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CONTROL OF ADULT NEURAL STEM CELL PROPERTIES (ANSCS) BY THE RETINOBLASTOMA PROTEIN,PRB

by
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AN ABSTRACT OF THE THESIS OF

Dayana Joseph El Hayek for Master of Science
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Title: Control of Adult Neural Stem cell Properties (aNSCs) by the Retinoblastoma Protein, pRb.

Neurogenesis is an ongoing developmental process throughout life in the adult mammalian brain; however, adult Neural Stem Cells (aNSCs)/progenitors display a low proliferative potential and restricted differentiation capacity *in vivo*. During development, the tumor suppressor gene, pRb, regulates distinct aspects of neurogenesis including neuronal proliferation, differentiation and migration. We have recently investigated the role of Rb in adult neurogenesis and found that it regulates the rate of proliferation of aNSCs/progenitors that are found in the adult subventricular zone (aSVZ) without affecting subsequent neuronal differentiation in the olfactory bulb. Here, we examined how Rb controls the properties of aNSCs/progenitors in culture including their proliferation rate, self-renewal capacity and differentiation potential. To do this, we induced a temporal deletion of Rb specifically in aNSCs/progenitors in 8 week-old mice using a Nestin-CreERT2-YFP Tamoxifen-inducible system and Rbfloxed/floxed mice. 5 days following treatment, we dissected and dissociated the aSVZ tissue and performed neurosphere assays from Rb+/- (control) and Rb-/- (mutant) animals. Cells were cultured at low and high densities in media supplemented with FGF2 and EGF for 6 days before passaging, and, cell sorting was performed toisolate recombined or green fluorescent cells only. In addition, we conducted birth-dating experiments*in vivo* using Bromodeoxyuridine (BrdU) to study the self-renewal property of aNSCs in the absence of Rb. Our results showed that:**1)**Rb controls the proliferation of adult progenitors as evidenced by the significant increase in the total number and the average size of primary neurospheres generated from Rb-/- NSCs versus Rb+/- NSCs. This difference was even more pronounced among recombined cells isolated by cell sorting.**2)**Rb does not seem to regulate the self-renewal capacity of aNSCsince the number of secondary neurospheres generated from these cells in culture did not change in the absence of Rb. This was further confirmed by long-term labeling experiments using BrDU incorporation in which we did not detect any change in the rate of division of aNSCs in the aSVZ.**3)**Loss of Rb does not affect the multipotential capacity of aNSCs/progenitors *in vitro*. Rb-null NSCs/progenitors were thus able to differentiate into astrocytes and neurons similar to Rb+/- NSCs. Altogether, these results are consistent with our *in vivo* findings and demonstrate that loss of Rb leads to enhanced neurogenesis both *in vivo* and *in vitro*. This data have direct implications for the potential manipulation of the Rb pathway to expand the pool of progenitors derived from NSCs for regenerative purposes in the future.

CONTENTS

ILLUSTRATIONS

TABLES

LIST OF ABBREVIATIONS

%	percent
+	positive
ABAM	Antibiotic-antimycotic
Asc1/Mash1	Aschaete-Scute
ANP	amplifying neural progenitors
BMP	bone morphogenetic protein
BP	basal progenitor
BrdU	5-bromo-2'-deoxyuridine
CB	calbindin
Cdks	cyclin dependent kinases
cDNA	complementary deoxyribonucleic acid
CNS	central nervous system
CNTF	ciliary neurotrophic factor
CP	cortical plate
CR	calretinin
Cre	Cre recombinase enzyme
Ct	control
d	day
DCX	doublecortin
DG	dentate gyrus
Dlx	*Distal-less* related mouse homologue
DNA	deoxyribonucleic acid
E	embryonic day
EGF	epidermal growth factor

ECM	extracellular matrix
E2F	E2 transcription factor
ERT2	modified estrogen receptor
FGF-2	fibroblast growth factor-2
FL	fiber layer
GABA	gamma-aminobutyric acid
GCL	granule cell layer
GCs	granule cells
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
GL	glomerular layer
GLAST	glutamate-aspartate transporter
hr	hour
IL-6	interleukin-6
Jak	Janus kinase
Kg	kilogram
Ki67	Nuclear antigen present in nuclei of cycling cells
mg	milligram
min	minute
ml	milliliter
Mut	mutant
MZ	marginal zone
NEP	neuroepithelial progenitors
NeuN	neuronal nuclear protein
NeuroD	neuronal differentiation

Neurog	Neurogenin
Ngn	Neurogenin
NSC	neural stem cells
OB	olfactory bulb
ºC	Degrees Celsius
P	post-natal day
PP	preplate
$p27^{kip1}$ /p27	tumor protein 27
PBS	phosphate buffered solution
PCR	polymerase chain reaction
PFA	paraformaldehyde
PNS	Peripheral nervous system
pRb	retinoblastoma protein
Rb	retinoblastoma
RG	radial glia
RMS	rostral migratory stream
SGZ	subgranular zone
Shh	Sonic hedgehog
Smo	Smoothened
SP	subplate
SVZ	subventricular zone
TH	tyrosine hydroxylase
Ttp53/p53	tumor protein 53
VZ	ventricular zone
YFP	yellow florescent protein

μl	microliter
μm	micron

CHAPTER I

INTRODUCTION

A. Neurogenesis

1. Definition and historical overview

Neurogenesis is the process of generating new and functional neurons from neural stem cells (NSCs). It has been traditionally perceived as a mechanism that occurs only during embryonic and perinatal stages in mammals in order to generate all the neurons that the central and peripheral nervous systems (CNS and PNS) are comprised of (Cajal, 1913). Moreover, once differentiated, neurons become post-mitotic cells, a process that is permanent and irreversible (Cajal, 1913). Thus, nerve regeneration in the adult brain has been long denied and this has been a central dogma in modern neuroscience since the time of its founders, Santiago Ramon y Cajal and Camillo Golgi (Cajal, 1913). In the second half of the twentieth century, Altman and colleagues made an intriguing discovery using [H^3]-thymidine labeling and identified new born cells in the adult rat hippocampus, yet the identity of these cells remained elusive (J Altman & Das, 1965) and these reports did not leave much impact among the scientific community at the time. Interestingly, about three decades later, neuroscientists discovered the existence of multipotent adult neural stem cells (aNSCs) in the adult mammalian brain (B. Reynolds & Weiss, 1992; Richards, Kilpatrick, & Barlett, 1992). These are astrocyte-like and quiescent cells derived from radial glia during development and found in two mains regions in the brain: the subgranular zone (SGZ) in the dentate gyrus in the hippocampus and the adult subventricular zone (aSVZ) lining the walls of the lateral ventricles(C Lois & Alvarez-Buylla, 1993; Rousselot, Lois, & Alvarez-Buylla, 1995). aNSCs give rise to specific types of neurons in both

regions under normal physiological conditions (Chojnacki & Weiss, 2008; Morshead, Craig, & van der Kooy, 1998)but were shown to possess a much broader differentiation potential *in vitro*(B. Reynolds & Weiss, 1992). Thereafter, it is now widely accepted that neurogenesis is a developmental process that is ongoing throughout lifein the mammalian brain albeit at a lower rate (for review; Brus et al. 2013), and this is true in rodents(Eriksson et al., 1998; Kokoeva, Yin, & Flier, 2005; B. Reynolds & Weiss, 1992) and infants in humans (Eriksson et al., 1998; Kukekov, Laywell, Suslov, Davied, & Scheffler, 1999).

2. Neural tube formation and CNS development

During development, neural induction is the initial step that leads to the formation of the neuroectoderm from the developing ectoderm. The neuroectoderm further develops into a neural plate with neural folds, which give rise to the neural tube through a process called neurulation. In mice, the neural tube is formed around embryonic day 7.5 (E7.5); neurogenesis begins at the same time and ends around E17.5. Differentiation of the neural tube occurs on three different levels: first, at the *anatomical level* whereby the neural tube and its lumen tighten to form the distinct vesicles of the brain and spinal cord. Second, at the *tissue level*, distinct groups of cells position themselves to form functional regions of the brain and spinal cord, a process called regionalization. Finally, at the *cellular level* where neuroepithelial cells differentiate into either neurons or supportive cells known as glia (Gilbert et al., 2013, Developmental Biology, 10th edition). After the complete formation of the neural tube, three primary brain vesicles are generated: 1) The prosencephalon or forebrain which later gives rise to the telencephalon including the cortex, the basal ganglia, the

hippocampus and the olfactory bulbs (Yokoyama et al.), and, the diencephalon which includes the thalamus, hypothalamus and optic cups (retina), 2) The mesencephalon or midbrain and, finally, 3)the rhombencephalonor hindbrain which is comprised of the metencephalon that gives rise to the cerebellum and pons, and, the myelencephalon that forms the medulla. In addition, four ventricles are formed in the spaces between these subdivisions in the adult brain: the two lateral ventricles, the third and fourth ventricles (Purves et al., 2012, Neuroscience, 5th edition).

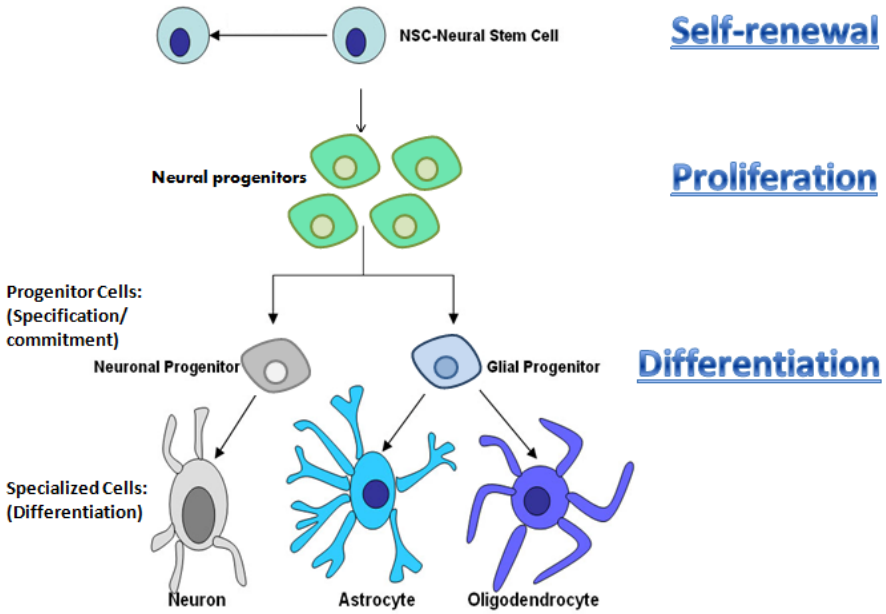


Figure1: The neural stem cell lineage. Neurogenesis includes three main steps: self-renewal, proliferation and differentiation. NSCs have the ability to self-renew and give rise to neural progenitors by asymmetrical divisions (Gage, 2000). These progenitors undergo several rounds of symmetrical divisions to generate specialized/committed cells including neuronal progenitors that will differentiate into neurons, and, glial progenitors that give rise to astrocytes and oligodendrocytes.

3. Embryonic development

Neurogenesis is a multistep process that includes cell proliferation, differentiation and migration (Figure 1) (Palmer et al., 1997; Gage, 2000). NSCs constitute a relatively slow-dividing population of multipotent cells that are capable of self-renewal and generation of neural progenitors. These early progenitors are rapidly-dividing and known as transit-amplifying cells that undergo several rounds of cell division to amplify the pool of progenitors. Late-born progenitors become more restricted in their lineage (Tri-, Bi or unipotent) as they commit to a specific fate (neuronal or glial). For instance, they give rise to neuroblasts or immature neurons that exit the cell cycle and initiate their differentiation program (Figure 1). Terminal differentiation is usually accompanied with cell migration to the final destination and/or a maturation step.During development, NSCs and progenitors show distinct patterns of gene expression and thus, are heterogeneous in space and time (S Temple, 2001). As such, a variety of signaling molecules derived from organizing centersin combination with the expression of distinct transcription factors promotes their differentiation into different subtypes of neurons, astrocytes and oligodendrocytes (Doetsch et al., 1999). Neurogenesis precedes oligodendrogenesis and astrogliogenesis in development (Figure 2, see next sections for detail) (Okano et al., 2009; Falk et al., 2009).

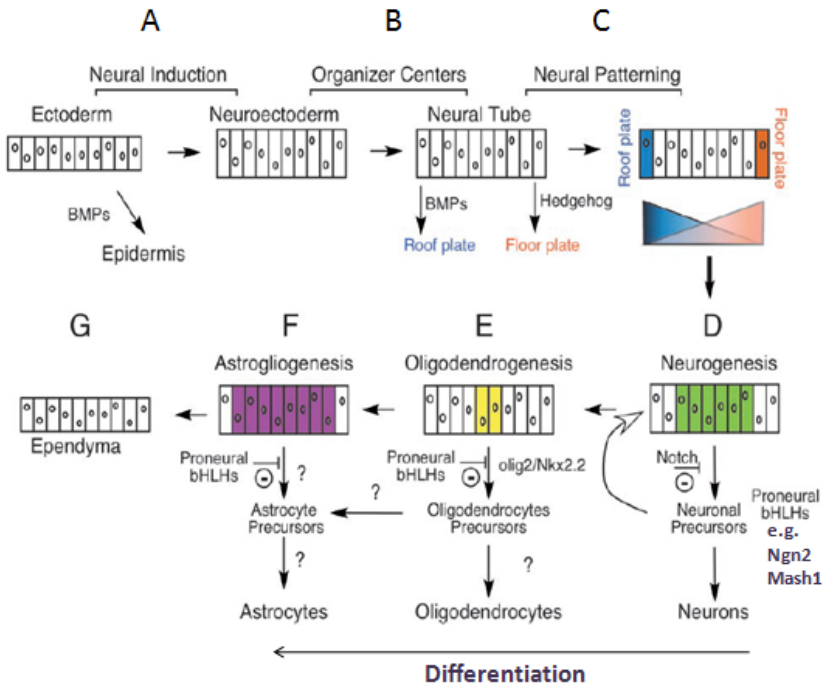


Figure2: Cellular and molecular mechanisms controlling neuronal and glial differentiation. Neural induction and patterning rely on the interaction between several signaling molecules such as Bone Morphogenetic Proteins (BMPs) and Sonic Hedgehog (Morshead et al., 1998; Morshhead et al., 1994), which are secreted by signaling centers such as the roof plate and floor plate, respectively. Neurogenesis is also regulated by the spatio-temporal expression of an array of transcription factors such as the pro-neural bHLHs genes (e.g. Ngn2, Mash1) and other signaling molecules such as the Notch/Delta pathway. At later developmental stages and following neurogenesis, distinct groups of transcription factors (e.g. Olig2/Nkx2.2) play a central role in oligodendrogenesis and astrogliogenesis (as modified from (Purves et al., 2012).

4. Embryonic NSCs and progenitors

As mentioned earlier, distinct populations of NSCs/progenitors are found in the developing telencephalon and generate the different types of neural cells according to a well-defined spatio-temporal gene regulation. For instance, the neuroepithelial progenitors (NEPs) are the first population of NSCs that lines the wall of the neural tube during early neurogenesis (E8-E11). These cells connected to the apical and basal surfaces of the single-layer neuroepithelium and divide symmetrically to generate two identical daughter cells found in the ventricular zone (VZ) (Figure 3 and 4a), or,

asymmetrically to produce a NEP, and, a VZ neuron or neuronal progenitor, which undergoes radial migration into the pre-plate(Utz, Dimova, Oppenlander, & Kerkhoff, 2010). Hence, the first wave of post-mitotic neurons occupies the PP during cortical development in rodents (Meyer et al., 2000), and, is produced by NEPs in the VZ, which is the major proliferative zone (Figures 3 and 4a). Starting around E13, NEPs give rise to a second class of NSCs known as radial glia (RG), which express specific markers such as GLAST (glutamate-aspartate transporter) and the glial fibrillary acidic protein (GFAP) (reviewed in(Conti & Cattaneo, 2010)). RG cells divide asymmetrically to generate a RG, and, either a post-mitotic neuron that migrates to the cortical plate, or, a basal progenitor (BP) which divides few more times in the subventricular zone (SVZ) and forms neuroblasts. The latter cells are immature neurons that exit the cell cycle and differentiate into cortical neurons (Figures 3 and 4b) (Haubensak et al., 2004; Noctor et al., 2004). Hence, the SVZ becomes the second proliferative zone as of E13(Smart, 1973) and from E15 onwards, the VZ starts to shrink referring to the end of neurogenesis (Smart and Smart, 1982).RG generate most projection neurons found in the cerebral cortex (Gotz and Barde, 2005; Malatesta et al., 2003). Moreover, their glial processes form scaffolds for newly-born and migrating neurons. BPs are lineage-restricted; they divide rapidly and symmetrically in the SVZ during the second wave of neurogenesis (E13-E17). Thus, they are characterized by their transit-amplifying potential leading to the production of the different populations of neuroblasts in a relatively short period of time (Figure 4b) (Haubensak et al., 2004; Noctor et al., 2004).Of note, the second wave of newborn neurons divide the PP into marginal zone (MZ), cortical plate(MacPherson et al., 2004) and subplate (SP) (Marin-Padilla, 1978; Smart and Mcsherry, 1982). The CP the mature cortex consisting of six

layers of cells formed in an inside-out manner (Figure 3) (Kostovic and Rakic, 1990). Finally, when neural development (including glia) is completed, residual RG give rise to adult NSCs which are astrocytes-like quiescent cells present in the aSVZ and SGZ of the dentate gyrus in the hippocampus (J. Altman & Bayer, 1990; Doetsch, 2003).

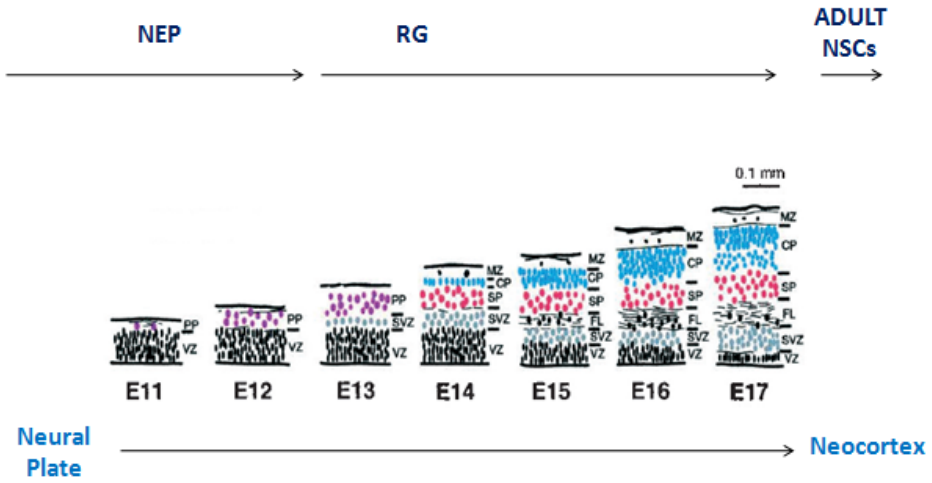


Figure 3: Cortical development in mice. After the formation of the neural tube around E7.5-8, the neocortex or cortical plate develops from a single-layer neuroepithelium between E11 and E17 and consists of six layers that are formed in an inside-out manner(MacPherson et al., 2004). Neuroepithelial cells (NEPs) are the first NSCs found in the ventricular zone (VZ), and later give rise to radial glia (RG) cells. Adult NSCs are derived from residual RG and reside in the adult SVZ.PP: preplate, SP: subplate, CP: cortical plate, MZ: marginal zone, FL: fiber layer Refer to text for detail (as modified from Molnar, 2006).

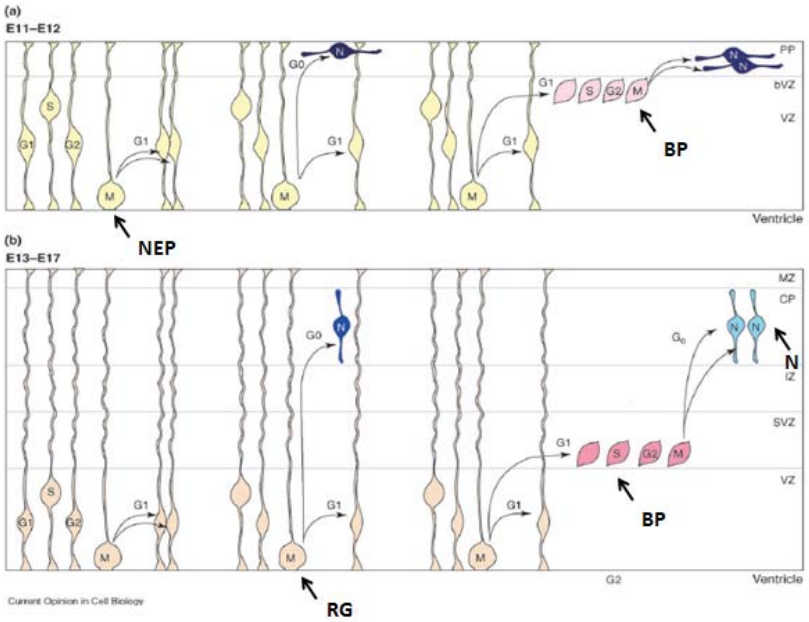


Figure4: NSCs and progenitors during cortical development. Several types of NSCs and progenitors contribute to cortical neurogenesis. (a)At E11-12, NEPs are the first stem cell population to reside in the VZ; they divide symmetrically to generate two identical NEP cells, or, asymmetrically to give rise to one NEP and a basal progenitor (BP) that differentiates into a neuron. (b) As neurogenesis continues (E13-E17), NEPs start expressing several radial glial markers and become RG cells that generate BPs occupying the SVZ. BPs further divide to generate neuroblasts that migrate to the cortical plate (Macpherson et al., 2004) along radial glia processes while they differentiate into mature neurons (as modified from Guillemot, 2005).

5. *Molecular mechanisms that control neurogenesis*

Neuroepithelial cells and glial cells in the brain have their apical processes exposed to ventricular fluid and basal processes facing the extracellular matrix (ECM). These cells are also laterally connected to each other by adherens and GAP junctions. Thus, cues originating from the basal and apical surfaces regulate neuroepithelial/radial glial (NE/RG) self-renewal and regenerative properties (Kosodo et al., 2004). It is particularly important to shed the light on the role played by the Wnt, Notch and Shh signaling pathways (Morshead et al., 1998; Morshhead et al.) in this regard. Previous work has thoroughly examined the role of Wnt signaling pathway either by reducing its ligand's level or eliminating a key element of the pathway such as β-catenin. In either

case, the number of NE/RG stem cells and derived progenitors was reduced while neuronal differentiation was boosted (Machon et al., 2003; Zechner et al., 2003). In contrast, induction of Wnt signaling induced stem cell's proliferation even during postnatal neurogenesis (Machon et al., 2007; Wexler et al., 2008) and this was coupled to an expansion in the pool of committed neuronal progenitors (Lie et al., 2005),demonstrating a key role for Wnt signaling in the control of NSCs self-renewal and proliferation. Notch signaling pathway was also found to regulate both adult NSCs and embryonic NE/RG cells development (Mizutani et al., 2007; Stump et al., 2002). In fact, elimination of several components of the Notch signaling pathway caused deregulation in the balance between proliferation and differentiation. Independent loss-of-function mutations in *Hes1, Hes3*and*Hes5*transcription factors, which are Notch signaling effectors, all caused reduction in the number of RG stem cells as well as premature neuronal differentiation in the mouse embryo (Yoshimatsu et al., 2006) and NSCs depletion in the adult brain (Gaiano, Nye, & Fishell, 2000; Hitoshi et al., 2002). Conversely, Notch activation inhibited neuronal differentiation in the embryonic cortex (by lateral inhibition) demonstrating that this pathway promotes NSCs self-renewal and progenitor proliferation (Chambers et al., 2001). Moreover, *in vitro* studies showed that NSCs generate more primary and secondary neurospheres upon Notch activation in culture while they retain the ability to differentiate into neurons, astrocytes and oligodendrocytes when transplanted *in vivo* (Mitzutani et al., 2007). In addition to Wnt and Notch, Shh is expressed in both the embryonic neuroepithelium (Lai et al., 2003) and adult hippocampus (Palma et al., 2005; Ahn and Joyner, 2005). Loss of function mutation in Smoothened (Smo) protein (Shh's ligand) blocked Shh signaling and resulted in the generation of less primary neurospheres from hippocampal tissue *in*

vitro (Machold et al., 2003). Studies have also showed that Shh regulates the transition from NSCs to fast-proliferating/committed progenitors in the retina, thus implicating Shh in the regulation of neural proliferation (Agathocleous et al., 2007). Besides signaling molecules, several key transcription factors are involved in the control of NSCs proliferation and differentiation. For instance, bHLH proneural genes including *Asc1*/*Mash1* (Achaete-scute), *Ngn1* and *Ngn2* (Neurogenin 1 and 2) are implicated in NSC properties specifically the acquisition of a neuronal fate and the induction of neuronal differentiation (specification/commitment) (Figure 3, Guillemot et al., 2007). Hence, *Ngn1* and *Ngn2*promote neurogenesis (Nieto, Schuurmans, Brz, & Guillemot, 2001; Sun et al., 2001) and directly specify the identity of dorsal telencephalic neurons by antagonizing the ventral pro-neuralgene*Mash1*(Fode et al., 2000). Another example is the homeodomain protein*Pax6*that is required for dorsal fate specification and subtype determination (Parras et al., 2004), however, *Pax6*function is distinct from *Ngn2* role (Hack et al., 2005).

In summary, substantial advance has been made in understanding the molecular mechanisms controlling neurogenesis. Accordingly, extensive molecular regulation of NSCs and their intrinsic properties allows progression into either a more proliferative state or toward a differentiation state (Purves et al., 2012, NEUROSCIENCE, 5th Edition). More work is still needed to uncover all regulatory mechanisms controlling nerve regeneration primarily in the adult brain.

6. Oligodendrogenesis

Oligodendrogenesis or formation of oligodendrocytes follows neurogenesis during development and is influenced by specific signals from distinct compartments of the neural tube. Hence, the notochord and the floor plate secrete Shh to form a morphogenetic gradient at the ventral side of the neural tube while the roof plate sends signals to the NEPs from the dorsal side that help determine their cell fate. Moreover, interplay between specific sets of homeobox genes such as *Olig2* and *Nkx2.2* triggers oligodendrogenesis (Figure 2E)(Briscoe, Pierani, Jessel, & Ericsson, 2000; El Waly, Macchi, Cayre, & Durbec, 2014; Kintner, 2002; Yun et al., 2010). *Olig2*, a gene required for generating motor neurons at early developmental stages, overlaps in expression with another patterning gene called *NKx2.2* in the neuroepithelium later in development. These two genes are responsible for oligodendrocytes formation. In contrast, proneural genes such as *Ngn2* were shown to counteract the function of Olig2 and NKx2.2 resulting in the inhibition of oligodendrogenesis in neuroepithelial cells. Conversely, differentiation into oligodendrocytes requires the inhibition of pro-neural gene function of Ngn2 by Olig2 and NKx2.2 (Sun et al., 2001; Zhou et al., 2001). Interestingly, oligodendrocytes formation is not only influenced by the type of genetic program at play but also indirectly by default differentiation, whenever neurogenesis is inhibited by Notch signaling for example (Figure 2D, Gaiano et al., 2000). On the other hand, *in vitro* studies have shown that NEPs removed from the cortex give rise to oligodendrocytes in the presence of fibroblast growth factor (FGF2), thus highlighting also a role for extrinsic signals in this differentiation program (Chandran et al., 2003; Kessaris et al., 2004).

7. *Astrogliogenesis*

Astrocytes were first defined by the prominent presence of cytoplasmic intermediate filaments (Vaughn and Peters, 1967; Choi and Lapham, 1978), one of which is the glial fibrillary acidic protein (GFAP) which became later on a hallmark of these cells (Bignami and Dahl, 1974). Similar to neurons and oligodendrocytes, astrocytes are also derived from NEPs; however they are formed indirectly via RG cells after neurogenesis (Figure 2F) (Choi et al., 1983; Barry and McDermott, 2005).Astrocytes specification is mediated by two main extrinsic cues:BMP signaling and the cytokines such as interleukin-e (IL-6) and ciliary neurotrophic factor (CNTF). Gross et al. (1996) suggested that the presence of BMPs in culture induced neural plate cells of the perinatal mouse SVZ to generate astrocytes. Subsequently, oligodendrocytes generation was strongly inhibited. Alternatively, CTNF and IL-6 both bind to the cell-surface receptor gp130 which promotes the activation of cytoplasmic tyrosine kinases of the Janus kinase (Jak) family. These kinases activate the Stat transcription factors which in turn trigger downstream effectors to activate astrocyte-specific gene expression (Darnell, 1997; reviews see Levy and Darnell, 2002).Other signaling pathways and molecules were implicated in astrogliogenesis such as the Notch signaling pathway which was involved in inducing astrogliogenesis by the activation of Notch intracellular domain (Tanigaki et al., 2001). It is still unclear, however, whether the Notch signaling pathway is independent or notof the Jak/Stat pathway. Finally the signaling molecule neureglin (Nrg) induced the formation of mature astrocytes in the developing brain (Purves et al., 2012, NEUROSCIENCE, 5th Edition).

B. Adult Neurogenesis

1. Adult NSCs: origin and location

Adult stem cells showed replication potential and regenerative capacity in several organs in mammals including the gut, the liver and the blood (Sage, 2012). In the adult brain, two neurogenic sites harbor adult quiescent NSCs: the SVZ lining the walls of the lateral ventricle (Garcion, Halilagic, Faissner, & ffrench-Constant, 2004; Lledo, Alonso, & Grubb, 2006; Pignatelli & Belluzzi, 2010) and the SGZ in the dentate gyrus of the hippocampus (Taupin P, 2002; Witman, 2009). The multipotency of aNSCs was extensively studied in long-term cultures where cells were expanded and passaged several times, then induced to differentiate into either glia or neurons. Moreover, when cultured in media enriched with growth factors, NSCs derived progenitors were able to acquire exclusive properties not found *in vivo* (Gabay, Lowell, Rubin, & Anderson, 2003; Morshhead et al., 1994; B. Reynolds & Weiss, 1992).

2. In vitro properties of aNSCs

Isolation and propagation of aNSCs in culture was problematic at first because the main factors needed to sustain these cells in their physiological niche were poorly understood. With the identification of two such growth factors, the Epidermal Growth Factor (EGF) and Fibroblast Growth Factor-2 (FGF2), cell culture conditions evolved to maintain prolonged cell division along with the NSCs properties (Laywell, Kukekov, & Steindler, 1999; B. Reynolds & Weiss, 1992). Reynolds et al. was the first to introduce the ‘neurosphere assay’ whereby a single NSC can generate free-floating aggregate of neural progenitors in culture called “Neurosphere” (Laywell et al., 1999; B. Reynolds & Weiss, 1992). Accordingly, adult SVZ tissue was isolated and

dissected; cells were plated in low-attachment tissue culture dishes in serum-free media rich in FGF-2 and EGF. NSCs and progenitors subsequently divided and formed free-floating primary neurospheres that can be dissociated and re-plated to produce secondary neurospheres (Figure 5) (Singec et al., 2006). However, only neurospheres derived from GFAP-positive NSCs (and not progenitors) were capable of long-term passaging in culture and this was directly associated with their unlimited self-renewal potential (Doetsch, Petreanu, Caille, Garcia-Verdugo, & Alvarez-Buylla, 2002). When the neurosphere protocol was first applied, about a thousand viablecells were plated and the number of neurospheres was estimated 6-7 days later (B. Reynolds & Weiss, 1992). More recent studies suggested that single-cell cultures(monoclonal) should be performed in order to confirm that the number of spheres generated reflect the true number of NSCs found in a tissue (Wachs, 2003). On the other hand, neurospheres can be dissociated and plated in monolayers then induced to differentiate into distinct neural lineages in the presence of serum and without growth factors (Figure 5).The neurosphere assay hence revealed the exclusive *in vitro* properties of (aNSCs) including their potential to proliferate, self-renew and differentiate into distinct mature cell types ((Klassen, Ziaeian, Kirov, Young, & Schwartz, 2004) reviewed in (Suh, Deng, & Gage, 2009)).

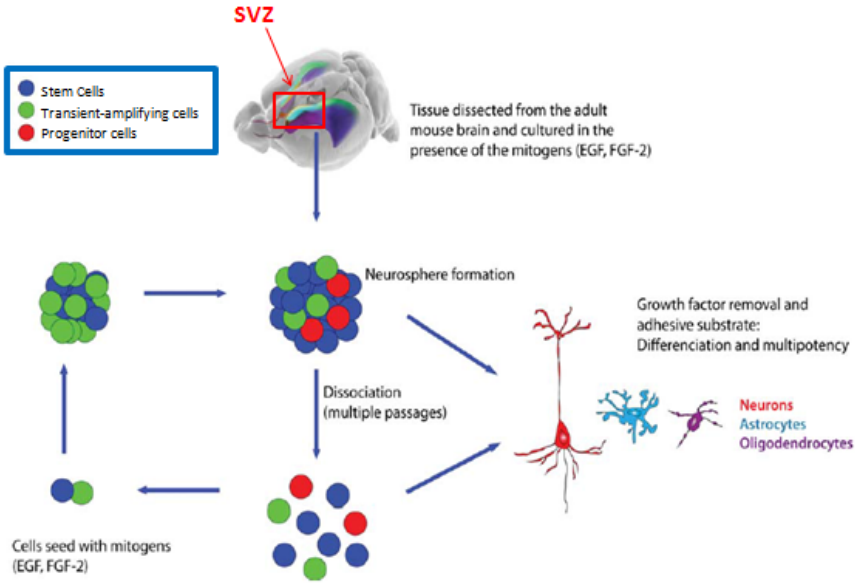


Figure 5: The neurosphere assay. The mouse SVZ tissue is dissected, then dissociated mechanically and chemically. NSCs/progenitors are isolated in single cells and plated in growth-factor rich media such as fibroblast growth factor-2 (FGF-2) and epidermal growth factor (EGF) to generate neurospheres (Gil-Perotin et al., 2013).Primary neurospheres derived from NSCs can be further dissociated and passaged multiple times whereas those derived from progenitors have a limited propagation potential. On the other hand, dissociated cells can be cultured on adhesive substrate in the presence of serum (without growth factors) to perform differentiation and multipotency tests (modified from (Gil-Perotin et al., 2013).

a. Proliferation potential

Neurospheres showed the ability to survive up to 60 passages in culture, however, when dissociated and cultured for more than 10 passages, they acquired tumor-like phenotypes due to accumulation of mutations and, in some cases, chromosomal instability (Vukicevic et al., 2010) and thus, had altered biological properties (Morshead, Benveniste, Iscove, & van der Kooy, 2002). When SVZ-derived tissue was dissociated and cultured in growth factors rich media, it generated a large number of neurospheres(B. A. Reynolds & Weiss, 1996). The proliferation potential was assessed by measuring the number and size of spheres generated after each passage (reviewed

in(Gil-Perotin et al., 2013). Nevertheless, the *in vitro* neurosphere assay is no longer adopted to test the clonality of the aNSCs since both stem cells and progenitors have the potential to produce neurospheres. After several passages, only stem cells will persist in culture since they have an unlimited proliferation potential contrary to the transient-amplifying progenitors which will stop proliferating during early passages (reviewed in (Gil-Perotin et al., 2013). In addition, the fact that each neurosphere reflects the proliferation potential of one cell can be debatable. Recent studies have used time-lapse imaging and showed that cells are motile in culture and tend to aggregate at high cell density which might produce experimental errors concerning the size and the number of neurospheres formed (Ladiwala, Basu, & Mathur, 2012). The proliferation potential of aNSCs is not the only property revealed in the neurosphere assay. One should assume that an individual neurosphere is made of a heterogenous cell population including stem cell(s) and progenitors and the only way to discriminate between them is by assessing the self-renewal potential of aNSCs (Figure 6)(Bez et al., 2003; Molofsky et al., 2003).

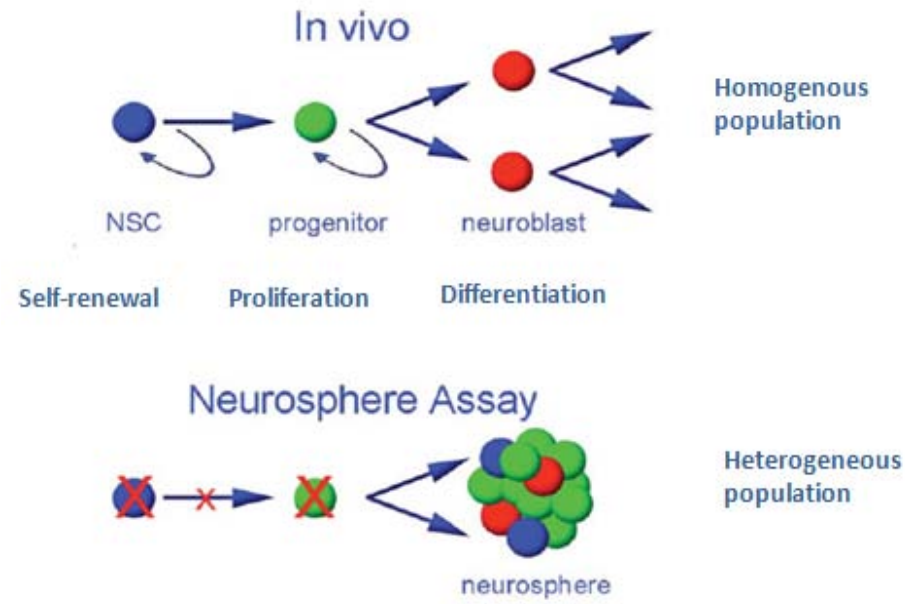


Figure 6: Limitations to the neurosphere assay.The progression from stem cells to mature neurons is well defined *in vivo*and leads to the generation of more or less homogenous neuronal populations or subpopulations. *In vitro*, however, a single neurosphere is rather a heterogeneous mixture of different cells at distinct developmental stages including stem cells, progenitors and neuroblasts. As such, the property of "stemness" is partially represented by the neurosphere assay in culture (as modified from (Gil-Perotin et al., 2013).

b. Self-renewal potential

Self-renewal potential is the ability of an individual neurosphere to generate a clone of neurospheres that can be propagated indefinitely. This property mainly indicates the presence of stem cells within aneurosphere. It also reflects the presence of NSCs in a specific niche, a concept known as "neurogenicity" of a region (reviewed in Gil-Perotin, 2013). The secondary neurosphere assay mentioned earlier is an indicator of 'stemness' thereby, directly related to the self-renewal potential of aNSCs (B. A. Reynolds & Weiss, 1996). However, one should always remember that a neurosphere is a mixture of heterogenous cell population including stem cells and progenitors (Young, Fogarty, Kessaris, & Richardson, 2007), and, only stem cells are capable of unlimited self-renewal after several passages (reviewed in (Gil-Perotin et al., 2013). Parker et al.

carried out a gene-expression profile and showed that neurosphere-derived from NSCs expressed genes related to differentiated cells more than the ones defining stem cells(Parker et al., 2005). Similarly, the expression profile of CNS derived-neurosphere showed notably enrichment in glial-associated genes (Jori et al., 2003; Schluter, Figiel, Rozyczka, & Engele, 2002)

c. Differentiation potential

aNSCs cultured *in vitro* are characterized by their ability not only to proliferate and self-renew but also generate several neural subtypes including neurons and glia (B. Reynolds & Weiss, 1992; Rietze & Reynolds, 2006). The methods used to differentiate aNSCs rely on the monolayer system where aNSCs are plated on poly-ornithin, laminin and/or fibronectin coated plates (Johe, Hazel, Muller, Dugich-Djordjevic, & McKay, 1996). In order to induce aNSCs differentiation; growth factors are removed from the media while fetal bovine serum and/or other substrates such as cytokines are added (Chojnacki & Weiss, 2008;Garcion et al., 2004; Grandbarbe et al., 2003). In such conditions, cells dissociated from neurospheres tend to adhere and differentiate into neurons, oligodendrocytes and astrocytes (B. Reynolds & Weiss, 1992; Rietze & Reynolds, 2006). *In vitro* differentiation assays were successfully performed on tissue derived from the adult mouse brain(Babu, Cheung, Kettenmann, Palmer, & Kempermann, 2007; Burrows, Wancio, Levitt, & Lillien, 1997; B. Reynolds & Weiss, 1992; Uchida et al., 2000)and the human hippocampus (Eriksson et al., 1998; Takahashi, Palmer, & Gage, 1999).

3. Neurogenesis in the adult SVZ

SVZ-aNSCs are a quiescent cell population present in a thin layer of the SVZ that extends along the walls of the LVs(Steindler, Kadrie, Fillmore, & Thomas, 1996; S. Temple & Alvarez-Buylla, 1999). Studies conducted on the SVZ ultrastructure and cell cycle length of SVZ cells revealed that the SVZ contains four different cell types (Doetsch, Garcia-Verdugo, & Alvarez-Buylla, 1997; C. Lois & Alvarez-Buylla, 1994; Peretto, Merighi, Fasolo, & Bonfanti, 1997). The first layer of cells that is lining the ventricle and in direct contact with cerebrospinal fluid is made of ependymal cells or type E cells. The adjacent cells are SVZ-NSCs referred to as type B cells. These are quiescent and slowly dividing and give rise to transient-amplifying progenitors or type C cells which divide rapidly (several times) to produce immature neurons or neuroblasts also called type A cells. Neuroblasts migrate to the OB along the rostral migratory stream (RMS), a route formed by the extended glial processes of astrocyte (C Lois, Garcia-Verdugo, & Alvarez-Buylla, 1996; Yokoyama et al.). In the OB, they dissociate from the RMS and spread radially to generate mature GABAergic neurons that integrate into pre-existing neuronal network primarily in the granule cell layer (GCL) and the periglomular layer(GL) (Figure 7)(reviewed by(Braun & Jessberger, 2014)). Newborn OB GABAergic neurons include distinct subtypes such as calretinin-(CR), calbindin-(CB) and tyrosine hydroxylase- (TH) expressing inhibitory neurons (reviewed by (Witman, 2009)). Of note, there is a high turnover of adult-born OB neurons which are replaced throughout adulthood, albeit at a lower rate(Imayoshi, 2008).

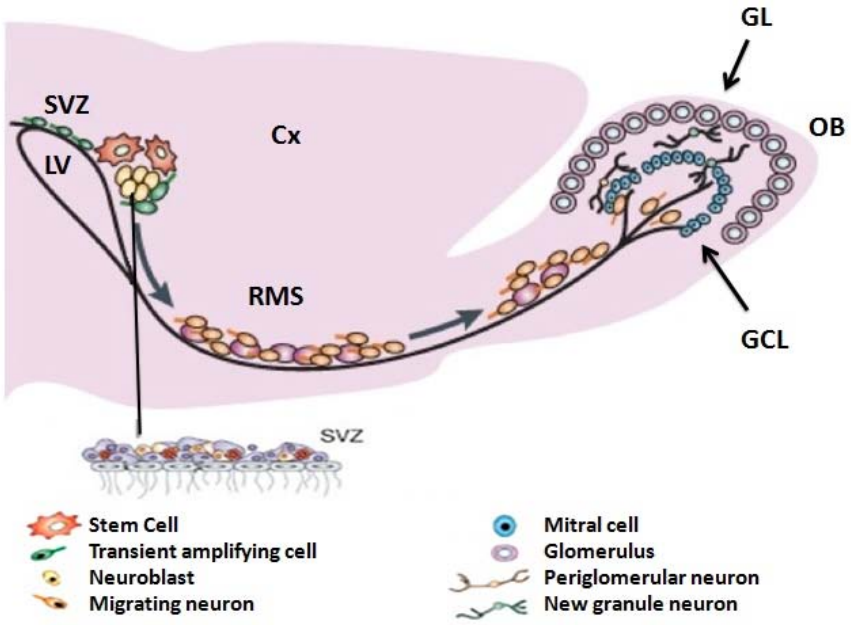


Figure 7: Neurogenesis in the adult SVZ. The SVZ harbors four types of cells: the ependymal cells called type E cells lining the walls of the LVs, the multipotent astrocyte-like stem cells also known as type B cells that generate transient-amplifying progenitor cells or type C cells, and, neuroblasts or type A cells that exit the cell cycle and migrate along the RMS to reach the OB where they integrate and differentiate into GABAergic interneurons in the periglomular layer (GL)(Lie et al., 2005)and the granule cell layer (GCL) (as modified from Dietrich, 2008).

In terms of function, newborn granule cells show long-term synaptic plasticity which disappears when these neurons become mature and fully differentiated, thus referring to the important role played by adult neurogenesis in synaptic plasticity inside the bulb(Nissant, Bardy, Katagiri, Murray, & Lledo, 2009). In addition, several behavioral studies showed that sensory experience regulates the survival rate of the new born neurons(Lledo et al., 2006; Yokoyama et al., 2011).On the other hand, blockage of neurogenesis was shown to alter specific odor tasks such as tough discrimination tasks between similar odors and short-term olfactory memory, proving that these functions are both dependent on newly-integrated neurons at least partially (Breton-Provencher, Lemasson, Peralta, & Saghatelyan, 2009). In summary, the above studies concluded that OB neurogenesis is involved in odor discrimination and olfactory memory (Bath et

al., 2008; G. Gheusi et al., 2000) and thus, is required for proper olfactory function and plasticity.

4. Molecular control of adult neurogenesis in the SVZ

Studies have shown that several key molecular pathways that control neurogenesis during development play a similar role during adult neurogenesis in the SVZ. For example, the interaction between cell surface ligands Delta and their receptors Notch is an important determination factor whether stem cells shall self-renew or generate post-mitotic neurons. This interaction activates a signal transduction pathway inside the cytoplasm and the nucleus whereby a fragment of notch receptor enters the nucleus and activates specific transcription factors such as the *Hes* genes, which in turn regulate the expression of key genes required for the terminal differentiation of neural cells. The most important among these genes are the bHLH neurogenic factors such as *NeuroD 1,2,4,6* (Neuronal differentiation), *Neurog1-3* (Neurogenin) and *Mash1*(Purves et al., 2012). Recent work showed that protein kinases such as Akt1 interacts with Notch signaling pathway and mediate neuronal differentiation (Cheng et al., 2013). Notch signaling is not the only pathway regulating fate determination in the adult brain. As a matter of fact, Sonic Hedghog (Morshead et al., 1998) signaling is also triggered in radial glia-like cells (Ahn & Joyner, 2005) and necessary for the maintenance of quiescence of aNSCs in the SVZ (Balordi & Fishell, 2007). Hence, the activation of Shh signaling pathway requires the cooperative binding of two surface receptor proteins smoothened and patched. Shh binds to patched and results in the accumulation of smoothened on the cell surface which triggers Gli1 (Glioma-associated oncogene homolog) to enter the nucleus and enhances the expression of neural identity genes

(Purves et al., 2012, Neuroscience, 5th edition). In contrast, Bone Morphogenetic Proteins (BMPs) inhibit neural differentiation by inducing glial differentiation in the adult SVZ(Bonaguidi et al., 2005; Lim et al., 2000). BMPs activate serine kinase receptors causing the phosphorylation of transcriptional regulators called SMADs which translocate into the nucleus and regulate the transcription of several target genes mainly involved in neural fate determination. The BMP action can be inhibited by endogenous antagonists such as Noggin and Chordin (Purves et al., 2012, Neuroscience, 5th edition). Lim et al. demonstrated that Noggin and Neurogenin-1 are expressed by SVZ ependymal cells which lead to an increase in neurogenesis (Lim et al., 2000). Thus, differentiation into either neuronal or glial type is a complex process that involves intrinsic and extrinsic cues in the adult brain.

5. Neurogenesis in the adult hippocampus and its function

The hippocampus constitutes the second neurogenic site found in the adult brain(Urban & Guillemot, 2014). Hence, neurogenesisis ongoing in the SGZ in the dentate gyrus where immature neurons migrate into the inner granule cell layer and generate excitatory granule cells(Zhao, Teng, Summers, Ming, & Gage, 2006).Later on, newly born neurons project their axons through the hilus towards the CA3 domain where they also project their dendrites (Zhao et al., 2006). Adult-born neurons thus integrate into existing neuronal network containing local interneurons, which initially release GABA to activate their function (Bhattacharyya, 2008; Ge, 2006). Soon afterwards, more GABAergic inputs and finally glutamatergic inputs are created to activate the new born neurons(Esposito et al., 2005; Ge et al., 2006; Overstreet-Wadiche, Bensen, &Westbrook, 2006). To note, molecular mechanisms and key regulators are conserved

in embryonic and adult neurogenesis in the hippocampus (Urban & Guillemot, 2014). Studies showed that neurogenesis in the hippocampus is directly involved in learning, memory and mood regulation(Lledo & Lazarini, 2007; Zhao, Deng, & Gage, 2008). For instance, X-irradiation and ablation of hippocampal neurogenesis led to a reduced performance in memory tasks that are dependent on the hippocampus (Winocur, Wojtowicz, Sekeres, Snyder, & Wang, 2006). Moreover, newly born granule cells showed exclusive physiological properties that made them capable to respond and integrate stimuli during memory formation (Bruel-Jungerman, Davis, & Laroche, 2007).

C. The Retinoblastoma Protein, pRB

1. The Retinoblastoma family: p107, pRb and p130

The Retinoblastoma protein (Jacks et al., 1992),pRb,is a member of the “pocket protein” family characterized by a highly conserved sequence called the ‘pocket domain’. This domain mediates the interactions of Rbwith cellular proteins such as viral onco-proteins produced by small DNA viruses or adenovirus, which showed an ability to alter cellular homeostasis (Felsani, Mileo, & Paggi, 2006). The mammalian “pocket protein” family is comprised of three proteins, pRb, p107 and p130, all of which primarily bind to E2F transcription factors through their pocket domain and regulate cell cycle related functions. Biochemical studies showed that pocket proteins have different binding preferences to E2Fs and thus, play distinct but complementary roles during development (Figure 8)(Beijersbergen et al., 1994; K. L. Ferguson & Slack, 2001; Stevaux & Dyson, 2002; Vairo et al., 1995). Despite this, they also display some overlapping functions and can compensate for one another in specific contexts as shown by several studies (Jacks et al., 1992; E. Y. Lee et al., 1992; E. Y. Lee et al.,

1994). For instance, when Rb is lost, p107 and p130 are able to partially compensate for its role in the nervous system and liver in the developing mouse (M. H. Lee et al., 1996; Lipinski & Jacks, 1999)

a. P107

P107 is normally expressed and active in undifferentiated cells including stem cells and early progenitors where it forms complexes with repressor E2Fs such E2F4 and 5 (Figure 8)(Beijersbergen et al., 1994; Ginsberg et al., 1994).P107 expression is thus restricted to the VZ in the developing brain where NSCs reside (K. L. Ferguson & Slack, 2001; Gill, Slack, Kiess, & Hamel, 1998). During neurogenesis, the levels of p107/E2F complexes start to decrease gradually in committed progenitors and post-mitotic neuroblasts (Gill et al., 1998) while the expression of Rb peaks in the same cells (Figure 8). In the developing and adult brain, Vanderluitet al. showed that p107 negatively controls the self-renewal potential of NSCs by analyzing the phenotype ofp107-null mice *in vivo* and performing primary and secondary neurospheres assays *in vitro*(Vanderluit et al., 2004). Later on, the same group demonstrated that p107 also regulates the rate of progenitor's commitment to a neuronal fate since p107-null mice revealed reduced number of cortical neurons (Vanderluit et al., 2007).

b. P130

P130 expression is mainly restricted to post-mitotic neurons (Jiang, Zacksenhaus, Gallie, & Phillips, 1997; Yoshikawa, 2000).In contrast top107 and pRb, p130 levels increase in newly differentiated and mature neurons as it is primarily implicated in neuronal survival and maintenance of the differentiated state (Callaghan et al., 1999;

K. L. Ferguson, Callaghan, O'Hare, Park, & Slack, 2000; Jiang et al., 1997). To carry this function, p130 preferentially interacts with the transcriptional repressors, E2F4 and5 (Vairo et al., 1995).

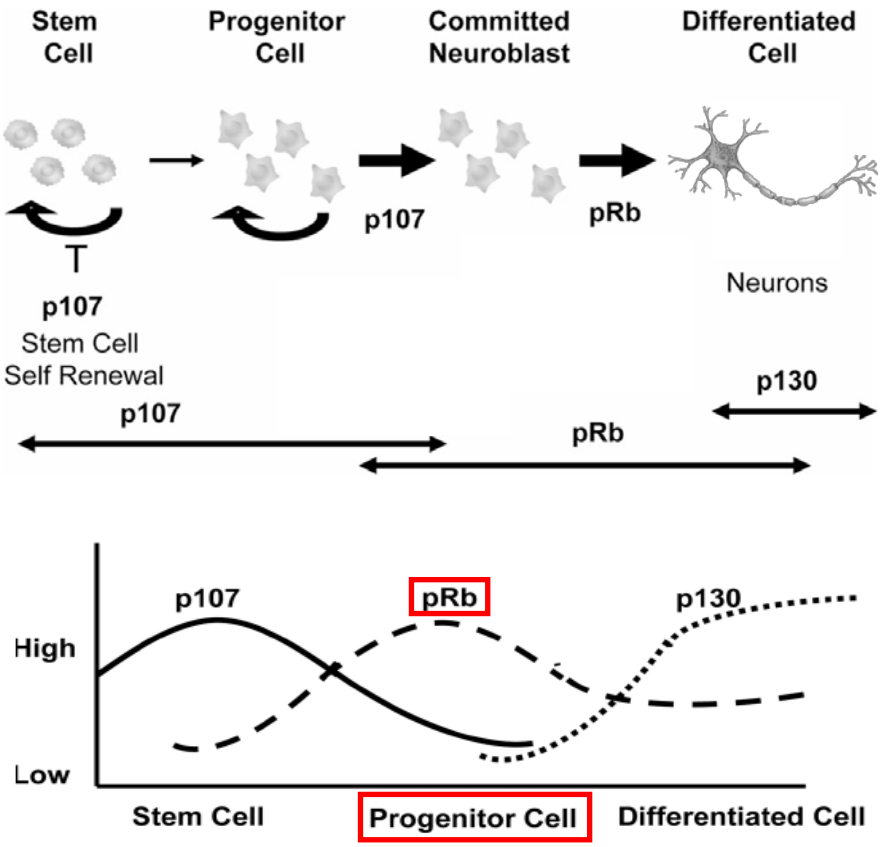


Figure 8: Distinct roles for pocket proteins during neurogenesis. The Rb family members control different stages of neurogenesis: p107 expression is highest in NSCs and early progenitors where it regulates self-renewal and commitment to a neuronal fate. In contrast, pRb is mainly expressed in committed progenitors and neuroblasts and controls cell cycle progression and terminal mitosis as well as cell differentiation and migration. p130 is primarily expressed in post-mitotic and mature cells and regulates cell survival (as modified from (Vanderluit et al., 2004).

2. ***Role of Rb in cell cycle control***

Rb is a tumor suppressor protein that interacts with the E2F transcription factors to control the cell cycle at the G1/S phase transition (Cobrinik, Dowdy, Hinds, Mittnacht, & Weinberg, 1992; Hamel, Gallie, & Phillips, 1992) and thus, primarily regulates cell proliferation. The E2F family members are divided into two categories: the transcriptional activators (E2F1, 2,3a) and the transcriptional repressors (E2F 3b, 4, 5, 6, 7and 8) (Blais & Dynlacht, 2004; Cam & Dynlacht, 2003; Dimova & Dyson, 2005). Unlike p107 and p130 which form complexes with repressor E2Fs such as E2F4 and 5, Rb mainly interacts with transcriptional activators including E2F1-3(Cam & Dynlacht, 2003; Dimova & Dyson, 2005). The function of pRb is directly associated with its phosphorylation state and the role played by cyclin-dependant kinase (CDK)/cyclin complexes (Buchkovich, Duffy, & Harlow, 1989; Wong & Weber, 2007). Hence, when hypo-phosphorylated, pRb binds to E2Fs and inhibits transcriptional activation of S phase genes (Buchkovich et al., 1989; Ludlow, Shon, Pipas, Livingston, & DeCaprio, 1990). Phosphorylation of Rb by CDK/cyclin complexes leads to its inactivation, thereafter freeing E2Fs and allowing the progression of the cell cycle (Lundberg & Weinberg, 1998).

3. Role of Rb during embryonic development

Rb is necessary for normal embryonic development; studies have shown that Rb-null (-/-) embryos have severe defects in the neural tube, skeletal muscle and placenta, and die by embryonic day 15 (E15)(Clarke et al., 1992; Jacks et al., 1992; E. Y. Lee et al., 1992; Zacksenhaus et al., 1996).Add to this, loss of Rb is associated with others impairments including ectopic cell proliferation, partial cell differentiation and

increased apoptosis(Clarke et al., 1992; Jacks et al., 1992; E. Y. Lee et al., 1992; E. Y. Lee et al., 1994).As stated earlier, the expression of Rb is up-regulated in proliferating progenitors including those that have initiated their differentiation program (committed progenitors). Therefore, Rb is necessary for terminal mitosis after commitment to neuronal fate (Figure 8) (Callaghan et al., 1999; K. L. Ferguson et al., 2000; Jiang et al., 1997; Slack, El-Bizri, Wong, Belliveau, & Miller, 1998; Slack, Hamel, Bladon, Gill, & McBurney, 1993).During development, loss of Rb causes ectopic cell proliferation and increased cell death in the proliferative zones of the developing cortex (Jacks et al., 1992; E. Y. Lee et al., 1992). Moreover, conditional Rb knock-out mice in the telencephalon exhibit ectopic cell proliferation, however, Rb-/- neuroblasts are able to differentiate and survive at least till birth, the time when these mice die (K. L. Ferguson et al., 2002). Similarly, other brain regions such as the retina showed increased proliferation and decreased cell survival upon loss of Rb(Chen et al., 2004; Marino, Hoogervoorst, Brandner, & Berns, 2003).

4. Role of Rb in cell differentiation and migration in the brain

Besides its main role in cell cycle control, *in vivo* and *in vitro* studies have shown that Rb plays an important role in the control of neuronal differentiation and migration in the brain. Conditional Rb mutant mice exhibit severe impairments in radial migration of cortical neurons during mid-gestation as well as tangential migration of interneurons to the cortex in a cell-autonomous manner (K. L. Ferguson et al., 2005). The control of cell migration by Rb is specifically mediated by E2F3 (K. A. McClellan et al., 2007)and affects directly the expression of genes involved in cell migration such as neogenin (Andrusiak et al. 2011). Using cell-type specific markers and birthdating

assays, a recent study detected the presence of differentiation and rostral migration defects of SVZ progenitors in the RMS to the OB(Ghanem et al., 2012).These defects are linked to decreased expression in*Dlx1* and *Dlx2*, two key transcription factors required for neuronal differentiation and migration. Moreover, the same study showed that, in the absence of Rb, E2F7 binds to E2F repressor sites present in the *Dlx1/2* enhancer and proximal promoter regions, and inhibits *Dlx* expression. Consequently, Rbis required to regulate the onset of differentiation of committed cells by controlling the level of repressors E2Fs (Ghanem et al., 2012). In the retina, loss of Rb caused similar defects in the development of cholinergic neurons and this was also linked to its control of cell differentiation (Chen et al., 2007; MacPherson et al., 2004).

5. Role of Rb in adult neurogenesis

As mentioned earlier, p107 was shown to negatively regulate the self-renewal property of aNSCs and is required for the commitment to a neuronal fate(Vanderluit et al. 2004 and 2007). Results showed that loss of p107 leads to an increase in slowly dividing/quiescent cells in the adult SVZ (type B) and in the number of primary and secondary neurospheres generated from p107-null NSCs and progenitors in culture (Vanderluit et al. 2004 and 2007).Recent work performed in our lab has investigated the role of Rb in the control of adult neurogenesis by inducing a temporal deletion of Rb specifically in adult NSCs and progenitors *in vivo*. To do this, Nestin-CreERT2 /YFP animals were crossed with Rbflox/flox animals. Results showed that loss of Rb causes increased cell proliferation in the aSVZ and RMS without affecting neuroblast's migration along the RMS. Using immunohistochemistry, Naser et al. 2015detected a 1.5-2 fold increase in the number of SVZ progenitors (BrdU+ and Ki67+) and

migratory neuroblasts inside RMS (DCX+; double cortin) in Rb-/- animals compared to Rb+/- controls. Interestingly, terminal differentiation and maturation of new born GABAergic interneurons are normal inside the OB despite the loss of Rb as indicated by the number of new-born neurons expressing NeuN specifically in the GL. Moreover, these neurons are able to integrate into the OB network and differentiate into distinct subtypes of interneurons expressing Calretinin- (CR), Calbindin- (CB) and Tyrosine Hydroxylase- (TH) (Naser R and Ghanem N, et al. 2015in preparation). Of note, Rb did not affect the rate of proliferation of aNSCs in the SVZ since their number did not change despite loss of Rb.

Altogether, our recent findings showed that Rb controls progenitor's proliferation and the rate of neurogenesis inside the OB without affecting the properties of aNSCs *in vivo*. Considering that aNSCs/progenitors possess low proliferation rate and restricted differentiation potential *in vivo,* we hypothesized that manipulation of the Rb pathway may help enhance the regenerative capacity of aNSCs and progenitors without affecting their differentiation capacity. Hence, the aim of the present study is to investigate how Rb controls the properties of aNSCs and progenitors *in vitr o*including their rate of proliferation, their self-renewal capacity and finally, their differentiation potential. If proven to be true, the potential expansion of the number of progenitors and hence mature neurons derived from aNSCs by deletion of Rb may have direct implications in regenerative medicine in the future.

CHAPTER II

RESULTS

A. *In vitro* neurosphere assays

1. Primary cultures of aSVZ tissue from Rb-null aNSCs versus Rb+/- aNSCs

The role of Rb in embryonic development and neurogenesis has been extensively studied using full and conditional knock-out mice models as described earlier(Clarke et al., 1992; K. L. Ferguson et al., 2005; K. L. Ferguson et al., 2002; Jacks et al., 1992; E. Y. Lee et al., 1992). In addition, our laboratory has recently characterized the role of Rb during adult neurogenesis and showed that Rb controls proliferation of aNSC/progenitors in the SVZ and the rate of neurogenesis in the OB without affecting neuronal differentiation and terminal maturation (Naser et al., in preparation; refer to introduction). In this complementary study, we have investigated how Rb regulates the properties of aNSCs *in vitro* including their self-renewal potential, their rate of proliferation and their differentiation capacity. Thus, we have induced a deletion of Rb by crossing Nestin-CreERT2; YFP/YFP; Rbflox/+ reporter mice and Rbflox/flox mice. As a result, we generated Nestin-CreERT2+/-; YFP/+; Rb +/+ (Rb wild type mice), Nestin-CreERT2+/-; YFP/+; Rb +/flox (Rb heterozygous mice), and, Nestin-CreERT2+/-; YFP+/-; Rb flox/flox (Rb mutant mice). Since heterozygous animals were perfectly normal, we used them as controls to study the phenotype of Rb-null animals and we referred to them thereafter as Rb+/- and Rb-/-, respectively. This approach ruled out any effect due to the insertion of the Cre cassette or the *loxP* sites. In this model, Rb was specifically deleted in aNSCs and progenitors in 8 week-old animals in a temporal manner by tamoxifen injections (Figure 9A and 9B). Animals were sacrificed and their

brains removed 5 or 12 days later. Then, the SVZ tissue was dissected, dissociated and plated to perform neurosphere assays (Figure 9C).

We first examined whether Cre recombination was successful in Rb+/- versus Rb-/- mice by assessing the number of green neurospheres obtained in primary cultures. To do this, we used an inverted fluorescent microscope and determined the percentage of recombined neurospheres (Greenough, West, & DeVoogd, 1978) among the total number of neurospheres in both genotypes. Results showed that 52-64% of all neurospheres were green at various plating densities and with no statistical difference between genotypes (Graph1).The ratios (Mutant/control) of green spheres were as follows: in 5000cells/mL r=1.07 (Rb+/-; 56.5 ± 7.7%vs Rb-/-.; 60.6 ± 2.88%, p=0.12), in 2500 cell/mL, r=0.86 (Rb+/-.; 64.2 ±2.6%vsRb-/-.; 55.04 ±3.17%, p=0.89), in 1250 cells/mL r=1.15 (Rb+/-.; 55.0 ±10.3% vsRb-/-.; 63.2 ±4.6%, p= 0.130) and in 625 cells/mL r=0.82 (Rb+/-.;62.6 ±3.3 vsRb-/-; 51.7 ±3.9, p=0.134).These results confirmed that the Cre recombination was successful *in vivo* as the percentage of recombined progeny reached an average of ~60% in our primary cultures and this was consistent in both genotypes.

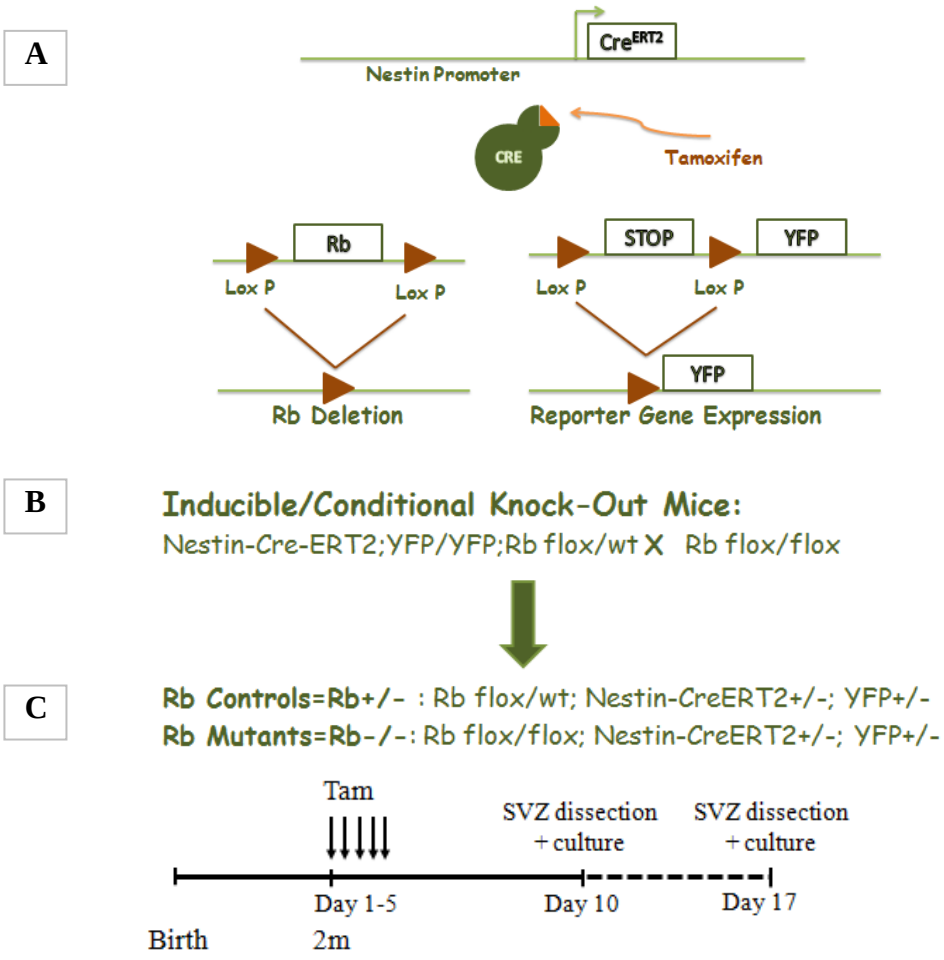
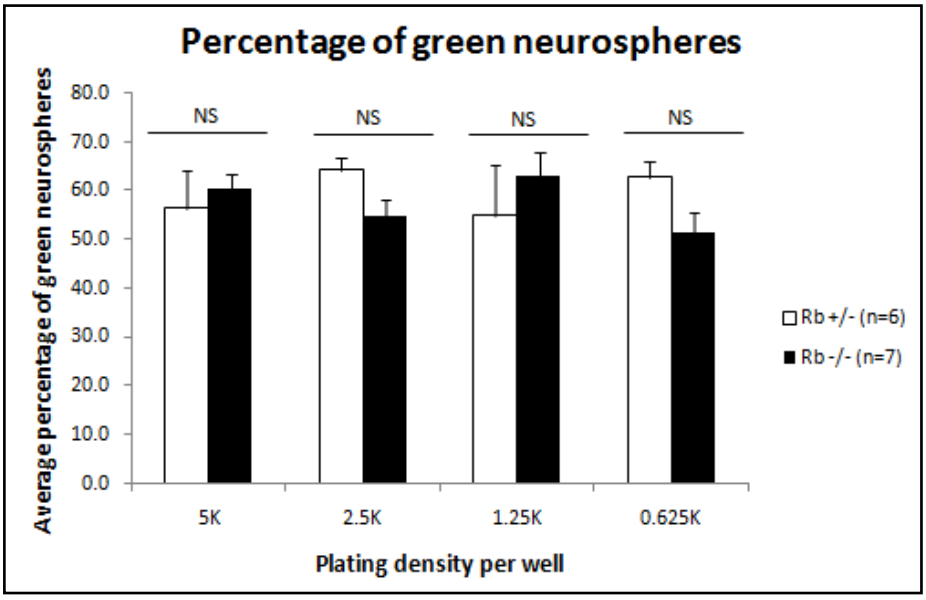


Figure 9: Mouse model used to perform an inducible deletion of Rb in aNSCs and progenitors.**(A)** Schematic representation depicting the Cre gene that is fused with a mutated estrogen receptor and under the control of the Nestin promoter. Nestin is an intermediate filament protein that is specifically expressed in NSCs and progenitors. Upon tamoxifen treatment, the Cre recombinase enzyme translocates to the nucleus and excises the DNA fragments flanked by two LoxP sites, thus deleting Rb and activating the expression of the YFP reporter gene. **(B)**Mating scheme performed to generate Rb control and mutant animals, thereafter referred to as Rb+/- and Rb-/-, respectively. **(C)** Experimental design for the neurosphere assays performed *in vitro*: 5d or 12d post deletion of Rb*in vivo*, SVZ tissues from Rb+/- and Rb-/- animals were dissected and cultured. wt: wild-type, flox: floxed allele.



Graph 1: Percentage of green/recombined neurospheres derived from aNSCs. 52-64% of the total number of neurospheres generated in primary cultures was green in Rb+/- and Rb-/- mice with no significant difference between genotypes, suggesting a successful Cre recombination *in vivo*. Error bars represent SD of measurements from n=6 Rb+/- and n=7 Rb-/-.K=1000, NS; not significant (t-tests).

2. Cell sorting and passaging of recombined neurospheres

Following the primary cultures, passages were done every 6-7 days by pooling together and dissociating both recombined and non-recombined spheres and the properties of Rb+/- aNSCs vs Rb-/-aNSCs were subsequently studied. Since the presence of wild type cells (Rb+/+) could interfere and underestimate the phenotype linked to loss of Rb, we performed a second set of cultures where dissociated cells were subjected to cell sorting at passage 2 in order to collect green/recombined cells only. Spheres derived from these cells were subsequently used to conduct the various *in vitro* tests/passages andall results were compared to those obtained from previous cultures (before cell sorting). A summary of all cultures performed in this study is shown in the table 1.

	Total n		ASSAY S performed					
	Rb+/-	Rb -/-	Primary Neurosphere	Secondary Neurosphere	Differentiation	Cell sorting	Passages	Amplification rate after P3
Culture #1	3	2	+	-	+	-	4	+
Culture #2	2	2	+	-	+	-	4	+
Culture #3	3	3	–	–	+	-	4	–
Culture #4	3	3	+	+	-	+	5	+
Culture #5	1	1	+	+	-	+	5	+

Table1 1: Table summarizing all cultures performed in the study. The assays performed in different cultures include primary neurosphere and/or secondary neurosphere assays and/or differentiation assays. Table indicates the number of mutant (Rb-/-) and control (Rb+/-) animals used, the number of passages and the amplification rate in each culture. (-) not performed, (+) performed.

B. Rb controls proliferation of aNSCs/progenitors by regulating the size and number of neurospheres generated *in vitro*

To assess the proliferation potential of aNSCs/progenitors in the absence versus the presence of Rb, we dissociated primary cultures derived from Rb+/- and Rb-/- animals and performed primary neurosphere assays by plating cells in24-well plates at four decreasing densities as follows: 5000 cells/mL, 2500 cells/mL, 1250 cells/mL and 625cells/mL. Five days later, neurospheres were measured and counted. Our results showed an increase in the number of spheres generated in Rb-/- compared with Rb+/- cultures and this increase was inversely proportional to the plating density (Figure 10A-B'). Hence, the ratios (Mutant/control) of number of spheres in various cell densities were: r=1.29in 5000 cells/mL (Rb+/-: 83.3±5.71 vsRb-/-.: 107.1 ±4.88, p=0.00032), r=1.31in 2500 cells/mL (Rb+/-.:40.9±4.29 vsRb-/-.; 53.7±6.83, p=0.011), r=1.47in 1250 cells/mL (Rb+/-.; 21.1±2.75 vsRb-/-.; 31.1±6.53, p=0.017) and r=1.50 in 625 cells/mL (Rb +/-;11.1±1.53 vsRb-/-; 16.7±2.83, p=0.168)(Graph2A). Interestingly, the difference in sphere number between genotypes was more pronounced in cultures

performed after cell-sorting and thus, the ratios (Mutant/control) ranged between 1.71 and 2.41 for the respective cell densities as follows: r=1.71 in 5000 cells/mL (Rb+/-.; 107.1 ± 4.03 vs Rb-/-., 183.33 ± 8.18, p=0.00083), r=1.90 in 2500 cells/mL (Rb+/-.; 42.1 ± 4.03 vs Rb-/-.; 80.0 ± 8.18, p=0.00034), r=2.15 in 1250 cells/mL (Rb+/-.; 22.9 ± 4.03 vs Rb-/-.; 49.11 ± 8.18, p=0.0019) and r=2.41 in 625 cells/mL (Rb+/-.; 8.4 ±4.03 vs Rb-/-,; 20.33 ± 8.16, p=0.045)(Figure 11 and Graph 2B).

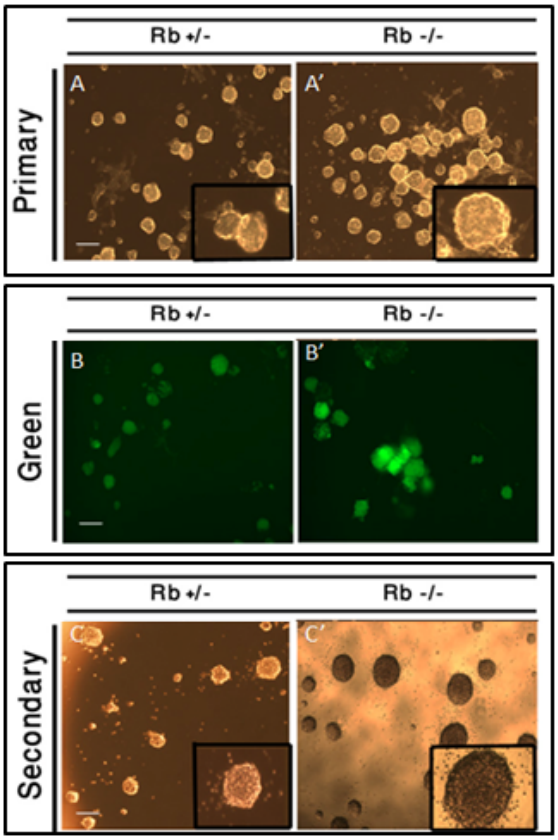
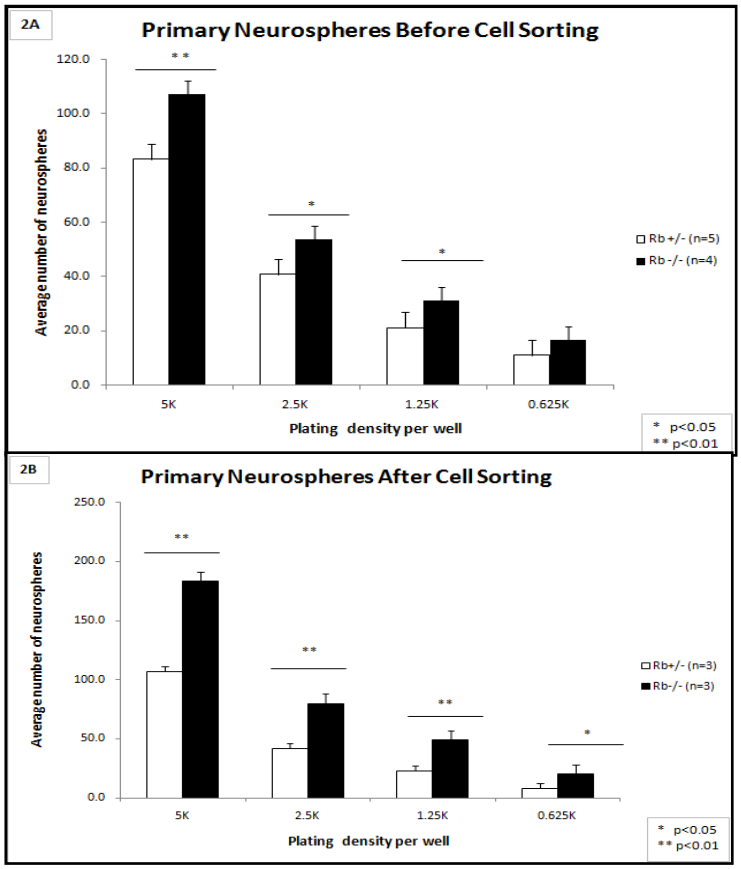


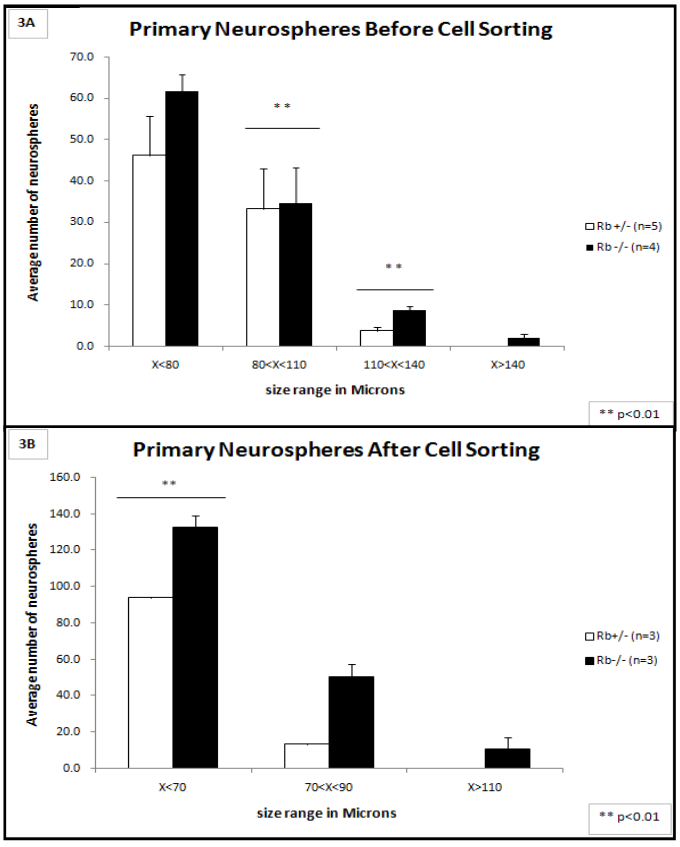
Figure 10: Loss of Rb causes an increase in proliferation of aNSCs/progenitors in culture. (A, A') Bright field images of primary neurosphere cultures showing increase in the size and number of neurospheres generated in Rb-/- versus Rb+/- (without cell sorting). This data is quantified in graphs 2A and 3A (see next sections). (B, B') Fluorescent images depicting the green neurospheres derived from recombined cells in the same cultures prior to cell sorting. (C, C') Bright field images of secondary neurosphere assays derived from Rb+/- versus Rb-/- cultures. Insets in A, A', C and C' represents higher magnification images of individual neurospheres. Scale bar = 100µm.



Graphs 2: Quantification of the total number of neurospheres derived from aNSCs in culture. Counts were performed on day 5 of primary neurosphere assays at different plating densities before (2A) and after cell sorting (2B). Results showed significant increase in average number of sphere generated in Rb-/- cultures compared with Rb+/-. Moreover, the ratio (mutant/control) of total spheres was inversely proportional to the plating density (see text): Of note, all spheres generated after cell sorting were green. Error bars represent SD of measurements from several animals as shown, and asterisks indicate a significant difference between genotypes using t-tests: (*) represents $p<0.05$ and (**) represents $p<0.01$.

Moreover, the size of neurospheres was significantly increased in Rb-null cultures compared with controls on average as more spheres of the different size ranges were generated in the absence of Rb (Fig. 12, compare A-C' and D-F', and, graphs 3A and 3B).Therefore, the ratios of (mutant/control) the number of neurospheres according to size in 5000 cells/mL plating density were: r=1.33 for x<80uM(Rb+/-.; 46.2 ± 9.44 vs Rb-/-.; 61.7 ± 4.09, p=0.139), r=1.04for 80uM<x<110uM(Rb+/-.;33.2 ± 9.81 vs Rb-/-

.;34.67 ± 8.5, p=0.842) and r=2.29 for 110uM<x<140uM (Rb+/-; 3.8 ± 0.92 vs Rb-/-.; 8.7 ± 1, p=0.001). Notably, the increase in sphere size was more remarkable in cultures after cell sorting and spheres with size ranging between 110µM and 140µM or higher were more frequently detected in Rb-null culture compared with controls; r=1.41 for x<70uM (Rb+/-.;93.9 ± 0.77 vs Rb-/-.; 132.8 ± 6.68, p= 0.0023), r=3.82 for 70uM<x<90uM (Rb+/-.;13.2 ± 0.77 vs Rb-/-.; 50.56 ± 6.68, p=0.324)(Graphs 3A and 3B).



Graphs 3: Quantification of the size of neurospheres derived from Rb+/- aNSCs versus Rb-/- aNSCs in culture. Measurements and counts were performed on day 5 of primary culture at 5k cells/well before (**3A**) and after cell sorting (**3B**). Results showed significant increase in the number of spheres of different sizes generated in Rb-/- compared with Rb+/-cultures. This was primarily true for sphere size above 110uM in 3A and for all spheres irrespective of size in 3B. Error bars represent SD of measurements from n=3 as indicated, and asterisks indicate a significant difference between genotypes using t-tests, (*) represents p<0.05 and (**) represents p<0.01.

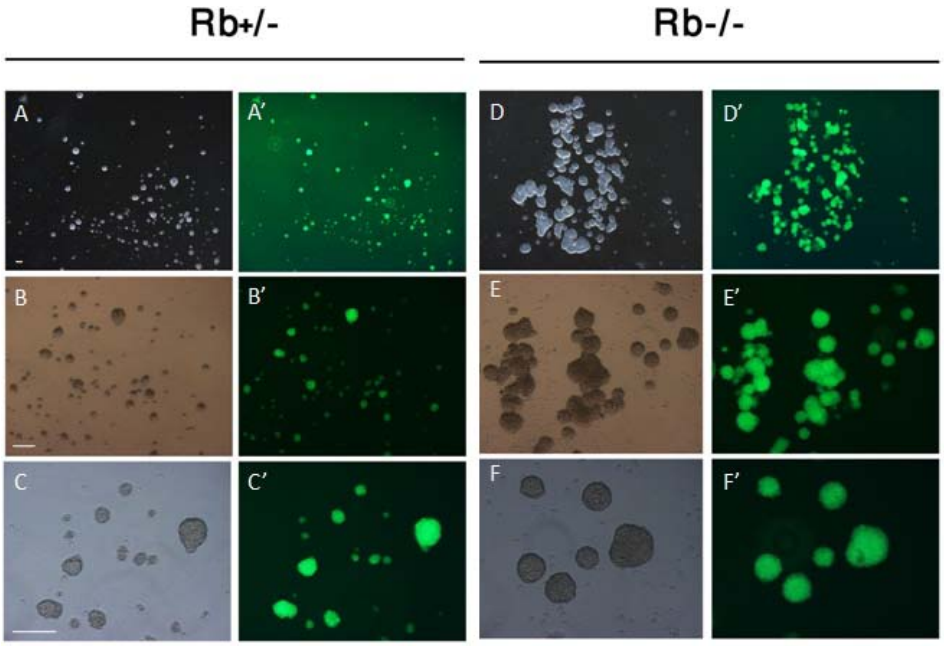
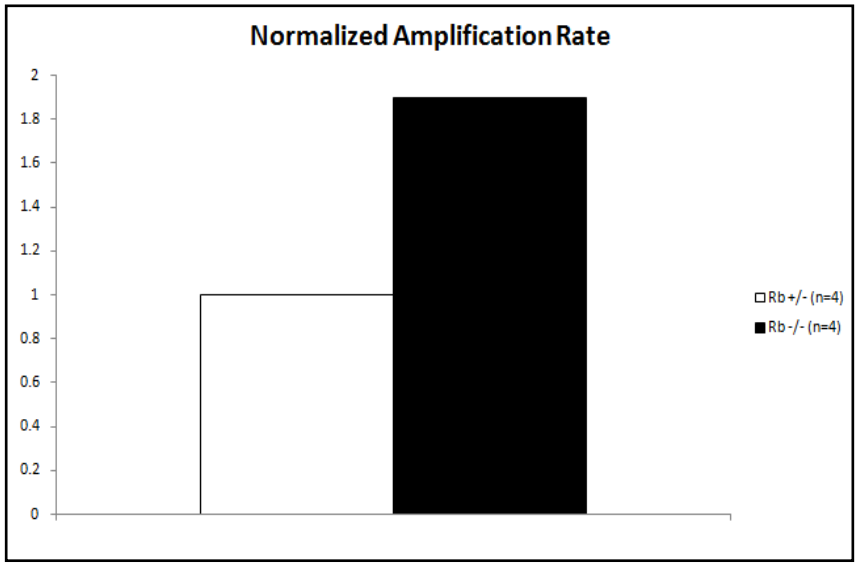


Figure 11: Loss of Rb leads to increased proliferation of aNSCs/progenitors *in vitro*. Bright field images (A-C and D-F) and corresponding fluorescent images (A'-C' and D'-F') from Rb +/- (A-C') and Rb-/- (D-F') cultures showing an increase in the size and number of green neurospheres generated after cell sorting. Images were taken at three increasing magnifications. Quantification of this data is shown in graphs 2B and 3B. Scale bar = 100µm.



Graph 4: The amplification rate (AR) representing the ratio of viable cells the total number of plated cells was calculated after each passage, every 6-7 days in culture. The average AR was normalized after three consecutive passages and showed a 1.90-fold increase in Rb-/- cultures compared with controls.

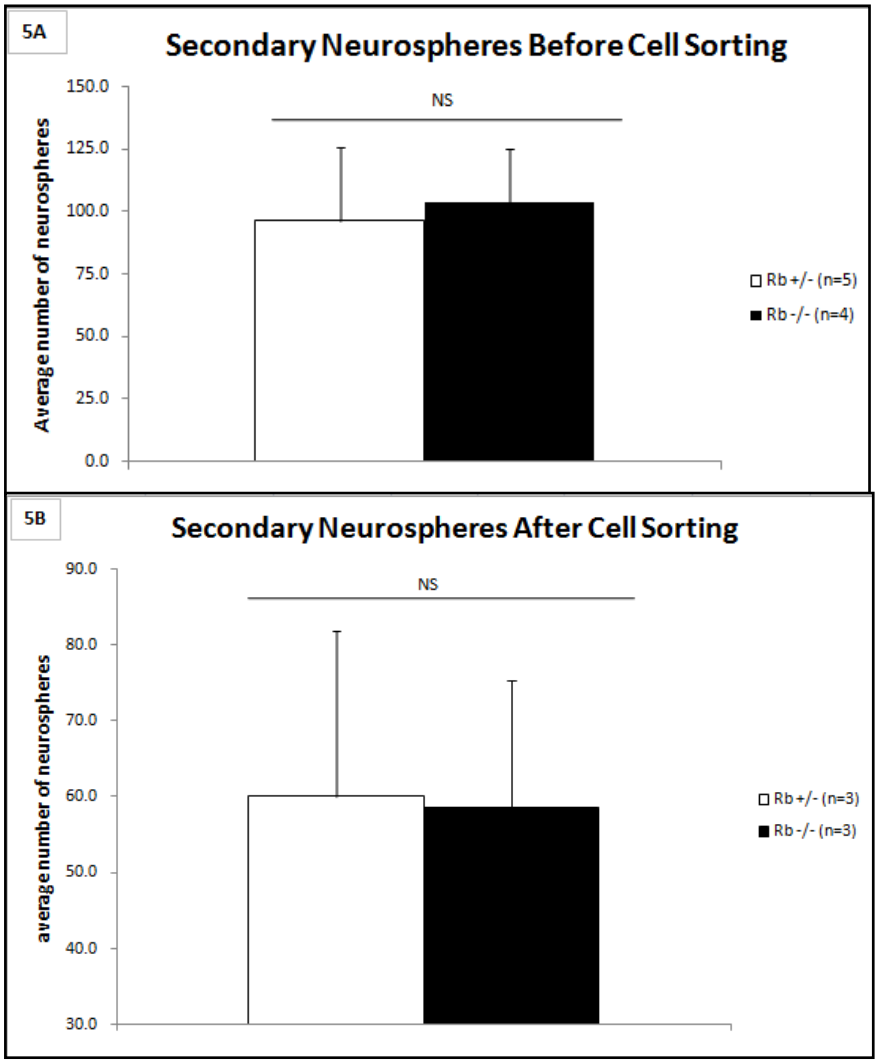
The effect of the loss of Rb on cell proliferation was further illustrated by the higher amplification rate observed in the absence of Rb. This rate represents the number of viable cells at each passage over the total number of plated cells. We found that, compared to Rb+/- cultures, Rb-null cultures showed a 1.90-fold increase in the normalized AR following three passages in culture(Graph4**)**.Altogether, our findings demonstrate that Rb controls the proliferation of aNSCs/progenitors by regulating the size and number of generated neurospheres in culture.

C. Rb is not required for the self-renewal potential of aNSCs

1. Secondary neurosphere assays performed in vitro

As previously described, a neurosphere consists of a heterogeneous population of cells including NSC(s) and/or progenitors. Both a NSC and a progenitor are capable of generating neurospheres but only NSC-derived spheres can be passaged indefinitely, and this is directly associated to their unlimited self-renewal property. To investigate whether Rb controls the self-renewal potential of aNSCs, we performed secondary neurosphere assays by dissociating single spheres of equal size derived from Rb+/- and Rb-/- primary cultures. Neurospheres were measured and counted on day 5. Results showed that the average size of secondary neurospheres was higher in Rb-/- culture compared with controls (Figure 10C-C'). However, there was no significant difference in the number of secondary neurospheres formed in both cultures and this was true for assays done before as well as after cell sorting (Graphs 5A and 5B). Hence, the ratio (Mutant/Control) of number of secondary neurospheres was ~1.08 (Rb+/-; 96.3 ± 29.64 vs Rb-/-; 103.8 ± 21.23, p=0.832; Graph 5A), and, ~1.07 (Rb+/-; 60.0 ± 21.8 vsRb-/-; 58.6 ± 16.6, p=0.821; Graph 5B). These findings indicate that Rb does not likely

control the self-renewal potential of aNSCs*in vitro;* however, further experiments e.g. generation of tertiary neurosphere assays, immunolabeling of aNSCs in culture may be required to validate this conclusion.

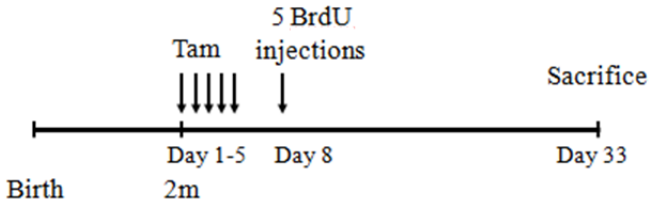


Graph 5: Secondary neurosphere assays were performed by dissociating and plating spheres of equal size. Quantification showed no significant difference (NS) in the numbers of secondary spheres generated from Rb+/- versus Rb-/- cultures and this was true before (5A) and after cell sorting (5B). Error bars represent SD of measurements from indicated 'n' and asterisks indicate no significant difference (NS) between genotypes using t-test.

2. *Long-term in vivo birthdating studies*

To confirm that loss of Rb does not affect the self-renewal potential of aNSCs, we conducted birth-dating studies to label aNSCs found in the aSVZ. To do this, tamoxifen- treated mice received five consecutive injections of BrdU (one injection every 2 hours over a 10h period) on day 8 after treatment and were sacrificed 28 days later (Figure 12A). In this experiment, only slow-proliferating cells including NSCs retained BrdU while fast-dividing cells diluted the label and/or migrated away from SVZ. As a result, we found no difference in BrdU incorporation in the SVZ in Rb-/- compared with Rb+/- mice (Figure 12B and graph 6: BrdU+: Rb+/- 2.52+0.88, Rb-/- 2.83+1.18, p=0.471,BrdU+;GFP+: Rb+/- 0.62+0.5 vsRb-/-1.33+0.47, p=0.210). This data further suggests that, while Rb regulates progenitor's proliferation, it is does not likely control proliferation of NSCs in the aSVZ and this is consistent with our *in vitro* data shown earlier as well as the role of Rb during embryonic development (Ferguson et al., 2002).

A.



B.

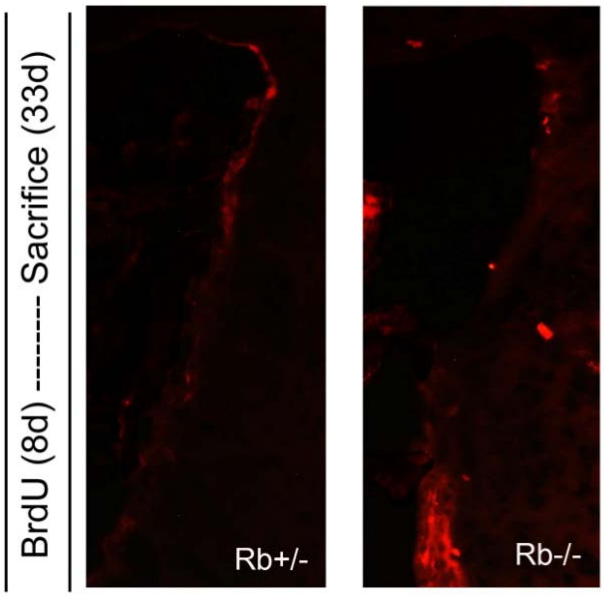
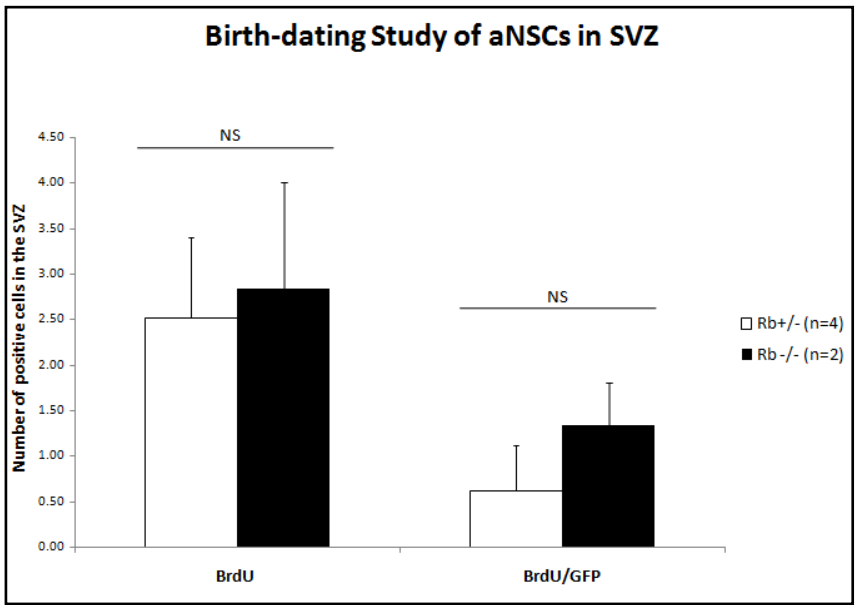


Figure 12: Rb does not regulate the self-renewal potential of aNSCs*in vivo*. **(A)**Experimental design for the birth-dating assays performed *in vivo*: 8d post deletion of Rb, 5BrdU injections were given to tamoxifen-treated mice and animals were sacrificed28 days later. **(B)** Immunohistochemistry on sagittal sections using anti-BrdU antibody and showing no difference in BrdU incorporation (Winner, Kohl, & Gage) in the SVZ in Rb+/- versus Rb-/- mice. Quantification of this data is shown in graph 6 (see next section).



Graph 6: Quantification of the birthdating data described in figure 13. Cells expressing BrdU- and GFP/BrdU-in SVZ were counted and showed no significant difference (NS) in number in Rb-/- compared to Rb +/- mice. . Error bars represent SD of measurements from n=4 Rb+/- and n=2 Rb-/- and asterisks refer to p values.

D. Rb-null NSCs display a normal differentiation potential compared to Rb+/- NSCs *in vitro*

As described earlier, aNSCs are multipotent cells that can differentiate into neurons astrocytes and oligodendrocytes in culture (Gabay et al., 2003). We assessed whether loss of Rb affects the differentiation potential of aNSCs by performing differentiation assays in culture. Hence, free floating neurospheres were dissociated and plated on monolayers to reach 70% confluency in the presence of growth factors. Then, cell differentiation was induced by removal of growth factors and addition of differentiation media supplemented with fetal bovine serum. 2d and 5d later, we analyzed the differentiated cells by immunostaining with cell-type specific markers. We thus double labeled cells with anti-Tuj-1or *β-III* tubulin which is specifically expressed in immature neurons, and, anti-GFAP or glial fibrillary acidic protein which is an astrocyte-specific

marker. We found that similar to Rb+/- cells, Rb-/- NSCs/progenitors cells primarily differentiated into astrocytes, which represented the majority of cells found in both cultures at day 2 and day 5 post-differentiation (Figure 13B-B', F-F'). Moreover, compared to controls, Rb-/- cells displayed a normal rate of neuronal differentiation at day 2 with no significant difference in the percentage of Tuj-1-positive cells found in both cultures after normalization to the total number of Hoechst-positive cells (Figure 13C-C' and graph 7:Rb+/-.; 1.08% ± 0.74 vsRb-/-; 1.83% ± 0.39, p=0.198). Similarly, at day 5 of differentiation, results showed no significant difference in the percentage of tuj1-positive cells in mutants compared to controls (Figure 13G-G' and graph 7;Rb+/-; 2.45% ± 1.24 vs Rb-/- 4.53% ± 2.53, p=0.272). Altogether, these results indicate thatRb null-NSCs retain a normal differentiation potential in culture compared to wild type cells and this is consistent with our *in vivo* data (Naser et al., in preparation).

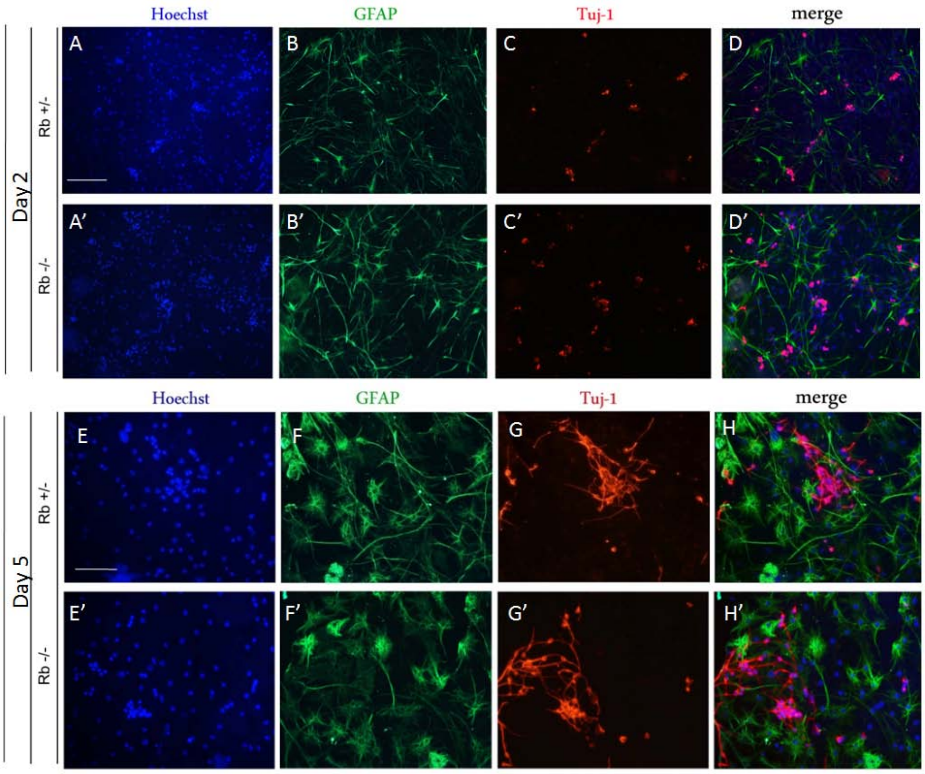


Figure 13: Rb-null progenitors display normal differentiation potential in culture. Differentiation assays were conducted by plating dissociated neurospheres in monolayers on coated dishes and then inducing their differentiation as described in the text. Double immunocytochemistry using anti-GFAP (green; mature astrocyte marker) (Babu et al., 2007) and anti-Tuj1 (red; early neuronal differentiation marker)antibodies were performed after 2d (A-D') and 5d (E-H') of differentiation in Rb+/- (A-H) and Rb-/-(A'-H') cultures. Compared to controls, Rb-null cells were able to differentiate into astrocytes (B,B', F and F') and showed normal neuronal differentiation potential after 2d and 5d in culture. Quantification of this data is shown in graph 7 (see next section).Scale bar = 100µm.

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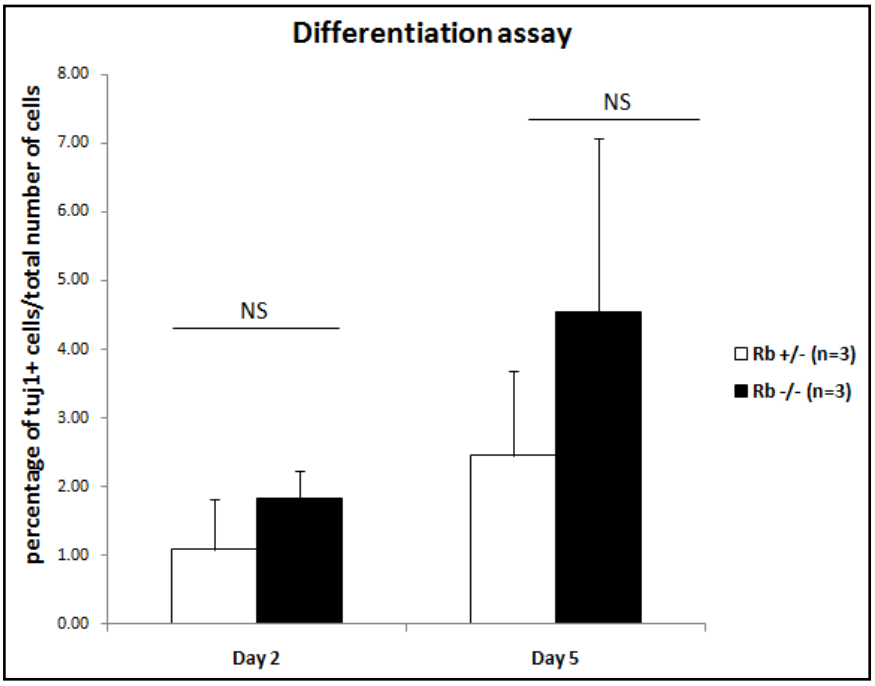

Graph 7: Quantification of Tuj-1 positive cells in culture following the differentiation assay described in figure 13. The numbers of Tuj1-positive cells were normalized over the total number of Hoechst nuclei and revealed no difference between genotypes. Error bars represent SD of measurements from n=3Ct and 3mut. (ns)indicates 'not significant' using t-tests.

CHAPTER III

DISCUSSION

Since the discovery of the regenerative capacity of the adult mammalian brain, extensive research has been conducted to better understand this property, which is directly associated with brain plasticity and adaptation under normal physiological conditions. Interestingly, the generation of new neurons and their ability to integrate into a pre-existing neuronal network in the adult brain underline the contribution of nerve regeneration to the ongoing processes of learning and memory in the hippocampus (Bath et al., 2008; Breton-Provencher et al., 2009; Bruel-Jungerman et al., 2007; G. Gheusi et al., 2000) as well as olfaction in the OB (Lazarini & Lledo, 2011).Much progress has been made to understand the properties of aNSCs and the supporting role attributed to their local environment/niche as well as the distinct steps of adult neurogenesis in rodents (Alvarez-Buylla & Lim, 2004; Duan, Kang, & al., 2008; Lledo et al., 2006) and humans (Curtis, Kam, & al., 2007; Eriksson et al., 1998; Sanai, Tramontin, & al., 2004). Moreover, the controlled expansion of aNSCs/progenitors followed by their targeted differentiation into desired lineages may lead to important therapeutic interventions. However, the restricted number of these cells and their low regenerative rate are still major obstacles facing this aim.

Cell cycle genes are key regulators of cell cycle progression and control the size of different neural populations in the brain in coordination with cell fate markers and differentiation genes (Beukelaers, Vandenbosch, & al., 2011; Cheffer, Tarnok, & al., 2013; K.A McClellan & Slack, 2006). For instance, the tumor suppressor gene, pRb, primarily regulates progenitor cells proliferation during brain development (K.L.

Ferguson, Vanderluit, & al., 2002)but is also involved in the control of neuronal migration(Andrusiak, McClellan, & al., 2011; K. L. Ferguson et al., 2005; K. A. McClellan et al., 2007)and differentiation during late embryogenesis (Ghanem et al. 2012). We have recently investigated the role of Rb in adult OB neurogenesis by inducing its temporal deletion specifically in NSCs and progenitors, and, showed that Rb promotes progenitor cells proliferation in the adult SVZ and this is associated with an enhanced neuroblast migration and differentiation *in vivo.* Notably, loss of Rb did not affect the self-renewal rate of NSCs, and, unlike during development, Rb-null progenitors differentiated normally and produce distinct subtypes of OB GABAergic interneurons that survived up to at least 4 months (Naser et al. 2015, in preparation). To further investigate the role of Rb in the control of aNSCs properties, we conducted here *in vitro* studies investigating the proliferation, self-renewal and differentiation properties of Rb+/-NSCs vsRb-/- NSCs in culture. We found that pRb regulates progenitor cells proliferation without affecting their differentiation capacity *in vitro,* and this is consistent with our *in vivo* results described above. Furthermore, we demonstrated that pRb does not seem to regulate the self-renewal property of aNSCs neither *in vivo* nor *in vitro* which explains the specificity of its function in the control of adult progenitor cells fate. Finally, we showed that loss of Rb leads to an enhanced neuronal differentiation of adult progenitors in culture despite the increase in their number. This study complements our previous *in vivo* findings and sheds the light on the role of the Rb pathway in the control of the generation of mature neurons from aNSCs outside the brain.

A. Conserved function for Rb in the control of progenitor cell proliferation in the adult versus embryonic brain.

We used the neurosphere system to assess the proliferation potential of aNSCs/progenitor cells originating from the adult SVZ following Rb deletion (Laywell et al., 1999; B. Reynolds & Weiss, 1992). We thus compared the rate of proliferation of Rb+/- NSCs and Rb-/- NSCs and found that loss of Rb leads to a significant increase in progenitor's proliferation as manifested by several observations in culture. First, the higher number of primary neurospheres derived from Rb-null SVZ tissue compared with those derived from the control tissue indicates that the pool of NSCs/progenitors capable of sphere formation is expanded in the absence of Rb (Figures 11A-A' and 12, and Graphs2A and 2B). Second, the increase in the average size of Rb-null neurosphere ssuggests that Rb-/- progenitors are undergoing more (possibly faster) cell divisions, thus, forming larger spheres compared with control cells (Figure 10 A-A' and 12 and Graph 3A and 3B). Third, the enhanced amplification rate (1.9 fold increase) in Rb-null culture vs Rb control confirmed that Rb indeed controls progenitor cells proliferation (Graph 4) but not NSCs (refer to next section).As a matter of fact, this control is directly associated with the tumor suppressor function of Rb and consistent with its role during embryonic brain development (Clarke et al., 1992, Ferguson et al. 2002, Ghanem et al. 2012). However, unlike during development where loss of Rb caused ectopic proliferation of cortical neuroblasts (Ferguson et al. 2002), Rb-null progenitor cells exited properly the cell cycle in culture and were able to differentiate normally (Figure 13, see next section), In summary, these findings indicate that Rb plays a conserved role in the control of progenitor cells proliferation in the adult vs embryonic brain.

B. Control of aNSCs properties by Rb in comparison with other cell cycle genes

As previously described, both stem cells and progenitors can generate neurospheres in culture, however, only stem cell-derived neurospheres are capable of unlimited self-renewal since this property is specific to stem cells and not progenitors (Chiasson, Tropepe, Morshead, & van der Kooy, 1999). We assessed the effect of loss of Rbon the self-renewal potential of aNSCs by performing: a) secondary neurosphere assays as initially designed by Reynolds et al. (B. Reynolds & Weiss, 1992), and, b) *in vivo* birthdating studies using BrdU. Our results showed that Rb does not seem to control the self-renewal potential of aNSCs because their rate of proliferation did not change following loss of Rb as assessed by long-term BrdU incorporation in the SVZ (Figure 12B). Add to this, the number of secondary neurospheres derived from Rb mutant vs control NSCs in culture was identical (Figure 10 C-C' and Graphs 5A and 5B). These findings suggest that Rb specifically controls progenitor's proliferation but may not play the same role in stem cells. This is in contrast to the role played by another Rb family member, p107, which was shown to negatively regulate the self-renewal of SVZ-NSCs in the adult brain (Vanderluit et al. 2004). In fact, unlike pRb-/- mice, p107-/- mice showed an expanded stem cell population and a striking increase in the number of neurosphere forming cells that exhibited enhanced capacity for self-renewal (Vanderluit et al. 2004). Besides pocket proteins, other cell cycle genes play distinct roles in the control of adult neurogenesis. For instance, loss of p53 increased the rate of division of slow- and fast-proliferating cells (type B and type C cells) and this was associated with rapid differentiation of type C into type A cells (Gil-Perotin et al. 2006). In comparison, loss of the cyclin dependent kinase inhibitor p27Kip1 had no effect on the number of stem cells but selectively increased the number of the transit-

amplifying progenitors concomitantly with a reduction in the number of neuroblasts in the SVZ (Doetsch et al. 2002). Combined loss of p53/p27Kip1 provided a proliferative advantage to SVZ populations and normal differentiation of neuroblasts (Gil-Perotin et al. 2011). Altogether, this work and other studies have showed that cell cycle genes play specific and critical roles during adult neurogenesis. Morevoer, the pocket proteins pRb and p107 have distinct but complementary roles in the adult brain.

C. Distinct roles for Rb in the control of neuronal Differentiation of adult versus embryonic progenitors

We examined how loss of Rb affects differentiation of aNSCs/progenitors by conducting differentiation assays and comparing the phenotype of Rb-null and Rb+/- cultures. We found that differentiation was normal in the absence of Rb taking into account the fact that Rb-null NSCs/progenitor cells exited properly the cell cycle (data not shown) and retained the potential to differentiate into neurons and astrocytes similar to Rb+/- NSCs (Figure 13and Graph 7). These findings are consistent with our *in vivo* data showing enhanced neurogenesis in the OB following loss of Rb.

In contrast to the data reported here and in the adult brain, previous work showed that Rb-null embryos display several developmental abnormalities accompanied by a partial failure in differentiation and increased apoptosis (Clarke et al., 1992; Jacks et al., 1992; E. Y. Lee et al., 1992; E. Y. Lee et al., 1994). Also, Rb conditional KO mice in the telencephalon exhibit severe migration and differentiation defects in the RMS and OB (Ghanem et al 2012). In fact, these defects were attributed to a decrease in the expression of Dlx1 and Dlx2 in the absence of Rb, which are two transcription factors necessary for early neuronal differentiation and migration (Ghanem et al., 2012).Since we did not identify any differentiation defects during adult neurogenesis in the absence

of Rb neither *in vivo* nor *in vitro*, we conclude that the Rb pathway plays distinct roles in NSCs/progenitor cells differentiation in the embryonic versus adult brain and, this may be linked to different spatio-temporal gene regulation. Alternatively, other cell cycle regulators e.g. p130 may compensate for the loss of Rb in the control of terminal differentiation, however, this possibility requires further investigation.

D. Rb and nerve regeneration: functional significance and potential therapeutic outcomes

Continuous neurogenesis is required for olfactory plasticity and sensory learning (Gilles Gheusi, Lepousez, & Lledo, 2012).In rodents, newly-born neuroblasts migrate along the RMS and reach the OB where they differentiate into GABAergic interneurons (reviewed in(Ernst et al., 2014; Sakamoto, Kageyama, & Imayoshi, 2014), however, not all of them integrate into the pre-existing neuronal network (Lledo et al., 2006). The fate of these neurons depend mainly on sensory activity; thus, deficiency in sensory input triggers neuronal apoptosis whereas olfactory enrichment and learning enhances neuronal survival (Petreanu & Alvarez-Buylla, 2002; Rochefort, Gheusi, Vincent, & Lledo, 2002). Thereafter, permanent generation of new neurons in the OB is essential for the acquisition of odor memory and odor discrimination (Lazarini and Lledo et al. 2011). Moreover, the generation and turnover of newly born neurons have critical roles not only in brain development and maintenance but also in neurodegenerative diseases such as Parkinson’s disease, Huntington disease and Alzheimer’s disease.In fact, specific alterations in neurogenic areas such as the dentate gyrus and SVZ/OB parallel the early or pre-motor symptoms that are detected in the early stages of these diseases such as depression, anxiety or olfactory dysfunction (reviewed in(Winner et al., 2011)). Also, new born neurons were reduced in patients

with Huntington's disease; thus, highlighting the importance of adult neurogenesis in cognition and motor coordination (Kohl et al., 2010).

On the other hand, aNSCs form a reservoir of undifferentiated cells that are able to proliferate and differentiate to replace lost neurons under normal physiological conditions or after brain injury or disease. Thereafter, the discovery of adult neurogenesis opened the door for potential new therapies to treat nerve degeneration. While much advancement has been accomplished in the understanding of the molecular mechanisms controlling aNSCs development and function, their limited number and low regenerative rate (mainly *in vivo*) are major obstacles facing their expansion and potential use for targeted differentiation therapies. Interestingly, this study has shown that Rb controls the proliferation of adult progenitors without altering their differentiation potential or the self-renewal of aNSCs. As such, future studies might aim to manipulate the Rb pathway in order to expand further the pool of progenitors without compromising their differentiation potential. This approach can be theoretically applied *in vivo* or *in vitro* and may present a promising tool for regenerative therapy. Progenitor cells hence generated *in vitro* could be potentially cryopreserved and used later on in regenerative medicine. Subsequently, if we are able to trigger cell differentiation using the right molecular cues according to desired program(s), neurosphere-derived neurons may be able to replace lost neurons in case of brain injury or neurodegenerative diseases. Yet, this possibility still encounters several problems. First, human therapy needs high number of differentiated cells, whereas neurosphere-derived cells are subjected to extrinsic and intrinsic stress that might cause cell death, thus resulting in insufficient numbers for human therapy. Second, the artificial environment in which aNSCs/progenitors are cultured does not mimic normal

physiological conditions which might alter the endogenous properties of derived progenitors. Finally, as discussed earlier, it is difficult to derive a pure population of progenitors using the neurosphere system since spheres are heterogeneous in nature, unless one performs complementary approaches such as cell sorting to enrich for specific cell population(s).

Despite the limitations that are still lying ahead, research on nerve regeneration in the light of adult neurogenesis has achieved tremendous progress and overcame several obstacles thus far. The future of regenerative medicine will definitely be very promising as new discoveries and applications unfold, thus bringing more hope for therapeutic interventions.

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