THE ROLE OF NOTCH1 IN ADULT HIPPOCAMPAL NEUROGENESIS AND FUNCTION

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DEDICATION

I dedicate this thesis to my family for their patience with my bookish ways; to my husband, Scott, for his open ears; to my wonderful summer volunteers, especially Allison, for all their helping hands; and to the many friends I have made along the way. I thank everyone for their encouragement and support.

THE ROLE OF NOTCH1 IN ADULT HIPPOCAMPAL NEUROGENESIS AND FUNCTION

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Abstract

Neurogenesis occurs throughout life in the hippocampal subgranular zone (SGZ) and is potently stimulated by exercise, but the underlying mechanisms are still poorly defined. Notch1 is a master regulator of developmental neurogenesis, yet its role in adult hippocampal neurogenesis is unclear. To test the hypothesis that cell-intrinsic Notch1 is critical to both basal and exercise-induced SGZ neurogenesis, we generated Nestin-creER^{T2}/R26R-YFP/Notch1$^{loxP/loxP}$ (Notch1 iKO) mice to inducibly ablate Notch1 in Nestin-expressing stem and progenitor SGZ cells. The total number of YFP+ SGZ cells increased over time in wild type

littermates, but not in Notch1 iKO mice. Morphological and phenotypic analyses revealed that fewer YFP+ DG neurons were generated over time in Notch1 iKO mice due to smaller pools of YFP+ stem-like and progenitor cells. Likewise, neural progenitors isolated from Notch1 iKO mice were incapable of forming new neurospheres with extended passaging. While non-running Notch1 iKO mice had fewer YFP+ SGZ cells relative to wild type littermates, Notch1 iKO mice given 30 days access to a running wheel had equal number of YFP+ SGZ cells relative to controls, suggesting that running rescued total YFP+ SGZ cell number independent of Notch1. However, running did not rescue YFP+ stem-like cell number in Notch1 iKO mice, suggesting that the putative stem-like SGZ cells make little contribution to adult hippocampal neurogenesis in these conditions. From these data, we conclude that Notch1 in Nestin+ stem and progenitor cells is critical to maintain basal adult hippocampal neurogenesis, but is not critical for exercise-induced neurogenesis. Neurogenesis has also been implicating in depression and behavioral response to antidepressants. To determine if reduced neurogenesis contributed to depression- or anxiety-related behavior, we assessed several measures of depression and anxiety in Notch1 iKO mice. We found that Notch1 iKO mice did not differ from WT mice in their behavior, suggesting that reduced neurogenesis is not associated with mood disturbances.

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LIST OF ABBREVIATIONS

AC3 – activated caspase-3

BDNF – brain-derived neurotrophic factor

BLBP – brain lipid binding protein

BrdU – bromodeoxyuridine

CREB – cyclicAMP response element biding protein

DCX – doublecortin

DG – dentate gyrus

EGF – epidermal growth factor

EPM – elevated plus maze

FGF – fibroblast growth factor

FST – forced swim test

GFAP – glial fibrilary acidic protein

GFP – green fluorescent protein

IHC – immunohistochemistry

i.p. – intraperitoneal

iNICD – inducible NICD overexpressing mouse

L/D – light/dark test

LH – learned helplessness

Nestin-GFP – nestin driven GFP transgenic mouse

NeuN – neuronal nuclei

NICD – Notch1 intracellular domain

Notch1 iKO – inducible Notch1 knockout mouse

NSC – Neural stem-like cell

OF – open field

PBS – phosphate buffered saline

SEM – standard error of the mean

SGZ – subgranular zone

SP – sucrose preference

SVZ – subventricular zone

TAM – tamoxifen

TrkB – tropomyosin related kinase B

WT – wild type

VEGF – vascular endothelial growth factor

YFP – yellow fluorescent protein

CHAPTER ONE

Introduction

Portions from invited review: Johnson MA, **Ables JL**, Eisch AJ. 2009. Cell-intrinsic signals that regulate adult neurogenesis *in vivo*: insights from inducible approaches. BMB Reports 2009 May 31;42(5):245-59.

Introduction

The birth of new neurons in the adult brain is a remarkable discovery that has gained increasing attention over the last forty years (Altman and Das, 1966; Kempermann et al., 2004). Interest has intensified with the discovery of neurogenesis in the adult human brain (Eriksson et al., 1998; Curtis et al., 2007; Roybon et al., 2009), by findings that link adult neurogenesis to normal brain function (Dupret et al., 2007; Imayoshi et al., 2008; Garthe et al., 2009) and disease (Eisch, 2002; Eisch et al., 2008; Kempermann et al., 2008; Vandenbosch et al., 2009), and by the tantalizing possibility of using adult neural stem cells in treatment of neurodegenerative and psychiatric disorders (Ormerod et al., 2008). Such intense research has revealed that adult neurogenesis occurs primarily in two brain regions, the subgranular zone (SGZ; Figure 1.1) and the subventricular zone (SVZ) (Ming and Song, 2005; Duan et al., 2008). This introduction will focus on SGZ neurogenesis. First we will discuss the differences between developmental and adult neurogenesis, followed by a discussion of the stages of

adult SGZ neurogenesis and what we know about the molecular basis of their regulation. After this, we will briefly discuss the dynamic regulation of neurogenesis by experience and environment. Then we will discuss the putative function of adult hippocampal neurogenesis and finally, we will end with a discussion of a specific gene involved in regulating neurogenesis, Notch1.

Developmental versus adult neurogenesis

Developmental neurogenesis differs from adult neurogenesis in several critical ways. Importantly, adult and developmental neurogenesis differ in the way in which neurogenesis proceeds. During development, neurogenesis is highly orchestrated, and neurons are generated in waves before sequential generation of glia (Kempermann et al., 2004). Large numbers of neural progenitors are generated and mature into neurons in concert. In contrast, neurogenesis in the adult is an individual process, with cells in various stages of becoming neurons and glia overlapping in both time and space (Johnson et al., 2009). Furthermore, neurogenesis in the adult brain proceeds in an environment that has specialized to support the generation of new neurons, or a neurogenic niche, whereas neurogenesis is globally promoted in the developing brain (Alvarez-Buylla and Lim, 2004). We have a firm understanding of the molecular mechanisms that regulate developmental neurogenesis from the study of numerous knock out mice. However, most of these KO mice are not viable, so it is unclear if the same genes that are critical for developmental neurogenesis play a similar role in the adult given the differences in timing and context.

Stages of adult hippocampal neurogenesis

Cells generated in the SGZ of the adult hippocampus predominantly become glutamatergic dentate gyrus (DG) granule cells (Duan et al., 2008). While much less prevalent, new glia are also generated in the adult hippocampus (Gage, 2000; Alvarez-Buylla et al., 2001; Ma et al., 2009). Life-long, new neurons are added to the DG via a remarkable "process" where the progeny of stem-like cells move through stages of proliferation, fate choice, migration and maturation (Kempermann et al., 2004; Lledo et al., 2006; Eisch et al., 2008). These stages are depicted in Figures 1.2 and 1.3, and are briefly described below.

Stem cells: Type-1 cells

Although the identity of the definitive stem cell in the SGZ remains unknown, current evidence suggests that Type-1 cells are the putative stem cells in the SGZ (Kempermann et al., 2004; Ming and Song, 2005; Duan et al., 2008; Ma et al., 2009). However, it has been difficult to demonstrate stem-cell properties of Type-1 cells *in vivo*. In theory, stem cells should be able to both self-renew and give rise to all cell types in the adult brain: neurons, astrocytes and oligodendrocytes. Evidence for these characteristics in Type-1 cells *in vivo* has hinged on the observation that Type-1 cells divide to reconstitute the SGZ after injury (Miles and Kernie, 2006; Yu et al., 2008) or pharmacologic ablation (Seri et al., 2001).

Despite convincing evidence of stem cell properties *in vivo* under basal conditions, Type-1 cells were identified as the putative stem cell in the SGZ by utilizing Nestin-GFP reporter mice (Yamaguchi et al., 2000; Kempermann et al., 2003). In these mice, Type-1 cells are GFP+ radial glial-like cells with a triangular cell body in the SGZ and a single process that extends through the granule cell layer, terminating within the inner molecular layer (Kempermann et al., 2004). Type-1 cells express markers of both stem cells (Nestin, Musashi, Sox2) and glia (glial fibrilary acidic protein [GFAP], high affinity astrocytic glutamate transporter [GLAST], brain lipid binding protein [BLBP, also known as FABP7]) (Kempermann et al., 2004; Duan et al., 2008). It is possible that Type-1 cells, like radial glial cells in the retina and adult stem cells in other tissues, are heterogeneous, expressing different combinations of markers and with differing capacities to give rise to multiple neural lineages (James et al., 2004; Suh et al., 2007; Graf and Stadtfeld, 2008; Ma et al., 2009). For example, not all GFAP+ radial glia in the SGZ of adult Nestin-GFP mice are GFP+ (Kempermann et al., 2004; Steiner et al., 2006) and Type-1 cells can be subdivided into several populations based on the presence or absence of neuronal markers such as Hu or Ascl1 (Seki et al., 2007). Furthermore, Nestin-expressing Type-1 cells generate neurons almost exclusively (Lagace et al., 2007), while GFAP-expressing Type-1 cells can generate neurons and glia (Ninkovic et al., 2007; Ma et al., 2009), suggesting that radial glial-like cells in the adult SGZ are not all the same. The heterogenity of Type-1 cells is the subject of intense research, including within the Eisch laboratory. As would be expected of an adult stem cell

(Fuchs, 2009), Type-1 cells are thought to be relatively quiescent and divide infrequently (Kempermann et al., 2004; Basak and Taylor, 2009; Ma et al., 2009). Radial glial-like cells in the SVZ divide approximately once every 15 days in the adult SVZ (Morshead et al., 1994). While Type-1 cells can be seen dividing after ablation of proliferation and after injury (Seri et al., 2001; Miles and Kernie, 2006, 2008), it is unknown how often Type-1 cells in the SGZ divide in the adult brain.

Transit amplifying progenitors: Type-2 cells

Despite a paucity of evidence on the dynamics of Type-1 cell division, current research suggests that Type-1 cells divide asymmetrically to self-renew and give rise to lineage-restricted Type-2 cells (reviewed in Kempermann et al., 2004; Ming and Song, 2005; Duan et al., 2008; Ma et al., 2009). Type-2 cells, also called transit-amplifying progenitor cells, are rapidly dividing progenitors with an average cell cycle time of about 12-16 hours (Nowakowski et al., 1989; Mandyam et al., 2004; Mandyam et al., 2007). Rapid proliferation of Type-2 cells allows them to expand their population before giving rise to more restricted progeny, hence the name “transit amplifying progenitor” (Duan et al., 2008). Type-2 cells are generally characterized by a lack of a process, and expression of a variety of markers of undifferentiated, proliferating cells (Sox2, Nestin, Tbr2, Ki67) as well as markers that indicate commitment to neuronal lineage (Ascl1 and Ngn2) (Hodge et al., 2008; Roybon et al., 2009). Type-2 cells can be further subdivided into Type-2a and -2b cells based on the presence or absence of the immature neuronal marker doublecortin (DCX; Kempermann et al., 2004).

Type-2 cells are the apparent link between radial glia and neuroblasts, also called Type-3 cells (Steiner et al., 2006), and appear to make the major contribution to SGZ neurogenesis. X-irradiation (Santarelli et al., 2003), genetic (Singer et al., 2009) and pharmacologic (Shors et al., 2002) ablation of dividing cells in the SGZ is sufficient to decrease neurogenesis, despite a relative sparing of Type-1 cells. In the case of genetic and pharmacologic ablation, Type-2 cells recover, presumably repopulated from Type-1 cells; however, this is in contrast to X-irradiation, where there is no recovery of neurogenesis in adult animals. Could Type-2 cells be the stem cells? One recent study suggests that they have some of the properties of stem cells (Suh et al., 2007). Clearly further research is needed to determine the relationship between Type-1 and Type-2 cells, and the contribution each makes to adult neurogenesis.

Immature and newborn neurons

Type-2 cells give rise to Type-3 cells, which no longer express Nestin and possess limited proliferative capacity (Kempermann et al., 2004; Roybon et al., 2009). Type-3 cells quickly exit the cell cycle and initiate the complex process of maturation into granule cells (Kempermann et al., 2004); however, little is known about the transition of mitotic Type-3 cells to post-mitotic immature neurons. Recent work from our lab and collaboration with the Hsieh lab indicates that CDK5 (Lagace et al., 2008) and NeuroD (Jenny Hsieh, personal communication)

may promote this transition, although more work is needed to understand if these genes truly promote this transition, or simply promote survival of new neurons.

To become fully mature, newborn neurons must send an axon to CA3 and dendrites into the molecular layer, where they make functional synapses (Duan et al., 2008; Toni et al., 2008). Full maturation in the adult mouse SGZ can take as long as 8 weeks, however not all newborn neurons survive this long (Ma et al., 2009). There is a critical period 2-4 weeks after neuronal birth, where immature neurons possess intrinsic excitability and enhanced LTP (Doetsch and Hen, 2005; Duan et al., 2008). The formation of synapses, or synaptogenesis may be a critical part of survival of new neurons. Increased excitability and enhanced LTP can both promote formation of synapses and functional integration (Doetsch and Hen, 2005). Much of the work on neurogenesis has focused on the maturation and survival of new neurons in the hippocampus, and a considerable amount is known about the molecular regulation of these new neurons, which is discussed in more detail in the next section. How and why specific neurons are selected for survival and integration over others, however, remains a mystery.

Regulation of adult hippocampal neurogenesis

To fully understand adult hippocampal neurogenesis, it is imperative to understand the molecular mechanisms that control where, when, how and to what extent adult neurogenesis occurs.

Type-1 cells, progenitors and proliferation

As the source of new neurons, Type-1 cells and proliferating progenitors are subject to tight molecular regulation. A variety of pathways converge to modulate proliferation of stem and progenitor cells in the SGZ (Figures 1.2 and 1.3; Duan et al., 2008; Zhao et al., 2008; Basak and Taylor, 2009). Epigenetics and chromatin remodeling are critical for stem cells to activate differentiation programs (Hsieh et al., 2004). Growth factors, such as heparin-binding epidermal growth factor (EGF) and fibroblast growth factor (FGF), have long been presumed to regulate adult neurogenesis (Goldman, 1998; Duan et al., 2008). FGF and EGF increase proliferation of NSCs and progenitors *in vivo* and *in vitro* (Reynolds et al., 1992; Jin et al., 2003; Pieper et al., 2005; Zhao et al., 2007; Zhao et al., 2008) and can even promote radial glial identity (Doetsch et al., 2002; Yoon et al., 2004). Vascular endothelial growth factor (VEGF) and the vascular niche contribute to promote proliferation (Palmer et al., 2000; Jin et al., 2002; Duan et al., 2008). Pathways critical for developmental neurogenesis are also critical for adult hippocampal proliferation. For example, β-catenin, a mediator of Wnt signaling, is expressed in progenitors and is critical for proliferation of adult SGZ progenitors (Madsen et al., 2003). Likewise, recent studies suggest that Notch1 signaling plays an important role in regulating proliferation with the adult SGZ (Breunig et al., 2007). Finally, neurotransmitters, including glutamate and GABA, link neurogenesis with excitation (Deisseroth et al., 2004; Joo et al., 2007), such that proliferation can be fine tuned to suit the current demands or activity in the hippocampus (Lehmann et al., 2005).

New neurons and survival

After proliferation, the next critical step in neurogenesis is the survival and maturation of newborn neurons (Duan et al., 2008; Ma et al., 2009). In further support of excitation-neurogenesis coupling, hippocampal activity seems critical to the selection and survival of newborn neurons (Deisseroth et al., 2004; Lehmann et al., 2005; Epp et al., 2007). As mediators of synaptic activity, neurotransmitters are obvious participants in the survival of newborn neurons. Indeed, GABA, glutamate, dopamine and serotonin have been implicated in modulating survival of new neurons (Overstreet Wadiche et al., 2005; Tozuka et al., 2005; Tashiro et al., 2006; Vicini, 2008; Zhao et al., 2008). For example, GABAergic signaling has been charged with dictating the “tempo” with which cells progress through stages of adult neurogenesis (Ge et al., 2007). Although not a neurotransmitter, BDNF is released from synapses (Binder and Scharfman, 2004) and has a well-known role in mediating survival of new neurons in the SGZ (Huang and Reichardt, 2001; Bergami et al., 2008). Furthermore, it appears that synaptogenesis, which facilitates both excitement and integration, is critically important for survival of new neurons (Song et al., 2005).

Molecular mechanisms underlying hippocampal neurogenesis

The pathways mentioned above appear to converge on several downstream effectors to regulate proliferation and survival. A common downstream effector of

growth factor signaling, cyclic-AMP response element binding protein (CREB) (Johannessen et al., 2004), is a likely candidate for mediating proliferation in response to growth factor stimulation. Proliferating progenitors and immature neurons in the SGZ present the phosphorylated form of CREB, and pharmacological activation of PKA/CREB signaling enhances neuronal proliferation (Nakagawa et al., 2002) while ablation of CREB decreases proliferation and neuronal survival (Dworkin et al., 2009). Furthermore, CREB is an important transcriptional activator of growth factors in the adult SGZ (Dworkin et al., 2009). Another candidate mediator of pathways that regulate proliferation is GSK3β, especially given its role in preventing cell cycle progression in the absence of growth factors (Stambolic and Woodgett, 1994). In an elegant study, Mao et al. found that DISC1, a gene linked with schizophrenia, lies at the crossroad of β-catenin and GSK3β (Mao et al., 2009). Knockdown of DISC1 decreased proliferation, with no change in cell death. Intriguingly, a GSK inhibitor prevented the decrease in SGZ proliferation, suggesting that DISC1 regulates proliferation through interaction with GSK3β. Finally, Akt, a protein implicated in stem cell survival and downstream of Notch signaling (Androutsellis-Theotokis et al., 2006), mediates neurogenesis in the adult SGZ, as administration of an Akt agonist increased proliferation in the SGZ (Shioda et al., 2008). This finding is especially interesting given that Akt regulates both CREB and GSK3β, suggesting that Akt may be a master regulator of proliferation and survival in the SGZ. While these studies have provided insight into possible mechanisms by which multiple pathways converge to neurogenesis in the SGZ, with the

exception of CREB, direct evidence of their involvement in mediating growth factor-induced proliferation is still lacking.

Dynamic regulation of adult hippocampal neurogenesis by environment and activity

Adult hippocampal neurogenesis is an incredibly plastic process, regulated by a wide variety of stimuli (Miles and Kernie, 2006; Eisch et al., 2008; Zhao et al., 2008). Aging, stress, injury, disease and drugs of abuse decrease hippocampal neurogenesis; while antidepressants, exercise, seizures and learning increase neurogenesis (Cameron and McKay, 1999; Gould et al., 1999; van Praag et al., 1999; Jessberger et al., 2005; Donovan et al., 2006; Arguello et al., 2008). Many of these stimuli regulate neurogenesis through changes in the neurogenic niche (Fig 1.3). For example, running increases growth and neurotrophic factors (Gomez-Pinilla et al., 1997; Russo-Neustadt et al., 2001; Fabel et al., 2003; Li et al., 2008; Trejo et al., 2008) and induces vascular remodeling (van Praag et al., 2005; Clark et al., 2009; Van der Borght et al., 2009), both of which are linked to increased progenitor proliferation and increased survival of new neurons (van Praag et al., 1999; van Praag, 2008). In addition to changes in the hippocampal niche, neurogenesis is extremely sensitive to changes in “activity” of the hippocampus (Deisseroth et al., 2004; Kempermann, 2008; Treves et al., 2008). For example, antidepressants and exercise increase the neuronal activity in the hippocampus (Airan et al., 2007), as does learning (Kempermann, 2008). These observations combined with findings that new neurons are more excitable than

their older counterparts led to the development of the activity-dependent hypothesis of neurogenesis. Few studies to date have examined the intrinsic requirements for activity- and exercise-induced neurogenesis, primarily due to lack of available tools. Utilizing inducible techniques, my studies expand on the current understanding of intrinsic signaling in the regulation of both basal and exercise-induced adult hippocampal neurogenesis (Chapter 3).

Function of adult hippocampal neurogenesis

Hippocampal neurogenesis is highly conserved, found in nearly all birds and mammals, and its conservation, as well as its lifelong duration, implies an important function (Goldman, 1998; Imayoshi et al., 2008; Treves et al., 2008). Several recent studies suggest that neurogenesis in the adult hippocampus is linked to learning and memory (reviewed in Gould et al., 1999; Treves et al., 2008; Zhao et al., 2008). The addition (and removal) of neurons in the dentate gyrus (DG) is critical for learning and recall (Shors et al., 2001; Dupret et al., 2007; Dupret et al., 2008; Imayoshi et al., 2008; Kempermann, 2008; Garthe et al., 2009). For example, in animals with abnormal Wnt signaling and reduced neurogenesis, long-term retention (>2 weeks) in the water maze was reduced (Jessberger et al., 2009). However, it appears that not all hippocampal dependent tasks rely on neurogenesis (Clark et al., 2008; Jessberger et al., 2009). In contrast, it appears as if neurogenesis might be required for memory consolidation (Zhao et al., 2007), pattern separation (Treves et al., 2008; Garthe et al., 2009), or memory clearance (Feng et al., 2001). These findings urge carful

consideration when choosing a learning and memory assay and call for the development of more neurogenesis-dependent tasks. Interestingly, learning also facilitates neurogenesis, suggesting that new neurons are functionally recruited by novelty (Leuner et al., 2006; Dalla et al., 2007; Sisti et al., 2007; Waddell and Shors, 2008). This finding provides additional support for the activity-dependent hypothesis of neurogenesis.

Adult hippocampal neurogenesis has also been implicated in regulation of mood, specifically depression and anxiety (Sahay and Hen, 2007; Eisch et al., 2008). Stress can precipitate depression and anxiety, especially chronically (Feder et al., 2009). Glucocorticoids are increased by chronic stress and are toxic to the hippocampus, leading to atrophy and reduced hippocampal volume (Bremner et al., 2000). Furthermore, stress decreases hippocampal neurogenesis (Duman et al., 2001; Hellsten et al., 2002). The finding that antidepressants increased neurogenesis in the SGZ (Malberg et al., 2000) coupled with an apparent requirement of neurogenesis for the behavioral effects of antidepressants (Santarelli et al., 2003) prompted the hypothesis that neurogenesis might be involved in depression, at least in recovery, if not in its etiology. In the past decade, several groups have sought to further explore the connection between neurogenesis and depression/anxiety. From this work, it has become increasingly clear that reduced or ablated neurogenesis is not sufficient to induce depression-related behaviors in animal models (Catts et al., 2008; David et al., 2009; Singer et al., 2009), but it can affect anxiety (Bergami et al., 2008; Perez et

al., 2009). However, after much research, the role that neurogenesis plays in behavioral response to antidepressants has become less clear, with some studies indicating a requirement (Li et al., 2008; Surget et al., 2008) while others do not (Singer et al., 2009). Perhaps the most recent study on the topic can reconcile these findings, as they suggest that some behaviors are neurogenesis-dependent while other are not (David et al., 2009). Other studies suggest that the effects of antidepressants may be age dependent (Navailles et al., 2008), further complicating the connection between neurogenesis and depression. Perhaps another reason for the discrepancies between these studies lies in the choice of and the wide variety of tests used to assess depression-related behavior. For example, the forced swim test is one of the most commonly used tests for screening depression-related behavior. However, this test is designed to predict the antidepressant efficacy of drugs and treatments, not depression (Porsolt et al., 1977; Cryan and Holmes, 2005). Clearly the relationship between neurogenesis and mood is complex and requires further research and the development of better models and tests of depression. Our Nestin-CreER^{T2} mouse model provides the perfect tool to specifically control neurogenesis for functional studies such as these (Chapter 5).

Notch1

Notch1 is a universally utilized signal integrator in stem cells, used to maintain self-renewal, regulate fate choices, and modulate proliferation (Artavanis-Tsakonas et al., 1999; Dumortier et al., 2005; Radtke et al., 2005; Yoon and

Gaiano, 2005; Wilson and Radtke, 2006). As a master regulator of stem cells, Notch1 is a perfect candidate gene for manipulation of adult hippocampal neurogenesis at its origin: Type-1 cells. Below, we will briefly discuss the role of Notch1 in stem cells in general and then in the setting of neurogenesis, both in development and in the adult.

Notch Signaling in Stem Cells

Notch signaling regulates the development of multicellular organisms as diverse as sea urchins and humans through short-range cell-cell interactions (Artavanis-Tsakonas et al., 1999). Notch signaling requires physical contact between cells to select between preexisting developmental programs, integrating both intrinsic and extrinsic cues to generate context-appropriate choices (Artavanis-Tsakonas et al., 1999; Wilson and Radtke, 2006; Kopan and Ilagan, 2009). The Notch family in mammals consists of four paralogs, Notch1-4, and several ligands, the most common being members of Delta-like (Dll) and Jagged families (Figure 1.4; Kopan and Ilagan, 2009). Notch signaling requires cell-cell interaction and typically follows this sequence of events (Figure 1.4): membrane-bound Notch binds to ligand (e.g. Delta) on an adjacent cell; a series of cleavage events leads to release of the Notch intracellular domain (NICD), which quickly translocates to the nucleus (Radtke et al., 2005; Bray, 2006); nuclear NICD interacts with mastermind-like to convert RBP-J, the required transcriptional cofactor of Notch, from a transcriptional repressor to an activator to promote expression of target genes, including members of the Hes and Herp families (Artavanis-Tsakonas et

al., 1999; Iso et al., 2003; Kageyama et al., 2005). Canonical Notch signaling results in RBP-J-dependent transcriptional activation, while non-canonical signaling does not depend on transcription (Androutsellis-Theotokis et al., 2006; Mizutani et al., 2007; Kopan and Ilagan, 2009). The mechanisms of non-canonical signaling are less well characterized, but may involve STAT3 and Akt (Androutsellis-Theotokis et al., 2006).

Notch signaling in stem cells amplifies and consolidates signals to select between preexisting developmental programs and promote or suppress a variety of outcomes, including proliferation, death, fate and differentiation (Androutsellis-Theotokis et al., 2006; Kopan and Ilagan, 2009). Cell-cell interactions implicate autonomous and non-autonomous effects if Notch signaling is disrupted. Indeed, disruption of Notch ligands leads to non-autonomous impairment of development, but disruption of Notch receptors leads to autonomous effects, indicating that signaling is one-way, with a signal-sending and signal-receiving cell (Androutsellis-Theotokis et al., 2006). However, this is an oversimplification, as a single cell can express both receptor and ligand. Intrinsic and extrinsic factors, as well as transcriptional feedback, regulate levels of ligand and receptor such that subtle differences are amplified and a signaling or receiving mode is established (Androutsellis-Theotokis et al., 2006). Thus, the level of receptor available dictates the ability of the cell to receive a signal. This is especially important given that each receptor can only be activated once, underscoring the dose-dependent nature of Notch signaling (Artavanis-Tsakonas et al., 1999;

Guentchev and McKay, 2006; Kopan and Ilagan, 2009). The mechanisms underlying selection of cells as signal senders or receivers is unknown, with some studies indicating that it may be stochastic (Johnston and Desplan, 2008), while others attribute it to oscillation of pathway components, especially Hes1 (Shimojo et al., 2008) or post-translational modifications that affect receptor affinity or availability (Justice and Jan, 2002; D'Souza et al., 2008).

Despite the varied and complex outcomes of Notch signaling, a clear role for this signaling “network” has emerged: to control fate choices between two cells (Androutsellis-Theotokis et al., 2006). Calling Notch signaling a “pathway” leaves out the cross talk with other pathways that is critical for its context dependent outcomes (reviewed in Hurlbut et al., 2007; Poellinger and Lendahl, 2008). In stem cells, Notch signaling has several modes by which it can select fate: lateral signaling and inductive signaling. Lateral signaling occurs between two relatively equivalent cells and functions to segregate lineages from clusters of progenitors, while inductive signaling occurs between two different types of cells, such that one cell specifies the fate of the other (Androutsellis-Theotokis et al., 2006; Wilson and Radtke, 2006). For example, in worms, lateral inhibition between two equivalent gonadal cells leads to the generation of an anchor cell and a ventral uterine precursor, while sensory organ precursors utilize inductive signaling to generate a hair-and-socket pair and a neuron-and-sheath pair (Artavanis-Tsakonas et al., 1999). Thus, a deceivingly simple signaling skeleton, composed of a ligand-receptor pair with no second messenger, can mediate a wide variety

of outcomes with multiple signaling modes and tailor stem cells and their progeny to the current context.

Notch Signaling in Developmental Neurogenesis

One of the first phenotypes observed in loss-of-function Notch mutants was an increase in neuronal differentiation (Androutsellis-Theotokis et al., 2006), suggesting that Notch signaling plays a specific role in regulating neurogenesis. Indeed, several human developmental diseases are caused by disruption of Notch signaling, including several with neurological manifestations, such as Alagille syndrome (Lasky and Wu, 2005) and Down syndrome (Fischer et al., 2005; Lockstone et al., 2007). We will briefly review what is understood about Notch signaling in development of the central nervous system (CNS), in order to understand what potential role Notch1 might play in adult neurogenesis. The reader is referred to these excellent reviews for a more thorough discussion of Notch signaling in neural development (Yoon and Gaiano, 2005; Louvi and Artavanis-Tsakonas, 2006).

In neural stem cells of the developing CNS, Notch is expressed on radial glia and activated by ligands expressed on neuroblasts (Casarosa et al., 1999; Yoon and Gaiano, 2005). To maintain an undifferentiated state, Notch activation promotes radial glial identity (Gaiano et al., 2000; Yoon et al., 2004) and expression of members of the Hes and Herp family that inhibit expression of proneural bHLH transcription factors, including Ascl1 (also known as Mash1) and Ngn2 (Iso et al.,

2003). Later in development, Notch activation promotes glial differentiation (Namihira et al., 2009). In neuroblasts, proneural bHLHs accumulate through unknown mechanisms and promote neuronal differentiation, as well as expression of the ligand Dll1 (Casarosa et al., 1999; Kageyama et al., 2005; Shimojo et al., 2008; Nelson et al., 2009). Ligand expression on neural progenitors is critical for stem cell maintenance (Nyfeler et al., 2005; Yeo and Chitnis, 2007). Hes1 and Hes5 are key among Notch targets and seem to mediate most of the canonical functions of Notch1 activation (Nakamura et al., 2000; Kageyama et al., 2005). While Hes5 is strictly regulated by Notch1, Hes1 is expressed before Notch1 and is also regulated by Shh signaling (Wall and Wallace, 2009), once again illustrating the integration of Notch signaling with other pathways.

In addition to the Hes/Herp family, Notch signaling activates targets that promote cell cycle entry and prevent cell cycle exit (Alexson et al., 2006; Guentchev and McKay, 2006; Dehay and Kennedy, 2007; Wall et al., 2009). Candidate targets include Fbxw7 (Ishikawa et al., 2008), an E3 ubiquitin ligase involved in the cell cycle, as well as CDK1 (Krejci et al., 2009), an important component of the mitosis promoting factor complex that regulates entry into and exit from mitosis (Salaun et al., 2008; Potapova et al., 2009). It is interesting to note that both Notch signaling and expression of cell cycle proteins are oscillatory (Shimojo et al., 2008; Potapova et al., 2009), underscoring the role of Notch in regulating proliferation. In addition to modulating progenitor number by regulating the cell

cycle, Notch signaling also affects apoptosis (Yang et al., 2004). Like proliferation, apoptosis is a critical aspect of determining the proper size and shape of the developing brain. The requirement for Notch1 in promoting proliferation and self-renewal is most evident in the neurosphere assay, where several studies have shown that intact Notch signaling is absolutely necessary (Hitoshi et al., 2002; Chevallier et al., 2005; Nyfeler et al., 2005; Alexson et al., 2006). While much is understood about Notch-dependent regulation of developmental neurogenesis, especially the core components, much remains to be determined because of the diversity of cross talk with other pathways and the pleiotropy of outcomes. What is certain, however, is the critical role that Notch signaling plays in maintaining a pool of undifferentiated stem and progenitors cells for generation of the full repertoire of cells in the CNS.

Notch Signaling in Adult Neurogenesis

Notch signaling has been demonstrated in regulating homeostasis and maintaining stem cells in several adult tissues with high turnover, including skin, hair, gut epithelium, blood vessels and the hematopoietic system (Radtke et al., 1999; Rangarajan et al., 2001; Limbourg et al., 2005; van Es et al., 2005; Vauclair et al., 2005; Wilson and Radtke, 2006). The neurogenic zones of the adult brain are another such self-renewing adult tissue and previous studies have demonstrated expression of *notch1* mRNA in the postnatal hippocampus by *in situ* hybridization and Notch1 protein by immunohistochemistry (IHC) (Stump et al., 2002; Nyfeler et al., 2005; Breunig et al., 2007), which we confirmed in the

adult hippocampus by IHC (Figure 1.5, see appendix for methods). However, due to limited tools and the critical role of Notch1 in development, few studies have examined the role Notch1 signaling in adult neurogenesis.

With the development of more sophisticated and inducible techniques (Chapter 2), recent studies have begun to address Notch1 signaling in the postnatal and adult neurogenesis. Using retroviral overexpression of NICD, Chambers et al. found that Notch signaling induced quiescence of progenitors in the early postnatal SVZ, inhibiting proliferation and differentiation into any cell type (Chambers et al., 2001). Reduced Notch1 signaling in Notch1/Jagged1 double hemizygous mice also reduced proliferation in the early postnatal SVZ (Nyfeler et al., 2005). Here, the defect in proliferation was associated with an inability of stem cells to self-renew as the authors demonstrated impaired secondary neurosphere formation after loss of either Jagged1 or Notch1 (Nyfeler et al., 2005). Because Notch1/Jagged1 double hemizygous mice did not survive longer than a few days, this study did not examine the role of Notch1 signaling in the adult SVZ. In the first study of Notch signaling in the adult lateral ventricle, manipulation of Notch signaling was restricted to the ependymal layer and not the SVZ (Carlen et al., 2009). Intriguingly, in ependymal cells lining the lateral ventricles of adult mice, Notch1 plays a previously unappreciated role in maintaining differentiation, rather than inhibiting differentiation. Here, Notch1 maintains the differentiated state of ependymal cells, and without Notch signaling, these cells generate neuroblasts (Carlen et al., 2009). Ependymal

cells however, are not stem cells, as they were incapable of self-renewal, emphasizing a new role for Notch signaling in a differentiated cell. To date, no studies have examined Notch signaling in neural stem cells of the adult SVZ. The field is ripe for such a study, especially given the advancement in techniques and the implications for SVZ-derived stem cells in regenerative therapies.

Like SVZ neurogenesis, study of Notch1 in the SGZ has only just begun. In the first and only study of Notch1 in the postnatal SGZ, loss of Notch1 signaling in GFAP+ stem cells shifted the phenotypic distribution of recombined cells from stem-like (GFAP+) to neuronal (DCX+), while overactivation of Notch1 signaling led to persistent GFAP+ stem-like cells and fewer DCX+ neurons. Loss of Notch1 signaling in SGZ stem-like cells and their progeny also increased cell cycle exit and decreased the progenitor pool, while overactivation of Notch1 signaling decreased cell cycle exit and increased the progenitor pool. These two findings are consistent with the role of Notch1 in inhibiting neuronal differentiation and maintaining stem cells during embryonic development (Yoon and Gaiano, 2005). Additionally, Breunig et al. found that loss of Notch1 led to stunted dendritic arbors and fewer varicosities, while overactivation of Notch1 signaling led to enlarged arbors and increased varicosities in DCX+ cells, a finding consistent with *in vitro* studies (Redmond et al., 2000; Salama-Cohen et al., 2006), but which had not been previously demonstrated *in vivo*. Instead of targeting Notch1 itself, some studies have manipulated targets of Notch signaling. Ascl1 promotes neuronal fate and differentiation, Dll1 expression and is negatively regulated by

Notch1 signaling (Kageyama et al., 2005; Kim et al., 2008). Regulation of Ascl1, most likely through Notch signaling, is a fundamental requirement for fate choice during neurogenesis in the adult SGZ. Lineage tracing indicates that Ascl1-expressing cells in the SGZ are predominantly progenitors that mature into neurons (Kim et al., 2007), however, overexpression of Ascl1 leads to oligodendrocyte differentiation and hilar migration (Jessberger et al., 2008). NeuroD is another proneural bHLH transcription factor that is further downstream from Notch than Ascl1 (Yoon and Gaiano, 2005). Our recent work using the Nestin-CreER^{T2} system (Chapter 2) has established that NeuroD is necessary and sufficient for granule cell differentiation in the adult SGZ (unpublished data, Jenny Hsieh). Several studies indicate that Notch ligands Jagged1 and Jagged2 are expressed in mature neurons in the DG (Stump et al., 2002; Breunig et al., 2007; Conboy et al., 2007), however no studies to date have examined which aspects of adult hippocampal neurogenesis are regulated by Jagged1/2. Evidence for Delta-like expression in the DG is lacking. The studies reviewed here have only revealed the tip of the iceberg. Notch signaling has many targets, and the effect of signaling on those targets is likely context dependent, urging further research in the role of Notch and its targets in adult neurogenesis.

It is important to note that most of the data in Breunig et al. comes from manipulation of GFAP+ stem-like cells in the early postnatal SGZ, a region distinct from the adult SGZ. However they also offer data that show Notch1 signaling regulates cell fate in the postnatal and adult SGZ similarly. In sum, this

seminal study demonstrated that Notch1 signaling is important not only in regulating cell cycle exit and fate choice in GFAP+ neural stem-like cells, but also in regulating choices at each subsequent stage of neurogenesis, even influencing maturation and survival (Figure 1.2). This work establishes that Notch1 signaling in the early postnatal and adult SGZ recapitulates embryonic Notch1 signaling, but these findings open up many avenues for future work. Is the Notch signaling in the adult SGZ context-dependent? Does Notch1 regulate activity-dependent neurogenesis? Given the growing evidence that adult SGZ neurogenesis influences hippocampal function and behavior (Santarelli et al., 2003; Bergami et al., 2008; Imayoshi et al., 2008; Zhang et al., 2008) what effect would disruption of Notch1 in SGZ stem-like cells and their progeny have on hippocampal function and hippocampal-dependent behaviors? Clearly many questions remain unanswered, and our inducible Nestin-CreER^{T2} mice are the perfect tool to dissect the cell-intrinsic role of Notch1 in adult SGZ neurogenesis and hippocampal function.

Organizing Hypothesis

Notch1 is a master regulator of neurogenesis. However, the role of Notch1 in generating neurons in the SGZ over the life of an adult remains unknown, primarily due to limitations in available techniques. Nor is it known if Notch1 is critical for unique aspects of adult neurogenesis, such as activity-dependent neurogenesis. We hypothesize that Notch1 is critical for maintenance of Type-1 cells in the SGZ, and thus for the generation of appropriate numbers of neurons

and response to running. To address this question, we generated mice with inducible inactivation of Notch1 in Nestin-expressing cells and follow the progeny of these cells over several months and after exposure to exercise. Because of the conflicting literature concerning the involvement of neurogenesis in depression, we further assess the functional impact of disrupted neurogenesis on mood-related behavior.

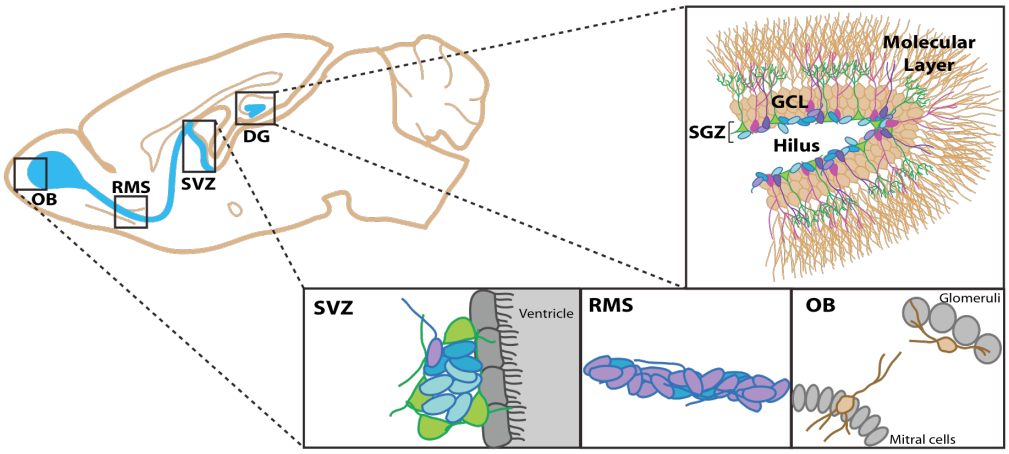

Figure 1.1: Adult neurogenesis occurs primarily in the subventricular zone (SVZ) and subgranular zone (SVZ).

A sagittal view of the adult mouse brain, the neurogenic regions are indicated in blue. In the SVZ, stem cells (green) reside in the wall of the lateral ventricle, just below the ependymal layer (gray), and give rise to transit amplifying progenitors (blue) and neuroblasts (purple). These neuroblasts migrate in chains along the rostral migratory stream (RMS) to reach the olfactory bulb (OB), where they mature into functionally integrated neurons. In the SGZ of the hippocampal dentate gyrus (DG), stem cells (green) clustered near the base of the hippocampal DG granule cell layer (GCL) give rise to transit amplifying progenitors (blue). These eventually give rise to immature (magenta) and mature (peach) granule cell neurons that primarily exist in the inner or hilar-half of the GCL but extend their processes out to the molecular layer to receive cortical input. (Figure from Johnson et al., 2009)

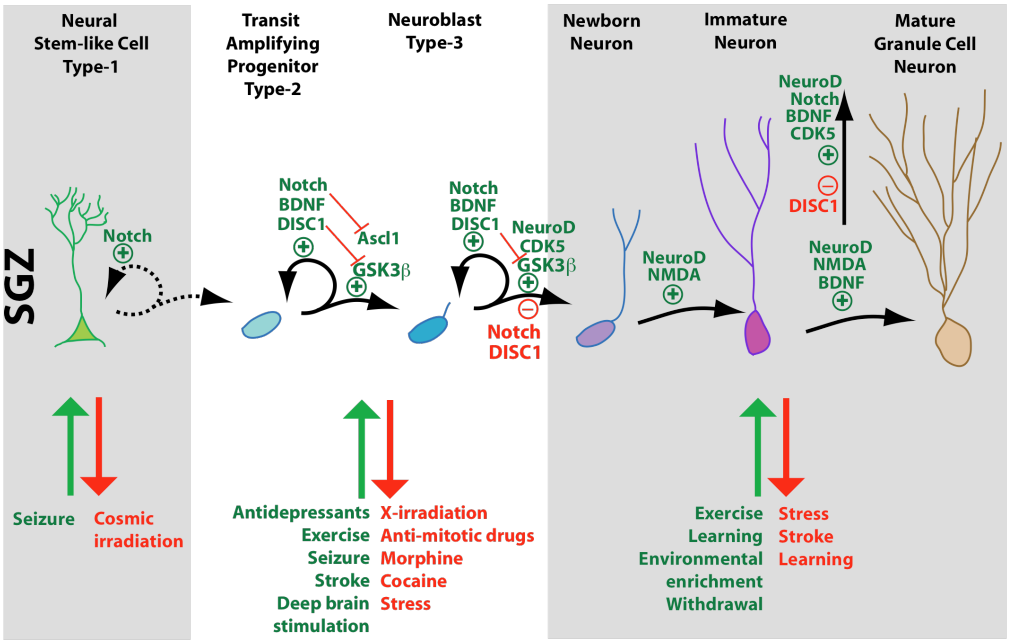

Figure 1.2: Adult neurogenesis is a process with distinct stages.

This schematic depicts the process of adult hippocampal neurogenesis. Stem cells on the left giving rise to rapidly dividing progenitors, which in turn develop into immature and eventually mature neurons on the right. Stem cells infrequently to self-renew and give rise to transit amplifying progenitors. Highly proliferative stages are indicated by the white box. Self-renewal in the SGZ may be dependent on Notch signaling. Transit amplifying progenitors give rise to lineage-restricted neuroblasts, both of which proliferate to expand their population. Several pathways converge to promote proliferation of these populations in the SGZ. Notch and DISC1 promote basal proliferation, while TrkB promotes proliferation in response to antidepressants. DISC1 may promote proliferation by inhibiting GSK3β and cell cycle exit. Notch signaling also negatively regulates

cell cycle exit of progenitors. Once SGZ neuroblasts exit the cell cycle, they differentiate into neurons and extend dendrites. Maturation and survival of newborn neurons is positively regulated by many pathways, including Cdk5, NMDAR, TrkB, and Notch, while DISC1 negatively regulates maturation. Each of the stages of neurogenesis is dynamically regulated by the environment and the experience of the animal, which translates to neuronal activity. Examples of stimuli that positively (green) and negatively (red) regulate each stage are indicated in the bottom row. (Figure adapted from Johnson et al., 2009)

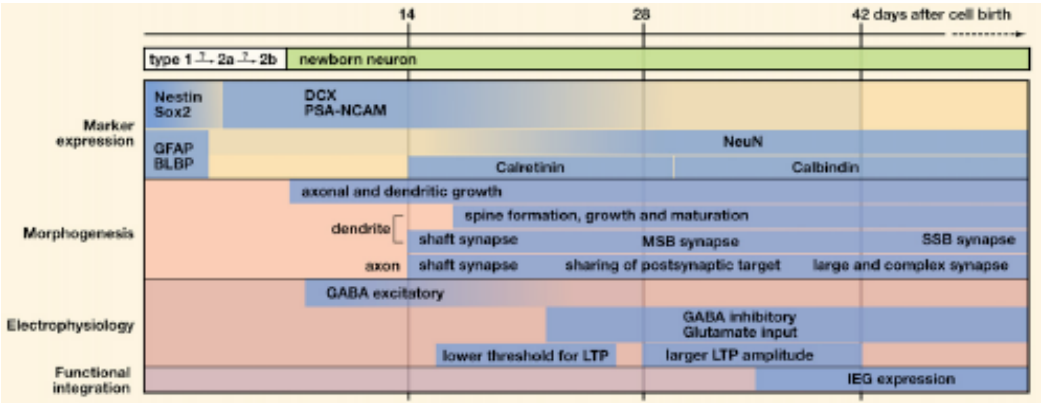

Figure 1.3: Timeline of development of newborn neurons in the SGZ.

Abbreviations: multiple synapse boutons (MSB); single synapse boutons (SSB); long-term potentiation (LTP); immediate early gene (IEG). (Figure from Zhao et al., 2008)

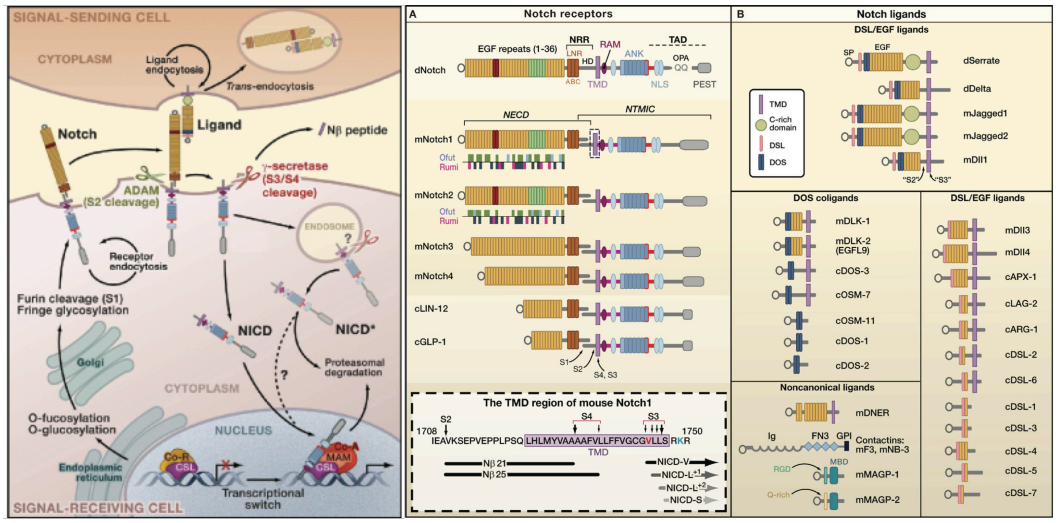

Figure 1.4: Core events and components of Notch signaling.

Left, The core Notch signaling pathway is mediated by regulated proteolysis. After translation, Notch is cleaved at S1 and glycosylated in the Golgi to produce a functional heterodimer. Notch1 heterodimer on the surface binds to ligand on the surface of an adjacent cell, exposing the S2 cleavage site. Cleavage at the S2 site is the critical step in activation of the receptor. The extracellular domain of Notch (NECD) is endocytosed with the ligand into the adjacent cell leaving the S3/S4 cleavage sites available for γ-secretase. Once cleaved at S3/S4, the intracellular domain of Notch (NICD) moves into the nucleus to convert the CSL complex from a transcriptional repressor to an activator, promoting expression of Notch target genes. ***Right,*** Domain organization of Notch pathway receptors and ligands. *A*, Notch1 receptors are large transmembrane proteins that contain multiple extracellular EGF-like repeats. EGF repeats in red and green mediate ligand binding. Glycosylation of EGF repeats modulates affinity for the different

types of ligands. The negative regulatory region follows the EGF repeats and prevents activation of the receptor in the absence of ligand. The NICD contains an RBPjκ association module (RAM) domain, nuclear localization sequences (NLS), ankyrin repeats (ANK), and a transactivation domain (TAD) that includes PEST domain for degradation. The inset shows detailed sequence of the transmembrane domain (TMD) of mouse Notch1, illustrating that γ-secretase cleavage leads to multiple isoforms of NICD, but only NICD molecules initiating at valine 1744 evade rapid degradation. Cleavage proceeds until a short Nβ peptide is left, similar to APP processing by γ-secretase. ***B,*** Known and putative Notch ligands can be subdivided based on their structure. Note that ligands in mammals are also transmembrane proteins with large extracellular EGF repeats and subject to some of the same cleavage events as the receptor. Abbreviations: DSL, Delta/Serrate/LAG-2; DOS, Delta and OSM-11 proteins; EGF, epidermal growth factor. (Figure adapted from Kopan and Ilagan, 2009)

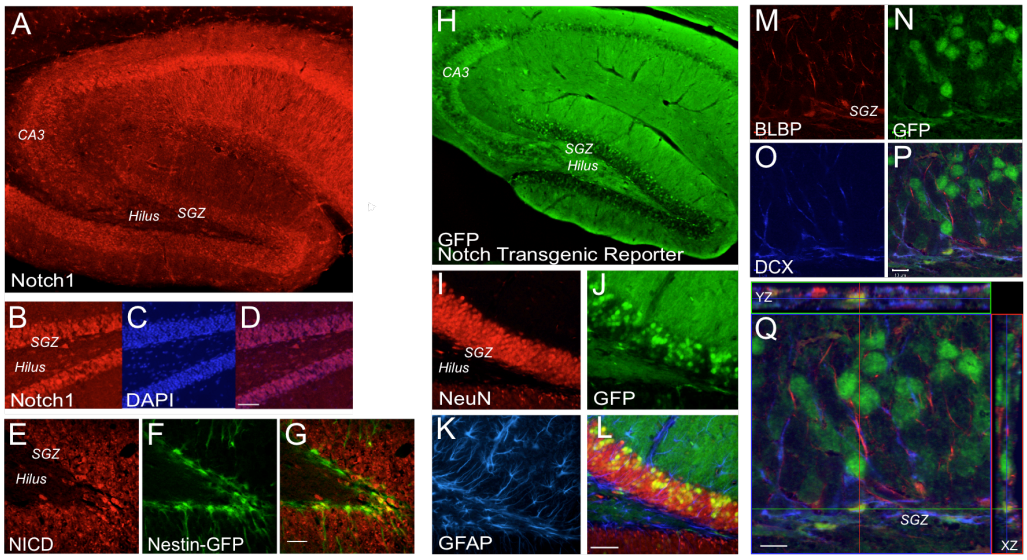

Figure 1.5: Notch1 is expressed and active in the adult hippocampus.

A-D, Full-length Notch1 is broadly distributed in the adult hippocampus, including the dentate gyrus. ***E-G,*** NICD, the cleaved and active form of Notch1, is found throughout the granule cell layer and in a subset of Nestin-expressing Type-1 cells in the SGZ. ***H-Q,*** GFP expression in the Notch transgenic reporter (Mizutani et al., 2007) indicates that Notch signaling is canonically active in the DG, including progenitors in the SGZ and mature neurons.

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CHAPTER TWO

Nestin-CreERT2/R26R-YFP mice: a valuable tool for studying basal and activity-induced neurogenesis

Portions 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 Nov 14;27(46):12623-9.

Introduction

Interest in adult neurogenesis has increased in recent years with the discovery that adult-generated granule cells integrate into the existing neural network of the dentate gyrus (DG) and have been linked with hippocampal function (Zhao et al., 2008). Furthermore, the process of neurogenesis is dynamically regulated by a variety of stimuli that also regulate hippocampal function, suggesting that neurogenesis and function are causally linked (Treves et al., 2008). For example, physical exercise potently increases proliferation, survival and net neurogenesis in the adult hippocampus, and improves mood and cognition, two functional domains of the hippocampus (Christie et al., 2008; van Praag, 2008). However, studies linking neurogenesis to hippocampal function have been hampered by an inability to specifically target and track neural progenitors in the adult SGZ.

Traditionally, studies of adult SGZ neurogenesis have relied on chemical methods to label cells in S-phase of the cell cycle (Altman and Das, 1965; Kee et al., 2002; Yokochi and Gilbert, 2007) or transgenic methods to label cells at discrete stages of adult neurogenesis (e.g. Yamaguchi et al., 2000). However, S-phase labeling of proliferating cells in the SGZ provides only a "snap-shot" of neurogenesis, and not all of the labeled cells retain the label over time, making long-term studies technically challenging. Like S-phase labeling, transgenic reporter lines only label cells when the promoter is active and they do not permanently label any individual cell. Neurogenesis is dynamically regulated by running, with proliferation increased after acute running and survival of new neurons increased by chronic running (van Praag, 2008). However, the majority of studies have used traditional methods to examine the dynamic regulation of neurogenesis, and have not been able to label and track cells to follow their response. While traditional techniques have laid a solid foundation upon which the field of neurogenesis had grown, their limitations have prompted the development of more sophisticated, inducible techniques.

Inducible transgenic mice allow permanent and selective manipulations of adult neurogenesis *in vivo*. Adult neural stem cells express a variety of stem cell and glial markers, including Nestin, the high-affinity glial glutamate transporter (GLAST), and glial fibrilary acidic protein and mice have been generated using each of these genes to drive an tamoxifen-inducible version of Cre recombinase (Feil et al., 2009), such as ER^{T2} or ER^{TM} (Garcia et al., 2004; Carlen et al., 2006;

Lagace et al., 2007; Ninkovic et al., 2007; Imayoshi et al., 2008; Li et al., 2008). Our Nestin-CreER^{T2} transgenic system allows us to label, track, and phenotype stem cells and their progeny in the adult subventricular zone (SVZ) and subgranular zone (SGZ), as well as remove genes specifically from Nestin-expressing cells after peripheral administration of tamoxifen (e.g. Lagace et al., 2008). Unlike inducible mice that utilize glial drivers (Ganat et al., 2006; Mori et al., 2006), our Nestin-CreER^{T2} mice display recombination only in neuronally-restricted progenitors, underscoring the utility of these mice for studies of the dynamic regulation and functional contribution of adult neurogenesis.

We report here on the first inducible Nestin-CreER^{T2} mouse that can be used to label, track, and phenotype stem cells and their progeny in the adult SGZ. Using this mouse, we explore if adult-generated neurons are derived from the Nestin lineage. In addition, we quantify the diverse composition of labeled cells over months following recombination, as well as estimate the total contribution of stem cells and their progeny to adult mice. Our data provide unique long-term insight into the importance of stem cells to neurogenesis in the SGZ, and underscore the utility of this mouse in gene deletion from stem cells and their progeny in the adult brain. Furthermore, we demonstrate that recombined cells in this model respond to running.

Materials and Methods

Nestin-CreER^{T2}/R26R-YFPtransgenic 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. Nestin-CreER^{T2} mice were bred with R26R-yellow fluorescent protein (YFP) reporter mice resulting in bigenic mice heterozygous for both transgenes (Figure 2.1a). Mice were genotyped by PCR using genomic DNA and primers previously published for Cre (Indra et al., 1999) and R26R reporter mice (Soriano, 1999). Data shown here are from the *k* line.

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 (i.p.; dissolved in 10% EtOH/90% sunflower oil) with minimal lethality (<5%). To examine the impact of TAM on survival of adult-generated neurons, mice were given BrdU (150 mg/kg, i.p.) 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.

Voluntary physical activity

To address how physical activity regulated adult hippocampal neurogenesis in this novel mouse model, Nestin-CreER^{T2}/R26R-YFP mice were single housed in modified cages with a running wheel (Coulbourn Instruments, Whitehall, PA, Cat# ACT-551 & ACT-552) with *ad lib* access to the wheel, water and food. The wheel was either locked (unable to turn; control group) or open (running group)

for 7 days beginning 12 days post-TAM. Revolutions were monitored and activity analyzed using ClockLab (ActiMetrics Software, Wilmette, IL). Mice were sacrificed after 7 days and YFP+ SGZ cells were visualized and quantified as described below.

Immunohistochemistry (IHC)

Tissue Sectioning and Preparation. 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 mm 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., 2004; Mandyam et al., 2007), with the exception that Sox-2/GFAP staining was performed using free-floating IHC.

Antibodies. 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 monoclonal anti-Glial fibrilary acidic protein (GFAP; Chemicon, Cat # MAB360; 1:3000); rabbit polyclonal anti-Green fluorescent protein (GFP, to detect YFP; 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).

IHC. 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 3% serum (donkey and/or horse) 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). 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 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) or red Nissl (Invitrogen, Eugene, OR, Cat # N-21482; 1:200). All slides were dehydrated and coverslipped using DPX.

Microscopic analysis and quantification

Quantification of cell number within the hippocampus was performed at 400x using an Olympus BX-51 microscope by an observer blind to experimental groups. 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). Data are presented as total YFP SGZ counts in Figure 2.2 and as YFP SGZ counts in each section across the anterior-posterior axis in Figure 2.3. Phenotypic analysis of YFP+ cells (50-150 cells/mouse, n=4-6 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 630X). Scanning and optical sectioning in the Z plane was performed as

described previously (Mandyam et al., 2004; Mandyam et al., 2007) and fluorescently labeled confocal images presented here were taken from one optical slice and imported into Photoshop (Adobe Systems). Verification of colocalization in the SGZ was achieved by importing stacks of Z images into a 3D reconstruction program, Volocity (Improvision), and performing rotation, transient modification and 3D rendering.

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. 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

While we have extensively characterized the contribution of Nestin-expressing cells in the SVZ to adult neurogenesis (Lagace et al., 2007), we will focus on SGZ neurogenesis in this chapter.

Nestin-CreER^{T2}/R26R-YFP mice display TAM-induced recombination limited to 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 tamoxifen (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 2.1a). The *k* line demonstrated the highest level of YFP expression out of five founder lines and all data presented here are from this line. Nestin-CreER^{T2}/R26R-YFP mice had YFP+ in neurogenic regions after TAM (Figure 2.1b) but there was no appreciable number of YFP+ cells in non-neurogenic regions, such as the cortex, thalamus, and cerebellum or in the absence of TAM (Figure 2.1c). Also, Nestin-CreER^{T2}/R26R-YFP mice had no Cre-induced abnormalities, contrary to previous reports (Forni et al., 2006). YFP+ cell number in the SGZ was similar between male and female mice (e.g. Figure 2.1d-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 (Figure 2.1f-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 effectively targets adult stem-like cells and their progeny in the well-characterized regions of adult neurogenesis, including the SGZ.

Recombined cells in the SGZ results in a "wave" 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 2.1d-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 2.2a: $F_{(4,42)}=15.5$, $p<0.005$). There was no significant change between 30-65 days and 65-100days post-TAM, indicating that the number of YFP+ cells reaches a plateau at 30 days (~10-12 weeks of age), similar to other models in the SGZ (Ninkovic et al., 2007; Imayoshi et al., 2008). 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. Together the results indicate that recombination in Nestin-expressing cells in the SGZ leads to a wave of labeled cells, rather than a continuous stream.

Over time, labeled stem-like cells and their progeny gave rise to mature dentate gyrus GCL neurons. At 30 days and beyond, the majority of YFP+ SGZ cells displayed a long process extending up into molecular layer capped by a highly arborized dendritic tree (Figure 2.2d). 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 2.2e-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 2.2f). YFP+ astrocytes were rare

(YFP+/S100B+/GFAP+ and astrocytic morphology), providing additional support that Nestin-expressing stem cells give rise to neurons, not astrocytes. Taken together, these data underscore a link between *nestin* expression and neuronal fate in the adult SGZ.

Recombined cells in the SGZ progress through distinct stages to become mature neurons

As would be expected if labeling stem-like cells and their progeny, Nestin-lineage YFP+ cells were morphologically heterogeneous post-TAM (Figure 2.1d-e, 2.2b). We classified YFP+ cells into non-exclusive phenotypic categories: stem (Sox2+/GFAP+/morphology), stem/progenitor (Nestin+), dividing (Ki67+), immature neurons (DCX+) or postmitotic neurons (DCX+/CR+, Figure 2.2b) (Kempermann et al., 2004; Ming and Song, 2005; Hattiangady et al., 2007). There was a significant difference in the proportion of recombined cells in these categories (Figure 3c: $F_{(4,60)}=53.18$, $p<0.000$) that changed post-TAM (Figure 3c; $F_{(3,60)}=5.87$, $p<0.005$). At 1 day post-TAM, 96% of YFP+ cells in the SGZ were Nestin+, indicating faithful targeting of the Nestin-expressing population. In addition, these mice provide a high efficiency of recombination in the SGZ; 12 days post-TAM, 97% of stem-like cells (GFAP+/Sox2+/radial glial morphology) were recombined (Lagace et al., 2007). Between 1 and 12 days, YFP+ cells matured from being dividing, stem-like and/or progenitor cells into immature or postmitotic neurons (Figure 2.2b-c). In contrast, between 12-30 days and 30-65 days, the proportion of YFP+ cells that expressed the different phenotypes

remained constant (post hocs >0.05) suggesting that neurogenesis from labeled cells had reached homeostasis by 65 days post-TAM. Despite a plateau in YFP+ cell number at 30 days post-TAM, stem-like YFP+ cells were clearly evident at the latest time points examined (Figure 2.2d, and 100 days post-TAM; N. DeCarolis unpublished data). However, the proportion of proliferating cells dropped over time (up to 65 days post-TAM as shown in Figure 2.2d, but almost zero at even later time points; N. DeCarolis unpublished data) suggesting that proliferating YFP+ cells are not maintained over time. Taken together, these data suggest that we are efficiently targeting a stable stem cell population in the SGZ and 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. Yet, the data also suggest that neurogenesis from a labeled cohort of Nestin-expressing cells is not continuous, but rather gives rise to a "wave" of progenitors and new neurons.

Recombined cells in the SGZ are dynamically regulated by physical activity

Physical activity increases proliferation and survival of S-phase labeled cells in the SGZ of adult mice (van Praag et al., 1999; Kronenberg et al., 2006; van Praag, 2008). To determine if labeled cells in our inducible mouse model were dynamically regulated by activity, we gave mice access to running wheels for 7 days, beginning 12 days post-TAM. Seven days of free access to a running wheel significantly increased the number of YFP+ SGZ cells (Figure 2.3a-c;

bregma *X* treatment interaction, $F_{(11,110)}$=2.647, p<0.01). This suggests that recombined progenitor cells or their progeny can respond to neurogenic stimuli. Considering mice were placed on the running wheels 12 days post-TAM, a time point when YFP+ cells present diverse cellular phenotypes (Figure 2.2c), 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 stimuli (Kronenberg et al., 2003). These data indicate that our Nestin-CreERT2/R26R-YFP mice label neural progenitors in the SGZ that are dynamically regulated, and demonstrate the utility of these mice in studying the effects of activity on discrete cohorts of newborn neurons.

Discussion

Inducible transgenic lines offer clear benefits over traditional approaches for studying adult neurogenesis, such as labeling of and genetic control over large numbers of cells in neurogenic regions of the adult brain. Here we demonstrate that TAM effectively drives recombination in Nestin-expressing stem cells in the SGZ during young adulthood. Furthermore, we show that Nestin-expressing cells give rise to predominantly neurons and that recombined cells are regulated by exercise. These data support that this mouse offers a potent tool for gene ablation studies in stem cells and their progeny and for studies of the dynamic regulation of large cohorts of adult-generated cells by activity.

Contribution of Nestin-expressing cells to the adult DG

Our Nestin-CreER^{T2}/R26R-YFP mouse allows one of the first quantifiable assessments of the long-term contribution of Nestin-expressing stem cells to adult neurogenesis in the DG. In the SGZ we estimate the contribution of stem cells is 1%, which is strikingly similar to previous estimates of the contribution of rapidly dividing cells (Doetsch and Hen, 2005). Our findings, however, are in contrast with those from another Nestin-CreER^{T2} inducible mouse in which they find that Nestin expressing cells contribute as much as 10% to the adult DG (Imayoshi et al., 2008). This discrepancy could be due to several technical differences, such as differences in TAM administration route, age, and dose, or may be due to differences in expression levels of Cre between lines. In our mouse, Cre is only faintly detectable by IHC (A. Arguello, data not shown), while Imayoshi et al. demonstrate clear Cre immunoreactivity within Nestin-expressing cells and nuclear translocation upon TAM administration (Imayoshi et al., 2008). These results suggest that their mouse could target more Nestin-expressing cells. This explanation is unsatisfying, however, since we have good efficiency in our mouse line (high proportion of stem-like cells that are YFP+ at early time points). In addition, since Imayoshi et al. do not examine the proportion of Nestin-expressing cells that are recombined in their paper, direct comparison between the two lines is difficult. Some researchers are directly comparing both lines in their laboratory (Paul Frankland, personal communication), and this side-by-side comparison will be helpful in understanding the discrepancy in the absolute percentage of DG neurons thought to be generated in the adult. Minimally, however, we can say that both papers support the fact that adult-hippocampal

neurogenesis contributes to a relatively small proportion of DG neuron formation.

In addition to determining its quantitative contribution, we show that *nestin* expression ultimately results in neuronal, but not astrocytic, progeny in the adult SGZ. While striking, this finding is in contrast to the multi-lineage role for Nestin in the embryo (Beech et al., 2004; Yu et al., 2005; Carlen et al., 2006; Imayoshi et al., 2006; Kuo et al., 2006; Burns et al., 2007), most notably in our Nestin-CreER^{T2}/R26R-YFP mouse (Battiste et al., 2007). However, these findings agree with those of Imayoshi et al., suggesting a distinct role for Nestin in the adult brain (Lagace et al., 2007), and other work that suggests that once Nestin is expressed in the adult, fate may be restricted to the neuronal lineage (Lagace et al., 2007; Imayoshi et al., 2008). Future studies should determine if Nestin-lineage restriction is due to intrinsic properties of aging stem cells or extrinsic properties of the neurogenic niche.

Recombination in Nestin-expressing cells labels a discrete cohort of new neurons

We find that our Nestin-CreER^{T2}/R26R-YFP mice demonstrate faithful and efficient recombination in adult SGZ stem/progenitor cells. At 1 day post-TAM, 96% of YFP+ cells in the SGZ were Nestin+, indicating faithful targeting of the Nestin-expressing population. In further support of this conclusion, *in vivo* recombination led to YFP+ neurospheres *in vitro* (Lagace et al., 2007), and, as expected from labeled stem cells, maturing YFP+ progeny were increasingly

evident after TAM. Unexpectedly, however, YFP+ cells increased only up to 30 days post-TAM, after which their number reached a plateau. We were surprised to find a plateau in the number of YFP+ SGZ neurons, as we expected an increase based on BrdU-labeling survival studies (Dayer et al., 2003; Kempermann et al., 2003) and the persistence of labeled stem cells. In agreement with our findings, studies of several other neural stem cell specific inducible transgenic lines also indicate that the number of recombined cells in the SGZ increases before reaching a plateau (Ninkovic et al., 2007; Imayoshi et al., 2008).

What then could explain the steady state of YFP+ cell number in the SGZ? It is unlikely 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, based on our analysis in the SVZ (Lagace et al., 2007) and the robustness of genes in the Rosa26 locus (Soriano, 1999). Another possibility is that the proportion of precursor and/or immature cells decreases with age (Rao et al., 2006; Hattiangady et al., 2007). The presence of YFP+ radial glial cells 100 days post-TAM suggests that over time there is not an exhaustion of recombined stem-like cells. However, analysis of precursor number 65 days and longer post-TAM revealed a decrease in proportion (Figure 2.2c) and then an almost complete reduction in proliferating YFP+ cells (N. DeCarolis, unpublished data). The plateau in YFP+ cells corresponds to a peak in the number of proliferating YFP+ progenitors, indicating that progenitors first expand

their population and then mature into neurons. This further suggests that recombined stem-like cells generate a bolus of progeny shortly after TAM, but become quiescent and do not continue to contribute to neurogenesis at extended times after TAM. The implications of this indicate that discrete cohorts of neurons are generated at any given time.

Recombined cells are regulated by activity

New neurons in the adult hippocampus are dynamically regulated by a variety of stimuli, ranging from stress to physical activity to drugs of abuse (Eisch et al., 2008), seemingly through the common mechanism of regulating hippocampal activity, thus coupling excitation with neurogenesis (Deisseroth et al., 2004; Kempermann, 2008). Physical activity, or exercise is one of the most robust inducers of proliferation and neurogenesis, with a peak effect on proliferation between 3 and 10 days of running (Kronenberg et al., 2006; van Praag, 2008). However, we find a relatively modest increase in total YFP+ cells in the SGZ of Nestin-CreER^{T2}/R26R-YFP mice after 7 days of running. This is consistent with a specific effect of running on proliferation, as proliferating YFP+ cells at 12 days post-TAM are only a subset of YFP+ cells. Perhaps a longer duration of running would produce a more dramatic effect on total YFP+ cell number (Chapter 3). The result demonstrate that recombined cells in the Nestin-CreER^{T2} mouse are amenable to manipulation by running, and suggests that this mouse will be a useful tool to assess the impact of neurogenic stimuli on the entire life of a cohort of neurons.

Conclusion

The Nestin-CreER^{T2} mouse will clearly be useful in studies that assess the role of neurogenesis in hippocampal function (Chapter 5), especially given the possibility of specific gene deletion or progenitor ablation in a discrete temporal cohort of new neurons. Our Nestin-CreER^{T2} mouse is extremely consistent between litters, emphasizing its usefulness for gene ablation studies (Chapters 3 and 4). We demonstrate the utility of this mouse model in functional studies by showing that YFP+ cells are increased by known neurogenic stimuli. In conclusion, our data provide novel insights into the dynamic contribution of Nestin-expressing stem-like cells and their progeny to dentate gyrus neurogenesis. The data strongly implicate that Nestin expression is coincident with neuronal fate restriction and that labeled stem cells give rise to a finite cohort of new neurons.

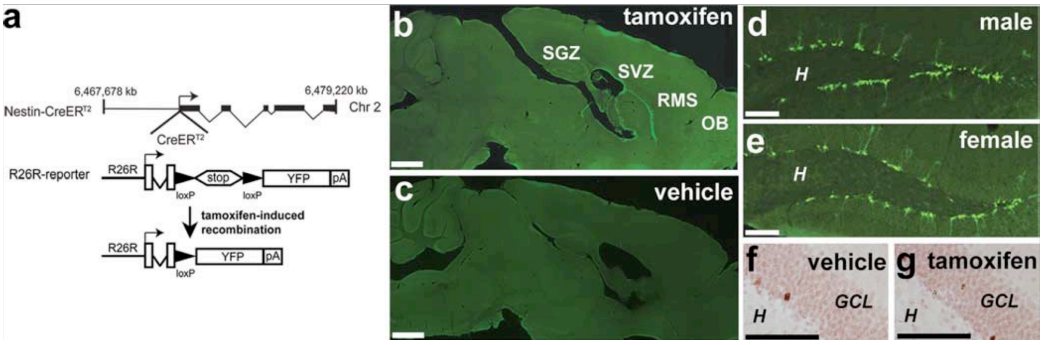

Figure 2.1. Recombination in Nestin-CreER^{T2}/R26R-YFP mice is induced by tamoxifen (TAM) and is specific to neurogenic regions.

(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.

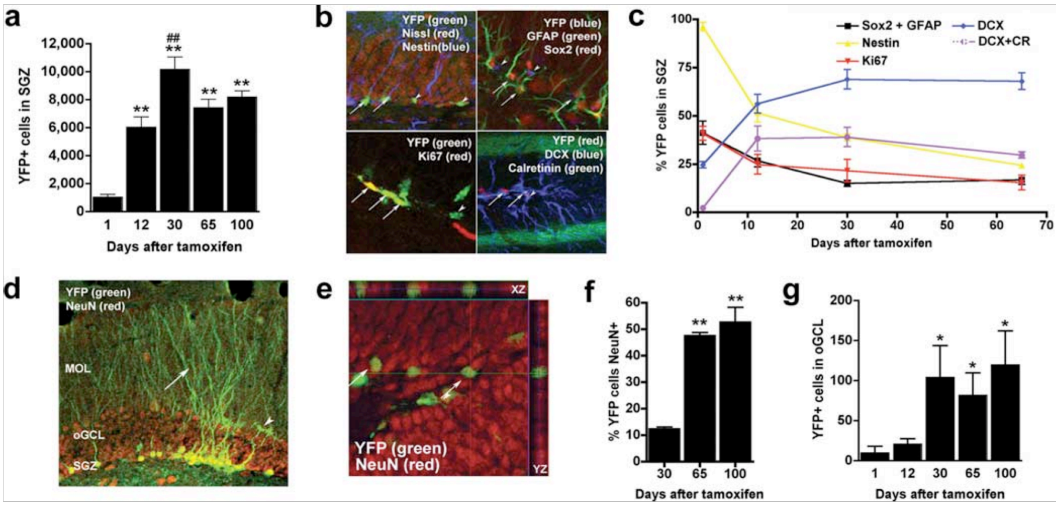

Figure 2.2. **Neurogenesis in the SGZ following TAM**.

At increasing time points following TAM **(a)** 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 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).

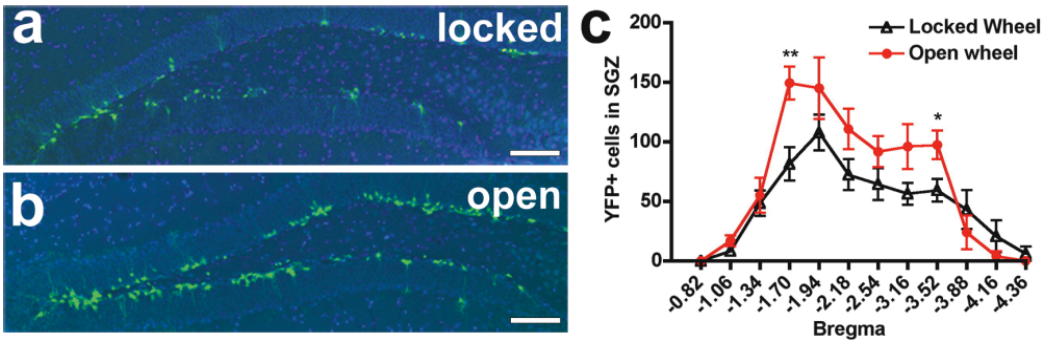

Figure 2.3. Physical activity increases YFP+ cells in Nestin-CreERT2/R26R-YFP mice.

(a-c) The number of YFP+ cells in the SGZ of running mice increased compared to mice with access to a locked wheel after 7 days.

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CHAPTER THREE

Notch1 in Nestin-expressing neural progenitors regulates proliferation but is not required for activity-dependent neurogenesis in the adult hippocampus

Portions adapted from: **Ables JL**, Johnson MA, Rivera PD, Gao Z, Cooper DC, Radtke F, DeCarolis NA, Hsieh J, Eisch AJ: Notch1-dependent maintenance of the adult hippocampal stem cell reservoir. In preparation for submission to Nature Neuroscience.

Introduction

Depression is a major illness, currently estimated as the second-leading cause of disability among young adults world-wide (Gaynes et al., 2008). Exercise holds great promise for treating depression and improving mental health. Physical activity improves hippocampal function, such as learning and memory (Christie et al., 2008; Eisch et al., 2008; Fabel and Kempermann, 2008) and increases adult hippocampal neurogenesis (van Praag et al., 1999). Neurogenesis occurs throughout adulthood in the hippocampal subgranular zone (SGZ) of the mammalian dentate gyrus (DG) (Imayoshi et al., 2008) and is linked with mood and hippocampal function (Doetsch and Hen, 2005; Dupret et al., 2007; Clark et al., 2008; Garthe et al., 2009). While this process of SGZ neurogenesis, or the

development of neurons from stem-like cells, and its dynamic regulation by a variety of stimuli, including physical activity, have been the focus of intense research, the cell-intrinsic molecular mechanisms that underlie SGZ neurogenesis and its regulation by physical activity remain largely unknown.

A reasonable candidate to consider as a cell-intrinsic regulator of basal and physical activity-dependent SGZ neurogenesis is Notch1. A member of a highly conserved pathway that regulates cellular (Artavanis-Tsakonas et al., 1999; Radtke et al., 2005) and hippocampal plasticity (Costa et al., 2003; Wang et al., 2004; Conboy et al., 2007), Notch1 is well known to regulate self-renewal and fate in embryonic neural stem cells (NSCs; Yoon and Gaiano, 2005; Corbin et al., 2008; Basak and Taylor, 2009). A seminal study in the postnatal and adult SGZ by Breunig et al. showed that in GFAP+ stem-like cells, Notch1 promotes radial glial identity, and negatively regulates cell cycle exit and neuronal differentiation (Breunig et al., 2007). However, in manipulating a GFAP+ population and in only examining a single point in time, Breunig et al. was unable to address the consequence of impaired Notch1 signaling in neuronally-restricted Nestin+ NSCs on the number of adult generated neurons over time. Thus, the long-term consequences of disrupted Notch1 signaling in adult Nestin+ SGZ NSCs and their progeny are unknown. In addition, while Notch1's cell surface expression makes it uniquely situated to integrate cues from the neurogenic niche to regulate neurogenesis (Artavanis-Tsakonas et al., 1999), no studies to date have

examined what role intrinsic Notch1 plays in physical activity-dependent neurogenesis.

We hypothesized that cell-intrinsic Notch1 signaling is critical for generating appropriate numbers of new neurons in the adult SGZ, both basally and after physical activity. To address this, we generated Nestin-creER^{T2}/R26R-YFP/Notch1$^{loxP/loxP}$ mice, referred to as Notch1 inducible knockout (iKO) mice. Tamoxifen induced-recombination in young adulthood allowed us to ablate Notch1 from Nestin-expressing NSCs and their progeny in the adult SGZ and to track the recombined cells and their progeny via yellow fluorescent protein (YFP). By assessing YFP+ cell number, morphology, proliferation, markers of neuronal development, and cell death in the SGZ of adult wild type (WT) and Notch1 iKO mice over three months under both basal and running conditions, we determined that Notch1 is critical for basal proliferation but is not required for physical activity-dependent proliferation.

Materials and Methods

Notch1 iKO mice

Animals were housed in a specific pathogen-free Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC)-approved facility at UT Southwestern on a 12-h light/dark cycle. All animal use procedures and husbandry were in strict accordance with the National Institutes of Health and Guide for the Care and Use of Laboratory Animals, and were approved by

the UT Southwestern Medical Center Animal Care and Use Committee. Care was taken to minimize the number of animals used and to diminish pain and suffering. Nestin-creER^{T2} and R26R-YFP mice (Lagace et al., 2007) maintained on a C57BL/6J background were crossed with Notch1^{loxP} mice (Radtke et al., 1999) maintained on an ICR (CD1) background. Only F3 intercross offspring were examined to a) ensure that all littermates examined were heterozygous for both *cre* and *YFP* and varied only for the Notch1^{loxP} allele, for which appropriate Mendelian ratios were observed, and b) to control for gene dosage from the different backgrounds, as running activity and neurogenesis are both highly strain dependent (Kempermann et al., 2006). Mice were genotyped by PCR using genomic DNA and primers previously published for Cre (Indra et al., 1999), R26R reporter (Soriano, 1999) and floxed Notch1 mice (Radtke et al., 1999).

Tamoxifen treatment

WT and Notch1 iKO mice (4-5 weeks old) were injected daily for 6 days with 180 mg/kg, i.p. tamoxifen (30 mg/ml in 10%EtOH/sunflower oil; Sigma-Aldrich, St. Louis, MO, Cat# T5648, S5007). Tamoxifen lethality was <5% at this age, dose and duration. Mice were perfused 13, 30, 60 or 90 days after the last tamoxifen injection.

Neurosphere isolation

Neurospheres were isolated from the subventricular zone as previously described (Pacey et al., 2006) approximately 40 days after *in vivo* tamoxifen treatment. Briefly, dissections from two WT or Notch1 iKO mice were pooled and

dissociated enzymatically (papain, deoxyribonuclease, and dispase in dissociation buffer) for 40 min at 37°C and plated at equal density. The experiment was carried out in duplicate, with 4 WT and 3 Notch1 iKO cultures in each. Neurospheres were maintained in serum-free culture media supplemented with N2, B27, bFGF, EGF and heparin and passaged when confluent (about every 7-10 days) using trypsin-EDTA. Secondary spheres were counted at confluence using a 1mm gridded dish. Genomic DNA was isolated from passage 4 neurospheres and subjected to PCR to confirm genomic recombination. Forward primer: 5'-ctg act tag tag ggg gaa aac, reverse primer: 5'-tac tcc gac acc caa tac ct.

Voluntary physical activity

For running experiments, mice were single housed in modified cages with a running wheel (Coulbourn Instruments, Whitehall, PA, Cat# ACT-551 & ACT-552) with *ad lib* access to the wheel, water and food. The wheel was either locked (unable to turn; control group) or open (running group) for 30 days beginning 30 days post-TAM or for 5 days beginning 60 days post-TAM. Revolutions were monitored and activity analyzed using ClockLab (ActiMetrics Software, Wilmette, IL). There was no statistical difference between mice on a locked wheel for 30 days and naïve group-housed mice on any measure 60 days post-TAM, so data from these two non-runner groups were combined and compared to the running group for Figures 3.5 and 3.6.

Immunohistochemistry (IHC)

Tissue Preparation. Animals were deeply anesthetized and transcardially perfused with cold 0.1M PBS for 5 minutes, followed by 4% paraformaldehyde (PFA) for 15-20 minutes. Brains were extracted and post-fixed overnight at RT in the same fixative before cryoprotection in 30% sucrose with 0.1% NaN_3 at RT overnight. Brains were stored at 4°C in the same cryoprotection solution until sectioning on a freezing microtome. Brains were sliced coronally 30µm thick and stored free-floating in 1x PBS with 0.1% NaN_3 at 4°C until stained.

Antibodies. The following primary antibodies were used: rabbit polyclonal anti-GFP (1:3000; Invitrogen, Eugene, OR, Cat# A11122), chicken polyclonal anti-GFP (1:500, Aves Labs, Tigard, OR, Cat# GFP-1020), rabbit monoclonal anti-Ki67 (1:500, Lab Vision/NeoMarkers, Thermo Fisher Scientific, Fremont, CA, Cat# RM-9106-S), rabbit polyclonal anti-cleaved caspase-3 (1:500, Cell Signal, Danvers, MA, Cat# 9661), and goat polyclonal anti-DCX (1:100, Santa Cruz Biotechnology, Santa Cruz, CA, Cat # sc-8066).

IHC. Staining was performed as previously described (Lagace et al., 2007). Briefly, for antigen retrieval, sections were mounted on slides and incubated in 0.01M citric acid (pH 6.0, 100°C) for 15 min. Following antigen retrieval sections were incubated with blocking solution (3% normal donkey serum, 0.3% Triton X-100 in TBS) for ≥20 min. Sections were then incubated with primary antibodies in carrier (3% normal donkey serum, 0.3% Tween20 in TBS) overnight at RT. Antibody staining was revealed using either species-specific fluorophore-

conjugated secondary antibodies (1:200 in TBS, Cy2, Cy3, and Cy5, Jackson ImmunoResearch, West Grove, PA) or detected with biotinylated secondary antibodies (1:200 in TBS, Jackson ImmunoResearch, Westgrove, PA) and revealed using ABC Elite kit (Vector Laboratories, Burlingame, CA, Cat# PK-6100) followed by TSA Renaissance fluorescent amplification kit (1:50, PerkinElmer Life Sciences, Boston, MA, Cat# NEL701). To remove endogenous peroxidase activity, sections were incubated with 0.03% H_2O_2 for 30 min before ABC and TSA. Slides were counterstained with DAPI (1:5000, Roche Applied Science, Indianapolis, IN, Cat# 236276) before they were dehydrated and coverslipped with DPX (Sigma-Aldrich, Cat# 44581). Omission of primary or secondary antibodies resulted in no staining and served as a negative control.

Quantification of YFP+ cells

Quantification of immunoreactive hippocampal cells was performed with an Olympus BX-51 microscope (400x) as previously described (Mandyam et al., 2004; Donovan et al., 2006; Lagace et al., 2007; Lagace et al., 2008). Briefly, an observer blind to experimental groups counted immunoreactive cells of every 9th 30µm coronal section throughout the SGZ (-0.82 mm to -4.24 mm from bregma) via the optical fractionation method. The total number of YFP+ SGZ cells was counted, and then the same slides were counted for YFP+ SGZ cells presenting Type-1 or neuronal morphology. Dividing the number of YFP+ cells with either morphology by the total number of YFP+ cells in the SGZ yielded the proportion of YFP+ cells with either morphology (Donovan et al., 2008; Imayoshi et al., 2008).

Phenotypic analysis of YFP+ cells

The phenotypic stage of YFP+ cells was examined by triple immunofluorescence-labeling of YFP, Ki67 and DCX. The percentage of 100-150 YFP+ cells per animal (n≥3 per group) was determined with a Zeiss Axiovert 200 and LSM510 confocal microscope (emission wavelengths 488, 543, and 633, 630x). Colocalization of signals was determined by scanning and optical sectioning in the Z plane. Images of a single Z-plane were imported in Adobe Photoshop CS2 and levels adjusted using the auto levels function for presentation only. Total number of YFP+ cells that were immunoreactive for a given marker (Ki67 or DCX) was determined by multiplying total YFP+ counts by the proportion that expressed the marker.

Statistical analysis

All data are presented as mean±SEM. If two groups were compared, an unpaired t test was applied. Otherwise, data were subjected to two-way ANOVA, followed by Bonferonni *post-hoc* test to determine significant differences between WT and Notch1 iKO groups. Statistical significance was set at $p<0.05$. Statistics were reported as an interaction between two variables (variable1 *X* variable2) followed by the F value ($F_{\text{degress of freedom, sample size}}$), which indicates the size of the difference between the means of each group, followed by the *p* value for the interaction.

Results

Generation of Notch1 iKO mice

Nestin-creER^{T2} and R26R-YFP (Lagace et al., 2007) mice were crossed with floxed Notch1 mice (Radtke et al., 1999) to generate viable and developmentally normal adult Notch1 inducible knockout (iKO) mice (Figure 3.1A). In these mice, a large portion of the rat *nestin* gene, including 5.8kb of the promoter and the second intronic enhancer, drives expression of a fusion protein of Cre recombinase and the modified estrogen receptor (CreER^{T2}). Upon injection of estrogen receptor ligand tamoxifen (TAM), CreER^{T2} moves from the cytoplasm into the nucleus where it recombines DNA at loxP sites, resulting in removal of the promoter and first exon of *notch1*, including the initiation codon, and the "stop" signal of *R26R-YFP.* This leads to ablation of Notch1 and expression of YFP in SGZ Nestin-expressing NSCs and progenitors. PCR was used to confirm that the *notch1* locus was recombined after TAM in Notch1 iKO (*notch1*$^{fl/fl}$) mice but not their wild type (WT) (*notch1*$^{+/+}$) littermates (Figure 3.3C).

Notch1 iKO mice have fewer YFP+ cells in the adult SGZ

To ensure maximal recombination in adult Nestin-expressing NSCs, 4-5 week old mice were injected with TAM (180 mg/kg/d, i.p., 6 days) and perfused 13, 30, 60 or 90 days post-TAM. As previously described (Lagace et al., 2007; Lagace et al., 2008), YFP+ cells were confined to the adult neurogenic niches (the subventricular zone and SGZ) regardless of the time post-TAM. YFP+ cells at all times post-TAM presented morphologies reminiscent of various stages of

neurogenesis, from Type-1 cells to mature neurons (e.g. Figures 3.1C and 3.2B; Lagace et al., 2007). YFP+ astrocytes or oligodendrocytes were extremely rare (data not shown), consistent with our previous finding that progeny of Nestin-expressing cells in the adult brain are primarily neurons (Lagace et al., 2007).

To determine the effect of Notch1 ablation on the number of adult-generated cells, we first quantified the number of recombined YFP+ cells in the SGZ of both Notch1 iKO mice and WT littermates. The number of YFP+ cells increased over time in WT mice, as previously shown (Lagace et al., 2007), but not in Notch1 iKO mice (Figure 3.1B; genotype *X* time interaction, $F_{3,52}$=9.552, p<0.0001). Representative confocal images of the DG reveal fewer YFP+ cells in the SGZ of Notch1 iKO mice 60 and 90 days post-TAM (Figure 3.1C). The lower number of YFP+ cells in Notch1 iKO mice at extended times post-TAM suggests that Notch1 is necessary for continuous generation of new cells in the SGZ of adult mice.

YFP+ NSCs and progenitors are decreased in the SGZ of adult Notch1 iKO mice

The current model of SGZ neurogenesis proposes that Nestin+ Type-1 adult NSCs give rise to rapidly-dividing, transit-amplifying progenitors/Type-2 cells (Kempermann, 2002) and Doublecortin (DCX)-expressing neuroblasts in the process of generating functionally integrated DG granule cells (Kempermann, 2002; Ming and Song, 2005).To determine which specific developmental stages

of adult SGZ neurogenesis were affected by ablation of Notch1 in Nestin-expressing cells (Johnson et al., 2009), we examined YFP+ cells based on a combination of their morphology and expression of markers related to dividing Type-2 cells (Ki67) and immature neurons (DCX) (Figure 3.2A). Type-1 NSCs, with their triangular body and single projection through the granule cell layer, and mature neurons, with their large round soma and branching dendritic tree, were evident in both WT and iKO mice (Figure 3.2B). YFP+ cells were further categorized as transit-amplifying progenitors (Figure 3.2C; Ki67+, arrow) and neuroblasts (DCX+, arrowhead).

In WT mice, YFP+ Type-1 cells represent a stable pool of NSCs, with no change in their number over time (Figure 2D; $F_{3,24}$=1.04, p=ns). In contrast, the number of YFP+ Type-1 cells in Notch1 iKO mice declined over time ($F_{3,17}$=3.44, p=0.04), suggesting that ablation of Notch1 impairs the maintenance of Type-1 cells. While not significant, there was a strong trend for an interaction between genotype and time in YFP+ Type-1 cell number ($F_{3,34}$=2.49, p=0.08). We confirmed this *in vivo* decline in stem-like cells *in vitro*: NSCs isolated from the subventricular zone of Notch1 iKO mice generated 70% fewer secondary spheres compared to those isolated from WT mice (p=0.001, Figure 3.3A). After 5 passages, NSCs isolated from Notch1 iKO mice could no longer generate spheres, while NSCs from WT mice continued to generate neurospheres with each passage (Figure 3.3B). Together, the *in vivo* and *in vitro* data suggest that

NSCs from Notch1 iKO mice have lost the ability to self-renew and indicate that Notch1 regulates maintenance of Nestin-expressing NSCs in the adult SGZ.

We next examined the transit amplifying population of YFP+ cells. While proliferation was not grossly disrupted in Notch1 iKO mice (as determined by total SGZ Ki67+ cell number; Figure 3.4A; genotype *X* time interaction, $F_{3,39}$=0.56, *p*=ns), the number of YFP+ cells that were Ki67+ was significantly reduced in Notch1 iKO mice (Figure 3.2E; genotype *X* time interaction, $F_{3,19}$=8.66, *p*=0.0008). To determine if loss of Notch1 affected neuroblasts, we examined the number of YFP+ cells that were DCX+. Like transit amplifying progenitors, the number of YFP+ cells that were DCX+ was significantly reduced >60 days post-TAM (Figure 3.2F; genotype *X* time interaction, $F_{3,19}$=10.29, *p*=0.0003). To confirm that fewer neurons were generated over time in Notch1 iKO mice compared to WT littermates, the number of YFP+ cells with neuronal morphology was quantified. Again, we found significantly fewer YFP+ neurons in Notch1 iKO mice compared to WT littermates (Figure 3.2G; genotype *X* time interaction, $F_{3,35}$=3.06, *p*=0.04). Together, the results suggest that Notch1 maintains the earliest stem-like and progenitor stages, and that fewer stem-like and progenitor cells leads to fewer neurons in the adult SGZ.

Notch1 is critical for proliferation in the SGZ

Because Notch1 is known to maintain neural stem cells by inhibiting neuronal differentiation (Yoon and Gaiano, 2005; Breunig et al., 2007), we examined the

proportion of YFP+ cells in each stage of adult hippocampal neurogenesis (Kempermann et al., 2004). While the number of YFP+ Type-1 cells decreased with time post-TAM in Notch1 iKO mice (Figure 3.2D), the proportion of YFP+ cells with Type-1 morphology did not differ at any time post-TAM between WT and Notch1 iKO mice (Figure 3.2H; genotype *X* time interaction, $F_{3,35}$=0.53, *p*=ns). Likewise, there was no difference over time between WT and Notch1 iKO mice in the proportion of YFP+ cells with neuronal morphology, (Figure 3.2K; genotype *X* time interaction, $F_{3,33}$=0.22, *p*=ns). However, the proportion of YFP+ cells that were Ki67+ transit-amplifying cells was decreased in Notch1 iKO mice compared to WT littermates (Figure 3.2I; genotype *X* time interaction, $F_{3,19}$=3.51, *p*=0.04). The proportion of YFP+ cell that were DCX+ neuroblasts did not differ between genotypes over time (Figure 3.2J; genotype *X* time interaction, $F_{3,19}$=0.57, *p*=ns); however, there was a strong trend of genotype, with Notch1 iKO mice trending toward a smaller proportion of neuroblasts >60 days post-TAM ($F_{1,19}$=3.90, *p*=0.06). Together the data suggest that Notch1 regulates neurogenesis by specifically promoting proliferation of Nestin-expressing progenitors in the adult SGZ.

Notch1 iKO mice do not have increased apoptosis in the SGZ

The generation of fewer neurons in Notch1 iKO could also be caused by decreased survival or increased death of recombined cells. To determine if cell death was increased in Notch1 iKO mice, we stained the SGZ for the apoptosis marker activated caspase-3 (AC3). The total number of AC3+ cells did not differ

between the two genotypes at any time after TAM (Figure 3.4B; genotype *X* time interaction, $F_{3,31}$=1.07, *p*=ns). Furthermore, when total AC3+ cells were normalized to total YFP+ cells (Lagace et al., 2008), there was no difference between genotypes (data not shown). These data suggest that loss of Notch1 in Nestin-expressing cells does not increase cell death in the SGZ.

30 days of running increases proliferation of SGZ progenitors in Notch1 iKO mice

Voluntary physical activity is one of the most potent stimulators of adult hippocampal proliferation and neurogenesis (van Praag, 2008). Because Notch1 iKO mice primarily exhibit impairment in proliferation (Figures 3.2E and 3.2I), we hypothesized that physical activity could rescue proliferation, and thus the number of labeled neurons in Notch1 iKO mice. To test this hypothesis, WT and Notch1 iKO littermates were given access to a running wheel for 30 days beginning 30 days post-TAM (Figure 3.5A).

It was imperative to first determine if WT and Notch1 iKO mice would run and could mount a neurogenic response to physical activity, as these are both highly linked with strain (Kempermann et al., 2006; Bednarczyk et al., 2009). WT and Notch1 iKO runners both increased the amount they ran over the first 10 days, after which they reached a steady state, and did not differ in the amount they ran in any given day (Figure 3.5B; genotype *X* running day interaction, $F_{29,290}$=1.13,

p=ns). As expected after 30 days of running in CD1 mice (Bednarczyk et al., 2009), the total number of Ki67+ cells was increased by physical activity in both WT and Notch1 iKO mice (Figure 3.5C; $F_{1,27}$=31.77, *p*<0.001). Together the data indicate that Notch1 iKO mice will run voluntarily and can respond to physical activity.

SGZ neurogenesis after physical activity is not dependent on intrinsic Notch1 signaling

To determine if Notch1 modulates physical activity-dependent neurogenesis, mice were placed in cages with running wheels 30 days post-TAM, a time when total YFP+ cell number was not different between genotypes (Figure 3.1B). Mice were allowed to run for 30 days, and at 60 days post-TAM YFP+ cell number was compared between the running and control mice. 30 days of running increased total YFP+ cells in WT mice and, strikingly, increased the total number of YFP+ cells in the SGZ of Notch1 iKO mice to WT levels (Figure 3.6A; genotype *X* running interaction, $F_{1,27}$=4.41, *p*=0.045). While we found that running could rescue total YFP+ cell number, running had no effect on the number of YFP+ Type-1 cells in either WT or Notch1 iKO mice (Figure 3.6B; genotype *X* running interaction, $F_{1,26}$=0.34, *p*=ns). Representative confocal images illustrate that the number of YFP+ cells is increased in both WT and Notch1 iKO runner mice (Figure 3.6C), but Notch1 iKO runners still display a paucity of Type-1 cells.

To determine if running rescued a specific stage of neurogenesis, we again utilized the markers Ki67 and DCX to label the populations of dividing and immature neurons, respectively. 30 days of running was sufficient to restore the number of YFP+ cells that were Ki67+ in Notch1 iKO mice to WT levels (Figure 3.6D; genotype *X* running interaction, $F_{1,22}$=8.49, p=0.008). Consistent with an increase in DCX+ cells after running in previous reports (Kronenberg et al., 2006; Koehl et al., 2008; Bednarczyk et al., 2009), 30 days of running increased the number of YFP+ cells that were DCX+ in both genotypes (Figure 3.6E; genotype *X* running interaction, $F_{1,22}$=2.09, p=ns; running, $F_{1,22}$=23.89, p<0.0001), although there was no interaction. Similar results were observed for the proportion of YFP+ cells that were Ki67+ or DCX+: 30 days of running was sufficient to restore proliferation in Notch1 iKO mice to WT levels (Figure 3.6F; genotype *X* running, $F_{1,21}$=21.15, p=0.0002) with no significant effect on neuroblasts (Figure 3.6G; genotype *X* running, $F_{1,21}$=0.77, p=ns). Together these data suggest that running rescues the number of labeled neurons in Notch1 iKO mice by increasing the pool of proliferating progenitors. Furthermore, these data demonstrate for the first time that wheel running for 30 days does not affect the number of Type-1 cells in SGZ.

Discussion

Adult hippocampal neurogenesis is a life-long and dynamic process where functionally integrated neurons are proposed to emerge from NSCs and progenitors in the SGZ (Kempermann et al., 2004). Each stage in the

development of an adult SGZ granule neuron is subject to regulation: proliferation, migration, survival, and extension of dendrites and axons (Ming and Song, 2005). Altering any of these stages can lead to changes in the net number of new neurons added to the DG, and indeed, each stage is discretely regulated by a variety of intrinsic and extrinsic factors (Ming and Song, 2005; Basak and Taylor, 2009). Here we provide several lines of evidence that intrinsic Notch1 signaling in Nestin-expressing cells maintains an adequate pool of stem-like and progenitor cells in the adult SGZ. We found that the number of YFP+ neurons was reduced by almost 50% in Notch1 iKO mice compared to their WT littermates. Surprisingly, given the role of Notch1 in promoting basal proliferation of progenitors, we found that wheel running rescued YFP+ progenitor proliferation and the number of YFP+ neurons in Notch1 iKO mice. However, there were still fewer YFP+ Type-1 cells in Notch1 iKO runners. Our data confirm that running has little effect on the proliferation Type-1 cells (Suh et al., 2007) (Kronenberg et al., 2003; Steiner et al., 2008), but extend on these findings to demonstrate that the number of Type-1 cells is not affected by running. These data further suggest that Nestin-expressing Type-1 cells do not contribute significantly to physical activity-dependent neurogenesis. Importantly, our data demonstrate that intrinsic Notch1 signaling is critical for basal proliferation, but not for proliferation of SGZ Nestin-expressing progenitors in response to running.

Notch1 promotes proliferation of Nestin-expressing cells in the adult SGZ

In our inducible mouse model (Lagace et al., 2007), YFP+ cells are predominantly Type-1 and transit-amplifying cells shortly post-TAM. As neurogenesis progresses in WT mice, the progenitor pool first expands its population before giving rise to neuroblasts that mature into functional neurons at longer intervals post-TAM. Phenotypic analysis of WT YFP+ cells revealed strikingly similar dynamics of neurogenesis to our original report (Lagace et al., 2007). Expansion of the transit-amplifying progenitor pool corresponds with increasing YFP+ cells, while the plateau of YFP+ cells corresponds with a decline in the recombined transit-amplifying progenitor pool in WT mice. In Notch1 iKO mice, however, YFP+ cells did not increase over time due to an inability of YFP+ cells to proliferate, which is critical to both stem-cell self-renewal and progenitor expansion (Androutsellis-Theotokis et al., 2006; Guentchev and McKay, 2006; Krejci et al., 2009).

Here we provide evidence that Notch1 is regulates both the maintenance of Type-1 cells and transit-amplifying progenitor expansion, with the result that the fewer net neurons are generated in Notch1 iKO mice. We confirmed *in vitro* that NPCs from Notch1 iKO mice were impaired in their ability to self-renew and proliferate. The number of apoptotic cells in Notch1 iKO mice was not increased, suggesting that YFP+ cells did not die with increasing frequency without Notch1. However, we cannot rule out impaired survival of recombined cells, as current methods of detecting cell death are not very sensitive. Our results are consistent

with previous reports demonstrating that Notch1 inhibits cell cycle exit of progenitors (Breunig et al., 2007), however, we expand on these findings and demonstrate that Notch1 is critical in the earliest stages of neurogenesis for the generation of appropriate numbers of progenitors and thus new neurons in the adult SGZ.

Notch1 ablation appears to specifically impair proliferation in Nestin-expressing cells, while the proportion of YFP+ cells in other stages of neurogenesis are relatively undisturbed. In fact, the only group of YFP+ cells that decreased in both number and proportion after Notch1 ablation was the proliferating progenitors. It is intriguing that the number of YFP+ Type-1 cells decreases in Notch1 iKO mice, while their proportion is unchanged compared to WT mice at any time post-TAM, nor is there a corresponding increase in the proportion of YFP+DCX+ neuroblasts or YFP+ cells with neuronal morphology. This is in contrast to recent findings, where ablation of Notch1 in the adult SGZ and SVZ decreased the GFAP+ stem-like proportion and increased the DCX+ proportion of recombined cells (Breunig et al., 2007; Carlen et al., 2009), and in contrast to the well-established role of Notch1 to inhibit neuronal differentiation (Yoon and Gaiano, 2005).

There are several possible reasons why we did not observe an increase in the proportion of neurons in Notch1 iKO mice. First, while all Type-1 cells in the SGZ are GFAP+, not all Type-1 cells are Nestin+ (Kempermann et al., 2004; Steiner

et al., 2006; Ehninger and Kempermann, 2008). The data presented here and in our previous study (Lagace et al., 2007) indicate that progeny of Nestin-expressing Type-1 cells are neuronally-restricted. Thus, if the fate of recombined cells is pre-determined, Notch1 ablation would have little effect on neuronal differentiation. However, one would still expect an increase in the proportion of YFP+ neurons if progenitors exit the cell cycle prematurely, and this is consistent with the data presented here. While phenotypic analysis of other stages did not reveal any gross abnormalities, it is possible that maturation and progression through the stages of neurogenesis is disrupted in Notch1 iKO mice. Second, it is important to note that the phenotypic groups in this study are not mutually exclusive. YFP+ cells that are Ki67+ comprise mitotic Type-1 (although rare), transit-amplifying progenitors and neuroblasts, while YFP+ cells that are DCX+ comprise proliferating and post-mitotic neuroblasts and immature neurons. Likewise, YFP+ neurons can be DCX+ or DCX-. This overlap of phenotypic groups could obscure an increase in neuronal differentiation. Indeed, if we extrapolate the proportion of mature neurons using a combination of morphological and immunohistochemical phenotyping, we find that neuronal differentiation is increased in Notch1 iKO mice (data not shown). Third, perhaps more time is needed to see the stem and progenitors fully exhausted before an increase in neuronal differentiation is observed. Despite the lack of increased neuronal differentiation, it is clear from our data that Notch1 promotes proliferation of Nestin-expressing cells in the SGZ and that this proliferation is critical for progenitor expansion and the net number of new neurons.

Notch1 and Nestin+ Type-1 cells are not required for physical activity-dependent neurogenesis

Voluntary physical activity is one of the most potent inducers of neurogenesis in the adult SGZ (van Praag et al., 1999; van Praag, 2008) and increases the number of new neurons primarily by increasing progenitor proliferation (Kronenberg et al., 2003). For this reason and because Notch1 mice demonstrate a specific impairment in proliferation, we hypothesized that running would either have no effect or it would rescue proliferation and neurogenesis in Notch1 iKO mice. Indeed, we found that 30 days of physical activity increased neurogenesis in WT mice and fully rescued both proliferation and the number of YFP+ neurons in Notch1 iKO runner mice to WT runner levels, suggesting that running-induced neurogenesis is independent of Notch1 signaling. Perhaps growth factors and neurotrophic factors that are increased after physical activity, such as BDNF, VEGF, β-endorphin and endocannabinoids (Fabel et al., 2003; Bjornebekk et al., 2005; Koehl et al., 2008; Hill et al., 2009), are sufficient to increase proliferation in the absence of Notch1 signaling. Our findings urge further investigation of the distinct mechanisms that regulate proliferation in basal and physical activity-dependent neurogenesis.

While 30 days of running rescued proliferation and increased neurogenesis in runner Notch1 iKO mice, like other studies (Kronenberg et al., 2003; Steiner et al., 2008), we found that physical activity had no effect on the number of YFP+

Type-1 cells in either the WT or Notch iKO mice. This is surprising if one believes that Type-1 cells are the source of Type-2 transit-amplifying cells (Seri et al., 2001; Kempermann et al., 2004; Miles and Kernie, 2006), but supports previous findings that running does not affect proliferation of Type-1 cells (Kronenberg et al., 2003; Steiner et al., 2008). Recovery of neurogenesis in Notch1 iKO 30-day runners without recovery of Type-1 cells suggests that Type-1 cells make only a minor contribution to adult SGZ neurogenesis. How can this be if the Type-1 cell is the stem cell? Perhaps running expands the transit-amplifying progenitors independently of Type-1 cells, or perhaps the Type-1 cell is not the SGZ stem cell. Clearly, our findings here call for intense research on the identity of the adult neural stem cell and the contribution it makes to adult neurogenesis.

Caveats

While we limited our assessment of neurogenesis to YFP+ cells, we have not been able to demonstrate that these cells no longer express Notch1. Recombination at the *R26R-YFP* locus does not indicate that both *notch1* alleles are recombined. We attempted to ensure that recombination was as efficient as possible by administering TAM for 6 days. Certainly the difference in YFP+ cell dynamics in the two genotypes suggests that there is an effect of Notch1 removal. However, it could be that without Notch1, YFP+ cells die or rapidly differentiate, and the remaining YFP+ cells that we examine at extended times post-TAM are WT. We have attempted to demonstrate efficient recombination by IHC for Notch1 or NICD in YFP+ cells in both WT and Notch1 iKO mice;

however, we have not been able to demonstrate specificity of currently available antibodies for Notch1. Isolation of DNA from neurospheres generated from the SVZ of WT and Notch1 iKO mice indicates that genomic recombination occurs at the *notch1* locus only in Notch1 iKO mice, but it does not correlate YFP expression with Notch1 ablation. Studies utilizing techniques to enrich the YFP+ population (e.g. FACS or laser capture followed by RT-PCR) are under way to determine if Notch1 ablation is faithfully reported by YFP expression.

By the nature of its cell surface expression and requirement of cell-cell interaction, ablation of Notch1 could have both cell-intrinsic and -extrinsic effects. Our studies here focused on the YFP+ population, and presumably the cell-intrinsic effect of Notch1 signaling. However, assessment of the total number of Ki67+ cells in the hippocampus suggests there may be both intrinsic and extrinsic effects of manipulating Notch1. For example, the fact that the total number of Ki67+ cells in the SGZ did not differ between genotypes suggests that YFP-, and presumably unrecombined, cells are able to proliferate and compensate for fewer YFP+Ki67+ cells in Notch1 iKO mice.

Finally, neurogenesis differs across the developmental stage of the animal, with Nestin-expressing cells in embryonic, postnatal and adult neurogenesis having unique characteristics. Adolescence marks a period of transition from developmental to adult neurogenesis. In mice, adolescence and sexual maturity occurs between 3-8 weeks of age (Hayashi et al., 2008). During this same time,

the magnitude of net neurogenesis in the SGZ decreases dramatically, suggesting that neurogenesis is sensitive to this critical developmental period. Therefore, one could argue that neural stem cells in this transitional period may possess different characteristics than stem cells present later in life. While it is clear that the magnitude of neurogenesis in the hippocampus declines with age (Cameron and McKay, 1999; Garcia, et al., 2004; Rao, et al., 2006; Alhenius, et al., 2009), the differentiation potential of stem cells in the aged versus young hippocampus is less clear. Some report that neuronal differentiation decreases with age (van Praag, et al., 2005), while others report no change (Rao, et al., 2006; Alhenius, et al., 2009). Indeed there are differences in expression of genes important for stem cell function and differentiation in young adult versus aged stem cells (Garcia, et al., 2004; Alhenius, et al., 2009), and yet young adult and aged stem cells have similar differentiation *in vitro* (Alhenius, et al., 2009). While we assessed neurogenesis of YFP+ cells in young adult mice (7-19 weeks old), we induced recombination in adolescent mice (4-5 weeks old). We utilized this transitional period to label the maximum number of neural stem cells and their progeny in the young adult. For example, TAM injection between 4 and 6 weeks of age gives rise to approximately 10,000 YFP+ immature neurons at 9-10 weeks of age (Chapters 2 and 3), while TAM injection at 10 weeks of age results in about half the number of labeled cells (personal communication, N.A. DeCarolis). In interpreting the results in this thesis, it is important to remember that Nestin-expressing cells at 4-5 weeks of age may have different characteristics and potential than Nestin-expressing cells >8 weeks of age and the observed

phenotype here could represent an adolescent or transitional phenotype that may be different from that observed later in adult life. Studies are currently underway in the Eisch lab to determine the potential age-related differences in Nestin-expressing neural stem cells.

Conclusion

This study is the first quantifiable assessment of how intrinsic Notch1 signaling in Nestin-expressing stem-like and progenitor cells affects their contribution to adult SGZ neurogenesis over an extended period of time and to physical activity-dependent neurogenesis. This is the first study to demonstrate that a specific gene regulates the number of Type-1 cells in the adult SGZ. While others have reported that a particular gene or pathway is required for activity-dependent neurogenesis (Fabel et al., 2003; Kitamura et al., 2003; Hunsberger et al., 2007; Koehl et al., 2008; Li et al., 2008; Trejo et al., 2008; Ma et al., 2009), this is the first study to demonstrate that a gene is required for basal but not physical activity-dependent proliferation.

We propose a model where Notch1 maintains a baseline level of neurogenesis by permitting proliferation of Nestin-expressing cells (Figure 3.10A). Without Notch1, Nestin-expressing cells exit the cell cycle and differentiate into neurons. Self-renewal and expansion of Nestin-expressing cells is disrupted and the net number of new neurons is decreased (Figure 3.10B). Running increases new neurons in WT and Notch1 iKO mice by increasing progenitor proliferation

possibly through the upregulation of growth and neurotrophic factors (Figure 3.10C-D). Our findings suggest that Notch1 plays a permissive role to maintain a minimum level of proliferation in the adult SGZ. Our findings that Notch1 is not necessary for proliferation after running urge further research on the distinct mechanisms that regulate SGZ proliferation in basal and activity-dependent neurogenesis.

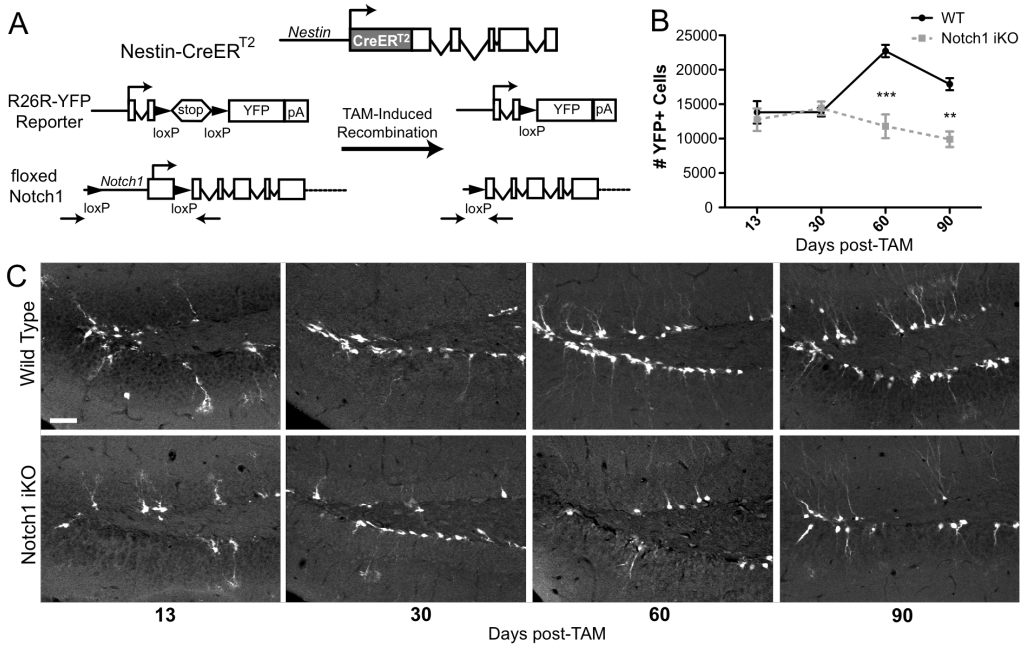

Figure 3.1. Ablation of Notch1 from Nestin-expressing adult neural progenitors decreases YFP+ cell number in the SGZ.

***A*:** Schematic of the 3 mouse lines used to generate Notch1 iKO mice following tamoxifen (TAM)-induced genomic recombination. Arrows indicate the primers used to verify genomic recombination of the *notch1* locus by PCR.

***B*:** Quantification of total YFP+ cell number in the SGZ. *C*: Representative confocal images of YFP+ cells in the SGZ of WT and Notch1 iKO mice at indicated days post-TAM. $^{**}p<0.01$ and $^{***}p<0.001$ vs. WT, Bonferroni *post-hoc*; n=5-11 per group. Scale bar in *C*, 50 μm.

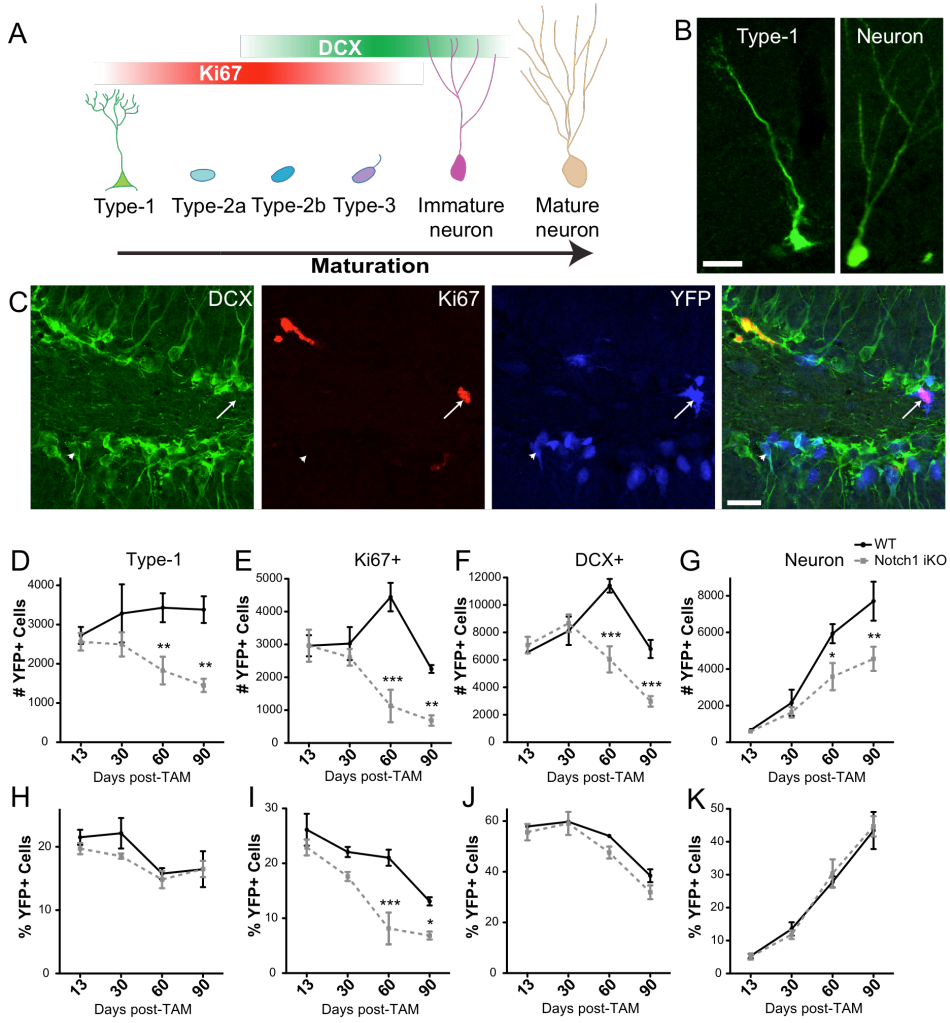

Figure 3.2. Ablation of Notch1 in Nestin-expressing cells impairs proliferation and reduces neurogenesis.

***A*:** Schematic of stages of granule cell development in the adult SGZ. Type-1 cells are the putative stem cell. Ki67 is expressed by transit-amplifying progenitor cells, while doublecortin (DCX) is expressed in neuroblasts and immature neurons. ***B*:** Representative confocal images of YFP+ Type-1 and neuron morphology with the granule cell layer. The dendrites of the neuron extend off the

image here and into the molecular layer (not shown). ***C:*** Representative confocal image of DCX, Ki67 and YFP in the SGZ 60 days post-TAM. The arrow indicates a YFP+Ki67+ cell and the arrowhead indicates a YFP+DCX+ cell. ***D:*** Quantification of number YFP+ cells with Type-1 morphology. ***E*****:** Quantification of number Ki67+/YFP+ cells. ***F*****:** Quantification number of DCX+/YFP+ cells. ***G*****:** Quantification number of YFP+ cells with neuronal morphology. ***H:*** Quantification of proportion of YFP+ cells with Type-1 morphology. ***I*****:** Quantification of proportion of Ki67+/YFP+ cells. ***J*****:** Quantification of proportion of DCX+/YFP+ cells. ***K*****:** Quantification of proportion of YFP+ cells with neuronal morphology. **p*<0.05, ***p*<0.01 and ****p*<0.001 vs. WT, Bonferroni *post-hoc*; *n*=3-7 per group. Scale bars in *B* and *C*, 20 μm.

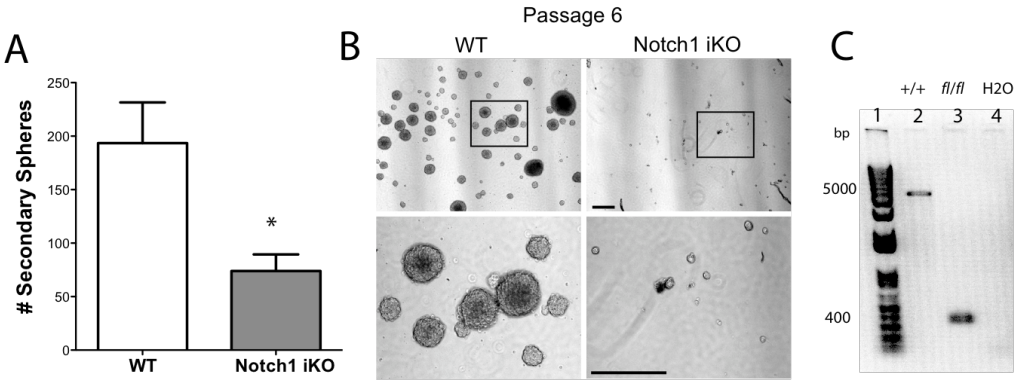

Figure 3.3. Neurosphere generation from SVZ NPCs is impaired in Notch1 iKO mice.

***A*:** Quantification of secondary spheres generated per SVZ in WT and Notch1 iKO mice. Primary spheres were harvested 40 days post-TAM. ***B*:** Representative images from passage 6 neurosphere cultures of SVZ NPCs in WT and Notch1 iKO mice. Bottom row is a closer view of the boxed region in the image above. Scale bar, 100 μm. ***C*:** PCR of genomic DNA from passage 4 neurosphere cultures of SVZ NPCs in WT (+/+) and Notch iKO (*fl*/*fl*) mice.

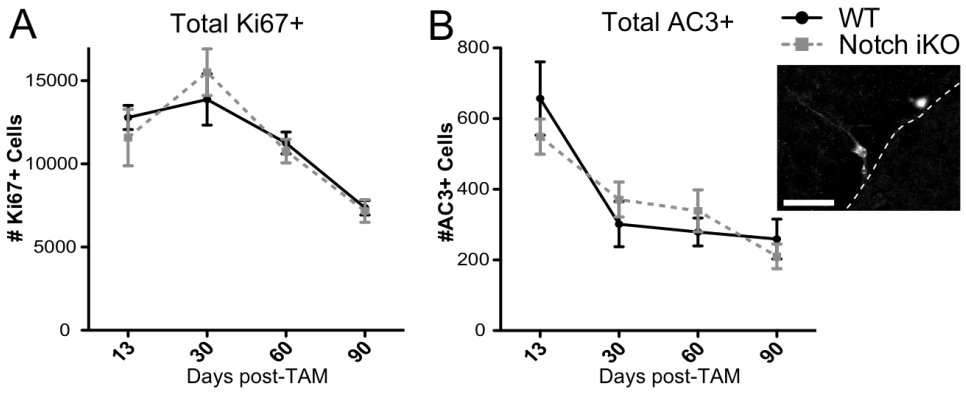

Figure 3.4. Notch1 ablation does not affect total SGZ proliferation or death.

***A*:** Quantification of the total number (YFP+ and YFP-) of Ki67+ cells in the SGZ.

***B*:** Quantification of the total number (YFP+ and YFP-) of activated caspase-3 (AC3)+ cells in the SGZ. The inset shows a confocal image of two representative AC3+ cells. Scale bar, 20 μm. *n*=4-9 per group.

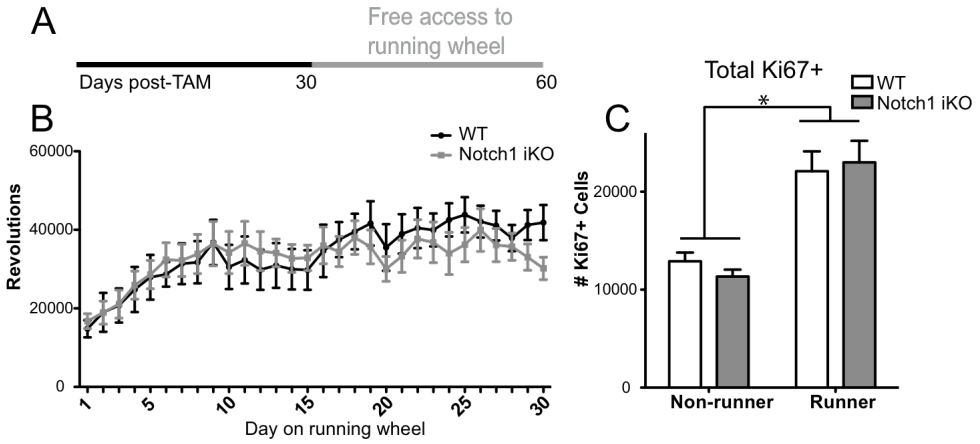

Figure 3.5. Both WT and Notch1 iKO mice respond to 30 days of voluntary physical activity.

A*:** WT and Notch1 iKO mice ran for 30 days, beginning 30d post-TAM and were perfused 60 day post-TAM. ***B: Quantification of revolutions per day in WT and Notch1 iKO runner mice. ***C*:** Quantification of total Ki67+ cells in the SGZ of WT and Notch1 iKO non-runner and runner mice. *n*=5-11 per group.

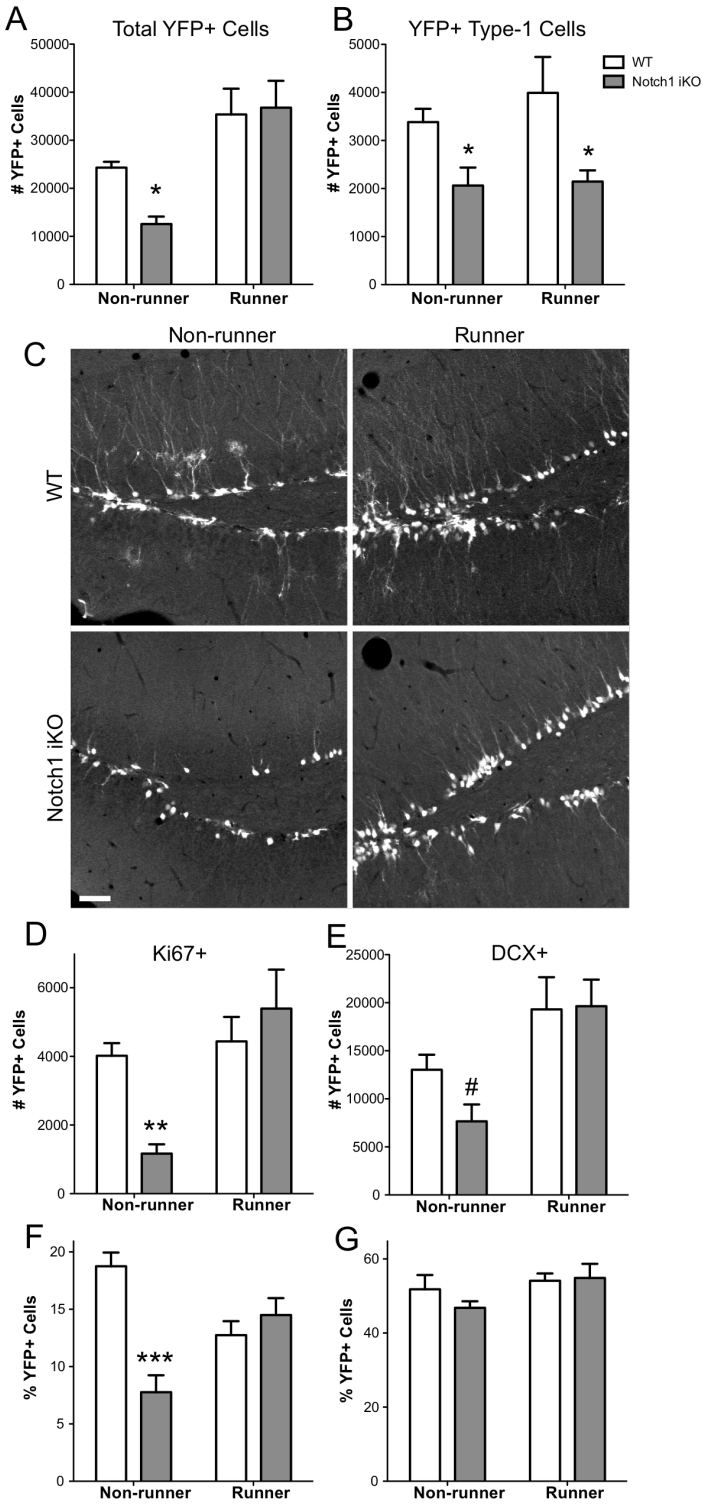
A
Total YFP+ Cells
YFP+ Cells
50000
40000
30000
20000
10000
0
*
Non-runner
Runner
B
YFP+ Type-1 Cells
WT
Notch1 iKO
YFP+ Cells
5000
4000
3000
2000
1000
0
*
*
Non-runner
Runner
C
Non-runner
Runner
WT
Notch1 iKO
D
Ki67+
YFP+ Cells
6000
4000
2000
0
**
Non-runner
Runner
E
DCX+
YFP+ Cells
25000
20000
15000
10000
5000
0
#
Non-runner
Runner
F
% YFP+ Cells
20
15
10
5
0
***
Non-runner
Runner
G
% YFP+ Cells
60
40
20
0
Non-runner
Runner

Figure 3.6. 30 days of running rescues proliferation and neurogenesis but not Type-1 cells in Notch1 iKO mice.

***A*:** Quantification of total YFP+ cells in SGZ of WT and Notch1 iKO mice after 30 days of running. ***B*:** Quantification of total YFP+ Type-1 cells in SGZ of WT and Notch1 iKO mice after 30 days of running. ***C*:** Representative confocal images of YFP+ cells in the SGZ of WT and Notch1 iKO non-runner and runner mice 60 days post-TAM. Scale bar, 50 μm. ***D*:** Quantification of number YFP+Ki67+ cells. ***E*:** Quantification number of YFP+DCX+ cells. ***F*:** Quantification of proportion of YFP+ cells that are Ki67+. ***G*:** Quantification of proportion of YFP+ cells that are DCX+. *p<0.05, **p<0.01 and ***p<0.001 vs. WT, Bonferroni *post-hoc*. n=4-11 per group.

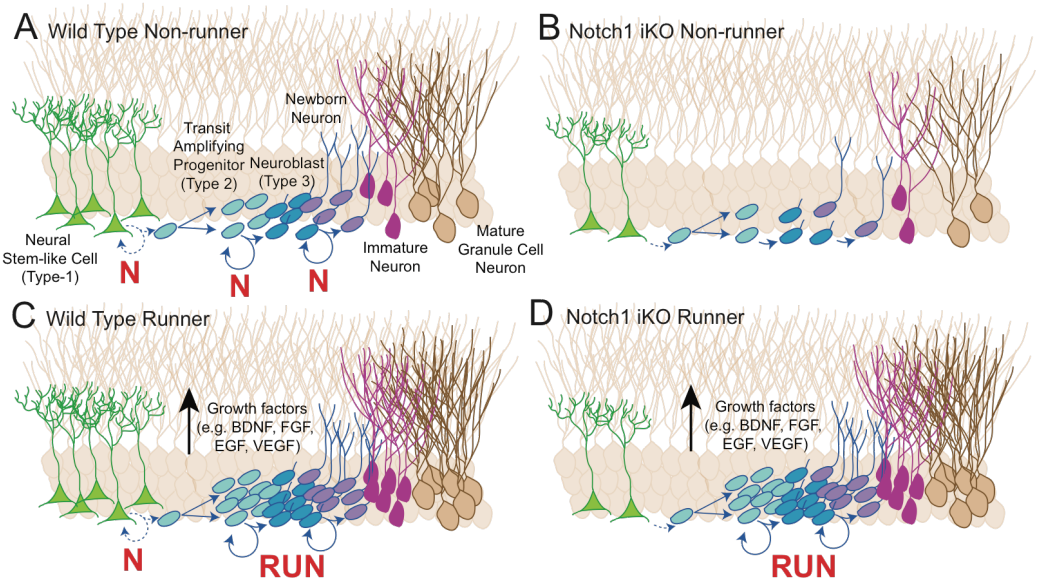

Figure 3.7. Working model of the role of Notch signaling in the adult SGZ at baseline and after running.

A: At baseline, Notch1 signaling (red "N") maintains a stable pool of Type-1 and progenitor cells. ***B:*** Ablation of Notch1 leads to an inability to maintain Type-1 cells and keep progenitors in the cell cycle, resulting in decreased neurogenesis. ***C:*** Running promotes proliferation of progenitors and results in increased net neurogenesis. ***D:*** In Notch1 iKO mice, running increases proliferation and rescues neurogenesis, but does not restore Notch1-dependent maintenance of Type-1 cells.

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CHAPTER FOUR

Inducible activation of Notch1 in adult hippocampal stem/progenitor cells

Introduction

We have found that ablation of Notch1 in Nestin-expressing cells in the adult subgranular zone (SGZ) leads to decreased neurogenesis, primarily via decreased proliferation (Chapter 3). Several studies indicate that Notch1 can both promote and inhibit proliferation (Ishikawa et al., 2008; Boggs et al., 2009; Guo et al., 2009), and that the outcome of Notch1 signaling is dose-dependent (Guentchev and McKay, 2006), context-dependent (Wilson and Radtke, 2006; Kopan and Ilagan, 2009) and temporally-dependent (Kageyama et al., 2008; Shimojo et al., 2008). For example, during central nervous system development, sustained expression of Hes1, an essential downstream effector of Notch1, inhibits proliferation and differentiation of neural progenitors by downregulating proneural bHLHs and Notch1 ligands (Baek et al., 2006). However, oscillatory expression of Hes1, as seen in proliferating progenitors, maintains Notch signaling and proliferation, while ablation of Hes1 leads to neuronal differentiation of progenitors (Shimojo et al., 2008). The adult brain provides a vastly different context than that of development. It is unclear if Notch signaling in the adult Nestin-expressing context differs from development.

Recent literature suggests that Notch1 also promotes proliferation in the adult central nervous system, as overexpression of the active intracellular domain of

Notch1 (NICD) in GFAP+ radial glia increases SGZ proliferation (Breunig et al., 2007). Furthermore, NICD overexpression prevents cell cycle exit and differentiation of dividing SGZ progenitors, while loss of Notch1 increases exit and promotes differentiation. These complementary findings suggest that Notch1 signaling in the context of GFAP+ cells in the adult SGZ is dose-dependent, but not temporally-dependent. While Breunig and colleagues demonstrated that inhibition of cell cycle exit was accompanied by reduced neuronal differentiation, their study did not determine the quantitative impact of NICD overexpression on adult hippocampal neurogenesis, nor is it known if neuronally-restricted Nestin-expressing cells would have the same response to overexpression of NICD as GFAP+ radial glia.

The finding that NICD promotes proliferation of adult neural progenitors is in contrast to NICD overexpression in developmental neurogenesis, where it leads to apoptosis of Nestin+ stem cells (Yang et al., 2004). Perhaps the differing results in these two studies are due to differences in the recombined population, as Nestin+ stem-like cells in the adult may represent a unique subset of GFAP+ neural stem cells with distinct characteristics (Pevny and Rao, 2003; Kempermann et al., 2004; Steiner et al., 2006; Graf and Stadtfeld, 2008) or perhaps it is due to age, as the pluripotency of Nestin-expressing cells changes with age (Battiste et al., 2007; Lagace et al., 2007). Either scenario could provide a distinct context for Notch1 signaling (Wilson and Radtke, 2006) with a distinct outcome. The ability of NICD to promote apoptosis in the adult SGZ is unknown.

We hypothesized that iNICD mice would have disrupted neurogenesis, due to several possible outcomes. First, iNICD might have fewer YFP+ cells because these cells are not able to proliferate or differentiate because of sustained Notch1 activation (Shimojo et al., 2008). Second, there could be more YFP+ proliferating progenitors, but fewer differentiated YFP+ cells (Breunig et al., 2007). Third, there could be no YFP+ cells in iNICD mice due to apoptosis (Yang et al., 2004). To test our hypothesis, we generated Nestin-CreERT2/R26R-YFP/NICD mice (inducible NICD mice or iNICD), to overexpress the active intracellular domain of human Notch1 (NICD) and evaluated the number of YFP+ cells at increasing intervals post-TAM using the same paradigm as before (Chapter 3). While these experiments require additional animals to reach a clear conclusion, the pilot data presented here suggest that like Notch1 deletion, NICD overexpression can robustly influence adult hippocampal neurogenesis.

Materials and Methods

Nestin-creERT2/R26R-YFP/NICD (iNICD) transgenic mice and tamoxifen administration

Animals were housed in a specific pathogen-free Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC)-approved facility at UT Southwestern on a 12-h light/dark cycle. All animal use procedures and husbandry were in strict accordance with the National Institutes of Health and Guide for the Care and Use of Laboratory Animals, and were approved by

the UT Southwestern Medical Center Animal Care and Use Committee. Care was taken to minimize the number of animals used and to diminish pain and suffering. Nestin-creER^{T2}/R26R-YFP mice (Lagace et al., 2007) were crossed with hemizygous floxed-stop NICD mice (C57BL/6J-Tg(ACTB-NOTCH1)1Shn/J, JAX Stock#: 006481), both maintained on a C57BL/6J background. The human Notch1 intracellular domain (NICD) (amino acid residues 1762–2304; including the RAM23 domain, CDC10/Ankyrin repeats, and two nuclear localization signals), as well as a SV40 late polyadenylation signal was inserted downstream of a chicken beta-actin-*loxP*-stop-*loxP* sequence. The resulting transgenic mice carry 10-20 copies of the transgene at a single insertion site. WT and NICD mice (4-5 weeks old) were injected daily for 5 days with 180 mg/kg, i.p. tamoxifen (30 mg/ml in 10%EtOH/sunflower oil; Sigma). Tamoxifen lethality was <5% at this age, dose and duration. Mice were perfused 13, 30, 60 or 90 days after the last tamoxifen injection (apporoximately 10 mice of each genotype/time point).

Immunohistochemistry (IHC)

Tissue Sectioning and Preparation. Mice were anesthetized and transcardially perfused with cold 0.1M PBS for 5 minutes, followed by 4% paraformaldehyde (PFA) for 15-20 minutes. Brains were extracted and post-fixed overnight at RT in the same fixative before cryoprotection in 30% sucrose with 0.1% NaN_3 at RT overnight. Brains were stored at 4°C in the same cryoprotection solution until sectioning on a freezing microtome. Brains were sliced coronally 30µm thick and stored free-floating in 1x PBS with 0.1% NaN_3 at 4°C until stained.

Antibodies. The following primary antibodies were used: rabbit polyclonal anti-GFP (1:3000; Invitrogen, Eugene, OR, Cat# A11122), chicken polyclonal anti-GFP (1:500, Aves Labs, Tigard, OR, Cat# GFP-1020), rabbit monoclonal anti-Ki67 (1:500, Lab Vision/NeoMarkers, Thermo Fisher Scientific, Fremont, CA, Cat# RM-9106-S), rabbit polyclonal anti-cleaved caspase-3 (1:500, Cell Signal, Danvers, MA, Cat# 9661), and goat polyclonal anti-DCX (1:100, Santa Cruz Biotechnology, Santa Cruz, CA, Cat # sc-8066).

IHC. Staining was performed as previously described (Lagace et al., 2007). Briefly, sections were mounted on slides and incubated in 0.01M citric acid (pH 6.0, 100°C) for 15 min for antigen retrieval. Following antigen retrieval, sections were incubated with blocking solution (3% normal donkey serum, 0.3% Triton X-100 in TBS) for ≥20 min. Sections were then incubated with primary antibodies in carrier (3% normal donkey serum, 0.3% Tween20 in TBS) overnight at RT. Antibody staining was revealed using either species-specific fluorophore-conjugated secondary antibodies (1:200 in TBS, Cy2, Cy3, and Cy5, Jackson ImmunoResearch) or detected with biotinylated secondary antibodies (1:200 in TBS, Jackson ImmunoResearch) and revealed using ABC Elite kit (Vector Laboratories, Burlingame, CA, Cat# PK-6100) followed by fluorescein-conjugated TSA Renaissance fluorescent amplification kit (1:50, PerkinElmer Life Sciences, Boston, MA, Cat# NEL701). To remove endogenous peroxidase activity, sections were incubated with 0.03% H_2O_2 for 30 min before ABC and TSA. Slides were counterstained with DAPI (1:5000, Roche Applied Science, Indianapolis, IN, Cat# 236276) before they were dehydrated and coverslipped with DPX (Sigma-

Aldrich, Cat# 44581). Omission of primary or secondary antibodies resulted in no staining and served as a negative control.

Quantification of YFP+ cells

Quantification of immunoreactive hippocampal cells was performed with an Olympus BX-51 microscope (400x) as previously described (Mandyam et al., 2004; Donovan et al., 2006; Lagace et al., 2007; Lagace et al., 2008). Briefly, an observer blind to experimental groups counted immunoreactive cells of every 9th 30µm coronal section throughout the SGZ (-0.82 mm to -4.24 mm from bregma) via the optical fractionation method.

Statistical analysis

All data are presented as mean±SEM and were subjected to two-way ANOVA, followed by Bonferonni *post-hoc* test. Statistical significance was set at $p<0.05$.

Results

iNICD mice display TAM-induced recombination in neurogenic brain regions

Inducible NICD transgenic mice were generated by crossing hemizygous NICD mice with Nestin-CreER^{T2}/R26R-YFP mice. In iNICD mice, TAM treatment leads to Cre-ER^{T2} translocation into the nucleus and excision of the STOP codons in both YFP and NICD cassettes, allowing for subsequent YFP and NICD expression (Figure 4.1). There was no appreciable number of YFP+ cells in non-neurogenic regions, such as the cortex, thalamus, and cerebellum. YFP+ cells

were limited to the SVZ, SGZ (Figure 4.1) and corpus callosum, consistent with other studies using this Cre-driver line (de Chevigny et al., 2008; Lagace et al., 2008), including my own (see Figures 2.1, 2.2 in Chapter 2 and Figures 3.1, 3.2 in Chapter 3).

iNICD mice do not have reduced YFP+ neurogenesis

To determine the dose-dependent role of Notch1 signaling in adult Nestin-expressing NSCs, we used the same experimental paradigm as for the Notch1 iKO mice (see Chapter 3): 4-5 week old mice were injected with TAM (180 mg/kg/d, i.p., 5 days) and perfused 13, 30, 60 or 90 days post-TAM. 30 days post-TAM, WT and iNICD mice do not differ in the number of YFP+ cells in the SGZ, while 60 days post-TAM, iNICD mice have almost no YFP+ cells (Figure 4.2). However, iNICD animals at 90 days have the same number of YFP+ cells as WT littermates. Our findings, however, did not reach statistical significance due to a large amount of variation within both genotypes, especially 90 days post-TAM (genotype *X* time interaction, $F_{2,32}$=0.31, *p*=ns; genotype, $F_{1,32}$=1.54, *p*=ns). Because of the large degree of variability in the data 90 days post-TAM, we verified the genotype of each animal. PCR from brain tissue confirmed that our original tail-snip genotyping was correct. We further examined whether litter or gender had any effect on the number of YFP+ cells and found no correlation in either WT or NICD animals. To confirm that recombination was effective, we stained for NICD. However, we did not observe any staining above endogenous levels in iNICD mice (data not shown). Thus, to account for the large degree of

variability in these studies and to clarify the results, more animals should be examined. Together the pilot data suggest that NICD overexpression in Nestin-expressing cells in the adult SGZ does not affect neurogenesis.

iNICD mice do not have increased cell death in the SGZ

Several studies have indicated that Notch1 regulates neural stem cell survival and death (Androutsellis-Theotokis et al., 2006; Ishikawa et al., 2008; Boggs et al., 2009). Overexpression of NICD in Nestin-expressing cells in the embryo leads to neural stem-cell apoptosis (Yang et al., 2004). To investigate whether NICD overexpression in adult Nestin-expressing SGZ cells increases cell death, we counted activated caspase-3 (AC3+) cells in the SGZ of WT and iNICD littermates. We found no difference in the number of AC3+ SGZ cells between genotypes at 30 or 60 days post-TAM (Figure 4.3, genotype *X* time interaction, $F_{1,15}$=0.68, *p*=ns; genotype, $F_{1,15}$=0.008, *p*=ns). If apoptosis was responsible for the apparent decline in YFP+ cells in iNICD mice 60 days post-TAM (Figure 4.2), we would expect to see an increase in AC3+ cells, either before or at 60 days post-TAM. Intriguingly, the number of AC3+ cells was quite low at 60 days post-TAM in both genotypes. Since we did not observe an increase in apoptotic cells, our data suggests that NICD overexpression in Nestin-expressing stem and progenitor cells in the adult SGZ does not induce cell death; however, further research is needed to clarify this finding and the phenotype of iNICD mice. Should iNICD mice have increased apoptosis of Nestin+ stem-like cells in the

adult SGZ, they would be a valuable tool for studying inducible ablation of neurogenesis and the functional contribution of Nestin-expressing stem-like cells.

Discussion

We have previously demonstrated that Notch1 is a critical positive regulator of proliferation of Nestin-expressing cells in the adult SGZ (Chapter 3). Several studies indicate that regulation of the cell cycle by Notch1 is dose-dependent (Guentchev and McKay, 2006; Wilson and Radtke, 2006), so to investigate the dose-dependent role of Notch1 in adult hippocampal neurogenesis, we generated iNICD mice and utilized the same experimental paradigm as for Notch1 iKO mice (Chapter 3). These mice provide a valuable tool in which we can overexpress the active intracellular domain of human Notch1 and examine its effects on proliferation, differentiation and survival in the discrete stages of neurogenesis over a period of young adulthood.

We hypothesized that iNICD mice would have disrupted neurogenesis, due to several possible outcomes. More proliferating progenitors (Breunig et al., 2007) should lead to more YFP+ cells in the SGZ; however, we found no change in the number of YFP+ cells between genotypes. Both apoptosis (Yang et al., 2004) and inhibition of proliferation (Baek et al., 2006; Shimojo et al., 2008) would lead to fewer YFP+ cells. While we did observe a transient decrease in YFP+ cells in iNICD mice at 60 days post-TAM, it was not statistically significant, and YFP+

cells appear to recover 90 days post-TAM. Increased apoptosis might explain why we observed so few YFP+ cells in iNICD mice 60 days post-TAM, yet total number of AC3+ cells was not increased at any time in iNICD mice. However, the window in which we can detect dying cells is extremely small (Harburg et al., 2007), so it is possible that we simply have missed the critical time point.

Due of the large degree of variability in both WT and iNICD mice, it is difficult to make any conclusions about the dynamics of neurogenesis in these mice from the present data. To account for variability, we are examining more animals. Perhaps examination of an earlier time, such as 13 days post-TAM, would reveal increased apoptosis or proliferation that has normalized by 60 and 90 days post-TAM. In fact, this is a possible explanation for some degree of variability. Perhaps NICD overexpression does induce apoptosis of Nestin-expressing cells, leading to fewer cells, and yet it is possible that some cells survive or are not recombined at the *NICD* transgene. Differences in the number of surviving cells at early times post-TAM could lead to even greater disparity at later times as progenitors mature. If there were enough remaining YFP+ cells, they could account for the normalization of YFP+ cell number at 90 days post-TAM. However, this seems unlikely, as a large degree of variability is also present in WT littermates, suggesting that it is not linked to NICD overexpression.

The preliminary data here provide compelling evidence that Notch1 signaling in the adult SGZ may be dose-dependent, and they evoke many more questions.

For example, NICD overexpression in the adult SGZ might differentially affect the distinct stages of neurogenesis. Disruption of Notch signaling oscillation seems to inhibit proliferation of progenitors and push them to a quiescent stem cell state, while ablation also leads to defects in proliferation, due to massive differentiation (Kageyama et al., 2008; Shimojo et al., 2008). If NICD overexpression induces apoptosis, is it limited to a discrete stage, or does it affect all stages equally? If NICD does not cause cell death, do the remaining cells differentiate? If so, do they become neurons, or is NICD overexpression sufficient to change the fate of Nestin-expressing cells in the adult SGZ? Likewise, formation of extended passage neurospheres is impaired in neural precursors isolated from Notch1 iKO mice. If NICD overexpression promotes proliferation, as demonstrated by Bruenig et al., then neurosphere formation would be enhanced from iNICD mice. However, if NICD overexpression in Nestin-expressing cells promotes apoptosis, as demonstrated by Yang et al., then neurosphere formation would be impaired. Finally, we have demonstrated that Notch1 is not required for exercise-induced proliferation or neurogenesis (Chapter 3). If exercise-induced neurogenesis is truly Notch1-independent, then iNICD mice should demonstrate increased neurogenesis after running. However, if iNICD mice have no recombined cells remaining due to apoptosis, exercise will not be able to rescue YFP+ neurogenesis. Alternatively, NICD overexpression may promote proliferation and inhibit differentiation, leaded to decreased net neurogenesis, despite increased numbers of precursors. If this is the case, then iNICD mice may not demonstrate increased neurogenesis to running, and would suggest that exercise-induced

neurogenesis is dependent on Notch1 signaling in a dose-dependent manner.

While the results using these iNICD mice require additional animals to clarify if and how neurogenesis is changed during the three months after overexpression, it is clear that these mice represent a valuable tool for assessing the role of Notch1 in adult neurogenesis. Furthermore, if NICD overexpression does lead to apoptosis, these iNICD mice represent a novel tool for inducible ablation of adult neurogenesis and the study of the functional contribution of adult hippocampal neurogenesis.

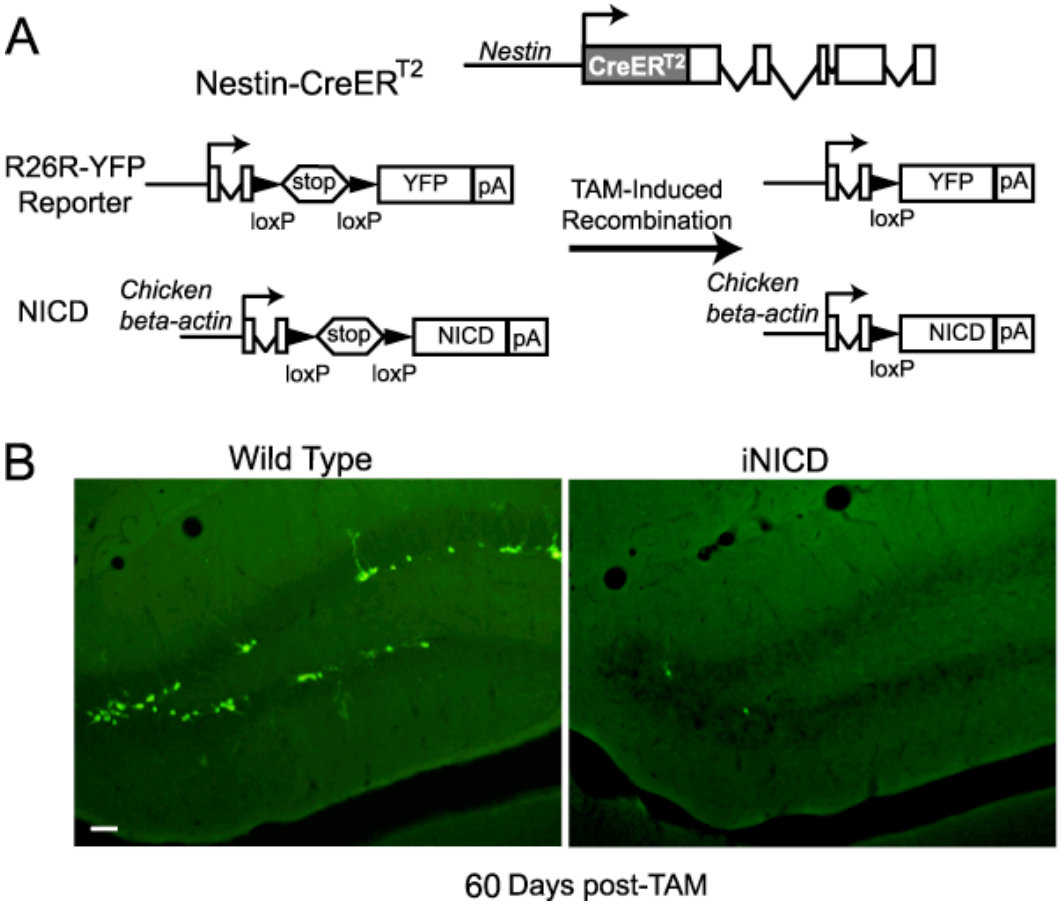

Figure 4.1. Recombined cells are limited to neurogenic regions in the adult brain of iNICD mice.

A. Schematic of the mouse lines used to generate iNICD mice **B.** Representative epifluorescent images of the DG of WT and iNICD mice 60 days post-TAM. Scale bar 20 μm.

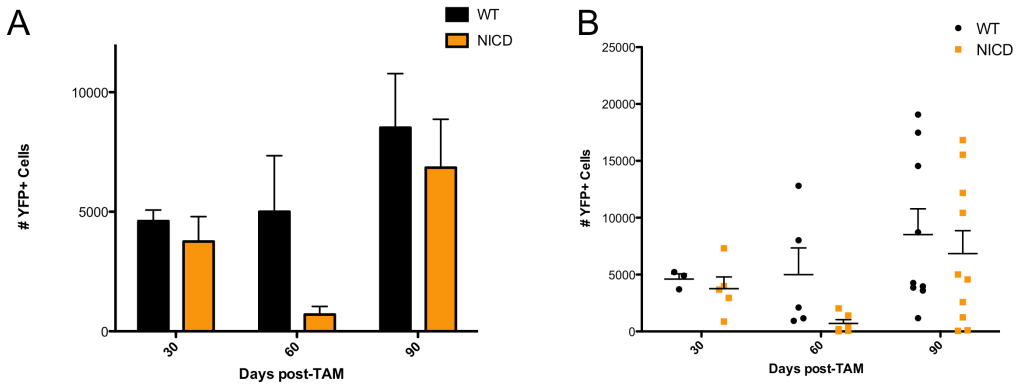

Figure 4.2. **The effect of NICD overexpression on SGZ neurogenesis is unclear.**

A. Quantification of the total number of YFP+ cells in the SGZ of WT and iNICD mice. **B.** The distribution of total number of YFP+ cells in the SGZ of WT and iNICD mice. Note the large degree of variability in both WT and iNICD groups.

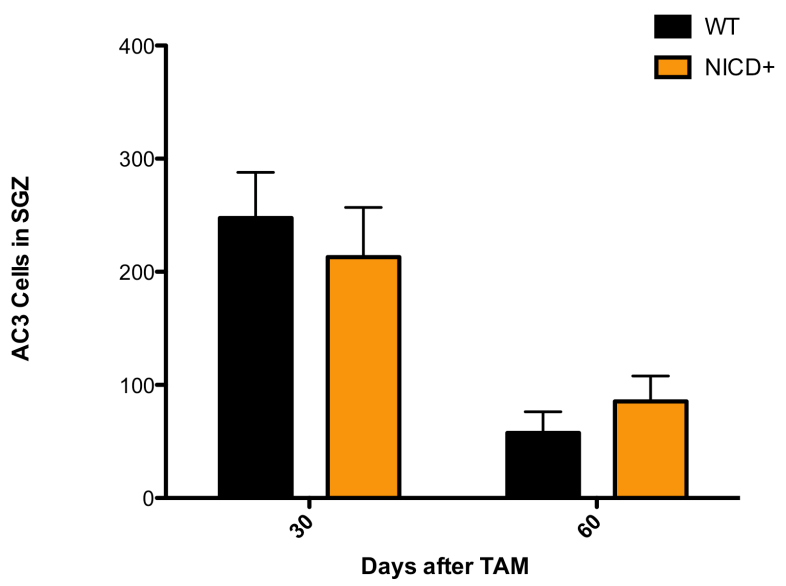

Figure 4.3. Apoptosis is not increased in iNICD mice.

Genotype has no effect on the total number of AC3+ cells in the SGZ at any time post-TAM. Note, however, the paucity of AC3+ cells at 60 days post-TAM in this line of mice.

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CHAPTER FIVE

Mood-related behavior is not affected in a mouse model of decreased neurogenesis

Introduction

Depression is a major illness, accounting for nearly 10% of primary care office visits, and is the second-leading cause of disability among young adults world-wide (Gaynes et al., 2008). Antidepressant medications, most commonly selective serotonin reuptake inhibitors (SSRIs), are the first line of treatment; however, remission rates with SSRIs or antidepressant monotherapy are only about 30% (Gaynes et al., 2008), urging the development of more effective therapies. Effective antidepressant treatments, including antidepressant drugs, exercise, and electroconvulsive therapy (ECT), all increase neurogenesis in the hippocampus (Malberg et al., 2000; Hellsten et al., 2002; Ernst et al., 2006; Eisch et al., 2008). This observation led to the development of the neurogenic hypothesis of depression, which suggests that hippocampal neurogenesis is critical for the behavioral effects of antidepressants (Santarelli et al., 2003; Sahay and Hen, 2007). Understanding the link between adult hippocampal neurogenesis and antidepressant efficacy is critical for the development of better animal models of mood disorders and more effective treatments for depression.

While there is some debate about the role of hippocampal neurogenesis in the pathophysiology of mood disorders, there is good evidence that increased

hippocampal neurogenesis is linked to behavioral response to antidepressants (Santarelli et al., 2003; Li et al., 2008; David et al., 2009). Several recent studies suggest that neurogenesis in the adult hippocampus is also linked to learning and memory, as well as mood (reviewed in Gould et al., 1999; Treves et al., 2008; Zhao et al., 2008). The addition and removal of neurons in the dentate gyrus (DG) is critical for learning and recall, especially in distinct but similar contexts (Shors et al., 2001; Dupret et al., 2007; Kempermann, 2008). Learning facilitates neurogenesis, suggesting that new neurons are functionally recruited by novelty (Leuner et al., 2006; Dalla et al., 2007; Sisti et al., 2007; Waddell and Shors, 2008). Furthermore, cognition is often impaired in depressed patients (Austin et al., 2001; Shors, 2006). In fact, one of the hallmarks of depression is memory impairment that reduces plasticity, and one could speculate, ability to cope with stress and novelty (Sapolsky, 2004). Increased neurogenesis after exercise (Bjornebekk et al., 2005; Ernst et al., 2006) or antidepressant administration (Malberg et al., 2000) could contribute to treatment of depression and cognitive improvement by increasing hippocampal and behavioral plasticity (Christie et al., 2008) and thereby facilitating recovery. However, the mechanisms underlying improved hippocampal function, especially the contribution of cell-intrinsic signaling within newborn adult DG neurons, are unknown.

I have previously demonstrated that Notch1 iKO mice have a 50% reduction in hippocampal neurogenesis (Chapter 3) and thus lack a full repertoire of new

neurons, making them a useful tool to assess the contribution of SGZ neurogenesis to hippocampal function, specifically mood. Despite the many links between neurogenesis and depression and intense effort, no studies to date have demonstrated a specific role for new neurons in depression or anxiety. Perhaps decreased neurogenesis in Notch1 iKO mice leads to impaired hippocampal plasticity and to depression-related behavior. To test this theory, we examined the behavior of Notch1 iKO and iNICD mice and their WT littermates on a variety of mood-related behaviors. However, we found that on a variety of measures of anxiety and depression, neither Notch1 iKO nor iNICD mice behaved differently than their WT littermates, suggesting that decreased neurogenesis does not underly the pathology of depression or anxiety.

Materials and Methods

Nestin-CreER^{T2}/floxed Notch1 (Notch1 iKO) and Nestin-CreERT2/NICD (iNICD) transgenic mice

Animals were housed in a specific pathogen-free Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC)-approved facility at UT Southwestern on a 12-h light/dark cycle. All animal use procedures and husbandry were in strict accordance with the National Institutes of Health and Guide for the Care and Use of Laboratory Animals, and were approved by the UT Southwestern Medical Center Animal Care and Use Committee. Care was taken to minimize the number of animals used and to diminish pain and suffering. Nestin-CreER^{T2} (Lagace et al., 2007) mice were bred with either floxed

Notch1 (Radtke et al., 1999) or floxed stop NICD (Yang et al., 2004) mice resulting in bigenic mice. Mice were genotyped by PCR using genomic DNA and primers previously published for Cre (Indra et al., 1999) and floxed Notch1 mice (Radtke et al., 1999) or NICD mice (JAX website, http://jaxmice.jax.org/pub-cgi/protocols/protocols.sh?objtype=protocol &protocol_id=1266).

Tamoxifen (TAM)

Nestin-CreER^{T2}/floxed Notch1 mice (4-5 weeks old) were administered TAM at 180 mg/kg/day for 6 days (i.p.; dissolved in 10% EtOH/90% sunflower oil) with minimal lethality (<5%). Nestin-CreER^{T2}/floxed stop NICD mice (4-5 weeks old) were administered TAM at 180 mg/kg/day for 5 days (i.p.; dissolved in 10% EtOH/90% sunflower oil) with minimal lethality (<5%). To examine the impact of TAM on hippocampal function, mice were run through a battery of behavioral assays at 30, 60 or 90 days post-TAM.

Behavioral Assays

Experimental design. Cohorts of 10-20 mice were subjected to either sucrose preference, or learned helplessness (LH), or a series of behavioral tests at 30, 60 or 90 days post-TAM. Data at each indicated time post-TAM is from a combination of 3 cohorts. The series of tests was performed in the following order, 1 test per day over 5 days: locomotion, open field, elevated plus maze (EPM), light/dark (L/D), and forced swim test (FST). In some instances (e.g. sucrose preference at 30 or 60 days post-TAM), a cohort was subjected to sucrose preference 30-60 days following the series of behavioral tests in order to minimize the number of animals used in this study (Figure 5.1). For each test

except sucrose preference, mice were habituated to the testing room for at least an hour prior to any testing and all tests were performed under red-light conditions, unless otherwise indicated. Mice were euthanized after behavioral assays. No tissue was collected as these mice did not carry the *R26R-YFP* reporter gene.

Locomotor. Locomotor testing was performed as previously described (Krishnan et al., 2008a). Briefly, mice were placed into a novel, empty standard cage within a photobeam box under red light, and beam breaks were measured in 10-minute intervals over a period of two hours by software (Photobeam Activity System, San Diego Instruments, San Diego, CA).

Open Field. Open field testing was performed as previously described (Krishnan et al., 2008a). Briefly mice were placed into the corner of a 1 x 1 m box under dim light (40 lux) conditions and allowed to freely explore for 5 min. Movement and duration spent in the periphery versus the center of the box was tracked using Ethovision software (version 3.0, Noldus, Leesburg, VA).

EPM. EPM testing was performed as previously described (Lutter et al., 2008b). Briefly, mice were placed in the center of an EPM (arms are 33 cm x 5 cm, with 25 cm tall walls on the closed arms) under red light and their behavior was tracked using Ethovision software (version 3.0, Noldus, Leesburg, VA) for 5 minutes to determine time spent in the closed and open arms, as well as the frequency of entries.

L/D. Light-dark testing was performed as previously described (Krishnan et al., 2008a). Briefly, animals were habituated to the dark side of a 2 chamber,

photobeam box for 2 minute, after which a door was opened to the brightly illuminated (100 lux) side. Mice were allowed to freely explore for 10 minutes, and behavior tracked by software that monitored beam-break (Med Associates, St. Albans, VT).

FST. FST testing was performed as previously described (Lutter et al., 2008a). Briefly, mice were tested for 6 minutes in a 4L Pyrex glass beaker containing 3L of water (24±1°C). A trained observer, blind to genotype, manually scored the last 4 minutes of the test from video of each session. Immobility was defined as no movement except for single limb paddling to maintain flotation.

LH. Learned helplessness testing was performed as previously described (Duman et al., 2008). Briefly, mice received two days of inescapable shock training (180 random foot shocks, 0.3 mA shock amplitude, 2 sec duration, 30 sec average interval) followed by one day of active avoidance testing. Mice were given 30 shuttle escape trials (0.3 mA foot shocks, 25 sec maximum duration, average interval of 30 sec). The shuttle door opened at the beginning of the shock and each trial was terminated when the mouse crossed into the adjacent "non-shock" side. Latency to escape and escape failures were recorded by the software controlling the shuttle boxes (Med Associates, St. Albans, VT).

Sucrose Preference. Sucrose preference testing was performed as previously described (Krishnan et al., 2008b). Briefly, mice were single-housed with a standard water bottle for 2 days prior to beginning the test. Mice were then given 2 50mL conical vials filled with water; the left vial designated "A" and the right "B". On the third day, both vials were filled with a 1% sucrose solution. On the fifth

day, bottle “A” was filled with 1% sucrose, and bottle “B” was filled with water. Mice were given a total of 4 days of choice between sucrose and water. Each day, the volume in each vial was marked and the position switched to control for side-bias.

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. All statistical analyses were performed using Prism (version 5.0) software. Statistical significance was defined as $p<0.05$. All statistics are presented in Tables 5.1 and 5.2.

Results

Locomotion is unaffected in inducible Notch1 transgenic mice

Because many of the tests used to assess mood-related behaviors are based on the movement of the animal (Harro, 2004; Cryan and Holmes, 2005), it was imperative to demonstrate that locomotion was not affected by disrupting Notch1 signaling in Nestin-expressing cells. We placed Notch1 iKO and iNICD, as well as their WT littermates, into normal cages within a photobeam box and measured locomotor activity over a two-hour period at 30, 60 and 90 days post-TAM. We found that both WT and inducible Notch1 mutant mice habituated to the new cage and locomotor activity decreased over the test period (Figure 5.2, see Tables 5.1 and 5.2 for all statistics). No effect of genotype was found at any time post-TAM (90 days post-TAM; not shown). From these data, we conclude that

disruption of Notch1 in Nestin-expressing cells in the adult brain for three months does not affect locomotion and will not confound further behavioral studies.

Anxiety-related behaviors are unaffected in inducible Notch1 transgenic mice

Anxiety is often co-morbid with depression and is responsive to antidepressant therapies (Bessa et al., 2009). Anxiety has also been linked to adult hippocampal neurogenesis (Bergami et al., 2008; Salam et al., 2009). For these reasons, we tested our inducible Notch1 mutant mice on three paradigms of anxiety to determine if reduced hippocampal neurogenesis was associated with increased anxiety-related behaviors. We found that Notch1 iKO mice did not have a significant behavioral phenotype at any time post-TAM on three independent measures: open field, elevated plus maze, and light/dark tests, although there was a strong trend ($p=0.08$) for an interaction of genotype and area on light/dark at 30d post-TAM (Figure 5.3). Like Notch1 iKO mice, iNICD had no overall anxiety-related behavioral phenotype, although WT and iNICD mice differed significantly ($p=0.007$) at 30d post-TAM on open field, but not on any other measure of anxiety (Figure 5.4). Together, these preliminary data suggest that a 50% reduction hippocampal neurogenesis caused by disrupted Notch1 signaling does not increase anxiety-related behavior over a period of three months following the manipulation.

Depression-related behaviors are unaffected in inducible Notch1 transgenic mice

To determine if Notch1 signaling plays any role in depression, we tested Notch1 iKO and iNICD mice on three independent measures of depression: forced swim, sucrose preference and learned helplessness tests. Each of the depression-related tests is designed to assess different aspects of depression. The forced swim and learned helplessness tests are behavioral models of despair, while the sucrose preference test can detect anhedonia (Cryan and Holmes, 2005). Similar to the anxiety-related tests, we found no statistically significant differences between genotypes on any test of depression-related behavior, although iNICD mice showed a strong trend in latency to immobility on FST ($p=0.09$; Figures 5.5 and 5.6). It is important to note, however, that the latencies we observed are quite low compared to previous reports, perhaps making interpretation difficult due to a floor effect. Together, these preliminary data indicate that a 50% reduction in hippocampal neurogenesis secondary to disrupted Notch1 signaling does not contribute to depression-related behaviors in the three months following recombination.

Discussion

The neurogenic hypothesis of depression suggests that mood-related behaviors, such as depression and anxiety, are linked to levels of adult hippocampal neurogenesis (Kempermann and Kronenberg, 2003; Sahay and Hen, 2007; Kempermann et al., 2008). We have developed a mouse model with a specific

reduction in adult hippocampal neurogenesis due to loss of stem and progenitor cells (Chapter 3). To determine if reduced neurogenesis contributes to abnormal mood-related behavior, we tested two lines of mice with inducible disruption of Notch1 signaling. There is some debate on whether hippocampal neurogenesis contributes to the pathology of depression, or if it simply facilitates recovery (Sapolsky, 2004). Here we provide preliminary data that anxiety- and depression-related behaviors are not affected by a 50% reduction in neurogenesis in inducible Notch1 mutant mice.

There are several possible explanations why we did not have any positive behavioral findings. First, there may not be any gross changes in neurogenesis. Assessment of total Ki67+ cells in the Notch1 iKO mice suggests compensation by non-recombined cells (Figure 3.4). Second, our findings confirm that decreased/ablated neurogenesis is not sufficient to produce a depression-related phenotype. The first study used X-ray irradiation to permanently and completely ablate neurogenesis and found that depression- and anxiety-related behavior was unaffected (Santarelli et al., 2003). More sophisticated genetic models of reduced (my own work; Li et al., 2008) or ablated neurogenesis (Singer et al., 2009) have provided further evidence that neurogenesis is not required for normal baseline depression-related behavior (Airan et al., 2007). On the other hand, some studies suggest that adult hippocampal neurogenesis does play a role in anxiety-related behavior (Bergami et al., 2008; Fuss et al., 2009; Salam et al., 2009). Third, timing is critical in assessing the contribution of neurogenesis to

hippocampal function. The newly born neurons have a critical period at about two weeks of age in which they are extremely plastic (Doetsch and Hen, 2005). Perhaps we did not assess our mice at the appropriate time, or perhaps we did not wait long enough. This seems unlikely, given that DCX+ recombined cells are similar in Notch1 iKO and WT mice up to 30 days post-TAM, after which they are decreased. We determined that behavior was indistinguishable at any time post-TAM, even during the critical DCX+ period of recombined cells.

Finally, perhaps a key component is missing in these studies of mood-related behavior: stress. Depression and anxiety in humans are often precipitated or worsened by stress. Glucocorticoids, including corticosterone in rodents and cortisol in humans, are released during stressful situations and are chronically elevated in depressed patients (Feder et al., 2009). The hippocampus is incredibly sensitive to glucocorticoids, and high levels are linked to reduced hippocampal volume (Bremner et al., 2000) and neurogenesis (Hellsten et al., 2002; Eisch et al., 2008). To mimic this hypercortisolemia, many animal models of depression include a component of stress (Cryan and Holmes, 2005; Berton et al., 2006; LaPlant et al., 2009) or administer corticosterone (David et al., 2009) to rodents to induce depression-related behaviors. While many of the tests that we administered to the mice were acutely stressful, especially FST and learned helplessness, the mice in this study did not experience chronic stress. Chronic social defeat is a stress-based model of anxiety and depression that is persistent and reversible upon chronic antidepressant treatment (Berton et al., 2006). This

model is unique in that it can also be utilized to assess resilience to stress (Krishnan et al., 2007). Would a larger proportion of Notch1 iKO mice display depression-related behavior after social defeat, indicating a susceptibility to stress? Future studies should assess the resilience of Notch1 iKO and iNICD mice to determine if decreased neurogenesis leads to susceptibility to stress and increased depression- and anxiety-related behaviors (Feder et al., 2009).

Perhaps the strongest piece of evidence in the neurogenic hypothesis of depression is the finding that increased hippocampal neurogenesis is linked to behavioral response to antidepressants and exercise (Santarelli et al., 2003; Bjornebekk et al., 2005; Li et al., 2008; Trejo et al., 2008; David et al., 2009; Salam et al., 2009). Future studies should determine the behavioral response to chronic antidepressant treatment or running. We have found that Notch1 iKO mice can increase neurogenesis after running (Chapter 3), which is antidepressant (Bjornebekk et al., 2005; Hunsberger et al., 2007), suggesting that these mice may still be sensitive to the behavioral effects of antidepressant treatment; however, not all behavioral responses to antidepressants are neurogenesis-dependent (David et al., 2009). Furthermore, it is important to determine if decreased neurogenesis is necessary for behavioral response in stress-based models of depression. Does stress prevent increased neurogenesis and behavioral response in Notch1 iKO mice? Understanding the relationships between stress, behavioral improvement and neurogenesis is critical for developing better treatments and prevention strategies.

Improved cognition, which increases an individuals ability to cope and adapt, is likely critical for prevention and recovery from depression. Mood, stress and cognition are intimately linked (Sapolsky, 2004; Bessa et al., 2009) and adult neurogenesis seems to lie at the intersection of these functional domains of the hippocampus. Therefore, is important to also address whether adult generated DG neurons are critical for proper memory formation. The inducible Notch1 mutant mice that we developed are the perfect tools to assess the role of neurogenesis and Notch1 in hippocampal function. Does decreased neurogenesis in inducible Notch1 mutant mice lead to reduced hippocampal plasticity, and learning and/or memory impairments? Several recent elegant genetic studies have indicated that adult neurogenesis is important for specific types of memory (Imayoshi et al., 2008; Zhang et al., 2008), often involving discrimination between two very similar contexts (Treves et al., 2008). It would be critical to dissect out the nature of any learning defect in Notch1 iKO mice. Is it specific to the stage of the affected cell? For example, would Notch1 disruption in stem and progenitor cells in the DG differ from Notch1 disruption only in mature granule cells? Notch1 is critical in mature granule cells for long-term hippocampal-dependent memory and synaptic plasticity (reviewed in Costa et al., 2005) as well as synaptic structure (Redmond et al., 2000; Salama-Cohen et al., 2006; Breunig et al., 2007). So perhaps new neurons that are deficient in Notch1 signaling do not make appropriate functional connections or are less excitable than new neurons with intact Notch1 signaling, leading to reduced plasticity and

impaired hippocampal function.

Currently, the prevailing hypothesis is that decreased neurogenesis contributes little to the actual depression-like behavior. Rather, increased neurogenesis is hypothesized to be critical for recovery (Santarelli et al., 2003). Our preliminary data supports this hypothesis, as we found no anxiety- or depression-related behaviors in inducible Notch1 mutant mice. While the most recent research suggests that not all of the behavioral effects are dependent on neurogenesis (Singer et al., 2009), it is clear that some effects are neurogenesis-dependent (David et al., 2009). Future work should assess the contribution of adult hippocampal neurogenesis in behavioral response to stress and to antidepressants. Finally, neurogenesis, mood and cognition are inter-related. In interpreting any mood-related behavioral assessments, it will be important to know if reduced neurogenesis is sufficient to disrupt learning and memory. The inducible Notch1 mutant mice that we developed represent a promising tool for the advancement of our understanding of the molecular biology of depression.

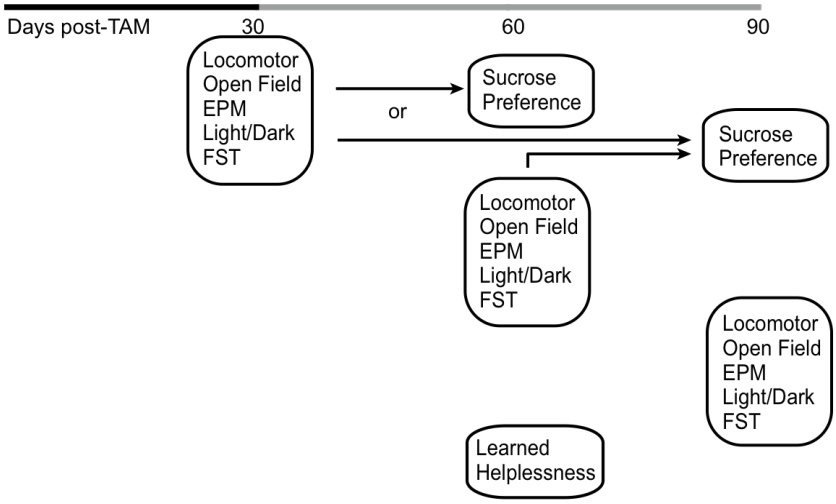

Figure 5.1. Experimental design..

Three cohorts of 10-20 mice each (box indicates the combined cohort of 30-60 animals) were assayed on several measures of anxiety and depression at a single time post-TAM: 30, 60 or 90 days. Cohorts were subjected to only one of the following: a series of behavioral tests (locomotor, open field, EPM, light/dark and FST; 1 test/day over 5 days), learned helplessness (2 days of training, 1 day of testing), or sucrose preference (4 days of training, 4 days of testing). In some cases, a cohort was administered sucrose preference 30-60 days following the behavioral series (arrow).

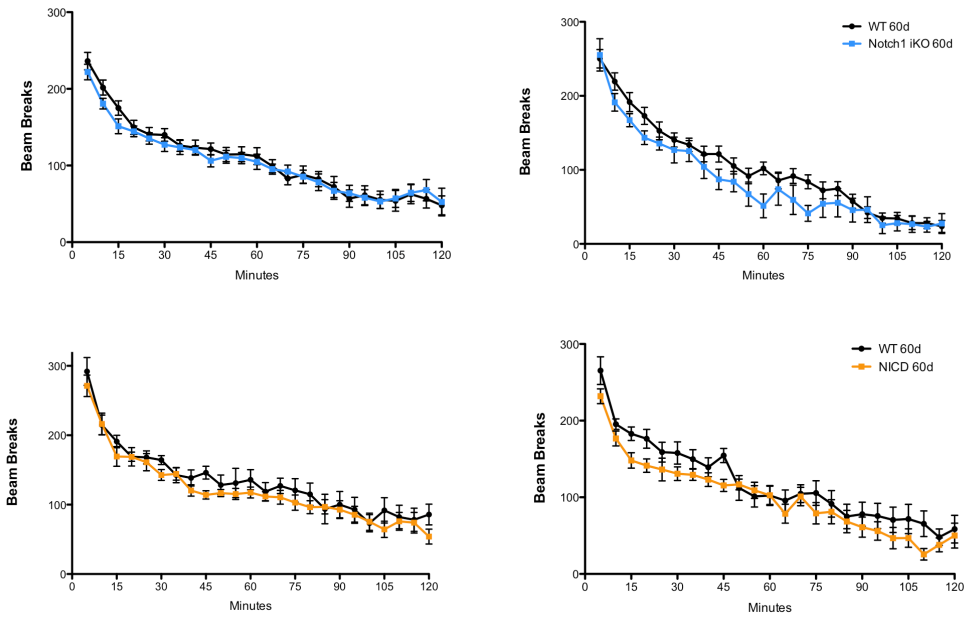

Figure 5.2. Locomotion is not affected by Notch1 mutations in Nestin-expressing cells.

Top, Locomotion of Notch1 iKO mice is not significantly different from WT littermates at 30 days (left) or 60 days (right) post-TAM. **Bottom,** Locomotion of iNICD mice is not significantly different from WT littermates at 30 days (left) or 60 days (right) post-TAM.

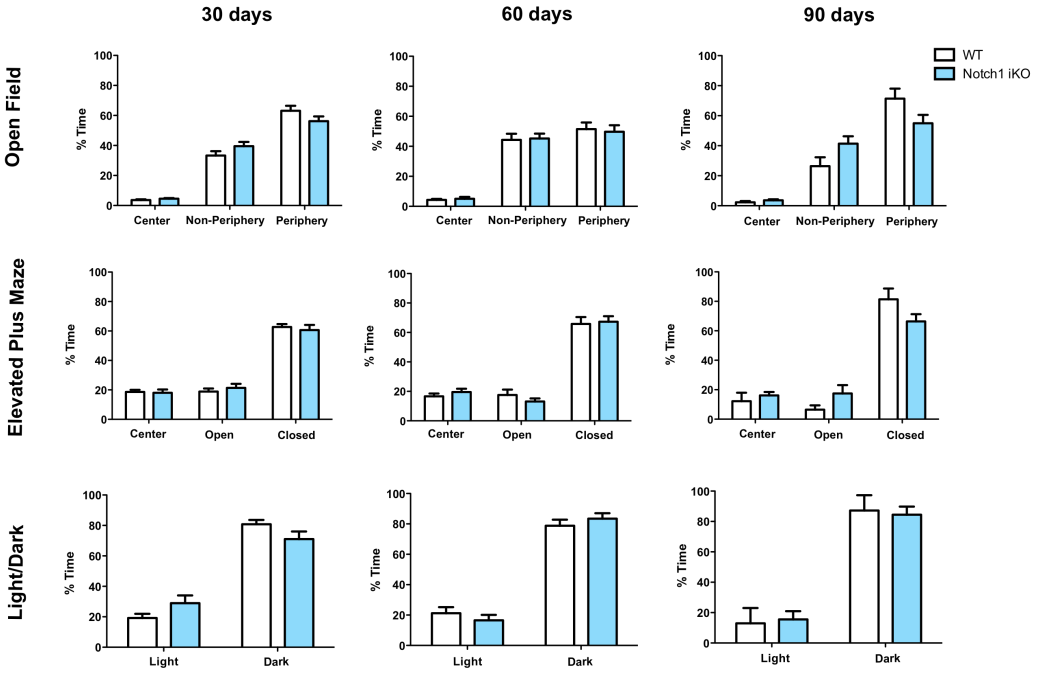

Figure 5.3. **Measures of anxiety are not changed in Notch1 iKO mice**.

Notch1 iKO behavior was not significantly different from WT littermate behavior on the open field, elevated plus maze or light/dark tests at any time post-TAM.

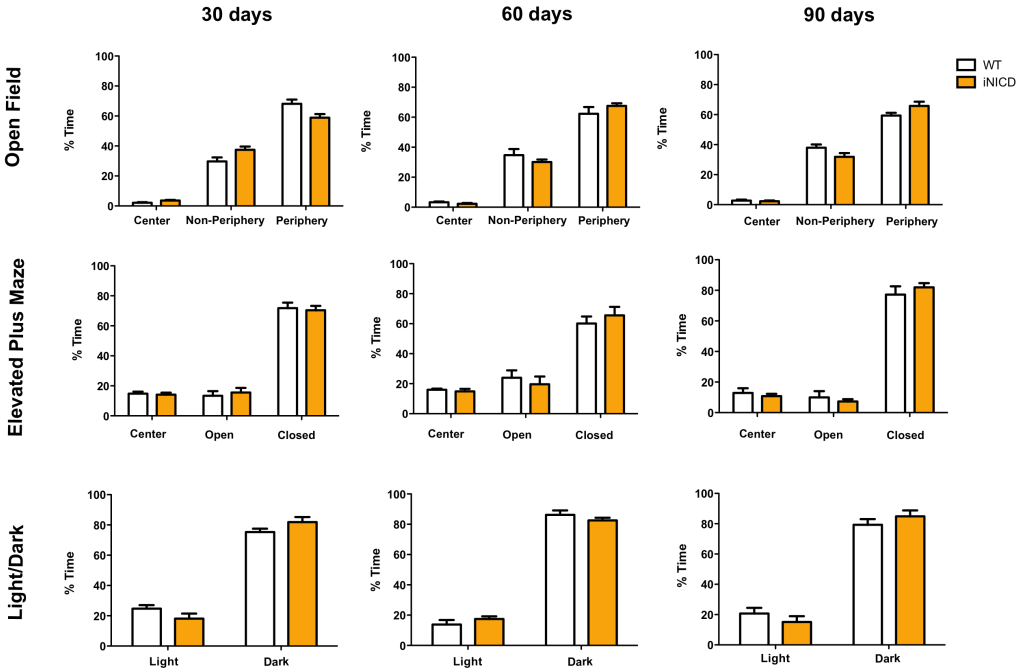

Figure 5.4. **Measures of anxiety are not changed in iNICD mice**.

iNICD behavior was not significantly different from WT littermate behavior on the open field, elevated plus maze or light/dark tests at any time post-TAM.

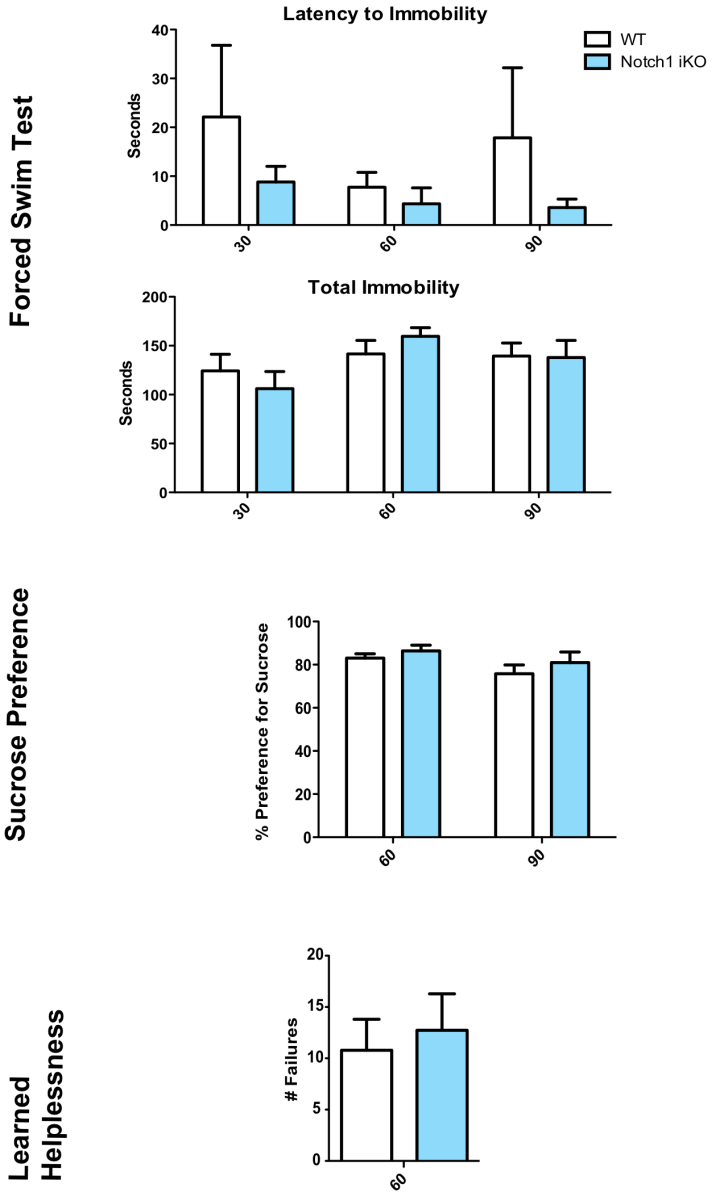

Figure 5.5. **Measures of depression are not changed in Notch1 iKO mice**. Notch1 iKO behavior was not significantly different from WT littermate behavior on the forced swim, sucrose preference or learned helplessness tests at any time post-TAM.

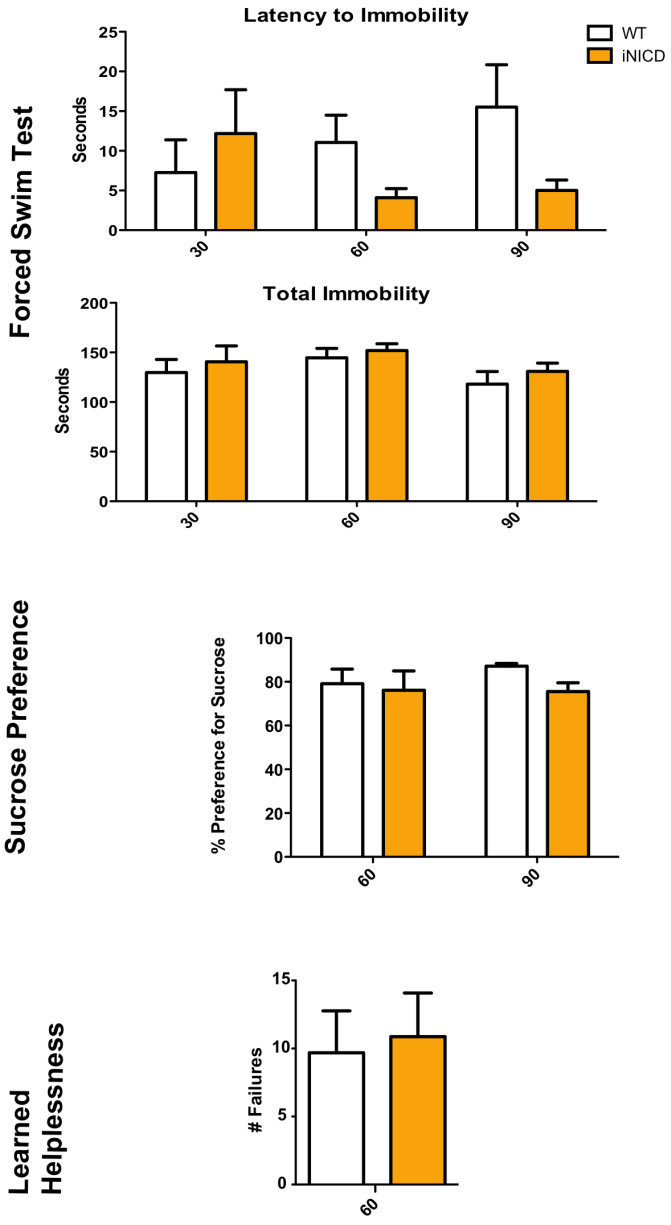

Figure 5.6. **Measures of depression are not changed in iNICD mice**. iNICD behavior was not significantly different from WT littermate behavior on the forced swim, sucrose preference or learned helplessness tests at any time post-TAM.

Group	Test	Days post-TAM	Statistics	p value
Notch1 iKO	Loco	30	Interaction: F(23,575)=0.50 Genotype: F(1,575)=0.21 Time: F(23,575)=58.16	0.96 0.65 <0.0001*
		60	Interaction: F(23,529)=1.04 Genotype: F(1,529)=2.32 Time: F(23,529)=73.76	0.41 0.14 <0.0001*
	Open Field	30	Interaction: F(2,50)=2.08 Genotype: F(1,50)=0.04 Area: F(2,50)=149.6	0.14 0.84 <0.0001*
		60	Interaction: F(2,38)=0.44 Genotype: F(1,38)=1.29 Area: F(2,38)=60.04	0.65 0.27 <0.0001*
		90	Interaction: F(2,20)=2.47 Genotype: F(1,20)=0.31 Area: F(2,20)=36.39	0.11 0.59 <0.0001*
	EPM	30	Interaction: F(2,50)=0.34 Genotype: F(1,50)=0.46 Area: F(2,50)=152.6	0.71 0.50 <0.0001*
		60	Interaction: F(2,38)=0.48 Genotype: F(1,38)=0.35 Area: F(2,38)=104.0	0.62 0.56 <0.0001*
		90	Interaction: F(2,18)=1.75 Genotype: F(1,18)=1.08 Area: F(2,18)=47.96	0.20 0.33 <0.0001*
	Light/Dark	30	Interaction: F(1,25)=3.34 Genotype: F(1,25)=-3.33 Area: F(1,25)=93.15	0.08 1.0 <0.0001*
		60	Interaction: F(1,19)=0.76 Genotype: F(1,19)=-3.94 Area: F(1,19)=133.6	0.39 1.0 <0.0001*
		90	Interaction: F(1,9)=0.06 Genotype: F(1,9)=3.94 Area: F(1,9)=44.89	0.81 0.08 <0.0001*
	FST (Latency)	30-90	Interaction: F(2,78)=0.24 Genotype: F(1,78)=1.93 Days post-TAM: F(2,78)=0.57	0.79 0.17 0.57
	FST (Total Immobility)	30-90	Interaction: F(2,78)=0.92 Genotype: F(1,78)=0.0003 Days post-TAM: F(2,78)=4.20	0.40 0.96 0.02 *
	Sucrose Preference	60-90	Interaction: F(1,25)=0.05 Genotype: F(1,25)=1.21 Days post-TAM: F(1,25)=2.72	0.82 0.28 0.11
	LH	60	N/A – Student's t-test	0.68

Table 5.1: Notch1 iKO Behavior Statistics

Group	Test	Days post-TAM	Statistics	p value
iNICD	Loco	30	Interaction: F(23,391)=0.50 Genotype: F(1,391)=1.36 Time: F(23,391)=44.09	0.97 0.26 <0.0001*
		60	Interaction: F(23,460)=0.83 Genotype: F(1,460)=2.65 Time: F(23,460)=47.98	0.69 0.12 <0.0001*
	Open Field	30	Interaction: F(2,34)=5.83 Genotype: F(1,34)=2.08 Area: F(2,34)=289.6	0.007* 0.17 <0.0001*
		60	Interaction: F(2,40)=1.30 Genotype: F(1,40)=1.21 Area: F(2,40)=202.2	0.28 0.28 <0.0001*
		90	Interaction: F(2,30)=2.45 Genotype: F(1,30)=0.67 Area: F(2,30)=227.1	0.10 0.42 <0.0001*
	EPM	30	Interaction: F(2,34)=0.16 Genotype: F(1,34)=0.02 Area: F(2,34)=189.1	0.86 0.89 <0.0001*
		60	Interaction: F(2,40)=0.43 Genotype: F(1,40)=0.83 Area: F(2,40)=47.05	0.66 0.37 <0.0001*
		90	Interaction: F(2,28)=0.66 Genotype: F(1,28)=0.40 Area: F(2,28)=250.7	0.53 0.54 <0.0001*
	Light/Dark	30	Interaction: F(1,17)=2.31 Genotype: F(1,17)=0.38 Area: F(1,17)=172.5	0.15 0.55 <0.0001*
		60	Interaction: F(1,20)=1.24 Genotype: F(1,20)=-1.64 Area: F(1,20)=433.4	0.29 1.0 <0.0001*
		90	Interaction: F(1,12)=1.02 Genotype: F(1,12)=-2.61 Area: F(1,12)=133.9	0.33 1.0 <0.0001*
	FST (Latency)	30-90	Interaction: F(2,77)=2.46 Genotype: F(1,77)=2.33 Days post-TAM: F(2,77)=0.45	0.09 0.13 0.64
	FST (Total Immobility)	30-90	Interaction: F(2,77)=0.04 Genotype: F(1,77)=1.34 Days post-TAM: F(2,77)=2.93	0.96 0.25 0.06
	Sucrose Preference	60-90	Interaction: F(1,47)=0.60 Genotype: F(1,47)=1.72 Days post-TAM: F(1,47)=0.44	0.44 0.20 0.51
	LH	60	N/A – Student's t-test	0.79

Table 5.2: iNICD Behavior Statistics

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CHAPTER SIX
Conclusions and Future Directions

Conclusions

Notch1 plays a well-characterized role in maintaining neural stem cells during central nervous system development for future neurogenesis. This dissertation expands on previous work by demonstrating that intact Notch1 is required in Nestin-expressing cells for appropriate levels of hippocampal neurogenesis in adult mice. We provide evidence that Notch1 maintains Type-1 cells and promotes proliferation of Type-2 progenitors in the subgranular zone (SGZ). While necessary for proliferation at baseline, we found that Notch1 is not required for physical activity-dependent proliferation and neurogenesis. While preliminary, our data on the overexpression of Notch1 intracellular domain (NICD) in stem cells and progeny suggests that overactivation of Notch1 signaling may also decrease neurogenesis. Increased adult hippocampal neurogenesis has been linked to behavioral recovery in animal models of depression, but not in the etiology of depression itself. We provide further evidence that specifically reducing adult neurogenesis is not sufficient to induce depression- or anxiety-related behaviors. Here we will review the pertinent finding from each section and the implications these findings have for the field, as well as suggest some future experiments.

Chapter 2: *Nestin-CreER^{T2}/R26R-YFP mice: a valuable tool for studying basal and activity-induced neurogenesis*

Research into the molecular mechanisms underlying adult neurogenesis has led to the development of new techniques that allow us to label, manipulate and track large populations of neural stem and progenitor cells in the SGZ. To this end, we generated Nestin-CreER^{T2}/R26R-YFP mice. We found that, unlike other inducible transgenic mice that targeted neural stem cells using glial drivers (Garcia et al., 2004; Ninkovic et al., 2007), recombination in Nestin-CreER^{T2}/R26R-YFP mice was strictly limited to progenitors that give rise to neurons (Lagace et al., 2007). YFP+ cells were initially composed of stem and progenitor cells that developed into neurons with time. Furthermore, we found that Nestin-CreER^{T2}/R26R-YFP mice allowed us to manipulate a large but specific cohort of stem cells in the adult SGZ that give rise to neurons, giving us temporal control over adult neurogenesis. Nestin-CreER^{T2}/R26R-YFP mice also allowed us to assess dynamic regulation of adult hippocampal neurogenesis. For example, YFP+ cells in the SGZ are increased after running. In sum, these mice provide a critical and necessary tool for studying the regulation and function of adult neurogenesis.

Future Directions, Chapter 2

Lineage restriction of Type-1 cells: Our findings in these mice raise some additional interesting questions that are the focus of continued research in the Eisch laboratory. Are Nestin-expressing Type-1 cells a more restricted subset of

adult SGZ radial glial cells? Or is this an artifact of Nestin overexpression in our transgenic mouse? The finding that other Nestin-CreER^{T2} mice are restricted to the neuronal lineage suggests that there is a correlation with Nestin expression and lineage restriction (Imayoshi et al., 2008).

Plateau in labeled cells: If Type-1 cells are the stem cells, it is intriguing that we, and others (Ninkovic et al., 2007; Imayoshi et al., 2008), see a plateau in the number of neurons generated in the SGZ in inducible transgenic mice. The plateau in labeled cells is not due to loss of Type-1 cells, as they persist at long intervals post-TAM (Lagace et al., 2007), rather my results suggest it is because the proliferating progenitors mature into neurons and are not replenished by labeled Type-1 cells. Are Type-1 cells programmed to give rise to progenitors only at a specific time during adulthood, after which they become quiescent? Currently, Nathan DeCarolis in the Eisch Laboratory is investigating the dynamics of Type-1 contribution to SGZ neurogenesis. Utilizing both our Nestin-CreER^{T2} (Lagace et al., 2007) and the GLAST-CreER^{T2} mice (Ninkovic et al., 2007), he is administering TAM at 5 weeks of age, and again 2 or 3 months later, to determine if there is an additive effect on the number of labeled cells. If Type-1 cells are programmed to give rise to distinct temporal cohorts in the adult SGZ, then we should observe an additive effect of double TAM administration. If we see more than a doubling of labeled cells, this suggests that we are recruiting both new and already labeled Type-1 cells. The results of these studies are

critical for understanding the dynamics of the contribution that adult stem cells make to neurogenesis.

If labeled Type-1 cells are quiescent at extended periods of time, can we recruit them to contribute significantly to SGZ neurogenesis by ablating proliferating progenitors? Again, Nathan is actively pursuing this line of investigation. Here, he is administering AraC to Nestin-CreER^{T2}/R26R-YFP mice to ablate progenitors and determine if labeled cells contribute to recovery. If Type-1 cells are stem cells, then they should contribute labeled progenitors during recovery. If they do not contribute to recovery, it suggests that Nestin-expressing Type-1 cells are not stem cells. Of course, that kind of result begs the next question: If the Type-1 cell isn't the neural stem cell, then what is, and what function do Type-1 cells serve? Are they simply support cells that provide a permissive neurogenic niche? Or do they play a role in modulating activity in the structure, much like Bergmann and Muller glia in the cerebellum and retina, respectively? These are the same questions the field has been asking for the past decade and remain the ultimate challenge and goal of research in adult neurogenesis. By administering the same TAM treatment paradigm to both our Nestin-CreER^{T2} (Lagace et al., 2007) and the GLAST-CreER^{T2} mice (Ninkovic et al., 2007), Nathan is investigating the identity of the adult neural stem cell. He is directly comparing the dynamics of YFP+ cells counts and the phenotypic markers expressed in YFP+ cells in identically treated Nestin- and GLAST- CreER^{T2} mice. I would expect that recombination leads to all lineages in GLAST-CreER^{T2} mice, while Nestin-

CreER^{T2} mice generate only neurons, further supporting that Nestin-expressing cells are lineage restricted. The combined results of these two studies have the potential to finally identify the adult neural stem cell and its dynamic ability to contribute to neurogenesis under normal conditions and in times of need. The implications for this in stem cell biology are astounding. This is a critical aspect that must be understood before stem cells can be used therapeutically to treat neurodegenerative disorders.

Chapter 3: *Notch1 promotes proliferation of Nestin-expressing neural progenitors but is not required for activity-dependent neurogenesis in the adult hippocampus*

In order to understand if Notch1 is critical for adult hippocampal neurogenesis, as is suggested by its role as a master regulator of embryonic neurogenesis, we generated Notch1 iKO mice to ablate Notch1 specifically in neuronal lineage-restricted Nestin+ stem and progenitor cells. I found that although Notch1 was required for Type-1 maintenance and progenitor proliferation, running fully rescued proliferation and neurogenesis in Notch1 iKO mice. However, 30 days of running was not sufficient to rescue Type-1 cells. These findings suggest that Notch1 plays distinct roles in stem and progenitor cells in the SGZ, and that Type-1 cells make little contribution to exercise-induced neurogenesis. While these studies shed light on the regulation of adult neurogenesis by Notch1, they raise several more questions, discussed in more detail below.

Future Directions, Chapter 3

Demonstration of Notch1 ablation: What if some of the YFP+ cells still have intact Notch1 signalling? While technically challenging, it is important that we demonstrate that Notch1 is ablated in the YFP+ cells, as this assumption affects how we interpret the data. Studies are currently under way to demonstrate loss of Notch1 or NICD in YFP+ cells by immunohistochemistry. First, we must demonstrate that the antibodies are specific for Notch1, however. To do this, we have injected AAV-CreGFP virus into the hippocampus of homozygous floxed Notch1 mice to remove Notch1 from infected cells. Thus far, we have been unsuccessful in demonstrating sufficient specificity in the currently available antibodies, as GFP+Cre+ cells are still immunoreactive for Notch1 and NICD. Studies are ongoing to optimize the staining protocol. Alternatively, we can isolate and purify YFP+ cells from the SGZ and look for Notch1 mRNA. Our first attempts at FACS for YFP+ cells from adult SGZ have not been successful, however, collaborators utilizing our Nestin-CreERT2 mice have reported recent success.

Stem cell maintenance: While we provide evidence that YFP+ Type-1 cells decrease with time after loss of Notch1, we did not assess their “stemness” or the markers that identify them as stem cells, nor did we determine what impact loss of YFP+ Type-1 cells had on the remaining YFP- Type-1 cells. Future studies should determine by immunohistochemistry if colocalization of the stem

cell markers Nestin, GLAST, Sox2, Musashi1, BLBP and/or GFAP is decreased in the remaining YFP+ Type-1 cells in Notch1 iKO mice compared to WT mice. I would expect that as a Notch1 target, the proportion of YFP+ Type-1 cells that are BLBP+ would be decreased in Notch1 iKO mice compared to WT mice, indicating decreased stem cell characteristics, consistent with a role for Notch1 in maintaining stem cells. Further, we cannot rule out the possibility that Type-1 cells have impaired survival in Notch1 iKO mice. We can assess survival signaling in Type-1 cells by immunohistochemical assessment of the phospho-Akt levels in Type-1 cells of WT vs. Notch1 iKO mice (Androutsellis-Theotokis et al., 2006).

Future studies after a much longer interval following TAM are critical to truly address the maintenance of Type-1 cells. Studies are currently underway examining the number of YFP+ Type-1 cells 215 days post-TAM in WT and Notch1 iKO mice. In WT mice, I would expect that YFP+ cells would be made up of Type-1 cells and neurons, based on studies in Chapter 2. However, if Notch1 is critical for maintenance of Type-1 cells in the adult SGZ, then I would expect that YFP+ cells in the Notch1 iKO mice 215 days post-TAM would be almost exclusively neurons, with almost no YFP+ Type-1 cells left. However, if the number of YFP+ Type-1 cells are unchanged between 90 and 215 days post-TAM, this might suggest that the remaining YFP+ cells may have intact Notch1 signaling. Finally, we have not truly addressed the context-dependent role of Notch1 in adult neurogenesis, however, our data does suggest that there may be

differences in the effect of Notch1 signaling depending on the cell type in which it is activated. Breunig et al. found that Notch1 signaling in GFAP+ radial glia in the SGZ regulates fate determination, whereas we found that fate (as determined by proportion of YFP+ cells with Type-1 or neuronal morphology) was not altered in progeny of Nestin-expressing cells. Is this due to intrinsic differences in the hypothesized subsets of stem cells in the SGZ? Or could this be due to differences in the ages of animals in the two studies?

Contribution of Type-1 cells to neurogenesis: One of the most intriguing aspects of these studies is the finding that YFP+ cells were largely unaffected until 60 days post-TAM, at which point, YFP+ cells in all stages of neurogenesis were decreased in Notch1 iKO mice. Where or when, then, does the decrease start? Are Type-1 cells decreased first, and this leads to fewer progenitors and fewer neurons? I feel its more likely that both Type-1 and Type-2 cells are impaired in their ability to proliferate without Notch1, and the combined decrease in both stem and progenitor cells leads to fewer new neurons. Future studies using a more detailed time course after TAM could shed more light on which cell type is affected first. However, it is possible that both Type-1 and Type-2 cells are intimately regulated by each other and thus it would be impossible to determine which cell type is responsible for reduced neurogenesis.

Stem cells and activity-dependent neurogenesis: If Type-1 cells do not contribute significantly to neurogenesis after running, are they the stem cells? Or are there

enough proliferating progenitors left that can expand their population in response to running? Perhaps longer running would eventually exhaust Type-1 cells as they give rise to Type-2 cells in Notch1 iKO mice, lending support to their identity as stem cells. Described in more detail below, we attempted to address this question by allowing WT and Notch1 iKO animals to run when YFP+ progenitors were quite low in Notch1 iKO mice to determine if running placed more demand on Type-1 cells, or if Type-2 cells could expand independently of Type-1 cells.

5 day of running: We found that 30 days of running was sufficient to rescue neurogenesis in Notch1 iKO mice (Figure 3.6). We hypothesized that this was due to the exercise-induced increase in proliferation of Type-2 cells. To test this we gave WT and Notch1 iKO mice access to running wheels when proliferation was significantly different between genotypes (Figure 3.2). Mice were allowed to run for 5 days, and at 65 days post-TAM YFP+ cell number was compared between the running and control mice (Figure 6.1A). Unlike Notch1 iKO mice and WT 30-day runners, WT mice did not increase the amount that they ran over the 5 days on the wheel. While not significant, there was a strong trend for an interaction (Figure 6.1B, revolutions *X* genotype interaction, $F_{4,44}$=2.44, p=0.06). Despite less running than expected, there was a significant proliferative response to 5 days of running in both WT and Notch1 iKO mice as indicated by total Ki67+ cells in the SGZ, with a larger increase in proliferation in Notch1 iKO mice (Figure 6.1C; genotype *X* running interaction, $F_{1,29}$=6.42, p=0.02).

Because WT mice in this set of experiments did not run as much as much as Notch1 iKO mice, it is difficult to make any strong conclusions as to the effect of acute running on YFP+ cells. However, the preliminary data on the 5-day running group does suggest some interesting findings. Simply placing animals on the running wheels for 5 days significantly increased the total number of YFP+ cells in both WT and Notch1 iKO mice, regardless of whether the wheel was locked or open, compared to naïve group-housed animals at 60 days post-TAM (Figure 6.2A, genotype *X* treatment interaction, $F_{2,22}$=1.16, *p*=ns; treatment, $F_{2,22}$=3.65, *p*=0.04). Likewise, there was a strong trend for an increase in YFP+ Type-1 cells after simply placing animals on the wheels for 5 days (Figure 6.2B; genotype *X* treatment interaction, $F_{2,22}$=0.99, *p*=ns; treatment, $F_{2,22}$=3.24, *p*=0.06). However, the large degree of variation in the number of YFP+ cells and YFP+ Type-1 cells in both WT and Notch1 iKO runners means these conclusions should be considered preliminary until more animals can be added.

We next examined the phenotype of YFP+ cells in 5-day runners, to determine if a short duration of running was sufficient to affect proliferation or neuronal differentiation. There was no significant effect of running on the number of YFP+ cells that were Ki67+ (Figure 6.2C; genotype *X* treatment interaction, $F_{2,12}$=2.33, *p*=ns; genotype, $F_{1,12}$=2.25, *p*=ns; treatment, $F_{2,12}$=1.04, *p*=ns). However we did find a significant interaction on the proportion of YFP+ cells that were Ki67+ (6.2E; genotype *X* treatment interaction, $F_{2,12}$=4.96, *p*=0.02), although none of the post-hocs revealed any significant differences between genotypes under any

condition. While 5 days of running had little effect on the number of YFP+ cells that were DCX+ (Figure 6.2D; genotype *X* treatment interaction, $F_{2,12}$=0.63, *p*=ns; genotype, $F_{1,12}$=8.22, *p*=0.01; treatment, $F_{2,12}$=0.95, *p*=ns), there was a strong trend on the proportion of YFP+ cells that were DCX+ (Figure 6.2F; genotype *X* treatment interaction, $F_{2,14}$=0.90, *p*=ns; genotype, $F_{1,14}$=1.36, *p*=0.07; treatment, $F_{2,14}$=2.90, *p*=0.08). Together these preliminary data suggest that exposure to the novel environment for only 5 days is sufficient to change neurogenesis in both WT and Notch1 iKO mice. However, these data on 5 day running in particular are preliminary, and further research is needed to clarify these results.

In an attempt to shed more light on what we hypothesized was a proliferative rescue after running, we gave WT and Notch1 iKO mice access to running wheels for only 5 days when proliferation was significantly different between genotypes (60 days post-TAM). Our preliminary data indicate that even acute running is sufficient to rescue proliferation in Notch1 iKO mice, supporting our hypothesis; however, there are several caveats to these experiments. First, and most surprisingly, we found that the running wheel itself, whether locked or unlocked, significantly affected total number of YFP+ cells and YFP+ Type-1 cells compared to naïve mice. This is especially surprising given that 30 days of access to a locked wheel did not affect neurogenesis. Does the novel running wheel environment transiently stimulate proliferation of Type-1 cells? This would explain why we see an effect on Type-1 cells after 5 days of running, but not after 30 days. To see if this is true, we could examine the number of mitotic Type-1

cells after 5 days of novel environment. Second, total YFP+ counts in both WT and Notch1 iKO mice are variable. This could be secondary to the variability that we saw in the amount that these mice ran, especially WT mice, which did run as much as we have previously demonstrated. The promising preliminary data calls for addition of more animals to the study, and incorporation of appropriate group-housed controls to determine the effect of environment on Type-1 cells. Our preliminary finding here have implications as quick and simple manipulation to increase Type-1 proliferation.

Cell cycle dynamics, downstream effectors: Does Notch1 regulate stem cell self-renewal and proliferation of progenitors by directly regulating critical proteins in cell cycle progression? Proliferation is critical for both self-renewal of Type-1 cells and expansion of progenitors. Does Notch1 regulate Type-1 cell maintenance by regulating the ability of Type-1 cells to proliferate, even if only rarely, or does it regulate asymmetry between the two daughter cells to generate a new Type-1 cell? A more careful examination of the number of YFP+ Type-1 cells that are Ki67+ might reveal that fewer YFP+ Type-1 cells are proliferating in Notch1 iKO mice. However, the rarity of this population might make it difficult to assess *in vivo*.

To examine how Notch1 modulates proliferation in the SGZ, future work should focus on manipulating various downstream targets of Notch1 such as Hes1/5, Ascl1, or cell cycle proteins, in combination with neurogenic stimuli to shed light

on the relationship between proliferation at baseline and activity-induced proliferation. Specifically, cell cycle proteins are likely candidates, as Notch1 has been shown in several other tissues to regulate key proteins at each of the cell cycle transitions (Wang et al., 2006; Ishikawa et al., 2008). In mice and in Drosophila, Notch1 directly regulates transcription of key proteins in the cell cycle transitions including Fbxw7, MCM, c-myc, p53 and CDK1 (Noseda and Karsan, 2006; Ishikawa et al., 2008; Nagao et al., 2008; Guo et al., 2009; Krejci et al., 2009). Notch1 can also indirectly, perhaps non-canonically, regulate the cell cycle and stem cell survival through Akt activation (Martinez Arias et al., 2002; Androutsellis-Theotokis et al., 2006; Gutierrez and Look, 2007; Mizutani et al., 2007).

CDK1 is an extremely attractive candidate for several reasons: 1) it is a direct target of Notch1 (Krejci et al., 2009), 2) it is regulated by a variety of converging pathways, and 3) it is critical for regulating cell cycle exit and entry (Pacek et al., 2004; Potapova et al., 2009). Examination of YFP+ Type-1 and progenitor cells in Notch1 iKO mice might reveal that CDK1 transcript and/or protein is decreased compared to WT mice. Further, I would expect that levels of this protein should be restored in Notch1 iKO runners, where proliferation is rescued, possibly by signaling though other pathways. Findings that support these predictions would clarify that Notch1 plays a permissive role in regulating proliferation in the adult SGZ, but that in cases of demand, such as increased activity after running, other pathways can send a Notch-independent mitogenic signal.

Notch1 and growth factor cross talk: Are the increased levels of growth and neurotrophic factors after running responsible for rescue of proliferation and thus neurogenesis? Future studies should also consider cross talk between growth factor and Notch1 signaling as a possible mechanism underlying disruption of proliferation under basal conditions and Notch1-independent proliferation after running (Figure 6.1; James et al., 2004; Yoon et al., 2004; Nagao et al., 2007; Krejci et al., 2009). The FGF pathway is directly regulated by Notch1 (Yoon et al., 2004; Krejci et al., 2009) and promotes proliferation of NSCs (Zhao et al., 2007). Furthermore, both Notch1 and growth factors, including FGF and EGF, are required for active proliferation of NSCs in culture (Nagao et al., 2007), but FGF is not sufficient to promote proliferation of NSCs in the absence of Notch1 signaling (Yoon et al., 2004). Running increases several growth and neurotrophic factors in the DG, including FGF, VEGF, IGF and BDNF (Gomez-Pinilla et al., 1997; Fabel et al., 2003; Fabel and Kempermann, 2008). It is tempting to speculate that running increases growth factor levels such that they can promote proliferation in the absence of Notch1.

Utilizing the tissue that we already have, we can begin to assess growth and neurotrophic signaling in Notch1 iKO runners by IHC for phospho-CREB (pCREB). Phosphorylation of cyclic-AMP reseponse element binding protein (CREB) is a common downstream event of many growth and neurotrophic factors as well as Akt (Johannessen et al., 2004). Studies of pCREB indicate that

it is found in and promotes proliferation of cells in the SGZ (Nakagawa et al., 2002a; Nakagawa et al., 2002b). Ongoing studies in the Eisch laboratory should examine changes in growth factor signaling in the SGZ by IHC for pCREB to determine if growth factor signaling is disrupted in Notch1 iKO mice and rescued by running. If this is the case, I would expect that levels of pCREB would be decreased in YFP+ cells in the SGZ of non-runner Notch1 iKO mice compared to WT mice (runner or non-runner) and runner Notch1 iKO mice. While these results would suggest that growth factors play a role in rescuing proliferation in Notch1 iKO runners they are not directly conclusive.

To directly determine if growth factors are responsible for rescued proliferation in Notch1 iKO mice, we should administer FGF or VEGF to Notch1 iKO mice and quantify YFP+ cells in the SGZ. VEGF would be more amenable to this sort of manipulation, as it can be administered peripherally; FGF, however, would require cannulation and central administration. If either of these growth factors is able to restore proliferation and/or neurogenesis of YFP+ cells in Notch1 iKO mice it would indicate that Notch1 is necessary for basal proliferation, but that a more “supportive” niche with increased levels of growth factors can sustain proliferation in the absence of Notch1 iKO.

Chapter 4: *Inducible activation of Notch1 in adult hippocampal stem/progenitor cells*

In order to understand how Notch1 overactivation might affect Nestin-expressing stem and progenitor cells, and thus neurogenesis, I generated inducible NICD (iNICD) mice and counted the number of YFP+ cells in the SGZ at increasing intervals post-TAM. I found that iNICD mice displayed strikingly a similar pattern of total YFP+ cells as Notch1 iKO mice, with no observable difference at 30 days post-TAM and decreased YFP+ cells at 60 days post-TAM. Due to a large degree of variation, I was unable to interpret the results at 90 days post-TAM and reach a definitive conclusion about the effect of NICD overexpression in Nestin-expressing cells on adult hippocampal neurogenesis. Yet, these preliminary findings suggest that neurogenesis in the SGZ is sensitive to both positive and negative Notch1 modulation.

Future Directions, Chapter 4

Future studies of iNICD mice should first determine what is happening to the number of YFP+ cells over time. To this end, I have generated several sets of animals to complete this study (13d: 9WT, 11iNICD; 30d: 6WT, 14 iNICD; 60d: 6WT, 6iNICD; 90d: 9WT 5iNICD). Once we have a grasp on the dynamics of YFP+ cells in iNICD mice, we should determine the expression of phenotypic markers in YFP+ cells. This would allow us to determine if NICD overexpression promotes proliferation (Ki67), inhibits neuronal differentiation (NeuroD and/or DCX expression) and/or promotes glial differentiation (S100b or GFAP expression) (Gaiano et al., 2000; Breunig et al., 2007; Namihira et al., 2009). The time post-TAM that we will examine will depend on the dynamics of total YFP+

cells. If proliferation and total YFP+ cells are increased at extended times post-TAM, this would indicate that Notch1 signaling is dose-dependent in Nestin-expressing cells. If so, then I would expect that neurosphere formation would be increased in iNICD mice (Guentchev and McKay, 2006; Breunig et al., 2007). However, an increase in YFP+ cells in iNICD mice likely will not mean increased numbers of new neurons. I would expect that there would be an expansion and accumulation of transit-amplifying cells. NICD overexpression would prevent their exit from the cell cycle and thus differentiation. If YFP+ cells in iNICD mice were able to differentiate, I would expect them to become astrocytes (Gaiano and Fishell, 2002).

If YFP+ cells are decreased at extended times post-TAM in iNICD brains, as our preliminary data suggests, it would support the hypothesis that oscillation of Notch signaling is critical for its function (Shimojo et al., 2008), and that disruption of oscillation, either by ablation or sustained activation, is sufficient to impair the generation of new neurons. Testing this oscillation hypothesis would require the development of live cell imaging of Notch activation or Hes1/5 expression in adult mice, a technique that is new to the Eisch lab. However, we have established a collaboration with Kageyama's group and could pursue this line of investigation in conjunction with their lab.

Fewer YFP+ cells in iNICD mice could also support the hypothesis that NICD overexpression promotes apoptosis of neural stem cells (Yang et al., 2004), thus

effectively cutting neurogenesis off at the source. To determine if apoptosis in increased in iNICD mice, we can quantify the number of AC3+ cells in the SGZ. Our preliminary data indicates that AC3+ cells do not differ between genotypes, however, detecting apoptosis, which is extremely transient, can be difficult. Using alternative methods of detection, such as counting pyknotic nuclei or TUNEL staining (Harburg et al., 2007) will be critical to fully assess cell death in these mice. If NICD overexpression does not disrupt neurogenesis, this would indicate that neural stem and progenitor cells are not sensitive to the dose of Notch1, rather any amount of Notch1 signal is enough to maintain proliferating progenitors. This seems unlikely given the evidence in the literature (Guentchev and McKay, 2006; Breunig et al., 2007).

Finally, future work with iNICD mice should examine their ability to respond to exercise. To do this, iNICD mice will be given access to running wheel for 30 days beginning at a time when YFP+ cells do not differ between genotypes. If NICD overexpression inhibits neuronal differentiation, iNICD runners may have more proliferating cells than WT mice and iNICD non-runner mice, but they would have fewer YFP+ neurons than WT mice.

Chapter 5: *Mood-related behavior is not affected in a mouse model of decreased neurogenesis*

Adult hippocampal neurogenesis lies at the intersection of cognition and mood (Bessa et al., 2009). In order to understand how decreased neurogenesis

affected base-line mood-related behavior, we tested Notch1 iKO and iNICD on a three measures of anxiety and three measures of depression. We found, that like other studies (Santarelli et al., 2003; Li et al., 2008; Singer et al., 2009), decreased neurogenesis was not sufficient to induce behavioral abnormalities on these tests. However, stress is a critical component of depression and anxiety (Feder et al., 2009). Therefore many questions remain unanswered in regards to this project, the most important being: Would mice with reduced neurogenesis be more or less resilient to the depression- and anxiety-inducing effects of stress? Are Notch1 iKO mice capable of both a behavioral and cellular response to antidepressants? These are discussed in detail below.

Future Directions, Chapter 5

To determine if Notch1 iKO mice are susceptible to stress, two experiments should be conducted in the future. One is to expose Notch1 iKO and WT mice to chronic unpredictable stress before testing for anxiety- and depression-related behavior (Cryan and Holmes, 2005; LaPlant et al., 2009). A second is to expose Notch1 iKO and WT mice to social defeat stress (Berton et al., 2006). Both of these experiments would determine if decreased neurogenesis increases susceptibility to stress, and both experiments would require a large number of animals (20-40 animals of each genotype). Further, to determine if the stress-induced behavioral changes were persistent, we should test the mice again one month later. While, I would not expect that a 50% reduction in neurogenesis would increase susceptibility to stress (Vollmayr et al., 2003), it may alter the

memory for the stressful experience, suggesting that neurogenesis-mediated learning is important in consolidation of emotional, as well as spatial memory.

To further expand on the role of neurogenesis in regulating mood, future studies in the Eisch lab should determine if Notch1 iKO mice respond behaviorally to antidepressants (Santarelli et al., 2003; David et al., 2009). Ideally, depression-related behavior would be induced first by stress, as described above. WT and Notch1 iKO mice would then be assessed for stress-induced depression-related behaviors. Those that demonstrated depression-related behaviors after stress would receive 28 days of fluoxetine injections, and then would be reassessed for depression-related behavior to determine if neurogenesis is critical for a behavioral response to antidepressants. If neurogenesis is critical for the behavioral effects of antidepressants I expect that Notch1 iKO mice would respond to antidepressant treatment, because Notch1 iKO mice respond cellularly to running, a neurogenic stimulus that is cellularly and behaviorally similar to antidepressants. This study would be quite challenging however. First, timing would be critical. Chronic stress would be started 30 days post-TAM, when the number of new neurons does not differ between genotypes, and antidepressant treatment should begin around 60 days post-TAM, when Notch1 iKO mice have fewer neurons.

Finally, future studies in the Eisch lab should also examine the impact of reduced neurogenesis on cognitive hippocampal function. However, the design of these

experiments should be carefully considered, as learning and memory deficits associated with decreased hippocampal neurogenesis are subtle and very specific (Zhang et al., 2008; Garthe et al., 2009). Thus, to determine if reduced neurogenesis in Notch1 iKO mice leads to learning and memory deficits, future experiments in the Eisch should focus on careful testing of learning and re-learning on Water maze as previously described (Garthe et al., 2009). An important caveat of all these behavioral studies is the timing of training or stress and antidepressant treatment relative to TAM. Knowing that new neurons have a critical window about 2-6 weeks after birth suggests that we should aim for 30-60 days post-TAM, but it is unclear if we should assess behavior at this time or train/stress at this time.

Summary

This dissertation has determined that Notch1 is critical in Nestin-expressing stem and progenitor cells for their maintenance and the generation of a full repertoire of neurons. Furthermore, we found that running was sufficient to fully rescue neurogenesis, by rescuing proliferation of progenitors but not stem cells. Future work manipulating various factors downstream targets of Notch1 such as Hes1/5, Ascl1, or cell cycle proteins in combination with neurogenic stimuli will help to shed light on the relationship between proliferation at baseline and activity-induced proliferation. Future work using Notch1 iKO mice as a model of reduced neurogenesis in behavioral assays will further our understanding of adult

hippocampal neurogenesis and its possible role in hippocampal function and the relationship of neurogenesis with the etiology and treatment of depression.

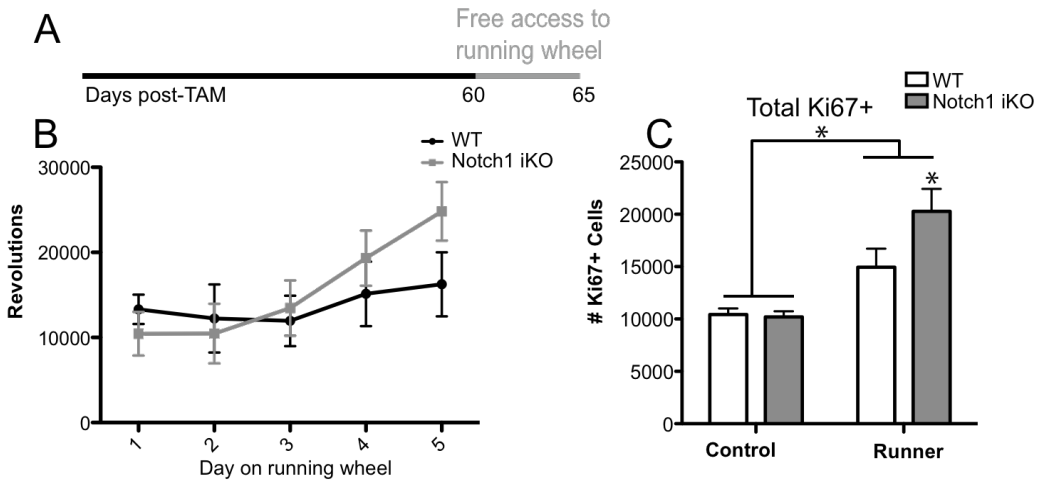

Figure 6.1. Both WT and Notch1 iKO mice respond to 5 days of voluntary physical activity.

A: WT and Notch1 iKO mice had access to a locked (control) or open (runner) wheel for 5 days, beginning 60d post-TAM and were perfused 65 days post-TAM. ***B****:* Quantification of revolutions per day in WT and Notch1 iKO runner mice. WT mice did not increase the amount they ran over the 5 days, unlike their Notch1 iKO littermates (strong trend, p=0.06) and WT 30 day runners (Figure 3.5). ***C***: Quantification of total Ki67+ cells in the SGZ of WT and Notch1 iKO non-runner and runner mice. Notch1 iKO runners have significantly more Ki67+ cells than WT runners. *p<0.05 vs. WT, Bonferroni *post-hoc*. n=5-6 per group.

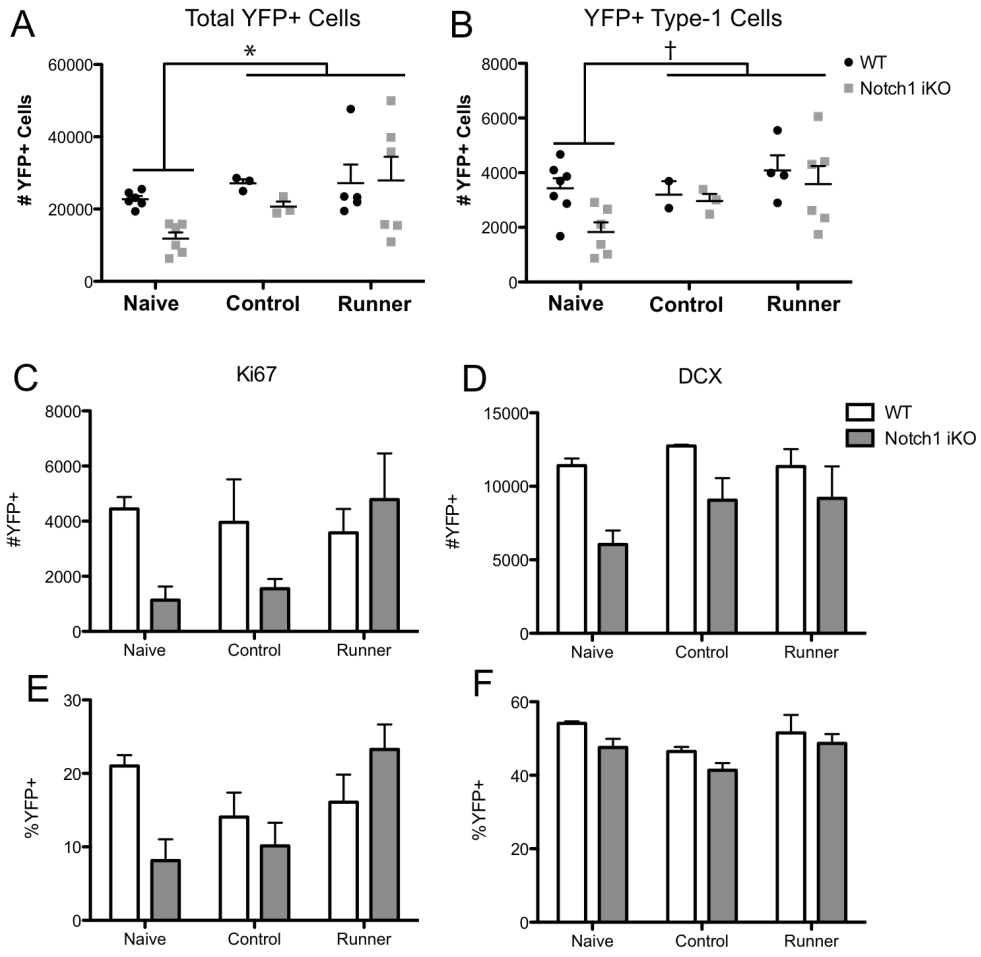

Figure 6.2. The response in YFP+ cells to 5 days of voluntary physical activity in both WT and Notch1 iKO mice is unclear.

A: Quantification of total YFP+ cells in the SGZ of WT and Notch1 iKO mice. Naïve mice are the original group-housed data presented in Figure 3.1. Control mice were single-housed with a locked wheel, and runners with an open wheel for 5 days. Note the large degree of variability in YFP+ cell number after 5 days of running in both WT and Notch1 iKO mice. ***B****:* Quantification of total YFP+ Type-1 cells in the SGZ of WT and Notch1 iKO mice. Note that there is no

difference in the number of YFP+ Type-1 cells between WT and Notch1 iKO mice given a running wheel, locked or open, as well as the large degree of variability in the runner group. ***C***: Quantification of the number of YFP+ cells that are Ki67+ in the SGZ of WT and Notch1 iKO non-runner and runner mice. ***D***: Quantification of the number of YFP+ cells that are DCX+ in the SGZ of WT and Notch1 iKO non-runner and runner mice. ***E***: Quantification of the proportion of YFP+ cells that are Ki67+ in the SGZ of WT and Notch1 iKO non-runner and runner mice. ***F***: Quantification of the proportion of YFP+ cells that are DCX+ in the SGZ of WT and Notch1 iKO non-runner and runner mice. *n*=3-6 per group.

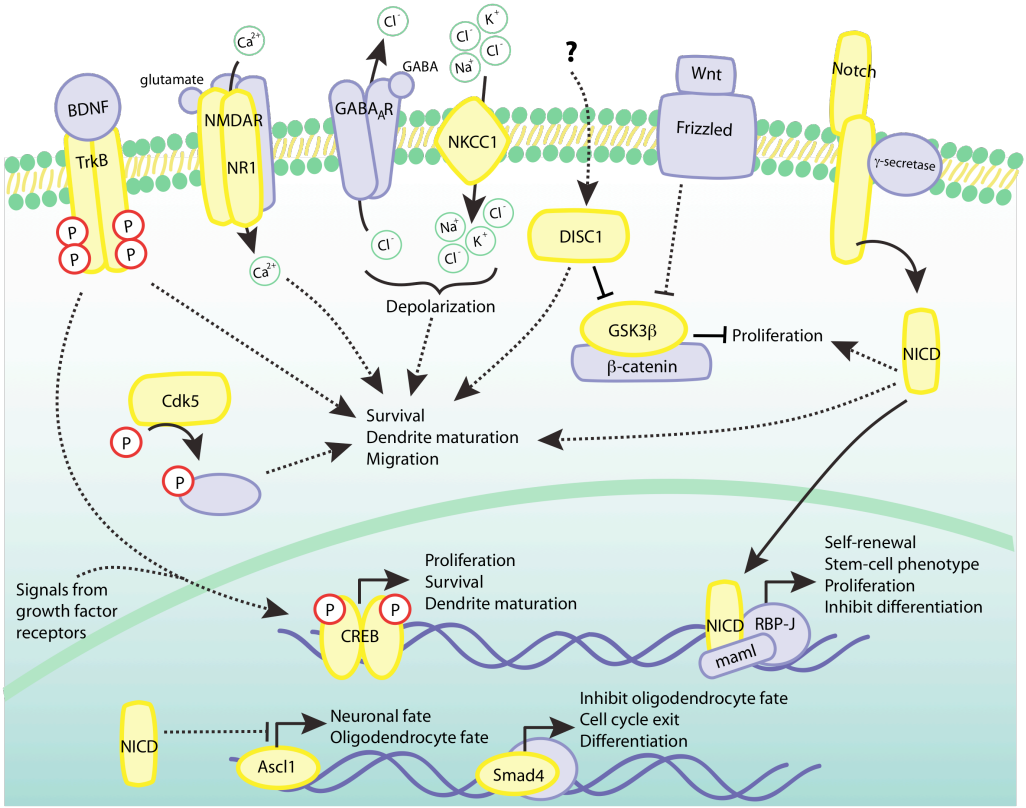

Figure 6.3. Cross talk of Notch1 signaling with other pathways that regulate proliferation in neural stem and progenitor cells.

Notch1 is extensively integrated with many pathways that converge to regulate proliferation of neural stem and progenitor cells. (Figure adapted from Johnson et al., 2009)

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APPENDIX

NICD and Notch1 IHC Methods and Attempted Studies

NICD Immunohistochemistry (IHC)

Tissue Sectioning and Preparation. Mice were anesthetized and transcardially perfused with cold 0.1M PBS for 5 minutes, followed by 2% paraformaldehyde (PFA) for 10 minutes. Brains were extracted and post-fixed 2 hours at RT in the same fixative before cryoprotection in 30% sucrose with 0.1% NaN_3 at RT overnight. Brains were stored at 4°C in the same cryoprotection solution until sectioning on a freezing microtome. Brains were sliced coronally 30µm thick and stored free-floating in 1x PBS with 0.1% NaN_3 at 4°C until stained. **Note: IHC will occasionally work with 4% PFA perfused tissue, but works better the lighter the fix.**

Antibodies. The following primary antibodies give the most comparable and most consistent results: rabbit polyclonal anti-NICD (gift from Alain Isreal; located in 4° box, 0.5 mL tube with blue dot on top, 1:100-1:500); rabbit polyclonal anti-NICD (Upstate Millipore, Billerica, MA, Cat # 07-220; located in -20° box, 1:100-1:500).

IHC. **This antigen is particularly difficult to access and requires significant efforts at retrieval.** Antigen retrieval on slide-mounted sections was performed using 0.01M Citric Acid (pH ~2.5, do not adjust pH of citric acid) at 100°C for 15 minutes, followed by 10 minutes in TBS at RT or in Target Retrieval Solution (Dako North America, Carpinteria, CA; Cat # S1700) at 100°C for 25 minutes, followed by cool-down in the same solution to RT, followed by permeabilization

with trypsin for 8 min at RT and denaturing in 2N HCl in TBS for 25 min at RT (see Eisch Lab IHC protocols for full trypsin and HCl pretreatment methods). 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 3% normal donkey serum and 0.3% Triton-X in TBS 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 at RT for two nights. For single labeling of NICD, primary antibody incubation was followed by labeling with a biotin-tagged donkey anti-rabbit secondary antibody for 1.5 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). **Note: Reuse primary from A. Isreal!** For double or triple labeling, you must complete antigen retrieval and staining for other antigens (e.g. YFP) first, followed by antigen retrieval for NICD. This may mean doing citric acid twice, the first time at pH 6, then at pH ~2.5 for NICD. Visualization with TSA will typically survive the subsequent rounds of retrieval (although FITC to a lesser extent), while fluor-conjugated secondaries may need fixation prior to antigen retrieval for NICD. **Do NOT do trypsin or HCl twice!** All slides were counterstained with a nuclear counterstain, DAPI (Roche Applied Science, Indianapolis, IN, Cat # 236276; 1:5000) or red Nissl (Invitrogen, Eugene, OR, Cat # N-21482; 1:200). All slides were dehydrated and coverslipped using DPX.

Notch1 Immunohistochemistry (IHC)

Tissue Sectioning and Preparation. Mice were anesthetized and transcardially perfused with cold 0.1M PBS for 5 minutes, followed by 2% paraformaldehyde (PFA) for 10 minutes. Brains were extracted and post-fixed 2 hours at RT in the same fixative before cryoprotection in 30% sucrose with 0.1% NaN_3 at RT overnight. Brains were stored at 4°C in the same cryoprotection solution until sectioning on a freezing microtome. Brains were sliced coronally 30µm thick and stored free-floating in 1x PBS with 0.1% NaN_3 at 4°C until stained. **Note: Full-length Notch1 IHC does not work well with 4% PFA perfused tissue.**

Antibodies. The following primary antibody gives the most consistent results: goat polyclonal anti-Notch1 (Santa Cruz Biotechnology, Santa Cruz, CA; located in 4° box, Cat # sc-6014; 1:50-1:100).

IHC. **This antigen is particularly sensitive to detergent and antigen retrieval.** Staining was performed on free-floating sections according to M. Donovan's method for TrkB (see Eisch Lab IHC protocols or my notebooks for detailed methods). 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 3-5% normal donkey serum in TBS 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 3-5% serum at RT overnight or at 4° C for two nights. For single labeling of Notch1, primary antibody incubation was followed by labeling with a biotin-tagged donkey anti-goat secondary antibody for 1.5 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 double or triple labeling, you must complete staining for Notch1 first. Visualization with TSA will ensure that signal survives the addition of detergent needed for subsequent rounds of staining. All slides were counterstained with a nuclear counterstain, DAPI (Roche Applied Science, Indianapolis, IN, Cat # 236276; 1:5000). **Note that dehydration with citrosolv will often decrease signal. Skip citrosolv or use the VWR Xylene substitute.**

Other Attempted Studies

1. Embryo studies:
    a. Summer 2007
    b. Methods:
        i. Treated pregnant moms with TAM at E9.5 or E11.5 and sacrificed 48 hrs later. Embryos fixed according to Johnson lab protocol (see JA protocols notebook) and sectioned on cryostat. Stained for Ki67, Mash1, Nestin.
    c. Results:
        i. No observable phenotype. Like adult studies, total Ki67 appeared unchanged.
    d. Caveats:
        i. No YFP included.

2. Pup studies:
    a. Summer 2007
    b. Methods:
        i. Treated mom (see notebook for details)
    c. Results:
        i. Pups very small.
        ii. No observable phenotype. Like adult studies, total Ki67 appeared unchanged.
    d. Caveats:
        i. No YFP included.
        ii. Tried dripping in mouth of pups like Shveta/Diane
            1. Spring 2009 (see notebook for methods)
            2. Mom died 1 wk post TAM to pups
            3. Used for FACS but no YFP+ cells detected
3. FACS:
    a. Winter 2008-Spring 2009
    b. Methods:
        i. Harvested NSC from SVZ of weanling-adult KxYxfN animals
    c. Results:
        i. Not able to detect YFP acutely after dissection
            1. Tons of debris

2. Jen in Kernie lab suggests using only papain for dissection, not papain/dispase/DNAse like Hsieh lab
    a. Quality of sample better with only papain
        i. Less debris on sort
    b. Younger animals give less debris and more viable cells

ii. YFP detected in neurosphere cultures (passage 2-3)

iii. Stained for YFP in EtOH fixed cells

1. Some signal in a few samples
2. Still not able to consistently detect YFP+ cells