TRANSCRIPTIONAL REGULATION OF ADULT NEUROGENESIS

BY NRSF/REST AND NEUROD1

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For I am only studying His handiwork.

# TRANSCRIPTIONAL REGULATION OF ADULT NEUROGENESIS BY NRSF/REST AND NEUROD1

by

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Neurogenesis in the adult brain is a complex and lifelong process that is regulated by multiple pathways and is sensitive to many external stimuli. Two critical regulatory factors in this process are NRSF/REST and NeuroD1. NRSF/REST, a transcriptional repressor that binds a specific NRSE site and recruits corepressors and chromatin remodeling machinery to repress its target genes, is critical for maintenance of the neural stem cell pool and for proper pacing of neuronal differentiation. NeuroD1, a bHLH transcription factor, is necessary for the terminal differentiation, maturation, and survival of newborn neurons. In addition, both factors are necessary for the neurogenic response to both physiological and pathological stimuli, which may induce neurogenesis through different pathways. Thus, NRSF/REST and NeuroD1 are necessary for neurogenesis to occur correctly, to persist throughout the organism's lifespan, and to respond to external stimuli.

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## Prior Publications

1. Sebastian Jessberger, Kinichi Nakashima, Gregory Clemenson, Eunice Mejia, Emily Mathews, **Kerstin Ure**, Shiori Ogawa, Christopher M. Sinton, Fred H. Gage, and Jenny Hsieh. Epigenetic modulation of seizure-induced neurogenesis and cognitive decline. 2007. *Journal of Neuroscience*, 27: 5967-75.

2. Zhengliang Gao*, **Kerstin Ure***, Jessica L. Ables, Diane C. Lagace, Klaus-Armin Nave, Sandra Goebbels, Amelia J. Eisch, and Jenny Hsieh. Essential role of NeuroD in the survival and maturation of newborn neurons in the adult mammalian brain. 2009. *Nature Neuroscience* 12(9): 1090-2.
   *Equal author contribution

3. Andrew A. Pieper, Shanhai Xie, Ema Capota, Sandi Jo Estill, Jeannie Zhong, Jeffrey M. Long, Ginger L. Becker, Paula Huntington, Shauna E. Goldman, Ching-Han Shen, Maria Capota, Jeremiah K. Britt, Tiina Kotti, **Kerstin Ure**, Daniel J. Brat, Noelle S. Williams, Karen S. MacMillan, Jacinth Naidoo, Lisa Melito, Jenny Hsieh, Jef De Brabander, Joseph M. Ready, and Steven L. McKnight. Discovery of a pro-neurogenic, neuroprotective chemical. 2010. *Cell*, 142(1): 39-51.

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## List of Abbreviations

°C: degrees Celsius
A,G,C,T: adenine, guanine, cytosine, thymine
AAALAC: Association for Assessment and Accreditation of Laboratory Animal Care
AC3: activated caspase 3
AraC: arabinofuranosyl cytidine
Ascl1: achaete-scute-like 1
BDNF: brain derived neurotrophic factor
bHLH: basic helix-loop-helix
ESC: embryonic stem cell
BMP: bone morphogenic protein
bp: base pair
BrdU: bromodeoxyuridine
β-TRCP: β-transducin repeat-containing protein
CA: cornus Ammon
cAMP: cyclic adenosine monophosphate
CBP: CREB binding protein
cKO: conditional knockout
CREB: cAMP response element-binding
CSF: cerebrospinal fluid
CtBP: C-terminal binding protein
DAPI: 4',6-diamidino-2-phenylindole
Dcx: doublecortin
DG: dentate gyrus
DISC1: disrupted in schizophrenia
Dlx2: distal-less homeobox 2
DMEM: Dulbecco's Modified Eagle Medium
DNA: deoxyribonucleic acid
dsRNA: double stranded RNA
EGF: epidermal growth factor
EGFR: EGF receptor
FACS: fluorescent activated cell sorting
FGF: fibroblast growth factor
GABA: γ-Aminobutyric acid
Gadd45b: growth arrest and DNA-damage-inducible, beta
GFAP: glial fibrillary acidic protein
GFP: green fluorescent protein
GLAST: glutamate aspartate transporter

GluR2: glutamate receptor subunit 2
GSK3β: glycogen synthase kinase 3 beta
HAT: histone acetyltransferase
HCN: hippocampal neuronal progenitors
HDAC: histone deacetylase
Hes5: hairy and enhancer-of-split 5
HVC: high vocal center
i.p.: intraperitoneally
IRES: internal ribosome entry site
KA: kainic acid
LEF: lymphoid enhancer factor
LTP: long-term potentiation
MeCP2: methyl CpG-binding protein
mg/kg: milligrams per kilogram
miRNA: micro ribonucleic acid
mM: millimolar
Mrna: messenger ribonucleic acid
mTOR: mammalian target-of-rapamycin
NADH: nicotinamide adenine dinucleotide
NCAM1: neural cell adhesion molecule
NDS: normal donkey serum
NeuN: neuronal nuclear antigen
ng/ml: nanogram/milliliter
NMDA: N-methyl-D-aspartate
NR1: NMDA receptor subunit 1
NRSE/RE1: neuron restrictive silencing element/restrictive element 1
NRSF/REST: neuron restrictive silencing factor/*RE-1* silencing transcription factor
NSC: neural stem cell
PAGE: polyacrylamide gel electrophoresis
PBS: phosphate-buffered saline
PCNA: proliferating cell nuclear antigen
PFA: paraformaldehyde
Prox1: prospero homeobox protein 1
PVA/DABCO: polyvinyl alcohol/1,4 diazabicyclo [2.2.2]octane
RNA: ribonucleic acid
SE: status epilepticus
sem: standard error of mean
SGZ: subgranular zone
Shh: sonic hedgehog

shRNA: short hairpin ribonucleic acid
SVZ: subventricular zone
SWI/SNF: switch/sucrose nonfermentable
TAM: tamoxifen
TBS: tris-buffered saline
TUNEL: terminal deoxynucleotidyl transferase dUTP nick end labeling
VPA: valproic acid
WT: wildtype
YFP: yellow fluorescent protein

# CHAPTER 1

## Introduction to the Literature

The human brain represents one of the last great frontiers for biologists. Despite multiple millennia of philosophy and two centuries of hypothesis-driven research, we are still far from understanding how a simple seven-pound organ can be responsible for the scope of human achievement, from the music of Rachmaninoff to the movement of the big toe.

The immense possibility of the human brain has for decades been attributed to synaptic plasticity, rather than an expansion of cell number. Since the groundbreaking work of Ramon y Cajal, neuroscientists operated under the assumption that there was no ongoing neurogenesis in the adult brain. Therefore, all learning and memory that occurs postnatally must be due to the creation, maintenance, and selective deletion of synapses. The discovery in the 1960s that there were in fact proliferating cells that could generate new, functional neurons after birth was initially highly controversial and is still the subject of intense research and discovery. This chapter will describe the discovery of this adult neurogenesis as well as the strides made in uncovering the regulatory mechanisms underlying the process, as well as the effect of pathological and physiological induction on neurogenesis.

### Discovery and Description of Adult Neurogenesis

In 1913, Ramon y Cajal stated that all neurons in the brain were generated from embryonic development, with no additional neurons being added after birth [1]. This statement quickly became dogma, and for fifty years there was little question that the brain was a static organ. However, in 1962, Joseph Altman was characterizing glial proliferation two months after traumatic brain injury when he noted the presence of $^{3}$[H]thymidine labeling in cells outside of the injury zone, particularly in the hippocampus [2]. By morphology, the labeled cells appeared to be multiple cell types,

including neurons. Altman deduced that these cells might represent the progeny of an proliferating, undifferentiated cell [2]. He confirmed this hypothesis with a series of elegant pulse-chase experiments, where rats were injected with $^{3}$[H]thymidine, sacrificed at different time points, and analyzed for $^{3}$[H]thymidine incorporation in granule neurons. By two months after injection, the vast majority of labeled cells had the stereotypical granule cell morphology. However, Altman described a group of "small cells with darkly staining nuclei" [3] found within the subgranular zone (SGZ) that incorporated $^{3}$[H]thymidine at high rates four days after injection but declined over time. Altman suggested that these small cells were his originally proposed undifferentiated cells, which proliferated and over time differentiated into mature granule neurons [3]. Today, these cells are known as adult neural stem cells.

Due to its refutation of dogma, Altman's work was highly controversial and was essentially ignored for several years. Confirmation of Altman's observation came from a surprising quarter. Fernando Nottebohm had been instrumental in describing the regions of the canary brain necessary for the learning, retention, and manipulation of birdsong, which he named the high vocal center (HVC). He and his lab noted that female canaries, who tend to sing very little, adopted masculine birdsongs when treated with testosterone. In addition, the size of the HVC in these birds increased significantly. They hypothesized that this increase was due to the addition of new cells in these testosterone-treated females, and that this process may underlie the assumption of new birdsongs and the ability to modulate songs in adulthood [4]. To test this, they treated birds with $^{3}$[H]thymidine to label dividing cells. As expected, they observed $^{3}$[H]thymidine-labeled neurons in testosterone-treated birds. Unexpectedly, labeled neurons were also noted in untreated animals; birds that had been sacrificed 48 hrs after $^{3}$[H]thymidine administration had a large population of labeled cells lining the ventricle lying between the hippocampus and the HVC. Based on this data, Nottebohm argued that neurogenesis was occurring in songbirds on a regular basis, and these newly-generated neurons originated from some type of precursor cells based in the ventricular zone [5]. Furthermore, electrophysiological recording of these labeled neurons confirmed that they

were functional and were capable of integrating into the surrounding circuitry [6], strongly suggesting that adult neurogenesis was likely a critical part of ongoing brain plasticity and not simply an interesting phenomenon.

While ongoing neurogenesis had been confirmed in songbirds and rodents, this process may be an evolutionary holdover that had been eliminated in higher order animals. If, indeed, adult neurogenesis could be confirmed in humans, the process might be necessary for some critical brain mechanism. The first suggestion of continued proliferation in the human brain came from Fred Gage's lab in 1998 [7]. Human brain tissue from cancer patients treated with BrdU as a diagnostic measure was stained for BrdU. Significant numbers of BrdU-labeled cells were found in all five patients examined, with cells predominantly being found in the dentate gyrus (DG) and the SVZ. Interestingly, the number of labeled cells decreased over time, with patients with shorter survival times harboring more cells than those with longer survival times. In addition, these BrdU cells colabeled with NeuN, suggesting that, as in lower order vertebrates, humans also have resident proliferating progenitors that can form new neurons.

If indeed there were neural stem cells resident in the adult brain, they should be able to be isolated and maintained in culture conditions that mimic the *in vivo* brain environment. Furthermore, the difficulty with *in vivo* studies necessitated the generation of an *in vitro* cell culture model that could be used to more easily tease out the mechanics of these adult neural stem cells. To this end, Fred Gage's lab succeeded in isolating a population of proliferating cells from adult rat hippocampus [8]. These cells could be maintained in culture with the addition of the growth factor Fgf-2 and could be induced to form neurons, oligodendrocytes, and astrocytes with the addition of fate-specifying factors. These cells could be successfully transplanted back into the rat hippocampus, where they migrated into multiple regions of the brain and differentiated into all three lineages [8]. This cell culture system, known as HCNs (hippocampal neural progenitors), is commonly used throughout the adult neurogenesis field and is still being maintained as of this writing.

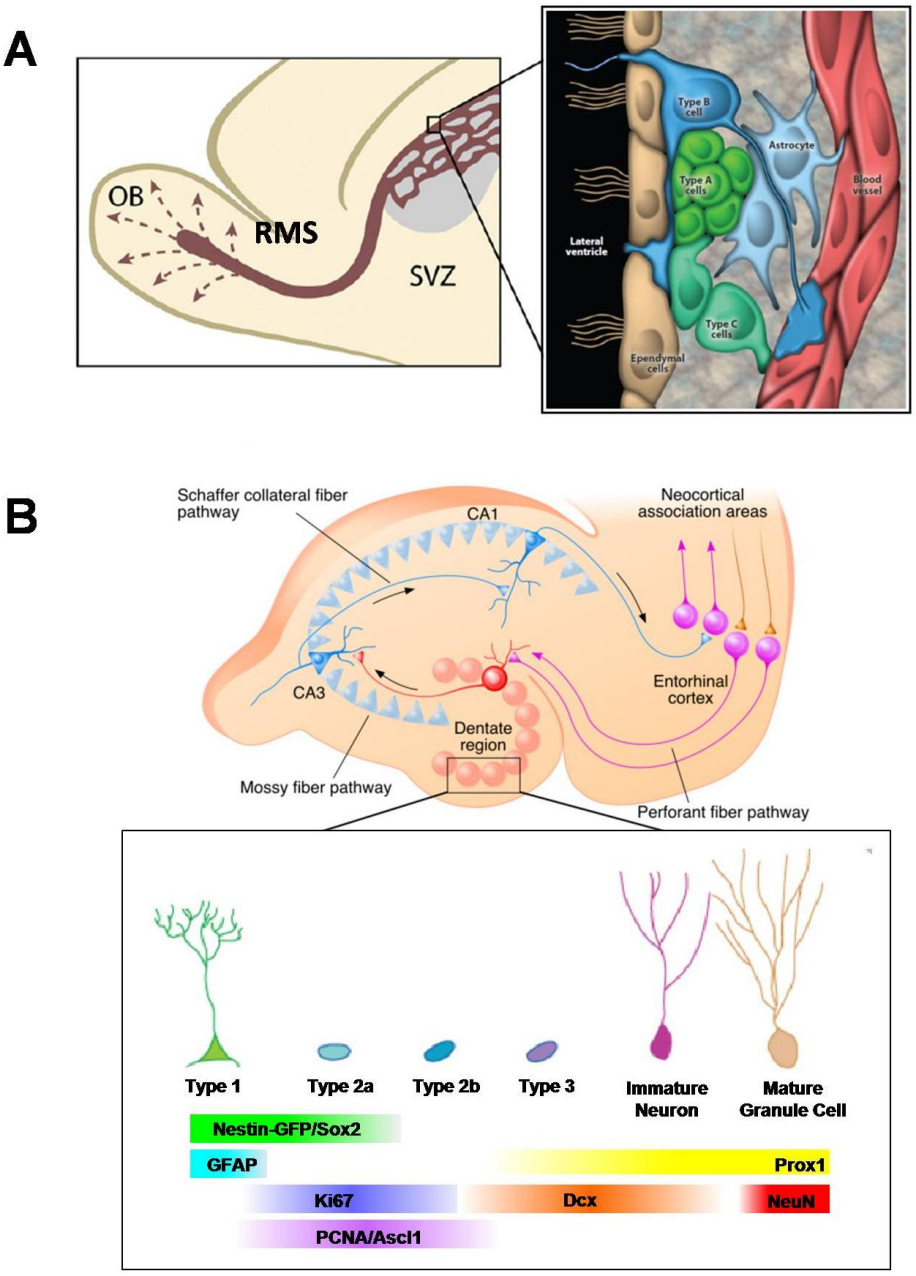


**Figure 1.1. Neurogenic regions of the adult mouse brain.** (A) Diagram of the neurogenic niche in the subventricular zone. (B) Diagram of the structure of the hippocampus and the cell-type specific markers for each stage of subgranular zone neurogenesis. Modified from Hsieh and Eisch 2009 and Suh 2009.

## Neurogenesis in the subventricular zone

One of the two neurogenic regions in the adult brain is the subventricular zone (SVZ) of the lateral ventricle. The lateral ventricle runs through both hemispheres of the brain, circulating cerebrospinal fluid (CSF) throughout the brain. Along the lamina of the lateral ventricle is a layer of ependymal cells with cilia extending into the circulating CSF. A second population of cells, the Type B cells, sits just medially of the ependymal layer and extends short processes through the layer to make contact with the interior of the ventricle (Figure 1.1A). These Type B cells express GFAP and are capable of differentiation into a more highly proliferative Type A neuronal progenitors, which in turn differentiate into neuroblasts. These still-maturing new neurons begin to migrate along the rostral migratory stream and continue maturing until they reach the olfactory bulb, where they integrate into the surrounding circuitry [9].

Neurogenesis in the SVZ is a complex process that encompasses its own body of literature. This thesis will focus exclusively on neurogenesis in the subgranular zone (SGZ); therefore, I will leave a more comprehensive description of this region to others and focus on the SGZ.

## Neurogenesis in the subgranular zone

The second neurogenic region of the adult brain is found in the SGZ of the DG in the hippocampus (Figure 1.1B). The DG is made up of two leaves of predominantly granule cell neurons which form a tooth or arrowhead shape surrounding the hilus, through which the axons from the granule neurons project to the pyramidal neurons of the CA3. The inner region of the two leaves makes up the SGZ, in which reside Type 1 neural stem cells. These cells have a striking radial morphology, with a triangular-shaped cell body, a single thick process extending through the granule cell layer, and a small tuft of processes extending into the molecular layer. These Type 1 cells are quiescent, dividing only very rarely to produce a Type 2a daughter cell. These Type 2a cells are

highly proliferative, with a cell cycle length of only 14 hours [10], and have only very short horizontal processes that are often difficult to see [11]. Type 2a cells begin to mature into less proliferative Type 2b and Type 3 cells, which are neuronally fated. These Type 3 cells eventually cease all proliferation and assume an immature neuronal morphology, with a rounded cell body and a large dendritic tree. The entire process completes with a mature granule neuron with mature morphology being generated 28 days after the initial differentiation signal [9].

A major difficulty in the field of adult neurogenesis is the lack of a single specific cell marker for each cell type along the process. Many markers will label multiple stages of the differentiation process or may be specific for other cell lineages, necessitating the use of a panel of markers to specifically identify each stage along the neurogenic process (Figure 1.2B). Type 1 cells label for stemness markers, such as nestin and Sox2, but also for GFAP, a well-known marker for some astrocytes. Because of their quiescent nature, they very rarely colocalize with markers of proliferation, such as BrdU, PCNA, and Ki67. Their highly proliferative Type 2a daughter cells, however, are commonly positive for proliferation markers. In addition, these cells lose GFAP expression while retaining nestin and Sox2 and begin expressing markers of differentiation, such as Ascl1. As they differentiate into Type 2b cells, stemness and proliferation marks disappear to be replaced by more neuronally fated markers, particularly doublecortin (Dcx). By the Type 3 stage, Ascl1 and proliferation markers have disappeared and are replaced only by markers of neuronal fate. At the immature neuron stage, the cells begin expressing markers of a neuronal identity, including Prox1 and calretinin. Once they are fully mature, markers of immaturity, such as Dcx and calretinin, are replaced by markers of maturity, including NeuN and calbindin [9, 12-13].

Many other models of stem cell biology house the stem cell population in a discrete niche, including the Drosophila ovary and mammalian epithelial and hematopoietic stem cells [14]. Little is known about the microenvironment necessary for neural stem cell maintenance. However, there is evidence that proliferating progenitors form clusters that are localized near capillaries in the SGZ. These clusters include

proliferating neuroblasts, some of which express VEGF receptors that would make them sensitive to signaling from endothelial cells, and some cells that themselves express endothelial markers [10, 15]. The exact relationship of this angiogenic niche to stem cell maintenance, proliferation, and neuronal differentiation remains unclear but is likely to hinge on the delivery of metabolic substrates and signaling molecules to the stem cell pool.

Interestingly, cell death plays a significant role in neurogenesis, with two waves of death eliminating first progenitors and then immature neurons. This first wave was first indicated by a significant loss of BrdU+ cells by 7 days after labeling [11]. This loss was pinpointed to within the first four days after a proliferation event, with unactivated, ramified microglia clearing newborn Type 2 and 3 cells. However, it is unclear what criteria determines which progenitors survive and which are cleared [16]. The second wave is assumed to occur during the immature neuron stage, between 7 and 28 days [17], when activity from the surrounding mature granule neurons and directly sensed by the newborn neuron determines its survival [18-19]. There is significant evidence that both glutamatergic and GABAergic signaling is linked to the regulation of neurogenesis and the survival of the resulting neurons, a topic that will be discussed more deeply below, but how the cell dies and is cleared is virtually unknown. Part of this process is mediated by the proapoptotic factor Bax, as Bax knockout animals have significantly less death and more newborn neurons retained, although these cells migrate ectopically and appear to never fully mature [20].

Those cells that survive are capable of forming functional granule neurons that morphologically and electrophysiologically resemble mature, existing granule cell neurons by 4 weeks after birth [21-22]. As these neurons develop, they project dendrites that reach the molecular layer by 10 days after birth. These dendrites form a complex tree that is very similar to mature neurons by 21 days after birth. Spines are generated by 16 days and functional synapses from the perforant path and to hilar interneurons and pyramidal neurons in the CA3 are present shortly after [23-25]. These newborn neurons tend to be more excitable than preexisting granule neurons [26], as they depolarize with

less stimuli and can generate a strong LTP with a longer decay rate that may be able to overcome the inhibition of more mature neurons [27-28]. Interestingly, newborn neurons exhibit tonic GABA activation even before they show a mature morphology. They are then innervated first by GABAergic signaling and then by glutamatergic synapses, much as developmentally generated neurons mature [29].

The generation of these new functional neurons in the hippocampus has been linked to many processes, from physiological to pathological. The hippocampus is critical to short-term memory formation and consolidation, as shown in human cases such as the famous Patient H.M., who after removal of both his temporal lobes as a treatment for intractable epilepsy was no longer able to form short term memories while showing less impairment in the retrieval of memories formed before the surgery [30]. There is a large body of research that suggests that the effectiveness of the hippocampus is due to its neurogenic potential. For instance, simple ablation of neurogenesis by genetic models, chemical agents, and irradiation has been linked to deficits in spatial learning and memory [31-33], although there is some suggestion that not all forms of hippocampal memory are associated with neurogenesis[34]. Interestingly, however, neurogenic deficits can actually amplify other forms of memory, as ablation of new neurons by irradiation and genetic means enhances working memory tasks with high interference, suggesting that the inability to form new neurons in response to new stimuli blocks overlap between two contradictory pieces of information. In short, because the mouse can not remember the first memory, it can not interfere with the second memory [35]. Correlating with the ablation data, the learning process itself has been linked to increased proliferation and neuron generation [36], and six week old neurons are more likely to be activated during Morris water maze probe training than their counterparts in a CaMKII mutant model of spatial learning deficiency that is not neurogenesis-dependent [37]. Taken together, it is legitimate to assume that a large part of hippocampal learning and memory is rooted in the neurogenic process, although the extent of its contribution still remains to be elucidated.

Research into adult neurogenesis has also shed light into other processes not usually ascribed to the hippocampus. Animal models of chronic stress and depression have been linked to lower rates of proliferation and neurogenesis in the DG [38-39], while chronic treatment with antidepressants has been shown to significantly increase neurogenesis while ameliorating the mental disorder [40]. Indeed, constant SGZ neurogenesis is likely necessary to gain the full benefit of antidepressant use [41]. Furthermore, there is increased suggestion that neurogenesis may be important in female mating behaviors, as females exposed to male pheromones have a strong increase in neurogenesis in both the SVZ and the SGZ. Ablation of neurogenesis by AraC eliminated the normal female preference for dominant males, suggesting the neurogenesis may be needed for the choice of the fittest mate [42].

While these findings strongly implicate adult SGZ neurogenesis in hippocampal function, particularly in learning and memory, the exact mechanism underlying the contribution of adult neurogenesis remains elusive. One interesting theoretical model suggests that the neurons formed by ongoing neurogenesis are critical to prevent catastrophic interference in the face of new and novel stimuli. Wiskott postulates that the DG acts as an encoder for new memories, which are actually stored in the CA3. The CA1 retrieves memories from the CA3 and decodes them for transfer into the cortex. New neuron integration in the DG creates new network points that must be encoded, thus expanding the CA3 network and allowing efficient retrieval of the information without interference from other, unrelated network patterns [43]. In addition, neurons generated at similar timepoints may signal to similar cohorts of CA3 neurons, producing overlapping patterns, while a set of neurons generated at a later timepoint will likely signal to a completely different set of CA3 neurons, generating an entirely different pattern. Thus, when a memory is triggered due to one event, such as a scent or a song, other memories that occurred at roughly the same time or place will also be activated, resulting in overlapping memories being retrieved as well as the initial memory[44].

As adult neurogenesis has been established as an important aspect of cognition, one would think that it would be preserved over the life of the animal. However, this is

not the case, as rates of proliferation and neuronal differentiation significantly decrease over time. In the rodent model, BrdU incorporation drops significantly throughout life, with year-old animals incorporating only 10% the BrdU their two month old counterparts take up. Interestingly, the largest drops occur before the first year of life; older animals maintain a more steady, although significantly lessened, rate of proliferation [45-46]. The reason for the drop is not currently clear. It is possible that over time NSCs lose proliferative capability and either senesce or die off as a natural part of aging, but currently these studies have yet to be published. There does appear to be a population of NSCs that are reactive to neurogenic induction, as voluntary exercise can induce proliferation and differentiation in aged animals [47]. Furthermore, housing animals in an enriched environment for their entire lifespan can prevent some of the proliferation loss, although not completely [48].

**Regulation of Adult Hippocampal Neurogenesis**

Neurogenesis in the developing embryo makes use of several major regulatory pathways to direct the development of the brain, particularly Notch, Wnt, Sonic Hedgehog (Shh), and Bmp signaling. While adult neurogenesis is a fundamentally different process than embryonic neurogenesis, it is not beyond reason that both processes have similar regulatory mechanisms.

The Notch pathway in particular has emerged as a critical pathway for the maintenance of the stem cell pool. Notch is known to signal to both Type 1 and Type 2a cells [49], and loss of Notch signaling leads to acceleration of differentiation and depletion of the NSC population in both the adult SVZ [50] and SGZ [51-52]. Conversely, overexpression of Notch signaling maintains stem cell identity and proliferation [52]. Interestingly, Notch may have an effect on dendritogenesis in newly born neurons. Cells devoid of Notch have sparse dendrites, while neurons with constitutive Notch signaling have significantly larger and more complex dendritic trees [52]. This regulation of dendritogenesis may be due to a regulation of the RhoA-associated serine/threonine kinases Rock 1 and 2 in parallel with p53 [53]. In addition, a

very recent study suggest that EGF and Notch signaling may be linked, as EGFR signaling in progenitors can repress Notch signaling and deplete the stem cell pool in the SVZ [54]. Interestingly, this finding also posits a feedback loop from more differentiated cells that can regulate the proliferation and maintenance of NSCs.

The Wnt pathway, both canonical and non-canonical, has also been implicated in adult neurogenesis. For instance, Wnt3 expression induces differentiation, while blockade of Wnt signaling nearly completely blocks neurogenesis [55]. In addition, lithium chloride, one of the earliest and most widely used mood stabilizers, induces progenitor proliferation [56] and blocks differentiation, possibly by elevating β-catenin and antagonizing GSK3β to preserve Wnt signaling [57]. This process may be mediated in part by DISC1, which has also been implicated in maturation and integration of newborn neurons [58-59].

The Sonic Hedgehog (Shh) pathway has also been implicated in adult neurogenesis, although its exact function is not as well understood. Shh is known to be necessary for proliferation and neurogenesis in the adult SVZ [60], and quiescent NSCs are sensitive to Shh signaling [61]. Interestingly, Shh is critical for the formation of the DG in the embryo, likely by utilizing the primary cilia of NSCs to transduce its signaling [62]. Nestin+ stem cell deletion of Sox2, which marks both Type 1 and Type 2a cells [63], results in loss of the stem cell pool and of neurogenesis shortly after birth, likely due to the misregulation of Shh, which is a direct target of Sox2. Treatment of Sox2 conditional knockout animals with a Shh agonist partially rescued this phenotype. There is also some suggestion that Wnt signaling may be partly regulated by Sox2, as Wnt3a mRNA was lost during embryonic development [63], suggesting that there might be crosstalk between the Wnt and Shh pathways.

While not as well studied as other pathways in the SGZ, the BMP pathway has been implicated in the maintenance of the stem cell pool as well. BMP signaling from the SVZ ependymal layer blocks neurogenesis [64]. In the SGZ, loss of Bmpr1a by genetic ablation or blockade with Noggin increases proliferation of Type 1 NSCs and

decreases neurogenesis, while treatment with BMP preserved quiescence [65-66]. Interestingly, BMP has been implicated in astrocytic fate choice, possibly by signaling to NRSF, a repressor of neuronal genes (see below), to restrict neuronal fate [67].

There is significant evidence that epigenetic regulation, particularly chromatin remodeling factors, may be deeply involved in the regulation of neurogenesis [68]. Valproic acid (VPA), a broad-spectrum histone deacetylase (HDAC) inhibitor, is a commonly used means of inducing differentiation in NSC cell cultures, possibly by inducing NeuroD expression [69]. Furthermore, the histone methyltransferase Mll1 is necessary for NSC fate choice in the SVZ, an effect that is likely mediated by the transcription factor Dlx2 [70] At the transcriptional level, Tlx, a nuclear receptor critical for NSC proliferation and maintenance [71], interacts with various HDACs to repress its target gene p21, thus preventing differentiation and mediating the maintenance of NSC proliferation [72]. p21 is also the target of Bmi-1, a polycomb family member known to recruit epigenetic machinery to repress its target genes [73]. Bmi-1, like Tlx, is also necessary for NSC self-renewal [74]. Interestingly, polycomb factors have been implicated in astrocyte vs neuron fate choice in the embryo and may have a similar function in the adult NSC [75].

An emerging field of investigation is small RNA regulation of neurogenesis, another means of epigenetically regulating gene expression. Although only a few years old, this direction has already isolated several microRNAs that are critical for neurogenesis. For instance, the small RNA miR-124 has been shown to induce differentiation in the SVZ by its repression of Sox9, the Notch ligand Jagged 1, and the transcription factor Dlx2 [76]. Conversely, miR-137, which is regulated by MeCP2 and Sox2, promotes proliferation at the expense of neuronal differentiation by repressing the polycomb family member Ezh2 [77]. MicroRNAs may also be necessary for later stages of neurogenesis, as miR-137 is necessary for dendritogenesis in immature neurons [78]. Additionally, miRNAs may be regulated by and themselves regulate transcription factors critical to neurogenesis. For instance, miR9 forms a negative feedback loop with Tlx to regulate proliferation and differentiation [79], and other similar relationships are likely.

The presence of a neurogenic region in the adult brain suggests that the hippocampus needs some means of remodeling itself due to extra-hippocampal activity. Thus, it is logical to assume that neuronal activity has some means of signaling to the NSC pool. While the exact mechanism still remains elusive, there is tantalizing evidence that this is indeed the case. For instance, knockout of gelsolin, a calcium-activated actin severer found in Type 1 NSCs, in nestin+ cells results in increased proliferation and Dcx expression, although cells in culture showed no difference in either parameter. The increase may be due to increased excitatory neurotransmitter release, and increased blood flow and vasculature in the hippocampus, which likely affects the putative vascular niche [15]. Increased activity of the surrounding neurons and a stabilized and expanded neurogenic niche may induce the noted higher rates of proliferation and differentiation [80]. Furthermore, the deletion of cAMP response element-binding protein (CREB) at the NSC level blocks differentiation, dendritogenesis, and survival of newborn neurons, likely due to a loss of sensitivity to GABA excitation [81], which is itself critical for the integration of new neurons into the surrounding circuitry [29]. In addition, newly generated neurons lacking a receptor for NMDA die off during maturation, suggesting that the ability to sense activity and form functional synapses is critical to the survival of adult-born neurons [82].

In addition to likely ensuring the survival of newly generated neurons, neuronal activity may itself be enough to induce proliferation and neurogenesis. LTP induction is enough to strongly drive proliferation of neuronal precursors, whether induced through the perforant path [83-84] or the mossy fiber afferents [85]. This process is likely dependent in some part on signaling through NMDA receptors [84]. Furthermore, there appears to be a critical period between seven and ten days after birth when LTP activation is necessary to ensure cell survival [86]. Regulation of neurogenesis by LTP and general neuronal activity may provide the link between the observed neurogenic effects of certain learning tasks and enriched environments [87], as LTP is generally considered to be a critical mediator of learning and memory [88]. In addition, GABAergic signaling may also play a role driving neuronal differentiation by signaling directly to Type 2 progenitors [89].

As discussed in this section, adult neurogenesis is a highly complex process with a multitude of factors influencing each stage of the process. The next two sections will introduce two important transcription factors, NRSF/REST and NeuroD, whose contributions to neurogenesis will be discussed in Chapters 3 through 5.

**NRSF/REST**

Neuron-restrictive silencer factor (NRSF), also known as RE-1 silencing transcription factor (REST), is a Krüppel-like transcription factor that binds a 21-23 base pair (bp) region, the NRSE/RE1, that is found in a multitude of genes throughout the genome [90-92]. NRSF's protein structure includes an N terminal repressive domain, an eight zinc finger DNA binding domain, and a C terminal repressive domain that includes a ninth zinc finger motif [93]. These domains recruit a wide variety of corepressors, such as Sin3A [94] and CoREST [95], and chromatin remodeling factors, including HDACs [94], the SWI/SNF complex [96], and possibly certain polycomb factors [97]. In addition, the genomic sequence codes for six exons, which can be spliced into at least five isoforms along with the functional full-length protein [93].

NRSF/REST (hereafter referred to as NRSF) was initially identified in parallel by both David Anderson's group [98] and Gail Mandel's group [99] in 1995. Anderson's group also generated a total knockout, which resulted in complete embryonic lethality of homozygotes by E11.5 [100]. Interestingly, the heterozygotes survived past birth with no apparent difference from wildtype animals. NRSF is ubiquitously expressed in the embryo at E8.5 to E9.5; by E9.5 the NRSF knockout embryos were already noticeably smaller and had begun to show widespread apoptosis. In addition, ectopic expression of the NRSF target gene Tuj1 was noted in the developing heart, myotome, spinal ganglia, and limb bud along with the widespread cell death. Although the early death of the embryo makes any neurological analysis difficult, its repression of neuronal genes in development suggests that NRSF is a critical factor in neurogenesis.

NRSF has traditionally been considered a repressor of neuronal genes in non-neuronal cells, as most of the target genes identified early on were neuronal [92, 98].

Indeed, NRSF is highly enriched on binding sites of neuronal genes in astrocytes [67]. However, as more sensitive means of genome mining were developed, including microarray and deep sequencing techniques, NRSF's function began to take on a far more complex role. NRSEs have been found in many non-neuronal genes, including genes with functions in pancreatic islet formation [90], cell adhesion [91], and mitosis [101]. In addition, NRSF can bind to various nucleotide permutations of the NRSE binding site with different affinities, which are occupied and actively repressed based on the expression levels of NRSF in the cell at that time [102]. These sites can be bound with different affinities for NRSF, allowing easy or difficult clearance depending on the strength of the interaction between NRSF and the site [103]. Furthermore, NRSF can bind a noncanonical site made up of the two halves of the canonical NRSE separated by a 17 bp spacer that is found in at least 20 binding sites [90]. This wide array of binding sites suggests that NRSF may play a more complex regulatory role than just simple transcriptional repression, particularly in light of the discovery of a dsRNA that can shift NRSF function from repression to activation [104], and argues that NRSF may have a role in far more functions than simple neurogenesis. This possible additional function has already been hinted at, as NRSF has been implicated in oncogenesis in a variety of tissues, including colon [105] and lung [106].

NRSF's function in embryonic development is obvious from the knockout phenotype [100], and NRSF occupancy of its target genes has been noted in mouse embryonic stem cells (ESCs), multipotent NSCs, and mature neurons, although this occupancy varies by individual cell type [107]. Interestingly, a highly-publicized report from Sadhan Majumder's group found that deletion of NRSF from ESCs blocks proliferation and drives differentiation into multiple lineages, suggesting that NRSF is critical to ESC identity and maintenance. In addition, it posited that NRSF maintained pluripotency by regulating the expression of several miRNAs, particularly miR-21 [108]. However, this report has been highly controversial, with several reports from others in the field that the data is not reproducible [109-110]. A second report suggested that while a cohort of NRSF target genes are precociously upregulated after NRSF deletion in ESCs, none of these disregulated genes are involved with ESC maintenance or differentiation.

NRSF knockout ESCs continue to proliferate and begin to differentiate into multiple lineages normally, directly contradicting Majumder's initial study [111]. While NRSF may have a function in some aspect of ESC function, this role remains elusive.

NRSF's role in neuronal differentiation from ESCs is much less controversial. NRSF occupancy and evacuation of its binding sites has been strongly linked to neuronal differentiation during the transition from ESC to mature cortical neuron [112]. In ESCs, NRSF binds its cohort of target genes and allows only a minimum of expression. As an ESC transitions to a neuronal progenitor, NRSF is selectively degraded [112], possibly by the E3 ubiquitin ligase β-TRCP, which can bind NRSF, ubiquitinate it, and send it to the proteosome for degradation [113]. This process may be antagonized by the telomere cap protein TRF2, which binds NRSF and prevents its degradation [114]. This degradation allows the full complement of neuronal genes to be expressed, allowing the transition to a more restricted cell type. However, there is likely a second class of genes, including BDNF and calbindin that are regulated by multiple factors, including MeCP2 and CoREST. After NRSF has been detached from its NRSE, these other factors continue repressing the target gene until another stimulus, such as membrane depolarization, allows greater expression of the otherwise repressed gene [112]. Based on this data, it is clear that, while NRSF may be an important regulator of many genes, it does not act in a vacuum. Instead, NRSF may be part of a much larger and complex network that is capable of very subtle changes in gene expression based on the current situation.

While the complexity of the downstream targets of NRSF is increasingly well understood, the upstream regulators of NRSF are barely known. Little work has been done to determine what may directly act on NRSF in the adult neural stem cell context. It is clear that NRSF is directly upregulated by the Bmp pathway in embryonic neural progenitors to block neurogenesis and induce gliogenesis [67]. Furthermore, the canonical Wnt pathway drives expression of NRSF during spinal cord development in the chick, although this has not been replicated in rodent models [115]. NRSF is also directly downstream of the Notch pathway, as Hes1 is able to bind and repress NRSF expression in cell culture systems [116]. Again, this has not been replicated in rodent

neuronal tissue, leaving many questions unanswered about how exactly NRSF is regulated and how this control translates in to downstream effects.

**NeuroD**

NeuroD1 (hereafter referred to as NeuroD) is a member of the basic helix-loop-helix (bHLH) family of transcription factors, a family with other members implicated in the regulation of neurogenesis, such as Mash 1 [117]. bHLH proteins interact with E proteins, which are expressed ubiquitously, to bind to E boxes found throughout the genome; inhibitory bHLH proteins can disrupt the protein-protein interaction, causing displacement of the complex from the genome [118].

NeuroD was originally cloned by Jacqueline Lee in 1995 [119]. Its expression in the developing embryo begins at E9.0, roughly the same time as the beginning of brain development. In addition, when NeuroD is overexpressed in *Xenopus* embryo ectoderm, a tissue that is not fated to generate neuronal tissue, the cells begin to express neuronal markers and adopt neuronal morphology [119]. Furthermore, knockout of NeuroD in retinal progenitors leads to an expansion of glia at the expense of neurons, accompanied by an increase in dying cells in the early postnatal retina [120]. These findings, along with the fact that many of NeuroD's demonstrated and predicted target genes are involved in neuronal fate choice or function [121], strongly suggest that NeuroD may have a function in cell fate and differentiation.

A total knockout of NeuroD was generated by the lab of Ming-Jer Tsai in 1997. The resulting mice were severely diabetic and died shortly after birth. Interestingly, postmortem analysis of the mice revealed that the pancreas was able to form. However, there was a severe reduction in β-cell number, and pancreatic islets were unable to form [122]. This postnatal lethality made it difficult to study the mouse brain, and the original knockout paper did not include an analysis of neuronal phenotypes. To remedy this, Jacqueline Lee's group generated a second knockout line with a knocked-in transgene carrying the NeuroD coding region driven by the insulin promoter, allowing NeuroD expression in the developing pancreatic islets but not in the developing brain [123].

These mice survived to adulthood but were much smaller and severely ataxic. Much of the brain appeared grossly normal except for the hippocampus and cerebellum, where the population of granule cells was severely depleted. The hippocampus in particular showed a complete loss of the entire dentate gyrus layer, while all other regions, including all CA regions, were otherwise normal. This loss of the DG was accompanied by a loss of proliferation, as shown by BrdU incorporation, and a massive increase in cell death, as determined by TUNEL assay [123]. Interestingly, the Lowenstein group later bred the NeuroD total knockout from 129/SvEv mice to the C57Bl/6J mouse line and confirmed the original embryonic lethality phenotype. However, when they bred the two lines together to form a hybrid, 60-70% of the mice survived to adulthood and replicated the phenotype of Lee's transgenic line. In addition, these mice developed spontaneous seizures [124]. Based on this phenotype, it is clear that NeuroD plays an important role in the generation of the hippocampal neurogenic niche during embryonic development. It also hints at a possible function during adult neurogenesis. However, this model was unable to suggest what the exact nature of that role might be.

Studies of NeuroD function in the neurogenic regions of the developing and adult brain have shed some light on what this role might be. In the SVZ, NeuroD is found in differentiating neuronal progenitors that have begun to cease proliferation and migrate through the RMS. Interestingly, overexpression of NeuroD in NSC cultures strongly induces neuronal differentiation and neurite outgrowth [125]. A similar result is seen in the SGZ, where NeuroD is found in Type 2a and 3 cells: overexpression of NeuroD in the developing brain strongly induces differentiation into mature granule neurons that can be visualized after birth [126]. This NeuroD-induced neuritogenesis is particularly interesting from a functional perspective, as neural activity induces CaMKII phosphorylation of NeuroD, which in turn drives dendritogenesis in the adult hippocampus [127]

How NeuroD is regulated in the adult brain is essentially unknown. There is strong evidence that the Wnt pathway activates NeuroD expression by alleviating Sox2 repression at the Sox/LEF site in NeuroD's promoter [128]. However, little else is

known as to regulation by other pathways. As a bHLH protein, it is likely that NeuroD is downstream of the Notch signaling pathway, although this has not been demonstrated experimentally. There is some suggestion that Notch may signal to NeuroD through the regulation of another bHLH protein, Neurogenin [129]. How exactly NeuroD is regulated is a potentially fruitful future avenue of study.

## Induction of Neurogenesis

One of the most interesting aspects of adult neurogenesis is its sensitivity to external stimuli, both physiological and pathological. A large body of work has documented the effect of enriched environment [87], voluntary exercise [130], stress [38], ischemia [131], traumatic brain injury [132], depression and antidepressant treatment [40], and epileptic seizures [133] on either enhancement or depression of SGZ neurogenesis. Chapter 6 will deal in depth with the effect of the deletion of two transcription factors in two models of induced neurogenesis, voluntary running and epileptic seizures, which will be discussed in detail here.

### *Voluntary exercise-induced neurogenesis*

Some of the earliest induction experiments involved placing animals in an enriched environment, which included toys, nesting material, and running wheels meant to provide a more interactive, mentally-stimulating living environment for the mice [87]. Interestingly, placing a running wheel in a normal housing cage devoid of enrichment also induced a strong burst of neurogenesis in the SGZ at both early and late timepoints after exposure, while enrichment alone was only able to induce proliferation at late timepoints [130], strongly suggesting that voluntary physical activity alone is a highly neurogenic stimulus. Furthermore, this effect seems to be SGZ-specific, with no effect being noted in the SVZ [134]. In keeping with this neurogenesis hypothesis, voluntary exercise has been linked to better performance in the Morris water maze [135] and on fine pattern separation tests [136], to stronger LTP [135], and to amelioration of depression-like rodent behaviors [137].

Several factors can modulate the neurogenic effect of voluntary exercise. Stress, which on its own can depress neurogenesis [38], can antagonize the neurogenic effect of running. In particular, Stranahan et al found that social isolation during the running period can lessen the proliferative effect in rats, an effect that can be reversed by blocking the increase in glucocorticoids [138]. Furthermore, Naylor et al suggested that the beneficial effect of running may be transient, as prolonged running in a spontaneously hypertensive rat model actually has a depressive effect on proliferation, again possibly due to increased production of corticosterone [139]. Interestingly, Naylor's findings directly contradict those of Stranahan, who found that singly housed rats actually had higher numbers of BrdU-positive cells after nearly twice the running time as in Naylor's studies. This discrepancy may be due to the models used in each of these papers. Regardless of the reason, however, these findings strongly suggest that induction of neurogenesis must maintain a fine balance between the beneficial effect of the stimulus and the stress incurred by the stimulus.

The mechanism by which voluntary exercise induces proliferation and differentiation is still largely unexplored. Cell type-specific marker studies have suggested that exercise predominantly affects proliferation in Type 2 cells [11, 13], although there is suggestion that there may be quiescent NSC involvement. For instance, running was able to partially rescue the Type 1 NSC pool in irradiated mice while increasing the number of neurons generated and normalizing radiation-induced behavioral alterations in an open-field test [140]. In addition, physical exercise may signal specifically to Notch-sensitive Type 1 cell, as shown by Hes5-reporter expression [49]. However, while running is able to rescue the loss of new neurons in the Notch cKO animal by increasing proliferation of early neuroblasts, the Type 1 neural stem cell pool depletion is unaffected [51]. Based on the totality of these findings, it is likely that the bulk of the effect of exercise on neurogenesis is mediated by the proliferating Type 2 cells. This does not rule out the possibility that Type 1 cells are sensitive to the effects of exercise, as increased proliferation of neuronal progenitors may encourage increase proliferation of the NSC pool [63], particularly in an injury situation like radiation treatment.

The regulatory mechanisms underpinning exercise-induced neurogenesis are not well understood. It is likely that many of the factors regulating uninduced neurogenesis also function here, which is supported by the finding that overexpression of BMP4 in NSCs can block the neurogenic effects of running [141]. However, there are likely a few fundamental differences, perhaps in the speed of the differentiation cascade or in the survival of exercise-generated neurons. For instance, voluntary exercise may accelerate the formation of spines during neuronal maturation [23]. In addition, running increases cell death, particularly after nearly two weeks of running, which can be reversed by deletion of the NMDA receptor ε1 subunit. This death may be particularly high in preexisting granule neurons, although the identity of these dying cells was not well established [142]. At the same time, proliferation after running can be blocked by loss of the NMDA receptor ε1 subunit [143]. This activity-linked regulation of induced neurogenesis may be an insight into how the voluntary running stimulus translates into neurogenesis.

Studies in wild mice have added an interesting wrinkle to the voluntary exercise story. While inbred laboratory animals show a robust proliferative response to physical activity, wild-caught long-tailed wood mice do not show any neurogenic response to either normal laboratory housing or to voluntary running [144]. Long-tailed wood mice (*Apodemus sylvaticus*) are one of the common house mouse's (*Mus musculus)* closest genetic relatives and show robust spatial memory and intelligence in the wild. While it is possible that inbreeding in lab mice has somehow resulted in an otherwise abnormal neurogenic reaction to exercise, the presence of this effect in multiple strains suggests that something more physiological may be at work. It is more likely that neurogenic induction may have a ceiling; in animals that are constantly active and mentally engaged, higher rates of neurogenesis become normal. Furthermore, the consistent experience may make these rates persist even after the loss of the stimulus, something that is not seen in their lab mice counterparts [143]

*Seizure-induced neurogenesis*

Temporal lobe epilepsy, a disorder of recurrent seizures, affects over two million people in the United States. Early anatomical studies of the epileptic human brain noted multiple abnormalities associated with chronic epileptic seizures, including increased mossy fiber sprouting and abnormal migration of granule neurons [145-147]. In animal models of induced seizures, there is pronounced proliferation and neurogenesis in the SGZ. However, these neurons are profoundly abnormal, as they sprout mossy fibers and migrate into the hilus and molecular layer [133, 148] Mice induced with pilocarpine, a chemoconvulsant, are more likely to develop anxiety-like behaviors and learning and memory impairments but do not show depressive-like behaviors [149]. Furthermore, a single induced seizure event will usually lead to spontaneous recurrent seizures roughly one month after the initial event [150].

These abnormalities hint that neurogenesis after seizures may follow a different path than basal or exercise-induced neurogenesis. The increased proliferation may be due to a strongly neurogenic stimuli, but the effect is amplified by a shorter cell cycle time [151]. Furthermore, seizure-born neurons have more dendrites, integrate more quickly, and integrate into the surrounding circuitry earlier than non-seizure born neurons [152]. Interestingly, when compared to animals induced with voluntary exercise, seizure-born neurons exhibited synaptic characteristics suggesting they were less excitable [153]. Seizure-born neurons also sprout basal dendrites and migrate abnormally, while pre-existing neurons that also experienced seizure activity do not [154-155]. Interestingly, this abnormal migration may be due to the disregulation of reelin [156]. Blocking seizure-induced neurogenesis prevented ectopic granule neurons from being formed. However, mossy fiber sprouting was still observed [157-158], likely from newborn neurons that had ceased proliferation but were not yet mature at the time of seizure induction [155]. While the reorganized circuitry may contribute to the observed epileptogenesis, it is likely that the ectopic migration of seizure-born neurons plays a particularly large role, as larger numbers of ectopic hilar neurons correlated with more frequent spontaneous behavioral seizures in the months after seizure induction [148].

The causative factor of seizure activity on neurogenesis is largely unknown. Several studies have attempted to identify what differentiation stage is most susceptible to seizure activity. There is a strong suggestion that the Type 1 cells are particularly affected, as they proliferate more after seizures [13, 159], but Type 2a neuroblasts[49] and Dcx positive neuronal progenitors [160] have also been implicated. What is most likely is that seizure activity affects every stage of differentiation, inducing proliferation in cells still capable of dividing and generating abnormalities in immature neurons that have not yet integrated into the surrounding circuitry.

The transcriptional aspects of seizure-induced neurogenesis are equally hazy. As would be expected, seizure activity upregulates many immediate early genes, including NGFI-A, NGFI-B, NGFI-C, egr-1, egr-2, Nurr1, c-fos, and c-jun in the DG within 30 minutes of the initial induction [161-162]. CREB expresses strongly and transiently in the DG within eight hours of status epilepticus, and it is likely that it signals to downstream activity-induced regulators of neurogenesis [163]. Both the Wnt and the Shh pathways have been implicated in the proliferative effect of seizure activity, but no definitive role has been deduced [164-165]. There is differential expression of the mRNA of several bHLH transcription factors after seizures, with Mash 1 and Id2 increasing, the Notch downstream effector Hes5 decreasing, and NeuroD and NeuroD2 remaining relatively unchanged [166]. The neurogenic effect is also likely partly mediated by Gadd45b, deletion of which can partly block seizure-induced proliferation and dendritogenesis [167]. Similarly, the mTOR pathway is activated during seizure activity, and continual inhibition of the pathway with rapamycin is able to prevent mossy fiber sprouting [168-169]. There have also been several microarray studies comparing control and seizure-affected tissue [170-173], but there is very little overlap between the gene lists of each of these studies [174]. Taken together, these findings are still disjointed, with no unifying theory of the neurogenic response to seizure activity.

One area of regulation that has gained traction in the last few years is the contribution of epigenetic mechanisms, particularly histone modifications. A well-known anticonvulsant, valproic acid (VPA), is also a broad-scale HDAC inhibitor [175-

176]. Treatment with VPA after seizures blocks much of the seizure-induced proliferation, neurogenesis, basal dendrite sprouting, and cognitive impairment [177-178]. In addition, within a few hours of the initial insult, markers of active chromatin, particularly acetylated histone 4 and phosphorylated histone 3, and the histone acetyltransferase (HAT) CBP, have begun to increase throughout the DG [162]. These marks are particularly found at neuronal genes, such as GluR2 and BDNF [179-180]. Interestingly, both of these genes are targets of NRSF. NRSF itself is upregulated after seizures [93, 177] and has been implicated in the repression of BDNF in concert with the NADH-binding corepressor CtBP in a model of epilepsy that can be modulated by metabolic interventions [181]. This, plus the differential expression of bHLH factors, including NeuroD, after seizures, makes NRSF and NeuroD an attractive axis to investigate in the context of induced neurogenesis, both physiological (running) and pathological (seizures).

**Summary**

Adult neurogenesis is a complex process underlying many critical aspects in the day-to-day functioning of the brain. While there is some understanding of the regulatory processes governing neurogenesis, there is still much that is not understood, including how NSCs are maintained, how cell fate choice is made, how differentiation progresses, and how these new neurons contribute to the surrounding circuitry. The next three chapters will provide insight into these questions by focusing on two transcription factors, NRSF and NeuroD, and how they function in normal and induced neurogenesis.

## Chapter 2

## Materials and Methods

### Animal Handling

All animals used for the studies described in this dissertation were housed in the University of Texas Southwestern NG animal resource center, an Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC)-accredited facility. The facility was kept on a 12 hour light/dark cycle. Animals used in the NeuroD study were fed a 4% protein chow, while NRSF animals were fed a 16% protein diet. The difference in feed between the NeuroD and NRSF proje cts was due to a change in animal feed protocols adopted by the NG animal facility before the beginning of the NRSF project.

NeuroD inducible conditional knockout (cKO) mice were generated by crossing a nestin-CreERT2/R26R-YFP line with mice carrying a floxed NeuroD locus (generously provided by Dr. Amelia Eisch and Dr. Klaus Armin-Nave, respectively). NFSF inducible cKO mice were generated by crossing the same nestin-CreERT2/R26R-YFP line with mice with a floxed NRSF locus, generated by Dr. Zhengliang Gao in the lab of Jenny Hsieh. All genotyping of offspring was done using PCR from genomic DNA isolated from tail snips; genotyping of NeuroD offspring was done according to protocols developed in the Nave lab, while NRSF genotyping was done using primers specific for the NRSF locus. Animals were used if they were heterozygous for Cre and YFP and homozygous for either the wildtype allele or for the floxed NRSF or NeuroD allele (henceforth designated for both lines as WT and cKO , respectively),

For both NRSF and NeuroD studies, four to six-week-old WT and cKO animals were injected daily with 150 mg/kg tamoxifen intraperitoneally (i.p.) for five to six days. The tamoxifen was dissolved in 10% ethanol and 90% sunflower oil and kept from light until use. Animals were allowed to survive for a desired time in normal housing.

**Tissue Preparation for Immunohistochemistry**

On the day of sacrifice, all animals were injected with 150 mg/kg BrdU i.p. two hours prior to sacrifice, unless specifically mentioned in the text. Mice were anesthetized with choral hydrate and transcardially perfused first with ice cold 0.1M PBS for seven minutes and then with ice cold 4% paraformaldehyde (PFA) in 0.1M PBS for fifteen minutes. Brains were removed from the perfused animals, postfixed overnight in 4% PFA in 0.1M PBS overnight at 4°C, and then incubated in 30% sucrose in 0.1M PBS until the brains sank to the bottom of the storage tube, also at 4°C. Brains were then bisected laterally along the central fissure. One half was mounted in water on a freezing microtome and sectioned coronally into 30µm slices, which were stored in 0.1M PBS with 0.1M sodium azide at 4°C until staining. For the NeuroD project, sections were divided serially into 9 vials; for the NRSF project, 12 tubes were used.

**Immunohistochemistry**

Brain sections were stained using one of two methods: free-floating or slide-mounted.

Sections stained using the free-floating protocol were stained in 12-well plates. Sections were submerged in three 1X TBS washes, blocked for one hour in 3% normal donkey serum (NDS) with 0.3% TritonX-100 in 1X TBS, and incubated in primary antibody with 0.5% NDS and 0.5% TritonX-100 in TBS from 24-48 hours. After primary antibody incubation, sections were washed three times and left in either a biotin- or a flourophore-conjugated secondary antibody overnight. Sections were checked for successful stains, counterstained with DAPI for five minutes, mounted on charged glass slides (Fisher SuperFrost), and coverslipped with PVA/DABCO.

Sections stained using the slide-mounted protocol were initially mounted on charged glass slides (Fisher SuperFrost) and allowed to dry for at least fifteen minutes. All slides were pretreated with an antigen retrieval step. For the citric acid pretreatment, 0.01M citric acid in deionized water was heated in a standard microwave to near boiling (~95°C). Sections were submerged in the hot citric acid solution for fifteen minutes, with

an additional twenty seconds in the microwave every three minutes to maintain the temperature. Sections were the plunged into a 1X TBS wash to cool the slides and then incubated in fresh 1X TBS for ten minutes. For the hydrochloric acid pretreatment, sections were incubated in a 0.1% trypsin/0.1% $CaCl_2$/deionized water solution for 8-10 minutes and washed three times. After pretreatment, the slides were blocked in 3% NDS with 0.3% TritonX-100 in 1X TBS for 30 minutes to 1 hour. Sections were then incubated in primary antibody overnight. After three 1X TBS washes, the slides were incubated with a fluorescent secondary antibody (1:200, Jackson Immunoresearch) from two hours to overnight. If the primary antibody required amplification, the sections were incubated in a biotinylated secondary antibody instead of a fluorescent antibody for the same amount of time. Endogenous peroxidases were quenched by a thirty minute incubation with 0.3% hydrogen peroxide. The sections were then incubated in Vectastain ABC (Vector Laboratories), which attached a strep-avidin complex to the biotin, for one hour and then treated with Tyramide-Plus (1:50, PerkinElmer) to add the fluorophore. All sections were checked for successful staining, counterstained with DAPI, and coverslipped as with the free-floating protocol.

For sections that were stained with NRSF, the initial protocol (Figure 4.1) required the same pretreatment as BrdU. After primary antibody treatment, the stain was amplified as just described. The optimized protocol (Figure 4.2) used free-floating sections that were frozen on dry ice twice for 15 minutes each time and then fixed with ice-cold methanol for 10 minutes at -20°C. The staining protocol was then continued as usual, with no amplification necessary.

Table 2.1 summarizes the antibody concentrations, pretreatments, method of staining, and need for amplification for the immunohistochemistries described.

**Analysis of Data**

Staining patterns and YFP+ cell numbers and morphology were analyzed with an Nikon TE2000-U inverted epifluorescent microscope (Nikon, Inc.) using either a 10X or 20X objective, with images being captured using Nikon's NES-Elements software.

Colocalization of cell markers was done using a Leica scanning confocal microscope (emission wavelengths 488, 543, and 633) with a 40X oil immersion objective. Analysis, image overlay, and Z-stacks were done using Leica's confocal software. Every 12$^{th}$ or 24$^{th}$ section of each animal was analyzed while the investigator was blinded to genotype. Unless noted in the text, all analysis was done by the author.

**Table 2.1. Antibodies used in immunohistochemistry analyses.**

| Antibody | Species | Company | Conc. | Pretreat | Mode | Amp? |
|---|---|---|---|---|---|---|
| AC3 | Rabbit | Cell Signaling | 1:500 | HCl | Slide | No |
| Ascl1 | Mouse | RDI Fitzgerald | 1:750 | CA | Slide | No |
| BrdU | Rat | Accurate | 1:500 | HCl | Slide | No |
| Dcx | Goat | Santa Cruz | 1:500 | CA | Slide | Yes |
| | | | 1:5000 | N/A | FF | No |
| GFAP | Guinea pig | Advanced Immuno-chemical | 1:4000 | CA | Slide | No |
| GS | Mouse | AbCam | 1:500 | CA | Slide | No |
| GSTπ | Mouse | BD Transduction | 1:3000 | CA | Slide | No |
| Ki67 | Rabbit | Neomarkers | 1:500 | CA | Slide | No |
| NeuN | Mouse | Millipore | 1:1000 | N/A | FF | No |
| NeuroD | Goat | Santa Cruz | 1:1000 | CA | Slide | No |
| NRSF | Rabbit | Upstate | 1:500 | BrdU | Slide | Yes |
| | Rabbit | AbCam | 1:100-200 | Freeze | FF | No |
| PDGFR | Rat | Santa Cruz | 1:500 | CA | Slide | No |
| Prox1 | Rabbit | Millipore | 1:1000 | N/A | FF | No |
| Sox2 | Rabbit | Chemicon | 1:500 | CA | Slide | No |
| YFP | Chicken | Aves | 1:8000-1:12000 | CA | Slide | Yes |
| | | | 1:2000 | None | FF | No |

Conc: concentration; Pretreat: pretreatment; Amp: amplification

**Statistics**

Cell counts for each animal were converted to hippocampal amounts by multiplying by the number of sections containing dentate gyrus and by the number of wells sections were collected in. Based on the hippocampal numbers, the average number of cells per section were calculated and used for statistical evaluation. For colocalization studies, all values were normalized by the animal's total YFP+ cell numbers. All animals of each genotype and/or treatment were averaged together. Animals with fewer than ten YFP+ cells or with values beyond two standard deviations of the mean were eliminated from consideration. Statistical significance was calculated using a Student's t test with Bonferroni post-tests. All error bars are expressed as ± standard error of mean (s.e.m.). Values of $p < 0.05$ were considered significant. All statistical analyses were carried out using Graphpad Prism 5.

**Generation and Analysis of NRSF shRNA**

The four NRSF shRNA constructs were designed according to guidelines that have been previously published [182]. In brief, the mRNA sequence of rat NRSF was searched for sequences matching an AAG(N18)TT pattern with ~50% GC content and no runs of more than four A or T. Identified sequences were blasted against the rat genome for homology to other regions. Unique sequences that met these conditions were used for the shRNA construct, which consisted of the mRNA sequence and its palindrome separated by a nine nucleotide linker. Designed constructs were ordered from Integrated DNA Technologies, with sense and antisense constructs ordered at 100nM concentration with PAGE purification and 5' phosphate modification. Each sense and antisense oligo was ligated together using annealing buffer (350mM TrisHCl pH 7.5, 50mM $MgCl_2$, 500mM KCl) and an annealing PCR program (95°C for 30s, 60°C for 10 min). The double-stranded shRNA constructs were then blunt-end cloned into an Hpa1-linearized pLLU2G shRNA plasmid. Correctly ligated plasmids were confirmed by DNA sequencing. To generate lentivirus, the constructs were transfected by lipofectamine 2000 into 293T cells. Media was collected for four days and spun at

19400rpm at 4°C for two hours to pellet the virus, which was resuspended in 1XPBS and frozen at -80°C until use. For electroporation, 2.5µg of each construct was electroporated into HCN cultures using an Amaxa Nucleofactor II and a rat NSC-specific kit, also from Amaxa.

For RT-PCR, cells were collected in Trizol. RNA was extracted using a phenol-chloroform extraction protocol, and genomic DNA contamination was removed using a DNA-Free kit (Ambion). RNA amounts were normalized between samples and RT-PCR was carried out using a Single-Strand Reverse Transcriptase kit (Invitrogen) using oligoDT primers. The resulting cDNA was subjected to PCR using target gene-specific primers and run on a 1.5% agarose gel. Densitometry of the bands was carried out on Image J software using the gel analysis function.

**Tissue Culture**

Unless otherwise noted, all *in vitro* analyses were done using HCNs [8], which are maintained by plating on laminin/polyornithine-coated plates in serum-free high glucose DMEM media from Omega supplemented with N2 and 20ng/ml Fgf-2 and kept in a 5% $CO_2$ chamber at 37°C. Exogenous constructs were introduced by either the addition of lentivirus directly into the media or by electroporation with an Amaxa kit.

For cell staining experiments, NSCs were plated in 4-well chamber slides after electroporation and treated as described in the text. The cells were fixed with 4% PFA for 20 minutes and then washed three times with 1X PBS. Cells were blocked with 3% NDS with 0.3% TritonX-100 in TBS for two hours and incubated in primary antibody in the same blocking solution overnight at 4°C while gently shaking. Primary antibodies used with this protocol were Tuj1 (1:500) and Map2ab (1:500). GFP could be seen without staining. After primary incubation, cells were washed three times in 1X TBS for fifteen minutes each and then with 1X TBS with 0.3% TritonX-100 for one hour. They were then incubated in secondary antibodies (1:250, Jackson Immunoresearch) in 1X TBS with 0.3% TritonX-100 for two hours, after which the slides were washed with 1X TBS three times for fifteen minutes each. After assuring the stain was successful, the

chambers were removed and the slides counterstained with DAPI and then coverslipped with PVA/DABCO.

**Induction of Seizures**

Animals with pilocarpine-induced seizures were initially treated with 1mg/kg of methylscopolamine 30 minutes before an i.p. injection of pilocarpine at doses varying from 250-300 mg/kg. After pilocarpine induction, animals were observed for the development of status epilepticus (SE). If no sign of significant seizure activity was noted within 45 minutes of the initial injection, the animals were boosted with 50% of the original dose.

For kainic acid (KA)-induced seizures, animals were injected i.p. with 20-35 mg/kg of KA. As with pilocarpine, the animals were observed for SE. If no signs of seizure activity were noted within 45 minutes, a boost of 50% of the original dose was injected.

In all induction models, animals were observed for at least two hours for development of SE. Behavioral seizures were scored according to J. Racine's 1972 paper, which set up a five-grade system of seizure nomenclature: (1) Mouth and facial movements, (2) Head nodding , (3) Forelimb shaking, (4) Rearing and shaking without falling, (5) Rearing and falling, with all loss of voluntary muscle control [183]. Animals that experienced at least two instances of a Grade 5 seizure were considered in SE.

All seizure experiments were carried out in accordance with an IACUC-approved protocol.

**Voluntary Running Procedure**

Mice were housed in the running facility maintained by the Eric Olson lab. All running data was collected using Dataquest A.R.T. by Gaile Vitug, a technician in the Olson lab. In short, mice were singly housed in a shoebox cage with a freely-moving running wheel connected to a recording device to record wheel revolutions. Mice were

allowed to run ad libitum for two weeks. Control mice were group housed in the same room.

# CHAPTER 3

## NeuroD regulation of basal adult neurogenesis

The data presented in this chapter have been published as a brief communication in *Nature Neuroscience* [184].

NeuroD is a bHLH transcription factor that has previously been used as a reporter gene for induction of neurogenesis and as a cellular marker for neuronally-committed progenitors. However, little is known about the exact role of NeuroD in neural stem cells (NSCs) and in neuronal fate choice. To attempt to answer this question, a NeuroD total knockout was generated by Ming-Jer Tsai's lab in 1997 [185]. As decribed in Chapter 1, the mouse showed perinatal lethality due to pancreatic islet malformation leading to postnatal diabetes and was thus unable to display an obvious brain phenotype. By generating a NeuroD knockout mouse with a NeuroD transgene driven by an insulin promoter, the Lee lab was able to evade the lethality phenotype by ensuring proper pancreatic formation while preserving the effects of NeuroD deletion in the remainder of the mouse. Interestingly, these NeuroD transgenic mice had almost complete loss of the granule cell populations in the brain. Of particular interest, the dentate gyrus of these mice was completely ablated, with a concurrent loss of proliferating cells and neurogenesis [186]. This phenotype strongly suggests that NeuroD plays a critical role in embryonic neurogenesis and the development of the dentate gyrus. Furthermore, it implies that NeuroD may also have a function in adult hippocampal neurogenesis.

Based on this existing literature, an inducible NeuroD conditional knockout mouse line with a nestin promoter-driven Cre-ERT2 and ROSA26R YFP reporter was generated by crossing the nestin-Cre-ERT2/R26R-YFP mouse line with another line carrying a floxed NeuroD allele. This cross was carried out by a postdoc in the lab, Dr.Zhengliang Gao. In these mice, Cre fused to a mutated estrogen receptor (Cre-ERT2) was produced in nestin-positive cells but was unable to enter the nucleus until bound by

the estrogen ligand tamoxifen, which was administered in daily doses for five days. Tamoxifen induction resulted in recombination and deletion of the floxed NeuroD allele.in NeuroD flox/flox animals (hereafter referred to as cKO) as well as expression of the YFP reporter gene in both wildtype and cKO mice.

To validate the mouse model, tamoxifen was injected for five days and the animals were sacrificed at 1, 4, 6, 8, 10, 18, and 40 days after the final injection. The animals were perfused and the brains harvested and sectioned. Sections were stained by immunohistochemistry for the YFP reporter and for endogenous NeuroD. YFP+/NeuroD+ cells were counted and normalized to the total number of YFP+ cells in the total dentate gyrus. The percentage of YFP/NeuroD double-positive cells steadily increased from 10% at 1 day after tamoxifen and eventually reached ~40% by 8 days (Figure 3.1A). The population remained steady at 40 days, the last time point analyzed. Based on this data, we can expect that at any time point, NeuroD is required by a large subset of neuronal progenitors, laying the groundwork for analysis of the consequences of removing NeuroD in these cells. In addition, we can predict that the vast majority of YFP+ cells in cKO brains will be null for NeuroD. This assumption was confirmed in the cKO 40 days after the end of tamoxifen administration (Figure 3.1B), as most of the YFP+ cells in the cKO were easily identified as NeuroD negative. Colocalization of YFP with NeuroD was significantly decreased in cKO animals when compared to WT (Figure 3.1C); concurrently, we observed a nearly 50% reduction in NeuroD positive cells in the entire dentate gyrus of cKO animals (Figure 3.1D). This finding confirms that NeuroD is deleted within YFP positive cells in the cKO and allowed us to confidently proceed with phenotyping of the cKO.

To determine the effect of an NSC-specific deletion of NeuroD, YFP+ cell numbers in the dentate gyrus were compared between wildtype and cKO littermates at 6 days and 40 days after the last tamoxifen injection. No change in YFP+ cell numbers was noted between the two genotypes at the 6 day time point. However, YFP+ cell counts were significantly decreased in cKO animals at 40 days after tamoxifen, (Figure 3.2). To determine the specific cell type lost in the cKO brain, YFP+ cells were assessed for morphology and for cell type-specific cell markers.

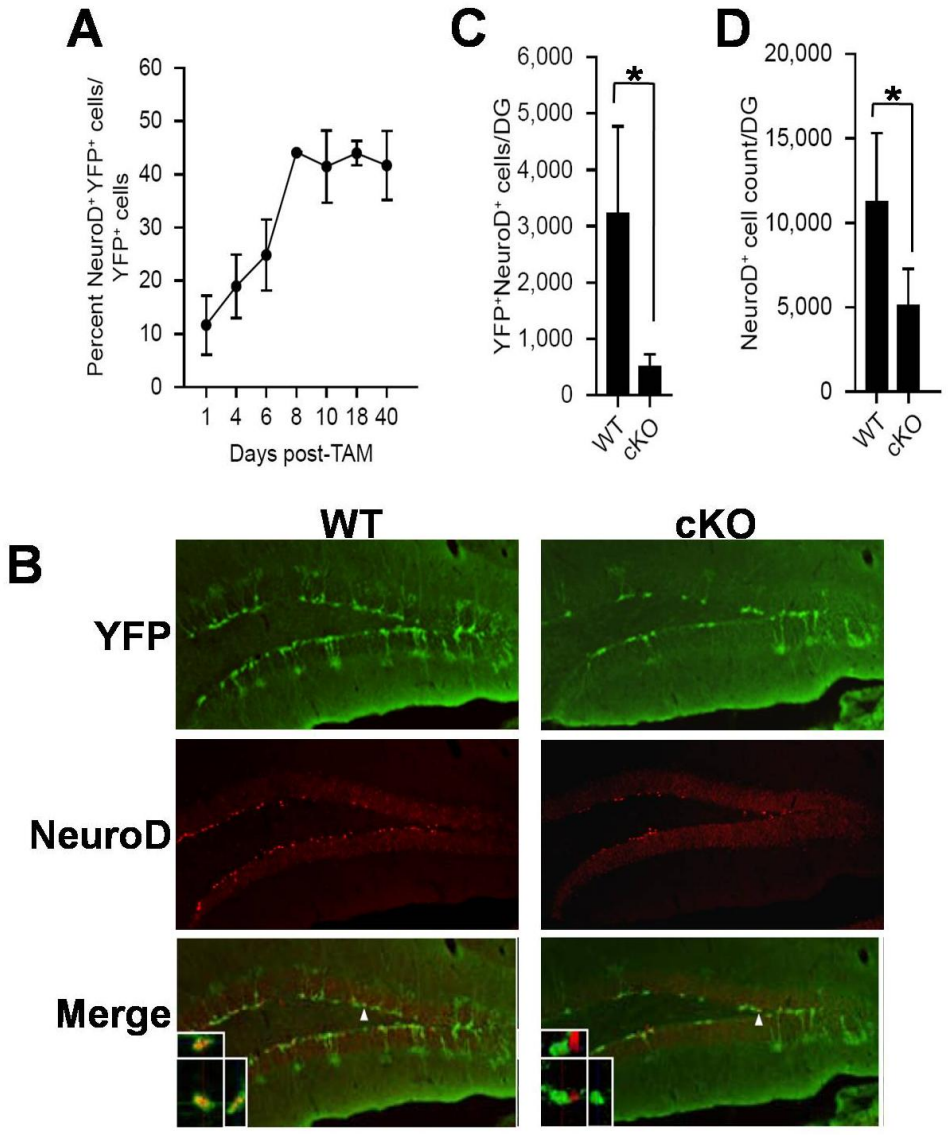


**Figure 3.1. NeuroD deletion in cKO dentate gyrus.** (A) Colocalization of YFP and NeuroD plateaus at 8 days after tamoxifen. (B) Representative images of NeuroD deletion from YFP+ cells in 40d cKO animals. (C) Tamoxifen-induced recombination in cKO animals significantly decreases YFP/NeuroD double positive cells when compared with wildtype. (D) Total NeuroD+ cells also decrease in the cKO dentate after tamoxifen treatment . *P < 0.0001

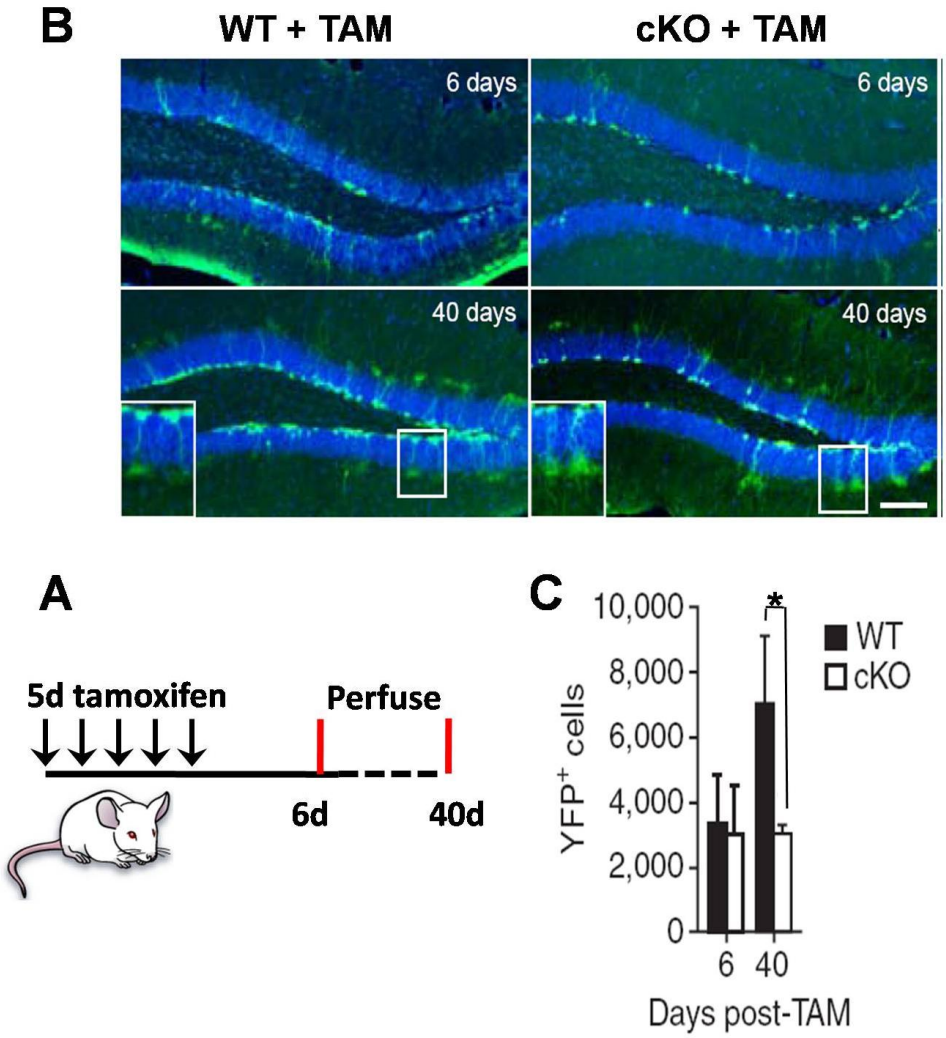


**Figure 3.2. Deletion of NeuroD in SGZ NSCs results in a loss of YFP-positive cells at 40 days after tamoxifen.** (A) Timeline of NeuroD cKO experiment. (B) Representative images of WT and cKO animals at 6 days and 40 days after tamoxifen. Insets are Type 1 neural stem cells, identified by morphology. (C) YFP+ cells are maintained at 6 days after tamoxifen but are significantly decreased at 40 days. *P<0.001

As discussed in Chapter 1, differentiating neuronal progenitors undergo a series of dramatic morphological changes as they progress from Type 1 NSCs to mature granule cell neurons. In the NeuroD inducible conditional knockout, the YFP reporter fills the entirety of the cell soma, allowing classification of the cell by its morphology. YFP+ cells were grouped into three classes: Type 1, characterized by a triangular cell body projecting a thick extension through the granule cell layer ending in a tuft of processes (Figure 3.3A arrow), Type 2/3, which have only a round cell body with no processes (Figure 3.3A arrowheads), and immature/mature neurons, identified by a round cell body ending in a long dendritic tree projecting into the molecular layer (Figure 3.3B). Total numbers of Type 1 and Type 2/3 cells did not significantly change; however, there was a nearly 50% loss of immature/mature neurons in the cKO (Figure 3.3C). This resulted in a proportional increase in Type 1 cells in the YFP+ cell population concurrent with a proportional loss of immature/mature neurons (Figure 3.3D).

To confirm the morphological data, we stained 40d WT and cKO sections with panels of cell-type specific markers (Figure 1.1B). Type 1 NSCs were identified by colocalization of YFP with GFAP and Sox2, while Type 2a cells were identified by the presence of Sox2 and the absence of GFAP. No significant loss of Type 1 or Type 2a cells was observed in cKO brains (Figure 3.4A,B). Furthermore, no change was noted in Ki67/YFP double-positive cells, indicating that the rapidly dividing Type 2a progenitors were not the source of the loss of YFP-positive cells in the cKO (Figure 3.4A,B). To assess the effect of NeuroD deletion on neuronally committed Type 3 progenitors and on immature and mature neurons, we stained 40d brain sections for Prox1 and NeuN. Type 3 and immature neurons were distinguished from mature neurons by colocalization of Prox1 and NeuN while mature neurons only stained for NeuN. YFP-positive Type 3/immature neurons were drastically decreased in the 40day cKO animals (Figure 3.4C,D), while mature neurons showed a trend toward loss that did not reach significance. This lack of significance is most likely due to the poor penetrance of the NeuN antibody, resulting in a small number of mature neurons present in 40d animals; better staining and later timepoints would almost certainly show a significant loss of mature neurons. We confirmed the loss of immature neurons by colocalizing YFP and

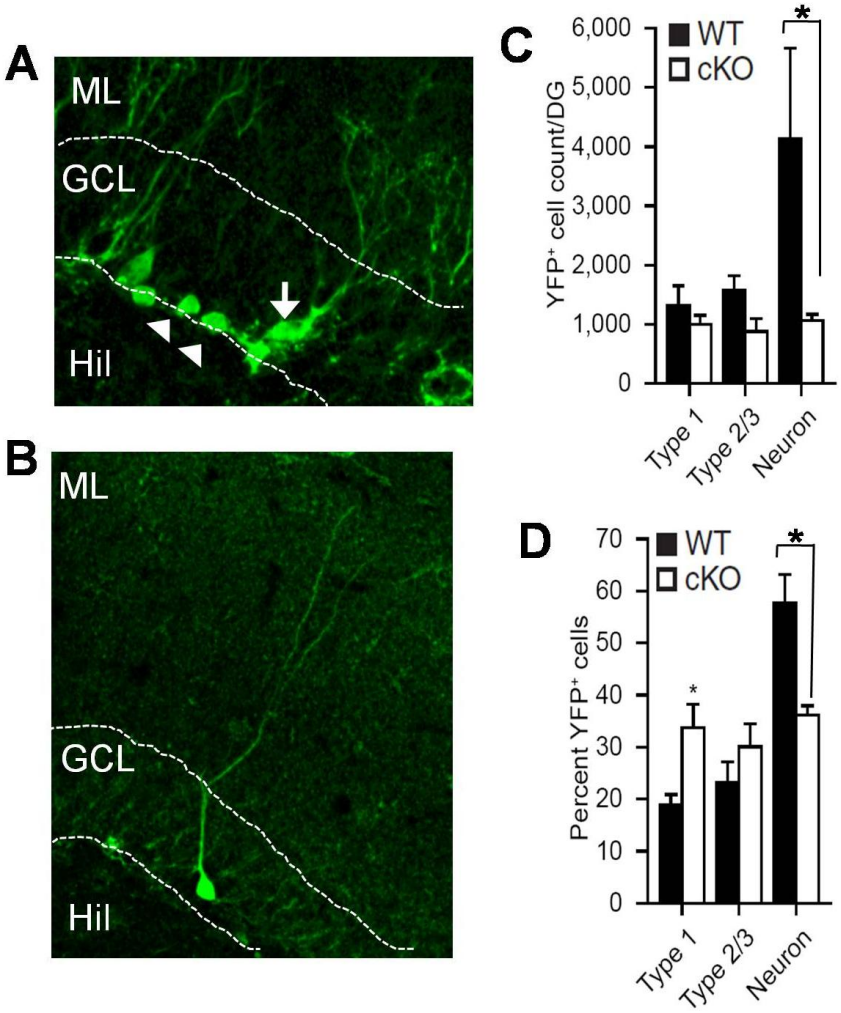


**Figure 3.3. Immature and mature neurons are significantly decreased in the cKO by morphological analysis.** (A) Examples of Type 1 (arrow) and Type 2/3 (arrowheads) morphologies. (B) Example of an immature/mature neuron morphology. (C) Total numbers of YFP+ neurons were significantly decreased in cKO animals 40 days after tamoxifen. (D) When normalized to total YFP+ cells, the proportion of immature/mature neurons is significantly decreased with a concurrent increase of Type 1 neural stem cells. *P < 0.001. ML: molecular layer. GCL: granule cell layer. Hil: hilus.

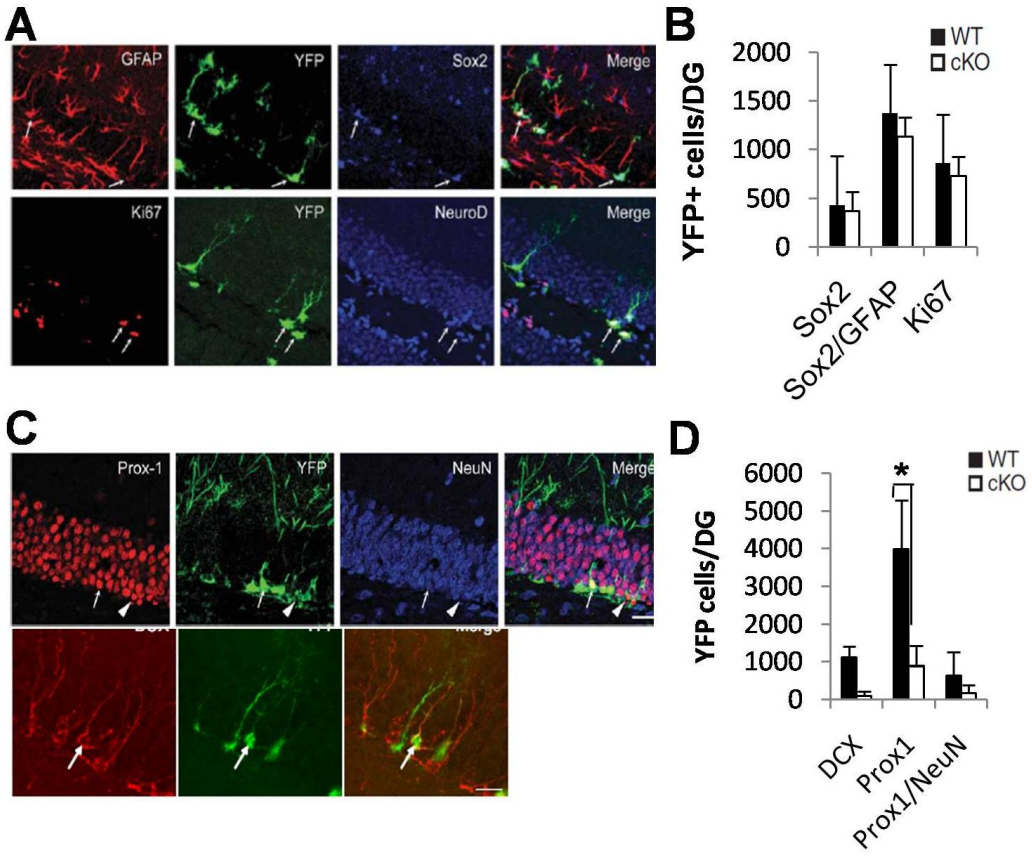


**Figure 3.4. Immature and mature neurons are significantly decreased in the cKO by cell-type specific marker analysis.** (A) Representative images of YFP/GFAP/Sox2 triple positive neural stem cells (top panel, arrows) and YFP/Ki67/NeuroD triple positive Type 2 neuronal progenitors (bottom panel, arrows). (B) Of the YFP+ population, neither Type 1 neural stem cells nor Type 2 neuronal progenitors are lost in the cKO at 40 days after tamoxifen. (C) Immature neurons were identified by YFP+/Prox1+/NeuN- staining (top panel, arrowhead) and by staining for Dcx (bottom panel). Mature neurons stained for YFP, Prox1, and NeuN (top panel, arrow). (D) YFP+ immature neurons are significantly decreased in cKO DGs 40 days after tamoxifen, with a strong trend toward a loss of mature neurons. *$P < 0.001$. Scale bars = 20µm.

doublecortin, another marker present in Type 3 and immature neurons that disappears as the new granule cells mature (Figure 3.4C,D). These findings suggest NeuroD has a critical functional role during the transition from early neuronal progenitors to mature, committed neurons.

The disappearance of YFP-positive cells in the cKO could be due to either a shift in cell identity to an astrocyte or oligodendrocyte fate or to cell death. The first possibility, a change in cell fate, is unlikely, as YFP-positive cells are not retained as other cell types with easily identifiable morphologies. In addition, YFP+/GFAP+/Sox2- cells, which could also be considered astrocytes, are not increased in cKO animals at either the 6 day or 40 day timepoint (data not shown). Furthermore, a postdoctoral researcher in the lab, Dr. Zhengliang Gao, has conducted *in vitro* studies using neurospheres infected with an adenovirus expressing Cre and have not noted any increase in GFAP+ astrocytes. The far more likely explanation is that the loss of NeuroD in newly-fated cells drives them to cell death. To determine if more cells were dying in the cKO animals, we stained for the cleaved form of caspase 3, also known as activated caspase 3 (AC3). AC3 is an upstream effector in the mitochondrial apoptosis pathway and is a commonly used marker for undergoing apoptosis in the brain [187]. We observed a significant increase in AC3-positive cells in cKO animals 40 days after tamoxifen, suggesting that there are indeed more apoptosing cells after NeuroD deletion (Figure 3.5). Thus, it appears that NeuroD may be necessary for the survival of newborn neurons.

Interestingly, deletion of NeuroD does not result in 100% lethality of new neurons. While it possible that the surviving YFP+ granule cells are simply cells that had successful recombination of the YFP reporter but not of the floxed NeuroD allele, it is also possible that some cells may be able to overcome the need for NeuroD and survive to a mature state. The latter explanation seems to be the case, as surviving YFP+ immature/mature neurons in the cKO have significantly shorter dendrites than their WT counterparts (Figure 3.6, analysis done by Zhengliang Gao). This finding is not entirely unexpected, as NeuroD's role as a transcription factor places it in a position to potentially regulate genes necessary for the development of neuronal characteristics, including

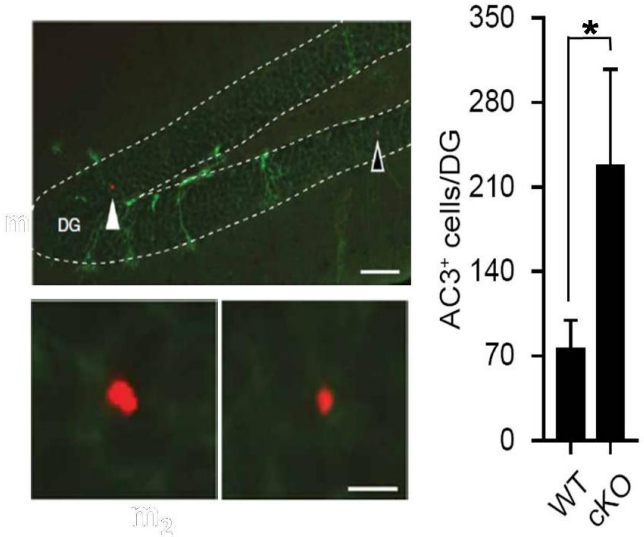


**Figure 3.5. Cell death is significantly increased 40 days after tamoxifen in cKO DGs.** Dying cells were visualized by activated caspase 3 (AC3) staining in the dentate gyrus. (sold arrowhead corresponds to left inset, empty arrowhead corresponds to right inset). cKO had significantly more dying cells than wildtype. *P<0.001. Scale bars = 20µm.

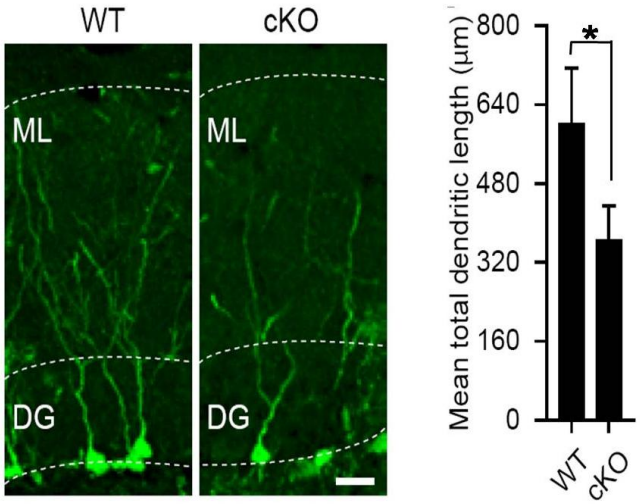


**Figure 3.6. Surviving neurons in cKO animals have dendritic abnormalities.** The few surviving neurons in cKO animals had significantly shorter, more broken-appearing dendrites than their wildtype counterparts. *P < 0.05. Scale bars = 20µm. ML: molecular layer. DG: dentate gyrus. Analysis done by Zhengliang Gao.

morphology and activity. NeuroD has been previously shown to be critical for dendritic formation in cerebellar slices [188], although what specific NeuroD regulatory targets are involved is difficult to say.

**Summary and Conclusions**

This chapter has described a critical need for NeuroD in the final stages of neuronal differentiation, particularly in the maturation and survival of newly born granule neurons. Interestingly, this study suggests that NeuroD is not required in the NSC or in the initial cell-fate choice. Whether this is due to redundant signaling and regulatory mechanisms that can compensate for the lack of NeuroD at this stage remains to be determined. What is likely is that there are other factors critical to NSC maintenance and fate choice that are upstream of NeuroD and may signal directly to NeuroD, including Wnt [128], Notch [129], and NRSF [91]. The next chapter will investigate the role of the third of these factors, NRSF, in the transition from NSC to mature granule cell.

# Chapter 4

## NRSF/REST regulation of adult neurogenesis

As described in Chapter 2, the transcriptional repressor NRSF/REST (hereafter referred to as NRSF) has long been suspected to be an active participant in the regulation of adult neurogenesis. While much of NRSF's mode of transcriptional repression has been described, including its cofactors and many of its actively bound target genes, the effect of NRSF's regulation on the neurogenic process still remains to be elucidated. This chapter will describe the careful analysis of the localization of NRSF protein expression in the adult hippocampus as well as the effect of its deletion in an *in vivo* and *in vitro* context. The data presented in this chapter has been submitted in manuscript form to *Journal of Neuroscience*.

### NRSF Immunohistochemistry

NRSF RNA is known to be found throughout the adult brain, predominantly in what appears to be neurons [93]. Due to the lack of a reliable antibody capable of generating reproducible staining patterns, little is known about the cell type-specific protein expression of NRSF. In order to determine in what cell types NRSF is found in the adult hippocampus, a commercially available NRSF antibody that stained specifically for NRSF in tissue was identified and a protocol for immunohistochemistry using that antibody was optimized.

Immunohistochemistry using other cell-type specific antibodies uses various methods of antigen retrieval, including a mild 0.1M citric acid boiling and a more stringent trypsin/2N hydrochloric acid (HCl) pretreatment. Of the NRSF antibodies on the market, only those carried by Upstate and by AbCam showed any successful staining with mild pretreatment, and these staining patterns were not consistently reproducible (Figure 4.1A). Stronger pretreatment with trypsin and HCl only produced staining with the Upstate NRSF antibody. While costaining with this antibody was difficult, it was possible to costain with the mature neuron marker NeuN and the astrocyte/NSC marker GFAP (Figure 4.1B). NRSF colocalized strongly with nearly all NeuN+ granule neurons

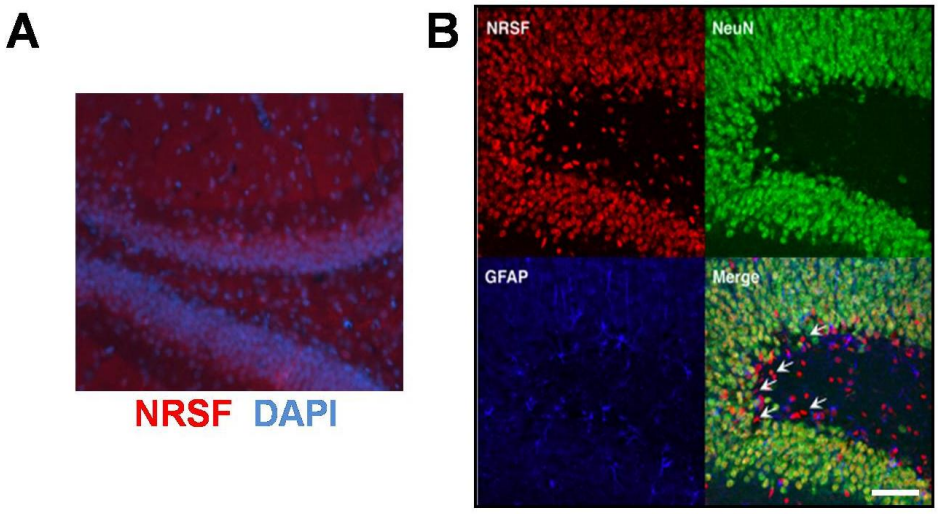


**Figure 4.1. Early NRSF immunohistochemistry attempts reveal NRSF expression in mature granule cells and progenitors.** (A) The AbCam NRSF antibody with citric acid pretreatment has faint granule cell expression. (B) HCl pretreatment and the Upstate NRSF antibody produces strong granule cell and GFAP+ NSC staining. Scale bars = 100µm.

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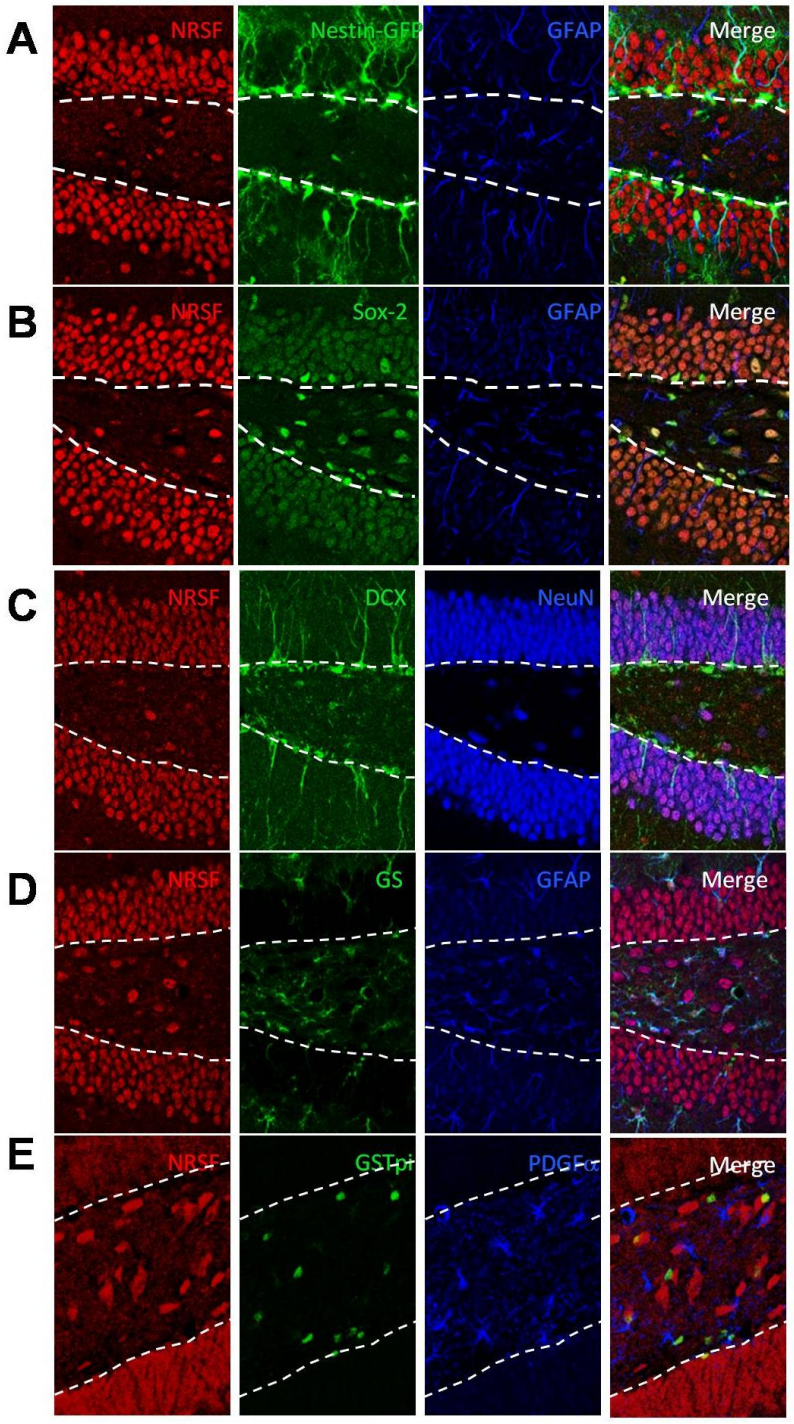
**Figure 4.2. NRSF expression patterns in wildtype adult brains.** NRSF colocalizes with stem cell markers nestin-GFP, GFAP, and Sox2 (A-B), with a population of Sox2+GFAP- neuronal progenitors (B), with Dcx+ immature and NeuN+ mature neurons (C), with astrocytes (D), and with some oligodendrocytes (E). IHC done by Mostafa Nashaat, images taken by Nashaat and author.


A
NRSF
Nestin-GFP
GFAP
Merge
B
NRSF
Sox-2
GFAP
Merge
C
NRSF
DCX
NeuN
Merge
D
NRSF
GS
GFAP
Merge
E
NRSF
GSTpi
PDGFα
Merge

in the dentate gyrus and with GFAP+ cells that appeared to be NSCs and astrocytes morphologically. Interestingly, numerous NRSF+ cells in the subgranular zone (SGZ) and the hilus did not stain for either NeuN or GFAP, suggesting that NRSF is present in some progenitor cells as well.

Work by others in the lab, including Dr. Zhengliang Gao and Mostafa Nashaat, further optimized the NRSF staining protocol by substitution of a freeze-thaw/methanol pretreatment for the trypsin/HCl process. This procedure worked well with both the AbCam and the Upstate NRSF antibodies and produced noticeable signal with other commercially available NRSF antibodies. In addition, the stain was reproducible and survived costaining with other cell-type specific markers. Using this new protocol, brain sections from perfused nestin-GFP reporter mice were stained for both NRSF and a panel of markers for NSCs, neuronal progenitors, and newborn neurons. NRSF was found in NSCs staining for the stem cell markers nestin, GFAP, and Sox 2 (Figure 4.2A,B) and in newborn neurons positive for Dcx (Figure 4.2C). NRSF also colocalized with NeuN in the mature granule neurons (Figure 4.2C), confirming the earlier staining pattern in the non-optimized protocol. In addition, NRSF was found in glutamine synthetase (GS) and GFAP double-positive astrocytes (Figure 4.2D) and in a subset of PDGFr and GST$\pi$ double-positive oligodendrocytes (Figure 4.2E), suggesting that NRSF expression may not be restricted to neuronal lineages.

To address the question of NRSF's presence in neuronal progenitors actively differentiating into new granule neurons, NRSF was costained with NeuroD. Interestingly, NRSF appeared to colocalize with a small cohort of NeuroD cells but not with the vast majority of them (Figure 4.3A). A closer examination revealed that NRSF expression seemed to be inverse to NeuroD expression: in short, where NRSF was high, NeuroD expression was low, and vice versa (Figure 4.3B). Quantification of NeuroD+ cells confirmed this, as most NeuroD+ cells did not costain with NRSF. The minority that were double positive showed a very clear inverse expression pattern (Figure 4.3C).

Based on this data, NRSF protein adopts a bimodal expression pattern in the adult hippocampus. It is expressed highly in Type 1 NSCs but is apparently

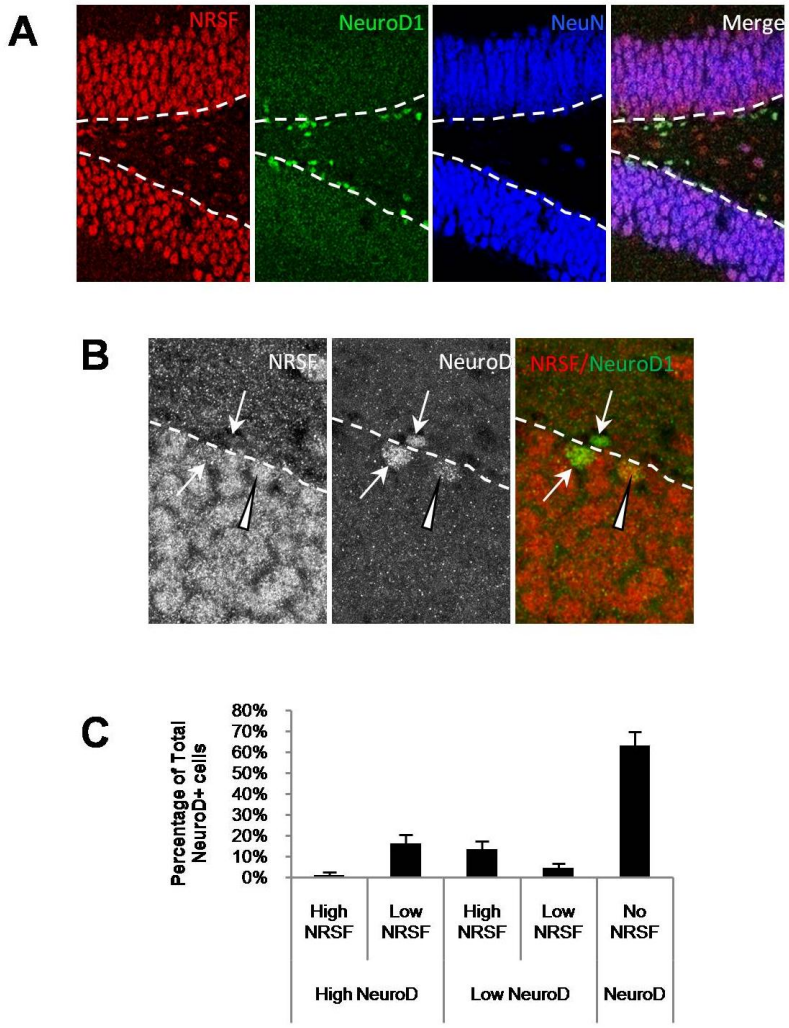



**Figure 4.3. NRSF and NeuroD are inversely expressed in the adult hippocampus. (**A). NRSF and NeuroD colocalize in only a subpopulation of NeuroD positive cells, while all NeuN+ cells express NRSF. (B) Cells with high NeuroD expression (arrows) are weakly NRSF+ or NRSF-. Cells with low NeuroD are strongly NRSF+ (arrowhead). (C) Quantification of NRSF expression levels in NeuroD+ cells, showing the inverse relationship between NRSF and NeuroD expression in the SGZ.

downregulated as these stem cells begin to differentiate into neuronally fated progenitors (ie Type 2 and 3 cells). At some point in this differentiation process, NRSF is again required and is upregulated as progenitors begin to adopt a neuronal morphology and complete the differentiation process.

**NRSF Inducible Conditional Knockout**

The NRSF expression data strongly suggests that NRSF plays an important role in the neurogenic process. The next logical step to determining the nature of this role was to generate a NRSF knockout mouse. The total knockout of NRSF in the developing mouse embryo results in complete lethality by E11.5 [100], preventing any analysis of the effect of the homozygous NRSF deletion on the postnatal mouse. In addition, no mouse with a NSC-specific deletion of NRSF has been generated. To determine the role of NRSF in postnatal hippocampal neurogenesis while circumventing the embryonic lethality of a germline knockout, a NRSF inducible conditional knockout (cKO) line was generated by Dr. Zhengliang Gao utilizing the nestin-CreERT2 driver mouse line used for the NeuroD knockout described in Chapter 3. In cells expressing nestin, the fourth exon of NRSF, which encodes the first four DNA-binding zinc fingers, was eliminated. The knockout strategy is illustrated in Figure 4.4.

*Effect of NRSF deletion on NSC quiescence and maintenance*

Four to six-week-old wildtype and NRSF cKO mice were injected with tamoxifen for five days, which induced recombination of the floxed allele in cells expressing nestin, and were then sacrificed at 10, 20, and 30 days after the last tamoxifen injection. Brains were sectioned and stained for the YFP reporter. YFP+ cells were counted and characterized for cell-type morphology, as described in Chapter 3. No increase in the total number of YFP+ cells was noted at any of the early time points (10d, 20d, and 30d after tamoxifen ) (Figure 4.5A). However, when YFP+ cells were categorized by morphology, the proportion of morphologically Type 1 NSCs significantly increased at 30 days after tamoxifen (Figure 4.5B), suggesting that the loss of NRSF may result in an expansion of the stem cell pool. This data was somewhat

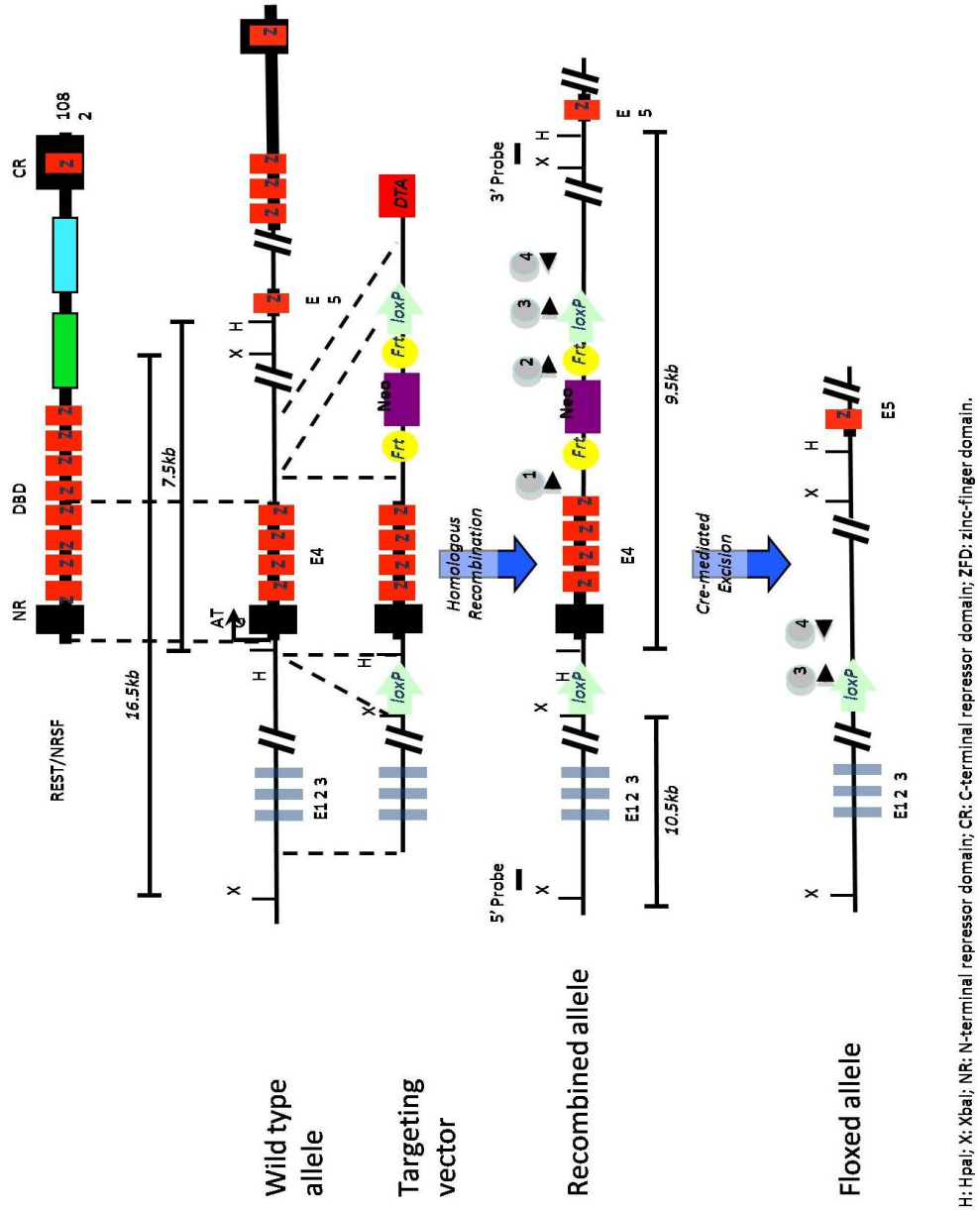



**Figure 4.4. Generation of NRSF conditional knockout allele.** The first four zinc fingers of NRSF were deleted by Cre recombination in nestin+ cells after treatment with tamoxifen.

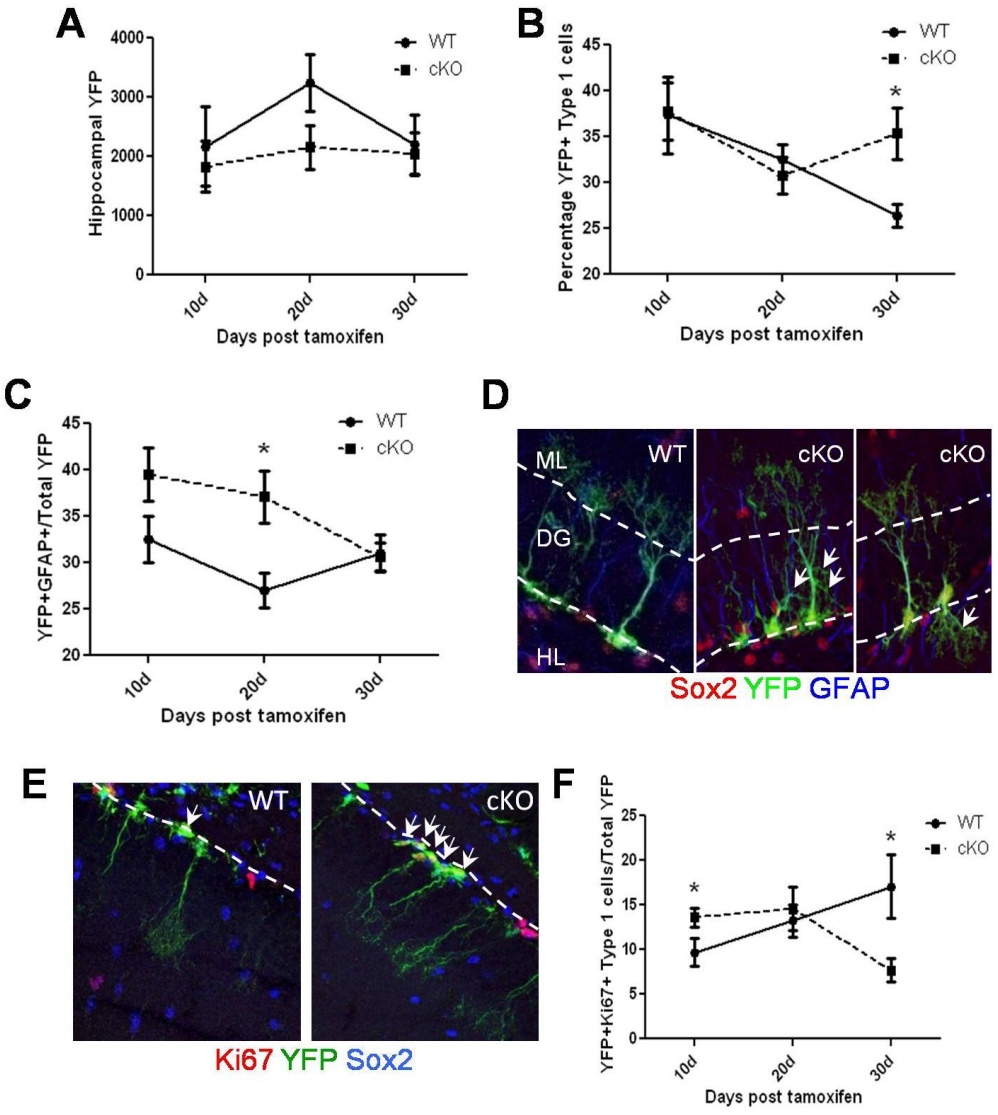



**Figure 4.5 Amplification of the Type 1 neural stem cell pool in the NRSF cKO.** (A) Total hippocampal YFP+ cell numbers do not change between wildtype and cKO animals 30d after tamoxifen. (B) The proportion of YFP+ Type 1 cells increases 30 days after tamoxifen. (C) GFAP+ cells increase by 20 days after tamoxifen. (D) cKO Type 1 cells have abnormal morphology (arrows). (E-F) The proportion of proliferating YFP+ Type 1 cells increases 10 days after tamoxifen and decreases at 30 days. * $P < 0.05$

complicated by the fact that YFP+GFAP+ cells were significantly increased at 20 days, but not at 30 days (Figure 4.5C). A trend toward an increase was noted at 10 days but did not reach significance. It is possible that this complication is due to a NSC population that retains GFAP expression while losing the stereotypical Type 1 morphology, possibly during cell division. As described the introduction to adult neurogenesis in Chapter 1, Type 1 cells are quiescent; proliferation is very slow and rare until the cell transitions to more highly proliferative Type 2a neuronal progenitor. If, however, the loss of NRSF led to increased proliferation of this population, an expansion of the morphologically Type 1 population might take longer to become noticeable, while expression of a cell type-specific marker such as GFAP that was retained during proliferation would become evident at an earlier time point. Interestingly, the cKO had a noticeable number of Type 1 stem cells with significant morphological abnormalities, including apparent migration away from the SGZ, misshaped tufts, early stem branching, hilar-targeted tufts, and small projections from the cell body, at 20 and 30 days (Figure 4.5D). These abnormal Type 1 cells could possibly be cells that are about to divide or that are assuming Type 1 morphology after dividing. It also suggests that the loss of NRSF causes something to be fundamentally wrong with the stem cell.

If indeed this increase in Type 1 cells in the cKO is due to increased proliferation, one would expect a higher proportion of Type 1 YFP+ cells to express markers of division, such as Ki67. To this end, brain sections were stained for YFP and Ki67 and were analyzed for the presence of Ki67 in cells with Type 1 morphology by a masters fellow in the lab, Mostafa Nashaat. As expected, Ki67+ proliferating Type 1 cells were increased in cKO animals (Figure 4.5E,F). Therefore, it is likely that the increase in NSCs after NRSF deletion is due to a loss of quiescence.

This data clearly demonstrates a role for NRSF in the maintenance of quiescence in the NSC pool. Without NRSF, NSCs begin to divide abnormally often, resulting in an expanded stem cell pool. Based on this conclusion, it is clear that NRSF is critical for the long term maintenance of NSCs in the adult hippocampus.

*Effect of NRSF deletion on neuronal differentiation and survival*

Based on NRSF's role in NSC maintenance, it is also logical to assume that this loss of quiescence and increase in proliferation would be accompanied by an acceleration of differentiation. To demonstrate this, brain sections from the 10, 20, and 30 day timepoints were stained for Ascl1 and NeuroD. Ascl1, a marker of early differentiation, was significantly more prevalent in cKO animals at 20 days after tamoxifen (Figure 4.6A,B), suggesting an acceleration in differentiation after NRSF deletion. There was also a trend toward an increase in Ascl1+ Type 1 cells at 20 and 30 days after tamoxifen, but the trend did not reach significance (Figure 4.6C). Interestingly, there was no change in the number of cells expressing NeuroD, which is expressed later during differentiation than Ascl1 (Figure 4.6D). This might be due to Ascl1's presence in predominantly Type 2 cells, while NeuroD is expressed from Type 2b progenitors to immature neurons. Without a day-by-day analysis of expression patterns, an increase in NeuroD expression in early progenitors would likely be obscured by later cell types. It is also possible that neurogenesis utilizes multiple pathways that act through pathway-specific target genes. NRSF may directly regulate Ascl1 but not NeuroD, which may be regulated either directly or indirectly by other transcriptional regulators of neurogenesis.

The acceleration in differentiation combined with the expansion of the neural stem pool likely would have profound effects on the number of new neurons being generated in the cKO. In addition, the bimodal expression pattern of NRSF protein suggests that it might also have a role in differentiation, maturation, and survival of newly-born neurons. In order to dissect this role, four- to six-week-old mice were treated with tamoxifen as before and sacrificed at 70, 120, and 200 days after the last tamoxifen injection. YFP+ cells were counted and assessed for morphology. While no differences were noted in Type 1 or Type 2/3 morphologies, there was a significant decrease in cells with neuronal morphology at 120 days after tamoxifen (Figure 4.7A,B). This was confirmed by colocalization with neuronal markers Prox1 and NeuN; a significant loss of YFP/Prox1/NeuN triple-positive neurons was noted at 120 days after tamoxifen (Figure

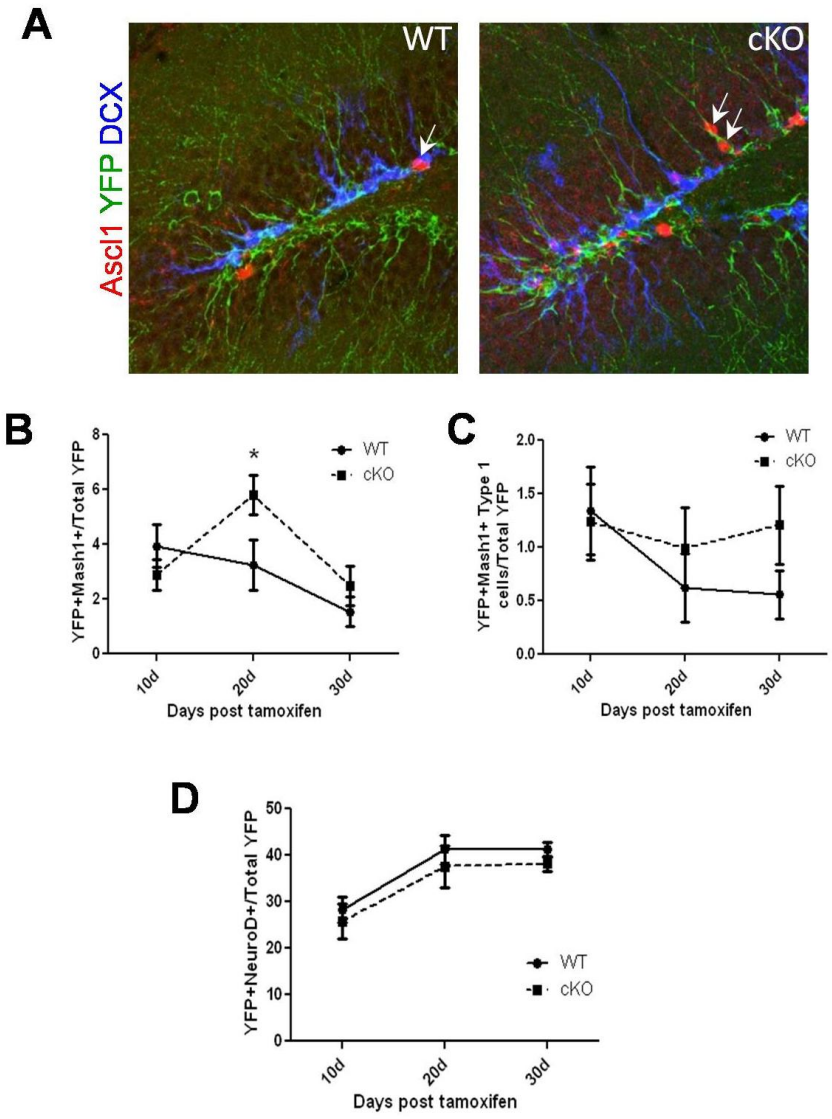



**Figure 4.6. NRSF cKO animals have more differentiating cells.** (A-B) Ascl1+YFP+ cells increase at 20 days after tamoxifen in cKO animals. (C) There is a strong trend toward more proliferating Type 1 cells at 30 days after tamoxifen in the cKO. (D) There is no change in NeuroD+ progenitors. * $P < 0.05$

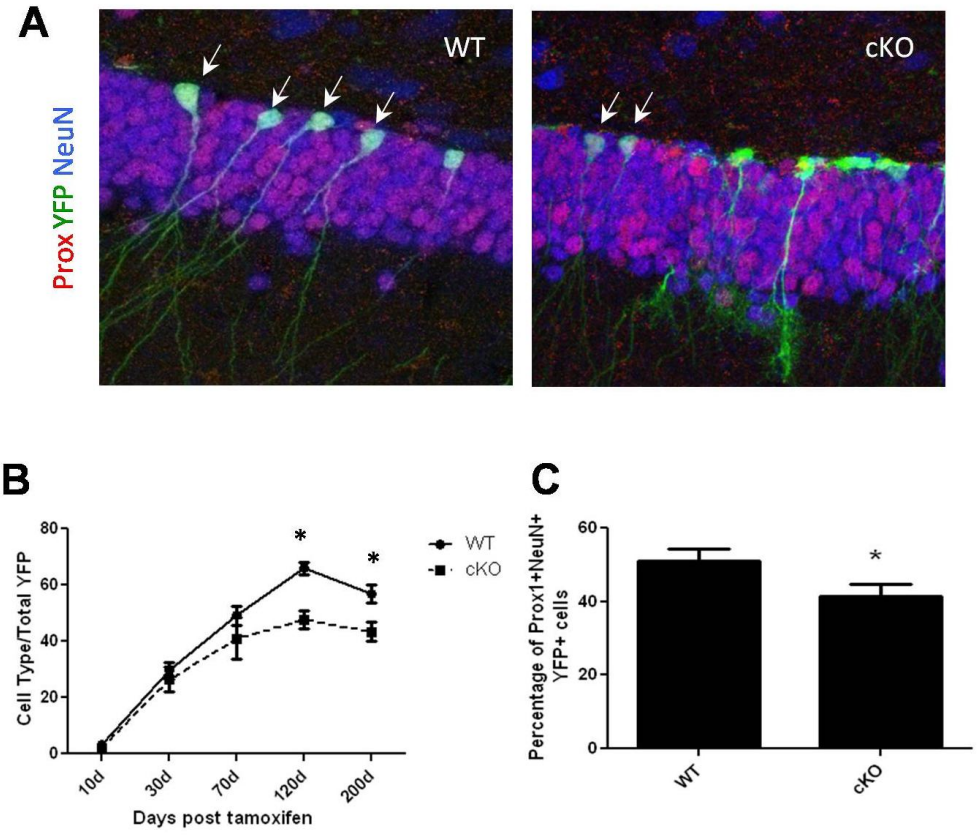



**Figure 4.7. NRSF deletion leads to the loss of new neurons by depletion of the stem cell pool.** (A) NRSF cKOs have fewer newborn granule neurons. (B) Fewer new neurons are born over time in the cKO, reaching a plateau earlier than their WT counterparts. (C) cKO animals have fewer Prox1+NeuN+ neurons at 70 days after tamoxifen. * $P < 0.05$

4.7C). This data suggests that NRSF is required for the late stages of differentiation and possibly for the survival of newborn neurons, but it is also possible that the loss of stem cell quiescence eventually leads to a depletion of the stem cell pool, which at long timepoints manifests as a loss of new neurons. This second interpretation is supported by an apparent plateau in new neuron formation beginning at 70d (Figure 4.7B). The WT animals continue to accumulate new neurons, particularly between the 70 and 120 day timepoints, eventually reaching nearly 80% neurons. In contrast, the cKO show a much slower growth of the neuronal population, only reaching 50% by the 120 day timepoint. Importantly, while there is a slight decrease in neuronal proportion at the 200 day timepoint in both groups, the fold difference between WT and cKO does not change. In addition, another lab member, Laura Yuan, demonstrated that there is no difference in AC3+ dying cells at any timepoint between the WT and the cKO (data not shown). If cKO neurons were simply dying off, a much larger fold difference would expected over time. In reality, the population does not change, suggesting that very few or no new neurons are being added. These two pieces of data support a model where the NSC pool in the cKO divides and differentiates itself to exhaustion, thus limiting the number of new neurons that can be generated at very late timepoints.

To better dissect out the dynamics of the observed neuronal acceleration, a BrdU pulse-chase experiment was designed that would label proliferating cells shortly after the loss of NRSF in order to track these cells through differentiation. Four week old mice were injected with five days of tamoxifen and then injected daily with 100mg/kg BrdU for fourteen days to label as many dividing cells as possible. The mice were then allowed to survive for 18 days to clear the BrdU (Figure 4.8A). Brain sections from these mice were stained for YFP and BrdU as well as for GFAP and Dcx to mark stem cells and newborn neurons, respectively. There was a strong trend of more BrdU+ cells in the cKO (Figure 4.8B), confirming the increased proliferation noted in the 10 day cKO animals (Figure 4.5E, F). As expected, only rare YFP+ cells in either genotype were BrdU/GFAP double-positive, with the vast majority already beginning to differentiate. Of these YFP+BrdU+ cells, significantly more of the cells in the cKO animals had a

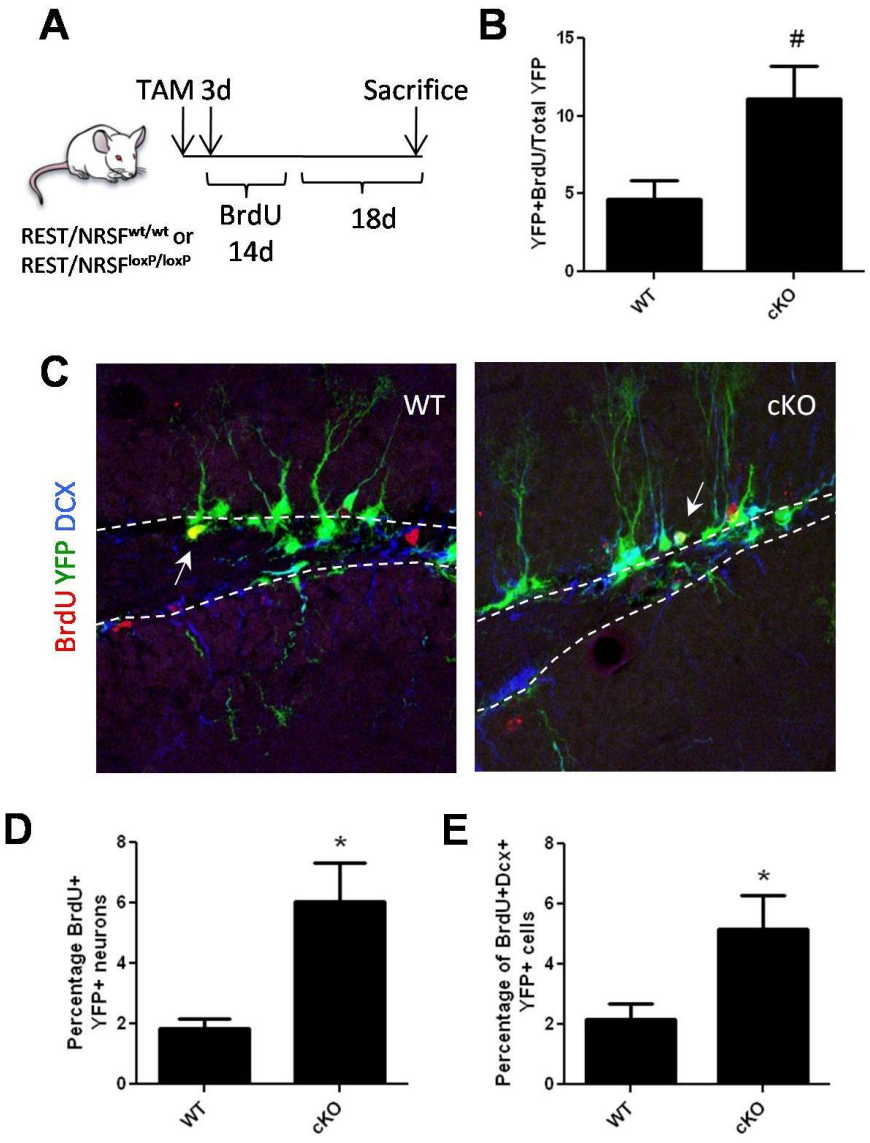


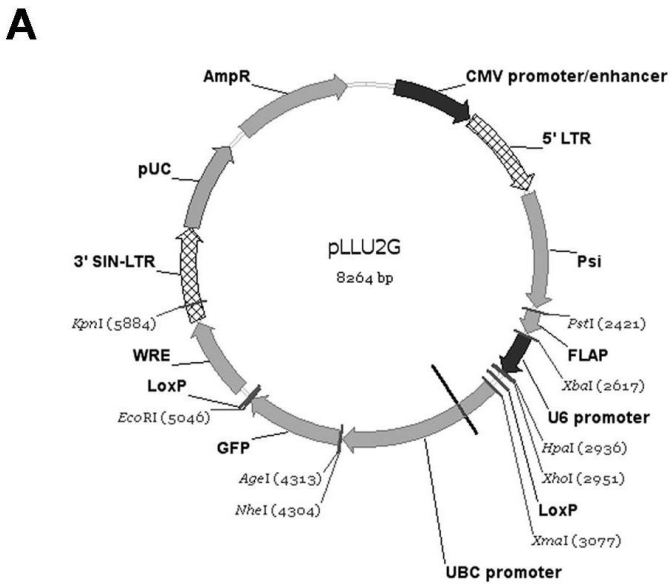

**Figure 4.8. Deletion of NRSF increases proliferation and accelerates differentiation.** (A) Experimental scheme of BrdU label-retaining experiment. (B) cKO animals strongly trend toward a significant increase in proliferation. (C) Proliferating cells in the cKO differentiate more quickly than WT cells, by both morphology (D) and by Dcx expression (E). * $P<0.05$, # $P<0.075$

neuronal phenotype (Figure 4.8C,D). There was a strong trend toward an increase in Dcx+ cells as well, but due to the low number of animals in the study set, this increase did not reach significance (Figure 4.8E). This data confirms that the loss of NRSF in NSCs leads to accelerated neuronal differentiation. Furthermore, the fact that NRSF cKO neurons were able to express neuronal markers and generate neuronal morphology suggests that NRSF may not be critical to survival of newly generated neurons.

### *In Vitro* Knockdown of NRSF by shRNA

While the NRSF inducible cKO is a powerful tool for elucidating NRSF's role in an *in vivo* context, the low number of recombined cells makes it difficult to ascertain the molecular mechanism underlying the cKO's phenotype. An *in vitro* method of NRSF knockout presents more opportunities to tease apart the effect of NRSF deletion. In order to begin delving into this mechanism, four small hairpin RNAs (shRNAs) were generated against four different mRNA sequences in the rat NRSF locus according to previously published methods [182]. Each sequence was cloned individually into a plasmid vector that included a U6 promoter to drive expression of the shRNA as well as an internal ribosome entry sequence (IRES) and a GFP reporter. The sequences of the four shRNAs and a schematic of the vector are shown in Figure 4.9A.

After cloning, each individual shRNA-containing plasmid was used to generate lentivirus, which was then used to infect HCNs [8] kept in growth conditions. Significant NRSF knockdown was noted after infection with each of the individual shRNA viruses, with constructs S1 and S3 showing the highest knockdown (Figure 4.9B). In addition, mRNA levels of two NRSF target genes, BDNF and SynapsinI, were increased by NRSF knockdown, as would be expected from NRSF's described function as a transcriptional repressor (Figure 4.10A). As expected, BDNF was upregulated by all four shRNA constructs. However, SynapsinI was only increased by S1 and S2 and seemed to be downregulated by S3 and S4. A possible explanation is that S3 and S4 code for different regions of the NRSF cDNA. It is possible that S1 and S2 knock down full length NRSF and the shorter isoforms, while S3 and S4 may only be knocking down full length NRSF




| | |
|---|---|
| S1: | GCGGAGTCTGAAGAACAGTT |
| S2: | AAGAACAGTTCGTACATCACA |
| S3: | GTGTAACCTGCAGTACCAT |
| S4: | GACCGTGCCAAGGAGAAGT |

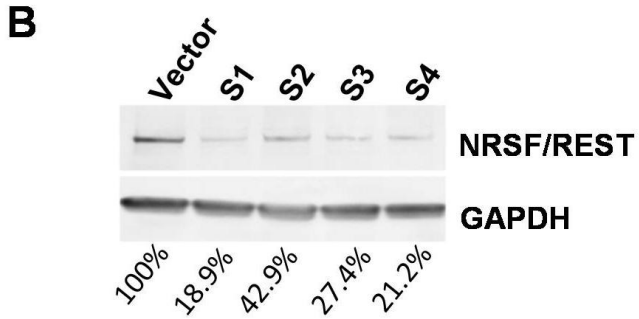



**Figure 4.9. Successful generation of the NRSF shRNA.** (A) Diagram of the pLLU2G shRNA plasmid and the sequence of the four shRNA constructs cloned into the plasmid. (B) All four constructs successfully knockdown NRSF in HCNs, with S1 and S3 producing the highest knockdown. Values below figure are percent decrease of each shRNA construct when compared to the vector, all normalized to their respective GAPDH bands.

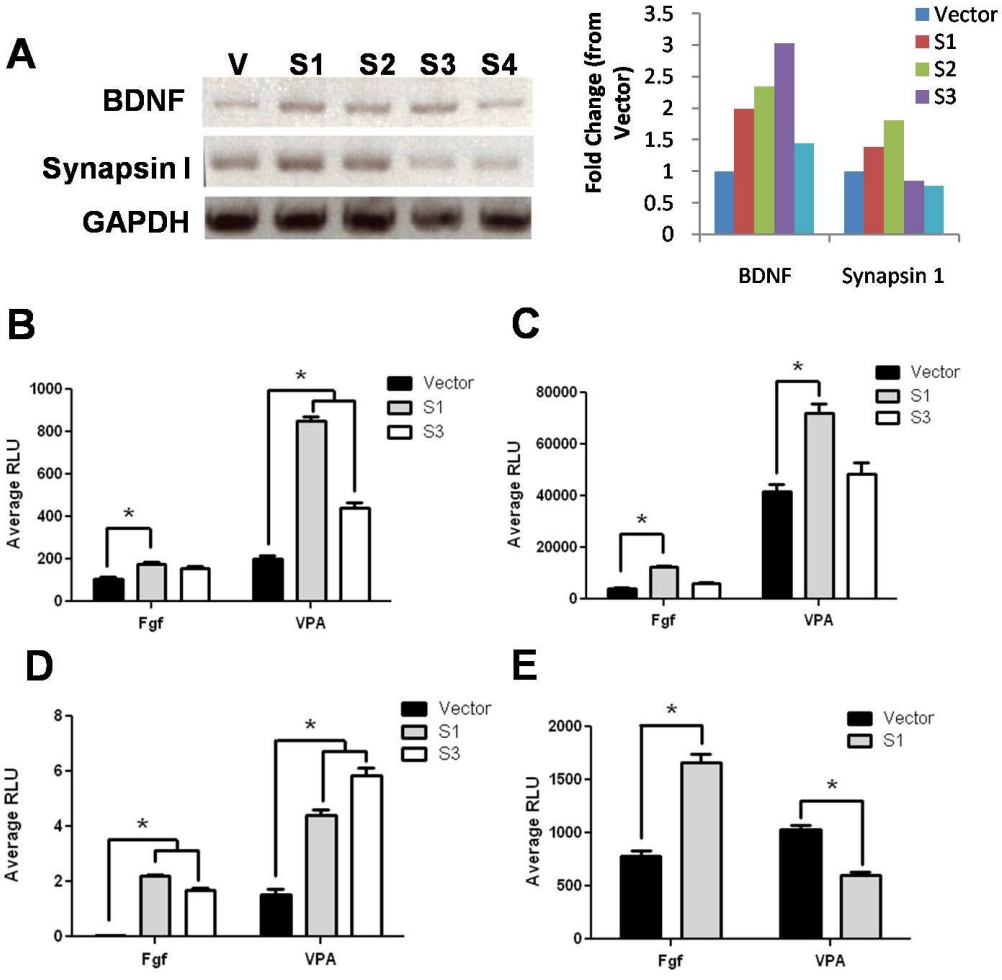



**Figure 4.10. Knockdown of NRSF *in vitro* primes HCNs for differentiation** (A) RNA expression of the NRSF target genes BDNF and SynapsinI after knockdown with the four NRSF shRNA constructs. Fold change based on the vector plasmid is graphed to the right. (B-D) Luciferase assays of BDNF (B), GluR2 (C), NR1 (D), and NeuroD (E) after knockdown with NRSF shRNA. Loss of NRSF increases the expression of these neuronal genes in both growth and differentiation conditions, except for NeuroD. *P<0.05

while leaving the isoforms intact. The isoforms may play a larger role in the downregulation of SynapsinI than full length NRSF.

To determine the effect of NRSF knockdown on the regulation of NRSF target genes, HCNs were electroporated with either the empty vector plasmid or one of shRNA constructs S1 and S3 as well as a target gene promoter driving a luciferase reporter. The cells were allowed to grow for 24 hours and then either treated with 1mM of valproic acid (VPA), a differentiation agent, or left in growth conditions for another 24 hours before the luciferase assay. Of the four target genes examined, all showed at least a slight upregulation in growth conditions (Figure 4.10B-E). Interestingly, in differentiation conditions, very strong upregulation of BDNF, GluR2, and NR1 occurred in cells infected with either S1 or S3 (Figure 4.10B-D). However, NeuroD was not upregulated, and in fact seemed to be significantly downregulated without NRSF (Figure 4.10E). This correlates with the *in vivo* data, which suggested that NRSF may not be the sole regulator of NeuroD and that its regulatory role at the NeuroD promoter may be more complicated than simple repression. When taken together, these findings seem to suggest that, when in growth conditions, the loss of NRSF primes NSCs to begin differentiating, an effect that is amplified when a neurogenic signal is detected.

In order to determine if this disregulation of NRSF target genes translates into increased differentiation, as seen *in vivo*, HCN cells were again electroporated with the vector plasmid, S1, or S3 and allowed to grow for 48 hours. The HCNs were then incubated with Fgf to maintain stem cell identity or with increasing concentrations (0.1mM, 0.3mM, and 1mM) of VPA for four days. Cells were then stained with Tuj1, an early indicator of neuronal differentiation, and Map2ab, a marker of more mature neurons [189]. Interestingly, cells electroporated with S1 had significantly larger proportions of Tuj1/Map2ab double positive GFP+ cells when compared with the cells electroporated with the control vector at the 0.1mM and 0.3mM concentrations, but at the 1mM concentration the proportion of differentiating cells actually decreased (Figure 4.11A, B). In addition, S3 did not show an increase in differentiating cells in any condition (Figure 4.11B). The increase at low concentrations of a differentiating agent supports the

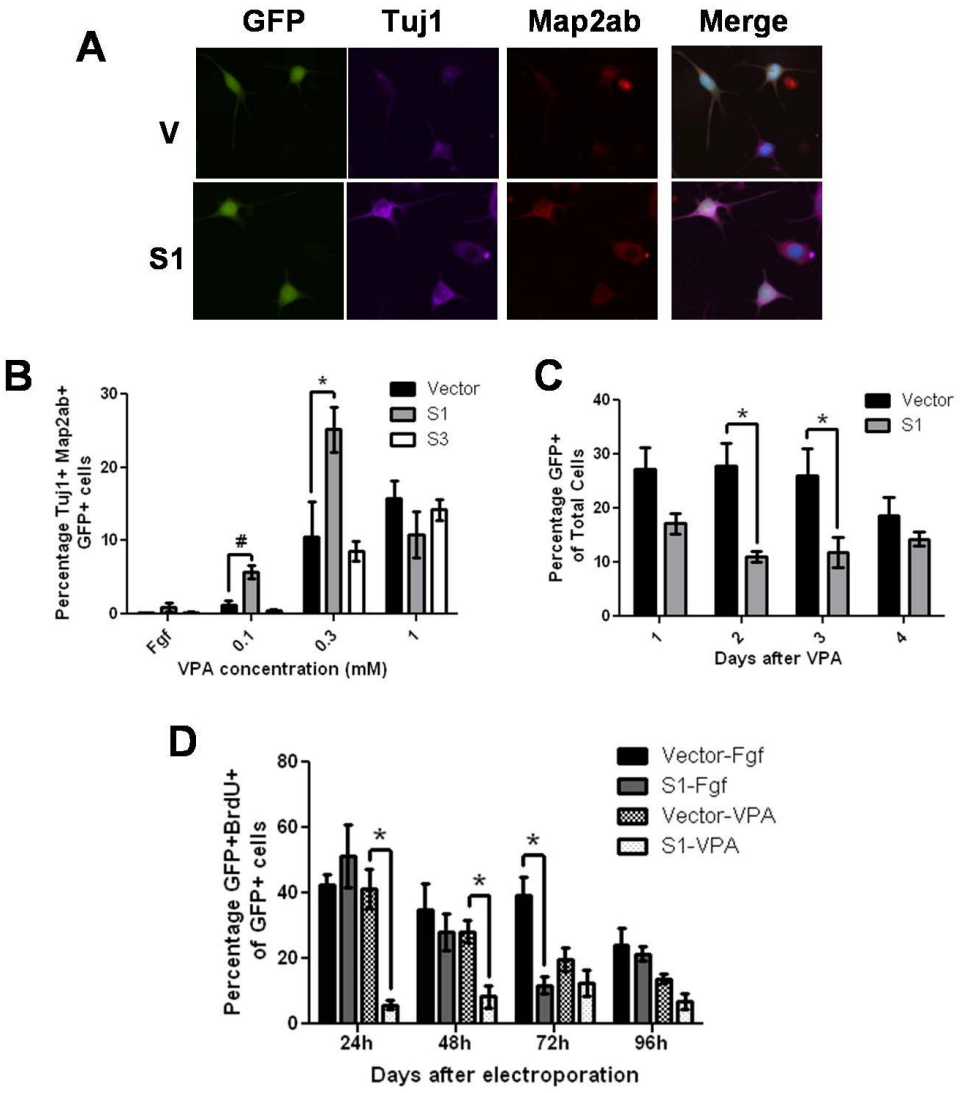



**Figure 4.11. Knockdown of NRSF *in vitro* accelerates neuronal differentiation in HCNs.** (A) After treatment with VPA, HCNs treated the NRSF shRNA S1 express more neuronal markers. (B) Neuronal markers increase in S1-treated cells after VPA treatment in a dose dependent manner. (C) S1-treated cells deplete over time after treatment with VPA. (D) S1-treated cells cease proliferation upon treatment with VPA. # P<0.1; *P<0.05

supposition that loss of NRSF primes stem cells for differentiation. The seeming loss of differentiating cells at high concentrations of VPA may be due to increased cell death in these cultures, especially as these cells were incubated in VPA for a longer period of time then the cells used for the analysis of target gene changes. To address this, the vector plasmid and S1 were electroporated into HCNs, incubated for 48 hours, and then treated with VPA. Cells were fixed every 24 hours after the addition of VPA. As expected, the proportion of GFP+ cells out of the total plated cells in the S1-treated cultures significantly decreased over time while the proportion of vector-treated cells remained relatively constant (Figure 4.11C). This raises the possibility that the loss of differentiating cells in the VPA dosage experiment may by due to cell death over time due to the loss of NRSF, either because NRSF is required for cell survival during differentiation (which is unlikely based on the *in vivo* data) or due to an increased susceptibility to toxicity from prolonged exposure to VPA. However, it is also likely that this decrease in GFP+ cells is due to an early cessation of proliferation, thus limiting the number of new cells generated over time.

To get a better idea of the effect of NRSF on proliferation over time, HCNs were electroporated as before, incubated for 48 hours before treatment with 1mM VPA, and fixed at 24, 48, 72, and 96 hours after VPA addition. BrdU was added 2 hours before fixation. As expected, BrdU incorporation decreased in both vector and S1-electroporated cells over time after treatment with VPA (Figure 4.11D), reflecting the transition from proliferating neuronal progenitor to terminally differentiated neuron. Interestingly, S1-treated cells showed little difference in proliferation from vector-treated cells until 72 hours after electroporation when kept in growth conditions. After VPA treatment, however, S1-treated cells had much less BrdU incorporation than vector-treated cells by 24 hours after VPA treatment. This confirms the previous *in vivo* and *in vitro* data suggesting that the loss of NRSF in neuronal progenitors primes the cells for differentiation, which accelerates in the presence of a differentiation signal. The noted loss of proliferating cells *in vitro*, unlike the increased proliferation noted shortly after NRSF deletion *in vivo*, is likely due to a shift in cell identity that occurs after isolation of stem cells from the brain culture. Others in the lab have noted this phenomenon, which

produces a highly proliferative but still fate-unrestricted cell, more like a Type 2 cell in the brain than a Type 1. This loss of proliferation may be due to this shift, which results in a replication of the later *in vivo* proliferation phenotype.

**Summary and Conclusions**

This chapter has described a role for NRSF in the maintenance of the NSC pool. Deletion of NRSF leads to a loss of NSC quiescence, with NSCs proliferating more often than in the WT and differentiating at an accelerated pace. This likely leads to an exhaustion of the NSC pool that at later timepoints is reflected in fewer new neurons being added to the DG. In addition, the *in vitro* data suggests that the loss of NRSF alone is not enough to drive this effect; a neurogenic stimulus may be necessary to initiate the differentiation cascade. The next chapter will investigate the effect of two neurogenic stimuli, pathological seizures and voluntary exercise, on neurogenesis in the context of the NRSF and the NeuroD cKO lines.

## CHAPTER 5

## Induction of neurogenesis in NRSF and NeuroD cKO

Chapter 3 and 4 dealt with transcriptional regulation of adult hippocampal neurogenesis in a physiological, baseline context. The animals used in those studies were housed in a normal cage environment with no environmental enrichment beyond social interaction with their cagemates. Thus, the neurogenic phenotypes described in these mice could be characterized as "basal" or "normal" neurogenesis. While an understanding of this basal neurogenesis is critical, there is a significant body of literature describing multiple means of inducing neurogenesis that adds a new complication to understanding adult neurogenesis. This literature is summarized and discussed in Chapter 1. Interestingly, the majority of these induction methods have correlates to human behavior and disease. Voluntary running, which correlates with voluntary exercise in humans, results in the generation of many new granule cell neurons in the hippocampus [130]. Seizure induction by chemoconvulsant administration and electroconvulsant shock also produces a large population of new neurons that are inherently aberrant, both by migration and activity [133, 152]. Temporal lobe epilepsy in humans also has a similar effect [145-146, 190].

This chapter will delve into two methods of neurogenic induction: voluntary running, a physiological stimulus, and kainic acid (KA)-induced seizures, a pathological stimulus. The NeuroD and NRSF cKO animals will be used as models to study the effect of these factors' deletion in these two models of induced neurogenesis.

### Optimization of mouse seizure model

Historically, the vast majority of rodent seizure studies have been conducted in rats, largely due to their hardiness, ease in handling, and high efficiency in reaching status epilepticus (SE) [191]. A mouse model of chemoconvulsant-induced seizures has been difficult to develop, as mice have more difficulty reaching SE without a high chance of mortality. In addition, there is marked strain variation regarding susceptibility to chemoconvulsants, latency to SE, mortality, and rates of seizure-induced neurogenesis

**Table 5.1. Incidence of seizures and mortality in the C57BL/6 x 129J/Sv strain of wildtype mice.**

| Convulsant (i.p.): | KA (25 mg/kg) | KA (30 mg/kg) | KA (35 mg/kg) | Pilo (250 mg/kg) | Pilo (300 mg/kg) |
|---|---|---|---|---|---|
| **SE incidence:** | 6/12 | 10/12 | 11/12 | 10/12 | 11/12 |
| **Percentage:** | 50% | 83% | 92% | 83% | 92% |
| **Mean latency to SE:** | 44±5 min | 32±2 min | 36±4 min | 31±3 min | 27±4 min |
| **Mortality:** | 2/12 | 4/12 | 5/12 | 6/12 | 8/12 |
| **Percentage:** | 17% | 33% | 42% | 50% | 66% |
| **KA**: kainic acid, **Pilo**: pilocarpine**, SE**: status epilepticus | | | | | |

[191]. However, as currently the generation of knockout rats is a highly inefficient process when compared to mice, it became imperative to optimize a seizure protocol for mice for use with the NRSF and NeuroD cKO animals.

Two commonly used chemoconvulsants are pilocarpine, a muscarinic acetylcholine receptor agonist [192], and kainic acid (KA), a glutamate receptor agonist [193]. Animals treated with pilocarpine are usually treated with methylscopolamine, a muscle relaxant, while KA is usually administered alone. To test which of these chemoconvulsants would produce high rates of SE with little mortality, untreated two month old mice from the NeuroD cKO line were treated with KA at 25, 30, and 35 mg/kg or with pilocarpine at 250 or 300 mg/kg after pretreatment with methylscopolamine (Table 5.1). The KA doses were chosen based on empirical data from previous attempts to induce seizures in the lab, while the pilocarpine doses were based on protocols from another lab (Helen Scharfman, personal communication). Twelve mice per condition were tested. While both tested doses of pilocarpine generated high rates of SE (83% and 92%, respectively), there was very significant mortality with both doses (50% and 66%, respectively). In contrast, while the 30 and 35mg/kg dose of KA had similar rates of SE (83% and 92%, respectively), these animals had much lower mortality rates (33% and 42%, respectively) In addition, pilocarpine-treated animals reached SE more quickly

than KA-treated animals and exhibited much more variability in the severity of their seizures, which likely contributed to the higher mortality. Based on these results, the 30mg/kg dose of KA was determined to be the best condition for seizure induction in the mouse .

**Effect of NRSF deletion on seizure-induced neurogenesis**

As described in Chapter 4, NRSF is required for the long-term maintenance of the NSC pool. Loss of NRSF results in a loss of stemness, increased proliferation, and accelerated neuronal differentiation that, over long periods of time, exhausts the stem cell pool in the adult hippocampus. However, even at late timepoints after NRSF deletion, Type 1 stem cells are still present in the SGZ. These cells may be remnants of the stem cell pool that have reached the end of their proliferative life and have become inert (Juan Encinas, personal communication). However, they may still be capable of functioning as NSCs if presented with a strong enough stimulus. In addition, NRSF mRNA is known to increase in the hippocampus after treatment with a chemoconvulsant [93], suggesting that NRSF likely has a function in seizure-induced neurogenesis. To explore this idea, four- to six-week old NRSF WT and cKO mice were treated with tamoxifen as before and allowed to survive for five months. Mice were then treated with a slightly lower dose of kainic acid (20-25 mg/kg) than the optimized protocol to prevent as much mortality as possible. Animals were observed for seizure activity and housed separately for four days until sacrifice. Brains were sectioned and stained for YFP and Ki67 to determine cell-type distributions by morphology and proliferation rates in the recombined cells. Any animals that did not reach status epilepticus (SE) were removed from analysis.

Morphological analysis of YFP+ cells revealed a significant increase in Type 1 cells in the cKO animals after seizures (Figure 5.1A) as well as a near significant decrease in cells with neuronal morphology (Figure 5.1B). This correlates with the 120 day data, which also showed a proportional increase in Type 1 cells (data not shown) and a decrease in neurons (Figure 4.7). Like the 120 day data, the absolute numbers of YFP+ Type 1 cells in the KA-treated animals did not change, while there was a large drop in the absolute number of neurons. Furthermore, there was no difference in YFP+Ki67+ cells

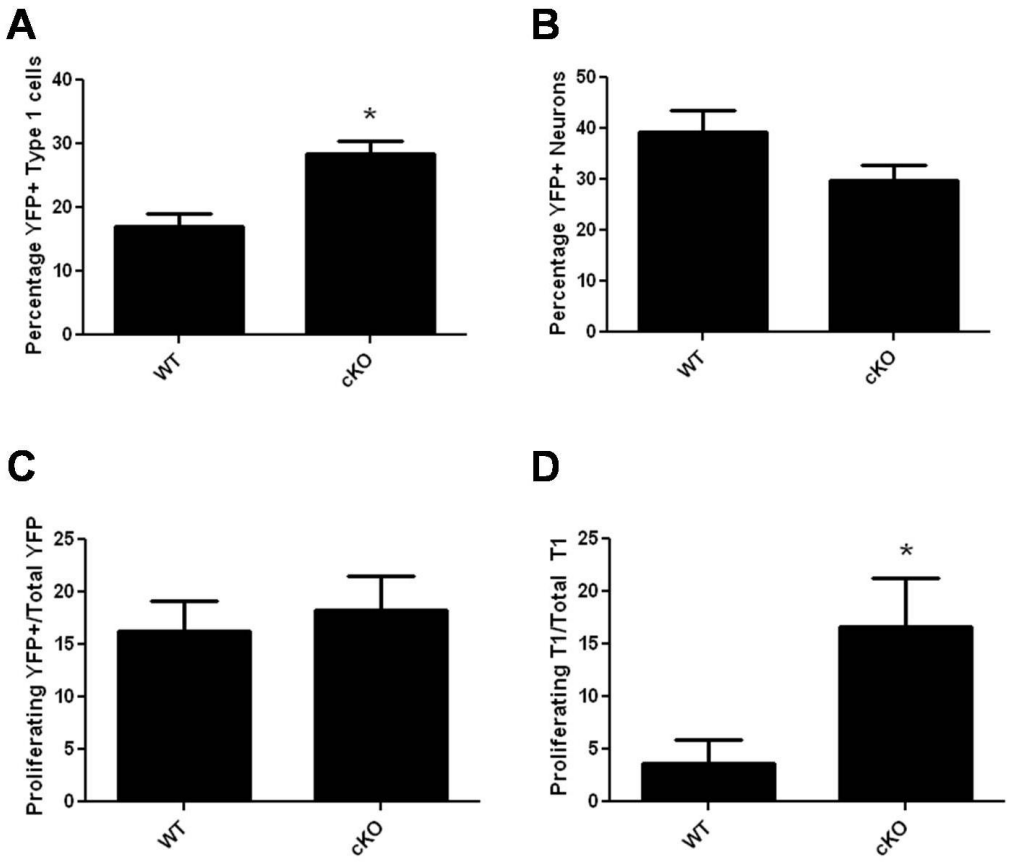



**Figure 5.1. Seizure induction in the NRSF cKO induces proliferation in Type 1 cells.** (A) After seizures, the proportion of Type 1 YFP+ cells increases in the NRSF cKO. (B) There is a strong but not significant trend toward a decrease in the proportion of cKO neurons. (C) There is no difference in Ki67+ proliferating cells after seizures between genotypes. (D) A significantly larger proportion of Type 1 cells are proliferating after seizures in the cKO. * $P<0.05$

between the WT and the cKO (Figure 5.1C). However, when YFP/Ki67 double-positive cells were analyzed for morphology, a significantly larger proportion of Type 1 cells in the cKO were proliferating four days after seizures (Figure 5.1D). Thus, within a short time after a SE event, the remaining cKO Type 1 cells had already been reactivated and had begun to proliferate, much as they do shortly after the initial deletion of NRSF. This suggests that a small population of NSCs may be able to survive the loss of NRSF and remain in a non-proliferative stem-like state, possibly by relying on other signaling pathways for rescue of the loss of NRSF's regulatory pathways. A second and more exciting possibility is that these survivor stem cells shift their identity to a more glial nature, which they can revert from by modulation of NRSF regulation and a strong stimulus, such as seizure activity.

**Effect of NeuroD deletion on pathological and physiological neurogenesis**

While the deletion of NRSF can provide an insight into the NSC reaction to environmental stimuli, the NeuroD cKO mouse line can reveal a the response of more commited progenitors as well as the effect these stimuli have on differentiation, maturation, and survival of these newly born neurons. Furthermore, the comparison of the effects of a pathological/deleterious stimuli, like seizures, and a physiological/beneficial stimulus, such as voluntary running, on neurogenesis after the loss of a necessary regulatory factor could provide an insight into how these stimuli function in the neurogenic process, resulting in the observed large burst of neurogenesis.

To this end, two month old NeuroD WT and cKO mice were treated with tamoxifen as before. Five days after tamoxifen, the mice were treated with the optimized KA seizure-induction method described in the first section of this chapter and housed separately for thirty days until sacrifice. Brains were sectioned and stained with YFP in order to determine the number of YFP+ cells and their morphologies. Interestingly, there was no difference in the total number of hippocampal YFP+ cells between WT and cKO non-seizure animals, but there was a very large drop in YFP+ cells in the cKO seizure animals (Figure 5.2A, B). However, there was no difference in the proportion of each morphology in the total YFP+ population (Figure 5.2C), meaning that this decrease in

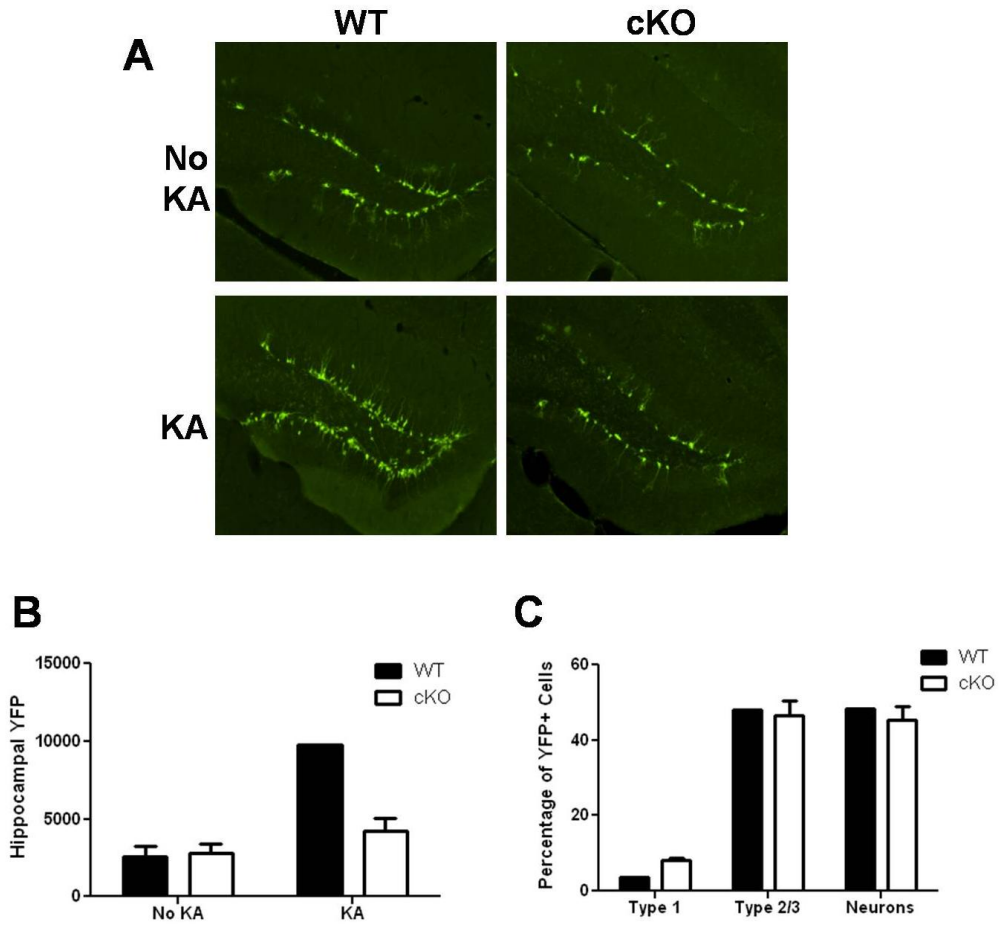



**Figure 5.2. Seizure induction rescues the loss of neurons in the NeuroD cKO .** (A-B) NeuroD cKO animals induced with seizures have fewer newborn neurons than WT animals. (C) There is no difference in the proportion of any cell type after seizures.

hippocampal YFP+ cells was due to a global decrease in cell number. This is a stark departure from the NeuroD cKO knockout, which showed a significant loss of newborn neurons at much the same timepoint. This data suggests that the seizure stimulus may bypass the need for NeuroD in the maturation and survival of new neurons. There are caveats to this study, as the number of animals used was very low, particularly in the WT KA-treated group. However, this study was repeated three times independently with modifications to the age of the animals and generated similar results each time (data not shown), lending credence to this finding.

To compare a more beneficial stimulus with the effect of the pathological seizure stimulus, a second cohort of four- to six-week old NeuroD WT and cKO animals were treated with tamoxifen. One week after the final injection of tamoxifen, the running group animals were singly-housed in cages with a running wheel. Mice were allowed to run ad libitum for two weeks, while control mice were kept in normal social housing in the same room. At the end of the running period, all animals were sacrificed and the brains were sectioned and again stained for YFP. In contrast to the seizure results, running animals showed no difference in total YFP+ cells by genotype (Figure 5.3A, B). Furthermore, there was a significant increase in the proportion of Type 2/3 cells and a similarly significant decrease in the proportion of new neurons in the cKO running animals (Figure 5.3C). The morphological phenotype closely matches the expected neuronal loss seen in the control cKO animals. The increased Type 2/3 cells are likely due to increased proliferation due to the running stimulus; cKO running animals had the same percentage of YFP/Ki67 double positive cells as their WT counterparts (Figure 5.3D). This proliferation is likely independent of NeuroD, but as the newly generated neuronal progenitors begin to differentiate, they are unable to overcome the loss of NeuroD and die off, as was seen in the original characterization of the NeuroD cKO.

**Summary and Conclusions**

This chapter described the effect of NRSF and NeuroD deletion on the neurogenic response to seizure stimuli and compared it with the effect of voluntary running in the NeuroD cKO. It reveals tantalizing clues into the nature of these two

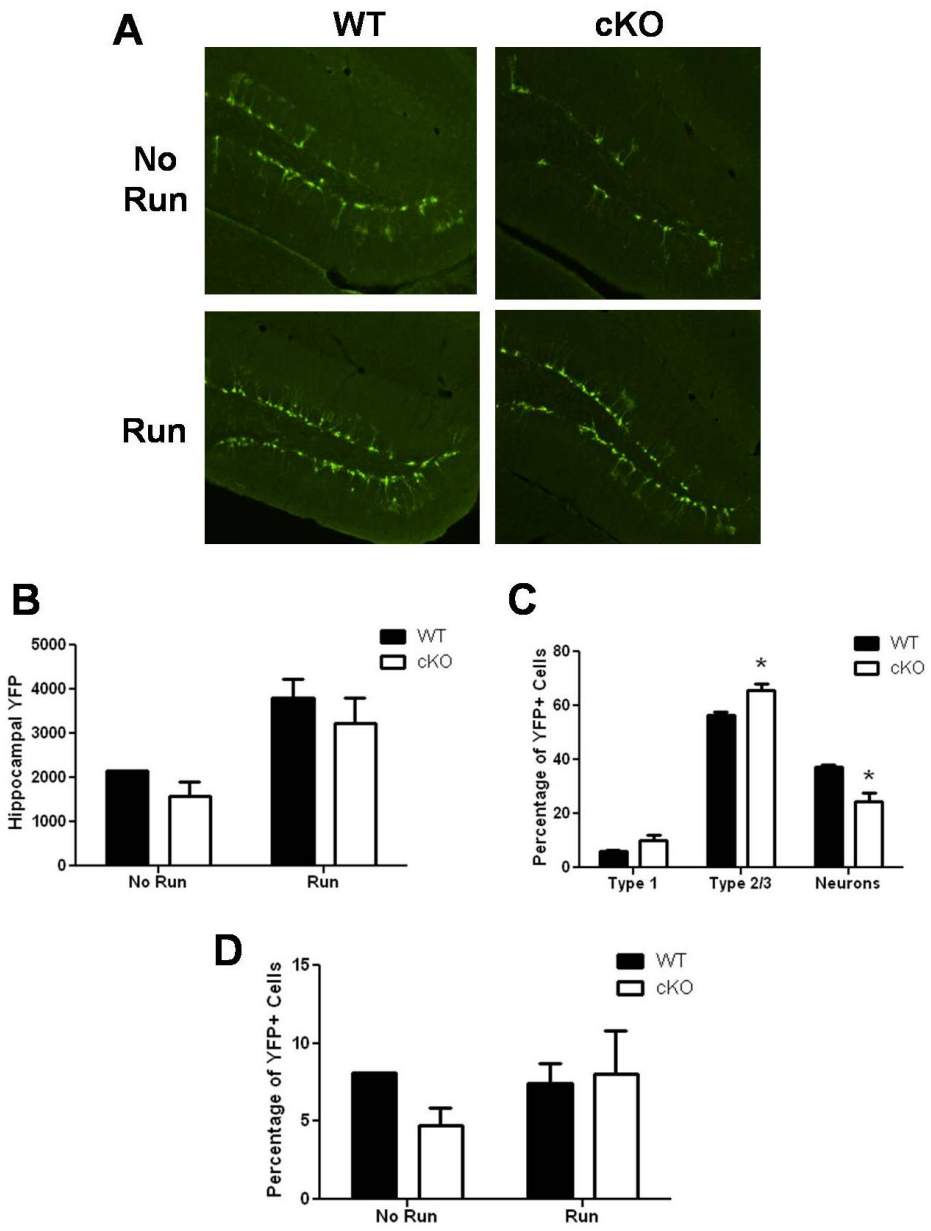



**Figure 5.3. Running increases neuronal progenitors but can not rescue the NeuroD cKO neuron loss.** (A-B) There is no difference in YFP+ cells between WT and cKO animals. (C) Running increases the proportion of Type 2/3 cells but can not overcome the need for NeuroD to form new neurons. (D) There is no difference in Ki67+ proliferating cells between genotypes. * $P < 0.05$

stimuli's signaling mechanisms in the hippocampal neurogenic zone, in particular suggesting that physiological and pathological neurogenesis may utilize separate pathways to produce new granule neurons.

# CHAPTER 6

## Discussion

The previous chapters have presented the data from several connected projects, all producing results that shed light on the larger concept of adult neurogenesis, both basal and induced. This chapter will present a unified explanation for the observed results as well as examine how this work advances the field. In addition, suggestions for future work will be presented.

### NeuroD is a critical factor in later stages of the neurogenic cascade

As reported in Chapter 3, NeuroD is critical for terminal differentiation, maturation, and survival of newborn neurons in the adult SGZ. This finding falls in line with the embryonic data, where NeuroD was necessary for the survival of granule neurons and for the development of the DG. What can not be answered in either of these studies is how exactly NeuroD functions in this process. The loss of NeuroD may prevent the neurogenic cascade from progressing correctly, resulting in fundamentally abnormal cells that can not survive. It is also possible that progenitors without NeuroD are able to differentiate normally but are unable to integrate into the surrounding circuitry and are thus eliminated. The answer may also lie in a combination of the two explanations.

When NeuroD is deleted *in vitro*, it blocks neuronal differentiation even after induction with a neurogenic factor. In addition, markers of neuronal fate, such as GluR2, Tuj1, and NR1 are significantly decreased, as well as a the pro-survival gene p21 [184]. This suggests that there is something fundamentally wrong with the neurogenic process, likely due to the loss of regulation at NeuroD's target genes, which include other neuronal genes like Prox1, Dcx, Synapsin III, and NCAM1 [121]. In addition, members of the Notch pathway, including Notch 1 and Hes5, have been identified as possible targets of NeuroD [121]. As there are suggestions that NeuroD itself is downstream of the Notch pathway [129], this suggests that NeuroD may play a role in mediating Notc

signaling at later points in differentiation. All together, NeuroD does likely play a role in ensuring normal differentiation while preventing cell death (Figure 6.1).

As discussed in Chapter 1, NeuroD also has been implicated as an important factor in dendritogenesis, particularly after neuronal activity [127]. The findings presented in Chapter 3 strongly argue for a similar role in the adult and set NeuroD as a direct effecter of dendrite growth as part of the maturation of new granule neurons, possibly by mediating Notch signaling [52]. The shortened dendrites in NeuroD cKO neurons are likely dysfunctional, although electrophysiological analysis of these cells would be necessary to confirm this. If indeed these neurons are unable to integrate into the hippocampal circuitry, they are likely eliminated, much as newborn neurons unable to respond to NMDA are lost [82]. This is useful from an evolutionary point of view, as unneeded cells generated in the adult could be eliminated in favor of neurons that are useful, thus conserving resources efficiently and blocking unnecessary noise that could interfere with the functioning of the hippocampal network.

Regardless of the exact explanation of NeuroD's function, it is increasingly clear that NeuroD is a critical part of the pathway. It stands as a possible downstream target of two of the major neurogenic pathways, Wnt [128] and Notch [129] and as an upstream regulator of multiple neuronal pathways. While not as important for neuronal fate determination, it is critical for the entire process to proceed smoothly.

**NRSF's role in neurogenesis**

As described in Chapter 4, NRSF is a critical regulator of NSC maintenance and differentiation. Without NRSF, the NSC pool begins to proliferate and differentiate at an accelerated rate, eventually depleting itself. Interestingly, however, neurogenesis is still able to proceed normally without NRSF until the proliferative ability of the resident NSCs is exhausted. Even more interestingly, there is no suggestion that the loss of NRSF allows cells to be more promiscuous in their cell fate choice, as there is no detectable increase in astrocytes or oligodendrocytes (data not shown). This partly disproves the

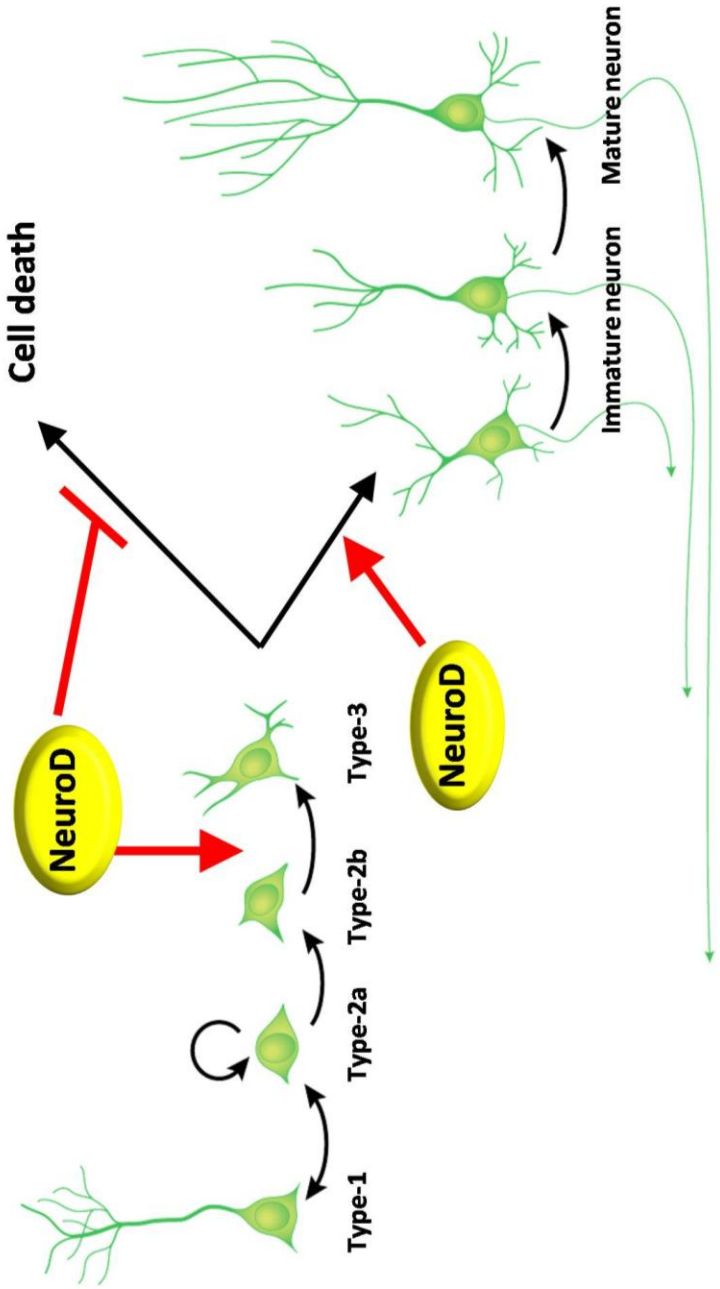



**Figure 6.1. Proposed model of NeuroD function in neurogenesis.**

widespread hypothesis that NRSF may function as a master regulator of neurogenesis but at the same time hints that NRSF's role may be much more complex than originally anticipated.

The vast number of NRSF's proven and predicted target genes suggest that the cKO phenotype might be due to a massive disregulation of its target genes, encompassing proliferation, differentiation, maturation, and survival pathways. Of particular interest to this thesis is the presence of an NRSE in NeuroD's promoter [91], suggesting that loss of NRSF repression, along with changes in other regulatory factors at the NeuroD promoter, may be one of the driving forces of later differentiation mediated by NeuroD. This might also partly explain the accelerated differentiation phenotype. However, it is odd that a transcriptional repressor with such a wide purview of regulation would not have a more robust phenotype. The fact that any neurons can be generated at all in the NRSF cKO suggests that there is some type of redundancy in the regulation of neurogenesis. NRSF may play an important role, but it is not the only factor.

While the regulatory activity of NRSF on its target genes has been studied extensively, there is very little work published on upstream regulators of NRSF itself in neural stem cells. As described in the introduction, a very recent report suggests that Bmp2 signals through NRSF to block differentiation and maintain stem cell identity [67]; additionally, both the Wnt and Notch pathways have been implicated in NRSF regulation, but only in different contexts [115-116]. Most of our understanding of how NRSF is regulated comes from posttranscriptional mechanisms, such as binding site affinity [103] and degradation [112-114]. Based on NRSF's function in the Type 1 NSC, it is not beyond imagining that NRSF could be a target of Notch, Wnt, Shh, and Bmp, or even another pathway that has yet to be identified. Furthermore, the downregulation of NRSF in NeuroD+ cells suggests that there must be some type of regulation that occurs as differentiation begins. This regulation may be mediated by NeuroD itself, as the NRSF regulatory sequence contains nine E-boxes. Furthermore, a close homolog of NeuroD, NeuroD2, has been shown to indirectly repress NRSF expression in an *in vitro* P19 cell culture system [194].

Based on the known function of NRSF, I propose a new hypothesis for its role in neurogenesis, not as a master regulator of neuronal fate but instead as a type of master integrator (Figure 6.2). In this model, NRSF is the target of multiple upstream regulatory pathways. At the same time, NRSF occupies NRSE sites at multiple target genes, with each site individually regulated by a complement of corepressors or activators. Based on the pattern of signaling to NRSF, it will recruit repression or activation machinery, which can include cofactors and chromatin remodeling factors, in a site-specific manner. NRSF may also vacate the NRSE completely, allowing other transcriptional regulators to control the expression of the target gene. By integrating the totality of signaling to the NSC, NRSF can quickly and precisely modify the transcriptional milieu of the cell to maintain stemness or promote differentiation.

This model does not preclude the presence of other factors that may act in the same manner as NRSF. As mentioned earlier, the NRSF cKO phenotype suggests that there is redundancy in the regulation of neurogenesis. The loss of NRSF's widespread regulation may be partially compensated for by other intermediaries, resulting in the cKO's subtle phenotype. One possible mediator could be NRSF's own cofactor, CoREST, which can also bind and regulate target genes independently of NRSF [195]. CoREST targets a large cohort of genes independently of NRSF, and many of these targets are involved in pluripotency, fate decision, and neuronal maturation. Interestingly, one of CoREST's targets is NeuroD [196]. In addition, each of the three options for regulation allows for other regulatory factors, such as MeCP2 or bHLH proteins, which could modulate the effect of NRSF regulation to add another level of complexity and sensitivity.

The identification of other master integrators presents a significant conceptual and technological problem. If, like NRSF, the individual knockout of a single master integrator produces only a subtle effect that is largely compensated for by other integrators, single knockouts will produce little useful data. Indeed, it is possible that both total and conditional knockouts of master integrators exist but have been largely ignored as having no phenotype. A better method for identifying master integrators may

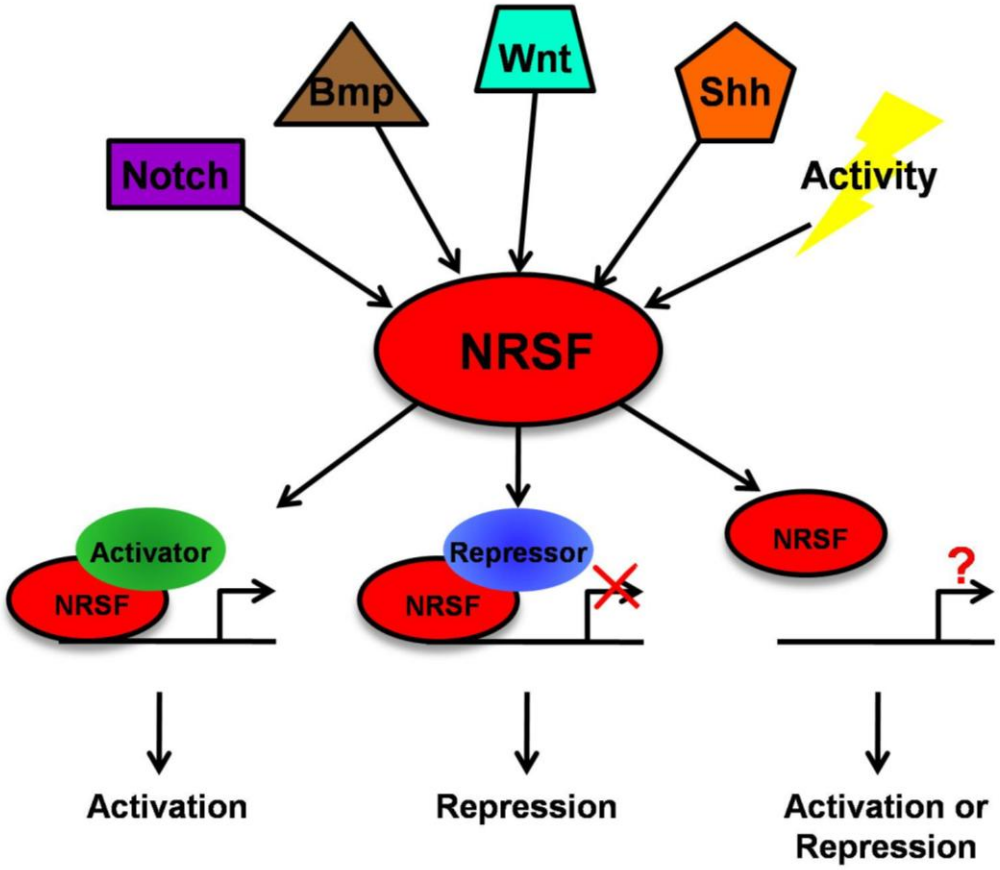



Figure 6.2. Proposed model of NRSF function in neurogenesis.

be to identify transcription factors that bind elements common throughout the genome or that form complexes at these sights. Candidate genes could then be knocked out genetically or knocked down using siRNA in cell culture systems, which would allow conditions to be modified to replicate proliferation and differentiation conditions. Cells collected from these conditions could then be used for deep sequencing techniques like RNA-seq and ChIP seq to identify the totality of changes in the entire genome. Deep sequencing would be a better method of approaching these changes than a microarray, which would bias the results to a small cohort of genes chosen by the investigator. Unfortunately, this method will be time consuming, but as deep sequencing technology progress over the next few years, the data generated from these assays would likely justify the time and financial investment.

**Type 1 senescence and seizure activity**

The NRSF cKO phenotype provides evidence of the finite proliferative capacity of the Type 1 NSC. Recent unpublished findings have suggested that an individual Type 1 cell can divide only a few times before losing its ability to proliferate and assuming more astrocytic-like qualities (Juan Encinas, personal communication). If this model is correct, then NRSF may be acting as a brake on this process. By slowing the proliferation of Type 1 cells and preventing differentiation, NRSF essentially buys time for the SGZ to remain neurogenic. In short, NRSF prevents the premature aging of the SGZ.

As described in Chapter 5, seizure activity in the NRSF cKO increases Type 1 proliferation. As the animals used in this experiment were nearly six months past tamoxifen induction of recombination, most of the YFP+ cells had ceased to proliferate and had likely changed to a more astrocytic identity. The reactivation of proliferation in these cells suggests that a strong enough neurogenic stimulus may be able to reprogram an otherwise senescent cell into a functioning Type 1 stem cell. How long this reprogramming lasts and if it is enough to generate a new neuron remains to be seen and would require additional time points after tamoxifen injections and after seizures to fully

analyze. In addition, while it would be neither practical nor advisable to attempt to reactivate neural stem cells with seizure activity in patients, this finding does at least suggest that senescence in the adult stem cell is not a permanent state and that a strong but non-pathological stimulus may be a viable means of reactivation.

It would be of particular interest to examine the transcriptional changes in this shift from quiescent Type 1 to astrocyte-like cell to seizure-reactivated Type 1. If these populations could be isolated from the hippocampus, an RNA-seq could reveal fascinating changes in transcription that might underlie stem cell identity and fate choice. As of this writing, the lack of a marker identifying each of these populations that would allow for easy FACS sorting makes this experiment technically very difficult and is thus beyond the scope of this thesis. However, as our understanding of the nature of the SGZ stem cell progresses, this experiment may become feasible.

An additional interesting aspect is the nature of the Type 1 cell division. Most work, including the projects presented here, has focused on an asymmetric division that results in a Type 2 daughter cell, but it is not inconceivable that the Type 1 NSC may divide symmetrically as well. These divisions are likely extremely rare and easily obscured by prolific asymmetric divisions that can be more readily visualized by proliferation markers and BrdU incorporation. If NRSF is indeed acting as a regulator of NSC proliferation, there may be an effect on this putative symmetric division, although what that effect may be is difficult to predict at this point. Answering this question will likely require the development of a live-imaging system that can monitor a large number of labeled cells for months, a system that is currently out of technological reach.

**Physiological and pathological neurogenesis may utilize different pathways**

One of the most fascinating aspects of neurogenesis is the ability of external stimuli to influence and fundamentally change the generation and integration of new neurons in the SGZ. On the surface, it would seem that any stimuli would induce neurogenesis using the same mechanism as basal neurogenesis, with the only differences being the speed and amount of neuronal differentiation. However, two different stimuli,

a physiological versus pathological induction, have profoundly different effects with the deletion of a single transcription factor, NeuroD. Voluntary exercise in the cKO does not increase the number of YFP+ cells but increases the proportion of neuronal progenitors while decreasing the proportion of neurons, while seizures in the cKO are able to increase the total number of YFP+ cells without changing the proportion of any cell type. This difference, when coupled with the basal neurogenesis phenotype of the NeuroD cKO, confirms the literature in suggesting that the two stimuli use separate pathways.

If indeed the two stimuli use different pathways, NeuroD could be a point of divergence or simply a readout of the two different pathways. The latter explanation seems the more likely, as it appears that exercise and seizures affect different cell types [13]. It is thus more likely that the diversion occurs early in the neurogenic cascade, with seizures able to bypass the need for NeuroD while running remains dependent on it. It is difficult to state with any certainty how exactly the two induction pathways differ, as there has been no careful comparison of the regulatory pathways involved. It is likely that the response to neurogenic stimuli is mediated by neuronal activity, particularly in light of the finding that animals deficient in NMDA receptors do not have the neurogenic response to running activity [143]. This activity probably has a spectrum of effects on multiple pathways, with the contribution of each pathway varying based on the type and strength of the neurogenic stimulus. If the proposal of NRSF function (Figure 6.1) is correct, this specialized activity could be translated into differing effects through the activity of these putative intermediaries, including NRSF.

Using the current tools, the next best step to investigate these potential divergent pathways is to repeat the running and seizure experiments in the NRSF cKO. NRSF's role in the Type 1 NSC may be a point of divergence, particularly if it is functioning as a master integrator that would respond differently to specific contexts. Furthermore, as NeuroD is a downstream target of NRSF, it would not be surprising to find the NeuroD results replicated in the NRSF cKO. However, if there are other factors besides NRSF involved, it is also likely that only a very subtle neurogenic effect may be noted. In

addition, these studies would need to be carried out in younger animals to avoid a reactivating effect on otherwise senescent Type 1s.

**The Type 1 controversy**

There is some controversy as to the nature of the Type 1 cell, namely whether it is a true stem cell or a specialized form of glia. The work of Arturo Alvarez-Buylla has strongly argued that Type 1 cells are not true neural stem cells but are instead radial glia, possibly descended from embryonic radial glia [197] There is some support for this, as the Type 1 cell expresses astrocyte markers, including GFAP and nestin, and its morphology does resemble the radial glia found during embryonic development, including the presence of a primary cilia in SGZ NSCs [62]. Furthermore, Type 1 cells have similar electrophysiological characteristics as astrocytes, while Type 2 cells seem to be an electrophysiological transition stage between astrocyte and neuron [198-199]. This provides an interesting discussion point in how the neurogenic niche forms embryonically. However, unlike astrocytes in other regions of the brain, these cells can divide and are multipotent. Furthermore, as discussed above, it is only after these cells have exhausted their proliferative capacity that they take on stereotypical astrocytic characteristics, suggesting that they are originally something else entirely.

While this question of astrocyte or not is intellectually interesting, it ultimately becomes a moot point when the reality of the cell's behavior is considered. If the NSC is truly glial in nature, then it is a highly specialized astrocyte with stem cell behavior. If the NSC is not a radial glia, then it is a stem cell that is remarkably similar to the surrounding astrocytes. The answer does not change the fact that the cell is multipotent and consistently produces new neurons, and continued argument serves only to increase confusion in the field.

A far more important and difficult question lies in the exact definition of the NSC. The traditional definition has been as described in Chapter 1: radial morphology, expressing GFAP, Sox2, and nestin, and quiescent. However, there is still no consensus on what cell is the NSC. A recent paper argued that there are actually two stem cell

populations, a radial and a horizontal population [49]. Others have argued that an even more primordial stem cell exists (Nathan DeCarolis, personal communication). These findings suggest that the neural stem cell pool may be heterogeneous; multiple specific cell types may all act as NSCs, or a single NSC may be able to take on different characteristics based on extrinsic factors, such as metabolic substrates or signaling from surrounding cells.

This controversy presents a problem for those who wish to explore neurogenesis using *in vivo* models. The generation of adult neural stem cell conditional knockouts requires a choice of a marker that will induce recombination specifically in NSCs, and that marker does not exist. Multiple Cre lines have been generated, including GFAP-Cre, GLAST-Cre, and nestin-Cre, as was used in this thesis. It is possible that a different phenotype could be observed simply by using a different Cre driver that targets a slightly different population of cell. Until an understanding of what exactly constitutes a NSC is formed, this controversy will continue.

**Neurogenesis and epileptogenesis: are the two processes linked?**

As discussed in the introduction, a single seizure event can result in spontaneously recurring epileptic seizures roughly a month after the initial insult [150]. It has been widely assumed that the seizure-induced neurogenesis occurring in the SGZ was at least partly responsible for this phenomenon due to the correlation in time span between the maturation of a new neuron and the development of seizures. In addition, seizure-generated neurons exhibit abnormal morphology, including basal dendrite (mossy fiber) sprouting, misdirected migration into the hilus and molecular layer, accelerated integration into the surrounding circuitry, and reduced excitability [133, 151-153], all of which could be contributing factors to the generation of spontaneous seizures.

How then can the generation of new but abnormal cells in one region of the brain create an effect that affects the entire brain? There are several possible explanations. First, the sprouting of mossy fibers that can make connections with surrounding neurons [200] may create a feedback look that can amplify abnormal activity, leading to the

generation of a new seizure. This explanation is likely improbable due to the hypoexcitable nature of the newborn neurons. In addition, a recent study reported that rats treated with rapamycin, an mTOR pathway antagonist, one day after seizure induction had significantly reduced aberrant mossy fiber sprouting with no change on cell death or neurogenesis. However, these animals still developed spontaneous seizures at the expected time point [168-169], suggesting that mossy fiber sprouting may not be enough to prevent epileptogenesis. Second, the abnormal migration of seizure-born neurons may result in both dendritic and axonal synapses forming on neurons that would normally never synapse with granule cell neurons, such as pyramidal neurons in the CA1. These abnormal connections could perturb the circuitry enough to generate a seizure under the right conditions. Third, the reduced excitability of seizure-born neurons could result in a net gain of excitability in the entire dentate gyrus. Granule cells are strongly GABAergic and may function as an inhibitory filter for signaling from the cortex [201]. If a large percentage of the granule cell layer is made up of neurons that are less likely to fire an inhibitory impulse upon activation, the net effect would be a loss of inhibition. Thus, the threshold to generate an epileptoform discharge would be lowered, allowing seizures to occur more frequently. With virtually no experimental data on the subject, it is difficult to predict which of these explanations is correct or if it is a combination of factors. It is also likely hippocampal adult neurogenesis is not the only factor involved in epileptogenesis; there are likely extrahippocampal causes, such as brain injury, neurotransmitter imbalances, or genetic defects that allow the initial seizure to occur and set the stage for following events. However, it is likely that seizure-induced neurogenesis plays an important role.

The best way to determine the exact contribution of seizure-induced neurogenesis is to ablate neurogenesis before seizures or to block the neurogenic response to seizure induction. While some attempt has been made to approach this question [157], it has proved to be a much more difficult proposition than expected. While there are several means of eliminating neurogenesis, such as irradiation, Ara-C treatment, or genetic models such as TK or diphtheria toxin-producing conditional alleles, no method can perfectly eliminate all new neuron formation in the SGZ. Methods that target dividing

cells, like irradiation or Ara-C, miss the slowly dividing Type 1 NSCs, which can later reestablish the progenitor pool after treatment has ended. Genetic methods may not be fully penetrant and depend on a stem cell-specific driver; as mentioned in the previous section, there is no known specific NSC marker and no exact definition of an NSC. In addition, these methods of ablation have the potential to produce a gliosis reaction that could confound attempts to understand a mechanism that itself causes damage to the surrounding neural tissue. Of these models, genetic means of ablation hold the greatest promise with fewer complications for delving into this question.

**The master integrator in neurogenesis and evolution**

In the fifty years since the existence of adult hippocampal neurogenesis was first discovered, great strides have been made in answering basic questions about the nature and maintenance of the NSC, the process of neuronal differentiation, the regulatory mechanisms underlying the entire process, and the functionality of the resulting neurons. However, the much larger question of why adult neurogenesis occurs at all remains virtually untouched. Why do stimuli like voluntary exercise, seizures, and learning events induce neurogenesis at all? How does an extracellular and even an external environmental stimuli produce a neurogenic reaction in the SGZ?

As described in the introduction, there is some suggestion that certain cell types are susceptible to certain stimuli. For instance, Type 1 NSCs seem to be sensitive to seizure induction ([13, 159] and Figure 5.1), while running preferentially activates the progenitor population [11, 13]. Virtually nothing is known about what cells react to means of inducing neurogenesis, such as enriched environments, or depressing neurogenesis, such as stress and drugs of abuse. However, knowing this information would only suggest a starting point for understanding how this induction or depression works. It is entirely possible that every stage along the neurogenic pathway is sensitive to stimuli but reacts in a different manner, either to promote or prevent proliferation and differentiation or in modulating survival, and it is very likely that surrounding glia cells are sensitive as well. Furthermore, these reactions may feed back onto other cell types,

either through the release of soluble factors that can activate the major regulatory pathways, like Wnt or Notch, or through modulation of metabolic factors. The incredible complexity of this process makes it even more extraordinary that any neurogenic effect can be seen through the noise.

The existence of a small group of master integrators, of which NRSF is likely one, presents an attractively simple way to translate the enormous volume of information being presented to a NSC into unique transcriptional modifications at a host of target genes (Figure 6.3). Furthermore, it allows the cell to translate signals from a multitude of sources into molecular change. For example, when a mouse is placed in a cage with a running wheel, it surveys its environment and recognizes the purpose of the running wheel, which likely involves accessing a memory connecting the moving wheel with the pleasurable experience of running. When the mouse is actively running, its heart rate will increase, its muscles increase their activity, and its brain will activate regions directly related to the process and the effects of running. This could result in hormone release and increased activity, as well as a possible increase in blood flow in the brain from the increased heart rate. All of these factors could be sensed by the NSC, either directly through the angiogenic niche or indirectly through changes in cell-extrinsic signaling. Intracellular effectors of extracellular signaling, such as NICD for the Notch pathway and β-catenin for the canonical Wnt pathway, and metabolic changes in the cell brought on by the gain or loss of growth factors from the niche are sensed within the nucleus by a master integrator. At a specific site like the NeuroD promoter, the regulatory effect of these downstream signals influence whether or not NRSF, the integrator, binds to the NRSE at the target gene as well as what cofactors are available for it to complex with, resulting in the upregulation of NeuroD and the induction of neurogenesis. At the same time, NRSF is repeating this process at hundreds of other genes, while other integrators or doing much the same thing at their specific target genes and with their specific cofactors. The combination of each gene-specific modification results in a net shift toward proliferation and neuronal differentiation.

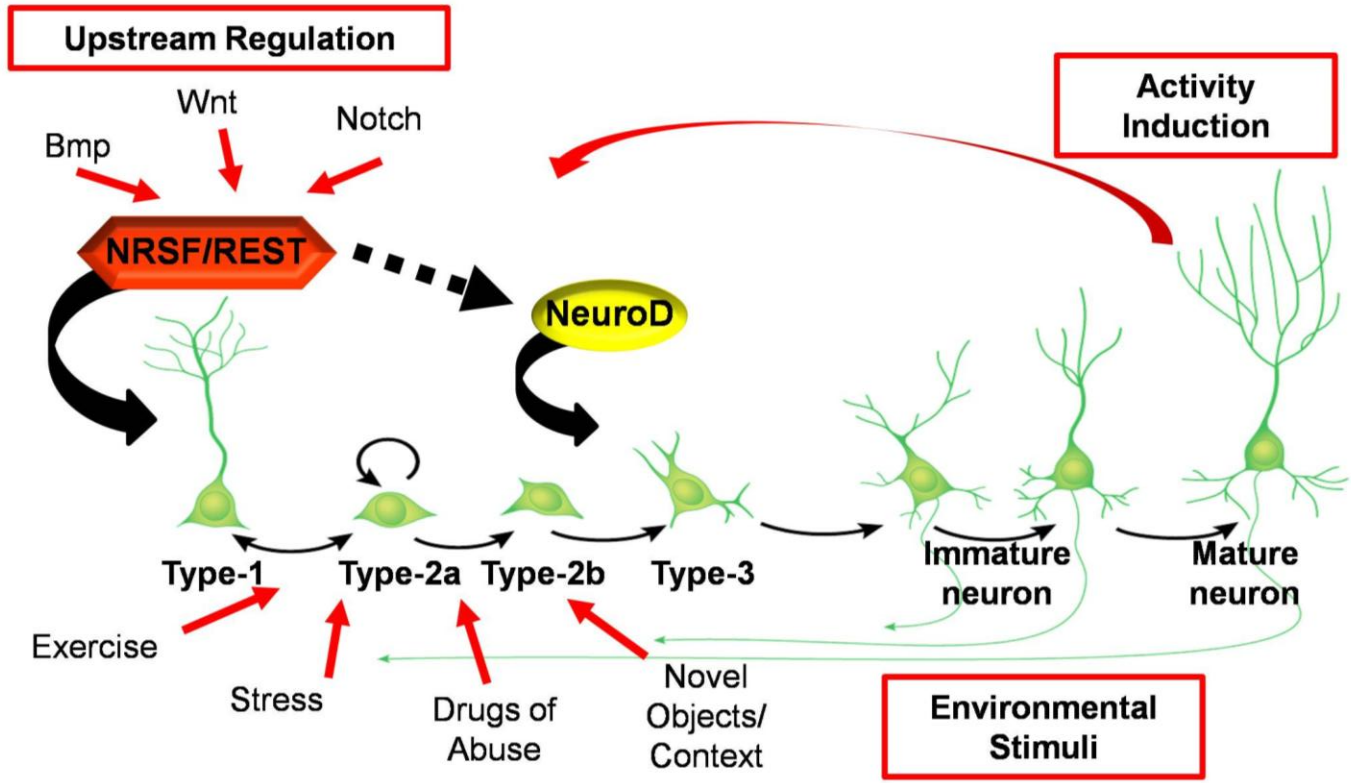



**Figure 6.3. A proposed model of neurogenesis where inductive signals are mediated through the NRSF/NeuroD regulatory axis..**

From an individual point of view, this model provides an exquisite level of customization: each method and instance of induction or depression produces a unique suite of regulatory modifications which in turn results in a similar but fundamentally specific neurogenic result. A single cell is dependent on what it is exposed to for its response, and since each NSC in the SGZ may see a different extracellular milieu, each NSC can react individually. For a single organism, this means that for a large-scale neurogenic response to occur, multiple factors must be present, and each factor determines the strength and the form of the response. From an evolutionary point of view, this provides a level of individuality that extends far beyond simple genetic polymorphisms. Fitness is dependent on an animal's ability to respond to its environment and how quickly it is able to adapt to changing circumstances. The presence of a core of master integrators allows customizable responses that may allow an animal to quickly coordinate a huge number of influences into a single adaptive response. Whether or not an organism makes a new neuron will thus depend not only on the act of running but on why the animal is running, how well fed it is, what its reproductive status is, whether it is in a familiar or novel environment, the state of the genomes it received from its parents, and a host of other conditions.

## Conclusion

As described throughout this thesis, adult neurogenesis is an incredibly complex process that is both practically and functionally important while being at the same time aesthetically beautiful. It is extraordinary that a process underlying so many functions critical to our ability to process and respond to our environments went undiscovered for so long. It is increasingly clear that neurogenesis under the correct conditions can allow us to form and retrieve memories, form connections with other memories that can later be a source of insight, and possibly choose proper mates. When neurogenesis is perturbed, we are more susceptible to depression, memory loss, and epileptogenesis. From an evolutionary standpoint, the energy needed to continue what is possibly a modified embryonic process throughout adulthood is perfectly acceptable, as it provides a competitive advantage to survival. It is probably for this reason that a process occurring

in our household pests also occurs in us. As the field matures in the future, it is likely that neurogenesis will prove to be a fruitful, exciting, and illuminating field that will inform our understanding of how our own brains work in ways we can not possibly imagine.

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