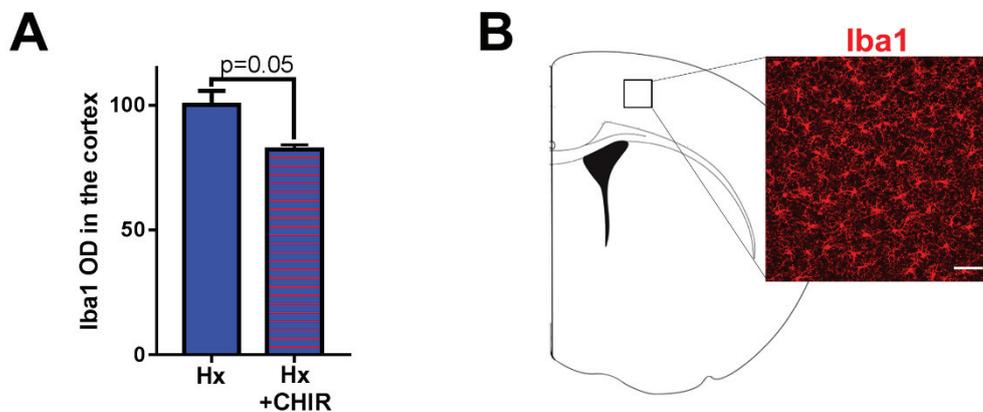
During my PhD we focused on the effect on Gsk3 $\beta$  inhibitors on Wnt/ $\beta$ -catenin signaling because of the extended literature highlighting their link (Voronkov and Krauss, 2013). I therefore assumed that the effect of CHIR99021 in the different context was due to its ability to activate Wnt/ $\beta$ -catenin pathway. However, Gsk3 $\beta$  is also involved in other signaling pathways. For instance, Gsk3 $\beta$  antagonizes sonic hedgehog (Shh) signaling pathway by promoting Gli protein degradation (Chen et al., 2011). Gsk3 $\beta$  is also implicated in Notch signaling as shown by its capacity to directly bind the intracellular subdomain of notch protein itself, therefore stabilizing the protein and activating Notch signaling (Foltz et al., 2002). As a consequence, Gsk3 $\beta$  inhibitors drive not only Wnt/ $\beta$ -catenin signaling activation, but also Shh activation and Notch signaling inhibition (R., 2013). Investigating the implication of these other pathways on the effect of CHIR99021 following chronic hypoxia would allow a better understanding of the molecular networks implicate in cortical regeneration.

### **b. Novel drugs will highlight novel molecular mechanism to influence subventricular zone neural stem cell fate**

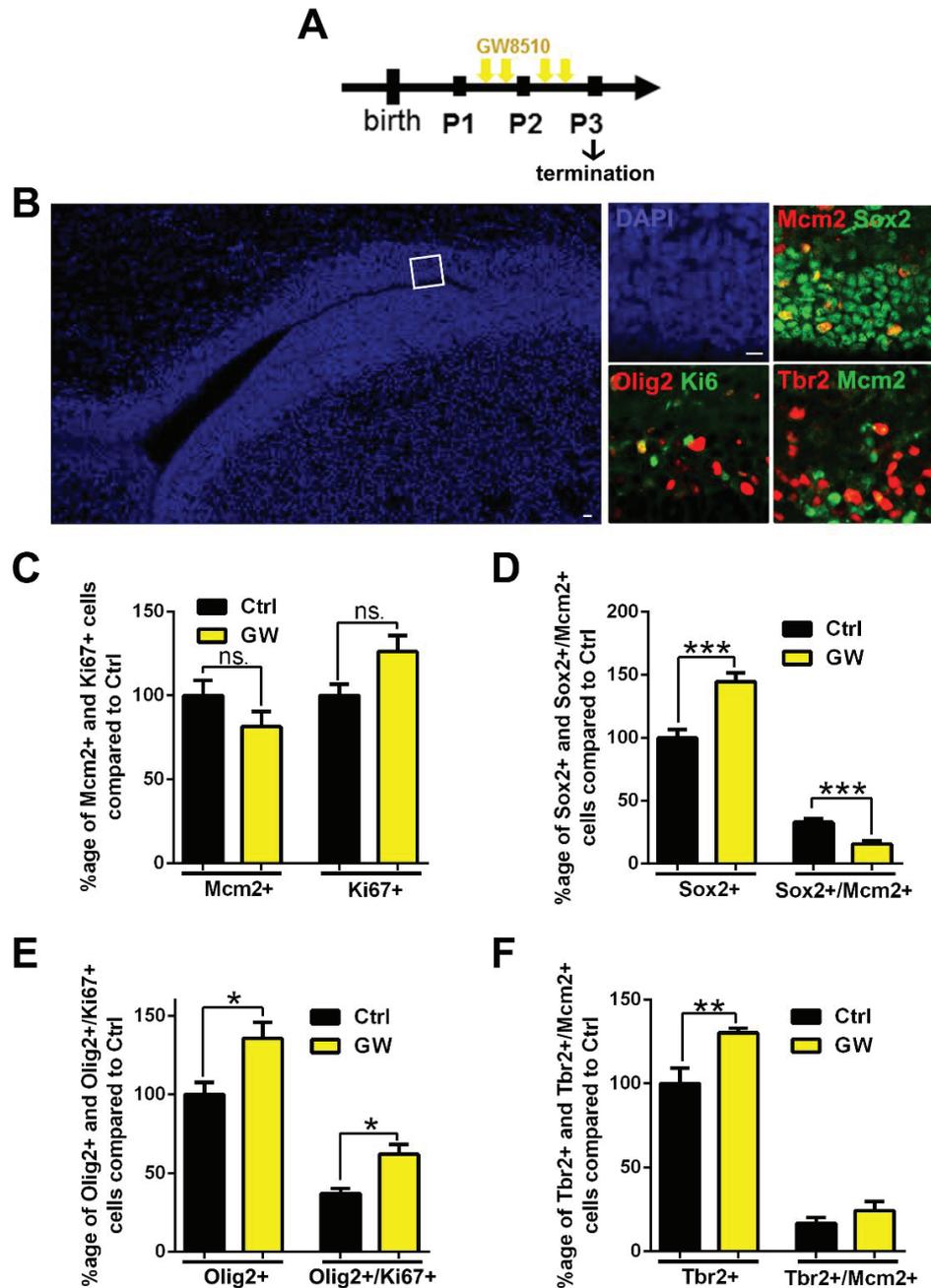
The pharmacogenomic approach developed by Kasum Azim highlighted several classes of small molecules. LY-294002, inhibitor of PI3K/Akt/mTor signaling, displayed in vivo pro-oligodendroglial properties accordingly to the prediction made in the bioinformatics analysis and could be used in the context of demyelinating injuries such as multiple sclerosis for instance (Lubetzki and Stankoff, 2014). We decided to focus our research mainly on Gsk3 $\beta$  inhibitors because of their rejuvenating and more particularly dorsalizing effects, which was of interest in the context of chronic hypoxia. In addition, Gsk3 $\beta$  inhibitors were already widely used notably for activating Wnt/ $\beta$ -catenin signaling, and previously studied in the context of postnatal CNS injuries

(Lambert et al., 2016). However, other small molecules seemed promising and could be used in a regenerative context.

Among others, we've tested the effect of the Cdk2 inhibitor GW8510, as a potential "dorsalizing" small molecule. Intraventricular administration of GW8510 promoted both populations of oligodendrocytes and glutamatergic neurons progenitors without affecting the pool of quiescent stem cells, while not affecting proliferation 24 hours after the last injection (**Fig SP2**). This preliminary result strengthens the bioinformatics analysis we performed and suggests that it is possible to promote postnatally dorsal lineages using different molecular mechanisms than the one we highlight in this manuscript, through Wnt/ $\beta$ -catenin signaling. Indeed, the literature about how influencing the cell cycle influences the commitment of VZ-SVZ neural stem cells is flourishing and it would be interesting to better understand the mechanisms of action of GW8510 at the molecular level.

**Supplementary data – Perspectives:**

**Figure SP1: CHIR99021 decrease inflammation in the cortex of hypoxic mice.** Mice were subjected to a hypoxic environment from P3 to P11 followed by intranasal administrations of CHIR99021 or vehicle. **(A)** Graph shows the percentage of the Iba1 staining optical density in hypoxic brains treated with CHIR99021 (Hx+CHIR) compared to controls (Hx). **(B)** Representative picture of Iba1 staining in the cortex, at the location of the insert on the coronal section diagram. Scale bar: 50um. Abbreviation: OD: optical density; Hx: hypoxic/Hx+CHIR: hypoxic with CHIR99021 treatment. p=p-value.



**Figure SP2: GW8510 promotes dorsal lineages of the subventricular zone.** (A) Experimental design. GW8510 was injected intraventricularly twice a day at P1 and P2 in OF1 mice before to be terminated at P3. (B) Overview of a subventricular zone on a coronal P3 with DAPI counterstaining. White insert shows the location of the microphotographies. Microphotographies show respectively DAPI, Mcm2 and Sox2, Olig2 and Ki67, Tbr2 and Mcm2 stainings in the dorsal subventricular zone. (C) Percentage of the density of proliferating cells (Mcm2+ and Ki67+) in the dorsal subventricular zone of mice treated with GW8510 (GW) compared to the controls (Ctrl). (D) Percentage of the density of stem cells (Sox2+ cells) in the dorsal subventricular zone of mice treated with GW8510 compared to controls and percentage of active stem cells (Sox2/Mcm2+) in the stem cell population. (E) Percentage of the density of oligodendrocyte progenitors (Olig2+ cells) in the dorsal subventricular zone of mice treated with GW8510 compared to controls and percentage of proliferating oligodendrocyte progenitors (Olig2+/Ki67+). (F) Percentage of the density of glutamatergic neuron progenitors (Tbr2+ cells) in the dorsal subventricular zone of mice treated with GW8510 compared to controls and

percentage of proliferating glutamatergic neuron progenitors (Tbr2/Mcm2+). Scale bars: 20um in B. Abbreviations: %age: percentage; P1/P2/P3: respectively 1, 2 and 3 days after birth; Ctrl: control; GW; treated with GW8510; ns=not significant. P-values: \* < 0.05; \*\* < 0.01; \*\*\* < 0.001.

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