EXPLORING A FUNCTIONAL DISCONNECT BETWEEN

NESTIN-EXPRESSING TYPE-1 CELLS AND

ADULT HIPPOCAMPAL NEUROGENESIS

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DEDICATION

I would like to thank my friends and family for their unwavering support and infinite patience during my graduate tenure. I would especially like to thank David Sauer, Scott Younger, Gregory Kunkel, Jon Self, and Calli Merkel for support in graduate school; and Chuck Krueger and Todd Faulk for providing a refuge.

EXPLORING A FUNCTIONAL DISCONNECT BETWEEN NESTIN-EXPRESSING TYPE-1 CELLS AND ADULT HIPPOCAMPAL NEUROGENESIS

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ABSTRACT

The subgranular zone of the hippocampal dentate gyrus generates new neurons throughout adulthood. The process of adult neurogenesis is well-described, but the source of proliferating progenitors is unknown. One potential source is the Type-1 cell, which is morphologically reminiscent of embryonic radial stem cells and expresses protein markers like nestin. However, there is no direct evidence that Type-1 cells are the source of neurogenesis. For this doctoral research, I asked two questions to gain more insight into the role of Type-1 cells in adult

hippocampal neurogenesis. First, how do stimuli that alter neurogenesis affect nestin-expressing Type-1 cells? Second, what is the contribution of Type-1 cells to neurogenesis under basal and experimental conditions? Using the constitutive nestin-GFP reporter mouse, I assessed how potent positive and negative neurogenic effectors, running, X-irradiation and stress, respectively, affect the number of Type-1 cells. Surprisingly, each stimulus induced little change in Type-1 cell number, though changes in neurogenesis were pronounced. While X-irradiation caused long-term proliferation deficits at all ages, Type-1 cells persisted with moderate, significant decreases in younger (6 weeks at irradiation) but not older mice (10-18 weeks), suggesting that Type-1 cells alone are not sufficient for neurogenesis. Running did not change the number of Type-1 cells but doubled proliferation under basal conditions and following X-irradiation. Additionally, neurogenic changes by social defeat stress were independent of changes in Type-1 cell number. Strikingly, these data also provide that survival of adult-born neurons is critical for long-term depression-like behavior. These data highlight a correlative disconnect between nestin-expressing Type-1 cells and neurogenesis. Additional functional data were derived from conditional (tamoxifen)-inducible, fate-tracking nestin-CreER^{T2}/R26R-YFP mice (Nes-YFP) in which Type-1 cells and their progeny are YFP+. While YFP+ cells produced neurons in the short-term, YFP+ progenitors were depleted at extended times, despite persistence of YFP+ Type-1 cells. In contrast, labeled cells in GLAST-CreER^{T2}/R26R-YFP mice (GL-YFP) maintained YFP+ progenitors and constitutive neurogenesis long-term. After proliferation was chemically ablated,

GL-YFP but not Nes-YFP mice showed recovery of YFP+ progenitors, suggesting that GL-YFP cells are stem-like while Nes-YFP cells are progenitor-like. This correlative and causative evidence that nestin-expressing Type-1 cells are not the source of neurogenesis significantly advances our understanding of the neurogenic process.

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ABC – Avidin-biotin complex reagent

AC3 – Activated caspase 3, also called cleaved caspase 3

ANOVA – Analysis of variance

AraC – Cytosine-β-D-arabinofuranoside, also cytarabine, cytosine arabinoside

BDNF – Brain-derived neurotrophic factor

BLBP – Brain lipid binding protein, also called brain fatty acid binding protein, a marker of radial glial and precursor cells

blbp – Gene for brain lipid binding protein

BrdU – Bromodeoxyuridine, a thymidine analog

CNS – Central nervous system

CORT – Corticosterone

CR – Calretinin

$CreER^{T2}$ – Bacterial cre-recombinase fused to modified estrogen receptor

DCX – Doublecortin, a marker of proliferative neuroblasts and immature neurons

DG – Dentate gyrus of the hippocampal formation

DNA – deoxyribonucleic acid

eGFP – Enhanced green fluorescent protein

FACS – Fluorescence-activated cell sorting

floxed – Flanked by loxP sites

GCL – Granule cell layer

GCV – Ganciclovir

GFAP – Glial fibrillary acidic protein, a marker of astrocytes and neural stem cells

gfap – Gene for glial fibrillary acidic protein

GFP – Green fluorescent protein

GLAST – Glutamate aspartate transporter, also EAAT1 or SLC1A3, a marker for astrocytes and neural stem cells

GL-YFP – GLAST-CreER^{T2}/R26R-YFP inducible transgenic mouse line

GOI – Gene of interest

ICV – Intracerebroventricular

IF – Intermediate filament

IHC – Immunohistochemistry

IP – Intraperitoneal

iPS – Inducible pluripotent stem cells

IR – Immunoreactive, cells detected by immunohistochemistry

IRR – Irradiation

Mol – Molecular layer

mRNA – Messenger RNA

NDS – Normal donkey serum

Nes-YFP – Nestin-CreER^{T2}/R26R-YFP inducible transgenic mouse line

NeuN – Neuronal nuclei, a marker for immature and mature neurons

OB – Olfactory bulb

oGCL – Outer granule cell layer

OPC – Oligodendrocyte precursor cell

PFA – 4% paraformaldehyde in 0.1M PBS

PBS – Phosphate-buffered saline

PCR – Polymerase chain reaction

qRT-PCR – Quantitative real-time polymerase chain reaction

R26R – Rosa26 reporter

RC – Radial cell with morphology similar to radial glia

RMS – Rostral migratory stream

RNA – Ribonucleic acid

RT – Room temperature

SDS – Sodium dodecyl sulfate

SEM – Standard error of mean

SGZ – Subgranular zone of hippocampal dentate gyrus

Sox2 – Sex-determining region on chromosome Y (SRY)-related high mobility group (HMG) box-2

sox2 – Gene for Sox2

SVZ – Subventricular zone of the lateral ventricles

TAM – Tamoxifen

TBS-T – Tris-buffered saline with 0.1% Tween-20

tk – Thymidine kinase

TSA – Tyramide signal amplification reagents

vim – Gene for vimentin

YFP – Yellow fluorescent protein

yfp – Gene for yellow fluorescent protein

CHAPTER ONE

Adult hippocampal stem cells *in vivo*: Hope or hype?

Adapted from: **DeCarolis NA**, Eisch AJ. "Adult hippocampal stem cells *in vivo*: Hope or hype?" *Manuscript in preparation for The Scientific World Journal.*

Abstract

Researchers from a broad range of fields are exploring the potential of the adult brain to grow new neurons in the hippocampus, a structure that regulates functions from memory formation to mood regulation and that is implicated in many psychiatric disorders. However, fundamental questions regarding the identity of the stem cells that are the putative source of these neurons remain unanswered. In this chapter, I review the important overlaps – and potential disconnects – between definitions of stem cells *in vivo*, *in situ,* and *in vitro.* To set the stage for the specific aims of my doctoral research, I also identify key questions that need to be answered to maximize the potential clinical impact of stem cell research for psychiatric and other neurological disorders.

Introduction

Few fields of science have moved as rapidly and held such translational promise as the field of adult neurogenesis. First discovered by Joseph Altman and his colleagues almost fifty years ago (Altman and Das, 1965; Altman and Das,

1967), the study of the birth of new neurons in the brain of sexually mature mammals has increased enormously. For example, the number of articles in PubMed that use the phrase “adult neurogenesis” has increased exponentially yearly from the 1980’s through present, with more than 600 publications expected in 2010 (**Figure 1.1**). While the field has been marked by controversy on the extent, location, and function of adult-generated neurons (Gould, 2007; Rakic, 1985; Rakic, 2002; Tanaka and Ferretti, 2009), a few key facts have emerged. As reviewed briefly below, at least two regions of the mammalian brain maintain the ability to generate new neurons throughout adult life (Boldrini et al., 2009; Eriksson et al., 1998; Manganas et al., 2007; Tonchev et al., 2005): the subgranular zone (SGZ) of the dentate gyrus of the hippocampal formation and the subventricular zone (SVZ) of the lateral ventricle. Both regions contain putative stem cells that proliferate and give rise to new neurons that are demonstrably important in memory, mood regulation, and other fundamental brain functions (**Figure 2A**) (reviewed in Abrous et al., 2005; Chojnacki et al., 2009; Duan et al., 2008; Johnson et al., 2009; Kriegstein and Alvarez-Buylla, 2009; Ming and Song, 2005).

As a relatively young field, it is striking how much impact research into adult neurogenesis has had in broad fields like biology, cancer, even neurology and psychiatry (Bao et al., 2006; Barani et al., 2007; DeCarolis and Eisch, 2010; Deleyrolle and Reynolds, 2009; Sutter et al., 2007; Vescovi et al., 2006). Not only has adult neurogenesis research expanded our understanding of normal brain

function (Fan et al., 2007; Hattiangady et al., 2005; Kempermann et al., 1997; van Praag et al., 2005), but also has helped to advance our understanding of pathological states like neurodegenerative diseases (Falk and Frisen, 2005; Greenberg and Jin, 2006; Lie et al., 2004; Lim et al., 2007; Miller, 2006; Yasuhara et al., 2006; Zhao et al., 2008) as well as psychiatric disorders (DeCarolis and Eisch, 2010; Eisch and Harburg, 2006; Encinas et al., 2006; Kempermann et al., 2008; Noonan et al., 2008).

While the study of adult neurogenesis likely helps pave the way to harnessing neuronal growth in regeneration and repair, a major obstacle has become increasingly obvious: researchers do not fully understand the identity, location, or characteristics of “stem cells” in the adult brain. Given the high-paced speed of adult neurogenesis research and the financial and intellectually resources already committed to this field, it is rather striking that this question – what is the adult stem cell *in vivo*? – remains unanswered. Therefore, it is timely to provide this perspective on what is known and what remains to be discovered about the putative source of adult neurogenesis. Here I briefly describe the process of neurogenesis in adult brain, focusing on hippocampal neurogenesis. I review the technical limitations that have hampered complete understanding of the function of hippocampal stem-like cells *in vivo*, and discuss recently developed tools and concepts that hold immense promise for expanding this understanding. I conclude by detailing fundamental questions that must be addressed before clinical hopes of regenerative medicine using hippocampal stem-like cells might

be realized, and thus set the stage for the specific aims that were the focus of my thesis research.

The process of adult neurogenesis: an overview

Within the subgranular zone (SGZ) of the hippocampal dentate gyrus (**Figure 1.2B**), cells with neurogenic potential are thought to progress through stages of development, including proliferation, maturation, and survival (**Figure 1.3**) (Encinas et al., 2006; Kempermann et al., 2004; Ming and Song, 2005; Overstreet-Wadiche and Westbrook, 2006). In the mouse, evidence suggests that putative hippocampal stem cells (called radial glia, Type-1 cells, Quiescent Neural Progenitors, or Type-B cells; hereafter called Type-1 cells) divide asymmetrically to produce a transiently-amplifying daughter cell with limited self-renewal (called Type-2 cells, Transiently Amplifying Neural Progenitors, or Type-D cells) (Mignone et al., 2004; Seri et al., 2004; Seri et al., 2001), although some recent papers have challenged the linearity of this relationship (Singer et al., 2009; Suh et al., 2007). These neural precursor cells give rise to neuroblasts, some of which produce immature neurons (Steiner et al., 2006). While neural precursors clearly divide, it remains unclear if a neural precursor cell can directly mature into a neuroblast, or if a neural precursor cell divides asymmetrically to produce a neuroblast (**Figure 1.3**). Similarly, it remains unknown if some neuroblasts begin differentiation and become post-mitotic, or if some neuroblasts divide to produce post-mitotic immature neurons that subsequently mature. Regardless, progeny of amplifying progenitors undergo fate selection, likely as

early as the precursor cell phase (Gao et al., 2009; Kuwabara et al., 2009; Roybon et al., 2009; Steiner et al., 2006). Early after cells become post-mitotic, they either die through apoptosis (Dayer et al., 2003; Kuhn et al., 2005; Sun et al., 2004) or undergo differentiation. As post-mitotic cells differentiate and mature, they extend processes into the molecular layer and into the hilus, which eventually become dendrites and nascent axons, respectively (Esposito et al., 2005; Overstreet-Wadiche and Westbrook, 2006; Steiner et al., 2008; Zhao et al., 2006). Over the course of weeks to months, adult-born neurons incorporate into hippocampal circuitry as mature dentate gyrus granule cells (van Praag et al., 2002b; Zhao et al., 2006). These stages are inherently dynamic, but few tools exist to follow an individual cell through its division and maturation. Even so, much information has been gained by assessing cell populations over the course of neurogenesis.

The focus of this review is the SGZ, but the other neurogenic region of the adult brain – the SVZ – deserves brief mention. SVZ neurogenesis has been the subject of several excellent reviews (Alvarez-Buylla and Garcia-Verdugo, 2002; Chojnacki et al., 2009; Whitman and Greer, 2009). The lateral walls of the adult ventricle contain astrocyte-like stem cells (called Type B cells), which produce transiently amplifying progenitors (called Type A cells; **Figure 1.2C**) (Doetsch et al., 1999a; Doetsch et al., 1999b). Type A progenitors give rise to neuronally-restricted Type C progenitors that migrate from the ventricular region towards the olfactory bulb (OB) along the rostral migratory stream (RMS), supported by a

network of blood vessels and ensheathed by Type B cells (**Figure 1.2D**) (Doetsch and Alvarez-Buylla, 1996; Lois and Alvarez-Buylla, 1994; Lois et al., 1996). Once the Type C progenitor cells reach the olfactory bulb, they migrate tangentially and differentiate into inhibitory granule cells or excitatory periglomerular cells (**Figure 1.2E**) (Beech et al., 2004; Loseva et al., 2009; Whitman and Greer, 2009). While the majority of adult-born neurons are olfactory bulb granular cells, the SVZ-RMS-OB network provides a unique area of study, because surviving progenitor cells purportedly must selectively differentiate into different neuronal cell types. Therefore, study of the OB facilitates deeper understanding in fate selection and differentiation.

Other regions of the adult brain reportedly give rise to new neurons as well, though no widespread consensus exists (Gould, 2007). Among the most controversial was the finding of adult-born neurons within the mammalian cortex, supported by some (Bedard et al., 2006; Bernier et al., 2002; Cameron and Dayer, 2008; Gould et al., 2001; Kaplan, 1981) but not others (Kornack and Rakic, 2001; Nowakowski and Hayes, 2000; Rakic, 1985; Rakic, 2002). Evidence suggests that cortical neurogenesis can occur after injuries like stroke (Gregorian et al., 2009; Gu et al., 2000; Zhang et al., 2006). Neurogenesis within the rodent hypothalamus has also been reported by the Flier group (Kokoeva et al., 2005; Kokoeva et al., 2007) and supported by others (Pierce and Xu, 2010; Xu et al., 2005). The subcallosal zone is another adult brain region with the capacity for neurogenesis, though the predominant cells generated are oligodendrocytes

(Laskowski et al., 2007; Seri et al., 2006). The cerebellum is another site that has recently been reported to have adult-born neurons (Chen et al., 2009; Ponti et al., 2008), an effect not observed by others (Lagace et al., 2007b). While these putative sites with adult-born neurons remain intriguing and noteworthy in hopes for future regenerative medicine, further experimental validation is necessary.

Type-1 cells: source of adult-generated hippocampal neurons?

The section above reviewed the great strides made in understanding the process of adult hippocampal neurogenesis (**Figure 1.3**). While much research has focused on the process of progenitor cells dividing, migrating, and ultimately differentiating into functional new neurons, researchers are only beginning to understand the stem cells that underlie neurogenesis in the adult brain. Here, I review the existing literature to support our claim that the putative hippocampal stem cell, the Type-1 cell, is poorly understood in its functional contribution to neurogenesis and in its cellular physiology, and lay out issues that need to be clarified or addressed in future research. Specifically, the major question plaguing researchers is the source of SGZ neurogenesis. While the Type-1 cell is the hypothesized stem cell, direct evidence linking it to neurogenesis is limited and remains largely correlative.

Type-1 cell characteristics and antigenicity

Within the SVZ, adult stem cells are radial glial cells that persist after embryonic development (Merkle et al., 2004). The little that is known about SGZ Type-1 cells also suggests that they maintain characteristics of embryonic radial glial cells, including **morphology** and **antigenicity**, which suggests an embryonic origin of these cells (Kriegstein and Alvarez-Buylla, 2009). Morphologically, Type-1 cells have an irregularly shaped or triangular soma and a vertical process that extends through the dentate gyrus granule cell layer to end in a tuft of fine processes (**Figures 1.2B, 1.3**) (Filippov et al., 2003; Liu et al., 2006; Mignone et al., 2004; Seri et al., 2001). The tuft of fibrils remains poorly understood, but may act as a sensor for signaling from the perforant pathway (Filippov et al., 2003; Seri et al., 2001). The electrophysiological properties of Type-1 cells have not been well studied, but preliminary analysis indicated that Type-1 cells generally have similar physiology to astrocytes (Filippov et al., 2003; Fukuda et al., 2003; Tozuka et al., 2005).

To understand Type-1 cells, researchers ideally study them within the unique, cellularly heterogeneous microenvironment of the SGZ *in vivo* (Lim et al., 2007; Melvin et al., 2007; Palmer et al., 2000). To do this, researchers need markers to label and track Type-1 cells *in vivo* or *in situ.* However, one of the greatest challenges to understanding neural stem cells has been the lack of unique markers (Pevny and Rao, 2003). While a number of markers have been characterized to delineate the stages of SGZ neurogenesis (**Figure 1.3**), Type-1

cells require labeling with multiple markers or morphologic criteria because all markers identified and characterized to date overlap other cell populations (Cai et al., 2003; Ernst and Christie, 2005; Jin and Galvan, 2007; Kempermann et al., 2004; Seri et al., 2004). Antigenically, Type-1 cells express a number of proteins found in two overlapping cell populations: astrocytes and progenitor cells. Most Type-1 cell markers have been characterized in the adult after their expression had been characterized during embryonic development. Supporting the hypothesis that Type-1 cells are of glial origins (Kriegstein and Alvarez-Buylla, 2009), several astrocytic and radial glial proteins are expressed in Type-1 cells, including glial fibrillary acidic protein (GFAP), glutamine-aspartate transporter (GLAST), vimentin, and brain lipid binding protein (BLBP, also called brain fatty acid binding protein, BFABP) (Anthony and Heintz, 2008; Anthony et al., 2004; Bachoo et al., 2004; Hartfuss et al., 2001; Mori et al., 2006; Namba et al., 2005; Seri et al., 2004; Seri et al., 2001; Steiner et al., 2006). In addition, Type-1 cells express proteins found in stem cell and early progenitor populations, including nestin, SRY-related HMG box gene-2 (Sox2), Pax6, and Musashi (Ferri et al., 2004; Graham et al., 2003; Heins et al., 2002; Kaneko et al., 2000; Lendahl et al., 1990; Sakakibara and Okano, 1997; Steiner et al., 2006). These latter markers persist into early progenitor cells, too, which may suggest that rapidly proliferating progenitors are also stem-like (Suh et al., 2007), though this awaits further confirmation.

Given the number of proteins that label Type-1 cells (**Figure 1.3**), the three markers have provided key insight into Type-1 cell function and neurogenesis: GFAP, Sox2, and nestin. The use and limits of each of these is discussed below, with an emphasis on the functional importance of the proteins in addition to its distribution in the adult brain. I note that the majority of information regarding markers of Type-1 cells comes from use of transgenic mice, highlighted in **Figure 1.3**. Because so many markers have been characterized for Type-1 cells, an assumption has emerged that all Type-1 cells are antigenically homogenous and include absolute overlap between various markers. However, there is accumulating evidence that indicates the heterogeneity of Type-1 cells. For example, while GFAP-expressing and Sox2-expressing (hereafter, GFAP+/Sox2+) cells with radial morphology are considered Type-1 stem cells (e.g. Lagace et al., 2007b; Naylor et al., 2008; Suh et al., 2007) and, similarly, nestin+/Sox2+ radial cells are Type-1 cells (e.g. Lagace et al., 2007b; Steiner et al., 2006), recent reports indicate that some GFAP+ radial cells are nestin-negative (Kempermann et al., 2004) and some nestin+ radial cells are Sox2-negative (Steiner et al., 2006). Recently, Seki and colleagues have noted differences in GFAP+ radial Type-1 cells (Liu et al., 2009; Seki et al., 2007), where a subpopulation of GFAP+ Type-1 cells express the neuronal marker Hu. The authors conclude that a subpopulation of radial glia may already be fated to produce neurons, even at the putative stem cell stage (Seki et al., 2007). Similarly, some SGZ radial glia express the proneural basic helix-loop-helix transcription factor Ascl1 (formerly Mash1)(Kim et al., 2007; Liu et al., 2009). One

explanation for disparities in cell populations may reflect incomplete detection via immunohistochemistry (IHC) of antigens *in situ* like Sox2 or nestin, or non-specific staining of antigens like Hu. Interestingly, recent studies defined heterogeneity of early SGZ progenitor subpopulations by staining for a broad panel of antigens (Roybon et al., 2009)(**Figure 1.3**), however, comprehensive characterization of Type-1 cell protein expression has not been undertaken.

While differences in marker expression in Type-1 cells remain largely unacknowledged, there is growing appreciation of antigenic and functional differences in other neural stem cell pools, including embryonic radial glia during neural development (Anthony and Heintz, 2008; Hartfuss et al., 2001; Kriegstein and Gotz, 2003; Pinto et al., 2008) and location-specific differences in the SVZ stem cell compartment (Alvarez-Buylla et al., 2008; Giachino and Taylor, 2009; Merkle et al., 2007). Studies using *in vitro* cultured stem cells in the neurosphere assay also support heterogeneity of SVZ stem-like cells (Suslov et al., 2002). However, similar functional exploration in the SGZ has been lacking but is much needed. Further, it remains unclear what the SGZ stem cell is *in vivo* and direct links between Type-1 cells and neurogenesis are limited.

Glial fibrillary acidic protein (GFAP): In the initial studies to explore the origins of adult neurogenesis within the mammalian SGZ and SVZ, GFAP was among the first proteins identified in putative stem cells (Doetsch et al., 1999a; Doetsch et al., 1997; Doetsch et al., 1999b; Seri et al., 2004; Seri et al., 2001). This Type-III

intermediate filament is mostly expressed within astrocytes, though there have been reports of expression in ependymal cells in the brain (Doetsch et al., 1997). Characterization of GFAP+ cells as putative stem cells comes from pioneering work by Doetsch and Alvarez-Buylla. In a series of elegant studies using chemical ablation of cell proliferation in the SVZ, GFAP+ cells are the only proliferating cells at the end of chemical treatment (Type B cells, **Figure 1.2C**)(Doetsch et al., 1999a; Doetsch et al., 1999b). Only after several days of recovery do intermediate progenitors emerge (Type C cells), appearance of which precedes the recovery of proliferative neuroblasts (Type A cells). The authors conclude that, because Type B cell proliferation precedes all other cell division in the SVZ that these Type B cells are likely the stem cells (Doetsch et al., 1999a; Doetsch et al., 1999b); similar studies in the SGZ identified GFAP+ radial cells as the first dividing cells whose proliferation preceded progenitor cells (Seri et al., 2001). Moreover, to confirm that new neurons were derived from GFAP+ cells in the SGZ, transgenic mice were infused with a specialized retrovirus, such that only GFAP+ cells could be transduced by the virus and be subsequently labeled. When the SGZ was assessed 4 weeks after viral infusion, new-born neurons were evident, confirming that GFAP+ cells were the origin for neuronal precursors (Seri et al., 2001). The results discuss labeled radial glia as well, but no labeled radial glia are shown, nor is the proximity of labeled neurons to radial glia discussed. Taken together, these data indicate that GFAP+ cells may provide a source for neurogenesis, but do not provide definitive proof that a single source of cells (GFAP+ radial glia) give rise to new neurons.

Other recent studies have assessed the contribution of GFAP+ cells to neurogenesis, providing direct evidence that new neurons are derived from GFAP+ cells. In a series of studies from Sofroniew and colleagues, two different transgenic lines were employed (Garcia et al., 2004). In the first line of mice, dividing GFAP+ cells were ablated chemically by ganciclovir, which resulted in nearly complete elimination of SVZ and SGZ neurogenesis *in vivo* and *in vitro* (**Table 1.1**)(Garcia et al., 2004; Imura et al., 2003). One interpretation proposed was that GFAP+ cells are the origin of adult neurogenesis, but another possibility that cannot be excluded from these studies is that dividing GFAP+ astrocytes provide critical support for neurogenesis. Alternatively, because only short time points were assessed (2 weeks after treatment), neurogenesis could return after long-term recovery, which could suggest a “pre-GFAP” stem cell and evidence exists for such a cell at least in the SVZ (Coskun et al., 2008). To address these limits, the authors used a second, fate-tracking transgenic mouse, in which cells derived from GFAP+ precursors are labeled with a reporter gene. Using thymidine-analog bromo-deoxyuridine (BrdU) and immunohistochemistry (IHC) for the reporter, authors found extensive colocalization of neuronal marker NeuN in both the dentate gyrus and the olfactory bulb (Garcia et al., 2004). These results support that a GFAP+ cell gives rise to adult-born neurons. Together, these results strongly support that adult-born cells are derived from a GFAP-expressing precursor, though it remains unclear if all GFAP+ cells can functionally contribute.

More support for the hypothesis that GFAP-expressing cells give rise to new adult-generated neurons comes from inducible fate-tracking transgenic mice, such as the hGFAP-CreER^{T2} mouse and the GLAST-CreER^{T2} mouse (Ganat et al., 2006; Garcia et al., 2004; Malatesta et al., 2003; Mori et al., 2006; Ninkovic et al., 2007). GFAP-driven fate tracking mice were used in which Cre-recombinase is driven by the *gfap* promoter (**Table 1.1**). Select GFAP+ cells undergo genetic recombination, allowing expression of a reporter gene in the recombined cells and progeny (Ganat et al., 2006; Garcia et al., 2004). The authors show that adult-generated neurons in both the OB and the SGZ were labeled and therefore are derived from GFAP+ cells. These data suggest that GFAP+ stem-like cells produce progenitors that become new neurons or GFAP+ cells eventually differentiate into mature neurons (Ganat et al., 2006; Garcia et al., 2004). Taken together, data on the proliferation of GFAP+ cells have provided the basis for current stance that SGZ stem cells are the source of adult-generated hippocampal neurons (Kempermann et al., 2004; Kriegstein and Alvarez-Buylla, 2009).

Consistent with this stance, *in vivo* ablation of dividing GFAP+ cells resulted in a 95% decrease in neurospheres (Morshead et al., 2003) and isolating GFAP+ cells enriched for sphere-forming cells (Imura et al., 2003; Imura et al., 2006; Pastrana et al., 2009). However, another study characterizing spheres found that less than 5% of primary neurospheres are GFAP+ (Kawaguchi et al., 2001).

Differences in the proportion of neurospheres that are GFAP+ may reflect more the progenitor nature of primary spheres (Doetsch et al., 2002) or may reflect that neurosphere-forming cells are heterogenous (Imura et al., 2006; Kim and Morshead, 2003; Suslov et al., 2002). Further, a recent series of studies indicate that GFAP+/GLAST+ astrocytes in the injured cortex may divide and regain the ability to generate neurospheres (Buffo et al., 2008). Together, most studies suggest that GFAP+ cells *in vivo* can form neurospheres *in vitro*, though these studies mostly rely on the SVZ instead of the SGZ.

While evidence has accumulated indicating that GFAP+ cells contribute to proliferation and give rise to neurons in the adult brain, studies on transgenic GFAP knock-out mice have yielded unexpected results, suggesting that GFAP is neither necessary nor sufficient for adult neurogenesis. Mice lacking GFAP (*gfap*$^{-/-}$ mice) develop grossly normally with relatively minor deficits in synaptic transmission and reactive astrocytosis (reviewed in Messing and Brenner, 2003). Strikingly, in mice lacking both GFAP and stem cell intermediate filament vimentin (*gfap*$^{-/-}$*vim*$^{-/-}$ mice), neurogenesis persists through adulthood, and knock-out mice actually maintain increased levels of SGZ neurogenesis compared to wild type (WT) controls (Larsson et al., 2004; Widestrand et al., 2007). Because stem-like cells maintain functional neurogenesis even in the absence of GFAP, these results suggest that GFAP is not necessary for constitutive neurogenesis (Morshead and van der Kooy, 2004). Other work has suggested that GFAP is not sufficient for neural stem cell activity, because not all GFAP+ cells in the brain

maintain characteristics of stem cells *in vitro* (Bonaguidi et al., 2005; Imura et al., 2006; Kim and Morshead, 2003). Therefore, GFAP may provide a marker for stem-like cells in the SGZ, but it appears that the protein itself is not essential for proliferation or maintenance of SGZ neurogenesis.

In addition to this under-appreciated fact that expression of GFAP alone is neither necessary nor sufficient for stem cell activity, a number of intriguing questions remain about the role of GFAP in regards to adult neurogenesis. For example, it is unclear if only a subpopulation of GFAP+ radial astrocytes maintain multilineage potential *in vitro* and *in vivo*. What makes some GFAP+ cells capable of generating neurospheres, whereas others cannot? Can all GFAP+ astrocytes resume proliferative activity during reactive gliosis?

Sex-determining region-related box-2 (Sox2): Another "marker" for SGZ Type-1 cells that deserves discussion for its role in neurogenesis is Sox2, a transcription factor that plays critical roles in maintenance and self-renewal of both embryonic (reviewed in Boiani and Scholer, 2005; Rizzino, 2009) and neural stem cells (Ferri et al., 2004; Graham et al., 2003). Constitutive knock-out of *sox2* results in early embryonic lethality, emphasizing its crucial role in mammalian development (Avilion et al., 2003). Sox2 is a "master regulator" in embryonic stem cells, and reintroduction of the *sox2* gene with other critical genes into somatic cells results in induced pluripotent stem cells (iPS cells), which have many characteristics of embryonic stem cells (Takahashi and Yamanaka, 2006; Yu et al., 2007). The

findings of these ground-breaking studies have been extended to generate human iPS cells and resulting in an explosion in high-profile research (e.g., reviewed in Pei, 2009; Pera, 2008), including advances in gene therapy and regenerative medicine beyond the scope of this review. In pluripotent embryonic stem and iPS cells, Sox2 acts synergistically with Oct4 (also called Pou5f1) and Nanog, other master stem cell transcription factors, to maintain self-renewal and pluripotent potential (reviewed in Boiani and Scholer, 2005; Boyer et al., 2006; Jaenisch and Young, 2008; Rizzino, 2009). Functionally, Sox2 binds to the promoter region and activates a number of embryonic stem cell-related genes, including *sox2, oct4,* and *nanog* genes to create a feed-forward loop to maintain pluripotency (Boyer et al., 2005; Chew et al., 2005; D'Amour and Gage, 2003; Loh et al., 2006). During differentiation, *oct4* is epigenetically silenced, leading to the down regulation of *sox2* and *nanog*, and the subsequent induction of cell-type specific genes (reviewed in Jaenisch and Young, 2008; Kashyap et al., 2009).

Similar to its role in maintaining embryonic pluripotent stem cells, Sox2 plays a critical role in maintaining the self-renewal and multipotency of neural stem cells both during fetal development and in the adult neurogenic regions (Favaro et al., 2009; Ferri et al., 2004; Graham et al., 2003). Sox2 is also expressed in SVZ stem cells (Alonso et al., 2008; Ferri et al., 2004). Within the SGZ, almost all Type-1 cells expressed Sox2, showing extensive colocalization with GFAP, BLBP, nestin, and Musashi (Steiner et al., 2006; Suh et al., 2007). Similarly, numerous studies have used Sox2 colocalization with GFAP as an index of

Type-1 cells in the SGZ (e.g. Favaro et al., 2009; Ferri et al., 2004; Komitova and Eriksson, 2004; Lagace et al., 2007b; Naylor et al., 2008; Suh et al., 2007). Moreover, most dividing cells in the adult SGZ and SVZ that label with exogenous BrdU or with endogenous cell cycle protein Ki67 are also Sox2+ (Komitova and Eriksson, 2004), though surprisingly, approximately 45% of dividing Sox2+ cells are negative for neurogenic markers like nestin or BLBP (Steiner et al., 2006). Recently, in an elegant series of studies involving limited viral transduction of Sox2-driven Cre recombinase within the mouse hippocampus, Gage and colleagues showed that Sox2+ cells are capable of generating neurons and astrocytes *in vivo*, and that Sox2+ cells maintain self-renewal capacity (Suh et al., 2007). However, these studies cannot discriminate the contribution of Type-1 cells and early progenitor cells and expression of Sox2 within Type-1 cells may only be coincidental to neurogenesis and the authors suggest that Sox2+ early precursors may actually give rise to Type-1 cells. Even so, the general consensus is that Sox2 provides an excellent marker of Type-1 and SGZ progenitor cells, and Sox2 expression is directly and functionally important for maintaining stem and progenitor cells *in vivo* and *in vitro*.

Not only does *sox2* gene expression correlate with Type-1 and progenitor cells, functional data also emphasize that Sox2 plays a critical role in neural stem cell maintenance. Assessing the functional importance of Sox2 in adult neurogenesis has been challenging because knockout of the *sox2* gene results in early embryonic lethality (Avilion et al., 2003). However, the critical importance of Sox2

in brain development has been recently demonstrated using a novel transgenic mouse line when a mutant *sox2* gene is neuronally restricted (Episkopou, 2005; Ferri et al., 2004). These mice develop and survive through birth, but postnatal survival is reduced, major brain malformations are evident, and neurons that do form show marked degeneration (Ferri et al., 2004). When *sox2* gene expression is eliminated in adult mice, Type-1 radial glia are quickly depleted and SGZ proliferation is significantly decreased (Favaro et al., 2009). Similarly, inhibition or knock-down of Sox2 results in cells leaving the cell cycle and differentiating (Graham et al., 2003; Tay et al., 2008) and selective elimination of the *sox2* gene from cells *in vitro* significantly reduces self-renewal of neurospheres (Favaro et al., 2009). Further, Sox2 expression positively correlates with neurosphere formation (Brazel et al., 2005; Suh et al., 2007; Zappone et al., 2000).

One potential mechanism by which Sox2 regulates stem cell function is through transcriptional activation. Indeed, Sox2 regulates expression of a number of multipotent neural stem cell genes including members of the Shh/Wnt/β-catenin signaling pathway and Notch family members (D'Amour and Gage, 2003; Favaro et al., 2009) as well as *blbp* and *nestin* (D'Amour and Gage, 2003; Jin et al., 2009; Tanaka et al., 2004). It is interesting that Sox2 positively regulates expression of genes that are markers of Type-1 cells like BLBP and nestin (**Figure 1.3**) while also inhibiting expression of genes involved in differentiation, like doublecortin and neurogenin (Boyer et al., 2005; D'Amour and Gage, 2003).

Given the important role of Sox2 in maintaining stem cells in the brain, it is surprising that Sox2 expression is pervasive through the brain. Several reports have characterized Sox2 expression in regions of the cortex, in the molecular layer of the dentate gyrus, and in the thalamus (Ferri et al., 2004; Komitova and Eriksson, 2004; Suh et al., 2007), in addition to localization within the neurogenic regions of the adult brain. Phenotypic characterization generally indicates that Sox2+ cells outside of the neurogenic niches are differentiated astrocytes, none of which proliferate under basal conditions (Hattiangady and Shetty, 2008; Suh et al., 2007). It remains to be seen if these Sox2+ astrocytes maintain proliferative potential under specific circumstances, or if Sox2 expression is coincidental and not functionally important. Further, to what extent do Sox2+ non-radial and Sox2+ Type-1 cells differ in their ability to produce progenitors and contribute to neurogenesis? Do all Sox2+ astrocytes maintain the ability to divide but remain quiescent? What are the dividing Sox2+ cells that do not express neurogenic markers like DCX or nestin?

Nestin: Nestin was initially characterized in embryonic radial glia and striated muscle (Hockfield and McKay, 1985; Lendahl et al., 1990), defining Type VI filaments, new class of intermediate filament (IF) because of its unique C-terminus and lack of sequence homology to other IFs (Lendahl et al., 1990). Unlike other IF classes, nestin cannot form homodimers and instead requires proteins from other IF classes, especially vimentin in embryonic radial glia and desmin in muscle progenitors (reviewed in Gilyarov, 2008; Michalczyk and

Ziman, 2005; Wiese et al., 2004). While the function of nestin remains unclear, nestin expression correlates in most cells with mitotic potential, such that most embryonic and postnatal cells expressing nestin maintain proliferative capacity whereas differentiating, post-mitotic cells lose nestin expression (e.g. Dahlstrand et al., 1995; Hockfield and McKay, 1985; Lendahl et al., 1990; Mignone et al., 2004; Wei et al., 2002). Because of this correlation between nestin expression and multipotency, nestin has become a widely used marker for neural and other stem cells.

In fitting with its putative localization in stem and progenitor cells, nestin is localized in the neurogenic regions of adult brain, and in the SGZ specifically, where both Type-1 and early progenitor cells are nestin-positive (**Figure 1.3**)(Filippov et al., 2003; Fukuda et al., 2003; Lagace et al., 2007b; Mignone et al., 2004). Nestin expression has also been characterized in other tissues outside the brain, including the retina, hair follicles, endothelial cells, and the heart (e.g. Gilyarov, 2008; Hoffman, 2006; Wiese et al., 2004) and nestin expression in these peripheral tissues is generally correlated with proliferative activity.

While the specific function of nestin remains largely unknown, new evidence suggests that nestin plays a critical role in a cell's proliferative capacity. Recent *in vitro* studies with antisense *nestin* mRNA or siRNA-mediated knock-down of *nestin* show decreases in cell proliferation (Daniel et al., 2008; Wei et al., 2008), lengthening of cell cycle time (Thomas et al., 2004), and decreases in survival

(Huang et al., 2009). Further, nestin expression is correlated with malignancy and aggressiveness in various CNS cancers (Ehrmann et al., 2005; Singh et al., 2004). Recently, nestin knock-out mice have been described (Park et al., 2009), but this study did not explore neurogenesis in the adult. More definitive studies of the functional role of nestin in SGZ neurogenesis are forthcoming.

Even without understanding of the functional importance of nestin, it is clear that nestin+ cells are important for ongoing neurogenesis. Ablation of dividing nestin+ progenitors *in vivo* using transgenic nestin-tk mice results in significant loss of progenitor cells from both the SGZ and the SVZ (**Table 1.1**)(Deng et al., 2009; Singer et al., 2009; Yu et al., 2008), indicating that progenitor cells express nestin. Interestingly, even after 6 weeks post-ablation, SGZ proliferation only recovered partially, though Type-1 cells still persisted (Singer et al., 2009). One interpretation of these data is that Type-1 radial glia cells may not divide or may not directly give rise to progenitor cells. In the same study, neurospheres were derived from the SVZ and results suggest that killing nestin+ cells eliminates most (but not all) neurospheres (Singer et al., 2009). These results suggest that most neurosphere-forming cells are nestin+ *in vitro* (and likely *in vivo*) but there are some neurospheres derived from nestin-negative precursor cells. These results fit well with other reports on the neurosphere forming potential of nestin+ cells in the adult SVZ, which is enriched by 5- to 7-fold over nestin-negative cells (Kawaguchi et al., 2001); another study found nestin+ cells enriched 1000-fold (Mignone et al., 2004). However, formation of multipotent neurospheres from

nestin-negative cells is also observed (Kawaguchi et al., 2001; Kukekov et al., 1997; Mignone et al., 2004; Singer et al., 2009). Together these studies suggest that while some nestin-negative cells may be neurogenic, the majority of neurosphere forming cells and new neurons in the brain are derived from nestin+ progenitors.

While nestin expression is well described in proliferating cells, there is also growing evidence that nestin is also expressed in non-neurogenic, non-proliferative cells. In most adult brain regions other than the SGZ and SVZ, nestin expression does not correlate with proliferative capacity (Dahlstrand et al., 1995; Ernst and Christie, 2005), with nestin protein expression characterized in tanycytes around the third ventricle as well as a number of other brain nuclei (Clarke et al., 1994; Ernst and Christie, 2005; Ernst and Christie, 2006; Xu et al., 2009). However, neurogenesis has recently been described in the third ventricle (Kokoeva et al., 2005; Kokoeva et al., 2007), though it remains to be seen if nestin-expressing tanycytes are the source. Interestingly, following brain injuries that induce reactive gliosis, differentiated nestin-negative astrocytes can revert to a proliferative state and coincidentally upregulate nestin (Clarke et al., 1994), though these cells are expressly not neurogenic (Alonso, 2005; Kronenberg et al., 2005; Lin et al., 1995). Therefore, while most nestin+ cells are capable of producing new neurons, there are a number of instances where nestin is expressed in non-proliferating cells and in cells that cannot produce neurons. Moreover, immunohistochemical (IHC) detection of endogenous nestin protein

labels a milieu of cellular phenotypes in the cortex, thalamus and prominently in many blood vessels in the brain and periphery (Amoh et al., 2004; Mokry et al., 2004; Tohyama et al., 1993). As a result, identifying nestin+ cells via IHC remains challenging, and transgenic reporter mice (described below) have allowed researchers to focus on subtypes of nestin+ cells, including Type-1 and early progenitors in the SGZ.

Technical hurdles in neurogenesis research

In vivo techniques

Even with the large number of proteins have been identified in Type-1 cells (**Figure 3**), the putative SGZ stem cell has proven very challenging to study within the physiological context of the rodent brain. In theory, asymmetric division of Type-1 cells produces a daughter progenitor cell while retaining the original Type-1 cell, though direct observation of dividing Type-1 cells is rare (Encinas et al., 2006; Seri et al., 2004). However, until recently it has been impossible to assess this proposed division pattern of Type-1 cells. In fact, much of our current understanding of neurogenesis comes from studies on cellular proliferation and maturation using two techniques: exogenously injected thymidine-analog **bromo-deoxyuridine (BrdU)** and targeted injection of **retroviruses** into the brain (e.g. Eisch and Mandyam, 2007; Imayoshi et al., 2009; Namba et al., 2005; Taupin, 2007; Valero et al., 2005; van Praag et al., 2005; Zhao et al., 2006). However, because neither of these techniques robustly target or label the slowly-dividing

Type-1 cells, Type-1 cells and their contributions to neurogenesis remain largely unknown.

BrdU is a thymidine analog that is incorporated into the DNA of cells in S-phase of mitosis. Consequently, at early points post-BrdU (e.g. 2 hrs), BrdU IHC identifies proliferating cells, whereas at extended times after BrdU (e.g. 4 weeks), it is possible to determine the fate and survival of dividing cells at the time of BrdU injection (e.g. Donovan et al., 2008; Lagace et al., 2010; Namba et al., 2005). One of the challenges of this approach is colocalization of BrdU with markers of Type-1 cells, many of which are filamentous or excluded from the nucleus (Taupin, 2007). As a result, the proportion of BrdU-IR cells that are Type-1 is varies between reports but is generally very low. Most proliferation studies that have specifically assessed Type-1 cells suggest that 2-5% of BrdU-immunoreactive (BrdU-IR) cells are Type-1 cells at a proliferation time point (e.g. Arguello et al., 2008; Huttmann et al., 2003; Kronenberg et al., 2003; Steiner et al., 2006). Indeed, a recent study by the Gage group found no BrdU-IR Type-1 cells under basal conditions (Suh et al., 2007). However, some groups have found considerably more BrdU-IR Type-1 cells, such that 15% of BrdU-labeled cells were Type-1 (Encinas et al., 2006), 30% (Fukuda et al., 2003), or even 60% (Seri et al., 2001). The variability in proportion of proliferating Type-1 cells is likely a direct result of multiple definitions of Type-1 cells and different stringencies used to colocalize markers. For example, the widely-used Type-1 marker GFAP is filamentous and staining is prominent in the radial process,

whereas BrdU is located within the nucleus. As a result colocalization requires three-dimensional analysis using confocal microscopy and rotational assessment (Seri et al., 2001; Suh et al., 2007; Taupin, 2007).

To overcome the difficulties of BrdU-colocalization, retroviral transduction of proliferating progenitors has also been used to studying neurogenesis. Similar to BrdU, retroviruses incorporate into the DNA of dividing cells and have allowed visualization of transduced cells with reporter genes like green fluorescent protein (GFP). In addition, retroviruses also allow researchers to genetically manipulate a subpopulation of SGZ dividing cells. In this way, researchers have been able to assess the fate of dividing cells (e.g. Seri et al., 2001; Zhao et al., 2006), express or inhibit specific genetic factors (e.g. Jessberger et al., 2008a; Lagace et al., 2008; Suh et al., 2007), and characterize the electrophysiological properties of cells that were dividing at the time of infusion (e.g. Alonso et al., 2008; Laplagne et al., 2006; van Praag et al., 2002b). However, retroviruses require invasive surgery, and the number of labeled cells is highly variable between animals, precluding quantification of labeled cells (van Praag et al., 2002b). In addition, because retroviruses incorporate into the DNA of dividing cells only after the breakdown of the nuclear membrane and after DNA replication, only one of the daughter cells retains retroviral labeling after cellular division (Noctor et al., 2001).

BrdU and retroviral labeling techniques have provided great insight into how SGZ proliferation, maturation and survival are dynamically regulated (Ferri et al., 2004; Hattiangady et al., 2005; Jessberger et al., 2005; Johnson et al., 2009; Mak et al., 2007; Pathania et al., 2010; Qu and Shi, 2009). However, while the dynamic regulation of neurogenic stages has been characterized, our understanding of Type-1 cell function *in vivo* remains limited. BrdU-labeling and retroviral techniques have limited use for exploring Type-1 cell function because these radial cells divide infrequently, precluding BrdU labeling and transduction by retroviruses (Kronenberg et al., 2003; Namba et al., 2005; Steiner et al., 2006; Zhao et al., 2006). There are only a few retroviral studies that report viral transduction of Type-1 cells, and transduced Type-1 cells were exceedingly rare (Suh et al., 2007) or were depicted but not discussed (van Praag et al., 2002b); it is unclear why the retroviruses used in these studies transducer Type-1 cells whereas other serotypes do not. As a result, studying SGZ Type-1 cells within the physiological context of the brain has been limited.

Recent advances in imaging are promising for studying neurogenesis within the brain's physiological context. Using **magnetic resonance imaging (MRI)**, Maletic-Savatic, Enikopolov and colleagues defined a unique biomarker signature in rodents unique to neural stem cells, allowing this population to be discriminated from neurons, glia, and other cell types (Manganas et al., 2007). The authors showed that intensity of the biomarker marker positively correlated with neural stem cell number *in vitro* and within the rodent brain in a region-

specific manner, allowing direct assessment of changes in neural stem cell activity within the brain's physiological context. Further, the authors extended these results and could identify neural stem cells within the human hippocampus, showing an age-related decline in the number of neural stem cells (Manganas et al., 2007). Imaging studies like this hold great promise for basic and clinical research, though one of the major limits to the technique so far is a very low signal-to-noise ratio, with almost no detectable signal in adult humans over the age of 30.

In vitro techniques

Considering the difficulties in studying stem cells within the context of the adult brain, researchers have devised *in vitro* methods to explore stem and progenitor cells, specifically isolating cells via **flow cytometry and fluorescence-activated cell sorting (FACS)** (Imura et al., 2006; Kim and Morshead, 2003) or culturing isolated cells as adherent monolayers or floating in the **neurosphere assay (NSA)**(Morshead et al., 1994; Palmer et al., 1997; Reynolds and Weiss, 1992; Rietze and Reynolds, 2006). The use of flow cytometry has generally been used in other stem cell fields, but its use in isolating stem-like cells from the brain is gaining support and has been used in conjunction with the NSA (Kim and Morshead, 2003). By combining both flow cytometry and the NSA, putative stem cells *in vivo* can be efficiently isolated and assessed for stem-like properties *in vitro,* and the general consensus in the field is that stem cells isolated from the brain maintain the potential to form neurospheres, called "neurosphere-forming

cells" for the purposes of discussion (Morshead et al., 1994; Reynolds and Weiss, 1992).

Flow cytometry and FACS have been indispensible tools for immunologists and stem cell biologists alike, though the application of FACS to neural stem cell biology has received relatively little use until recently. In essence, flow cytometry can quantify cells based on a number of physical attributes, including size, shape, and whether the cell is alive. FACS is a specific application of flow cytometry, whereby individual cells can be quantified and isolated based on surface marker expression. Using FACS and characterized surface markers, hematopoietic stem cells can selectively be isolated from bone marrow reproducibly and with relative ease (Morrison et al., 1996 and references therein). One unique property of purported stem cells (including hemapoietic and neural stem cells) is rapid dye efflux in FACS, known as "side population" isolation (e.g. Goodell et al., 1996; Goodell et al., 1997; Hulspas and Quesenberry, 2000; Kim and Morshead, 2003; Murayama et al., 2002). Stem-like cells contain active channels to pump out toxins, resulting in rapid efflux of dye (Goodell et al., 1996; Kim and Morshead, 2003; Zhou et al., 2001) and "side population" isolation results in enriching for neurosphere-forming cells. In addition, FACS has provided a powerful tool for isolating cells expressing fluorescent proteins from transgenic mice (**Table 1.1**), allowing more detailed *in vitro* characterization including as nestin-GFP or GFAP-GFP cells (Ma et al., 2006; Mignone et al., 2004; Pastrana et al., 2009). Recently, FACS has been

used to assess BrdU-labeled cells quantitatively (Balu et al., 2009), though the application to Type-1 cells is notably limited because of limited BrdU incorporation.

Use of FACS analysis has been useful for characterizing surface markers on stem-like cells. For example, some surface markers have recently been identified on stem cells, most notably Lewis X (LeX, also CD15/stage specific embryonic antigen-1, SSEA-1) in the SGZ, SVZ, and embryonic brain (Corti et al., 2005; Hermann et al., 2006; Imura et al., 2006; Kim and Morshead, 2003; Shi et al., 2004) and Prominin-1 (CD133/AC133) in the SVZ and embryonic brain but excluded from the SGZ (Corti et al., 2007; Coskun et al., 2008; Florek et al., 2005; Hermann et al., 2006; Mirzadeh et al., 2008; Pfenninger et al., 2007). In addition to enriching for neurosphere forming cells, FACS has been useful to purify specific cell populations of the neurogenic lineage and has facilitated gene expression profiles of diverse cell populations (Gilley et al., 2009; Hermann et al., 2006).

Flow cytometry has been coupled with the NSA to directly assess markers of sphere-forming cells. Specifically, the NSA has been a well-characterized *in vitro* tool to identify cells that likely maintained stem-like qualities *in vivo*, specifically self-renewal and multipotency (Chojnacki and Weiss, 2008; Jensen and Parmar, 2006; Marshall et al., 2008; Rietze and Reynolds, 2006). Further, the NSA has been used to show that the adult human hippocampus maintains a pool of

multipotent stem-like cells (Eriksson et al., 1998; Johansson et al., 1999; Kukekov et al., 1999; Roy et al., 2000). In essence, to culture cells for the NSA, acutely dissected tissue is minced and enzymatically dissociated to a single-cell suspension, which is subsequently grown under culture conditions to promote proliferation or differentiation of the cells. Self-renewal is assessed by the capacity of a single cell to grow new spheres over serial passages; multipotency is assessed by the ability of a sphere (derived from a single cell) to differentiate into neurons, glia, and oligodendrocytes (Morshead and van der Kooy, 2004; Reynolds and Weiss, 1992; Seaberg and van der Kooy, 2003).

While the NSA has provided a means of assessing stem cell potential *in vitro*, a number of limitations to the assay have been reported. For example, the NSA provides a means to assess subpopulations of stem cells and progenitors but inherently precludes study of the complex microenvironmental factors that comprise the vascular niche, known to significantly influence stem cell behavior (Golmohammadi et al., 2008; Louis et al., 2008; Pevny and Rao, 2003; Reynolds and Rietze, 2005; Seaberg and van der Kooy, 2003). Also, most spheres are rarely derived from a single cell and therefore are not clonal; this limits the assay's use for multi-lineage analysis unless stringent culture conditions are employed (Coles-Takabe et al., 2008; Singec et al., 2006). In addition, it has been reported that culturing cells *in vitro* changes some inherent properties of the cell, which may alter the cell's intrinsic properties and neurosphere-forming potential (Dromard et al., 2007; Gabay et al., 2003). A final cause for concern

with the NSA is that the number of spheres does not quantitatively reflect the actual stem cell number (Golmohammadi et al., 2008; Louis et al., 2008; Pevny and Rao, 2003). Further, reported results from the NSA may be difficult to interpret because the majority of spheres are derived from progenitor – not stem – cells (Doetsch et al., 2002; Reynolds and Rietze, 2005); stringent methodological criteria allow discrimination of stem versus progenitor cells, required for accurate interpretation of stem cells in culture (Doetsch et al., 2002; Reynolds and Rietze, 2005; Singec et al., 2006). To address many of the technical limitations of the NSA, a more stringent assay, called the neural colony forming cell assay, has been developed (Golmohammadi et al., 2008; Louis et al., 2008), though its use has been limited and still requires stem cells to be extracted from their *in vivo* niche for artificial culture conditions.

Further, results from *in vitro* assessments of the hippocampus have been controversial and the existence of a hippocampal neurosphere-forming cell has been hotly contested. Studies from the Gage group and others have determined that multipotent, self-renewing cells can be derived from the hippocampus and passaged serially, concluding that the hippocampus contains *bona fide* stem cells (Gage et al., 1995; Palmer et al., 1997; Walker et al., 2008). In direct contrast, van der Kooy and colleagues have asserted that the SVZ but not the hippocampus contains true stem cells with self-renewal and multipotency (Seaberg and van der Kooy, 2002; Seaberg and van der Kooy, 2003). Recent studies that used the neural colony forming cell assay found that cells from the

early post-natal and adult dentate gyrus cannot form colonies, which suggested the lack of "true" stem cells in the hippocampus (Bull and Bartlett, 2005; Seaberg et al., 2005). However, the same group found that large, clonal neurospheres could be produced under specific conditions (Warner-Schmidt et al., 2008), suggesting that latent stem-like cells might reside in the hippocampus but may not maintain stem-like features under most culture conditions.

Transgenic approaches to study hippocampal stem cells

To overcome technical limits associated with *in vivo* and *in vitro* assessment of stem cell potential within the SGZ, transgenic mice provided new tools and insight into the process and function of neurogenesis. Transgenic mouse lines have been generated for the last two decades (**Table 1.1**), and those lines that are useful for the study of adult neurogenesis include targeted knock-out mice like the *vimentin-/-* mouse (Colucci-Guyon et al., 1994) and random insertion of reporter constructs like the nestin-GFP mouse (Mignone et al., 2004; Yamaguchi et al., 2000). Recent advances in genome manipulation have paved the way for labeling and tracking cell progeny through bacterial Cre recombinase and its ability to selectively recombine target DNA sequences (*loxP*), allowing constitutive recombination in models like GFAP-Cre (Garcia et al., 2004; Gregorian et al., 2009) and inducible recombination like in the nestin-CreER^{T2} transgenic (Battiste et al., 2007; Carlen et al., 2006; Imayoshi et al., 2008; Lagace et al., 2007b). Another type of transgenic mice has greatly informed our understanding is the HSV-tk mouse in which expression of herpes simplex virus

thymidine kinase protein is driven by a gene of interest, such as the nestin-tk mouse (Singer et al., 2009; Yu et al., 2008). Other types of transgenic mice (including inducible doxycycline-driven TetOp effectors in double- and triple-transgenic systems (Yu et al., 2005)) have been used to a limited extent but will not be discussed here, in part because their use has been superseded by inducible fate-tracking Cre-driver mice.

To facilitate the broad utility of recently generated transgenic mice, here I provide a brief overview of discrete elements for transgenic mouse production which is discussed at length elsewhere (e.g. Gaveriaux-Ruff and Kieffer, 2007; Sauer, 1998; Wang, 2009; Yu and Bradley, 2001). First, because transgenic mice require manipulation of genes at the genomic level, the transgene must be incorporated into germline DNA and passed to offspring. Once the transgene DNA construct has been cloned and incorporated into a vector, the DNA must be integrated into the mouse genome. There are two general approaches for integrating the transgenic vector into the host genome: targeted and random. The two critical components of any transgenic animal are the driver and the transgene, which will be briefly discussed. Targeted transgenesis is more difficult than random insertion of a transgene because of the extensive span of native gene sequence required on both the 5' and 3' ends; however, extensive DNA flanking the endogenous gene facilitates genetic recombination and ensures specificity. Targeted genetic approaches either allow elimination of essential gene elements (in the case of a knock-out mouse) or to selectively change the

gene (in the case of flanking the gene with loxP sites ["floxed" gene] or targeted mutations). In knock-out mice, the gene of interest (GOI) is eliminated from all cells constitutively throughout embryonic and postnatal development, but knock-out mice have provided key insight into adult neurogenesis, demonstrating that GFAP and vimentin are neither necessary nor sufficient to maintain neurogenesis (Larsson et al., 2004; Widestrand et al., 2007). However, constitutive GOI knock-out mice are limited in that essential genes result in embryonic lethality (e.g. Sox2, Avilion et al., 2003) or compensation by other endogenous genes with redundant function (Brocard et al., 1997; Gaveriaux-Ruff and Kieffer, 2007).

One means to overcome these limitations is by generating targeted alleles that are floxed. By flanking critical *cis*-elements of the GOI with loxP sites, the intervening DNA sequence can that can be inducibly or selectively removed by Cre-mediated recombination. In this way, embryonic lethality of *sox2* knock-out mice can be overcome by elimination of floxed Sox2 in the adult brain *in vivo* (Favaro et al., 2009). An additional application is the selective expression of a GOI can be mediated by removal of a "stop" codon that precedes the GOI. In this selective reporter strategy, Cre mediates excision of the stop sequence and enables transcription of the GOI. Further, recombination occurs at the DNA level and is therefore permanent in both the cell that expresses Cre and its progeny. In this way, recombination in targeted reporter mice like the Rosa26-Reporter-LacZ and -YFP mice (Soriano, 1999; Srinivas et al., 2001) allows Cre-mediated fate

tracking of stem cells and progeny within the physiological context of the adult SGZ (e.g. Imayoshi et al., 2006; Lagace et al., 2007b).

In contrast to the targeted genetic manipulations described above, most transgenic mice generated to date have random genomic insertion, permitting transgene expression without altering the endogenous gene. Briefly, the two critical elements in generating mice with transgene insertion are the driver and the transgene. The driver is generally the essential promoter region and genetic elements from GOI, conferring the temporal and cellular specificity of transgene expression. In regards to the promoter or driver, the *nestin* gene contains a well-described second intronic enhancer that selectively limits expression to the central nervous system (Lothian et al., 1999; Xu et al., 2009; Yaworsky and Kappen, 1999; Zimmerman et al., 1994). Therefore, as a number of independent researchers developed nestin transgenic mice, inclusion of this *cis*-element into transgenic constructs helped confer specificity (Kawaguchi et al., 2001; Singer et al., 2009) though see (Betz et al., 1996). On the other hand, the transgene is functional component of the mouse, and examples include reporter genes or functional enzymes. Reporter constructs include genes like GFP and LacZ which localize expression of the GOI within the brain like nestin-GFP mice which overcome difficulty in staining endogenous nestin (Yamaguchi et al., 2000) and also facilitate isolation of reporter-expressing cells via FACS (e.g. Mignone et al., 2004). Functional enzymes include thymidine-kinase (tk), bacterial Cre

recombinase, or Cre recombinase conjugated to a modified estrogen receptors like CreER^{T2} (**Table 1.1**), which have provided key insight into neurogenesis.

Even though randomly integrating transgenic mice offer powerful new tools for exploring neurogenesis and other functions, there are several caveats and limits to transgenic approaches. Because the construct integrates randomly into the genome, a major variable that cannot be controlled within a single mouse line is the possibility of positional effects, inserting into heterochromatin, and more than one copy of the transgene integrated (e.g. Giraldo and Montoliu, 2001; Matthaei, 2007; Wilson et al., 1990). In addition, as transgenic lines are derived from a single founder mouse, it is critical to compare to multiple founders of the same transgene, each of which may have different expression patterns or strength of signal (Feng et al., 2000). Another cautionary note regarding transgenic mice derived from different laboratories: each construct and derived mouse line may ostensibly be the same but can be generated from different transgenes. In comparing different transgenic mice that are ostensibly the same (e.g. there are several lines of nestin-GFP transgenic mice made in different laboratories), the mice often differ in the driver construct. In the case of the nestin-GFP mouse, the nestin-eGFP mice from the Okano laboratory (Yamaguchi et al., 2000) and the Enikopolov laboratory (Mignone et al., 2004) both contain the *nestin* second intron but differ in regard to the extent of promoter included and location of the reporter gene. In the Okano mouse, the second intron enhancer precedes a minimal heat shock promoter to drive GFP expression (Yamaguchi et al., 2000),

whereas in the Enikopolov mouse, a large portion of the *nestin* promoter precedes the GFP gene, which is followed by the second intron (Mignone et al., 2004). While both transgenic mice have fairly comparable expression patterns within the brain, expression patterns the Enikopolov nestin-GFP mouse have been noted in the periphery, including hair follicle stem cells (Li et al., 2003; Mignone et al., 2007) and skeletal muscle cells (Day et al., 2007), emphasizing that promoter elements, transgene construct, and random integration all may result in differences between transgenic mice.

Even given theses caveats, randomly integrated transgenic mice like tk-driver lines have provided key insight into cellular contributions to neurogenesis. Driver-specific tk-lines have provided new information in regards to how ablation of dividing cells and subsequent recovery are mediated by specific cell types. When tk-expressing cells undergo cell division in the presence of the antiviral drug ganciclovir (GCV), toxic metabolites are produced that kill proliferating cells. These tk-transgenics allow spatial (in cells where the GOI is expressed) and temporal control (when cells expressing the GOI divide and when GCV is present; **Table 1.1**). Two most widely used drivers have been GFAP-tk (Bush et al., 1999; Garcia et al., 2004) and nestin-tk (Deng et al., 2009; Singer et al., 2009; Yu et al., 2008) and comparing results between these mice are both intriguing and puzzling. The same GFAP-tk mouse generated by Sofroniew and colleagues has been widely used to explore GFAP as a potential marker of neural stem cells (Bush et al., 1999; Bush et al., 1998; Garcia et al., 2004; Imura

et al., 2003; Imura et al., 2006; Morshead et al., 2003). In the presence of GCV, dividing GFAP-tk+ cells are eliminated; however, after GCV is no longer present, proliferation in both the SGZ and SVZ is virtually eliminated with little or no recovery (Garcia et al., 2004). Coincident with the elimination of proliferation *in vivo*, neurosphere-forming potential is also effectively abolished (Imura et al., 2003; Imura et al., 2006; Morshead et al., 2003). In the nestin-tk mouse, on the other hand, proliferation is similarly ablated but neurogenesis partially recovers at >6 weeks post-GCV (Singer et al., 2009). Interestingly, Parent and colleagues also note that Type-1 cells remain (Singer et al., 2009). However, a number of possibilities could explain the disparities between GFAP-tk and nestin-tk mice in the recovery of neurogenesis. One interpretation is that GFAP+ and nestin+ cell populations overlap, but GFAP+ cells are more “stem-like” and their elimination effectively abolishes neurogenesis whereas nestin+ cells are more “precursor” cells. Another explanation is that, at similar extended periods post-GCV which have not yet been explored in the GFAP-tk mice, similar partial or full recovery might be observed, which could suggest that all hippocampal cells are precursor-like or that some cells remain quiescent for long periods and are unable to be eliminated by GCV. It will be interesting to see if this partial recovery of neurogenesis is recapitulated in other nestin-tk mice (Deng et al., 2009; Yu et al., 2008), in other nestin-driven transgenic mice that ablate proliferation (Dupret et al., 2008; Imayoshi et al., 2008) or in other GFAP-tk mice (Delaney et al., 1996). Further, these possibilities warrant further research but may support that Type-1 cells are heterogeneous in their terms of progenitor production.

Other transgenic mice lines in which the fate of progeny can be tracked may potentially address the questions raised using different tk-driver mice. Using random targeting, mice can be generated in which which bacterial Cre recombinase is expressed under the control of a GOI, creating a Cre-driver line. When Cre is expressed in cells that contain a floxed gene or stop codon, Cre-recombinase targets loxP sites, which consist of two 13-base pair sequences of DNA with an intervening 8-bp sequence (Gaveriaux-Ruff and Kieffer, 2007; Lee and Saito, 1998; Sauer, 1998; Yu and Bradley, 2001). Therefore, Cre expression is temporally and spatially restricted, inasmuch as the GOI is similarly restricted (**Table 1.1**). However, unlike GOI-reporter mice, which recapitulate endogenous gene expression, Cre-mediated recombination at the genomic level results in permanent changes in recombined cells, which are subsequently inherited by progeny even after the GOI is no longer expressed. As such, Cre-mediated recombination allows fate tracking of Cre-driver cells, and a number of researchers have applied this strategy label a population of proliferating or stem cells, track progeny, and explore cell fate by providing spatial resolution at the level of GOI-expressing cells (e.g. Anthony et al., 2004; Garcia et al., 2004; Malatesta et al., 2003). These constitutive Cre-driver lines have provided insight into the process of neurogenesis, and importantly, have demonstrated *in vivo* that embryonic radial glia give rise to most of the brain's neurons and glia (Anthony et al., 2004; Malatesta et al., 2003). These striking genetic results confirm *in vivo* retroviral-mediated labeling (Noctor et al., 2001; Noctor et al.,

2002) and supply *in vivo* confirmation of hypotheses from *in vitro* cell culture (Hartfuss et al., 2001; Malatesta et al., 2000). However, because Cre-expression is present even during embryogenesis, these mice are of limited use in studying adult neurogenesis, particularly because adult stem cells in the SVZ are direct decedents of embryonic radial glia (Bonfanti and Peretto, 2007; Merkle et al., 2004). Evidence also exists to suggest that SGZ Type-1 cells are also likely derived from embryonic radial glia (Eckenhoff and Rakic, 1984). As a result of the extensive overlap of markers between embryonic radial glia and adult SGZ Type-1 cells (**Figure 3**), delineating the contribution of adult-born versus perinatal-born cells is virtually impossible in constitutive Cre-driver lines. Additionally, constitutive Cre expression has lead to unintended anatomic aberrations, including hydrocephaly in nestin-Cre mice (Betz et al., 1996; Forni et al., 2006).

To more selectively control Cre-driver expression in a more temporally- and spatially-restricted manner, Chambon and colleagues created and characterized inducible bacterial Cre-recombinase that has been attached to a modified estrogen receptor (Brocard et al., 1997; Feil et al., 1996; Metzger et al., 1995). Because Cre is fused to an estrogen receptor (ER), Cre is excluded from the nucleus, precluding recombination. To prevent activation by endogenous estrogen, the ER gene has been modified in tamoxifen- (TAM) or 4-hydroxy-tamoxifen (OH-TAM)-inducible Cre-ER^T and Cre-ER^{T2} constructs, leading to selective nuclear translocation where Cre can induce recombination (Brocard et al., 1997; Indra et al., 1999). Newly-generated inducible Cre-driver mice,

including nestin-CreER^{T2} (Lagace et al., 2007b) and GLAST-CreER^{T2} lines (Mori et al., 2006) crossed with floxed-stop reporter lines such as R26R-YFP (Soriano, 1999), have allowed researchers to specifically address questions regarding adult neurogenesis that were previously untenable. Further, cre/loxP-mediated selective elimination of genes from the adult animal also overcomes constitutive gene knock out that causes prenatal lethality, as is the case for Sox2 (Avilion et al., 2003; Favaro et al., 2009).

Questions remaining with regard to Type-1 stem cells *in vivo* and adult neurogenesis

New markers, new models of neurogenesis and new advances in transgenic technology have allowed researchers to address critical questions about adult neurogenesis that heretofore could not be addressed. For example, we know that the vast majority of adult-born cells that express nestin and survive in the dentate are going to become neurons (Lagace et al., 2007b), which provides new information on when progenitor cells undergo fate selection. New data have emerged to support the linear progression of early progenitor cells to post-mitotic neuroblast cells that mature into mature neurons has supported much of the Kempermann model for the putative stages of neurogenesis (Kempermann et al., 2004; Lagace et al., 2007b; Ninkovic et al., 2007) but also have provided evidence against the linearity of Type-1 cells giving rise to progenitors (Singer et al., 2009; Suh et al., 2007). Cell-intrinsic factors governing discrete stages of neurogenesis have been elucidated but also there is growing appreciation for

cell-extrinsic factors as well (e.g. Ahn and Joyner, 2005; Breunig et al., 2007; Gao et al., 2009; Jessberger et al., 2008a; Kuwabara et al., 2009; Lagace et al., 2008; Zhang et al., 2008). Long-standing controversies regarding the subependymal layer of the lateral ventricle as the source of olfactory bulb neurogenesis have largely been resolved (Johansson et al., 1999; Mirzadeh et al., 2008; Morshead et al., 1994).

However, this progress has also raised additional questions that need to be addressed. Given the growing appreciation that proliferation, differentiation, and survival of adult-generated cells can be discretely regulated at the molecular, cellular and physiological levels, it is striking that it remains unknown how effectors of neurogenesis alter Type-1 cells. In addition, recent studies question whether Type-1 cells are heterogeneous and whether Type-1 cells contribute to SGZ neurogenesis.

In Chapter Two, I explore how two effectors of neurogenesis, X-irradiation and running, independently and together alter Type-1 cells within the context of the hippocampus using a transgenic nestin-GFP reporter mouse. The effects of X-Irradiation and running on Type-1 cells have been inconclusive, even though both manipulations are well-characterized, physiologically relevant, and robust negative and positive regulators of neurogenesis, respectively. Thus, my work in Chapter Two significantly advances our knowledge of how Type-1 cells are altered by these effectors.

In Chapter Three, I explore how stress, which is a negative regulator of proliferation, alters survival of adult-generated neurons and the role of neurogenesis in social avoidance behavior. Under the larger framework of stress as a negative regulator of neurogenesis, I also explored how Type-1 cells are altered by stress in the nestin-GFP mouse.

In Chapter Four, I use an inducible fate-tracking nestin-CreER^{T2}/R26R-YFP mouse to address how nestin-expressing Type-1 and progenitor cells contribute to adult hippocampal neurogenesis. Recent evidence from previous studies in our own laboratory (Chapters Two and Three) and others raise the critical question of Type-1 contribution to neurogenesis and suggest for functional heterogeneity of Type-1 cells.

In Chapter Five, I directly test the contribution of Type-1 cells contribute to basal neurogenesis and test functional heterogeneity of Type-1 cells expressing different markers. Using nestin-CreER^{T2}/R26R-YFP and GLAST-CreER^{T2}/R26R-YFP mice, where the drivers overlap only in Type-1 cells, I test long-term maintenance of the proliferative progenitor pool basally. I also assess whether Type-1 cells contribute to recovery after the progenitor pool is chemically ablated.

In sum, while transgenic approaches, neurospheres, BrdU infusion, and retroviral labeling have all provide key insight into hippocampal neurogenesis, ultimately,

each technique is limited because the animal must be sacrificed. However, until researchers can identify a single cell within the brain and assess its division, maturation, and ultimately its survival over a prolonged period, many questions will remain speculative. My work described in this thesis combines multiple transgenic mouse lines to assess the Type-1 cells at the population level and at the level of individual cells.

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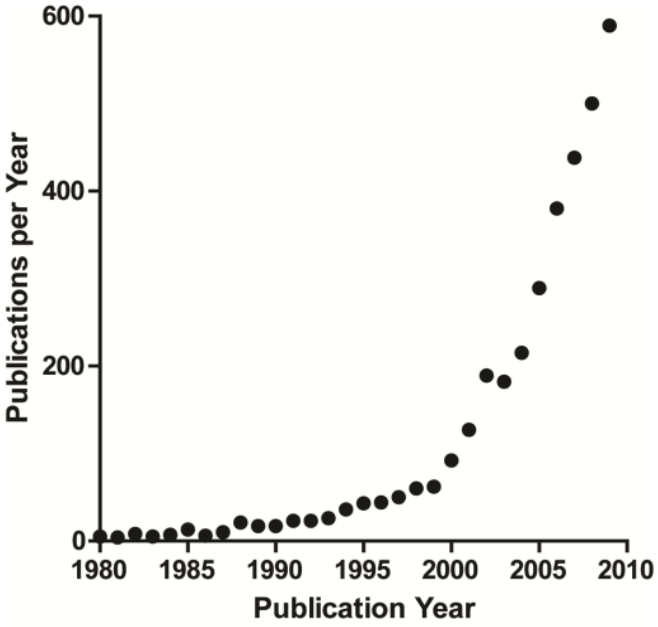

Figure 1.1. Rapid growth in the field of adult neurogenesis, 1980-2009. Results of Medline (http://www.ncbi.nlm.nih.gov) search for “adult neurogenesis” graphed by year of publication reveal tremendous growth in the field, particularly within the last decade.

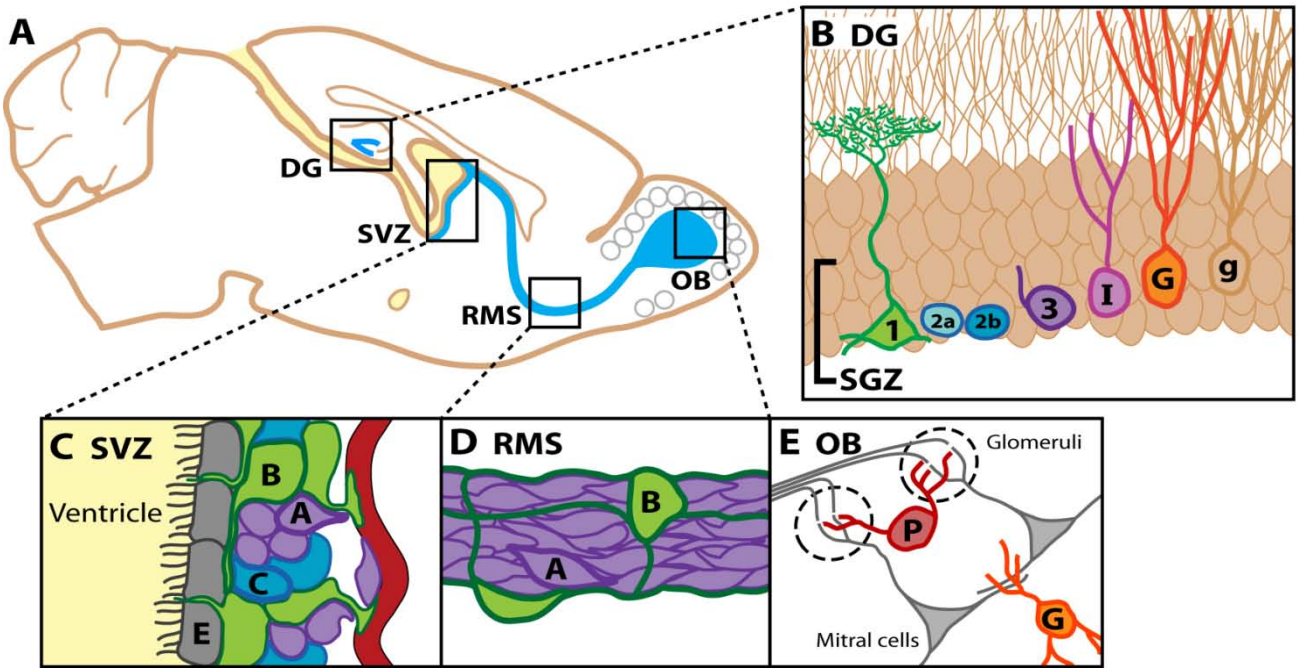

Figure 1.2. Neurogenesis in the adult brain. (A) Major neurogenic regions of the adult rodent brain are highlighted in blue on a sagittal brain cartoon. (B) In the DG, neurogenesis occurs in the SGZ along inner half of the granule cell layer bordering the hilus (white)(Kempermann et al., 2004). Type-1 cells (denoted 1 and shaded green) are the putative stem cells. Type-2a and -2b (blue) amplifying progenitors divide frequently and rapidly. Type-3 cells (violet) are late neuronal progenitors which still undergo mitosis. Immature neurons (I, fuschia) migrate a short distance into the granule cell and extend dendrites through the granule cell. Mature adult-born granule cells (G, orange) are morphologically and electrophysiologically identical to granule cells (g, brown) born during the early post-natal period. (C) In the SVZ, astrocyte-like stem cells (Type B cells, green)

give rise to early progenitors (Type C cells, blue), which give rise to amplifying progenitors (Type A cells, purple). (D) Type A cells migrate from the SGZ alonmg the RMS via a networks of astrocytic Type B. (E) Type A progenitors migrate tangentially from the RMS into the OB, where they ultimately differentiate into periglomerular cells (P, red) or granular cells (G, orange). DG, dentate gyrus; OB, olfactory bulb; RMS, rostral migratory stream; SGZ, subgranular zone; SVZ, subventricular zone.

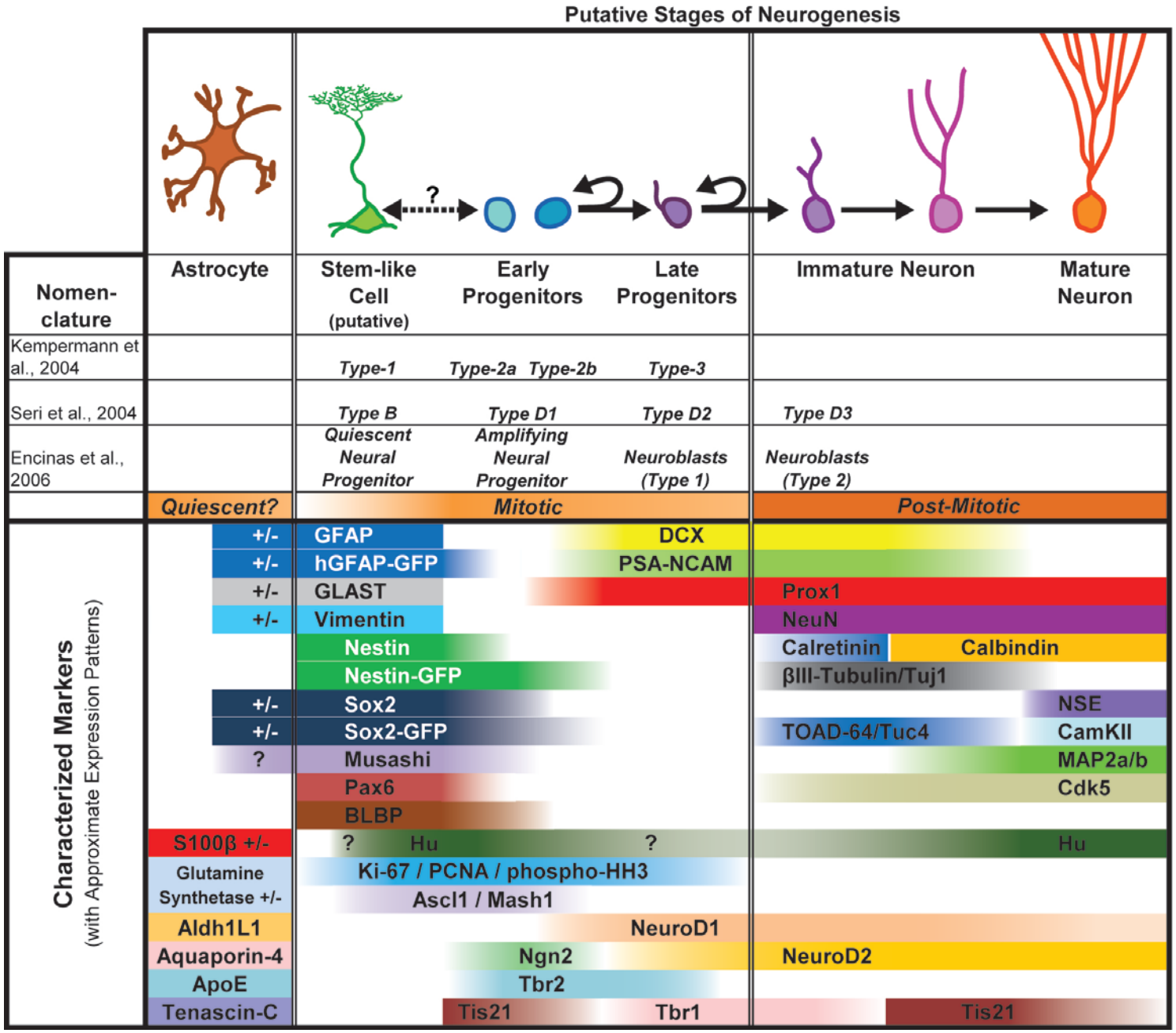
Putative Stages of Neurogenesis
?
Nomen-clature
Astrocyte
Stem-like Cell (putative)
Early Progenitors
Late Progenitors
Immature Neuron
Mature Neuron
Kempermann et al., 2004
Type-1
Type-2a
Type-2b
Type-3
Seri et al., 2004
Type B
Type D1
Type D2
Type D3
Encinas et al., 2006
Quiescent Neural Progenitor
Amplifying Neural Progenitor
Neuroblasts (Type 1)
Neuroblasts (Type 2)
Quiescent?
Mitotic
Post-Mitotic
Characterized Markers
(with Approximate Expression Patterns)
+/- GFAP
DCX
+/- hGFAP-GFP
PSA-NCAM
+/- GLAST
Prox1
+/- Vimentin
NeuN
Nestin
Calretinin
Calbindin
Nestin-GFP
βIII-Tubulin/Tuj1
+/- Sox2
NSE
+/- Sox2-GFP
TOAD-64/Tuc4
CamKII
? Musashi
MAP2a/b
Pax6
Cdk5
BLBP
S100β +/-
? Hu ?
Hu
Glutamine Synthetase +/-
Ki-67 / PCNA / phospho-HH3
Ascl1 / Mash1
Aldh1L1
NeuroD1
Aquaporin-4
Ngn2
NeuroD2
ApoE
Tbr2
Tenascin-C
Tis21
Tbr1
Tis21

Figure 1.3. Markers for putative stages of neurogenesis. Cartoons in the top row depict the putative stages of neurogenesis with nomenclatures put forth by various researchers. The Type-1 cell is the hypothesized stem cell but is poorly understood because of extensive overlap with astrocytes and progenitor cells (see main text). Astrocytes share many characteristic markers, overlapping considerably with Type-1 stem-like cells. However, while GFAP has been among the most widely used markers of astrocytes, the majority of astrocytes are GFAP- and most characterized markers are found only in a subset of astrocytes, denoted +/-. S100β is a marker of mature astrocytes that is excluded from Type-1 cells. It has not been determined if other astrocyte markers, including Aquaporin-4 and ApoE, are also expressed in Type-1 cells. Most markers of neurogenic stages are dynamically and transiently expressed, with the relative expression onset and offset indicated by color gradients. Because all markers overlap in multiple cell types, the hypothesized stages likely do reflect gradual maturation as opposed to discrete, independent stages.

Type of Mouse	**Knock-Out**	**Reporter driver**	**Thymidine-Kinase driver**	**Cre-driver: Constitutive**	**Cre-driver: Inducible**	**"Floxed" Gene**
Examples	GFAP-/- Vim-/-	hGFAP-GFP nestin-GFP	GFAP-tk nestin-tk	nestin-Cre hGFAP-Cre	GLAST-CreER^{T2} nestin-CreER^{T2}	Rosa26R-YFP Sox2$^{floxed/floxed}$
Genomic Integration	Targeted	Random	Random	Random	Random	Targeted
Effect	Eliminate GOI from all cells	Express reporter in GOI-expressing cells	Kills dividing TK-expressing cells, only when gancyclovir is present	Drive DNA recombination in cells expressing GOI	Drive DNA recombination in GOI-expressing cells, only when ligand is present	Recombine genomic DNA between loxP sites in Cre-expressing cells
Localization and Extent of Effect	Not restricted (All cells in all tissue)	Spatially restricted (All GOI-expressing cells)	Spatially and Temporally restricted (Kills GOI-expressing cells, under very restricted conditions)	Spatially restricted (All GOI-expressing cells and progeny)	Spatially and Temporally restricted (Chemically-induced subpopulation of GOI-expressing cells and progeny)	Spatially and Temporally restricted (Where and whenever Cre-recombinase is expressed)
Duration of Effect	Permanent	As long as GOI is expressed	Permanent (cells are eliminated)	Permanent in cells and progeny, even after GOI turned off	Permanent in cells and progeny, even after GOI turned off	Permanent in cells and progeny
Level of Effect	Organismal	Population of cells currently expressing GOI	Tissue or region after gancyclovir administration	Population and progeny of cells that have expressed GOI	Individual cells recombined	Individual cells recombined
Pros:	-Insight into overall importance of gene for development and function	- Relatively easy to generate - Useful characterization	- Eliminating GOI-expressing progenitor population	- Fate-tracking of cells driving GOI - Selective manipulation of DNA in population of GOI-expressing cells - Very good for developmental manipulations	- Same Pros as consitutive Cre-recombinase drivers - Good for selective manipulations in adult	- Selective knock-out of GOI or selective knock-in of GOI
Cons:	- Most difficult to generate - Potential compensatory effects from other genes, cells - Requires homozygous breeding	- Limited functional insight -No fate tracking	- Limited overall application - Costly drug administration - Non-dividing cells not directly affected	- Additional transgenic mouse lines with Cre-recognition sites ("floxed" gene) required - Sometimes difficult to interpret effects because of potential compensatory effects - Potentially "leaky" transgene	- Same Cons as constitutive Cre-recombinase drivers - Costly drug administration - Recombination only in subset of cells sometimes masks overall changes	- Requires Cre-driver for effect (by either crossing to a Cre-driver mouse or by viral infusion of Cre-virus) - Limited use on its own - May require homozygous breeding - Difficult to generate

Table 1.1. Transgenic mice for studying neurogenesis *in vivo*. Various “types” of transgenic mice used to assess Type-1 cells and neurogenesis within the body’s physiological context. **Rows:** Genomic integration: How the vector construct is incorporated into the founder genome, such as direct targeting of the endogenous GOI for replacement (in knock-out lines) or random integration of transgene into the founder’s genome, which can result in multiple copies. Effect: The defining principle behind the transgenic mouse, such as reporter gene expression eliminating gene expression constitutively in knock-out mice or inducibly in “floxed” mice. Localization and extent of effect: The spatial or temporal restriction of transgene effects. Duration of effect: Temporal duration of transgene effects. Level of effect: Extent of transgenic effect on the mouse. Pros and Cons: The benefits and limits/drawbacks of type of mouse. **Columns:** See text for more specific information on each type of transgenic mouse. GOI, gene of interest, which is generally the transgene, targets either “driving expression” from gene’s promoter elements and exons or alterations to the endogenous genomic locus.

CHAPTER TWO

Running after irradiation ameliorates the proliferation deficit but not the nestin-expressing Type-1 deficit in the adult hippocampal subgranular zone

Adapted from: **DeCarolis NA**, Ahn FA, Lagace DC, Ables JA, Chen B, Eisch, AJ. Running partially rescues X-ray-induced deficits in adult hippocampal neurogenesis, independent of changes in nestin-expressing Type-1 stem-like cells. *In review at Hippocampus*.

Abstract

X-irradiation and running are clinically-relevant manipulations that alter mammalian adult hippocampal neurogenesis. However, it is unclear if long-lasting deficits in hippocampal neurogenesis post-irradiation (post-IRR) are caused by loss of Type-1 cells (the putative hippocampal stem cell), if running changes Type-1 cell number, or whether running post-IRR normalizes adult neurogenesis. To address these and other knowledge gaps about differences in sex and age in Type-1 cell numbers, we quantified subgranular zone (SGZ) Type-1 cells in nestin-GFP male and female mice after IRR (5Gy whole-head), 7d of running, or combined exposure. Type-1 cell number, proliferation (assessed via BrdU), or radiation-induced changes in these measures were not sex-dependent. However, there was an age-dependent sensitivity of Type-1 cells post-IRR, with fewer cells in mice irradiated at 6 weeks vs. older ages. The

proliferation deficit seen 2 months post-IRR did not correlate with loss of Type-1 cells or persistent inflammation. Strikingly, running ameliorated the post-IRR proliferation deficit but not the Type-1 cell deficit. These data show that Type-1 cell number is differentially altered by X-irradiation, running and age. These data also underscore the importance of microenvironmental factors in the SGZ post-IRR and urge reevaluation of the role of Type-1 cells in neurogenesis.

Introduction

Radiation is a commonly used and effective treatment for CNS cancers in children and adults. However, radiotherapy is often accompanied by decreased brain health and learning problems later in life (e.g. Douw et al., 2009). Adult-generated hippocampal neurons are exquisitely sensitive to radiation (e.g. Mizumatsu et al., 2003; Wojtowicz, 2006) and deficits in neurogenesis may contribute to the cognitive deficits seen after irradiation (post-IRR) (e.g. Raber et al., 2004b; Wojtowicz et al., 2008). Thus, it has been proposed that efforts to rescue subgranular zone (SGZ) neurogenesis may attenuate further cognitive decline in human cancer survivors (Acharya et al., 2009; Naylor et al., 2008). Interestingly, running potently increases the proliferation and survival of adult-generated hippocampal neurons (e.g. van Praag et al., 1999a) and attenuates radiation-induced deficits in cognition and neurogenesis in young animals (Naylor et al., 2008). However, more research is needed to understand how running,

irradiation, and running and irradiation together influence the complex process of adult neurogenesis (Kempermann et al., 2004).

In particular, it is controversial how such positive (running) and negative (irradiation) effectors of neurogenesis influence Type-1 cells, the putative hippocampal stem cells (Kempermann et al., 2004; Seri et al., 2001). The effect of running on the number of Type-1 cells is unclear, with studies reporting increases (Naylor et al., 2008) or no change (Suh et al., 2007) in Type-1 cell number. Similarly, it is unclear how running influences Type-1 cell proliferation, with reports of increases (Steiner et al., 2004; Suh et al., 2007) or no change (Kronenberg et al., 2003; Steiner et al., 2008) in Type-1 proliferation. The effect of X-irradiation on Type-1 cells is also unclear. While it has been suggested that a reduction in the number of Type-1 cells underlies the long-lasting deficits in adult neurogenesis post-IRR (McGinn et al., 2008), and Type-1 cell number is reduced when irradiation occurs in early life (P9; e.g. Hellstrom et al., 2009; Naylor et al., 2008), the effect of X-irradiation on Type-1 number in adulthood is not known. Therefore a primary goal of this study is to clarify how running and irradiation independently and together alter Type-1 cell number in the adult SGZ.

A secondary goal of this study is to assess sex differences in number of Type-1 cells basally and in response to stimuli like running and irradiation. Basally, there are conflicting reports on whether proliferation or survival of adult-generated SGZ

cells differ in male versus female mice (Lagace et al., 2007a; Silasi et al., 2004), and notably, no studies have examined whether there is a basal sex difference in Type-1 cell number. It is also unclear whether there is a sex difference in neurogenic response to running or irradiation, likely because studies on running and neurogenesis typically use females (e.g. van Praag et al., 1999b), while studies on irradiation and neurogenesis typically use males (e.g. Mizumatsu et al., 2003) or combine males and females (e.g. Naylor et al., 2008; Rola et al., 2004). The knowledge gap regarding the potential importance of sex in running and radiation urged us to examine sex differences in both SGZ proliferation and Type-1 cell number.

Methods and results

To quantify basal Type-1 cell number and the influence of irradiation and running on proliferation of SGZ progenitors and stem cells, we used adult male and female nestin-GFP mice (6, 10, or 18 weeks of age (wks)) (Yamaguchi et al., 2000), which constitutively express cytoplasmic green fluorescent protein in SGZ Type-1 stem and early progenitor cells. Experiments were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* and approved by the Institutional Animal Care and Use Committee. To label proliferating cells (Lagace et al., 2007a), mice were administered bromo-deoxyuridine (BrdU, Boehringer Mannheim; 150 mg/kg, IP) 2hr before intracardial perfusion. Hippocampal sections (30-µm thick, 1:9 series) were

mounted onto charged slides and stained for cell types as follows: nestin-GFP+ cells were visualized with rabbit anti-GFP (1:3000; A11122, Invitrogen); astroglia and Type-1 cells with mouse anti-GFAP (1:500; MAB360, Millipore); proliferating cells with rat anti-BrdU (1:400; OB0030, Accurate Chemical). Free-floating sections were stained with microglial marker rabbit anti-Iba1 (1:10,000; 016-20001, Wako Chemicals). Staining patterns were similar to those previously described (BrdU (Lagace et al., 2007a); GFP (Yamaguchi et al., 2000); GFAP (Naylor et al., 2008); Iba1 (Hellstrom et al., 2009)) and omission of primary antibody resulted in absence of staining. Cells were quantified using stereological methods (Lagace et al., in press). Data are presented as mean ± SEM. Graphs were prepared and data were analyzed using GraphPad Prism (version 5) using a two-tailed *t*-test or Two-Way ANOVA with Bonferroni post-hoc test, unless otherwise noted. Statistical significance was set at $p<0.05$.

As expected (e.g. Kempermann et al., 2004), radial glia-like Type-1 cells expressed GFP, presented a radial glial-like morphology (irregularly-shaped SGZ soma with a radial process, **Figure 2.1A1-A3**), and were visible throughout the anterior-posterior axis of the dentate gyrus. The central process of GFP+ Type-1 cells extended through the granular cell layer (GCL) and into the inner molecular layer, ending in a dense tuft of fibrils, while the soma of nestin-GFP+ progenitor cells lacked processes. Also as expected (Kempermann et al., 2004; Steiner et al., 2006), nearly all nestin-GFP+ Type-1 cells also stained for GFAP, but not all

GFAP+ radial processes co-labeled with GFP (arrowheads, **Figure 2.1A**). Using these criteria – irregular GFP+ soma, radial process extending through the GCL – GFP-expressing Type-1 cells could be reliably identified and quantified.

To assess the effects of sex and X-irradiation on SGZ proliferation and Type-1 cells, nestin-GFP mice (6wks) were cranially-irradiated with a clinically-relevant dose of X-irradiation (5Gy) (Mizumatsu et al., 2003). Briefly, male and female mice (n=6-8 mice/group) were anesthetized with ketamine/xylazine (9 mg/kg; 1 mg/kg in saline, IP) prior to placement in an X-RAD 320 self-contained irradiation system (Precision X-Ray, Inc) equipped with a custom-synthesized collimator for delivery of a 10mm diameter X-ray beam to head (source-to-skin distance 88 cm; 250 kV, 15mA; 1.08 Gy/min, 4.5min= 5Gy)(Lagace et al., in press). Sham-irradiated controls were handled similarly but the irradiator was not turned on. When mice were 19wks of age (>2 months post-IRR), mice were given BrdU 2hr before brains were extracted. Confirming previous work that irradiation produced long-lasting decrease in proliferation (Mizumatsu et al., 2003; Wojtowicz, 2006), the number of proliferating BrdU-immunoreactive (BrdU-IR) cells was decreased by ~75% compared to controls (**Figure 2.2A**, $F_{(1,18)}$=20.31, *p*=0.003). There was no basal sex differences in BrdU-IR cell number, consistent with previous reports (Lagace et al., 2007a), or in BrdU-IR cell number after irradiation (**Figure 2.2A**). Quantification of nestin-GFP+ Type-1 cells revealed a significant decrease (~35%) in irradiated mice compared to controls (**Figure 2.2B**, $F_{(1,24)}$=16.95,

p=0.004). No difference in Type-1 cell number was observed by sex in either irradiated or sham mice (**Figure 2.2B**). Thus, X-irradiation caused a long-lasting decrease of proliferating and Type-1 SGZ cells. Because there were no sex differences in Type-1 or BrdU-IR cells basally, and no differences between sexes in irradiation-induced changes, mice of both sexes were used for the remaining studies and data were collapsed.

In the early post-natal period, progenitor cells show an age-dependent sensitivity to irradiation (Fukuda et al., 2005). To examine if this is also true in adulthood, mice were exposed to 5Gy X-irradiation at 6, 10, or 18wks of age, then all groups were sacrificed at 19wks (>2 months post-IRR for animals irradiated at 6 and 10wks, n=3-7 mice/group). Type-1 cells were quantified and compared to controls (sham at 6wks (8628 ± 831) and 18wks (8370 ± 65), combined due to lack of statistical difference (*p*=0.83)). Consistent with other experiments (Mizumatsu et al., 2003; Wojtowicz, 2006), proliferation of BrdU-IR cells was robustly decreased all irradiated mice, regardless of age at irradiation (data not shown). In two independent experiments, there was a significant effect of age at irradiation on Type-1 cell number (**Figure 2.3**, $F_{(3,17)}$=4.487, *p*<0.05). Mice irradiated at 6wks had ~30% fewer Type-1 cells than controls (*p*<0.05), a decrease comparable to our initial studies (**Figure 2.2B**). However, mice irradiated at 10 or 18wks had statistically similar Type-1 cell numbers relative to controls, with a trend for a fewer cells at 6 vs 18wks (*p*=0.0596). Thus, Type-1

cell number was only decreased by irradiation at 6wks was decreased by X-irradiation. For the remaining studies, mice were irradiated at 6wks.

To assess whether the irradiation-induced decrease in SGZ proliferation could be reversed by running, nestin-GFP mice were sham- or X-irradiated and allowed to recover for 2 months (**Figure 2.1C**; n=5-11 mice/group). Mice were then singly-housed with *ad libitum* access to a running wheel (Coulbourn Instruments, Whitehall, PA, USA, Cat# ACT-551, ACT-552), water and food. Mice had access to either a locked (unable to turn; control group) or open (running group) wheel for 7d to increase SGZ proliferation (Holmes et al., 2004). In preliminary experiments, there was no difference in running between irradiated and sham mice and no difference in pattern or amount of running between sexes (data not shown), consistent with previous reports (Clark et al., 2008). There were significantly fewer SGZ BrdU-IR cells in irradiated mice compared to controls (**Figure 2.1D-G**, $F_{(1,39)}$=62.81, p<0.0001), and there was a significant effect of wheel running ($F_{(2,39)}$=15.37, p<0.0001). Irradiated mice on locked wheels (Irradiated-Locked) had significantly fewer BrdU-IR cells compared to Sham-Locked controls (p<0.001). Similarly, Irradiated-Open mice had significantly fewer BrdU-IR cells compared to Sham-Locked controls (p<0.01). Strikingly, Irradiated-Open mice had significantly more BrdU-IR cells than Irradiated-Locked mice (p<0.05). Thus, running post-irradiation in adulthood attenuated radiation-induced deficits in SGZ proliferation.

As Type-1 cells have been proposed to mediate recovery of proliferation after ablation (e.g. Seri et al., 2001), we explored the hypothesis that running ameliorated the post-IRR proliferation deficit via an increase in Type-1 cell number. Consistent with earlier findings (**Figures 2.2; 2.3**), X-irradiation significantly decreased Type-1 cell number compared to controls (**Figure 2.1A-B,G**, $F_{(2,15)}$=8.040, p<0.01). Mice from both Irradiated-Locked and Irradiated-Open groups had significantly fewer Type-1 cells than Sham-Locked mice (**Figure 2.1G**; p's<0.05). There was no difference in Type-1 cell number between irradiated mice with access to locked or open wheels. Thus, running ameliorated post-IRR proliferation deficits but did not alter the number of Type-1 cells.

Inflammation is associated with decreased hippocampal proliferation in rats (Monje et al., 2003), and physical activity has anti-inflammatory effects (Cotman et al., 2007). Thus, we next explored the hypothesis that running ameliorated the post-IRR proliferation deficits via anti-inflammatory effects, which could be implied by altered presence of Iba-1-IR microglia. Contrary to our hypothesis, there were no obvious changes in Iba-1-IR cell number between Sham-Locked, Irradiated-Locked, and Irradiated-Open groups 2 months post-IRR (**Figure 2.4**). This suggested that radiation-induced inflammation normalized in mice within 2 months, as previously reported in mice irradiated in very early life (Hellstrom et

al., 2009). Therefore, we cannot conclude whether running post-IRR ameliorated the post-IRR proliferation deficits.

Because irradiation alone decreased Type-1 cell number (**Figure 2.1G**), it was important to assess running-induced changes in non-irradiated mice. Nestin-GFP mice (18wks) were individually housed with locked or open running wheels for 7d, mirroring the timecourse of previous studies but without irradiation (**Figure 2.1C**). As expected, running caused a significant increase in BrdU-IR cell number (**Figure 2.5C**, $t_{(6)}$=3.594; p<0.05). Strikingly, there was no change in Type-1 cell number after running (**Figure 2.5A-C**, $t_{(6)}$=0.7464; p=ns). These results confirmed that running increased proliferation independently from changes in Type-1 cell number.

Discussion and conclusions

One of the most notable findings presented here is that although proliferating SGZ cells doubled with running post-IRR, Type-1 cell number did not change. This result supports that an increase in Type-1 SGZ cells is not necessary for running to ameliorate irradiation-induced proliferation deficits. In the more general context of adult neurogenesis, this finding also might imply that physiological manipulations which alter proliferation or neurogenesis occur independently from changes in Type-1 cell number. Several possibilities might explain this apparent disparity. First, running might specifically target progenitor

cells instead of Type-1 cells (Kronenberg et al., 2003; Steiner et al., 2008). Second, running might increase asymmetrical Type-1 cell division, as seen with an NMDA antagonist (Namba et al., 2009). Third, the nestin-expressing radial glia cells may not make a substantive contribution to neurogenesis (Singer et al., 2009; Suh et al., 2007). The latter is particular controversial given that Type-1 cells have been implicated in repopulating hippocampal progenitors (Seri et al., 2001). These hypotheses and the current work highlight the need for our lab and others to delineate the molecular and cellular mechanisms underlying running-induced neurogenesis and to clarify the role of Type-1 cells in ongoing neurogenesis.

An additional notable finding presented here is that irradiation in early adulthood (P42) was not associated with an increase in Type-1 cell number following running. This is in contrast to previous work where irradiation in the early postnatal period (P9) is associated with an increase in Type-1 cell number following running (Naylor et al., 2008). The disparity in our results may result from length of running (7d here versus 1 month in Naylor et al.). Additionally, the age at irradiation likely plays an important role, given that the P9 dentate gyrus is still undergoing rapid development (e.g. Fukuda et al., 2005; Hellstrom et al., 2009; Naylor et al., 2008). It is likely that both microenvironmental and cell-intrinsic factors in the more mature adult dentate gyrus are less permissive of a running-induced increase in Type-1 cells post-IRR (Fike et al., 2007; Fukuda et

al., 2005). In fact, we find that running alone – without irradiation – also did not change Type-1 cell number, in agreement with previous reports in adult mice (Kronenberg et al., 2003; Naylor et al., 2008; Suh et al., 2007). It is possible that adult Type-1 SGZ cells express different receptors than early postnatal SGZ cells, allowing distinct response to microenvironmental neurogenic cues. Alternatively, the microenvironment between the young and adult SGZ may be less responsive or less permissive. Exploring cell-intrinsic and extrinsic factors that alter neurogenesis with age is an area that warrants additional vigorous research.

The lack of change in Type-1 cell number following running is consistent with previous work showing either no changes (Kronenberg et al., 2003; Steiner et al., 2008) or only modest increases (Suh et al., 2007) in the proportion of Type-1 cells dividing. Taken together with my data, this suggests that potent effectors of proliferation and neurogenesis generally are modestly or not correlated with changes in total Type-1 cells. This hypothesis is supported by recent findings that chronic social stress significantly reduces proliferation in the absence of changes in Type-1 cell number (Lagace et al., in press). Indeed, there is no correlation between the number of Type-1 cells and the number of BrdU-IR cells in sham mice or irradiated mice with access to an open running wheel (data not shown). While the limits of the nestin-GFP mouse as a constitutive reporter model preclude us from drawing conclusions about whether Type-1 cells play a

functional role in on-going neurogenesis, my data emphasize the need for more research to critically examine how Type-1 cells contribute to constitutive neurogenesis *in vivo*.

Given the growing appreciation that neurogenesis plays a role in certain types of learning (Imayoshi et al., 2008; Wojtowicz et al., 2008), my data support the hypothesis that even partial rescue of adult neurogenesis post-IRR may contribute to behavioral improvements in children and young adults previously exposed to radiation (Acharya et al., 2009; Monje, 2008; Raber et al., 2004b). These results are also exciting in that radiation-induced deficits in proliferation can still be rescued partially, even after prolonged recovery (>2 months post-IRR). Moreover, these data surprisingly show that proliferation can recover independently from the Type-1 stem cell pool. More work is needed to explore the functional and behavioral recovery following radiation-induced damage, as well as cellular and molecular mechanisms underlying the complex interplay between neurogenesis, stem cells, and cognitive performance.

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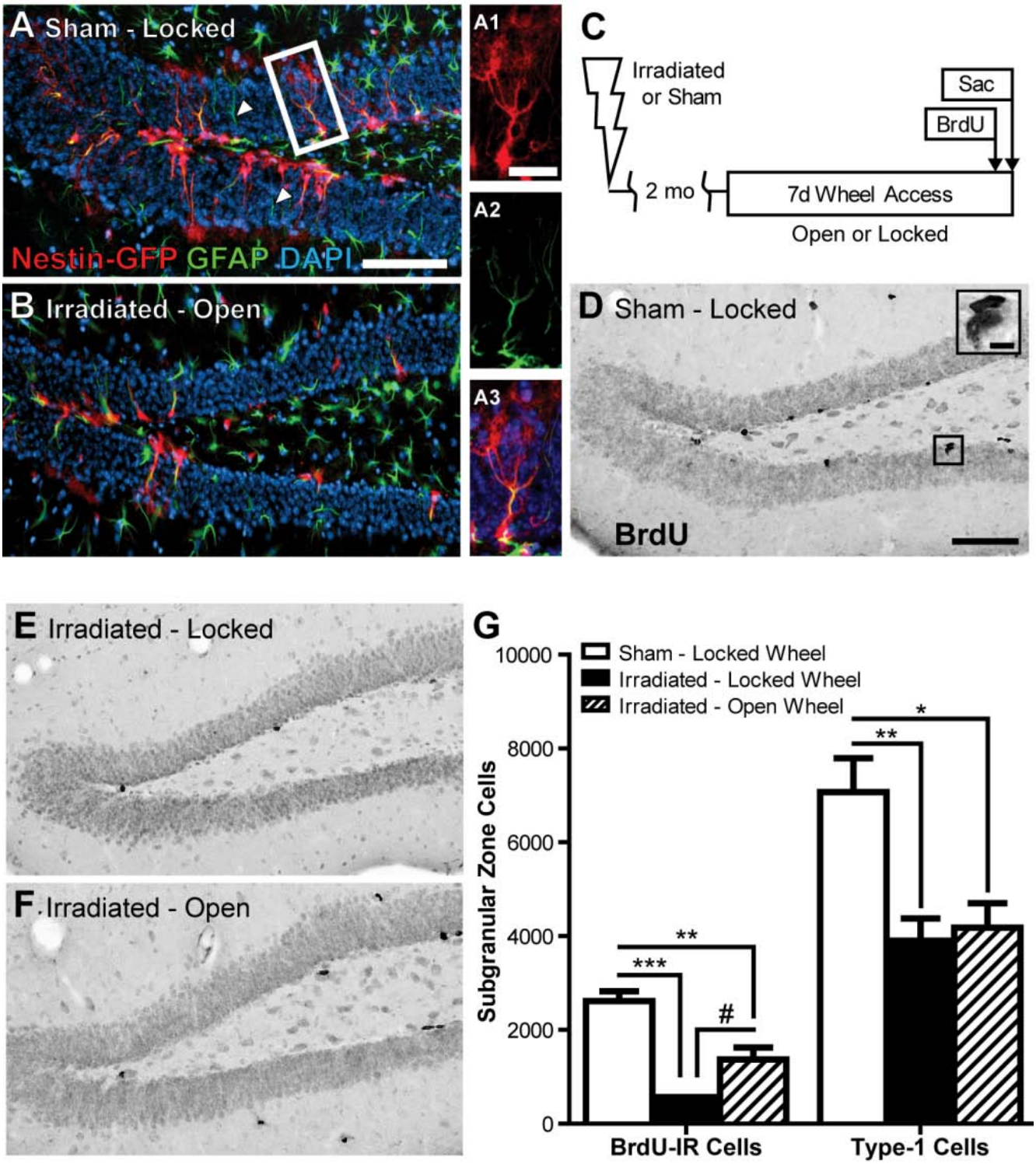
A Sham - Locked
Nestin-GFP GFAP DAPI
A1
A2
A3
B Irradiated - Open
C
Irradiated
or Sham
Sac
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2 mo
7d Wheel Access
Open or Locked
D Sham - Locked
BrdU
E Irradiated - Locked
F Irradiated - Open
G
Sham - Locked Wheel
Irradiated - Locked Wheel
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8000
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BrdU-IR Cells
Type-1 Cells

Figure 2.1. Running partially ameliorates radiation-induced deficits in SGZ proliferation without changing the number of Type-1 cells. (A-B) Representative photomicrographs stained for nestin-GFP (red), astroglial and stem cell marker GFAP (green) and nuclear counterstain DAPI (blue) from the dentate gyrus of (A) sham mouse with access to a locked wheel; (B) irradiated mouse with access to a locked wheel. Arrowheads in (A) highlight nestin-GFP-/GFAP+ radial glia. Inserts highlight a Type-1 stem-like cell with characteristic radial glial morphology of GFP (A1), expression of GFAP in radial processes (A2) and merged (A3). (C) Schematic for experimental procedures. Nestin-GFP mice were sham- or X-irradiated (IRR) with 5 Gy localized to the head at 6 weeks of age. After 2 months of recovery post-IRR, mice had 7d access to running wheels that were either locked or open. At the end of wheel access, all mice were administered BrdU (150 mg/kg, IP) to label proliferating cells and sacrificed 2hr later. (D-F) Representative photomicrographs highlight BrdU-IR cells (black) from the dentate gyrus of (D) sham mouse with access to a locked wheel; (E) irradiated mouse with access to a locked wheel; (F) irradiated mouse with access to an open running wheel. Inset in (D) shows a cluster of BrdU-IR cells at higher magnification. (G) Quantification of BrdU-IR and Type-1 cells in sham mice on locked wheels (white bar), irradiated mice on locked wheels (black) or irradiated mice with access to open running wheels (hatched). X-irradiation causes a significant decrease in proliferating BrdU-IR cells compared to sham controls, whereas running on an open wheel (hatched bars) causes a significant increase

in BrdU-IR cells in irradiated mice compared to irradiated mice on locked wheels. Radiation causes a significant decrease in the number of Type-1 cells compared to sham control, regardless of activity. Scale bar in (A) is 50 μm applies to (A-B); scale bar in (A1) is 15 μm and applies to (A1-A3). Scale bar in (D) is 50 μm applies to (D-F); scale bar in inset of (D) is 5 μm. Mean ± S.E.M. 5-11 mice/group. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ compared to Sham-Locked group; #, $p<0.05$ compared to Irradiated-Open group. Mean ± S.E.M, n=5-7 mice/group.

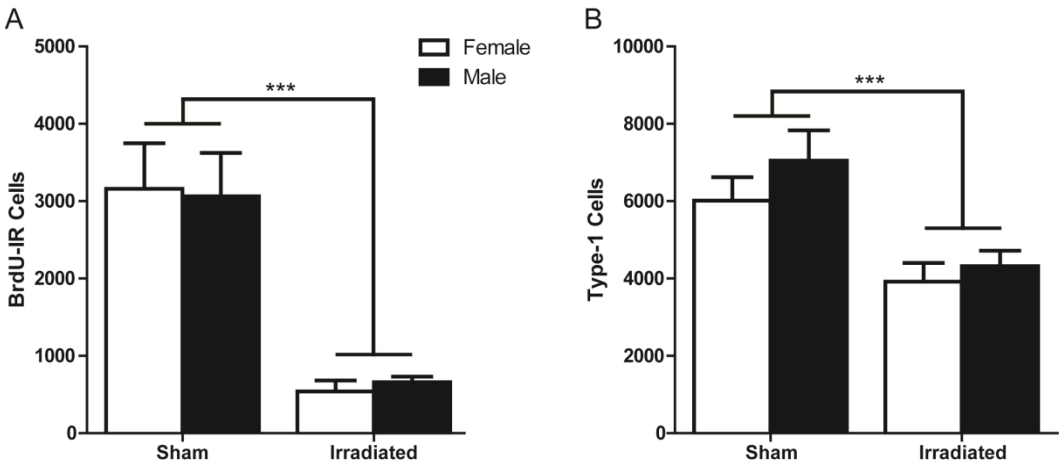

Figure 2.2. There are no basal sex differences in BrdU-IR or Type-1 cells, independent of X-irradiation. Nestin-GFP mice at 6 weeks of age received sham or 5 Gy X-irradiation; 2 months later mice received a single dose of BrdU (150 mg/kg) and were sacrificed 2 hrs later. (A) There is no difference in number of BrdU-IR cells between female (white) and male (black). 5 Gy X-irradiation causes a significant decrease in the number of BrdU-IR cells compared to sham-irradiated controls. (B) 5 Gy X-irradiation significantly decreases the number of Type-1 cells, independent of sex. Mean ± SEM, 6-8 mice/group. ***, $p < 0.001$.

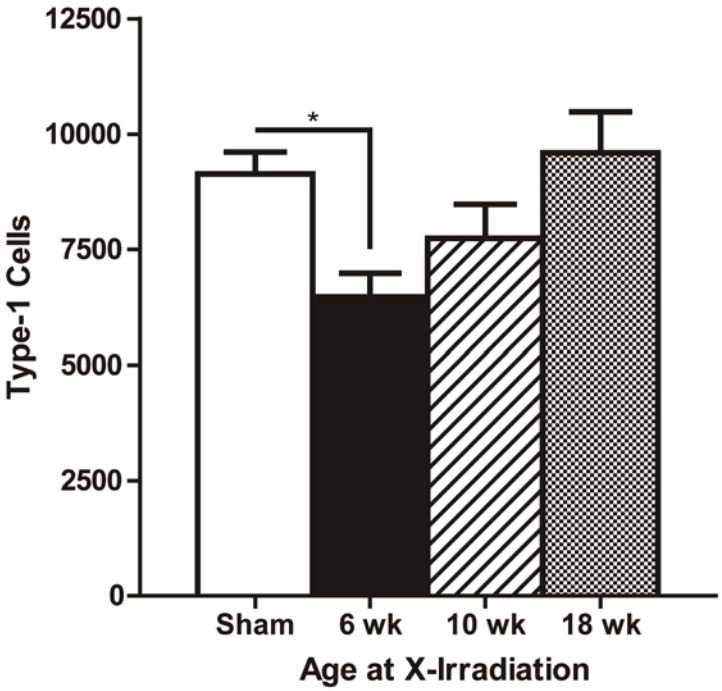

Figure 2.3. X-irradiation causes an age-dependent decrease in the number of Type-1 cells. Nestin-GFP mice received sham (6 or 18 weeks of age) or 5 Gy X-irradiation (6, 10, or 18 weeks of age). All mice were sacrificed at 19 weeks of age. X-irradiation at 6 weeks of age (black bar) causes a significant decrease in number of Type-1 cells compared to sham-irradiated controls (white). Irradiation does not significantly decrease the number of Type-1 cells when mice are 10 weeks (hatched) or 18 weeks of age (checked). There is a strong (non-significant) trend toward a decreased between mice irradiated at 18 weeks and 6 weeks of age. Mean ± SEM, 3-7 mice/group. *, $p < 0.05$.

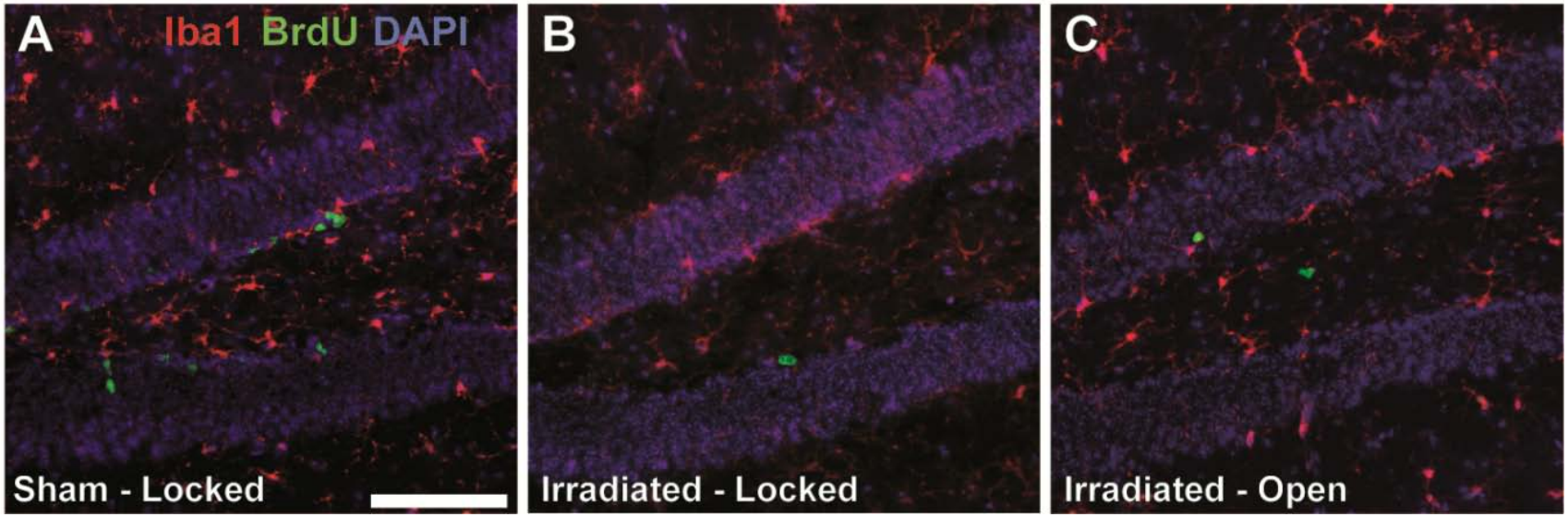

Figure 2.4. Radiation-induced inflammation normalizes within two months. Representative epifluorescent photomicrographs from mice irradiated at 6 weeks of age, allowed to recover for 2 months, then given access to locked or open wheels for 7d. (A) Sham-Locked mice; (B) Irradiated-Locked mice; and (C) Irradiated-Open mice. Representative sections are stained for microglial marker Iba-1 (red), exogenous S-phase marker BrdU (green) and nuclear counterstain DAPI (blue). There are no overt differences in microglial density or activation between groups. Scale bar in (A) is 50 µm applies to (A-C).

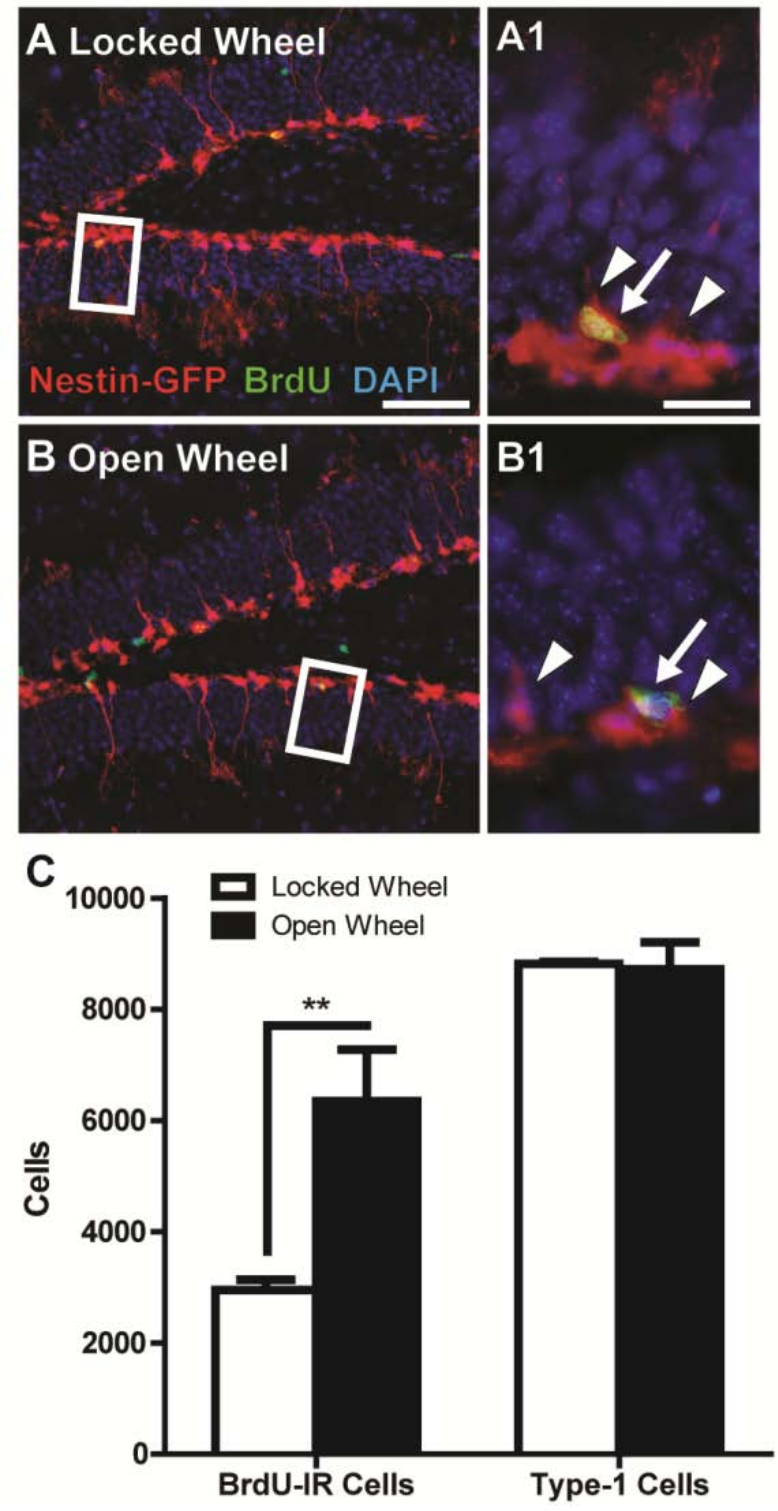
A Locked Wheel
A1
Nestin-GFP BrdU DAPI
B Open Wheel
B1
C
Locked Wheel
Open Wheel
**
Cells
10000
8000
6000
4000
2000
0
BrdU-IR Cells
Type-1 Cells

Figure 2.5. Voluntary running increases proliferation without changing Type-1 cell number in nestin-GFP mice. (A-B) Representative epifluorescent photomicrographs from mice with 7d access to either (A) locked or (B) open running wheel. Sections are stained for nestin-GFP (red), exogenous S-phase marker BrdU (green), and counterstained with DAPI (blue). Insets in (A1) and (B1) highlight Type-1 cells with radial glial morphology (arrowheads) and BrdU-IR cells (arrows) at higher magnification. Note a Type-1 cell that is also BrdU-IR in A1. (C) Access to an open wheel (black bars) causes a 2-fold increase in the number of proliferating BrdU-IR cells (green in A, B) compared to control mice on locked wheels (white bars). However, running does not change the number of Type-1 stem cells. Scale bar in (A) is 25 μm and applies to (A-B); scale bar in inset is 10 μm and applies to (A1-B1). Mean ± SEM, 3-4 mice/group. **, $p < 0.01$.

CHAPTER THREE

Adult hippocampal neurogenesis is functionally important for stress-induced social avoidance

Adapted from: Lagace DC, Donovan MH, **DeCarolis NA**, Farnbauch LA, Malhotra S, Berton O, Nestler EJ, Krishnan V, Eisch AJ. 2010. "Adult hippocampal neurogenesis is functionally important for stress-induced social avoidance. Proceedings of the National Academy of Science, [E-pub ahead of print].

Abstract

The long-term response to chronic stress is variable, with some individuals developing maladaptive functioning while others are resilient. Stress reduces neurogenesis in the adult hippocampal subgranular zone (SGZ), but it is unknown if stress-induced changes in neurogenesis contribute to individual vulnerability. Using a chronic social defeat stress model, we explored whether the susceptibility to stress-induced social avoidance was related to changes in SGZ proliferation and neurogenesis. Immediately after social defeat, stress-exposed mice (irrespective of whether they displayed social avoidance) had fewer proliferating SGZ cells labeled with the S-phase marker BrdU. The decrease was transient, as BrdU cell numbers were normalized 24hrs later. The survival of BrdU cells labeled prior to defeat stress was also not altered.

However, four weeks after the last stress, mice that displayed social avoidance had more surviving dentate gyrus neurons. Thus, dentate gyrus neurogenesis is increased after social defeat stress selectively in mice that display persistent social avoidance. Supporting a functional role for adult-generated dentate gyrus neurons, ablation of neurogenesis via X-ray irradiation robustly inhibited social avoidance. These data suggest the period of time after cessation of stress is a critical period for the establishment of persistent cellular and behavioral responses to stress, and show that a compensatory enhancement in neurogenesis is related to the long-term individual differences in maladaptive responses to stress.

Introduction

A central component of optimal functioning and survival is allostasis, or an individual's dynamic response to internal and external sources of stress required to maintain physiological and behavioral homeostasis (Joels et al., 2007). For some individuals, exposure to chronic stress leads to pathologically-enhanced allostatic response ("allostatic overload") (McEwen, 2007), which ultimately results in disease states like major depressive disorder or post traumatic stress disorder. Interestingly, the vast majority of stress-exposed individuals do not develop stress-related psychopathology (Charney and Manji, 2004; Yehuda and LeDoux, 2007). Recently, neuroadaptations in the mesolimbic reward system were identified that contribute to the susceptibility to stress (Krishnan et al., 2007), raising the question as to whether neuroadaptations occur in other brain

regions that also influence individual responses to stress. Because stress potently modulates learning and memory, and the hippocampus is intimately linked to these processes, it is critical to evaluate how hippocampal structure and function are altered by stress.

One notable stress-induced hippocampal neuroadaptation is inhibition of cell proliferation in the subgranular zone (SGZ), a region that gives rise to adult-generated dentate gyrus granule cell neurons (Joels et al., 2007; Pittenger and Duman, 2007). This inhibition generalizes across species and stress paradigms and may represent a long-lasting, maladaptive response to chronic stress (Joels et al., 2007; Pittenger and Duman, 2007). Previous attempts to link SGZ proliferation to stress-induced behavioral response have failed (Malberg and Duman, 2003; Vollmayr et al., 2003). However, neurogenesis is a complex multi-stage process involving proliferation, neuronal differentiation, and ultimately survival and integration into circuitry (Kempermann et al., 2004) that requires at least ~2-4 weeks (Kee et al., 2007; Zhao et al., 2008). While previous studies have focused on short-term effects of stress on proliferation, it remains unclear if there are longer-lasting stress-induced changes in neurogenesis that may influence behavior.

To examine the behavioral significance of stress-induced changes in hippocampal neurogenesis, we employed a social defeat stress paradigm that produces cohorts of mice that can be classified as “susceptible” or

"unsusceptible" to stress (Krishnan et al., 2007). Susceptible mice show a long-lasting social avoidance behavior and a depressive-like phenotype, characterized by anhedonia, social anxiety, heightened sensitization to psychostimulants, and circadian changes, which together suggest a maladaptive phenotype (Krishnan et al., 2007). In contrast, unsusceptible mice are indistinguishable from controls in these measures and thus are interpreted as being resilient in the face of social adversity. Because both susceptible and unsusceptible mice experience the same degree of stress, it is not surprising that they both exhibit some similar stress-related physiological responses like elevated corticosterone (CORT) levels.

Given the persistent changes in social behavior one month following defeat (Berton et al., 2006; Krishnan et al., 2007), we wondered whether the proliferation and survival of adult-born neurons contributed to this phenotype. We report here that independent of susceptibility to stress, there is a robust and transient reduction in proliferation immediately after stress with no change in long-term survival of cells that were born prior to the stress. However, examination of neuronal survival after cessation of stress reveals that susceptible mice have enhanced dentate gyrus neurogenesis. Moreover, ablation of neurogenesis shows that adult hippocampal neurogenesis is required for stress-induced social avoidance, emphasizing a functional role for newly born neurons.

Methods

Mice, social defeat, and social interaction protocol. Adult (5-8 weeks of age) male mice expressing GFP under the control of the nestin promoter (Yamaguchi et al., 2000) were the subjects and CD1 retired breeders (Charles River) were the aggressors. Social defeat was performed daily for 10 days similar to previously published reports (Berton et al., 2006; Krishnan et al., 2007; Tsankova et al., 2006) and in Extended Methods, below. Social interaction testing comprised of 2 trials for 150sec each and is described previously (Berton et al., 2006; Krishnan et al., 2007; Tsankova et al., 2006). Briefly, for interaction testing, in the first trial a mouse is placed into an open field box and allowed to explore a plastic enclosure placed within the pre-defined interaction zone. In the second trial the mouse is returned to the open field arena containing a plastic enclosure, now holding an awake CD1 aggressor mouse. An interaction score is calculated for each animal, defined as the ratio of time spent in the interaction zone with the aggressor mouse present divided by the time spent in interaction zone without an aggressor present. Control mice tend to interact with a social target and generally have an interaction ratio>100; “susceptible” mice are defined as mice having undergone social defeat with an interaction ratio<100.

Ablation of neurogenesis using X-ray irradiation. Ionizing radiation was carried out using X-RAD 320 self-contained irradiation system (Precision X-ray Inc).

BrdU injection, tissue collection and processing. Mice received an IP injection of

BrdU (150 mg/kg; Boehringer Mannheim, Mannheim, Germany) prior to sacrifice to label proliferating cells in the S-phase of the cell cycle. The time between BrdU injection and sacrifice varied across experimental groups (+1min-P, +24hrs-P, -12hrs-S, +24hrs-S; see Figs 3A, Figure 4A). Using stereological assessment (Eisch et al., 2000; Gould et al., 1999), BrdU-, Ki67-, DCX- and GFP-IR cells were counted in every ninth section of the hippocampus at 400x magnification. Colocalization of fluorescence was determined via confocal microscopy (630x magnification) using multi-track scanning. Colocalization was verified by 3D reconstruction.

Results

Chronic social defeat stress produces a specific avoidance of a potential aggressor. Mice were categorized as susceptible or unsusceptible to defeat stress based on the social interaction test, which measures the time a mouse spends interacting in the absence or presence of an unfamiliar mouse (Berton et al., 2006; Krishnan et al., 2007). Twenty-four hours following 10 days of social defeat, stressed (susceptible and unsusceptible) and control mice spent comparable time exploring the corners or interaction zone in the absence of a social target (**Figure 3.1A**). However, in the presence of a social target, susceptible mice spent significantly more time in the corners and less time in the interaction zone (**Figure 3.1B**). Thus, susceptible mice had a significantly reduced interaction ratio (ratio of time spent in interaction zone in the absence vs presence of social target; **Figures 3.1C; 3.5**). One day of defeat stress did not

reduce the interaction ratio, but 5 or 10 days did (**Figure 3.1D**). Because 10 days of defeat stress reliably produced ~50% susceptible mice, this protocol was used for remaining studies.

We previously demonstrated that avoidance behavior is associated with behavioral abnormalities associated with depression and anxiety, and thus can be interpreted as maladaptive (Krishnan et al., 2007). The avoidance behavior also generalizes to non-aggressor mice and is reversed by antidepressants (Berton et al., 2006; Krishnan et al., 2007). However, another interpretation is that avoidance is adaptive and may be associated with enhanced learning or memory to avoid a potential threat. We tested whether susceptible mice were different from controls in avoidance of a non-social adverse stimuli, social interaction, or memory. We observed no significant differences in passive avoidance behavior (**Figure 3.1E**), suggesting susceptible mice do not have specific differences in avoidance of non-social stimuli or alterations in short-term memory. Similarly, there were no differences in social interaction between the groups as assessed in the juvenile interaction test (**Figure 3.1F**). Thus avoidance of the aggressor mouse is a specific behavior that occurs in susceptible mice in the presence of other maladaptive behaviors, and does not generalize to deficits in avoidance, social interaction, or short-term memory.

Stress transiently reduces number of S-phase SGZ cells in susceptible and unsusceptible mice. The number of cells in S-phase of the cell cycle in dentate

gyrus subregions (**Figure 3.6**) was examined by quantifying BrdU-immunoreactive (BrdU-IR) cells immediately (+1min-P) and one day after stress (+24hrs-P; **Figure 3.2A**, Extended Methods). The frequency of susceptible mice was 52% and 44% for the +1min-P and +24hrs-P groups, respectively (**Figure 3.7**). Both susceptible and unsusceptible mice had fewer BrdU-IR SGZ cells immediately after defeat, which normalized 24hrs after defeat (**Figure 3.2B-C**). The reduction occurred across the septotemporal axis of the SGZ and but not in other dentate gyrus regions (**Figures 3.6; 3.8A-D**), emphasizing the restriction of this significant decrease to the neurogenic region of the dentate gyrus. There was also no correlation between BrdU-IR cell number and interaction ratio in the +1min-P (r=0.28, *p*=ns) or +24hrs-P (r=0.07, *p*=ns) mice.

While stress resulted in an immediate decrease in SGZ cells in S-phase (**Figure 3.2C**), there was surprisingly no difference in total number of proliferating cells between the groups as measured by number of Ki67-IR cells in SGZ (**Figure 3.2D**)(Eisch and Mandyam, 2007; Kee et al., 2002). To assess if stress altered cell cycle dynamics, the colocalization of Ki67-IR and BrdU-IR cells was used to determine the percentage of total cycling cells that were in the S-phase of the cell cycle (**Figure 3.2E**)(Eisch and Mandyam, 2007). Susceptible and unsusceptible mice had a significantly lower proportion of cells in S-phase immediately after the last defeat (**Figure 3.2F**), an effect that was normalized 24hrs later (**Figure 3.7E**). This suggests the transient, stress-induced decrease in BrdU-IR cell

number reflects a decreased S-phase cohort or a stalling of cells in S-phase, but not a general decrease in the number of proliferating cells.

When CORT was assessed immediately after stress, there was a significant increase in CORT levels in both susceptible and unsusceptible mice (**Figure 3.2G**). However, 24hrs after stress, CORT levels normalized in both susceptible and unsusceptible mice relative to controls (**Figure 3.8F**). There was also a significant inverse correlation between levels of CORT and number of BrdU-IR SGZ cells ($r=-0.43$, $p<0.05$). In agreement with others (Cameron and Gould, 1994), a decrease in BrdU-IR SGZ cell number was associated with higher CORT levels. Thus, an increase in CORT was concurrent with fewer cells in S-phase immediately after defeat stress irrespective of behavioral phenotype, and these changes did not distinguish the mice based on their susceptibly to stress.

Mice with long-term susceptibility to stress have enhanced dentate gyrus neurogenesis after stress. To determine if susceptibility to stress was related to neurogenesis, cell survival was measured for cells born prior to defeat (BrdU given 12hrs prior to stress initiation [-12hrs-S]) and cells born after 10 days of defeat (BrdU given 24hrs after cessation of stress [+24hrs-S], **Figure 3.3A**). 58% and 46% of the -12hrs-S and +24hrs-S mice were susceptible to defeat stress, respectively (Figures 3.3B, 3.9). Consistent with previous findings (Krishnan et al., 2007), avoidance behavior in susceptible mice was stable and persistent with

a significant positive correlation between interaction ratios 24hrs and 4 weeks following defeat (Figure 3.9, r=0.5, $p<0.05$).

When S-phase cells were labeled with BrdU prior to initiation of stress (a time when BrdU will label equivalent number of cells in all groups) and examined 4 weeks after completion of the 10 days of defeat stress, there was no difference in surviving BrdU-IR cells (-12hrs-S, **Figure 3.3A, C**). In sharp contrast, when S-phase cells were labeled with BrdU one day after the cessation of stress (a time the stress-induced decrease in proliferation is normalized, **Figure 3.2**), susceptible mice had significantly more surviving BrdU-IR cells 4 weeks later compared to both control and unsusceptible mice (+24hrs-S, Figure 3.3C). There was also a significant increase in BrdU-IR cells in the dentate gyrus molecular layer and a non-significant trend for increase in cells in the outer granule cell layer (**Figure 3.10**). Differentiation of surviving BrdU-IR SGZ cells was not significantly different between stressed and control mice, with over 75% of BrdU-IR cells expressing the mature neuronal marker NeuN (**Figure 3.3D-E**).

Enhanced dentate gyrus neurogenesis in susceptible mice is associated with altered number of transient amplifying progenitors but not with altered BDNF signaling or cell death. One potential mechanism for the increased survival of adult-generated dentate gyrus neurons in susceptible mice is increased hippocampal levels of brain-derived neurotrophic factor (BDNF) (Sairanen et al., 2005). Regional hippocampal dissections and subsequent immunoblotting

revealed no change in protein levels between controls and either group of stressed mice for BDNF, its receptor TrkB (full-length or truncated), or downstream signaling molecule ERK (**Figure 3.11**). Thus the increased neurogenesis in susceptible mice is not caused by grossly altered hippocampal BDNF signaling.

To determine if the enhanced survival of BrdU-IR cells was related to either cell death or the number of cells at the different stages of maturation at time of BrdU labeling (+24hrs-P), both the number of activated caspase-3 (AC3-IR) SGZ cells and phenotype of BrdU-IR cells was determined. There was no significant difference in number of AC3-IR cells (susceptible 227±33, unsusceptible 230±31, controls 242±28 cells, $F_{(2,17)}$=0.07, *p*=ns; n=5-7 mice/group) suggesting that the enhanced survival in susceptible mice was not due to a difference in apoptosis. To assess the proportion of SGZ cells in distinct stages of neurogenesis (Kronenberg et al., 2003; Yamaguchi et al., 2000), BrdU-IR cells were phenotyped based on colocalization of BrdU with the immature neuron marker doublecortin (DCX) and green fluorescent protein (GFP) in the nestin-GFP reporter mouse. Using morphology of DCX and GFP, four distinct cell types were identified that represent stages and a potential lineage of hippocampal neurogenesis (**Figure 3.12A**, Extended Methods)(Kempermann et al., 2004; Kronenberg et al., 2003). Briefly, stem-like cells (Type-1; GFP+/DCX-, radial glial-like morphology) and early progenitors (Type-2a; GFP+/DCX-, compact morphology) give rise to late progenitors (Type-2b, GFP+/DCX+, compact

morphology) and then to maturing progenitors/neuroblasts (Type-3, GFP-/DCX+). Susceptible and unsusceptible mice did not differ in the proportion of BrdU-IR cells that were Type-1 (**Figure 3.12B**) or the number of Type-1 cells (**Figure 3.12C**), or in the percentage of BrdU-IR cells that were Type-3 (mature progenitor, **Figure 3.12B**). Interestingly, susceptible mice had a higher proportion of BrdU-IR cells that were Type-2a cells and a lower proportion of Type-2b cells (**Figure 3.12B**). These data suggest that the enhanced neurogenesis in susceptible mice is in part due to greater numbers of transient-amplifying progenitors immediately after stress.

X-ray irradiation prior to social defeat attenuates the percentage of mice that have a susceptible phenotype. To explore the hypothesis that enhanced neurogenesis might contribute to behavioral susceptibility to stress, we ablated neurogenesis via cranial X-ray irradiation (X-irradiation) four weeks prior to social defeat and assessed effects on behaviors relevant to social interaction and short-term memory (**Figure 3.4A**). Consistent with previous reports (Wojtowicz, 2006), X-irradiation ablated proliferation and neurogenesis four weeks post-irradiation, with irradiated mice displaying severe deficits in BrdU-, Ki67- and DCX-IR cells compared to sham controls (**Figure 3.4B-C**). In addition, X-irradiated mice displayed no significant differences in social avoidance, passive avoidance, or juvenile interaction behavior (**Figure 3.4A, D-F**). Together these data support that cranial irradiation drastically reduced proliferation and neurogenesis without any overt changes in behavioral measures.

Sham- and X-irradiated mice underwent 10 days of social defeat stress and were tested for social interaction one day or 4 weeks later (**Figure 3.4G**). Surprisingly, irradiated mice were unsusceptible to defeat stress and did not display social avoidance, resulting in a significantly increased interaction ratio when compared to sham-irradiated defeated mice (**Figure 3.4H**). The percentage of irradiated mice that displayed social avoidance following defeat stress was 25% and 37% at day 11 and 4 weeks, respectively. Importantly, control non-defeated mice that were sham- or X-irradiated had no significant difference in interaction ratios, supporting our previous finding that X-irradiation did not alter social interaction behavior (**Figure 3.4D, H**). Four weeks after defeat, irradiated mice had very few SGZ cells labeled with BrdU, Ki67, or DCX (**Figure 3.4I**), and had significantly fewer BrdU-IR cells that survived 4 weeks later compared to sham-irradiated mice (sham/defeat=249±42, X-ray/defeat=23±9; $t_{(10)}$=4.4, p<0.005; n=5-7/group). Thus, ablation of adult neurogenesis via X-irradiation decreases the proportion of mice that are susceptible to stress-induced avoidance.

Discussion

The social defeat paradigm is a robust model of stress-induced social avoidance that produces multiple features of stress susceptibility in approximately one-half of the subjects (Krishnan et al., 2007). As such, this is an ideal model in which to probe the neural mechanisms contributing to an organism's response to chronic, severe stress. Here we explore the potential links between adult hippocampal

neurogenesis and the “memory” of severe stress. It is well known that stress decreases SGZ proliferation, and we show that after social defeat stress significantly but transiently reduces SGZ proliferation immediately after the last stress independent of a mouse’s susceptibility. Surprisingly, we show that mice susceptible to defeat stress have significantly enhanced survival of dentate gyrus neurons that were generated after – but not before – defeat stress compared to either unsusceptible mice or controls. Thus, increased hippocampal neurogenesis is related to the long-term maintenance of social avoidance. When neurogenesis was ablated by X-ray irradiation, significantly fewer mice displayed social avoidance. Thus, hippocampal neurogenesis appears to be involved in persistent social avoidance behavior. These data suggest that a compensatory enhancement in hippocampal neurogenesis is related to the long-term individual differences in maladaptive responses to stress.

Stress is generally associated with decreased SGZ proliferation resulting in decreased survival and neurogenesis (Joels et al., 2007; Pittenger and Duman, 2007). Our current results concur with the preponderance of literature demonstrating decreased cell proliferation during or immediately after stress (reviewed in Banasr and Duman, 2007; Gould and Tanapat, 1999; Schmidt and Duman, 2007; Zhao et al., 2008). Our findings are also consistent with reports of no change in the number of cells that survive if cells are labeled with BrdU before stress (Heine et al., 2004). In contrast, when we label cells with BrdU one day after stress, we find enhanced cell survival, which mirrors the enhanced survival

seen post-ischemia (Jin et al., 2001; Raber et al., 2004a). Importantly, we find that enhanced neuronal survival is associated with sustained behavioral alterations post-stress: the number of surviving BrdU-IR cells is increased only in mice with persistent social avoidance. When we analyze all defeated mice in this study, regardless of the differences in susceptibility to stress, there are no differences in survival post-defeat. The lack of decrease in neurogenesis post-stress between stressed and control mice is consistent with no change in cell survival one day following chronic unpredictable stress in rats (Heine et al., 2004), and the normal granule cell layer volume reported in many stress studies (Czeh et al., 2001; Jayatissa et al., 2006; Yap et al., 2006). However, by analyzing the susceptibility to stress through behavioral outcomes we have uncovered the striking significant increase in survival in BrdU-IR cells in those mice that are specifically susceptible to stress. These findings urge the use of a similar behavioral analysis in other stress models in order to clarify the neuroadapations associated with the diverse behavioral coping mechanisms utilized after stress.

Mechanistically, we hypothesized that the increased cell survival in susceptible mice might be mediated by BDNF since this neurotrophic factor has been linked with enhanced dentate gyrus neurogenesis (Sairanen et al., 2005) and plays a critical role in the nucleus accumbens of susceptible mice (Krishnan et al., 2007). However, hippocampal levels of key components of BDNF signaling were not different from controls at any time point post-defeat. These data highlight the

regional specificity of BDNF actions within the nucleus accumbens following defeat. Cell death also does not account for the enhanced cell survival in susceptible mice. Because few factors are known that alter the survival of adult-generated hippocampal neurons independent of changes in proliferation, ongoing studies with gene profiling will likely provide previously undescribed mechanisms that could account for increased survival in mice susceptible to defeat stress.

Unlike the specific increase in neurogenesis observed in mice susceptible to stress, mice that were either susceptible or unsusceptible to stress had a transient decrease in BrdU-IR cell number immediately post-defeat. We show that this decrease was restricted to the neurogenic SGZ, suggesting a region-specific mechanism for this regulation. This finding agrees with the majority of literature showing fewer BrdU-IR cells immediately after chronic stress paradigms (Czeh et al., 2001; Czeh et al., 2002; Gould et al., 1997; Gould et al., 1998; Simon et al., 2005; Yap et al., 2006). We demonstrate the reduction in BrdU-IR cells immediately following stress does not generalize to an overall decrease in the total number of proliferating cells, assessed by immunoreactivity of the endogenous cell cycle protein Ki67. This may be surprising given that BrdU- and Ki67-IR cell numbers are often used as interchangeable indices of proliferation (Kee et al., 2002; Wojtowicz and Kee, 2006). Our current data and other work (Tanapat et al., 1999) caution against this methodology, emphasizing that cells in discrete phases of the cell cycle can be differentially influenced by stimuli (Eisch and Mandyam, 2007; Taupin, 2007) or that cells can be temporarily

stalled in S-phase by stress (e.g. Flint et al., 2007). Our further analysis of BrdU-IR cells in nestin-GFP mice revealed that one day post-stress, susceptible mice had a specific increase in the proportion of BrdU-IR cells that were early progenitors (Type-2a) compared to unsusceptible mice. Although several studies have utilized nestin-GFP mice to determine the neurogenic stage affected by stimuli that increase neurogenesis, such as running (reviewed in Kempermann et al., 2004), the current data reveal how stress affects different stages of dentate gyrus neurogenesis. We show that following chronic stress, the populations of stem-like Type-1 and mature progenitor Type-3 cells remain largely unaffected, consistent with work suggesting that rapidly-dividing Type-2 cells modulate neurogenesis (Kempermann et al., 2004).

Ablation of neurogenesis prior to social defeat inhibited social avoidance, which could be interpreted as an antidepressant-like effect (Berton et al., 2006). It seems counterintuitive that antidepressant treatment which enhances neurogenesis inhibits social avoidance (Krishnan et al., 2007), while ablation of neurogenesis also inhibits stress-induced social avoidance. It could be that the effect of antidepressants on social avoidance may be mediated via other neural circuits (Berton et al., 2006). Alternatively, regulation of neurogenesis may be required for hippocampal-based plasticity, whether adaptive or maladaptive in nature. Social avoidance behavior appears to be mediated by situational encoding of information, because avoidance occurs in the presence of an awake CD1 mouse but does not generalize to an anaesthetized CD1 aggressor mouse

(Berton et al., 2006; Krishnan et al., 2007). The avoidance behavior exhibited by susceptible mice is also specific to potentially threatening stimuli because avoidance did not generalize as deficits in passive avoidance, social interaction, or short-term memory. A role for hippocampal neurogenesis and learning and memory in stress-induced social avoidance is consistent with the naturally important role of strong memory formation in the presence of a threatening stimulus (Shors, 2006). However, stress does not consistently enhance learning and memory, likely due to the various types and durations of stress, as well as its predictability (Sandi and Pinelo-Nava, 2007).

We show that ablating neurogenesis via x-ray irradiation decreased social avoidance in defeated mice but did not change basal levels of social interaction in non-defeated controls. Although these data agree with work showing that ablation of neurogenesis does not alter behavioral measures associated with memory (Airan et al., 2007; Santarelli et al., 2003; Surget et al., 2008; Wang et al., 2008), our data uniquely suggest that alterations in hippocampal neurogenesis provide synaptic plasticity required for social avoidance. This work also highlights that the period of time after cessation of stress is a critical period for the establishment of persistent cellular and behavioral responses to stress, and thus is likely amenable to therapeutic intervention.

Author contributions: D.C.L., M.H.D., N.A.D., O.B., V.K., and A.J.E. designed research; D.C.L., M.H.D., N.A.D., L.A.F., S.M., O.B., V.K., and A.J.E. performed research; D.C.L., M.H.D., N.A.D., V.K., and A.J.E. analyzed data; and D.C.L., M.H.D., N.A.D., L.A.F., O.B., E.N., V.K., and A.J.E. wrote the paper.

Extended Methods

Social Defeat and Social Interaction Protocol. The social defeat and interaction testing were performed as described previously (Berton et al., 2006; Krishnan et al., 2007; Tsankova et al., 2006). A CD1 aggressor mouse was housed on one side of a cage that was partitioned with a plastic divider throughout the 10 days of defeat stress. A test mouse was housed on the other side of the divider. The defeat was performed 2-3 hrs prior to the onset of the dark phase of the light cycle. During the 5 min defeat period, the test mouse was moved to the side of the cage housing the aggressor, which would physically attack the test mouse. Test mice were rotated so that after each defeat period, test mice were housed in a new cage living opposite a new aggressor. Control mice were housed in identical partitioned cages to those used for defeat, but instead of being housed beside an aggressor they were housed opposite to other control mice. Control mice were handed for each day at the same time of day as when the defeat was performed throughout the 10 days.

Social interaction was measured in the morning between 800-1200 hr and consisted of two trials lasting 150 sec each. Using Ethovision software, the

movements of the test mouse were tracked within a white open field box (45 cm long x 45 cm wide x 30 cm high). Within the box, an interaction zone (26 cm wide x 14 cm long) was defined and a plastic enclosure (10 cm x 6 cm) was placed in the center of the interaction zone against the wall of the box. In each trial, the mouse was placed randomly into either one of the corners of the box on the side opposite to the interaction zone. In the first trial, the test mouse was placed into the open area with the empty enclosure in the interaction zone. The time that the mouse spent in either the interaction zone or corners opposite from the interaction zone was quantified. In the second trial, the same measurements were taken, but unlike the first trial, an unfamiliar CD1 aggressor mouse was placed into the enclosure. The interaction ratio was calculated as (time spent in interaction zone with aggressor mouse / time spent in interaction zone with empty enclosure present) multiplied by 100. Immediately after testing for social interaction all mice were singly housed until sacrifice.

Ablation of Neurogenesis Using X-Ray Irradiation. Ionizing radiation was carried out using X-RAD 320 self-contained irradiation system (Precision X-Ray Inc). The X-RAD 320 irradiator is equipped with a custom-synthesized collimator that delivered an x-ray beam of 10 mm in diameter at rate of 1.08 Gy per min (250 kV, 15mA) for 4.5 min to achieve the cumulative desired dose of 5 Gy. Mice were anesthetized with ketamine/xylazine (9 mg/kg; 1 mg/kg in saline) before being positioned in the irradiator with their skulls located under collimator for whole-head irradiation. For sham-irradiated controls, anesthetized mice were

positioned into the irradiator but the device was never turned on. Following irradiation, mice recovered on a heating pad and were returned to previous housing conditions when fully awake.

Corticosterone (CORT) Measurements. Blood samples were centrifuged (1000x g for 15 min) and plasma was frozen at -20°C until assayed for corticosterone levels using the mouse OCTEIA CORT competitive enzyme immunoassay (Immunodiagnostic System)(Lagace et al., 2006).

BrdU Injection and Collection. Mice received an IP injection of BrdU (150 mg/kg; Boehringer Mannheim, Mannheim, Germany) prior to sacrifice to label proliferating cells in the S-phase of the cell cycle. The time between BrdU injection and sacrifice varied across experimental groups (+1min-P, +24hrs-P, -12hrs-S, +24hrs-S; see Figs 3A, Figure 4A). For proliferation analyses this time was under the estimated length of G2 (4-5hrs) (Cameron and McKay, 2001; Hayes and Nowakowski, 2002); thus it is not surprising that there is no difference in BrdU-IR cell number between 30min and 2hrs (Mandyam et al., 2007). BrdU injection occurred in each group 10hrs following social interaction testing, except for +1min-P, when mice were injected immediately after defeat and sacrificed 30 min later. For the +24hrs-P and +24hrs-S, BrdU was given 24hrs following the last defeat and mice were sacrificed either 2hrs or 4 weeks following BrdU injection, respectively.

Tissue Collection and Processing. All mice were euthanized between 1600 and 1900 hours. Mice were decapitated, blood was collected for CORT analysis, and brains were cut along the midsagittal sulcus. One hemisphere was immersion fixed for immunihistochemistry (IHC; Figure 3.12), and the other was used for hippocampal dissection and subsequent protein content analyses via Western blot (Fig. 3.11). For immersion fixation, hemispheres were placed in 4% paraformaldehyde (PFA, wt/vol) in 0.1M phosphate-buffered saline (PBS) for 3 days with PFA changed daily. Immersion-fixed tissue was viable for all IHC applications and there were no differences between immersion-fixed and perfused tissue in any measure of IR cells (Figure 3.13).

Tissue Processing and Immunohistochemistry (IHC). After immersion fixing in PFA, brains were subsequently cryoprotected in 30% sucrose (wt/vol) in 0.1M PBS and 0.1% sodium azide (NaN_3) and sectioned coronally on a freezing microtome (Leica, Wetzlar, Germany) at 30 µm through the hippocampus. Nine serial sets of sections were stored in 0.1% NaN_3 in 1XPBS at 4^{o}C until processing. For staining, one series of sections were ordered from rostral to caudal, mounted on glass slides (Fisher Superfrost/Plus, Hampton, NH), and dried overnight. Slides were coded to ensure objectivity, and the code was not broken until after data collection was complete.

For BrdU IHC, pretreatment consisted of: antigen retrieval (0.01M citric acid, pH 6.0, 95°C, 10 min), membrane permeabilization (0.1% trypsin in 0.1M Tris and

0.1% $CaCl_2$, 10 min), and DNA denaturation (2N hydrochloric acid in 1XPBS, 30 min). Following pretreatment, nonspecific staining was blocked by incubation with 3% normal donkey serum (NDS, vol/vol) in 0.1% TritonX-100 in 1XPBS. Sections were incubated in rat anti-BrdU primary antibody (1:300, Accurate Chemical, Westbury, NY) in 3% NDS, 0.1% Tween-20 in 1XPBS overnight. The following day, sections were incubated with biotinylated-donkey anti-rat secondary antibody (1:200; Sigma Laboratories, St. Louis, MI) in 1.5% NDS for 60 min, 0.3% hydrogen peroxide (Sigma Laboratories) for 30 min, and avidin-biotin complex (Vector Laboratories) for 90 min. Staining of BrdU-immunreactive (BrdU-IR) cells was visualized using DAB (Pierce, Rockford, IL) for 20-30 min. Nuclear Fast Red (Vector Laboratories) was used as a counterstain. All slides were dehydrated and coverslipped.

For BrdU/NeuN/GFAP triple-labeling immunofluorescent staining, sections were pretreated as described above and nonspecific labeling was blocked with 3% NDS for 60 min before overnight incubation with rat anti-BrdU, mouse anti-neuronal nuclei (NeuN, 1:50, Chemicon International, Temecula, CA) and rabbit anti-glial fibrillary acidic protein (GFAP, 1:500; DAKO, Glostrup, Denmark). Sections were then incubated in fluorescent CY2-donkey anti-rat, CY5-donkey anti-rabbit, and CY3-goat anti-mouse secondary antibodies (1:200; Jackson ImmunoResearch, West Grove, PA) for 3 hrs, counterstained with DAPI (1:5000; Roche, Basel, Switzerland), dehydrated, and coverslipped.

For BrdU/Ki67 double-labeling fluorescent IHC, sections underwent antigen retrieval (first step of pretreatment) followed by blocking of nonspecific staining in 3% NDS for 60 min before overnight incubation with rabbit anti-Ki67 (1:1000, Vector Laboratories, Burlingame, CA). Sections were then incubated in biotinylated-donkey anti-rabbit (1:200; Vector), and amplified by ABC (Vector) and CY3-tyramide signal amplification (TSA; Perkin-Elmer, Norton, Ohio) followed by membrane permeabilization and DNA denaturation (the second and third pretreatment steps), blocking again in 3% NDS for 60 min and a second overnight incubation with rat anti-BrdU, detected as above with fluorescent CY2-donkey anti-rat.

Similarly, for BrdU/GFP/Dcx triple-labeling fluorescent IHC, sections underwent antigen retrieval, blocking of nonspecific staining in 3% NDS for 60 min and overnight incubation with rabbit anti-GFP (1:3000; Abcam, Cambridge, UK) and goat anti-Dcx (1:5000; Santa Cruz, Santa Cruz, CA; 1:5000). This was followed by sequential incubation in 0.3% hydrogen peroxide, biotinylated-horse anti-goat (1:200; Vector), ABC (Vector) and CY5-TSA (Perkin-Elmer), then 0.3% hydrogen peroxide again, biotinylated-donkey anti-rabbit (1:200; Jackson ImmunoResearch), ABC (Vector) and CY3-TSA (Perkin-Elmer). Finally, the sections were incubated again in 3% NDS for 60 min and a second overnight incubation with primary rat anti-BrdU antibody, and signal was detected on day three with fluorescent CY2-donkey-anti-rat antibody.

Stereological Cell Counting. Bright-field or epifluorescent staining of section on coded slides was visualized and quantified with an Olympus BX-51 microscope. Staining was examined and quantified in several regions of the dentate gyrus (DG) of the hippocampus (bregma -0.82 to -4.24), including the SGZ, molecular layer (Mol), polymorphic layer (hilus), and outer granular cell layer (oGCL)(Franklin and Paxinos, 2004; Mandyam et al., 2004). The SGZ has been shown to give rise to cells with neurogenic potential, and therefore was the focus of these studies for BrdU-, Ki67-, DCX-, and AC3-IR as well as for Type-1 cell counts. The other hippocampal regions were assessed to control for bioavailability of the exogenous marker BrdU (Noonan et al., 2008), to augment our previous work on the proliferative capacity and regulation of proliferation of these neighboring dentate gyrus regions (Eisch and Mandyam, 2007), and thus to explore the regional specificity of stress-induced effects. The SGZ was defined as a region straddling the border of the granule cell layer (GCL) and the hilus: three GCL cell widths into the hilus and the inner half of the GCL adjacent to the hilus. The Mol was defined as the region between the superior limb of the GCL and the hippocampal fissure, and the inferior limb of the GCL and the ventral and medial borders of the DG; inner and outer Mol results were combined for this study. A cell on the border in the middle of the GCL was considered in the SGZ, whereas a cell touching the GCL on the border of the Mol was considered in the oGCL. A schematic of the anterior hippocampus (-2.18 from bregma) depicting the four DG regions analyzed is shown in Figure 3.6. However, the hippocampus across the septotemporal axis (-0.82 to -4.24 from bregma) was analyzed for this

study.

The quantification of immunoreactive cells (IR) in the SGZ, Mol, oGCL, and hilus was performed using stereology (Eisch et al., 2000; Mouton, 2002) by an examiner blinded to treatment. Using an Olympus BX-51 microscope at 400x magnification and continual adjustment through the depth of the section, IR cells in each dentate gyrus subregion were counted in every ninth section through the septotemporal aixs of the hippocampus. The section sampling fraction was 1/9, and the resulting number of cells per dentate gyrus was multiplied by 9 to obtain an estimate of the total number of cells per dentate gyrus subregion. As these are rare population, the all IR-labeled cells in the given region were counted (area sampling fraction of 1). Based on the previous work from our labs and others (Dayer et al., 2003; Eisch et al., 2000; Gould et al., 1997; Lister et al., 2005), the height sampling fraction was set at 1. As previously described by our lab and others (Mandyam et al., 2007; Olariu et al., 2007), BrdU- and Ki67-IR cells within clusters were resolved by distinguishing nuclear borders with an objective with appropriate working distance. For Type-1 cell analysis, GFP-IR cells with a radial process perpendicular to the SGZ were quantified by a single observer blind to experimental condition. In addition to estimating the total number of IR cells across the entire hippocampus, the distribution of cells across the longitudinal axis of the hippocampus was analyzed as different distances from bregma, and data are presented as total number of cells in the SGZ per section at each septotemporal point (Franklin and Paxinos, 2004).

IR-Cell Phenotyping. Colocalization of immunofluorescence was determined with a confocal microscope (Zeiss Axiovert 200 and LSM510-META, Carl Zeiss, Oberkochen, Germany; emission wavelengths 488, 543, and 633 nm) at 630x using multi-track scanning and an optical section thickness of approximately 0.5 μm in the *Z*-plane. To guard against false positives (Raff, 2003), colocalization was verified by importing *Z*-stacks of images into a 3D reconstruction program (Volocity, Improvision, Lexington, MA). The 3D renderings were rotated and examined from multiple perspectives to ensure colocalization. For presentation, images were imported into Photoshop (Adobe Systems 7.0, Carlsbad, CA) and adjustments were made only via the "level" function. To assess the putative stages of neurogenesis, we examined the morphology and colocalization of BrdU-IR cells DCX and GFP to identify four distinct cell types that represent putative stages and potential lineage of hippocampal neurogenesis (Donovan et al., 2008; Kempermann et al., 2004). We identified stem-like cells (Type-1) as GFP-IR/DCX- with radial glial-like morphology; early progenitors (Type-2a) as GFP-IR/DCX- with compact morphology; late progenitors (Type-2b) as GFP-IR/DCX-IR with compact morphology; and progenitors/neuroblasts (Type-3) as GFP-/DCX-IR. Figure 3.12 provides examples of these cell types.

Protein Extracts and Western Blot Analysis. The hippocampus from the remaining hemisphere of each mouse was rapidly dissected and frozen at -80°C until processed. Extracts were homogenized by sonication and boiled in 1%

sodium dodecyl sulfate (SDS) with 50mM sodium fluoride. Protein concentrations were determined by bicinchoninic acid assay (Pierce). Forty micrograms of total protein from each sample was electrophoresed on precast 4%-20% SDS gradient gels (BioRad). Following transfer, PVDF membranes were washed for 1 hr in 1xTris-buffered saline with 0.1% Tween-20 (TBS-T), and blocked in 5% (wt/vol) milk for 1 hr at 25°C. The membrane was then incubated in a solution of the appropriate primary antibody overnight at 4°C. Antibodies used included: anti-BDNF (N-20, 1:200; Santa Cruz), anti-TrkB (07-225, 1:4000; Upstate), anti-ERK 1/2 (4695, 1:1000; Cell Signaling), anti-ERK1/2 (4377, 1:1000; Cell Signaling). On the second day, blots were washed and then incubated with peroxidase-labeled secondary antibody at 25°C for 1 hr, and bands were visualized by enhanced chemiluminescence (Amersham, GE Healthcare, Buckinghamshire, UK). Results were quantified using National Institutes of Health image software.

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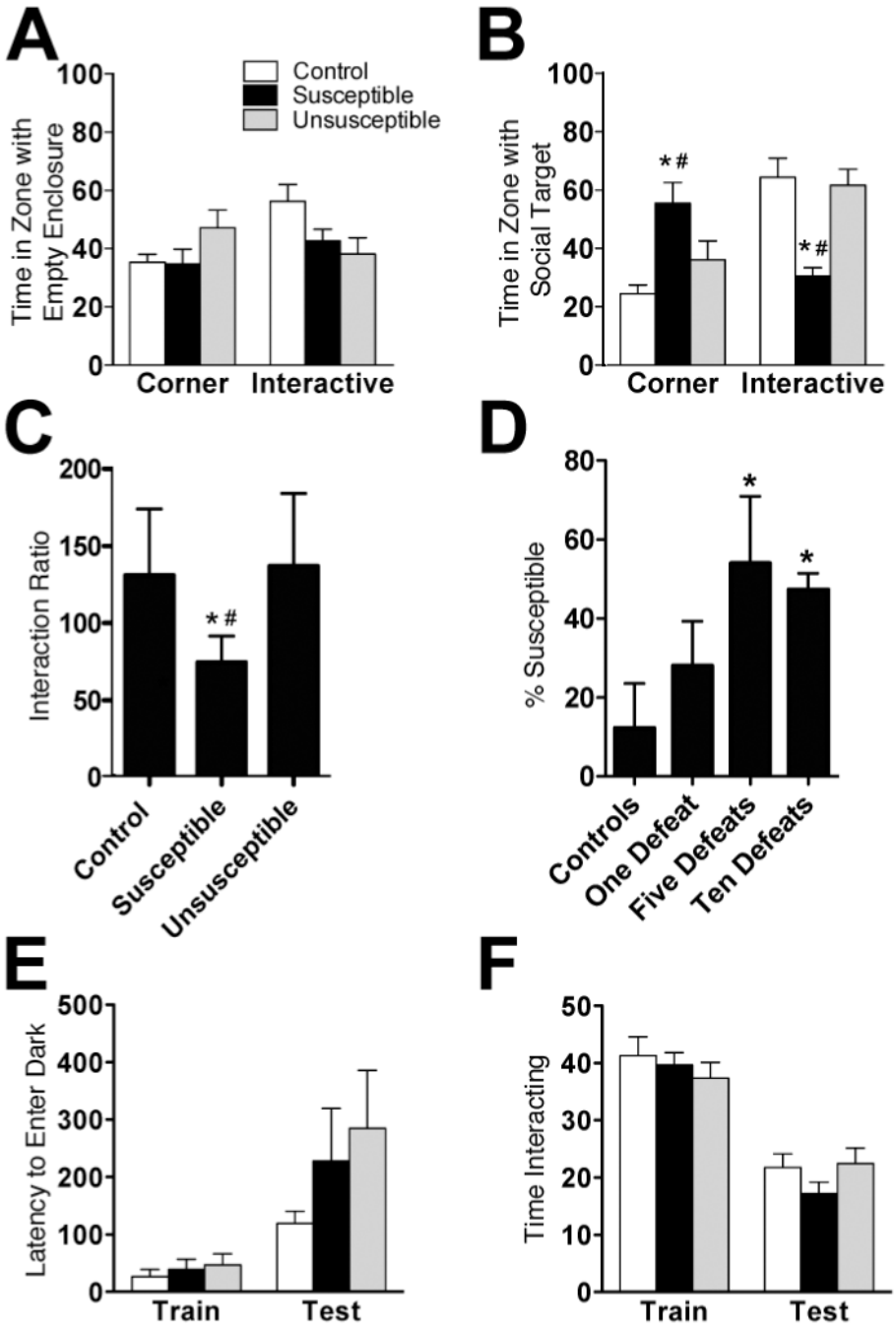
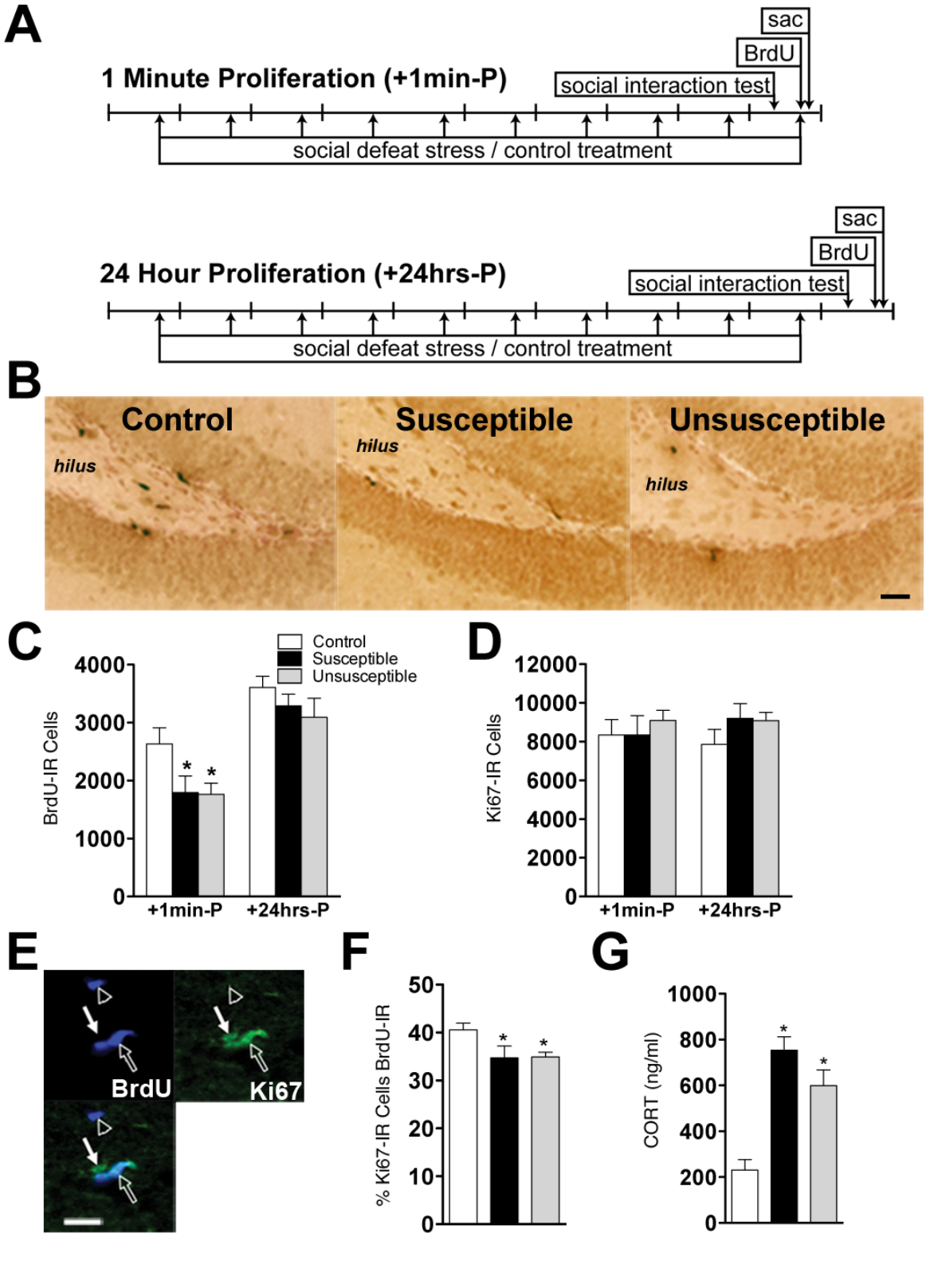


Figure 3.1. Chronic social defeat stress produces specific social avoidance of an aggressor. (A) In the presence of an empty enclosure, time (sec) spent in corners

opposite the interaction zone ($F_{2,37}$=1.8, *p*=ns) and interaction zone ($F_{2,37}$=2.6, *p*=ns). (B) In the presence of a social target, time spent in corners ($F_{2,37}$=4.8, *p*<0.05) and interaction zone ($F_{2,37}$=7.5, *p*<0.005). (C) Interaction ratio determined by proportion of time in presence versus absence of social target ($F_{(2,39)}$=6.6, *p*<0.005). (n=18-31/group for A-C). (D) Proportion of mice with a susceptible phenotype with an interaction ratio <100 after 1, 5 or 10 days of defeat stress ($F_{(3,9)}$=8.0, *p*<0.05; n=5-36/group). (E) Passive avoidance performance expressed as latency (sec) to enter the dark, punished compartment at training and testing (group, $F_{(2,36)}$=1.4, *p*=ns; time, $F_{(1,36)}$=11.4, *p*<0.005; n=6-8/group). (F) Social behavior expressed as time (sec) interacting with juvenile during first exposure (training) and exposure to same juvenile 3 days later (test) (group, $F_{(2,84)}$=0.7, *p*=ns; time $F_{(1,84)}$=77.4, *p*<0.001; n=6-8/group). * *p*<0.05; $^{\#}$ *p*<0.05 compared to control and unsusceptible mice in Bonferroni *post-hoc* test, respectively, mean ± SEM.


A
1 Minute Proliferation (+1min-P)
sac
BrdU
social interaction test
social defeat stress / control treatment
24 Hour Proliferation (+24hrs-P)
sac
BrdU
social interaction test
social defeat stress / control treatment
B
Control
hilus
Susceptible
hilus
Unsusceptible
hilus
C
Control
Susceptible
Unsusceptible
BrdU-IR Cells
4000
3000
2000
1000
0
+1min-P
+24hrs-P
D
Ki67-IR Cells
12000
10000
8000
6000
4000
2000
0
+1min-P
+24hrs-P
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Ki67
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% Ki67-IR Cells BrdU-IR
50
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1000
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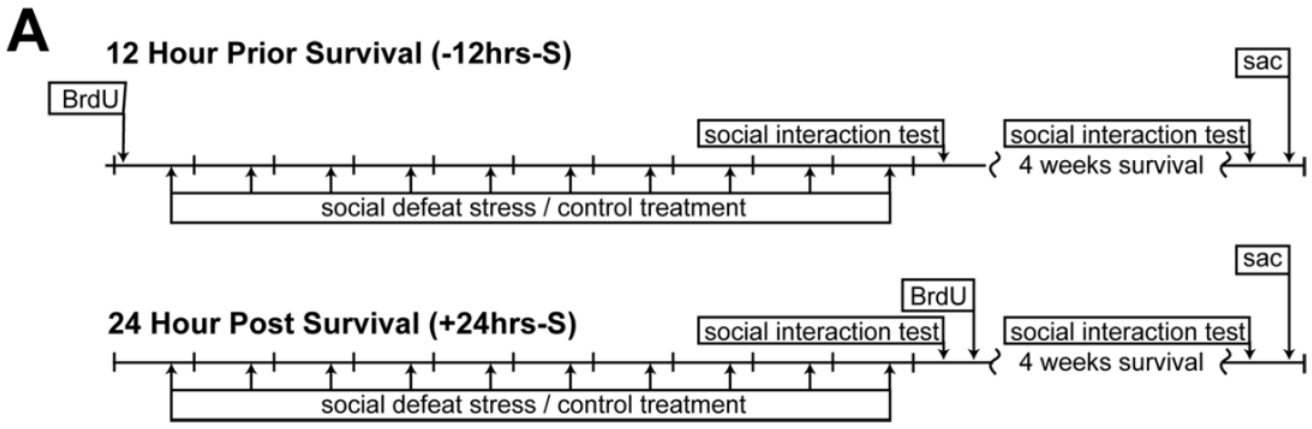
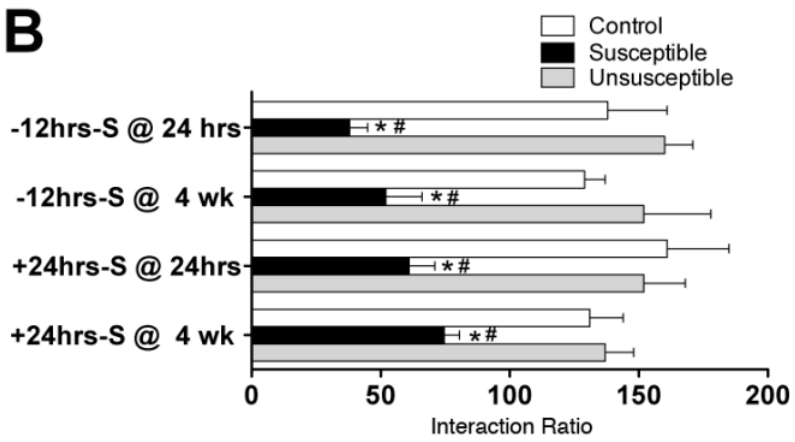
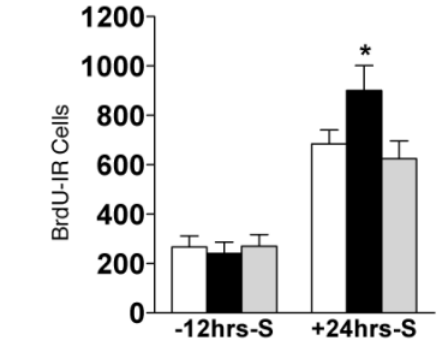
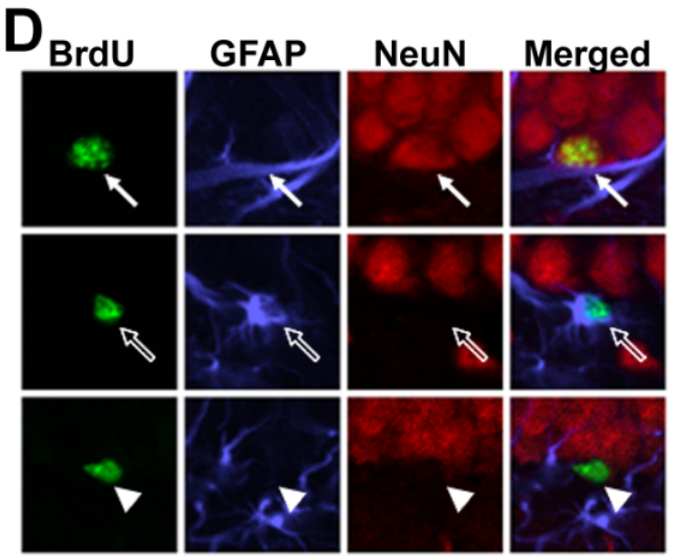
600
400
200
0

Figure 3.2. Defeat stress results in a transient decrease in number of BrdU-IR cells regardless of susceptibility. (A) Schematic of study groups. “+1min-proliferation” (+1min-P) group received BrdU immediately after the last defeat episode (n=9-18/group). “+24h-proliferation” (+24hrs-P) group received BrdU 24hrs after the last defeat episode (n=7-12/group). (B) BrdU-IR cells in stressed mice (susceptible and unsusceptible) compared to control mice (+1min-P) (Scale bar 100μm). (C) BrdU-IR cell number in SGZ ($F_{(2, 54)}$=4.3, p<0.05). (D) Ki67-IR cell number in SGZ ($F_{(2, 51)}$=0.9, p=ns). (E) IHC reveals BrdU-IR cells (open arrowhead), Ki67-IR cells (closed arrow) and cells that were both BrdU-IR and Ki67-IR (open arrow) (Scale bar 25 μm). (F) Percent of Ki67-IR cells that are BrdU-IR in +1min-P ($F_{(2, 29)}$=5.1, p<0.05, n=7-13/group). (G) CORT levels in mice 30 min (+1min-P) after last defeat stress ($F_{(2,26)}$=23.2, p<0.001; n=6-13/group). * p<0.05 compared to control mice in Bonferroni *post-hoc* test, mean ± SEM.


A
12 Hour Prior Survival (-12hrs-S)
BrdU
social interaction test
social interaction test
sac
4 weeks survival
social defeat stress / control treatment
24 Hour Post Survival (+24hrs-S)
BrdU
social interaction test
social interaction test
sac
4 weeks survival
social defeat stress / control treatment


B
Control
Susceptible
Unsusceptible
-12hrs-S @ 24 hrs
-12hrs-S @ 4 wk
+24hrs-S @ 24hrs
+24hrs-S @ 4 wk
* #
0
50
100
150
200
Interaction Ratio


C
BrdU-IR Cells
1200
1000
800
600
400
200
0
*
-12hrs-S
+24hrs-S


D
BrdU
GFAP
NeuN
Merged

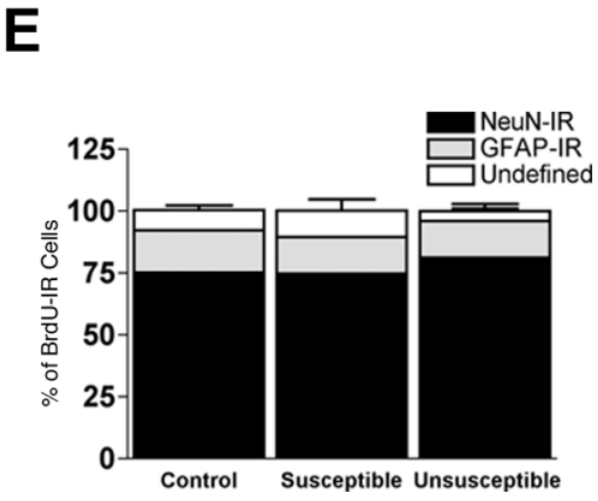

E
NeuN-IR
GFAP-IR
Undefined
% of BrdU-IR Cells
125
100
75
50
25
0
Control
Susceptible
Unsusceptible

Figure 3.3. Increased neurogenesis in susceptible mice after cessation of stress. (A) Schematic of experiments in which “-12hrs-survival” mice (-12hrs-S, top line) were given BrdU 12hrs prior to first defeat episode (n=7-14/group). “+24hrs-survival” mice (+24hrs-S, bottom line) were given BrdU 24hrs after the last defeat episode (n=8-15/group). Both groups were perfused 4 weeks after the last defeat stress. (B) Interaction ratios 24hrs and 4 weeks after stress (group, $F_{(2,97)}$=24.8, p<0.001; time $F_{(3,97)}$=0.3, p=ns). (C) Number of surviving BrdU-IR cells in SGZ ($F_{(1, 39)}$=61.7, p<0.001). (D) BrdU-IR cells were classified as neurons (BrdU-IR [green] and NeuN-IR [red], closed arrow), glial (BrdU-IR [green] and GFAP-IR [blue], open arrow) or undefined (BrdU-IR only, arrowhead). (E) Phenotypic analysis of surviving BrdU-IR cells ($F_{(2,81)}$=228.3, p<0.001; n=7-15/group). * p<0.05; $^{\#}$ p<0.05 compared to control and unsusceptible mice in Bonferroni *post-hoc* test, respectively, mean ± SEM.

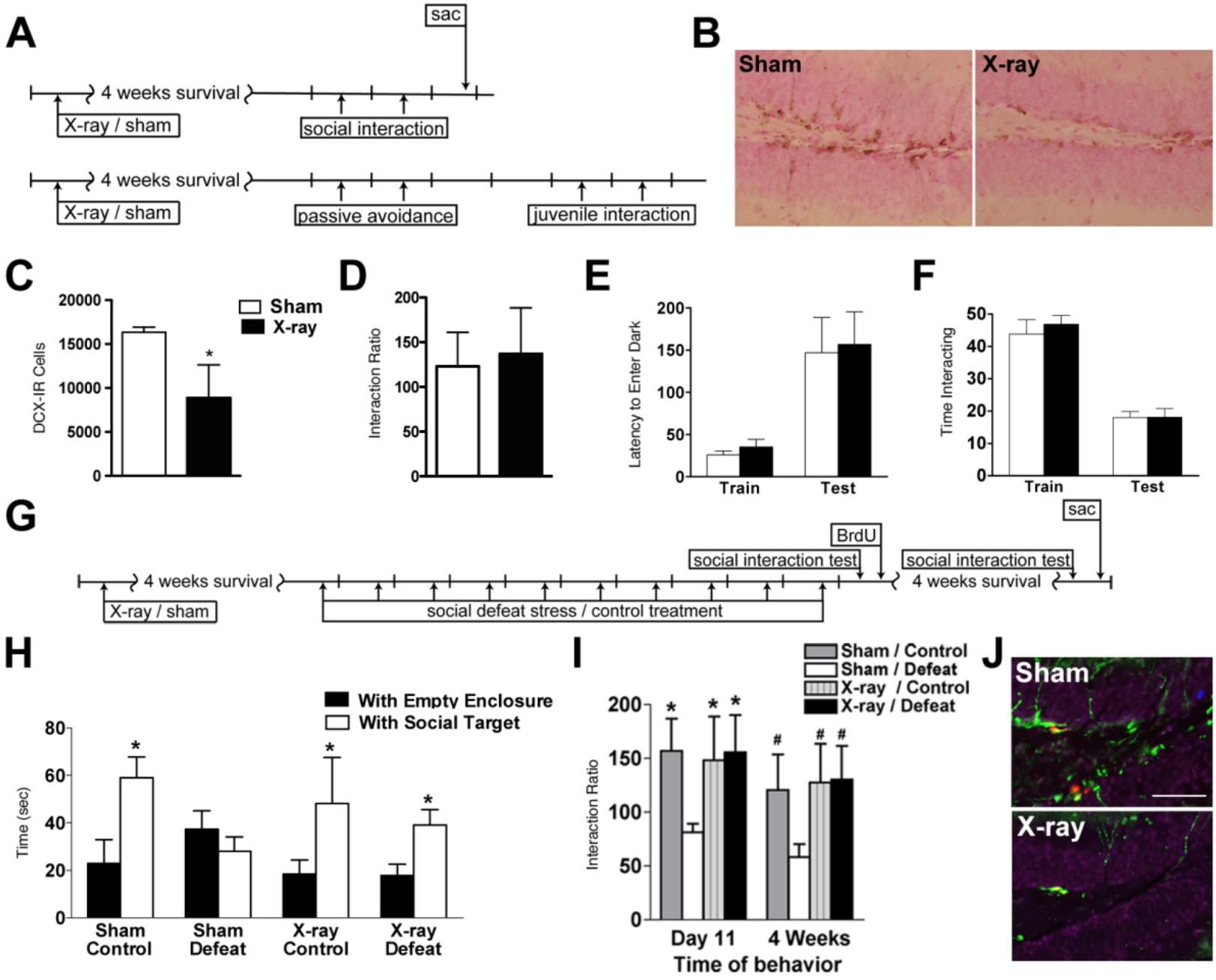

A
sac
4 weeks survival
X-ray / sham
social interaction
4 weeks survival
X-ray / sham
passive avoidance
juvenile interaction
B
Sham
X-ray
C
DCX-IR Cells
20000
15000
10000
5000
0
Sham
X-ray
*
D
Interaction Ratio
200
150
100
50
0
E
Latency to Enter Dark
200
150
100
50
0
Train
Test
F
Time Interacting
50
40
30
20
10
0
Train
Test
G
sac
BrdU
social interaction test
social interaction test
4 weeks survival
4 weeks survival
X-ray / sham
social defeat stress / control treatment
H
With Empty Enclosure
With Social Target
Time (sec)
80
60
40
20
0
*
*
*
Sham Control
Sham Defeat
X-ray Control
X-ray Defeat
I
Sham / Control
Sham / Defeat
X-ray / Control
X-ray / Defeat
Interaction Ratio
200
150
100
50
0
*
*
*
#
#
#
Day 11
4 Weeks
Time of behavior
J
Sham
X-ray

Figure 3.4. Ablation of neurogenesis prior to stress reduces susceptibility to social avoidance behavior. (A) Mice received 5 Gy X-ray or sham treatment 4 weeks before assessment of (top line) social interaction and number of BrdU-IR cells present 2hr following injection (n=6/group) or (bottom line) passive avoidance and juvenile interaction testing (n=12/group). (B) DCX-IR cells in the dentate gyrus of sham and X-irradiated mice 4 weeks post-irradiation. (C) DCX-IR cell number in SGZ ($t_{(8)}$=4.4, p<0.01). (D) Social interaction analysis expressed as interaction ratio ($t_{(9)}$=0.5, p=ns). (E) Passive avoidance performance expressed as latency (sec) to enter the dark, punished compartment at training and testing (group, $F_{(1,44)}$=0.1, p=ns; time, $F_{(1,44)}$=17.8, p<0.001). (F) Social behavior expressed as time (sec) interacting with juvenile during first exposure (training) and exposure to same juvenile 3 days later (test) (group $F_{(1,44)}$=0.3, p=ns; time $F_{(1,44)}$=78.4, p<0.001). (G) Mice received 5 Gy X-ray or sham treatment 28 days prior to 10 days of social defeat (n=8-11/group). (H) Interaction ratio from social interaction testing completed 24hrs (Day 11) and 4 weeks following 10 days of defeat stress ($F_{(3, 61)}$=3.2, p<0.05). (I) DCX-IR (green), Ki67-IR (red) and BrdU-IR (blue) cells 4 weeks after sham or X-ray exposure. (Scale bar 50 μm). * p<0.05, compared to Sham/Defeat Day 11 mice; $^{\#}$ p<0.05, compared to control 4 week mice in Bonferroni *post-hoc* test, mean ± SEM.

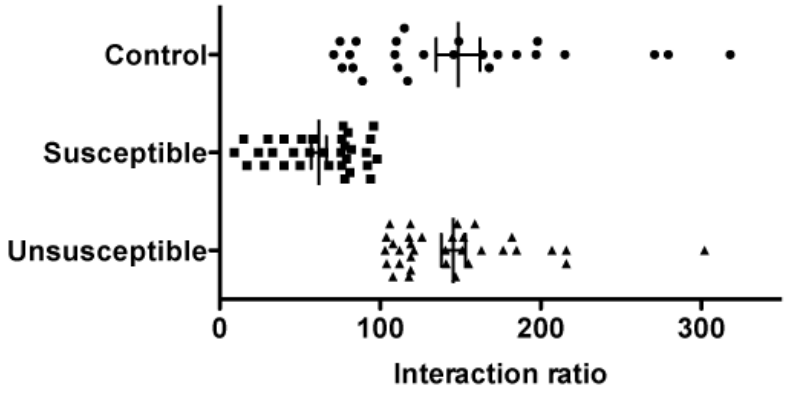


Figure 3.5. Horizontal scatter plot depicting the distribution of interaction ratios 24 hr following social defeat stress for control, susceptible, and unsusceptible mice over multiple social defeat experiments [combined mice included in Figure 3.1C and 3.1E (24 hr-P group).

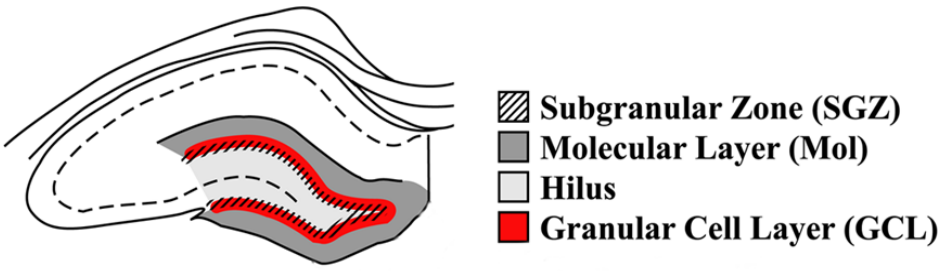


Figure 3.6. Four regions of the dentate gyrus (-1.5 to -6.3 mm from bregma) were examined as previously described (Mandyam et al., 2004). These include the molecular layer (Mol), subgranular zone (SGZ), outer portion of the granule cell layer (GCL is shaded red, while oGCL is non-striped red portion), and hilus (H). See main text and SI methods for details of regional assessment of cell counts.

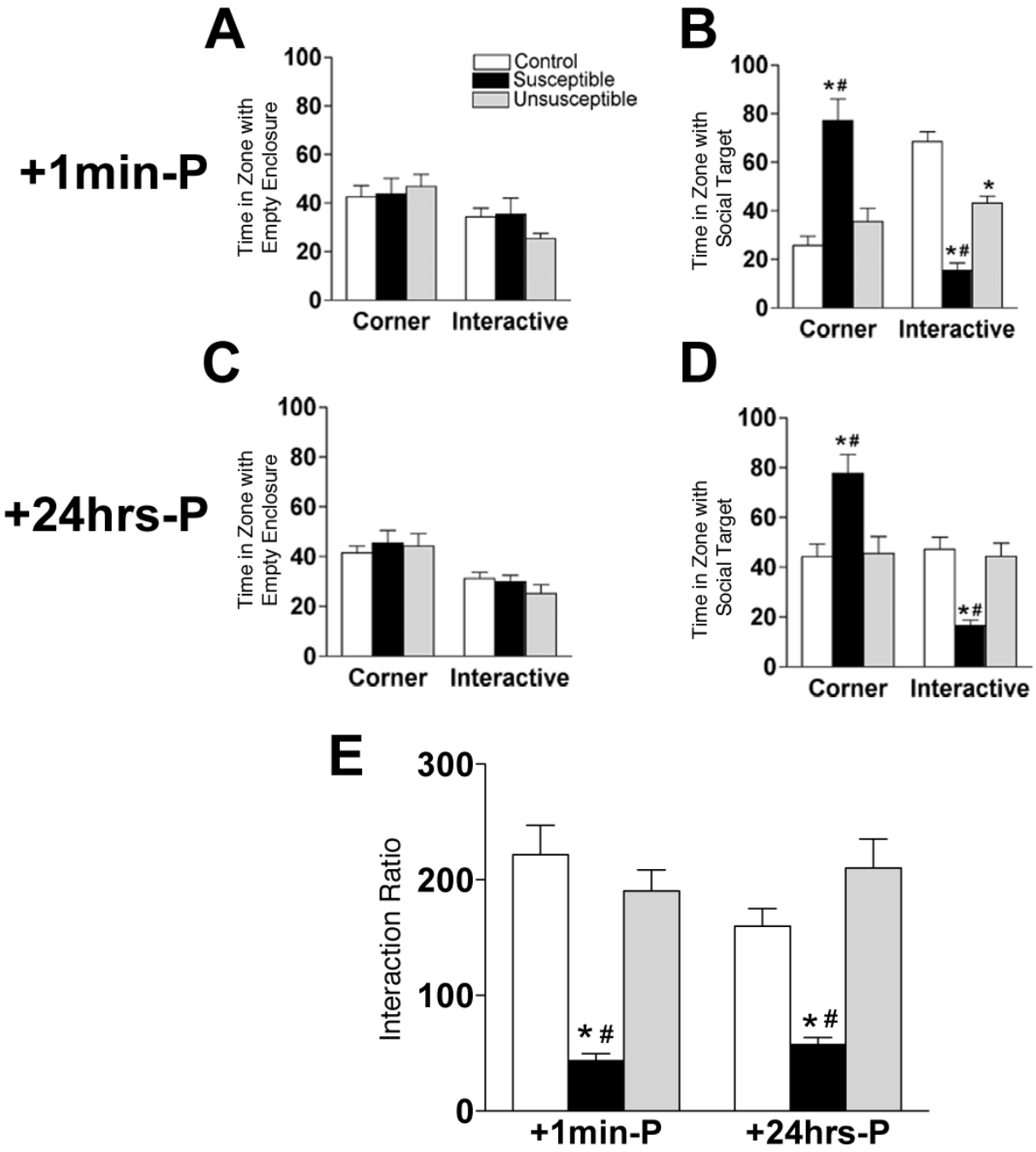


Figure 3.7. Mice susceptible to defeat stress display similar social interaction 12 hours prior to (A, B: +1min-P, n=9-18/group) or 12 hours after (B, C: +24hrs-P,

n=7-12/ group) day 10 of defeat. (A) Time (sec) spent in the corner ($F_{2,37}$=0.20, *p*=ns) and within the interaction zone ($F_{2,37}$=2.42, *p*=ns) in +1min-P group in the presence of a empty enclosure. (B) Time (sec) spent in the corners ($F_{2,37}$=16.5, *p*<0.001) and within the interaction zone ($F_{2,37}$=48.3, *p*<0.001) in +1min-P group in the presence of a social target. (C) Time (sec) spent in the corner ($F_{2,64}$=0.3, *p*=ns) and within the interaction zone ($F_{2,64}$=1.0, *p*=ns) in +24hrs-P group in the presence of a empty enclosure. (D) Time (sec) spent in the corners ($F_{2,64}$=7.8, *p*<0.005) and within the interaction zone ($F_{2,64}$=12.1, *p*<0.001) in +24hrs-P group in the presence of a social target. (E) Interaction ratio in both the +1min-P and +24hrs-P experiments ($F_{2,101}$=35.1, *p*<0.001).

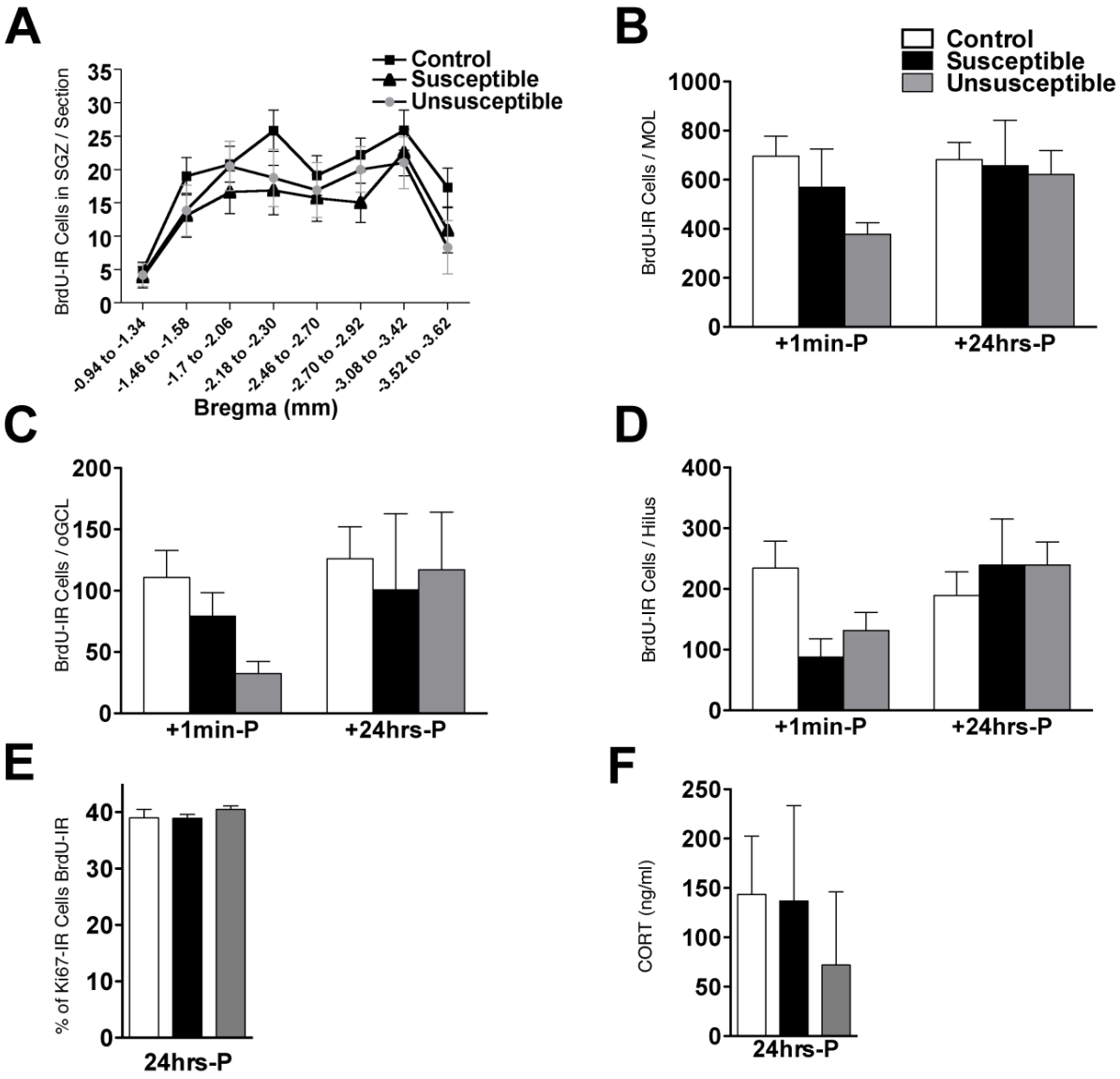


Figure 3.8. Examination of BrdU-IR cells throughout the hippocampus immediately (+1min-P) and 24 hours after (+24hr-P) defeat stress. (A) BrdU-IR cell counts at different septotemporal locations throughout the dentate gyrus ($F_{(2,22)}$=2.2, *p*=ns; n=6-11/group). (B) BrdU-IR cell counts in Mol ($F_{(2,50)}$=1.5, *p*=ns). (C) BrdU-IR cell counts in oGCL ($F_{(2,50)}$=1.1, *p*=ns). (D) BrdU-IR cell

counts in Hilus ($F_{(2,50)}$=0.6, *p*=ns). (E) Percent of Ki67-IR cells that are BrdU-IR in +24hrs-P ($F_{(2, 20)}$=0.4, *p*=ns, n=5-10/group). (F) Serum CORT levels in mice 24 hrs after last defeat ($F_{(2,22)}$=0.25, *p*=ns; n=6-10/group).

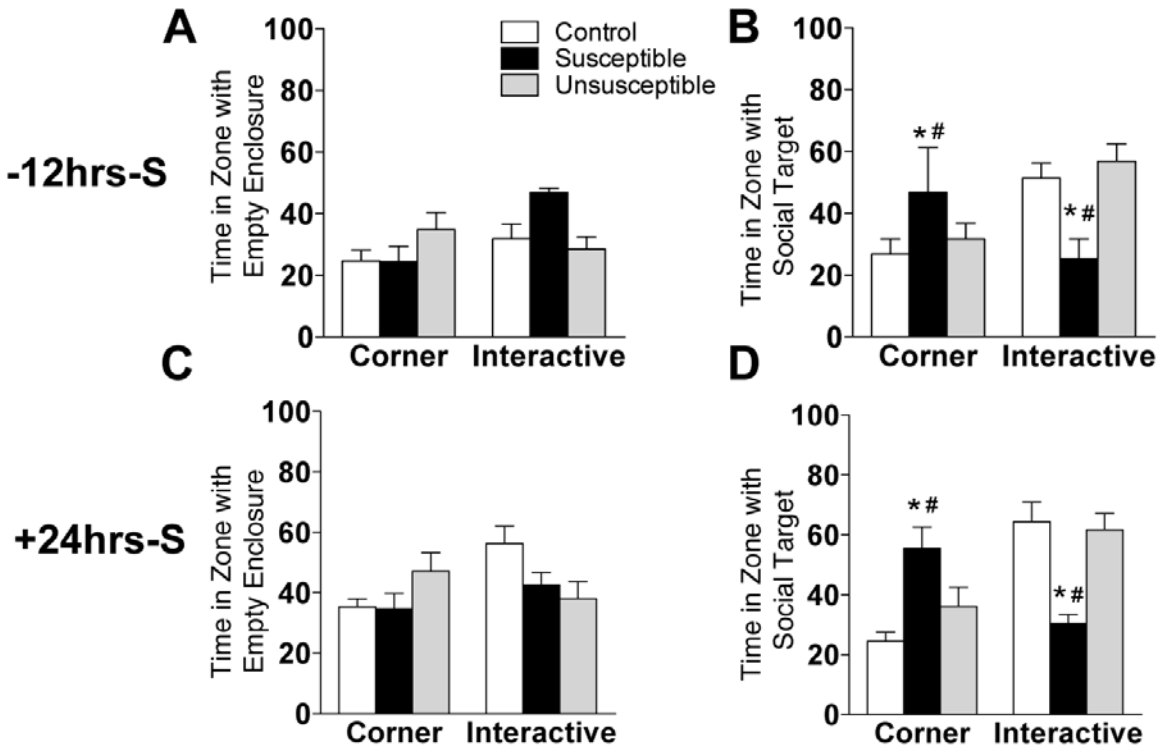


Figure 3.9. Mice susceptible to defeat in -12hrs-S (A, B, n=7-10 per group) and +24hrs-S (C, D, n=8-15/group) groups display similar social interaction 4 weeks after day 10 of defeat stress. (A) Time (sec) spent in either corner ($F_{2,39}$=0.6, p=ns) and within the interaction zone (F2,39=2.6, p=ns) in -12hrs-S group in the presence of an empty enclosure. (B) Time (sec) spent in the corners (F2,39=6.9, p<0.01) and within the interaction zone (F2,39=10.7, p<0.01) in -12hrs-S group in the presence of a social target. (C) Time (sec) in the corners ($F_{2,56}$=0.2, p=ns) and within the interaction zone ($F_{2,56}$=4.0, p=ns) in +24hrs-S group in the presence of an empty enclosure. (D) Time (sec) spent in the corners ($F_{2,56}$=8.2, p<0.005) and within the interaction zone ($F_{2,56}$=10.2, p<0.001) in +24hrs-S group in the presence of a social target.

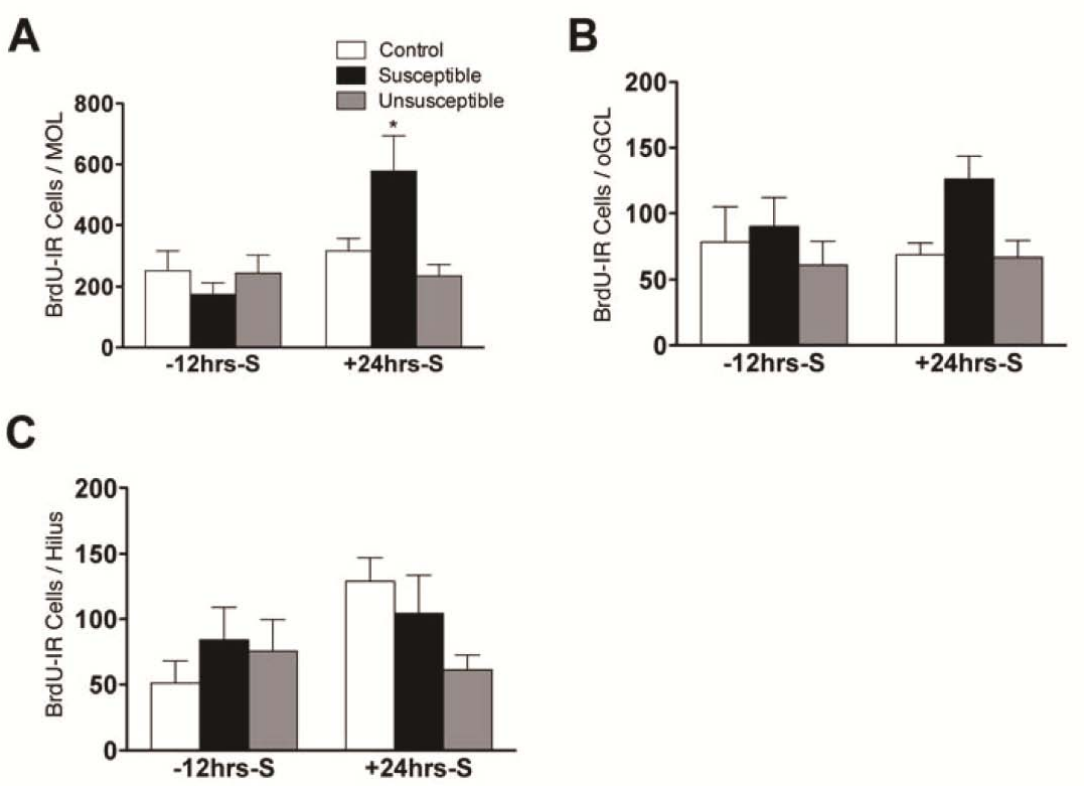


Figure 3.10. Examination of surviving BrdU-IR cells labeled immediately prior to (-12hrs-S, n=7-10 per group) or one day after defeat (+24hrs-S. n=8-15/group). (A) BrdU-IR cell counts in Mol ($F_{(2,54)}$=6.0, p<0.05) (B) BrdU-IR cell counts in oGCL ($F_{(2,54)}$=2.5, p=ns). (C) BrdU-IR cell counts in Hilus ($F_{(2,54)}$=0.8, p=ns).

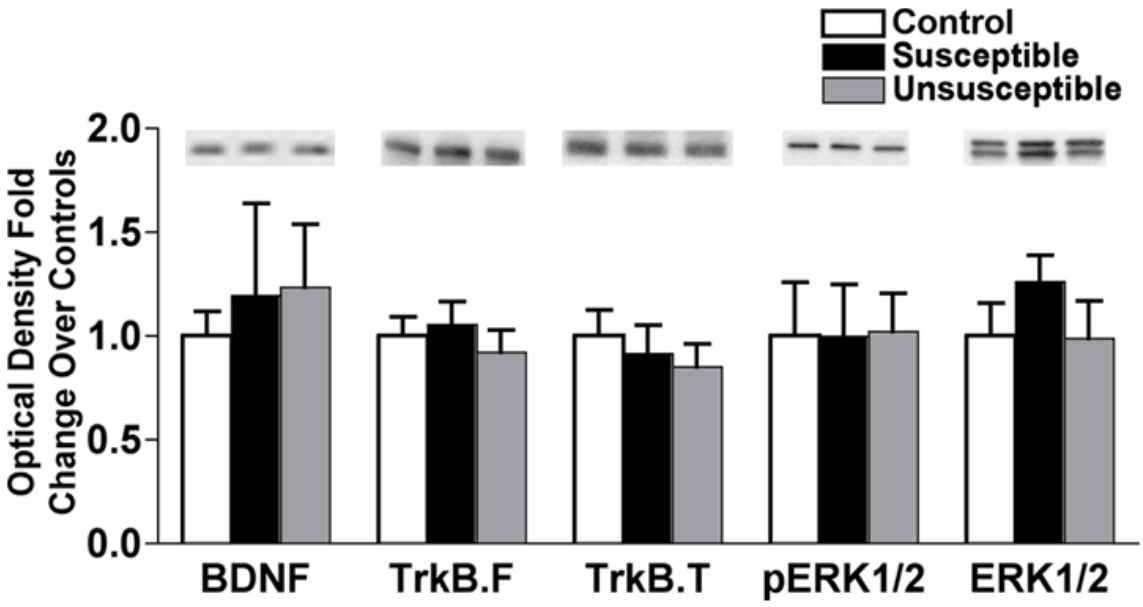


Figure 3.11. Representative western blots for BDNF and its receptor (TrkB) and one of its downstream signaling protein (ERK) in total hippocampal extracts 4 weeks following defeat (+24hrs-S). There are no significant differences between control versus defeated animals in proteins levels for components of the BDNF signaling pathway (main effect group: $F_{(2, 115)}$=0.3, *p*=ns; main effect protein: $F_{(4,115)}$=0.6, *p*=ns; group x protein: $F_{(8, 115)}$=0.3, *p*=ns; n=7-10/group).

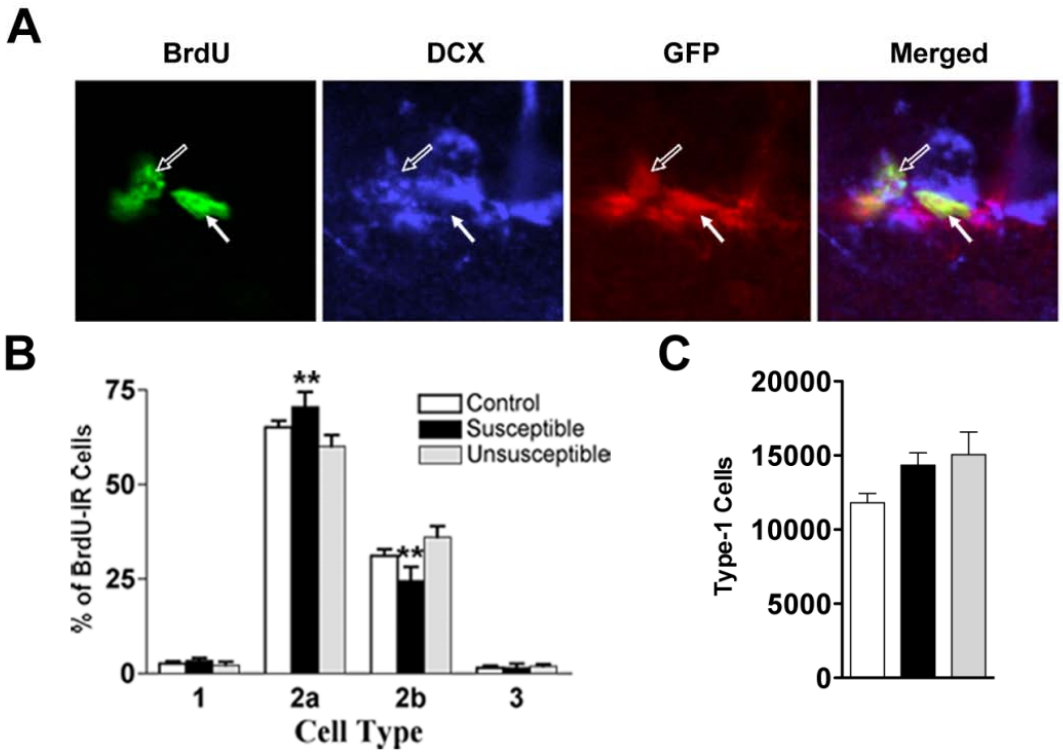


Figure 3.12. Analysis of BrdU cells that are in distinct stages of neurogenesis (Type-1, Type-2a, Type-2b, and Type-3) in the Nestin-GFP mice 24hrs after defeat stress (+24hr-P). (A) Representative staining for BrdU-IR (green), DCX-IR (blue), and GFP-IR (red) with arrows highlighting Type-2a (GFP-IR/DCX-, closed arrow) and Type-2b cells (GFP-IR/DCX-IR, open arrow). (B) Susceptible mice had a significantly greater percentage of BrdU-IR cells that were in Type-2a and lower percentage of cells that were Type-2b compared to unsusceptible mice (main effect group: $F_{(2, 80)}$=0.0, *p*=ns; main effect phenotype: $F_{(3,80)}$=692.9, *p*<0.0001; group x phenotype interaction: $F_{(6, 80)}$=5.3, *p*<0.01.; n=6-10/group). (C) Total number of Type 1 cells is not significantly different between the groups ($F_{(2,14)}$=3.4, *p*=ns).

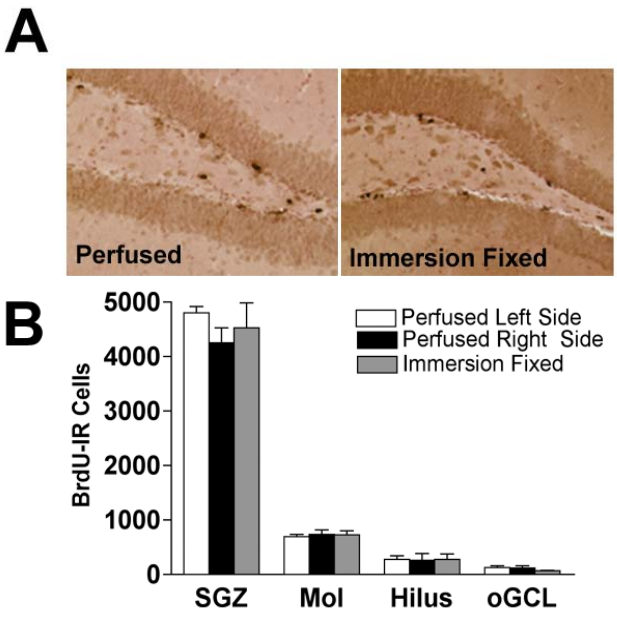


Figure 3.13. Fixation (intracardial perfusion vs. immersion fixation) does not influence dentate gyrus BrdU-IR cell number. (A) Representative microphotograph of BrdU-IR cells in perfused versus fixed tissue. (B) Number of BrdU-IR cells in the left or right side of a perfused brain when compared to a immersion fixed brain (main effect group: $F_{(2, 21)}$=1.1, *p*=ns; main effect region: $F_{(3, 21)}$=302.1, *p*<0.001; group x region: $F_{(6, 21)}$=1.5, *p*=ns; n=4/group). Mean ± SEM.

CHAPTER FOUR

Nestin-CreER^{T2}/R26R-YFP mouse is a powerful tool to assess the contribution of nestin-expressing cells to adult neurogenesis

Adapted from: Lagace DC, Whitman MC, Noonan MA, Ables JL, **DeCarolis NA**, Arguello AA, Donovan MH, Fischer SJ, Farnbauch LA, Beech RD, DiLeone RJ, Greer CA, Mandyam CD, Eisch AJ. 2007. Dynamic contribution of nestin-expressing stem cells to adult neurogenesis. Journal of Neuroscience. 27:12623-9.

Abstract

Understanding the fate of adult-generated neurons and the mechanisms that influence them requires consistent labeling and tracking of large numbers of stem cells. We generated a nestin-CreER^{T2}/R26R-YFP mouse to inducibly label nestin-expressing stem cells and their progeny in the adult SVZ and SGZ. The estrogen ligand tamoxifen specifically induced recombination in stem cells and their progeny in nestin-CreER^{T2}/R26R-YFP mice, as 97% of SGZ stem-like cells (GFAP+/Sox2+ with radial glial morphology) expressed YFP; YFP+ neurospheres could be generated *in vitro* after recombination *in vivo;* and maturing YFP+ progeny were increasingly evident in the olfactory bulb (OB) and dentate gyrus (DG) granule cell layer. Revealing an unexpected regional dissimilarity in adult neurogenesis, YFP+ cells accumulated up to 100 days post-

TAM in the OB, but in the SGZ, YFP+ cells reached a plateau 30 days post-TAM. In addition, most SVZ and SGZ YFP+ cells became neurons, underscoring a link between nestin and neuronal fate. Finally, quantification of YFP+ cells in nestin-CreER^{T2}/R26R-YFP mice allowed quantitative estimation, for example, that stem cells and their progeny contribute to no more than 1% of the adult DG granule cell layer. In addition to revealing the dynamic contribution of nestin-expressing stem cells to adult neurogenesis, this work highlights the utility of the nestin-CreER^{T2}/R26R-YFP mouse for inducible gene ablation in stem cells and their progeny *in vivo* in the two major regions of adult neurogenesis.

Introduction

In the postnatal brain, evidence suggests progeny of glial-fibrillary acidic protein-(GFAP) and nestin-expressing stem cells become neurons and astrocytes by progression through distinct stages (Doetsch et al., 1999a; Ganat et al., 2006; Garcia et al., 2004; Kempermann et al., 2004). In the subventricular and hippocampal subgranular zones (SVZ, SGZ), the stage at which progeny are restricted to a neuronal fate is unknown. In addition, it is unclear in the adult how many of these cells integrate into the existing neural network. Studies addressing these questions typically use thymidine analogs such as bromdeoxyuridine (BrdU) that target rapidly dividing cells, or transgenic reporter mice that label discrete stages of adult neurogenesis. In general, these techniques are of limited use for stem cell analysis since their markers either dilute with cell division or provide only transient labeling (Dayer et al., 2003; Yamaguchi et al., 2000). While

viral-mediated fluorescent labeling can tag stem cells and does not dilute, the necessity of intracranial injection and the restricted spread of virus make it nearly impossible for viruses to consistently label large number of cells, particularly in the SGZ (e.g. van Praag et al., 2002a). It is clear that questions of fate restriction and cellular contribution require a different approach to label and track large populations of stem cells and their progeny in the adult brain.

To address this challenge in studying neurogenesis, the Eisch laboratory and others created conditional and inducible nestin-driven transgenic mice (Beech et al., 2004; Burns et al., 2007; Carlen et al., 2006; Imayoshi et al., 2006; Kuo et al., 2006; Yu et al., 2005). While many of these mouse models are highly effective in labeling and tracking the multipotent lineage of nestin-recombined cells in the embryo, only three of these models have demonstrated efficient labeling within the adult SVZ (Burns et al., 2007; Carlen et al., 2006; Yu et al., 2005) and none have labeled the large pool of adult SGZ stem and progenitor cells.

This transgenic mouse is the first inducible nestin-CreER^{T2} mouse that can be used to label, track, and phenotype stem cells and their progeny in the adult SVZ and SGZ. Using this mouse, researchers can explore if adult-generated neurons are derived from the nestin lineage. In addition, the diverse composition of labeled cells is quantified over months following recombination, as well as the total contribution of stem cells and their progeny to adult mice is estimated. These data provide unique long-term insight into the importance of stem cells to

neurogenesis in the SVZ and SGZ, and underscore the utility of this mouse in gene deletion from stem cells and their progeny in the adult brain.

Methods

Generation and genotyping of nestin-CreER^{T2}/R26R-YFP transgenic mice. Animal experiments were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* and approved by the UT Southwestern Animal Care and Use Committee. The CreER^{T2} sequence (Indra et al., 1999 generous gift from Pierre Chambon, IGBMC, France) was cloned into the *Sal*I and *Nhe*I sites of Nestin Xh5 plasmid (generous gift from Weimin Zhong, Yale University). The Xh5 plasmid has been previously published in other mouse models and contains similar elements to Nes/PlacZ/3 introns (Beech et al., 2004; Zimmerman et al., 1994). The nestin-CreER^{T2} founder mice were generated by pronuclear injection of *Sma*I digest of nestin-CreER^{T2} into C57Bl/6J fertilized eggs. Five independent lines were generated (*g, h, i, j, k*), with four lines *(g, i, j, k)* having robust Cre mRNA in brain as determined by qRT-PCR (data not shown). Nestin-CreER^{T2} mice were bred with R26R-YFP reporter mice resulting in bigenic mice heterozygous for both transgenes (Figure 4.1a). Mice were genotyped by PCR using genomic DNA and primers previously published for Cre (Indra et al., 1999) and R26R reporter mice (Soriano, 1999).

Tamoxifen (TAM) and BrdU administration. Nestin-CreER^{T2}/R26R-YFP mice (5-7 weeks old) were administered TAM at 180 mg/kg/day for 5 days (IP; dissolved in

10% EtOH/90% sunflower oil). This dosage was based on preliminary studies with hydroxytamoxifen (up to 1 mg/day for 5 days) and TAM (up to 300 mg/kg/day for 10 days) that demonstrated 5 days of 180 mg/kg/day TAM produced the maximal recombination with minimal lethality (<5%). To examine the impact of TAM on survival of adult-generated neurons, mice were given BrdU (150 mg/kg, IP) one-day prior to TAM and sacrificed 28 days later. For phenotypic analysis, mice were sacrificed 1 day (n=7), 12 days (n=11), 30 days (n=10), 65 days (n=9), and 100 days (n=6) post-TAM.

Immunohistochemistry (IHC). Mice were anesthetized and transcardially perfused with cold 4% paraformaldehyde in 0.1M PBS. Brains were removed from the skull, postfixed in 4% paraformaldehyde overnight, and then transferred to 30% sucrose in 0.1M PBS. Brains were sectioned 30- or 40-µm thick on a freezing microtome in either the coronal or sagittal plane. Sections were stored in 1x PBS with 0.01% sodium azide (Donovan et al., 2006). All IHC was completed on tissue mounted onto charged slides as previously published (Mandyam et al., 2007; Mandyam et al., 2004), with the exception that Sox-2/GFAP staining was performed using free-floating IHC.

The following primary antibodies were used: rat monoclonal anti-BrdU (Accurate, Westbury, NY, Cat # OBT0030; 1:300); mouse monoclonal anti-calretinin (CR; Swant, Bellinzona, Switzerland, Cat # 6B3; 1:1000); goat polyclonal anti-doublecortin (DCX; Santa Cruz Biotechnology, Santa Cruz, CA, Cat # sc-8066;

1:1000 or 1:5000); mouse anti-GAD65/67 (Stressgen, Victoria, BC, Cat # MSA-225; 1:1000); mouse anti-calretinin (CR; Chemicon, Temecula, CA, Cat # MAB1568; 1:1000); mouse monoclonal anti-glial fibrillary acidic protein (GFAP; Chemicon, Cat # MAB360; 1:3000); rabbit polyclonal anti-green fluorescent protein (GFP; Invitrogen, Eugene, OR, Cat # A11122; 1:500 or 1:3000); rabbit polyclonal anti-Ki67 (Novocastra Laboratories, Norwell, MA, Cat # NCL-Ki67p; 1:500); rabbit anti-SRY-related HMG-box gene 2 (Sox-2; Chemicon, Cat # AB5603; 1:3000); mouse anti-nestin (BD Pharmigen, USA, Cat # 60051A; 1:2000).

To quench endogenous lipofuscin fluorescence, some sections (OB sections for DCX/YFP) were stained with 1% Sudan Black in 70% methanol for 5 minutes, cleared in 70% ethanol, rinsed in PBS, and then mounted. Antigen retrieval on slide-mounted sections was performed using 0.01M Citric Acid (pH 6.0) at 100°C for 15 minutes, followed by 10 minutes in PBS at RT. To remove any endogenous peroxidase activity, all sections were incubated with 0.3% H_2O_2 for 30 min. Non-specific binding was blocked with 2-3% serum (donkey, horse or bovine serum albumin) and 0.3% Triton-X in PBS for 30-60 min. Antibody specificity was determined by lack of staining after omission of primary or secondary antibodies.

Incubation with the primary antibody was done with 3% serum and 0.3% Tween-20 overnight. For double or triple labeling, some primary antibodies were

simultaneously incubated (DCX/CR/YFP; Sox2/GFAP; nestin/YFP; GAD65-67/YFP). For Ki67/YFP IHC, incubation with each antibody was done separately. Slides were first incubated with the YFP antibody and staining was completed, followed by fixation of the stained slides in 4% paraformaldehyde for 20 to 60 min, prior to incubation with the primary antibody for Ki67. Similarly, for Sox2/GFAP/YFP IHC, slides were stained for both the Sox2 and GFAP simultaneously, and then the stained slides were fixed in 4% paraformaldehyde for 20 to 60 min prior staining for YFP.

For single labeling of YFP, primary antibody incubation was followed by labeling with a biotin-tagged donkey anti-rabbit secondary antibody with 1.5% serum for 1 hr (Jackson ImmunoResearch, West Grove, PA; Cat # 711-065-152; 1:200). Sections were then incubated in ABC for 1 hr (Vector Laboratories, Burlingame, CA, Cat # PK-6100; 1:50) and staining was visualized with Tyramide-Plus signal amplification (TSA, PerkinElmer Life Sciences, Boston MA, Cat # SAT705A; 1:50). For DCX IHC in the OB, primary antibody incubation was followed by incubation for up to 4 hours with an Alexa-conjugated secondary (Molecular Probes, Carlsbad, CA; 1:1000). For CR, Sox2, and GFAP staining, primary antibody incubation was followed by incubation for up to 4 hours with a fluorescent-tagged secondary antibody (Cy2, Cy3, or Cy5 conjugated IgG antibodies (Jackson ImmunoResearch, Cat # 715-225-150, 711-165-152, 711-065-152; 1:200). Alternatively, for YFP, DCX, Ki67, and nestin staining in the SGZ, primary antibody incubation was followed with an appropriate biotin-tagged

secondary, followed by ABC and tyramide signal amplification, just as for single YFP staining.

All slides were counterstained with a nuclear counterstain, DAPI (Roche Applied Science, Indianapolis, IN, Cat # 236276; 1:5000), red Nissl (Invitrogen, Eugene, OR, Cat # N-21482; 1:200) or DRAQ5 (Alexis Biochemicals, Cat # BOS-889-001; 1:1000). All slides were dehydrated and coverslipped using DPX.

Microscopic analysis and quantification. Quantification of cell number within the hippocampus was performed at 630x using an Olympus BX-51 microscope by an observer blind to experimental groups. Slides were coded during IHC and the code was not broken until after analysis was complete. For the analysis in hippocampus, YFP+ cells were quantified in every 9th coronal section throughout the SGZ and outer portion of the granule cell layer of the dentate gyrus (bregma -0.82 mm to -4.24 mm) as previously described (Mandyam et al., 2007).

The number of YFP+ cells per OB glomerulus was quantified as previously reported (Merson et al., 2006). Briefly, YFP+ cells were counted in 50 glomeruli per mouse in 5 matched serial sections of the mouse olfactory bulb (10 glomeruli per section, glomeruli chosen as described in Merson et al., 2006). The average number of YFP+ cells per glomerulus is reported. For analysis of the number of YFP+ cells in the OB GCL, 4 matched serial OB sections were analyzed. YFP+ cells in the OB 1 day post-TAM were not quantified due to the very low number.

The volume of the OB GCL excluding the RMS was obtained via optical fractionation using StereoInvestigator software (MBF Bioscience, Williston, VT). A grid (500 x 500 μm^2) was superimposed over each section, and YFP+ cells were counted at 400x in 50 x 50 x 6 μm^3 sample volumes with an upper and lower guard distance of 1 µm.

Phenotypic analysis of YFP+ cells (50-150 cells/mouse for the OB and SGZ, n=4-6 mice per time point; 100-600 cells/mouse for the RMS and GCL, n=2-3 mice per time point) was performed using a confocal microscope (Leica TCS SL confocal and Zeiss Axiovert 200 and LSM510-META; emission wavelengths 488, 543, and 633, magnification of X630 to X1000). Scanning and optical sectioning in the Z plane was performed as described previously (Mandyam et al., 2007; Mandyam et al., 2004) and fluorescently labeled confocal images presented here were taken from one optical slice and imported into Photoshop (Adobe Systems) for composition purposes or into Metamorph for RMS and OB GCL phenotypic analysis. Verification of colocalization in the OB and SGZ was achieved by importing stacks of Z images into a 3D reconstruction program, Volocity (Improvision), and performing rotation, transient modification and 3D rendering.

Neurosphere assay. Nestin-CreER^{T2}/R26R-YFP mice were utilized for the neurosphere assay 12 days post-tamoxifen, a time point when YFP+ cells are readily visible in the postnatal neurogenic regions (e.g. Figures 4.1-4.3). Dissected lateral ventricles were dissociated (Seaberg and van der Kooy, 2002),

cell viability assessed by trypan blue exclusion (Sigma), and viable cells plated at 10,000 cells/ml in growth media (either Neurobasal-A (Gibco) media supplemented with B27 (Gibco), or DMEM/F12 (Gibco) media supplemented with N2; both medias augmented with EGF (20 ng/ml) and bFGF (10 ng/ml)). Spheres were counted at day 7 *in vitro*, and spheres were assayed for self-renewal via passaging (Tropepe et al., 1999). Quantification of recombined (YFP+) spheres as percent of total spheres was determined by three independent experiments done in quadruplicate. A minimum of three different fields of view was evaluated at 4x to assess a minimum of 25 neurospheres in each field of view. Genotype did not alter neurosphere formation *in vitro,* as similar number of spheres was generated from mice negative for both genes (n=2-3 per genotype; Figure 4.4).

Statistical analyses. The data are reported as mean ± SEM. Statistical analyses were performed using a multiple variable analysis of variance (ANOVA) followed by a Bonferroni post-hoc test. Post-hoc analyses were performed using the Bonferroni comparison. All statistical analyses were performed using either SPSS (version 11.0.2) or Prism (version 4.0) software. Statistical significance was defined as $p<0.05$.

Results

Nestin-CreER^{T2}/R26R-YFP mice display TAM-induced recombination in neurogenic brain regions. The nestin-CreER^{T2} inducible transgenic mouse was generated using 5.8kB of the *nestin* promoter and exons 1-3 of the nestin gene. Recombination efficacy was tested by administering TAM to nestin-CreER^{T2}/R26R-YFP mice. TAM binds to the mutated estrogen receptor (ER^{T2}) allowing Cre-ER^{T2} to translocate into the nucleus and excise the STOP codon, allowing for subsequent YFP expression (**Figure 4.1A**). Adult mice from four founder lines (*g, i, j, k)* had YFP+ cells in the SVZ, RMS, and OB, while two founder lines (*i, k*) had YFP+ cells additionally in the SGZ (**Figure 4.1B**). Data presented in this paper are from the *k* line. In all lines, there was no appreciable number of YFP+ cells in non-neurogenic regions, such as the cortex, thalamus, and cerebellum. Also, no line had Cre-induced abnormalities, contrary to previous reports (Forni et al., 2006). At all time points examined, no YFP+ cells were detectable in nestin-CreER^{T2}/R26R-YFP mice in the absence of TAM (**Figure 4.1C**). YFP+ cell number was also similar between male and female mice (e.g. **Figure 4.1D-E**: 12 days after TAM, number of YFP+ cells in SGZ in male=3654±278 vs. female=4239±414). TAM did not alter the survival of adult-generated cells, as demonstrated by quantification of number of surviving BrdU-labeled cells (e.g. **Figure 4.1F-G**: 28 days after BrdU injection, number of BrdU+ cells in SGZ in vehicle=2718±278 vs. TAM=2788±238). Taken together, these data suggest that the nestin-CreER^{T2} system targets adult stem cells and their progeny in the well-characterized regions of adult neurogenesis.

Recombination in stem cells and their progeny in the SVZ results in an increasing number of newly formed mature neurons in the RMS and OB. The time course of the appearance of recombined (YFP+) cells in the SVZ, and subsequently in the OB GCL and glomerular cell layer (OB GL), revealed a dynamic addition of newly born cells in the adult (**Figure 4.2A-C**). One day after TAM, nestin-lineage (YFP+) cells were evident in the SVZ, with few in the RMS and none in the OB (**Figure 4.2A**). In agreement with recombination taking place in stem/progenitor cells, YFP+ cells were evident in the SVZ at all time points examined (**Figure 4.2A**) and YFP+ neurospheres could be propagated *in vitro* from the SVZ of nestin-CreER^{T2}/R26R-YFP mice given TAM *in vivo* (**Figure 4.4A-D**). Both the OB GCL and OB GL showed an accumulation of YFP+ cell through 100 days post-TAM (**Figure 4.2B**: GCL, $F_{(4,24)}$=11.9, p<0.0001; **Figure 2C**: GL, $F_{(3,25)}$=12.2, p<0.005). The vast majority of YFP+ cells were in the OB GCL rather than in the GL (note difference in *y* axis in **Figure 4.2B-C**). Within the OB GCL, YFP+ cells were most dense in the deep internal portions of the GCL throughout the longitudinal axis of the OB, consistent with previous reports in adult mice using BrdU to assess OB neurogenesis (Lemasson et al., 2005; Mandairon et al., 2006). This quantification allowed estimation of the contribution of YFP+ cells to adult OB. Since the adult mouse OB GCL has ~ 410,000 cells/mm^3 (Parrish-Aungst et al., 2007), YFP+ cells represented 0.3%, 1.1%, and 2.6% of the total OB GCL density at 30, 65, and 100 days post-TAM, respectively. In contrast, since each glomerulus is estimated to be surrounded by ~100 periglomerular cells (data not shown and Merson et al., 2006), YFP+ cells represented 2.7%,

3.1%, and 4.9% of total periglomerular cell number at 30, 65, and 100 days post-TAM, respectively.

Consistent with the significant increase in YFP+ cells over time, phenotypic analysis revealed increasing maturity of YFP+ cells in the RMS/OB. There was a significant difference in the percent of neuroblasts recombined (YFP+/doublecortin+ (DCX), Brown et al., 2003) at increasing times post-TAM (**Figure 4.2D**: $F_{(2,14)}=6.1$, $p<0.05$), as well as among the caudal RMS, RMS in OB, and OB GCL (**Figure 4.2D**: $F_{(2,14)}=28.3$, $p<0.0001$). Over time, in the caudal RMS the percent of YFP+ that were neuroblasts increased, while in the OB the percent of YFP+ that were neuroblasts decreased. This correlates with the significant increase in the percent of YFP+ cells in the GCL that did not express DCX between 12 and 30 days or between 30 and 65 days (**Figure 4.2E**; phenotype by time interaction: $F_{(4,18)}=70.8$, $p<0.0001$). These changes are expected due to the differentiation of YFP+ cells and their progeny. Indeed, over 75% of YFP+ cells in the OB GCL expressed the mature neuronal marker NeuN from 65 and 100 days post-TAM (**Figure 4.2F-G**: $F_{(2,11)}=10.8$, $p<0.005$), in agreement with BrdU survival studies demonstrating ~80% of OB GCL BrdU+ cells label with NeuN (Mandairon et al., 2006). As expected, many YFP+ OB GCL cells were also GABAergic, and a proportion were also calretinin (CR)+ (**Figure 4.2H-I**)(Lois and Alvarez-Buylla, 1994).

Recombination in stem cells and their progeny in the SGZ results in an increasing number of newly formed mature neurons in the hippocampal granule cell layer. At all time points post-TAM, YFP+ cells were prominent within the dentate gyrus, with over 90% of cells residing within the SGZ relative to the other dentate gyrus regions (**Figure 4.1D-E**). There was a significant difference in number of SGZ YFP+ cells at increasing times post-TAM, with significantly more cells between 1 day and all other time points, as well as between 12 and 30 days (**Figure 4.3A**: $F_{(4,42)}=15.5$, $p<0.005$). There was no significant change between 30-65 days and 65-100 days post-TAM, indicating that the number of YFP+ cells reaches a plateau at 30 days. As the adult mouse has ~1 million dentate gyrus GCL cells (Abusaad et al., 1999; Harburg et al., 2007), YFP+ cells are estimated to represented 1.0%, 0.75%, and 0.82% of the total dentate gyrus GCL cells 30, 65, and 100 days post-TAM, respectively. The plateau in the SGZ is likely not due to inefficient labeling, since at 12 days post-TAM 97.1%±1.6% of stem-like SGZ cells were recombined. In this analysis, stem-like cells were identified by their immunoreactivity for both Sox2 (which labels astrocyte stem-like cells, rapidly dividing precursor cells, and mature astroglial cells) and GFAP (which labels astrocyte stem-like cells and mature astroglial cells) in combination with assessment for stem-like radial glial morphology using confocal analysis (Graham et al., 2003; Hattiangady and Shetty, 2008; Komitova and Eriksson, 2004; Pevny and Rao, 2003; Seri et al., 2004; Seri et al., 2001). Moreover, this plateau was not likely due to TAM- or YFP-induced disruption of gross cellular function, since neurogenic stimuli, such as free access to a running wheel,

significantly increased the number of YFP+ SGZ cells (**Figure 4.5A-C**; bregma by treatment interaction, $F_{(11,110)}$=2.647, p<0.01). This suggests that at least some of the recombined progenitors cells or their progeny can respond to neurogenic stimuli. Considering mice were placed on the running wheels at a time point when YFP+ cells present diverse cellular phenotypes (**Figure 4.5C**), the relatively modest effect of running in our data compared to others likely results from the specific sensitivity of Type-2 cells to this neurogenic stimulus (Kronenberg et al., 2003).

As would be expected if labeling SGZ stem cells and their progeny, nestin-lineage YFP+ cells were morphologically heterogeneous post-TAM (**Figures 4.1D-E, 4.3B**). Using IHC, morphological analyses and confocal microscopy, we classified YFP+ cells into non-exclusive phenotypic categories: stem (Sox2+/GFAP+), stem/progenitor (nestin+), dividing (Ki67+), immature neurons (DCX+) or postmitotic neurons (DCX+/CR+, **Figure 4.2B**)(Hattiangady and Shetty, 2008; Kempermann et al., 2004; Ming and Song, 2005). There was a significant difference in the proportion of recombined cells in these categories (**Figure 4.3C**: $F_{(4,60)}$=53.18, p<0.000) that changed post-TAM (**Figure 4.3C**; $F_{(3,60)}$=5.87, p<0.005). Between 1 and 12 days, YFP+ cells matured from being dividing, stem-like and/or progenitor cells into immature or postmitotic neurons (**Figure 4.3B-C**). In contrast, between 12-30 days and 30-65 days, the proportion of YFP+ cells that expressed the different phenotypes remained constant (*post hoc's* >0.05). Taken together, these data suggest that nestin drives expression in

stem-like and progenitor cells in the SGZ. This is supported by the presence of stem-like recombined cells with radial glial morphology in the SGZ at all time points, including 100 days post-TAM.

Over time, labeled stem cells and their progeny gave rise to mature dentate gyrus GCL neurons. At 30 days and beyond, the majority of the YFP+ SGZ cells displayed a long process extending up into molecular layer capped by a highly arborized dendritic tree (**Figure 4.3D**). At 65 days and beyond, YFP+ fibers, presumably mossy fibers from YFP+ granule cells, densely innervated CA3. In addition, there was a significant increase in the proportion of recombined cells that expressed NeuN between 30 days and subsequent time points (**Figure 4.3E-F**: $F_{(2,8)}$=41.4, p<0.0005). By 65 days post-TAM, the percent of YFP+ cells that expressed NeuN reached a plateau with approximately 50% of YFP+ cells being neurons at 65 and 100 days (**Figure 4.3F**). YFP+ astrocytes were rare (YFP+/S100β+/GFAP+ and astrocytic morphology), providing additional support that nestin-expressing stem cells give rise to neurons, not astrocytes. There was also a significant increase in YFP+ cells that had a mature neuronal morphology in the outer granule cell layer (oGCL; **Figure 4.3G**: $F_{(4,42)}$=2.9, p<0.05). However, the cells in the oGCL at all time points represented less than 1% of all recombined cells, supporting that most maturing granule cells do not migrate from the SGZ into the outer granule cell layer (Kempermann et al., 2003). Taken together, these data underscore a link between nestin expression and neuronal fate.

Discussion

Multiple lines of evidence support our conclusion that TAM effectively drives recombination in nestin-expressing stem cells in the SVZ and SGZ during young adulthood. In nestin-CreER^{T2}/R26R-YFP mice, 96% of YFP+ cells in the SGZ were nestin+ 1-day post-TAM, demonstrating that recombination occurs in nestin-expressing stem/progenitor cells. At 12 days post-TAM, 97% of stem-like cells (GFAP+/Sox2+/radial glial morphology) were recombined, and that even at 100 days post-TAM these cells were present. In addition, *in vivo* recombination led to YFP+ neurospheres *in vitro* and, as expected from labeled stem cells, maturing YFP+ progeny were increasingly evident after TAM. These data support that this mouse offers a potent tool for gene ablation studies in stem cells and their progeny.

The nestin-CreER^{T2}/R26R-YFP mouse allows the first quantifiable assessment of the long-term contribution of nestin-expressing stem cells to adult neurogenesis in both the OB and DG. For example, in the SGZ the estimated contribution of stem cells is 1%, which is strikingly similar to previous estimates of the contribution of rapidly dividing cells (Doetsch and Hen, 2005) and validates that stem cells give rise to the rapidly dividing progenitor cells in the SGZ. In addition, the intriguing dynamics of YFP+ accumulation in the OB versus SGZ is revealed in part. YFP+ cell density in the OB, which is dependent on the influx of YFP+ RMS neuroblasts and their surviving progeny, increased up to 100 days post-TAM. The increase in density is due to the accumulation of maturing cells in the

OB GCL and GL. This finding suggests the addition of new OB cells outpaces new cell turnover, and might be expected since the volume of the murine OB increases in the first two years of life (Mirich et al., 2002). In contrast to the cell accumulation in the OB, the number of YFP+ cells in the SGZ reached a plateau 30 days post-TAM. It is surprising to find a plateau in the number of YFP+ neurons, as an increase was expected based on BrdU-labeling survival studies (Dayer et al., 2003; Kempermann et al., 2003). What could explain the steady state of YFP+ cell number in the SGZ? One possibility is that the proportion of stem, precursor, and/or immature cells decreases with age (Hattiangady and Shetty, 2008; Rao et al., 2006). However, the presence of YFP+ radial glial cells 100 days post-TAM and the steady state of the diverse population of recombined cells suggest that over time there is not an exhaustion of recombined stem cells. A second possibility is that analysis of longer times post-TAM would reveal an eventual reduction of precursor number or activity, leading to a corresponding increase in the proportion of mature neurons. A third possibility is that the YFP transgene is silenced over time, leading to an underestimation of YFP+ cell number in the SGZ, and producing the plateau seen 65-100d post-TAM. While this is important to evaluate directly, this possibility is not supported by the increase in YFP+ cell number in the OB in this mouse, the similar steady state dynamics seen in the SGZ of another transgenic mouse with an astrocyte-related gene driver of recombination (GLAST-CreER^{T2}; personal communication Magdelena Götz), and the general robustness of genes in the *rosa* locus. These and other possibilities are important to consider in the ongoing and necessary

evaluation of the hypothesis that stem cells and their progeny achieve homeostasis in the adult mouse SGZ.

In addition to revealing the distinct accumulation dynamics in the SVZ and SGZ, these data also address the role of nestin-expressing cells in adult neurogenesis: most progeny of nestin-expressing YFP+ cells are fated to become neurons. These data are in agreement with links between nestin expression and a specific lineage in many non-neuronal and oncogenic stem cells (Wiese et al., 2004). However, they are in contrast to the multi-lineage role for nestin in the embryo (Beech et al., 2004; Burns et al., 2007; Carlen et al., 2006; Imayoshi et al., 2006; Kuo et al., 2006; Yu et al., 2005), most notably in this same nestin-CreER^{T2}/R26R-YFP mouse (Battiste et al., 2007). This underscores the importance of the permissive neurogenic microenvironment in determining the ultimate phenotypic fate of the progeny of nestin-expressing cells.

Nestin expression can ultimately result in neuronal, but not astrocytic, progeny. It is striking that inducible mouse models utilizing astrocyte-related gene drivers ((GFAP, GLAST) Garcia et al., 2004; Mori et al., 2006) drive expression in astrocytes throughout the brain, yet also give rise to neurons in the OB and SGZ. Interestingly, Type-1 radial glial cells in the SGZ have recently been divided into categories of GFAP+/nestin- and GFAP+/nestin+ (Kempermann et al., 2004; Seki et al., 2007). Thus, the ability of this particular nestin-CreER^{T2}/R26R-YFP mouse to generate YFP+ neurons in the OB and SGZ urges research on factors that

guide a GFAP+ or GLAST+ cell to become nestin+, in order to provide the much-needed information on neuronal fate restriction in the adult brain.

The nestin-CreER^{T2} mouse will clearly be useful in gene deletion or progenitor ablation studies. While elegant, viral-mediated Cre manipulation does not allow quantification of labeled cells along the longitudinal axis of the SGZ due to limited viral diffusion and often variability in titer between viral preparations (van Praag et al., 2002a). This nestin-CreER^{T2} mouse is extremely consistent between litters, emphasizing its usefulness for gene ablation studies. Similarly, it can be used to inducibly drive cell death of neural stem cells in the adult brain, offering an alternative to the current progenitor ablation strategies (e.g. Garcia et al., 2004). As the nestin-CreER^{T2} mouse is inducible, it may also allow for future comparison of the present findings in young adulthood with neurogenesis in older mice, as the dynamics of neurogenesis may shift with age.

In conclusion, these data provide unique insights into the dynamic contribution of stem cells and their progeny to OB and dentate gyrus neurogenesis. The data strongly implicate that nestin expression is coincident with neuronal fate restriction. Furthermore, the quantification of the cellular and temporal specificity in the adult SVZ and SGZ validates this model as a valuable tool to guide future research identifying the contribution of stem cells and adult neurogenesis to neural circuitry and function.

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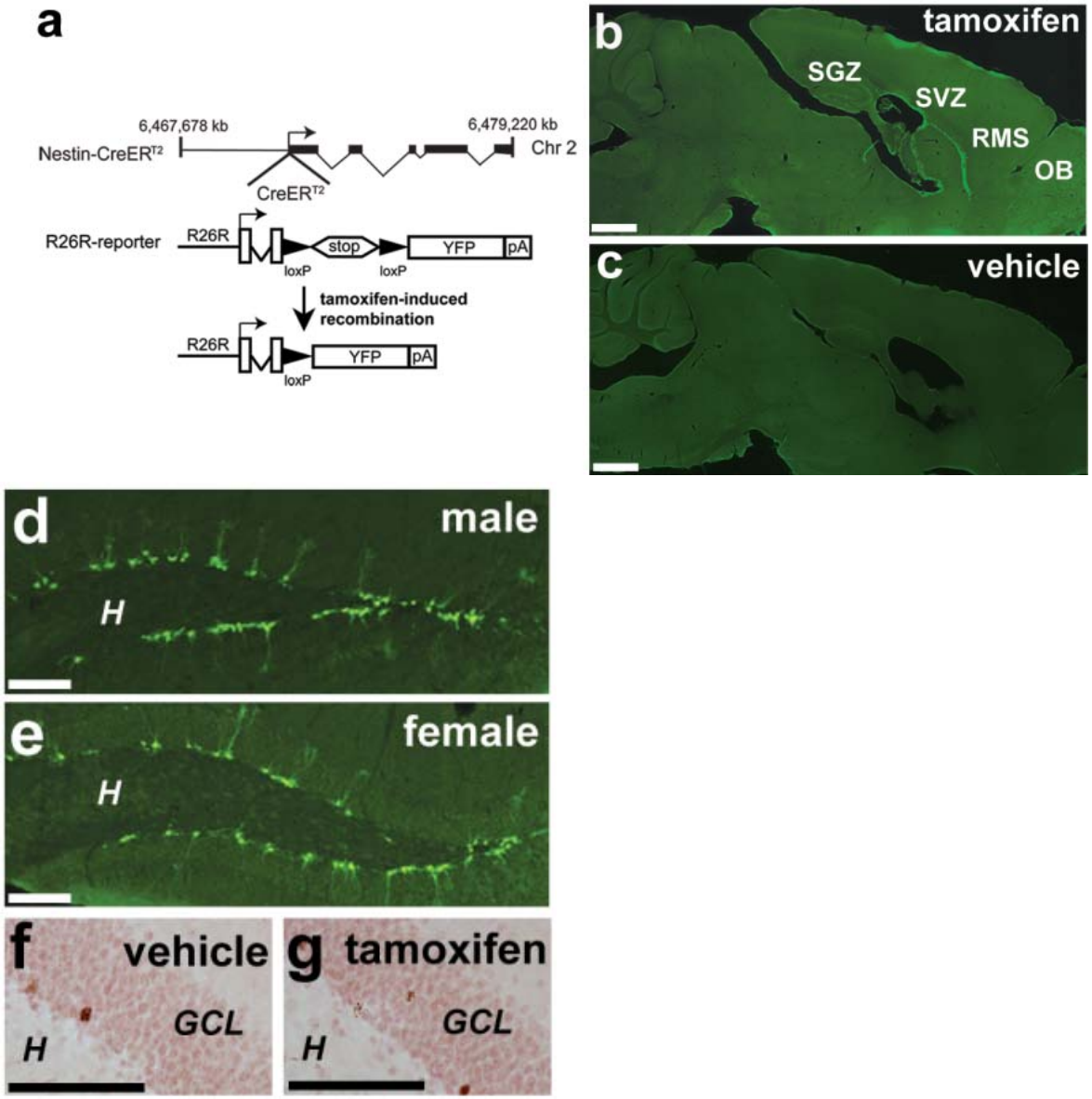
a
6,467,678 kb
6,479,220 kb
Nestin-CreER^{T2}
Chr 2
CreER^{T2}
R26R-reporter
R26R
stop
YFP
pA
loxP
loxP
tamoxifen-induced recombination
R26R
YFP
pA
loxP
b
tamoxifen
SGZ
SVZ
RMS
OB
c
vehicle
d
male
H
e
female
H
f
vehicle
GCL
H
g
tamoxifen
GCL
H

Figure 4.1. Recombination in nestin-CreER^{T2}/R26R-YFP mice is induced by tamoxifen (TAM) and is specific to neurogenic regions. (a) The nestin-CreER^{T2} construct has 5.8kB of the *nestin* promoter and exons 1-3, including the 2nd intronic enhancer. (b, c) YFP+ cells are evident 12 days after TAM in the SVZ, RMS, OB GCL, and SGZ of nestin-CreER^{T2}/R26R-YFP mice given TAM but not vehicle. (d,e) Recombination efficiency in the SGZ is similar in male and female mice 12 days after TAM (male=3654±278 vs. female=4239±414). (f,g) Mice given BrdU one day prior to TAM or vehicle and sacrificed 28 days later have similar numbers of SGZ BrdU cells (vehicle=2718±278 vs. TAM=2788±238). Scale bar=1 mm (b,c). Scale bar=100 µm (d-g). SGZ=subgranular zone; RMS=rostral migratory stream; SVZ= subventricular zone; OB=olfactory bulb; H=hilus of dentate gyrus; GCL=granule cell layer of dentate gyrus.

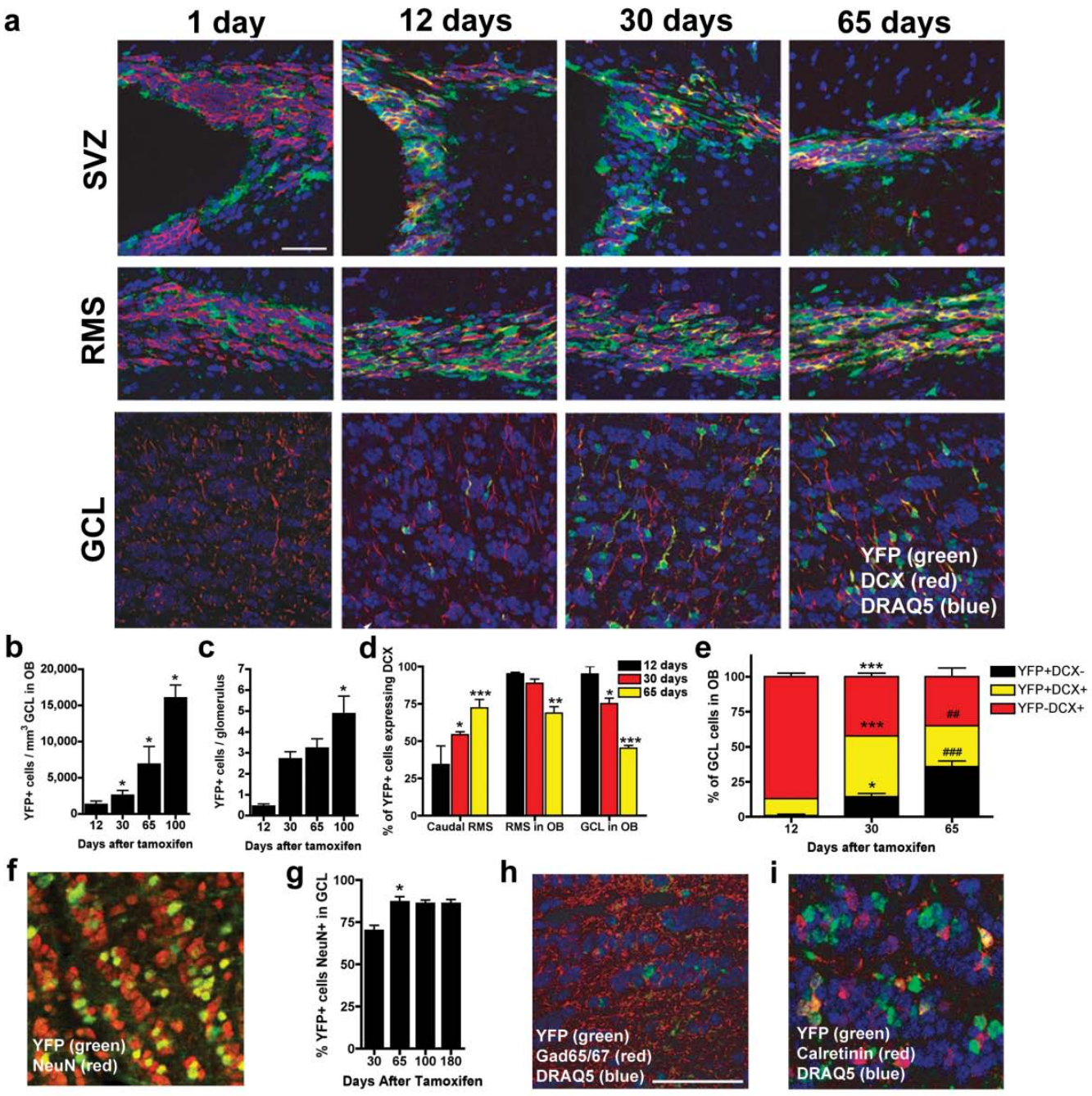
a
1 day
12 days
30 days
65 days
SVZ
RMS
GCL
YFP (green)
DCX (red)
DRAQ5 (blue)
b
YFP+ cells / mm3 GCL in OB
Days after tamoxifen
c
YFP+ cells / glomerulus
Days after tamoxifen
d
% of YFP+ cells expressing DCX
12 days
30 days
65 days
Caudal RMS
RMS in OB
GCL in OB
e
% of GCL cells in OB
YFP+DCX-
YFP+DCX+
YFP-DCX+
Days after tamoxifen
f
YFP (green)
NeuN (red)
g
% YFP+ cells NeuN+ in GCL
Days After Tamoxifen
h
YFP (green)
Gad65/67 (red)
DRAQ5 (blue)
i
YFP (green)
Calretinin (red)
DRAQ5 (blue)

Figure 4.2. Neurogenesis in the SVZ/RMS/OB following TAM (a) Time course for the appearance of YFP+ cells in the SVZ, RMS, and OB GCL 1, 12, 30 and 65 days after tamoxifen. Recombined cells (YFP+, green) are present one day following tamoxifen within the SVZ, whereas fewer YFP+ cells are present in the RMS and virtually no recombined cells are present within the GCL. With increasing time after tamoxifen, there are many recombined cells in each of these areas with a variable proportion of these cells being neuroblasts as demonstrated by coexpression of YFP and DCX (red). (b, c) YFP+ cells in the OB GCL (a) and OB GL (b) significantly increased up to 100 days (*$p<0.05$ vs. 12 days); (d) the proportion of YFP+ cells that are migrating neuroblasts (DCX+) increased in the caudal RMS, but decreased in the RMS in OB and GCL in OB (* $p<0.05$, **$p<0.01$, ***$p<0.005$ vs. 12 days); (e) the proportion of YFP+ cells (black and yellow bars) that are not migrating neuroblasts (black bars) increased in the OB GCL (* $p<0.05$, ****$p<0.005$ vs. 12 days; $^{\#\#}$ $p<0.01$, $^{\#\#\#}p<0.005$ vs. 30 days). (f, g) 30 days after tamoxifen, most recombined cells in the GCL were NeuN+ (*$p<0.05$ vs. 30 days) as well as (h, i) GABAergic interneurons, as assessed via colabeling with YFP and GAD65/67 (h; counterstain DRAQ5) and calretinin (i). Scale bar=50 µm (a,h,i). SVZ: Subventricular Zone; RMS: Rostral Migratory Stream; GCL: granule cell layer of the olfactory bulb.

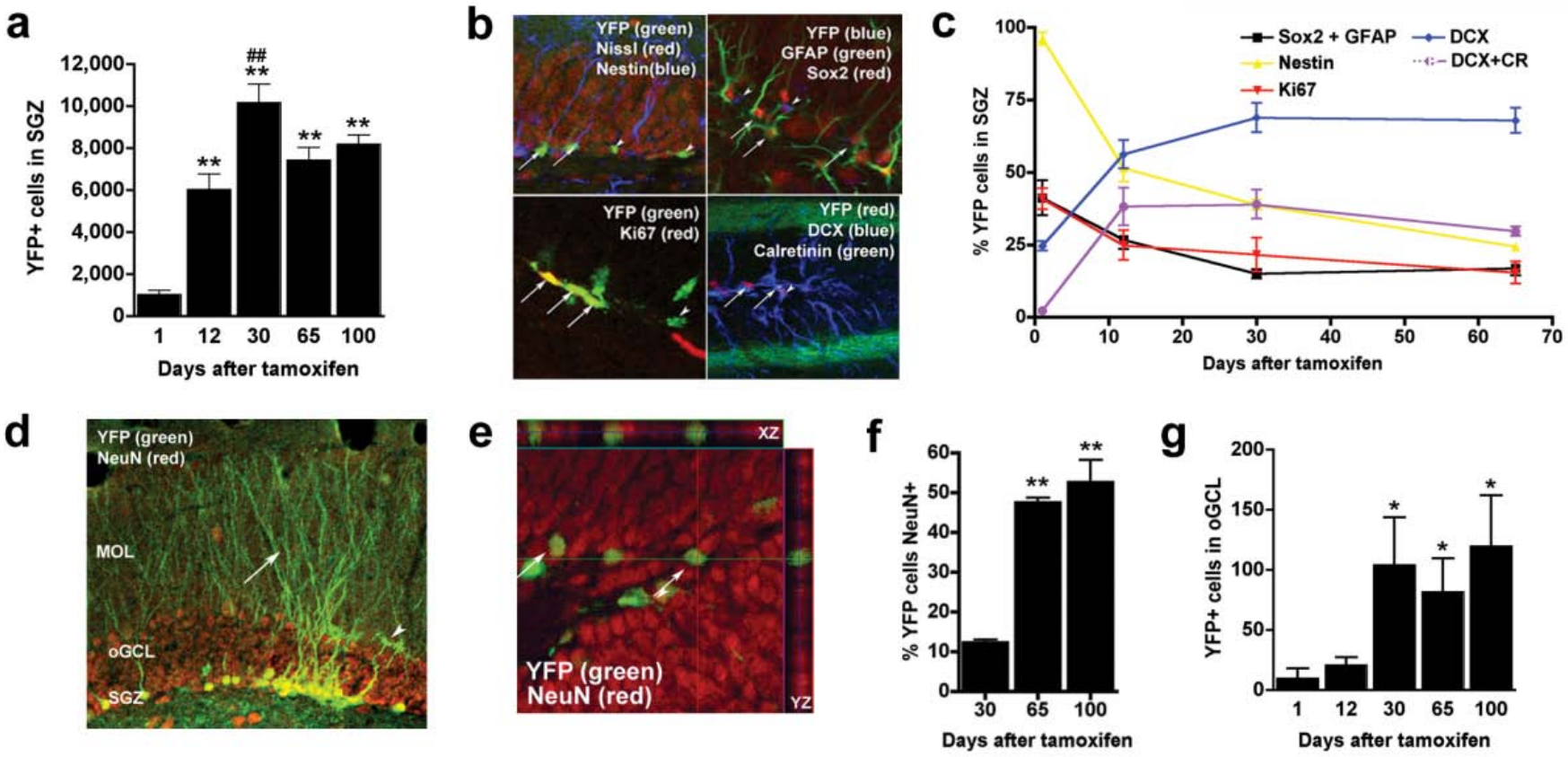
a
YFP+ cells in SGZ
12,000
10,000
8,000
6,000
4,000
2,000
0
1
12
30
65
100
Days after tamoxifen
##
**
b
YFP (green)
Nissl (red)
Nestin(blue)
YFP (blue)
GFAP (green)
Sox2 (red)
YFP (green)
Ki67 (red)
YFP (red)
DCX (blue)
Calretinin (green)
c
% YFP cells in SGZ
Sox2 + GFAP
DCX
Nestin
DCX+CR
Ki67
Days after tamoxifen
d
YFP (green)
NeuN (red)
MOL
oGCL
SGZ
e
XZ
YFP (green)
NeuN (red)
YZ
f
% YFP cells NeuN+
Days after tamoxifen
g
YFP+ cells in oGCL
Days after tamoxifen

Figure 4.3. Neurogenesis in the SGZ following TAM. (a) At increasing time points following TAM, YFP+ cell number in the hippocampal SGZ increased up to 30 days (**p<0.01 vs. 1 day, ## p<0.01 vs. 12 days); (b,c) the proportion of YFP+ cells in SGZ expressing immature markers decreased, while those expressing mature markers increased (arrow: YFP+/Nestin+, YFP+/GFAP+/Sox2+, YFP+/Ki67+, YFP+/DCX+/CR+; arrowhead: YFP+/Nestin-, YFP+/GFAP-/Sox2+); (d-f) an increasing percentage of YFP+ cells in the GCL of the dentate gyrus have a mature phenotype with branched processes (arrow in d) extending into the molecular layer and colocalization with the NeuN (**p<0.01 vs. 30 days, ## p<0.01 vs. 65 days) (arrow: YFP+/NeuN+ in e); and (g) YFP+ cell number in the outer portion of dentate gyrus granule cell layer (oGCL) increased (*p<0.01 vs. 12 days).

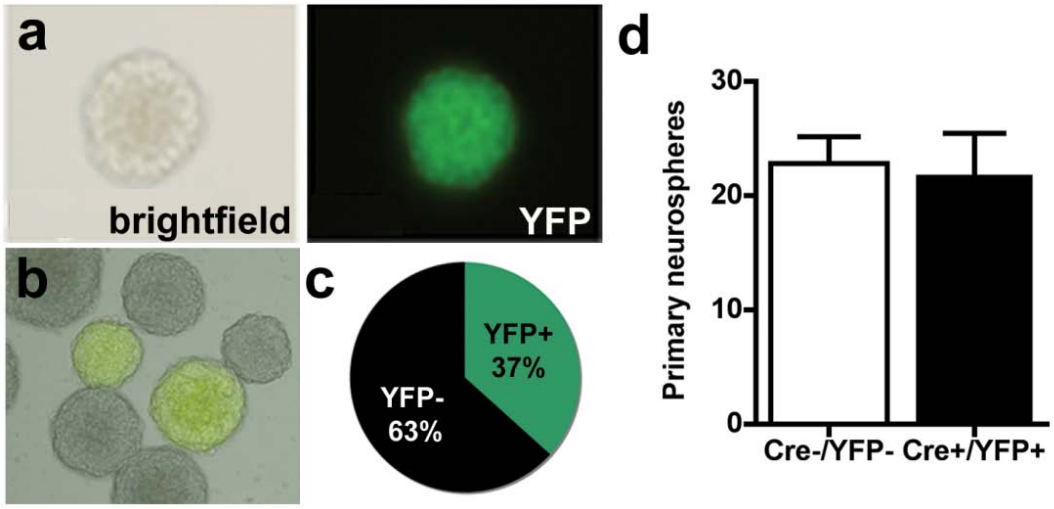

Figure 4.4. After tamoxifen-induced recombination *in vivo*, SVZ cells from postnatal nestin-CreER^{T2}/R26R-YFP mice form primary and secondary neurospheres *in vitro*. (a, b) Neurospheres were evident via brightfield microscopy and many of them were YFP+. (c) After one week in culture with mitogens, 36.5±5% of the derived spheres were YFP+. This percentage reflects that many cells non-stem cells can generate neurospheres in the adult (Pevny and Rao, 2003). These other cells therefore likely dilute the proportion of total neurospheres *in vitro* that are YFP+ at the time point examined (12 days post-tamoxifen *in vivo*). A similar percentage of secondary spheres were YFP+, and all cells within the YFP+ spheres were YFP+ (data not shown; n=2, 25 spheres per mouse). (d) Equal number of neurospheres was generated from nestin-CreER^{T2}/R26R-YFP mice and mice negative for both genes ($p>0.05$).

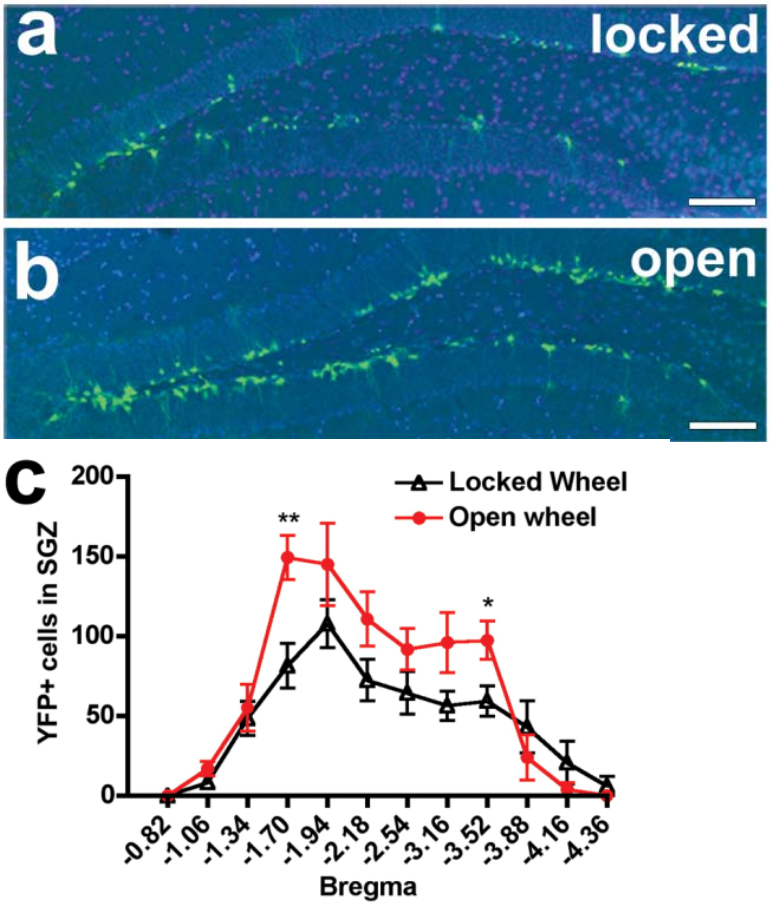

Figure 4.5. Recombined cells can respond to neurogenic stimuli. Nestin-CreER^{T2}/R26R-YFP mice were individually housed with open or locked running wheels for 7 days on the 12th day after tamoxifen. (a,b) Mice with locked wheels (a) had notably fewer YFP+ cells in the dentate gyrus SGZ than mice with open wheels (b). Scale bar=100 µm (a-b). (c) Significantly more YFP+ cells were evident in the SGZ in mice that ran on the open wheels (bregma by treatment interaction, $F_{(11,110)}=2.647$, $p<0.01$).

CHAPTER FIVE

GLAST-expressing cells contribute to constitutive adult hippocampal neurogenesis whereas nestin-expressing cells do not

DeCarolis NA, Mechanic M, Petrik D, Ables JA, Malhotra S, Lagace DC, Eisch, AJ. GLAST-expressing cells contribute to constitutive adult hippocampal neurogenesis whereas nestin-expressing cells do not. *Manuscript in preparation.*

Abstract

Radial astrocyte-like cells (RCs) are the hypothesized source of adult hippocampal neurogenesis, but *in vivo* experimental evidence is largely correlative. Using inducible Cre-driver transgenic mouse lines to track RCs and progeny, we compare the neurogenic contribution from GLAST- and nestin-expressing cells. We find that GLAST-expressing cells contribute both to long-term hippocampal neurogenesis and recovery after ablating proliferation, whereas nestin-expressing cells make only limited contributions. These data emphasize the heterogeneity of adult RCs and suggest a new model for the lineage progression for adult hippocampal neurogenesis.

Introduction

Discrete regions of the adult brain make neurons throughout life, including the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG) and the anterior subventricular zone (SVZ, Ming and Song, 2005). The progression of a progenitor cell through maturation and survival into a dentate gyrus granule cell has been well described (Ming and Song, 2005), but the putative stem cell that gives rise to these progenitors is poorly defined. One hypothesis is that GFAP+ radial cells with glia-like properties (RCs, also Type-1 cells, Type B cells, or Quiescent Neural Precursors) divide asymmetrically to produce rapidly dividing progenitors (Seri et al., 2004; Seri et al., 2001). However, recent studies have challenged the linearity of this hypothesis (Singer et al., 2009; Suh et al., 2007), drawing into question whether RCs are actually the source of hippocampal neurogenesis.

RCs in the adult resemble radial glia during embryonic neurogenesis. Morphologically, both cell types have a long central process, which in adult RCs extends through the hippocampal dentate gyrus and terminates in the inner molecular layer in characteristic arborous tuft (Mignone et al., 2004; Seri et al., 2001). SGZ RCs also express markers of embryonic radial stem cells including nestin, glutamate-aspartate transporter (GLAST), glial fibrillary acidic protein (GFAP), brain lipid binding protein (BLBP), and Sox2 (Kempermann et al., 2004; Pinto and Gotz, 2007; Steiner et al., 2006; Suh et al., 2007). However, none of these markers is unique for RGs though, with overlapping expression either in astrocytes or progenitor cells. As a result, most studies use intersectional

labeling with multiple markers to identify RCs, under the assumption that the RC population is homogenous (Mignone et al., 2004; Seri et al., 2004). Recent reports, however, suggest that the RC pool is heterogeneous and that commonly used markers to identify putative stem cells are identify subpopulations of SGZ stem-like cells (Liu et al., 2009; Seki et al., 2007; Steiner et al., 2006). While the heterogeneity of SGZ RCs has remained unappreciated, accumulating evidence indicates functional and phenotypic heterogeneity of other neural stem cell pools, including embryonic and adult SVZ cells (reviewed in Alvarez-Buylla et al., 2008; Kriegstein and Gotz, 2003). Therefore, determining the identity and functional contribution of the hippocampal stem cell is timely and of broad interest.

Methods and results

To assess the source of hippocampal neurogenesis and explore the contribution of potential RGC subpopulations to adult neurogenesis, we used two tamoxifen (TAM)-inducible Cre-driver transgenic mouse lines, nestin-CreER^{T2} and GLAST-CreER^{T2}, in which YFP expression only overlaps in RCs (Lagace et al., 2007b; Ninkovic et al., 2007). By independently crossing these lines with the Rosa26R-YFP reporter mice, we produced offspring heterozygous for both transgenes (referred to as Nes-YFP and GL-YFP, respectively) in which genetic recombination was selectively induced by TAM administration. Young adult mice (5-6 weeks) receive TAM (180 mg/kg/day IP for 5 days) at 30 mg/ml dissolved in 10% EtOH in sunflower seed oil, as previously described (Lagace et al., 2007b).

Following TAM administration, a subset of nestin- and GLAST-expressing cells expressed the *yfp* gene, which allowed us to track recombined cells and progeny.

To assess the progeny and fate of recombined cells, Nes-YFP and GL-YFP mice were killed at various times post-TAM to allow analysis of the number and phenotype of YFP+ cells. These studies expanded on previous findings (Lagace et al., 2007b) by exploring the YFP+ progenitor pool up to 180d post-TAM and quantifying changes in the number and phenotype of YFP+ cells in adult GL-YFP mice. Qualitative examination revealed that Nes-YFP mice have more YFP+ cells at 30d post-TAM compared to 100d post-TAM (**Figure 5.1A**) though there were more YFP+ cells with neuron-like morphology; interestingly, very few YFP+ progenitor cells are evident at extended times (100d+ post-TAM). Qualitative assessment in GL-YFP mice suggests that the number of YFP+ cells increases between 30d and 100d (**Figure 5.1B**) and that there are increasingly more YFP+ neurons over time (**Figure 5.1C-D**). Quantitatively, in Nes-YFP mice, the number of YFP+ cells peaked 30d post-TAM (**Figure 5.1A, top**) and then reached a plateau. At 100d post-TAM (**Figure 5.1A, bottom**) YFP+ progenitors depleted and there was no net change in cell number or phenotype between 100 and 180d post-TAM (**Figure 5.1E**), extending our previous results from 12d through 100d post-TAM (**Figure 4.3A**)(Lagace et al., 2007b) and consistent with other nestin-CreER^{T2}/R26R-YFP mice (Imayoshi et al., 2008). In contrast, our preliminary data quantifying YFP+ cells in GL-YFP mice (**Figure 5.1F**) suggest that YFP+ cells did not peak but approached an asymptote. However, these results are

preliminary (n=2/time point) and more animals are being added to more definitively assess changes in YFP+ cell number over time.

One possible explanation for the plateau in YFP+ cell number in both Nes-YFP and GL-YFP mice is that progenitor proliferation equilibrated with maturation and cell death. Therefore, we assessed the phenotype of YFP+ cells using markers for RCs and progenitor cells, including GFAP, BLBP, and Sox2, which allowed us to discriminate RCs by their radial process and identify progenitor cells by BLBP and/or Sox2 expression (**Figure 5.1C**) (Lagace et al., 2007b; Pinto and Gotz, 2007; Steiner et al., 2006). Previous reports indicate near-absolute colocalization of GFAP and BLBP in radial cells in the adult SGZ (Steiner et al., 2006), but it was important to confirm these previous findings. GFAP+/Sox2+ or BLBP+/Sox2+ RCs are morphologically distinguishable from progenitors by a central, perpendicular process through the granule cell layer (GCL, **Figure 5.1C**). Using confocal microscopy, we determined that in both Nes-YFP and GL-YFP mice, the proportion of YFP+ cells that were RCs was highest at early times post-TAM, preceding the accumulation of maturing neurons (**Figure 5.1C, G-H**). Also, as expected, the proportion of YFP+ cells that were RCs declined over time, as the proportion of YFP+ cells that were neuronal increased (**Figure 5.1D, G-H**). Strikingly, however, after 100d in Nes-YFP mice, there were virtually no detectable progenitor cells, but the proportion of YFP+ RCs remained around 20%. Similarly, after 100d, the proportion of YFP+ cells that were neuronal remained constant, when assessed either morphologically (70%, **Figure 5.1G**) or

via colocalization with neuronal marker NeuN (75%, data not shown). In contrast, in GL-YFP mice, YFP+ progenitor cells persisted at 100d post-TAM and the proportion of YFP+ neurons continued to rise across time points. These data suggest that GLAST-expressing cells can contribute to the long-term maintenance of neurogenesis, though additional animals at each time point and extended times post-TAM must be assessed to confirm the maintenance of the YFP+ progenitor pool. However, the depletion of YFP+ progenitors in Nes-YFP mice suggests that labeled nestin-expressing cells and progeny make a substantial contribution to neurogenesis at early time points but not to persistent, constitutive neurogenesis. Thus far our results suggest that GLAST-expressing and nestin-expressing cells may make different contributions to on-going SGZ neurogenesis. However, these data cannot specifically assess the contribution of RCs because multiple cell types are labeled in both mice.

To directly assess whether GLAST+ cells and nestin+ RCs contribute to hippocampal neurogenesis, we chemically ablated proliferation via central nervous system administration of an antimitotic drug and assessed recovery of proliferation and YFP+ cells. We hypothesized that if YFP+ RCs contributed to neurogenesis, then the number of YFP+ progenitor cells would increase as proliferation recovered. At 25d post-TAM, mice were surgically implanted with intracerebroventricular (ICV) cannulae attached to osmotic minipumps that for 6d infused either cytotoxic cytosine-β-D-arabinofuranoside (AraC, 2% w/v) or saline as control (**Figure 5.2A**), essentially as described (Mak et al., 2007). Mice were

given BrdU (150 mg/kg, IP) and killed 2hrs later, either at 0d or 7d recovery post-infusion, which allowed us to assess YFP+ and BrdU-IR cells (**Figure 5.2A**) (Mak et al., 2007; Seri et al., 2001). As expected, in both Nes-YFP and GL-YFP mice, after 6d of AraC infusion (0d recovery), the number of proliferating BrdU-IR cells was significantly decreased, which normalized after 7d recovery (**Figure 5.2B-F**), consistent with previous reports (Seri et al., 2004; Seri et al., 2001). Because of basal differences in proliferation and number of YFP+ between Nes-YFP and GL-YFP strains (**Figure 5.3**), data are presented as percent of saline-treated controls at 0d (**Figure 5.2C-D, F-G**). There was a significant effect of AraC on the total percent of YFP+ cells by Two-Way ANOVA in both Nes-YFP ($F_{(1,18)}$=21.53; p<0.001) and GL-YFP mice ($F_{(1,12)}$=7.54; p<0.05), supporting that many YFP+ cells at this time were progenitors (**Figure 5.1B-D**). In Nes-YFP mice at 0d recovery from AraC, there was a significant 50% decrease in YFP+ cells (saline, 100.0%±14.6 vs AraC, 52.4%±8.55). Strikingly, however, there was no recovery of YFP+ cells in the Nes-YFP mice (**Figure 5.2B, D**) and the percent of YFP+ cells remained significantly decreased compared to controls at 7d recovery (saline, 121%±15.4 vs AraC, 54.8%±10.0)(**Figure 5.2D**). Importantly, YFP+ RCs were abundant in Nes-YFP mice, regardless of AraC or saline infusion, and the proportion of YFP+ RCs was significantly increased at 0d and 7d recovery compared to controls, supporting that progenitor cells (and not RGs) were ablated (**Figure 5.4**).

In contrast, while AraC administration significantly decreased YFP+ cells in GL-YFP mice at 0d recovery by ~40% (saline, 100.0%±8.9 vs AraC, 68.7%±7.3), deficits in YFP+ cells in the GL-YFP mice normalized after 7d (saline, 87.5%±6.0 vs AraC, 76.4%±8.1), suggesting that GLAST-expressing cells and their progeny contributed to the repopulation of SGZ progenitors (**Figure 5.2E-G**). Consistent with this, YFP+/BrdU+ cells were evident at 7d recovery in GL-YFP mice administered AraC (**Figure 5.2E**, arrows and inset).

Discussion and conclusions

Together, these data suggest functional differences between GLAST-expressing and nestin-expressing cells in their respective contribution to neurogenesis. Recombined, YFP+ progenitor cells persist at extended times post-TAM in GL-YFP mice, whereas almost no progenitor cells are evident at 100d or 180d post-TAM in Nes-YFP mice. Second, when YFP+ progenitor cells are chemically ablated with AraC, total YFP+ cells recover in GL-YFP mice whereas deficits in YFP+ cells persist in Nes-YFP mice, even though the total number of proliferating, BrdU-IR cells normalized by 7d post-infusion in both lines.

There are two potential hypotheses that could explain these results (**Figure 5.5**). First, the RC population may be heterogenous, such that a subset of RCs that is GLAST+ is the source of neurogenesis whereas nestin+ RCs do not give rise to progenitors. Alternatively, GLAST-expressing astrocytes may directly give rise to progenitors. Experimental data presented here cannot discriminate these

possibilities, however there is literature supporting the first hypothesis and refuting the second.

Heterogeneity of radial cells within the hippocampus has been acknowledged, but is not well accepted. For example, GFAP+/nestin- and GFAP+/nestin+ radial Type-1 cells have been previously observed using the nestin-GFP reporter mouse, reported by our lab (**Figure 2.1A**) and others (Kempermann et al., 2004; Steiner et al., 2006). As an extension to the studies here, GLAST, BLBP, and GFAP are ubiquitously co-expressed in radial cells of the adult brain and during last embryogenesis (Mori et al., 2006 and references therein; Platel et al., 2009). Heterogeneity of stem cell populations has been previously described in the embryonic and adult brains (Hartfuss et al., 2001; Merkle et al., 2007; Pinto et al., 2008). Importantly, differences in expression of GLAST, BLBP, and the RC2 antigen distinguish subclasses of embryonic radial glia (Hartfuss et al., 2001), and the RC2 radial glia antigen is product of the *nestin* gene (Park et al., 2009). Taken together, these reports indicate functional differences in GLAST+ and nestin+ cells in the developing brain (Kriegstein and Gotz, 2003)and heterogeneity of astrocytes more broadly (Bachoo et al., 2004). Additional evidence for functional differences between nestin+ and GFAP+ cells comes from genetic ablation studies with thymidine-kinase, whereby ablation of dividing GFAP+ cells causes a permanent elimination of neurogenesis (Garcia et al., 2004; Morshead et al., 2003) whereas ablation of dividing nestin+ cells are partially reversed over time (Singer et al., 2009). Therefore, it may not be

surprising that functional differences between GFAP+/GLAST+ cells and nestin+ RCs persist in the adult, as demonstrated here.

However, we cannot exclude the possibility that the differences observed in contribution to neurogenesis are not the result of the non-overlapping populations, specifically that GLAST+ astrocytes are the source of progenitors. However, maturation of astrocytes correlates with cell-cycle exit and loss of stem-like capacities (Raponi et al., 2007), and S100β-expressing astrocytes, which are a subset of mature astrocytes (Wang and Bordey, 2008), are non-proliferative (Kempermann et al., 2003; Raponi et al., 2007; Steiner et al., 2004) though see (Namba et al., 2005; Seri et al., 2004).

Alternatively, the observed differences might reflect limits of the experimental approaches. Used a single Nes-YFP line to compare to a single GL-YFP line, and differences could reflect off-target effects resulting from random transgenic insertion (discussed in Chapter One of this thesis). While we cannot entirely exclude this possibility, we do have evidence supporting functional differences. For example, other lines of inducible nestin-CreER^{T2} mice reach a static point beyond which new neurons are no longer added (Imayoshi et al., 2008), though this is reported at 6 mo post-TAM, compared to 4 mo post-TAM reported here.

It is also possible that our conclusions are premature, because the YFP+ progenitor pool in GL-YFP mice might deplete at longer time-points. In addition to

adding later time points to exclude this possibility, we also show that GL-YFP cells contribute to recovery after AraC. Additional experiments are needed to confirm that more YFP+ cells are proliferating with BrdU- or Ki67-colabeling (**Figure 5.2E**) and that YFP+ cells are in the neuronal lineage with colabeling of immature and mature markers like doublecortin or NeuN.

Additionally, it will be important for us to validate that the proportion of YFP+ RCs increases in both Nes-YFP (**Figure 5.3**) and GL-YFP mice after AraC, by phenotyping YFP+ cells with radial markers like BLBP and GFAP. We also may not have waited long enough post-AraC to observe recovery in Nes-YFP mice. While proliferation normalized 7d post-AraC, it remains possible that the restoration of proliferating cells does not indicate a functional return of neurogenesis. Alternatively, our ablation scheme may have altered the microenvironment to preclude nestin-cell division in response.

It will be important to assess a positive regulator of neurogenesis as well. At an extended time post-TAM, when there are no more YFP+ progenitors in the Nes-YFP SGZ, stimulating neurogenesis might “jump start” YFP+ RCs to proliferate. For example, while running stimulates proliferation of early progenitors likely by symmetric division (**Figure 2.3**), it is possible that RCs may divide asymmetrically to expand the progenitor pool (Suh et al., 2007).

These studies provide the first insight into functional differences in cellular contribution to neurogenesis. Future studies are needed to clarify if radial glia-like cells in the SGZ are indeed proliferative. In addition, it is remains unclear what the function of RCs is, if they are not in fact the source of neurogenesis. Given their glial-like properties, including end-feet associated with the vasculature, it is possible that RCs play an instructive or supportive role in neurogenesis, beyond directly contributing by cellular division. It is also interesting that some nestin-expressing RCs do divide (e.g. Arguello et al., 2008; Kronenberg et al., 2003).

Our data indicate that functional heterogeneity of adult RCs recapitulates embryonic development. While more functional data are warranted, these results challenge the prevailing view that RCs are homogeneous and functionally identical. These results also indicate that some cells (such as GLAST-expressing RCs in the GL-YFP mouse) may be more “stem-like” than nestin-expressing cells. Alternatively, these differences may not be functionally important *in vivo* but reflect differences between transgenic lines and an ostensible need to characterize “stemness” (Lander, 2009).

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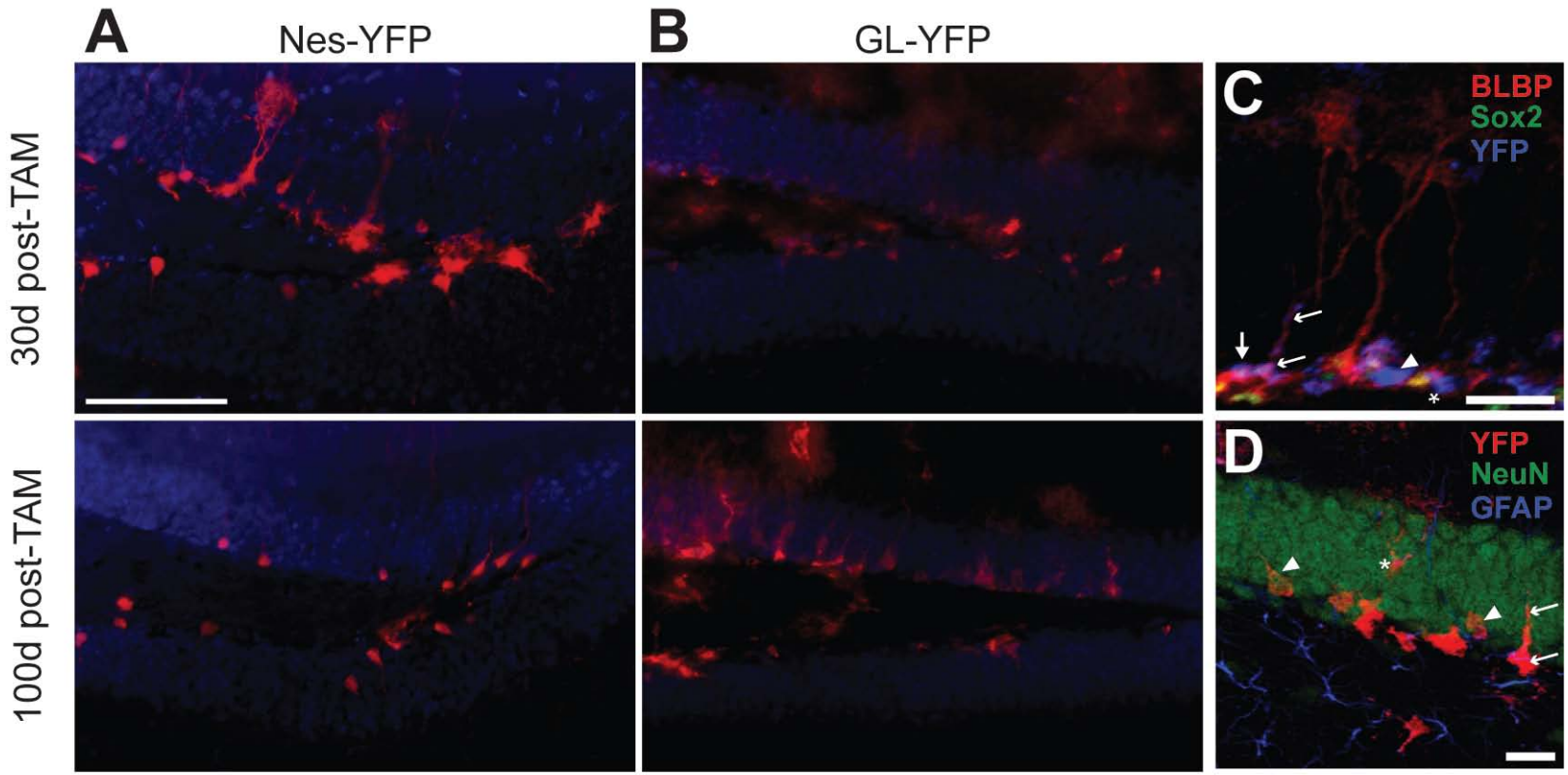
A
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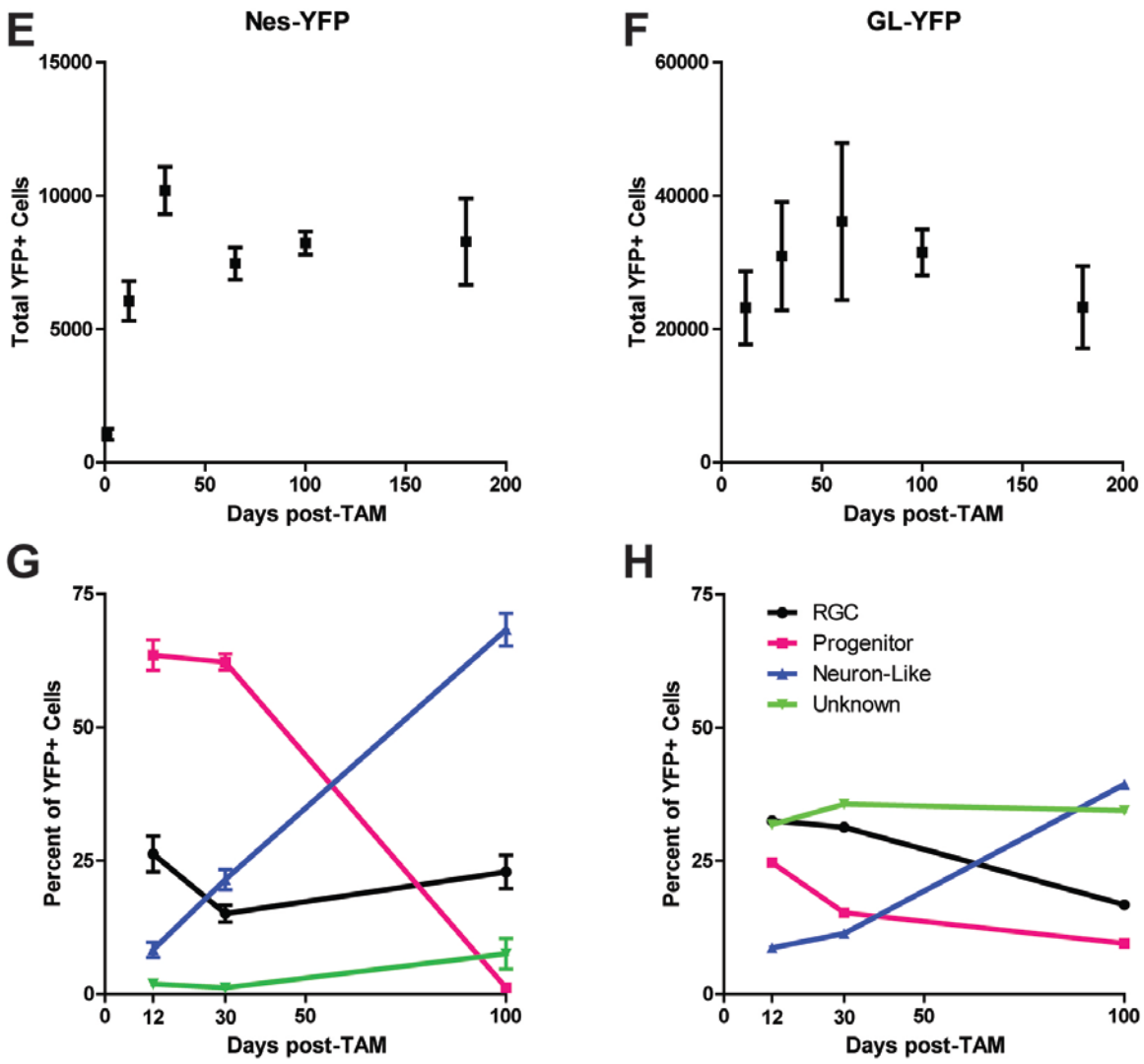
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GL-YFP
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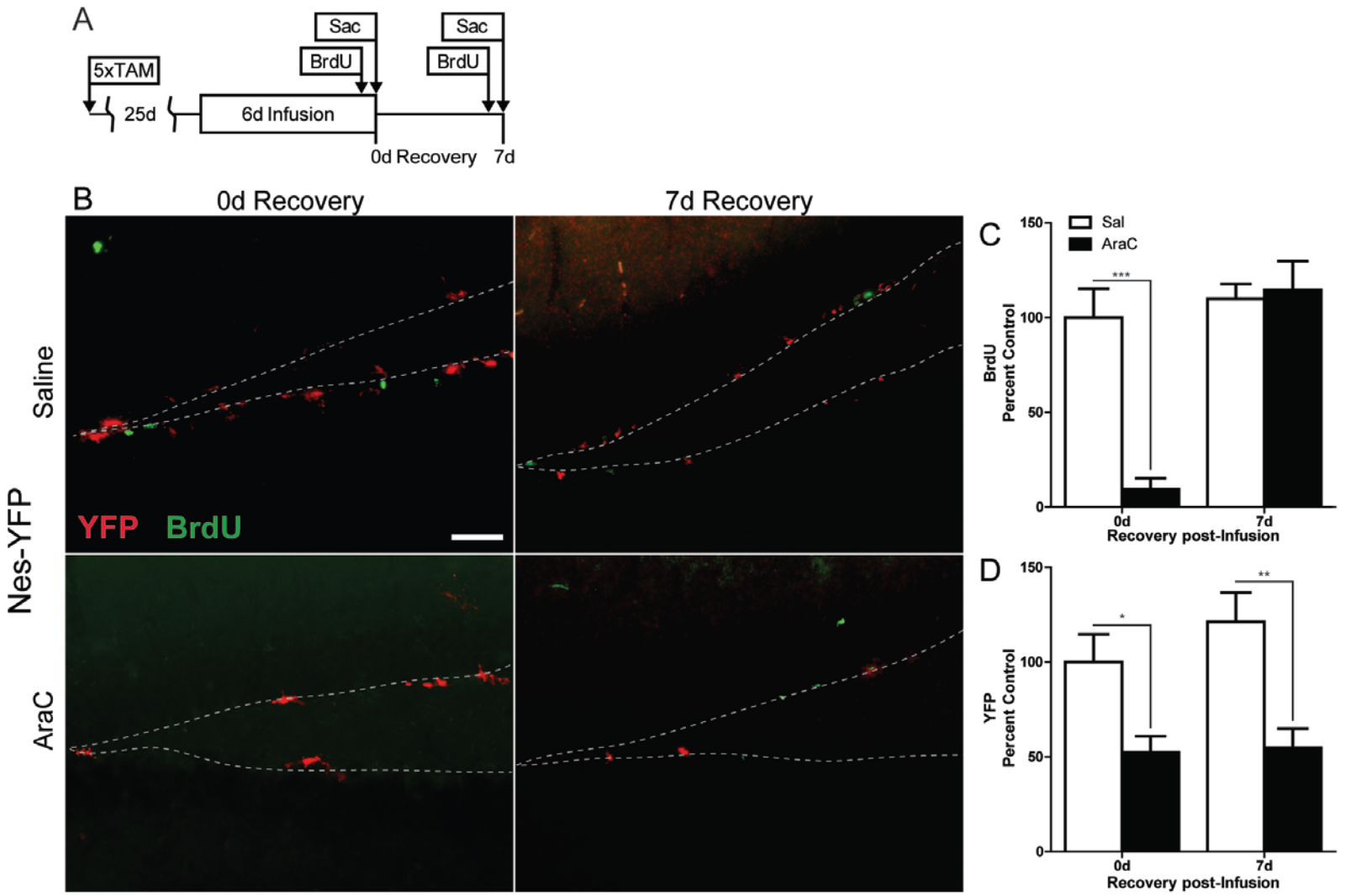
60000
40000
20000
0
0
50
100
150
200
Days post-TAM
G
Percent of YFP+ Cells
75
50
25
0
0
12
30
50
100
Days post-TAM
H
Percent of YFP+ Cells
RGC
Progenitor
Neuron-Like
Unknown
75
50
25
0
0
12
30
50
100
Days post-TAM

Figure 5.1. SGZ neurogenesis in Nes-YFP and GL-YFP mice after TAM. Representative pictomicrographs between (A) Nes-YFP and (B) GL-YFP mice at 30d (top) and 100d (bottom) post-TAM. Note that in the Nes-YFP mice, the number of YFP+ cells goes down with time, whereas in GL-YFP mice, the number of YFP+ cells increases. (C) Single confocal z-plane of a GL-YFP section collected 30d post-TAM, stained for BLBP (red), Sox2 (green), and YFP (blue). The small arrows identify a RC with its characteristic triangular soma and central process extending into the GCL; the cell is BLBP+/Sox2+/YFP+. The wide arrow indicates a labeled progenitor cell that is BLBP+/Sox2+/YFP+ but lacks distinctive morphology. A YFP+ neuroblast (arrowhead) is identified as BLBP-/Sox2-/YFP+ with a small process, consistent with an immature neuron. Asterisk indicates a BLBP-/Sox2-/YFP+ cell with morphology reminiscent of an astrocyte. (D) Single confocal z-plane of a GL-YFP section collected 100d post-TAM, stained for YFP (red), NeuN (green), and GFAP (blue). The small arrows indicate a YFP+/NeuN-/GFAP+ RC with its characteristic process that colocalizes with GFAP. Arrowheads indicate YFP+/NeuN+/GFAP- mature neurons. Asterisk identifies a YFP+/NeuN-/GFAP+ astrocyte in the outer granule cell layer. (E) The total number of YFP+ cells in Nes-YFP mice over time post-TAM. The number peaks at 30d and then reaches a plateau between 60d and 180d post-TAM. (F) Preliminary data on the number of YFP+ cells in GL-YFP mice over time post-TAM, representing n=2 mice per time point. (G, H) The phenotypic changes in YFP+ cells in (G) Nes-YFP and (H) GL-YFP mice over time post-TAM. Nes-YFP mice were analyzed with GFAP/Sox2/YFP whereas GL-YFP mice were analyzed

with BLBP/Sox2/YFP. Preliminary analysis indicated that all GFAP+ RC were also BLBP+. In Nes-YFP mice, the proportion of YFP+ progenitor cells (GFAP-/Sox2+/YFP+) was very high at early times post-TAM, whereas at 100d post-TAM, the YFP+ progenitor pool was depleted. The proportion of YFP+ neurons increased over time until it reached a steady state at 100d, at which time almost all YFP+ cells were either neuronal or RCs. Similarly, in GL-YFP mice, the proportion of YFP+ neurons increased over time. However, YFP+ progenitor cells were evident at 100d post-TAM as were YFP+ RCs. Scale bar in (A) upper left panel is 50 µm and is the same for all panels in A and B. Scale bar in (C) is 20 µm; bar in (D) is 15 µm.


A
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B
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AraC
Nes-YFP
YFP
BrdU
C
Sal
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Percent Control
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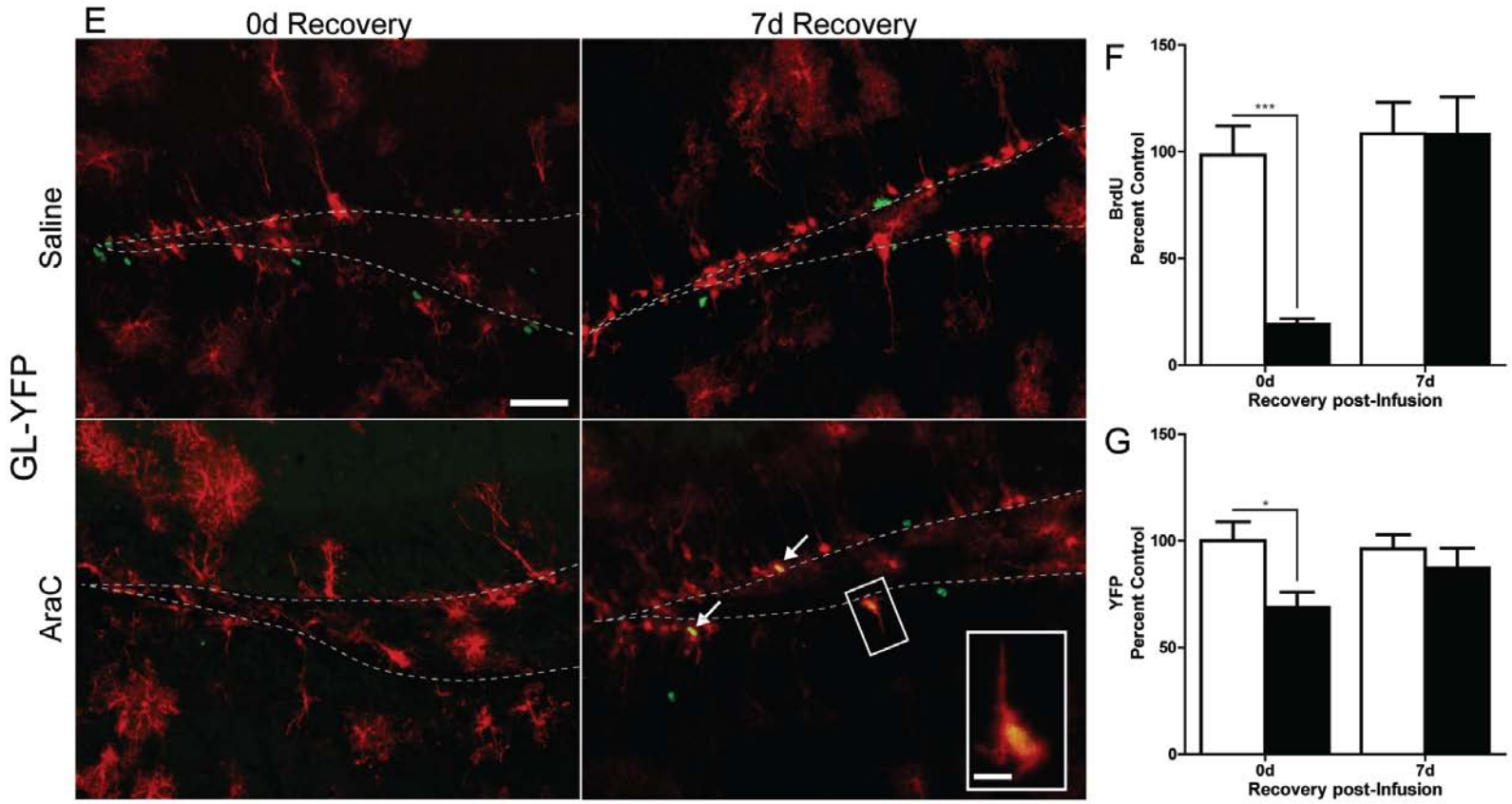
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Recovery post-Infusion

Figure 5.2. Recombined cells from the GLAST lineage facilitate proliferative recovery after chemical ablation of neurogenesis whereas nestin-expressing cells do not. (A) Schematic of experimental design. Nes-YFP or GL-YFP mice were administered TAM around 5-6 weeks of age. 25d later, all mice were implanted cannula attached to osmotic minipumps (0. 5 µl/hr, 7d) to deliver AraC (2% v/w in saline) or vehicle alone for 6d. At 0d or 7d recovery post-infusion, mice received BrdU (150 mg/kg, IP) and were perfused 2 hrs later. (B-G) AraC-induced ablation of proliferation and recovery in Nes-YFP mice (B-D) and GL-YFP mice (E-G). (B) Representative micrographs of Nes-YFP stained for YFP (red) or BrdU (green) in the mouse dentate gyrus after saline (top row) or AraC infusion (bottom) at 0d (left column) and 7d (right) recovery post-infusion. Grey, dashed lines indicate the border between the granule cell layer and the hilus. (C) Infusion of AraC results in almost complete ablation of proliferating BrdU-IR cells compared to saline-infused controls. By 7d post-infusion, proliferating BrdU-IR cells normalized to control values. (D) AraC infusion significantly decreases the number of YFP+ cells in the SGZ compared to controls. The significant decrease persists at least 7d post-infusion of AraC, and there is no change in the number of YFP cells 0d or 7d post-AraC. (E) Representative micrographs of GLxY mice stained for YFP (red) or BrdU (green) in the dentate gyrus. Arrows indicate colabeled BrdU-IR/YFP+ cells. Inset in lower right panel shows higher magnification of boxed region, depicting a proliferating BrdU-IR RC. (F) Infusion of AraC significantly decreased proliferating BrdU-IR cells compared to saline-infused controls, which normalized by 7d post-infusion. (G) Immediately after AraC infusion, there is a significant decrease in the number of YFP+ cells in the

SGZ compared to controls. However, at 7d post-infusion, there is no difference in YFP cells between saline- and AraC-infused mice. Data presented as percent change from 0d saline control (mean ± SEM, n=3-8 mice/group). *, p<0.05; **, p<0.01; ***, p<0.001 compared to controls by Bonferoni post-test. Scale bars in B, E represents 100 µm. Scale bar in inset of E represents 5 µm.

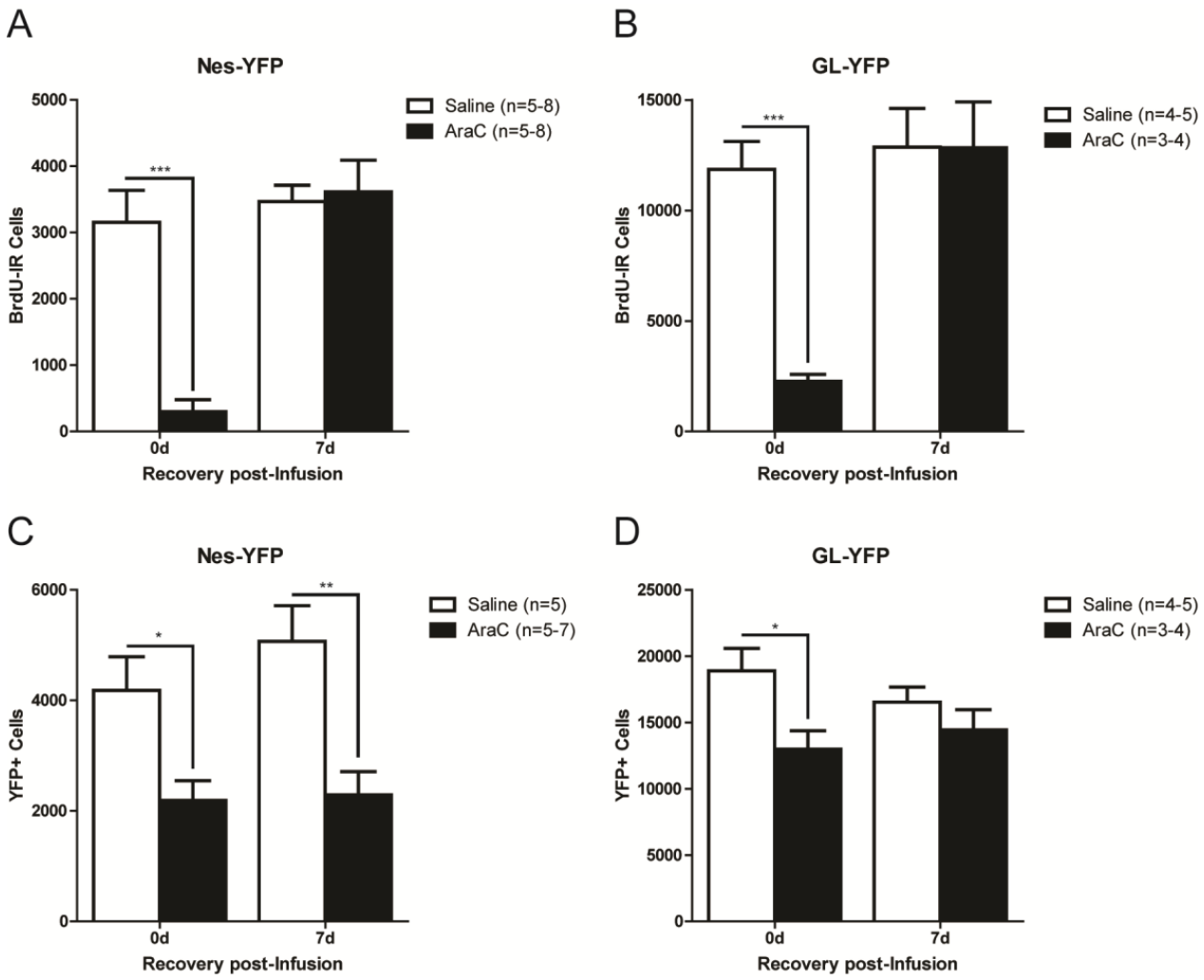

Figure 5.3. Quantification of BrdU-IR cells and YFP+ cells indicate significant basal disparities between Nes-YFP and GL-YFP transgenic lines. (A-B) The number of BrdU-IR cells in Nes-YFP mice is largely consistent with previous findings reported here (e.g. Figures 2.1G; 2.2C; 3.2C) whereas the number of BrdU-IR cells in GL-YFP is approximately 3 times higher. (C-D) The absolute number of YFP+ cells in Nes-YFP mice around 30d post-TAM is approximately 4 times lower than GL-YFP mice, likely reflecting differences between populations labeled and differences in recombination efficacy.

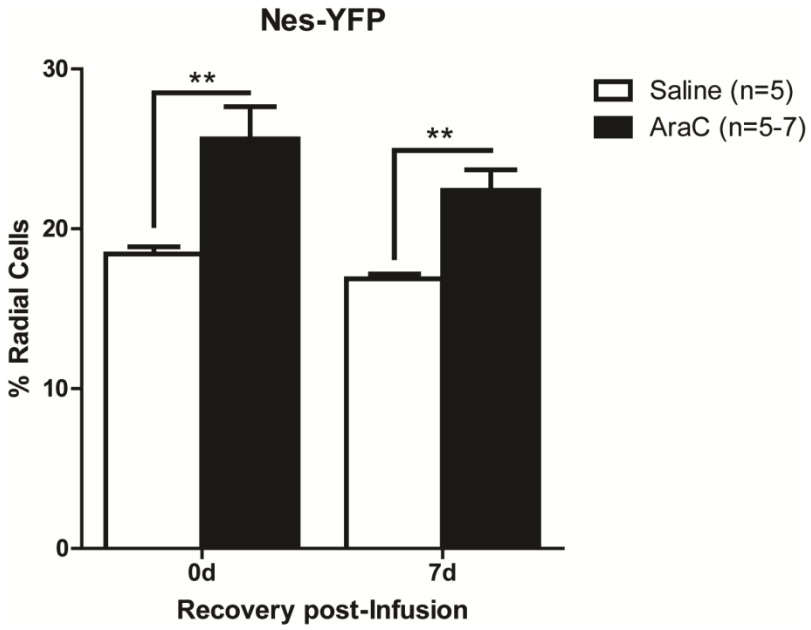

Figure 5.4. The proportion of YFP+ cells that are radial glia-like increases significantly in Nes-YFP mice after ablation of progenitor cells. YFP+ radial cells were identified morphologically. For each mouse, the number of YFP+ RCs was divided by the total number of YFP+ cells to determine the proportion of radial cells. Consistent phenotypic analysis of RCs with GFAP/Sox2 around 30d post-TAM (Figure 4.3), approximately 18% of YFP+ cells are RCs. **, $p<0.01$.

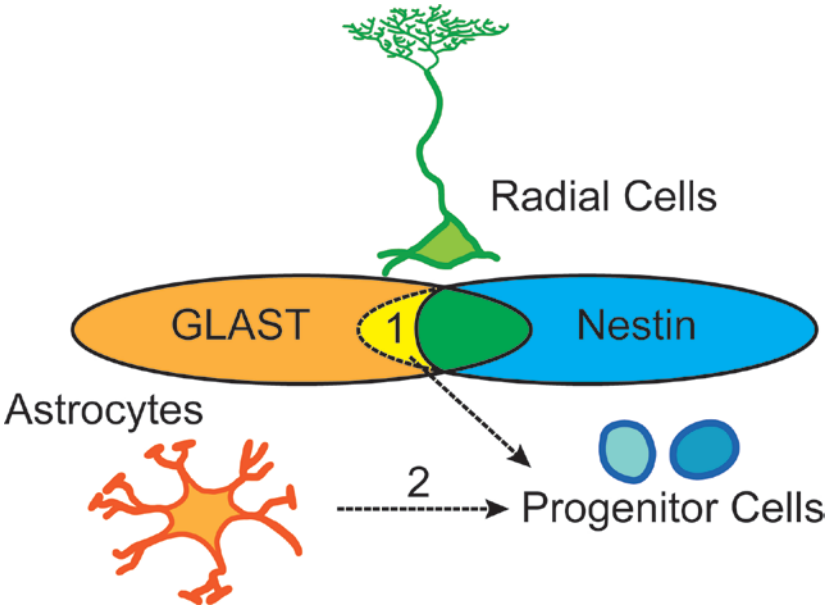

Figure 5.5. Two alternate hypotheses on how GLAST-expressing but not nestin-expressing cells contribute to constitutive neurogenesis. GLAST protein expression (represented by orange oval) overlaps with two primary populations in the adult SGZ: astrocytes (orange) and radial cells (green). Nestin protein expression (represented by blue oval) overlaps with two primary populations in the adult SGZ: early neurogenic progenitor cells (blue) and radial cells (green). Hypothesis 1: RCs are a heterogeneous population of cells, such that GLAST+/nestin- radial cells (overlapping region in yellow) give rise to progenitor cells, whereas GLAST+/nestin+ radial cells (overlapping green region) do not give rise to progenitors. Hypothesis 2: GLAST+ astrocytes directly give rise to progenitors, independent of GLAST+/nestin+ radial cells.

CHAPTER SIX

Conclusions and discussion

Overall Conclusions

The experiments presented here explore the connections between Type-1 cells and constitutive adult neurogenesis in the SGZ. Functional evidence indicating that Type-1 cells are the putative stem cell has been lacking, prompting me to ask two critical questions: How do effectors of neurogenesis change Type-1 cells? What is the contribution of Type-1 cells directly to neurogenesis? The first question is addressed in Chapters Two and Three, with detailed rationale, data, and conclusions suggesting that changes in neurogenesis (from effectors like stress, running, and X-irradiation) occur independently from changes in the number of nestin-GFP+ Type-1 cells. The second question is explored in greater detail in Chapters Four and Five, with the data supporting a disconnect between constitutive hippocampal neurogenesis and Type-1 cells. Strikingly, though, these data also demonstrate functional heterogeneity of Type-1 cells labeled in GLAST-CreER^{T2}/R26R-YFP (GL-YFP) and nestin-CreER^{T2}/R26R-YFP (Nes-YFP) mice and lead us to this conclusion: GLAST+ but not nestin+ cells make long-lasting contributions to neurogenesis. Here I review the main conclusions and future directions for each of my thesis chapters thus far, and then introduce my new model for how to think about Type-1 cells in future experiments.

Chapter Two Conclusions: ***Running after irradiation ameliorates the proliferation deficit but not the nestin-expressing Type-1 deficit in the adult hippocampal subgranular zone***

We explored how two potent effectors of neurogenesis (running and X-irradiation) altered nestin-GFP+ Type-1 cells *in vivo*. We found that X-irradiation caused a significant decrease in the total number of nestin-GFP+ Type-1 cells only when the mice were young adult (6 weeks of age) at the time of radiation; at other ages at irradiation (10 or 18 weeks), we did not observe any significant decrease in Type-1 cell number, though X-irradiation produced a long-term and robust reduction in SGZ proliferation. In contrast, running produced a long-term and robust enhancement in SGZ proliferation, but again the number of Type-1 cells was not changed. When the two effectors were combined, running partially reversed X-ray-induced deficits in proliferation without significantly changing the number of Type-1 cells. We also showed that deficits in proliferation after radiation were likely not mediated by persistent inflammation, because we did not observe overt differences in inflammation markers between sham- and X-irradiated mice, regardless of wheel running. In conclusion, we observe a potential disconnect between Type-1 cell number and persistent neurogenesis.

<u>Future directions, Chapter Two</u>

A number of questions should be addressed to conclusively demonstrate that observed changes in proliferation are independent of changes in Type-1 cells as a result of running and X-irradiation. First, it will be critical to assess the

proliferative state of Type-1 cells after each effector. So far, we have quantified the total population of Type-1 cells without regard to individual differences in proliferation. It will be important to assess colocalization of GFP/BrdU/GFAP to clearly identify Type-1 cells and assess the proportion that are undergoing division. Another critical facet that must be addressed quantitatively is inflammation. While the general microglial marker Iba1 does not show over changes at the sectional level, more detailed analysis is required to assess immunoreactivity (for example, by pixel density or intensity). Alternatively, immune activation should be assayed by CD68 expression, or by immunoblotting from dentate gyrus dissections. Another possible mechanism by which running attenuates radiation-induced deficits is through vascular changes, which can be assessed with endoglin IHC or other blood vessel markers. It will be important to assess long-term changes to the vascular niche as a result of running and radiation, both independently and together.

One of the larger questions that remains following the studies presented in Chapter Two is how long-lasting are the effects of running on recovery. It is of particular interest to explore whether running induces a transient recovery of proliferation or if the effects are long lasting. For example, if irradiated mice run for 7d, as in these studies, but are returned to normal housing conditions, does the partial rescue of proliferation persist long-term (suggesting a reversal of radiation-induced damages)? If the running-mediated recovery of proliferation is only temporary and, after running ends, proliferation in irradiated mice returns to

very low levels, then this might suggest that running and irradiation alter proliferation via discrete mechanisms, challenging researchers to develop better techniques and models to separate the functional differences between effectors.

Chapter Three Conclusions: *Adult hippocampal neurogenesis is functionally important for stress-induced social avoidance*

The studies in Chapter Three demonstrated an important role for neurogenesis in stress-mediated behavioral responses. In addition to defining this novel functional role for neurogenesis, these studies also showed that – contrary to the long-accepted paradigm that stress decreases proliferation – stress-induced decrease in proliferation is short-lived and can in some mice (those that are susceptible to a stressful experience) lead to a long-term increase in the number of surviving SGZ cells. Moreover, these studies highlight a disconnect between Type-1 cells and neurogenesis, such that changes in neurogenesis in stress- and time-dependent manner occur independently from overt changes in the population of Type-1 cells.

<u>Future experiments: Chapter Three</u>

The studies presented and published in Chapter Three (Lagace et al., 2010) exhaustively explore the importance of neurogenesis in social defeat stress. However, these results remain to be explored in other models of stress and depression. Moreover, it will be important to assess whether neurogenesis is required for antidepressant reversal of the social avoidance behavioral

phenotype (Berton et al., 2006; Krishnan et al., 2007). Recent evidence suggests that neurogenesis is required for antidepressant efficacy and that BDNF is critical in mediating these behavioral effects (e.g. Berton et al., 2006; Santarelli et al., 2003). However, our results suggest that the behavioral phenotype observed is independent of overt changes in BDNF protein levels (Lagace et al., 2010). Therefore, additional work is needed to address this potential disconnect.

Chapter Four Conclusions: *The nestin-CreER^{T2}/R26R-YFP mouse is a powerful tool to assess the contribution of nestin-expressing cells to adult neurogenesis*

The studies presented in Chapter Four (Lagace et al., 2007b) highlight that nestin-expressing cells make a dynamic contribution to neurogenesis. In other words, in nestin-CreER^{T2}/R26R-YFP (Nes-YFP) the phenotype of labeled YFP+ cells changes over time as more neurons are added to the hippocampus and the olfactory bulb. One of the conclusions from the studies is that the dynamics our inducible fate-tracking Nes-YFP mice confirm the predicted contribution of adult-born cells from previous BrdU studies. Interestingly, we also showed that the number of recombined YFP+ progeny peaks very early post-TAM (30d), then reaches a plateau.

<u>Chapter Four, Future Directions</u>

While ground-breaking, the studies presented in Chapter Four have some shortcomings. For example, they did not address the long-term contribution to

neurogenesis, specifically at extended times (e.g. 180d+) post-TAM. In addition, these studies did not explore the long-term maintenance of the YFP+ progenitor pool, which would be expected if nestin-expressing cells are in fact stem cells. Many of these studies have been addressed in Chapter Five of this thesis.

Chapter Five Conclusions: *GLAST-expressing cells contribute to constitutive adult hippocampal neurogenesis whereas nestin-expressing cells do not*

Studies presented in Chapter Five present interesting and important results that potentially challenge our understanding of nestin-expressing Type-1 cells as the source of persistent adult hippocampal neurogenesis. Our preliminary data on differences between nestin-CreER^{T2}/R26R-YFP (Nes-YFP) and GLAST-CreER^{T2}/R26R-YFP (GL-YFP) mice indicate that the YFP+ progenitor pool is depleted in Nes-YFP mice at late times post-TAM, whereas the labeled progenitor pool persists in GL-YFP mice. This suggests either heterogeneity of Type-1 cells or that GLAST+ astrocytes provide a previously unappreciated source of YFP+ progenitors. We directly tested whether YFP+ Type-1 cells in the Nes-YFP or GL-YFP mice can functionally support the recovery of neurogenesis after progenitor cells are chemically ablated via AraC. Strikingly, we found that YFP-labeled cells in GL-YFP mice can participate in the functional recovery, whereas in Nes-YFP mice there are no additional YFP+ cells once proliferation normalizes post-AraC. These results suggest that a GLAST+ but not nestin+ cell is likely the constitutive source of on-going adult SGZ neurogenesis.

Chapter Five: Future directions

While the results presented for Nes-YFP are largely complete, much additional work is required to draw definitive conclusions regarding a GLAST+ stem cell as the putative source of SGZ neurogenesis. For example, we need more thorough analysis of both the number of YFP+ cells and their phenotype in GL-YFP mice at times post-TAM. As it stands, we have preliminary indications but cannot draw definitive conclusions. It will also be important for us to extend our current phenotypic analysis beyond BLBP/Sox2/YFP triple labeling of RG and progenitor cells to include more thorough assessment of labeled neurons (likely YFP/DCX/NeuN or YFP/Prox1/NeuN triple labeling) and astrocytes (likely YFP/GFAP/S100β labeling). Our prediction that the YFP+ progenitor pool persists in GL-YFP mice awaits further confirmation at more protracted times post-TAM, including up to one year.

Another finding in Chapter Five that needs further examination is the data showing that YFP+ cells in GL-YFP mice contribute to the recovery of proliferation after AraC whereas YFP+ cells in Nes-YFP mice do not. We will need to demonstrate this functional disparity in at least two critical ways. First, it will be important to quantify the proportion of dividing YFP+ cells in both Nes-YFP and GL-YFP mice. Our prediction would be that more YFP+ cells colocalize with proliferation markers like BrdU or Ki67 in GL-YFP mice if YFP+ cells contribute to recovery. It is also important to demonstrate that the YFP+ cells in GL-YFP mice during recovery post-AraC are actually neurogenic. Because of the

induced pathology during the chemical ablation of all cell proliferation, including neurogenesis, gliogenesis, microgliosis, and angiogenesis, it is reasonable that the increase in YFP+ cells in GL-YFP mice are actually glia, given that GFAP+/GLAST+ astrocytes can revert to a proliferative state during reactive gliosis (Clarke et al., 1994; Lin et al., 1995). Therefore, we must assess the proportion of YFP+ cells that are immature neurons (DCX+/Prox1+) in both Nes-YFP and GL-YFP at 0d and 7d recovery post-AraC, with our hypothesis that there is an increase in the proportion of YFP+/DCX+/Prox1+ neurons in GL-YFP mice 7d post-AraC, whereas there is no change in this proportion in Nes-YFP mice.

It will also be important to assess other effectors of neurogenesis in GL-YFP and Nes-YFP mice. For example, we have already prepared tissue to assess whether running can drive proliferation of YFP+ cells in GL-YFP and Nes-YFP mice. While we have previously presented data that running increases proliferation of YFP+ cells in Nes-YFP mice at 30d post-TAM (**Figure 4.5**)(Lagace et al., 2007b), these results can be interpreted as a direct effect of running on the YFP+ early progenitors. To directly assess how YFP+ RCs contribute to running-mediated proliferation, Nes-YFP or GL-YFP mice had unlimited access to open or locked running wheels starting ~120d post-TAM, a time point when there are almost no YFP+ progenitor cells in Nes-YFP mice. After 2 or 4 weeks of running, mice are sacrificed and tissue has been processed but not analyzed. It should be noted that in the GL-YFP mice, we cannot draw direct conclusions regarding YFP+ RCs

from these studies, in large part because the YFP+ progenitor pool persists at this time point. Therefore, we cannot conclude whether a robust increase in YFP+ cells in the GL-YFP mice is a direct contribution from YFP+ RCs. In Nes-YFP mice, however, we will be able to interpret this result, in large part because the only YFP+ cells remaining in the SGZ at ~120d are Type-1 cells and post-mitotic neurons (**Figure 5.1G**).

When Chapters Two through Five are considered together, they highlight that radial glia-like cells are likely a heterogeneous population that can differentially contribute to adult SGZ neurogenesis. These results have prompted me to re-evaluate the prevailing Kempermann model of neurogenesis (**Figure 6.1**) and to propose a new model against which future experiments can be tested (**Figure 6.2**).

Type-1 cell heterogeneity: A new view of radial glia in the SGZ?

Based on the data presented here and supported by other key publications (Singer et al., 2009; Suh et al., 2007), I offer revisions to the prevailing Kempermann model of neurogenesis (**Figure 6.1**) to explain how differences in radial cells can alternatively support neurogenesis and gliogenesis within the physiological context of the brain (**Figure 6.2**). The prevailing Kempermann model assumes that all SGZ radial cells (RCs) are functionally the same and defines Type-1 cells GFAP+/nestin+ cells with a radial morphology. However, GFAP+/nestin- RCs have been observed here (**Figure 2.2A**) and elsewhere

(Kempermann et al., 2004; Steiner et al., 2006), though these nestin-negative cells purportedly lack proliferative activity and neurogenic function (**Table 6.1**). My proposed model suggests two subtypes of radial glia-like cells (RCs) in the SGZ, which are morphologically similar but functionally and antigenically distinct: the Type-0 cell (pronounced “Type Naught”) and the Type-1 cell. I propose that the Type-0 cell is a multipotent “pre-nestin” stem cell with the capacity to produce the three neural lineages *in vivo* (**Figure 6.2A**; neurons, astrocytes, and oligodendrocytes). While under basal conditions, only astrocytes and neurons are born in the adult SGZ, the presence of oligodendrocyte precursors (Thallmair et al., 2006) and the formation of oligodendrocytes after genetic manipulations suggest that the SGZ can produce oligodendrocytes (Jessberger et al., 2008b). I propose that Type-0 cells in the adult are similar to embryonic radial glia, having a similar distinctive morphology and expressing markers like GLAST, GFAP, BLBP, Sox2, and vimentin (Hartfuss et al., 2001; Pinto and Gotz, 2007). However, my model holds that Type-0 cells do not express nestin (**Figure 6.2A**). Cells like this – RC morphology, GFAP+/nestin- – have been previously noted but grouped with Type-1 cells, leaving them largely appreciated (**Figure 2.1**; e.g. Kempermann et al., 2004; Steiner et al., 2006). As described in detail in the next section and in **Tables 6.1** and **6.2**, I contrast the prevailing Kempermann model and my revised Type-0 model and highlight how my revised Type-0 model explains the persistence of both gliogenesis and neurogenesis in the SGZ and more clearly defines the lineages of both processes within the physiological context of the adult SGZ.

My model proposes that Type-0 cells can divide asymmetrically to produce two different precursors (**Figure 6.1B, H**). I propose that once nestin expression is initiated by currently unknown factors but potentially from environmental changes like Notch and Wnt signaling, Type-0 stem cells transform into the classically described Type-1 cells in Kempermann's prevailing model (**Figure 6.1**). Correlated with the expression of nestin, Type-0 cells lose their multi-potency, maintain expression of other RC markers (GLAST, etc), and potentially become fate-restricted so that its progeny are neurons (**Figure 6.2C-G**), detailed below.

However, if nestin expression is suppressed and the Type-0 cell divides with a self-renewal capacity, the daughter cell likely corresponds to the oligodendrocyte precursor cell (OPC, **Figure 6.2H-J**). OPCs defined in part by expression of the chondroitin sulfate proteoglycan NG2 have previously been characterized in the brain and SGZ (**Figure 6.J**) but NG2+ OPCs have not been shown to produce neurons *in vivo* (Belachew et al., 2002; Thallmair et al., 2006). The preponderance of NG2+ cells become either oligodendrocytes or astrocytes *in vitro* and *in vivo* (**Figure 6.2K-L**), but can also give rise to neurons when cultured (Aguirre et al., 2004; Belachew et al., 2002; Zhu et al., 2008). Within the SVZ, GFAP+/GLAST+/NG2+ cells give rise to astrocytes and oligodendrocytes but are not neurogenic (Platel et al., 2009), though it remains to be determined if GLAST+/GFAP+ cells directly give rise to NG2+ cells and oligodendrocytes or if GLAST and NG2 coexpression is simply coincident. My Type-0 model suggests that Nes-YFP cells will not become oligodendrocytes or astrocytes within the

SGZ (**Table 6.2**). Previous results with the Nes-YFP indicate there negligible numbers of astrocytes produced within the SGZ (Lagace et al., 2007b), though there are abundant YFP+ astrocytes and oligodendrocytes found in the white matter. These data inform my Type-0 model but remain largely speculative.

Several facets of my revised model, specifically regarding gliogenesis, warrant further research, especially with transgenic tools available (**Table 6.2**). For example, do GLAST+ cells directly produce NG2+ cells, or is their co-expression correlative (Aguirre et al., 2004; Belachew et al., 2002; Zhu et al., 2008)? To what extent are YFP+ SGZ oligodendrocytes produced in the GL-YFP mouse (Platel et al., 2009)? The prevailing Kempermann model does not explain the prospective origins of adult-born astrocytes nor the potential for adult-born oligodendrocytes (**Table 6.1**).

Evidence for functionally distinct Type-0 and Type-1 cells *in vivo*

Of more specific interest to this thesis and of broader interest to the research and medical communities is clarification of the source of neurogenic precursors (**Figure 6.2A-D**). In the Nes-YFP mouse, almost all YFP+ SGZ cells at 100d post-TAM are either neuronal or Type-1 cells (**Figures 4.3; 5.1**)(Lagace et al., 2007b), suggesting that progeny of nestin-expressing cells are likely fated into the neurogenic lineage. While Sox2 expression is found basally in astrocytes, RCs (Type-0 and Type-1 cells), and progenitor cells (Type-2 progenitors, **Figures 1.3; 6.2**)(Ferri et al., 2004; Steiner et al., 2006; Suh et al., 2007), recent

work highlights that Sox2+ cells can give rise to neurons and astrocytes in the adult brain. Use of retroviral-Cre to label dividing Sox2+ via recombinase-induced YFP-labeling led to YFP+ astrocytes and neurons in the SGZ (Suh et al., 2007). As mature astrocytes are likely not transduced by retroviruses because they rarely divide under basal conditions, Gage and colleagues concluded that Sox2-expressing cells are multipotent in the SGZ (Suh et al., 2007). This is in sharp contrast to our work, where we find that nestin-expressing cells are unipotent in the SGZ (**Figure 4.3; 5.1**)(Lagace et al., 2007b). The contrasting results between the multipotency of Sox2+ cells *in vivo* and the potentially fated nestin+ cells suggests that Sox2 is likely expressed in Type-0 and Type-1 cells and provides functional evidence for differences between nestin-negative Type-0 and nestin+ Type-1 cells (**Figure 6.2**).

Additional evidence for functionally heterogeneous Type-0 and Type-1 cells comes from our experiments using AraC in Nes-YFP and GL-YFP mice. Intracerebroventricular (ICV) infusion of AraC has been used previously to ablate proliferation temporarily, such that the number of BrdU-IR progenitor cells normalizes within 7d (**Figure 5.2**)(Mak et al., 2007; Seri et al., 2004; Seri et al., 2001). We hypothesized that, if YFP+ radial glia contribute to neurogenesis, then there would be more YFP+ cells at 7d recovery after AraC. In Nes-YFP and GL-YFP mice, there was a significant decrease in the number of YFP+ cells immediately after AraC, confirming a number of YFP+ cells were progenitors when AraC was infused. Surprisingly, though, at 7d recovery when proliferation

returned to baseline after AraC infusion, there was no recovery of YFP+ cells in Nes-YFP mice, though the number of YFP+ cells in GL-YFP mice was not significantly different from saline-treated controls (**Figure 5.2**). These results suggest that there are functional differences between nestin+ RCs and GLAST+ RCs (**Figure 6.2**).

Other recent work highlights subpopulations of RCs that can give rise to fated neuronal precursors. Seki and colleagues recently described subpopulations of GFAP+ RCs that are GFAP+/Hu- and GFAP+/Hu+ (Liu et al., 2009; Seki et al., 2007). These observations are notable because Hu is marker for neurons, and the authors conclude that the Hu+ subset of GFAP+ radial cells are likely neuronally-fated (Liu et al., 2009; Seki et al., 2007). These observations of fated RCs fit with my revised model, such that GFAP+/Hu- RCs are likely the Type-0 cells, whereas GFAP+/Hu+ RCs may fit into the Type-1 population (**Figure 6.2**). If GFAP+/Hu+ cells are also nestin+, then these results would fit well with our observation that YFP+ cells in Nes-YFP mice become neurons or remain Type-1 (Lagace et al., 2007b). Additional experiments are needed to confirm these observations. First, are all YFP+ Type-1 cells in the Nes-YFP mouse also Hu+? We might predict that a subset of YFP+ RCs in the GL-YFP mouse would be Hu+ (corresponding to Type-1 cells) whereas other YFP+ Type-0 cells would be Hu-negative. It will be interesting to test if Hu expression in nestin+ Type-1 cells is, in

fact, linked (causatively or correlatively) with the birth of fated Type-2 neuronal precursor cells.

Further correlative evidence for Type-0 and Type-1 functional heterogeneity comes from the YFP progenitor pool in Nes-YFP and GL-YFP mice. My revised model predicts YFP+ progenitor cells in the Nes-YFP would deplete over time because YFP+ Type-1 cells are not the constitutive source of progenitors (**Figure 6.2; Table 6.2**). On the other hand, my proposed model predicts that in GL-YFP mice, where YFP+/GLAST+ Type-0 cells are the putative source for progenitors, the pool of YFP+ progenitors would be maintained. Indeed, phenotypic analysis of the progenitor pool in Nes-YFP versus GL-YFP mice suggests that at 100d post-TAM, there are no longer YFP+ progenitors in Nes-YFP mice but YFP+ progenitor cells persist in GL-YFP (**Figure 5.1**). It is important to note, while analysis in Nes-YFP mice is thorough and complete, these data on GL-YFP mice are pilot data only, based on n=2 mice per time point. More thorough analysis is ongoing and awaits further confirmation.

Changes in RC nestin expression over time

Moreover, one of the critical assertions of my revised model (**Figure 6.2**) that distinguishes it from the prevailing Kempermann model (**Figure 6.1**) is that nestin+ Type-1 cells do not sustain multiple rounds of cell division (**Tables 6.1; 6.2**). In my model, Type-1 cells are minimally proliferative, likely dividing around

the same time as nestin becomes expressed but likely only one time, based on the inability of YFP+ Type-1 cells in the Nes-YFP model to recover after AraC (**Figures 5.2; 5.3**). In this way, Type-1 cells can produce progenitors but are not the constitutive source. In our Nes-YFP mouse, recombination is induced in Type-1 cells and early progenitors. Over time, the YFP+ progenitors cells divide symmetrically but only a limited number of times, as has been previously described (**Figure 6.1; 6.2D**)(reviewed in Kempermann et al., 2004; Ming and Song, 2005). Therefore, my model predicts that YFP+ Type-2 cells eventually deplete in Nes-YFP mice (**Table 6.2**). New progenitor cells are born but are hypothesized to come from a YFP-negative Type-0 cell which potentially acquires nestin-expression long after TAM-induced recombination (**Figure 6.2B-D**). My model predicts that the proportion of nestin-expressing Type-1 cells labeled with YFP at early times post-TAM would be high but that the proportion of YFP+/nestin+ Type-1 cells would decrease over time as more RCs acquire nestin expression. Strikingly, this is observed in the Nes-YFP mouse, where there is a statistically significant difference in the proportion of RCs that are YFP+ over times post-TAM by One-Way ANOVA, $F_{(5,20)}=17.77$, $p<0.001$. At 12d post-TAM, nearly 97% of GFAP+/Sox2+ cells are also YFP+ (Lagace et al., 2007b), whereas at 180d post-TAM, only 40% of Type-1 cells are YFP+ (**Figure 6.3A**). In contrast, when the total number of YFP+ radial cells is quantified in Nes-YFP mice over time, there was no statistical difference in the number of YFP+ RCs over time by One-Way ANOVA ($F_{(5,17)}=1.743$, $p=0.1788$), confirming that the number of YFP+ Type-1 cells is constant over time (~1500 cells between 12d

and 180d post-TAM, **Figure 6.3B**). One interpretation of these results is that, over time nestin+ RCs are present that were either nestin-negative RCs at the time of TAM-induced recombination, or that new nestin+ RCs are added to the pool over time.

We have performed an experiment to directly assess whether new nestin+ cells are added to the SGZ over time by administering multiple rounds of TAM to Nes-YFP mice. Our prediction was that the number of YFP+ RCs would additively increase if new nestin+ RCs are added over time. Indeed, when we treated Nes-YFP mice with TAM at 6 wks, 14 wks, or both 6 & 14 wks of age (**Figure 6.4A**), we saw a synergistic (super additive) increase in the total number of YFP+ cells (**Figure 6.4B**) whereas there was only an additive increase in the number of YFP+ Type-1 cells (**Figure 6.4C**). Interpretation of these results is complicated, which has precluded us from discussing them before. We would expect that if there are more nestin+ Type-1 cells over time, then there would be more YFP+ Type-1 cells in mice administered TAM 14 weeks compared to mice given TAM at 6 wks. However, we saw fewer YFP+ Type-1 cells in mice labeled at 14 wks compared to 6 weeks. Additionally, we would have expected comparable numbers of YFP+ Type-1 cells between mice administered TAM at 14 wks and at 6 & 14 wks, under the assumption that there would have been equal numbers of nestin+ Type-1 cells to undergo recombination. Again, we saw significantly fewer Type-1 cells labeled in mice administered TAM at 14 compared to mice receiving TAM at both time points. These disparities might be best explained by

decreasing recombination efficacy with age, such that fewer Type-1 cells are recombined in older mice. Further, the results are complicated because TAM stimulates proliferation, leading to more BrdU-IR cells in mice administered TAM at 14 wks and at 6 & 14wks, compared to mice given TAM only at 6 wks. To avoid TAM-mediated increases in proliferation, we also sacrificed mice at 30d post-TAM (data not shown). Because there are still a significant proportion of YFP+ progenitor cells at 60d post-TAM (**Figure 5.2**), our results are confounded because the proliferation of YFP+ progenitors is likely stimulated by TAM in the same way that proliferating BrdU-IR cells are stimulated. To overcome these caveats, we repeated the experiment with 100d between the first round of TAM administration and the second (instead of 60d), though tissue has not been processed or analyzed.

Additional correlative support for my revised Type-0 model

My revised Type-0 model set forth also ties other correlative data regarding effectors of neurogenesis and RCs (Type-0 and Type-1)(**Figure 6.2**). Because different sets of markers (Sox2+/GFAP+ RG, Sox2+ RG, or nestin-GFP+ RG) have been used to identify RCs under the assumption that this population is homogeneous, the effects of running on RC proliferation and total number have been ostensibly in conflict. Even though the number of RCs is not changed by running (**Figure 2.1, 2.4**; Naylor et al., 2008; Suh et al., 2007), running results in an increase in the number of dividing Sox2+/GFAP+ RCs (Naylor et al., 2008) or total Sox2+ RCs (Suh et al., 2007), which reflect both Type-0 and Type-1 cell

pools in my revised Type-0 model (**Figure 6.2**). However, reports that specifically examine the proliferation of nestin+ Type-1 cells show no change in Type-1 proliferation (Kronenberg et al., 2003; Steiner et al., 2008). If nestin-negative Type-0 cells are distinguished from nestin+ Type-1 in these reports, then one interpretation is that Type-1 cells do not proliferate more after running, whereas running stimulates Type-0 cell division. These data are consistent with my revised model because Type-1 cells do not show extensive proliferation (**Figure 6.2C**) whereas Type-0 cells putatively can undergo repeated cell divisions (**Figure 6.2H**). Correlatively, gliogenesis is also increased by running (Steiner et al., 2008), which could explain how Type-0 cells undergo proliferation without a resultant increase in number of nestin+ Type-1 cells (**Figure 2.1, 2.4**). Alternatively, while the proportion of Type-0 cell undergoing division might be significantly increased by running compared to non-running Type-0 proliferation, a net change in the total number of Type-0 or Type-1 might not be detectable quantitatively, in part because numerically so few Type-0 cells are dividing at any time and because there are so many Type-1 cells.

My revised model also accounts for uncharacterized observations that have not been described or quantified. For example, I have observed a many isolated YFP+ Type-1 cells in the Nes-YFP that are not positioned close to other YFP+ cells in the SGZ. This could suggest that at the time of TAM-induced recombination, these cells had already undergone their limited proliferation and therefore no labeled progenitors were produced. Similarly, a number of YFP+

neurons are observed in locations that are not close to YFP+ Type-1 cells; this may be explained by YFP+ progenitor cells labeled at the time of TAM but the Type-1 parent was not recombined.

Predictions from my revised Type-0 model

One important characteristic of any model is that predictions can be made which must be further tested (**Tables 6.1; 6.2**). First, my Type-0 model asserts that the SGZ RC pool is heterogeneous. While functional data in Chapter Five and observational data in Chapter Two support a heterogeneous RC pool in the SGZ, another interpretation of these data is that the effects are transgenic mouse-specific. Would similar observations be made in all inducible nestin-Cre mice, such as the depletion of the labeled progenitor pool and lack of labeled progenitors following recovery post-AraC? Alternatively, if we waited longer times post-AraC in Nes-YFP mice, would we see any recovery of YFP+ cells? If we observed longer times post-TAM in GL-YFP mice, would we observe similar depletion of YFP+ progenitor cells? Questions like these require more experimental data.

My Type-0 model makes other predictions that are directly testable within the systems at hand. First, we might predict that there is a greater proportion of GLAST+ RCs early in a mouse's life that are nestin-negative (corresponding to more Type-0 cells relative to Type-1 cells), and that with increased aging, more GLAST+ RCs cells are nestin+ Type-1 cells and fewer are Type-0. The gradual

conversion of Type-0 stem cells into Type-1 cells could provide an underlying mechanism for the age-related decline in progenitor cell proliferation, consistent with some observations in other laboratories (J. Encinas and G. Enikopolov, personal communication). My revised Type-0 model would also predict that the number of nestin+ Type-1 cells would increase with age, assuming there are no other physiological effectors of Type-1 cells independent of neurogenesis.

A final prediction from my model is that ablating nestin+ cells would result in a temporary reduction in neurogenesis with gradual recovery over time, whereas ablating GLAST+ cells would result in permanent elimination of neurogenesis. Data from nestin-tk mice support the gradual and partial recovery of neurogenesis (Singer et al., 2009), despite the persistence of Type-1 cells in the SGZ. Similarly, ablation of GLAST+ Type-0 cells in GFAP-tk results in a long-term loss of neurogenesis (Garcia et al., 2004). However, direct comparison of tk-mice lines following the same experimental procedures and time course is warranted and informative. However, as with all transgenic experiments, there are caveats and interpreting result might be challenging, specifically because only dividing cells that are tk-expressing in the presence of gancyclovir (GCV) would be ablated. If GLAST+ Type-0 cells are quiescent throughout GCV administration, this potential source of neurogenesis would persist and could facilitate recovery long-term, leading to the premature assertion that the Type-0/Type-1 model of SGZ stem cells is wrong. Similarly, if nestin+ Type-1 cells are quiescent but maintain proliferative capacity, then gradual recovery of

neurogenesis in nestin-tk mice might result in premature acceptance of this model. Until it can be conclusively demonstrated that nestin+ Type-1 cells do not undergo repeated cell division, it is difficult to interpret recovery in nestin-tk mice.

Given the predictive value in my model of Type-0 and Type-1 cells (**Figure 6.2**), there are a number of questions that must be addressed beyond the scope of this research. For example, what makes Type-0 and Type-1 cells functionally different beyond nestin-expression? Why do nestin+ early progenitors divide frequently and symmetrically, whereas expression of nestin in Type-1 cells is correlated with limited cell division? Further, what is the functional role of Type-1 radial cells? If these are demonstrably not the source of neurogenesis as my model predicts and supported by other observations (Singer et al., 2009; Suh et al., 2007), then why do these cells persist in adulthood? Clearly, many more questions emerge than have been answered as a result of exploring the functional disconnect between nestin-expressing Type-1 cells and adult hippocampal neurogenesis.

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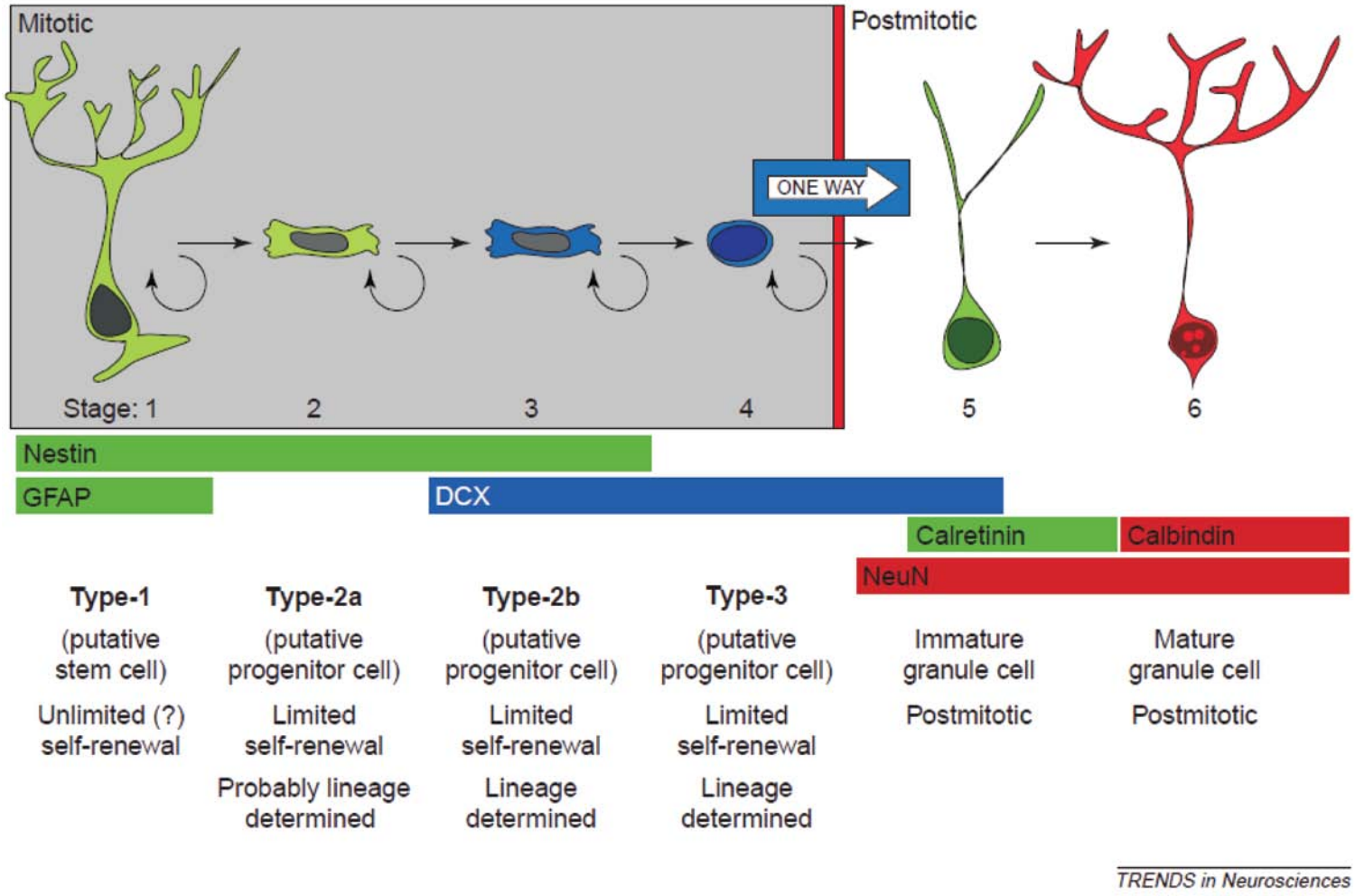
Mitotic
Postmitotic
ONE WAY
Stage: 1
2
3
4
5
6
Nestin
GFAP
DCX
Calretinin
Calbindin
NeuN
Type-1
(putative stem cell)
Unlimited (?) self-renewal
Type-2a
(putative progenitor cell)
Limited self-renewal
Probably lineage determined
Type-2b
(putative progenitor cell)
Limited self-renewal
Lineage determined
Type-3
(putative progenitor cell)
Limited self-renewal
Lineage determined
Immature granule cell
Postmitotic
Mature granule cell
Postmitotic
TRENDS in Neurosciences

Figure 6.1. Prevailing Kempermann model of SGZ neurogenesis (Kempermann et al., 2004). The first model to delineate the putative stages or “milestones” of adult neurogenesis in the SGZ was established by Kempermann and colleagues (Kempermann et al., 2004). While several populations of proliferating cells can be detected in the adult SGZ, the proposed lineage of proliferating cell types in the SGZ indicates that any maturing and surviving cells become neurons. Proliferating cell types in this model (grey box) are based on morphology and staining of makers including GFP (green bar) and doublecortin (DCX, blue bar) in the nestin-GFP transgenic mouse made by Yamaguchi (Yamaguchi et al., 2000). While the precise relationships among cells are not clear, cells are thought to form a lineage, where Type-1 cells (nestin-GFP+/GFAP+/DCX-) divide infrequently to replenish the rapidly dividing Type-2a population (GFP+/GFAP-/DCX-). Type-2a cells in turn divide to produce Type-2b cells (GFP+/GFAP-/DCX+), and Type-2b cells divide to produce Type-3 cells (GFP-/GFAP-/DCX+), eventually yielding postmitotic immature neurons.

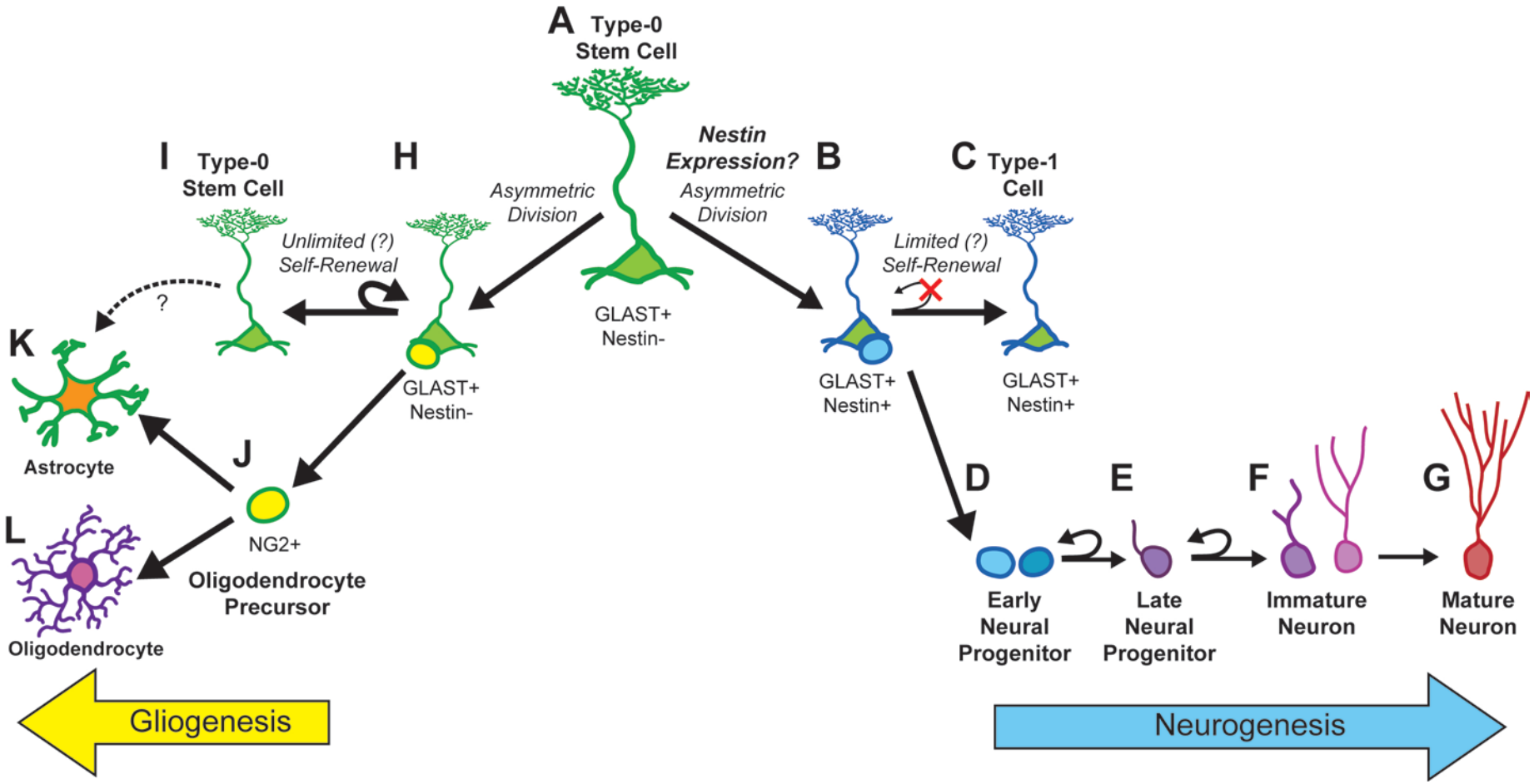
A Type-0 Stem Cell
Nestin Expression?
Asymmetric Division
Asymmetric Division
GLAST+ Nestin-
I Type-0 Stem Cell
Unlimited (?) Self-Renewal
?
H
GLAST+ Nestin-
B
GLAST+ Nestin+
C Type-1 Cell
Limited (?) Self-Renewal
GLAST+ Nestin+
K
Astrocyte
J
NG2+
L
Oligodendrocyte Precursor
Oligodendrocyte
D
Early Neural Progenitor
E
Late Neural Progenitor
F
Immature Neuron
G
Mature Neuron
Gliogenesis
Neurogenesis

Figure 6.2. My proposed Type-0 model of SGZ neurogenesis. My model builds on the prevailing model established by Kempermann et al. (2004), depicted in Figure 6.1. To avoid confusion, I use nomenclature consistent with the Kempermann model. My model asserts that the population of radial glial-like cells within the adult dentate gyrus is functionally and antigenically heterogenous. I propose that nestin-negative stem cell (A) can undergo two different types of asymmetric cell division depending on whether nestin is expressed, to produce precursor cells that eventually result in either gliogenesis (H-L, yellow arrow) or neurogenesis (B-G, blue arrow). (A) A nestin-negative stem cell is termed the Type-0 cell (pronounced “Type naught”) and is morphologically indistinguishable from the nestin-expressing Type-1 cell in the Kempermann model. Type-0 cells express GLAST and likely other markers of radial glia cells, including Sox2, vimentin, BLBP, and GFAP, but are negative for nestin. In my proposed model above, GLAST+ cells are filled with light green; nestin+ cells are outlined in dark blue; GFAP+ cells are outlined in dark green. (B) When a Type-0 divides when nestin is expressed (depicted as cells with dark blue outlines), I propose that the Type-0 cell likely undergoes asymmetric cell division to produce a nestin+ Type-1 cell and a nestin+ early progenitor. (C, D) The Type-1 cell undergoes asymmetrical cell division likely only once to produce a neuronally-restricted early progenitor cell (depicted in the schematic by light blue filling). Following a single cell division, the Type-1 cell likely becomes post-mitotic or may be quiescent. (D-G) Early neural progenitors cells (blue, comparable to the Type-2a and -2b cells in the Kempermann model) can repeatedly divide symmetrically to expand the progenitor pool and (E) produce late neural progenitors (purple, equivalent to the Type-3 cell in the Kempermann model) that can still divide and give rise to (F)

post-mitotic immature neurons (pink), some of which differentiate and incorporate into hippocampal circuitry as mature neurons (red). The Type-1 cells (C) is comparable to the Kempermann Type-1 cell, except that in my proposed model, Type-1 cells do not undergo repeated cell divisions and are not the main source of early progenitor cells. The stages of neurogenesis in my proposed model (D-G) are virtually identical to the Kempermann model's milestones of neurogenesis (detailed above in Figure 6.1). (H-L) An alternative process that Type-0 cells can drive is gliogenesis. (H-J) When Type-0 cells divide asymmetrically with self-renewal, I propose that (I) the Type-0 cell is maintained and (J) daughter is an oligodendrocyte precursors (filled with yellow), which expresses the proteoglycan NG2. OPCs have previously been described to be bi-potent, capable of producing (K) astrocytes (filled orange) or (L) oligodendrocytes (purple). Type-0 cells may be able to directly produce astrocytes (dashed arrow). The prevailing Kempermann model does not address the origins of astrocytes and oligodendrocytes in the SGZ.

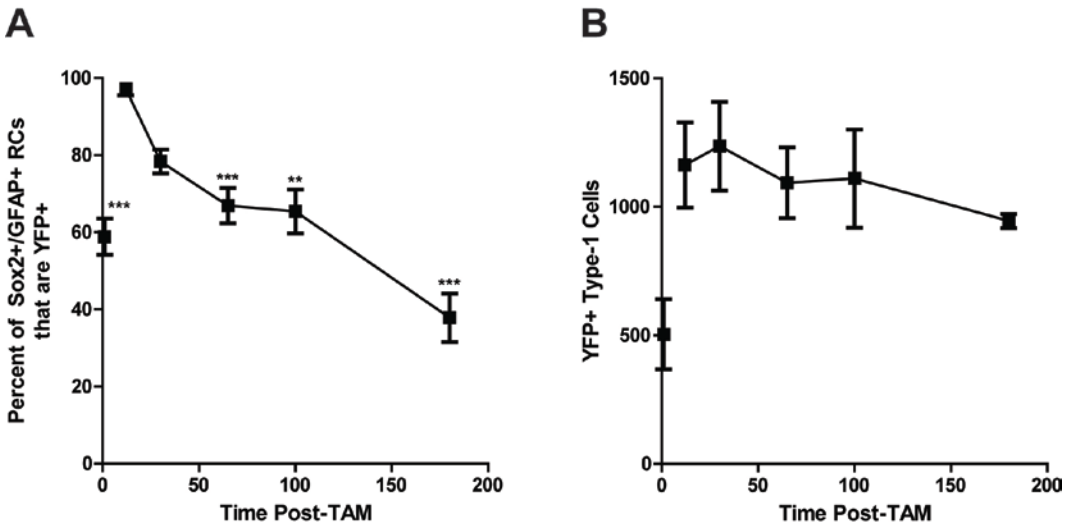

Figure 6.3. The proportion of YFP+ RCs decreases over time in Nes-YFP but the number of YFP+ RCs remain constant. (A) When the proportion of Sox2+/GFAP+ cells (Type-0 or Type-1) that were YFP+ was assessed at various times post-TAM, the relative proportion declines dramatically, suggesting that there are proportionally fewer YFP+ RCs compared to YFP-negative RCs over time. (B) When the number of YFP+ RCs was quantified based on RC morphology, the absolute number remained constant from 12d to 180d post-TAM. Data represent the mean ± SEM. In A, n=3-6 mice per time point, 50-100 RCs per mouse; in B, n=3-6 mice per time point across the anterior-posterior extent of the hippocampus in 1:9 series. **, $p<0.01$; ***, $p<0.001$, each compared to 12d values by Bonferroni's multiple comparison post-hoc test.

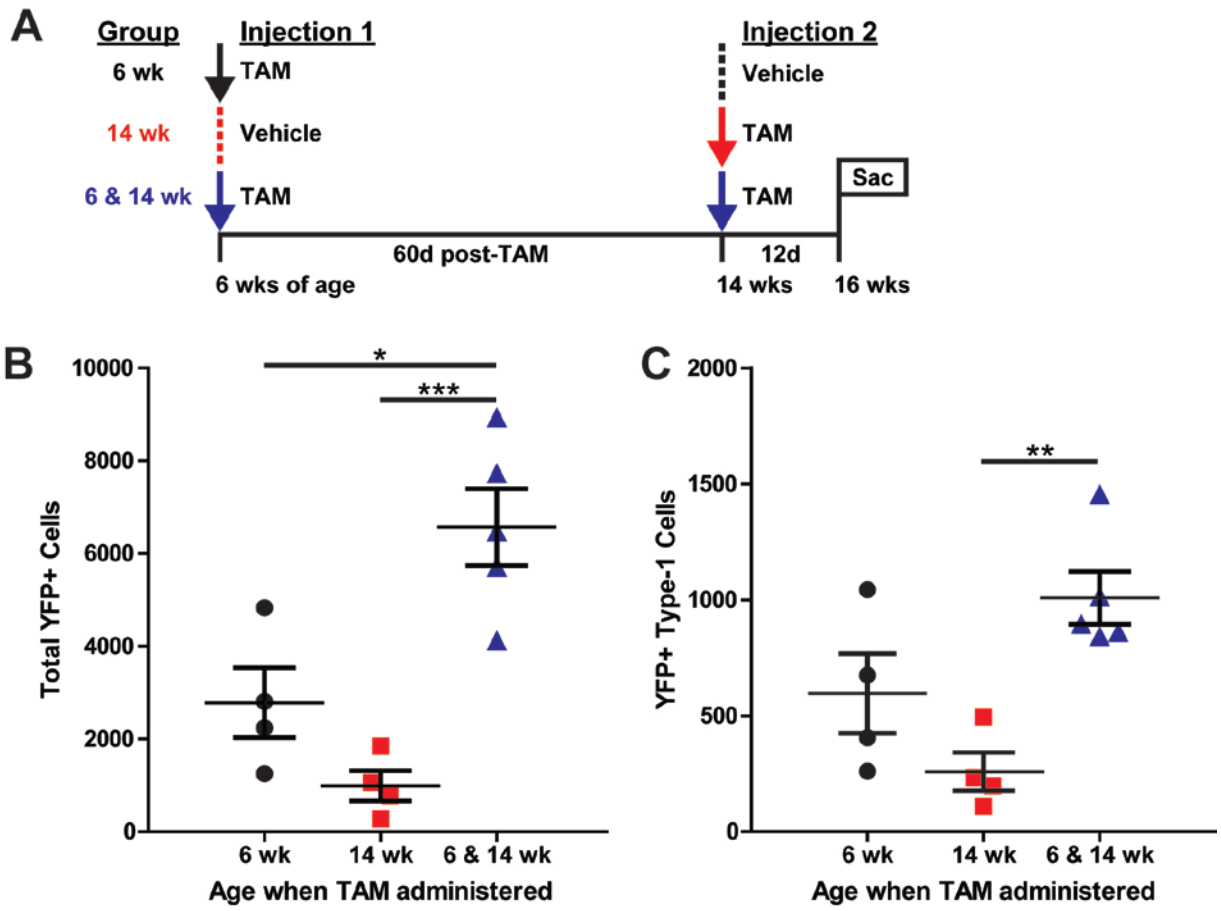

Figure 6.4. Multiple rounds of TAM administration in Nes-YFP mice result in increased number of YFP+ cells and YFP+ Type-1 cells. (A) Schematic of experimental procedure in which Nes-YFP were divided into 3 groups based on when they were administered TAM: either at 6 weeks (black arrow), 14 weeks (red arrow), or both 6 & 14 weeks (blue arrows). To minimize variability associated with injection stress and handling, mice that did not receive TAM were administered vehicle (dashed lines). At 12d post-2nd TAM, all mice were sacrificed. (B) The total number of YFP+ cells increases more than additively in mice administered TAM at both 6 & 14 wks, compared to either time alone. (C) The number of YFP+ Type-1 cells, identified by morphology, was additively

increased in mice receiving TAM at both 6 & 14 wks. Bars represent the mean±SEM of individual replicates. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$, compared to mice administered TAM at 6 & 14 wks.

The prevailing Kempermann model explains:
how early progenitors mature progressively into mature neurons.
why most adult-born progenitor cells become neurons.
why so few Type-1 cells are labeled with proliferation markers like BrdU and endogenous Ki67.
the progressive electrophysiological maturation of adult-born progenitor cells and neurons.
why progenitor cells and neurogenesis recover after ablating proliferation.

The prevailing Kempermann model fails to explain:
differences between GFAP+/nestin+ Type-1 cells and GFAP+/nestin- RCs. Several reports (including the original description of the Kempermann model) note the presence of GFAP+ radial cells which are not nestin-GFP positive, though the model fails to describe what these cells are or their contribution to neurogenesis.
what Type-1 cells functionally do.
why nestin+ Type-2 cells divide frequently and symmetrically, whereas nestin+ Type-1 undergo very limited division.
why YFP+ Type-1 cells in Nes-YFP mice are located alone and distant from YFP+ neurons.
why adult-generated YFP+ neurons are not always in close physical proximity to YFP+ Type-1 cells in Nes-YFP mice.
why some GFAP+ RCs are Hu+; why GFAP+/Hu+ cells have some proliferative capacity; and why GFAP+/Hu+ RCs may be neuronally-restricted.
where adult-born astrocyte and oligodendrocyte precursors originate in the adult SGZ.
how effectors of neurogenesis relate to effectors of gliogenesis.
why there is a gradual decrease in the proportion of YFP+ Type-1 cells in Nes-YFP, whereas the number of YFP+ Type-1 cells remains constant.
decreases in neurogenesis with age.

The prevailing Kempermann model predicts that:
RCs are a homogenous cell pool.
transgenic mouse lines whose driver expression only overlaps in the Type-1 cell pool would have comparable (or equivalent) long-lasting alterations in SGZ neurogenesis.
progenitor cell fate is likely correlated with onset of DCX expression (Type-2b cell stage).
ablating nestin+, GFAP+, or GLAST+ cells results in permanent elimination of neurogenesis.

Table 6.1. This table details the things that prevailing Kempermann model (Kempermann et al., 2004) explains or fails to explain, and the predictions that emerge from this model. The prevailing model is delineated in Figure 6.1, above. See text for more thorough explanation of each point.

My Type-0 model explains:
why almost all YFP+ cells at late times post-TAM in Nes-YFP mice are neurons or RCs.
why there is no recovery of YFP+ cells from YFP+ Type-1 cells post-AraC in Nes-YFP mice, whereas there is recovery in GL-YFP mice.
why some GFAP+ RCs are Hu+; why GFAP+/Hu+ cells have some proliferative capacity; and why GFAP+/Hu+ RCs may be neuronally-restricted.
why the YFP+ progenitor pool eventually depletes in Nes-YFP mice.
why solitary Type-1 cells are sometimes found in Nes-YFP mice, or why Type-1 cells are sometimes found in close proximity to adult-generated neurons.
why new YFP+ neurons are not always in close physical association with Type-1 cells.
where oligodendrocyte precursor cells originate.
why neurogenesis decreases over time through the gradual transition of GLAST+/nestin- cells to GLAST+/nestin+ cells.
why there is a gradual decrease in the proportion of YFP+ Type-1 cells in Nes-YFP, whereas the number of YFP+ Type-1 cells remains constant.

My Type-0 model fails to explain:
what induces nestin-expression in "Type-0" cells.
what is the function of Type-1 cells.
why nestin+ progenitors divide frequently and symmetrically, whereas nestin+ Type-1 undergo very limited division.

My Type-0 model predicts that:
RCs are a heterogeneous cell pool.
early in life, there is a greater proportion of GLAST+/nestin- RCs (Type-0 cells), whereas with aging, there are more GLAST+/nestin+ cells (Type-1 cells).
the number of nestin+ Type-1 cells would increase with age, unless there are age-related influences on nestin+ Type-1 cells that have not been defined.
in Nes-YFP mice, the proportion of YFP+ RCs (Type-1 cells) decreases over time, whereas in GL-YFP mice, the proportion of recombined YFP+ RCs (Type-0) remains constant.
ablating nestin+ cells results in a temporary (not permanent) reduction in neurogenesis, whereas ablating GLAST+ cells results in permanent elimination of neurogenesis.

Table 6.2. This table details what my Type-0 model explains or fails to explain, and what predictions emerge from this model. My Type-0 model is delineated in Figure 6.2, above. See text for more thorough explanation of each point.