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The lineage and diversity of postnatal neural stem cells

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The Lineage and Diversity of Postnatal Neural Stem Cells

by

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DISSERTATION

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For my parents,

Hellmut and Hildegard Merkle.

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Abstract

The lineage and diversity of postnatal neural stem cells

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Florian T. Merkle

Neural stem cells (NSCs) generate the diverse types cell types that make up adult brain. NSCs persist in the subventricular zone (SVZ) of the adult rodent brain, where they generate astrocytes, oligodendrocytes, and multiple types of olfactory bulb (OB) neurons. They have astrocyte-like characteristics and act as stem cells in culture, but little is known about their origin or potential *in vivo*. In this work, I describe a technique to specifically and permanently label radial glial cells in the neonatal mouse brain and demonstrate that as a population, these cells act as multipotent NSCs. Labeled radial glia generate adult NSCs that are neurogenic *in vivo* and that individual radial glia and their adult SVZ progeny act as multipotent stem cells *in vitro*. Therefore, throughout development, neural stem cells have glial characteristics. Second, I show that the adult NSCs is a unique cell type that shares features of radial glia and differentiated astrocytes. Third, I show that NSCs persist in the dorsal wall of the lateral ventricle (LV), the anterior medial wall of LV, and in the rostral migratory stream (RMS). Thus, adult neurogenesis occurs in a significantly larger region than has been appreciated. Fourth, I show that NSCs labeled in different neurogenic subregions produce different types of olfactory bulb neurons. Thus, adult NSCs consist of different populations of restricted progenitors segregated into different domains. Finally, I show that the neurogenic potential of these progenitors is maintained during postnatal development and after being

cultured or heterotopically grafted, suggesting that adult NSC potential is established during embryonic development and maintained by a cell-autonomous mechanism. Together, these findings suggest that embryonic NSCs transform into adult NSCs that maintain embryonic characteristics and regional identity, but are restricted in their neurogenic potential.

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List of Symbols, Abbreviations and Nomenclature

Symbol	Definition
AChE	Acetylcholinesterase
Ad:Cre	Adenovirus expressing Cre
Ad:GFAP-Cre	Adenovirus expressing Cre under the murine GFAP promoter
AEP	Anterior Entopeduncular area
AP	Anterior-Posterior Alkaline Phosphatase
APP	Amyloid Precursor Protein
AraC	Cytosine Arabinoside
BABB	Benzyl Alcohol Benzyl Benzoate clearing solution
BCIP	5-Bromo-4-Chloro-3-Indolyl-Phosphatate
bFGF/FGF_2	basic Fibroblast Growth Factor
BMP	Bone Morphogenetic Protein
BPE	Bovine Pituitary Extract
C	Cortical
Cas	Castor transcription factor
CalB	Calbindin calcium binding protein
CalR	Calretinin calcium binding protein
CaMKIV	Calcium/calmodulin dependent Kinase IV
CB	Cerebellum
CC	Corpus Callosum
CH	Cortical Hem
ChAT	Choline Acetyltranferase
CldU	5-Chloro-2'-deoxyuridine
CNS	Central Nervous System
CNTF	Ciliary Neurotrophic Factor
CP	Cortical Plate
Cre	Cre recombinase protein from bacteriophage P1
CSF	Cerebrospinal Fluid
Ctx	Cortex
D	Dorsal
DAB	Diaminobenzidine
DAPI	4’,6-diamidino-2-phenylindole
Dcx	Doublecortin
DiI	1,1'-Dioctodecyl-3,3,3',3'-tetramethylindocarbocyanine
DG	Dentate Gyrus
DIV	Days In Vitro
dLGE	dorsal Lateral Ganglionic Eminance
Dlx1/2/5/6	Distalless-related transcription factor 1/2/5/6
DMEM	Dulbecco's Modified Eagle's Medium
DMEM/F12	Dulbecco's Modified Eagle's Medium/Ham’s F12 50:50 mix

DMSO	Dimethyl Sulfoxide
DP	Dorsal Pallium
E	Embryonic day
EDTA	Ethylenediamine Tetraacetic Acid
EGF	Epidermal Growth Factor
eGFP	enhanced Green Fluorescent Protein
EGFR	Epidermal Growth Factor Receptor
EM	Electron Microscopy
Emx1/2	Empty spiracles homolog transcription factor 1/2
EPL	External Plexiform Layer
Er81	Ets variant gene 1 transcription factor
FACS	Fluorescence Activated Cell Sorting
FBS	Fetal Bovine Serum
FCS	Fetal Calf Serum
FGF	Fibroblast Growth Factor
Foxg1	Forkhead box G1 transcription factor
GABA	Gamma-Aminobutyric Acid
GC	Granule Cell
GFAP	Glial Fibrillary Acidic Protein
GFP	Green Fluorescent Protein
GL	Glomerular Layer
Gli1/2/3	Glioma-associated oncogene homolog 1/2/3 zinc finger transcription factors
GRL	Granule Cell Layer
Gsh1/2	GS homeobox transcription factor 1/2
Hb	Hunchback transcription factor
HRP	Horseradish Peroxidase
HSV-TK	Herpes Simplex Virus Thymidine Kinase
ICC	Immunocytochemistry
IdU	5-Iodo-2'-deoxyuridine
IHC	Immunohistochemistry
IP	Intraperitoneal
IPC	Intermediate Progenitor Cell
Kr	Krüppel transcription factor
L15	Leibovitz's L-15 Medium
LoxP	Locus of crossing over in phage P1
LacZ	Beta-galactosidase encoding gene from *Escherichia coli*
LGE	Lateral Ganglionic Eminance
Lhx6/7	LIM homeobox protein 6/7

LP	Lateral Pallium
LV	Lateral Ventricle
M	Medial
Mash1	Murine achaete-scute complex homolog-like 1
MBP	Myelin Basic Protein
MC	Mitral Cell
MCL	Mitral Cell Layer
MGE	Medial Ganglionic Eminance
M/T	Mitral and Tufted Cells
Myc	Myelocytomatosis oncogene
N	Neuron/Neuroblast
NBT	4-Nitro Blue Tetrazolium chloride
NE	Neuroepithelium
NEC	Neuroepithelial Cell
NC	Neurocalcin
NG2	NG2 chondroitin proteoglycan
Ngn1/2	Neurogenin transcription factor 1/2
Nkx2.1	thyroid transcription factor 1
Nkx2.2	NK2 transcription factor related, locus 2
Notch1	Notch gene homolog 1
NSC	Neural Stem Cell
Numb	Numb gene homolog
O4	Oligodendrocyte marker, clone O4, reacts with POA
OB	Olfactory Bulb
OE	Olfactory Epithelium
Olig2	Oligodendrocyte transcription factor 2
ONL	Olfactory Nerve Layer
OPC	Oligodendrocyte Precursor Cell
OSN	Olfactory Sensory Neuron
P	Postnatal day
Pax6	Paired box domain transcription factor 6
PB	Phosphate Buffer
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline + 0.1% Triton X-100
PCR	Polymerase Chain Reaction
PDK	Poly-D-Lysine
Pdm	Drosophila POU domain transcription factor
PDGF	Platelet-Derived Growth Factor
PDGFRalpha	Platelet-Derived Growth Factor Receptor alpha
PFA	Paraformaldehyde
PGC	Periglomerular Cell
POA	Proligodendrocyte Antigen (sulfated glycolipid)

PSA-NCAM	Polysialic Acid-Neural Cell Adhesion Molecule
PV	Parvalbumin
RC2	Radial Cell 2 antigen
RMS	Rostral Migratory Stream
RPM	Rotations Per Minute
RT	Room Temperature
S phase	Synthesis phase
SAC	Short Axon Cell
SCZ	Subcallosal Zone
SD	Standard Deviation
SEM	Standard Error of the Mean
SGZ	Subgranular Zone
Shh	Sonic hedgehog
Sox29/10/11	SRY-box containing gene 2/9/10/11
Sp8	trans-acting transcription factor 8
Stri	Striatum
SVZ	Subventricular Zone
TC	Tufted Cell
TF	Transcription Factor
TGFalpha	Transforming Growth Factor alpha
TH	Tyrosine Hydroxylase
TrkB	neurotrophic tyrosine kinase receptor, type 2
Tuj1	Beta III tubulin
TX	Triton X-100 detergent
UA	Uranyl Acetate
V	Ventral
Vax1	Ventral anterior homeobox containing gene 1
vLGE	ventral Lateral Ganglionic Eminance
VP	Ventral Pallium
VZ	Ventricular Zone
Wnt	Wingless
Zic1	Zinc finger protein of the cerebellum 1

Chapter 1

Introduction

Overview and statement of objectives

The brain is arguably the most complex organ in the body. It is composed of billions of neurons (Chklovskii et al., 2004), connected and arranged in precise patterns. Together, these cells control essential bodily functions and allow an animal to sense and respond appropriately to its environment. The complexity of the brain is derived from a small number of neural stem cells (NSCs) that proliferate, acquire regional identities, and produce different cell types. Understanding NSCs therefore is central to understanding brain development.

NSCs are neural cells that maintain the potential to generate multiple neural cell types over long periods of time. They exist not only in the embryonic brain, but also in restricted regions of the adult brain. Most adult neurogenesis occurs in two germinal zones: the subgranular zone (SGZ) of the dentate gyrus (DG) of the hippocampus and the subventricular zone (SVZ) of the lateral wall of the lateral ventricle, (reviewed in (Temple, 2001)). Over the last several years, adult NSCs in these regions have been shown to correspond to an astrocyte-like cell (Doetsch et al., 1999a; Imura et al., 2003; Morshead et al., 2003; Seri et al., 2001). It is not known how these astrocyte-like cells differ from astrocytes elsewhere in the brain, nor is their developmental cell-of-origin known. Furthermore, it is not clear how far the SVZ extends in the brain, how stem cells

are organized in the SVZ, or if they constitute a homogeneous or diverse population of progenitors.

Adult neurogenesis is a robust phenomenon; thousands of neurons are born every day in the SVZ. These newborn neurons, or neuroblasts, migrate long distances to reach the olfactory bulb (OB), where they differentiate into many different types of interneurons to replace dying cells. These interneurons regulate the brain activity that underlies olfaction, the sense of smell. However, the extent of interneuron diversity and mechanism by which diversity is generated is unclear.

In this work, I will address several questions: 1) What is the developmental origin of adult SVZ NSCs? 2) What is the spatial extent of the SVZ? 3) Are SVZ NSCs equivalent or diverse? 4) Are SVZ NSCs plastic or committed? To address these questions, I will examine neural stem cells and their progeny throughout postnatal development. First, I will review the mechanisms that pattern the developing brain and generate cell diversity. Second, I will review the identity, potential, and function of adult NSCs and the cells they produce. Third, I will review the structure of the olfactory bulb, the diversity of postnatally born neuron types, and the possible functional role of adult neurogenesis. Finally, I will briefly state how I addressed the questions listed above.

Section 1: Embryonic brain development

Neural specification and brain patterning

Every animal begins its life cycle as a single totipotent cell. In early embryonic development, this cell divides rapidly and its daughter cells organize into three different germ layers: the endoderm, mesoderm, and ectoderm. The central nervous system (CNS) is derived from a region of the ectoderm (Gallera, 1971), a sheet of cells known as the neuroepithelium (NE) (Schoenwolf and Alvarez, 1991). Through beautifully coordinated movements, the edges of this sheet fold together to form the neural tube, the fluid-filled center of which later becomes the ventricular system and spinal canal (Karfunkel, 1989; Morriss-Kay et al., 1994; Schoenwolf, 1991) (Fig. 1A-D). As neural tissue is induced and undergoes dramatic morphological transformations, it is patterned by gradients of morphogens secreted in “organizing centers” (Alvarez and Schoenwolf, 1991; Monuki and Walsh, 2001; Wilson and Rubenstein, 2000) (Fig. 2). NSCs respond to these morphogens by expressing or silencing different sets of genes, thereby acquiring regional identity (Shimamura et al., 1997). Notably, such genes include transcription factors (TFs) that regulate the expression of many other genes (Fig. 3). By this mechanism, the CNS is divided into different dorsoventral and rostrocaudal domains, in which NSCs produce different progeny (Jessell and Sanes, 2000; Krumlauf et al., 1993; Rubenstein and Beachy, 1998; Shimamura et al., 1997). This organization is refined by cell migration, (Hatten, 1999; Marin and Rubenstein, 2003), the establishment of connectivity (Aoki and Siekevitz, 1988; Cohen-Cory, 2002; Hensch, 2005; Isacson and Deacon, 1997;

Kalil, 1989) and the selective pruning of cells and their connections (Gao et al., 1999; Gordon, 1995; Hensch, 2005).

Primary progenitors in embryonic development

The brain is derived from a sheet-like neuroepithelium (NE) composed of neuroepithelial cells (NECs). These cells are radially elongated and contact both the apical (ventricular) and basal (pial) surfaces (Fig.1E). Interestingly, they divide at the ventricular surface but pull their nucleus toward the pial surface during interphase (Bhide, 1996; Noctor et al., 2004; Sheth and Bhide, 1997; Smart, 1976). This process is known as interkinetic nuclear migration. Initially, NECs divide symmetrically to increase the pool of stem cells but later divide asymmetrically, producing a stem cell that remains at the ventricular surface and a daughter cell that migrates radially outward (Huttner and Brand, 1997; Huttner and Kosodo, 2005). Some of these daughter cells are postmitotic neurons, but other cells continue to proliferate just above the ventricle in the subventricular zone (SVZ), as will be discussed below. As daughter cells accumulate, progenitors dividing at the ventricular surface form an identifiable pseudostratified epithelium known as the ventricular zone (VZ). The accumulation of daughter cells leads to a thickening of the early brain, which radially stretches NECs. By the onset of neurogenesis at E10-E11 in the mouse forebrain, (Caviness et al., 1995), NECs disappear to be replaced by radial glial cells (Anthony et al., 2004).

Like NECs, radial glia maintain contact with ventricular surface via a short endfoot, but also with the pial surface via a thin, radially projecting basal process.

Though they appear to be distinct cell types, NECs and radial glia share many characteristics (reviewed in (Gotz and Huttner, 2005; Malatesta et al., 2003)), and it has been suggested that NECs transform directly into radial glia, though this has yet to be proven. Radial glia are characterized not only by their unique radial morphology, but also by their expression of the intermediate filament associated protein RC2 (Misson et al., 1988), and the intermediate filament proteins nestin (Frederiksen and McKay, 1988; Hockfield and McKay, 1985) and vimentin (Pixley and De Vellis, 1984). More recently, it has been shown that radial glia express surface markers found on astrocytes, including brain lipid binding protein (BLBP) (Feng and Heintz, 1995), the glutamate transporter GLAST (Shibata et al., 1997), and tenascin-C (Peretto et al., 2005). Interestingly, BLBP is heterogeneously expressed in space and time (Anthony et al., 2004), supporting previous observations indicating that radial glia are functionally heterogeneous (Kriegstein and Gotz, 2003). In primates, radial glia express the astrocyte marker GFAP (Levitt and Rakic, 1980).

The long, basal process of radial glial cells establishes specialized contacts with blood vessels (Bass et al., 1992; Mission et al., 1991) and guides the migrating neuroblasts born in the VZ toward the pial surface (Campbell and Gotz, 2002; Rakic, 1985; Rakic, 1995) (Fig. 4). For many years, it was thought that radial glia were merely a scaffold for migrating neurons that were generated by an unidentified progenitor near the ventricular surface. However, recent work has shown that radial glia undergo interkinetic nuclear migration and generate neurons at the VZ via asymmetric division in the embryonic mouse brain (Gotz et

al., 2002; Gregg et al., 2002; Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2001; Noctor et al., 2002; Noctor et al., 2004). Though radial glia were initially thought to generate only a subset of CNS neurons, (Malatesta et al., 2003), they are now recognized as the principal, if not the only NSC of the embryonic mammalian forebrain (Anthony et al., 2004). Mammalian radial glial cells are thought to transform into astroglial and ependymal cells near the end of gliogenesis (Edwards et al., 1990; Schmechel and Rakic, 1979; Spassky et al., 2005; Takahashi et al., 1990), reviewed in (Gotz and Barde, 2005). However, neurogenic radial glia are retained in some adult songbirds (Alvarez-Buylla et al., 1990), lizards (Garcia-Verdugo et al., 2002), turtles (Russo et al., 2004), and fish (Zupanc, 2006) (reviewed in (Tramontin et al., 2003)).

At early stages in development, a NSC division may result in a neuron that inherits the radial glial fiber, which it uses to pull itself up to the cortical plate (Miyata et al., 2001; Nadarajah, 2003; Nadarajah et al., 2001). This mode of neural migration, called somal translocation, may be unique to the early brain. At later developmental stages, radial glial cells retain their radial process (Noctor et al., 2001) and neurons generated at the ventricular surface often migrate radially along radial glial processes (reviewed in (Kriegstein and Noctor, 2004)).

Tangential migration and the generation of OB interneurons

Though many neurons migrate radially, several neuronal populations migrate tangentially through the brain. These neurons include inhibitory interneurons, which express the neurotransmitter GABA and constitute

approximately 20% of all cortical neurons (Hendry et al., 1987; Meinecke and Peters, 1987; Parnavelas et al., 1977) (reviewed in (Markram et al., 2004)). These cells do not respect regional forebrain boundaries as they migrate, and disperse over a wide area before maturing (Heffron and Golden, 2000; Letinic et al., 2002). Genetic and embryological evidence suggests that these cells are derived not from the cortex, but from subcortical structures.

Around mid-gestation, the embryonic mouse brain contains two prominent subcortical structures: the medial ganglionic eminance (MGE) and the lateral ganglionic eminance (LGE) (Fig. 3) (Smart and Sturrock, 1979). When these structures were experimentally separated from the cortex in slice culture, the cortex received fewer cortical interneurons (Anderson et al., 1997), implying that interneurons were generated in the ganglionic eminences. MGE or LGE- derived cells were shown to have a different migratory potential when dissected and grafted; MGE-derived cells dispersed widely in the cortex, whereas LGE-derived cells were able to migrate to the olfactory bulbs (Wichterle et al., 1999). This confirmed earlier studies in which LGE cells were labeled with ^{3}H and shown to migrate to the OB (De Carlos et al., 1996), and in which MGE cells were labeled with the lipophilic dye 1,1'-dioctodecyl-3,3,3',3'-tetramethylindocarbocyanine (DiI) and traced into the cortex (Lavdas et al., 1999).

Genetic fate-mapping of genes expressed in the MGE and/or LGE showed that TFs regulate the generation of cortical and olfactory bulb interneurons (Puelles et al., 2000; Stenman et al., 2003), notably Dlx1/2 (Anderson et al., 1997; Pleasure et al., 2000a), Pax6 (Dellovade et al., 1998; Hack et al., 2005;

Kohwi et al., 2005; Toresson et al., 2000; Yun et al., 2001), Gsh2 (Corbin et al., 2000; Toresson and Campbell, 2001; Toresson et al., 2000; Yun et al., 2003; Yun et al., 2001), Nkx2.1 (Pleasure et al., 2000a; Sussel et al., 1999), Sp8 (Waclaw et al., 2006), and Zic1 (Inoue et al., 2007) (Fig. 3). These data were confirmed by in vivo fate mapping of transplanted MGE and LGE cells (Wichterle et al., 2001) and by culturing LGE or MGE cells and staining the neurons they produce for cell-type specific markers (Xu et al., 2004). Together, these experiments demonstrate that the cells of the embryonic LGE and adult lateral ventricular wall both generate olfactory bulb neurons, reside in the same anatomical location, and express similar TFs. Thus, it is thought that the adult SVZ is derived from the LGE, although other embryonic structures may also contribute to the adult SVZ.

Intermediate progenitors

Primary NSCs divide asymmetrically in the VZ of the developing brain to produce another NSC and a more committed daughter cell. This daughter cell may be a neuron or glial cell, but may also be an intermediate progenitor or transit/transient amplifying cell that continues to divide in the SVZ (Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004) (Fig 5). More than 90% of neurons are thought to be generated by symmetrically dividing SVZ progenitors, which amplifies the number of neurons produced by a single NSC division (Haubensak et al., 2004; Miyata et al., 2004). This amplification step may be an important determinant of brain size; species with a larger cortex have a larger

pool of intermediate progenitors and a larger SVZ (Martinez-Cerdeno et al., 2006). Symmetric and asymmetric divisions can be distinguished by the plane of division or the localization of cytoplasmic determinants such as EGFR (Sun et al., 2005) and Numb (Zhong et al., 1996) (reviewed in (Huttner and Kosodo, 2005)). However, much of what we have learned about the behavior of primary and intermediate progenitors comes from studies using confocal time-lapse imaging of fluorescently labeled cells in embryonic slice cultures. This technique revealed that when intermediate progenitors reach the SVZ, they send out numerous processes as if sensing local cues (Noctor et al., 2004). Interestingly, there is evidence from work in Drosophila that the neural progenitor cleavage plane is determined by its interaction with epithelial cells (Siegrist and Doe, 2006). Thus, it is possible that environmental factors regulate the behavior of mammalian intermediate progenitors as well as NSCs.

Temporal regulation of NSC potential

The neurogenic potential of NSCs is regulated by temporal as well as spatial factors. Clear evidence for this was provided by birthdating studies, in which newborn cells are labeled and traced over time. Proliferating cells can be labeled by an injection of radioactive (tritiated) thymidine (^{3}H), or the thymidine analog bromodeoxyuridine (BrdU), which incorporates into DNA as it is being synthesized, thereby labeling cells that are preparing to divide. Through this approach and later work, it was shown that neurogenesis begins in the forebrain at around E10 in mice and peaks from E14-E16 (Caviness et al., 1995). At E17,

neurogenesis ceases in most regions and gliogenesis, which begins around E12.5, becomes the prominent (reviewed in (Rowitch, 2004)). Furthermore, different neuron types are born at different ages. This has been clearly demonstrated in the cerebral cortex, a six-layered structure populated by morphologically and electrophysiologically distinct neuron types. Birthdating studies showed that layer 1 develops first but layers 2-6 appear to develop sequentially in an inside-out fashion; layer 6 (the deepest layer) neurons are generated second and layer 2 neurons are generated last. In this system, later-born neurons migrate past earlier born cells to reach their target location (Ignacio et al., 1995).

By heterochronically grafting cortical progenitors, McConnell and colleagues demonstrated that progenitors that would normally produce layer 4 cells could appropriately produce later-born (layer 2/3) cell types when grafted into an older brain. However, older progenitors only produced later-born cell types, even when placed in a younger environment (Desai and McConnell, 2000; McConnell, 1988; McConnell, 1990). Furthermore, it was shown that younger progenitors had to be in S phase in order to be respecified by an older environment, suggesting that their response to environmental cues was cell-cycle dependent (McConnell and Kaznowski, 1991). Similarly, heterochronic transplantation of cerebellar progenitors confirmed that younger, but not older progenitors are capable of making all major cerebellar types, and heterotopic transplantation demonstrated that they are committed to the cerebellar fate (Grimaldi et al., 2005; Klein et al., 2005). Interestingly, clonal cultures of neural

stem cells were shown to generate layer specific neurons at the same developmental time points as their counterparts *in vivo* (Shen et al., 2006). This suggests that mammalian neural stem cells contain a timing mechanism that persists in isolation from environmental signals. The basis for this mechanism in mammals is unclear, but work in Drosophila has uncovered a temporal code for neuron identity at the molecular level.

Chris Doe and colleagues have shown that neural identity is determined by the sequential activation of the transcription factors (TFs) Hunchback (Hb), Krüppel (Kr), Pdm, and Castor (Cas) in neuroblasts (neural progenitors) (Isshiki et al., 2001) (Fig. 6A). These TFs are expressed in both the neuron and neuroblast at the time the neuron is born, but only the neuron maintains high levels of TF expression. The loss of TFs expressed early in the lineage, such as Hb, results in the loss of early born cell types, whereas overexpression of Hb forces later-born neurons to adopt an Hb fate. However, if Hb expression is induced progressively later, neuroblasts lose the ability to produce early-born neuron types (Pearson and Doe, 2003) and become increasingly insensitive to respecification (Grosskortenhaus et al., 2005). A similar phenomenon has been appreciated in the mammalian brain; the transcriptional repressor Foxg1 represses the production of early born cell types. When it is knocked out, early cell fates persist, and when conditionally knocked out later in development, early-born neuron types can be ectopically produced in an older brain (Hanashima et al., 2004) (Fig 6B-D). In summary, a growing body of evidence suggests that local cues specify mammalian NSCs, resulting in the generation of temporally

and spatially appropriate cell types. As NSCs respond to these cues, they become increasingly insensitive to respecification (reviewed in (Pearson and Doe, 2004; Temple, 2001)).

Summary

Embryonic mammalian NSCs are glial-like cells that generate neurons and glia, sometimes via an intermediate progenitor. NSCs generate the tremendous diversity in cell types by acquiring regional and temporal identity. However, the potential and plasticity of NSCs diminishes over time, so that by the time of birth, NSCs have largely disappeared. However, as we will see in the next section, a remarkable subset of glial-like neural stem cells continues to generate neurons in the adult brain.

Section 2: Adult neural stem cells

A historical perspective on adult neurogenesis

The assumption that mammalian neurons were exclusively born in the embryo was not seriously challenged until the 1960s, when Joseph Altman presented a series of papers describing the generation of neurons in the adult mammalian brain. He treated rats and cats with tritiated thymidine (^{3}H) to label proliferating cells, and found evidence for widespread proliferation (Altman, 1962; Altman, 1963). Eventually, he (Altman, 1967; Altman and Das, 1965) and others (Kaplan and Hinds, 1977) identified two main proliferative regions in the adult mammalian brain: the lateral walls of the lateral ventricle (LV), and the dentate gyrus (DG) of the hippocampus (Fig. 7). These regions were confirmed to be the primary sites of adult neurogenesis (Kaplan and Bell, 1983; Lois and Alvarez-Buylla, 1993; Reynolds and Weiss, 1992a; van Praag et al., 2002), reviewed in (Alvarez-Buylla and Garcia-Verdugo, 2002; Gage, 2000). The new neurons were identified as “microneurons” or interneurons (Altman, 1967; Kaplan et al., 1985; Stanfield and Trice, 1988), of which large numbers appeared to be migrating to the olfactory bulbs (Altman, 1969; Bayer, 1985).

However, these findings were not widely accepted until the functional integration of new neurons was convincingly shown in songbirds (Paton and Nottebohm, 1984). The discovery of adult neurogenesis led to a new set of questions: 1) Why does neurogenesis persist in the adult brain? 2) Why does neurogenesis only persist in certain regions? 3) What is the functional role of the

new neurons? 4) Where do the stem cells that generate the new neurons reside? 5) What is the identity of the adult neural stem cells? Below, I will discuss the work of others that has answered some of these questions. I will concentrate on the stem cells that generate OB interneurons, though hippocampal progenitors and their progeny have been studied extensively (Altman and Bayer, 1990; Doetsch, 2003; Eves et al., 1992; Lennington et al., 2003; Palmer et al., 2000; Pleasure et al., 2000b; Seaberg and van der Kooy, 2002; Seri and Alvarez-Buylla, 2002; Seri et al., 2004 (reviewed in (Hatten, et al. 1999; Seri et al., 2001; van Praag et al., 2002))

The SVZ neural stem cell

Neurogenic SVZ progenitors were first isolated by culturing dissociated cells from different regions of the adult rodent brain in the presence of the growth factors EGF and FGF (Morshead et al., 1994; Reynolds and Weiss, 1992a). Some cells proliferated under these conditions, forming free-floating balls of cells called neurospheres. These neurospheres could be passaged and differentiated into multiple cell types (neurons, astrocytes, and oligodendrocytes), thereby demonstrating self-renewal and multipotency *in vitro*. These criteria have been used to define the adult NSC *in vitro* (Capela and Temple, 2002; Geschwind et al., 2001; Tropepe et al., 1999). Since the lateral wall of the LV contains many neurosphere-forming cells it was thought that this region might constitute the entire SVZ germinal zone. However, other studies have suggested that neural stem cells might also reside in the RMS (Bonfanti et al., 1997; Gritti et al., 2002; Liu and Martin, 2003; Pencea et al., 2001), the medial wall of the LV (Doetsch et

al., 1999a; Morshead et al., 1994), the dorsal wall of the LV (Kohwi et al., 2007; Ventura and Goldman, 2007), and in the subcallosal zone (SCZ) (Seri et al., 2006). The SCZ is a germinal zone caudal and dorsal to the SVZ, just beneath corpus callosum and adjacent to the hippocampus where the LV has collapsed due to the growth of the hippocampus (Seri et al., 2006) (Fig. 8). Other groups have found neurosphere forming cells throughout the ventricular neuraxis (Weiss et al., 1996a).

The lack of a unique stem cell marker or a technique to directly label NSCs has made it difficult to determine whether these regions contain multipotent stem cells or merely restricted neural progenitors. Culturing progenitors may alter their potential (Doetsch et al., 2002; Gabay et al., 2003) and retrovirus injected *in vivo* will infect all dividing cells, including neuroblasts that may be migrating through the injection site (Miller et al., 1990). Therefore, the true *in vivo* potential of NSCs and the spatial extent of the adult germinal zone remains obscure.

The lateral wall of the lateral ventricle contains at least four different cell types, distinguished by their morphology, ultrastructure and marker gene expression (Doetsch et al., 1997) (Fig. 9). They comprise a sheet just a few cell layers thick and adjacent to the ventricle. This region is therefore called the subventricular zone (SVZ) or subependymal zone (SEZ). Though it shares many properties with the embryonic SVZ, the adult SVZ is unique since it contains primary progenitors as well as intermediate progenitors. The principal cell type contacting the LV is the multiciliated ependymal (type E) cell. Though it has been

suggested that these cells may be stem cells (Johansson et al., 1999), careful analysis has suggested that they do not divide in the adult brain and therefore could not act as progenitors (Chiasson et al., 1999; Doetsch et al., 1999a; Laywell et al., 2000; Spassky et al., 2005). The remaining cell types, called type A, B and C cells, were described by immunohistochemistry and electron microscopy (Doetsch et al., 1997). Type A cells correspond to neuroblasts that have a migratory morphology and express the markers Tuj1, Dcx and PSA-NCAM. Within the SVZ, they migrate in chains that merge at the rostral tip of the LV to form a single pathway called the rostral migratory stream (RMS), which extends into the OB core (Lois and Alvarez-Buylla, 1994). These chains are often surrounded by the GFAP+ processes of astrocytes, some of which correspond to type B cells. Type B cells share many features with astrocytes, including GFAP expression, complex processes, intermediate filaments, specialized contact with blood vessels, and glycogen granules. C cells have a relatively simple morphology with scant cytoplasm and express the markers Mash1 (Parras et al., 2004) and Dlx2.

The adult NSC was identified based on its ability to regenerate the cell types of the SVZ. Though A, B and C cells are all proliferative, A cells have a limited self-renewal potential in culture (Lim and Alvarez-Buylla, 1999), whereas B and C cells can be passaged multiple times (Doetsch et al., 2002), suggesting that B or C cells can act as stem cells in vitro. Type B cells were suggested to be the primary precursor *in vivo* in a study where mice were treated with the antimitotic drug cytosine arabinoside (AraC), which eliminates rapidly dividing

cells. This treatment effectively removed type A and C cells from the SVZ but largely spared type E and B cells. After withdrawal of AraC, B cells proliferated and first produced type C cells, which then generated type A cells (Doetsch et al., 1999b). Furthermore, GFAP+ SVZ cells specifically targeted with an avian virus (RCAS) carrying GFP resulted in labeled SVZ astrocyte-like cells, neuroblasts, and OB neurons. These experiments showed that B cells were sufficient to generate OB neurons.

Additional experiments ablated B cells to show that they were also necessary for adult neurogenesis. Neurogenesis was effectively abolished in adult mice conditionally expressing the toxin herpes simplex virus thymidine kinase (HSV-TK) under the control of the GFAP promoter (Imura et al., 2003). Additional work *in vivo* and *in vitro* demonstrated that most, if not all adult-born OB neurons were derived from GFAP+ cells, which tended to have a relatively simple unipolar or bipolar morphology (Garcia et al., 2004). Therefore, there is now a general consensus that adult mammalian NSCs are GFAP+ astrocyte-like cells. This seems to hold true not just for the SVZ, but also for the hippocampal dentate gyrus (Gage et al., 1998) where astrocyte-like NSCs with radial morphology give rise to granule neurons (Filippov et al., 2003; Fukuda et al., 2003; Seri et al., 2004; Seri et al., 2001). Furthermore, radial astrocyte-like Müller glia are thought to generate glutamatergic retinal neurons (Bernardos et al., 2007; Fischer and Reh, 2001). Since embryonic and adult NSCs have glial characteristics, it has been suggested that NSCs lie within the NEC -> radial glia

-> astrocyte lineage (Alvarez-Buylla et al., 2001), though this hypothesis has not been tested experimentally (Fig. 10).

Intermediate progenitors, oligodendrocytes, and brain tumor stem cells

Not only do primary progenitors appear to share common features, but they also appear to generate intermediate progenitors throughout development. Adult SVZ stem cells produce type C cells, which proliferate rapidly and produce immature neurons (Doetsch et al., 1999a). Interestingly, some intermediate progenitors express the transcription factor Olig2 and produce oligodendrocytes (Menn et al., 2006). Neurons and Olig2+ progenitors may be derived from SVZ astrocytes expressing PDGFRα, a receptor for platelet-derived growth factor (PDGF) (Jackson et al., 2006). This receptor is also expressed by the widely distributed NG2+ oligodendrocyte precursor cells (OPCs), which generate oligodendrocytes in the adult brain (Polito and Reynolds, 2005). Whereas oligodendrocyte-generating SVZ stem cells may be multipotent (Menn et al., 2006) adult OPCs appear to be committed to oligodendrogenesis, though some NG2+ cells may produce astrocytes in response to injury.

Interestingly, PDGFRα+ cells hyperproliferate *in vivo* in response to PDGF and form tumor-like masses (Assanah et al., 2006; Jackson et al., 2006). The majority of cells in these masses express Olig2, which is a marker for many human brain tumors (Lu et al., 2001). These findings are of particular interest since brain tumors may be propagated by a cancerous “tumor stem cell” (Singh et al., 2004). The identity of the precursor to the tumor stem cell is currently

unknown, but it may be a NSC or an intermediate progenitor; some brain tumors appear to arise near neurogenic niches (Sanai et al., 2005). Understanding the role of NSCs in tumor formation may lead to more effective treatment for brain tumors. It may also shed light on stem cell plasticity in the normal and diseased brain.

Plasticity vs. commitment

It is unclear how much plasticity adult NSCs retain and how much they might regain under the right circumstances. Some vertebrates can completely regenerate their spinal cord from CNS tissue following tail amputation (Gargioli and Slack, 2004) and planarians can regenerate their entire nervous system from multipotent neoblasts (Reddien and Sanchez Alvarado, 2004). Mammals lack this regenerative potential, though they do mount a limited response to brain injury by stimulating local progenitors, which form glial scars and remyelinate lesions (Hagg and Oudega, 2006; Sofroniew, 2005). It is important to determine why mammalian stem cells appear to be more restricted, since plastic stem cells could be used therapeutically to replace lost tissue.

About 15 years ago, investigators began to graft embryonic and adult NSCs into the intact or diseased nervous system and reported functional engraftment and production of region-appropriate cell types. Cerebellar progenitors rendered multipotent by virally mediated transduction with avian myc (Ryder et al., 1989) were shown to generate neurons and glia with cerebellar phenotypes after being grafted into the developing cerebellum (Snyder et al.,

1997). It was then suggested that these cells could functionally integrate into other brain regions and ameliorate disease phenotypes such as mucopolysaccharidosis VII (Sly disease), a lysosomal storage disorder (Snyder et al., 1995), Tay-Sachs disease (Lacorazza et al., 1996) and dysmyelination in shiverer mice lacking myelin basic protein (MBP) (Yandava et al., 1999). Further experiments claimed that human NSCs could replace specific cell populations in the mouse brain (Flax et al., 1998) and that grafted NSCs have the ability to home in on sites of injury and initiate regeneration and repair (Aboody et al., 2000). Another group showed that NSCs isolated from the adult hippocampus (Suhonen et al., 1996) or spinal cord (Shihabuddin et al., 2000), produced region-appropriate neuron types when cultured and grafted into other adult germinal niches. These studies suggested that NSCs can be plastic, and that environmental rather than intrinsic factors determine which cell types they produce.

This interpretation contrasts with work demonstrating the regional and temporal commitment of NSCs to a particular neurogenic potential, as discussed in Section 1. The conflicting results might be reconciled by considering the different experimental techniques used. Respecification was observed when NSCs are cultured for long periods of time in the presence of growth factors, or when they were transformed with the oncogene myc. On the other hand, NSCs cultured for short periods of time displayed fate restriction similar to cells *in vivo* (Shen et al., 2006). Also, NSCs isolated from the SVZ and grafted into other brain regions soon after isolation remained effectively neurogenic only in adult

neurogenic niches (Herrera et al., 1999). As techniques to label and identify grafted cells and their progeny have improved, evidence for dramatic NSC plasticity has not been reproduced, whereas evidence for their commitment is mounting. To date, the promise of inducing stem cells to produce functional, region-appropriate neurons at the site of injury has not been realized.

Molecular regulation of neurogenesis

Adult germinal zones are thought to be unique environments that support neurogenesis, sometimes referred to as “neurogenic niches”. This idea derives from the observation that adult SVZ stem cells generate OB neurons when transplanted into the SVZ but not when transplanted to other regions (Doetsch and Alvarez-Buylla, 1996; Herrera et al., 1999; Lim et al., 1997; Lois and Alvarez-Buylla, 1994). Molecules that maintain the niche environment and regulate neurogenesis have been examined in many studies. Many developmentally important signaling pathways seem to regulate adult neurogenesis in the SGZ, and possibly also in the SVZ (reviewed in (Alvarez-Buylla and Lim, 2004; Hagg, 2005; Lennington et al., 2003)). In the SVZ, these have been shown to include: APP (Caille et al., 2004), BMP4 and Noggin (Lim et al., 2000), CNTF (Emsley and Hagg, 2003; Shimazaki et al., 2001), EGF (Kuhn et al., 1997; Mahanthappa and Schwarting, 1993; Reynolds and Weiss, 1992a), Ephrin-A2 (Holmberg et al., 2005), FGF2 (Kuhn et al., 1997; Zheng et al., 2004), PDGF (Jackson et al., 2006), Notch1 (Gaiano et al., 2000; Hitoshi et al., 2002), Shh (Machold et al., 2003; Palma et al., 2005), and TGF α (Enwere et al., 2004). Furthermore, nitric

oxide (Cheng et al., 2003; Packer et al., 2003) and the neurotransmitters serotonin (5HT1a receptor) (Banasr et al., 2004; Mattson et al., 2004), dopamine (D2/3 receptor) (Baker et al., 2004; Hoglinger et al., 2004; Kippin et al., 2005), and GABA (GABAA receptor) (Antonopoulos et al., 1997; Liu et al., 2005) regulate SVZ progenitor proliferation, as do the hormones prolactin (Shingo et al., 2003) and thyroid hormone (Lemkine et al., 2005). Finally, there is growing evidence that interaction with a vascular environment is essential for the long-term maintenance of adult neurogenesis (Shen et al., 2004; Yang et al., 2005).

In addition to these exogenous factors, TFs have been shown to regulate progenitor potential and neural specification. Removal of both of the homeobox transcription factors Dlx1 and Dlx2 results in the dramatic loss of OB interneurons (Anderson et al., 1997; Bulfone et al., 1998), whereas Vax1 (Soria et al., 2004), Sox2 (Ferri et al., 2004; Graham et al., 2003; Wegner and Stolt, 2005), Zic1/3 (Inoue et al., 2007), and the basic helix-loop-helix (bHLH) transcription factor Mash1 (Parras et al., 2004) have been suggested to regulate progenitor self-renewal, proliferation of differentiation. As more factors are discovered, our understanding of neurogenesis increases but it becomes difficult to identify the fundamental aspects of NSC regulation. For this, we need a more complete understanding of NSC organization and potential.

Summary

Adult mammalian NSCs reside in the SVZ and have the potential to produce astrocytes, oligodendrocytes and neurons. They are phenotypically

astrocyte-like cells, but differ from astrocytes elsewhere in the brain since they retain the ability to generate neurons. However, adult NSCs require the unique environment of the germinal zone to remain neurogenic. Within this germinal zone, they are regulated by a combination of environmental and intrinsic factors. The neurogenic potential of adult NSCs appears to be restricted, though long periods of time in culture may alter their potential. SVZ stem cells produce neuroblasts that migrate in chains to the olfactory bulb, where they differentiate into several types of interneurons. In the next section, I will examine the production and function of these neurons.

Section 3: Postnatally born olfactory bulb neurons

Olfaction and OB organization

The SVZ produces interneurons that modulate olfaction. The process of olfaction begins when inhaled odor molecules pass through the nostrils and into the turbinate bones of the sinus cavity (Fig. 11). There, they bind specific odorant receptors on the surface of olfactory sensory neurons (OSNs), residing in a germinal olfactory epithelium (OE). Remarkably, though a huge family of genes encodes the odorant receptors (Buck and Axel, 1991), each OSN expresses only one of these genes (Chess et al., 1994; Malnic et al., 1999). OSNs expressing the same gene send axons from their diverse locations in the OE to the same location in the OB (Mombaerts et al., 1996). Their axons terminate in spherical structures of dense neuropil called glomeruli where they form synapses with neurons that project to olfactory cortex (Axel, 2005; Shepherd and Greer, 1998). OB interneurons modulate both the input to and output of these projection neurons. The first stage in olfactory processing thus occurs in the OB and is strongly influenced by interneurons (Shepherd, 1972; Shepherd and Greer, 1998).

The strikingly beautiful organization of different OB cell types was documented by the pioneering neuroanatomists Golgi and Ramon y Cajal almost 100 years ago (Golgi, 1989; Ramón y Cajal, 1995). From the core of the olfactory bulb to its surface, its layers consist of: the granule cell layer (GCL), internal plexiform layer (IPL), mitral cell layer (MCL), external plexiform layer (EPL),

glomerular layer (GL) and olfactory nerve layer (ONL) (Fig. 12). These layers form a characteristic three layered allocortex and like other cortical regions, it contains two main types of neurons: projection neurons and local interneurons.

Diversity among OB neurons

The projection neurons, mitral and tufted (M/T) cells, extend a primary dendrite to a single glomerulus where they receive synaptic input from OSNs and extend secondary dendrites laterally within the EPL. The cell bodies of mitral cells form the well-defined MCL, whereas tufted cell bodies are dispersed in the EPL. M/T cells send axons to the piriform (olfactory) cortex where further processing likely leads to behavioral responses and the conscious perception of odors.

Local interneurons are a more numerous and diverse group of cells consisting of granule cells (GCs) and short axon cells (SACs), which include periglomerular cells (PGCs) (Fig. 12). Postnatally born cells consist of approximately 97% GCs and 3% PGCs (Kaplan et al., 1985; Winner et al., 2002), although it is unclear if other OB neurons are also generated postnatally. In the mouse, all GCs and PGCs are GABAergic (Kohwi et al., 2007; Shepherd and Greer, 1998), though a subpopulation of PGC is also dopaminergic and expresses tyrosine hydroxylase (TH) (Kosaka et al., 1985), the rate-limiting enzyme in the synthesis of dopamine.

As their name implies, PGCs surround glomeruli and extend processes into or between glomeruli where they form dendrodendritic synapses with OSN

axons and M/T cell dendrites. They may also project a short axon to a neighboring glomerulus. Therefore, PGCs modulate the input that M/T cells receive from OSNs. PGCs can be divided into several non-overlapping groups based on their expression of TH or the calcium binding proteins calbindin (CalB) and calretinin (CalR) (De Marchis et al., 2007; Kohwi et al., 2007; Kosaka and Kosaka, 2005). These cells participate in different circuits: TH-expressing (TH+) cells form contacts with both OSNs and M/T cells, whereas CalB+ and CalR+ cells preferentially contact M/T cells (reviewed in (Kosaka et al., 1998)).

Granule cell bodies are found in the GRL and, more rarely, in the MCL. They lack axons and interact with M/T dendrites via reciprocal dendrodendritic synapses in the EPL. Interestingly, the dendrites of different GCs arborize in different parts of the EPL. The dendrites of deep GCs, named for the location of their cell bodies in the deep GRL (close to the OB core) ramify extensively in the deep EPL, whereas the dendrites of superficial GCs arborize primarily in the superficial EPL (Mori et al., 1983; Price and Powell, 1970). This organization positions deep and superficial GCs to interact with the secondary dendrites of mitral and tufted cells, respectively (Orona et al., 1983; Scott, 1986). This specificity suggests that deep and superficial GCs form unique elements of the olfactory bulb circuitry (Shepherd and Greer, 1998). Supporting this notion, these cells have been shown to be electrophysiologically distinct (Carleton et al., 2003). Another GC subtype expresses CalR (Jacobowitz and Winsky, 1991) and another has processes that arborize in both the deep and superficial EPL (Mori et al., 1983; Price and Powell, 1970).

In addition to these interneurons, there is another class of poorly characterized interneurons collectively known as short axon cells (SACs). In addition to PGCs, these cells include Blanes cells, Golgi cells, Cajal cells, horizontal cells, Van Gehuchten cells, and superficial SACs. The role of these cells in olfactory processing is unclear. The ongoing discovery of marker genes suggests that known interneuron types can be further subdivided raising the possibility that new functional elements of the OB circuitry will be discovered. The calcium/calmodulin-dependent protein kinase IV (CaMKIV) was shown to be expressed mostly in deep GCs while the Ets transcription factor Er81, which is known to regulate CaMKIV expression, was weakly expressed in deep GCs but highly expressed in superficial GCs and in TH+ PGCs (Saino-Saito et al., 2007). Furthermore, a diverse but restricted population of OB neurons including SACs expresses nitric oxide synthase (NOS) (Kosaka and Kosaka, 2007) choline acetyltransferase (ChAT) and acetylcholinesterase (AChE) (Porteros et al., 2007), parvalbumin (PV) (Jia and Halpern, 2004) and neurocalcin (NC) (Alonso et al., 2001).

In summary, there are two main types of well-known olfactory bulb interneurons, which can each be split into three subtypes: periglomerular cells (TH+, CalB+ and CalR+) and granule cells (deep, superficial, and CalR+). The adult production of these different OB interneuron types raises several questions. Why are so many different cell types replaced in the adult brain? How are they produced in the proper ratios? How to they contribute to proper olfactory bulb

function? Where are they generated? Are they all produced from one stem cell or from different progenitors?

Molecular specification of OB interneurons

It is clear that OB interneurons are a diverse population of cells, but how is this diversity generated? Since the field of mouse genetics has matured, some of the molecular determinants of OB neuron type are now known. For example, calretinin (CalR) expressing OB interneurons were dramatically reduced in mice lacking the zinc finger transcription factor Sp8 (Waclaw et al., 2006). The homeobox transcription factor Pax6 has been shown to regulate the production of OB neurons (Dellovade et al., 1998), particularly TH+ periglomerular cells (Hack et al., 2005; Kohwi et al., 2005) and superficial granule cells (Kohwi et al., 2005). TH+ periglomerular cell production is also thought to be regulated by the homeobox transcription factors Dlx1 and Dlx2 (Saino-Saito et al., 2003). Furthermore, Er81+ neurons were dramatically reduced in mice lacking Gsh1/2 or Gsh2 (Stenman et al., 2003). In many of these studies, the potential of these transcription factors have been identified based on the gene knockout phenotypes in mice. These studies may not always reveal the direct function of the gene, since its absence may indirectly affect other genes, or other pathways may compensate for its loss. Their roles in olfactory neuron fate specification should therefore be confirmed in gain of function experiments and *in vivo* linage tracing in mice containing the intact gene.

The functional role of postnatal neurogenesis

Thousands of OB interneurons are replaced each day (Bayer, 1983; Petreanu and Alvarez-Buylla, 2002). The functional role of this constant replacement is not known, but it has been suggested that interneuron turnover increases plasticity in the OB to enhance olfactory discrimination (Cecchi et al., 2001; Gheusi et al., 2000), reviewed in (Doetsch and Hen, 2005; Lledo et al., 2006). New neurons might enhance olfactory discrimination by modulating the spatial or the temporal activity of OB projection neurons.

Interneurons could refine the activity of M/T cells by lateral inhibition. When a mitral cell is activated by sensory input, its dendrites depolarize, stimulating inhibitory granule cells though dendrodendritic synapses. This should cause the granule cell to release GABA and inhibit nearby mitral cells, inhibiting them and lowering the background activity (Mori et al., 1999; Yokoi et al., 1995). However, a recent study in which channelrhodopsin-expressing mitral cells were activated by different sized spots of light did not find evidence for such lateral inhibition (Arenkiel et al., 2007).

A second hypothesis is derived from the observation that representations of similar odors in insect antenna lobes become increasingly dissimilar over time. It has been suggested that odor discrimination may require this temporal evolution of neural activity, which is influenced by the interaction of interneurons with projection neurons (Laurent and Davidowitz, 1994; Laurent et al., 1996; Lemon and Getz, 2000; Stopfer et al., 1997). Interneurons might trigger different projection neuron firing modes by disinhibiting calcium channels (Av-Ron and

Vibert, 1996). However, these observations have been difficult to reproduce (Vetter et al., 2006) and since the temporal signal may take 500 ms or more to evolve, it cannot explain rapid odor detection and behavioral responses such as predator evasion.

Fundamentally, these hypotheses remain insufficient to explain why adult neurogenesis exists. Lateral inhibition in the retina and temporal coding in the auditory system are robust in the absence of constant interneuron turnover. However, since the continual addition of new neurons to the olfactory bulb has been apparently conserved over 600 million years of evolution (reviewed in (Alvarez-Buylla and Lois, 1995; Cayre et al., 2002; Garcia-Verdugo et al., 2002)), is likely to play an important functional role. Perhaps the targeted, functional manipulation of postnatally born interneuron populations will shed light on this mystery.

Summary

The olfactory bulb processes and relays olfactory information to the cortex. The constant replacement of adult born interneurons is likely to play an important role in olfaction, but it is unclear why this turnover is necessary for olfactory processing. The diversity of interneuron types also suggests that replacing different elements of the olfactory bulb circuit is important for this role. Some of the molecular mechanisms regulating OB interneuron production and diversity have been identified, but the mechanisms by which adult NSCs produce diverse OB neuron types are obscure.

Section 4: Objectives and approach

In the remainder of the work, I will address the questions outlined in the overview of this chapter. Here, I restate these questions and briefly discuss the approaches I will take to address them.

The neural stem cell lineage

It is not known what distinguishes SVZ astrocyte-like stem cells and astrocytes elsewhere in the brain. Similarly, the developmental origin of adult neural stem cells remains obscure. To address these questions, I hypothesized that adult NSCs are derived from neonatal radial glia. I developed a novel technique to permanently and specifically label these radial glia, and followed their progeny *in vivo*.

The extent of the SVZ and the diversity of adult neural stem cells

Though it is known that the lateral wall of the LV harbors adult NSCs, it is not clear how far this germinal niche extends into adjacent regions. The organization and potential of stem cells within this niche is also unclear – it is commonly assumed that adult NSCs are equivalent and multipotent, but they could also be a heterogeneous population of more restricted progenitors. To address these questions, I systematically targeted NSCs in different subregions of the SVZ and traced their fate *in vivo*.

The potential and plasticity of adult neural stem cells

Experiments in which cultured progenitors from hippocampus of the spinal cord were grafted into the SVZ suggest that NSCs are plastic and can make region-appropriate cell types (Shihabuddin et al., 2000; Suhonen et al., 1996). To determine if NSCs are plastic or committed, I cultured them or grafted them from one region of the SVZ to another and examined the cell types they produced. I also developed a technique to specifically label adult NSCs in different regions to determine if the neurogenic potential of NSCs changes over the course of postnatal development.

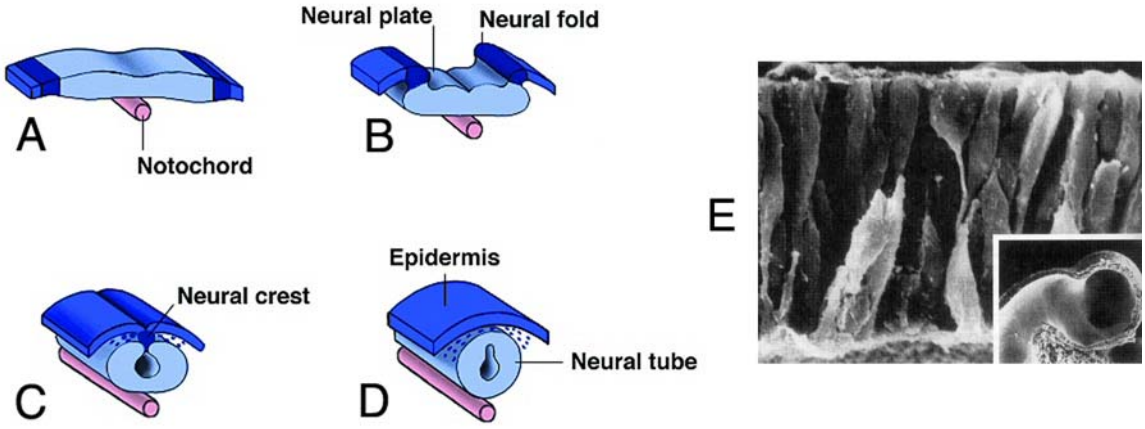


Figure 1. Formation of the CNS in early development.

(A) The nervous system begins as an epithelial sheet of neural stem cells, known as the neuroepithelium. Ventral identity is induced in part by Shh, which is highly expressed in the notochord. **(B)** The neuroepithelium, also known as the neural plate at this stage, invaginates. (**C** and **D**) The dorsal edges of the neural plate join to form the neural tube, which is then covered by the epidermis. (**E**) A scanning electron micrograph of a cross section of the neural tube reveals the bipolar, radial morphology of neuroepithelial cells (NECs).

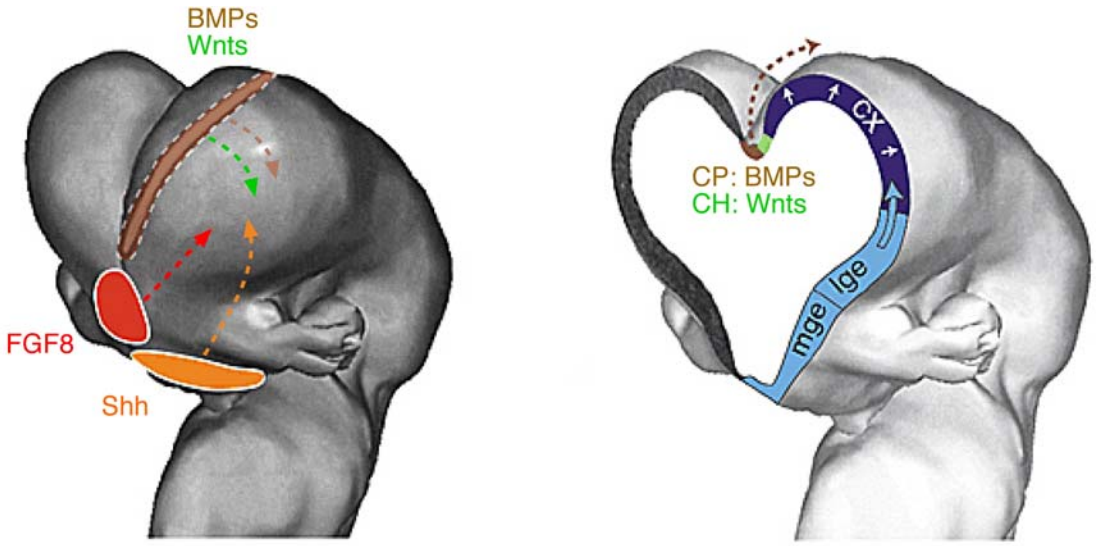


Figure 2. Gradients of signaling molecules pattern the brain.

Signaling molecules such as Shh, FGF8, Wnts and BMPs are released in “signaling centers”. The local release of these factors produces concentration gradients along the rostrocaudal, dorsoventral, and medial-lateral axes. NSCs respond to these factors and acquire regional identity based on the coincidence and concentration of these signals in space and time. This regional identity patterns the brain into different domains containing NSCs that produce different cell types. Adapted from (Monuki and Walsh, 2001). BMP, bone morphogenetic protein; CX, cortex; FGF8, fibroblast growth factor 8; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; Shh, sonic hedgehog, Wnt, wingless

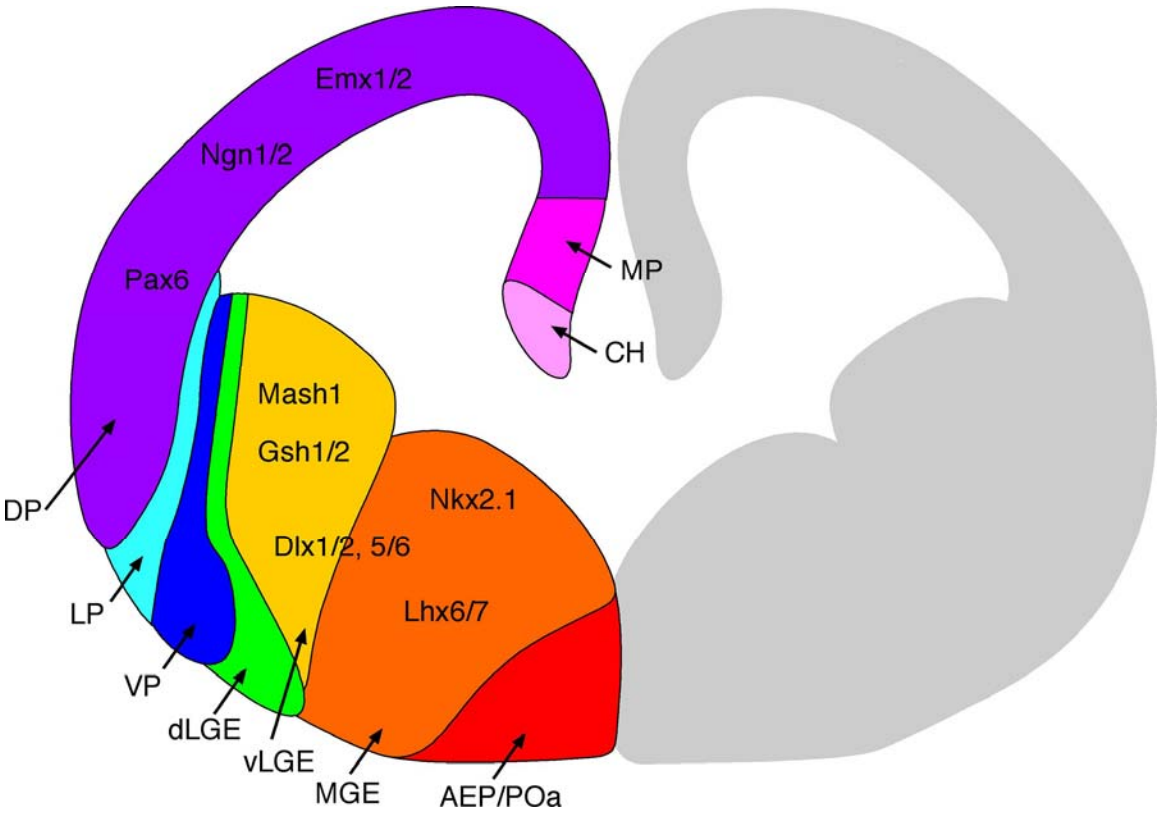


Figure 3. The region-specific expression of transcription factors defines domains of the developing mouse brain.

This schematized cross section of an embryonic mouse brain illustrates morphologically and genetically defined regions. Two principal regions shown here, the pallium and subpallium, are further divided into subregions. Different regions express different combinations of transcription factors. Of note are the transcription factors Pax6, Gsh2, and Nkx2.1, which define NSC populations in the LGE and MGE and interact to form boundaries between different regions. AEP, anterior entopeduncular area; dLGE, dorsal LGE; CH, cortical hem; DP, dorsal pallium; LGE, lateral ganglionic eminance; LP, lateral pallium; MGE, medial ganglionic eminance; MP, medial pallium; vLGE, ventral LGE; VP, ventral pallium. Adapted from (Schuurmans and Guillemot, 2002).

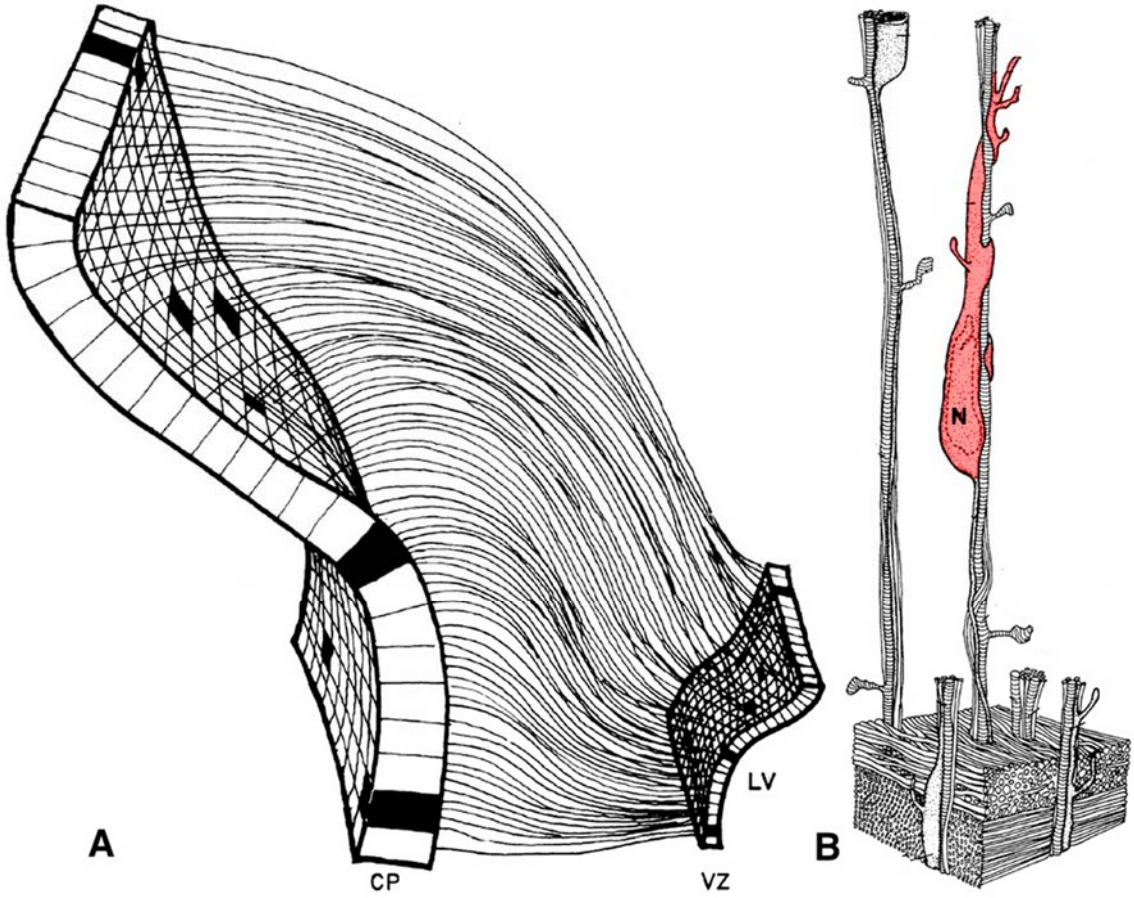


Figure 4. Radial glial cells are NSCs that guide the radial migration of neuroblasts.

(**A**) Radial glial cells send a radial process to the brain surface from their cell bodies at the ventricular zone (VZ) where, in this case, they contact the lateral ventricle (LV). (**B**) These radial process (two long vertical processes) guide the radial migration of newly born neuroblasts (N, red). CP, cortical plate. Adapted from (Rakic, 2003).

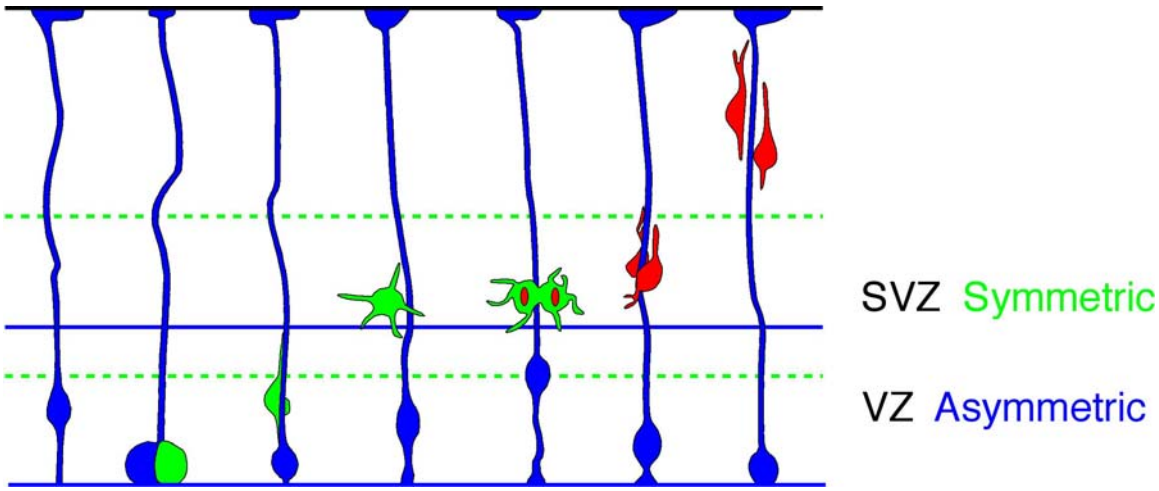


Figure 5. Intermediate progenitors divide symmetrically in the SVZ to generate equivalent progeny.

This illustration shows sequential frames (left to right) of a time-lapse movie of an embryonic NSC and its progeny in the embryonic mouse cortex. Radial glia (blue) are primary progenitors that undergo interkinetic nuclear migration in the VZ (solid blue lines). They divide asymmetrically at the ventricular surface to produce neurons (red) that migrate up the radial process (right), but can also give rise to intermediate progenitors. Intermediate progenitors (green) can migrate up the radial process and divide symmetrically one or more times in the SVZ (dashed green lines) to generate equivalent progeny, in this case neuroblasts (red). These divisions in the SVZ are thought to amplify the number of progeny from a single NSC division in both the embryonic brain and in the adult SVZ. Adapted from (Noctor et al., 2004).

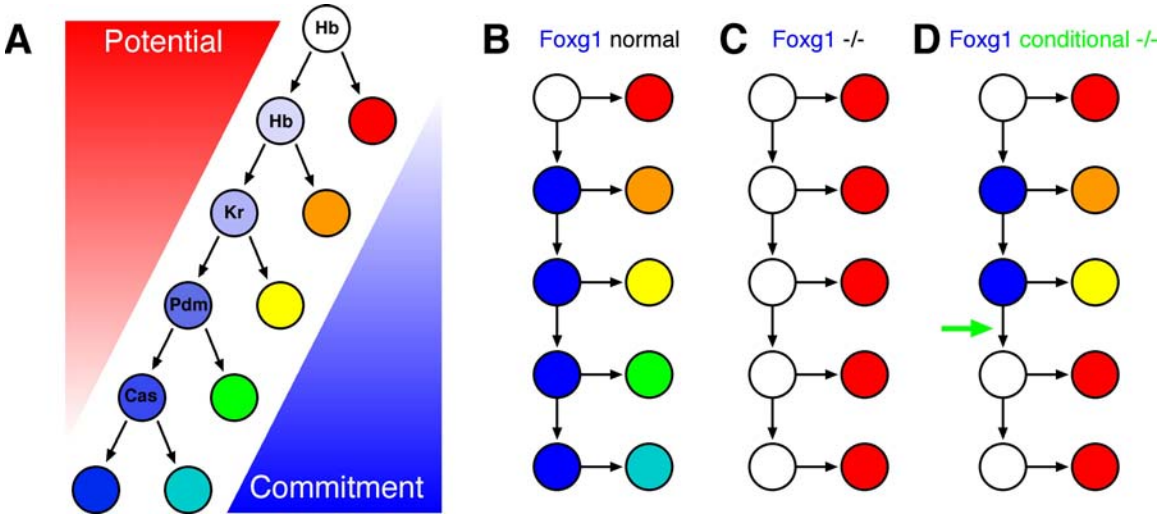


Figure 6. Temporal regulation of NSC neurogenic potential.

(**A**) Neural progenitors in Drosophila (white to blue shading, left) express a temporally variant sequence of transcription factors: Hb -> Kr -> Pdm -> Cas. The temporal expression of transcription factors leads to the generation of diverse progeny (colored circles, right). Over time, these progenitors lose the potential (red shading) to produce earlier-born neuron types and are increasingly committed (blue shading) to producing later born cell types. (**B**) In mammals, a cortical NSC (white circle) produces diverse neuronal progeny in a well-defined temporal order. The transcription factor Foxg1 (blue shading) is expressed after early-born neuron types are generated. (**C**) When Foxg1 is eliminated, only early-born cell types are produced. (**D**) When Foxg1 is conditionally eliminated later in development (green arrow), NSCs are able to continue producing early-born cell types. It is unclear if even older progenitors would retain this potential. Cas, Castor; Foxg1, forkhead homeobox transcription factor G1; Hb, Hunchback; Kr, Krüppel; Pdm, POU domain containing transcription factor. Adapted from (Hanashima et al., 2004; Pearson and Doe, 2004).

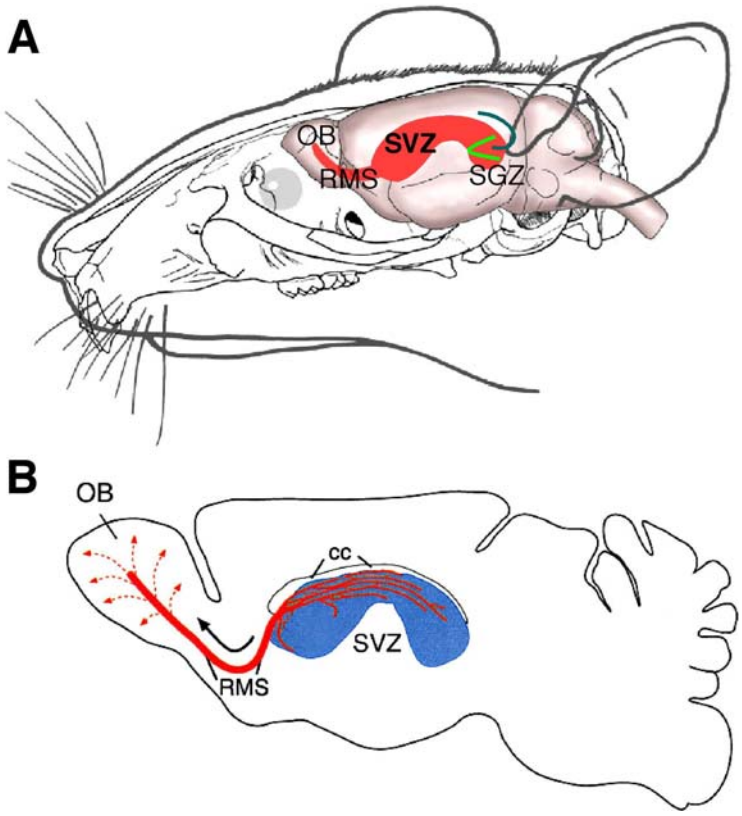


Figure 7. Neurogenic zones in the adult mouse brain.

(**A**) The adult mouse brain (pink) is shown positioned in the head and skull, illustrating the location of the olfactory bulb (OB) in relation to the nasal cavity. Superimposed on the brain are the two principle adult neurogenic regions: the subventricular zone (SVZ) on the lateral wall of the lateral ventricle (red), and the subgranular zone (SGZ) of the dentate gyrus of the hippocampus (light green). The rostral migratory stream (RMS) by which neuroblasts reach the OB is also illustrated (red). (**B**) The RMS is composed of chains of neuroblasts (red) that originate in the SVZ (blue). Upon reaching the olfactory bulb, neuroblasts migrate radially (dashed red arrows) and differentiate into OB interneurons. Adapted from (Alvarez-Buylla and Garcia-Verdugo, 2002; Lennington et al., 2003).

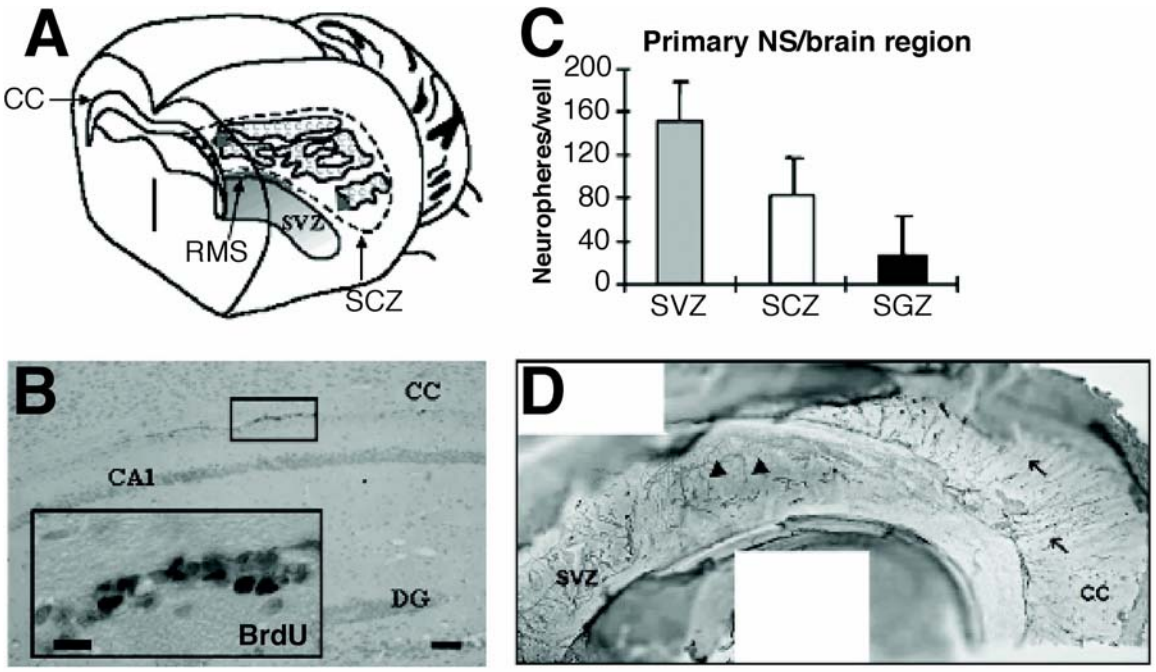


Figure 8. Proliferation in the subcallosal zone.

(**A**) The subcallosal zone (SCZ) is situated between the corpus callosum (CC) and the hippocampus and is anatomically continuous with the subventricular zone (SVZ) of the lateral wall of the lateral ventricle. (**B**) After administration of BrdU to label cells in S phase, clusters of BrdU+ cells can be seen in the SCZ (inset, higher magnification), demonstrating the SCZ contains proliferative cells. CA1, CA1 region of the hippocampus; DG, dentate gyrus of the hippocampus. (**C**) When cultured, the SCZ generated more primary neurospheres than the subgranular zone (SGZ) of the DG, but not as many as the SVZ. This indicates the SGZ contains a progenitor that proliferates in response to EGF and FGF. (**D**) A whole mount preparation of the lateral ventricular wall and SCZ illustrates the presence of doublecortin+ cells in the SCZ, suggesting that neurons may be produced locally. Adapted from (Menn et al., 2006; Seri et al., 2006).

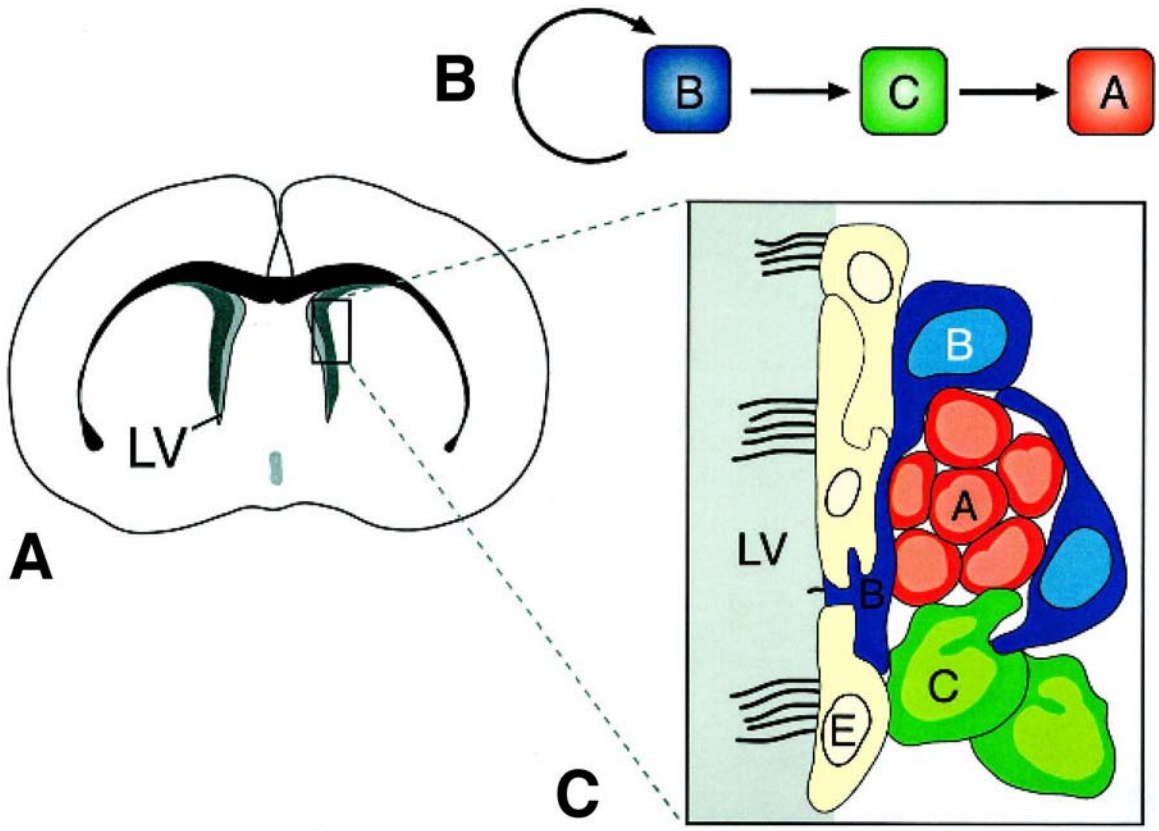


Figure 9. Cell types and organization of the SVZ.

(**A**) Tracing of a coronal section of an adult mouse brain, showing the SVZ (dark gray) adjacent to the lateral ventricle (LV, light grey). (**B**) The SVZ contains three proliferative cell types (A, B and C cells). B cells divide, generating another B cell and a C cell. The C cell divides rapidly, producing A cells. (**C**) Organization and morphology of SVZ cell types. B cells (blue) are astrocyte-like cells that surround chains migrating neuroblasts, or A cells, shown here in cross section. B cells sometimes contact the ventricle, into which they extend a primary cilium. C cells (green) have a spheroid morphology and are closely associated with A and B cells. Adapted from (Alvarez-Buylla and Garcia-Verdugo, 2002).

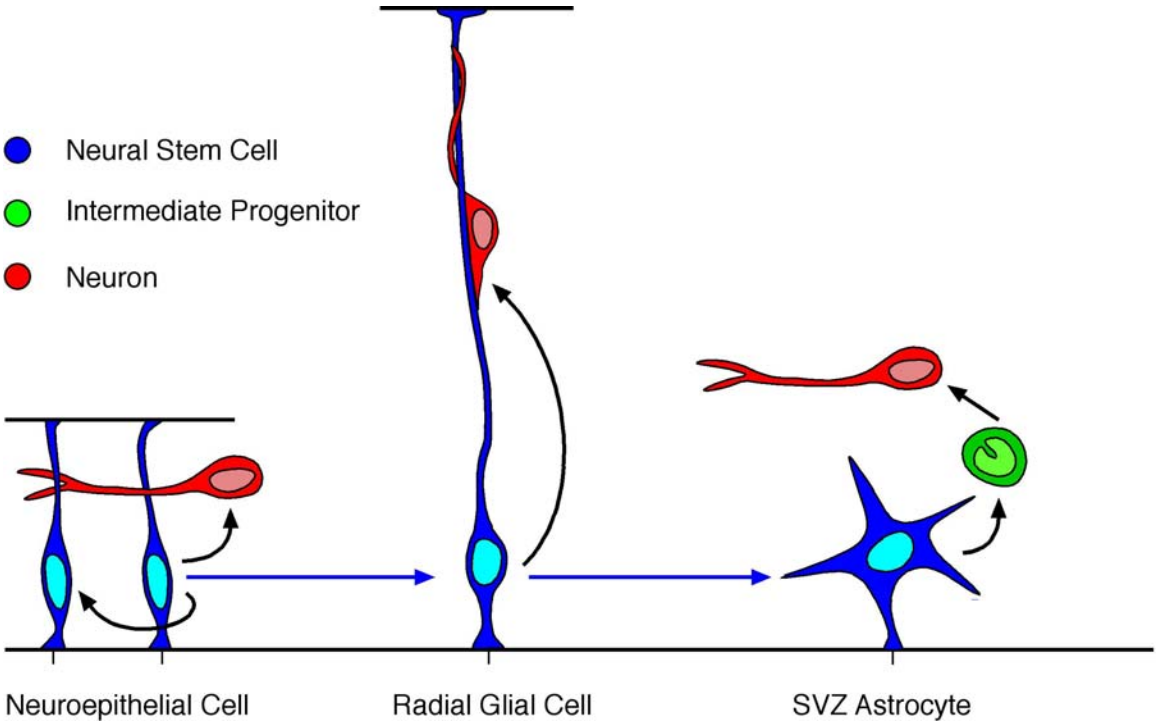


Figure 10. Model of the NSC lineage in development.

NSCs were shown to be glial cells in the adult and in the developing brain. However, the link between embryonic and adult NSCs has not been proven. In this model of NSC lineage, neuroepithelial cells (NECs) transform into radial glial cells, some of which then transform into astrocyte-like adult SVZ stem cells. Adapted from (Doetsch, 2003).

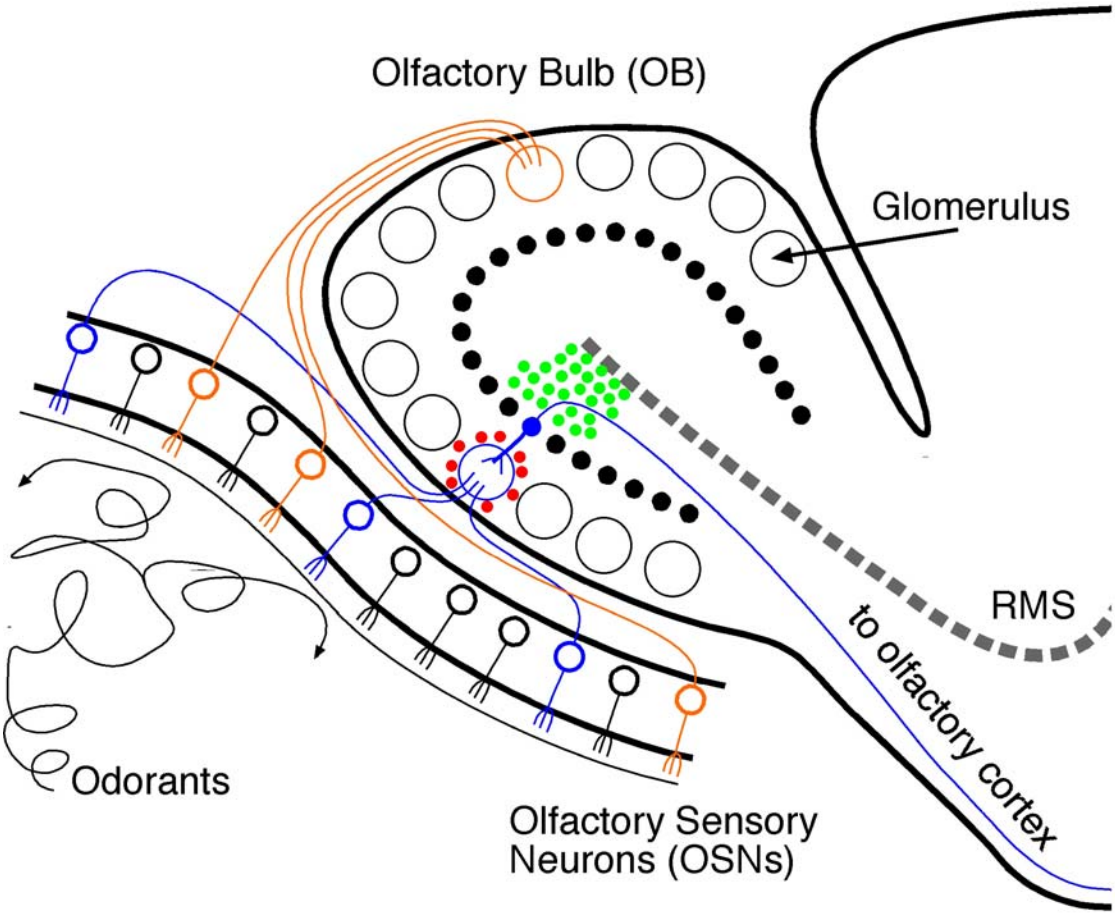


Figure 11. The olfactory system.

The early steps of olfactory processing are illustrated in this simplified schematic diagram of the olfactory system. Odorants enter through the nostrils into sinus cavities containing turbinate bones. The turbinates are covered by the olfactory epithelium containing olfactory sensory neurons (OSNs). Each OSN expresses only one out of approximately 1000 olfactory receptor genes. OSNs expressing the same receptor are widely distributed, but their axons project to the same location in the olfactory bulb (OB). This location is known as a glomerulus, a dense region of neuropil where the axons of OSNs form synapses with the primary dendrites of mitral cells (MC) and tufted cells (not shown). Mitral cells project from the OB to olfactory cortex, where olfactory perception is thought to take place. OB interneurons modulate the activity of mitral and tufted cells and include granule cells (green) and periglomerular cells (red).

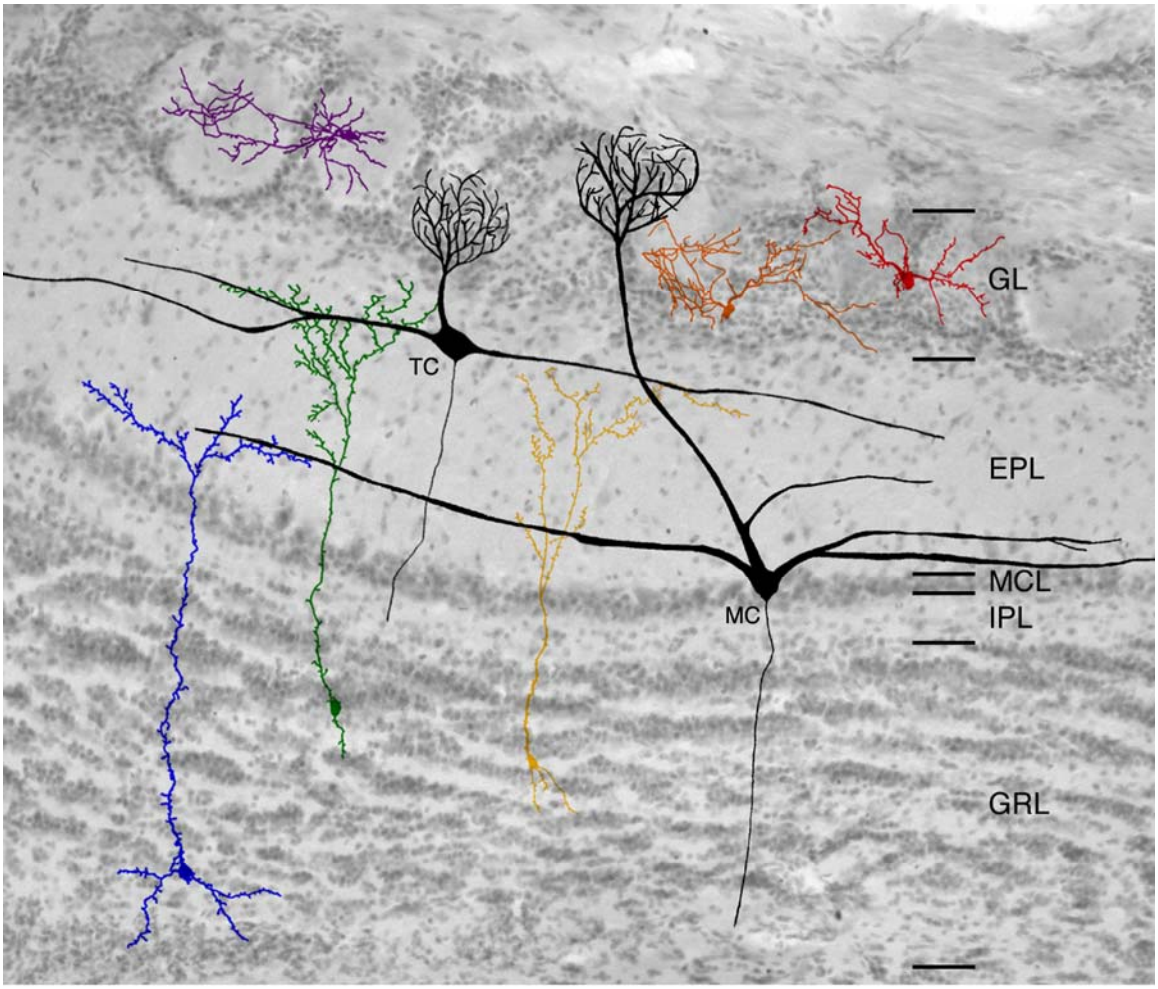

GL
TC
EPL
MCL
IPL
MC
GRL
Deep GC
Superficial GC
Calretinin+ GC
Calretinin+ PGC
TH+ PGC
Calbindin+ PGC

Figure 12. Organization and some cell types of the olfactory bulb.

Camera lucida traces of different OB cell types were color coded and superimposed in the appropriate layer of a hematoxylin stained coronal section of the adult mouse OB. Only a portion of the OB is shown; superficial is up and deep (core) is down. The layers of the OB are the granule cell layer (GRL), internal plexiform layer (IPL), mitral cell layer (MCL), external plexiform layer (EPL), and glomerular layer (GL). The two major types of projection neurons, mitral cells (MC) and tufted cells (TC), project primary dendrites to a single glomerulus and project secondary dendrites in the EPL. OB interneurons – granule cells (GC) and periglomerular cells (PGC) – can be divided into six different types based on their morphology and marker gene expression. Deep GCs preferentially contact MCs, whereas superficial GCs preferentially contact TCs. Marker genes include calbindin, calretinin, and tyrosine hydroxylase (TH).

Chapter 2

Materials and Methods

Overview

Essential techniques for experiments are described with the exception of standard techniques, which are referenced where appropriate. Relevant solutions and materials are included. Credit for techniques and protocols is given where possible and appropriate. This chapter is organized into eight sections: 1) Equipment and tools, 2) Animals and genotyping, 3) Cloning, 4) Histology and immunohistochemistry, 5) Tissue dissection and culture, 6) Surgical and animal procedures, 7) Terminal procedures, and 8) Data analysis.

Section 1: Equipment and tools

Production of customized head molds

To facilitate stereotaxic injection of neonatal mouse pups (see "stereotaxic injection of neonatal pups" below), head molds were made to stabilize the brain by cradling the side of the anesthetized mouse head. First, molds of P0 pups were made by immersing lethally anesthetized pups in a 4% solution of low melting point agarose. Once the agarose had hardened, pups were removed and the resulting cavity was filled with liquid dental acrylic to create a detailed positive mold of the pup. Once hardened, the head and neck of the acrylic mold was pressed onto oven-bake clay (Sculpey, Polyform Products Company, Elk Grove Village, IL), which was then shaped to ensure correct alignment of the brain and baked to ensure the mold was durable enough for reproducible injections. The resulting mold was then surrounded with modeling clay to make further adjustments to head position and to allow the mold to be firmly attached to the platform of a customized stereotaxic injection rig.

Production of pulled glass micropipettes for viral injection and cell grafting into the neonatal and adult mouse brain

Pulled glass micropipettes were used to inject virus or cells into the mouse brain with minimal injury. Pipettes were pulled from 5 μm glass capillary tubes (Drummond WIRETROL 5 μl, case of 100 disposable micropipettes with Dispenser Top, Drummond Scientific Company, Broomall, PA) using a vertical

pipette puller (David Kopf Instruments, model 700C). The heating element and solenoid of the puller were adjusted to achieve the desired shape: a smoothly tapered point extending about 6 mm from the beginning of the taper to the tip. Pipette tips that were pulled too thin and straight were discarded, since these may clog with cells or break off in the animal. For the particular pipette puller used, the optimal current to heat the heating element to achieve the proper pipette shape was empirically determined to be 18 Amps. After the pipette was pulled, a 30-degree bevel was ground on the tip to sharpen it like a hypodermic needle. This modification facilitated penetration of the neonatal skin and skull and significantly reduced injury, and ensured that the pipette had and appropriately sized outer diameter. For viral injections, an outer diameter of 30 μm was used, whereas an outer diameter of 50 μm was used for cell grafts. The diameter was measured by a reticule in the eyepiece of a microscope used to confirm the outer diameter and angle of the bevel. To grind the bevel, compressed air was blown through the pipette to prevent the entry of glass fragments, and the pipette was held against a rotating disc covered in diamond dust abrasive at an angle of 30 degrees.

Section 2: Animals and genotyping

All experiments were performed on mice, which were housed and treated according to the guidelines put forth by the University of California, San Francisco Laboratory Animal Care and Use Committee. Mouse strains used in this study (ActB-GFP, CD1, R26R, Z/AP and Z/EG) are described in Table 1.

Table 1. Mouse stains used and their relevant information.

Strain	Full name	Source	Description	Ref.
ActB-GFP	STOCK Tg(ACTB-Bgeo/GFP) 21Lbe/J	Jackson Labs	These transgenic mice constitutively express lacZ under the control of the CMV enhancer/chicken actin promoter. Used as donor animals for cell grafting experiments.	(Hadjantonakis et al., 1998)
CD1		Charles River Labs	Outbred wild-type strain used as background for breeding, as wild-type control, and as host for grafting experiments.	N/A
R26R	B6.129S4-Gt(ROSA) 26Sortm1Sor/J	Jackson Labs	Transgenic mice containing floxed STOP cassette inserted in the Rosa26 locus. Excision by Cre results in constitutive lacZ expression. Maintained as homozygotes on a mixed C57/Bl6:CD1 background (outbred to CD1 for at least 3 generations). Homozygous males were crossed to CD1 females to obtain heterozygous pups for experiments.	(Soriano, 1999)
Z/AP	STOCK Tg(ACTB-Bgeo/GFP) 21Lbe/J	Jackson Labs	Transgenic mice containing floxed marker gene (lacZ) and translational STOP cassette under control of the CMV enhancer/chicken b actin promoter. Excision by Cre replaces lacZ expression with hPLAP expression in most lineages, but stronger in glia than astrocytes, weak in oligodendrocytes. Maintained as heterozygotes on a CD1 background.	(Lobe et al., 1999)

			Heterozygous males were crossed to CD1 females to obtain heterozygous pups for experiments (homozygous mice have abnormal OB development).	
Z/EG	STOCK Tg(ACTB-Bgeo/GFP) 21Lbe/J	Jackson Labs	Transgenic mice containing floxed marker gene (lacZ) and translational STOP cassette under control of the CMV enhancer/chicken b actin promoter. Excision by Cre replaces lacZ expression with eGFP expression in most lineages, but stronger in glia than astrocytes, weak in oligodendrocytes. Maintained as heterozygotes on a CD1 background. Heterozygous males were crossed to CD1 females to obtain heterozygous pups for experiments (homozygous mice have abnormal OB development).	(Novak et al., 2000)

Genotyping by β-gal histochemistry

Mice carrying the lacZ transgene (Z/AP and Z/EG) were genotyped by β-gal histochemistry (see "β-gal staining" below). A 1-2 mm piece from the tip of a newborn to adult mice tail was placed in 100 µl β-gal solution in an Eppendorf tube and incubated for 30 minutes or more at 37°C. Tails of mice homozygous or heterozygous for the Z/AP or Z/EG transgene developed a blue precipitate.

Rapid PCR genotyping (Modified from Jeremy Reiter)

Other transgenic mouse lines were genotyped by PCR. There are two protocols for extracting DNA for genotyping: a rapid and a standard protocol. For many mouse strains and PCR conditions, the rapid protocol provides DNA of sufficient purity for PCR genotyping.

A 1-2 mm piece of tail from newborn to adult mice was digested for 30 minutes to 2 hours in 100 µl of 25 mM NaOH with 2 mM EDTA in a 1.5 ml Eppendorf tube and placed on a heating block at 100°C. Tissue was dissolved by gentle trituration with a fresh 1 ml pipette tip. To stabilize DNA, 100 ml 40 mM Tris-HCl pH 7.5 was added to each tube and mixed by hand or by gentle trituration. 1-2 µl of this solution was used directly as a source of DNA for PCR. Note: DNA in this state is stable for up to 48 hours at 4°C.

Volumes and reagents for a single 20 µl PCR varied slightly for different primers, but were usually the following: 1.5 µl DNA solution, 2 µl complete 10x buffer, 0.2 µl forward primer, 0.2 µl reverse primer, 0.2 µl Platinum Taq (Invitrogen), and 15.9 µl autoclaved ddH_2O. Complete 10x buffer contains 10x Platinum Taq buffer (Invitrogen) supplemented with BSA, dNTPs and MgCl2 to final concentrations of 1 mg/ml, 2 mM each, and 15 mM, respectively. Reactions were run on a PTC-200 thermal cycler (MJ Research) for 30-35 cycles. Cycle number, primer design, and annealing and extension temperatures are published (Lobe et al., 1999; Novak et al., 2000; Soriano, 1999). 10 µl of the resulting product was separated by electrophoresis on a 1% agarose gel, and visualized with ethidium bromide. Mice whose DNA produced a PCR band of the expected size were selected for experiments.

Standard PCR genotyping (modified from Nicholas Willis)

For standard PCR genotyping, DNA was extracted 1-2 mm tissue from the tails of newborn to adult mice. Tails were digested in 400 µl DNA lysis buffer in

an Eppendorf tube overnight at 55°C. A 50 ml volume of lysis buffer contained 5 ml 1M Tris pH 8.0, 2 ml 5M NaCl, 500 µl 0.5M EDTA, 500µl, 20% SDS, 250 µl 20 mg/ml proteinase K (added fresh each time) and ddH_2O to bring the volume to 50 ml. Tissue was then triturated gently (not vortexed) until dissolved. DNA was purified by chloroform extraction: 40 µl 3M NaOAc and 600 µl chloroform were added and tubes were incubated at -20°C for at least 20 minutes. Note: DNA can be stored in this form for up to two weeks at -20°C. Next, tubes were spun at maximum speed (13,000 RPM) in a tabletop Eppendorf centrifuge for 20 minutes for juveniles and adult tissue and 30 minutes for embryos and neonatal tissue. The supernatant was transferred to a clean Eppendorf tube and 600 µl 100% ethanol chilled to -20°C was added and mixed by hand. DNA was pelleted by centrifuging at maximum speed for 15 minutes at 4°C. The supernatant was removed and the pellet was washed with 70% ethanol chilled to -20°C, then spun again at maximum speed for 15 minutes at 4°C. The pellet was then air dried and resuspended in 100 µl ddH_2O (150 µl ddH_2O if pellet is visibly big). 1-2 µl of this purified DNA was used for PCR (see “Rapid PCR genotyping” above).

Section 3: Cloning

Generation of Ad:GFAP-Cre virus

To construct this adenovirus, we placed Cre under the control of the mouse GFAP promoter (GFAPp) previously confirmed to be specifically active in GFAP+ cells (Doetsch et al., 1999a). The NLSCre fragment was removed from ßactin-Cre plasmid (Lewandoski et al., 1997) with SalI and EcoRI and ligated into a SalI and EcoRI digested pCI vector (Promega). NLSCre was then released from pCI vector with SalI and XhoI and ligated into XhoI digested adenoviral transfer plasmid pAd5-GFAPp (Doetsch et al., 1999a), which contains the 2.5 kb mouse GFAP promoter region. The resulting plasmid was cut with NotI, then filled in and partially digested with EcoRV to release the GFAPp-NLSCre construct, which was then ligated into XmnI digested pENTR11 plasmid (Invitrogen) 5' of a previously inserted BGH polyA sequence. The resulting GFAPp-NLSCre-pA fragment was then transferred from pENTR11 into pAd/PL-DEST using LR clonase (Invitrogen). This created pAd/PL-GFAPp-NLSCre-pA, which contains the wild-type Ad5 genome deleted for E1 and E3. This vector was then transfected into HEK293 cells to produce replication-defective adenovirus (pAd5-GFAPp-NLSCre or Ad:GFAP-Cre), which was purified twice by cesium chloride banding. The titer was 1×10^{12} infections particles/ml.

Section 4: Histology and Immunohistochemistry

Immunohistochemistry

Mice were perfused (see "Transcardial perfusion for immunohistochemistry" below) to obtain well-fixed brain tissue. Fixed and washed brains were sectioned at the Vibratome (Leica model VT 1000S). Adult brains were blocked by placing them in a stainless steel brain mold (ASI instruments model RBM 2000C) and coronally cut at the desired level with a razor blade. Neonatal and early postnatal brains were blocked manually with a razor blade. Brains were dabbed on a paper towel to remove excess liquid and attached to a metal block with superglue for Vibratome sectioning. Brains were embedded in 2% agarose in phosphate buffered saline (PBS), and sectioned at 50 μm. Sections were collected in a 1/6 series in 24 well tissue culture plates in PBS.

To prepare cultured cells for immunohistochemistry, culture medium was gently removed from culture wells and 4% paraformaldehyde (PFA) was gently added and incubated with the cultures for 30 minutes at room temperature (RT). PFA was then removed and cultures were gently rinsed three times with PBS using a plastic transfer pipette.

The addition of sodium azide (NaN_3) to a final concentration of 0.1% allowed sections and cultures to be stored at 4°C for more than a year. Some antigens deteriorated over time, but most could be visualized by immunostaining many months later.

Free-floating sections and cultures were immunostained with primary antibodies directed against antigens listed in Table 2. These antibodies were visualized with secondary antibodies specific to the host and serotype of the primary antibody and conjugated to fluorophores or biotin (followed by streptavidin), as listed in Table 3.

Table 2. Primary antibodies

Specificity/Antigen	Host	Supplier	Dilution
Calbindin D-28K (CalB)	rabbit polyclonal IgG	Cell Signaling Technology	1:1000
	rabbit polyclonal IgG	Chemicon	1:500
	rabbit polyclonal IgG	Swant	1:1000
Calretinin (CalR)	rabbit polyclonal IgG	Swant	1:1000
β-galactosidase (β-gal)	mouse monoclonal IgG2a	Promega	1:500
BrdU	rat monoclonal IgG2a	AbCam	1:10
	mouse monoclonal IgG	Chemicon	1:50
heat stable antigen (CD24)	rat mono IgG2b/kappa	BD Pharmingen	1:500 – no TX
Cre recombinase	mouse monoclonal IgG1kappa	Chemicon	1:1000
Dlx2	rabbit polyclonal IgG	generous gift from David Eisenstat and Stuart Anderson	1:1000
	rabbit polyclonal IgG	Abcam	1:400
Doublecortin	guinea pig polyclonal IgG	Chemicon	1:1000
GABA	rabbit polyclonal IgG	Sigma	1:2500
GFAP	rabbit polyclonal IgG	DAKO	1:1000
	mouse monoclonal IgG1	Chemicon	1:1000
GFP	chicken polyclonal IgY	Aves Labs Inc	1:1000
	rabbit polyclonal IgG	Novus Biologicals	1:500
	sheep polyclonal IgG	Biogenesis	1:500
Glutamate transporter (Glt-1)	guinea pig antiserum	Chemicon	1:1000
HuC/HuD	mouse monoclonal IgG	Molecular Probes, Invitrogen	10 ug/ml
Mash1	mouse monoclonal IgG1	BD Pharmingen	1:200
Nestin	mouse monoclonal IgG1	Chemicon	1:100
Neuronal nuclei (NeuN)	mouse monoclonal IgG1	Chemicon	1:250
Nkx2.1	rabbit polyclonal IgG	Biopat, Italy	1:1000
O4	mouse monoclonal IgG	Chemicon	1:100 – no TX
Olig2	mouse monoclonal IgG	generous gift from	1:20,000

		David Rowitch	
Pax6	mouse monoclonal IgG1/kappa	generous gift from Atsushi Kawakami	1:500
Pbx3	rabbit polyclonal IgG	Santa Cruz Biotechnology	1:50
PSA-NCAM	mouse monoclonal IgM	AbCys, France	1:400
RC2 (radial cell 2)	mouse monoclonal IgM as antiserum	Developmental Studies Hybridoma Bank, deposited by Miyuki Yamamoto	1:5
S100-β	rabbit polyclonal IgG1/kappa	DAKO	1:1000
β-tubulin type III (Tuj1)	mouse monoclonal IgG2a	Covance	1:500
Tyrosine hydroxylase (TH)	rabbit polyclonal IgG	Pel-Freez Biologicals	1:1000
Vimentin (clone LN-6)	mouse monoclonal IgM	Sigma	1:1000

Table 3. Secondary antibodies and conjugates used to visualize primary antibodies

Specificity	Host	Conjugation	Supplier	Dilution
Biotin		Streptavidin 350	Molecular Probes, Invitrogen	1:200
		Streptavidin 488	Molecular Probes, Invitrogen	1:200
		Streptavidin 594	Molecular Probes, Invitrogen	1:200
Chicken IgY	Goat IgG	Biotin	Jackson ImmunoResearch	1:200
	Goat IgG	Alexa Fluor 488	Molecular Probes, Invitrogen	1:500
Guinea Pig IgG	Donkey IgG	Biotin	Jackson ImmunoResearch	1:200
	Goat IgG	Alexa Fluor 594	Molecular Probes, Invitrogen	1:500
	Goat IgG	Alexa Fluor 633	Molecular Probes, Invitrogen	1:500
Mouse IgG	Goat IgG	Biotin	Jackson ImmunoResearch	1:200
	Donkey IgG	Alexa Fluor 488	Molecular Probes, Invitrogen	1:500
	Goat IgG	Alexa Fluor 488	Molecular Probes, Invitrogen	1:500
	Goat IgG	Alexa Fluor 555	Molecular Probes, Invitrogen	1:500
	Donkey IgG	Cy3	Jackson ImmunoResearch	1:200
	Donkey IgG	Alexa Fluor 594	Molecular Probes, Invitrogen	1:500
	Goat IgG	Alexa Fluor 594	Molecular Probes, Invitrogen	1:500
	Goat IgG	Alexa Fluor 647	Molecular Probes, Invitrogen	1:500
Mouse IgG1	Goat IgG	Alexa Fluor 488	Molecular Probes, Invitrogen	1:500
	Goat IgG	Alexa Fluor 633	Molecular Probes, Invitrogen	1:500
Mouse IgG2a	Goat IgG	Biotin	Jackson ImmunoResearch	1:200
	Goat IgG	Alexa Fluor 488	Molecular Probes, Invitrogen	1:500
	Goat IgG	Alexa Fluor	Molecular Probes,	1:500

		555	Invitrogen	
	Goat IgG	Alexa Fluor 647	Molecular Probes, Invitrogen	1:500
Mouse IgM	Goat IgG	Biotin	Jackson ImmunoResearch	1:200
	Goat IgG	Alexa Fluor 594	Molecular Probes, Invitrogen	1:500
Rabbit IgG	Goat IgG	Biotin	Jackson ImmunoResearch	1:200
	Goat IgG	Alexa Fluor 350	Molecular Probes, Invitrogen	1:500
	Donkey IgG	Cy3	Jackson ImmunoResearch	1:200
	Goat IgG	Alexa Fluor 488	Molecular Probes, Invitrogen	1:500
	Donkey IgG	Alexa Fluor 594	Molecular Probes, Invitrogen	1:500
	Goat IgG	Alexa Fluor 647	Molecular Probes, Invitrogen	1:500
Rat IgG	Donkey IgG	Biotin	Jackson ImmunoResearch	1:200
	Goat IgG	Alexa Fluor 488	Molecular Probes, Invitrogen	1:500
	Donkey IgG	Alexa Fluor 594	Molecular Probes, Invitrogen	1:500
	Goat IgG	Alexa Fluor 647	Molecular Probes, Invitrogen	1:500

Primary and secondary antibodies were diluted in blocking solution containing the detergent Triton X-100 (TX) unless otherwise indicated. Blocking solution contained 8% normal serum from the host animal of the secondary antibody, 2% BSA, 0.1% TX and 0.1% NaN_3 diluted in PBS. This solution was passed through a 0.22 µm sterile filter and stored at 4°C. Tissue (brain sections or cultured cells) was briefly rinsed with PBS before primary antibody in blocking solution was added, and incubated on an orbital shaker in the dark overnight (or longer) at 4°C. Then, primary antibody was removed and saved at 4°C for later

use: antibodies for most antigens were recycled many times and kept fresh in the presence of 0.1 % NaN_3 for up to 6 months. Tissue was then rinsed briefly 3 times in PBS followed by a 1-hour wash in PBS. Then the appropriate secondary antibody was added in blocking solution and incubated on an orbital shaker in the dark overnight (or longer) at 4°C. Following this treatment, tissue was rinsed twice with PBS, and counterstained with 4',6-diamidino-2-phenylindole (DAPI) at a final concentration of 10 mg/ml for 5 minutes if fluorescently conjugated secondary antibodies were used. After two more 30 minute washes in PBS, sections were then floated on PBS and mounted on Superfrost Plus glass slides (Fisher). After drying on the slides, fluorescently labeled sections were rehydrated for 30 seconds in ddH_2O and mounted with Aqua Poly/Mount mounting medium (Polysciences Inc., Warrington PA) using Fisher brand glass coverslips. Slides were dried in the dark at RT overnight, then transferred to slide boxes and stored in the dark at 4°C. Omission of primary antibodies eliminated staining.

Sections or cultures treated with a biotin-conjugated secondary were visualized with Alexa Fluor conjugated streptavidin for 30 minutes at RT after rinsing off the secondary antibody. Following an additional 3 rinses in PBS, tissue was counterstained with DAPI and mounted as described above. Biotin was also sometimes visualized with horseradish peroxidase (HRP) conjugated streptavidin followed by diaminobenzidine (DAB) (see “DAB staining of cultures and Vibratome sections” below).

DAB staining of cultures and Vibratome sections

Before incubation with primary or secondary antibodies, the endogenous peroxidase activity of tissue to be stained with DAB was quenched with 1% H_2O_2 in PBS for 2 hours at RT on an orbital shaker, followed by two 15 minute washes in PBS. After this treatment, tissue was immunostained (see "Immunohistochemistry" above) using a biotinylated secondary antibody. After being rinsed in PBS, tissue was incubated in fresh ABC solution prepared according to the manufacturer's instructions (Vectastain Elite ABC kit, PK-6100 Standard) for 30 minutes in the dark. HRP and DAB are light-sensitive, so care was taken not to unnecessarily expose tissue to light until DAB was developed. Next, tissue was rinsed 3 times in PBS and incubated for five minutes in 1 ml DAB solution (0.167 mg/ml DAB powder in PBS). Next, 1 µl 30% H_2O_2 was added to each well to initiate staining. Staining was monitored periodically until well developed (2 minutes to overnight) and was stopped by three washes with PBS.

Sections were floated on PBS, mounted on Superfrost Plus slides, and dried for 1 hour or more at RT in the dark. Slides were rehydrated for 30 seconds in ddH_2O, counterstained with hematoxylin to visualize nuclei if desired (see "Hematoxylin staining" below), and dehydrated in an ethanol series (5 minutes each in 70%, 90%, 95%, 100%, 100%). Slides were then drip-dried, cleared in BABB (2 parts benzyl alcohol to 1 part benzyl benzoate) for 5 minutes, then briefly drip-dried and mounted with DPX neutral mounting medium (Sigma).

Slides were air-dried in the dark for 48 hours, placed in slide boxes and stored at 4°C.

Hematoxylin staining

Slide-mounted sections were counterstained for hematoxylin by rehydrating mounted sections for 30 seconds in ddH_2O, and placing them in filtered hematoxylin solution (Surgipath, Harris formula) for 2 minutes. Slides were then placed in running tap water for 2 minutes or until all traces of hematoxylin were gone, rinsed in ddH_20 for 30 seconds, and de-stained by three dips in acid alcohol (1 ml 10N HCl in 400 ml 70% ethanol). Slides were again placed in running tap water for 1 minute, then into ddH_20 for 30 seconds, and transferred to bluing solution (6.16 g lithium bicarbonate in 400 ml ddH_2O) for 1 minute or until the color of the stain changed from purple to blue. Slides were then placed in running tap water for 1 minute, followed by ddH_20 for 30 seconds, and were dehydrated, cleared in BABB, and mounted as described above (see "DAB staining of cultures and Vibratome sections").

β-gal staining

Perfused brains were cut on the Vibratome at 50 µm and collected into PBS, or cut at 200 µm and collected into PB in clean glassware for electron microscopy (EM). Alternatively, freshly cut mouse tails were placed in Eppendorf tubes for genotyping (see "Genotyping by β-gal histochemistry" above). Tissue was then transferred to X-gal buffer (120 µl X-gal solution, 20 µL MgCl solution,

0.5 ml K3 solution, 0.5 ml K4 solution, brought to 10 ml 0.1M PB). X-gal solution was made filter-sterilizing 200 mg X-gal dissolved in 5 ml dimethylformamide (stored in dark at -20°C. $MgCl_2$ solution was made by dissolving 0.9521 g $MgCl_2$ in 10 ml ddH_2O and 0.2 µm filter-sterilizing (stored in the dark at RT). K3 and K4 solutions were made by filter-sterilizing 1.646 g of K^+ ferricyanide or 2.112 g K^+ ferrocyanide dissolved in 50 ml 0.1M PB, respectively (stored in the dark at RT). Sections were incubated in the dark at 37°C and monitored until darkly stained but before blue precipitate has diffused (30 minutes to overnight). Staining was stopped by three washes with PB or PBS. Sections were then mounted on Superfrost Plus slides (Fisher) or processed for electron microscopy (EM) (see "Tissue preparation for electron microscopy" below). Mounted sections were coversliped with Aqua Poly/Mount.

Alkaline phosphatase (AP) staining

Vibratome sections from Z/AP mice were cut at 50 µm and stored in PBS without NaN_3. When not being used immediately, PBS on stored sections was changed at least every three days. Sections were then heated 65°C for 30 minutes to inactivate endogenous AP. After two rinses in PBS, sections were transferred to sterile filtered AP buffer. AP buffer consisted of 400 ml dH_2O, 50 ml 1M Tris-HCl pH 9.5 (100mM final), 10 ml 5M NaCl (100mM final), 2.5 ml 1 M $MgCl_2$ (5mM final) brought to a pH of 9.5 with HCl and a volume of 500 ml with ddH_2O. After 10 minutes, AP buffer was removed and active AP buffer was added in the dark. 10 ml active AP buffer contains 10 ml AP buffer, 100 µl 4-nitro

blue tetrazolium chloride (NBT) (Boehringer Mannheim, cat # 85244223), and 20 µl 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) (Boehringer Mannheim, cat # 85266220). Staining was monitored until well developed (5 minutes to overnight) and stopped by rinsing several times with PBS. Sections were mounted in PBS on chromalum or silanated slides (see "Chromalum slides" and "Silanated slides" below), and coversliped with Aqua Poly/Mount. After drying in the dark at RT overnight, slides were placed in slide boxes and stored at 4°C.

Chromalum slides

Chromalum slides are particularly sticky and can be used to mount sections when Superfrost Plus sections fail. Slides were prepared by first soaking Superfrost slides in hot tap water with microdetergent for several hours, then rinsing them in warm tap water for 30 minutes. They were then soaked in 5% HCl for 5 minutes and rinsed in running ddH_2O for 30 minutes. They were then completely dried in a 110°C oven for 30 or more minutes and slowly dipped in chromalum solution. To prepare chromalum solution, 500 mg chromium potassium sulfate was added 600 ml ddH2O to make chromium solution. This was then added to 5 g (300 bloom) gelatin completely dissolved in 400 ml boiling ddH_2O. After being dipped in chromalum solution slowly to prevent any bubbles from forming, they were slowly removed and dried overnight in a hood, protected from dust with a paper towel. Slides were then transferred to a box and stored at RT (good for a month or more).

Silanated slides

Silanated slides are particularly sticky and can be used to mount sections when Superfrost Plus fails. To make these slides, a fresh pack of frosted or Superfrost Plus slides (Fisher) was soaked in methanol for two minutes to remove trace oil and water. Next, slides were transferred to acetone for 2 minutes to remove water and residual oil and dirt. They were then transferred to silane solution (10 ml 3-aminopropyltriethoxysilane in 490 ml acetone) for two minutes. They were then rinsed in running ddH_2O for 5 minutes taking care not to expose the slides to any detergents, completely dried in a 110°C oven for 30 or more minutes, and transferred to a box at RT for storage (good for a month or more).

Antigen recovery and processing for BrdU staining (adapted from Bettina Seri)

For double-labeling of BrdU, free-floating 50 µm Vibratome sections in 24 well tissue culture plates were immunostained for antigens as described above (see "Immunohistochemistry"). Before counterstaining with DAPI or antigen recovery for BrdU, which destroys many antigens, secondary antibody was washed off three times with PBS and fixed to the section with 4% PFA for 15 minutes at RT. Sections were then rinsed twice with PBS and incubated in 2N HCl for 30 minutes at 37°C to denature DNA, thereby exposing the BrdU antigen for immunostaining. Following this treatment, sections were rinsed in boric acid buffer (6.82 g orthoboraic acid and 8.55 g sodium tetraborate brought to 1 liter in ddH2O and adjusted to pH 8.5) for 10 minutes. Sections were then washed 3

times in PBS, incubated in primary and secondary antibodies for BrdU, and counterstained for DAPI as described above (see "Immunohistochemistry"). Sections were mounted and stored as described above.

Immunostaining of whole mount preparations of the lateral ventricular wall (adapted from Fiona Doetsch, Bene Menn, and Zaman Mirzadeh)

Whole mounts were prepared to visualize the distribution of labeled stem cells on the lateral ventricular walls. Whole mounts were prepared by sacrificing neonatal or adult animals, removing their brains and immediately dissecting out the lateral ventricular wall in warm Liebovitz's L15 (L15) medium under a dissecting scope as previously published (Doetsch and Alvarez-Buylla, 1996). Whole mounts were drop fixed in cold 4% PFA containing 0.1% TX and fixed overnight. They were then rinsed in PBST (PBS containing 0.1% TX) and primary antibody in blocking solution was added as described above (see "Immunohistochemistry"), except that the blocking solution contained 2% TX rather than 0.1% TX. Whole mounts were incubated for 72 hours at 4°C, then washed gently 3x in PBST at RT for 1 day before being incubated in secondary antibody for an additional 72 hours at 4°C. Secondary antibody was washed off by multiple rinses with PBST and whole mounts were counterstained with DAPI if desired. After staining, the SVZ was carefully dissected from the whole mount and mounted on a Superfrost Plus slide with Aqua Poly/Mount. Slides were coversliped and stored as described above (see "Immunohistochemistry"). Once stained and mounted, whole mounts could be dehydrated in an ethanol series,

cleared with BABB, and mounted in DPX neutral mounting medium as described above in "DAB staining of cultures and Vibratome sections".

Tissue preparation for electron microscopy (EM)

To prepare samples for transmission EM, brains were fixed (see "Perfusion for standard and β-gal electron microscopy" below), collected in clean glassware, and rinsed in five times for ten minutes each rinse in PB at 4°C before storage. 50-200 µm sections were cut in PB at the Vibratome and collected into clean glassware containing PB. If appropriate, tissue was stained for β-gal as described above (see "β-gal staining" above) and rinsed with PB. Tissue was then transferred to 1% osmium tetroxide (OsO_4) with 7% glucose in 0.1M PB (0.50 ml 0.2M PB, 0.25 ml 4% osmium tetroxide, 0.25 ml H_2O, 0.07 g glucose for each vial containing 7-10 full sections) for 1 hour in the dark at RT. Sections were then removed from OsO_4 and transferred to maleic acid. Maleic acid contained 25 ml component A, 27 ml component B and 48 ml ddH2O and was adjusted to a pH of 7.4 with HCl. Component A contained 12.1 g Tris-HCl and 11.6 g maleic acid in 500 ml ddH_2O, and component B contained 0.8 g NaOH in 100 ml dH_2O. After three 15 minute rinses in maleic acid, sections were transferred to freshly made 2% uranyl acetate (UA) at 4°C in the dark. UA is dissolved in maleic acid by sonication and must be used immediately. Sections were incubated in 2% UA in maleic acid for 3 hours at 4°C in the dark. Next, sections were rinsed three times for 10 minutes in maleic acid and dehydrated in an ethanol series at 4°C for 5 minutes with 25%, 50%, 70%, three times for five minutes in 95% and three

times for 10 minutes in 100%. Sections were then rinsed twice for 10 minutes with propylene oxide at RT in a fume hood and transferred directly to araldite on a shaker to include overnight at RT.

Araldite was prepared by mixing 10 ml component A/M (Durcupan ACM Fluka) with 10 ml component B (Durcupan ACM Fluka) in a specimen cup, then adding 0.4 ml component D (Durcupan ACM Fluka) and mixing well, and adding 0.4 ml component C (Durcupan ACM Fluka) and mixing well. Before sections were added, bubbles were removed by placing the araldite in a vacuum chamber. Araldite was then transferred to foil cups and placed on a shaker for one hour before sections were added and incubated overnight. After incubation, foil cups containing sections were placed on a slide warmer for 5 minutes at 60°C. Sections were carefully removed and placed between two clean sheets of overhead transparency paper and incubated at 60°C for two hours. Sections were then flattened by placing a weight on top of the transparency paper and were transferred to a 60°C oven for three days.

Further processing for transmission EM was performed as previously described (Herrera et al., 1999). Cell counting in the neonatal ventricular zone using electron microscopy was performed as previously described (Doetsch et al., 1997). The ultrastructural criteria to identify radial glia and adult SVZ cell types are published (Doetsch et al., 1997; Hinds and Ruffett, 1971).

Section 5: Tissue dissection and culture

Dissection of SVZ and other brain regions from neonatal and adult mice

Brain regions were dissected from mice using the following tools: one sharp, blue microdissection knife (Sharppoint 22.5 degree microsurgical knife, Surgical Specialties Corporation, Reading PA 19606, REF 72-2201), two sharp 45 degree forceps, one pair of stout, curved forceps for retracting the skull, one pair of small, stout scissors for cutting through the skull and one pair of scissors for decapitation. All tools were sterilized by autoclaving or immersion in hot beads (240°C) and were sprayed with 70% ethanol prior to use. Outside of the tissue culture room, P0-P7 mice were killed by rapid decapitation and older mice were killed by overdose of tribromoethanol (Avertin) followed by cervical dislocation. After the head was liberally sprayed with 70% ethanol, the brain was dissected (see "Dissection of fresh or fixed brains" below) and placed in ice-cold Liebovitz's L-15 medium or pipes buffer (120mM NaCl, 5mM KCl, 25mM glucose, 20mM Pipes (pH 7.5), 0.0005% phenol red).

For various experiments, dissected regions included RMS, dorsal, ventral, anterior or posterior SVZ, the anterior medial ventricular wall, cortex and striatum. Relative to bregma in an adult mouse, RMS was taken anterior to -3.5, SVZ and medial wall from between -1.5 and 2, and cortex and striatum from between -0.5 and 1.5. Meninges were removed from all regions with forceps. To obtain RMS tissue, the core of the olfactory bulb was dissected by cutting away more superficial layers. SVZ, cortex, and striatum were dissected by cutting a

coronal slab and turning the slab caudal-side-up, making it easy to visualize the ventricles. SVZ, medial wall, and SVZ sub-regions were obtained by making two cuts through the brain flanking the desired piece of tissue, creating a wedge-shaped piece of tissue. The desired region was then trimmed off the end of the wedge and collected. Dorsal SVZ was taken from the region between the corpus callosum and the striatum, lateral to the lateral ventricle. Cortex was obtained by cutting above the corpus callosum from the dorsal midline, lateral to the insular cortex. Striatum was dissected from the coronal slab by liberally trimming away all surrounding tissue. Tissue pieces were collected in ice-cold Liebovitz's L-15 medium or pipes buffer.

Cell dissociation (adapted from Dan Lim)

The SVZ or other brain regions were dissected from neonatal to adult mice as described above (see "Dissection of SVZ and other brain regions from neonatal and adult mice"). These regions were then digested in 250 µl 0.25% Trypsin-EDTA for 30 minutes at 37°C. 750 µl fetal bovine serum (FBS) was then added to stop digestion and tissue was pelleted by centrifugation for two minutes at 1000 RPM on a Beckman tabletop centrifuge. The supernatant was removed and tissue was triturated in 1 ml fresh medium by 15-20 passes through a 1 ml pipette tip. Cells were then pelleted for five minutes at 1000 RPM, and the supernatant was aspirated and replaced with fresh medium. Cells were counted on a hemocytometer and plated at the desired density (10-20 cells per microliter

for clonal experiments or 30-50,000 cells per microliter for expansion) on 24, 12 or 6 well plates in 0.5, 1, or 3 ml medium, respectively.

Cortical or SVZ astrocyte monolayer cultures, grown on glass coverslips (adapted from Dan Lim, Tony Tramontin, and from (Schacher, 1992))

These cultures were used to expand astrocytes in culture for transplantation or other experiments such as the viral diffusion assay (see below). Cortex was dissected from P0-P7 animals and SVZ was dissected from neonatal to adult animals as described above (see "Dissection of SVZ and other brain regions from neonatal and adult mice"). Tissue was then digested for 30 minutes in 250 µl 0.25% Trypsin-EDTA solution at 37°C and triturated in the presence of an additional 750 µl astrocyte complete medium and 1 mg/ml DNAse. Astrocyte complete medium was made by sterile filtering 44 ml DMEM, 5 ml fetal calf serum (FCS), 0.5 ml 100x antibiotic/antimycotic, and 0.5 ml 200 mM L-glutamine. Cells were the pelleted by centrifugation at 1000 RPM for 5 minutes on a Beckman tabletop centrifuge, the supernatant was carefully aspirated and the pellet was resuspended in 1 ml astrocyte complete medium. Cells were plated at 1-2,000,000 per T-25 flask in 3 ml astrocyte complete medium, and placed in an incubator at 37°C and 5% CO_2. Medium was replaced every 3 days, and cultures took 7-12 days to reach confluence.

Upon reaching confluence, cultures were purified for strongly adherent astrocytes by placing the closed flask on an orbital shaker overnight at 260-300 RPM (Levison and McCarthy, 1991). Cells were then washed three times with

fresh astrocyte complete medium, released from the plate by incubation with 0.25% Trypsin-EDTA for 5-10 minutes at 37°C, and triturated, pelleted, and resuspended in astrocyte complete medium as described above. To passage cultures, cells were counted and plated at a density of 20,000 cells/cm^2 (about 1:4 split). Passaged cells reached confluence in 3-7 days and could be frozen for later use (see "Freezing and thawing cells" below).

Cells to be differentiated or used for experiments were counted and plated at 500,000 cells per well of a 24 well plate containing a coated glass coverslip. Glass coverslips were prepared by washing them in acetone, then in ethanol, then flame sterilizing them and transferring one round coverslip to one well of a 24 well plate. Coverslips were then treated for one hour with 0.4 ml of 0.1 mg/ml poly-D-lysine (PDK) in ddH_20 at 37°C, then thoroughly rinsed four times with ddH_2O and incubated with 0.4 ml of 0.01 mg/ml laminin in PBS for 1 hour or more @ 37°C. Upon reaching confluence, cultures were fixed and stained as described above (see "Immunohistochemistry").

Freezing and thawing cells

Astrocyte and neural stem cell cultures were frozen for later use. Cells were harvested in growth phase, which was achieved by splitting an adherent, confluent culture 1:2 the previous day. Cells were released from the plate, dissociated and pelleted as described above. The cell pellet was then resuspended in 880 µl ice-chilled astrocyte complete medium containing 10% FCS. To this suspension, 120 µl of ice-chilled DMSO containing 10% FCS was

added drop-wise. The cell suspension was thoroughly mixed after the addition of each drop to minimize heating and osmotic shock. The suspension was then chilled on ice for 30 minutes, transferred to a freezing tube pre-chilled at -80°C placed in a well-insulated box, and placed at -80°C overnight for slow freezing. Cells were stored at -80°C for use within a month and transferred to liquid nitrogen for long-term storage.

Cells were thawed rapidly in a 37°C water bath, transferred to a 50 ml conical tube, and 9 ml medium warmed to 37°C was slowly added to minimize osmotic shock. Cells were then pelleted at 1000 RPM for 5 minutes, resuspended in fresh warm medium, plated at high density (100,000 or more cells/cm^2).

Neurosphere cultures (adapted from Tony Tramontin)

Tissue was dissected and collected in pipes buffer (120mM NaCl, 5mM KCl, 25mM glucose, 20mM Pipes (pH 7.5), 0.0005% phenol red) as described above, except that tissue was digested in 1 ml activated papain in 4 ml pipes buffer rather than 0.25% trypsin-EDTA. 1M Pipes was made by sterile filtering 15.12 g Pipes dissolved in 45 ml ddH_20, adjusted to pH 7.5 with NaOH and brought to 50 ml wit ddH2O. Activated papain was generated by sterile filtering 3 mg papain dissolved in 1 ml ddH2O containing 1.1 mM EDTA and 5.5 mM L-cysteine/HCl. Tissue was incubated in activated papain at 37°C for 30 minutes, then the supernatant was removed and 1 ml control medium containing 1 mg/ml tissue culture grade DNAse was added. The tissue was triturated as described

above and pelleted by centrifugation for 5 minutes at 1000 RPM.

500 ml of neurosphere control medium was made by filter sterilizing 415 ml DMEM/F12, 10 ml 30% glucose (30 g D-(+)-glucose in 100 ml ddH2O, mixed by sonication and sterile filtered), 7.5 ml 7.5% $NaHCO_3$, 2.5 ml 1 M HEPES, 5 ml 200 mM L-glutamine, 50 ml 10x Hormone mix and 5 ml 100x antibiotic/antimycotic. Hormone mix contained 400 ml DMEM/F12, 8 ml 30% glucose, 6 ml 7.5% $NaHCO_3$, 2 ml 1 M HEPES, 400 mg apo-transferrin, 40 ml insulin solution, 40 ml putrescine solution, 40 µl 200 µM progesterone and 40 µl 3 mM sodium selenite, filter-sterilized. Insulin solution contained 100 mg insulin in 4 ml sterile 0.1 N HCl and 36 ml sterile ddH_2O, and putrescince solution contaied 38.6 mg putrescine in 40 ml sterile ddH_2O. Complete medium was made by sterile filtering 49.7 ml control medium containing 200 mg BSA and 100 µl heparin solution before adding 250 µl EGF (stock = 4 µg/ml) 20 µl bFGF (stock = 25 µg/ml). Heparin solution was made by filter sterilizing 38,000 units heparin dissolved in 100 ml ddH_2O.

Cells were then resuspended in 1 ml neurosphere complete medium, passed through a cell strainer and counted. They were then plated at clonal density (10-20 cells/µl) in 6 well plates (ultra low attachment Corning) in 2-3 ml neurosphere complete medium and incubated at 37°C in 5% CO_2. When neurospheres reached a mean diameter of 100 µm, they were collected into a 15 ml conical tube and pelleted by gravity. The supernatant was carefully aspirated and replaced with 1 ml fresh complete medium. Cells were triturated to a single cell suspension, passed through a cell strainer, and pelleted by centrifugation at

1000 RPM for 5 minutes. Cells were then resuspended in 1 ml fresh neurosphere complete medium, counted, and plated at 5-20 spheres per well.

Neurospheres were differentiated by collecting them in a conical tube as described above, and gently resuspending them in neurosphere differentiation medium #1. This medium contains 49.9 ml control medium, 100 µl heparin solution and 20 µl bFGF. After neurospheres had settled, the supernatant was aspirated and they were again resuspended in neurosphere differentiation medium #1. Individual spheres were picked and transferred, or a desired concentration of spheres was transferred to wells filled with differentiation medium #1 in wells or poly-D-lysine (PDK)-treated coverslips coated with Matrigel that had been diluted 1:100 in control medium. Neurospheres were differentiated in this medium for two days, then washed once with control medium, and incubated in neurosphere differentiation medium #2, containing 49 ml control medium and 1 ml fetal bovine serum (FBS). Following differentiation for 5 days at 37°C, 5% CO_2, medium was gently aspirated and cultures were fixed with 4% PFA for 15 minutes at RT, then gently rinsed three times with PBS. Cultures were then immunostained for O4, Tuj1, GFAP and GFP.

Clonal generation of neurospheres (modified from Tony Tramontin and (Doetsch et al., 1999a))

To determine whether radial glia or their adult progeny could form neurospheres, Z/EG animals were injected with Ad:Cre at P0 and the lateral ventricular wall of P2, P10 and P90 animals was dissected, dissociated, and

placed in neurosphere medium as previously reported (Doetsch et al., 1999a) (see also "neurosphere cultures" above). After amplification in culture, cells were sorted for eGFP by fluorescent activated cell sorting (FACS) on a MoFlo Cytomation (Freiburg, Germany) cell sorter at approximately 100 cells/second. The fluorescence of sorted cells was bimodally distributed; the mean fluorescence of the eGFP+ cells was approximately two orders of magnitude greater than the peak for eGFP- cells. Visual inspection confirmed strong eGFP fluorescence in more than 98% of sorted cells. Cells were sorted a second time using the same parameters to achieve an approximately 99.9% pure population of eGFP+ cells. Visual inspection confirmed that all neurospheres formed by these cells were strongly green fluorescent. Upon plating at clonal density (20 cells/μL), these cells generated neurospheres that were individually differentiated and stained for eGFP, O4, Tuj1, and GFAP as previously described (Doetsch et al., 1999a).

Neural stem cell monolayer cultures (adapted from Dan Lim and from (Scheffler et al., 2005))

Tissue from neurogenic regions was dissected as described above and collected in ice-cold DMEM/F12+Glutamax (Gibco). Tissue was then incubated 0.25 ml 0.25% Trypsin-EDTA for 30 minutes at 37°C and triturated and pelleted as described above. Cells were then resuspended in 2 ml N5 medium and plated onto one well of a 12 well tissue culture plate. N5 medium contained 2.5 ml fetal bovine serum (FBS), 0.5 ml 100x antibiotic/antimycotic, 0.5 ml N2 supplement

(Gibco), 87.5 µl bovine pituitary extract, bFGF (20 ng/ml final concentration) and EGF (20 ng/ml final concentration) in DMEM/F12+Glutamax (Gibco) at a final volume of 50 ml. Cells were transferred to an incubator overnight at 37°C and 5% CO_2.

One day following plating, the plate was rinsed with its own medium to wash off weakly adhering cells, which were then transferred to a fresh well of a 12 well plate. This procedure removed strongly adhering cells including non-stem cell astrocytes and microglia, enriching the culture for neural stem cells that grow as an adherent monolayer. Cultures were returned to the incubator and N5 medium was replaced every 3 days. Upon reaching confluence, cells were split 1:2 or 1:4 as described above.

After three passages, cells were frozen as described above, or collected for heterotopic grafting (see below) or differentiation. For differentiation, cells in growth phase were collected, pelleted, and resuspended in N5 medium at 300 cells/µl in 100 µl in a 96 well tissue culture plate and returned to the incubator. After cells had attached to the plate overnight, N5 medium was gently aspirated, washed twice with N6 medium, and replaced with N6 medium. N6 medium contained 49 ml DMEM/F12+Glutamax (Gibco), 0.5 ml antibiotic/antimycotic, 0.5 ml N2 supplement (Gibco) and 87.5 µl bovine pituitary extract. Cultures were returned to the incubator and differentiated for 7 days in N6, which was replaced every 3 days. Following differentiation, cultures were fixed and immunostained (see “Immunohistochemistry” above)

Neural stem cell cultures for in vitro potential determination

Anterior (i/iiM), dorsal (ii/iiiD) and ventral (iiV/iiiV) brain regions were microdissected from wild-type perinatal mice and dissociated as described above. Regions are named as in Chapter 4, Figure 1H. Cells were then grown as previously described (see (Scheffler et al., 2005) and "Neural stem cell monolayer cultures" above) on 6 well plates and passaged twice over 7 days. After the second passage, cells were transferred to 96 well plates and differentiated for an additional 7 days. Following differentiation, cells were fixed for 30 minutes at RT in 4% PFA and immunostained for GFAP, Olig2, doublecortin, calbindin and calretinin as described above (see "Immunohistochemistry").

Neural stem cell cultures for heterotopic grafting

Forty-eight hours after being labeled with Ad:Cre at P0, GFP+ radial glia from the anterior dorsal (iiiD) or ventral (iiiV) SVZ were dissected, dissociated and cultured as described above (see "Neural stem cell monolayer cultures"). Regions are named as in Chapter 4, Figure 1H. Additionally, cells were isolated and cultured from P0 CD1 of ActB-GFP mice (Hadjantonakis et al., 1998), which constitutively express GFP, from regions iiM, iii/ivD, iii/ivV and vD/V. After 14 days in culture and three passages, cells were collected, washed in L15 and grafted into regions iiD, iiiD, iiiV or vD/V of neonatal CD1 mice (see "Heterotopic grafting of radial glia" below).

Section 6: Surgical and animal procedures

All protocols and procedures followed the guidelines of the Laboratory Animal Resource Center at the University of California, San Francisco.

BrdU administration

Injections for short survivals were performed 1 hour prior to sacrifice. Mice were injected intraperitoneally (IP) with BrdU stock solution (15 mg/ml BrdU in sterile saline) to achieve a final concentration of concentration of 50 mg/kg. For long survival pulse chase experiments, adult mice (≥P30) were given BrdU in the drinking water (deionized, distilled water) at a concentration of 1 mg/ml for 3 followed by a survival of 2 weeks, or for 7 days followed by a 3 week survival.

Stereotaxic injection of neonatal pups

Newborn (P0, 1.5-1.75 g) R26R, Z/AP or Z/EG mice were anesthetized by hypothermia for 60 seconds and placed onto the platform of a stereotaxic injection rig where their heads were stabilized by a customized head mold (see "Production of customized head molds" above). They were injected with 20 nl of adenovirus (Ad:Cre) with a beveled pulled glass micropipette (see "Production of pulled glass micropipettes for viral injection and cell grafting into the neonatal and adult mouse brain" above) with a 30 μm diameter tip, back-filled with mineral oil and front-loaded with 1-2 μl virus. Unless otherwise noted, the injection needle was positioned at an angle of 45 degrees and pups were placed on their sides in

custom-made head molds, so that the needle entered the brain from below at an angle of 45 degrees, along the trajectory of radial glial fibers.

Injection coordinates were determined based on immunostaining for radial glial fibers in Vibratome sections and confirmed by dye injection. Using the center of the eyeball to zero the x (anterior-posterior) and y (medial-lateral) coordinates, and the skin surface to zero the z (depth) coordinate, the injection coordinates were (x, y, z in mm): region i (0.25, 1.6, -2.5), region iiM (0.75, 3.5, -4.0), region iiD (0.75, 0.9, -1.5), region iiV (0.75, 2.0, -2.85), region iiiD (1.25, 1.0, -1.65), region iiiV (1.25, 2.5, 2.75), region ivD (1.75, 1.25, -1.75), region ivV (1.75, 2.5, -2.75), region vD (2.25, 1.15, -1.75), region vV (2.25, 2.0, -2.9), and region vi (2.75, 1.75, -2.15). Regions are named as in Chapter 4, Figure 1H.

To limit trauma from injecting though the eyeball, regions i and iiD can also be targeted from above at an angle of 45 degrees at (0.6, 2.5, -1.6) and (1.1, 3.25, -1.2), respectively. This trajectory was also used for to target radial glia cells with striatal injections in experiments to determine the developmental origin of adult neural stem cells at (1.6, 2.8, -1.6). For these injections, pups were placed ventral side down in a customized head mold and coordinates were zeroed as follows: x (anterior-posterior) centered on the eyeball as above, y (medial-lateral) centered on the midline, and z (depth) centered on the skin surface. These same landmarks were used to zero coordinates for targeting of cortical radial glia from straight above. The injection coordinates were: region iiC (1.1, 0.8, -0.3), region iiiC (1.6, 1.0, -0.3), region ivC (2.0, 1.0, - 0.3), region vC

(2.4, 1.0, -0.3). In all cases, trajectories were chosen to specifically label radial glial cells and avoid direct labeling of the SVZ or ventricular system.

Following injections, pups were returned to their mothers and monitored until they had resumed nursing. Cannibalism was not observed with CD1 animals, but pups from C57/Bl6 and other strains were lightly rubbed with urine from the mother before being returned to mitigate cannibalism. Animals were sacrificed 2 hours to 150 days later.

Grafting of labeled cells to determine stem cell identity

To obtain labeled radial glia, heterozygous Z/EG pups were injected at P0, sacrificed at P3, and dissected under a fluorescent dissecting scope to expose the lateral wall of the lateral ventricle. This allowed the injection site and a well-defined patch of eGFP+ radial glial cell bodies in the VZ to be visualized. Labeled cells in the VZ (n = 6 grafts) and the injection site (n = 4 grafts) were microdissected and stereotaxically grafted into the VZ/SVZ of wild-type pups, which were sacrificed at P30. In a separate experiment, labeled cells from the injection site (n = 6) were grafted into the ventrolateral striatum. Radial glial grafts, but not grafts from cells labeled locally at the injection site, produced all the cell types seen from the injection alone.

Heterotopic grafting of radial glia

To obtain labeled radial glial cells, heterozygous Z/EG mice were injected at P0 with 20 nl Ad:Cre to target levels ii or iv for anterior/posterior grafts or

regions iiiD or iiiV for dorsal/ventral grafts as described above. Regions are named as in Chapter 4, Figure 1H. Two hours after injection, the animals were sacrificed and the targeted SVZ subregions were microdissected in ice-cold Liebovitz's L-15 medium (UCSF Tissue Culture Facility) to obtain virally infected radial glial cell bodies, which were then mechanically triturated to a single cell suspension. Cells were then washed and resuspended in L-15 medium, and front-loaded into a beveled pulled glass micropipette with a 50 μm diameter tip (see "Production of pulled glass micropipettes for viral injection and cell grafting into the neonatal and adult mouse brain" above). Neonatal CD1 pups were injected with 100-200 nl of cell suspension (~2000 cells). Injections were performed at an angle of 45 degrees from above with axes zeroed as described above at the following coordinates: region iiD (1.1, 2.5, 1.5), region iiiD (1.6, 2.1, 1.3), region iiiV (1.6, 3.0, 2.2) or region vD (2.4, 3.5, 1.0). Grafted pups were returned to their mothers and monitored until they had resumed nursing. Animals were sacrificed 40 days after grafting.

Heterotopic grafting of cultured neural stem cells

Forty-eight hours after being labeled with Ad:Cre at P0, GFP+ cells from the anterior dorsal (iiiD) or ventral (iiiV) SVZ were dissected, dissociated and cultured as adherent monolayers as described above (Scheffler et al., 2005). Regions are named as in Chapter 4, Figure 1H. Additionally, cells were isolated and cultured from P0 CD1 of ActB-GFP mice (Hadjantonakis et al., 1998), which constitutively express GFP, from regions iiM, iii/ivD, iii/ivV and vD/V. After 15

days in culture and three passages, cells were collected, washed in L15 and grafted into regions iiD, iiiD, iiiV or vD/V of neonatal CD1 mice as described above. Animals were sacrificed 28 days after grafting.

Stereotaxic injection of adult mice

Heterozygous adult (P60) Z/EG mice were anesthetized with 250 mg/kg tribromoethanol (Avertin) until pedal reflex was abolished, and placed in ear bars on a customized stereotaxic rig. After making a small incision, a hole was drilled in the skull at the injection coordinate to expose the brain surface. The brain was then injected with 20 or 40 nl adenovirus (Ad:GFAP-Cre) from a beveled pulled glass micropipette (see "Production of pulled glass micropipettes for viral injection and cell grafting into the neonatal and adult mouse brain" above). Injection coordinates were determined from a stereotaxic mouse brain atlas and confirmed with dye injections. The x and y coordinates were zeroed at bregma, and the z coordinate was zeroed at the brain surface. Injections of regions iiiD and iiiV were performed at an angle of 45 degrees ensure regionally specific labeling at the following coordinates: region iiiD (-0.5, 3.5, -1.8) and region iiiV (-0.5, 4.5, -3.6). Regions i and vC were injected from straight above with 20 nl Ad:GFAP-Cre for region i at (-3.3, 0.82, -2.9) as described previously (Hack et al., 2005) and with 40 nl Ad:GFAP-Cre at region vC at (1.5, 2, -0.5). The incision was closed with silk suture, and animals were placed on a warm surface and monitored until they resumed feeding and grooming activity. Animals were sacrificed after 28 days. Regions are named as in Chapter 4, Figure 1H.

Section 7: Terminal procedures

Transcardial perfusion for immunohistochemistry

Juvenile and adult animals were deeply anesthetized with tribromoethanol (Avertin) and monitored until the pedal reflex was abolished. Neonatal and early postnatal (P0-P5) mice were deeply anesthetized by hypothermia. Mice were then transferred to a perfusion tray and the abdominal and thoracic cavities were opened. The diaphragm was transected and the pericardium was carefully removed with forceps. An incision was made in the right atrium to provide a low resistance path for fluid to leave the circulatory system, and a perfusion needle was inserted into the left ventricle. Adult and juvenile mice were transcardially perfused with 20 ml room temperature (RT) 0.9% saline followed by 20 ml cold (4°C) 4% paraformaldehyde (PFA) using a perfusion pump (Watson Marlow model 323). Neonatal and early postnatal mice (P0-P10) were manually perfused with 5 ml 0.9% saline followed by 5 ml 4% PFA using 10 ml syringes. For some perfusions, 0.9% saline also contained 1% heparin to prevent blood clotting. Before PFA was administered, we ensured that blood was effectively removed from the circulatory system by verifying that no visible blood was leaving the right atrium and that the liver had cleared. The quality of fixation was determined by monitoring the movement of the tail in response to initial PFA administration and the stiffness of muscles and the liver. Following perfusion, brains were dissected as described below and postfixed in 4% PFA overnight at 4°C, then given three brief rinses in phosphate buffered saline (PBS) at followed by a PBS wash for

one or more hours hour at RT. Brains were stored at 4°C in PBS + 0.1% NaN_3.

Perfusion for standard and β-gal electron microscopy (adapted from Jose Manuel Garcia Verdugo and Tony Tramontin)

Mice were dissected and perfused as described above, but instead of cold 4% PFA, mice were perfused with a solution containing 2.5% glutaraldehyde and 2% PFA. This solution was made by mixing 10 g paraformaldehyde in 200 ml dH_2O, and heating it to ≤ 55°C under constant stirring. 10N NaOH was added drop-wise until PFA was completely dissolved. After cooling slightly, 50 ml 0.2M monobasic phosphate buffer (PB) and 200 ml 0.2M dibasic PB were added, adjusted to pH to 7.4 with HCl and filtered. Then, 50 ml of 25% glutaraldehyde (EM sciences catalogue #16210) was added. Mice were perfused with 0.9% saline with 1% heparin (at 37°C) until no blood could be seen leaving the right atrium (about 3 minutes) and with 2.5% glutaraldehyde and 2% PFA (at 37°C) for 5-10 minutes. Following fixation, the fixed brain was left in the skull for 30 minutes before dissection and postfixation overnight in 2.5% glutaraldehyde and 2% paraformaldehyde in clean glassware. The following morning, brains were rinsed 5 times, 30 minutes each rinse, in large volumes of 0.1M PB at RT. Brains were stored in very clean glassware in 0.1M PB in clean glass vial at 4°C.

Dissection of fresh or fixed brains

The head was sprayed liberally with 70% ethanol decapitated with a pair of large, sharp surgical scissors. With a pair of fine serrated scissors (Iris ToughCut, Fine Science Tools), an incision was cut through the skin along the midline from back of skull midline to tip of nose. The skin was then laterally retracted past the eyeballs and held in place with the left hand. Next, one blade of the scissors was inserted completely through the orbital foramen, allowing the skull to be cut just rostral to the olfactory bulbs. Next, a careful caudal to rostral midline incision was made through the skull, all the way up to the previous cut rostral to the olfactory bulbs. Next, horizontal cuts were made in the ventral skull bilaterally from the brainstem to about halfway to the olfactory bulbs. After the meninges covering the olfactory bulbs were carefully removed, the skull was peeled back with a pair of forceps and held in place by the left hand. The brain was then removed by lifting the olfactory bulbs with a pair of blunt forceps, which were then used to transect the optic and cranial nerves. The brain was then dropped into ice-cold medium or fixative.

Section 8: Data analysis

Quantification and image processing

Images of DAB stained tissue and images for granule cell quantification were taken on an Olympus AX70 microscope with a Retiga 2000R digital camera (Qimaging Corporation) on a G5 Macintosh computer (Apple Computer Inc.) running Openlab 5.0.1 or 5.0.2 (Improvision Inc.). Fluorescent images of immunostained brain sections were taken on an Olympus Fluoroview FV1000 confocal microscope. Fluorescent images of neural stem cell cultures were taken on a Zeiss Aviovert 200M microscope with a Hamamatsu Orca-ER digital camera (Hamamatsu Photonics). Images were processed for brightness and contrast using Adobe Photoshop 7.0 (Adobe Systems Inc.). Quantification of double-labeled cells was performed manually at 40x on an Olympus AX70 microscope. Granule cell position within the granule cell layer was measured from photomicrographs using ImageJ 1.37v (National Institutes of Health). Only injections resulting in specific labeling of the targeted region were included for analysis. For each injection, graft or culture, an average of 509 ($\pm$ 390) cells in three to eight independent experiments were analyzed.

Chapter 3

The neural stem cell lineage

Overview

Neural stem cells with the characteristics of astrocytes persist in the subventricular zone (SVZ) of the juvenile and adult brain. These cells generate large numbers of new neurons that migrate through the rostral migratory stream (RMS) to the olfactory bulb. The developmental origin of adult neural stem cells is not known. Here I describe a lox-Cre based technique to specifically and permanently label a restricted population of striatal radial glia in newborn mice. Within the first few days after labeling, these radial glial cells gave rise to neurons, oligodendrocytes and astrocytes, including astrocytes in the SVZ. Remarkably, after this labeling the RMS contained labeled migratory neuroblasts at all ages examined, including 150-day-old mice. Labeling dividing cells with the S-phase marker BrdU showed that new neurons continue to be produced in the adult by precursors that were derived from radial glia. Furthermore, both neonatal radial glia and radial glia-derived cells in the adult lateral ventricular wall generated self-renewing, multipotent neurospheres. These results demonstrate that radial glial cells not only serve as progenitors for many neurons and glial cells soon after birth, but also give rise to adult SVZ stem cells that continue to produce neurons throughout adult life. This work identifies - and provides a method to genetically modify - the lineage that links neonatal and adult neural stem cells.

Introduction

The mammalian brain retains neural stem cells in the subventricular zone (SVZ) of the lateral ventricular wall. These cells generate new neurons (Gage, 2000; Lois and Alvarez-Buylla, 1993; Morshead et al., 1994; Temple, 2001) that migrate along the rostral migratory stream (RMS) to the olfactory bulb where they differentiate into granular and periglomerular interneurons (Alvarez-Buylla and Garcia-Verdugo, 2002; Luskin, 1998). Although they are actively neurogenic, adult SVZ stem cells express glial fibrillary acidic protein (GFAP) and have the morphology and ultrastructure of astrocytes (Doetsch et al., 1999a; Imura et al., 2003; Laywell et al., 2000; Skogh et al., 2001). These findings challenge the classical view that all astrocytes are terminally differentiated glial cells belonging to a lineage separate from that of neurons (Alvarez-Buylla et al., 2001; Doetsch et al., 1999a). What is the developmental origin of these astrocytic stem cells?

Here, I tested the hypothesis that adult SVZ neural stem cells are derived from radial glia. Radial glial cells have a long, RC2 positive (Misson et al., 1988) basal process that extends from their cell bodies in the ventricular zone (VZ) through the parenchyma towards the brain surface (Noctor et al., 2002). Radial glia have anatomical features of cells in the astroglial lineage including endfeet on blood vessels, intermediate filaments and glycogen granules. Near the end of histogenesis, radial glia differentiate into parenchymal astrocytes (Schmechel and Rakic, 1979; Voigt, 1989). For these reasons, radial glia were classically viewed as immature glial cells that guide neuronal migration and function as scaffolding for brain development (Mission et al., 1991; Rakic, 1972). However,

studies in songbirds (Alvarez-Buylla et al., 1990), where radial glia persist into adult life, and work in the developing rodent brain (Anthony et al., 2004; Gotz et al., 2002; Li et al., 2004a; Malatesta et al., 2000; Miyata et al., 2001; Noctor et al., 2001; Tamamaki et al., 2001) indicate that radial glia also function as neural progenitors. We (Alvarez-Buylla et al., 2001), and others (Campbell and Gotz, 2002; Doetsch, 2003; Kriegstein and Gotz, 2003) have hypothesized that neural stem cells may be contained within what was classically considered the macroglial lineage (i.e. neuroepithelial cells - radial glia - astrocytes).

In the present study, I genetically tagged a restricted population of radial glia that persist in the lateral wall of the lateral ventricle of newborn mice (Tramontin et al., 2003). I show that these radial glial cells give rise to neurons, astrocytes, ependymal cells and oligodendrocytes. More importantly, I show that these neonatal radial glial cells give rise to the SVZ astrocytes that maintain neurogenesis in the adult mammalian brain. This work identifies the neonatal origin of adult SVZ neural stem cells.

Results

Maturation of the SVZ

I began my investigation of adult NSC origins with Anthony D. Tramontin by studying the composition of the lateral ventricular wall at different ages. At P0, the striatal VZ contains primarily radial glial cell bodies and a few immature ependymal cells, but no astrocytes (Tramontin et al., 2003). The cell bodies of subcortical (striatal) radial glia line the lateral wall of the lateral ventricle, and their long RC2+ basal processes curve through the striatum to contact the brain surface (Fig. 1A). To determine the time course of astrocyte formation in the SVZ, I immunostained for GFAP (Doetsch et al., 1997) and RC2 (Misson et al., 1988) to label astrocytes and radial glia, respectively, at postnatal days 0, 2, 4, 6, 8, 10, and 60 (n = 4 per age group). The disappearance of radial glia from the lateral ventricular wall correlates with the appearance of GFAP+ astrocytes (at P6) directly below the VZ in the developing SVZ (Fig. 1B-F). We hypothesized that striatal radial glia in the neonatal brain give rise to the neurogenic astrocytes found in the adult SVZ.

Radial glial targeting and lineage tracing

To test this hypothesis, we developed a technique to trace the progeny of a restricted population of radial glia. We found that radial glia in P0 mice can be labeled by a replication incompetent, eGFP-expressing adenovirus (Ad:GFP). To ensure that we were specifically labeling radial glia and not other cells in the VZ

or its vicinity, we infected radial glia at their distal basal processes by stereotaxically injecting Ad:GFP (20 nl) into ventrolateral striatum of P0 mice near the border to the piriform cortex (Fig. 1G). Two days after infection, we observed labeling at the injection site and in a restricted region of the dorsolateral striatal VZ, 1.10 ± 0.13 mm (mean ± SEM) away from the injection site (Fig. 1H; 3K). Labeled cells in the VZ had the distinctive morphology of radial glial cells and had RC2+ processes that traversed the injection site (see below). Thus, radial glial cell bodies can be retrogradely labeled with adenoviruses. The rapid infection, short half-life, and limited diffusion of adenoviruses through brain parenchyma (Peltekian et al., 1997) results in rapid, localized labeling. However, the adenoviral genome is not incorporated into cellular DNA and becomes diluted with cell division. Therefore, we used a Cre-lox based strategy to trace the long-term progeny of radial glia. We used the same targeting strategy to infect the distal processes of radial glia, but injected P0 Z/AP (Lobe et al., 1999), Z/EG (Novak et al., 2000), or R26R (Soriano, 1999) mice with an adenovirus expressing Cre recombinase (Ad:Cre) (Anton and Graham, 1995). Z/AP mice express alkaline phosphatase (AP), Z/EG mice express eGFP, and R26R mice express LacZ upon Cre-mediated recombination, so infected radial glia and their progeny are permanently labeled.

As with Ad:GFP injected mice, Ad:Cre injected Z/AP, Z/EG or R26R mice had AP, eGFP or LacZ positive cells, respectively, at the injection site and in the VZ. I confirmed that all labeled cells in the VZ were radial glia by immunostaining for RC2 (Fig. 1I) and by electron microscopy (Fig. 1J, 527 cells analyzed) two

days after injection. I never detected labeled cells in the medial ventricular wall or on the contralateral side, indicating that the Ad:Cre did not leak into the ventricular lumen.

To determine whether adenovirus injected in the ventrolateral striatum could be diffusing through the striatum and infecting cells at or near the VZ, I injected Ad:Cre into the lateral striatum of wild-type (CD1) P0 pups together with bromophenol blue to visualize the injection site. At various time points after injection (5 min, 20 min, 1 hr, 4 hr, 8 hr, 24 hr), I sacrificed the animals and microdissected equally sized explants of the injection site, the ipsilateral ventricular wall, and the striatum halfway between these two sites (Fig. 2A). I then cultured these explants for three days on top of monolayers of Z/EG (eGFP Ad:Cre reporting) SVZ astrocytes (n=3 per condition). I found eGFP+ cells in monolayers cultured with the injection site explant but never from monolayers cultured with the ventricular wall explant. Furthermore, the number of cells infected by each explant rapidly decreased over time; after 4 hours I could not detect any infectious particles (Fig. 2B). Therefore, cells were not nonspecifically labeled at or near the VZ by viral diffusion through the parenchyma, and all labeled cells in the VZ correspond to radial glia infected at their distal processes.

Radial glia produce all four major classes of brain cells

I then examined the progeny of radial glia in Z/AP, Z/EG and R26R mice that survived 4, 7, 10, 15, 30, 45, 90, or 150 days ($n \geq 4$ per age group) after Ad:Cre injection. At all time points, I observed labeled cells in the VZ and SVZ,

at the injection site in the ventrolateral striatum, and scattered stellate astrocytes in the striatum. I identified radial glial progeny by staining for AP to reveal the Golgi-like morphology (Fig. 5C-L), by immunofluorescence for cell-type specific markers, and by electron microscopy. At short survivals (P4-P10), I observed radial glia retracting their basal process from the injection site and transforming into striatal (Fig. 1K) and SVZ astrocytes (Fig. 1L). From P4 on, the SVZ and RMS contained many migratory neuroblasts (Fig. 1M; 3A,B) and from P10 on, the ipsilateral olfactory bulb contained large numbers of labeled granular (Fig. 1M,N,O; 3C) and periglomerular neurons (Fig. 1M,N,P). In addition, I identified labeled ependymal cells lining the lateral ventricular wall (Fig. 3E, F) and oligodendrocytes in the corpus callosum and striatum (Fig. 3G, H). I provide a more detailed description of the generation of ependymal cells from radial glia in a separate study (Spassky et al., 2005). Importantly, I also observed labeled type B cells, the astrocytic stem cells of the adult SVZ (Doetsch et al., 1999a; Imura et al., 2003; Laywell et al., 2000; Skogh et al., 2001), (Fig. 3I, J) and type C cells, the neurogenic transit amplifying cells that are produced by SVZ astrocytes (Doetsch et al., 1999a; Doetsch et al., 1997; Doetsch et al., 2002) (Fig. 3D), strongly suggesting that radial glia give rise to adult neural stem cells.

Radial glia, not striatal cells, act as progenitors

To demonstrate that these cell types are exclusively derived from radial glia, I microdissected either labeled radial glia from the VZ (Fig. 3K, arrow) or labeled cells from the injection site (Fig. 3K, arrowheads) and stereotaxically

grafted them into the VZ/SVZ of wild-type mice. The pure population of labeled radial glia produced all cell types I observed in Ad:Cre injected animals, including SVZ astrocytes, neuroblasts, and olfactory bulb interneurons (Fig. 3L). However, cells grafted from the injection site into the VZ/SVZ or into the ventrolateral striatum never produced ependymal cells, oligodendrocytes, neuroblasts or olfactory bulb neurons. In a separate experiment, I found that radial glia could be labeled by adenoviral injection into the lateral ventricle of P0 Z/AP mice, demonstrating that they contact the ventricular surface. These labeled cells gave rise to all progeny I observed with injection into the ventrolateral striatum, including type B, C and A cells and olfactory bulb interneurons (Fig. 5J-L). Thus, the progenitor cells in this system are those that have the defining characteristics of striatal radial glia: cell bodies in the VZ that contact the ventricular surface and RC2+ processes that project through the striatum.

Radial glia produce adult neural stem cells

Type B, C and A cells compose the neurogenic lineage of the adult brain (Doetsch et al., 1999a; Herrera et al., 1999). Type C and A cells compose transient populations that disappear when neurogenesis is eliminated by treatment with antimitotic agents (Ahn and Joyner, 2005; Doetsch et al., 1999a; Parras et al., 2004). We observe large numbers of type A and C cells in adult (up to P150) mice after labeling radial glia in the neonatal mouse. This suggests that labeled radial glia generate the actively neurogenic adult stem cell lineage.

To directly test this inference, I injected Z/EG mice with Ad:Cre in the ventrolateral striatum at P0 and allowed them to survive for 30 days. At P30, I added 1 mg/ml of the S-phase marker 5-bromo-2'-deoxyuridine (BrdU) to their drinking water for three days, as previously described (Doetsch et al., 1999a). I then sacrificed these animals at P33 (n=8) or at P45 (n=9) and immunostained for eGFP and BrdU. In the brains of the P33 mice, I found BrdU and eGFP double-labeled astrocytes in the SVZ (Fig. 4A,B) and neuroblasts throughout the RMS (Fig. 4C). In P45 mice, most of these double-labeled cells had migrated to the olfactory bulb and matured into interneurons (Fig. 4D), but some remained in the SVZ and RMS. Thus, radial glia generate a population of cells that continue to produce neurons in the adult brain long after radial glia have disappeared. These results indicate radial glia give rise to the adult neural stem cell linage in vivo.

To determine whether these adult cells can function as stem cells in vitro, I generated neurospheres from the lateral wall of the lateral ventricle of P2, P10 and P90 Z/EG animals injected with Ad:Cre in the ventrolateral striatum at P0. A neurosphere is a floating cell mass that results from the expansion of a single cell grown in dissociated culture (Gage et al., 1995; Morshead et al., 1994; Reynolds and Weiss, 1992b; Weiss et al., 1996b). Neurospheres are thought to be formed by stem cells since they are self-renewing (neurospheres form from dissociated neurospheres) and multipotent (neurospheres have the ability to differentiate into multiple cell types) (Alvarez-Buylla and Temple, 1998; Gage, 1998; McKay, 1997; Morrison et al., 1997; Weiss et al., 1996b), though they may also be

produced by C cells (Doetsch et al., 2002). Immediately after isolation from the ventricular wall, all age groups I examined generated primary eGFP+ neurospheres (Fig. 4E) that differentiated into neurons, oligodendrocytes and astrocytes. This demonstrates that radial glia and their progeny are multipotent *in vitro*. To demonstrate self-renewal and to ensure a pure population of eGFP+ cells, I passaged primary neurospheres three or more times and then purified eGFP+ cells by FACS. I then passaged these cells at least three more times, sorted again by FACS, and grew them at clonal density (20 cells/μl) for three passages. At each passage, I ensured a single-cell suspension by visual inspection after passage through a cell strainer. After these passages, I hand picked individual neurospheres and transferred them to separate wells for differentiation. These clonally generated, radial glia-derived neurospheres from P2, P10 and P90 animals differentiated into neurons, astrocytes and oligodendrocytes (Fig. 4F-K). These experiments demonstrate that individual radial glia and their progeny in the adult brain can act as stem cells *in vitro*. Since radial glial derived cells were also shown to be actively neurogenic in the adult brain *in vivo*, this work suggests that individual radial glia and their progeny belong to a lineage of stem cells that produces neurons and glia in the postnatal brain.

Discussion

I demonstrate that radial glia in the neonatal ventricular wall produce multiple classes of brain cells: astrocytes, oligodendrocytes, ependymal cells and neurons. Importantly, I also show that these radial glia give rise to adult SVZ stem cells that maintain the neurogenic lineage in the adult brain. This conclusion is based on four separate observations. First, infected radial glia produced GFAP+ SVZ astrocytes, which have been identified as the neurogenic stem cells in the adult SVZ (Doetsch et al., 1999a; Imura et al., 2003; Laywell et al., 2000; Skogh et al., 2001). Second, after radial glial labeling, the adult RMS was full of labeled migratory neuroblasts that could only have been produced in the adult brain from neurogenic adult stem cells, since radial glia disappear from the brain soon after birth. Third, I confirmed the generation of neurogenic progeny from radial glia derived cells by double-labeling SVZ astrocytes, neuroblasts and olfactory bulb neurons with BrdU. Finally, I demonstrated that radial glia and their progeny isolated from the P2, P10 and P90 brain clonally generate self-renewing, multipotent neurospheres. I conclude that striatal radial glia give rise to SVZ astrocytes that continue to generate neurons in the adult brain. This finding extends evidence that radial glia can generate neurons (Alvarez-Buylla et al., 1990; Anthony et al., 2004; Gotz et al., 2002; Li et al., 2004a; Malatesta et al., 2000; Miyata et al., 2001; Noctor et al., 2001; Tamamaki et al., 2001) and supports the hypothesis (Alvarez-Buylla et al., 2001; Campbell and Gotz, 2002; Doetsch, 2003; Kriegstein and Gotz, 2003), that the stem cells

of the adult mammalian brain are derived from radial glia, which are ultimately derived from neuroepithelial cells (Fig. 6).

The origin of adult hippocampal progenitors

This finding confirms that NSCs of the SVZ are glial-like cells throughout development. However, it is not known if adult NSCs in other regions are derived from radial glia. The SGZ of the hippocampus, the other major germinal zone of the adult mammalian brain, contains astrocyte-like cells that generate granule neurons (Filippov et al., 2003; Fukuda et al., 2003; Seri et al., 2004; Seri et al., 2001). These astrocytes have a radial morphology and have been called “radial astrocytes” or Type I progenitors (Fukuda et al., 2003; Kronenberg et al., 2003).

The developmental origin of these SGZ radial astrocytes is unknown. To determine their origin, one could fate-map hippocampal radial glia prior to their embryonic translocation. This could be accomplished by genetically tagging cells with an inducible hippocampal specific marker, or by using ultrasound guided *in utero* viral injection or electroporation.

Several studies have claimed that while SVZ progenitors act as multipotent stem cells, hippocampal stem cells are restricted progenitors (Bull and Bartlett, 2005; Seaberg and van der Kooy, 2002) but the reason for this difference is not known. One hypothesis is that it is due to the different developmental histories of the SVZ and SGZ. SGZ progenitors become isolated from the ventricle when the hippocampus “rolls in” during development (Smart, 1961) and translocate to the DG (Eckenhoff and Rakic, 1984), whereas SVZ

progenitors remain closely apposed to the ventricle. To test this hypothesis, one could transplant embryonic hippocampal progenitors to the SVZ and vice versa to determine if continued contact with the ventricle influences the neurogenic potential or multipotency of adult NSCs.

The identity of the spinal cord progenitor

The adult mammalian spinal cord has also been shown to contain cells that act as neural progenitors *in vitro* (Kehl et al., 1997; Shihabuddin et al., 1997; Weiss et al., 1996a), but their identity is unclear. Some groups have suggested that they are ependymal cells (Johansson et al., 1999), whereas other groups have called them radial tanycytes (Mori et al., 1990; Prieto et al., 2000; Rafols and Goshgarian, 1985). The adult NSC of the mammalian spinal cord might be identified by specifically labeling and tracing the fate of neonatal spinal cord radial glia with Ad:Cre injections.

Heterogeneity vs. temporal shift in potential

The production of ependymal cells and parenchymal astrocytes in the neonatal but not in the adult brain has two possible explanations. Either adult NSCs have lost the potential to produce these cell types, or a heterogeneous population of radial glia was labeled, of which only a subpopulation generated adult stem cells. To distinguish between these two possibilities, we treated pregnant mice with BrdU showed that most ependymal cells were generated embryonically (Fig. 7A) and were directly derived from radial glia (Fig. 7B-F)

(Spassky et al., 2005). This suggests that a subset of radial glia were committed to producing ependymal cells at the time of labeling at P0. It is also likely that the parenchymal astrocytes were generated by the terminal differentiation of radial glia (Schmechel and Rakic, 1979; Voigt, 1989), which would remove them from the progenitor pool. These data strongly suggest that neonatal striatal radial glia are a heterogeneous population.

However, not all cell types and subtypes generated by radial glia and adult NSCs were examined, so it is possible that their potential changes over postnatal development. A recent study in which embryonic, neonatal, and adult SVZ cells were labeled suggested that different PGC types are made at different times in development (De Marchis et al., 2007), though this study and others do not support this conclusion (Kohwi et al., 2007), (see Chapter 5). To address this issue directly, small numbers of neonatal radial glia could be labeled with Ad:Cre, labeled with the thymidine analog 5-chloro-2'-deoxyuridine (CldU) and then labeled again in the adult with a second thymidine analog, 5-iodo-2'-deoxyuridine (IdU) (Vega and Peterson, 2005). This dual labeling strategy would allow stem cell progeny generated at distinct developmental times to be identified by IHC. The biased or exclusive production of a particular cell type at a particular time point would imply a temporal shift in progenitor potential.

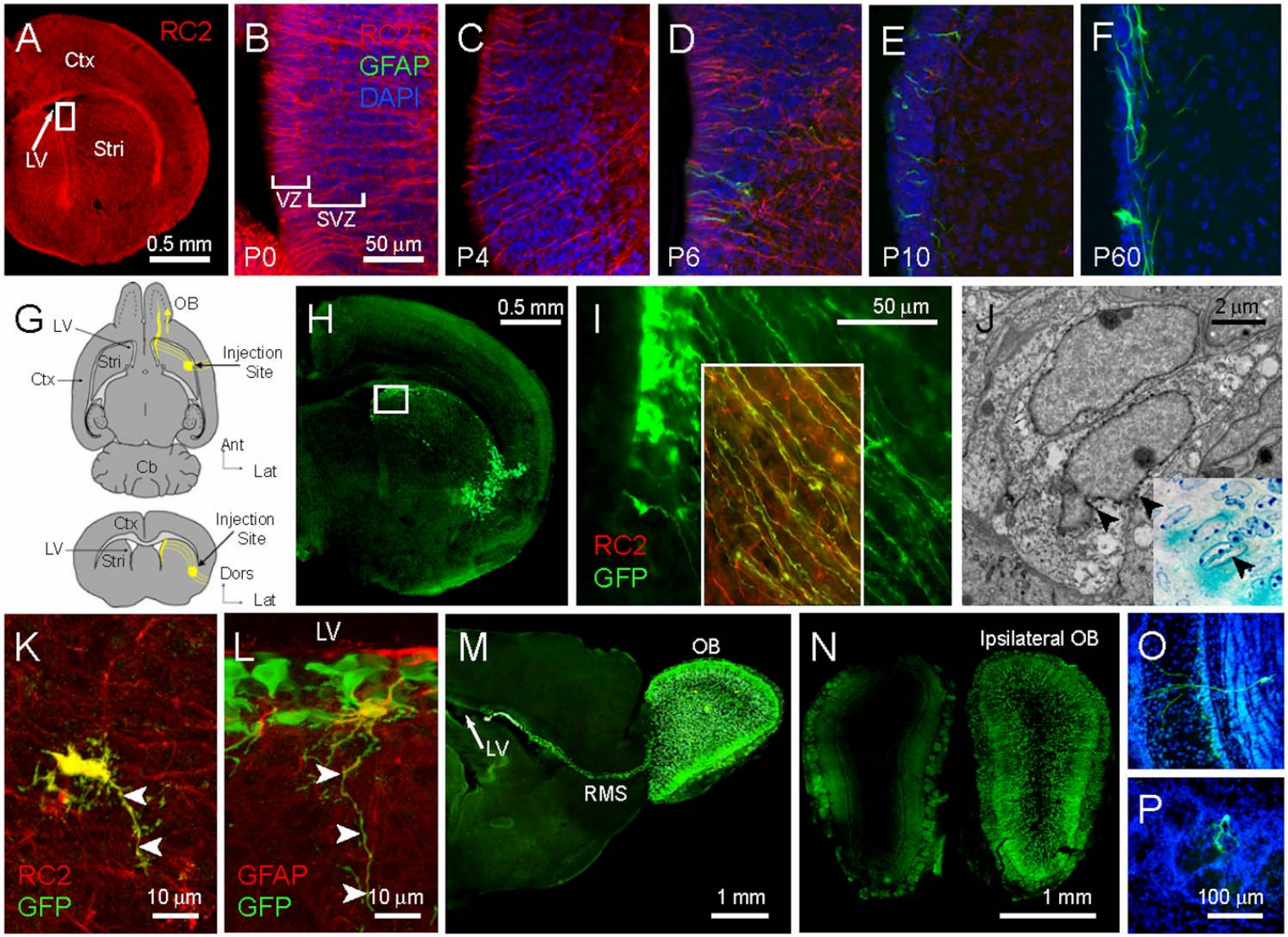
The embryonic character of adult NSCs

The transformation of radial glia into astrocyte-like cells raises the possibility that adult NSCs retain radial glial characteristics that distinguish them

from astrocytes elsewhere in the brain. Interestingly, the administration of EGF into the lateral ventricle induces SVZ cells to adopt a prominent radial morphology and express the radial glial specific marker RC2 in vivo (Gregg and Weiss, 2003). Furthermore, SVZ astrocytes were shown to display electrophysiological properties intermediate between radial glia and astrocytes (Liu et al., 2006). Another group has found that the ventral adult SVZ contains radial astrocyte-like cells (Sundholm-Peters et al., 2004), suggesting that astrocyte-like adult stem cells may physically resemble their radial glial precursors.

Preliminary work (done in collaboration with Zaman Mirzadeh) confirms and extends this finding. The adult SVZ contains radial GFAP+ processes that can be seen in lateral wall whole mounts (Fig. 8A,B). At least some of these process-bearing cells are derived from radial glia, since they are seen in the adult brain after P0 radial glial targeting (Fig. 8C, D). These cells appear to retain contact with the lateral ventricle, as has been reported for some adult NSCs (Doetsch et al., 1999a), and can be labeled by intraventricular injections of Ad:GFAP-Cre (Fig. 8E-G) an adenovirus expressing Cre recombinase under the murine GFAP promoter (see Chapter 2, Section 3, “Generation of Ad:GFAP-Cre virus”, and Chapter 5). After 28 days, many neurons can be seen in the olfactory bulb contralateral to the injected ventricle, suggesting that these radial astrocytes are neurogenic (Fig. 8H). These preliminary findings raise the possibility that the adult germinal zone is maintained by ventricle-contacting stem cells that establish a modified VZ in the adult brain. Thus, the organization of the

mammalian SVZ may resemble the neurogenic zones of species that retain an adult VZ formed by radial glial-like neural stem cells (reviewed in (Doetsch, 2003; Garcia-Verdugo et al., 2002)). Together, these findings support the conclusion that over development, the NSC lineage progresses from neuroepithelial cell -> radial glial cell -> specialized radial glial cell/astrocyte-like cell.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100 µm

Figure 1. Radial glia are specifically labeled in the neonatal mouse brain.

(**A**) RC2+ radial glial processes extend from the VZ to the brain surface. (**B-F**) In the region boxed in (A), the disappearance of RC2+ radial glia correlates with the appearance of GFAP+ SVZ astrocytes. (**G**) Radial glial processes can be infected by an adenovirus injected into the ventrolateral striatum. (**H, I**) This results in labeling of cells at the injection site and in the dorsolateral VZ. VZ cells (boxed region in H) have long radial processes (I) that express RC2 (boxed region in I), and extend from the VZ through the injection site. (**J**) These cells have the ultrastructure of radial glia. Insert shows X-gal precipitate (arrowheads) in the same cells (from P2 R26R mice) at the light microscope. (**K, L**) In the early postnatal brain, radial glia retract their processes (arrowheads) as they transform into astrocytes in the striatum (K) and in the SVZ (L). (**M-P**) Their progeny, however, continue to generate large numbers of neuroblasts in the P90 brain (M) that migrate along the RMS to the olfactory bulb (N) and differentiate into and granular (O) and periglomerular cells (P). Cb, cerebellum; Ctx, cortex; LV, lateral ventricle; RMS, rostral migratory stream; Stri, striatum; SVZ, subventricular zone; VZ, ventricular zone.

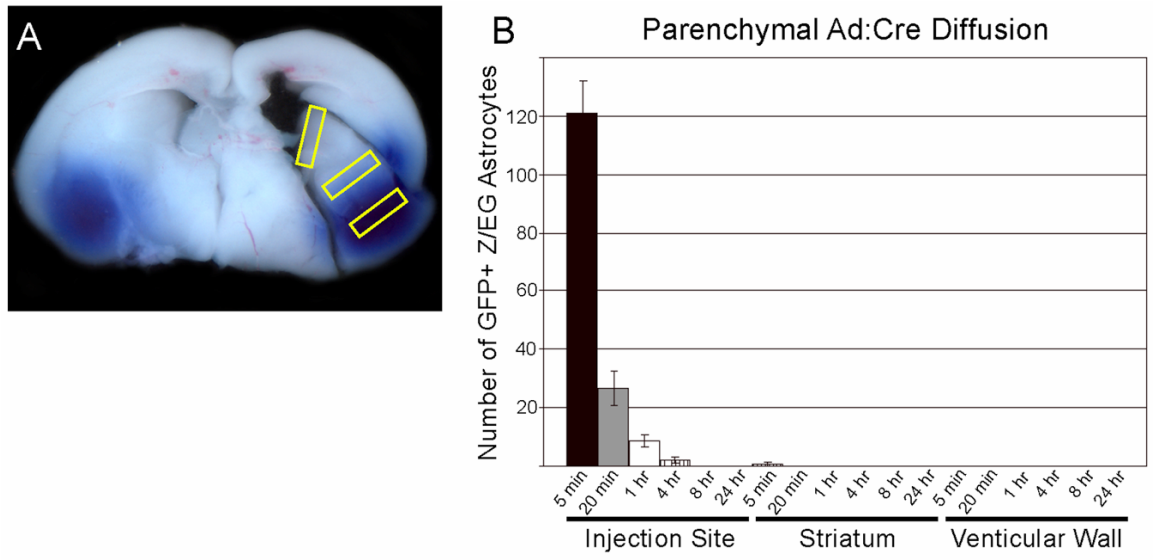


Figure 2. Adenovirus does not diffuse outside of the injection site and infects cells within 8 hours.

(**A**) Co-injection of Ad:Cre with bromophenol blue allowed the injection site to be visualized. Explants of the injection site, as well as the ipsilateral ventricular wall and the striatum were microdissected (boxes in A) and placed on monolayers of Z/EG astrocytes to assay for infectious particles. Infectious particles diffused out of the injection site explant and induced eGFP expression in monolayer astrocytes. **(B)** Virus never diffused to the ventricular wall and after 4 hours, no infectious particles were detectable in any region tested.

A
Doublecortin
10 µm
B
E
A
2 µm
G
Olig2
10 µm
H
Olig
4 µm
C
NeuN
10 µm
D
C
2 µm
I
GFAP
10 µm
J
B
2 µm
E
CD24
10 µm
F
2 µm
K
1 mm
L
RMS
GCL
200 µm

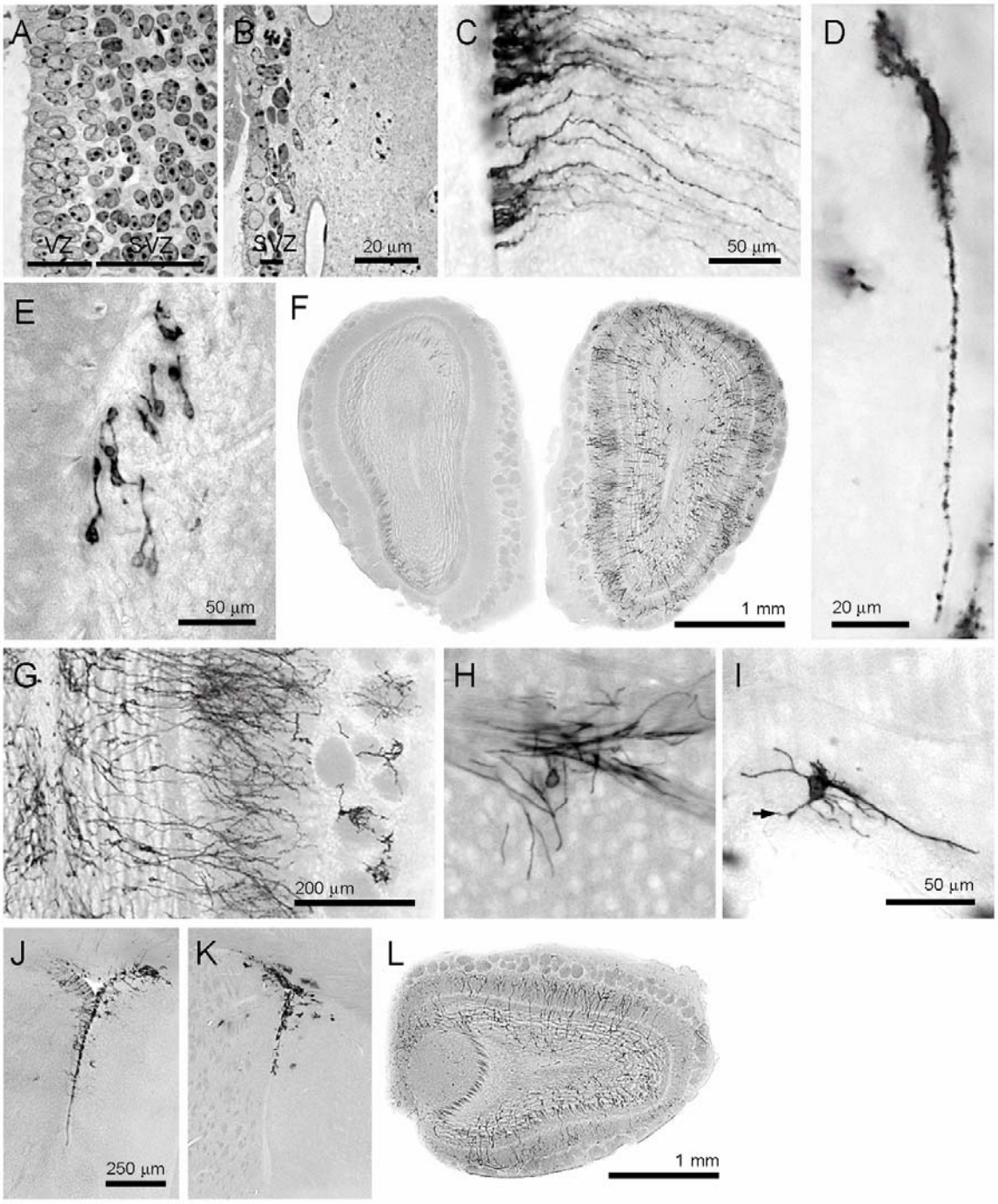
Figure 3. Radial glia produce all classes of brain cells.

Radial glial progeny were identified at the confocal microscope by eGFP expression in Z/EG mice and at the electron microscope by dark, perinuclear grains of X-gal precipitate in R26R mice (black arrowheads). (**A-C, E-H**) The progeny include doublecortin+ neuroblasts (Type A cells) (A, B), NeuN+ neurons (C), ciliated (arrows) CD24+ ependymal cells (E, F) and Olig2+ oligodendrocytes whose processes (arrows) intercalate between myelinated axons (G, H). (**D, I, J**) Radial glia also produce GFAP+ SVZ astrocytes or type B cells (I, J, intermediate filaments shown in insert) and neurogenic transit amplifying type C cells (D). (**K**) A whole mount of the lateral ventricular wall reveals the injection site deep in the tissue at a deeper focal plane (yellow arrowheads) and a well-defined patch of labeled radial glial cell bodies (arrow). (**L**) When grafted into a wild-type mouse, radial glial cell bodies (but not cell bodies from the injection site) produce all cell types obtained by viral injection alone, including neuroblasts (arrow) and olfactory bulb neurons (yellow arrowhead) in the adult brain. RMS = rostral migratory stream, GCL = granule cell layer.

A
BrdU
GFP
GFAP
5 µm
B
C
50 µm
D
100 µm
E
100 µm
F
GFP
200 µm
G
Tuj1
H
GFAP
O4
I
Tuj1
10 µm
J
GFAP
K
O4

Figure 4. Radial glia-derived adult stem cells remain actively neurogenic in vivo.

(**A, B**) Radial glia-derived (eGFP+) GFAP+ astrocytes in the adult (P33) SVZ are labeled by the S-phase marker BrdU (A) (GFAP eGFP double labeling shown at higher magnification in B). (**C**) Radial glial labeling also resulted in many double-labeled migratory neuroblasts. (**D**) These adult-born neuroblasts migrate to the olfactory bulb and differentiate into interneurons. (**E**) Radial glia and their adult progeny are self-renewing and multipotent in vitro. eGFP+ cells isolated from the ventricular wall of P2, P10 and P90 Z/EG mice injected with Ad:Cre in the ventrolateral striatum at P0 clonally produce primary neurospheres. (**F-H**) These eGFP+ neurospheres were sorted by FACS and grown again at clonal density for several passages to ensure clonality and self-renewal. All cells in clonally grown neurospheres are eGFP+ (F) and differentiate into Tuj1+ neurons (visualized by DAB, dark brown spots in G), GFAP+ astrocytes and O4+ oligodendrocytes (H). (**I-K**) (I) shows a higher magnification of a Tuj1+ neuron (box in G) and (J) and (K) show GFAP+ processes of astrocytes and an O4+ oligodendrocyte, respectively (box in H). The differentiated neurosphere shown in (F-K) was isolated from a P90 mouse, but similar results were obtained from all ages examined, demonstrating that both radial glia and their progeny are multipotent, self-renewing stem cells. F-H and I-K share the same scale bar.


A
VZ
SVZ
B
SVZ
20 µm
C
50 µm
D
20 µm
E
50 µm
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1 mm
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200 µm
H
I
50 µm
J
250 µm
K
L
1 mm

Figure 5. Development of the SVZ and morphology of NSCs and their progeny.

(**A**) The P0 VZ is several cell bodies thick and consists mostly of radial glia cell bodies, while the SVZ is about twice as thick and consists mainly of radial glia and neuroblasts. (**B**) The mature VZ is a monolayer of ependymal cells and the SVZ is only 2-3 cells thick, consisting of astrocytes, neuroblasts and type C cells. (**C, D**) Ad:Cre injection into the ventrolateral striatum of Z/AP mice results in Golgi-like alkaline phosphatase staining that reveals the morphology of radial glia at P2 (C), some of which retract their cell bodies into the striatum (D) and differentiate into astrocytes. (**E-G**) At P30, there is continued production of migratory neuroblasts (E) that differentiate into granular and periglomerular olfactory bulb interneurons on the injected side (F), shown enlarged in (G). (**H, I**) Initially labeled radial glia also give rise to oligodendrocytes (H) and SVZ astrocytes (I) that occasionally contact the ventricle (arrow). Radial glia are also labeled when Ad:Cre is injected into the lateral ventricle. (**J, K**) The staining pattern at P2 reveals cells with a radial morphology (J), but these processes are less visible by P30 (K). (**L**) At P30, the olfactory bulb of these injected animals is filled with neuroblasts and granular and periglomerular interneurons. Panels H and I, and J and K share the same scale bar.

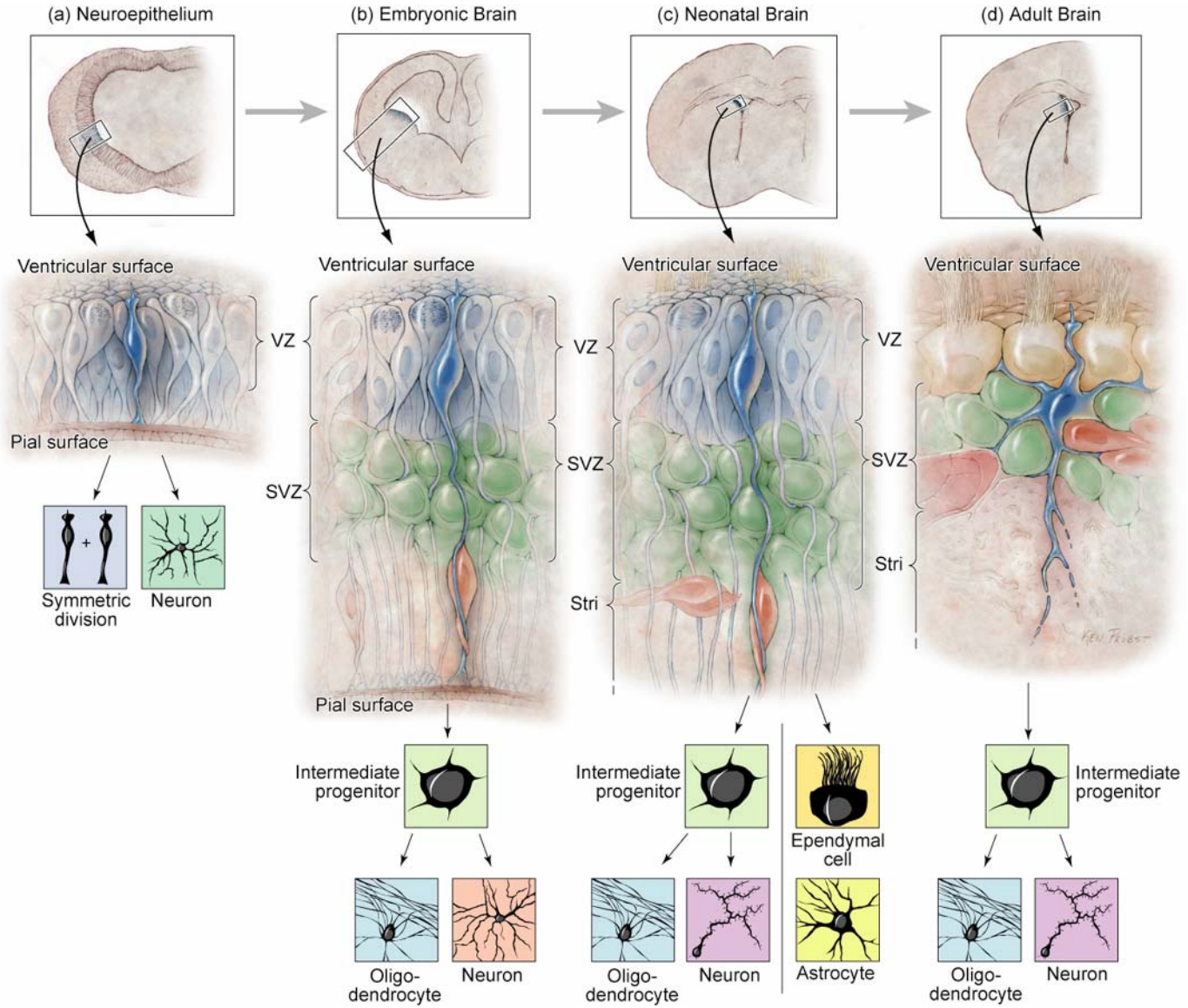

(a) Neuroepithelium
(b) Embryonic Brain
(c) Neonatal Brain
(d) Adult Brain
Ventricular surface
Ventricular surface
Ventricular surface
Ventricular surface
VZ
SVZ
Pial surface
VZ
SVZ
Stri
Pial surface
VZ
SVZ
Stri
Symmetric division
Neuron
Intermediate progenitor
Intermediate progenitor
Ependymal cell
Intermediate progenitor
Oligo-dendrocyte
Neuron
Oligo-dendrocyte
Neuron
Astrocyte
Oligo-dendrocyte
Neuron

Figure 6. Neural stem cells and their progeny in the developing forebrain.

The NSCs (shown in blue) of the lateral ventricular wall change their shape and produce different progeny as the brain develops. They begin as NECs and transform into radial glial cells, which mature into astrocytes. NSCs maintain contact with the ventricle, into which they project a cilium. The potential of an individual stem cell *in vivo* is not known and the progeny shown in this schematic are produced by the NSC population. Stem cells produce progeny either directly or via an intermediate progenitor (shown in green), which has been either included or omitted for clarity. Different types of progeny may be produced by different intermediate progenitors, though just one is shown here. **(A)** At early developmental stages the CNS is a tubular structure. It is made up of NECs, which divide symmetrically at the ventricular surface to expand the stem cell pool. At this time, some early-born neurons such as Cajal-Retzius cells are produced. **(B)** NECs cells most likely differentiate into embryonic radial glia, which divide to generate striatal neurons and oligodendrocytes either directly or via an intermediate progenitor in the SVZ. The radial processes of radial glia support the migration of neuroblasts (shown in red). **(C)** Radial glia persist in the neonatal brain, where they generate oligodendrocytes and olfactory bulb interneurons, and ependymal cells. They also generate astrocytes, some of which remain stem cells in the adult. **(D)** In the adult brain, neurogenic astrocytes often retain a radial process and contact both the ventricle and the basal lamina of blood vessels. They generate oligodendrocytes and olfactory bulb interneurons. Abbreviations: NEC = neuroepithelial cell; NSC = neural stem cell; RGC = radial glial cell; Stri. = striatum; SVZ = subventricular zone; VZ = ventricular zone.

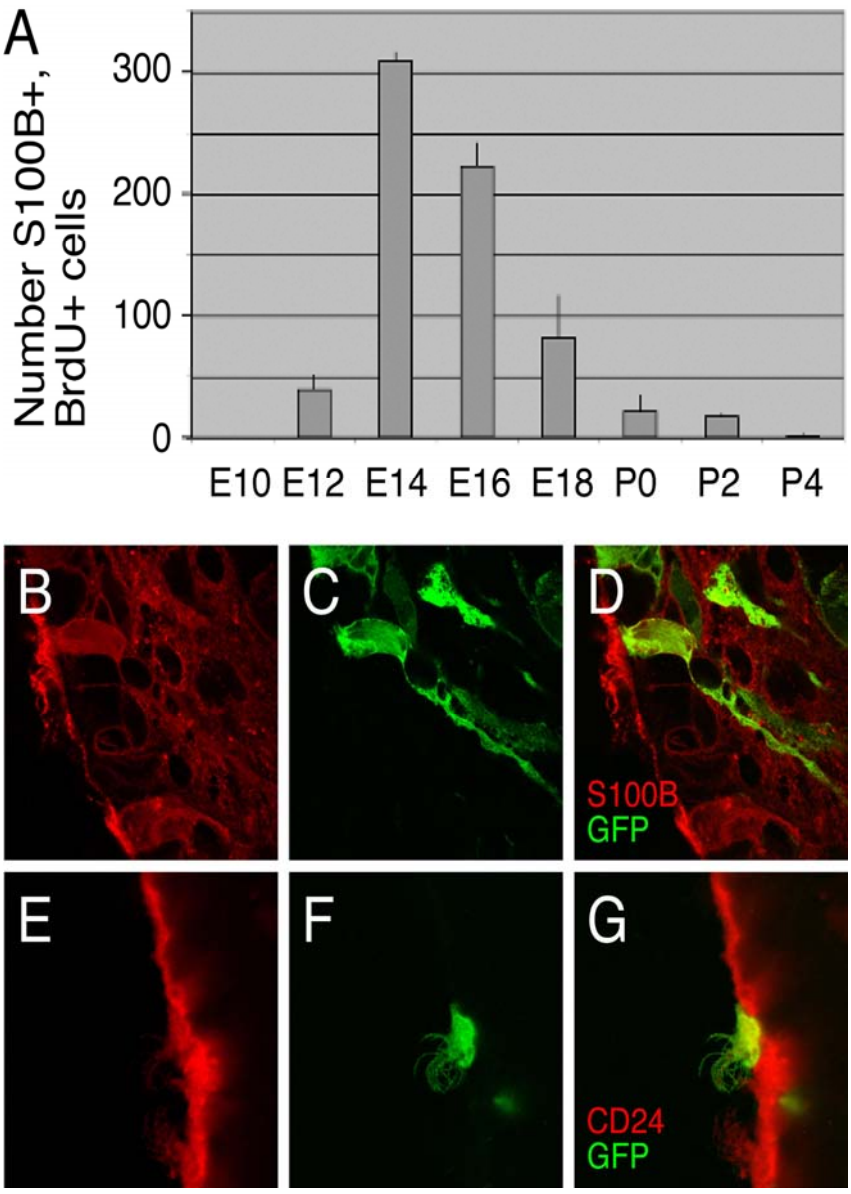


Figure 7. Ependymal cells are postmitotic in the adult and are derived from radial glia. **(A)** BrdU birthdating shows that most ependymal cells are born in the embryo. **(B-D)** At early postnatal ages, radial glia labeled at P0 show both a radial process and the cuboidal morphology and S100B gene expression characteristic of adult ependymal cells. **(E-G)** In the adult brain, some radial glia targeted at P0 have transformed into mature, CD24+ multiciliated ependymal cells.

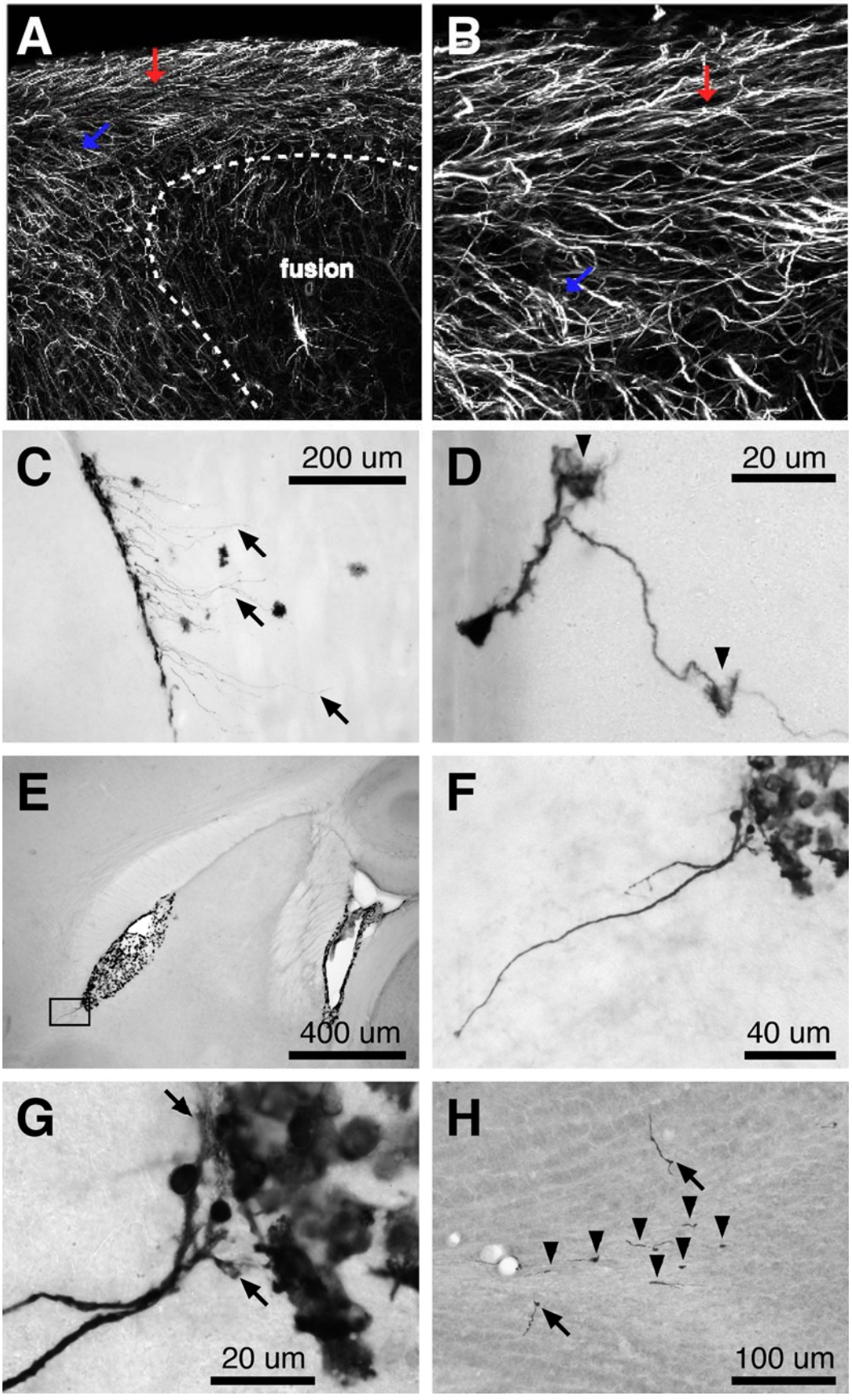

A
fusion
B
C
200 um
D
20 um
E
400 um
F
40 um
G
20 um
H
100 um

Figure 8. Neurogenic, ventricle-contacting astrocytes with radial morphology persist in the adult mouse brain.

(A, B) Confocal photomicrographs of whole-mount staining for GFAP reveals prominent GFAP+ processes that are aligned and project from the ventricular surface into the parenchyma at an angle. Cells indicated by blue and red arrows in (A) are shown at higher magnification in (B). **(C, D)** Targeting of neonatal radial glia generated cells in the adult brain that retain long radial processes (arrows in C) that often make specialized contacts with blood vessels (arrowheads in D). **(E-G)** Injection of Ad:GFAP-Cre into the lateral ventricle of Z/EG mice labels ependymal cells (E), but also radial astrocyte-like cells (boxed region in E shown at higher magnification in F). These cells appear to contact the ventricle and extend processes in the SVZ (arrows in G). **(H)** Four weeks after lateral ventricular injection, neuroblasts (arrowheads) and immature neurons (arrows) can be seen in the olfactory bulb contralateral to the injection site, indicating that ventricle-contacting cells are neurogenic. Panels (C-H) show photomicrographs of cells visualized by immunoperioxidase immunochemistry for GFP.

Chapter 4

The extent of the SVZ and the diversity of adult neural stem cells

Overview

Neural stem cells are known to reside in the SVZ of the lateral wall of the lateral ventricle and generate at least six different subtypes of olfactory bulb interneurons. The specific origin of these interneuron types within the SVZ has remained obscure. To address this question, I improved the targeting technique described in Chapter 3 and systematically labeled spatially restricted populations of radial glia in neurogenic regions of the neonatal brain. I show that regions of cortex and septum adjacent to the lateral wall of the lateral ventricle also contain long-lived progenitors that produce olfactory bulb interneurons in the postnatal brain. I also show that each targeted cortical, septal or SVZ region contains progenitors that generate different subtypes of olfactory bulb interneurons. These findings suggest that neural stem cells are restricted in their potential to generate multiple interneuron types and populate different domains of an extensive periventricular germinal zone.

Introduction

Since the discovery of the SVZ, it has been unclear why OB neurons are generated in such an extensive germinal zone. Initially, it was though that neurogenesis was restricted to the anterior-most portion of the SVZ (Lois and Alvarez-Buylla, 1994; Luskin, 1993), but later work showed that neurons were born throughout the lateral ventricular wall (Doetsch and Alvarez-Buylla, 1996). Newly-born neurons (neuroblasts) therefore have to migrate over long distances; it takes approximately 2-6 days for a neuroblast to reach to the OB after being born in the SVZ (Lois and Alvarez-Buylla, 1994; Petreanu and Alvarez-Buylla, 2002). Long-distance migration is not a feature of other adult neurogenic regions, including the hippocampus (Gage et al., 1998; Seri et al., 2004), olfactory epithelium (Calof et al., 1996; Morrison and Costanzo, 1992), and possibly the hypothalamus (Kokoeva et al., 2005) and retina (Bernardos et al., 2007; Fischer and Reh, 2001). It is unknown why OB interneurons generated so far away from their destination rather than simply being generated in the OB.

Neuroblasts integrate and mature into several distinct cell types upon reaching the olfactory bulb (Alvarez-Buylla and Garcia-Verdugo, 2002; Hack et al., 2005; Kohwi et al., 2005). The two principal types of adult-born olfactory neurons - periglomerular cells and granule cells - are interneurons that modulate the activity of neurons that project to olfactory cortex. Periglomerular cells can be subdivided into three non-overlapping populations of cells: calretinin- (CalR+) and calbindin-expressing (CalB+) cells, and tyrosine hydroxylase-expressing (TH+) dopaminergic cells (Kosaka et al., 1995). Granule cells include deep,

superficial, and CalR+ cells (Fig. 4A) (Jacobowitz and Winsky, 1991; Price and Powell, 1970). These subtypes are thought to be distinct functional elements of the olfactory bulb circuitry (Kosaka et al., 1995; Price and Powell, 1970; Shepherd and Greer, 1998). For a more detailed description of different OB interneuron types, see Chapter 1, Section 3, “Diversity among OB neurons”.

It is not known where in the SVZ these different cell types are generated. Interestingly, the regional origin of CalR+ cells, which constitute roughly 20% of OB interneurons (Jacobowitz and Winsky, 1991; Kosaka et al., 1998), has not been determined. A recent study has suggested that the transcription factor Sp8 controls CalR+ cell identity, since about half of these cells are absent in mice lacking Sp8 in Dlx5/6+ regions (Waclaw et al., 2006). During development, Sp8 is expressed not only in the lateral wall, but also in the RMS, medial wall, and cortical (dorsal) wall of the lateral ventricle, so CalR+ cells may be generated outside of the lateral wall.

It is commonly assumed that multipotent NSCs are able to produce each of these OB neuron types (Fig. 1A). However, recent evidence suggests that neural stem cells may be more restricted than was previously thought (see Chapter 1, Section 2, “Plasticity vs. commitment”), and that neuroblasts are specified to a particular fate before reaching the olfactory bulb (Hack et al., 2005; Kohwi et al., 2005; Saino-Saito et al., 2004). I therefore hypothesized that stem cells are not equivalent and that they may specify the fate of the neurons they produce (Fig. 1B). To test this hypothesis, I labeled stem cells in different regions and followed their progeny *in vivo*.

Results

I have previously shown (see Chapter 3, Results) that adult neural stem cells are derived from radial glia present in the neonatal (P0) mouse brain (Merkle et al., 2004). Radial glia, which are now recognized as the principal stem cell of the embryonic and early postnatal mouse brain (Anthony et al., 2004; Noctor et al., 2002), have a unique morphology that allows them to be targeted specifically. Their cell bodies line the ventricles and they send a long, radial process to the brain surface. Adenoviruses readily infect these processes and are transported to the cell body. When an adenovirus expressing Cre recombinase (Ad:Cre) is injected into GFP reporter (Z/EG) mice (Novak et al., 2000), infected radial glia and their progeny become permanently labeled with GFP (Merkle et al., 2004). Since adenoviruses do not diffuse readily in the brain, the localized injection of a small volume (20 nl) of Ad:Cre labels a spatially restricted patch of neural stem cells whose processes passed through the injection site.

Regional targeting of neonatal radial glia

To label radial glia in a regionally specific manner, I developed a method to stereotaxically inject Ad:Cre in neonatal (P0) mice (Fig. 1E). Rather than injecting radial glia at a trajectory that passed through a large area of parenchyma, injections were performed at a trajectory parallel to radial glial processes (see Chapter 2, Section 6 “Stereotaxic injection of neonatal pups”). Injections resulted in the reproducible labeling of spatially segregated patches of

radial glia (Fig. 1C, D) and the adult SVZ stem cells they generate (Fig. 1F, G). In contrast, cells labeled locally at the injection site do not give rise to olfactory bulb neurons or neural stem cells (Merkle et al., 2004). By systematically varying the injection location (Fig. 1H), I targeted 15 different populations of radial glial cells at six different rostrocaudal levels (i-vi). I targeted the dorsal (D) or ventral (V) lateral wall of the lateral ventricle at four different rostrocaudal levels (ii-v) and the dorsoventral extent of the lateral wall at level vi. I also targeted the RMS (i), the medial (septal) wall (iiM), and the cortical wall of the lateral ventricle (iiC-vC) since these regions had been suggested to contain neural progenitors (Doetsch et al., 1999a; Morshead et al., 1994; Seri et al., 2006; Ventura and Goldman, 2007).

When I analyzed the brains of mice four weeks after Ad:Cre injection, I observed a patch of labeled cells in the same anatomical location as the targeted radial glial cell bodies, indicating that neural stem cells do not disperse tangentially (Fig. 2). Figure 1F and G show examples of SVZ labeling after neonatal targeting of dorsal and ventral SVZ radial glia, respectively.

Production of OB interneurons from labeled radial glia.

After labeling different neurogenic subregions in the neonatal mouse, I waited 28 days and examined the mature GFP-labeled neurons in the olfactory bulb. Olfactory bulb interneurons were produced from all labeled regions including regions i, iiM and iiC-vC, which extend beyond the lateral wall of the lateral ventricle, the accepted boundary of the neurogenic adult SVZ.

Remarkably, each region only gave rise to a very specific subset of interneuron subtypes. Anterior and dorsal regions produced periglomerular cells (Fig. 3A-D; 4B), though the highest percentage was produced in region iiM (Fig. 3A, B; 4B). Periglomerular cell subtypes were also produced in a region-specific manner. Dorsal regions (iiC-vC) produced the highest percentage of TH+ cells (Fig. 4C, 5A-C) whereas CalB+ cells were produced mainly ventrally, in regions iiV and iiiV (Fig. 4D, 5D-F). CalR+ periglomerular cells were less frequently observed when these regions were targeted, but were frequently labeled with targeting of regions i and iiM (Fig. 4E, 5G-I). Each targeted region produced granule cells (Fig. 4F), though region iiM produced relatively few (Fig. 3A, B; 4B). Dorsal regions (iiC-vC, iiD, iiiD) tended to produce superficial granule cells while ventral regions (iiV-vV) produced mostly deep granule cells (Fig. 3E-H; 4F). Interestingly, CalR+ granule cells were produced mostly from the anterior regions (i and iiM) that also produced many CalR+ periglomerular cells (Fig. 4G, 5J-L). A recent study suggested that CalR+ OB interneurons are derived Sp8+ cells in the dorsal LGE (Waclaw et al., 2006). However, grafted LGE cells do not produce CalR+ cells (Kohwi et al., 2007) suggesting they are produced by Sp8+ cells in regions i and iiM (Waclaw et al., 2006) rather than the dorsal LGE.

The data presented demonstrate that SVZ neural stem are restricted in the types of neurons they generate in vivo. I conclude that postnatal neural stem cells are diverse and are organized in an intricate mosaic in an extensive postnatal germinal zone (Fig. 6).

Discussion

Spatial extent of adult neurogenesis

The continued presence of neuroblasts in each of the targeted regions (Fig. 3B, D, F, H) suggests that radial glia transform into long-lived neurogenic progenitors not only in the periventricular striatum (Merkle et al., 2004), but also in the RMS and periventricular cortex and septum. It remains to be seen if these regions contain multipotent stem cells or merely neurogenic progenitors. However, it is clear that postnatal neurogenesis occurs in an extensive zone that includes the anterior medial wall and a region of cortex that appears to span the rostrocaudal axis of the lateral ventricle. This finding is consistent with several recent studies suggesting that cortical (Kohwi et al., 2007; Ventura and Goldman, 2007; Willaime-Morawek et al., 2006) and septal (Inoue et al., 2007; Kohwi et al., 2007) progenitors contribute to olfactory bulb neurogenesis.

I found that different regions produced different OB neuron types, but that the borders between these regions, defined by cell type production, could be sharp or diffuse. For example, CalB+ PGCs (Fig. 4D) and CalR+ PGCs (Fig. 4E) were almost exclusively produced in the ventral and anterior SVZ, respectively, suggesting that these domains are well-defined. On the other hand, TH+ PGCs (Fig 4C) and superficial GCs (Fig 4F) are produced in a dorsoventral gradient that extends from cortical regions down into the lateral ventricular wall. The dorsal domain might therefore have a “fuzzier” border. However, no domain existed in isolation; at domain boundaries I obtained mixed cell populations (Fig. 4B-G. This implies a functional continuity of progenitors in a single, anatomically

continuous adult neurogenic zone (Fig. 7B).

The boundaries of this zone have not been thoroughly established. I did not observe OB neuroblasts when targeting the medial cortex or the septum posterior to region iiM (data not shown). The extent of the neurogenic zone could be more solidly established by targeting small patches of radial glia close to the supposed boundary between neurogenic and non-neurogenic regions. Additionally, the expression of a combination of markers could help define the boundaries. BrdU-incorporating and Dcx+ cells were found in all regions that produced neuroblasts after targeting, but not in non-neurogenic regions. This suggests that the borders of the neurogenic niche could be identified histologically and later confirmed by specific targeting of progenitors and lineage tracing. It has also been shown that the SVZ and SGZ receive both serotonergic and dopaminergic projections, and that the overlap of these projections appears to be unique to neurogenic regions (Hagg, 2005). Thus, overlapping neurotransmitter patterns may help define the extent of the neurogenic zone.

It is not known why some regions of the brain retain NSCs while they disappear from other regions during development. It would be interesting to determine the developmental history of the entire neurogenic zone by adapting methods that have been applied to the neonatal SVZ. This could be accomplished by fate-mapping radial glia labeled in specific regions of the embryo by viral injection or by electroporation. This approach would shed light on the developmental histories of neural progenitors in different regions, which would inform future work to elucidate the mechanisms involved in maintaining

NSCs in the adult brain. For example, cells at either side of the border of the neurogenic zone could be compared by gene chip analysis or by immunostaining for candidate genes thought to be relevant for stem cell maintenance, such as Notch1 (Gaiano and Fishell, 2002; Hitoshi et al., 2002) or Shh (Machold et al., 2003).

Diversity of neural progenitors and the control of adult neurogenesis

The generation of different cell types in different regions of the neurogenic niche suggests that neural progenitors are diverse. This has important implications both for the organization of adult neurogenesis and the nature of adult NSCs. First, it clarifies why neurons are born in such a large germinal zone and migrate over such long distances to reach the OB: many different regions contribute to the diversity of OB interneurons. This may provide a mechanism for the brain to fine-tune the OB circuitry by dynamically stimulating or inhibiting neurogenesis in different regions to regulate interneuron production. Progenitors in different regions could respond to neurotransmitters or through specific signaling pathways. For example, serotonergic projections are topographically organized (Molliver, 1987) and cells with active Gli1, a downstream target of Shh, may be concentrated in the ventral SVZ ((Ahn and Joyner, 2005) and unpublished observations).

This discovery also raises the possibility that changes in the size of a particular neurogenic domain or in the activity of its progenitors might influence olfactory discrimination. Since olfaction is a critically important sense for many

animals, these changes would be under selective pressure and might influence the evolution of the brain regions in which adult NSCs reside. It will be interesting to determine if the adult human brain, which has been shown to contain cells that can act as neural progenitors *in vitro* (Sanai et al., 2004), also contains a germinal zone that is divided into different domains. If this is the case, it might be possible to harvest progenitors of a desired cell type from a particular region of the neurogenic zone, expand their numbers in vitro to obtain a particular neuron type, and transplant them back into patients.

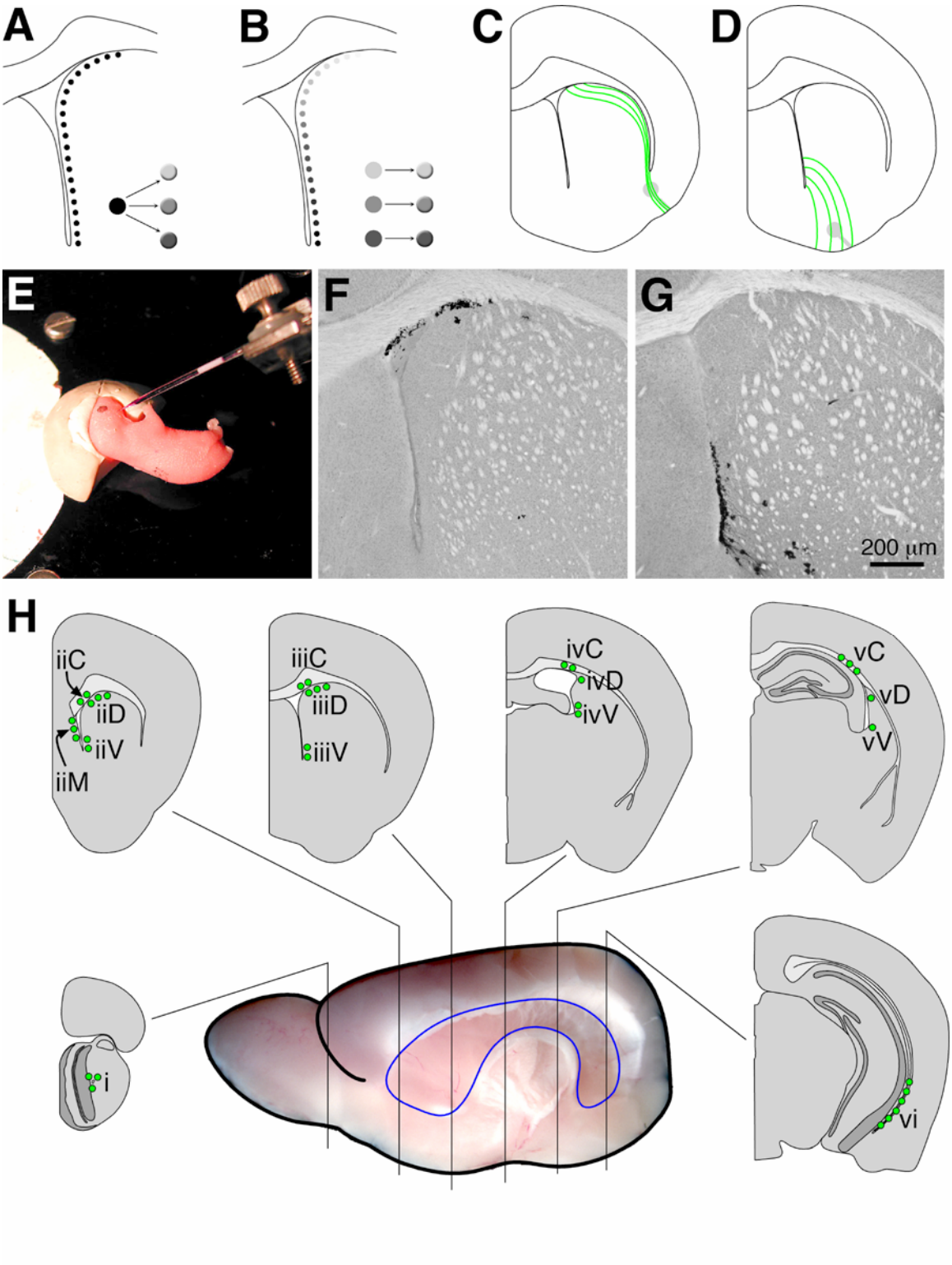
The function of adult SVZ neurogenesis

The regionalized production of different OB interneuron types provides a unique opportunity to investigate the function of adult neurogenesis. By targeting progenitors in a certain region, either genetically or with a virus, one could genetically modify a particular subset of OB interneurons. It is now possible to reversibly activate or inactivate targeted elements of brain circuitry with light (Arenkiel et al., 2007; Chambers et al., 2006; Gorostiza et al., 2007; Kramer et al., 2005; Szobota et al., 2007). The effect of activating or silencing these cells on the OB circuit activity could be studied electrophysiologically *in vivo* or in slice cultures in the presence or absence of light. If an effect is seen, the behavioral consequences such as olfactory discrimination or olfactory conditioning could be studied *in vivo*. These studies might clarify the relative contributions of each of these cells to the OB circuitry and might shed light on why so many different cell types are replaced throughout adulthood. Perhaps many different elements of the

circuit must be modified in parallel to achieve the desired functional result. This model could have implications for cell-based therapies for brain repair; transplantation of a combination of different cell types, rather than a single cell type, might be required to functionally repair damaged circuits.

The adult NSC: a restricted progenitor?

Finally, the apparent restriction of different progenitors to a small subset of different OB interneuron types changes our perception of adult NSCs. Whereas they are commonly thought to be homogeneous and plastic, the data presented above suggest that they are committed to producing only few different cell types. This finding resonates with the growing body of evidence that NSCs are heterogeneous and become increasingly restricted over the course of development (see Chapter 1, also reviewed in (Pearson and Doe, 2004; Temple, 2001)). Though it is not yet known if NSC potential is intrinsic or dictated by the environment (see Chapter 5), it is clear that *in vivo* they exist as a functionally diverse and topographically organized population of cells.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vC
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i
vi

Figure 1. Specific regional targeting of neural stem cells.

(**A** and **B**) In the traditional model of SVZ stem cell potential (**A**), equivalent stem cells (black dots) generate multiple neuron types, which are produced by a diverse stem cell population in the proposed model (**B**). (**C** and **D**) Diagram of a neonatal mouse brain showing targeting of dorsal (**C**) or ventral (**D**) radial glia (green) with virus deposited at the injection site and along the needle tract (gray circle and bar). (**E**) Stereotaxic setup showing an acrylic model of a neonatal pup positioned in a customized head mold for viral injection parallel to radial glial processes. This allows specific labeling of small groups of radial glia without contaminating other areas by virus deposited along the needle tact. (**F** and **G**) Photomicrograph of a P28 Z/EG brain injected at P0 to target dorsal (**F**) or ventral (**G**) radial glia, visualized with immunoperoxidase staining for GFP. (**H**) Diagram of brain regions targeted. Representative frontal sections of the right hemisphere traced from the adult brain are shown relative to a photomicrograph of an adult lateral ventricular wall whole mount outlined in blue (Doetsch and Alvarez-Buylla, 1996). Targeted regions, indicated by green dots, are named for their anterior-posterior level (i-vi) followed by the location within that level, where C is cortical, M is medial, D is dorsal and V is ventral.

A
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ivC
J
ivD
K
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L
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M
vD
N
vV
O
vi

Figure 2. Region-specific labeling of radial glia derived cells.

(**A-O**) Photomicrographs of P28 Z/EG brains showing labeled cells visualized by immunoperoxidase for GFP. Cells were labeled by stereotaxic injection of Ad:Cre at a trajectory parallel to radial glial processes, resulting in cells labeled locally at the injection site (arrows) and radial glial cells in neurogenic zones distant from the injection. Some progeny of labeled radial glia remain in germinal zones (arrowheads), where they continue to produce olfactory bulb interneurons . In (**O**), the injection site is in a different plane.

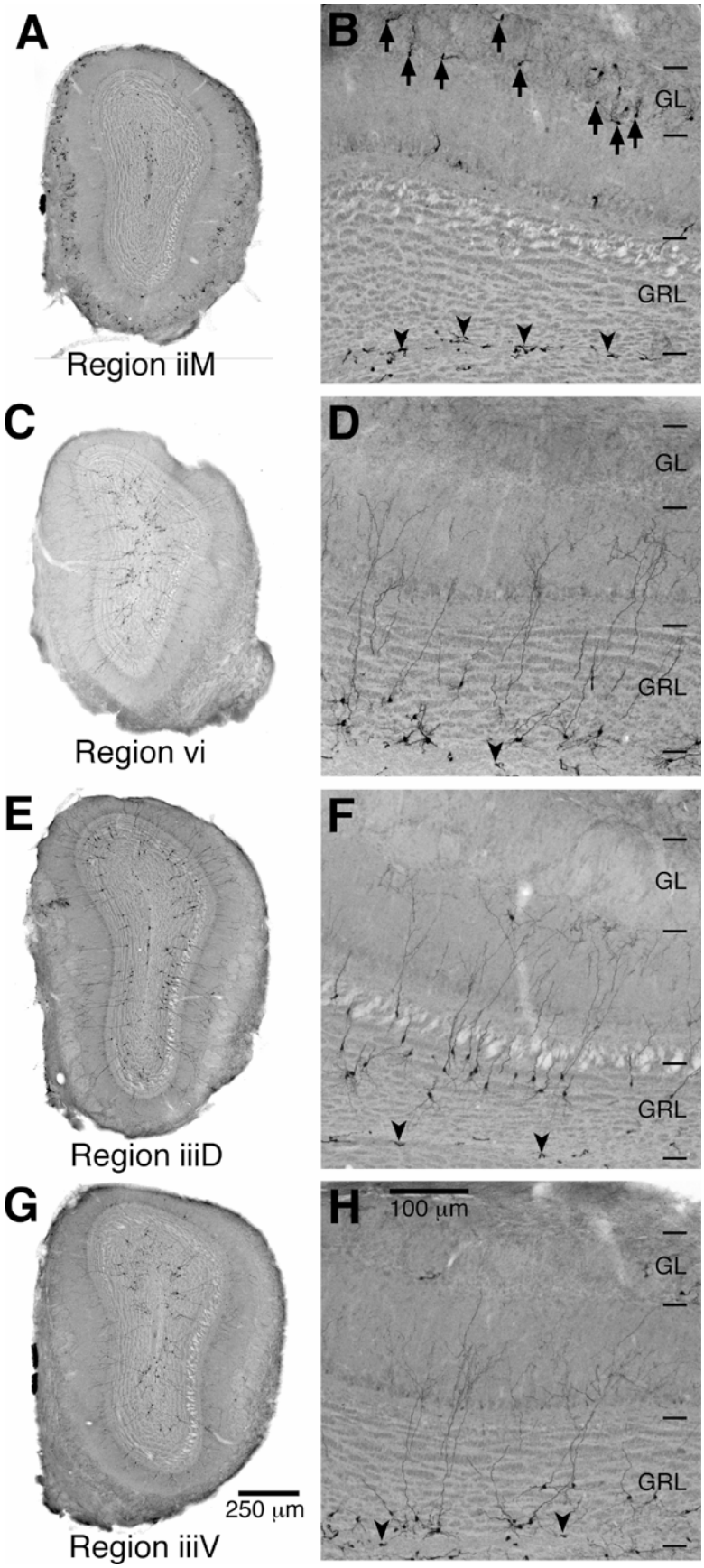
A
Region iiM
B
GL
GRL
C
Region vi
D
GL
GRL
E
Region iiiD
F
GL
GRL
G
250 μm
Region iiiV
H
100 μm
GL
GRL

Figure 3. Production of olfactory bulb neurons from different neurogenic subregions.

All images are photomicrographs of olfactory bulb frontal sections from P28 Z/EG mice injected with Ad:Cre at P0, visualized by immunoperoxidase staining for GFP. (**A** and **B**) When region iiM is targeted, low magnification reveals that most olfactory bulb cells are found in the glomerular layer (GL) (A), and almost all cells with mature cells have periglomerular cell morphology at higher magnification (B). (**C** and **D**) Only granule cells are produced when region vi is targeted. (**E** to **H**) Neonatal targeting of region iiiD leads to superficial granule cell production (E and F), whereas targeting region iiiV (G and H) leads to deep granule cell production. Note the position of the cell bodies within the granule cell layer (GRL). The persistence of neuroblasts in the olfactory bulb core (arrowheads) in all labeled regions suggests the continued presence of a radial glial-derived adult neural stem cell.

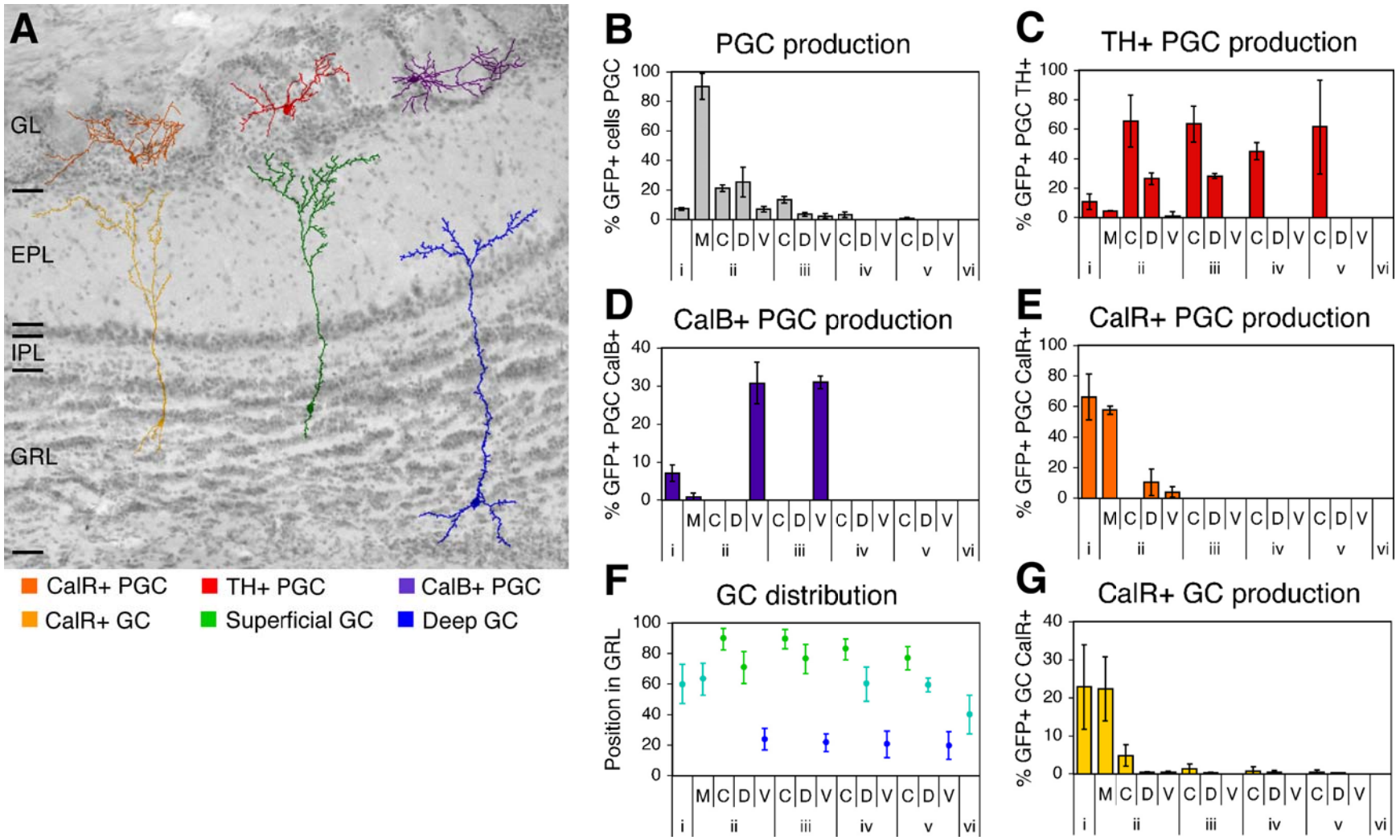
A
GL
EPL
IPL
GRL
CalR+ PGC
TH+ PGC
CalB+ PGC
CalR+ GC
Superficial GC
Deep GC
B
PGC production
% GFP+ cells PGC
C
TH+ PGC production
% GFP+ PGC TH+
D
CalB+ PGC production
% GFP+ PGC CalB+
E
CalR+ PGC production
% GFP+ PGC CalR+
F
GC distribution
Position in GRL
G
CalR+ GC production
% GFP+ GC CalR+
M C D V C D V C D V C D V
i ii iii iv v vi

Figure 4. **Regional production of postnatally born interneuron subtypes.** (**A**) Color-coded camera lucida traces of analyzed interneuron subtypes superimposed over a photomicrograph of the olfactory bulb. EPL, external plexiform layer; IPL, internal plexiform layer; GC, granule cell; GL, glomerular layer; GRL, granule cell layer; PGC, periglomerular cell. (**B**) Percentage of labeled (GFP+) olfactory bulb neurons that are periglomerular cells. All analysis was performed 28 days after stem cell labeling and targeted regions are named as in Fig. 1H, where M is medial, C is cortical, D is dorsal and V is ventral. Error bars represent standard deviation of the mean, except in (**F**) where they represent standard error of the mean. (**C-E**) Percentage of labeled periglomerular cells immunopositive for TH (**C**), CalR (**D**), or CalB (**E**). (**F**) Position of labeled granule cells within the GRL. Cell distributions that could not be definitively classified as superficial (green) or deep (blue) are indicated in teal. (**E**) Percentage of labeled granule cells immunopositive for CalR.

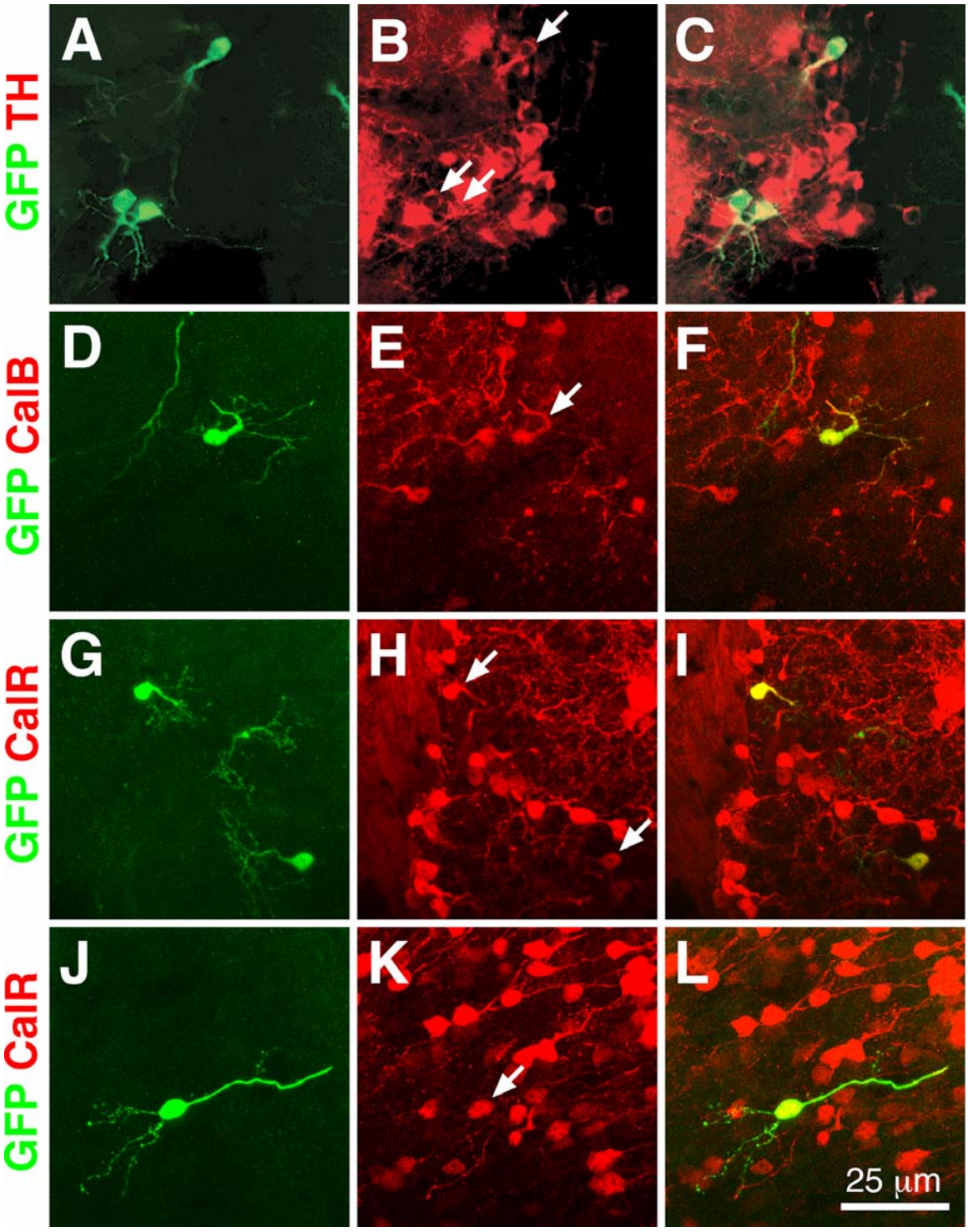
A
B
C
GFP TH
D
E
F
GFP CaIB
G
H
I
GFP CaIR
J
K
L
GFP CaIR
25 µm

Figure 5. Confocal identification of NSC-derived OB interneuron subtypes . Confocal images show P28 olfactory bulb frontal sections from P0 targeted Z/EG mice. Double-label immunofluorescence for GFP (**A**, **D**, **G** and **J**) and subtype specific markers tyrosine hydroxylase (**B**), calbindin (**E**) and calretinin (**H** and **K**), show radial glia-derived TH+ (**C**), CalB+ (**F**) and CalR+ (**I**) periglomerular cells and CalR+ granule cells (**L**) in the olfactory bulb. Arrows show the location of GFP+ cells.

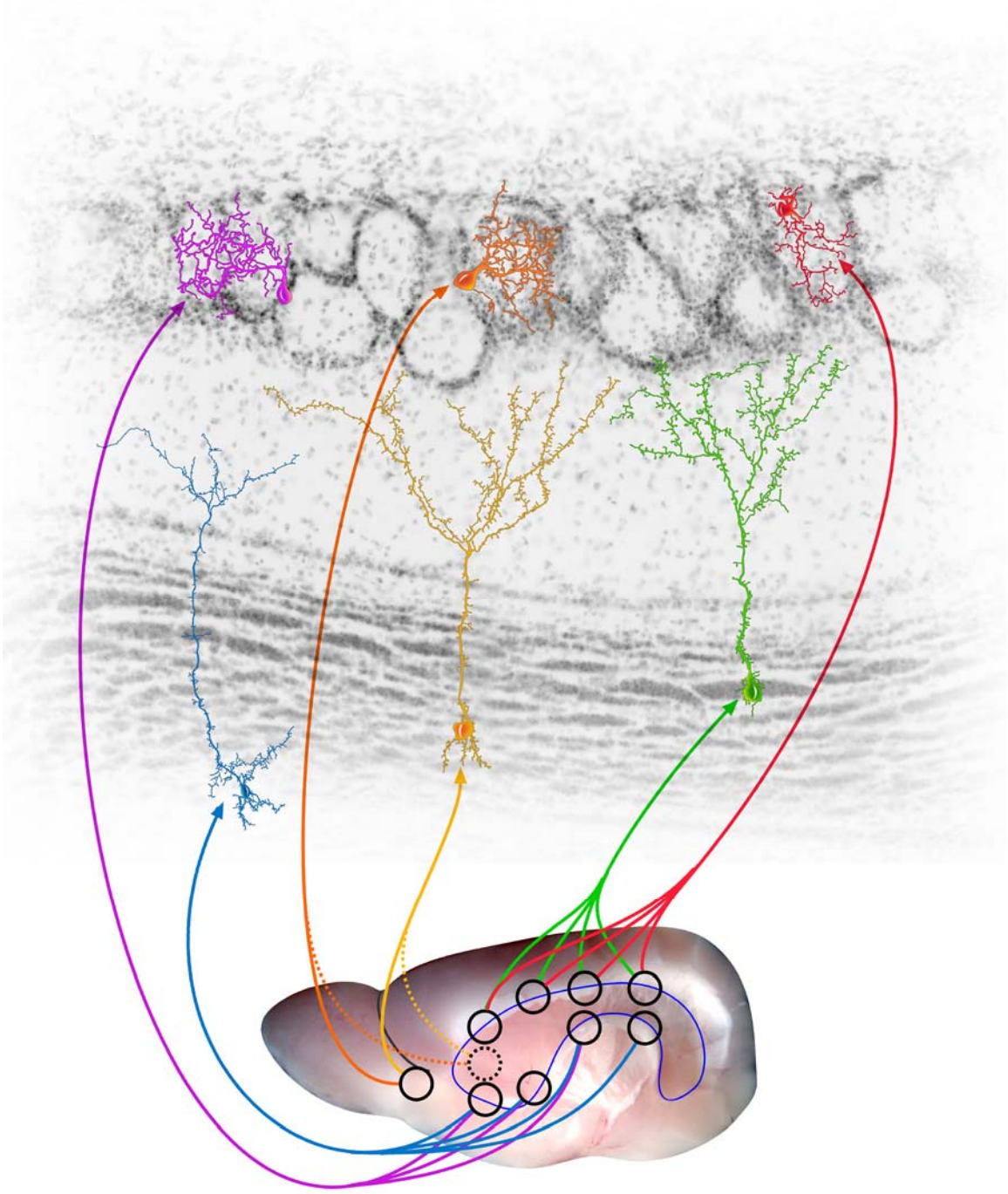

Figure 6. Schematic fate map of different OB interneuron subtypes.

The region-specific production of OB interneuron subtypes is illustrated. A whole mount preparation (outlined in blue, below) exposes the lateral wall of the lateral ventricle, but neurogenic regions in the RMS, septum and cortex cannot be seen. The generation of different OB interneuron subtypes (above) is indicated by arrows originating from analyzed regions on the lateral wall of the lateral ventricle (solid circles). Neuron generation from the septum (region iiM) is indicated by a dashed circle. The morphologies of different neuron types were traced manually, color-coded, and superimposed on their appropriate location in the adult mouse olfactory bulb. Each neuron type is generated in a particular domain of the adult germinal niche, suggesting that stem cells are heterogeneous and restricted, not homogeneous and plastic as widely assumed. Illustration by Florian T. Merkle and Kenneth Xavier Probst.

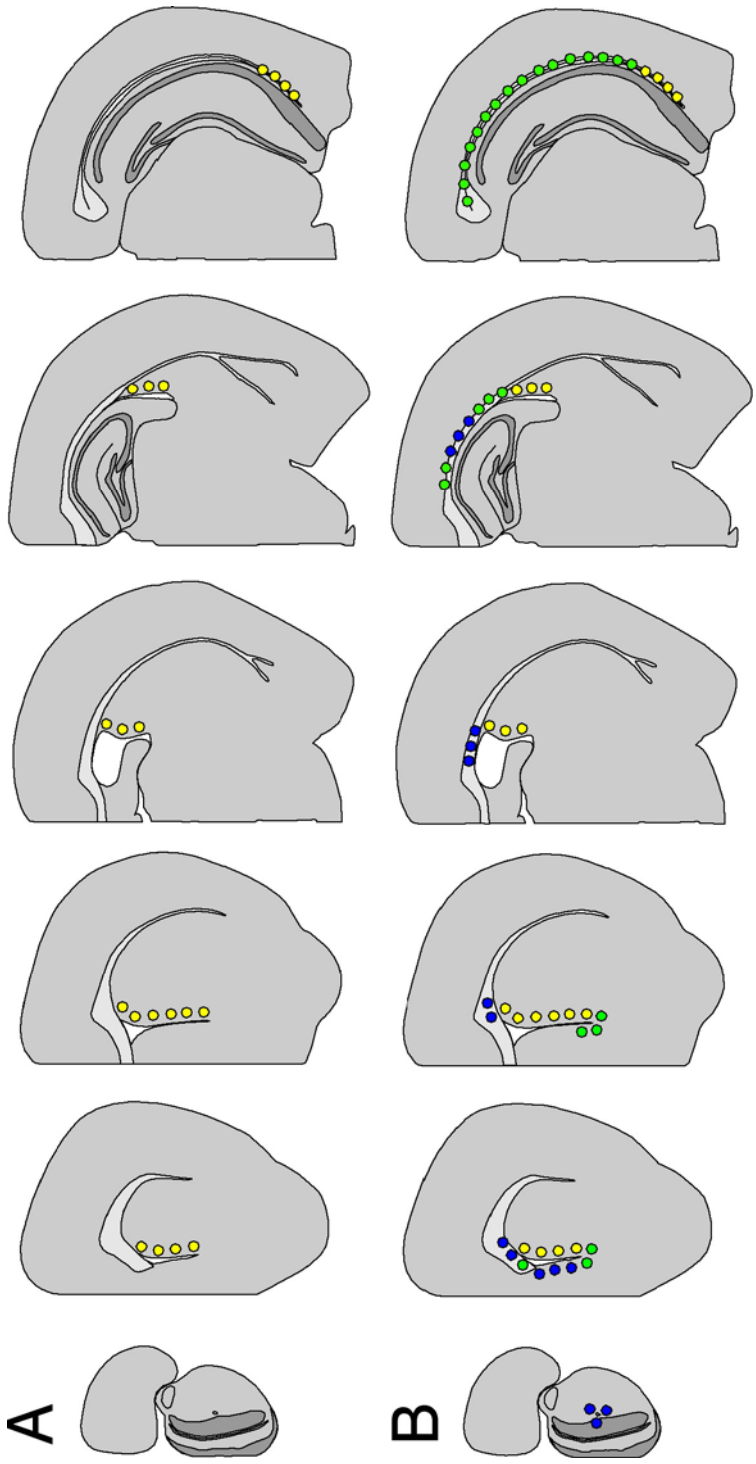
A
B

Figure 7. Known and proposed extent of the forebrain neurogenic zone. Neural stem cells may reside over a wide area of the forebrain, spanning the rostrocaudal extent of the embryonic lateral ventricle. **(A)** Schematic diagram of showing the regions of neurogenesis in the lateral ventricular wall (yellow circles) on traced sections of the right hemisphere of an adult mouse brain. **(B)** Additional regions in which adult NSCs were discovered by neonatal and adult targeting are indicated in blue circles. Green circles indicate putative neurogenic areas that have not been experimentally verified to contain NSCs.

Chapter 5

The potential and plasticity of adult neural stem cells

Overview

The discovery that NSCs are organized into different domains and appear to be restricted in their neurogenic potential in vivo suggests that they are a diverse group of cells. To determine if adult NSCs share the same regionally specific neurogenic potential, I developed a technique to specifically label adult NSCs in different subregions of the SVZ. I found that NSCs targeted in the same region of the neonatal or adult brain generated similar OB neuron types, suggesting that NSC neurogenic potential is relatively stable during postnatal development and that NSCs are diverse. However, it is unclear if this diversity is due to factors intrinsic to progenitors or environmental factors distributed in different spatial patterns. To distinguish between these possibilities, I isolated progenitors from different neurogenic subregions and grew them *in vitro*. Rather than producing a variety of neuron types, cultured progenitors generated only a subset of region-appropriate neuron types, much like progenitors *in vivo*. Next, I heterotopically grafted progenitors from one SVZ subregion to another to determine if the neurogenic potential of progenitors would be respecified by environmental factors. However, grafted neural progenitors continued to produce cell types appropriate to their region of origin. These findings suggest that rather

than being homogeneous and highly plastic, neural stem cells are a restricted and diverse population of progenitors.

Introduction

Our understanding of NSC potential is strongly influenced by the *in vitro* techniques with which they were first isolated (Morshead et al., 1994; Reynolds and Weiss, 1992a). These results led to the widely held assumption that neural stem cells are a homogeneous population of multipotent, plastic progenitors. However, when I specifically labeled radial glia in the neonatal brain, I found that different regions of the SVZ produced only restricted subsets of OB interneuron types. It was not clear if neuroblasts were being specified by factors intrinsic to stem cells, or if environmental factors specified stem cells or neuroblasts after they were generated. Furthermore, I found that each targeted region continued to produce neuroblasts in the adult brain, suggesting the persistence of an active neural stem cell population (see Chapter 4, Results, Fig. 1H; 4B, D, F, H). Since neonatal radial glia convert into SVZ astrocytes that express GFAP and function as the adult neural stem cells (Doetsch et al., 1999a; Imura et al., 2003; Merkle et al., 2004), I hypothesized that radial glia in different neurogenic subregions generate GFAP+ adult neural stem cells that are restricted in their potential to produce different types of olfactory bulb interneurons.

Results

Maintenance of stem cell potential over postnatal development

To test whether adult neural stem cells in different regions produced different neuron types, I developed a technique to specifically target primary progenitors expressing GFAP. We accomplished this by constructing an adenovirus that expresses Cre under the murine GFAP promoter (Ad:GFAP-Cre). This virus induces recombination in GFAP-expressing cells, including adult neural stem cells, but not the more differentiated cells they give rise to (Fig. 1; 2A-C). I injected P60 Z/EG mice with Ad:GFAP-Cre in regions i, iiiD, iiiV and vC (Fig. 2D-G) and sacrificed the animals 28 days later to examine the olfactory bulb cell types produced. Each targeted region produced olfactory bulb neurons and neuroblasts (Fig. 3A-D), suggesting that they contain long-lived populations of GFAP+ neurogenic progenitors. This finding corroborates previous work suggesting the presence of a neurogenic progenitor in the adult RMS (Fukushima et al., 2002; Gritti et al., 2002; Hack et al., 2005) and subcallosal zone (regions ivC and vC) (Seri et al., 2006; Ventura and Goldman, 2007).

Furthermore, these labeled progenitors produced superficial and deep granule cells as well as TH+, CalB+ and CalR+ cells in a manner similar to the region-specific pattern observed after neonatal labeling. Neonatally targeted and adult-targeted progenitors in region i produced CalR+ GCs (Fig. 2F), whereas progenitors in regions iiiD and vC produced superficial GCs (Fig. 2A-E) and TH+ PGCs (Fig. 2F), and progenitors in region iiiV produced deep GCs (Fig. 2A-E) and CalB+ PGCs (Fig. 2F; Chapter 4, Fig. 4). A recent study suggests that the

potential of progenitors to produce different types of periglomerular cells changes over development (De Marchis et al., 2007). Our results raise the possibility that this work may have inadvertently examined progenitors and tangentially migrating neuroblasts from different regions at different ages. In contrast, our technique targets only primary progenitors either by targeting the processes of radial glial cells in the neonatal brain or by specifically recombining GFAP+ cells in the adult brain. The apparent maintenance of stem cell potential over postnatal development suggests that the factors specifying neural progenitors in the SVZ are maintained throughout development in a regionally specific manner.

Postnatal NSC potential is determined by a cell-intrinsic mechanism

Factors that specify stem cell or neuroblast fate could be either environmental or intrinsic to stem cells. To distinguish between these two possibilities, I challenged neonatal stem cells by heterotopic transplantation. If environmental factors specify the fate of newborn neurons, grafted (donor) stem cells and their progeny should respond to these factors and generate cell types appropriate to the host region.

To obtain labeled neural stem cells, neonatal radial glia were infected with Ad:Cre as described above, microdissected after two hours, dissociated to a single cell suspension, and homotopically or heterotopically grafted into the littermates of donor animals (Fig. 4A). I then analyzed the olfactory bulb cell types produced 40 days after grafting and found that labeled radial glia continued to produce the cell types appropriate to their region of origin; ventral progenitors

grafted into the dorsal or ventral SVZ produced deep GC (Fig. 4B). Furthermore, progenitors from the anterior SVZ continued to produce PGCs when grafted into the posterior or anterior SVZ, whereas posterior progenitors never did (Fig. 4C).

In order to determine if progenitors would maintain their region-specific potential in the absence of any environmental cues, I cultured anterior, dorsal, or ventral progenitors under adherent conditions that recapitulate postnatal SVZ neurogenesis (Scheffler et al., 2005) (see Chapter 2, Section 5, "Neural stem cell monolayer cultures" and "Neural stem cell cultures for in vitro potential determination"). Cultures were expanded for two weeks, differentiated for 7 days, (Fig. 5A) and immunostained for the cell type specific markers CalR and CalB (Fig. 6). Like progenitors targeted in vivo, cultured cells from anterior regions produced CalR+ cells, whereas ventral progenitors produced CalB+ cells (Fig. 5B). Thus, neural stem cells maintained their region-specific potential despite being removed from their complex environment and exposed to a cocktail of growth factors.

I confirmed this result by targeting dorsal or ventral radial glia with Ad:Cre in the neonatal brain. After two days, targeted cells were cultured as described above (see Chapter 2, Section 5, "Neural stem cell monolayer cultures" and "Neural stem cells cultured for heterotopic grafting"). Cultured cells were then grafted into the dorsal or ventral SVZ of wild-type neonatal mice (Fig. 7A) (see Chapter 2, Section 6, "Heterotopic grafting of cultured neural stem cells"). Again, grafted cells produced cell types appropriate to their region of origin, not their grafted location (Fig. 7B).

The above experiments suggest that neural stem cells are not readily respecified by environmental factors present in the postnatal brain. However, I cannot exclude the possibility that unlabeled cells grafted alongside labeled stem cells could be carrying factors such as secreted or membrane-bound proteins from the donor environment into the host graft site. Therefore, I microdissected progenitors from different regions of neonatal wild-type or ActB-GFP mice (Hadjantonakis et al., 1998) and cultured them for three passages over two weeks. To ensure that grafted cells were surrounded by foreign environmental cues, I mixed GFP+ cells from the donor region with a 10:1 excess of unlabeled wild-type cells from the host region that had been cultured in parallel to the GFP+ cells. The cell mixture was then grafted into wild-type neonatal mice (Fig. 10A). Four weeks later, grafted brains contained labeled astrocyte-like cells at the graft site (Fig. 8A, C, E, and G) and mature neurons in the olfactory bulbs (Fig. 8B, D, F, and H). Since grafted cells and targeted primary progenitors produced similar proportions of neuroblasts (Fig. 9), grafted cells were most likely stem cells and not intermediate progenitors. Once again, heterotopically grafted stem cells produced neuronal subtypes with the same regional specificity observed by *in vivo* lineage tracing (Fig. 10B-E).

The lack of evidence for even partial respecification indicates that grafted cells behaved as a unified population that was resistant to environmental respecification. However, I cannot discard the possibility that some environmental factors were not totally eliminated in our experiments, or that neural stem cell potential could be altered by factors not included in our culture

conditions or after extended periods of time *ex vivo*. For example, membrane-bound signaling molecules would be diluted but not eliminated upon progenitor expansion in culture. These environmental factors could maintain the regional specification of progenitors for short periods of time. Therefore, repeated passages or long-term exposure to growth factors might reveal the latent multipotency of NSCs.

Previous studies have suggested that adult hippocampal or spinal cord progenitors might be respecified when heterotopically grafted (Shihabuddin et al., 2000; Suhonen et al., 1996). Though these findings are inconsistent with my data, the results might be reconciled considering that the hippocampal cells of Suhonen et al. were cultured for over two years. These culture conditions, which I did not test in our study, could result in re-specification. Furthermore, rat hippocampal progenitors might be more plastic than mouse SVZ progenitors. Finally, our present results and previous work (Doetsch and Alvarez-Buylla, 1996; Seri et al., 2006) demonstrate that there are progenitors of OB neurons immediately adjacent to the hippocampus. These progenitors may have contaminated the hippocampal cultures of Suhonen et al. and produced OB neurons when grafted into the SVZ.

Thus, the most parsimonious interpretation of the data presented above is that the neurogenic potential of NSCs in the SVZ is intrinsically restricted. I conclude that postnatal neural stem cells are diverse and are organized in an intricate mosaic in the postnatal germinal zone.

Discussion

In Chapter 4, I demonstrated that progenitors in the SVZ are organized into domains in an extensive periventricular germinal zone. Here, I demonstrate that the potential of these progenitors is restricted and cell-intrinsic. These findings suggest that, as during seen during embryonic brain development (Campbell, 2003; Guillemot, 2005; Rubenstein and Beachy, 1998), the potential of postnatal neural stem cells is determined by a spatial code. Since stem cells do not appear to migrate tangentially as they mature from radial glia into astrocyte-like adult cells, they must have integrated positional information at some point in development. One possibility is that this positional information becomes encoded in the progenitors, perhaps by expression of a transcription factor code, and maintained into adulthood. This insight is a key step toward understanding the molecular mechanisms of neural stem cell potential.

The production of similar OB interneuron types from similar regions of neonatal and adult SVZ suggests that stem cell potential does not change significantly over postnatal development. However, only certain cell types were examined with a limited set of morphological criteria and marker genes. Thus, it remains possible that the potential of NSCs to produce astrocytes, oligodendrocytes or other types of OB interneurons changes over time. Furthermore, only P0 and P60 NSCs were compared and it remains possible that certain cell populations are produced within the first few months of life but not later. To more carefully determine if there is a temporal shift in NSC potential during postnatal development, a greater variety of cell types should be examined

at a greater number of time points. If it is possible to specifically label NSCs in the embryonic brain (see Chapter 6, "A technique to specifically label embryonic NSCs"), this analysis could be extended to the embryonic brain.

I dissociated and cultured NSCs to demonstrate that they retain the ability to generate region-specific cell types in the absence of environmental cues. After multiple passages and two weeks in culture, soluble and membrane-bound factors should be significantly diluted. Contaminating cell types might still be present, but unless they are as proliferative as NSCs and their progeny, they would be at a competitive disadvantage and would be diluted after two weeks in culture. Therefore, the continued presence of environmental factors in cultured cells cannot be completely discounted. However, since no change in region-specific cell type production was observed, the most parsimonious explanation is that NSC potential is cell-autonomous.

Another potential concern about culturing progenitors is that both NSCs and intermediate progenitors have been reported to respond to growth factors and act like NSCs under neurosphere conditions (Doetsch et al., 2002). Therefore, cultures may include both primary and transformed intermediate progenitors. However, when removed from these conditions and grafted back into the SVZ, neurospheres formed from intermediate progenitors are inefficient at producing OB interneurons (Isabel Caille, unpublished observations). Therefore, I did not grow neurospheres but used an adherent culture system reported to more closely recapitulate adult neurogenesis (Scheffler et al., 2005). After being cultured and grafted, these cells generated as many neuroblasts as

directly targeted primary progenitors, suggesting that cultures consisted mostly of primary progenitors. However, I cannot discount the possibility that under these conditions, intermediate progenitors acquire and maintain the ability to generate neurons for long periods of time, even after being grafted back into the SVZ. To directly test this possibility, C cells could be purified from Mash1^{GFP} mice (Wildner et al., 2006) and cultured under adherent conditions to determine if they are able to acquire multipotency under neurosphere and adherent conditions. After a certain amount of time in culture, these cells could then be grafted into the SVZ to determine if C-cell derived cultures exhibit the same levels of neurogenesis after grafting as directly targeted primary progenitors.

Furthermore, the genetic basis of NSC potential could be examined by labeling stem cells in different regions, purifying them by FACS, and comparing them by gene chip analysis. Shared genes might may be involved in maintaining the neural stem cell state, whereas differentially expressed genes might may be involved in specifying different cell types. With this approach, it would be possible to identify the set of genes required to make, for example, a dopaminergic OB interneuron. Together with the appropriate inductive signals, one could activate these genes in embryonic stem (ES) cells or immature neural stem cells (embryonic radial glia) to guide them towards making these cell types in vitro. This approach has been successfully used to generate motor neurons from ES cells (Miles et al., 2004; Wichterle et al., 2002). Alternatively, these genes could be activated in adult stem cells that do not normally express them. This would test whether a single gene or set of genes is sufficient to drive adult NSCs toward

a particular phenotype, shedding light on the mechanisms of NSC commitment. One could also conditionally remove these genes or knock down their activity to determine if they are necessary for specifying a particular fate. There is evidence that the transcription factors Pax6 and Olig2 regulate the production of TH+ PGCs and oligodendrocytes, respectively, in the adult brain when overexpressed or knocked down (Hack et al., 2005; Kohwi et al., 2005). These results have not been repeated, but they suggest that while NSCs may not be sensitive responsive to environmental factors present in the neonatal brain, their potential can still be altered by genetic manipulation.

One approach to identify other genes that may specify different types of OB interneurons is to examine TFs and other genes implicated in OB neurogenesis or expressed in regions of the embryonic brain thought to give rise to the adult SVZ. In addition to Pax6 and Olig2, such candidate genes include Dlx1/2/5/6 (Kohwi et al., 2007; Saino-Saito et al., 2003; Xu et al., 2004), Emx1/2 (Kohwi et al., 2007), Er81 and Gsh1/2 (Stenman et al., 2003), Gli1/2/3 (Ahn and Joyner, 2005; Palma et al., 2005; Ruiz i Altaba et al., 2003), Mash1 (Parras et al., 2004), Ngn1/2 (Schuurmans and Guillemot, 2002; Toresson et al., 2000), Nkx2.1/2.2 (Backman et al., 2005; Sussel et al., 1999; Xu et al., 2004), Notch1 (Chambers et al., 2001; Givogri et al., 2006; Irvin et al., 2001; Stump et al., 2002; Tokunaga et al., 2004), Sox2/9/10/11 (Wegner and Stolt, 2005; Zappone et al., 2000), Sp8 (Waclaw et al., 2006), and Zic1 (Inoue et al., 2007).

A second approach is to screen through a gene expression database for genes differentially expressed in different regions of the SVZ. These databases

include the Allen Brain Atlas (Lein et al., 2007), which contains detailed photomicrographs of adult mouse brain sections stained for many different genes by in situ hybridization and the GENSAT (Gong et al., 2003; Heintz, 2004) database of BAC transgenic mice immunostained with DAB for GFP. I screened through several thousand transcription factors and genes involved in the major signaling pathways to identify novel candidates that might regulate OB neurogenesis. The results of this screen are summarized in Table 4.

In conclusion, my findings suggests that we should abandon the idea that NSCs are a homogeneous population of multipotent progenitors. The potential of an individual stem cell *in vivo*, the molecular basis of its potential, and the plasticity of this cell in response to genetic and environmental manipulation will have to be determined in future studies. I will discuss these issues and techniques to address them in Chapter 6.

Table 4. Genes that may direct OB interneuron production by SVZ stem cells.

Gene	Expression in SVZ	Expression in OB	Description from literature	Reference
Dlx6os1	SVZ specific, including ventral medial wall. Specifically dorsal in posterior areas. Patchy staining high in RMS suggests neuroblast expression	Expression in core, superficial GRL, scattered cells in MCL, EPL, GL	Ultraconserved noncoding RNA present in embryonic LGE and interacts with local TF. Induces neurons expressing it to differentiate in response to Shh. Whole mount in situ hybridization shows that it's expressed at high levels in the branchial arches, ventral forebrain, olfactory bulb, and limbs. Expression is linked to Shh and the Dlx family of proteins.	(Faedo et al., 2004; Kohtz and Fishell, 2004)
Foxp2	Expressed in striatum and scattered SVZ cells, particularly in anterior ventral regions	Clear dark expression in PGCs, some cells in very superficial GRL, IPL, MCL	Only transcription factor directly linked to human speech, commonly studied TF in songbird system. Overlap with Emx genes in zebrafish in early development but later expression is highest in optic tectum and vestibulocerebellum.	(Ferland et al., 2003; Li et al., 2004b)
Og9x	Highly expressed, strongest in scattered cells in dorsal SVZ and RMS	High in PGC, scattered GC	Maternally expressed homeobox TF. Widely expressed in embryo, localized to epidermis in neonates and maturing oocytes and fertilized eggs in adults. In the brain, it's expressed in the cerebral cortex, hippocampus, pontine nuclei, choroid plexus, and the cerebellum.	(Cinquanta et al., 2000)
Pbx3	Expressed strongly in patches, especially in the ventral tip, wedge area, and RMS, stronger anterior than posterior	GC only	Homeobox transcription factor, three amino acid loop extension (TALE) family, embryonically expressed in brain and epithelia including olfactory bulb epithelium. Developmentally important (also found in PNS, limb buds, etc.) but not as widely expressed as Pbx1 and Pbx2. Possibly overexpressed in human brain tumors. Has homology to proto-oncogene.	(Di Giacomo et al., 2006)
Rax	Weak, dorsal-specific expression	Scattered cells in GL, GRL, MCL	Homeobox TF essential for retinal development. Sometimes co-expressed with Pax6 and Six3 in culture and in developing retina. Could regulate or be regulated by	(Mikkola et al., 2001; Tabata et al., 2004)

			Pax6, expression in SVZ is similar.	
Tbr2	Expressed in type C and A cells	Neuroblasts and projection neurons	Downstream of pax6 (pax6->tbr2->tbr1) in radial glial differentiation, present in SVZ cells and adult DG.	(Englund et al., 2005)
Tcf4	Strongly SVZ specific, patchy staining	Clusters of GC, PGC	Wnt target gene, downregulated in brain of Shh mutants (bmp4 upregulated), directly binds beta-catenin and is highly expressed in embryonic CNS and limb buds. Under control of Pax6. Absent in Pax6 mutants.	(Matsunaga et al., 2000)
Tiam1	Weak expression in SVZ, RMS	Expression strong in core, very superficial GRL, MCL	Guanine nucleotide exchange factor that regulates numerous biologic properties including migration and invasion and reduces cellular adhesion. Important for neuroblast migration? Cells overexpressing Tiam1 increase nestin expression. Presence of cells in superficial GRL may indicate a role in differentiation. Activated by TrkB. Directly binds to c-myc.	(Kawauchi et al., 2003; Leeuwen et al., 1997; Miyamoto et al., 2006; Tolias et al., 2007; Zhang and Macara, 2006)
Tshz1	Strongly expressed ventrally	GC and PGC	Teashirt TF similar to Drosophila, knockout/overexpression induces homeotic mutations including ectopic eyes	(Manfroid et al., 2004)
Wfs1	Expressed in ventral striatum immediately adjacent to ventral tip of SVZ	Scattered cells with bias to deep GRL	Encodes transmembrane protein that modulates (Ca2+) concentration in ER, regulated by Sp1 and Sp3 TF. Gene knockout results in Wolfram syndrome, a recessive neurodegenerative disease accompanied by optic and autic atrophy.	(Takei et al., 2006; Yamaguchi et al., 2004)
Zbtb20	SVZ specific, higher in dorsal and ventral tips, scattered cells	OB core and PGC only	TF specifically expressed in early hippocampal neurons, cerebellar granule cells, gliogenic progenitors and differentiated glia. Closely related to BCL-6, and may be involved in hematopoiesis, oncogenesis, and immune responses and pyramidal neuron production.	(Nielsen et al., 2007)
Zfhx2as	SVZ specific, patchy expression	Scattered cells, mostly in GC	Zinc finger homeobox 2 TF, expressed in many different organisms but of unknown function.	(Komine et al., 2006)

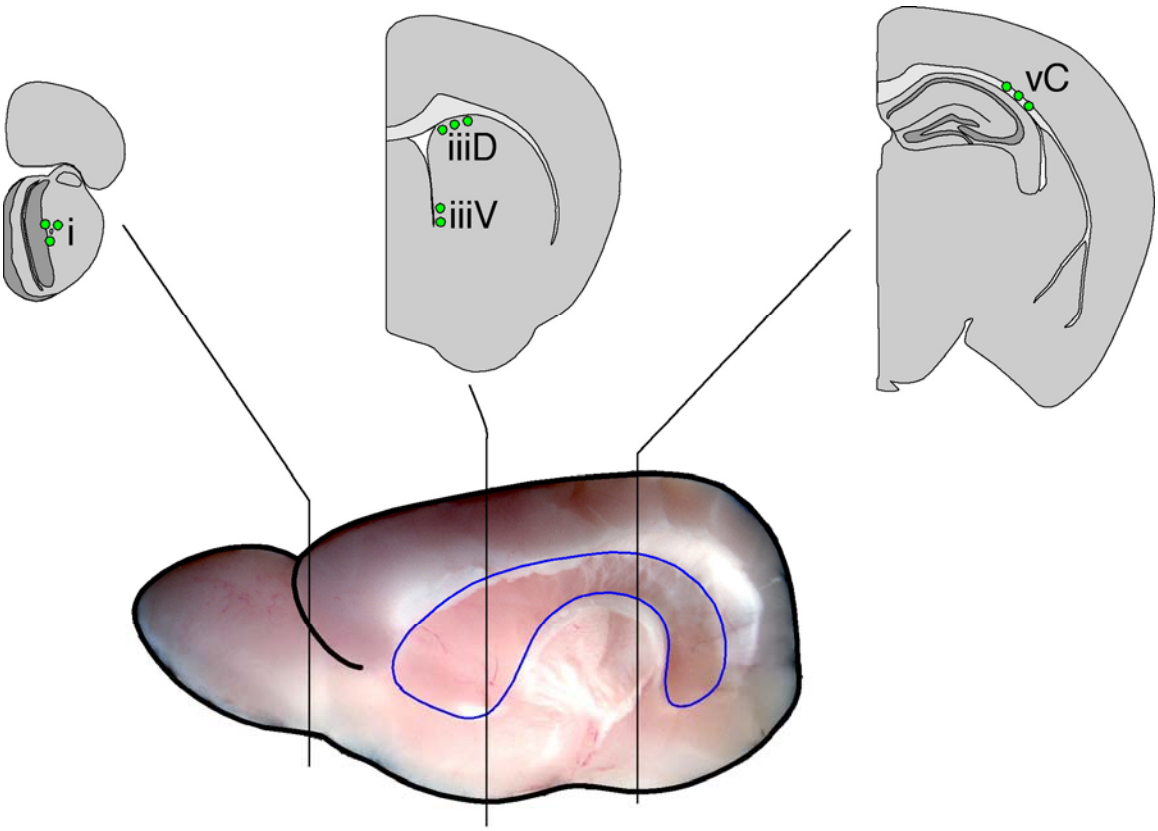

Figure 1. Specific regional targeting of neural stem cells.

Diagram of adult brain regions targeted. Representative traces of frontal sections from the right hemisphere or an adult mouse brain are shown relative to a photomicrograph of an adult lateral ventricular wall whole mount (Doetsch and Alvarez-Buylla, 1996). Targeted regions, indicated by green dots, are named for their anterior-posterior level (i-vi) followed by the location within that level, where C is cortical, D is dorsal and V is ventral.

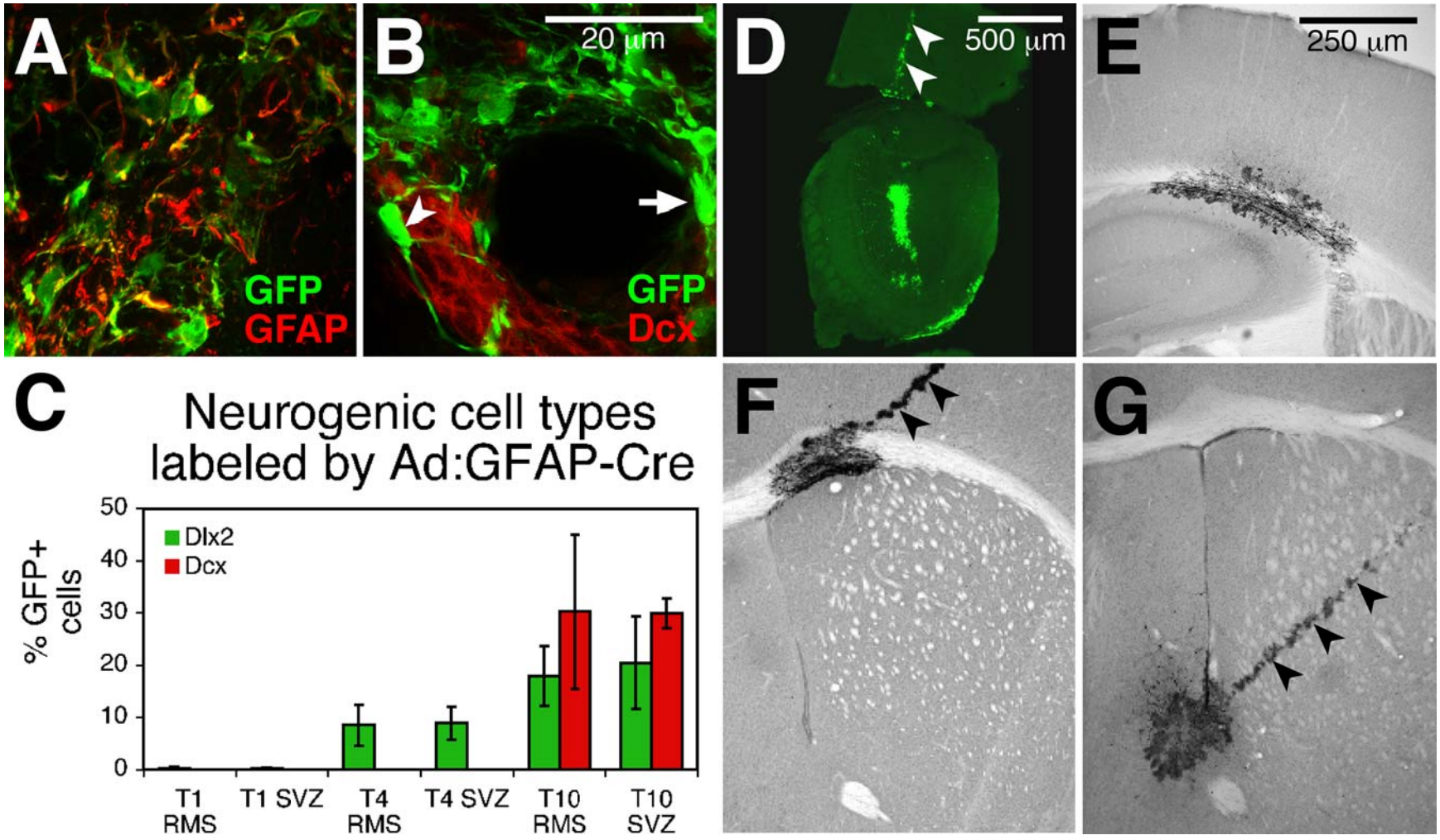
A
GFP
GFAP
B
20 μm
GFP
Dcx
D
500 μm
E
250 μm
C
Neurogenic cell types
labeled by Ad:GFAP-Cre
% GFP+ cells
50
40
30
20
10
0
Dlx2
Dcx
T1 RMS
T1 SVZ
T4 RMS
T4 SVZ
T10 RMS
T10 SVZ
F
G

Figure 2. Ad:GFAP-Cre specifically induces recombination in GFAP-expressing cells in adult mice.

(**A**) One day after injection of Ad:GFAP-Cre into the P60 SVZ, there are many recombined (GFP expressing) GFAP+ cells. (**B**) These GFP+ cells display astrocytic morphology, often contact blood vessels (arrow), and surround doublecortin (Dcx)-expressing neuroblasts (arrowhead). Neuroblasts are not recombined. (**C**) Quantification of double-labeled cells one (T1), four (T4) or ten (T10) days after injection of Ad:GFAP-Cre into the SVZ or RMS reveals that Dlx2+ intermediate progenitors and Dcx+ neuroblasts, are not directly recombined but are generated by GFAP+ neural stem cells. (**D-G**) Photomicrographs of frontal sections showing labeled cells at the injection site and along the injection tract (arrowheads) 28 days after Ad:GFAP-Cre injection. Cells are visualized by fluorescent immunostaining for GFP for region i (**D**) or immunoperoxidase staining for GFP in regions vC (**E**), iiiD (**F**) or iiiV (**G**). Since viral diffusion is limited, these regions can be specifically targeted with Ad:GFAP-Cre when approached at a trajectory (arrowheads) that avoids other neurogenic regions.

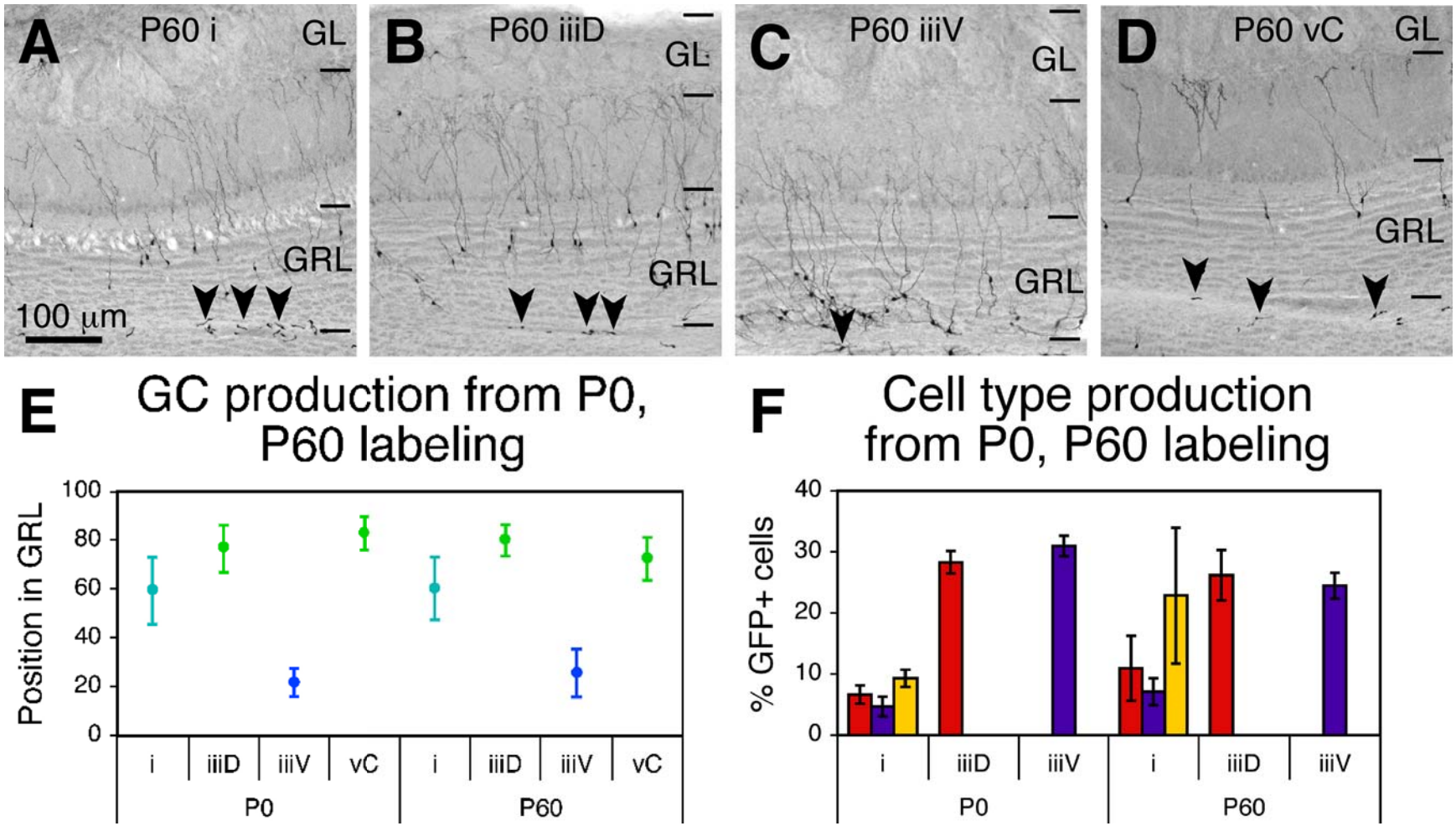
A
P60 i
GL
GRL
100 μm
B
P60 iiiD
GL
GRL
C
P60 iiiV
GL
GRL
D
P60 vC
GL
GRL
E
GC production from P0, P60 labeling
Position in GRL
100
80
60
40
20
0
i
iiiD
iiiV
vC
i
iiiD
iiiV
vC
P0
P60
F
Cell type production from P0, P60 labeling
% GFP+ cells
40
30
20
10
0
i
iiiD
iiiV
i
iiiD
iiiV
P0
P60

Figure 3. As in the neonatal brain, different subregions of the adult brain generate different types of OB interneurons.

(**A-D**) Photomicrographs of olfactory bulbs of Z/EG mice injected with Ad:GFAP-Cre 28 days earlier at P60 in region i (**A**), iiiD (**B**), iiiV (**C**), or vC (**D**), with cells visualized by immunoperoxidase staining for GFP. Note the distribution of granule cells within the granule cell layer (GRL) and continued presence of neuroblasts in the olfactory bulb core (arrowheads). (**E** and **F**) Quantification of labeled granule cell position in the GRL (**E**) and TH, CalB and CalR periglomerular cell production (**F**) 28 days following radial glial (P0) or adult (P60) stem cell targeting. Note the similarity in region-specific cell production from regions targeted at P0 or P60.

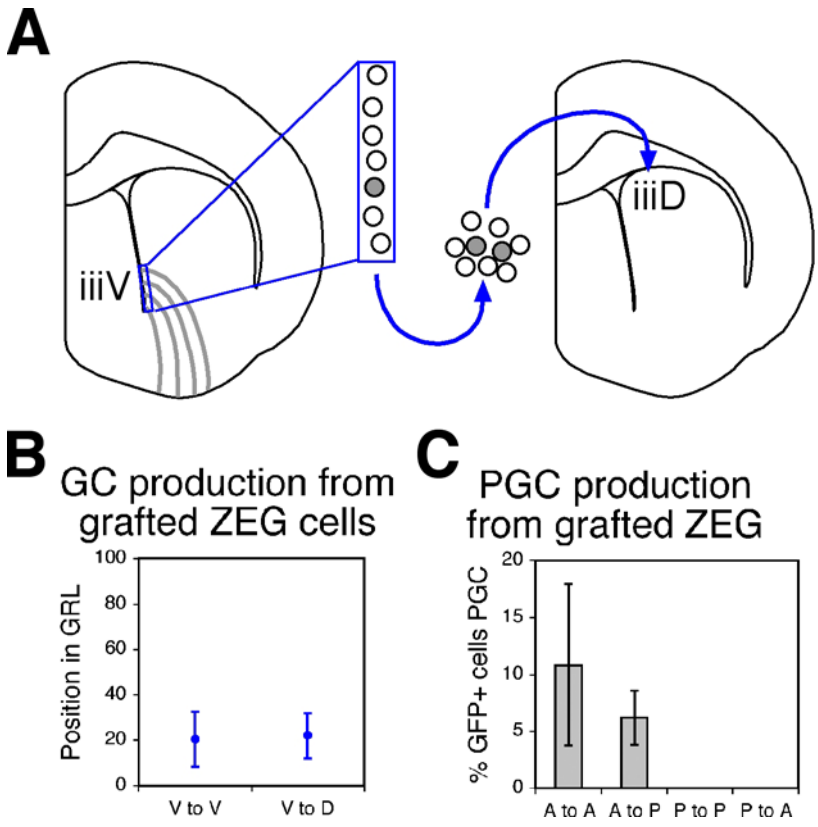

Figure 4. **Neural stem cells maintain their potential when heterotopically grafted.**

(**A**) Radial glia were targeted with Ad:Cre and microdissected from different regions two hours after viral infection. In previous experiments (data not shown) it was determined that Ad:Cre infects radial glial processes and is retrogradely transported to the cell body in 30-60 minutes. Infected radial glia were dissociated and grafted into wild-type neonatal mice. (**B** and **C**) Distribution of GFP+ granule cells in the granule cell layer from dorsal/ventral grafts (B) and the percentage of mature GFP+ OB neurons that were periglomerular cells from anterior/posterior grafts (C) 40 days after ventral/dorsal grafting.

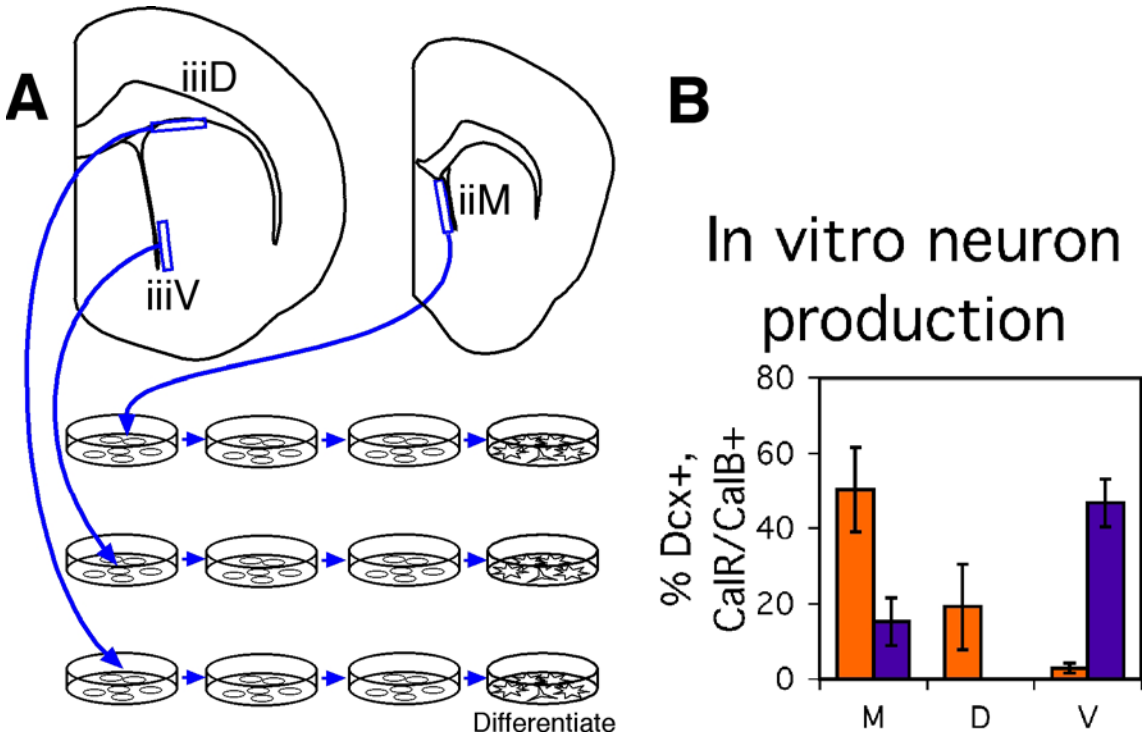

Figure 5. Neural progenitors maintain their potential in culture.

(**A**) Schematic diagram showing the isolation and culturing of progenitors from different neurogenic regions. Cells were cultured for 7 days and were passaged 2 times. (**B**) Percentage of neurons expressing CalR (orange) or CalB (purple) after 7 days of differentiation *in vitro*. The immunostaining of different cell types in culture is shown in Fig. 6.

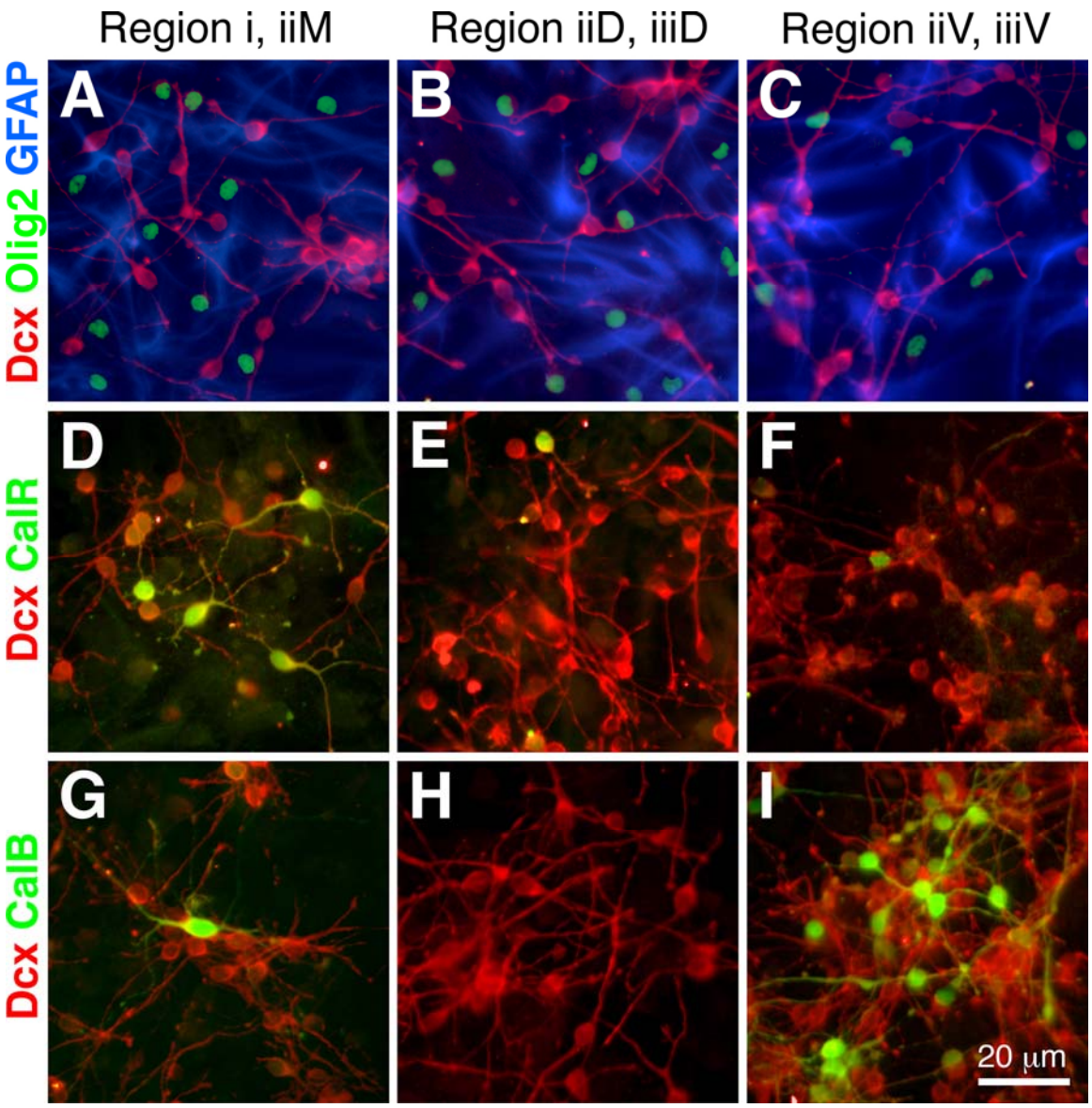
Region i, iiM
Region iiD, iiiD
Region iiV, iiiV
Dcx Olig2 GFAP
Dcx CalR
Dcx CalB
A
B
C
D
E
F
G
H
I
20 μm

Figure 6. Neural stem cells maintain their region-specific potential in culture.

(**A**-**C**) Photomicrographs of differentiated neural stem cell cultures immunostained to identify neurons (Dcx+), oligodendrocytes (Olig2+) and astrocytes (GFAP+). Progenitors dissected from regions i/iiM (A), ii/iiiD (B), and ii/iiiV (C) were cultured for 7 days, passaged twice, and differentiated for 7 days. (**D-I**) When stained for neuron type specific markers CalR (D-F) and CalB (G-I), cultures exhibited region-specific neuron production, with most of the CalR+ cells produced in regions i and iiM (D) and most of the CalB+ cells produced in regions iiV and iiiV (I). See Fig. 5B for quantification.

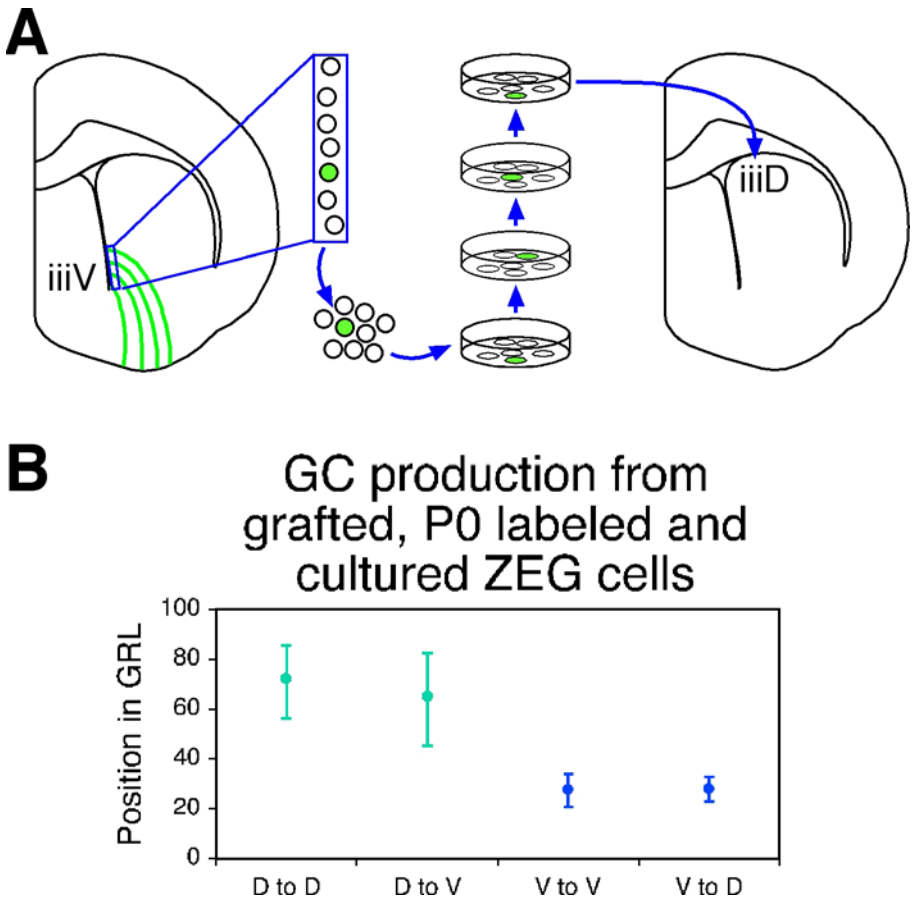

Figure 7. Neural stem cells are resistant to respecification after culture and heterotopic grafting.

(**A**) Schematic diagram showing the targeting, culture and grafting of radial glia from neonatal Z/EG mice to neonatal CD1 mice. Radial glia in regions iiiD and iiiV were specifically targeted with Ad:Cre, microdissected after two days, and cultured for two weeks and three passages. Cultured cells were then grafted into regions iiiD or iiiV. (**B**) The distribution of ventral/dorsal graft-derived granule cells in the granule cell layer demonstrates that cells were not respecified.

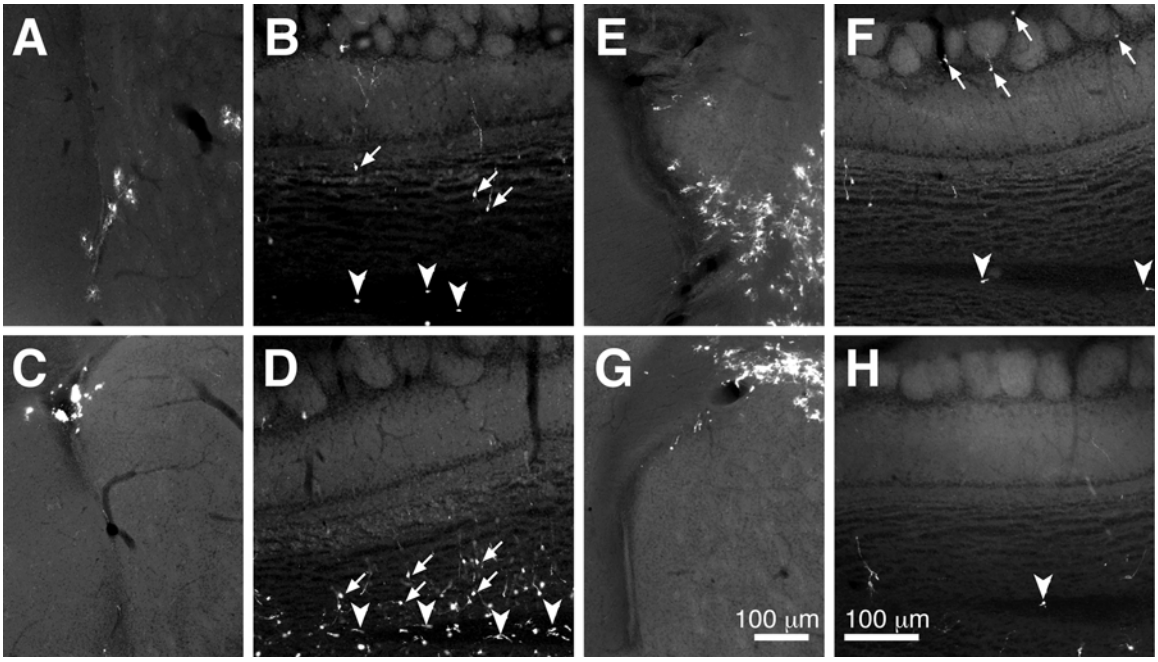

Figure 8. Cultured neural stem cells remain neurogenic and maintain region-specific potential after heterotopic grafting.

Images are fluorescent photomicrographs of GFP-labeled cells in recipient brains four weeks after heterotopic grafting. (**A** and **B**) Cells grafted from the dorsal to the ventral SVZ (A), produce mostly superficial olfactory bulb granule cells (arrows in B). The presence of migrating neuroblasts in the olfactory bulb core (arrowheads), suggests that grafted cells are long-lived neurogenic progenitors. (**C** and **D**) Ventral cells grafted to the dorsal SVZ (C) produce neuroblasts (arrowheads in D) and deep granule cells (arrows). (**E-H**) Anterior cells grafted to the posterior SVZ (E) produce neuroblasts (arrowheads in F) and many periglomerular cells (arrows) while posterior cells grafted to the anterior SVZ (G) produce neuroblasts (arrowhead in H) and very few periglomerular cells. After being cultured and heterotopically grafted, progenitors produce cell types similar to those observed from in vivo targeting.

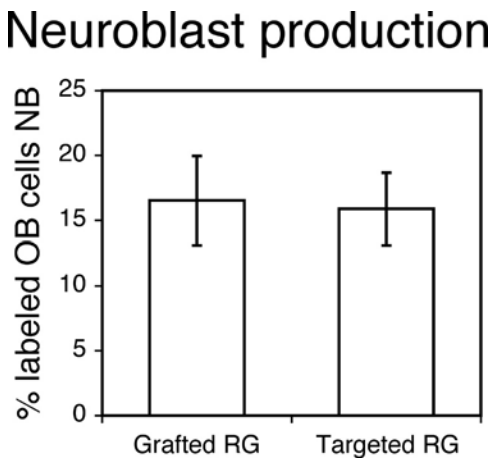

Figure 9. Grafted cells remain actively neurogenic.

Graph showing the quantification of neuroblast production four weeks after cultured cells were grafted or radial glia were specifically labeled with Ad:Cre. For each grafted or targeted region, neuroblasts in the olfactory bulb core and mature olfactory bulb neurons were counted for three separate mice, and the ratio of neuroblasts to all counted olfactory bulb cells was determined. Error bars show standard error of the mean. Grafted cells produced as many neurons as radial glia-derived neural stem cells, suggesting that grafted cells were long-lived neural stem cells.

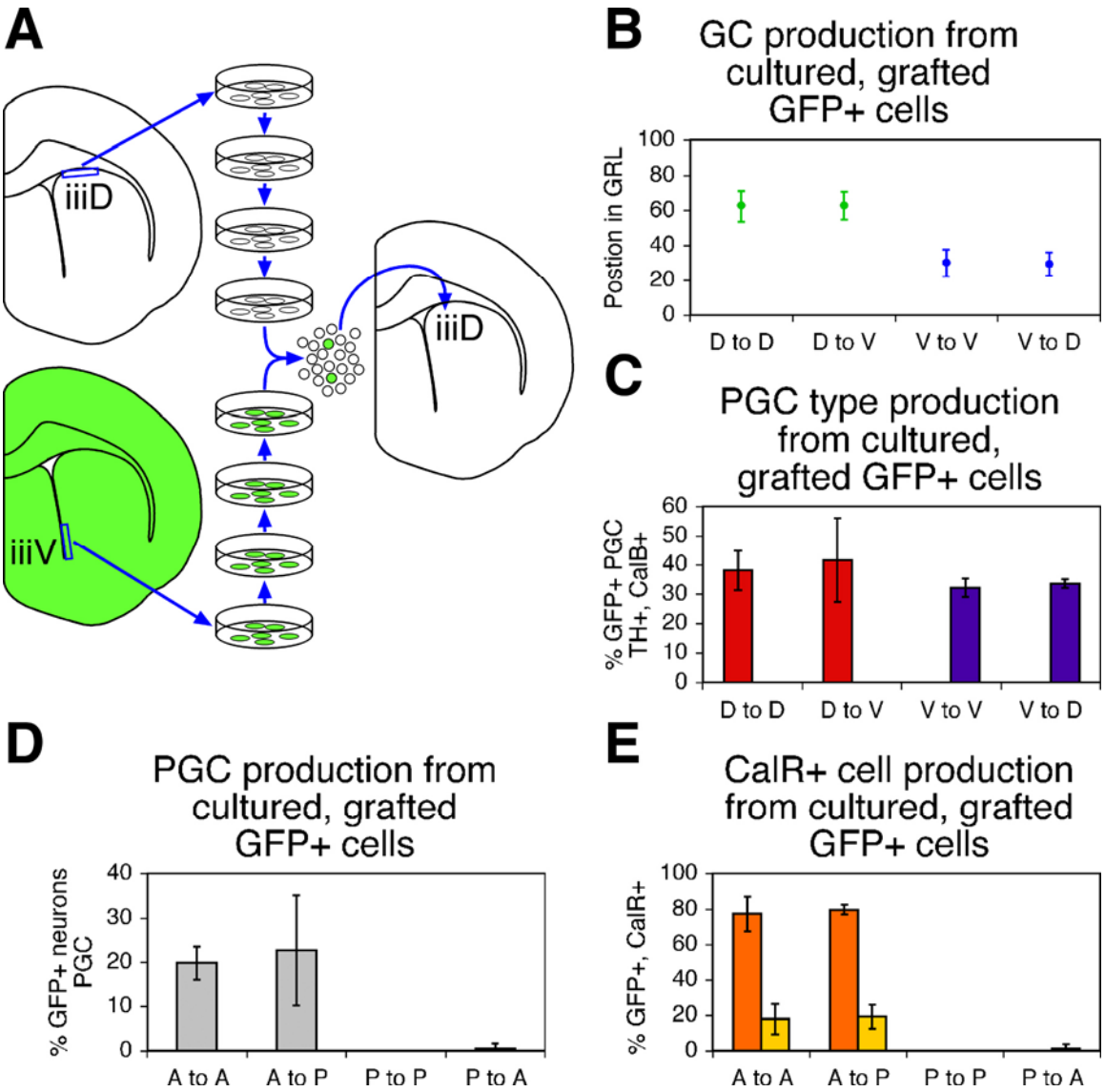
A
iiiD
iiiV
iiiD
B
GC production from cultured, grafted GFP+ cells
Postion in GRL
100
80
60
40
20
0
D to D
D to V
V to V
V to D
C
PGC type production from cultured, grafted GFP+ cells
% GFP+ PGC TH+, CalB+
60
50
40
30
20
10
0
D to D
D to V
V to V
V to D
D
PGC production from cultured, grafted GFP+ cells
% GFP+ neurons PGC
40
30
20
10
0
A to A
A to P
P to P
P to A
E
CalR+ cell production from cultured, grafted GFP+ cells
% GFP+, CalR+
100
80
60
40
20
0
A to A
A to P
P to P
P to A

Figure 10. Neural stem cell potential is cell-intrinsic.

(**A**) Neonatal SVZ progenitors from GFP+ and wild-type mice were cultured for multiple passages, mixed 1:10, and grafted into the SVZ of wild-type P0 host. (**B** and **C**) Quantification of granule cell distribution in the granule cell layer (**B**) and of TH+ (red) or CalB+ (purple) periglomerular cell production (**C**) by GFP+ cells from regions iii/ivD or iii/ivV grafted heterotopically and homotopically into regions iiiD or iiiV. (**D** and **E**) Quantification of periglomerular cell production (**D**) or CalR+ periglomerular (orange) and granule cell (gold) production (**E**) by GFP+ cells from regions iiM or vD/V grafted into regions iiD or vD.

Chapter 6

Concluding remarks and future directions

Overview

Four principal conclusions are presented in this work: 1) adult NSCs of the SVZ are derived from radial glia, 2) the adult SVZ includes not only the lateral wall of the lateral ventricle but also the RMS and portions of the cortex and septum 3) NSCs in different regions of the postnatal SVZ generate different types of OB interneurons, and 4) NSCs of the postnatal SVZ are a diverse set of restricted progenitors that are not respecified when heterotopically transplanted or cultured. Below I discuss some of the implications of these conclusions and suggest further experiments based on these findings that may expand our understanding of NSCs.

Our understanding of the *in vivo* potential of NSCs has been limited by the lack of techniques to specifically label them. To study NSC behavior, investigators have cultured them *in vitro*, grafted them, or nonspecifically labeled them *in vivo* with retroviruses. However, *in vitro* studies place NSCs in a foreign context and test the potential of a progenitor to respond to factors in the culture medium in the absence of normal environmental cues, rather than revealing the true potential of an NSC *in vivo*. Studies in which progenitors are grafted or labeled with retrovirus are complicated by the fact that a mixture of different cell types are labeled and traced. Therefore, these techniques also obscure the true

potential of NSCs *in vivo*. Based on data obtained from using these methods, investigators have assumed that NSCs are able to generate many different neuron types *in vivo*. However, this assumption is inconsistent with the results of my work, in which NSCs from both the neonatal and adult brain were specifically targeted and followed *in vivo*.

From the work I presented above, we have gained a deeper understanding of stem cell potential in the postnatal SVZ, but we still know relatively little about the embryonic NSCs that generate postnatal NSCs. When do embryonic NSCs acquire regional identity? When do they become committed to a particular fate and resistant to respecification? How does their neurogenic potential change over time? Which embryonic NSCs contribute to the adult NSC population?

A technique to specifically label embryonic NSCs

To address these questions, I propose that the radial glial targeting technique used to label neonatal NSCs technique could be adapted to target the radial processes of NSCs in the embryonic mammalian brain. Others have shown that it is possible to visualize and inject specific regions of the embryonic mouse or rat brain *in utero* with the help of ultrasound backscatter microscopy (Butt et al., 2005; Olsson et al., 1997; Turnbull et al., 1995; Wichterle et al., 2001). To permanently label cells, Ad:Cre could be targeted to the distal processes of radial glia in embryonic Cre reporter mice. Alternatively, radial glia of embryonic Cre reporter mice could be labeled by injecting a Cre-expressing

plasmid DNA construct into the parenchyma and inducing transfection by electroporation. Electroporation allows additional flexibility, since it does not require a viral vector and allows the introduction of constructs to overexpress or knock down a gene of interest with relative ease.

One potential drawback to targeting embryonic NSCs is that embryonic brains are smaller than postnatal brains, making it more challenging to cleanly target a specific area. Furthermore, the distance between the injection site and the radial glial cell body would be smaller than in the neonatal brain, raising the possibility that NSCs might be labeled nonspecifically by diffusion of the virus or plasmid to the VZ. To enhance the specificity of the technique, radial glia could be labeled combinatorially, from both the parenchyma and the ventricle. For example, a wild-type brain could be injected with Ad:Cre targeted to the radial process and a retrovirus containing a floxed reporter gene targeted to the lateral ventricle. Only dividing cells would be infected by the retrovirus, which would integrate into the genome, and only cells also infected by Ad:Cre would recombine the reporter gene in the retroviral genome. This approach would specifically label dividing, ventricle-contacting cells with long radial processes in a restricted spatial location. Lentiviruses, which integrate in the host cell genome but can also infect non-mitotic cells, could be used rather than retrovirus to increase the efficiency of this technique if too few labeled cells are obtained.

Once cells have been labeled *in vivo*, injected animals would be sacrificed at the desired time points and labeled cells could be examined. It would also be

possible to microdissect labeled cells at different time points and culture or graft them. Potential applications of this technique are described below.

The developmental origin of the neurogenic zone

The origins of the adult neurogenic zone are unclear. However, it is necessary to understand the developmental origins of different adult NSCs in order to determine how neurogenic niches are formed, how NSCs are maintained into adulthood, and what genes regulate their neurogenic potential. Based on anatomic, genetic and functional similarities, it is thought that embryonic LGE gives rise to the adult lateral ventricular wall (see Chapter 1, Section 1, "Tangential migration and the generation of OB interneurons"). However, data presented here and in recently published work (Kohwi et al., 2007; Ventura and Goldman, 2007) demonstrates that pallial and septal regions also contribute to OB neurogenesis in the postnatal brain. Therefore, the adult SVZ is likely derived from the LGE as well as other regions of the embryonic telencephalon. These other embryonic regions could be identified by systematically targeting NSCs in discrete locations of the embryonic brain as described above, allowing injected mice to survive into adulthood, and determining which labeled regions continue to generate OB interneurons in the adult brain. Based on these experiments, a fate map of the adult SVZ could be constructed.

This fate map could have well-defined borders early in embryonic development, or it could evolve gradually by the progressive loss of progenitors that produce OB interneurons. To distinguish between these possibilities,

different regions of the embryonic mouse brain could be targeted as described above, and mice could be sacrificed different time points after injection. Once the origin of each neurogenic subregion is known, progenitors within and without the developing SVZ could be compared by gene chip analysis to identify genes that may specify adult NSCs or regulate self-renewal.

The heterogeneity of NSC lineages

The discovery that cortical radial glia generate OB interneurons is surprising, particularly since cortical interneurons are derived from the MGE rather than the cortex. Additionally, cortical NSCs were previously thought to be depleted at the end of gliogenesis when radial glia terminally differentiated into parenchymal astrocytes (Schmechel and Rakic, 1979; Voigt, 1989) (Fig. 1A). Cortical GFAP+ cells produced OB interneurons but did not appear to migrate tangentially when targeted with Ad:GFAP-Cre in the adult brain, but their developmental origin is unclear. It has been suggested that embryonic cortical progenitors can migrate into the SVZ (Willaime-Morawek et al., 2006), so one possibility is that subcortical progenitors can also migrate into the cortex. However, this hypothesis is not supported by work demonstrating that neonatally targeted NSCs do not migrate tangentially (Ventura and Goldman, 2007) (see Chapter 4). To directly test this hypothesis, subcortical radial should be specifically targeted in the embryonic brain and traced into the adult brain.

An alternative hypothesis is that cortical NSCs do not migrate but are heterogeneous; many cortical NSCs may disappear at the end of histogenesis

but some may display a neurogenic potential that resembles that of subcortical NSCs over long periods of time postnatally (Fig. 1B). These OB interneuron-producing cortical progenitors may express transcription factors thought to be specific to subpallial structures. For example, cells expressing both the Emx1, Dlx2 and Dlx5/6+ are found at the rostral pallial/septal boundary (Kohwi et al., 2007). This overlap of gene expression becomes more pronounced at the anterior pallial/subpallial boundary as the SVZ transitions into the RMS (John Rubenstein and colleagues, personal communication). Certain genes, such as Pax6 are expressed at high levels in the cortex but their expression spills down into LGE, albeit at lower levels. It is possible that a transcription factor that plays an important role in determining adult NSC identity and OB interneuron fate is expressed at high levels subcortical structures and at lower levels in the pallium. Such a transcription factor has not been identified and the expression of subcortically-specific genes such as Dlx1/2/5/6 and Gsh1/2 drops sharply at the pallial/subpallial boundary. However, perhaps even low or transient expression of these genes at early developmental time points is sufficient to specify some pallial cells to produce OB interneurons.

To test the latter hypothesis, known subcortically-expressed genes could be overexpressed in the embryonic cortex along with a marker gene, and traced into adulthood *in vivo*. This experiment would determine if these genes are sufficient to specify OB interneuron production and to maintain these progenitors as adult NSCs. Also, these genes could be overexpressed at different developmental time points to determine when progenitors are specified to remain

postnatal NSCs. Finally, cortical progenitors, subcortical progenitors, and cortical progenitors expressing subcortical transcription factors could be cultured and followed by time lapse video microscopy. This would allow their lineages to be reconstructed and directly compared. The results of these studies would inform future efforts to experimentally direct NSCs to produce particular neuron types.

NSC potential restriction and plasticity

It is widely accepted that the neurogenic potential of NSCs changes over time (see (Temple, 2001) and Chapter 1, Section 1, "Temporal regulation of NSC potential"). However, the data presented here demonstrate that the potential of neonatal radial glia to produce different types of OB interneurons does not change appreciably as they transform into adult astrocyte-like stem cells. As discussed above (see Chapter 3, Discussion, "Heterogeneity vs. temporal shift in potential"), it is likely that neonatally-labeled radial glia are a heterogeneous population of cells. Some radial glia are postmitotic and committed to producing ependymal cells or parenchymal astrocytes, and some remain neurogenic in the neonate and the adult. Transplantation and adult labeling experiments suggest that neurogenic neonatal radial glia are committed to generating certain cell types and maintain this commitment into adulthood. It should be noted that the OB interneurons types were distinguished by morphology and marker genes which may not have captured the respecification of NSCs to other OB interneuron subtypes or indeed other cell types such as astrocytes or oligodendrocytes, which were not examined. NSC potential is likely to be

dynamic in the embryo as the brain is being actively patterned. However, together my findings suggest that the potential of adult NSCs to produce the OB interneuron types examined has been restricted before birth.

It is not known when in development this restriction takes place or what mechanisms are involved. To determine how the potential of a NSC changes over time, embryonic NSCs in a particular region could be specifically labeled at different time points and their progeny could be followed *in vivo*. To determine if their potential at a particular time point is restricted or plastic, labeled NSCs from a particular region could be challenged by transplanting them into the same region of a younger or older brain. This analysis may elucidate the temporal profile of NSC lineage restriction. Furthermore, it may pinpoint a window in time when NSCs can be manipulated by a particular environmental or intrinsic factor. For example, it has been shown in culture that NSCs are able to generate earlier-born cell types when the transcription factor Foxg1 is knocked down at E12 but they seem to be committed by E15 (Shen et al., 2006). This finding supports the idea that mammalian NSCs gradually become more committed and lose the ability to be respecified.

Finally, it may be possible to identify genes involved in regulating NSC potential by labeling NSCs in a particular region, microdissecting labeled cells at different time points, and comparing their gene expression profiles by gene chip analysis. The effect of candidate genes could then be determined by overexpression or knockdown in vivo, and lineage tracing of targeted NSCs.

The *in vivo* potential of an individual NSC

The potential of an individual NSC is not known. NSCs may produce astrocytes, oligodendrocytes, and neurons *in vivo* (Fig. 2A) as they do in the presence of high levels of growth factors *in vitro*. However, these culture conditions can alter their potential such that they no longer resemble NSCs *in vivo* (Gabay et al., 2003). Alternatively, NSCs may be a heterogeneous population of restricted progenitors *in vivo* and only appear to be homogeneous because of their combined potential (Fig. 2B). Yet another possibility is that they are truly multipotent *in vivo*, but only produce certain cell types in response to physiologically relevant environmental stimuli (Fig. 2C).

To sort out these possibilities, individual NSCs and their progeny must be labeled and tracked *in vivo*. This has previously been accomplished by infecting progenitors with a library of viruses, where each virus carries a marker gene and a unique genetic tag (Cepko et al., 1995; Walsh and Cepko, 1992). Cells labeled by the marker gene were then dissected, and their DNA was extracted and purified to identify the genetic tag by PCR. However, this process is laborious and problematic since viral particles sometimes clump together, leading to multiple infection of individual clones.

A new tool has recently emerged that may facilitate *in vivo* clonal analysis: the Brainbow mouse, generated by Josh Sanes and Jeff Lichtman (personal communication with Jeff Lichtman). Brainbow transgenic mice carry multiple copies of a cassette containing four different colored fluorescent proteins, each flanked by a loxP site. Cre recombinase stochastically recombines

these sites, generating diverse combinations of fluorescent proteins. Up to 70 different colors can be reliably distinguished by the human eye (Jeff Lichtman, personal communication), so the recombination of a small number of progenitors would allow several unique NSCs and their progeny to be identified simultaneously. This would allow lineages of stem cells in different regions to be reconstructed and compared. The current strain of Brainbow mice express fluorescent proteins under the Thy1 promoter, which becomes active postnatally in only a subset of neurons. Therefore, another Brainbow strain that uses an embryonically expressed ubiquitous promoter must be developed in order to carry out the proposed lineage tracing experiments.

A model of postnatal NSC potential

Though specific labeling of NSCs in the neonatal and adult brain, it is now clear that different regions of the SVZ generate different types of OB interneurons. Since populations rather than individual labeled cells were examined, no conclusions can be drawn about the potential of individual NSCs except that they do not behave like a homogeneous population of multipotent stem cells (Fig. 3A). Therefore, NSCs are likely a diverse population of more restricted progenitors, although it remains unclear whether they are restricted to producing only one type (Fig. 3B) or several types (Fig. 3C) of OB interneuron.

After specific labeling of neonatal radial glia or adult GFAP+ progenitors, three domains can be appreciated: a rostral/septal (R) domain where CalR+ GCs and PGCs were produced, a dorsal (D) domain where superficial GC and TH+

PGCs were produced, and a ventral (V) domain where deep GCs and CalB+ PGCs were produced (see Chapter 4, Fig. 6). In each of these regions, both GCs and PGCs are produced. This suggests that each domain may contain progenitors capable of producing both GCs and PGCs. These progenitors will be referred to as type R, type D and type V progenitors, named for the SVZ domain they occupy (Fig. 3C). This interpretation is supported by evidence that superficial GCs express mRNA for TH (Baker et al., 2001; Saino-Saito et al., 2004), though the functional enzyme cannot be detected by immunohistochemistry. Thus, superficial GCs and TH+ PGCs - which are produced in the same domain - share a common set of genes and may be derived from a common (type D) progenitor.

This model, however, cannot explain why so many PGCs are produced in the medial wall (region iiM) or why so few, if any, PGCs are produced in caudal and ventral regions. It remains possible, though, that environmental factors encountered earlier in development biased progenitors in different regions to alter their production of PGCs. Environmental factors in the neonatal SVZ are unlikely to regulate the production of PGCs, since NSCs grafted from rostral regions continued to produce PGCs when grafted to the caudal SVZ, and caudal progenitors did not gain the ability to make PGCs when grafted to the rostral SVZ. To determine which model most accurately describes NSC potential in the postnatal brain, it will be necessary to label and trace the lineage of individual NSCs.

Conclusion

The data presented in this dissertation challenge the traditional view that neural stem cells are homogeneous, plastic, and multipotent progenitors. NSCs in different regions of the postnatal neurogenic niche appear to be restricted and committed to producing different neuron types. Although this finding narrows our view of NSC potential, it brings us a step closer to an accurate understanding of their *in vivo* capabilities. With this new insight, we may be able to design more effective therapeutic strategies based on the true in vivo capabilities of NSCs. Also, with the techniques introduced to label and genetically manipulate NSCs, we are now armed with new tools to examine the molecular mechanisms regulating stem cell potential and plasticity. Ultimately, this knowledge may lead to a more comprehensive understanding of neural stem cell biology and provide a foundation for future efforts to manipulate stem cells.

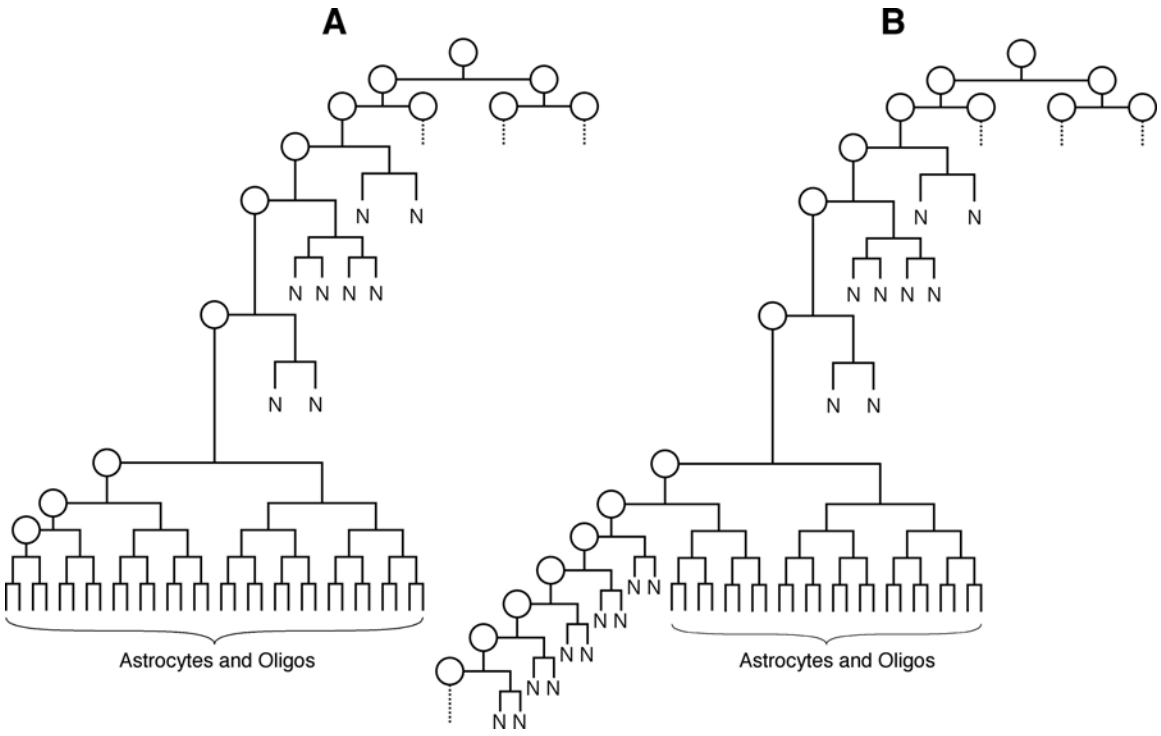

Figure 1. Schematic cell linage of cortical and adult neurogenic cortical progenitors. **(A)** Schematic of the cell linage of NSCs (white circles) of the cerebral cortex. NSCs first divide symmetrically, then divide asymmetrically to generate neurons (N), then generate astrocytes and oligodendrocytes (oligos) and finally are removed from the stem cell pool by terminally differentiating into astrocytes. Adapted from (Temple, 2001). **(B)** Proposed cell linage of a cortical NSC that continues producing OB interneurons in the adult. Rather than differentiating into a parenchymal astrocyte, the NSC (white circles) remains neurogenic. The actual lineage of cortical NSCs remains to be determined.

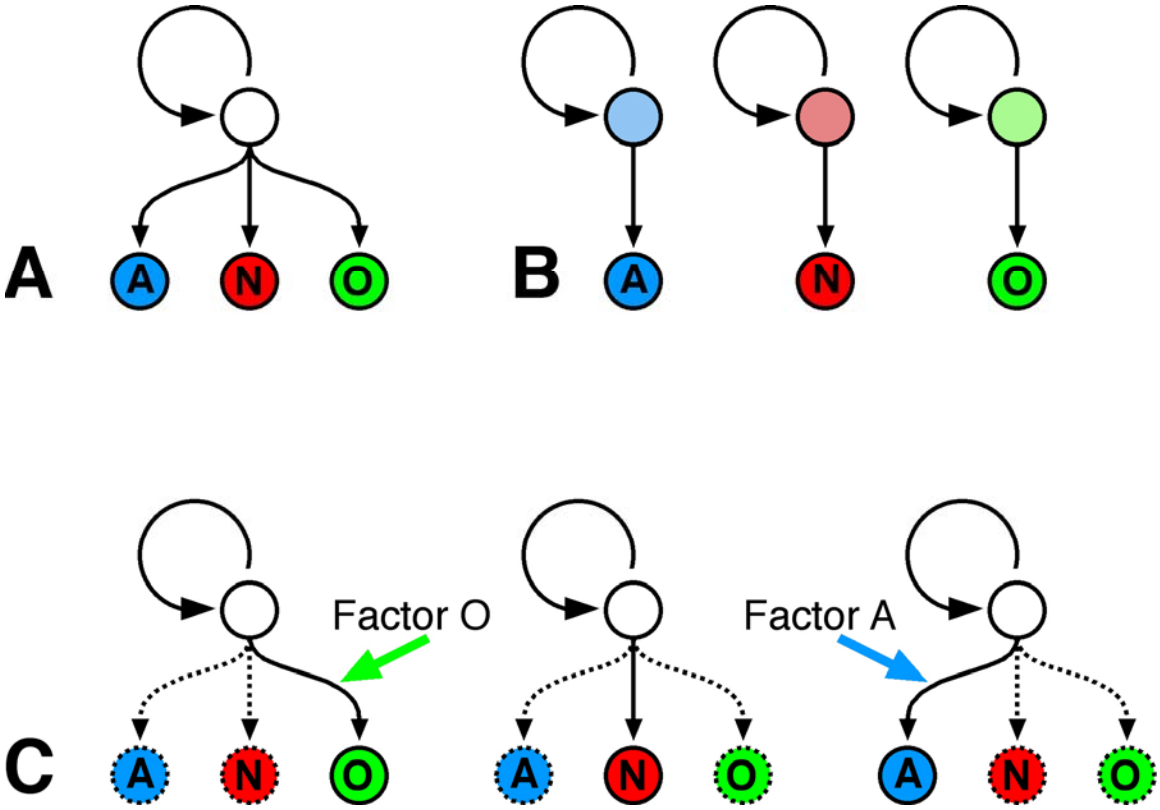

Figure 2. Models of NSC potential in vivo.

(A) NSCs (white circle) may retain the potential to produce astrocytes (A, blue), neurons (N, red), and oligodendrocytes (O, green) under normal conditions in vivo. **(B)** NSCs may be a heterogeneous population of restricted progenitors in vivo, each of which produces only one cell type. Exposure to growth factors in vitro may induce these progenitors to become multipotent. **(C)** NSCs may produce restricted cell types under normal conditions in vivo, but retain the potential to produce astrocytes, neurons, and oligodendrocytes. Exposing NSCs to growth factors in vitro may reveal this latent potential. NSCs may be biased toward a particular lineage by endogenous factors.

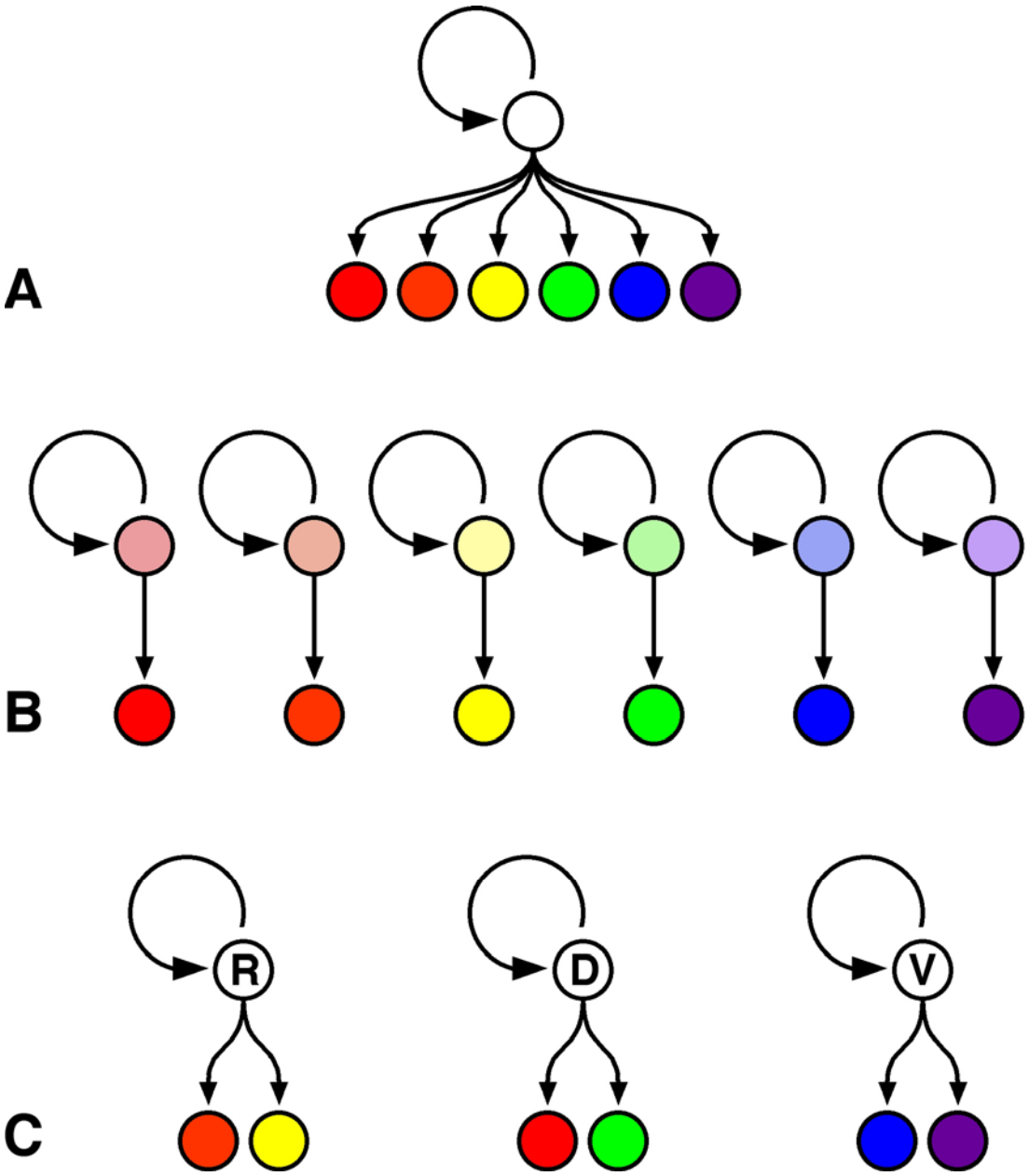

Figure 3. Models of NSC potential to produce OB interneurons.

(A) It is widely thought that NSCs (white circle) are able to produce TH+ PGCs (red), CalR+ PGCs (orange), CalR+ GCs (yellow), superficial GCs (green), deep GCs (blue), and CalB+ PGCs (purple). **(B)** NSCs may be a heterogeneous population of restricted progenitors, each of which produces only one neuron type. **(C)** NSCs in rostral/septal (R), dorsal (D), and ventral (V) domains may have the potential to produce both GCs and PGCs.

Chapter 7

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